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CD8 T cell differentiation during immune responses

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FACULTÉ DE MEDECINE-site Necker

INSERM U1020 (ex U591)

THÈSE

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DOCTEUR

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École Doctoral: Gc2iD

Présentée et soutenue publiquement par

Sara Sofia de Campos Pereira Lemos

Le 23 Mai 2014

CD8 T cell differentiation during immune responses

Jury:

Dr. Jacqueline MARVEL	Rapporteur
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Dr. Benedita ROCHA	Directeur de these

To a wonderful person, an extraordinary woman and an amazing mother...

my mother, Arlete Pereira

"The first precept was never to accept a thing as true until I knew it as such without a single doubt."

René Descartes (French philosopher and scientist)

"We live where no one knows the answer
and the struggle is to figure out the question"

Joshua Schimel (in "*Writing Science*" book).

" To be great, be whole;
Exclude nothing, exaggerate nothing that is not you.
Be whole in everything. Put all you are
Into the smallest thing you do.
So, in each lake, the moon shines with splendor
Because it blooms up above."

Fernando Pessoa (Portuguese poet)

"The beauty of life is not about finding something big enough,
but rather on finding something deep enough!

Sara Lemos (a dreamer!)

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Abbreviations

AIRE Autoimmune regulator
AP-1 Activator protein 1
APC Antigen presenting cell
ATP Adenosine triphosphate
BAFF B cell-activating factor
Bcl-6 B-cell lymphoma 6
BCR B cell receptor
BIR Baculovirus inhibitor repeats
Blimp1 B-lymphocyte induced maturation protein
C Carboxyl terminal region
C3,4,5 Complement fragments 3,4,5
Ca^{2+} Calcium
CaMK Ca2+-camlodulin-dependent kinase
CARD Caspase activation and recruitment domains
CD Cluster of differentiation
cDC conventional Dendritic Cell
CDRs Complementary-determining regions
CFSE Carboxyfluorescein diacetate succinimidyl ester
Clb Casitas B-lymphoma
CLP Common myeloid progenitors
CLR C-type lectin receptor
CMP Common myeloid progenitors
CpG cytidine-phosphate-guanosine
CRAC Ca2+ release-activation Ca2+ channels
Csk C-terminal Src kinase
cSMAC Central supramolecular activation cluster
CTL Cytotoxic T lymphocyte
CTLA-4 Cytotoxic T lymphocyte antigen-4
DAG Diacylglycerol
DAMP Damage-associated molecular patterns
DC Dendritic cell
DN Double negative
DNA Deoxyribonucleic acid
ds double strand
EMC Extracellular matrixes
Eomes Eomesodermin
ER Endoplasmic reticulum
Erk Extracellular signal-regulated kinase
ETP Early T-lineage progenitor
Fc Fragment crystallizable region
FOXP3 Forkhead box protein 3
FPR1 N-formyl peptide receptor
FRC Fibroblastic reticular cells
GM-CSF Granulocyte-macrophage colony stimulating factor
GP33 Glycoprotein 33 (residues 33 to 41)
GRK2 G protein-coupled receptor kinase2
Gzm Granzyme
HA Hyaluronic acid
HEV High endothelial venules
HSC Hematopoietic stem cell
HSV Herpes simplex virus
HY Male-specific antigen
ICAM Intercellular adhesion molecule-1
IEL Intraepithelial lymphocytes
IFN Interferon
Ig Immunoglobulin
IkB Inhibitor of NF-kB
IKKi Inducible inhibitor of NF-kB [ikB] kinase
IL Interleukyne
iNOS inducible nitric oxide synthase
IP-10 Interferon gamma-induced protein 10 (CXCL10)
IP3 Inositol 1,4,5-triphosphate
IRAK Interleukin-1 Receptor-Associated Kinase
IRF Interferon regulatory transcription factor
IS Immunological synape
ITAM Immunoreceptor tyrosine-based activation motif
ITIM Immunoreceptor tyrosine-based inhibition motif
JNK c-Jun NH2-terminal kinase
KLRG1 Killer cell lectin-like receptor G-1
KO Knock out
LAT Linker for activation of T cells
Lck Lymphocyte-specific Tyrosine Kinase
LCMV Lymphocytic choriomeningitis virus
LFA-1 Lymphocyte function-associated antigen1
LM *Listeria Monocytogenes*
LNs Lymph nodes
LPS Lipopolysaccharide
LY6C Lymphocyte antigen 6C
Lyve-1 Lymphatic vessel endothelial hyaluronan receptor 1
MAC1 Macrophage receptor 1
MAPK Mitogen-activated protein kinase
MDP Muramyl depeptide
MHC Major histocompatibility complex
MIP Macrophage Inflammatory Proteins
MPECs Memory precursor effector cells
MPP Multipotent progenitors

mTOR	Mammalian target of rapamycin
MyD88	Myeloid differentiation primary response gene 88
NADPH	Nicotinamide adenine dinucleotide phosphate-oxidase
NET	Neutrophil extracellular traps
NFAT	Nuclear factor of activated T cells
NFkB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NK	Natural killer cell
NK T	Natural killer-T cell
NLR	Nod-like receptor
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain
OT-1	OVA-trangenic
OVA	Ovalbumin
p38	P38 mitogen-activated protein kinases
PAMP	Pathogen associated molecular patterns
PD-1	Programmed death-1
pDC	plasmacytoid Dendritic Cell
PDGF	Platelet-derived growth factor
PECAM1	Platelet endothelial cell adhesion molecule
PGE/PGI	Prostaglandins E and I
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol 4,5-bisphosphate
PKC	Protein kinase C
PLC	Phospholipase C
PMN	Polymorphonuclear leucokytes
PNAd	Periheral node addressins
poly I:C	polyinosinic-polycytidycic acid
PPs	Peyer' patches
Prf1	Perforin
PRR	Pathogen recognition receptor
PSGL1	P-selectin glycoprotein ligand 1
pSMAC	Peripheral supramolecular activation cluster
PTK	Protein with tyrosine kinase activity
pTα	pre-TCRα chain
PYD	Pyrin domain
RAG	Recombination activating gene
RANTES	Regulated on activation, normal T cell expressed and secreted (CCL5)
RIP2	Receptor interacting protein-2
RLR	RIG-I-like receptors
RNA	Ribonucleic acid
ROS	Reactive oxygen species
S1P	Sphingosine-1-phospate
S1P1	S1P-receptor 1
SCID	Severe combined immunodeficiency
Ser	Serine
SHP1	SH2 domain-containing protein tyrosine phosphatase
SLECs	Short-lived effector cells
SLO	Secondary lymphoid organs
SLP-76	Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa
SP	Single positive
SPHK	Sphingosine kinases
ss	single strand
STAT	Signal transducer and activator of transcription
TAP	Transporter associated with antigen processing
TBK	TANK binding kinase-1
T_{CM}	Central memory T cell
TCR	T cell receptor
TD	T cell-dependent
TEC	Thymic ephitelial cell
T_{EM}	Effector memory T cell
Tfh	Follicular helper T cell
Tg	Transgenic
TGF	Transforming growth factor
tGPI	trypomastigote glycosylphosphatidylinositol mucin
Th	T helper cell
Thr	Threonine
TI	T cell-independent
T_{IM}	Inflammatory memory T cell
Tip-DC	TNF- and iNOS-producing DC
TIRAP	Toll-interleukin 1 receptor (TIR) domain containing adaptor protein
TLR	Toll like receptor
TN	Triple negative
TNFα	Tumor-necrosis factor-α
TRAM	TRIF-related adaptor molecule
Treg	Regulatory T cells
TRIF	TIR-domain-containing adapter-inducing interferon-β
T_{RM}	Resident memory T cell
Tyr	Tyrosine
V	amino-terminal variable region
VLA4	very late antigen 4
WT	wild type
ZAP-70	ζ-Associated Protein of 70-kDa

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Summary

CD8 T cells are essential for the elimination of intracellular pathogens and tumor cells. Understanding how naïve CD8 T cells differentiate into effector cells capable of eliminating pathogens and to generate adequate memory cells during immune responses is fundamental for optimal T cell vaccine design.
In this PhD thesis work we addressed two central questions:

1) What are the mechanisms by which early effector T cells could act as pro-inflammatory effectors? And what is their role in the immune response?

2) How heterogeneous are CD8 responses? Could different pathogens modulate CD8 T cell differentiation programs and be responsible for CD8 cell-to-cell heterogeneity? Could they also generate memory cells with different protection capacities?

To address these questions related to the diversity of CD8 T cell differentiation during immune responses, we used the single cell RT-PCR technique to detect *ex vivo* expression of mRNA in each individual cell, and Brefeldin A injected mice to detect *ex vivo* intracellular proteins. As experimental system to evaluate *in vivo* cell activation we used T cell receptor transgenic (TCR-Tg) CD8 T cells.

Since the use of TCR-Tg cells to study immune responses has been subjected to criticism (due to high frequency of naïve-precursor transfers), in a first Ms. we compared the behavior of TCR-Tg and endogenous (non-transgenic and present at low frequency) cells in the same mouse. We found fully overlapping behavior between these two cell populations, which reinforced the advantage of using TCR-Tg cells to study CD8 immune responses. In addition, we concluded that the frequency of naïve-precursors do not induce diversity on CD8 T cell differentiation patterns.

In a second Ms. we evaluated the impact of different pathogens in the diversity of CD8 T cell properties during two different immune responses: OT1 TCR-Tg cells (specific for OVA antigen) in the response to LM-OVA (*Listeria Monocytogenes* expressing OVA) infection; and P14 TCR-Tg cells (specific for GP33 epitope) in the response to Lymphocytic choriomeningitis vírus (LCMV) infection. We found that OT1 and P14 cells had different properties. As this difference could also be attributed to the different TCR avidity between OT1 and P14 cells, we then compared the behavior of P14 and OT-1 cells in the same mouse, co-injected with LM-OVA and LM-GP33. Since no differences were then detected, these results demonstrated that priming with different pathogens generates CD8 T cells with different characteristics that are not determined by TCR usage, but rather by the infection context. In addition, when looking for the protection capacity of endogenous CD8 memory cells generated in bacterial or viral context, we found that memory cells generated after LCMV priming were more efficient in responding to a second challenge, than memory cells generated after LM-GP33 priming. We also found that this better protection is associated with a T cell effector memory (T_{EM}) phenotype associated with the LCMV infection, in contrast with a T cell central memory (T_{CM}) phenotype generated after LM-OVA infection. These results demonstrate that different pathogens are responsible for diversity of CD8 T cell differentiation patterns and that even when distinct pathogens are efficiently eliminated during the primary immune response the quality of the memory generated may differ.

In a third Ms. we studied the mechanisms by which effector CD8 T cells attracted other cell types in the early days of an immune response. We used two experimental systems: the response of OT1 TCR-Tg cells to LM-OVA infection; and the response of anti-HY TCR-Tg cells to male cells ("sterile"-non infectious context). In both cases we found that immediately after activation, CD8 T cells expressed high levels of pro-inflammatory cytokines and chemokines (such as TNFα, XCL1, CCL3 and CCL4). We also confirmed the expression of these earlier mediators in a small fraction of activated endogenous cells, which could still be identified by pMHC multimers. A local injection of CD8 pro-inflammatory effectors in the ear induced: hypertrophy of the draining lymph node (DLN); recruitment of several leucokytes (B, T, NK, Monocytes, PMNs and DCs) into the DLN; and increased S1P levels in the DLN responsible for a cell egress block. This inflammatory potential was also detected after intranodal injection of a physiological number of CD8 pro-inflammatory effectors. In contrast with the classic cytotoxic CD8 T cell functions, the pro-inflammatory mediator's expression declined with cell division and when antigen was still abundant. The rapid loss of CD8 inflammatory effector functions was correlated with an extensive TCR down-regulation at the cell surface, as well as with a down-regulation of the TCR signaling pathways (MAPkinases). These results demonstrated for the first time that CD8 responses involve two distinct effector phases with opposite rules (inflammatory and cytotoxic), and also that cognate antigen stimulation is sufficient to induce the CD8 inflammatory effector phase necessary for maximal screen of rare APCs first presenting the antigen.

In conclusion, our studies revealed diversity on CD8 T cell functions (inflammatory and cytotoxic) during immune responses and that different pathogens induce distinct CD8 T cell differentiation patterns. These results are thus crucial to predict and to efficiently evaluate CD8 T cell responses.

Introduction

I. The Immune System

A key feature of the immune system is the ability to induce protective immunity against pathogens while maintaining tolerance towards self and innocuous environmental antigens, and it has evolved to **protect organisms from diseases**. It allows the host to detect and eliminate a diversity of pathogenic organisms that are themselves constantly evolving (virus, bacteria, and worms) and it also helps the host to eliminate toxic or allergic substances, and tumor cells. To mobilize a response against these threats, the host's immune system needs to **distinguish self from non-self**, needs to distinguish **foreign and harmful from the own healthy cells.** This discrimination is essential in order to efficiently eliminate the threat without an excessive damage of self-tissues. Classically, the immune system has been divided in two categories: the innate immune system and the adaptive immune system.

1. Innate Immune System

Innate immune system acts as the first line of resistance against pathogen invasion and includes: i) **physical barriers** such as epithelial cells layers, secreted mucus overlaying the epithelium in the respiratory, gastrointestinal and genitourinary tracts, and the epithelial cilia that sweep away the mucus layer; ii) **soluble proteins** constitutively present in biological fluids (complement proteins, defensins and ficolins) or those released from activated cells (cytokines that regulate the function of other cells, chemokines that attract inflammatory leukocytes, lipid mediators of inflammation, reactive free radical species, and bioactive amines and enzymes that contribute to tissue inflammation); iii) membrane bound and cytoplasmic **receptors** that are expressed broadly on a large number of cells. These receptors are encoded, in their mature functional forms, by **conserved and limited germ-line genes** of the host, which enable the **recognition of molecular patterns shared** by many invading environmental signals (Rahman *et al.* 2008; Cui *et al.* 2011).

2. Adaptive Immune System

Unlike innate system, the adaptive immune system has **restrict recognition** for its target antigens, and is based primarily on **antigen-specific receptors** expressed on the surfaces of T- and B-lymphocytes. T cell receptors (TCR) and immunoglobulin (Ig) B cell receptors (BCR) are encoded by genes that are assembled by **somatic rearrangement of germ-line gene elements**. These rearrangements permit the formation of a vast **diversity of receptors** able to recognize virtually any type of antigen. The mechanisms governing the assembly and the selection of the B and T cell antigen receptors allows for a properly functioning repertoire of receptor-bearing cells. Besides the

specificity, another hallmark of adaptive immune responses is the production of long-lived **(memory)** cells that persist and can rapidly express effector functions after a second time encounter with their specific antigen.

In spite of being described as separate arms of the immune response, innate and adaptive systems **act together** with the innate response being the first line of host defense and the adaptive response becoming prominent after several days. Several components of the innate system contribute to **acute inflammation** induced by microbial infection or tissue damage, and also for the **activation of adaptive antigen-specific cells** essential for an effective immune response (Dutko and Oldstone 1983; Panus *et al.* 2000; Jang *et al.* 2009).

3. Cellular elements of immune system

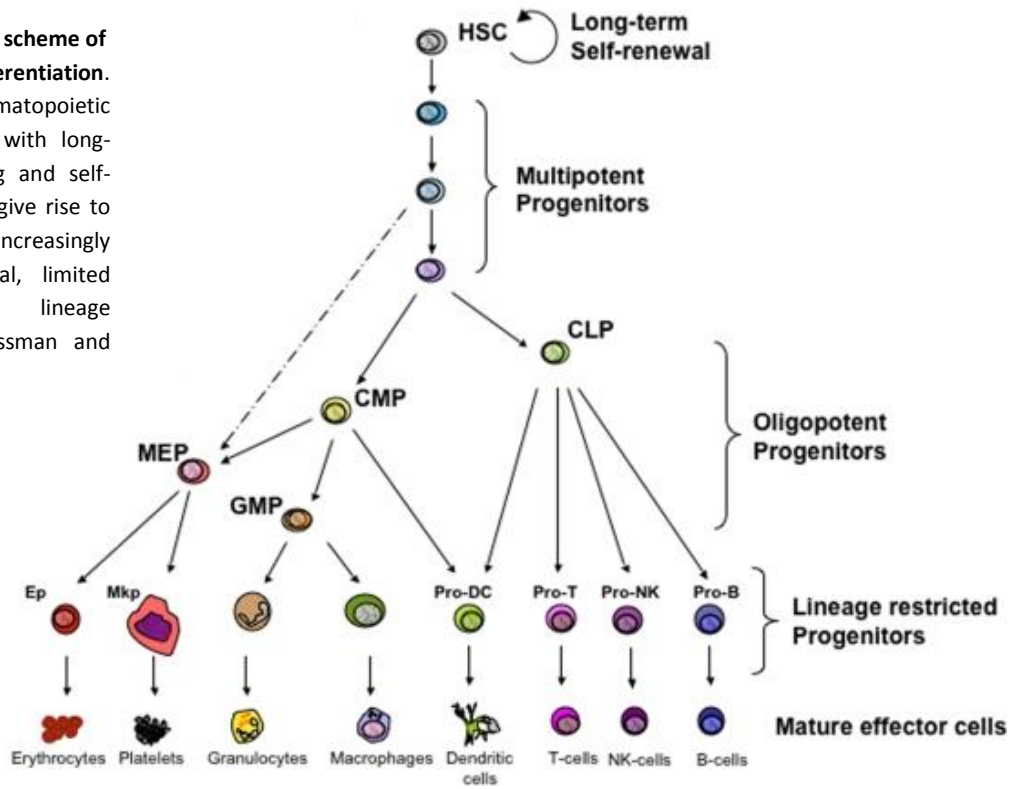
3.1. Hemathopoiese

An immune response includes contributions from many subsets of leukocytes. Different leukocytes can be discriminated morphologically and by differentiation antigens on their membrane surfaces, also named as cluster of differentiation (CD).

All blood cells, including mature circulating lymphocytes, differentiate from the same progenitor cells, the **hematopoietic stem cells (HSC)**, which are found in bone marrow, peripheral blood and placenta. These pluripotent HSCs are capable of self-renewal and multilineage differentiation, giving rise to all types of blood cells throughout an individual's life by generating precursors of increasingly limited potential and lineage-bias. HSCs differentiate in **multipotent progenitors (MPP)**, which have lost their self renewing capacity, and then further differentiate into **common myeloid progenitors (CMP)** or **common lymphoid progenitors (CLP)** (Fig. 1).

CMPs give rise to erythrocytes, platelets, macrophages and to distinct forms of granulocytes. Granulocyte lineage cells include neutrophils, eosinophils/mast cells and basophils. CLPs further differentiate into mature lymphocytes: T cells, B cells, natural killer (NK) cells (Weissman and Shizuru 2008).

Figure 1. Simplified scheme of hematopoietic differentiation. Pluripotent hematopoietic stem cells (HSCs) with long-term reconstituting and self-renewing capacity give rise to precursors with increasingly limited self-renewal, limited potential and lineage commitment (Weissman and Shizuru 2008).



3.2. T cell development: thymocytes

The development of functional T cells is essential for mounting a protective immune response against diverse threats. Whereas the majority of hematopoietic lineages mature in the bone marrow, T cell development takes place in a specialized organ: the thymus. This primary lymphoid organ is responsible for the **generation and selection of T cells** bearing a **diverse T cell receptor (TCR) repertoire**: restricted to self-major histocompatibility complexes (MHC) and tolerant to self-antigens. Besides the differentiation of distinct T cells: CD4, CD8α/β and γδ T cells, the thymus also supports the differentiation of NKT cells, regulatory T cells (T_{reg}), and intraepithelial lymphocytes (IEL) (Weinreich and Hogquist 2008).

Due to the scope of this thesis in study CD8 T cell differentiation during immune responses (cells whose TCR is composed of an α and β chains: TCRαβ), only the TCRαβ-cell development will be described below, and in subsequent chapters, TCRαβ T cells will be simply designated as T cells.

The thymus is colonized by hematopoietic progenitors derived from HSC cells that then become committed towards a T cell differentiation program. T cell differentiation relies on multiple signals provided by the **thymic stroma** that is composed of dendritic cells, macrophages, endothelial cells, fibroblasts and thymic epithelial cells (TEC). Those signals include: growth factors (c-Kit ligand, FLT3L

and IL-7), chemokines (CCL25, CxCL12, CCL19 and CCL21) and cell surface receptor ligands (Notch ligand and peptide-MHC ligands) that sustain thymocyte survival, proliferation, migration and differentiation (Alves *et al.* 2009a). Among these, the cytokine IL-7, which is predominantly produced by TECs (Alves *et al.* 2009b), has a crucial role in promoting survival and expansion of early T cell precursors (Peschon *et al.* 1994).

The identity of the cell progenitor, which initially seeds the thymus, is still open to debate, however it is established that the **early T-lineage progenitor (ETP)** is the most immature T-cell precursor within the thymus (Bhandoola *et al.* 2007; Benz *et al.* 2008). The majority of intrathymic precursors do not express CD4 nor CD8 surface markers, and hence, they are designated as **double-negative (DN) thymocytes** (in addition, as they do not express CD3 either, they are also nominated as triple-negative (TN) thymocytes). DN thymocytes are subdivided into 4 subpopulations according the surface expression profiles of CD44 (an adhesion receptor) and CD25 (the α chain of IL-2 receptor): DN1 ($CD44^{+}CD25^{-}$), DN2 ($CD44^{+}CD25^{+}$), DN3 ($CD44^{-}CD25^{+}$) and DN4 ($CD44^{-}$ $CD25^{-}$) (Godfrey *et al.* 1993). Early thymocytes possess multilineage potential, which is progressively restricted as cells transit through the DN stages of T-cell development (Fig. 2).

DN1 thymocytes constitute a heterogeneous population with different potential to generate T cells and different maturation and proliferation capacities. This population can be further subdivided according to the IL-7Rα and c-Kit expression, where $CD44^{+}$ $CD25^{-}$ c-Kit^{hi} IL-7$R\alpha^{-}$ are the most immature thymocytes, also known as ETPs (Allman *et al.* 2003). The ETPs retain yet the potential to develop NK, DCs, B and some myeloid cells. They do not express the recombination-activating gene (*Rag*) or the *CD3ε* genes at detectable levels, and they do not have D-J rearrangements of TCRβ locus. However, they still keep a strong proliferative capacity. In contrast, the most mature DN1 subpopulation expresses IL-7Rα and it expresses CD3ε and have D-J rearrangement of TCRβ locus (Porritt *et al.* 2004).

DN1 cells become committed to the T lineage upon Notch1-Delta-like four (Notch1-DL4) interactions on the thymic stroma, resulting in transition to the **DN2 stage**, which is characterized by the upregulation of CD25. DN2 population has lost the B cell potential but some cells retain yet NK and myeloid potential (Balciunaite *et al.* 2005). During the DN2- DN3 stages, lymphocyte and T-cell-specific factors such as Rag-1, Rag-2, pTα (pre-TCRα chain), CD3ε and IL-7R are up regulated (Taghon *et al.* 2005).

DN3 thymocytes undergo intense V-DJ rearrangement of the TCRβ locus. Those cells that have generated a functionally rearranged TCRβ chain associate it with the invariant pre-TCRα and CD3 signaling molecules to assemble the pre-TCR on cell surface. This process is called β selection as thymocytes that fail to generate an in frame/functional TCRβ chain are not selected for further differentiation to αβ lineage and thus may die by apoptosis. Signaling through the pre-TCR promotes: proliferation, induce differentiation into DN4 cells, and inhibit further TCRβ rearrangement (allelic

exclusion) by negative regulation of Rag genes (reviewed in (von Boehmer *et al.* 1998; Ciofani and Zuniga-Pflucker 2007)).

In contrast with DN2 stage where T cell precursors express IL-7Rα and c-Kit, in late DN3 stage both of these receptors are downregulated, rendering DN3 cells dependent on pre-TCR and Notch1-DL signaling for survival (Ciofani and Zuniga-Pflucker 2005).

In mice lacking *CD3ε* or *Rag-2* gene (TCRs-V(D)J recombination abrogated), thymocytes do not develop beyond the DN3 stage (Shinkai *et al.* 1992; DeJarnette *et al.* 1998). When IL-7 signaling is impaired (caused by loss of either α or common γ chains of the IL-7R) there is an arrest in the development at the DN2 stage (Peschon *et al.* 1994; Cao *et al.* 1995; Moore *et al.* 1996). Moreover, absence of IL-7 induces apoptosis of DN1, DN2 and DN3 thymocytes, and IL-7 assures survival of these thymocytes by increasing the intercellular levels of anti-apoptotic Bcl-2 and reduction of pro-apoptotic Bax (Kim *et al.* 1998).

Following β selection, DN3 cells express CD27 and progress to the **DN4 stage**, characterized by the loss of CD25 expression. The expression of CD4 and CD8 coreceptors is initiated and these thymocytes become designed as **double positive (DP):** $CD8^+CD4^+$ cells.

DP cells represent the majority of the thymocytes, they highly express Rag-1 and Rag-2, and they also initiate *Tcrα* gene rearrangements, resulting in the surface expression of TCRαβ/CD3 complexes. Due to the random nature of TCR α and β loci rearrangements, a vast diversity of TCRs is generated to cope with an immense variety of antigens. However, not all TCRs are capable to effectively interact with peptide-MHC (pMHC) complexes, while some will strongly recognize self-peptides. In order to generate useful and safe mature TCRαβ cells, DP thymocytes pass through a positive and negative selection processes. The fate of a DP cell is dependent on the signaling mediated by the interaction of the TCRαβ with self-peptides MHC class I and II, highly expressed by thymic ephitelial cells.

Positive selection: DP thymocytes bearing TCRαβ that fail to productively interact with self peptide-MHC class I or class II complexes die by neglect within few days. Thus, only thymocytes that receive signals from the TCR engagement are selected (positive selection). The TCR engagement is responsible not only for the survival but also for the maturation of double positive (DP) thymocytes on single positive (SP) cells. Based on the appropriate degree of interaction between the TCR and the pMHC complexes expressed on thymic epithelial cells, and depending on the class of MHC molecule recognized, thymocytes are positively selected either to a $CD4^+$ or to a $CD8^+$ single-positive (SP) cell fate. CD4 and CD8 molecules are coreceptors that bind to MHC class I and class II, respectively, favoring CD8+ and CD4+ T-cell MHC restriction. Their cytoplasmic domain binds to the tyrosine kinase Lck that, when in close vicinity to the TCR complex, initiates TCR signaling. Therefore, CD4 and CD8 coreceptors provide yet an extra guard against the selection of non-MHC reactive cells (coreceptors not engaged) as they sequester the Lck far from the TCR complex.

Negative selection: SP thymocytes carrying TCRαβ with high avidity for self pMHC (strong TCR signaling) undergo TCR-induced programmed cell death. Thus, only thymocytes that do not express TCR with high affinity for self antigens are selected (negative selection). This involves the exposure of thymocytes to peripheral tissue-specific antigens ectopically expressed by thymic epithelial cells under the control of the transcription factor AIRE (autoimmune regulator). This selection leads to the elimination of self-reactive T cells (reviewed in (Takahama 2006; Carpenter and Bosselut 2010)).

These selection processes ensure that only **self-MHC-restricted** and **self-tolerant T cells** survive and leave the thymus as mature T cells that continuously circulate between blood and lymph through secondary lymphoid organs in search of invading pathogens/cognate antigens interactions. **Survival and homeostatic proliferation** of individual **naïve T cells in the periphery** is dependent on continuous TCR signaling (interaction with self-pMHC ligands) (Tanchot *et al.* 1997) plus IL-7R signaling (Schluns *et al.* 2000), which occurs in secondary lymphoid organs.

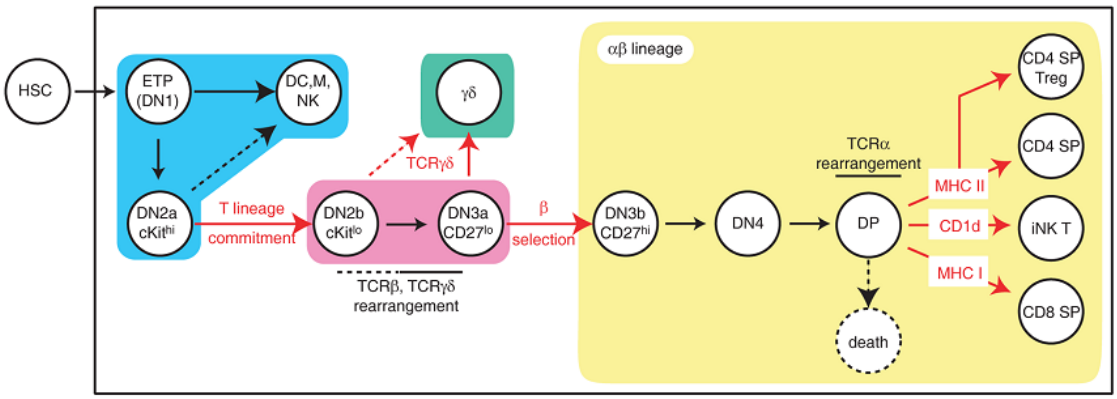


Figure 2. Overview of T cell development in thymus: from an ETP to a mature CD8 T cell.
The earliest thymic precursors entering in the thymus derive from bone marrow hematopoietic stem cells (HSCs) and are designated as early T cell progenitors (ETPs). Expression of CD4 and CD8 defines DN (double negative) and DP (double positive) thymocytes. Expression of CD44 and CD25 defines four DN subtypes: DN1, DN2, DN3 and DN4. During the DN1-DN4 progression, thymocytes lose their potential to develop dendritic (DC), natural killer (NK) or myeloid (M) lineages and gain specific characteristics of T cell lineage commitment. β selection engages TCRαβ commitment of DN3 cells, and TCRαβ signaling upon MHC class I recognition induces differentiation of DPs into mature $CD8^+$ single positive (SP) cells, which are released into the periphery and join the pool of naïve CD8 T cells (Carpenter and Bosselut 2010).

3.2.1. Thymocytes as short-lived cells (a notion to reconsider)

Surgical removal of the thymus demonstrated the requirement of this organ in the generation of T cells (Miller 1961). Likewise, **thymus grafts** have also been used to correct deficiencies of the thymus epithelium (Markert *et al.* 2007; Markert *et al.* 2011). However, they are **not used to correct intrinsic T cell deficiencies**, as it is believed that thymocytes are **short-lived**, and thus, continuous T cell differentiation in the thymus depends on constant supply of lymphocytes progenitors from bone

marrow (Berzins *et al.* 1998). This occurs even when the host is T cell deficient. Transplantation of wild-type thymi into hosts which are unable to generate mature T cells (SCID or Rag2-/- hosts) also show that, in the transplants, **donor T cells are substituted by incompetent precursors from the host bone marrow** within few weeks (Frey *et al.* 1992; Takeda *et al.* 1996).

3.3. Innate and adaptive cells of the immune system

Cellular elements of the innate system are: mast cells, basophiles, eosinophils, neutrophils, macrophages, dendritic cells (DC) and natural killer cells (NK). The major functions of the innate system cells include:

1) the initial recognition of foreign substances;
2) the recruitment of additional immune cells to the sites of infection and inflammation though the production of inflammatory mediators;
3) the elimination of microorganisms by phagocytosis, reactive oxygen species production, type I IFNs, or complement cascade activation;
4) triggering of the adaptive immune system through antigen presentation.

These cells bear pattern recognition receptors (PRRs) that recognize a broad molecular patterns found on pathogens: PAMPs (pathogen associated molecular patterns).

Cellular elements of adaptive immune system are the T and B lymphocytes. Both of these cells recognize specific targets trough a vast diversity of receptors generated by genetic recombination of antigen receptor gene segments, associated to several other mechanisms, as reported above.

Activated **B cells** are responsible for antigen-specific antibodies secretion and they provide an important line of defense against infection through the neutralization and/or elimination of extracellular pathogens or foreign substances. In addition, B cells also function as antigen presenting cells, they produce multiple cytokines and they can suppress inflammatory responses that occur during autoimmune diseases or that can be caused by unresolved infection. B cells recognize and capture external antigens through their B cell receptor (BCR), a cell surface immunoglobulin (Ig) receptor which recognizes antigens directly without need for antigen processing. Upon binding to the BCR, the antigen is internalized by receptor-mediated endocytosis and it is processed by degradation into peptides. These antigenic peptides are displayed by B cells on their surface MHC class II molecules, where they can be recognized by antigen-specific T helper cells. This B and T cell interaction (T cell-dependent (TD) activation of B cells) provides the co-stimulatory signal required for B cells to differentiate into high-affinity antibody-producing plasma cells and to develop into memory B cell populations. B cells can also be activated and produce antibodies in a T-cell independent (TI) mode through: signaling of their TLRs (Toll like receptors), BCR crosslinking, and help signals provided by bone-marrow-derived myeloid cells. However, the nature of antigens

recognized and the outcome of antibody responses are distinct between TD and TI B cell activation (Mauri and Bosma 2012; Vinuesa and Chang 2013; Yuseff *et al.* 2013).

T cells are divided in two major subsets: $CD8^+$ cytotoxic (CTLs) and $CD4^+$ helper T (Th) cells.

In general, **CD8 T cells** respond to intracellular infections with virus, protozoa and intracytoplasmatic bacteria, and also to tumor cells. Upon antigen recognition through interaction of the TCR and the processed pathogen/malignant-derived peptide bound on MHC class I of an APC, CD8 T cells specifically kill the infected or tumoral cells by production of cytotoxic molecules. They also produce cytokines, chemokines and microbicidial and anti viral molecules to combat infection (Harty *et al.* 2000).

CD4 T cells play an important role in coordinating acquired immune responses. There are several Th cell subsets (Th1, Th2, Th17, Th21, Treg), and each of which has specialized functions to control immune responses. These subsets emerge from naïve CD4 T cells after specific recognition of antigen-derived peptide displayed on MHC class II molecules of APCs and B cells. Both the TCR-mediated stimulation and the cytokine environment influence the fate decision of naïve CD4 T cells towards distinct Th subsets. Each Th cell subset expresses a unique set of transcription factors and produce hallmark cytokines that promote: CD8 T cell or B cell differentiation and memory establishment; enhance innate immune components' action; or even suppress the immune response (Yamane and Paul 2013).

II. Innate immune response

The innate immune system not only monitors the host for microbes but also the health of the host's own cells. In response to pathogens and cell injury, the innate immune system **alerts the adaptive immune system** to a potential problem in order to generate a specific response through antigen presentation. However, in parallel, the innate immune system also **rapidly mobilizes innate defenses** to the site of injury through the generation of an inflammatory response.

1. Inflammation: the key component of innate responses

Inflammation is an immediate response that is triggered by noxious stimuli and conditions, such as infection and tissue's injury (classical acute or chronic inflammatory response), or such as tissue's stress or malfunction (para-inflammatory response). The defining features of inflammation are redness, swelling, heat, pain, and loss of tissue function which have as physiological basis the local and transient: vasodilatation, leakage of plasma soluble molecules, and migration of leukocytes out of blood vessels into the surroundings of the affected tissue.

The physiologic purpose of inflammation is to restore the homeostasis, resulting in the elimination of the infectious agents and/or the repair of tissue injury. A typical inflammatory response consists of four components: i) the inflammatory inducers, ii) the sensors that detect them, iii) the inflammatory mediators induced by the sensors, and iv) the inflammatory effectors/target tissues that are affected by the inflammatory mediators (Fig. 3) (Nathan 2002; Medzhitov 2010).

Inflammation is triggered when innate immune cells residing in tissues (macrophages, mast cells and dendritic cells) or non professional immune cells (epithelial cells, endothelial cells and fibroblasts), as well as circulating monocytes and neutrophils, recognize pathogen invasion or cell damage through their intracellular or surface-expressed pattern recognition receptors (PRRs). These receptors (**inflammatory sensors),** directly or indirectly detect **inflammatory inducers** like pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs). DAMPs are nuclear or cytosolic host molecules that when released outside the cell, or exposed on cell surface following tissue injury or cell necrosis, can contribute to a sterile (noninfectious) inflammation. They include ATP, the cytokine IL1α, uric acid, calcium-binding proteins, DNA-binding nuclear proteins, amyloid β fibrils, heat shock proteins, defensins, phagocyte-specific proteins, etc. By contrast, PAMPs are exogenous molecules of both pathogenic and non-pathogenic origin; they are shared by a large group of microorganisms and are often conserved products essential for microbial survival. They include bacterial and viral nucleic acids, fungal β-glucan and α-mannan cell wall components, bacterial protein flagellin, components of the peptidoglycan bacterial cell wall, lipopolysaccharide (LPS) from Gram-negative bacteria, etc (Medzhitov 2008; Newton and Dixit 2012).

Inflammatory mediators are responsible for vascular alterations and for the recruitment of leukocytes.

Vasoactive amines (histamine and serotonin) are produced in an all-or-none matter by mast cells and platelets degranulation, and they are responsible for increased vascular permeability and vasodilatation. Vasoactive peptides can be stored in an active form in secretory vesicles (like substance P) or generated by proteolytic processing of inactive precursors in the extracellular fluid (for example, kinins, fibrinopeptide and fibrin degradation products). Substance P is released by sensory neurons and promotes itself mast-cell degranulation. Other vasoactive peptides are generated through proteolysis by the Hageman factor, thrombin or plasmin and cause vasodilatation and increased vascular permeability. The Hageman factor activates the kallikrein-kinincascate resulting in bradykinin production which has a pain-stimulating effect. The complement fragments C3a, C4a and C5a (known as anaphylatoxins) are produced via several pathways of complement activation. C5a, in a higher extension than C3a and C4a, promote granulocyte and monocyte recruitment and induce mast cell degranulation, therefore affecting the vasculature. Lipid mediators, (such as eicosanoids and platelet-activating factors) are derived from phosphatidylcholine, a phospholipid present in the inner leaflet of cellular membranes. After activation by intracellular Ca^{2+} ions, cytosolic phospholipase A_2 generates arachidonic acid and lysophosphatidic acid. Arachidonic acid is metabolized to form eicosanoids either by cyclooxygenases, which generate prostaglandins and thromboxanes, or by lipoxygenases, which generate leukotrienes and lipoxins. The prostaglandins PGE_2 and PGI_2 cause vasodilatation and PGE_2 induce high sensitivity to pain and fever, and can stimulate DCs and promote IL-12 production, which is necessary for efficient antigen presentation and T ell activation. Lipotoxins (and dietary ω3-fatty-acid-derived resolvins and protectins) inhibit inflammation and promote resolution of inflammation and tissue repair. Platelet-activating factors are generated by the acetylation of lysophospatidic acid and induce recruitment of leukocytes, vasodilatation and vasoconstriction, increase vascular permeability and platelet activation. Proteolytic enzymes (including elastin, chathepsins and matrix metalloproteinases) through degradation of extra cellular matrix and basement-membrane proteins are involved in host defense, tissue remodeling and leukocyte migration (reviewed in Medzhitov 2008).

Inflammatory cytokines (tumor-necrosis factor-a (TNF-a), IL-1, IL-6 and many others are produced by many cell types, most importantly by macrophage and mast cells. They promote leukocyte extravasation by increasing the levels of leukocyte adhesion molecules on endothelial cells. In addition, they can have systemic effects. They induce hepatocytes to produce acute phase proteins such as C-reactive protein and coagulation factors, and they activate brain endothelium to produce PGE_2. Activated dendritic cells, macrophages, and neutrophils, remove foreign particles or host debris by phagocytosis and they also secrete cytokines that shape the lymphocyte-mediated adaptive immune response.

Depending on the type of infection (bacterial, viral, or parasitic), the sensors, mediators, and target tissues vary such that the appropriate type of inflammatory response is induced. For example, viral infections induce the production of type-I interferons (IFN-α and IFN-β) by infected cells and the

activation of cytotoxic lymphocytes, whereas infections with parasitic worms lead to the production of histamines, IL-4, IL-5, and IL-13 by mast cells and basophils.

Chemokines (e.g. CCL2 and CXCL8) are produced by many cell types in response to inducers of inflammation and they control leukocyte extravasation and chemotaxis towards the affected tissues (reviewed in Medzhitov 2010; Newton and Dixit 2012).

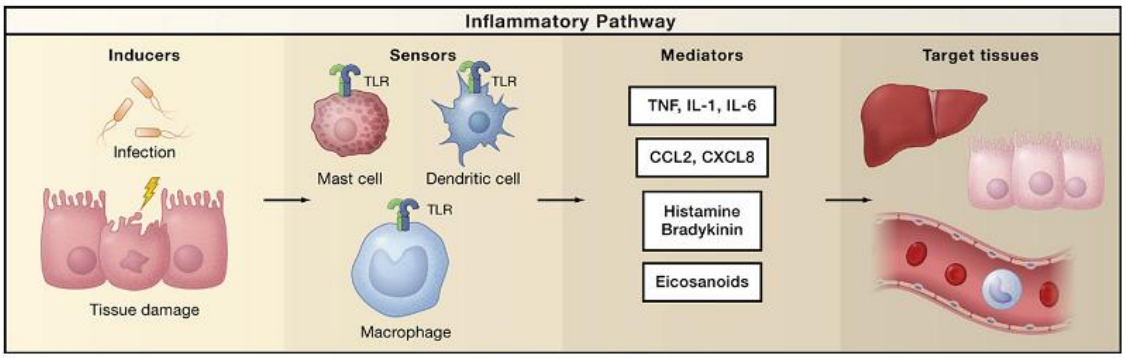


Figure 3. Simplified inflammatory pathway components.
Inducers (infection or tissue damage) initiate the inflammatory response and are detected by sensors like Toll-like receptors (TLRs) which are expressed on sentinel cells, such us tissue-resident macrophages, dendritic cells and mast cells. They induce the production of mediators, including cytokines, chemokines, bioactive amines, eicosanoids, and products of proteolytic cascades, such us bradykinin. These inflammatory inducers act on several target tissues to elicit vascular alterations and circulating leuckocyte recruitment to the site of injury (Medzhitov 2010).

2. Pattern recognition receptors (PRRs): innate immune recognition

Pattern recognition receptors (PRRs) are not only involved in sensing pathogen invasion but also in sensing damaged cells. PPRs include Toll-like receptors **(TLRs)**, Nod-like receptors **(NLRs)**, RIG-I-like receptors **(RLRs)**, and C-type lectin receptors **(CLRs)**.

2.1. Toll-like receptors (TLRs)

TLRs were the first PPRs family members to be identified and are one of the best-characterized. They are type I transmembrane proteins expressed either on cell surface or associated with intracellular vesicles. TLRs are characterized by N-terminal leucine-rich repeats on their ectodomain that mediate the recognition of a wide range of PAMPs, and also by a cytoplasmic Toll/IL-1R homology (TIR) domain that activate downstream signaling pathways.

Ten TLRs have been identified in humans and 12 in mice, with TLR1-TLR9 being conserved in both species. Mouse TLR10 is not functional because of a retrovirus insertion, and TLR11, TLR12 and TLR13 have been lost from the human genome. Depending on their cellular localization and respective PAMP ligands, TLRs are divided in two subgroups: 1) TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11, which are expressed on cell surfaces and recognize mainly microbial membrane components such as lipids, lipoproteins and proteins; 2) TLR3, TLR7, TLR8 and TLR9, which are exclusively expressed in intracellular vesicles (endoplasmic reticulum, endosomes, lysosomes and endolysosomes) and recognize microbial nucleic acids. (Kawai and Akira 2010).
Different TLRs recognize different molecular patterns of microorganisms and self-components (Table 1).

Table 1. Pattern recognition receptors (PRRs): TLRs, RLRs, NLRs, CLRs, and their ligands (Takeuchi and Akira 2010).

PRRs	Localization	Ligand	Origin of the Ligand
TLR			
TLR1	Plasma membrane	Triacyl lipoprotein	Bacteria
TLR2	Plasma membrane	Lipoprotein	Bacteria, viruses, parasites, self
TLR3	Endolysosome	dsRNA	Virus
TLR4	Plasma membrane	LPS	Bacteria, viruses, self
TLR5	Plasma membrane	Flagellin	Bacteria
TLR6	Plasma membrane	Diacyl lipoprotein	Bacteria, viruses
TLR7 (human TLR8)	Endolysosome	ssRNA	Virus, bacteria, self
TLR9	Endolysosome	CpG-DNA	Virus, bacteria, protozoa, self
TLR10	Endolysosome	Unknown	Unknown
TLR11	Plasma membrane	Profilin-like molecule	Protozoa
RLR			
RIG-I	Cytoplasm	Short dsRNA, 5′triphosphate dsRNA	RNA viruses, DNA virus
MDA5	Cytoplasm	Long dsRNA	RNA viruses (Picornaviridae)
LGP2	Cytoplasm	Unknown	RNA viruses
NLR			
NOD1	Cytoplasm	iE-DAP	Bacteria
NOD2	Cytoplasm	MDP	Bacteria
CLR			
Dectin-1	Plasma membrane	β-Glucan	Fungi
Dectin-2	Plasma membrane	β-Glucan	Fungi
MINCLE	Plasma membrane	SAP130	Self, fungi

TLR4 forms a complex with MD2, and together they serve as the main LPS -binding component (lipopolysaccharide, a component of the outer membrane of Gram-negative bacteria known to be a cause of septic shock). The formation of a receptor homodimer composed of two copies of the TLR4-MD2-LPS complex (Park *et al.* 2009) initially transmits signals for the early-phase activation of NF-kB by recruiting the TIR domain-containing adaptors TIRAP (Mal) and MyD88 (MyD88-dependent pathway). The TLR4-MD2-LPS complex is then internalized and retained in the endosome, where it triggers signal transduction by recruiting TRAM and TRIF, which leads to the activation of IRF3 (for induction of type I interferon) and the late-phase NF-kB (TRIF-dependent pathway). Both early- and late-phase activation of NK-kB is required for the induction of inflammatory cytokines (Kawai and Akira 2010). In addition to binding LPS, TLR4 is involved in the recognition of respiratory syncytial

virus fusion proteins, mouse mammary tumor virus envelop proteins, Streptococcus pneumonia pneumolysin and the plant-derived cytostatic drug paclotaxel (Akira *et al.* 2006). TLR4 is also involved in the recognition of viruses by binding to viral envelope proteins, and it modulates pathogenesis of H5N1 avian influenza virus infection by recognizing a DAMP rather than the virus itself (Imai *et al.* 2008). Concerning host cell ligands, TLR4 recognizes fibrinogen, hyaluronic acid, and heparin sulfate fragments, as well as several heat shock proteins (also secreted by bacteria) (Akira and Takeda 2004).

TLR2 recognize lipopeptides from bacteria, peptidoglycan and lipoteichoic acid from Gram-positive bacteria, lipoarabinomannan from mycobacteria, zymosan from fungi, tGPI-mucin from Trypanossoma cruzi, the hemagglutinin protein from measles virus, and HSP70 from host cells (Akira *et al.* 2006). TLR2 generally forms heterodimers with TLR1 or TLR6. Different lipid-binding pockets formed with TLR1 or TLR6 are responsible for the discrimination between lipoproteins. TLR2-TLR1 heterodimer recognizes triacylated lipopeptides from Gram-negative bacteria and mycoplasma, whereas TLR2-TLR6 heterodimer recognizes diacylated lipopetides from Gram-positive bacteria and mycoplasma. Although it was believed that TLR2 agonists mainly induce the production of inflammatory cytokines and not type I interferon by macrophages and dendritic cells, it was also shown that it can trigger the production of type I interferon by inflammatory monocytes in response to infection with vaccinia virus (Barbalat *et al.* 2009). This suggests that cellular responses to TLR2 ligands differ depending on the cell types involved.

TLR2 and TLR4 engagement also results in recruitment of mitochondria to macrophage phagosomes and increased production of mitochondrial ROS that have been implicated in mouse macrophage bactericidal activity (West *et al.* 2011).

TLR5 recognizes the flagellin protein component of bacterial flagella. $CD11c^{+}CD11b^{+}$ lamina propia DCs (LPDCs) in the small intestine have high expression of TLR5. Lamina propia DCs are unique in promoting the differentiation of IL-17-producing helper T cells (Th17 cells) and T helper type 1 (Th1) cells, as well as the differentiation of naïve B cells into immunoglobulin A-producing plasma cells in response to flagellin (Uematsu *et al.* 2008).

TLR11 is a relative of TLR5, it is expressed in mouse's kidney and bladder, it recognizes urophatogenic bacterial components, and TLR11-deficient mice are susceptible to infection with these bacteria (Zhang *et al.* 2004). TLR11 also recognizes the profilin-like molecule derived from the intracellular protozoan Taxoplasma gondii (Yarovinsky *et al.* 2005).

TLR3 recognizes a synthetic analog of double-strand RNA (dsRNA), poly I:C (polyinosinic-polycytidylic acid), which mimics viral infection and induces antiviral immune responses. TLR3 triggers antiviral immune responses through the production of type I interferon and inflammatory cytokines with an essential role in preventing virus infection. TLR3-deficient mice are susceptible to lethal infection with murine Cytomegalovirus (Tabeta *et al.* 2004), and TLR3 deficiency in humans is

associated with susceptibility to herpes simplex virus type 1 (HSV-1) (Zhang *et al.* 2007). In the endolysosome, TLR3 also recognizes the genomic RNA of reoviruses, dsRNA produced during replication of single strand RNA (ssRNA) and certain small interfering RNAs (Akira *et al.* 2006; Bell *et al.* 2006). Ligand binding dimerizes two TLR3 molecules (Choe *et al.* 2005).

TLR7 was originally identified as recognizing imidazoquinoline derivates and guanine analogs such as loxoribine, which have antiviral and antitumor properties. It recognizes ssRNA derived from RNA viruses such as vesicular stomatitis virus, influenza A virus and human immunodeficiency virus (Kawai and Akira 2006). TLR7 also recognizes synthetic poly (U) RNA and certain small interfering RNAs (Hornung *et al.* 2005). TLR7 is highly expressed on plasmacytoid DCs (pDCs), which are able to produce large amount of type I IFN after virus infection, and cytokine induction that in response to RNA virus are totally dependent of TLR7 (Kawai and Akira 2006). In addition, TLR7 expressed on conventional DCs (cDCs) senses RNA from group B Steptococcus bacteria and induces type I IFN (Mancuso *et al.* 2009). TLR7 senses virus that are internalized and recruited to the endolysosomes, and also virus that enter the cytoplasm via autophagy (in which self-proteins and damaged organelles are degraded in double-membrane vesicles: autophagosomes). pDCs show constitutive autophagy formation, and pDCs lacking the autophagy-relate protein Atg5 show defects in the production of interferon-α after infection with vesicular stomatitis virus (Lee *et al.* 2007).

TLR9 recognizes unmethylated 2'-deoxyribo CpG (cytidine-phosphate-guanosine) DNA motifs that are frequently present in bacteria and viruses but are rare in mammalian cells. Synthetic CpG oligodeoxynucleotides function as TLR9 ligands and directly activate DCs, macrophages, B cells, and drive strong Th1 responses There is high expression of TLR9 in pDCs and it serves as a sensor of DNA virus infection (like murine cytomegalovirus, HSV-1 and HSV-2) (Akira *et al.* 2006). TLR9 also recognizes the insoluble crystal hemozoin, which is generated on the detoxification process after digestion of host hemoglobin by the malaria parasite Plasmodium falciparum (Coban *et al.* 2010).

Individual **TLRs trigger** specific biological responses. TLR3 and TLR4 generate both type I interferon and inflammatory cytokine responses, whereas cell surface TLR1-TLR2, TLR2-TLR6 and TLR5 induce mainly inflammatory cytokines (Fig. 4). These differences are due to TIR domain-containing **adaptor molecules: MyD88, TIRAP, TRIF and TRAM**, which are recruited by distinct TLRs and activate distinct signaling pathways. **MyD88** was the first member of the TIR-family to be discovered and is universally used by all TLRs except TLR3, and activates the transcriptional factor NF-kB and mitogen-activated protein kinases (MAPKs) to induce inflammatory cytokines. In contrast, **TRIF** is used by TLR3 and TLR4, and induces alternative pathways that lead to activation of the transcription factor IRF3 and NF-kB and the consequent induction of type I interferon and inflammatory cytokines. TIRAP is an adaptor that recruits MyD88 to TLR2 and TLR4, whereas TRAM is an adaptor that recruits TRIF to TLR4.

TLR4 is the only TLR that recruits four adaptor proteins and activates two distinct signaling pathways: the MyD88-dependent and the TRIF-dependent pathways (Fig. 4). These two pathways

have different kinetics. TLR4 initially recruits TIRAP and MyD88, which recruits IRAKs, TRAF6 and the TAK1 complex, leading to early-phase activation of NF-kB and MAP kinases (ERK, JNK and p38). Signaling via the MyD88-dependent pathway leads to up-regulation of pro-inflammatory cytokines like IL-6, IL-12 and TNFα. TLR4 is then endocytosed and delivered to intracellular vesicles to form a complex with TRAM and TRIF. This complex then recruits TRAF3 and the protein kinases TBK1 (TANK binding kinase-1) and IKKi (Inducible inhibitor of NF-kB [ikB] kinase), which catalyze the phosphorylation of IRF3, leading to the expression of type I IFN. TRAM-TRIF also recruits TRAF6 and TAK1 to mediate late-phase activation of NF-kB and MAP kinases. Signaling via the TRIF pathway also leads to secretion of chemokines such as RANTES and IP-10 (Kawai *et al.* 2001; Hirotani *et al.* 2005) and up-regulation of costimulatory molecules (Hoebe *et al.* 2003; Yamamoto *et al.* 2003). Whereas activation of the TRIF-dependent pathway is sufficient for type I IFN induction, activation of both the MyD88- and TRIF-dependent pathways is required to drive robust NK-kB and MAP kinase activation and the subsequent induction of inflammatory cytokines (Table 2) (reviewed in Kawai and Akira 2010; Kawai and Akira 2011).

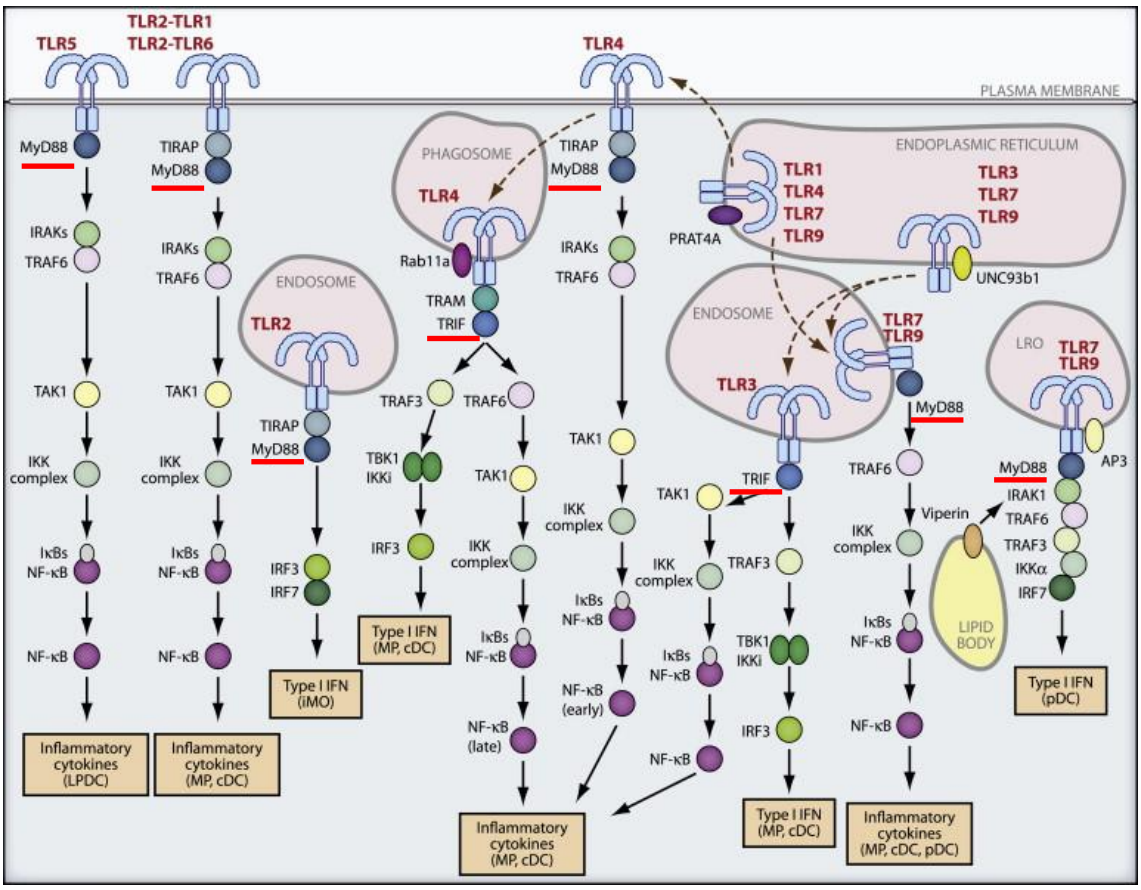


Figure 4. TLR trafficking and signaling.

Individual TLRs initiate overlapping and distinct signaling pathways in various cell types such as macrophages (MP), convensional DC (cDC), plasmacytoid DC (pDC), lamina propia DC (LPDC), and inflammatory monocytes (iMO). PAMP engagements induce conformational changes of TLRs that allow homo- or heterophilic interactions of TLRs and recruitment of adaptor proteins such as MyD88 (universally used by all TLRs except TLR3), TIRAP, TRIF and TRAM. (LRO, lysosome-related organelle) (Kawai and Akira 2011).

Table 2. Functional outputs of some of the genes upregulated by TLR4 (Newton and Dixit 2012).

Output	Genes
Leukocyte recruitment	*Ccl2, Ccl3, Ccl4, Ccl5, Cxcl1, Cxcl2, Cxcl5, Cxcl10, Ccrl2*
Cell adhesion	*Icam1, Vcam1*
Cell survival	*Bcl2a1, Cflar*
Remodeling of extracellular matrix	*Mmp13*
Vascular effects	*Edn1*
Synthesis of inflammatory mediators	*Hdc, Nos2, Ptges, Ptgs2*
Inflammatory cytokines	*Il1a, Il1b, Il6, Il18, Tnf*
Antiviral response	*Ifnb*
Intracellular signaling (positive)	*Birc2, Birc3, Casp4, Mefv, Nfkbiz*
Intracellular signaling (negative)	*Bcl3, Dusp1, Nfkbia, Socs3, Tnfaip3, Zc3h12a*
PRRs	*Fpr1, Nlrp3*
Regulators of adaptive immune response	*Ch25h, Icosl, Il10, Il12a, Il12b, Il15, Tnfsf9*

2.2. NOD-like receptors (NLRs)

NLRs are a family of molecules that sense a wide range of ligands (PAMPs, non-PAMP particles and cellular stresses) within the cytoplasm of cells. This family comprises 23 members in humans and approximately 34 in mice. The NLR family is composed of three domains: a C-terminal leucine –rich repeats involved in the recognition; a N-terminal domain that harbors protein-binding motifs, such as CARD, a pyrin domain (PYD), and a baculovirus inhibitor repeats (BIRs) in most of NLRs; and an intermediate domain consisting of nucleotide-binding and oligomerization (NACHT) domains, which are required for ligand-induced, ATP-dependent oligomerization of the sensors and formation of active receptor complexes and consequent downstream signaling (Kanneganti *et al.* 2007; Kumar *et al.* 2011).

NOD1 and NOD2 (also known as CARD4 and CARD15, respectively) are the best characterized NLRs and they comprise an N-terminal domain containing either one (NOD1) or two (NOD2) CARDs. These receptors are mainly expressed in the cytosol of various cells, however, their expression on the plasma membrane has also been reported (Barnich *et al.* 2005; Kufer *et al.* 2008). NOD1 and NOD2 recognize structures of bacterial peptidoglycans. NOD1 recognizes γ-D-glutsmyl-mesodiaminopimelic acid (iE DAP) from Bacillus subtilis, Listeria monocytogenes, Echerichia coli, Pseudomonas aeruginosa, Helicobacter pylori, etc. NOD2 recognizes muramyl dipeptide (MDP) from Streptococcus pneumonia, Mycobacterium tuberculosis, Listeria monocytogenes, Salmonella typhimurium, etc. (Kumar *et al.* 2011). NOD2 is also important for the defense against pathogenic protozoal parasites, such as Toxoplama gondii (Shaw *et al.* 2009), and is involved in 5'-triphosphate RNA-induced type I IFN production and host defense against respiratory syncytial virus infection (Sabbah *et al.* 2009). PAMP recognition initiates oligomerization of these receptors, which subsequently recruit CARD-containing adaptor protein known as RIP2 and activates NF-kB and MAP kinases to induce the transcription of inflammatory cytokines (Park *et al.* 2007). NOD1 and NOD2 also have been shown to stimulate autophagy independently of RIP2 (Travassos *et al.* 2010).

Stimulation of macrophages and DCs with microbial PAMPs also initiates the assembly of a protein complex known as the **inflammasome**, which is composed of NLR members (e.g. NLRP3, NLRC4, NLRP1). Inflammasome initiates the proteolytic cleavage (or maturation) of various caspases, resulting in the maturation and production of inflammatory cytokines, such as IL-1β and IL-18, or initiates cell death. The NLRP3 inflammasome is the most widely studied and it recognizes ATP, uric acid crystals, heat shock proteins, viral RNA, bacterial DNA and bacteria muramyl dipeptide (Mogensen 2009; Kumar *et al.* 2011).

3. Monocytes/Macrophages

Monocytes are derived from precursors in the bone marrow and traffic via bloodstream to peripheral tissues. During both homeostasis and inflammation, circulating monocytes leave the bloodstream and migrate into tissues where, depending on local growth factors, pro-inflammatory cytokines and microbial products, they may differentiate into macrophages or dendritic cells populations. The ability of monocytes to mobilize and traffic to where they are needed is central for their pro-inflammatory and antimicrobial roles, and also for patrolling and tissue repair functions (Shi and Pamer 2011).

In mice, expression of Ly6C and CD11b identifies a monocyte subset that expresses high levels of CC-chemokine receptor 2 (CCR2) but low levels of CX_3-chemokine receptor 1 (CX_3CR1). These granular and large monocytes are also called inflammatory or $LY6C^{high}$ monocytes, represent 2-5% of circulating white blood cells in uninfected mouse, and are rapidly recruited to sites of infection and inflammation.

CCR2 has a crucial role in the trafficking of these monocytes as CCR2 deficiency reduces $LY6C^{high}$ monocyte trafficking to sites of inflammation. CCL2 and CCL7 chemokines (CCR2 ligands) mediate this recruitment. CCL2 is expressed by nucleated cells in response to activation by pro-inflammatory cytokines or stimulation of innate immune receptors by a range of microbial molecules. CCL7 is also induced by bacterial infection (Jia *et al.* 2008). During *Listeria Monocytogenes* (LM) infection in mice, bone marrow stromal cells which express TLRs, respond to very low concentration of TLR ligands by producing CCL2 (Shi *et al.* 2011). In addition to MyD88/TLR signals, type I IFNs ligands also contribute to CCL2 induction. In the absence of either MyD88 or type I IFN receptor signaling, monocytes egress from bone marrow (monocytosis) and recruitment to infected spleen are maintained. However, the combined loss of MyD88 and IFNAR signaling results in a dramatic reduction in early monocyte recruitment from the bone marrow and also results in diminished accumulation of Tip-DCs (TNFα - and iNOS-producing DCs) in the infected spleen (Jia *et al.* 2009). Both TNFα and iNOS (inducible nitric oxide synthase) contribute to innate defense against LM and are produced by Tip-DCs, a cell population derived from $LY6C^{high}$ monocytes that are recruited to the foci of infection (Serbina *et al.* 2003). Recruited NK cells in proximity of recruited $LY6C^{high}$ monocytes in the spleen produce

interferon-γ (IFNγ), and thus drive monocyte differentiation into Tip-Dcs by a MyD88 dependent pathway (Serbina *et al.* 2003; Kang *et al.* 2008).

Monocytes also express CCR1 and CCR5 chemokine receptors (shared ligands: CCL3 and CCL5), but unlike CCR2, CCR1 and CCR5 are expressed by multiple cell types (Shi and Pamer 2011).

Besides chemokines, integrins and other adhesion molecules are also involved in monocyte trafficking. LY6C^{high} monocytes in mice express L-selectin (CD62L), P-selectin glycoprotein ligand 1 (PSGL1), lymphocyte function-associated antigen 1 (LFA1); also known as αLβ2 integrin), macrophage receptor 1 (MAC1; also known as αMβ2 integrin), platelet endothelial cell adhesion molecule (PECAM1) and very late antigen4 (VLA4; also known as α4β1 integrin), which all contribute to leukocyte adhesion and migration (Ley *et al.* 2007). Following recruitment to the inflamed tissue, LY6C^{high} monocytes up regulate CD11c and MHC class II and migrate to draining lymph nodes, where they can promote T cell proliferation, suggesting that this subset of monocytes differentiates into DCs. LY6C^{high} monocytes have a short transit time in the bloodstream and are not recovered from peripheral tissues in the absence of inflammation, but instead home to the bone marrow (Geissmann *et al.* 2003; Serbina *et al.* 2008).

A second subset of circulating monocytes in mice expresses high levels of CX3CR1 and low levels of CCR2 and Ly6C (also referred as CX3CR1^{high} or LY6C^{low} monocytes), they are less prevalent than LY6C^{high} monocytes and they circulate for long time periods. These monocytes adhere and migrate (patrolling process) along the luminal surface of endothelial cells that line small blood vessels and traffic into peripheral tissues under non inflammatory conditions. These cells give rise to tissue macrophages and DCs and are referred to as resident monocytes (Auffray *et al.* 2007; Shi and Pamer 2011).

Macrophages express many receptors that mediate their diverse function. The opsonic receptors include complement receptors (integrins) and Fc receptors (Ig superfamily), and they function in phagocytosis and endocytosis of complement- or antibody – opsonised particles, respectively. Another group of phagocytic/endocytic surface receptors are the non Toll-like receptors (NTLRs), which include the family of scavenger receptors and the C-type lectins. Scavenger receptors include CD36, SREC and LOX-1 and they have also been shown to collaborate with TLR to NF-kB induction (Gordon 2007).

Following tissue injury or infection, the first-responder macrophages usually secrete TNFα (tumor necrosis factor) NO (nitric oxide) and IL-1, which participate in the activation of various antimicrobial mechanisms, including oxidative processes to kill invading organisms. Activated macrophages also produce IL-12 and IL-23, which influence the polarization of Th1 and Th17 cells. In addition to the innate pro-inflammatory role, phagocytic activity and role in antimicrobial immunity (M1 macrophage response: triggered by LPS or IFNγ), macrophages also exhibit an anti-proinflammatory and wound healing response to restore tissue homeostasis (M2 macrophage response: following exposure to IL-4 and/or IL-13) by production of TGFb1 (transforming growth factor), PDGF (platelet-derived growth factor), IL-10 and specific chemokines that recruit fibroblasts, Th2 and Treg cells (Murray and Wynn 2011).

4. Neutrophils

Neutrophils are polymorph nuclear (PMN) leucocytes (together with eosinophils and basophils) and are the major effectors of acute inflammation. During inflammation the number of neutrophils in tissues increases and, with time, these cells die by apoptosis and are removed by macrophages and DCs (Stark *et al.* 2005). In humans, 50-70% of circulating leukocytes are neutrophils, whereas the representation of these cells in mice is only 10-25% (Mestas and Hughes 2004).

Neutrophils are also considered the first leucocytes to be recruited to an inflammatory site after the initial detection of PAMPs and DAMPs by macrophages and patrolling monocytes. Endothelial cells near the inflammation site are stimulated by inflammatory signals (chemokines, lipid mediators, TNFa, IL-1b and IL-17, etc) to express adhesion molecules on their luminal site: P-selectin, E-selectins and ICAMs (intercellular adhesion molecules) (Borregaard 2010). On the surface of neutrophils, PSGL-1 (P-selectin glycoprotein ligand-1) and L-selectin are constitutively expressed proteins that engage the P and E-selectins of endothelial cells, resulting in selectin-mediated rolling of neutrophils along the endothelium. Engagement of PSGL-1 and L-selectin on neutrophils activates a variety of kinases that change the neutrophil biology and lead to β2-integrins activation and clustering on the surface. β2 integrins (LFA-1 and Mac-1/CD11b) engage the endothelial ligand (ICAM-1 proteins) resulting in the firm adhesion state to prepare trans-endothelial migration. In the interstitial space, neutrophils follow a hierarchy of chemotactic gradients towards host- or pathogen- derived chemoattractants (reviewed in Amulic *et al.* 2012).

Pro-inflammatory cytokines produced by macrophages, such as TNFα, IL-6, CXCL8 (IL-8), CCL3 and CCL4 promote recruitment of neutrophils. A multitude of chemokines can direct neutrophil recruitment, through the two main chemokine receptors expressed on neutrophils, CCR1 and CXCR2. Proteases produced by neutrophils, macrophages and endothelial cells cleave and increase the chemotactic activity of CXCL1, CXCL8 and CXCL5, all ligands of the neutrophil receptor CXCR2 (reviewed in Soehnlein and Lindbom 2010).

Concomitantly, activation of pattern-recognition receptors (PRRs) contributes to further activation of neutrophils, e.g., induction of the oxidative burst. In neutrophils, all TLRs are constitutively expressed, except TLR3 (Sabroe *et al.* 2005). Other PRRs that induce neutrophil chemotaxis and functional activation is the N-formyl peptide receptor (FPR1). The production of formylated proteins is restricted to bacteria and mitochondria (Zhang *et al.* 2010). Leukotriene B4, a lipid mediator, synthesized by monocytes and macrophages, in addition to its chemotactic effect, it also induces secretion of neutrophil granule proteins and ROS (reactive oxygen species)(Schoenberger 2003).

Neutrophils encapsulate microorganisms in phagosomes through Fc- and C3 complement-receptor's engagement and kill them by NADPH oxygenase-dependent mechanisms (reactive oxygen species: ROS) or by antibacterial proteins (cathepsons, defensins, lactoferrin and lysozyme). Besides being released into phagosomes, the antibacterial proteins can also be released into the extracellular milieu, a process called degranulation. In addition, highly activated neutrophils can also eliminate

extracellular pathogens by releasing neutrophil extracellular traps (NETs): NETs are composed of decondensed DNA attached to histones, proteins (lactoferrin and cathepsins) and enzymes (elastase) that are released from neutrophil granules as an active form of cell death. NETs prevent spreading of pathogens through their immobilization and also facilitate phagocytosis of trapped microorganisms (Brinkmann *et al.* 2004).

After the influx of neutrophils to the inflammation locus, neutrophils recruit monocytes by expressing CCL2, CCL3 (MIP-1α), CCL20 (MIP-3α), and CCL19 (MIP-3β) chemoattractants. Their granule proteins also induce monocyte extravasation (Scapini *et al.* 2001). Subcutaneous inoculation of *Leishmaniasis major* triggered a massive and rapid infiltration of neutrophils that secrete CCL3, recruiting DCs to the site of inoculation and initiating a protective Th1 response (Peters *et al.* 2008; Charmoy *et al.* 2010). Activated neutrophils can also induce DCs maturation in vitro (van Gisbergen *et al.* 2005). Infection of mice with *Legionella pneumophila* triggers production of IFNγ by NK cells, which is dependent on both neutrophil-derived IL-18 and DC-derived IL-12 (Sporri *et al.* 2008).

Neutrophils can also regulate adaptive immune responses. In spleen, neutrophils release BAFF (B cell-activating factor) and CD40 ligand, and promote help to B cell secretion of IgM and IgG (Puga *et al.* 2012). After intradermal injection of modified vaccinia Ankara virus, neutrophils carry virus from the skin to the bone marrow, where they interact with resident myeloid APCs, resulting in an alternative source of primed CD8 T cells (Duffy *et al.* 2012). Moreover, Nets formed by neutrophils prime CD4 T helper cells by reducing their activation threshold. (Tillack *et al.* 2012). Neutrophils also produce IFNγ, often at early stages of Listeria infection, which promote adaptive immunity through effects on MHC expression and T helper cell development (Yin and Ferguson 2009).

5. Natural killer cells

Natural killer cells (NKs) survey host tissues for signs of infection, transformation or stress, and they kill target cells that have become useless or are detrimental to the host. NK cells have been classified as a component of the innate immune system, however, they also share some attributes with T and B cells of the adaptive immune system.

NK cells are originated from a common lymphoid progenitor and depend on signals mediated by the common IL-2 receptor γ-chain (γ_c) for survival and homeostasis. In the mouse, the earliest lineage-committed precursors are characterized by the expression of IL-2R and IL-15Rβ. Next stages of maturation involve sequential acquisition of NK1.1 and CD94-NKG2 receptors and integrin α_v subunit. NK cells then express Ly-49 and c-kit, followed by an NK cell expansion stage that is characterized by up-regulation of DX5.

During basal homeostasis, mature peripheral NK cells reside in the blood, spleen, liver, lung and other organs. NK cells are found only at low frequency in the lymph nodes.

Although not fully characterized, many chemokines have been implicated in NK cells migration between the blood, lymphoid organs and non-lymphoid organs. These include CCL3 (MIP1α), CCL4 (MIP1β), CCL5, CCL19, CXCL12, CXCL16 and CX_3CL1. In addition, during NK cell development and homeostasis, the expression of α2, α4, and αMb2/MAC1 integrins and chemokine receptors (CCR1, CCR5, CCR7, CXCR3, CXCR4, CXCR6 and CX_3CR1) are regulated.

During infection, NKs become activated through the balance of inhibitory and activating receptor stimulation, and by pro-inflammatory cytokines (such as IL-12 and type I IFNs), and as a result they produce large amounts of IFNγ, perforin and granzymes. In addition, NK cells are an important source of inflammatory cytokines that regulate adaptive immune system in host protection from tumors and viruses, via their cross talk with APCs like DCs, monocytes and macrophages (reviewed in Kim *et al.* 2002; Degli-Esposti and Smyth 2005; Sun and Lanier 2011).

6. Dendritic cells

Dendritic cells (DCs) are designated as professional antigen-presenting cells (APCs) and comprise several subsets. Differences in location, life cycle, and intrinsic ability to capture, process and present antigens on their MHC class I and class II molecules – a prerequisite for T cell priming – enable each DC subset to have distinct roles in immunity to infection and in the maintenance of self tolerance.

The most common method to generate mouse DCs in vitro involves culturing bone marrow or spleen precursors in medium supplemented with granulocyte/macrophage colony stimulating factor (GM-CSF), with or without IL-4. However, the DCs generated by this method are homogeneous and resemble monocyte-derived DCs, which almost do not correspond with any lymphoid-organ-resident DCs subsets found in vivo. This highlights the importance of analyzing the properties of DCs in vivo, where anatomical location of the DCs, the accessibility of the antigen to that location, and the effect of pathogens exerted on the DCs further influence their properties (Shortman and Naik 2007).

There are two main subtypes of DCs in mouse secondary lymphoid organs: conventional DCs (cDC) and plasmacytoid DCs (pDC). **cDC** are short-lived cells expressing high levels of CD11c and are subdivided into resident DCs and migratory DCs. **Resident DCs** differentiate in the lymphoid organs from blood-born precursors and can be divided into CD8a-expressing DC ($CD8^+$ DC) and $CD8^-$ DC. These subsets are the only ones found in the spleen. **Migratory DCs** develop from earlier precursors in peripheral tissues and travel through the afferent lymphatics to reach the local draining lymph nodes. This group of DCs is absent from the spleen and thymus and it includes epidermal Langerhans cells, epithelial cells, and their dermal counterparts. **cDCs** have an enhanced ability to sense tissue injuries, capture environmental- and cell-associated antigens and process and present phagocytosed antigens to T cells due to: i) their critical location in non-lymphoid tissues and in the spleen marginal zone in the steady sate, where they constantly acquire tissue and blood antigens; ii) their superior antigen processing and presentation machinery; iii) their superior ability to migrate loaded with

tissue antigens to the T cell zone LNs in the steady state and inflamed state; iv) their superior ability to prime naïve T cells responses. **pDC** are relatively long-lived cells, they circulate through the blood and lymphoid tissues, they express low levels of MHC class I and co-stimulatory molecules and low levels of the integrin CD11c in the steady state. They also express a narrow range of pattern-recognition receptors (PRRs) that include TLR7 and TLR9 and are thus specialized to respond to viral infection. Upon recognition of foreign nucleic acids, they have the unique capacity to secrete large amounts of type I interferons and acquire the capacity to present foreign antigens (reviewed in Segura *et al.* 2007; Merad *et al.* 2013).

When a microbe infects a tissue, immature DCs sense the microbe through the different types of pattern recognition receptors (PRRs) and then capture the microbe or its products by the actin-dependent process of phagocytosis (for particulate antigens) and receptor-mediated endocytosis or macropinocytosis (for soluble antigens). To be presented, the antigens have to access the compartments where proteolytic degradation generate peptide ligands for MHC class I or II molecules (peptide processing). Peptides derived from proteins degraded mainly in the cytosol by the proteasome are presented by MHC class I molecules, whereas peptides derived from proteins degraded in endosomal compartments by the cathepsins and other hydrolytic enzymes are presented by MHC class II molecules. Antigens can be classified as endogenous (when synthesized by the antigen-presenting cells themselves) or exogenous (when synthesized by other cells). Any endogenous polypeptide can occur in the cytosol as a functional protein or as a defective ribosomal product, so DCs continually present peptides that are derived from endogenous proteins on MHC class I molecules. Similarly, endogenous proteins that access endosomal compartments of DCs are efficiently presented on their own MHC class II molecules (like components of the endocytic pathway, membrane proteins and cytosolic proteins that are transferred into endosomes by autophagy). Therefore all DCs constitutively present peptides that are derived from their own components on MHC class I and II molecules. When cells become infected with virus, the endogenous viral antigens are incorporated into their antigen presentation pathways, and DCs are no exception. However, participation of DCs in antiviral responses can be compromised due to multiple viruses' mechanisms to interfere with antigen-presentation pathways. The presentation of exogenous antigens relies on the ability of cells to deliver the antigens to the correct processing compartment. These antigens must be endocytosed by pinocytosis, phagocytosis or receptor-mediated endocytosis in order for antigens to become readily accessible to endosomal proteases and so be presented by MHC class II molecules. In addition, some cells can present these antigens via MHC class I molecules, a process known as cross-presentation. This pathway is of particular relevance in DCs because they appear to be the main cell population that cans cross-present antigens in vivo. Among the lymphoid-organ-resident DCs, the $CD8^+$ DCs are the most efficient in the phagocytose of dead cells and, consequently, at MHC class II presentation and MHC class I cross-presentation of cellular antigens (Villadangos and Schnorrer 2007).

$CD8^+$ DCs hold the machinery to efficiently **cross-present antigens** (present exogenous antigens on their MHC class I molecule). When purified DC subsets were cultured with antigen-specific CD8 T cells ex vivo, resident $CD8^+$ DCs were shown to be the sole antigen cross-presenting cells after intravenous infection with LM, intravenous or intraperitoneal infection with LCMV (Lymphocytic choriomeningitis virus) (Belz *et al.* 2005), and after cutaneous infection with HSV (Allan *et al.* 2006). In addition to $CD8^+$DCs, migratory lung and dermal DCs can cross-present antigen in vivo. $CD8^+$ DC can also efficiently present antigens to $CD4^+$ T cells during infection (Sponaas *et al.* 2006; Mount *et al.* 2008) and they are the only subtype capable of presenting Toxoplasma gondii profilin-like molecules, owing to their unique expression of TLR11 (Yarovinsky *et al.* 2006). $CD8^+$ DCs and $CD103^+$ cDC (migratory lung epithelial subset) are the only cDCs with the double-stranded viral RNA sensor -TLR3. Both subsets express high levels of CD26 scavenger receptor, which binds to dead cells; high levels of C-type lectin Clec9A, which senses necrotic bodies; and the chemokine receptor XCR1) (Dorner *et al.* 2009; Sancho *et al.* 2009; Davey *et al.* 2010).

For productive immunity to occur, DCs must present not only peptide-MHC complexes but also additional costimulatory signals (such as molecules of the B7 family, including CD80 and CD86) to T cells. The interaction between CD80 or CD86 and CD28 on T cells results in the up-regulation of CD40 ligand on T cells. The T cells may then engage CD40 on DCs and trigger a burst of cytokine expression, including IL-12, which induces IFN-γ in T cells. Signaling through CD40 also up-regulates numerous other co-stimulatory molecules, which may play distinctive roles in tuning the immune response (Banchereau and Steinman 1998; Pulendran *et al.* 2001).

III. Acquired immune response

CD8 T cell response

CD8 T cells responses are necessary for the control of a variety of intracellular bacterial and viral infections, and tumors.

Upon cognate antigen recognition through their TCRs, naïve CD8 T cells initiates multiple intracellular signals with complex molecular associations that lead to a cellular response. This includes changes in cell surface phenotype, extensive division, acquisition of effector functions (such as cytokine secretion and cytotoxicity) and altered survival requirements to constitute the memory pool.

1. TCRαβ structure

The antigen receptor of T cells (TCR) is a multimeric transmembranar complex -**TCR/CD3 complex** - composed of six different polypeptide chains, organized into an eight-chain structure: a clonotypic and polimorphic heterodimer constituted by TCRα-TCRβ chains, responsible for specific ligand-binding; and three nonpolymophic signaling dimmers (CD3εCD3δ and CD3εCD3γ heterodimers, and ζζ homodimers chains), which are non-covalently bound to each other (Fig. 5).

TCRα and TCRβ polypeptides consist of an amino-terminal variable (V) region and a carboxyl-terminal constant (C) region. **The diversity of the T cell repertoire** is mostly determined by the complementary-determining regions (CDRs), on both TCR Vα and Vβ domains, which are generated by programmed rearrangement of germline [V-(D)-J] gene segments during T cell development. However, due to short cytoplasmic tails, TCR-α and -β chains lack inherent signaling activity. Thus, signaling activity is depending on their association with transducing CD3 and ζζ dimmers (the CD3 complex).

Unlike αβ chains, nonpolymorphic chains (**CD3εCD3δ, CD3εCD3γ and ζζ dimmers**) have long cytoplasmic tails and are required for **receptor assembly**, **cell-surface expression**, and **signaling transduction** (Weiss and Stobo 1984).

Assembly of TCRαβ/CD3 complex begins in the endoplasmic reticulum (ER) with formation of ζζ dimmers and the TCRαβ/CD3δεγε hexamer. In the absence of any individual component of the CD3 complex, the other chains are synthesized at normal rates, but they are largely retained in the ER and degraded instead of being transported to the cell surface (Delgado and Alarcon 2005). All of the components are joined into a full octameric receptor in the Golgi compartment when a TCRαβ/CD3δεγε complex associate with ζζ dimmers (Minami *et al.* 1987b; Wegener *et al.* 1992; Dietrich *et al.* 1999).

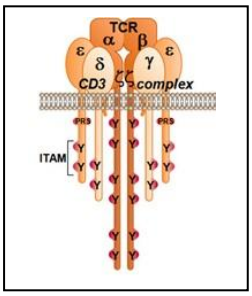


Figure 5. The TCRαβ/CD3 complex of T cells: it is composed of six different chains organized in eight-chain structure (TCRα-TCRβ, CD3εCD3δ, CD3εCD3γ and ζζ chains) with 10 ITAMs (immunoreceptor tyrosine-based activation motifs). YY: tyrosine residues (Guy *et al.* 2013).

The crucial regions on cytoplasmic domains of signaling chains are the immunoreceptor tyrosine-based activation motifs (ITAMs) that are phosphorylated upon TCR ligation. Each ζ chain has three ITAMs whereas each CD3 chain has only one ITAM (Weissman *et al.* 1988). When phosphorylated, CD3ε δ, ε γ, and ζζ chains serve as docking sites for recruitment of proteins with tyrosine kinase activity (PTK) that initiate a cascade of phosphorylation events (Weiss and Littman 1994) (Wang and Reinherz 2012).

Hereafter, the term "TCR" is used to refer to the TCR αβ heterodimer /CD3 complex.

2. T-cell co-receptor: CD8αβ

CD4 and CD8 are transmembrane glycoproteins that bind to the TCR-engaged pMHC molecule and are involved in optimal TCR recognition and T-cell activation.

CD4 co-receptor comprises four Ig-like domains, whereas CD8 co-receptor is a disulfite-linked heterodimer encoded by two distinct chains: **CD8α and CD8β**, each consisting of a single Ig-like domain. CD8α chain can also form homodimers (CD8αα) that may be expressed in several cell types: DC, activated CD4 and CD8 T cells, γδ T cells, NK cells and intraepithelial lymphocytes.

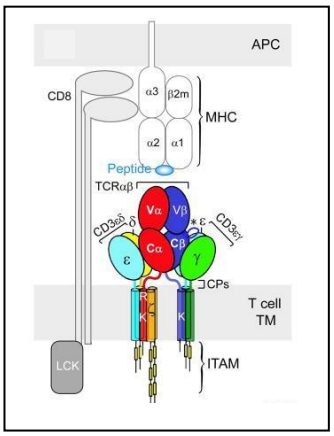


Figure 6. TCR complex and CD8αβ heterodimer interactions with a pMHC class I molecule on an antigen presenting cell (APC): cooperative trimeric interaction (Wang and Reinherz 2012)

CD8αβ heterodimer is only expressed in thymus derived $CD8^+$ T cells, thus CD8β is commonly used as the marker to define thymus derived conventional CD8 T cells.

T-cell co-receptors CD4 and CD8 **facilitate the adhesion/binding to the pMHC**. CD4 binds to MHC class II molecules, whereas CD8 binds to MHC class I, which favor the $CD4^+$ and $CD8^+$ T cell restriction to their respective MHC (Doherty and Zinkernagel 1975).

Besides favoring the trimeric interaction CD4 and CD8α also deliver **the Lck** (Lymphocyte-specific tyrosine kinase) **in close vicinity to the TCR-pMHC interaction** so that exposed ITAMs on CD3 complex-cytoplasmic tails can be phosphorylated on tyrosine residues, allowing Zap-70 recruitment and consequential downstream signaling (Fig. 6). (reviewed in Wang and Reinherz 2012).

3. Immunological synapse and TCR triggering

T cell activation occurs in the context of close contact between an APC and T cell, referred to as immunological **synapse** (IS). The IS is an organized structure composed of two concentric regions: the TCR-rich central supramolecular activation cluster (cSMAC), surrounded by the integrin-rich peripheral SMAC (pSMAC).

Engagement of the TCR by pMHC triggers profound reorganization of the protein-lipid compositions of the IS, leading to the formation of **signaling competent protein microclusters** that contain TCR complexes associated with signaling molecules. These intracellular signaling clusters initiate: (i) actin cytoskeletal rearrangements that results in clusters translocation toward the cSMAC; (ii) polarization of T cell where the microtubular organizing center (MTOC) moves toward the T cell/APC contact side; and (iii) are responsible for subsequent signaling and activation of T cells, including the induction of a characteristic cascade of tyrosine phosphorylation events, Ca^{2+} release, and target gene expression (Fig. 7a) (Billadeau *et al.* 2007).

How the ligation of the TCR is translated into the first intracellular signals remains controversial. Current models suggest that TCR aggregation, conformational changes within the TCR complex and exclusion of inhibitory molecules (segregation) are all required for signal initiation.

Interestingly, in resting T cells, the CD3ζ and/or CD3ε tails are tightly associated with the lipid-rich inner leaflet of the plasma membrane, rendering them **inaccessible to Lck phosphorylation** but, after TCR ligation, they are released from the membrane and available to be phosphorylated (Xu *et al.* 2008).

TCR aggregation is supported by the existence of preformed TCR aggregates on nonactivated T cells and by the inability of soluble monomeric pMHC to activate T cells. **Conformational changes** after crosslinking of TCR/CD3 complexes with multimeric pMHC enables close contact and transphosphorylation between the CD3 tails and associated PTKs, and it also may result in competition for membrane lipids between the CD3 chains, resulting in dissociation of the cytoplasmic domains from the membrane and subsequent ITAM phosphorylation. It has also been demonstrated that short extracellular domains of adhesion molecules contribute to a close contact between an APC and T cells and that **inhibitory phosphatases with long extracellular domains such CD45 are excluded** from the contact zone because of their size, allowing TCR signaling (Davis and van der Merwe 2006; Smith-Garvin *et al.* 2009; van der Merwe and Dushek 2011).

4. TCR downstream signaling: MAPKs (Erk, p38, JNK), NF-kB, and AKT pathways

Signal transduction pathways within cells rely on multiple and complex cascades of phosphorylation and desphosphorylation executed by kinases and phosphatases, which leads to the activation of several transcription factors that then guide gene transcription programs. MAP kinases are among the most ancient signal transduction pathways, and they are involved in many cellular programs such as cell proliferation, differentiation, movement and death. In immune responses, MAP kinases pathways are involved in the initiation phase of innate immunity (i.e. Toll like receptor signaling), activation of adaptive immunity (i.e. TCR signaling, cytokine receptors) and in cell death. MAP kinase family members are divided in three mains subgroups: ERK, p38 and JNK.

The earliest step in intracellular signaling following TCR ligation is the activation of the Src-family kinase **Lck** which is associated to the **CD8 or CD4 co-receptors**. When activated, Lck phosphorylate the tyrosine residues (YY) in **ITAMs on TCR/CD3 complex**. Phosphorylated ITAMs promote the recruitment of the Syk-family kinase **ZAP-70** (ζ-Associated Protein of 70-kDa) that are then able to phospholyrate the transmembrane **LAT** (Linker for Activation of T cells) and the cytosolic **SLP-76** (Src homology 2 domain-containing leukocyte phosphoprotein of 76 kDa) adaptor proteins. These two adapters form the backbone of the proximal signaling complex (Fig. 7a) that organizes effector molecules in the correct spatiotemporal manner allowing activation of multiple distal signaling pathways (Fig. 7b) (Sommers *et al.* 2004).

a) <u>p44/p42 MAPKs and NF-kB phatways</u>

Phosphorylated **LAT** leads to the recruitment and activation of **PLCγ1** (PhophoLipase C, gamma 1) that hydrolyzes the membrane lipid PIP2, producing the second messengers IP3 (Inositol 1,4,5-triphosphate)and DAG (dyacylglycerol).

Production of **DAG** results in the activation of two major pathways involving Ras and PKCθ (protein kinase C).

Ras is required for Raf-1 activation which initiates a mitogen-associated protein kinase (MAPK) phosphorylation activation cascade: Raf-1 is a MAPKKK that phosphorylates and activates MAPKKs, which in turn phosphorylate and activate <u>p44 and p42 MAPK's</u>, also named extracellular signal-regulated kinase (<u>Erk1 and Erk2</u> respectively). When dually phosphorylated they enter into the nucleus and regulate the <u>Fos and STAT3</u> (signal transducer and activator of transcription 3) expression.

PKCθ is activated after DAG binding and regulates the IkB (Inhibit*or* of NF-kB) degradation and the release of <u>NK-kB</u>, resulting in NF-kB nuclear localization where it activates genes involved in the effector function, survival, and homeostasis of T cells.

IP3 stimulates Ca^{2+}-permeable ion channel receptors (IP3R) on the endoplasmic reticulum (ER) membrane leading to the release of ER Ca^{2+} stores into the cytoplasm. Depletion of ER Ca^{2+} triggers a sustained influx of extracellular Ca^{2+} through the activation of plasma membrane CRAC (Ca^{2+} release-activation Ca^{2+} channels). Increase of intracellular Ca^{2+} levels results in activation of phosphatase Calcineurin and CaMK (Ca^{2+}-calmodulin-dependent kinase). Then, calcineurin dephosphorylates members of the nuclear factor of activated T cells (NFAT) family leading to its translocation to the nucleus, where it forms cooperative complexes with a variety of other transcription factors resulting in different gene expression patterns and function outcomes, depending on the context of the TCR signal. The most well-studied interaction integrates Ca^{2+} and Ras signals (NFAT/AP-1) which results in IL-2 expression (Fig, 7b). (reviewed in Smith-Garvin *et al.* 2009).

NFAT activity in the absence of AP-1 (Activator protein 1) activation induces a pattern of gene expression that results in T cell anergy and lack of IL-2 production (Macian *et al.* 2002). FOXP3 (the regulatory T cell lineage specific transcription factor forkhead box protein 3) also cooperates with NFAT and antagonizes NFAT/AP-1 gene transcription, resulting in Treg functional gene expression and lack of IL-2 production (Wu *et al.* 2006). NFAT family members can also cooperate with STAT proteins to induce either Th1 or Th2 differentiation through T-bet or GATA3 expression, respectively (Savignac *et al.* 2007).

b) p38 MAPK pathway

T cell signaling is mainly mediated by the specific pathway involving LAT activation, however there is an alternative pathway, linked to TCR signaling, that is independent of LAT and that circumvent, i.e. evade the classical MAPK cascade that it is also regulated by stress conditions.

In response to a variety of growth factors, cytokines and stress signals p38 kinase is activated by dual phosphorylation of a conserved Trh 180-X-Tyr182 motif through a MAPK cascade (MAPKKK-MAPKK-MAPK pathway).

In T cells, it has been generally assumed that p38 is regulated via the same MAPKKK-MAPKK pathway; however, it was demonstrated that after TCR ligation in CD28 coligation context, the **ZAP-70** kinase can directly phosphorylates **p38** (Salvador *et al.* 2005). The directly phosphorylation of **p38** by **ZAP-70** occurs on a previously unrecognized site, at Tyr323, that in turn induces p38 autophosphorylation. Both T and B cells activate p38 but only the TCR activation induces p38 autophosphotylation. It remains to be determined whether the two modes of p38 activation activate different targets and thus specifically regulate T cell functions (Rudd 2005). p38 MAP kinase signaling pathway controls IFN-γ production in both CD4 and CD8 T cells but only regulates apoptosis selectively in CD8 T cells and not in CD4 T cells (Merritt *et al.* 2000).

c) JNK MAPK pathway

Three members of the c-Jun NH2-terminal kinase (JNK) family have been identified (JNK1, JNK2, and JNK3). JNK3 is primarily expressed in brain, heart, and testis, but not in hematopoietic cells. JNK1 and JKN2 are expressed more ubiquitously, but their expression levels in T and B cells are very low prior to cell activation, being induced after antigen stimulation. JNKs are activated by dual phosphorylation on Thr183 and Tyr185 by MKK4 and MKK7.

In vitro studies have shown that the loss of Jnk2 causes hyperproliferation of CD8 T cells, which is due to an increased production of IL-2. Accordingly, upon infection with LCMV, an increase in the expansion of antigen (virus)-specific CD8 T cell in Jnk2$^{-/-}$ mice has also been shown *in vivo*. Distinctly, the absence of Jnk1 seems to have the opposite effect on CD8 T cells. CD8 T cells that lack JNK1 are hypoproliferative *in vitro* due to impaired expression of IL-2Ra (CD25). Upon LCMV infection, Jnk1$^{-/-}$ mice have a decreased expansion of virus-specific CD8 T cells. Although no effect of JNK1 deficiency in the survival of CD8 T cells is reported *in vitro*, increased apoptosis of virus-specific CD8 T cells was found *in vivo*. (Conze *et al.* 2002) (Arbour *et al.* 2002) (Rincon and Davis 2009). JNK1 is required for expansion of CD8 T cells by regulating the levels of growth factor receptors as well as the sensitivity of these cells to activation-induced death. According, Jnk1$^{-/-}$ mice exhibit major defects in tumor immunosurveillance associated with a decreased cytotoxic T-cell function and a reduced expression of T-bet, eomesodermin, and perforin (Gao *et al.* 2005).

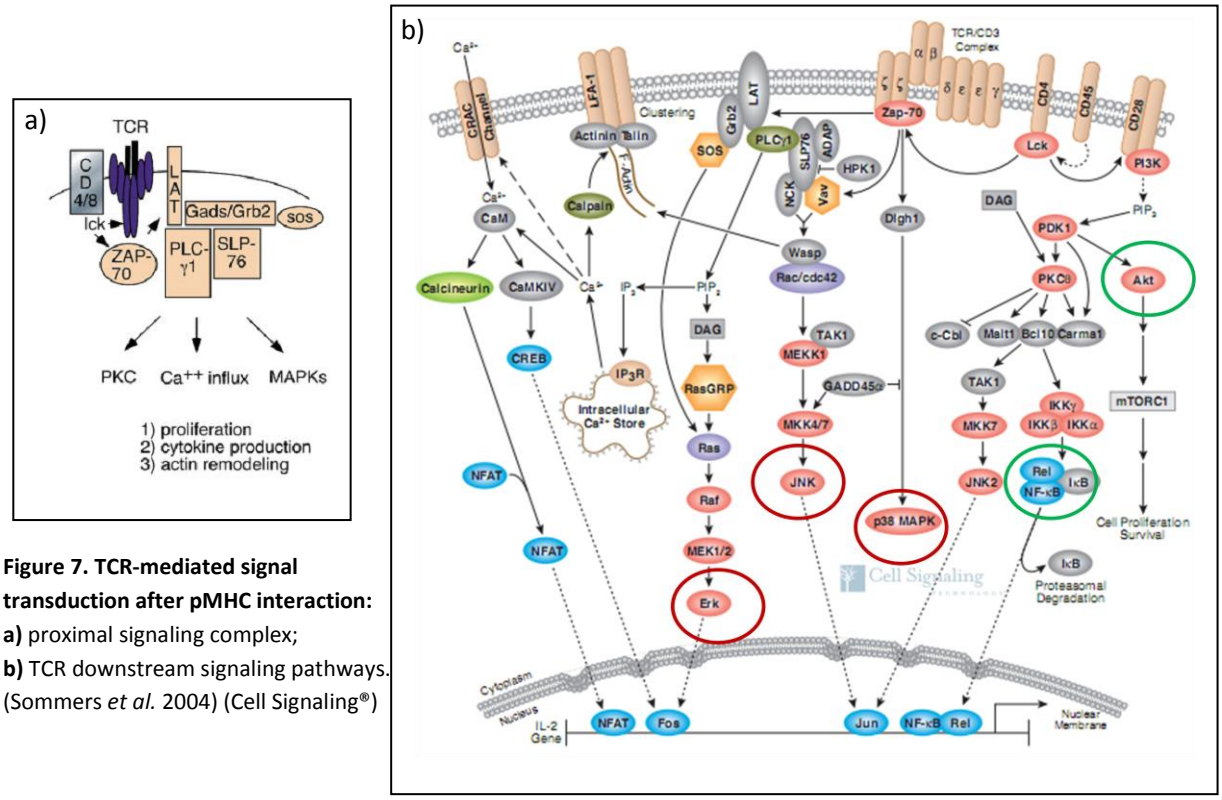


Figure 7. TCR-mediated signal transduction after pMHC interaction:
a) proximal signaling complex;
b) TCR downstream signaling pathways.
(Sommers *et al.* 2004) (Cell Signaling®)

d) AKT/CD28 co-stimulation pathway

pMHC recognition through the TCR is not sufficient **for full T cell activation**, and a variety of co-stimulatory ligand-receptor interactions are also required to provide additional positive or negative signals. CD28 is one of the receptors expressed on T cells that provide positive co-stimulatory signals. In human T cell clones that express ~30000 TCRs, the threshold number of triggered TCRs necessary for T cell activation is reduced from ~8000 (in absence) to ~1000 TCRs in the presence of CD28-mediated co-stimulation. Thus, the role of CD28 co-stimulation is not to increase the number of triggered TCRs, but rather to amplify the signal transmitted in order that a cellular response can be achieved at lower number of triggered TCRs (Viola and Lanzavecchia 1996). **CD28 enhances TCR signaling** more robustly than other co-stimulatory molecules and it promotes T cell proliferation, cytokine production, cell survival, and controls cellular metabolism (Acuto and Michel 2003).

Following the binding of CD28 to its **ligands, CD80 or CD86 on APCs**, the p85 regulatory subunit of PI3K (Phosphoinositide 3-kinase) associates with a pYMNM motif on the cytoplasmic tail of CD28. This regulatory subunit recruits the p110 catalytic subunit of **PI3K**, which converts **PIP2 to PIP3** at the membrane. Localized PIP3 serves as docking site for **PDK1** (3-phosphophoinositide-dependent protein kinase 1) and its target **Akt** (also known as PKB). TCR signaling has also been shown to synergize with IL-2 receptor, besides with CD28 receptor, to activate the PI3K-dependent kinase, Akt.

Akt accomplishes this function by associating with CARM1 and facilitating the assembly of the CMB (CARD11-BCL-10-MALT1) complex, which is critical for NF-kB activation (Narayan *et al.* 2006). Activated Akt enhances the nuclear translocation of NF-kB, which induces the expression of prosurvival genes including Bcl-xl. Akt has also the ability to inhibit transcription factors that promote cell cycle arrest, which results in Akt-driven cell survival and proliferation. In addition, Akt also affects optimal transcription of NFAT-regulated genes, such as IL-2 (Beals *et al.* 1997). TCR/CD28 colligation also regulates T cell metabolism by increasing the cell surface expression of the insulin transporter Glut1, leading to increased glucose uptake and glycolysis, which is also mediated by Akt (Frauwirth *et al.* 2002; Jacobs *et al.* 2008). Another characteristic of CD28-mediated signaling is the enhanced Ca^{2+} flux after Lck binding to a CD28 tail motif (Heyeck *et al.* 1997).

The magnitude of the response induced by TCR ligation is considerably augmented with CD28 colligation; however, other costimulatory receptors can also influence immune responses: ICOS (inducible costimulator), OX40 and 4-1BB. Indeed, CD28 also promotes expression of these three costimulatory receptors that prolong and sustain an immune response by themselves. In the case of OX40 and 4-1BB, these co-receptors are also involved in memory T cell formation (Watts 2005).

Activation of **mTOR pathway** (mammalian target of rapamycin), which is composed of mTORC1 and mTORC2 complexes is another critical event during T cell activation and it is suggested to lie downstream of the kinase Akt, although it remains to be determined how exactly mTOR is activated downstream of the TCR. Both mTORC1 and mTORC2 are activated within minutes after TCR stimulation, and the magnitude of mTOR activation is directly correlated with the duration of the T cell-DC interaction and the dose of the cognate antigen. The activity of mTOR is further shaped by co-

stimulatory signals: CD28-mediated co-stimulation is a classic activator of the PI3K-Akt pathway, which further upregulates the mTOR activity induced by the TCR to facilitate productive T cell activation. mTOR can also be activated by nutrient sensing and cytokine signals. mTOR complexes regulate a wide range of processes including autophagy, metabolism and the expression of transcription factors such as T-bet, Eomesodermin and FoxO. mTOR complexes control the switch from catabolic to anabolic metabolism in effector CD8 T cells, and the switch back to catabolic metabolism in memory CD8 T cells. In addition, mTOR pathways play a critical role in the activation of naïve CD8 T cells to effector cells and they also negatively regulate the development of CD8 memory T cells. (reviewed in Chi 2012; Powell *et al.* 2012).

The use of a mTOR inhibitor, rapamycin, promotes the generation of protective T cell memory in models of infection with LCMV and LM. Mechanistically, this has been associated with the induction of a metabolic switch from glycolysis to fatty acid oxidation, in the presence of rapamycin (Araki *et al.* 2009; Pearce *et al.* 2009). Akt-inhibited T cells reveal expression of memory-associated genes including IL-7R, CCR7 and CD62L, while the expression effector-associated genes including IFNγ, granzyme B and perforin were reduced (Zehn *et al.* 2012).

5. Negative regulation of TCR signaling

Signaling through the TCR activate multiple effector pathways. To ensure that T cells respond to the appropriate ligands for the proper duration, activation of these pathways has to be regulated. Similar to the positive regulation of T cell activation, negative regulation is also mediated by TCR-generated signals and other cell surface receptors.

Even the most proximal TCR signaling events are actively regulated. For example, **Csk** (C-terminal Src kinase) is responsible for phosphorylating Lck on its inhibitory tyrosine residue (Y505) and thus maintaining Lck in an inactive state (Vang *et al.* 2004). **CD45** phosphatase also limits Lck activity by desphosphorylating its active site. However, CD45 is also able to dephosphorylate the inhibitory site of Lck, allowing for Lck autophosphorylation and activation. Whether CD45 negatively or positively impacts TCR signaling is likely to be controlled by CD45 proximity to TCR-stimulated effector molecules during TCR engagement and by its enzymatically favorable conformation (Vang *et al.* 2004). In the case of weak or antagonistic TCR ligation, **SHP1** (SH2 domain-containing protein tyrosine phosphatase) desphosphorylates the active site of Lck, resulting in cessation of the TCR signal. On the other hand, in the presence of strong or agonistic TCR ligation, Erk is rapidly activated and phosphorylates Lck on Ser59. This activity is thought to prevent SHP1 binding, thus keeping Lck active to sustain TCR signals and further amplify Erk activity (Stefanova *et al.* 2003). However, the extent to which this regulatory loop operates *in vivo* on the context of agonist stimulation awaits further analysis.

TCR-generated regulatory signals are also aided by co-receptor signals. **CTLA-4** (cytotoxic T lymphocyte antigen-4) and **PD-1** (programmed death-1) are two examples of co-receptors that limit the expansion and activation of TCR-triggered T cells. These molecules are found on activated T cells with their peak of expression at 24-48h after stimulation. Genetic studies have also documented the importance of both for maintaining self-tolerance.

Both CTLA-4 (inhibitory co-receptor) and CD28 (costimulatory co-receptor) share the ligands CD80 (B7.1) and CD86 (B7.2) on APCs. One of the mechanisms to inhibit T cell responses by CTLA-4 is through the competition or sequestration of ligands, as a result of the high affinity of CTLA-4 for B7 ligands (Thompson and Allison 1997; van der Merwe *et al.* 1997).

PD-1 receptor belongs to the CD28/CTLA-4 family and negatively regulates TCR signaling upon engagement of one of its ligands PD-ligand 1 (B7-H1) and PD-ligand 2 (B7-DC) (Sharpe and Freeman 2002). Mice deficient in PD-1 display multiple autoimmune defects and loss of peripheral tolerance. The ligands for PD-1 are members of the B7 co-stimulatory molecules family, and are expressed on APCs, endothelial and epithelial cells, and on activated lymphocytes (Latchman *et al.* 2001). PD-1 is transiently up-regulated in activated T cells during antigen presentation, and its ligation to PD-ligand 1 recruits SHP1 and 2, that desphosphorylates effector molecules associated with TCR, leading to termination of TCR signal transduction (Chemnitz *et al.* 2004; Sheppard *et al.* 2004).

During chronic infections, PD-1 is highly expressed on virus-specific CD8 T cells, and it is also correlated with an “exhausted” T cells phenotype that is reversed upon PD-1 neutralization (Barber *et al.* 2006). The PD-1 pathway can also compromise CD8 T cell responses during some acute infection and contributes to the functional impairment of “helpless” CD8 T cell (Brown *et al.* 2010). It was also shown that PD-1 impedes accumulation of T_{EM} phenotype memory CD8 T cells by promoting their apoptotic death (Charlton *et al.* 2013).

Cbl (Casitas B-lymphoma) proteins also play an important role as negative regulators of T cell signaling. Two family members, c-Cbl and Cbl-b, are immune modulators, as deletion of each gene *in vivo* leads to hypercellularity and, in the case of Cbl-b, spontaneous multiorgan infiltration. Both c-Cbl and Cbl-b facilitate the ubiquitination of proteins, targeting them to degradation. Following stimulation with antigen peptides, Cbl proteins mediate the downregulation of the TCR, and are also involved in the dissipation of the early signaling complex. (Murphy *et al.* 1998; Naramura *et al.* 2002; Balagopalan *et al.* 2007).

6. TCR downregulation

The capacity of T cells to develop and exert their function critically depends on signals mediated through the TCR. The TCR is a **spontaneously cycling receptor** (Krangel 1987; Minami *et al.* 1987a), and its cell surface expression level is the result of a dynamic equilibrium maintained by: the

membrane expression of newly synthesized TCR, the receptor internalization, the recycling to the cell surface, and its degradation (Geisler 2004). In steady state condition, a balance between the rate of internalization and recycling is tightly maintained. Following T cell activation and phosphorylation of the various TCR subunits, degradation of TCR is strongly favored leading to a decrease in recycling that results in downregulation of cell surface levels (D'Oro *et al.* 1997; Valitutti *et al.* 1997).

TCR downregulation occurs in response to MHC/peptide stimulation, anti-TCR antibody binding, or to treatment of T cells with activators of protein kinase C (PKC) like phorbol esters. **At least two distinct pathways** exist to explain TCR downregulation. One pathway is dependent on tyrosin kinase activity (Lck) and leads to TCR ubiquitination and degradation. The other pathway is dependent on PKC-mediated activation of the di-leucine-based motif found in the CD3γ chain of the TCR and it leads to TCR recycling (Lauritsen *et al.* 1998). Recognition of the TCR subunit CD3γ by PKC, with subsequent phosphorylation of CD3γ, exposes the CD3γ leucine-based internalization motif to which AP-2 (a clathrin-coated vesicle adaptor protein) binds to promote TCR endocytosis (Dietrich *et al.* 1997). The TCR complex has two different internalization motifs: tyrosin-based and dileucine-based. It is also proposed that ζ chains have a major role in shielding the internalization motifs on CD3γ chains. Internalization motifs are also present on CD3δ, CD3ε and ζ chains (Szymczak and Vignali 2005). Recently, it has been shown that the interaction between PD ligand 1 on DCs and PD-1 receptor on CD8 T cells contributes to ligand-induced TCR downmodulation and that this is mediated via the up-regulation of Cbl-b E3 ubiquitin ligase in CD8 T cells. Accordingly, interference with PD-1 signaling inhibits TCR downregulation leading to hyper-activated CD8 T cells (Karwacz *et al.* 2011).

Conflicting data have been published on the downregulation and degradation rates of the individual TCR subunits, as well as several divergent models for TCR downregulation have also been suggested. Most studies found that TCR downregulation is caused by an increase in the endocytic rate constant after TCR triggering, however some other studies indicated that TCR ligation induces TCR downregulation by a reduction in the exocytic rate constant rather than by an increase in the endocytic rate constant. Divergent models indicated that the degradation rate constant increases after TCR triggering and that all TCR subunits are degraded in parallel, where others found that TCR triggering transiently deviates TCR-α and -β chains from degradation and that TCR subunits segregate and are degraded with distinct degradation rate constants (reviewed in Geisler 2004; von Essen *et al.* 2004).

Most of these studies have been done using T cell lines or primary cells activated *in vitro*. As *is vitro* activation conditions not always mimic *in vivo* environments, further research into *in vivo* systems is need in order to reach full consensus about the mechanisms that mediate TCR downregulation.

Internalization of signal-transducing receptors is thought to produce a **dual effect**. First, it may contribute to signal transducing by favoring the encounter between receptor and intracellular signaling molecules. In addition, TCR internalization may play an important role during T cell

activation by allowing serial triggering of multiple TCR complexes by few pMHC complexes. Indeed, downregulation of unengaged TCR receptors has also been reported to occur with activation. Second, it may contribute to the termination of cellular responses by reducing the number of receptors at the cell surface or by uncoupling receptors from membrane-anchored signaling molecules (San Jose *et al.* 2000).

7. Cell surface phenotypic modifications after T cell activation

CD69 and CD44

CD69 is a C-type lectin surface receptor, with unknown ligand. T cells rapidly express CD69 upon stimulation by TCR (Testi *et al.* 1989) hence, CD69 has been used as an activation marker (Ziegler *et al.* 1994) and it is known as the very early activation marker. CD69 is also up regulated in T cells by exposure to type I IFN (IFNα/β) and other inflammatory mediators within the first hours of T cells arriving in an inflamed LN (Shiow *et al.* 2006; Grigorova *et al.* 2010).
CD69 also affects the migration of immune cells. It directly interacts with S1P1 receptor on the lymphocyte surface inducing down-regulation of S1P1 expression (a receptor required for lymphocyte egress in lymphoid organs). Consequently, CD69 mediates the retention of activated lymphocytes in the secondary lymphoid organs (Shiow *et al.* 2006).

CD44 is a surface glycoprotein with multiple isoforms and it binds to a common component of extracellular matrixes (ECM): the hyaluronic acid (HA). The interaction between CD44 receptor and its ligand promotes cell adhesion and migration. Differential glycosylation of CD44 influences its binding to HA, and additional ligands include osteopontin, serglycin, collagens, fibronectin, and laminin. CD44 is found constitutively on the surface of many cell types, including resting T cells, which express the invariant or standard form. This molecule localizes in lipid rafts with TCR and is upregulated early after TCR engagement (DeGrendele *et al.* 1997b). Once upregulated on responding T cells, CD44 expression is sustained on effector cells (promotes T cell extravasation into inflammatory site) as well as on memory cells. Thus, CD44 is the most widely used marker to distinguish prior antigen-exposure cells ($CD44^{high}$) from naïve cells ($CD44^{low}$), although that this distinction is restricted to CD8 T cells in the B6 mice strain (DeGrendele *et al.* 1997a)

CD62-L and CCR7

CD62-L and CCR7 are homing receptors expressed by most naïve T cells as well as by a subset of previously activated T cells.

CD62-L is also known as L-selectin and it is a member of the selectin adhesion receptor family required for lymphocyte homing to peripheral lymph nodes. Ligands for CD62L are collectively referred to as peripheral node addressins (PNAd), and are highly expressed in the high endothelial venules (HEV) (Hemmerich *et al.* 2001). Upon cell activation, CD62-L is rapidly shed from lymphocytes and neutrophils by L-selectin sheddase and metalloproteinases, which mediate the release of CD62L ectodomains from the cell surface (Preece *et al.* 1996).

CCR7 is a chemokine receptor with significant role in the homeostatic trafficking of naïve T cells to secondary lymphoid organs (SLO). Binding of its ligands, CCL19 and CCL21 on the luminal surfaces of HEVs, mediates the entry of T cells into resting lymph nodes (von Andrian and Mackay 2000; Guarda *et al.* 2007).

Naïve CD8 T cells move in a linear fashion from the blood to the lymph nodes, and then into lymphatics and back into the systemic circulation via the thoracic duct in order to survey the entire body for DCs presenting cognate antigens, which will result in their activation. Owing to the shear stress within vessels and the high avidity/low affinity nature of CD62-L – PNAd interactions, naïve CD8 T cells roll across the HEV endothelium. During this rolling process, CCR7 on T cells interact with CCL19 and CCL21 on the surface of the HEV and this induces conformational changes in LFA-1 (an integrin that function as adhesion molecule). The enhanced interaction between LFA-1 and ICAM-1 expressed on HEV endothelium firmly attaches the T cells to the endothelium enabling it to transmigrate the HEV and enter the lymph node to screen for DCs presenting cognate antigens.

After activation, CD8 T cells lose expression of both CD62-L and CCR7, which prevent these cells from gaining access to lymph nodes through the HEV. Instead, activated CD8 T cells gain expression of a new cohort of trafficking molecules to inflamed tissues.

Following activation, extensive clone expansion, acquisition of effector functions and antigen elimination, the majority of effector CD8 T cells die and only 5-10% of the responding antigen-specific CD8 T cells persist as memory cells. As memory cells are formed, CD62-L and CCR7 are re-expressed and the percentage of cell expressing these receptors continues to increase over time. (reviewed in Nolz *et al.* 2011).

The levels of CD62-L and CCR7 expression, along with other surface markers, have been used to distinguish naïve, effector and memory T cells. In particularly, CD62-L and CCR7 molecules have been used to sub-divide CD8 memory population into **central memory (T_{CM})** or **effector memory (T_{EM})**.

T_{CM} and T_{EM} subdivision was first described in **human** PBMCs according to their potential of migration, proliferative capacity, and effector function. T_{CM} express CCR7, are predominant in secondary lymph nodes, produce IL-2 and proliferate extensively, and require previous re-stimulation to mediate direct effector functions. T_{EM} do not express CCR7, have the capacity to migrate to inflamed tissues/peripheral compartments, are less proliferative and produce effector cytokines such as IFNγ. CD62-L analysis in these subpopulation revealed an enrichment of CD62-L^{high} cells in the $CCR7^{+}$ central memory population (89% to 98% are CD62-L^{high}), and enrichment of CD62-L^{low} in the $CCR7^{-}$ effector memory population (16% to 20% are CD62-L^{high}). (Sallusto *et al.* 1999).

In mice, "T_{CM}" and "T_{EM}" were firstly named based on their location: lymphoid and non-lymphoid organs, respectively (Masopust *et al.* 2001); while others have named them based on the phenotype (Wherry *et al.* 2003). The attempt to report the clearly defined T_{CM} and T_{EM} populations in humans to the mice system has been extensive but, never fully and truly achieved. Indeed, several groups have analyzed murine "T_{CM}" and "T_{EM}" in different models, as defined by both location and CD62-L expression, and actually arrived to different conclusion regarding the functional properties of these subsets and/or the precursor-relationship between them. (Kaech and Ahmed 2001; Masopust *et al.* 2001; Wherry *et al.* 2003; Kedzierska *et al.* 2007). Additionally, in humans T_{CM} and T_{EM} are considered disparate lineages, with no $T_{EM} \rightarrow T_{CM}$ conversion, and with $T_{CM} \rightarrow T_{EM}$ conversion only during T_{CM} activation. In mice, both conversions are frequently seen in the course of the immune response, with the conversion kinetics of CD62-$L^{low} \rightarrow$ CD62-L^{high} being used to characterize memory subsets. The kinetic of this conversion is highly variable between systems. Experimental factors like antigen load (Wherry *et al.* 2003), frequency of antigen-specific naïve cells (Marzo *et al.* 2005; Badovinac *et al.* 2007; Wirth *et al.* 2009), and duration of the infection (Sarkar *et al.* 2007; Sarkar *et al.* 2008) influence this conversion.

Thus, many **controversial** issues remain in the field of memory CD8 T cells, and conclusions of many studies in the field must be taken with caution because of: i) the different systems and models used; ii) the broad and imprecise definition of a "memory" T cell; iii) the possible existence of heterogeneous memory populations; and iv) the distinct markers/criteria used to define memory subtypes.

IL-7R and KLRG1

Earlier after antigen stimulation, different receptors have been shown to be differentially expressed in distinct subsets of CD8 T cells, such as IL-7R (Kaech *et al.* 2003), KLRG1 (Joshi *et al.* 2007), CD27 (Hikono *et al.* 2007), and IL-2Rα (Kalia *et al.* 2010; Pipkin *et al.* 2010). (reviewed in Belz and Kallies 2010).

IL-7 is an essential cytokine during lymphocyte development and it also modulates peripheral T cell homeostasis and memory cell generation. The IL-7 receptor consists of a common cytokine receptor γ-chain (γ_c) and a unique IL-7R α-chain, and **IL-7R** signaling results in the maintenance of cell survival by promoting a favorable balance of Bcl-2 family members (Schluns *et al.* 2000).

Both naïve and memory T cells express high levels of IL7-R, and IL-7 is required for their survival. Following antigen stimulation, most of T cells downregulate IL-7R and only a subpopulation of these cells will regain IL-7R expression during the course of the response/transition to memory. The proportion of cells that downregulate IL-7R and the rate of IL-$7R^{- \rightarrow +}$ conversion also varies according

the system studied (Schluns *et al.* 2000; Bachmann *et al.* 2005; Huster *et al.* 2006; Badovinac *et al.* 2007; Badovinac and Harty 2007; Sarkar *et al.* 2008; Pearce *et al.* 2009).

IL-7R^{high} cells phenotypically resemble memory cells but they also express effector molecules such Gzmb and IFNγ, similar to their IL-7R^{low} counterparts. However, IL-7R^{high} cells have a high potential to become long-lived memory T cells, indicating that the **precursors of high-quality memory** cell are among the IL-7R^{+} population. This was shown when collected IL-7R^{+} or IL-7R^{-} cells (from primary responses) were injected into naïve hosts, and the former revealed increased survival, and enhanced proliferative and protective capacities upon re-infection. IL-7R^{low} cells appear to represent **end-stage effector CD8 T cells**, which cannot progress into the memory compartment (Kaech *et al.* 2003; Huster *et al.* 2004). Thus, IL-7Rα expression has been used as a marker that can distinguish effector cells that are short lived from those that will develop into functional memory cells. However, it is not clear if IL-7R re-expression is directly implicated in the process of memory generation: ectopic expression of IL-7R was not sufficient to rescue the survival of short-lived effector T cells (Hand *et al.* 2007). In addition, non infectious contexts of T cell activation, like peptide immunization, can generate uniform IL-7R CD8 T cells, but many of these cells do not become long-lived memory cells (Lacombe *et al.* 2005; Castellino and Germain 2007).

The killer cell lectin-like receptor G-1 (**KLRG-1**) is a C-type lectin-like receptor that contains an ITIM (immunoreceptor tyrosine-based inhibition motif). KLRG-1 recognizes E-, N- and R-cadherins, which are transmembrane glycoproteins that mediate Ca^{2+} dependent cell-cell adhesion. E-cadherin is expressed on epithelial cells, Langerhans cells and on peripheral blood cells, notably on myeloid DCs.

<u>Following infection</u>, KLRG-1 is strongly upregulated on murine effector CD8 and effector memory CD8 T cells (Robbins *et al.* 2003; Thimme *et al.* 2005). Expression of KLRG-1 by T cells is associated with attenuation of effector responses and with prevention of auto-reactivity during immune responses (Colonna 2006).

The extent of KLRG-1 expression is linked to the amount of inflammatory cytokines such as IL-12 and type I IFNs, and to T-bet expression, in a dose-dependent manner (Joshi *et al.* 2007; Keppler *et al.* 2009). Differences coming from this features may likely be responsible for differences in the frequency of KLRG-1^{+} CD8 T cells detected in different infection: LCMV, influenza virus and LM (Kallies *et al.* 2009; Keppler *et al.* 2009; Rutishauser *et al.* 2009). KLRG-1^{low} cells show increased survival and proliferative potential (Joshi *et al.* 2007; Sarkar *et al.* 2008). In addition, CD8 T cells expressing low or intermediate levels of KLRG-1 also express low levels of IL-2Rα and display a higher capacity to give rise to CD62-L^{high} IL-7R^{high} memory cells. Accordingly, IL-2Rα^{high} T cells express high levels of KLRG-1 and Annexin V, suggesting that these cells form terminally differentiated effector cells with increased apoptosis (Kalia *et al.* 2010). The KLRG-1's ITIM motif mediates its effects through the recruitment of SHIP-1 and SHP-2 phosphatases that degrade PIP3 to PIP2, and thus contribute to a defect in Akt phosphorylation and proliferative dysfunction of high differentiated CD8 T cells (Henson and Akbar 2009; Henson *et al.* 2009).

Heterogeneity in KLRG-1 expression has been suggested to identify effector CD8 T cells with distinct memory lineages fates. When associated with low expression of IL-7R, KLRG-1 has been used as marker of short-lived effector cells (**SLECs**). By contrast, KLRG-1^{low} and IL-$7R^{high}$ are considered as memory precursor effector cells (**MPECs**) (Joshi *et al.* 2007; Sarkar *et al.* 2008).

8. Lymphocyte traffic

An efficient CD8 T cell response to infection conceptually relies on two primary goals: i) generation of large number of CTLs (cytotoxic effector T lymphocytes) to immediately eliminate the present infection, and ii) retaining a cell subset with enhanced longevity to protect against future encounters with the same pathogen. To achieve these goals, naïve CD8 T cells must **encounter their cognate antigen**. However, several factors limit that encounter:

i) Pathogens generally enter the body at peripheral sites such as the skin, gastrointestinal tract or lung ephitelium areas, where naïve T cells are largely excluded;

ii) Naïve CD8 T cells specific for a given cognate antigen are rare and continuously circulate all over the body into blood and lymph fluids;

iii) Immediately after infection, APCs presenting cognate antigens are also rare. As an example, it was shown that immediately after aerosol infection with L50 of influenza virus, only four infectious particles were transmitted to the host (MacInnes *et al.* 2011).

To maximize the encounter with foreign invaders, lymphocytes continuously survey **secondary lymph organs** (SLO) such as spleen, lymph nodes (LNs) and Peyer'patches (PPs) for the presence of foreign antigens. The spleen is specialized to present blood-borne antigens; whereas LNs filter lymph draining from skin or mucosal surfaces; and PPs obtain antigen by transepithelial transport from the intestinal lumen.

To continue antigen **surveillance**, lymphocytes must **enter**, **transit** and **egress** SLO. The entry of lymphocytes into LNs occurs through high endothelial venules (HEVs), whereas in the spleen, as there are no HEV, lymphocytes directly pass from the blood to the red pulp, and subsequently migrate to the T cell zones (white pulp). The egress from spleen occurs into blood, whereas egress from LN occurs into lymph that carries cells back to the blood via the thoracic duct.

8.1) LN entry and transit

The entry of lymphocytes into LNs **trough HEVs** is initiated by the lymphocyte homing receptor CD62L, a L-selectin that mediates tethering and rolling of lymphocytes along the HEV endothelial cells, which express mucin-like glycoproteins (CD62L ligands). Naïve B and T cells rolling HEV walls then enter lymph nodes trough a multistep adhesion and migration cascade that depends on chemokine-induced activation of integrins on lymphocytes' cell surface.

Naïve T cells express CCR7 and CXCR4 chemokines receptors, whereas naïve B cells express CXCR5 in addition to the former two. The CCL21 chemokine (ligand of CCR7) is abundantly expressed by murine HEV endothelial cells, whereas CXCL12 (ligand of CXCR4) and CXCL13 (ligand of CXCR5) are produced by lymph node stromal cells and transcytosed to the luminal surface of HEV endothelial cells.

Signaling through CCR7 together with the shear force of blood flow induces conformal changes in the LFA-1 (lymphocyte function-associated antigen1) integrin. LFA-1 then mediates firm biding to ICAM1 and ICAM2 adhesion molecules expressed on HEV endothelial cells, contributing for successful extravasations. (reviewed in Girard *et al.* 2012).

After leaving HEVs, T cells rapidly **migrate along the fibroblastic reticular cells (FRC)** network and CCL21 and CCL19 chemokines regulate intranodal motility and migration velocity of T cells. CCL21 is abundantly expressed by T cells zone FRCs, and CCR7-deficient T cells exhibit a reduced motility in LNs. As CCR7-deficient T cells exit lymph nodes more rapidly than wt T cells, CCR7 and FCR-derived CCL21 and CCL19 are also crucial for retention and accumulation of T cells in LN's paracortical T cell areas. Thus CCR7 is a master regulator of T cell trafficking in LN: it promotes T cell entry, motility, compartmentalization and retention.

Although the main function of HEV is to recruit large numbers of naïve B and T cells into LNs, other cell types can use this path. In addition to naïve T cells, Treg, T_{CM} cells, pDCs and pre-cDCs also enter LNs through HEVs in steady state conditions (entry also mediated by the expression of CD62L and CCR7). (reviewed in Girard *et al.* 2012)

Effector memory T cells and DCs can also enter lymph nodes **through afferent lymphatic vessels**. The entry of DCs into terminal lymphatics, in both steady-state and inflammatory conditions, is dependent on CCR7 expression at their surface as well as the CCL21 secretion on terminal lymphatic vessels. (reviewed in Girard *et al.* 2012).

8.2) LN egress

After exploring a given lymph node for several hours (approximately 8-12 hours for a naïve T cells, or 24 hours for a naïve B cell), naïve lymphocytes that do not encounter their target antigen leave the lymph node through **efferent lymphatic vessels**.

In the past few years the S1P gradients (in blood and lymph), and the S1P-receptor 1 (S1P1) expressed on lymphocytes' surface have been intensely explored as a mechanism required for: lymphocyte homing to lymphoid organs, and lymphocyte egress into blood and lymph (reviewed in Cyster 2005; Schwab and Cyster 2007; Rivera *et al.* 2008; Cyster and Schwab 2012).

a) S1P and S1P-receptors

S1P (sphingosine-1-phospate) is a **sphingolipid metabolite** and is derived from sphingosin, the backbone of most sphingolipids. Sphingolipids are essential plasma-membrane lipids and are concentrated in lipid rafts or cholesterol-enriched membrane microdomains. Following stimulation of

various plasma-membrane receptors (tyrosin-kinase, G-protein-coupled, cytokine and ITAM-bearing receptors) sphingolipids can be rapidly metabolized through the activation of enzymatic cascades, which convert spingolipids such as sphingomyelin to ceramide, and subsequently to sphingosine. Two sphingosine kinases then phosphorylate sphingosine to generate S1P: SPHK1 and SPHK2. (Spiegel and Milstien 2003; Chalfant and Spiegel 2005; Rivera *et al.* 2008).

S1P is **synthesized** by SPHKs in most eukaryotic cells, and it can be **exported,** irreversibly **degraded** by intracellular S1P lyase, or **dephosphorylated** by S1P phosphatase. So, in most tissues, including lymphoid tissues, S1P levels are extremely low. Notable exceptions are the blood and lymph.

In blood, erythrocytes are the major source of plasma S1P: they generate S1P but lack S1P-degrading enzymes (Ito *et al.* 2007; Pappu *et al.* 2007). In addition, platelets, which lack S1P lyase, contain large amount of S1P in microvisicles that can be released in circulation upon activation by trombin (Yatomi *et al.* 2001). It is also reported that mast cells secrete S1P when activated by IgE-bounded antigen (Olivera *et al.* 2007). However, neither platelets nor mast cell seem to have a role in regulating the homeostatic levels of S1P in the blood. In addition, *in vitro* studies have shown that vascular endothelium can act as a source of plasma S1P through the actions of SPHKs in endothelial cells (Venkataraman *et al.* 2008).

In lymph, S1P is not supplied by plasma, although lymph is essential transduced plasma, and the carriers of S1P (albumin and high-density lipoproteins) transit from plasma to lymph. Initially it had been shown that lymph S1P comes from a radio-resistant source, probably the endothelium (Pappu *et al.* 2007). Recently it has been demonstrated that lymphatic endothelial cells are an *in vivo* source of S1P. This study reported that mice with Lymphatic vessel endothelial hyaluronan receptor 1 (Lyve-1) CRE-mediated ablation of Sphk1, and also lacking Sphk2, have a loss of S1P in lymph while maintaining normal levels plasma S1P (Pham *et al.* 2010).

S1P has dual role. As intracellular signaling molecule regulates diverse biological functions, such as cell proliferation, survival and secretion. As extracellular signaling molecule is implicated in cell migration, angiogenesis, inflammatory responses by innate immune cells, and lymphocyte migration (reviewed in Spiegel and Milstien 2003).

Secreted S1P signals through five members of the **S1P-receptor family**: S1P1-S1P5: These mediate diverse cellular functions through differential coupling of the receptor to heterotrimeric G-proteins (α_i, α_q or $\alpha_{12/13}$), and through the heterogeneity in their constitutive and inducible expression (Sanchez and Hla 2004). S1P1 was originally identified in endothelial cells, and is expressed by most immune cells. T cells express S1P1 and S1P4, whereas mast cells and macrophages express S1P1 and S1P2. S1P5 is expressed by DCs and NK cells. (reviewed in Rivera *et al.* 2008).

b) <u>S1P and S1P-receptors in lymphocyte trafficking</u>

In the early 1990s, the **involvement of a $G_{\alpha i}$-coupled receptor in lymphocyte egress** was suggested after a study showing that expression of the pertussis toxin catalytic subunit ($G_{\alpha i}$ inactivation) inhibited the export of mature T cells from the thymus (Chaffin and Perlmutter 1991).

The next critical insight indicating involvement of a G protein-coupled receptor emerged from natural products screening for immunosuppressive drugs. That search identified the fungal metabolite myriocin, and chemical modification of myriocin generated the FTY720 compound, which prevented skin allograft rejection. The **FTY720 (Fingolimod) drug removed lymphocytes from blood and lymph**, preventing them from reaching the skin graft (Brinkmann and Lynch 2002). FTY720-treated mice then showed that LN medullary sinus were emptied of lymphocytes, which suggested that cells could not access egress structures (Mandala *et al.* 2002). The breakthrough in the S1P and S1P-receptors mechanism involved in lymphocyte trafficking came with the realization that FTY720 was a sphingosine analogue that could be phosphorylated by sphingosine kinase to produce a S1P-receptor ligand with potent effects (such as S1P-receptor agonism (for S1P1, S1P3, S1P4 and S1P5), and downregulation of S1P-receptor expression) (Brinkmann *et al.* 2002; Matloubian *et al.* 2004).

The steps involved in lymphocyte egress have been most completely defined in LNs. This reflects the fact that LN entry and exit sites are distinct, allowing the two processes to be measured separately, and that LNs are the most accessible lymphoid organs for intravital microscopy.

c) <u>S1P gradients and S1P1 in LN T cell egress</u>

S1P gradients are important for LN egress: i) sphingosine kinases inactivation in lymphatic endothelial cells markedly reduce S1P levels in lymph and impair lymphocyte egress from LNs (Pham *et al.* 2010); ii) the inhibition of S1P lyase (by treatment mice with THI) increases total levels of S1P in LNs, and results in LN-lymphocyte sequestration (Schwab *et al.* 2005).

T cell egress from LN is also dependent on the receptor S1P1: i) upon adoptive transfer into a WT host, *S1pr1*$^{-/-}$ T cells enter LNs but cannot egress into lymph, and *S1pr1*$^{+/-}$ T cells exit more slowly than *S1pr1*$^{+/+}$ T cells (Schwab and Cyster 2007; Pham *et al.* 2008); ii) one third of wt but not S1P1-deficient T cells were able to crossed the lymphatic endothelium and entered the sinus at multiple locations (Grigorova *et al.* 2009).

It was also shown that S1P1 signaling appears to act principally by **overcoming retention mediated by Gαi-coupled receptors** (like CCR7 and CXCR5). In the absence of S1P-S1P1 signaling, T cell treatment with pertussis toxin (which inactivates Gαi) restores their egress through cortical sinuses (Pham *et al.* 2008; Pham *et al.* 2010).

Direct analysis of S1P1 receptor expression on recirculating lymphocytes revealed that S1P1 is downregulated in the blood, upregulated in the lymph node parenchyma (which contain low levels of extracellular S1P) and downregulated again in the lymph (Matloubian *et al.* 2004; Boscacci *et al.* 2010). Associated with this, high levels of S1P are found in the blood and lymph, which have been shown to induce the rapid downregulation/internalization of S1P1 receptor (Lo *et al.* 2005). It is thus proposed that a cyclic **ligand-induced modulation of S1P1 expression** is required for lymphocyte recirculation:

i) High levels of S1P in blood downregulate S1P1 expression in naïve lymphocytes allowing them to entry LNs through HEVs.

ii) Near the cortical sinus S1P1 signaling overrides CCR7-mediated retention in the T cell areas allowing T cells to cross the lymphatic endothelium and enter the lymph node sinus system (reviewed in Cyster and Schwab 2012; Girard *et al.* 2012). (Fig. 8)

Recently, G protein-coupled receptor kinase2 (GRK2) has been shown to contribute to the downregulation of S1P1 on blood lymphocytes. GRK2-deficient T cells in the blood have high levels of S1P1 at cell surface and had a defect in homing to LNs, whereas no defect in LN entry of GRK2-deficient or WT lymphocytes was observed in mice lacking blood S1P. This indicated that GRK2-mediated downregulation of S1P1 on blood lymphocytes is required to overcome their attraction to S1P in the blood (Arnon *et al.* 2011).

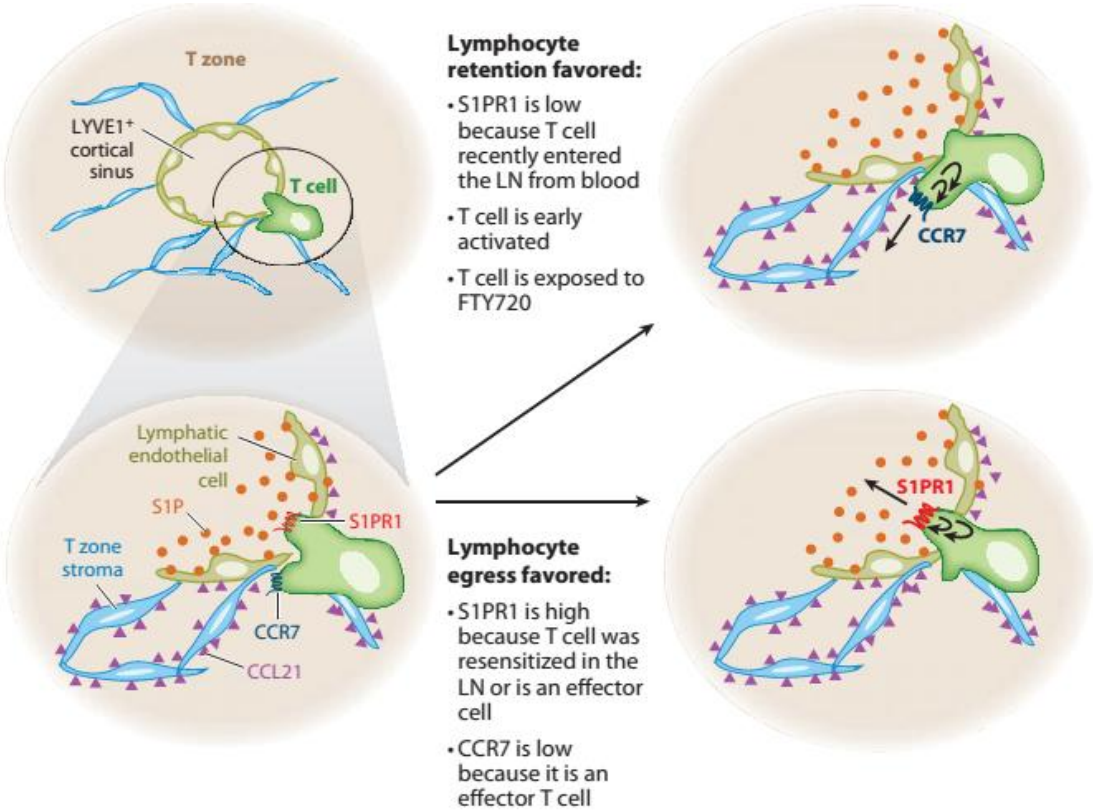


Figure 8. Model of events occurring during lymph node egress decision making. The T cell (green) express CCR7 and S1P1, and the relative signaling strength of these two Gi-coupled receptors in the different regions of the cell may dictate the cell's egress into cortical sinus. (Cyster and Schwab 2012).

8.3) <u>During inflammation</u>

During inflammation, many changes occur in lymph nodes.

Early studies on the traffic within afferent and efferent lymphatic vessels, both in sheep and mouse, have already reported that local recruitment of antigen-specific cells was always preceded by

profound modification of cell migration affecting all lymphosytes. It was shown that shortly after antigen administration, the influx of cells in the draining LN increases affecting both antigen-specific and non-specific T cells, whereas egress was totally blocked for 1-3 days. This phenomenon was named as “non-specific trapping” or “lymph node shut-down phase”, and it was considered crucial to allowing every lymphocyte a chance to encounter their cognate antigen. (Hall and Morris 1965; Zatz and Lance 1971; Cahill *et al.* 1976).

It was also demonstrated that following this “lymph-node shut down phase” of lymphocyte trapping, there was a sudden reestablishment of lymphocyte egress, where antigen-specific lymphocytes that interacted with antigen-presenting cells were retained in the draining LN, while cells with other specificities left. (Sprent *et al.* 1971; Sprent and Miller 1976).

Recently, soon after the initial inflammatory challenge, it was shown that LNs undergo substantial remodeling, which includes increases in size, in cellularity, and in the expression of primary feed arterioles and HEV networks (von Andrian and Mempel 2003; Drayton *et al.* 2006; Bajenoff *et al.* 2007).

The flow of afferent lymphatic vessels also increases, and large numbers of mature CCR7-expressing DCs are transported to the inflamed lymph node (von Andrian and Mempel 2003; Lammermann *et al.* 2008).

Pro-inflammatory cytokines up regulate cell adhesion molecules expression on HEVs (such as P-selectin and E-selectin), and CCL2 and CXCL9 are also accumulated in the HEV lumen (von Andrian and Mempel 2003; Miyasaka and Tanaka 2004). As consequence, naïve B and T cells, activated T cells, NK cells and monocytes migrate through HEVs into inflamed LNs, strongly increasing the input of immune cells into the draining LN of an inflamed tissue.

Some evidences support different mechanisms that may condition lymphocyte egress block from the inflamed LN during the shut-down phase of lymphocyte trapping (reviewed in Cyster and Schwab 2012):

i) <u>CD69 mediated (post-translational S1P1 regulation</u>). CD69 associates with S1P1, it inhibits S1P1 signaling function and promotes protein complex internalization and degradation, limiting egress during immune responses (Shiow *et al.* 2006). CD69 is induced by TCR activation, by exposure to type I IFNs and a number of other inflammatory mediators (Grigorova *et al.* 2010).

ii) <u>Transcriptional S1P1 regulation</u>. Within a day of activation in the LN, cognate T cells markedly downregulate *S1P1* transcript levels (Matloubian *et al.* 2004). In T cells, KLF2 binds and activates S1P1 promoter and promotes CD62L, CCR7 and β7-integrin expression (Carlson *et al.* 2006; Bai *et al.* 2007). In activated T cells, KLF2 transcripts are much reduced (Carlson *et al.* 2006).

iii) <u>Ligand distribution (S1P concentration)</u>. Many inflammatory stimuli increase the expression of Sphk1 in DCs and other myeloid cells (Jung *et al.* 2007; Hammad *et al.* 2008; Puneet *et al.* 2010), and allergic stimuli can provoke S1P release from mast cells (Spiegel and Milstien 2011). However, whether these contributions result in significant increases in interstitial S1P concentration or in a change in lymphocyte retention remains to be tested *in vivo*.

Thereby, both the increase in cell entry and the blockade of cell egress contribute to the rapid accumulation of recirculating cells at the inflamed LN to ensure that a large and rare repertoire of antigen-specific lymphocytes will have enough time to move among resident cells until meeting the rare APCs first presenting antigen. It is also suggested that as many as 5000 naïve T cells can visit a single DC per hour (Bousso and Robey 2003; Miller *et al.* 2004).

Cells that recognize the cognate antigen then need to be retained for longer periods to undergo clonal expansion and receive instructive signals before exiting as effector cells and travelling to the site of infection to mediate their protective functions

9. Effector functions: cytolysis, chemokine and chemokine production

CD8 T cells produce a range of effector molecules that mediate the defense against pathogens. Direct **cytolysis** of target cells (mediated by perforin and granzyme releases), and Fas signaling top that list. CD8 T cells also secrete **cytokines** such as tumor necrosis factor (TNFα) and interferon-γ (IFN-γ), which play important roles in the antimicrobial defense. In addition, pathogen-specific CD8 T cells also express **chemokines** that attract inflammatory cells to sites of infection. (reviewed in Harty *et al.* 2000; Wong and Pamer 2003).

a) Cytotoxicity: (FasL, Prf1 and Gzmb)

The classical effector functions associated to CD8 T cells are the production of IFNγ and the cytotoxic activity (CTL). This cytolysis capacity of activated CD8 T cells occur by two distinct molecular pathways: i) receptor-mediated induction of apoptosis or ii) delivery of cytotoxic granules into target cells. (reviewed in Russell and Ley 2002) (Kagi *et al.* 1994c).

i) Fas ligand (FasL) is a type-II transmembrane protein and it signals through Fas receptor (FasR), TRAILR or TNFRI expressed on target cells. The receptor-mediated pathway is initiated by the adaptor molecule FADD (Fas- associated via death domain) recruitment. Caspase-8 is then activated and the downstream pathway involves caspase-3 and CAD (caspase-dependent DNase) leading to DNA fragmentation and apoptosis.

ii) The delivery of cytotoxic granules relies on the use of **perforin (Prf1)** to potentiate the traffic of **granzymes (Gzm**) into the target cells, where they consequently initiate cell death through several mechanisms, such as activation of caspase-independent mitochondrial and nuclear cell-death pathways, disruption of the plasma membrane, and damaging of DNA.

After TCR-mediated signaling, cytotoxic granules containing the pore-forming protein, perforin, and the serine proteases granzymes are delivered to the target cell at the synapse region. The change in [Ca^{2+}] and pH activates perforin to form hydrophilic pores on target cell's membrane and it favors activation of granzymes A and B. Granzyme B (Gzmb) can induce both caspase-independent and caspase-dependent death, and acts quickly by cleaving molecules like procaspase-3. Granzyme A (Gzma) induces cell death with a slower kinetics, and in a caspase-independent mechanism that induces DNA fragmentation and mitochondrial depolarization.

$Prf1^{-/-}$ mice are dramatically sensitive to infection with ectromelia virus (Mullbacher *et al.* 1999), fail to clear LCMV infection long after WT mice do it (Kagi *et al.* 1994b; Walsh *et al.* 1994), and are unable to control *T. gondii* infection (Denkers *et al.* 1997). Perforin-deficient CD8 T cells are also less efficient than WT cells to clearing LM infection, although they may clear this pathogen through a mechanism independent from FasL, but requiring TNFα (Kagi *et al.* 1994a; White and Harty 1998).

Both cytolytic pathways and IFNγ secretion are crucial to the main effector functions of a CD8 T cell -kill a target cell-, and these effector functions are induced after extensive division.

b) Cytokines: (IFNγ, TNFα)

IFNγ

Besides modulating innate immune responses and CD4 T cell differentiation, IFNγ triggers direct antiviral mechanisms in many cells types (Boehm *et al.* 1997), and it is also considered to be a signature cytokine expressed by antigen-experienced CD8 T cells upon antigen re-stimulation (Murali-Krishna *et al.* 1998). Analysis of immune responses in IFNγ- or IFNγR-deficient mice, or under the influence of IFNγ-neutralizing antibodies have also shown the importance of IFNγ in the resistance to different pathogens (*Plasmodium,* Toxoplasmosis*, Clamydia, Listeria* (LM), LCMV and HBV).

CD8 T cell-mediated protection to several infections has also been shown to be dependent of IFNγ. Transfer of activated CD8 T cell clones, or CD4-depleted splenocytes, from immunized mice were able to increase resistance to *Plasmodium* infection (Weiss *et al.* 1992), Toxoplasmosis (Suzuki and Remington 1990) and Clamydia (Starnbach *et al.* 1994), and that resistance was abrogated when injecting IFNγ-neutralizing antibodies.

During LM infection, IFNγ is produced in the first few days following infection by NK and dendritic cells, and later, as adaptive immunity develops, IFNγ is produced by CD4 Th1 and CD8 T cells (Conlan *et al.* 1993; Busch *et al.* 1999; Frucht *et al.* 2001).

As many cell types can produce IFNγ after infection, sometimes it is not clear if IFNγ production by CD8 T cells is required and essential to protect from infection. During LM infection, it was

demonstrated that WT CD8 T cells were more effective in clearing this intracellular bacteria than IFNγ$^{-/-}$ CD8 T cells, when compared on a per cell basis (Messingham *et al.* 2007). IFNγ also has an essential role in activating listericidal activity in macrophages at the early phases of the immune response (Portnoy *et al.* 1989).

Production of IFNγ is also important for the control of LCMV infection, and the requirement of this cytokine in the clearance of acute infection is strongly influenced by the tropism and invasiveness of the infectious virus strain (Moskophidis *et al.* 1994; Nansen *et al.* 1999).

It is also reported that IFNγ upregulates: gene expression of several proteasomal subunits and proteasomal regulators implicated in antigen degradation; the TAP transporter proteins (transporter associated with antigen processing); and MHC class I molecules, rendering intracellular pathogens more susceptible to CD8 adaptive immune responses (Fruh and Yang 1999). In addition, IFNγ may influence the strength and/or duration of TCR signaling as it is involved in the upregulation of the CD8 molecule (Apte *et al.* 2008).

TNFα

TNFα is a cytokine produced by many cell types, including CD8 and CD4 T cells, NK cells, macrophages, dendritic cells and epithelial cells, and it also has a variety of effects on cells expressing one or both the TNF receptors, ranging from activation to death.

As already stressed before in the Inflammation section (Chapter II), TNFα is a potent inflammatory cytokine and it functions to recruit and/or activate microbial activities of macrophages and neutrophils.

TNFα binds to two receptors, TNFR 1 and 2, of which TNFR2 is the predominant receptor on CD8 T cells. TNFα exists in two forms, a membrane bound form (mTNF) and a soluble form (sTNF). mTNF triggers TNFR1 and TNFR2, whereas sTNF has preferential effects in TNFR1 over TNFR2. (reviewed in Harty *et al.* 2000; Wong and Pamer 2003).

The role of TNFα in CD8 T cell responses appears to be context dependent. In LCMV and influenza virus infections, absence of TNFα signaling has been shown to enhance CD8 T cells responses against these viruses (Turner *et al.* 2004; Suresh *et al.* 2005; Damjanovic *et al.* 2011). In contrast, in Listeria infection, TNF-and TNFRI-deficient mice are extremely susceptible to primary LM infection and, TNFα binding to TNFR2 is costimulatory for T cells and can prolong T cell responses (Kim *et al.* 2006).

TNFα derived from CD8 T cells may also participate in antilisterial immunity by other mechanisms: i) it might contribute to the maximal activation of anti-microbial capacities of macrophages; ii) besides activation of proinflammatory genes, signaling through TNFR1 can also activate caspase cascasdes and induce apoptosis, suggesting a CD8 T cell-TNFα dependent death of

the infected cell (this would release LM to extracellular space in order to be available for macrophages and neutrophils phagocytosis); iii) TNFα is also able to up regulate adhesion molecules expression on endothelial cells, promoting recruitment of accessory cells to the infection site (reviewed in Harty *et al.* 2000; Wong and Pamer 2003).

c) <u>Chemokines</u>: (CCL2, CCL3, CCL4, CCL5, XCL1)

Chemokines are small heparin-binding proteins and were originally studied because of their role in inflammation. Nowadays chemokines and their receptors are known to play a crucial role in directing the movement of mononuclear cells throughout the body, contributing to the initiation of T cell responses. They are needed to: attract monocytes and immature dendritic cells (DCs) to the site of inflammation; to direct maturing antigen-presenting cells (APCs) to lymphatic vessels; to bring T cells and APCs together within the draining lymphoid organ; and they are also responsible for the homing of effector T cells to sites of inflammation.(Luther and Cyster 2001).

Some chemokines are expressed constitutively and are involved in the organization of lymphoid tissue (homeostatic chemokines such as CCL19 and CCL21). Other are typically induced *de novo* in response to infection, and are responsible for effector-cells recruitment to the inflammation/infection site (inflammatory chemokines such as CCL2, CCL3, CCL4, CCL5 and XCL1).

Chemokines have been classified in four main families:

i) The largest family consists of CC chemokines, so named because the first two of the four cysteine residues in these molecules are adjacent to each other. The most thoroughly characterized member of the CC subfamily is the monocyte chemoattractant protein 1 (MCP-1), also known as CCL2. Other CC chemokines include the macrophage inflammatory protein MIP-1α (CCL3) and MIP-1β (CCL4), and CCL5 (RANTES).
ii) The CXC family is characterized by a single amino acid residue between the two canonical cysteines. Among several members, CXCL8 (IL-8) is the prototype, and it attracts PMN cells to acute inflammation sites and induce their granule exocytosis. It also activates monocytes (phogocytosis) and recruits these cells to vascular lesions. CXCL8 acts through the receptors CXCR1 and CXCR2 expressed on neutrophils and monocytes.
iii) The CX_3C family only has one member: CX_3CL1. This chemokine is both secreted and tethered to the surface membrane, serving as both chemoattractant and adhesion molecule. A TNFα-converting enzyme can cleave CX_3CL1 from the cell membrane, freeing the cytokine to function as a soluble chemoattractant.
iv) A fourth family is characterized by a single cysteine residue, and it includes only two members: lymphotactin-α (XCL1) and lymphotactin- β (XCL2). (reviewed in Moser and Loetscher 2001; and in Charo and Ransohoff 2006).

Chemokine **expression** is controlled by activation of transcription factors that depend on **MAP Kinase signaling pathways**. For example: HIV-1 up-regulates production of CCL2 via AP-1 (Lim and Garzino-Demo 2000); a virus infection of human bronchial ephitelial cells leads to expression of CCL5 through a mechanism dependent on p38 and JNK (Hashimoto *et al.* 2000); CXCL8 is expressed during viral infection through an AP-1-dependent mechanism and in cooperation with NF-kB (Mori *et al.* 1998); in DCs activated with proteins derived from bacterial cultures, CCL2, CCL5, and CXCL8 production is dependent on NF-kB and MAPK-p38 and -JNK pathways (Bernatoniene *et al.* 2008); gp120 stimulated macrophages secrete CCL2 and CCL4 in a manner dependent of p38 and JNK (Del Corno *et al.* 2001); in monocytes activated by GM-CSF, CCL3 production is dependent on MAPK-ERK1/2 (Wang *et al.* 2005).

Chemokines exert their effects through the **binding to G-protein-coupled receptors** on cell surfaces, leading to **cell activation and migration**. Once activated, these receptors trigger IP3 formation, intracellular calcium release and PKC activation that ultimately regulate directional motion of the cell. Chemokine-receptor binding also activates Rho family proteins, which are guanosine triphosphate-binding proteins with the capacity to influence cell motility through regulation of actin-dependent processes such as pseudopod formation and membrane ruffling. (O'Hayre *et al.* 2008).

CCL2 and CCL5

CCL2 is secreted by fibroblast, endothelial cells, vascular smooth muscle cells, monocytes, T cell, and other cell types that mediated the influx of cells to sites of inflammation. Thus, its expression has been observed in a large number of tissues during inflammation dependent disease progression (atherosclerosis, arthritis and cancer), where the influx of macrophages into these tissues has been suggested to exacerbate these diseases. CCL2 expression is regulated at the transcriptional level by stimulatory factors such as TNFα, IFNγ, PDGF and stress factors, and in many of these regulatory responses, the pro-inflammatory NF-kB transcription factor is a key mediator (Kumar and Boss 2000).

CCL2 triggers chemotaxis recruitment of CCR2-expressing cells: CCR2 is mainly expressed by monocytes, but also by dendritic cells, memory T cells, and basophiles (Charo and Ransohoff 2006). CCL2-CCR2 interaction also induces upregulation of β2 integrin in monocytes (promoting adhesion of monocytes to extracellular matrix proteins), as well as degranulation of basophils and eosinophils (Melgarejo *et al.* 2009). In addition, CCL2-CCR2 also have a role in Th cell differentiation (Gu *et al.* 2000) (Traynor *et al.* 2000) and memory CD8 T cell generation and function (Wang *et al.* 2008).

CCL5 binds to CCR1, CCR3 and CCR5, and it mediates the trafficking and homing of T cells, monocytes, basophils, eosinophils, DCs, NK and mast cells. Increased CCL5 expression has been associated with a range of inflammatory disorders and pathologies including allogeneic transplant

rejection, atherosclerosis, arthritis, atopic dermatitis, delayed-type hypersensitivity reactions and asthma. In these pathologies, CCL5 is thought to act by promoting leukocyte infiltration to sites of inflammation (Charo and Ransohoff 2006). CCL5 also plays a key role in the immune response to viral infection. Virus-specific CD8 T cells degranulate CCL5 along with perforin and granzyme A (Levy 2009).

CCL5 is expressed by several cell types such as endothelial cells, epithelial cells, or monocytes within hours of exposure to proinflammatory stimuli like TNFα, IFNγ, viruses and LPS. In contrast, *ccl5* mRNA expression in naïve T lymphocytes is only appearing 3 to 4 days after priming. Unlike naïve, resting memory CD8 T cells produce CCL5 immediately after TCR triggering, which is associated to an increased half-life of its mRNA, as well as to a constitutively transcription of *ccl5* gene (Marcais *et al.* 2006).

CCL3 and CCL4

CCL3 and CCL4 are constitutively expressed at low levels in most mature haematpoietic cells including monocytes/macrophages, T, B and NK cells. After appropriated stimulation by LPS, IL-1, IFN-α or IFNγ, their expression can be induced in other cell types such as vascular smooth muscle cells and microglia (Zeremski *et al.* 2007).

Both chemokines use CCR5 as their common receptor, whereas CCL3 also binds to CCR1. Through these receptors, MIP-1 family members orchestrate acute and chronic inflammatory responses at sites of injury or infection, by recruiting pro-inflammatory cells. MIP-1 chemokines are also involved in transendothelial migration of monocytes, DCs, and NK cells, and in promoting T-cell migration to inflamed tissues (Charo and Ransohoff 2006).

CCL3 recruits IFNγ activated neutrophils, a small subpopulation of CCR1 expressing eosinophils, and it is critical for macrophages chemoattractant in cutaneous wound repair promoting healing, basophile chemotaxis, histamine release, and eosinophilia in a allergic asthma model (Maurer and von Stebut 2004).

CCL3 and CCL4 gradients are also responsible for guiding naïve CD8 T cells to sites of CD4 T cell-dendritic cell interactions in immunogen-draining LNs. This occurs by upregulation of the receptor for these chemokines (CCR5) on naïve CD8 T cells (Castellino *et al.* 2006; Bajenoff *et al.* 2007).

Besides the chemoattractant function of MIP-1 chemokines, triggering of CCR1 and CCR5 by them also induce activation of immune cells, like degranulation of basophils (Kuna *et al.* 1992) and eosinophils (Alam *et al.* 1993), activation of T cells (Bacon *et al.* 1995), production of TNFα and radical oxygen intermediates by mononuclear phagocytes and neutrophils (Narni-Mancinelli *et al.* 2007). In a model of murine listeriosis, MIP-1s, CCL5 and XCL1 chemokines cooperate with IFNγ to the up-regulation of CD40, IL-12 and TNFα , which are central for macrophages effector function (Dorner *et al.* 2002). CD8 T cell-mediated protection against Listeria is also dependent on CCL3 production (Narni-Mancinelli *et al.* 2007).

XCL1

XCL1 is expressed by activated CD8 T cells (Dorner *et al.* 1997; Hedrick *et al.* 1997), CD4 T cells, NK cells, NKT cells, γδ T cells and thymic medullary ephitelial cells (reviewed in Lei and Takahama 2012). It has been reported as being induced by stimulation through TCRs in Th1 cells, but not in Th2 cells, and also co-secreted to a high degree with IFNγ by activated Th1 cells (Dorner *et al.* 2002). XCL1 is yet secreted by islet-specific Th1 cells in autoimmune diabetes and detected in mononuclear cells of peripheral blood from patients with multiple sclerosis (Jalonen *et al.* 2002).

XCL1 has originally been described as a chemoattractant for thymocytes and T lymphocytes (Kelner *et al.* 1994), ant it elicits its chemotactic functions by binding to its only receptor, XCR1.

Early studies suggested that XCR1 mRNA was present in many cell types. In mice, it was detected in $CD8^+$ cells, $NK1.1^+$ cells, B cells, $CD4^+$ T cells and neutrophils. However, these early detections of XCR1 mRNA seemed to include false-positive signals based on PCR primers designed within the exon 2. Since a XCR1-specific mAb was not available at the time, the detection of XCR1 expression had been done by RT-PCR, assuming the existence of only a single-exon coding for XCR1 (a system prone to false positive results). Later on, it was discovered that the murine XCR1 gene contained two exons, and the use of an "intro-spanning" RT-PCR revealed that XCR1 mRNA is selectively expressed in conventional DCs, and not in resting or activated T cells, B cells, NK cells, or plasmocytoid DCs. A detailed analysis showed that only $CD8^+$ DCs (70-85% of splenic CD8+ Dcs) and a small proportion of $CD8^-$DCs express XCR1 mRNA (Dorner *et al.* 2009) (reviewed in Kroczek and Henn 2012; Lei and Takahama 2012). In addition, a reporter mouse with LacZ expression under the control of XCR1 promoter showed that, in the spleen, XCR1 signal was associated with $CD8^+$DCs in the red pulp and in T cell zones. In lymph nodes, XCR1 expression was identified in paracortical areas and subcapsular sinuses (Dorner *et al.* 2009). XCR1 expression has also been fund to be a universal marker specifically expressed by the $CD8\alpha^+$ -type DCs: ovine CD26+DCs, mouse $CD8\alpha^+$ DCs, and human $BDCA3^+$ DCs (Crozat *et al.* 2010).

Concerning the role of XCL1-XCR1 in cytotoxic immune responses, Ovalbumin (OVA)-specific OT-1 TCR-transgenic CD8 splenic T cells produce XCL1 8-36h after encountering the OVA antigen presented by $CD8^+$DCs, and the ablation of XCL1 ($Xcl1^{-/-}$ OT1 adoptively transferred and activated in a $XCL1^{-/-}$ host) reduces the proliferation, IFNγ secretion, and cytotoxicity of OT-1 cells in vivo (Dorner *et al.* 2009). It was also reported that *Xcl1* mRNA is stored selectively in memory CD8 T cells, allowing them to rapidly produce high levels of these chemokine upon stimulation (Crozat *et al.* 2010).

In XCL1-deficient mouse, CD8 T cell response to LM infection is decreased and there is a higher bacterial load early in the infection, indicating that XCR1 promotes the ability of $CD8^+$ DCs to activate early CD8 T cell mediated defense against this intracellular pathogen (Crozat *et al.* 2010).

Thus XCL1/XCR1 interaction has a crucial role in the cross-talk between CD8 T cells and cross-presenting DCs, which is of major importance in the defense against viral and bacterial pathogens, and also in the recognition of tumor antigens (Huang *et al.* 1994).

10. Transcription factors: the intrinsic controls

Antigen-mediated activation of naïve CD8 T cells, though TCR stimulation, leads to the cooperation of essential transcription factors, as described previously: NFAT, AP-1, NF-kB, Fos and Jun. This results in the activation of multiple pathways, influencing cell cycle, survival, and expression of cytokines and cytotoxic molecules. The final outcome of individual cell activation is complex, and the fate of CD8 T cell differentiation is influenced by different factor such as: strength and duration of the TCR-mediated signaling, nature of the APC and co-stimulatory molecules, cytokine environment and CD4 cell-mediated help.

Other transcription factors have been implicated in the regulation of effector and memory CD8 T cell differentiation. These include: T-bet, Eomes, Bcl-6 and Blimp1. (reviewed in Belz and Kallies 2010).

10.1 T-bet and Eomes

T-bet and Eomes are members of a phylogenetically conserved family of genes that share a common DNA-binding domain - the T-box-, and their expression is induced after T cell activation.

a) <u>In CD4 T cells:</u>

T-bet (encoded by the *Tbx21* gene) was originally described as a T cell transcription factor that <u>regulates naïve CD4 T cell differentiation into T helper 1 (Th1</u>) cell lineage commitment and <u>controls IFNγ expression</u>, a hallmark Th1 cytokine.

T-bet is not expressed in naïve CD4 T cells but is quickly induced in response to TCR, IFNγR-STAT1 and IL12R-STAT4 signaling pathways. TCR and IFNγR signaling induce the first wave of T-bet expression, which is independent of IL-12R signaling caused by the TCR-mediated inhibition of IL-12Rβ2 subunit expression. Cessation of both TCR stimulation and IL-2R-STAT5 signaling induces IL-12Rβ2 subunit expression, which thus enables IL-12R signaling. Via STAT4, IL-12 induces a second wave of T-bet expression required for the Th1 cell phenotype stabilization.

Different mechanisms by which T-bet modifies chromatin state have been described. T-bet-mediated chromatin changes are primarily dependent on the ability of T-bet to recruit enzymes that generate chromatin modifications associated with either gene activation (histone H3 or H4 acetylation, and H3 lysine 4 (H3K4) dimethylation) or gene repression (H3K27 trimethylation) to the T-bet-regulated gene loci. In addition, T-bet directly activates the *Ifng* gene by binding to the *Ifng* promoter and to multiple distal regulatory elements located upstream and downstream of the *Ifng*

gene (most of them serving as T-bet-dependent enhancers). T-bet-mediated transactivation of *Ifng* gene is further enhanced by HLX and RUNX3 transcription factors, which expression is induced by T-bet. Another T-bet key regulatory role is the organization of the *Ifng* locus three-dimensional architecture, by enhancing occupancy of the transcriptional repressor CTCF between the boundaries of the *Ifng* locus. This configuration promotes robust *Ifng* expression in Th1 cells.

In Th1 cells, 50% of the Th1 cell-specific genes are also directly activated by T-bet. This includes: *Ifng, Tnf, Xcl1, Ccl3, Ccl4, Cxcr3 and Ccr5*. T-bet not only regulates activation of Th1 cell-specific genes but also inhibit alternative Th cell differentiation pathways. T-bet interacts with GATA3 sequestrating it away from Th2 cell-specific genes (the *Il4, Il5* and *Il13* locus) and the T-bet-RUNX3 complex binding to the *Il4* silencer prevents the expression of Th2 cell-specific cytokine genes in developing Th1 cells. T-bet expression in Th precursor cells also inhibits commitment of the Th17 cell lineage by blocking RUNX1-mediated induction of the Th17 cell-specific transcription factor RORγt. Thus, differentiation of Tbx21$^{-/-}$ CD4 T cells under Th17-skewing condition produces a higher frequency of Th17 cells with high expression of IL-17A, IL-17F and IL-21. (reviewed in Lazarevic *et al.* 2013).

b) <u>In C8 T cells:</u>

In CD8 T cells, the acquisition of <u>effector functions</u> (i) and <u>the development of memory</u> cells (ii) are both crucially dependent on transcription events that are regulated by T-bet and EOMES.

(i) T-bet expression is rapidly induced by TCR and IL-12R signaling, and is required for the early production of IFNγ and Gzmb by antigen-specific CD8 T cells. EOMES is induced later in a RUNX3-dependent manner, can substitute for T-bet to promote IFNγ and Gzmb expression, and together with STAT5 regulates perforin gene expression in CD8 T cells. (Takemoto *et al.* 2006; Cruz-Guilloty *et al.* 2009; Pipkin *et al.* 2010)

CD8 T cells that lack either T-bet or EOMES show “minor” defects in their effector functions, which suggests that these two transcriptional factors have overlapping and partially redundant roles in establishing the differentiation programs on CD8 T cells. However, CD8 T cells deficient in both T-bet and EOMES lose their <u>IFNγ production and cytotoxicity</u>, in response to LCMV infection. It is also reported that these cells aberrantly produce large amounts of IL-17A causing severe autoimmune pathology with excessive neutrophil infiltration (Intlekofer *et al.* 2005; Intlekofer *et al.* 2008). Thus, the concerned action of T-bet and EOMES results in the development of fully differentiated effector CD8 T cells that migrate to tissues and that secrete IFNγ, TNFα, and/or lyse infected cells by releasing cytotoxic granules that contain granzymes and perforin. (Takemoto *et al.* 2006; Cruz-Guilloty *et al.* 2009; Pipkin *et al.* 2010).

(ii) During CD8 T cell priming, inflammatory signals define a T-bet-EOMES expression gradient, which consequently influence the fate of effector CD8 T cells. For instance, IL-12R signaling provides the instructive signal for the terminal differentiation of short-lived effector cell (SLECs) by enhancing and maintaining STAT4-mediated mTOR kinase activity, which function as a molecular switch to induce T-bet expression and to repress EOMES through the inhibition of FOXO1 transcriptional activity. Thus, T-bet expression is induced initially by TCR signaling, and amplified by IL-12-mediated signaling and mTOR activity in effector CD8 T cells, whereas Eomes expression is repressed by IL-12 and mTOR. Eomes expression seems to be induced subsequently to T-bet, in part, through an inhibitory effect of FOXO1 on T-bet expression (Takemoto *et al.* 2006; Joshi *et al.* 2007; Rao *et al.* 2010; Rao *et al.* 2012).

As CD8 T cells acquire a memory phenotype, EOMES expression increases whereas T-bet levels decrease (Intlekofer *et al.* 2007; Banerjee *et al.* 2010; Joshi *et al.* 2011). Nevertheless, low levels of T-bet are maintained in memory CD8 T cells, as T-bet and EOMES cooperate to sustain proper expression of IL-2Rβ (β-subunit of IL-2R and IL-15R) promoting the longevity and the homeostatic proliferation of memory CD8 T cells (Intlekofer *et al.* 2005).

Mice with both T-bet and Eomes mutations virtually lack memory CD8 T cells and NK cells, a phenotype that results from a direct role of both transcription factors in regulating IL-2Rβ expression (which enables IL-15R signaling). As IL-15 (and IL-7) signaling are required for memory CD8 T cell homeostasis and survival, T-bet and Eomes promote CD8 memory T cell generation and maintenance (Pearce *et al.* 2003; Intlekofer *et al.* 2005).

It is also reported that Eomes-deficient effector CD8 T cells, although efficiently generating KLRG1^{low} IL-7R^{hi} memory precursor cells, they are unable to generate memory cells with normal expression of IL-2Rβ, CD62L, CXCR3 and CXCR4, which are involved in IL-15-mediated signaling and homing to lymph nodes and bone marrow. Thus, memory CD8 T cells lacking Eomes contain fewer T_{CM} cells and have impaired homeostatic turnover and long-term persistence (Banerjee *et al.* 2010).

Accordingly, it is also reported that T-bet represses IL-7Rα transcription driving differentiation of effector and T_{EM} CD8 T cells at the expense of T_{CM} cells (Intlekofer *et al.* 2007).

Therefore, T_{EM} cells express higher levels of T-bet and seem to be more differentiated than T_{CM}. In contrast, the expression of EOMES is higher in T_{CM} than in T_{EM}.

Interestingly, memory CD8 T cells that differentiate in the absence of CD4 T cells help (a condition that is associated with defective memory formation (Bourgeois *et al.* 2002b)) seem to be more T_{EM} cell-like , as they are characterized by an overexpression of T-bet (Intlekofer *et al.* 2007).

In conclusion, in acute infection, the ratio of T-bet to Eomes expression is highest at effector cell stages and lowest at memory cell stages, which influences the phenotype, function and long-term fate of effector CD8 T cells: how this ratio is regulated it is still under intense scrutiny, but the balance of FOXO1 activity and exposure to pro-inflammatory cytokines (such us IL-12, type I IFNs and

IL-2) and other factors (such as IL-4, IL-10, IL-21 and WNT signaling) during infection are involved. (Takemoto *et al.* 2006; Zhou *et al.* 2010; Rao *et al.* 2012; Wiesel *et al.* 2012).

c) During chronic infections

CD8 T cell responses, during chronic infections, are quite different from those observed during acute infections (above reported). When exposed to persistent antigenic stimulation, CD8 T cells become dysfunctional, exhausted and fail to differentiate into effective memory CD8 T cells. The exhaustion of CD8 T cells is accompanied by the upregulation of inhibitory molecules (such as PD1, lymphocyte activation gene 3 protein (LAG3) and B- and T-lymphocyte attenuator (BTLA)), as well as defective proliferation, cytokine production and cytotoxicity. (Blackburn *et al.* 2009; Virgin *et al.* 2009). During chronic infection, it is also reported that T-bet expression decays over time in antiviral CD8 T cells as the degree of dysfunction and PD-1 expression increase. It was also shown that T-bet directly binds to *Pd1* gene repressing its expression. Thus, T-bet deficiency leads to an increase in PD1 expression and a decrease in CD8 T cell function and survival (Kao *et al.* 2011).

10.2 Bcl-6 and Blimp1

Bcl-6 and Blimp1 are another pair of antagonistic transcription factors that function as genetic switches for cell fate decisions in B and T cells (Crotty *et al.* 2010).

Bcl-6 (B-cell lymphoma 6) is a transcription factor that plays an important role in the late stages of CD8 T cell differentiation. Bcl-6-deficient mice are impaired in their ability to maintain CD8 T cell memory, while mice overexpressing it have increased numbers of memory cells, with Bcl-6 being crucial for the formation mature self-renewing of T_{CM} (Ichii *et al.* 2002; Ichii *et al.* 2004). Bcl-6 has also been shown to suppress Gzmb expression, which is consistent with its positive role in memory T cell generation (Yoshida *et al.* 2006). IL-10 and IL-21, signaling through STAT3 (signal transducer and activator of transcription 3), are possible candidates for sustaining or even increase Bcl6 expression in memory CD8 T cells after acute infection (Cui *et al.* 2011).

Blimp1 (B-lymphocyte induced maturation protein) is also a transcriptional factor that plays a crucial role in transcriptional networks guiding effector and memory T cell fates. It is robustly expressed by effector CD8 T cells and, it promotes CD8 T cell terminal differentiation and repress the acquisition of central memory T cell properties (Kallies *et al.* 2009; Rutishauser *et al.* 2009).

In vivo studies demonstrated that Blimp1 is required for the generation of short-lived effector cells (SLECs) in response to LCMV infection. In Blimp1-deficient mice, antigen-specific CD8 T cells fail to downregulate IL-7R and show impaired KLRG1 expression, adopting a memory precursor phenotype rather than an effector phenotype. Accordingly, in Blimp1-deficient mice, antigen-specific CD8 T cells also show a reduced downregulation of IL-2 secretion, impaired Gzmb expression and reduced cytotoxicity. (Kallies *et al.* 2009; Rutishauser *et al.* 2009).

Blimp1 also influences T cell localization in tissues by regulating the expression of several chemokines receptors including CCR7, CCR5 and CCR6. Thus, in the absence of Blimp1, antigen-specific CD8 T cells failed to home to infected tissues, such as the lung, and accumulated in draining lymph nodes. Blimp1-deficient effector CD8 T cells express lower level of T-bet but higher levels of Eomes than WT-cells. (Kallies *et al.* 2009; Rutishauser *et al.* 2009). Thus, it is suggested that Blimp1 and T-bet jointly regulate the terminal differentiation of effector T cells and also cooperate in the suppression of IL-2 production.

11. Differentiation programs in B cells, CD4, and CD8 T cells

The heterogeneity of T cell populations during infections provides a highly complex landscape for **CD8 T cell differentiation studies**. In parallel, a number of transcription factors have emerged with key roles in the differentiation of effector and memory T cells, and some of them also appear to fulfill similar roles in B cells. Thus, **enlightening the transcriptional networks** behind that diversity, in both T and B cells, is of major importance for a **rational vaccine design,** that should provide not only an efficient elimination of the pathogens at the first encounter, but also a lifelong protection for subsequent encounters.

a) B cells

Well established models for plasma cell and memory B cell differentiation have been supported by a series of stage-specific markers and well-defined transcriptional regulators that unambiguously define effector and memory B cell subsets: i) short lived plasmablasts, which secrete large amount of mostly low-affinity immunoglobulins (Igs); ii) long-lived isotype-switched plasma cells, producing high-affinity Igs for lasting protection; and iii) memory B cells, which upon Ag re-encounter rapidly proliferate and differentiate into Ig-secreting cells (reviewed in Nutt *et al.* 2007; Klein and Dalla-Favera 2008).

While many of the factors implicated in late B- and T cell-differentiation are lineage specific, a core group of transcription factors such as Blimp1, Bcl-6, and interferon regulatory factor-4, have

emerged as key players in plasmas B cell and memory B cell differentiation as well as in effector and memory T cell differentiation (Mittrucker *et al.* 1997; Calame 2006; Nutt *et al.* 2007). **Blimp1** has long been implicated in the differentiation of B cells into plasmablasts and plasma cells (effector population of the B cell lineage) and, among B cells, its expression is restricted to this subpopulation of cells (Shapiro-Shelef *et al.* 2003; Kallies *et al.* 2004). **Bcl-6,** on the other hand, is an essential regulator of germinal center B cells, which are the principal precursors of memory B cells (Fukuda *et al.* 1997).

b) CD4 T cells

CD4 T cell differentiation into effector cells, with different characteristics and functions, is also well described, and each subset is distinguished by a specialized gene expression program that is under the control of a lineage-defining transcription factor.

Resting naïve CD4 T cells are designed T helper (Th) cells, and they release very low level of cytokines. Early after stimulation by antigens and APCs, Th cells begin to produce IL-2 and are then designated Th0. As Th cells continue to respond to activating signals, they undergo clonal expansion, and progress to differentiate in well characterized Th1, Th2, Th17, T follicular help (T_{FH}) or peripheral derived regulatory (Treg) cells. The type of Th fade is depending on the nature of the cytokines present at the site of activation, which then induce the expression of lineage-defining transcription regulators (reviewed in Sallusto and Lanzavecchia 2009).

The CD4 T cells lineage-defining transcription factors are: **T-bet** for the Th1 cell lineage, **GATA3** for Th2 cell lineage, **RORγt** for the Th17 cell lineage, **Bcl-6** for T_{FH} cells, and **FOXP3** for Treg cells. Accordingly, CD4 Th subsets are also defined by: the cytokine signature expressed, their distinct homing properties, and their specialized effector function, which make them better prepared to target a particular class of pathogen (Fig. 9).

Differentiation towards Th1 cells is induced by IL-12 and IFNγ produced by macrophages or NK cells, and results in IFNγ production and effective activation of macrophages microbicidal function against intracellular bacteria, protozoa and virus, with Th1 cells supporting cell mediated immune responses.

Differentiation towards Th2 cells is induced by IL-4 produced by $NK1.1^+$ T cells, basophiles, or mast cells, and Th2 cells produce IL-4, IL-5, IL-9, IL-13 and GM-CSF, which are required for the elimination of extracellular parasites. Th2 cells support humoral and allergic responses.

Differentiation towards Th17 cells is induced by TGFβ and IL-6, and Th17 cells protect mucosal surfaces against extracellular bacteria and fungi through the production of IL-17A, IL-17F, IL-21 and IL-22. IL-17A is responsible for inducing a variety of proinflammatory mediators, such as IL-1, IL-6, TNFα, CXCL8 (IL-8, a neutrophil chemoatractant), granulocyte colony-stimulating factor (G-CSF), and

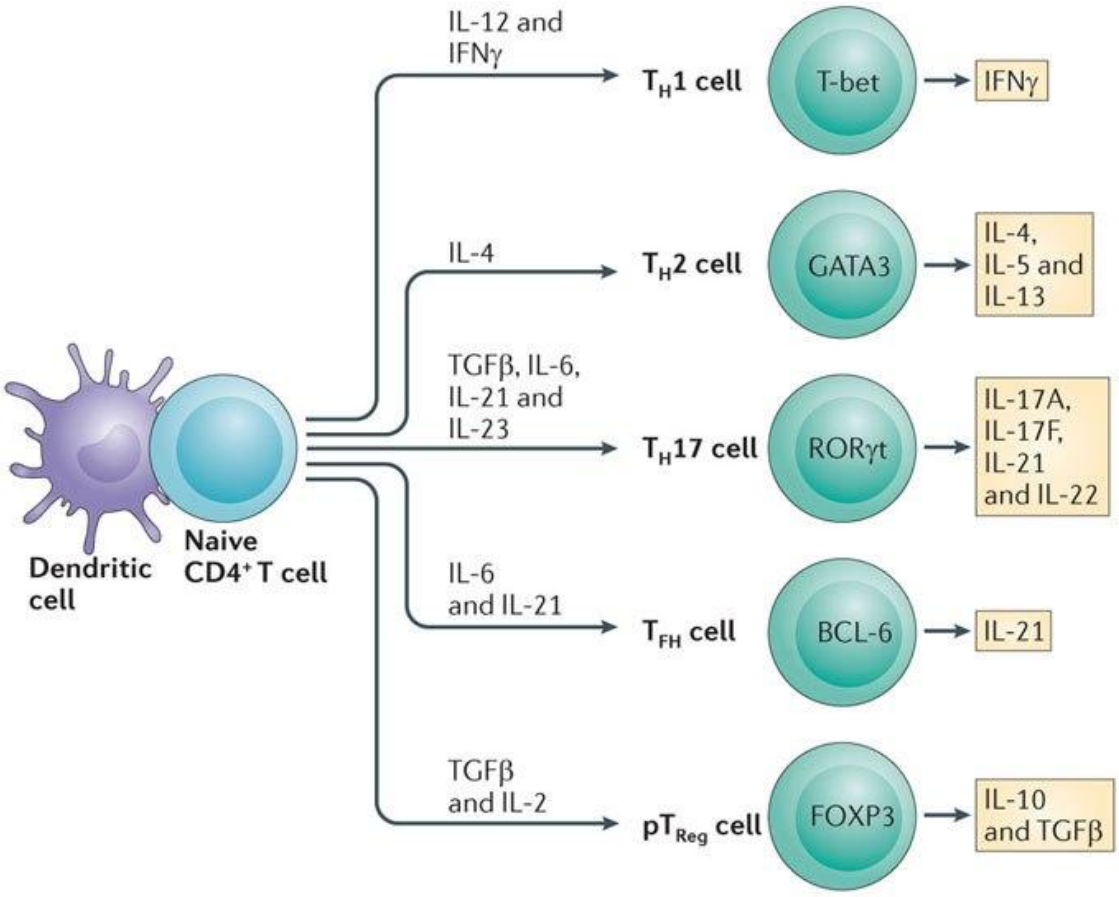


Figure 9. Differentiation of CD4$^+$ T cells into different T helper (Th) subsets: instructed cytokines, lineage-defining transcription factors, and signature expressed cytokines. (Lazarevic *et al.* 2013).

granulocyte-macrophage colony-stimulating factor (GM-CSF) by epithelial , endothelial, and other stromal cells, which ultimately leads to the recruitment and activation of neutrophils (Ouyang *et al.* 2008). Thus, Th17 cells are induced early in the adaptive response to extracellular bacteria and help to recruit neutrophils to eliminate these pathogens.

T_{FH} cells support antiviral humoral immunity by promoting antibody class-switching and affinity maturation in germinal-center B cells.

Another functionally distinct and differentiated subset of CD4 T cells is the subset of regulatory T cells (Treg). It is characterized by surface expression of CD4 and CD25, and by nuclear expression of the Foxp3 transcription factor that is essential for their development. These cells prevent inflammation-mediated tissue injury through the local secretion of suppressive cytokines such as IL-10, IL-35 and TGFβ, or through the cell-cell contact-mediated inhibition of CD4$^+$ effector cell proliferation. (reviewed in Sallusto and Lanzavecchia 2009; Lazarevic *et al.* 2013).

c) CD8 T cells

In contrast to B and CD4 T cells, CD8 differentiation is less well characterized. It should be noted that several events after activation of T cells have been revealed in CD4 T cells and then extrapolated to CD8 T cells; that the vast majority of effector function are evaluated after *in vitro* reactivation with pathogen epitopes and that *in vitro* re-stimulations have also shown to considerably modify *ex vivo* readouts; that different infection models have been used to characterize CD8 T cell differentiation; and yet, that distinct systems/techniques used to evaluate CD8 T cell immune responses are not always the most accurate way to unveil the outcome of CD8 differentiation *in vivo*.

Nevertheless, due to intense studies during the past decade in characterizing effector and memory CD8 T cell subsets, as well as the recent attention to the transcriptional circuits associated with them, a **graded activity of transcriptional programs** have been proposed to control effector and memory T cell differentiation (reviewed in Kaech and Cui 2012).

This **model** postulates that, in response to different levels of signal input, the differentiation of antigen-specific effector CD8 T cells occurs along a continuum: from cells that have greater memory cell potential, longevity and proliferative potential; to terminally differentiated effector T cells (Fig. 10a). These heterogeneous differentiation states of CD8 T cells can also be distinguished by the expression of several surface markers, such as KLRG1, IL-7Rα, CXCR3, CD62L and CCR7 (Fig. 10b).

The transcriptional programs controlling terminal effector cell differentiation and memory cell potential are proposed to be based on a graded expression or activity of certain competing sets of transcription factors with:

i) **T-bet, BLIMP1, ID2 and STAT4** being associated with effector cells terminally differentiated, reduced proliferative capacity and longevity;

ii) **EOMES, BCL-6, ID3 and STAT3** preventing terminal differentiation of effector cells and helping to maintain memory cell properties (Fig. 10c).

In addition, it is argued that this gradient model also provides a flexible way to manage the size and the quality of CD8 T cell populations during an infection. In particularly, by taking in consideration the unpredictable properties of an infection, which vary in intensity, tropism and duration. (reviewed in Kaech and Cui 2012).

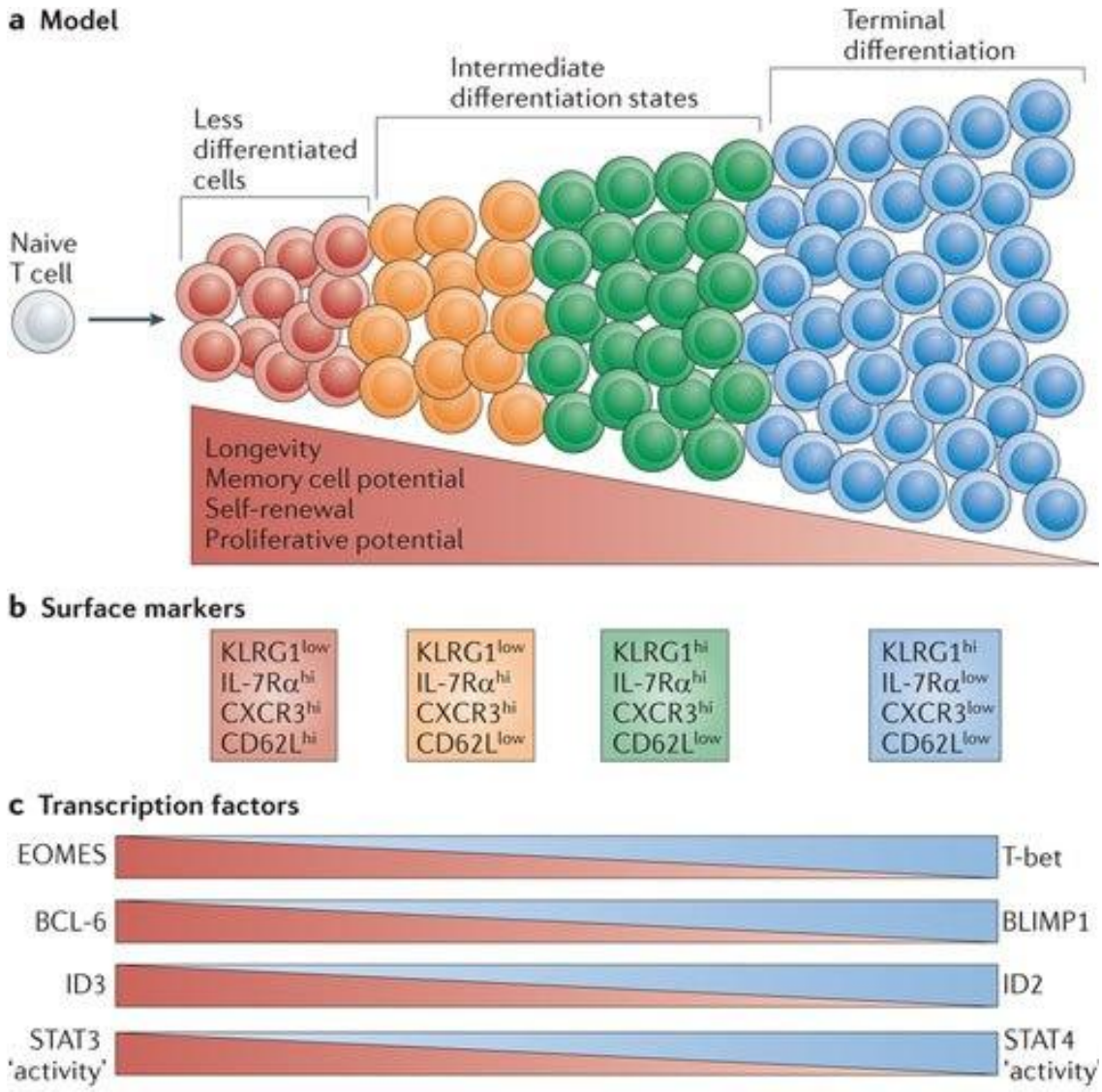


Figure 10. Model of transcriptional programs controlling differentiation of antigen-specific effector CD8 T cells: graded activity of transcription factors. **a)** In response to different levels of signal input, CD8 T cells differentiate into cells with greater memory cell potential, longevity and proliferative potential or into effector cells terminally differentiated. **b)** Heterogeneous differentiation states of CD8 T cells can be distinguished by the expression of several markers. **c)** Transcriptional programs controlling terminal effector cell differentiation and memory potential seem to be based on graded expression or activity of certain competing sets of transcription factors. (Kaech and Cui 2012).

12. Memory CD8 T cells and secondary immune responses

a) Cytotoxic T lymphocyte (CTL) response to acute infection

The development of CTL responses is necessary to control of a variety of bacterial and viral infections, and it is generally divided into four phases:

i) during effector phase, naïve CD8 T cell are primed, undergo dramatic expansion, acquire effector functions, travel to sites of infection, and mediate pathogen clearance by killing infected cells and secreting effector cytokines;

ii) during the contraction phase, most effector CTLs die, leaving behind 5-10% of the original burst size as long-lived memory cells;

iii) during the memory maintenance phase, memory CTL cells are maintained at stable levels through life;

iv) the rapid recall response of memory CTL following re-exposure to the pathogen provides enhanced protection to the host. (Fig. 11)

Thus, the primary goal of a CD8 T cell response is to simultaneously generate: i) an expendable pool of effector cells to combat the present infection, and ii) a pool of long-lived progenitors to combat future infections. These long-lived cells are designated as memory cells and are the base of the life-long acquired immunity after vaccination or infection. (reviewed in Williams and Bevan 2007).

b) Memory CD8 T cell definition

The definition of a "memory" CD8 T cell has changed over time. Initially memory CD8 T cells were defined as primed cells persisting *in vivo* after antigen elimination and that only such cells would be capable of inducing long-term protection. However it was subsequently shown that at least two situations did not fit on this definition. First, there are CD8 T cells able to eliminate the antigen and also to persist *in vivo*, but which do not have the capacity to mount efficient secondary responses or to protect from re-infection (e.g. when naïve CD8 T cells are primed in the absence of CD4 help). Secondly, this initial definition implies that CD8 T cells are "effectors" when antigen is present, and become "memory" when antigen is eliminated. However, not only some effector functions persist after antigen elimination, but "memory" cells also persist in the presence of antigen (like in chronic infections situations). As the broader-initial definition included heterogeneous CD8 T cell populations, with different properties and originated under different contexts, it was clear that the memory CD8 T cell definition should not be based neither on the phase of the immune response when CD8 T cells are recovered, nor on the degree of antigen load present. Instead, it should take into account functional properties (division, survival, differentiation in effector function and cytokine

secretion capacity), and also the capacity to induce protection. (reviewed in Rocha and Tanchot 2004a; Rocha and Tanchot 2004b).

c) Efficiency of secondary immune responses

Multiple mechanisms account for the efficiency of secondary immune responses. Prior to infection, the precursor frequency of CD8 T cells specific for particular antigen hovers in the range of 1 in 100000, or from 50-1000 of a given specificity. Following Ag exposure, antigen-specific memory cells are more abundant than naïve cells (Busch *et al.* 1998; Murali-Krishna *et al.* 1998; Blattman *et al.* 2002). *In vivo*, memory CD8 T cells have less stringent requirements to survive than naïve cells, they can expand in absence of cognate antigen and have higher RNA content and expression of perforin and FasL effector molecules. Yet, upon *in vivo* Ag-exposure, memory CD8 T cells start to divide sooner and faster, and also have a reduced loss rate during division. Cytokines are also produced at much higher amounts and can be detected much sooner in memory CD8 T cells than in naïve cells. (Tanchot *et al.* 1997; Tanchot *et al.* 1998; Freitas and Rocha 2000; Veiga-Fernandes *et al.* 2000; Veiga-Fernandes and Rocha 2004).

In conclusion, upon re-exposure to antigen, secondary responses are quicker, have much higher amplitude, and thus should lead to an efficient protection, which are the basic principles behind efficient vaccination protocols. (reviewed in Sprent and Surh 2002; Rocha and Tanchot 2004a).

d) CD4 T cell help to CD8 responses

Yet, to generate CD8 T cells capable of efficient recall responses to antigen, CD4 T cell help is absolutely required. This requirement was demonstrated in several different models: in monoclonal CD8 T cells specific for male antigen (Bourgeois *et al.* 2002a; Bourgeois *et al.* 2002b), in CD8 responses to cross-presented Ags or to a viral infection with LCMV (Janssen *et al.* 2003), in response to vaccine virus, expressing GP33 from LCMV (Shedlock and Shen 2003), and in response to LM expressing OVA or recombinant LCMV GP33 (Sun and Bevan 2003). In all these studies activation and differentiation of antigen CD8 T cells in the primary response did not require CD4 help but, CD8 T cells primed in the absence of CD4 help, performed poorly in secondary responses (lethargic cells).

CD8 T cells primed in absence of CD4 help proliferate poorly when re-challenged with antigen (Bourgeois *et al.* 2002a; Janssen *et al.* 2003; Shedlock and Shen 2003; Sun and Bevan 2003); they secret low levels of cytokines similar to those secreted by naïve cells (Bourgeois *et al.* 2002a; Bourgeois *et al.* 2002b); and they are unable to compete with naïve cells of the same specificity in

secondary responses (Shedlock and Shen 2003; Sun and Bevan 2003). CD40-CD40L interactions are involved in CD4 help to CD8 responses, either by direct CD40 engagement on CD8 T cells (Bourgeois *et al.* 2002a; Rapetti *et al.* 2008) or indirect CD40 engagement on APCs. (reviewed in Rocha and Tanchot 2004a).

e) Memory CD8 T cell subsets

As previously mentioned, memory CD8 T cells have been broadly divided into central memory (T_{CM}) and effector memory (T_{EM}) subsets in humans, and caution should be taken when applying the same classification to mouse and to different infection models. The criteria associated with these subsets distinction are the phenotype, proliferative capacity, function, and migration capacity. Therefore, **T_{CM}** are CD62L^{high} CCR7^{+}, reside in secondary lymph nodes, have a greater proliferative potential and do not express immediate lytic function. In contrast, **T_{EM}** are CD62^{low} CCR7^{-}, found in nonlymphoid tissue and constitutively display cytotoxicity effector functions. Interestingly, repetitive reactivation of memory CD8 T cells through vaccine boosters or successive infections cumulatively augments the effector-like properties of memory CD8 T cells and the frequency of T_{EM} in the memory pool (Jameson and Masopust 2009; Nolz and Harty 2011) (Fig. 11 and Table 3).

Recently, another subset of memory T cells has been proposed: tissue-resident memory T cells (**T_{RM}**). It has been described, that after infection had been cleared, there are memory cells that reside long-term in the brain and mucosal tissues (such as lungs, gut and skin) and that show limited levels of egress and recirculation. These memory cells have been designated tissue-resident memory T cells and have a characteristic CD103^{hi}CD69^{hi}CD62L^{low}CD27^{low} phenotype (Gebhardt *et al.* 2009; Masopust *et al.* 2010; Sheridan and Lefrancois 2011). (Table 3).

In contrast with memory CD8 T cells generated in response to infectious pathogens or in the presence of PAMP- innate signals, that mimic pathogens, it was also recently characterized a memory CD8 T cell subset generated under sterile inflammatory conditions designated as **T_{IM}** (inflammatory memory T cells). These cells are identified by their CD44/CD122 (IL-2Rβ)^{intermediate} phenotype. They are also present in unmanipulated wild-type mice and they display intermediate levels of several other memory traits (IFNγ, CCL5, T-bet and Eomes). It is thought that in absence of stimulation, these memory cells with intermediate phenotype might be lost with time, and that the capacity of T_{IM} cells to further differentiate and acquire high levels of CD122 are involved in the recall contact of hypersensitivity reaction. (Mbitikon-Kobo *et al.* 2009).

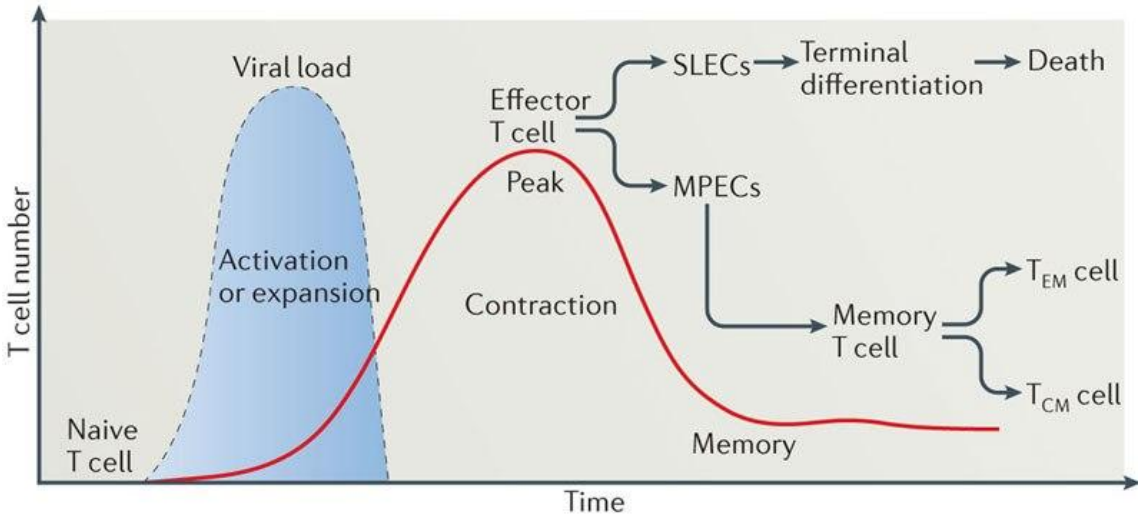


Figure 11. Different phases of a CD8 T cell response to an acute infection and heterogeneous CD8 T cell populations with different fates and memory potential. When a naïve CD8 T cell encounters an antigen, it undergoes a differentiation program that can be divided into three main developmental stages: clonal expansion and differentiation, contraction, and memory formation. Along with the immune response, heterogeneous CD8 T cell populations at the peak and memory stages can be distinguished: SLECs (short-lived effector cells), MPECs (memory precursor effector cells), T_{EM} (T effector memory cells) and T_{CM} (T central memory cells). (Lazarevic *et al.* 2013).

Table 3. Memory T cell subsets: T_{CM} (T cell central memory), T_{EM} (T cell effector memory) and T_{RM} (tissue-resident memory T cells). (Kaech and Cui 2012)

Cell type	Phenotype	Location	Functional properties
T_{CM} cell	$CD62L^{hi}CCR7^{hi}$	Lymph nodes, spleen, blood	↑ Proliferative potential ↑ IL-2 production ↑ Migration ↓ Effector functions and cytotoxicity
T_{EM} cell	$CD62L^{low}CCR7^{low}$	Spleen, blood, liver	↓ Proliferative potential ↓ IL-2 production ↑ Migration ↑ Effector functions and cytotoxicity
T_{RM} cell	$CD103^{hi}CD69^{hi}CD62L^{low}CD27^{low}$; expression of tissue-specific chemokine receptors and integrins*	Skin, lung, gut, brain	↓ Proliferative potential ↓ IL-2 production ↓ Migration ↑ Effector functions and cytotoxicity

f) Early markers for memory precursor cells

Concerning the identification of an early marker for memory precursor cells, as previously addressed, IL-7R and KLRG1 expression have been two strong candidates, however never absolute ones.

Another marker that has also been associated with memory T cell precursors is the CD8αα homodimer. This alternative complex of two CD8α molecules is induced in both $CD4^+$ and CD8αβ activated T cells, and in the course of primary responses to LCMV, CD8αα is transiently expressed in antigen-specific T cells and lost thereafter. When antigen-specific $CD8\alpha\alpha^-$ and $CD8\alpha\alpha^+$ were isolated at day 7 of LCMV infection and then injected into adoptive hosts, only $CD8\alpha\alpha^+$ cell were able to generate secondary memory precursors. In $E8_1^{-/-}$ mice that cannot generate CD8αα homodimers, it was also shown that transient expression of CD8αα is required for memory T cell generation (Madakamutil *et al.* 2004). However, identification of these CD8αα was performed by the use of TL-tet (thymus leukemia antigen-tetramers), which were lately reported to be "non-specific" and to fail indentifying CD8αα cells (Peaudecerf *et al.* 2011). In this context, it is unclear what cell types were identified by this methodology.

g) Models for effector and memory T cell heterogeneity generation

How effector CD8 T cell differentiation is modulated to originate cells with various phenotypes, functions and short- or long-term fates is still an open question. Recently, Kaech and Cui have summarized some of the models put forward to explain how heterogeneous pools of effector and memory CD8 T cells arise during infection (Fig. 12) (Kaech and Cui 2012):

a) Separate-precursor model: naïve T cells are pre-programmed to adopt certain differentiation states following activation based on information received during thymic development;
b) Decreasing-potential model: effector T cells adopt a variety of different states according to the cumulative history of signals that they encounter during infection. Repetitive stimulations with antigen (signal 1), co-stimulatory molecules (signal 2), and pro-inflammatory cytokines (signal 3) drive a greater effector cell proliferation and terminal differentiation state (which remain functional and cytotoxic, but lose memory cell properties such as enhanced longevity and proliferation potential);
c) Signal-strength model: the formation of heterogeneous population is dependent on the overall strength of the signals 1, 2 and 3 that are encountered early during the T-cell priming (a strong signals select out T cells that are competent to form memory and cause terminal effector T cell differentiation);
d) Asymmetric cell fate model: effector and memory T cell fates arise from a single precursor T cell through asymmetric cell division occurring as early as the first cell division after antigen stimulation (daughter cell that is closer to the APC/synapse contact adopts an effector cell fate).

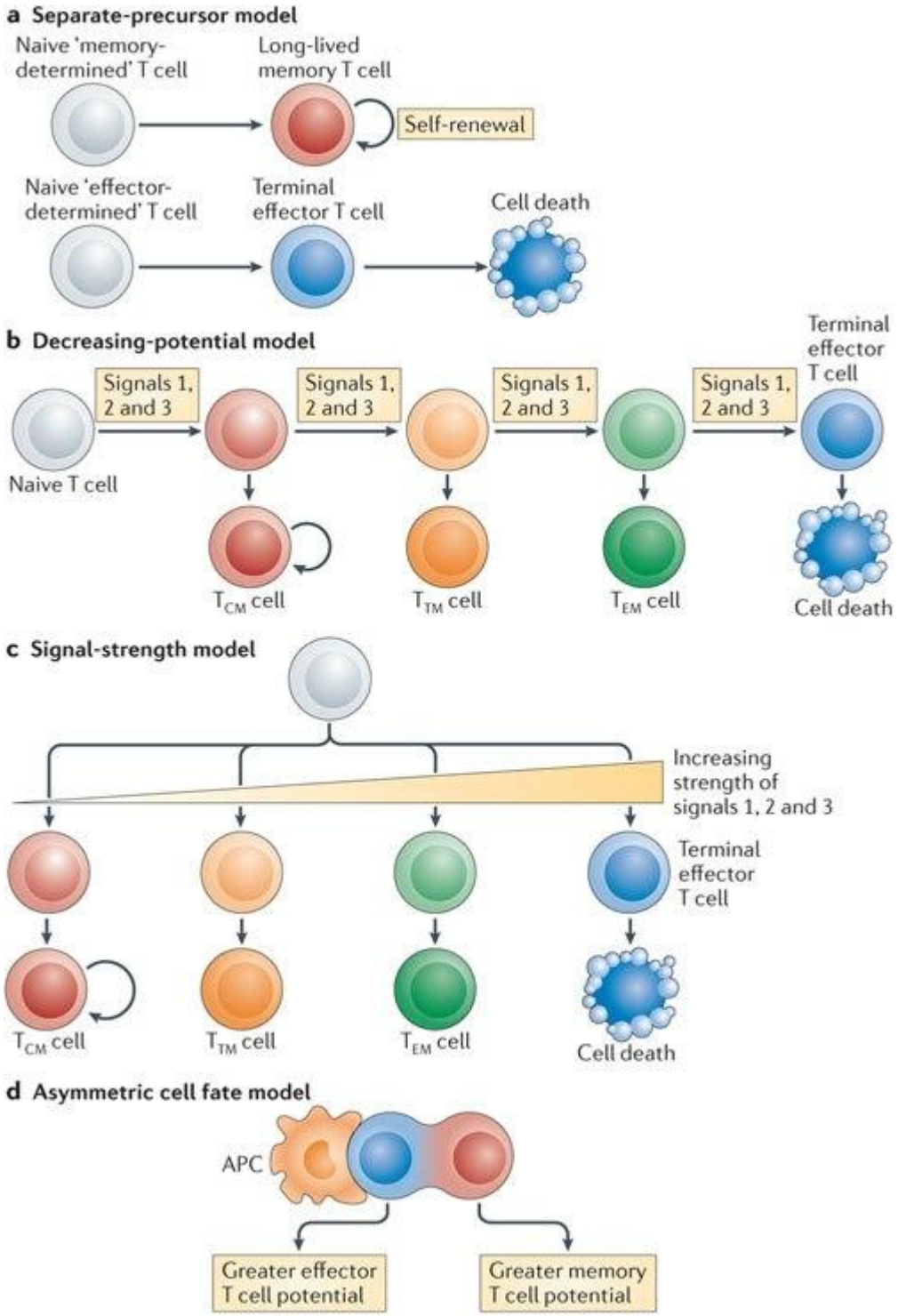


Figure 12. Models for generating effector and memory T cell heterogeneity (Kaech and Cui 2012).

IV. Methods and models to evaluate T-cell immune responses

1. Fluorencent-labeled pMHC multimers

T cells carry T-cell receptors (TCRs) recognizing specific MHC-peptide complexes displayed on the surface of antigen-presenting cells. By exploiting the specificity of this interaction, soluble multimeric forms of pMHC molecules (MHC multimers) have been designed to detect antigen-specific T cells.

As the affinity of an individual TCR for the peptide-loaded MHC is very low (when compared to the antibody-antigen interactions), the use of monomeric pMHC reagents to detect Ag specific T cells proved to be ineffective due to weak an transient binding. Thus, different ways of multimerizing pMHC complexes to improve their binding capacity were tried, and a site-specific **biotinylation method**, by which biotinylated pMHC complexes could be tetramerized with fluorescently labeled streptavidin molecules, has been widely adopted (Altman *et al.* 1996).

Fluorescent-labeled MHC multimers provide a powerful tool to monitor T cell-mediated immune responses through direct **detection** and **quantification** of antigen-specific T cells without any further *in vitro* manipulation. These multimers allow the detection of specific cells present either in *ex vivo* fluid samples, by flow cytometry, or *in situ* samples. In addition, the use of multimers constitutes a dominant utensil for physical **purification/isolation** of Ag-specific T cells for their further functional characterization and manipulation.

a) Improvements in multimers technology

Improvements in multimers techonology have not only increased their scope of application but also simplified their production procedure. Major differences between MHC multimers are related to: (i) the **valency/number of pMHC** complexes, (ii) the **expression system** through which proteins are produced, and (iii) the **peptide-loading strategy** used to assemble a desired peptide on the MHC biding groove (Bakker and Schumacher 2005).

To improve sensitivity of detection, both the valency of pMHC complexes and the valency of fluorochromes per molecule have been increased in order to augment the binding stability and brightness, respectively. The limited number of fluorophores available are circumvented by combinatorial staining strategies (e.g. stained with a mix of identical tetramers that are conjugated to different fluorophores) (Yi *et al.* 2010b) or by using streptavidin-coated with quantum dots (more stable than organic fluorochromes and with a narrow emission spectra) (Yi *et al.* 2010c). The use of two fluorophores to label the same epitope reduces the nonspecific staining (Hadrup *et al.* 2009).

Concerning the peptide-loading strategy, exchangeable peptides for one single batch of prepared MHC protein revolutionized the diversity and the rapid production of specific p-MHC

multimers. Ultraviolet light has been used to dissociate UV-sensitive ligands from MHC class I molecules (UV-mediated peptide-exchange technology). In contrast with the low pH method, this system does not change MHC structures when generating these "conditional" MHC class I ligands (Toebes *et al.* 2006).

b) Different types of MHC-peptide multimers

There are different types of MHC-peptide multimers: dimmers (multimer:IgG construct), tetramers, pentamers, streptamers (reversible bound multimers), dextramers, octamers and also pMHC coated vesicles (Casalegno-Garduno *et al.* 2010).

Tetramers: were first described by Altman et al. in 1996 for direct visualization and quantification of Ag-specific cytotoxic T cells; they are complexes of four biotinylated MHC molecules associated with a specific peptide that are tetramerized by a fluorescence-labeled streptavidin or avidin, which have the ability to bind four biotin molecules (Altman *et al.* 1996); due to the tetrahedral special configuration, no more than tree MHC-peptide complexes are available for TCR-binding.

Pentamers: were introduced in 2000 by ProImmune^{R}; they consist of five MHC-peptide complexes that are multimerized by a self-assembling coiled-coil domain; due to their planar configuration, all five MHC-peptide complexes are available for binding to complementary T cell receptors (higher avidity); each Pentamer also comprises up to five fluorescent or biotin tags (brighter signal).

Dextramers: were first introduced in 2005 by Immudex^{R} and are the next generation of fluorescent MHC multimers, carrying more MHC-molecules and more fluorochromes than conventional MHC multimers. These features increase their avidity for the specific T cell and enhance their staining intensity, respectively. Stability of the dextramers is ensured by the dextran polymer backbone that stabilizes the conformation of attached MHC-peptide complexes and fluorochromes. Minimal background staining is ensured by a novel method of production concerning the peptide-loading strategy. All these improvements significantly increase the detection resolution/sensitivity of T cell receptors with low affinity for MHC-peptide complexes, as well as the detection of extremely rare populations (Batard *et al.* 2006) (Jeannet *et al.* 2010).

c) MHC-peptide multimers applicability

p-MHC multimers have been widely used: for high-throughput analysis of Ag-specific T cells during vaccine trials in microarrays (Yi *et al.* 2010b) (Zhou *et al.* 2010); for high throughput epitope discovery (Sondergaard *et al.* 2009); for detection and purification of human cytotoxic T lymphocytes directed against tumor-associated and viral antigens (Yi *et al.* 2009); to compare vaccination strategies for their ability to enhance the frequency of high avidity clones, by quantifying the relative off-rates for TCR (Kaka *et al.* 2009); for *in situ* imaging to localize tumor cell or pathogen infected cells (Yi *et al.* 2010a; Cox *et al.* 2011); and for observation of TCR polarization during immunological synapse formation *in vivo* (Spolski *et al.* 2009).

Moreover, the use of MHC-peptide multimers has also been extended to enriching procedures. It overcomes limitations of techniques where cells that do not have proliferative potential or cytokine production could not be detected, allowing the detection of rare naïve or anergic antigen specific T cells (Parmigiani *et al.* 2011) (Monteleone *et al.* 2009).

Since the introduction of pMHC multimers two decades ago, many improvements have greatly increased their capacities to detect, quantify, isolate and manipulate antigen-specific T cells (Bakker and Schumacher 2005; Davis *et al.* 2011).

2. TCR Transgenic T cells

The genetic introduction in T cells of TCR α and β genes with a defined specify was initially developed to study the mechanisms of repertoire selection in the thymus (Kisielow *et al.* 1988), and our laboratory introduced this strategy to study T cell peripheral responses. It allowed to demonstrate that T cells could become tolerant outside the thymus (Rocha and von Boehmer 1991) and that antigen-specific T cells could have different fates, depending on the amount of antigen they encountered (Rocha *et al.* 1993; Tanchot *et al.* 1998). Thus, adoptive transfer of TCR-Tg T cells has been extremely valuable to study T-cell activation and differentiation of antigen-specific T cells *in vivo*.

a) HY specific TCR-Tg cells

The mouse minor histocompatibility (H) male-specific antigens (Y) (HY antigens), are expressed in male tissues, and are derived from different genes located on the Y chromosome. The Smcy gene was the first gene identified as encoding an MHC class I-restrict HY epitope, whereas the Dby was the first one identified for an MHC class II-restrict male epitope (Brandt *et al.* 2003; Zeng *et al.* 2005).

Different HY epitopes/peptides derived from the same male protein have also been identified, and distinct TCR-Tg mouse strains have been genetically engineered to express the corresponding specific TCR. Two broadly used Tg mouse strains bearing TCRs specific for male antigens are the (i) HY and (ii) Marilyn mouse trains. (i) HY mice enclose CD8 T cells expressing a transgenic TCR (VαT3.70 Vβ8.2) that binds specifically to the Smcy antigen-derived peptide (KCSRNQYL) presented on H-2D^b MHC class I molecules (Korn *et al.* 2007). (ii) Marilyn mice have CD4 T cells expressing a transgenic TCR that specifically recognize the Dby derived peptide (NAGFNSNRANSSRSS) presented on H-2A^b MHC class II molecules (Lantz *et al.* 2000). These models are particularly useful to study T cell differentiation in a "sterile" non-infectious context (where CD8 HY female cells are activated by male cells).

b) OVA specific TCR-Tg cells

The chicken egg albumin - Ovalbumin (OVA) - has also been widely used as an antigen model. Two broadly used Tg mouse strains bearing TCRs specific for OVA antigens are the: (i) OT-1 and (ii) OT-2 mouse strains. (i) The OT-1 mice harbor CD8 T cells expressing a transgenic TCR (Vα2 Vβ5) specific for the OVA$_{257-264}$ peptide (SIINFEKL) presented in the context of H-2K^b MHC class I molecules (Nembrini *et al.* 2006). (ii) The OT-2 strain ports CD4 T cells expressing a transgenic TCR specific for the OVA$_{323-339}$ peptide (ISQAVHAAHAEINEAGR) presented in H-2A^b MHC class II molecules (Bots *et al.* 2005).

c) GP33 specific TCR-Tg cells

The LCMV has been extensively used as an infection model subject to selective pressures due to pathogen/host coevolution. The P14 mouse strain harbors CD8 T cells expressing a transgenic TCR (Vα2 Vβ8.1) specific for the LCMV glycoprotein peptide GP$_{33-41}$ (KAVYNFATM) present in the context of H-2D^b MHC class I molecules (Pircher *et al.* 1989; White *et al.* 1999).

d) TCR-Tg mice in a Rag$^{-/-}$ background

The absence of allelic exclusion in the expression of the TCR-α chain initially caused a trouble in using TCR-Tg mice. In these mice, the majority of TCR-Tg cells, besides expressing the transgenic TCR-α chain, also co-expressed different levels of endogenous TCR-α chains (Bluthmann *et al.* 1988), resulting in different avidity for the antigen and complicating the readouts of the peripheral responses. To overcome this limitation, TCR-Tg mice were initially crossed to a *Scid* background, but this strategy was found inappropriate to evaluate peripheral responses (Rocha, personal communication): the inability of Scid cells to repair DNA prevented the peripheral expansion of TCR-Tg cells. Our laboratory thus developed the strategy to cross TCR-Tg mice into the Rag$^{-/-}$-background

in order to abolish endogenous TCR rearrangements (Rocha *et al.* 1993). Thereby, these mice are a source of naïve monoclonal T cell population, which bears a single and defined TCR.

A number of allelic variations of surface receptors (congenic markers) have been bred onto TCR-Tg mice to discriminate donor T cells from recipient's own cells. The most common congenic markers are the CD45 and CD90 and, different allelic forms of each, can be detected by specific antibodies. Alternative approaches to track donor TCR-Tg cells after adoptive transfer can be the use of: antibodies specific for TCR Vα or Vβ segments; CFSE (carboxyfluorescein diacetate succinimidyl ester) to distinguish donor cells in cases where they have not divided extensively; and pMHC multimers. (reviewed in Elsaesser *et al.* 2009).

3. Models of infection: LM and LCMV

The most commonly used models to study CD8 T cells differentiation rely on *Listeria monocytogenes* or LCMV infection.

a) Listeria monocytogenes (LM)

LM is a ubiquitous Gram-positive bacterium and it is a facultative intracellular pathogen able to infect a broad variety of mammalian cell types, including ephitelial cells, endothelial cells, hepatocytes, DCs and macrophages (Kayal and Charbit 2006).

The natural route of infection with LM is through the gastrointestinal tract. It infects intestinal epithelial cells by interaction of internalin A (expressed on bacterial cell surface) with epithelial cadherin (E-cadherin), which is expressed at the surface of ephitelial cells (Gaillard *et al.* 1991). However, mice are relatively resistant to intestinal infection with LM due to a single amino-acid difference between human and mouse E-cadherin (Lecuit *et al.* 2001). After traversing the epithelial cell layer, bacteria disseminate in the bloodstream to other organs, such as the spleen and liver, where they are internalized by macrophages. In the liver, LM enters hepatocytes by expressing internalin B, which binds to a hepatocyte growth factor receptor (Shen *et al.* 2000).

In laboratory studies to characterize immune responses, this pathogen is inoculated intravenous or intraperitoneal eliciting an immune response to systemic infection.

After cellular invasion, LM rapidly escapes from the primary phagosome by membrane lysis upon secretion of listeriolysin O (LLO) and phosphoinositide specific phospholipase C (Bielecki *et al.* 1990).

Invasion of the cytosol is fundamental for virulence and for triggering innate and adaptive immune responses. LM stains that lack LLO do not induce protective immunity in immunized mice (Berche *et al.* 1987).

Once in the cytosol, LM actively replicates and expresses other virulence factors such as ActA and SecA2.

ActA is a surface-enchored actin-assembly-inducing protein that induce polymerization of actin filaments to propel bacteria through the cytoplasm and into neighbouring cells (Domann *et al.* 1992; Kocks *et al.* 1992). ActA-mutant LM strains are greatly attenuated. This mutant is used at higher doses, does not spread, it reaches higher density within infected cells, does not increase the number of infected cells throughout the infection, and it is cleared more quickly. However, it induces innate immune responses and prime protective T cell responses (Goossens and Milon 1992).

SecA2 is involved in the secretion of a subset of proteins via the general secretory pathway (Sec system) (Lenz *et al.* 2003). SecA2-deficient LM does not induce protective secondary responses, although it generates normal numbers of Ag-specific CD8 T cells. These CD8 T cells also score normally when tested for cytolitic function and IFNγ, TNFα and GzmB production, but are unable to reduce the bacterial load. (Muraille *et al.* 2007; Narni-Mancinelli *et al.* 2007). Immunization with SecA2-deficient LM results in a defect in the CCL3 production by CD8 T cells. CCL3 is responsible for TNFα production by mononuclear phagocytic cells (MPCs), and CCL3 and TNFα are required for the production of reactive oxygen intermediates by MPCs and neutrophils (Narni-Mancinelli *et al.* 2007).

LM elicits a strong innate immune response, namely by triggering TLR2 and TLR5, which is essential to controlling the infection in its initial phase. Both TLR2, which recognize bacterial peptidoglycan, lipoteichoic acid and lipoproteins; and TLR5, which recognizes bacterial flagellins have been implicated in the recognition of LM (Hayashi *et al.* 2001; Seki *et al.* 2002). MyD88, an adaptor that mediates TLR signals, is also essential for innate immune defense against LM. Mice that lack MyD88 are more susceptible to LM infection than mice that lack either IFNγ or both IL-12 and IL-18 (Edelson and Unanue 2002; Seki *et al.* 2002). Mice lacking, TNFα, iNOS, or CCR2 are also more susceptible to LM infection (Pamer 2004).

It was also shown that after systemic LM administration, DCs transport bacteria to the white-pulp areas of the spleen, where they initiate secretion of chemokines required for NK and monocytes recruitment. DCs undergo MyD88-dependent activation and secrete IL-12 and IL-18 that in turn activate newly recruited NK cells to produce IFNγ. Recruited cells are then organized in clusters: monocytes are positioned in proximity to infected cells, and NK cells form a cuff at the periphery, where NK-derived IFNγ induces monocyte activation. This leads to MHC class II and iNOS upregulation and subsequent differentiation of monocytes in TipDCs. TipDCs sense microbial infection in a MyD88-dependent manner and secrete TNFα and NO, leading to bacterial replication restriction. (Kang *et al.* 2008; reviewed in Serbina and Pamer 2008).

Besides the MyD88-dependent pathway that emanates from the cell surface and phagosome leading to the expression of inflammatory and suppressive/regulatory cytokines such as TNF-α, IL-12,

and IL-10; two other innate pathways are induced by Listeria infection. A second pathway emanates from the cytosol and is dependent on STING (stimulator of interferon genes) and IRF3. STING is activated by cyclic di-AMP secreted by intracellular bacteria, and leads to the expression of IFN-β and coregulated genes. A third pathway is Caspase-1-dependent on inflammasome activation and results in the proteolytic activation and secretion of IL-1β and IL-18 and cell death. This pathway is known to be activated by LM trough the NLP3 (a NOD-like receptor that integrates the inflammasome complex). As mentioned before, Listeria also activates NOD1 and NOD2 (reviewed in Schuppler and Loessner 2010; and in Witte *et al.* 2012).

Thus, the innate signaling in LM infection is complex and involves multiple mediators.

Although LM has been widely used to investigate innate immunity to bacterial infection, it has been used even more extensively to characterize T cell-mediated immune responses. T cells mediate the clearance of LM after infection (McGregor *et al.* 1970), with humoral immunity providing only a small contribution to protective immunity. Among αβT cells, CD8 T cells provide a more substantial contribution to long-term protective immunity (Ladel *et al.* 1994). In vivo depletion of CD8 T cells impairs LM clearance both in primary and secondary responses (Mielke *et al.* 1988).

LM derived MHCIa-restricted peptides are generated from secreted proteins, many of which contribute to bacterial virulence. LLO is one of the most antigenic secreted proteins in terms of specific-CD8 T cell induction. Another antigen that induces substantial CD8 T cell responses is p60, a hydrolase involved in bacterial septation. Both LLO and p60 are rapidly degraded by the proteosome of the host cell (reviewed in Pamer 2004). In the spleen, most of the bacteria are present in $CD11b^{high}CD11c^{-}$ cells, but $CD11c^{high}$ cells are required for priming of CD8 T cells (Jung *et al.* 2002; Muraille *et al.* 2005).

b) Lymphocytic choriomeningitis virus (LCMV)

LCMV is a natural mouse pathogen and a prototypic member of Arenaviridae. It is an enveloped virus with helical nucleocapside, and is a negative-stranded RNA virus whose genome has two segments (small (S) RNA and large (L) RNA) encoding for only four proteins. The S RNA segment encodes the viral glycoprotein (GP) and nucleoprotein (NP), whereas the L RNA encodes the L and Z proteins (Sevilla *et al.* 2000).

The outcome of LCMV infection in mice depends on a number of factors including the mouse strain, mouse age, strain of the viral isolate, dose of virus, and route of infection (Roost *et al.* 1988). Thus, differences in viral and host determinants have allowed for the development of distinct LCMV model systems for studying efficient responses leading to virus elimination, or viral persistence and immunosupression (reviewed in Humphreys *et al.* 2008):

(i) In the carrier state model, WT mice are infected at birth or in the uterus with LCMV and become lifelong carriers of the virus. This model is associated with clonal deletion of LCMV-specific T cells (chronic infection).

(ii) Intravenous or intraperitoneal infections of adult WT mice with LCMV clone 13, or other highly virulent and fast growing LCMV strains, results in a vigorous expansion of virus-specific CD8 T cells, however virus persists for 90-150 days in most organs. This infection is associated with virus-specific immunosupression (progressive deletion of virus-specific CD8 T cells, and destruction and functional disruption of APCs)

(iii) WT mice being infected with slow growing isolates of LCMV, such the LCMV Armstrong strain, results in a robust CD8 T cell response, which mediates viral clearance from the spleen within 8-10 days. This infection model neither results in the attrition of virus-specific CD8 T cells nor in a generalized immunosupression, and it leaves APCs largely intact and unaffected (acute infection).

(It is worth to mention that unless stated differently, the term LCMV is always related to an acute infection, thus related to LCMV Armstrong infection, which actually is the strain subject of interest during this thesis).

LCMV primarily infects macrophages, lymphocytes, DCs and glial cells, and it mainly infects cells through the α-dystroglycan (α-DG) receptor, which is a ubiquitous cellular receptor for proteins of the extracellular matrix.

Armstrong 53b viral variant binds to α-DG with a lower affinity than Clone 13, and sequence comparison between these two variants showed that Armstrong 53b has an amino-acid substitution in the GP precursor, which is used for the virus binding to its cellular receptor. Armstrong LCMV infection is associated with: (i) viral replication in the red pulp of the spleen, (ii) minimal replication in $CD11c^+$ and $DEC\text{-}205^+$ splenic dendritic cells (among the immune system, these cells primarily express α-DG), (iii) a robust anti-LCMV CTL response that clears the acute infection.

Infection with low doses of either LCMV WE or LCMV Armstrong strains has been used over the past decades to investigate effector and memory CD8 T cell differentiation upon acute and resolved viral infection. Acute infection of mice with LCMV results in rapid viral growth that causes little host damage since LCMV is a noncytophatic virus.

The primary CD8 T cell response to LCMV Armstrong is not dependent upon the presence of CD4 T cells or B cells, although neutralizing antibodies are necessary to prevent viral reemergence and CD4 T cell help is necessary for long-term memory CD8 T cell generation. The perforin-dependent pathway and the IFNγ production are crucial for the control of LCMV infection.

The CD8 T cell response to LCMV infection is massive and, at the peak of the response, ~90% of activated splenic CD8 T cells are directed against 28 defined epitopes in $H\text{-}2^b$ mice. In C57BL/6 mice, three $H\text{-}2D^b$-restrict epitopes are the major targets of the CTL response: GP33 ($GP_{33\text{-}41}$), GP276 ($GP_{276\text{-}286}$), and NP396 ($NP_{396\text{-}404}$). These peptides are present at different densities on the cell surface MHC class I molecules (GP33>NP396 >GP276), and their abundance correlates with the magnitude of CTL responses to these peptides following LCMV infection. (reviewed in Wong and Pamer 2003).

c) Pathogen induced cytokine milieu

Inflammatory cytokines like IL-12 and type-I IFN (IFN-I) have long been known to promote early control of pathogens replication and to stimulate APC functions. However recent studies have demonstrated that inflammatory signals also act directly on T cells.

Activation of CD8 T cells depends on three signals: TCR engagement (signal 1), costimulation (signal 2) and an inflammatory stimulus (signal 3) via cytokines such as IL-12 or IFN-I.

In vitro, both IL-12 and IFN-I cytokines are involved in the expansion and effector function of CD8 T cells (Curtsinger *et al.* 1999; Valenzuela *et al.* 2002; Curtsinger *et al.* 2003; Curtsinger *et al.* 2005).

In vivo, **expansion and survival** of CD8 T cells is strictly dependent on IFN-I signaling during LCMV infection but less critical after vaccinia virus (VV) or LM infections (Kolumam *et al.* 2005; Aichele *et al.* 2006; Thompson *et al.* 2006). On the other hand, direct IL-12 signaling is mandatory for T cell expansion after LM infection but not after viral infection with LCMV, VSV (Vesicular stomatitis virus) or VV (Keppler *et al.* 2009). It is also know that high levels of IFN-I suppress IL-12 production (Cousens *et al.* 1997). Indeed, absence of IL-12 is characteristic of LCMV, but not in other viral infection (Orange and Biron 1996; Cousens *et al.* 1997).

Thus, using CD8 T cells lacking receptors for IL-12, IFN-I, or both, it was shown that CD8 T cell expansion depends on IFN-I for LCMV infection, IFN-I and IL-12 for LM and VSV infection, or is largely independent of the two cytokines for VV infection (Keppler *et al.* 2012).

In addition, CD8 T cells lacking IL-12 and IFN-I signals are impaired in cytokine production and **cytolytic activity** in the context of VSV and LM infection. These effector CD8 T cells fail to express KLRG1, thereby exhibiting a memory-like phenotype which correlate with the lower expression of T-bet and higher Eomes expression (Keppler *et al.* 2009; Keppler *et al.* 2012). Indeed, in LM infection IL-12 induces T-bet and represses Eomes (Takemoto *et al.* 2006).

IFN-I receptor- deficient P14 TCR-Tg CD8 T cells adoptively transferred to WT hosts exhibit a severe defect in their ability to expand and **generate memory** populations after LCMV infection (Kolumam *et al.* 2005; Aichele *et al.* 2006).

Overall, these results indicate that a variable interplay between IL-12 and IFN-I cytokines is mandatory for expansion, effector function or cell fate decision of CD8 T cells in the context of different infections.

Aims and experimental approaches

During immune responses, naïve CD8 T cells are called on to develop multiple activities required to control antigen load, as well as to generate memory cells able to efficiently respond to rechallenge. Thus, addressing how naïve CD8 T cells undergo functional programming that determinates critical aspects of their differentiation at both effector and memory stages is of great interest to understand the establishment of successful immunity.

Several approaches have been used to study CD8 T cell responses and technological advances in pMHC I multimers, intracellular staining protocols, TCR-Tg mice used for adoptive transfer studies, and gene profile analysis either by genetic arrays or at single-cell level have greatly contributed to uncover the phenotypic and function diversity within effector and memory T cell populations.

When studying CD8 T cell differentiation *in vivo*, through the analysis of simultaneously expressed genes in each individual cell during immune responses (by single-cell multiplex RT-PCR technique), our laboratory detected that: i) individual effector genes had different kinetics of expression/down-regulation, and several effector genes were co-expressed stochastically, which revealed a cell-to-cell heterogeneity in CD8 responses; ii) shortly after activation, CD8 T cells were found to be inflammatory effectors rather than presenting cytotoxic functions. Thus, both the markedly cell-to-cell heterogeneity inside a responding population and the diversity of CD8 T cell effector functions acquired on immune responses led us to focus on the diversity of CD8 T cell differentiation occurring during immune responses: what signals modulate this diversity of CD8 T cells, which cell differentiation transcripts control different CD8 T cell behaviors? What type of effector and memory cells can be formed and maintained during a CD8 response? What roles do they play in protective immunity?

The final outcome of individual T cell activation is complex and the fate of T cell differentiation is influenced by different factors such as: strength and duration of TCR signaling, nature of APCs and co-stimulatory signaling, and cytokine environment. Indeed, CD4 T cells differentiate into distinct effector subsets, and the fate of each subset is depending on the nature of the cytokines present at the site of activation, which then induce a coordinated co-expression of transcription factors and effector molecules. Thus, even if T cell activation is triggered by TCR engagement, the context in which antigen presentation occurs is also central for the final outcome of a T cell response. Thus, understanding how the diversity of CD8 T cells responses are induced and regulated to achieve different immune outcomes is fundamental for the designing of optimal T cell vaccines. Moreover, the impact of the early CD8 effector functions in generating protective response is still unknown.

When studying CD8 T cell differentiation, it must be taken in account that a CD8 T cell response is part of an ongoing immune response involving several cells and components of the immune system. Thus, most infectious agents induce inflammatory responses by activating innate cells, and these inflammatory reactions have a major role in recruiting additional immune cells to the sites of inflammation or infection, in order to trigger lymphocyte's activation and thus eliminate the

infectious agents. Thus, understanding which processes provide the properly encounter of naïve CD8 T cells with cognate antigen is also fundamental for mounting an efficient CD8 T cell response.

Therefore, the main scope of this thesis work was to **characterize the diversity of CD8 T cell differentiation occurring during immune responses**. In particularly we addressed the following questions:

I. Does the frequency of naïve-precursor cells have an impact on the diversity of CD8 T cell immune responses?
II. Can different pathogens modulate CD8 T cell properties and thus generate diversity on CD8 T cell responses?
III. What is the diversity of CD8 T cell effector functions and what are their roles during an immune response?

To answer these fundamental questions we used two main approaches:

IV. The adoptive transfer of TCR-Tg cells: a well established system to study *in vivo* T cell activation and differentiation;
V. The sensitive single - cell multiplex RT-PCR method: to analyze the complexity and the diversity of CD8 T cell responses

I. Does the frequency of naïve-precursor cells have an impact on the diversity of CD8 T cell immune responses?

Antiviral CD8 T cells respond to only a minute fraction of the large potential peptide determinants encoded by viral genomes and therefore, immunogenic epitopes are ordered into hierarchies based on the magnitude of the cognate CD8 T cell responses generated after infection. Immunodominant epitopes have been shown to be critical in eliminating infected cells and in contributing to the memory T cell pool. However, the extent to which immunodominance (naïve precursor frequency) guides CD8 differentiation is still unclear. Relate to this subject is the question of whether information provided by adoptive transfers of TCR-Tg cells at a high precursor frequency (artificially induced immunodominance/precursors introduced at high frequencies) could be representative or not, of endogenous populations present at rare frequencies.

It has been suggested that CD8 TCR-Tg cells originated from precursor introduced at unnaturally high frequencies reveal altered differentiation during infection (Badovinac *et al.* 2007) and that initial T cell frequency influences memory generation (Marzo *et al.* 2005). As these conclusions were made

by direct comparison of TCR-Tg cells with endogenous cells in different mice, and as it had also been revealed that adoptive transfers using high levels of precursors cells interfere with the kinetics of pathogen clearance (Sarkar *et al.* 2007), it remained unclear if:

A) The diversity of CD8 T cell behaviors attributed to the use of high precursor frequencies is the consequence of distinct differentiation pathways or merely the consequence of altered differentiation kinetics? So, what is the impact of the immunodominance in guiding the diversity of CD8 T cell responses?

B) Information provided by adoptive transfers of TCR-Tg cells at a high precursor frequency (artificially induced immunodominance) are representative or not of endogenous populations present at rare frequencies ?

To answer these questions we:

1.1) Evaluated if CD8 dominant and subdominant populations exhibit the same properties when generated from natural precursor's frequencies. For this, we analyzed two dominant vs one subdominant CD8 T cell clones naturally generated in a mouse responding to acute LCMV infection. In all three endogenous populations, and at several time points of the infection, we analyzed: (i) the expression of CD8 T cell associated with cytokine and cytotoxic effector genes (by single-cell multiplex RT-PCR); (ii) the expression of surface proteins associated with CD8 T cell differentiation and memory generation (by Flow Cytometry).

1.2) Evaluated if the establishment of a more pronounced immunodominance hierarchy, between two populations responding to the same epitope, influences CD8 T cell differentiation into effector and memory cells. We adoptive transferred high number of TCR-Tg CD8 T cells (P14 cells, specific for GP_{33-41}) into a host mouse and, after LCMV immunization, we studied both transgenic (P14) and endogenous GP33-specific populations (originated from abundant and rare precursor, respectively). We compared GP33-specific Tg and endogenous cells on the same infected mouse to guarantee the same kinetic of pathogen clearance for both populations. The same genes and surface markers as above were analyzed at several day post infection.

1.3) Evaluated the impact of TCR downregulation in pMHC-I multimer labeling at early stages of the immune response. As activated CD8 T cells strongly down regulate their TCRs, we compared the capacity of pMHC-I multimers vs congenic markers to track antigen-specific TCR-Tg CD8 T cells after their *in vivo* activation. In addition, we also analyzed multimer labeling capacity in CD8 endogenous activated cells and correlated the intensity of the labeling with functional properties.

II. Can different pathogens modulate CD8 T cell properties and thus generate diversity on CD8 T cell responses?

While studying differentiation of monoclonal TCR-Tg cell populations by evaluating the expression of 20 CD8 T cells associated genes at single cell level, our group noticed that, after *in vivo* activation, different genes had different kinetics of expression (were induced, transcribed and declined at different time points of the response), and that they were randomly associated. Thus, these results strongly emphasized a cell-to-cell heterogeneity among an activated population of CD8 T cells responding to a given epitope (Peixoto *et al.* 2007). These cell-to-cell heterogeneity challenged the notion of a "single differentiation pathway" for CD8 T cells (classically defined as IFNγ/CTL differentiation pathway), and thus raised the possibility of the existence of multiple programs of CD8 T cell differentiation and of the existence of CD8 T cells with different fates.

In our previous study to evaluate the impact of immunodominance on the diversity of CD8 T cells properties, we excluded that such heterogeneity could be due to different clonal abundance (Munitic *et al.* 2009). In addition, it has been suggested that pathogen biology is critical in determining the specific requirement for signal 3 (inflammatory cytokines) activation of antigen-specific T cells (Haring *et al.* 2006). Thus, we wondered if**:**

A) Different pathogens/infectious contexts can modulate CD8 T cell differentiation programs and therefore be responsible for CD8 cell-to-cell heterogeneity ?

Relate to this subject is the question whether different pathogens may or may not generate identical memory T cell types after an efficient primary immune response. Clarifying this issue is important for optimal T cell vaccine design, and several hypothesis are possible: should vaccines used attenuated pathogens in order to recapitulate the behavior of the pathogen that it aims to control?; should vaccines guarantee the generation of as many memory cells as possible, with different pathogens originating identical memory cell types?; or should vaccines guarantee the generation of a peculiar memory type, with pathogens originating distinct memory types? Therefore, we wondered if:

B) Different pathogens may generate memory T cell types with different capacities to confer protection ?

The progress in vaccine design and in the evaluation of protection requires the use of reliable methods and systems, not only to induce adequate immune responses, but also to monitor these responses in vaccinated individuals. Thus, it is fundamental to use accurate models and systems to unveil the complexity of CD8 T cell effector functions and cell heterogeneity *in vivo*.

In the majority of the cases the method used to access T cell effector functions is the *in vitro* T cell reactivation with pathogen epitopes. However, it has been demonstrated that *in vitro* re-stimulation considerably modifies the *ex vivo* readouts (Panus *et al.* 2000; Veiga-Fernandes *et al.* 2000), and also that *in vitro* readouts are unable to discriminate T cells that lead to abortive immune

responses from those leading to pathogen clearance and efficient memory generation (Tanchot *et al.* 1997; Kassiotis *et al.* 2002).

In addition, TCR-Tg cells have also been largely used as a system to study T cell differentiation during immune responses, but unfortunately also subject of severe criticisms, as already pointed out before. Thus, we wondered:

C) What would be the most advantageous experimental strategies to study the diversity of CD8 immune responses?

To answer these questions we:

2.1) Compared the readouts obtained from different approaches: we evaluate the diversity of CD8 T cell properties as they are induced during *in vivo* responses by

(i) *ex vivo* evaluation of mRNA (single-cell multiplex RT-PCR),

(ii) *ex vivo* evaluation of protein in Brefeldin A injected mice (Flow Cytometry),

iii) protein evaluation after *in vitro* re-stimulation (Flow Cytometry).

2.2) Evaluated the impact of TCR down-regulation in the assessment of CD8 T cell immune responses diversity. We studied the level of TCR down-regulation in: several immunizing conditions of Ag and TCR-Tg cell doses; at several days of the response; and according to cell division state.

2.3) Characterized CD8 T cell immune responses to two different infectious contexts. We compared OT-1 cells activated in the LM-OVA context vs P14 cells activated in the LCMV context (expression of several molecules involved in CD8's differentiation, effector function and memory generation were evaluated).

2.4) Excluded that possible differences between OT-1 and P14 cells (when activated under different infectious contexts) could arise from different TCR avidities between these two transgenic clones. We subsequently compared the response of OT-1 and P14 cells in the same infectious context (Listeria context): OT-1 and P14 cells were adoptively co-transferred into the same mouse that was subsequently immunized with LM-OVA and LM-GP33.

2.5) Evaluated the capacity of pathogen elimination between memory CD8 T cells generated in two distinct infectious contexts. Previously LM-GP33 (i) or LCMV (ii) immunized mice were challenged two months later with a lethal dose of LM-GP33 bacteria, and 24h later the bacterial loads were determined in both groups of mice (i and ii).

III. What is the diversity of CD8 T cell effector functions and what are their roles during an immune response?

While studying *in vivo* CD8 T cell differentiation, previous work in the lab has shown that early in the immune response (before the expansion peak) CD8 T cells did not expressed the classical CD8 effector molecules, but rather, they expressed pro-inflammatory cytokines such as *Tgfb* and TNFα. In addition, these cells did not express TGFβ receptor subunits necessary for signaling (*Tgfbr1* and *Tgfbr2*), suggesting that TGFβ could only act in *trans,* where it was described to be pro-inflammatory (Ludviksson and Gunnlaugsdottir 2003). Moreover, when transferred to normal mice, these early effector CD8 T cells*,* instead of killing antigen-loaded targets, they actually induced *in vivo* local retention of both antigen-loaded and non antigen-loaded targets at the site of effector's CD8 T cell injection. These early CD8 effectors cells were then named as "inflammatory effectors". Thus, these results suggested diversity on CD8 effector T cell properties during immune responses, where early CD8 effectors could be involved in cell recruitment events (Peixoto *et al.* 2007). Thus we wondered:

A) What are the mechanisms by which early CD8 effector T cells could act as pro-inflammatory effectors and recruit other cells?

B) What is the role of these pro-inflammatory effectors during an immune response?

To answer these questions we:

3.1) Characterized the pro-inflammatory role of CD8 T cells at early time points of the immune response:

a) Identifying other pro-inflammatory mediators (chemokines) that could also be expressed;

b) Correlating the gene expression of pro-inflammatory mediators with cell activation and division status;

c) Investigating the causes that restrict expression of pro-inflammatory mediator to early phases of the immune response;

We used the adoptive transfer of OT-1 cells to track antigen specific cells at very early time points of the immune response, activated them *in vivo* with LM-OVA immunization, recovered OT-1 cells at several days after infection, and analyzed mRNA expression (by single cell multiplex RT-PCR) or intracellular protein expression of pro-inflammatory mediators (Fig. 13A).

3.2) Examined if CD8 T cells also acquire pro-inflammatory effector functions in the absence of pathogen associated innate signals. We studied: i) anti-HY CD8 TCR-Tg cells after *in vivo* activation with male antigen ("sterile"/non infectious context); and ii) endogenous CD8 T cells from MyD88-deficient mice (majority of innate signal abrogated) after Listeria activation.

3.3) Determined the *in vivo* physiological role of pro-inflammatory CD8 effectors by:

a) Testing if early CD8 effectors are able to induce "*in vivo"* cell recruitment into lymph nodes. We isolated pro-inflammatory CD8 effectors (OT-1 or HY), injected them in the braquial lymph

nodes (BRLNs) of a host mouse, and then evaluated leukocyte's recruitment to that BRLN (Fig. 13B).

b) Testing if pro-inflammatory CD8 T cells are also involved in the blocking of LN egress.
We injected pro-inflammatory CD8 effectors in a BRLN of a normal host, and check if they could modify local S1P concentrations. S1P levels were evaluated by measuring their capacity to induce internalization of the corresponding receptor in the WEH123 cell line, which express a FLAG-tagged S1P receptor (Flag-S1P1).

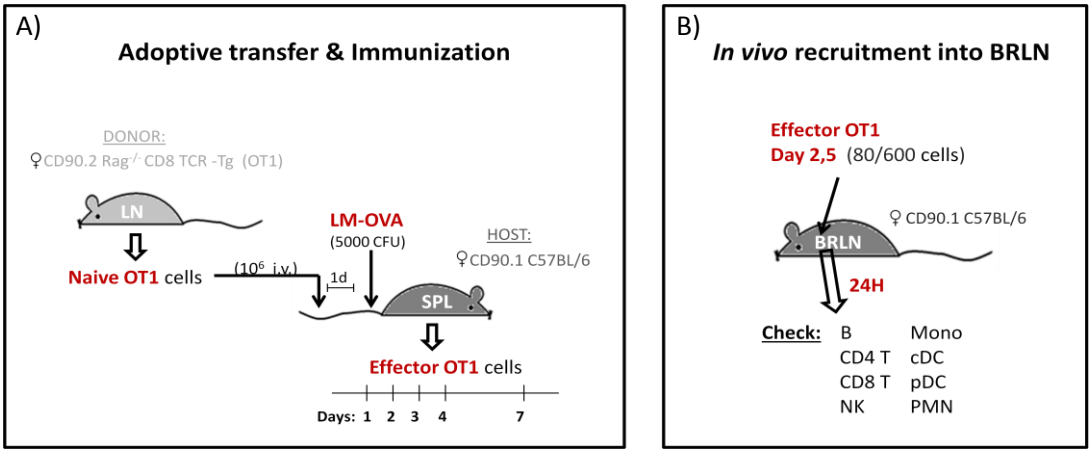


Figure 13. Used protocols: (A) to generate *in vivo* effector CD8 T cells, **(B)** to test cell recruitment capacity of pro-inflammatory effectors.
A) 10^6 naïve OT1 cells were isolated from lymph nodes (LN) of CD90.2 Rag1$^{-/-}$ CD8 TCR-Tg donors (OT1 mouse strain) and were intravenously injected into a host mouse with a different congenic marker (CD90.1 C57BL/6). OT1 cells harbor TCRs specific for $OVA_{257-264}$ peptide, and one day later, the host mouse was immunized with 5.000 CFU of *Listeria Monocytogenes* expressing OVA (LM-OVA). The spleen was recovery on different days of the immune response to study pro-inflammatory (day 1 to 4) and cytotoxic (day 7) effector function on OT1 cells.
B) Pro-inflammatory OT1 effectors isolated at day 2,5 post-infection were injected at numbers corresponding to expected physiological conditions (80 cells) or higher (600 cells) into a braquial lymph node (BRLN) of a host C57BL/6 mouse. 24h later, the presence of different types of leukocytes recruited to the BRLN was evaluated.

IV. Adoptive transfer of TCR-Tg T cells system

This system has several advantages. First, by transferring high numbers of antigen-specific cells into the host, it allows to **study the diversity of specific T cells responses at early time points**, when endogenous specific cells are rare and difficult to detect. Secondly, by using their **congenic marker**, it allows the identification of antigen-specific cells independently of TCR surface expression levels. Third, by bearing defined TCRs (monoclonal population), it ensures that putative heterogeneity that might arrive during immune response is generated independently of the TCR affinity differences, allowing to **study the same clone in different infectious contexts.**

Two main TCR-Tg CD8 mouse strains were used to study the diversity of *in vivo* CD8 T cell responses: **OT-1** and **P14**. Two different live pathogens were utilized to specifically activate them *in vivo*: the bacteria *Listeria monocytogenes* (LM) and the Lymphocytic Choriomeningitis virus (LCMV).

- Briefly, certain numbers of naïve monoclonal **TCR-Tg CD8 donor cells,** recovered from LNs of **OT-1** or **P14** mice (on $Rag^{-/-}$ background), were intravenously injected into syngenic **C57BL/6 host mice**, which expressed an allelic form of CD45 or CD90 distinct from the donor's cells. Afterwards, mice that had received OT-1 cells ($OVA_{257-264}$ TCR specific) were immunized with recombinant *Listeria monocytogenes* expressing OVA (**LM-OVA**), whereas mice that had received P14 cells (GP_{33-41} TCR specific) were immunized with **LCMV** (the glycoprotein GP33 is naturally expressed on this virus surface), or alternatively, they were immunized with recombinant *Listeria monocytogenes* expressing the LCMV's GP33 epitope (**LM-GP33**).

Additionally, we also used the **anti-HY** TCR-Tg CD8 **mouse strain** to investigate diversity of CD8 T cell responses in the absence of pathogen-derived innate signals (**pathogen free or sterile immunization protocol**).

- In brief, monoclonal TCR-Tg CD8 cells or TCR-Tg CD4 cells specific for the male antigen were recovered from LNs of **HY female mice** (on $Rag^{-/-}$ background) **(donors)**. Together with CD4 helper T cells (purified from a female C57BL/6), those TCR-Tg CD8 T cells (specific for male antigen) were intravenously injected into **syngenic $Rag2^{-/-}$ female mice (hosts)**, which were **previously reconstituted** with a mix of female and male cells. These reconstituting cells were obtained from bone marrow of a female and male $CD3^{-/-}$ mouse, in which the **male cells act as antigen providers/immunizing agent** (Peixoto *et al.* 2007).

V. Single - cell multiplex RT-PCR method

To monitor the complexity and the diversity of CD8 T cell responses to *in vivo* antigenic stimulation, we recovered CD8 T cells at several time points after infection, or male immunization, and we studied their gene expression profile by using the sensitive single-cell multiplex RT-PCR method developed in our laboratory. This method allows **simultaneous quantification** and comparison of the expression of 20 genes in the same cell, **without further *in vitro* manipulation** (Peixoto *et al.* 2004).

- Briefly, single-cells are sorted, lysed and the mRNA is retrotranscribed using specific 3'primers. A first round of PCR amplification follows in the presence of both the 3' and 5' primers for all 20 different genes in the same reaction (multiplex amplification). Amplified products are then split into individual wells, and a second seminasted real-time or qualitative PCR amplifies each individual gene separately (Fig. 14). To maintain the initial abundance relationships, this technique requires an accurate control of PCR-amplification's efficiency and competition. The former, to guarantee maximal and uniform amplification for all genes. The later, to avoid inhibition between primers and/or amplicons during multigene amplification.

Using this powerful technique we can determine the **frequency** of cells expressing each gene (evaluate cell heterogeneity inside a population), and also assess the gene **co-expression** in the same cell (indicative of their potential function). This technique is particularly useful when studying **rare populations** and when **antibodies are not available** for protein detection. Moreover, since this technique allows a precise quantification of the absolute number of mRNA molecules per cell, ranging from 2 to $1{,}28 \times 10^9$ for each individual gene, it permits a **clearly discrimination between expressing and non-expressing cells** when molecules are poorly expressed (clear cut between positive and negative cells for a given gene expression).

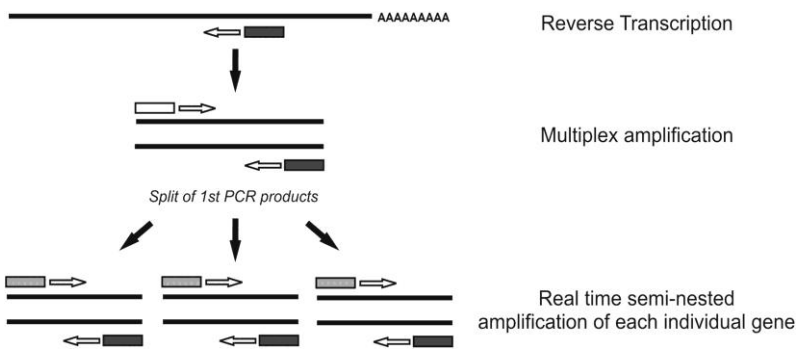


Figure 14. Outline of the quantitative single-cell multiplex RT-PCR technique (Peixoto *et al.* 2004).

Results

Article I

Epitope specificity and relative clonal abundance do not affect CD8 differentiation patterns during Lymphocytic Choriomeningitis Virus infection

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Epitope Specificity and Relative Clonal Abundance Do Not Affect CD8 Differentiation Patterns during Lymphocytic Choriomeningitis Virus Infection[▽]

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To evaluate the impact of immunodominance on CD8 T-cell properties, we compared the functional properties of dominant and subdominant populations in the response to lymphocytic choriomeningitis virus (LCMV). To improve functional discrimination, in addition to the usual tests of phenotype and function, we used a sensitive technique that allows the screening of all CD8 effector genes simultaneously in single cells. Surprisingly, these methods failed to reveal a major impact of clonal dominance in CD8 properties throughout the response. Aiming to increase clonal dominance, we examined high-frequency transferred P14 T-cell receptor transgenic (TCR Tg) cells. Under these conditions LCMV is cleared faster, and accordingly we found an accelerated response. However, when Tg and endogenous cells were studied in the same mice, where they should be subjected to the same antigen load, they showed overlapping properties, and the presence of P14 cells did not modify endogenous responses to other LCMV epitopes or a perturbed immunodominance hierarchy in the memory phase. Using allotype-labeled Tg cells, we found that during acute infection up to 80% downregulated their TCR and were undetectable by tetramer binding, and that tetramer-negative and tetramer-positive cells had very different features. Since Tg cells are not available to evaluate immune responses in humans and, in many cases, are not available from the mouse, the tetramer-based evaluation of early immune responses in most situations of high viremia may be incomplete and biased.

The lymphocytic choriomeningitis virus (LCMV)-induced immune cell response in mice is particularly impressive in its breadth, since at the peak of the response ~90% of activated splenic CD8$^+$ T cells are directed against 28 defined epitopes in H-2^b mice (23, 24, 27). The immunodominance hierarchy then observed may be determined by a variety of parameters, including epitope prevalence, antigen processing and/or its binding affinity to major histocompatibility complex (MHC), T-cell precursor frequency and/or recruitment, and T-cell receptor (TCR) affinity and avidity (55). However, the extent to which this immunodominance guides functional performance still is unknown. Related to this issue is the question of whether the information generated from studies involving artificially induced immunodominance by the adoptive transfer of TCR transgenic (Tg) cells at a high precursor frequency can be generalized to endogenous cells, which are present in small numbers (10^{-4} to 10^{-5}) and consist of polyclonal T-cell subpopulations with different avidities. It was suggested recently that CD8$^+$ TCR Tg cells originating from precursors introduced at unnaturally high frequencies exhibit altered differentiation during infection, as they were shown to reexpress CD62L and interleukin-7R (IL-7R) much sooner than endogenous cells (2, 26). However, it remains controversial whether these findings reflect, as suggested, major differences in differentiation pathways (2, 26) or whether the observed dissimilarities are due to differences in differentiation kinetics (35).

Cytotoxic effector CD8$^+$ T cells generated in various infectious models traditionally were regarded as uniform populations that could secrete gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α) upon in vitro restimulation and exert cytotoxic effects (12). However, the current methods used to study CD8 function during immune responses have several limitations. Cells producing cytokines usually are not detected directly ex vivo, because these proteins, once produced, are immediately secreted into the environment and do not accumulate inside the cell in amounts sufficient to be visualized by intracellular staining. Therefore, cytokine production currently is detected after in vitro restimulation, but under these conditions antigen-experienced cells from a normal response (where cells are not tolerized) all score very similarly, i.e., it is no longer possible to distinguish the expansion phase, effector peak, or memory cells' cytokine expression capacities; these tests similarly identify all antigen-specific cells throughout the

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response (29). Concerns were raised that the in vitro restimulation necessary for revealing many functional traits could result in the erroneous overestimation of the number and quality of effector cells present in situ at any given time. Indeed, it was shown that in vitro restimulation could induce major alterations in ex vivo readouts: IFN-γ mRNA expression frequencies of 10% evaluated ex vivo were shown to increase to 90% (47), and TNF-α expression increased from <1 to 100% after a 4-h peptide stimulation (31). These differences may be due to the organ's three-dimensional structure, which significantly modifies CD8 responses (38). In addition, cytokine secretion greatly depends on the strength of stimulation (16, 37). Therefore, the in vitro environment may fail to reproduce the in vivo cell interactions, the peculiar inflammatory environment induced by the infection, and the local amount of pathogen-derived peptides.

To monitor CD8 differentiation as it unfolds in vivo, we recently developed a sensitive reverse transcription-PCR (RT-PCR) method capable of measuring the expression of up to 20 genes simultaneously in the same cell without further in vitro manipulation. We showed that this method allows a much better discrimination of cell properties throughout the immune response compared to that of more conventional approaches (28, 32, 33). We could discriminate very different cytokine mRNA expression profiles at different phases of the response. These and other gene expression profiles predicted very different functional properties of CD8 T cells in early expansion, response peak, or memory phase that were confirmed by in vivo functional tests. Notably, we also found that the coexpression frequency of mRNAs coding for perforin and granzyme B in the same cell directly predicted CD8 T cells' cytotoxic capacity (32).

As the approach described above provided us with a more detailed analysis of the behavior of $CD8^+$ T cells during immune responses, we applied it together with other more conventional approaches to study the influence of clonal dominance in the behavior of CD8 T cells after infection. We studied endogenous cells responding to immunodominant (NP396 and GP33) and subdominant (GP276) LCMV epitopes and found they had similar properties, suggesting that the infectious environment rather than TCR specificity or relative clonal abundance had the major influence in shaping T-cell properties. To amplify differences in relative clonal abundance, we further compared high-frequency transferred TCR Tg cells specific for the GP33 epitope (P14) to the endogenous cells recognizing the same or other LCMV peptides. Surprisingly, we found that previously reported differences in Tg behavior (2) could be fully explained by differences in the response kinetics, since they were not found when Tg and endogenous cells were studied in the same mouse. Moreover, P14 transfers did not modify the endogenous response to other LCMV epitopes or the immunodominance hierarchy in the memory phase. Finally, in these adoptive transfer studies we could monitor the transferred Tg population by both allotype labeling and GP33 tetramer binding. We found that during the expansion phase, a substantial fraction of allotype-positive Tg cells downregulated TCR expression and could not be recognized by tetramer binding, and that tetramer-negative (tet^{neg}) and tetramer-positive (tet^{pos}) cells had very different properties. These results reveal that the evaluation of the early immune response in normal individuals by tetramer binding is incomplete and may be very biased. Thus, TCR Tg cells, because of their ease of detection by allotype markers, may provide the only means of accurately characterizing the entire spectrum of activated CD8 T cells in the early stages of the immune response.

MATERIALS AND METHODS

Mice. CD45.2 $Rag2^{-/-}$ P14 TCR Tg mice (P14) expressing a TCR specific for LCMV epitope GP33-41 (GP33) and backcrossed onto the C57BL/6 (B6) background were bred at the Centre de Distribution, Typage et Archivage (CDTA, Orléans, France). B6.CD45.1 and B6.CD45.2 mice were purchased from Charles River (Margate, United Kingdom) and the Jackson Laboratory (Bar Harbor, ME). Animal studies were carried out according to United Kingdom Home Office regulations or the University of Massachusetts Medical School, Department of Animal Medicine, regulations and were approved by the site ethical review committee.

Antibodies, MHC class I (MHC-I) tetramers, and other reagents. Labeled antibodies to CD8, CD45.2, CD45.1, TCRαβ, CD69, CD3, Ly6C, CD25, CD27, CD127, CD44, CD122, KLRG1, IFN-γ, TNF-α, and IL-2 and isotype-matched control antibodies were from either BD Biosciences (San Jose, CA) or eBioscience (San Diego, CA). Granzyme B was from Caltag. GP33-41 $H\text{-}2D^b$ (GP33), NP396-404 $H\text{-}2D^b$ (NP396), and GP276-286 $H\text{-}2D^b$ (GP276) tetramers were obtained from Beckmann Coulter (Marseille, France). 5-(6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) was obtained from Molecular Probes (Eugene, OR).

Viral growth and titration and infection of mice. LCMV strain Armstrong (clone 5.3b) was grown in BHK-21 cells, and infectious LCMV was quantitated by plaque assay on Vero cell monolayers as previously described (9). B6 mice were infected intraperitoneally with 2×10^5 PFU of LCMV Armstrong. Some animals were inoculated with 5×10^3 or 5×10^5 TCR Tg cells (prepared from the lymph nodes of P14 mice) 1 day prior to infection.

CFSE labeling, intracellular staining, and in vivo cytotoxicity assays. CFSE labeling was done by incubating cells for 10 min at 37°C with 1 μM CFSE in RPMI medium. Cells were labeled with CFSE by incubation for 10 min at 37°C with 1 μM CFSE in RPMI. For intracellular cytokine staining, splenocytes from LCMV-infected mice were incubated without peptide or with 0.2 to 0.4 μg/ml NP396, GP33, or GP276 peptide for 5 h in the presence of 10 μg/ml of brefeldin A, and then cytokine levels were determined. Granzyme B staining was performed without restimulation. Intracellular staining was performed using the Cytofix/Cytoperm kit (BD Biosciences, San Jose, CA) according to the manufacturer's instructions. In vivo cytotoxicity assays were performed as previously described (4). Briefly, a mixture of 10^7 GP33 or NP396 peptide-pulsed (1 μM) CFSE-labeled and 10^7 nonpulsed nonlabeled splenocytes was injected intravenously into LCMV-infected or control mice at 8 and 60 days postinfection. Peptide-specific cytotoxicity was determined in the spleen 12 h later and was calculated using the following formula: $100 - \{100 \times [(\%\ \text{peptide pulsed infected}/\%\ \text{peptide nonpulsed infected})/(\%\ \text{peptide pulsed control}/\%\ \text{peptide nonpulsed control})]\}$.

Single-cell purification and gene expression analysis. The purification and single-cell sorting of CD8 T cells was described previously (32). P14 cells were distinguished from endogenous GP33-specific cells by the expression of a congenic marker. Each individual cell was analyzed for the coexpression of mRNAs coding for TGF-β (*Tgfb1*), TNF-α (*Tnf*), IL-2 (*Il2*), IFN-γ (*Ifng*), perforin (*Prf1*), granzyme A (*Gzma*), granzyme B (*Gzmb*), FasL (*Fasl*), and CD3ε (*Cd3ε*), the latter to ensure CD8 sorting specificity. The accuracy and efficiency of the method were described previously (33).

Immunosuppression protocol. Two months after LCMV infection, mice were depleted of T cells by the intraperitoneal injection of 500 μg of anti-CD8α antibody (clone 53.6.7) and 500 μg of anti-Thy1.2 antibody (clone 30H12) twice per week for five consecutive weeks, and lung, lymph nodes, spleen, kidney, testes, brain, liver, and bone marrow were harvested for the determination of virus titers. Virus titers in spleen, serum, or other tissues were determined by plaque forming (49).

Statistical analysis. Associations or dissociations between the pattern of expression of different genes and differences in the expression of individual genes between different populations of cells were analyzed using a two-tailed Fisher's exact test. A P value of <0.05 was considered statistically significant.

Gene nomenclature. Abbreviations used for mRNAs were those recommended by the International Committee on Standardized Genetic Nomenclature for Mice.

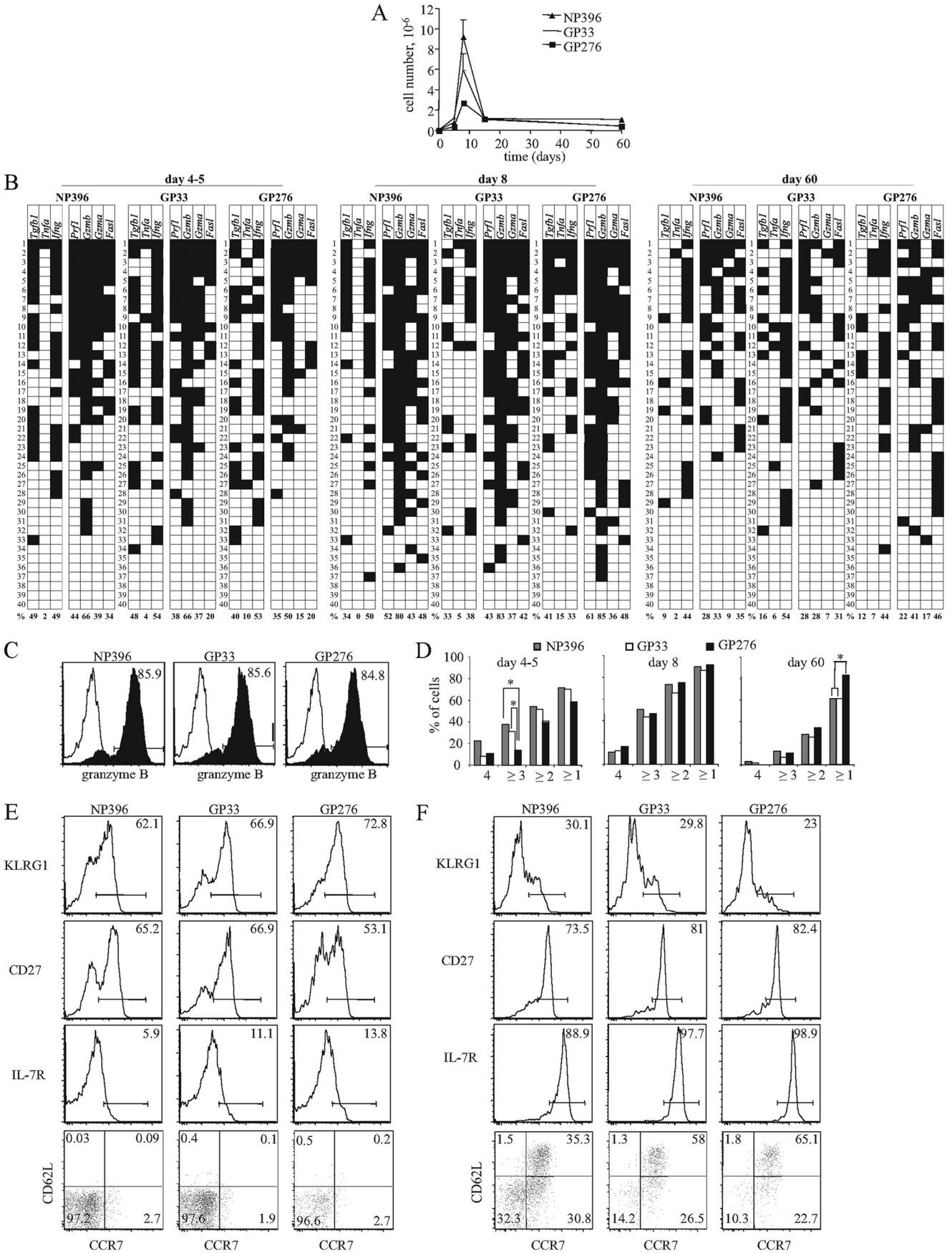
RESULTS

Endogenous CD8$^+$ T cells with different epitope specificities exhibit similar differentiation patterns after LCMV infection. Although a reproducible response hierarchy is found during LCMV infection in mice (15, 29), it still is unclear whether dominant and subdominant T cells are functionally distinct and/or whether their differentiation kinetics differ. To address this, first we screened responding cells for the expression of 14 effector genes known to be expressed by T lymphocytes (32). We found that during LCMV infection, only eight of these genes were expressed. We evaluated the pattern of these genes' expression in CD8$^+$ T-cell populations specific for two dominant LCMV epitopes (NP396 and GP33) and one subdominant epitope (GP276) following the infection of mice with LCMV Armstrong, which should cover all effector mediators during this response. The ratio between the most and the least abundant populations (NP396 and GP276 specific) was approximately 1:3 to 1:4, and the size of the GP33-specific population was between these levels (Fig. 1A), which is consistent with observations made in previous studies (29). At days 4 to 5 postinfection, ex vivo cytokine gene expression was identical between cells responding to all three epitopes, and approximately half of screened cells expressed *Ifng* and *Tgfb1* (although they were not always coexpressed), while *Tnf* was expressed in only a minority of cells (Fig. 1B). The frequency of cells expressing effector molecules with direct cytotoxic potential (*Gzma*, *Gzmb*, and *Prf1*) was indistinguishable between GP33- and NP396-specific populations. The only statistically significant difference between the two dominant and the subdominant population was that the latter expressed less *Gzma* (Fisher's exact test; $P = 0.0236$ for NP396 versus GP276 and $P = 0.0172$ for GP33 versus GP276). However, at day 8 the differences were abolished and all populations expressed the individual genes with a similar frequency (Fig. 1B). At the same time, all of the cytotoxic effector genes (*Prf1*, *Fasl*, *Gzma*, and *Gzmb*) reached the peak of their expression. Due to a lack of suitably optimized antibodies to evaluate native perforin, we could quantitate protein levels of only granzyme B at the single-cell level. Intracellular staining for granzyme B demonstrated that mRNAs were actively translated into large amounts of protein, as the percentages of cells expressing granzyme B mRNA and protein were approximately equal (Fig. 1C). To simplify the analysis of gene coexpression (and to provide an estimate of the cytotoxic potential of the cells in each population), we determined the number of cells that coexpressed cytotoxic genes (*Prf1*, *Gzmb*, *Gzma*, and *Fasl*) and calculated the cumulative proportion of cells expressing all four of these mRNAs, ≥ 3, ≥ 2, or ≥ 1 (Fig. 1D). On day 8 postinfection, approximately 90% of all cells expressed at least one of these cytotoxic effector genes, while 40 to 50% coexpressed three or more.

We have shown previously that during maturation from the effector to the memory phase, OT-1 and HY TCR Tg cells markedly reduced effector gene expression and coexpression (32). These findings were confirmed in all three endogenous populations analyzed here. At day 60, the frequency of the expression of the majority of effector genes was much lower than that at the peak of cell expansion, and the cells showed a low level of gene coexpression (less than 30% of cells expressed two or more genes together) (Fig. 1B and D). While a similar proportion of NP396- and GP33-specific cells expressed at least one of the cytotoxic effector genes, a slightly higher proportion of GP276-specific did so (Fig. 1D). Importantly, however, the percentage of cells expressing each individual gene was not significantly different in memory populations of different immunodominance. Overall, these results show that dominant and subdominant populations do not show major differences in effector gene expression patterns. Besides, cytokine expression after T-cell activation previously failed to discriminate between differences of cell populations recognizing different LCMV peptides (29).

We next determined if differences in immunodominance have an impact on the expression of cell surface markers associated with CD8 differentiation. At the peak of the response, LCMV-specific T cells had fully downregulated CCR7, CD62L, and IL-7R and upregulated CD27 and KLRG1 (Fig. 1E). While dominant NP396- and GP33-specific cells scored similarly for all of these parameters, GP276-specific cells showed a slight decrease in CD27 expression and an increase in KLRG1 expression. This difference could be due to the delayed kinetics of GP276-specific cells we already detected in our gene expression analysis and that disappeared in the memory phase. As described previously, LCMV-specific memory cells reexpressed CCR7, CD62L, and IL-7R, further upregulated CD27, and downregulated KLRG1 (Fig. 1F) (41, 50). Since, in contrast to what is found in human T cells, CCR7 and CD62L are not necessarily coexpressed in mouse memory cells (42), we evaluated the coexpression of these two markers. Indeed, we found a significant fraction of CCR7$^+$ CD62L$^{low/-}$ cells in all LCMV-specific memory populations, i.e., these cells could not be classified as either T-cell central memory (T_{CM}) or T-cell effector memory (T_{EM}) (Fig. 1F). Besides, both CCR7$^-$ and KLRG1$^+$ cells expressed IL-7R, in contrast to what is found in human cells. Therefore, the overall phenotype of LCMV-specific mouse memory cells contrasts to that found in human populations, where T_{CM} CCR7$^+$ cells express CD62L and the loss of CCR7 and KLRG1 correlates with IL-7R downregulation. It also must be noted that human T_{CM} and T_{EM} populations have very different functional properties and gene expression profiles even when studied using our single-cell strategy (25, 28), while the gene expression of the NP396-specific cell cohort, which had more CCR7$^-$ and CD62L^{low} cells, was similar to that found in other cell types (Fig. 1B). These results indicate that T_{CM} and T_{EM} human memory populations have no direct equivalent in the mouse.

The comparison of memory cell phenotypes showed variations both between individual mice studied in the same experiment and between experiments (see below). However, the NP396-specific cohort frequently had higher frequencies of CCR7$^-$ CD62L$^-$ cells and a slight reduction of CD27^{high} representation than cells with other LCMV specificities, but other phenotypes were equivalent. These results confirm that cells recognizing the NP396 epitope have a slower kinetics of CD62L upregulation (35). However, the reduced expression of CD62L in the dominant NP396-specific population contradicts the notion that more abundant clones preferentially upregulate CD62L (30).


A
cell number, 10^{-6}
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NP396
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day 4-5
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E
KLRG1
CD27
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CCR7
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58
14.2
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22.7

Long-term gene expression at the memory stage is not due to viral latency. As CD8$^+$ T-cell differentiation during LCMV Armstrong infection resulted in the generation of a substantial fraction of memory cells expressing mRNAs for at least one cytotoxic effector component, we considered the possibility that viral clearance is incomplete, and that although it is undetectable by plaque-forming assays, virus may be persisting at low levels, as has been found to occur with more pathogenic LCMV strains such as LCMV WE (7). CD8-deficient or CD8-depleted mice are unable to contain the virus (11), so we reasoned that if virus was latent, it would reappear if T cells were depleted. We thus rendered a group of mice that had resolved an acute LCMV infection immunodeficient by depleting them of T cells and tested them for the reemergence of virus. Plaque-forming assays carried out on lymphoid tissues and various peripheral organs (brain, kidney, testis, liver, and lung) of T-cell-depleted animals were all negative (data not shown), strongly arguing against the possibility that chronic low-level stimulation by LCMV was provoking long-term gene expression.

We have noted previously that the quantity of mRNA expression for each memory CD8 T cell was significantly below the levels found at the peak of the response (32). Moreover, memory cells did not express measurable levels of granzyme B protein (Fig. 2A) and did not secrete IFN-γ without restimulation (data not shown), confirming previous findings (51). Nevertheless, in contrast to naïve cells, they were capable of performing peptide-pulsed target elimination after 12 h, which was remarkably similar to target elimination by effector cells (Fig. 2B). This analysis confirmed our previous findings that mRNA profiles constitute sensitive means of predicting the cytotoxic potential of CD8 cells (32).

Monoclonal TCR Tg cells and the endogenous cells of the same specificity have identical differentiation patterns. As we did not observe any striking differences in the expression profiles of several cytokine and cytotoxic effector molecules between the dominant and subdominant populations, we asked if a more robust immunodominance hierarchy established upon the adoptive transfer of high numbers of TCR Tg cells, in which the latter would dominate the endogenous repertoire, would reveal the differences in effector and memory cell generation. We initially compared the functional properties of LCMV-specific memory cells generated after the adoptive transfer of large (5×10^5) and small (5×10^3) numbers of P14 cells. Given an estimated engraftment level of 10% (5), the low-dose adoptive transfer likely would have resulted in a precursor frequency approaching that of the endogenous GP33-specific precursors, while the high dose likely exceeded it by more than 100-fold (5, 24, 30). Importantly, memory P14 cells originating from large and small cell numbers had similar IFN-γ and TNF-α secretion potential upon in vitro restimulation (Fig. 3A), arguing against the previously suggested idea that a shifted CD8$^+$ T-cell maturation at the beginning of the response has a long-term effect on memory T-cell functions (2).

We next compared the early phases of the response. The

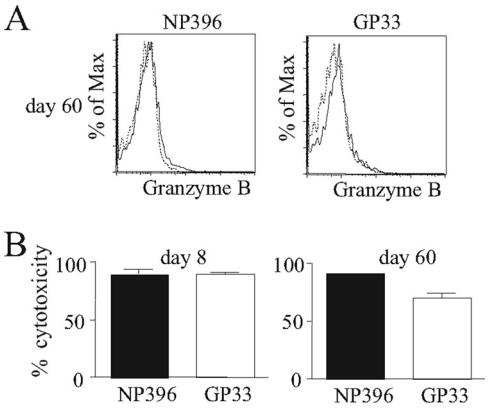

FIG. 2. Long-term gene expression in memory cells is not due to viral latency. B6 mice were infected with 2×10^5 PFU of LCMV Armstrong. (A) Intracellular granzyme B expression was analyzed on days 8 and 60 postinfection for the indicated epitope-restricted populations (black line). The isotype control staining of the same cell populations also is shown (dashed line). The profile shown is representative of results obtained from three individual mice. (B) An in vivo cytotoxicity assay also was performed on days 8 and 60 postinfection, and specific cytotoxicity is depicted; the results shown are the mean of data from three individual mice, and the error bars indicate standard errors of the means.

FIG. 1. Differentiation patterns of CD8 cells recognizing dominant and subdominant LCMV epitopes show marked similarity. B6 mice were infected with 2×10^5 PFU of LCMV Armstrong. (A) The number of NP396-tet$^+$, GP33-tet$^+$, and GP276-tet$^+$ cells in the spleen was analyzed at different time points after infection. The results shown are the means of values from three to six mice tested in two separate experiments, and the error bars indicate one standard error above the means (SEM). (B) Individual cells of each epitope specificity were recovered at the indicated times (days) postinfection from six individual mice in two independent experiments and were tested directly ex vivo for the coexpression of the indicated effector mRNAs. Forty-five to 90 cells of each specificity were evaluated per time point. Only wells that were positive for *CD3ε* (indicating that they contained a cell) were included in the analysis. Since we did not find significant variation between mice and between experiments, the data were pooled. Each horizontal row represents the pattern of gene expression in the same single cell; representative results from 40 cells are shown. Gene expression is indicated in black, and negative results are shown in white. Cells are ordered by the number of cytotoxic effector genes they expressed. The percentages at the bottom of each column represent the frequency at which the indicated gene was expressed in the whole population analyzed. (C) On day 8 postinfection, NP396-, GP33-, and GP276-reactive cells were identified by tetramer staining, and granzyme B expression in each population was analyzed directly ex vivo by intracellular staining. The filled histograms represent granzyme B staining, and the white histograms show the staining of the same populations with an isotype-matched control antibody. (D) The number of mRNAs for cytotoxic effector genes (*Prf1*, *Gzma*, *Gzmb*, and *FasL*) coexpressed by each cell was calculated (0 to 4). The results are expressed cumulatively as the percentage of cells specific for a given epitope that coexpressed mRNAs for ≥1, ≥2, ≥3, or 4 of these genes at the indicated times postinfection (a cell expressing two genes would be included in both the ≥2 and ≥1 categories. Statistically significant differences (as determined using Fisher's exact test) are marked (*, $P < 0.05$). (E) Phenotypes of LCMV- specific T cells at day 8 after infection. Graphs are from one individual mouse out of six mice studied in two independent experiments showing overlapping results. (F) Phenotypes of LCMV-specific memory cells. Results are from one mouse out of seven studied in three independent experiments. We found considerable variation in the expression of CD62L.

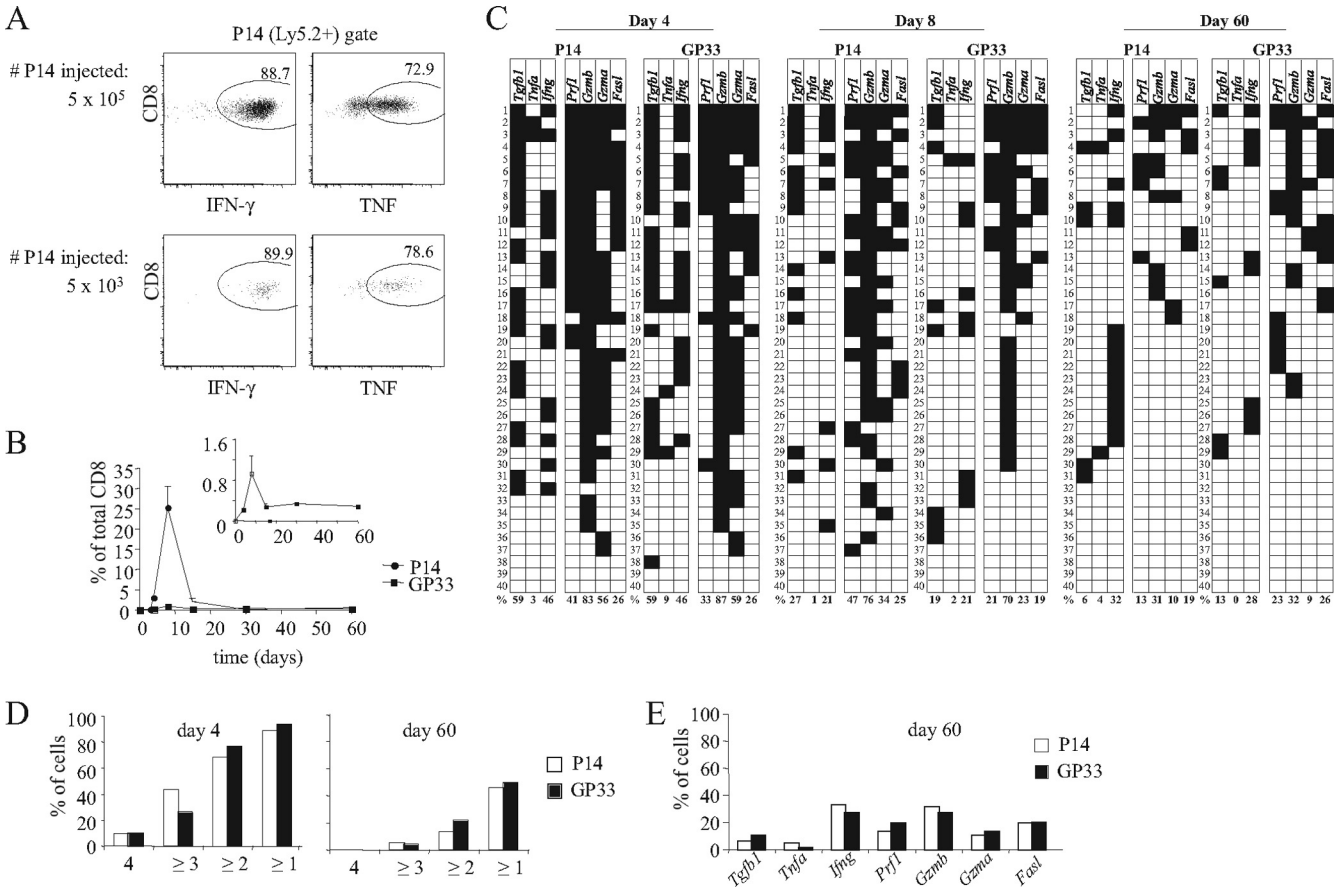

FIG. 3. Differentiation patterns of adoptively transferred TCR Tg cells and endogenous cells recognizing the same epitope in the same mice. (A) B6.Ly5.1 mice were injected with 5×10^5 or 5×10^3 P14 Tg cells (Ly5.2$^+$) and infected with 2×10^5 PFU of LCMV Armstrong. At day 90 postinfection, splenocytes were removed and restimulated in vitro with the GP33 peptide, and the proportion of P14 cells producing IFN-γ and TNF-α was analyzed by intracellular cytokine staining. The dot plots show representative results from one animal in each group and are gated on P14$^+$ cells. The percentage of P14$^+$ cells secreting each cytokine is indicated within the dot plots. (B to E) B6.Ly5.1 mice were injected with 5×10^5 P14 Tg cells (Ly5.2$^+$) and infected with 2×10^5 PFU of LCMV Armstrong. (B) The proportion of P14 and endogenous GP33-specific cells in the spleen was analyzed over time in the same mice. The mean results from three individual mice are shown, and the error bars indicate standard errors of the means. The inset shows a magnification of the endogenous cell graph. (C) Gene expression in individually sorted P14 and endogenous GP33-specific cells was analyzed; the results are presented as described for Fig. 1B. (D) The pattern of the coexpression of cytotoxic effector mRNAs also was analyzed and is presented as described for Fig. 1D. (E) The percentage of cells on day 60 expressing each of the indicated genes (cytotoxic effector genes as shown in Fig. 3B) was analyzed and is shown for 90 individually sorted P14 and 70 endogenous GP33-specific cells. The cells were obtained from three individual mice.

differentiation of CD8$^+$ T cells initially present at either high or low precursor numbers was analyzed previously in the different animals. However, adoptive transfers of $>10^5$ TCR Tg cells accelerate the speed of viral clearance (10, 56). It therefore was possible that the observed differences in T-cell differentiation kinetics (2, 26, 45) were attributable to the differences in the antigen clearance between mice left uninjected or injected with high frequencies of naïve cells. We therefore restricted our investigation to the comparison of P14 cells transferred at high precursor frequencies to endogenous cells present in the same mice to allow both populations to have an equal exposure to the infectious environment. The gene expression analysis of naïve P14 cells showed that rare cells expressed either *Tgfb1* or *Prf1* but none of the other effector genes (data not shown). At days 4 to 8 of the response, TCR Tg cells outnumbered endogenous GP33-specific cells by up to 30-fold (Fig. 3B), but the cytokine and cytotoxic effector genes transcribed in both populations were similarly represented. The resemblance between those populations also was apparent on the analysis of cytotoxic gene coexpression (Fig. 3D). Most importantly, memory TCR Tg and endogenous GP33-specific cells had the same expression frequencies for all screened genes (Fig. 3C and E). In summary, once putative differences in antigen loads are avoided, both effector and memory GP33-specific cells developing in the same mice from precursors initially present at widely disparate numbers had equivalent expression profiles for all genes tested.

It also was reported that high-dose naïve TCR Tg cell transfers induced the precocious upregulation of CD62L and IL-7R compared to that of equivalent populations injected at a low frequency (2). To determine if these differences also were due to accelerated response kinetics due to the faster resolution of the infectious stimuli, we compared the expression of these markers in normal mice and in P14-transferred mice infected

simultaneously with LCMV. One week after infection, the endogenous populations in normal mice had fully downregulated IL-7R and CD62L expression, while P14 Tg cells expressed higher levels of CD62L and IL-7R (Fig. 4A). However, this upregulation was not peculiar to populations present in high frequencies. In P14-injected mice, all LCMV-specific populations (either Tg or endogenous) also upregulated these markers. The abundant P14 and the rare GP33-specific endogenous populations of P14-injected mice expressed similar levels of IL-7R. Endogenous NP396- and GP276-specific cells also upregulated IL-7R expression, although they did so at slightly lower levels than those found in GP33-specific T cells. The CD62L expression was upregulated to equivalent levels in both Tg and endogenous cells of all peptide specificities. These results indicate that the precocious upregulation of these markers described after high-frequency adoptive transfers is not a property of dominant clones. Rather, it appears to be the consequence of accelerated response kinetics that are known to occur in these circumstances, since it affects all LCMV-specific populations present in the same mouse. In addition, we also failed to confirm that Tg memory cells expressed a predominantly CD62L^{hi} phenotype (Fig. 4B). Rather, we found a significant variation in different mice studied in the same experiment and between experiments.

Finally, we evaluated the impact of high-dose P14 adoptive transfers on endogenous response. As reported previously (5) and shown in Fig. 1, the absolute number of endogenous GP33-specific cells was reduced in P14-injected mice compared to that found in normal mice infected simultaneously (Fig. 4C). Surprisingly, P14 adoptive transfers did not substantially affect the T-cell responses to other LCMV epitopes (Fig. 4C and D). At both the response peak and at the memory phase, the number of NP396- and GP276-specific cells determined either by tetramer staining or their capacity to secrete cytokines after in vitro stimulation (Fig. 4D) was similar in P14-injected and normal mice. We conclude that the injection of P14 cells accelerates response kinetics, as shown by the modifications of IL-7R and CD62L in all cells responding to LCMV in P14 injected mice. Otherwise, it does not affect the properties of LCMV-specific cells or influence the endogenous responses to other LCMV epitopes.

TCR downregulation at the early stages of the response masks the detection of Ag-specific cells. A potential drawback to the use of tetramer staining to identify epitope-specific T cells for functional profiling is the TCR downregulation that follows T-cell activation. We tested whether tetramer staining is a reliable marker of Ag-specific cells during the immune response. The Tg cells that can be identified by an allotype marker showed a substantial loss of surface tetramer labeling during the expansion period. This effect was not immediate, sparing the first 2 days of the response, when TCR-Tg cells were activated but most had not divided (not shown). At day 3 of the LCMV response, we saw considerable mouse-to-mouse variability, with 30 to 70% of P14 cells being undetectable by tetramer labeling. By day 5, less mouse-to-mouse variation was observed, yet 25% of P14 cells did not label with tetramers (Fig. 5A). The comparison of tet^{neg} and highly tetramer-positive (tet^{hi}) populations showed that the former did not express CD3, confirming that the loss of tetramer binding was caused by TCR downregulation (Fig. 5B, upper row). We further tested if TCR downregulation could bias the evaluation of the properties of antigen-specific cells during the response. Indeed, tet^{neg} and tet^{hi} cells on day 3 of infection differed in CD69, CD27, CD25, and Ly6C expression levels (Fig. 5B, lower row), while IL-7R, CD122, and CD44 were expressed similarly (data not shown). To investigate if such downregulation was just an artifact induced by high-frequency transfers or could occur when antigen-specific naïve cells were present at a physiologic number, we studied Tg cells injected at low frequency. As expected, the kinetics of the Tg cell response was much slower. At day 4, TCR downmodulation was evident but Tg cells were very rare. We found a considerable TCR downmodulation even at day 5 of the response, when more than half of the Tg pool was failing to bind tetramers (Fig. 5C).

We aimed to investigate if TCR downregulation also could bias the evaluation of the normal endogenous response. Since tet^{neg} endogenous cells cannot be visualized, we compared tet^{hi} and tetramer-intermediate (tet^{int}) cells in normal mice (Fig. 5D). Importantly, tet^{int} cells had substantially higher granzyme B expression than tet^{hi} cells (Fig. 5D), directly linking the activation status measured by TCR downmodulation to a different effector profile. Thus, although we lack the means to test for the endogenous cells that are completely tet^{neg} (since these cells do not express TCR they also should not score as IFN-γ producers after in vitro stimulation), our results strongly suggest that our current methods of detection fail to identify a substantial fraction of antigen-specific cells during the expansion phase, and moreover, they introduce bias in the evaluation of the properties of antigen-specific cells from normal mice.

Cytokine and cytotoxic effector gene expression are not synchronized. As T cells do not reach lymphoid organs synchronously and are exposed to highly varied microenvironmental stimuli, cells at various differentiation stages are found at any given time. Having established that TCR Tg cells allow us to fully assess the early dynamics of the CD8 differentiation, we further subdivided their progression steps by a combination of CFSE and CD69 labeling. CFSE labeling allowed us to focus on the majority of P14 cells (>95%) that have divided four or more times by day 3 (Fig. 6A). The CFSE-low P14 population was further subdivided into more recently and less recently activated subsets on the basis of the differential expression of an early and transient T-cell activation marker, CD69 (34); less advanced CD69^{+} and more advanced CD69^{-} cells were sorted. When gene expression patterns were analyzed, cytokine gene expression was found to differ from the expression of cytotoxic effector genes, and in general, cytotoxic effector genes were transcribed longer than cytokine genes (Fig. 6B). Cytokine expression consistently peaked early (on day 3 or before), there was no difference between CD69^{+} and CD69^{-} cells (Fig. 6B), and a highly significant drop of expression occurred during the next day (Fig. 6C). On the other hand, the transcription of cytotoxic effector genes varied: *Gzmb* and *Prf1* peaked early, while *Gzma* and *Fasl* were present only in a minority of CD69^{+} cells, and a significant rise in their expression occurred as they progressed to the CD69^{-} stage (for *Gzma*, $P = 0.0001$; for *Fasl*, $P = 0.002$). Notably, the generation of *Il2* mRNA was found rarely in ex vivo P14 cells (less than 5% of total cells; data not shown) at any of the stages tested (days 3, 4, 8, 15, 30, and 60). The latter finding casts doubt on the physiological significance of reports that TCR Tg

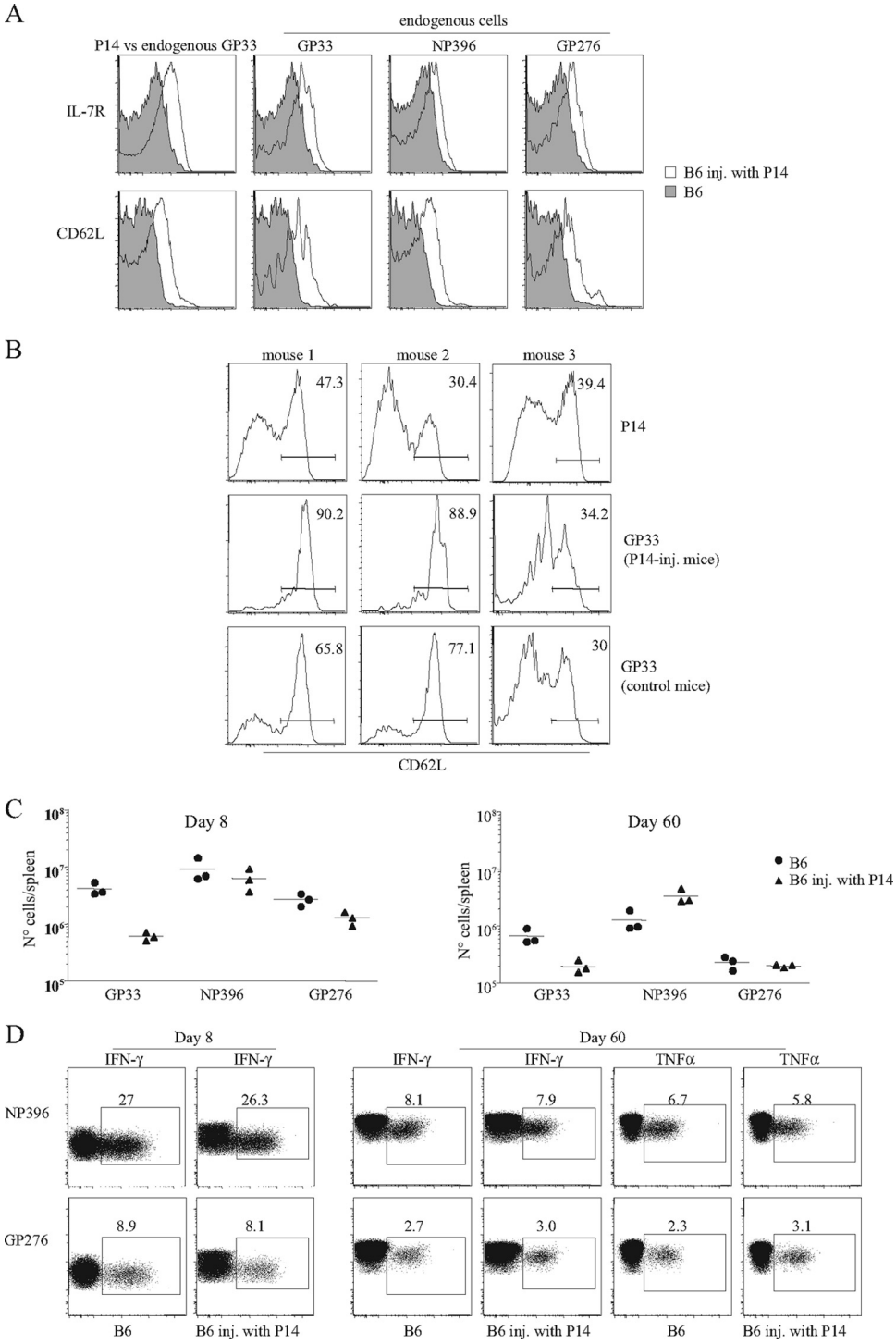

FIG. 4. Impact of high-dose naïve Tg transfers on the endogenous response. B6.Ly5.1 mice left untreated or were injected with 5×10^5 P14 Tg cells (Ly5.2$^+$) and were infected simultaneously with 2×10^5 PFU of LCMV Armstrong and studied at days 8 and 60 after infection. (A) CD62L and IL-7R expression in cells of with different peptide specificities at day 8 after infection. Histograms compare CD62L and IL-7R expression levels of CD8 cells with the indicated peptide specificities in 1 P14 injected (inj.) (open graphs) and 1 noninjected B6 mouse (gray) of 12 mice studied in two independent experiments. On the far left, P14 cells (open histogram) are compared to GP33-specific noninjected mice. (B) Variation of CD62L expression in GP33-specific cells 2 months after infection. Graphs compare Tg cells (upper) to endogenous cells present in the same mouse (middle). The lower graphs show endogenous cells in the mice that were not injected with P14 cells. (C) Absolute numbers of cells of different peptide specificity at day 8 (left) and 2 months (right) after infection. Results show individual mice from one experiment out of two with equivalent results. (D) IFN-γ expression after in vitro stimulation with NP396 and GP276 peptides at day 8 (left) and 2 months (right) after infection.

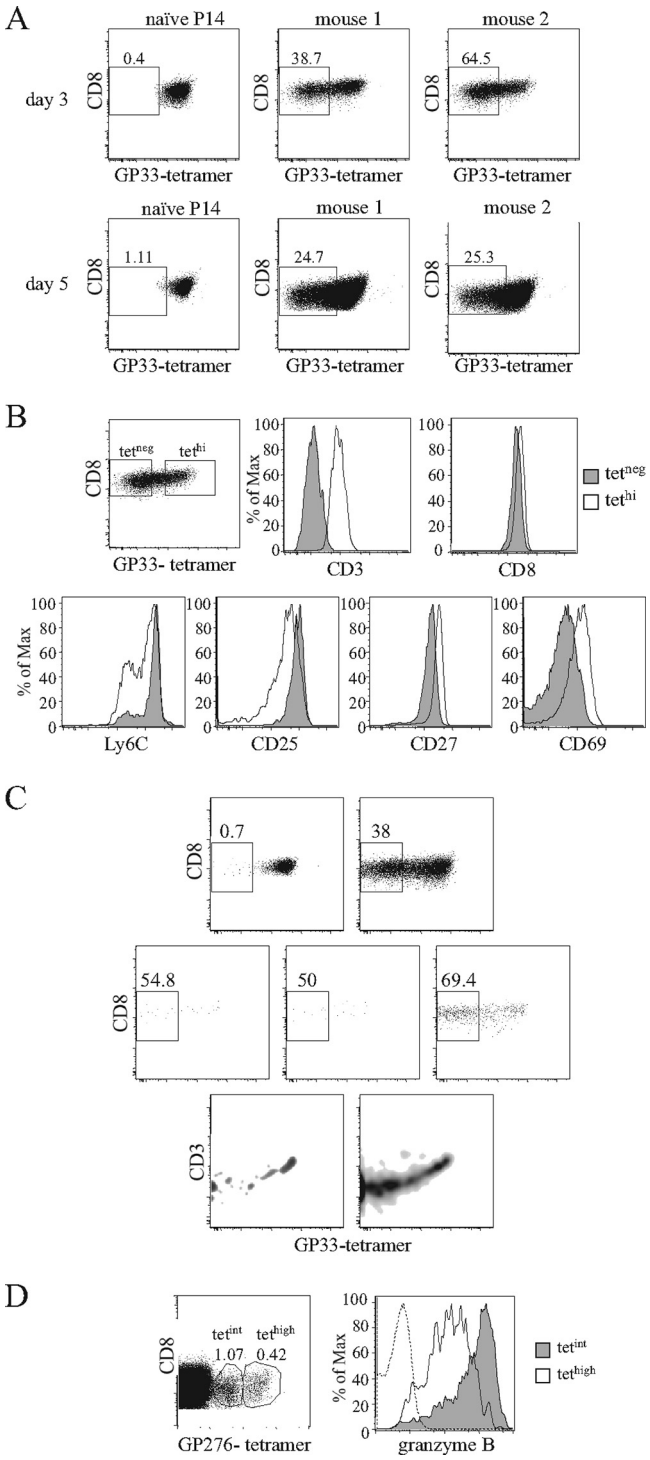

FIG. 5. MHC-I tetramer labeling during the expansion phase. B6.Ly5.1 mice left untreated or receiving Ly5.2$^+$ P14 Tg cells were infected with 2×10^5 PFU of LCMV Armstrong and studied at different time points after infection. (A and B) Mice were injected with 5×10^5 Tg cells. (A) Results compare GP33 tetramer (tet) binding in Ly5.2$^+$ P14 naïve cells and in P14 cells at on day 3 (top) and day 5 (bottom) after infection. Staining is from individual mice representative of four experiments with two to three mice per time point. (B) On day three after infection, P14 cells were arbitrarily subdivided into tet^{neg} (gray) and tet^{hi} (white) subsets, and each population was tested for the expression of the indicated cell surface molecules. Gates for tet^{neg} cells were established in noninfected B6 mice and for tet^{hi} in naïve Tg cells. (C) B6 mice were injected with either 5×10^5 or 10^4 Ly5.2$^+$ P14 Tg cells and studied at days 4 and 5 after infection. Results are for GP33 tetramer binding in P14 Tg cells. Naïve mice (upper left), cells from mice injected with 5×10^5 naïve cells (upper right), and cells from three individual mice injected with 10^4 naïve cells (lower graphs) are shown. At day 4, very few Tg cells were detected in the latter mice. (D) Granzyme B expression in cells expressing different tet binding intensity in normal mice. CD8 cells were recovered 5 days after infection, labeled with GP276 tetramers, and subdivided into tet^{int} and tet^{hi} populations. Results show the gates used for such subdivision and intracellular granzyme B staining for tet^{int} (gray) and tet^{hi} (white) populations; the dashed lines represent the staining of the same cells with an isotype control antibody.

effector CD8 cells arising from high precursor frequencies are more likely to secrete IL-2 upon in vitro stimulation (2). *Il7r* downregulation (Fig. 6C) followed the kinetics previously described on a protein level (19). Furthermore, we observed that *Ccr7* expression rapidly dropped between days 3 and 4, most likely participating in the release of more mature Ccr7$^-$ cells from T-cell-restricted areas of secondary lymphoid tissues. As we have demonstrated that gene expression in LCMV-specific cells evolves very fast over short periods of time and at defined differentiation milestones (such as the CD69$^+$→CD69$^-$ transition), the analysis of other markers of cell progression/fath (17) is expected to provide us with an even richer picture of gene differentiation dynamics and cell heterogeneity.

DISCUSSION

TCR Tg cells commonly are used in the assessment of the properties of T cells. They are easy to manipulate and to visualize. Their defined TCR expression allows one to monitor the same clone throughout the immune response (39, 40). This characteristic is fundamental to determine if the changes in population properties throughout the response are due to the selection of particular clones of antigen-specific cells. However, it was suggested recently that when TCR Tg cells are present at high precursor frequencies, their intraclonal competition for antigen leads to their suboptimal activation and abnormal differentiation (2, 26, 45). Several reasons prompted us to reexamine this claim in greater detail. First, several studies showed that a short-term contact with an antigen is sufficient to trigger a complete CD8 differentiation program (18, 46), and that extensive CD8 expansion is not a prerequisite for efficient memory generation (3). Second, in other studies effector CD8 numbers seem to hit a similar ceiling regardless of initial variability in precursor numbers or specificity (22), arguing for stimulation-tailored rather than T-cell-intrinsic differentiation pathways. Finally, other data suggested alternative explanations to the different behavior of high- and low-density TCR Tg cell transfers. The adoptive transfers of $>10^5$ TCR Tg cells have been shown to alter the kinetics of pathogen clearance and the timing of peak CD8$^+$ T-cell expansion (10, 35, 56). Since high- and low-dose transferred populations were studied systematically in different recipients where antigen loads and antigen clearance are known to be different, alterations in the course of infection could account for the different population properties in mice that received different numbers of TCR Tg cells.

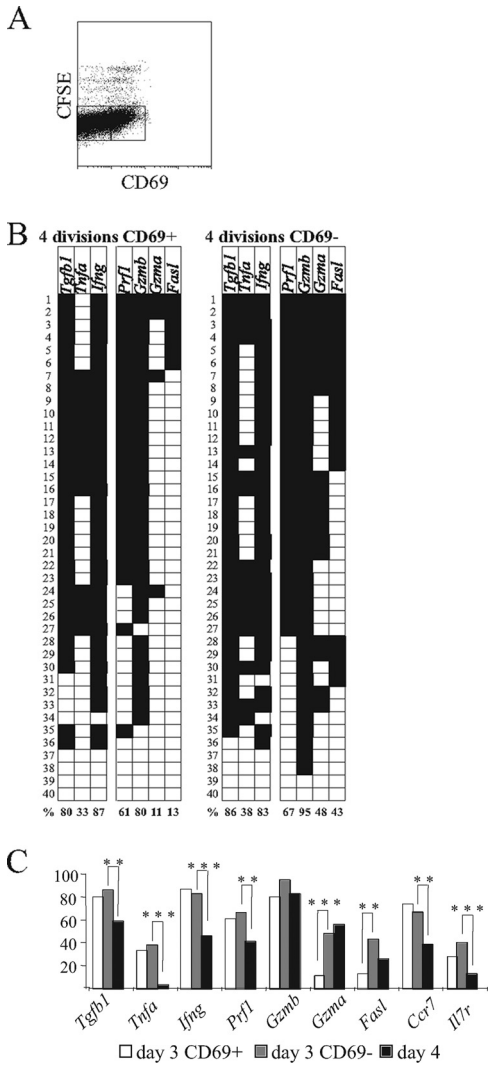

FIG. 6. Rapid progress of $CD8^+$ T-cell differentiation during early infection can be monitored using a combination of TCR Tg cells, CFSE division profiles, and CD69 expression. B6.Ly5.1 mice were injected with 5×10^5 P14 Tg cells ($Ly5.2^+$) and infected with 2×10^5 PFU of LCMV Armstrong. (A) At day 3 postinfection, splenocytes were stained with CD69, and division profiles were analyzed by the evaluation of CFSE expression. Cells that had divided four or more times were sorted into two subpopulations ($CD69^+$ and $CD69^-$) based on the indicated gates. (B) Gene expression in individually sorted $CD69^+$ and $CD69^-$ P14 cells was analyzed on day 3 postinfection; the results are presented in the same format as that used for Fig. 1B. (C) The progression of gene expression in individually sorted P14 cells was analyzed as the linear maturation of cells occurred (i.e., in the following sequence: day 3 $CD69^+$ cells [white]→ day 3 $CD69^-$ cells [grey]→ day 4 [black]). Statistically significant differences between progressive differentiation stages are marked (** and ***, $P < 0.01$ and $P < 0.001$, respectively.

Competition for antigen and clonal competition also occur in normal immune responses and contribute to the immunodominance hierarchy observed. A partial or complete compensation for a loss of a particular epitope by other specificities has been known to occur (1, 21, 24, 36, 44). In some circumstances it has been suggested that the cytokine-mediated active suppression of dominant clones over subdominant ones (immunodomination) occurs (48, 52), but the existence of such active immunosuppression still is disputed (24). Besides, although immunodominance has been studied widely in many infectious models, it still is unclear whether dominant and subdominant populations diverge in their functional capacities and protection capabilities (6, 20, 48). To address these issues, in addition to conventional tests, we performed a powerful single-cell multigene expression study of several antigen-specific populations during the course of LCMV infection in mice. When studied in the same infectious context in the same mice at the same time point of the response, the T-cell populations of different specificities and present at different frequencies showed remarkably similar features. Thus, except at the earliest stages of infection (days 4 to 5) when GP276-specific cells expressed less granzyme A mRNA than NP396- and GP33-specific cells, dominant and subdominant cell effector and memory had remarkably similar cytokine (*Ifng*, *Tnf*, and *Tgfb1*) and cytotoxic gene expression (*Prf1*, *Gzmb*, *Gzma*, and *Fasl*) and coexpression profiles. Previous comparisons of cytokine profiles after in vitro stimulation also failed to reveal major differences (49), and we found that cell surface markers' expression most frequently was overlapping. As an exception, the subdominant GP276-specific population showed some delay in CD27 upregulation and KLRG1 downregulation at day 8, but these differences disappeared in the memory phase, when these cells' phenotypes were equivalent to those found in GP33-specific cells. Conversely, the NP396-specific memory cohort usually had a larger fraction of $CCR7^-$ $CD62L^{low}$ cells than cell populations with other peptide specificities, but otherwise they expressed the same KLRG1 and IL-7R labeling, and it was reported previously that this cell type eventually also upregulates the expression of both of these ligands. Overall, these data directly argue against the hypothesis that dominant and subdominant populations follow disparate differentiation pathways. These findings were confirmed even when major differences in clonal abundance were introduced artificially by the adoptive transfer of Tg cells.

The differentiation profiles of monoclonal T-cell populations recently have fallen under scrutiny, since several reports suggested that the artificial introduction of TCR Tg CD8 cells in numbers exceeding those of endogenous cells of similar epitope specificity (5, 24, 30) resulted in the inadequate differentiation of TCR Tg cells (2, 26, 45). These reports, however, focused mainly on CD62L and IL-7R expression analysis, and functional assays were performed only at a single time point of the infection. These studies also did not take into consideration possible differences in response kinetics that could result from the introduction of a large cohort of naïve Tg cells. Indeed, abundant and rare clone behavior always was studied in different mice, where Tg cells could be submitted to different antigen loads and abundant and rare clone accumulation peaked at different time points (2). Supporting the notion that previously reported differences between high- and low-dose transfers can be explained by a different response kinetics, adoptive transfers of $>10^5$ precursors were shown to accelerate the kinetics of pathogen clearance and CD8 expansion (10, 35, 56).

Contrary to those studies, we compared TCR Tg and endogenous cells of the same epitope specificity from the same animals, where both faced exactly the same antigen exposure

and showed similar response kinetics. Moreover, when Tg cells are present, the endogenous GP33-specific population expands very little, which should prevent any TCR downregulation early in the response. We found that under these conditions, Tg and endogenous GP33-specific CD8 cells retrieved from the same mice always were remarkably similar. They not only had initiated IL-7R and CD62L upregulation precociously at day 8 but also showed similar phenotypes and gene expression profiles at the response peak. The analysis of CD8 T cells with other specificities in these transferred mice also supported the notion that high-dose transfers only accelerate response kinetics. Indeed, we found that in P14-injected mice both NP396- and GP276-specific populations also had initiated IL-7R and CD62L upregulation at day 8 after infection. Surprisingly, these cells appeared to be otherwise unaffected by the presence of high frequencies of TCR Tg cells. Their frequency and their capacity to produce IFN-γ was similar in mice left untreated or receiving P14 Tg cells. These results demonstrate that high-frequency adoptive transfers do not inhibit overall endogenous responses but only influence the expansion of T-cell populations with the same TCR specificity.

Our results also do not support the notion that high-frequency transfers induce major modifications in the properties of memory cells. We demonstrated that memory cells on day 90 that arose from 5×10^3 and 5×10^5 P14 cells did not differ in functional capacities such as stimulation-induced cytokine secretion. We did not find evidence for the predominant generation of CD62L$^+$ Tg memory cells in high-frequency transfers. In our hands, the GP33-specific endogenous memory cells frequently expressed more CD62L than the Tg memory cells. Differences between the present and previously published results (2, 26) could be due to mouse-to-mouse variability, as we found in our experiments, or to the fact that we always evaluated endogenous and Tg cells present in the same mouse. Our results suggest that studies showing a preferential expression of CD62L in high-frequency cells were not exhaustive, and that the conclusion that these cells only generate CD62L$^+$ T_{CM} (a major argument to suggest abnormal differentiation) is unreliable.

Overall, these data suggests that high-frequency adoptive transfers just accelerate response kinetics, and that Tg cells only compete with the endogenous cells that share the same TCR specificity. It is likely that such competition is greatly influenced by the relative avidity/cross-reactivity of the TCR Tg cells with respect to the average avidity/cross-reactivity of the endogenous antigen-specific cells. Different Tg CD8s populations were classified according to these parameters in the hierarchy OT1 > P14 > anti-HY (13), which appears to correlate directly with their inhibitory effect on endogenous responses. Indeed, the transfer of the high-avidity/cross-reactive OT-1 clone virtually abrogates endogenous responses, while P14 transfers have a smaller effect (2). In contrast, in high-frequency anti-HY Tg transfers to normal mice, the endogenous cells partially outcompete the Tg population. Both Tg and endogenous responses show reduced amplitude and become similarly represented in the overall anti-HY response (47).

TCR downregulation is a rapid and dose-dependent corollary of T-cell activation in vitro (43) but is rather transitory, lasting for about 24 h. TCR downregulation also was detected in acute infections in vivo (8, 54), but due to the lack of other markers to identify antigen-specific cells, these previous studies could not evaluate fully the extent of this phenomenon. Here, we established that Tg cells identified by an allogeneic marker, even when present at physiologic frequencies, downregulated TCR expression, and a major fraction fully lost TCR cell surface expression and failed to bind tetramers. This behavior is likely a common feature of CD8 immune responses, since we also found it in other infectious models and in other TCR Tg cells (P14 or OT-1 cells immunized with *Listeria*-expressing GP33 [LM-GP33] or LM-OVA, respectively; unpublished data).

Several aspects of this phenomenon must be emphasized. In contrast to the transient loss of TCR after in vitro activation, in vivo responding populations could remain TCR negative throughout a long time period during the expansion phase; activation status and tetramer binding were inversely correlated, allowing for the possibility that more activated cells could be rendered completely invisible by prominent TCR downregulation.

To summarize, the detailed analysis of CD8 T cells responding to different LCMV epitopes in the same infectious environment showed that relative clone abundance or TCR specificity did not alter substantially the properties of effector and memory cells. From this perspective, the current notion that high-frequency transfers of naïve Tg cells induce abnormal T-cell differentiation must be toned down. We found that differences in Tg behavior can be explained by a different response kinetics, that abundant Tg and rare endogenous cells with the same peptide specificity had overlapping properties, and that Tg cells did not affect the amplitude or the quality of the endogenous response to other LCMV peptides. It also was demonstrated recently that high-frequency transfers did not affect the quality of the memory responses (53). In contrast, the use of TCR Tg cells that can be recognized by allotype markers revealed that during acute infection, when high viral loads are present, a substantial fraction of responding cells downregulate their TCR and fail to bind MHC tetramers, and that tet^{pos} and tet^{neg} cells have different properties. Therefore, TCR-Tg mice may be fundamental for the evaluation of the entirety of the early immune response.

Finally, the important and long-lasting loss of TCR expression we found to occur during the expansion phase has major implications for our capacity to study early events in the vast majority of acute infections in the mouse (when Tg cells are not available) and, more importantly, in humans. Studies based on the tetramer binding identification and/or magnetic bead purification of antigen-specific cells likely are incomplete and biased (14, 30), since they select subpopulations with peculiar properties that do not represent the overall characteristics of the responding peptide-specific cohort. Moreover, it is at present unclear if any of the methods currently used to identify responding cells will be able to do so and in which circumstances. The failure to bind tetramers is due to TCR downregulation. It therefore is possible that the vast majority of tet^{neg} cells also are undetected through cytokine expression after in vitro stimulation with specific peptides, since these responses depend on the cell surface expression of the peptide-specific TCR. Moreover, we found that TCR downregulation increased when higher doses of virus were injected, suggesting

that the higher the viremia the more incomplete will be our assessment of the acute response. This important pitfall must be taken into consideration: we may fail to detect a major cohort of responding cells when high virus loads are present.

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REFERENCES

1. **Andreansky, S. S., J. Stambas, P. G. Thomas, W. Xie, R. J. Webby, and P. C. Doherty.** 2005. Consequences of immunodominant epitope deletion for minor influenza virus-specific CD8$^+$-T-cell responses. J. Virol. **79:**4329–4339.
2. **Badovinac, V. P., J. S. Haring, and J. T. Harty.** 2007. Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8$^+$ T cell response to infection. Immunity **26:**827–841.
3. **Badovinac, V. P., B. B. Porter, and J. T. Harty.** 2004. CD8+ T cell contraction is controlled by early inflammation. Nat. Immunol. **5:**809–817.
4. **Barber, D. L., E. J. Wherry, and R. Ahmed.** 2003. Cutting edge: rapid in vivo killing by memory CD8 T cells. J. Immunol. **171:**27–31.
5. **Blattman, J. N., R. Antia, D. J. Sourdive, X. Wang, S. M. Kaech, K. Murali-Krishna, J. D. Altman, and R. Ahmed.** 2002. Estimating the precursor frequency of naive antigen-specific CD8 T cells. J. Exp. Med. **195:**657–664.
6. **Busch, D. H., I. M. Pilip, S. Vijh, and E. G. Pamer.** 1998. Coordinate regulation of complex T cell populations responding to bacterial infection. Immunity **8:**353–362.
7. **Ciurea, A., P. Klenerman, L. Hunziker, E. Horvath, B. Odermatt, A. F. Ochsenbein, H. Hengartner, and R. M. Zinkernagel.** 1999. Persistence of lymphocytic choriomeningitis virus at very low levels in immune mice. Proc. Natl. Acad. Sci. USA **96:**11964–11969.
8. **Drake, D. R. R., R. M. Ream, C. W. Lawrence, and T. J. Braciale.** 2005. Transient loss of MHC class I tetramer binding after CD8+ T cell activation reflects altered T cell effector function. J. Immunol. **175:**1507–1515.
9. **Dutko, F. J., and M. B. Oldstone.** 1983. Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. J. Gen. Virol. **64:**1689–1698.
10. **Ehl, S., P. Klenerman, R. M. Zinkernagel, and G. Bocharov.** 1998. The impact of variation in the number of CD8$^+$ T-cell precursors on the outcome of virus infection. Cell Immunol. **189:**67–73.
11. **Fung-Leung, W. P., T. M. Kundig, R. M. Zinkernagel, and T. W. Mak.** 1991. Immune response against lymphocytic choriomeningitis virus infection in mice without CD8 expression. J. Exp. Med. **174:**1425–1429.
12. **Glimcher, L. H., M. J. Townsend, B. M. Sullivan, and G. M. Lord.** 2004. Recent developments in the transcriptional regulation of cytolytic effector cells. Nat. Rev. Immunol. **4:**900–911.
13. **Hao, Y., N. Legrand, and A. A. Freitas.** 2006. The clone size of peripheral CD8 T cells is regulated by TCR promiscuity. J. Exp. Med. **203:**1643–1649.
14. **Hataye, J., J. J. Moon, A. Khoruts, C. Reilly, and M. K. Jenkins.** 2006. Naive and memory CD4+ T cell survival controlled by clonal abundance. Science **312:**114–116.
15. **Homann, D., L. Teyton, and M. B. Oldstone.** 2001. Differential regulation of antiviral T-cell immunity results in stable CD8+ but declining CD4+ T-cell memory. Nat. Med. **7:**913–919.
16. **Itoh, Y., and R. N. Germain.** 1997. Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of CD4+ T cells. J. Exp. Med. **186:**757–766.
17. **Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin, and S. M. Kaech.** 2007. Inflammation directs memory precursor and short-lived effector CD8$^+$ T cell fates via the graded expression of T-bet transcription factor. Immunity **27:**281–295.
18. **Kaech, S. M., and R. Ahmed.** 2001. Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells. Nat. Immunol. **2:**415–422.
19. **Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh, and R. Ahmed.** 2003. Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells. Nat. Immunol. **4:**1191–1198.
20. **Kedl, R. M., W. A. Rees, D. A. Hildeman, B. Schaefer, T. Mitchell, J. Kappler, and P. Marrack.** 2000. T cells compete for access to antigen-bearing antigen-presenting cells. J. Exp. Med. **192:**1105–1113.
21. **Kedzierska, K., C. Guillonneau, S. Gras, L. A. Hatton, R. Webby, A. W. Purcell, J. Rossjohn, P. C. Doherty, and S. J. Turner.** 2008. Complete modification of TCR specificity and repertoire selection does not perturb a CD8+ T cell immunodominance hierarchy. Proc. Natl. Acad. Sci. USA **105:**19408–19413.
22. **Kemp, R. A., T. J. Powell, D. W. Dwyer, and R. W. Dutton.** 2004. Cutting edge: regulation of CD8+ T cell effector population size. J. Immunol. **173:**2923–2927.
23. **Kotturi, M. F., B. Peters, F. J. Buendia-Laysa, J. Sidney, C. Oseroff, J. Botten, H. Grey, M. J. Buchmeier, and A. Sette.** 2007. The CD8$^+$ T-cell response to lymphocytic choriomeningitis virus involves the L antigen: uncovering new tricks for an old virus. J. Virol. **81:**4928–4940.
24. **Kotturi, M. F., I. Scott, T. Wolfe, B. Peters, J. Sidney, H. Cheroutre, M. G. von Herrath, M. J. Buchmeier, H. Grey, and A. Sette.** 2008. Naive precursor frequencies and MHC binding rather than the degree of epitope diversity shape CD8+ T cell immunodominance. J. Immunol. **181:**2124–2133.
25. **Lanzavecchia, A., and F. Sallusto.** 2000. Dynamics of T lymphocyte responses: intermediates, effectors, and memory cells. Science **290:**92–97.
26. **Marzo, A. L., K. D. Klonowski, A. Le Bon, P. Borrow, D. F. Tough, and L. Lefrancois.** 2005. Initial T cell frequency dictates memory CD8+ T cell lineage commitment. Nat. Immunol. **6:**793–799.
27. **Masopust, D., K. Murali-Krishna, and R. Ahmed.** 2007. Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: it is even bigger than we thought. J. Virol. **81:**2002–2011.
28. **Monteiro, M., C. Evaristo, A. Legrand, A. Nicoletti, and B. Rocha.** 2007. Cartography of gene expression in CD8 single cells: novel CCR7 subsets suggest differentiation independent of CD45RA expression. Blood **109:**2863–2870.
29. **Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky, and R. Ahmed.** 1998. Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection. Immunity **8:**177–187.
30. **Obar, J. J., K. M. Khanna, and L. Lefrancois.** 2008. Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. Immunity **28:**859–869.
31. **Panus, J. F., L. J. McHeyzer-Williams, and M. G. McHeyzer-Williams.** 2000. Antigen-specific T helper cell function: differential cytokine expression in primary and memory responses. J. Exp. Med. **192:**1301–1316.
32. **Peixoto, A., C. Evaristo, I. Munitic, M. Monteiro, A. Charbit, B. Rocha, and H. Veiga-Fernandes.** 2007. CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response. J. Exp. Med. **204:**1193–1205.
33. **Peixoto, A., M. Monteiro, B. Rocha, and H. Veiga-Fernandes.** 2004. Quantification of multiple gene expression in individual cells. Genome Res. **14:**1938–1947.
34. **Sancho, D., M. Gomez, and F. Sanchez-Madrid.** 2005. CD69 is an immunoregulatory molecule induced following activation. Trends Immunol. **26:**136–140.
35. **Sarkar, S., V. Teichgraber, V. Kalia, A. Polley, D. Masopust, L. E. Harrington, R. Ahmed, and E. J. Wherry.** 2007. Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation. J. Immunol. **179:**6704–6714.
36. **Slifka, M. K., J. N. Blattman, D. J. Sourdive, F. Liu, D. L. Huffman, T. Wolfe, A. Hughes, M. B. Oldstone, R. Ahmed, and M. G. Von Herrath.** 2003. Preferential escape of subdominant CD8+ T cells during negative selection results in an altered antiviral T cell hierarchy. J. Immunol. **170:**1231–1239.
37. **Slifka, M. K., and J. L. Whitton.** 2001. Functional avidity maturation of CD8$^+$ T cells without selection of higher affinity TCR. Nat. Immunol. **2:**711–717.
38. **Surh, C. D., O. Boyman, J. F. Purton, and J. Sprent.** 2006. Homeostasis of memory T cells. Immunol. Rev. **211:**154–163.
39. **Tanchot, C., S. Guillaume, J. Delon, C. Bourgeois, A. Franzke, A. Sarukhan, A. Trautmann, and B. Rocha.** 1998. Modifications of CD8+ T cell function during in vivo memory or tolerance induction. Immunity **8:**581–590.
40. **Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas, and B. Rocha.** 1997. Differential requirements for survival and proliferation of CD8 naive or memory T cells. Science **276:**2057–2062.
41. **Unsoeld, H., S. Krautwald, D. Voehringer, U. Kunzendorf, and H. Pircher.** 2002. Cutting edge: CCR7+ and CCR7− memory T cells do not differ in immediate effector cell function. J. Immunol. **169:**638–641.
42. **Unsoeld, H., and H. Pircher.** 2005. Complex memory T-cell phenotypes revealed by coexpression of CD62L and CCR7. J. Virol. **79:**4510–4513.
43. **Valitutti, S., S. Muller, M. Cella, E. Padovan, and A. Lanzavecchia.** 1995. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. Nature **375:**148–151.
44. **van der Most, R. G., K. Murali-Krishna, J. G. Lanier, E. J. Wherry, M. T. Puglielli, J. N. Blattman, A. Sette, and R. Ahmed.** 2003. Changing immunodominance patterns in antiviral CD8 T-cell responses after loss of epitope presentation or chronic antigenic stimulation. Virology **315:**93–102.

45. **van Faassen, H., M. Saldanha, D. Gilbertson, R. Dudani, L. Krishnan, and S. Sad.** 2005. Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central ($CD62L^{high}CD44^{high}$) subset. J. Immunol. **174:**5341–5350.
46. **van Stipdonk, M. J., E. E. Lemmens, and S. P. Schoenberger.** 2001. Naive CTLs require a single brief period of antigenic stimulation for clonal expansion and differentiation. Nat. Immunol. **2:**423–429.
47. **Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean, and B. Rocha.** 2000. Response of naive and memory CD8+ T cells to antigen stimulation in vivo. Nat. Immunol. **1:**47–53.
48. **Von Herrath, M. G., B. Coon, and M. B. Oldstone.** 1997. Low-affinity cytotoxic T-lymphocytes require IFN-gamma to clear an acute viral infection. Virology **229:**349–359.
49. **Wherry, E. J., J. N. Blattman, K. Murali-Krishna, R. van der Most, and R. Ahmed.** 2003. Viral persistence alters CD8 T-cell immunodominance and tissue distribution and results in distinct stages of functional impairment. J. Virol. **77:**4911–4927.
50. **Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber, and R. Ahmed.** 2007. Molecular signature of $CD8^+$ T cell exhaustion during chronic viral infection. Immunity **27:**670–684.
51. **Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian, and R. Ahmed.** 2003. Lineage relationship and protective immunity of memory CD8 T cell subsets. Nat. Immunol. **4:**225–234.
52. **Whitmire, J. K., J. T. Tan, and J. L. Whitton.** 2005. Interferon-gamma acts directly on CD8+ T cells to increase their abundance during virus infection. J. Exp. Med. **201:**1053–1059.
53. **Wirth, T. C., N. L. Pham, J. T. Harty, and V. P. Badovinac.** 5 February 2009, posting date. High initial frequency of TCR-transgenic CD8 T cells alters inflammation and pathogen clearance without affecting memory T cell function. Mol. Immunol. doi:10.1016/j.molimm.2008.12.018.
54. **Xiao, Z., M. F. Mescher, and S. C. Jameson.** 2007. Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. J. Exp. Med. **204:**2667–2677.
55. **Yewdell, J. W., and J. R. Bennink.** 1999. Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses. Annu. Rev. Immunol. **17:**51–88.
56. **Zimmermann, C., A. Prevost-Blondel, C. Blaser, and H. Pircher.** 1999. Kinetics of the response of naive and memory CD8 T cells to antigen: similarities and differences. Eur. J. Immunol. **29:**284–290.

Article II

Equally efficient primary CD8 immune responses generate memory with different protection capacity

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Equally efficient primary CD8 immune responses generate memory with different protection capacity

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Abstract

Different pathogens likely represent distinct challenges to the host immune system, but if the same vaccination strategies are equally efficient at inducing protection against all pathogen types is unknown. Doubts have also been raised about the best methodology for immune response studies and identifying protection in vaccinated cohorts. Here, we show that CD8 T cell function can only be accurately evaluated by *ex vivo* approaches, as the current *in vitro* methods using T cell re-stimulation never mimic *in vivo* behavior. Major and persistent TCR down-regulation abrogates MHC-tetramer binding during the expansion phase, preventing the identification of a large fraction of endogenous antigen-specific cells. We also show that priming with different pathogens generates CD8 T cells with different characteristics that are not determined by TCR usage, but by the infection context. Finally, we report that two primary responses leading to complete pathogen elimination generate memory cells with different protection capacities, and that T_{EM} cells are more efficient at conferring protection than T_{CM} cells.

INTRODUCTION

Different infections may represent very different challenges to the host immune system. To meet such challenges, CD4 T cells generate various effector types (e.g., TH1, TH2, and TH17). Although CD8 T cells may generate equivalent subtypes under appropriate *in vitro* conditions, CD8 effector cells arising *in vivo* are usually regarded as following a single Ifn-γ/CTL differentiation pathway. The notion of "a single differentiation pathway" was challenged by our studies of the co-expression of multiple effector genes in several CD8 immune responses *in vivo*. Instead of the coordinated expression of effector molecules, individual effector genes had different kinetics of expression/down-regulation. Moreover, when their co-expression was evaluated at the single-cell level, they associated randomly in both TCR-Tg (Peixoto *et al.* 2007) and endogenous antigen-specific cells (Munitic *et al.* 2009). This picture of CD8 differentiation diverges considerably from the coordinated co-expression of transcription factors and effector molecules occurring during TH1/TH2/TH17 CD4 differentiation and suggests that each individual effector gene may have independent regulatory mechanisms, which could result in flexible and quite heterogeneous CD8 T cell responses.

Clarifying this issue is important for optimal T cell vaccine design. Several hypotheses are possible. First, each pathogen may induce a particular gene expression pattern that is optimal for the control of that infection and for the generation of efficient protection. In this case, each vaccine should recapitulate the behavior of the pathogen it aims to control as much as possible. The recent success of vaccination using a genetically attenuated malaria strain supports this hypothesis (Spring *et al.* 2013). Second, although gene expression patterns possibly differ in primary responses, different infections may generate identical memory T cell types with similar protection capacity. In these circumstances, all vaccination protocols should have identical efficiency, as long as they are able to generate abundant memory cells. Transcriptome analysis of different immune responses supports this hypothesis: it has shown different gene expression patterns in primary responses to different pathogens, but identical properties in all of the memory cells that are generated (Best *et al.* 2013). Third, different infections may generate memory cells with different capacities to confer protection. In the latter case, vaccination should aim to generate these protective memory types. This latter hypothesis is supported by recent success in inducing protection against and clearing the otherwise lethal SIV (Hansen *et al.* 2013). Immunization with RhCMV bearing SIV epitopes was able to confer protection and to clear the already established viremia. In humans, CMV

infection generates T_{EM} cells co-expressing IFN-γ, perforin, and granzymes which, when present, efficiently control CMV (Ribeiro-dos-Santos *et al.* 2012).

All of these possibilities raise a second issue of identifying the best method for following immune responses and determining protection. In the vast majority of cases, effector functions are evaluated after *in vitro* reactivation with pathogen epitopes, but doubts have been raised regarding whether these methods ever recapitulate *in vivo* cell properties. *In vitro* stimulation has clearly been demonstrated to induce considerable bias in humans (Almeida *et al.* 2009). In the mouse, *in vitro* re-stimulation has also been shown to modify *ex vivo* readouts considerably, with TNFα expression frequencies changing from <1% to 100% in the same populations after *in vitro* re-stimulation (Panus *et al.* 2000). Furthermore, *in vitro* readouts are unable to discriminate the properties of T cells in certain abortive immune responses from those leading to pathogen clearance and efficient memory generation (Tanchot *et al.* 1998; Kassiotis *et al.* 2002). Despite these data, *in vitro* re-stimulation tests have been argued to be reliable for scoring function because they always recapitulate the behavior of antigen-specific cells when directly confronted with the antigen *in vivo*, but this notion was never confirmed.

The relative reliability and possible biases of different experimental approaches to studying T cell differentiation during immune responses is also controversial. The use of monoclonal (Mo) TCR-Tg cells allows discrimination of any modification of T cell function during the immune response based on the selection of cells with particular TCR expression. However, TCR-Tg cells may not mimic the behavior of endogenous cells (Marzo *et al.* 2005; van Faassen *et al.* 2005; Badovinac *et al.* 2007). On the other hand, the study of endogenous responses may also have bias. TCR-Tg cells and endogenous cells down-regulate their TCR while responding to antigen (Valitutti *et al.* 1995; Drake *et al.* 2005; Xiao *et al.* 2007; Munitic *et al.* 2009), preventing their identification by MHC-peptide tetramers (Tet).

Here we address the following questions: What is the best strategy for studying immune responses and evaluating protection? Do different pathogens induce different CD8 differentiation programs? If so, can these differences be attributed to different TCR usage/affinity or to differences in infectious context? What is the best strategy for vaccinating: using the same atenuated pathogen or using methods that generate many memory cells or peculiar memory types?

RESULTS

1° Question: What is the best strategy to study immune responses?

a) Comparison of different strategies to evaluate T cell functionality

First, we attempted to determine the best approach for evaluating CD8 T cell properties induced *in vivo*, by comparing three different appoaches. We compared the detection of protein after *in vitro* reactivation, with an RT-PCR assay allowing us to score up to 20 different mRNAs in each individual cell, and the protein detection *ex vivo* in brefeldine A injected mice.

Protein detection after *in vitro* reactivation is the most frequently used strategy, allowing clear-cut discrimination between positive and negative cells. This method has been criticized as not reproducing *in vivo* cell behavior, with expression profiles being influenced by the *in vitro* re-stimulation conditions. However, *in vitro* reactivation tests are generally thought to reliably reproduce cell behavior when they encounter the antigen *in vivo*. RT-PCR has the advantage of studying cell properties *ex vivo* and obtaining considerable information from a reduced number of antigen-specific cells, such as those present in the early immune response. The method also clearly differentiates between positive and negative cells because it detects two mRNAs/cell for each tested gene. However, this method only detects mRNA and not protein. We also evaluated the detection of protein using specific antibodies to cell surface molecules and intracytoplasmic labeling for secreted molecules in mice previously injected with Brefeldin A, which blocks protein secretion. Similar to the RT-PCR method, antigen-specific cells were characterized *ex vivo* in the absence of further manipulation. This method detects proteins but may have reduced discriminative power when molecules are poorly expressed or when antibodies available have not enough quality. Moreover, the information available during early responses may be more restricted, as the number of cells required to obtain clear-cut data is relatively high and antigen-specific cells are infrequent in the beginning of the immune response.

We compared these three approaches by evaluating the *in vivo* production of IFN-γ by OT-1 Tg cells infected with live *Listeria monocytogenes* (LM) expressing ovalbumine (LM-OVA). Under our immunizing conditions, the CD8 response peaked 7 days after priming and declined thereafter. Maximum bacterial loads were reached 2-3 days after infection, and no antigen was detected by day 7. Protein expression *ex vivo* and after *in vitro* stimulation was measured in the same experiments. *Ex vivo* mRNA expression was measured in separate experiments.

We did not find any major differences between *ex vivo* mRNA and protein detection. Slight experimental variations accounted for minor variations in the percentage of positive cells (Fig. 1A).

When protein expression was low, as on days 5 and 7 after infection, mRNA detection provided a better clear-cut separation between negative and positive cells. In contrast, *in vitro* reactivation did not mimic *ex vivo* expression profiles in regards to the percentage of positive cells or the amount of protein detected based on MFI (Fig. 1B). These parameters varied with *in vitro* re-stimulation conditions. After *in vitro* stimulation, up to 87% of cells were detected as IFN-γ producers, but the maximal percentage of positive cells *ex vivo* (on day 2, when LM-OVA load peaked) was 41%. These differences were not due to a failure of the *ex vivo* detection method to visualize *ex vivo* IFN-γ expression. When infected mice were further injected with the OVA peptide, the frequency of *ex vivo* IFN-γ protein detection in Brefeldin A-treated mice was similar to the frequency after *in vitro* re-stimulation, and the protein amounts (MFI) were even higher (Fig. 1C). These data demonstrate that *in vitro* reactivation never recapitulates the behavior of T cells *in vivo*, even when these T cells are directly confronted with a high antigen dose.

Next, we correlated *ex vivo* mRNA and protein detection for the different molecules we studied by comparing the results of different experiments, each using one of these two methods (Fig S1-3). In all cases, the discrimination between positive and negative cells was clear-cut when using mRNA results, but protein labeling could not be as discriminative. The Pearson correlation coefficient approched 1 for all molecules, i.e., significant identity was found between mRNA and protein detection (Fig. 2).

We conclude that, for the molecules we studied, *ex vivo* mRNA detection correlates with *ex vivo* protein expression. Therefore, both these two methods reflect cell behavior *in vivo*. In contrast, methods involving *in vitro* reactivation do not recapitulate *in vivo* behavior, even when cells are in the presence of maximal antigen doses.

1° Question: What is the best experimental strategy to study immune responses?

b) Possible biases in the study of endogenous responses

The study of immune responses requires the identification of antigen-specific cells, either by the adoptive transfer of allotype-labeled cells expressing a transgenic TCR of known specificity, or by recognizing endogenous antigen-specific cells using multimers of Class I molecules binding specific peptides. Mo TCR-Tg mice have the advantage that CD8 T cells use the same TCR throughout the response. Therefore, differences found in cell properties throughout the response cannot be attributed to differences in the repertoire of individual mice, or to the selection of cells with different TCR affinity. However, it was suggested that the behavior of TCR-Tg cells may be different from that of endogenous cells (Marzo *et al.* 2005; van Faassen *et al.* 2005; Badovinac *et al.* 2007), though this conclusion has been downplayed (Sarkar *et al.* 2007; Munitic *et al.* 2009). On the other hand it is

known that T cells down-regulate their TCR during immune responses, losing the capacity to bind MHC tetramers (Tet) (Valitutti *et al.* 1995; Drake *et al.* 2005; Xiao *et al.* 2007; Munitic *et al.* 2009). This down-regulation was found in OT-1 and P14 TCR-Tg cells and in endogenous cells after both bacterial and viral infection. Recently, we quantified the extent of this down-regulation by comparing the number of TCR-Tg cells recognized by a different allotype to the number of these cells that bound Tet during the immune response. We found that 60-80% of responding cells failed to bind Tet during the expansion phase. To better understand the possible impact of TCR down-regulation, we studied the level of TCR down-regulation in several immunization conditions. We transferred different numbers of CD45.2$^+$ MoTg P14 cells into CD45.1$^+$ hosts, which were subsequently immunized with different doses of LM-GP33. To evaluate the extent of TCR down-regulation, we determined the gate position for tetramer-positive cells in each experiment by using both Tg P14 naïve cells labeled with Tet-GP33 (Fig. 3A, left) and total CD8$^+$ cells from an immunized mouse labeled with all fluorescent Abs minus (FM)-Tet-GP33 to control for background fluorescence (Fig. 3A, middle). Under these circumstances, all responding cells can be recognized by the expression of a different allotype, which is expressed throughout the response (Fig. 3A, right).

TCR down-regulation was inversely proportional to the number of transferred cells and increased with the antigen dose (Fig. 3B), suggesting that it was conditioned by the strength of antigen stimulation. Correlation of cell division with TCR expression/Tet binding showed that CD8 T cells lost TCR expression after division (Fig. 3C). Maximal Tg TCR down-regulation was present 2 days after infection, when LM concentrations peaked. Tg cells progressively regained Tet binding, but a significant fraction remained Tet^{neg} 6 days after immunization (Fig. 3D). We conclude that allotype labeling of TCR-Tg allows the assessement of all CD8-responding cells, whereas tetramer binding fails to identify a substantial fraction.

Overall, these results reveal pitfalls in the study of the endogenous response and suggest that, during early responses, a significant fraction of endogenous antigen-specific cells cannot be identified. As TCR down-regulation and the failure to bind tetramers persists throughout the expansion phase, the number and expansion rate of endogenous cells is underestimated. Because TCR down-regulation correlates to cell division, and cell division correlates to cell differentiation into effector functions, the study of the functional properties of endogenous cells is biased.

2° Question: Do different pathogens induce different CD8 differentiation programs?
If so, is this conditioned by different TCR usage?

We selected two systems with similar kinetics for the immune response and pathogen clearance. In both cases, the immune response peaked 7 days after priming and the presence of the pathogen was no

longer detected at this time point (not shown). Compared to P14 Tg cells responding to LCMV, OT-1 CD8 T cells responding to LM-OVA expressed higher frequencies of *Tgfβ*, *Il10r*, and *Il21r* throughout the response, and these cells reverted to the *Ccr7*$^{+}$ T_{CM} phenotype 2 months after priming. Roughly half of these memory cells expressed *Prf1* and *Fasl*. In contrast, approximately 50% of LCMV-specific cells exhibited persistent expression of *Ifng*; they maintained a relatively high frequency of *Ifng* expression at 2 months after priming, and most acquired a *Ccr7*$^{-}$ T_{EM} phenotype (Fig. 4).

We attempted to determine whether these different characteristics were due to different TCR expression or to differences in the immunization context. Therefore, we co-transferred OT-1 and P14 Mo Tg cells into the same mouse that was immunized with both LM-OVA and LM-GP33. In this context, both TCR-Tg populations behaved similarly, expressing relatively high frequencies of *Il10r* and *Il21r* and reverting to a T_{CM} phenotype characteristic of the LM-OVA response (Fig. 5). The only significant difference between P14 and OT-1 was a slightly higher expression of IL-7R in P14. Because the avidity of OT-1 Tg cells is higher than that of P14 (Kassiotis *et al.* 2003; Hao *et al.* 2006), these results indicate that TCR-specificity and avidity has little impact on the pattern of CD8 differentiation. These differentiation patterns are conditioned mostly by the characteristics/environment of the infection.

3° Question: What is the best strategy to vaccinate: use the same atenuated pathogen? use methods generating many memory cells? generate peculiar memory types?

To compare the protective capacity of the memory cells generated after different infections, B6 mice were primed with LCMV, LM-OVA, or LM-GP33. Two months later, we studied their capacity to handle a lethal dose (10^6 LM) of LM-OVA or LM-GP33. To validate these tests, we determined whether bacterial elimination requires the presence of antigen-specific memory cells. Indeed, this was the case: when boosted with LM-OVA, mice primed with LM-OVA had reduced bacterial loads, whereas mice primed with LCMV had bacterial loads identical to those of the naïve mice studied simultaneously (Fig. 6). Next, we compared the clearance of LM-GP33 in memory mice primed with either LM-GP33 or LCMV. We expected LM-GP33 priming to provide better protection than LCMV priming. The number of GP33-specific cells in the spleen of LM-GP33-primed mice (average $2x10^6$ cells) was slightly higher than that of LCMV-infected mice (average $1.6x10^6$). In addition to GP33-specific cells, the memory pool of LM-GP33-primed mice should also include memory cells specific for other unknown LM epitopes. Moreover, mice primed with LM should have pathogen-specific antibodies that contribute to LM clearance (Shen *et al.* 2003) and are absent in LCMV-primed mice. Surprisingly, this putative increase in memory pool sizes, antibodies, or pathogen-specific LM priming did not confer increased protection to LM-GP33-primed mice. Mice primed with LCMV eliminated LM-GP33 more efficiently than LM-GP33-primed mice (Fig. 6).

DISCUSSION

Progress in vaccine design and the evaluation of protection requires the use of reliable methods to induce adequate immune responses and to monitor the presence of these responses in vaccinated individuals. The present study reports several pitfalls that must be taken into consideration. First, we show that the *in vitro* reactivation tests currently used to study functionality do not correlate with the *in vivo* behavior of CD8 T cells. The lack of correlation between *in vitro* reactivation and *ex vivo* readouts was described previously for both CD4 (Panus *et al.* 2000) and CD8 T cells in the mouse (Veiga-Fernandes *et al.* 2000), but despite these studies, the simplicity of *in vitro* reactivation methods and the possibility of using them in large cohorts still make these popular tests for identifying T cell function. It has been argued that although these *in vitro* read-outs may not reflect *in vivo* cell behavior at all time points of the response, they would always show how lymphocytes react when confronted with the antigen *in vivo*. However, we clearly show that this is not the case. By comparing *ex vivo* and *in vitro* read-outs when T cells are in the presence of very high bacterial loads, we found that *in vitro* reactivation tests always induce much stronger responses than those found *ex vivo*, suggesting that the peptide concentrations presented *in vitro* may be much higher than those presented *in vivo*. By studying human T cell clones with different avidity, it was also shown that the number and the type of effector functions induced after *in vitro* reactivation depends on both the TCR avidity and the peptide concentration used for re-stimulation. For high affinity clones, a low antigen concentration was optimal to reveal effector functions, whereas higher antigen concentrations were required in low affinity clones (Almeida *et al.* 2009). This limitation handicaps the study of polyclonal T cell populations found in different individuals, which likely harbor T cells with different TCR avidity. The reactivation tests used to study polyclonal populations of antigen-specific cells in mice and humans risk stimulating some cells and not others, to be optimal for a limited and unknown fraction of all antigen-specific cells. However, although functionality cannot not be evaluated by *in vitro* tests, these tests may remain useful for determining the number and peptide specificity of antigen-specific memory cells present in each individual. The majority of CD8 T cells recovered Tet binding capacity in the memory phase (not shown), and reactivation tests and tetramer binding have been reported to have identical frequency read-outs (Wherry *et al.* 2007). We conclude that more laborious *ex vivo* methods must be pursued for the study of T cell function after vaccination. Although the toxicity of Brefeldin A prevents its use in humans, the good correlation we found between *ex vivo* protein and mRNA expression indicates that the quantification of mRNA expression may be useful for studying T cell functionality in human cohorts. Supporting this notion, we quantified mRNA expression to evaluate CD8 T cell function in healthy donors and HIV-infected patients. The two cohorts were different, but within each cohort different donors had the same read-outs (Ribeiro-dos-Santos *et al.*

2012). These results indicate that the reported heterogeneous behavior of human CD8 T cells is largely artifactual due to the *in vitro* reactivation methodology currently used to evaluate their function.

Though the identification of T cell functions may be improved by the use of *ex vivo* methodology, we could not find a better strategy for identifying endogenous antigen-specific cells during immune responses. As previously reported in both OT-1 and P14 Tg mice and in endogenous cells (Valitutti *et al.* 1995; Drake *et al.* 2005; Xiao *et al.* 2007; Munitic *et al.* 2009), we observed major TCR down-regulation and a loss of Tet binding throughout the expansion phase. We attempted to reverse this down-regulation by pre-incubating cells at 37°C, but this strategy did not improve Tet binding. Therefore, the number of endogenous antigen-specific cells, their expansion rates, the amplitude of the endogenous responses may be underestimated. The present study also showed that TCR down-regulation correlates with cell division and, after the transfer of low numbers of Tg cells (approaching the number of endogenous naïve cells), most cells have already divided and lost Tet binding by day 3. Because the differentiation of CD8 effector functions also increases with cell division, the effector cells generated during endogenous responses may also be underestimated.

These results revive previous discussions on the relative merit of TCR-Tg and endogenous cells in immune response studies. It was claimed that TCR-Tg cells in general, and when used in high dose transfers in particular, did not mimic the behavior of endogenous cells. The cells have been argued to have poor survival rates and express different markers, particularly IL-7R and CD62L (Marzo *et al.* 2005; Badovinac *et al.* 2007). This data led to the current conclusion that immune response studies using TCR-Tg cells are artifactual. However, a more careful comparison of endogenous and TCR-Tg responses tones down this conclusion considerably. The presence of high frequencies of naïve Tg cells induced a slight acceleration in the immune response kinetics, leading to more rapid pathogen elimination (Sarkar *et al.* 2007). The reported differences in IL-7R and CD62L expression between TCR-Tg and endogenous cells were due solely to different kinetics, as these studies compared expression on the same day in different mice. At this time point, endogenous cells were yet expanding and thus yet lacked the expression of IL-7R and CD62L while Tg cells (IL-7R+CD62L+) were already at the contraction phase. When Tg and endogenous cells were studied in the same mouse, their properties fully overlapped. In addition, when studied in the same mouse, Tg and endogenous cells generated the same number of memory cells with the same characteristics (Munitic *et al.* 2009). Overall, these results and the present data revise current notions on the relative merit of TCR-Tg versus endogenous T cell studies and reveal that important bias is also present in studies of the endogenous response, preventing the identification of a significant cohort of endogenous cells and underestimating effector cell numbers in the expansion phase. Therefore, a study of the full entity of an early immune response can only be achieved by following allotype- labeled TCR-Tg cells. Rather than being antagonistic, TCR-Tg and endogenous cell studies may both be required.

We also compared the impact of different infections on the properties of CD8 T cells. LM and LCMV infections induce the production of different innate cytokines, with LM responses being characterized by the production of IL-12, which is virtually absent in LCMV-infected mice (Orange and Biron 1996; Cousens *et al.* 1997). We were surprised to find no differences in IL-12R expression in these infections, and this cytokine may only influence early LM responses, as the expression of IL-12R by CD8 T cells is lost by day 6 after priming. In contrast, the majority of unique characteristics of the LM responses can be attributed to an effect of IL-21, although, to the best of our knowledge, this cytokine was not previously implicated in LM responses. In contrast to the CD8 T cell response to LCMV, the majority of OT-1 Tg cells responding to LM-OVA express the *Il21R* receptor, which is maintained in 40% of memory cells. IL-21 favors the response to IL-10 (Spolski *et al.* 2009), the abrogation of IFN-γ production (Casey and Mescher 2007), and increased perforin expression (Parmigiani *et al.* 2011). Moreover, IL-21 favors the generation of T_{CM} memory cells (Allard *et al.* 2007; Kaka *et al.* 2009). Thus, IL-21 signaling may explain the unique features of the LM responses, specifically the rapid decline in IFN-γ expression, the relatively high expression of *Il10R* and *Perf*, and the generation of T_{CM} memory cells. Another characteristic of the LM response is the very high expression of TGF-β. This cytokine contributes to the generation of TH17 cells (Bettelli *et al.* 2006; Veldhoen *et al.* 2006), which are the major source of IL-21 (Wei *et al.* 2007).

In regards to vaccination strategies, reproduction of the infectious context was not a pre-requisite for efficient memory generation in the two systems we used. These data indicate that the use of attenuated vaccines, which likely reproduce the behavior of the pathogen more closely, is not always a pre-requisite for the induction of efficient protection. Nonetheless, more complex pathogens, such as parasites, that evolve through different cycles in the host and frequently implicate different protection mechanisms, may be controlled more efficiently by attenuated vaccination, as has been found for malaria (Nganou-Makamdop and Sauerwein 2013; Teirlinck *et al.* 2013). Our results also show that not all immunizations have an identical capacity to confer protection; after LCMV immunization, protection was more efficient than that induced by LM priming. We did not find evidence that this increased protection capacity was due to an increased number of memory cells in the lymphoid organs. After LCMV infection, the number of GP33-specific memory cells in the spleen was slightly lower (average $1.6x10^6$) than after LM priming (average $2x10^6$), and their T_{EM} phenotype excludes them from the LNs. However, we cannot formaly exclude that GP33-specific memory cells are more abundant after LCMV immunization, as they may be dispersed in other tissues, where we cannot count them. However, we would expect that LM immunization should actually generate more memory cells and a more diverse repertoire than LCMV infection. In addition to GP33-specific cells, memory cells recognizing other LM epitopes should also be present, and LM immunization induces the production of specific Abs that would be absent in LCMV-primed mice. However, these putative

advantages of LM priming did not lead to an increased protection capacity, suggesting that the memory induced after LCMV infection is more efficient than those generated after LM priming.

This increased protection capacity of the LCMV infection may be related to the generation of cells with a T_{EM} phenotype. T_{EM}, which localize in tissues and have higher expression levels of *Tbet* and *Eomes* and effector functions in both mice (Masopust *et al.* 2001; Pearce *et al.* 2003; Intlekofer *et al.* 2005) and humans (Monteiro *et al.* 2007), have been proposed to be more efficient than T_{CM} in impairing pathogen replication at its earliest stage (Franchini 2009). This notion stems from the recent success in vaccination shedules inducing protection against and clearance of SIV (Hansen *et al.* 2009; Hansen *et al.* 2013). These vaccines use ShCMV bearing SIV epitopes. We and others previously showed that CMV infection generates T_{EM} with the same characteristics as LCMV memory cells (6, 45-46). Our present results, which confirm the higher protection capacity of T_{EM} cells in a different species and a different infection, strongly support that T_{EM} cells have a higher protection capacity. Comparisons of the protection capacity of T_{EM} and T_{CM} with the same specificity in other infections will be important to confirm and extend this notion.

MATERIALS AND METHODS

Mice and immunization protocols

All mice had the C57Bl/6 (B6) background. B6.CD90.1$^+$, B6.CD45.1$^+$, Rag2$^-$ Monoclonal (Mo) P14 CD90.2$^+$ TCR-Tg mice expressing the CD45.1 or CD45.2 allotype were obtained from our breeding colonies at the Center for the Development of Advanced Experimental Techniques (Orleans, France). Rag1$^-$ Mo OT-1 Tg mice were from the animal facilities of the Pasteur Institute, a kind gift from Antonio Freitas. In the majority of experiments, 10^6 lymph node cells derived from Mo P14 Tg mice or Mo OT-1 Tg mice were adoptively transferred to sex-matched 6 to 8-week-old CD45.1$^+$ or CD90.1$^+$ B6 mice. One day after this transfer, the mice were infected with LM or LCMV. The number of transferred Tg cells varied for some mice, and these cells could have been labeled with 5 µM CFSE (Molecular Probes, Eugene, OR) prior to injection. For immunization with LCMV, stocks of LCMV Armstrong 53b, a clone “triple plaque purified” from Arm CA 1371, were grown on baby hamster kidney cells and titers determined by plaque assay on Vero cells as described previously (Dutko and Oldstone 1983). Mice were infected intraperitoneally with 2 x 10^5 PFU of LCMV Armstrong strain 53b. LM-OVA or LM-GP33 were kind gifts from L. Lefrançois. For the LM infections, the bacteria were recovered during the exponential growth phase and the mice injected intravenously. Bacterial loads were evaluated one day after infection as CFU per spleen. All experiments were carried out according to the French Ethical Commission on Animal Experimentation.

Antibodies and tetramers for cytofluorometry and cell sorting

The following Abs were used for the identification of cell surface molecules: anti-CD45.1 (A20), anti-CD45.2 (104-2.1), anti-CD90.1 (OX-7), anti-CD90.2 (30-H12), anti-CD8β (H35-172), anti-CD69 (H1.2F3), anti-CD360/IL-21R (4A9), and anti-CD212/IL-12Rβ1 (114) from BD Bioscience (San Jose, CA); anti-CD44 (1M781), anti-KLRG1 (2F1), anti-CD127/IL-7R (A7R34), and anti-CD197/CCR7 (4B12) from eBioscience (San Diego, CA); and anti-CD210/IL-10R (1B1.3a) from BioLegend (San Diego, CA). Abs for intracytoplasmatic labeling were: anti-IFNγ (XMG 1.2) and anti-TNFα (MP6XT22) from BD Bioscience (San Jose, CA), and anti-granzyme B (16G6), anti-T-bet (4-B10), and anti-EOMES (Dan11mag) from eBioscience (San Diego, CA). All the above mentionated mAbs were directly coupled to FITC, PE, PerCPCy5.5, PECy7, APC, APCCy7, Pacific Blue, APC Alexa Fluor 780, or Alexa Fluor 647. Anti-TGFβ (56E4) (Cell Signaling Technology, Danvers, MA) was revealed with a goat anti-rabbit Alexa Fluor 488 secondary Ab (Invitrogen, Carlsbad, CA).

Secreted cytokines were detected by intracellular staining as described previously (Lowsky *et al.* 2005). For e*x vivo* detection, mice were injected with 0.25 mg of Brefeldin A (Sigma-Aldrich, St. Louis, USA) 4 h before detection. For *in vitro* reactivation, total spleen cells were incubated with the indicated doses of OVA peptide (SIINFEKL) (PolyPeptide Group, Strasbourg, France) and Brefeldin A. GP33 (KAVYNFATM) and OVA (SIINFEKL) loaded tetramers were obtained from Beckman Coulter (Marseille, France). Before tetramer labeling, spleen cell suspensions were depleted of non-CD8 T cells using a cocktail of mAbs (TER119, CD19, Mac-1, GR1, CD4, B220) and Dynabeads (Dynal AS, Oslo, Norway).

Evaluation of gene expression in individual cells

The expression of multiple genes was analyzed in individual cells as described in detail previously (Peixoto *et al.* 2004). Notably, this method requires multiple validation steps that we previously performed and reported, and for each gene it detected two mRNA molecules/cell. The primers selected for PCR reactions are listed in Supplemental Table 1.

Statistical analysis

Potential differences in the expression of different genes were studied using the two-tailed Fisher's exact test. One-way ANOVA was used for bacterial loads. A P-value < 0.05 was considered significant. Potential differences between mRNA and protein expression in each response was evalated by taking into account all points and all experiments in which these two parameters were studied using the Pearson correlation coefficient (r). All statistical

analyses were performed using Graphpad Prism (GraphPad Software). Perfect correlation (i.e., full identity between the two read-outs) corresponded to r=1. The P-values correspond to significant similarities and not to significant differences. A P-value <0.05 was considered to signify overlapping responses.

Gene nomenclature

Genetic nomenclature was used according to the guidelines from the International Committee on Standardized Genetic Nomenclature for Mice (http://www.informatics.jax.org). In this nomenclature, genes and mRNA have the same abbreviation. The mRNAs studied were perforin (*Prf1*), granzyme B (*Gzmb*), FasL (*Fasl*), IFNγ (*Ifng*), TGFβ1 (*Tgfb1*), TNFα (*Tnf*), IL10R (*Ilr10*), IL21R (*Ilr21*), IL12Rβ2 (*Il12rb2*), IL7R (*Il7r*), CCR7 (*Ccr7*), KLRG1 (*Klrg1*), eomesodermin (*Eomes*), T-box 21 (*Tbx21*), programmed cell death 1 (*Pdcd1*), and CD3ε (*Cd3e*).

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Author's contributions S.L., I.M., and C.E. performed the experiments. H.D. prepared the LCMV and immunized mice with LCMV. I.D. immunized mice with LM. A.C. contributed his expertise in LM infections. B. Rocha supervised the study and wrote the manuscript.

References

1 **Peixoto, A., Evaristo, C., Munitic, I., Monteiro, M., Charbit, A., Rocha, B. and Veiga-Fernandes, H.,** CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response. *J Exp Med* 2007. **204**: 1193-1205.

2 **Munitic, I., Decaluwe, H., Evaristo, C., Lemos, S., Wlodarczyk, M., Worth, A., Le Bon, A., Selin, L. K., Riviere, Y., Di Santo, J. P., Borrow, P. and Rocha, B.,** Epitope specificity and relative clonal abundance do not affect CD8 differentiation patterns during lymphocytic choriomeningitis virus infection. *J Virol* 2009. **83**: 11795-11807.

3 **Spring, M., Murphy, J., Nielsen, R., Dowler, M., Bennett, J. W., Zarling, S., Williams, J., de la Vega, P., Ware, L., Komisar, J., Polhemus, M., Richie, T. L., Epstein, J., Tamminga, C., Chuang, I., Richie, N., O'Neil, M., Heppner, D. G., Healer, J., O'Neill, M., Smithers, H., Finney, O. C.,**

Mikolajczak, S. A., Wang, R., Cowman, A., Ockenhouse, C., Krzych, U. and Kappe, S. H., First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers. *Vaccine* 2013. **31**: 4975-4983.

4 **Best, J. A., Blair, D. A., Knell, J., Yang, E., Mayya, V., Doedens, A., Dustin, M. L. and Goldrath, A. W.,** Transcriptional insights into the CD8(+) T cell response to infection and memory T cell formation. *Nat Immunol* 2013. **14**: 404-412.

5 **Hansen, S. G., Piatak, M., Jr., Ventura, A. B., Hughes, C. M., Gilbride, R. M., Ford, J. C., Oswald, K., Shoemaker, R., Li, Y., Lewis, M. S., Gilliam, A. N., Xu, G., Whizin, N., Burwitz, B. J., Planer, S. L., Turner, J. M., Legasse, A. W., Axthelm, M. K., Nelson, J. A., Fruh, K., Sacha, J. B., Estes, J. D., Keele, B. F., Edlefsen, P. T., Lifson, J. D. and Picker, L. J.,** Immune clearance of highly pathogenic SIV infection. *Nature* 2013. **502**: 100-104.

6 **Ribeiro-dos-Santos, P., Turnbull, E. L., Monteiro, M., Legrand, A., Conrod, K., Baalwa, J., Pellegrino, P., Shaw, G. M., Williams, I., Borrow, P. and Rocha, B.,** Chronic HIV infection affects the expression of the 2 transcription factors required for CD8 T-cell differentiation into cytolytic effectors. *Blood* 2012. **119**: 4928-4938.

7 **Almeida, J. R., Sauce, D., Price, D. A., Papagno, L., Shin, S. Y., Moris, A., Larsen, M., Pancino, G., Douek, D. C., Autran, B., Saez-Cirion, A. and Appay, V.,** Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity. *Blood* 2009. **113**: 6351-6360.

8 **Panus, J. F., McHeyzer-Williams, L. J. and McHeyzer-Williams, M. G.,** Antigen-specific T helper cell function: differential cytokine expression in primary and memory responses. *J Exp Med* 2000. **192**: 1301-1316.

9 **Kassiotis, G., Garcia, S., Simpson, E. and Stockinger, B.,** Impairment of immunological memory in the absence of MHC despite survival of memory T cells. *Nat Immunol* 2002. **3**: 244-250.

10 **Tanchot, C., Guillaume, S., Delon, J., Bourgeois, C., Franzke, A., Sarukhan, A., Trautmann, A. and Rocha, B.,** Modifications of CD8+ T cell function during in vivo memory or tolerance induction. *Immunity* 1998. **8**: 581-590.

11 **Marzo, A. L., Klonowski, K. D., Le Bon, A., Borrow, P., Tough, D. F. and Lefrancois, L.,** Initial T cell frequency dictates memory CD8+ T cell lineage commitment. *Nat Immunol* 2005. **6**: 793-799.

12 **van Faassen, H., Saldanha, M., Gilbertson, D., Dudani, R., Krishnan, L. and Sad, S.,** Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62LhighCD44high) subset. *J Immunol* 2005. **174**: 5341-5350.

13 **Badovinac, V. P., Haring, J. S. and Harty, J. T.,** Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection. *Immunity* 2007. **26**: 827-841.

14 **Valitutti, S., Muller, S., Cella, M., Padovan, E. and Lanzavecchia, A.,** Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* 1995. **375**: 148-151.

15 **Xiao, Z., Mescher, M. F. and Jameson, S. C.,** Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. *J Exp Med* 2007. **204**: 2667-2677.

16 **Drake, D. R., 3rd, Ream, R. M., Lawrence, C. W. and Braciale, T. J.,** Transient loss of MHC class I tetramer binding after CD8+ T cell activation reflects altered T cell effector function. *J Immunol* 2005. **175**: 1507-1515.

17 **Sarkar, S., Teichgraber, V., Kalia, V., Polley, A., Masopust, D., Harrington, L. E., Ahmed, R. and Wherry, E. J.,** Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation. *J Immunol* 2007. **179**: 6704-6714.

18 **Hao, Y., Legrand, N. and Freitas, A. A.,** The clone size of peripheral CD8 T cells is regulated by TCR promiscuity. *J Exp Med* 2006. **203**: 1643-1649.

19 **Kassiotis, G., Zamoyska, R. and Stockinger, B.,** Involvement of avidity for major histocompatibility complex in homeostasis of naive and memory T cells. *J Exp Med* 2003. **197**: 1007-1016.

20 **Shen, H., Whitmire, J. K., Fan, X., Shedlock, D. J., Kaech, S. M. and Ahmed, R.,** A specific role for B cells in the generation of CD8 T cell memory by recombinant Listeria monocytogenes. *J Immunol* 2003. **170**: 1443-1451.

21 **Veiga-Fernandes, H., Walter, U., Bourgeois, C., McLean, A. and Rocha, B.,** Response of naive and memory CD8+ T cells to antigen stimulation in vivo. *Nat Immunol* 2000. **1**: 47-53.

22 **Wherry, E. J., Ha, S. J., Kaech, S. M., Haining, W. N., Sarkar, S., Kalia, V., Subramaniam, S., Blattman, J. N., Barber, D. L. and Ahmed, R.,** Molecular signature of CD8+ T cell exhaustion during chronic viral infection. *Immunity* 2007. **27**: 670-684.

23 **Orange, J. S. and Biron, C. A.,** An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections. *J Immunol* 1996. **156**: 1138-1142.

24 **Cousens, L. P., Orange, J. S., Su, H. C. and Biron, C. A.,** Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection. *Proc Natl Acad Sci U S A* 1997. **94**: 634-639.

25 **Spolski, R., Kim, H. P., Zhu, W., Levy, D. E. and Leonard, W. J.,** IL-21 mediates suppressive effects via its induction of IL-10. *J Immunol* 2009. **182**: 2859-2867.

26 **Casey, K. A. and Mescher, M. F.,** IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype. *Journal of immunology* 2007. **178**: 7640-7648.

27 **Parmigiani, A., Pallin, M. F., Schmidtmayerova, H., Lichtenheld, M. G. and Pahwa, S.,** Interleukin-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells. *Hum Immunol* 2011. **72**: 115-123.

28 **Kaka, A. S., Shaffer, D. R., Hartmaier, R., Leen, A. M., Lu, A., Bear, A., Rooney, C. M. and Foster, A. E.,** Genetic modification of T cells with IL-21 enhances antigen presentation and generation of central memory tumor-specific cytotoxic T-lymphocytes. *J Immunother* 2009. **32**: 726-736.

29 **Allard, E. L., Hardy, M. P., Leignadier, J., Marquis, M., Rooney, J., Lehoux, D. and Labrecque, N.,** Overexpression of IL-21 promotes massive CD8+ memory T cell accumulation. *Eur J Immunol* 2007. **37**: 3069-3077.

30 **Veldhoen, M., Hocking, R. J., Atkins, C. J., Locksley, R. M. and Stockinger, B.,** TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells. *Immunity* 2006. **24**: 179-189.

31 **Bettelli, E., Carrier, Y., Gao, W., Korn, T., Strom, T. B., Oukka, M., Weiner, H. L. and Kuchroo, V. K.,** Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells. *Nature* 2006. **441**: 235-238.

32 **Wei, L., Laurence, A., Elias, K. M. and O'Shea, J. J.,** IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner. *J Biol Chem* 2007. **282**: 34605-34610.

33 **Teirlinck, A. C., Roestenberg, M., van de Vegte-Bolmer, M., Scholzen, A., Heinrichs, M. J., Siebelink-Stoter, R., Graumans, W., van Gemert, G. J., Teelen, K., Vos, M. W., Nganou-Makamdop, K., Borrmann, S., Rozier, Y. P., Erkens, M. A., Luty, A. J., Hermsen, C. C., Sim, B. K., van Lieshout, L., Hoffman, S. L., Visser, L. G. and Sauerwein, R. W.,** NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections. *J Infect Dis* 2013. **207**: 656-660.

34 **Nganou-Makamdop, K. and Sauerwein, R. W.,** Liver or blood-stage arrest during malaria sporozoite immunization: the later the better? *Trends Parasitol* 2013. **29**: 304-310.

35 **Masopust, D., Vezys, V., Marzo, A. L. and Lefrancois, L.,** Preferential localization of effector memory cells in nonlymphoid tissue. *Science* 2001. **291**: 2413-2417.

36 **Pearce, E. L., Mullen, A. C., Martins, G. A., Krawczyk, C. M., Hutchins, A. S., Zediak, V. P., Banica, M., DiCioccio, C. B., Gross, D. A., Mao, C. A., Shen, H., Cereb, N., Yang, S. Y., Lindsten, T., Rossant, J., Hunter, C. A. and Reiner, S. L.,** Control of effector CD8+ T cell function by the transcription factor Eomesodermin. *Science* 2003. **302**: 1041-1043.

37 **Intlekofer, A. M., Takemoto, N., Wherry, E. J., Longworth, S. A., Northrup, J. T., Palanivel, V. R., Mullen, A. C., Gasink, C. R., Kaech, S. M., Miller, J. D., Gapin, L., Ryan, K., Russ, A. P., Lindsten, T., Orange, J. S., Goldrath, A. W., Ahmed, R. and Reiner, S. L.,** Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin. *Nat Immunol* 2005. **6**: 1236-1244.

38 **Monteiro, M., Evaristo, C., Legrand, A., Nicoletti, A. and Rocha, B.,** Cartography of gene expression in CD8 single cells: novel CCR7- subsets suggest differentiation independent of CD45RA expression. *Blood* 2007. **109**: 2863-2870.

39 **Franchini, G.,** Choosing the right memory T cell for HIV. *Nat Med* 2009. **15**: 244-246.

40 **Hansen, S. G., Vieville, C., Whizin, N., Coyne-Johnson, L., Siess, D. C., Drummond, D. D., Legasse, A. W., Axthelm, M. K., Oswald, K., Trubey, C. M., Piatak, M., Jr., Lifson, J. D., Nelson, J. A., Jarvis, M. A. and Picker, L. J.,** Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge. *Nat Med* 2009. **15**: 293-299.

41 **Dutko, F. J. and Oldstone, M. B.,** Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains. *J Gen Virol* 1983. **64 (Pt 8)**: 1689-1698.

42 **Lowsky, R., Takahashi, T., Liu, Y. P., Dejbakhsh-Jones, S., Grumet, F. C., Shizuru, J. A., Laport, G. G., Stockerl-Goldstein, K. E., Johnston, L. J., Hoppe, R. T., Bloch, D. A., Blume, K. G., Negrin, R. S. and Strober, S.,** Protective conditioning for acute graft-versus-host disease. *N Engl J Med* 2005. **353**: 1321-1331.

43 **Peixoto, A., Monteiro, M., Rocha, B. and Veiga-Fernandes, H.,** Quantification of multiple gene expression in individual cells. *Genome Res* 2004. **14**: 1938-1947.

Footnotes:

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2. Abbreviations: LCMV, Lymphocytic Choriomeningitis Virus; LM, Listeria monocytogenes; LM-OVA, L. monocytogenes expressing ovalbumin; LM-GP33, LM expressing the LCMV peptide GP33-4; SIV, Simian immuno-defficiency virus; tet+, tetramer+cells; T_{EM}, T cell effector memory; T_{CM}, T cell central memory.

3. Current addresses: [1] Department of Medicine, University of Chicago, Chicago, IL, USA; [2] Department of Biotechnology, University of Rijeka, Rijeka, Croatia; [3] CHU Sainte-Justine Research Center, University of Montreal, Quebec, Canada

Figure Legends

Figure 1. **Comparison of IFN-γ expression *ex vivo* and after *in vitro* reactivation.** CD90.2 MoOT-1 Tg cells were transferred to CD90.1 syngeneic mice. After 24 h, these mice were infected with 5,000 LM-OVA expressing both the OT-1 and OT-2 epitopes. On different days (D) after infection, the expression of IFNγ by splenic CD90.2 Tg cells was determined using different methods. All histograms show intracytoplasmic staining with anti-IFN-γ Abs or an isotype control (gray) for one typical experiment out of the three performed. A) *Ex vivo* expression: IFNγ expression *ex vivo* in Brefeldine A injected mice, 4 h post- Brefeldine A injection. In brackets: the frequency of Tg cells expressing *Ifng*, determined by single-cell RT-PCR (mean ± SEM of three experiments). **B)** *In vitro* expression: IFNγ expression by Splenic CD90.2 Tg cells re-stimulated *in vitro* with the indicated doses of OVA peptide, in the presence of Brefeldin A for 4 h. **C)** Five days after infection, mice were injected with 5 µM OVA peptide and Brefeldin A and studied 4 h later.

Figure 2. Comparison between *ex vivo* mRNA and protein read-outs. CD90.2 $CD8^+$ Mo OT-1 Tg cells were immunized with LM-OVA, as described in Fig.1. At different days after infection, splenic CD90.2 Tg cells were either sorted as single cells to determine the expression frequency of different mRNAs, or labeled with different antibodies to evaluate protein expression. Results show mRNA (squares) and protein expression (circles). Each point represents the mean of two experiments or the mean ± SEM of three to five experiments. For all molecules, the Pearson correlation coefficient (r) was significant (*$p<0.05$, **$p<0.01$), indicating that the results overlap.

Figure 3. TCR down-regulation during immune responses. The indicated number of CD45.2 $CD8^+$ MoP14 Tg cells labelled or not with CFSE were transferred to CD45.1 syngeneic receipients. One day later these mice were infected with the indicated doses (CFU) of live LM-GP33. **A)** Left and Middle panels: The controls used in each experiment to define the gates identifying Tet^+ cells were Tet binding in naïve GP33-specific P14 Tg cells (left) and $CD8^+$ cells from an immunized mouse labelled with all fluorescence Abs except Tet, defining background fluorescence (midle). Right panel: Allotype labeling of $5x10^5$ transferred TCR-Tg cells 3 days after infection.. **B, D)** Tet binding in gated $CD45.2^+$ P14 cells on days 2 **(B)** and 6 **D)** after infection. **C)** Correlation of Tet binding with division 3 days after infection.

Figure 4. Comparison of gene expression frequencies in antigen-specific cells after infection with LM-OVA or LCMV. CD90.2$^+$ P14 or OT-1 MoTg cells were transferred to syngeneic CD90.1$^+$ hosts. After 24 h, these mice were infected with 2x10^5 PFU of LCMV-Armstrong or 5x10^3 CFU of LM-OVA, respecitvely. Spleens were recovered on different days after infection and activated TCR-Tg cells (CD69$^+$ or CD44$^+$) sorted as single cells. Each individual cell was tested for co-expression of the respective mRNAs. Point 0 corresponds to gene expression in naïve Tg cells. Data are frequency estimates calculated from 48 cells per time point/experiment/mouse and from 2-5 experiments in which 2-3 mice were studied). *p<0.05, **p<0.01, two-tailed Fisher's exact test.

Figure 5. Comparison of gene expression frequencies in different CD8 T cells immunized in the same mouse. The same number of CD90.2$^+$ CD45.1$^+$ P14 and CD90.2$^+$ CD45.2$^+$ OT-1Tg cells were co-transferred to CD90.1$^+$ CD45.2$^+$ syngeneic receipients. After 24 h, these mice were infected with 2,500 CFUs of LM-OVA and 2,500 CFUs of LM-GP33. Individual Tg cells were sorted at different time points after infection and studied as described in Figure 4. Data are presented as the mean ± SEM of three individual mice studied in two independent experiments. *p<0.05, two-tailed Fisher's exact test.

Figure 6. The protection capacity of different of the memory generated after different infections. B6 mice were infected with either 2x10^5 PFU of LCMV-Armstrong or 5x10^3 CFU of LM-OVA or LM-GP33. Two months later, naïve mice or different types of memory mice were infected with a lethal dose (10^6) of LM-OVA or LM-GP33 and bacteria loads studied one day later. Data are CFU/spleen, with each point representing an individual mouse studied in two independent experiments. *p<0.05, *** p<0.001, one-way ANOVA; ns, non significant.

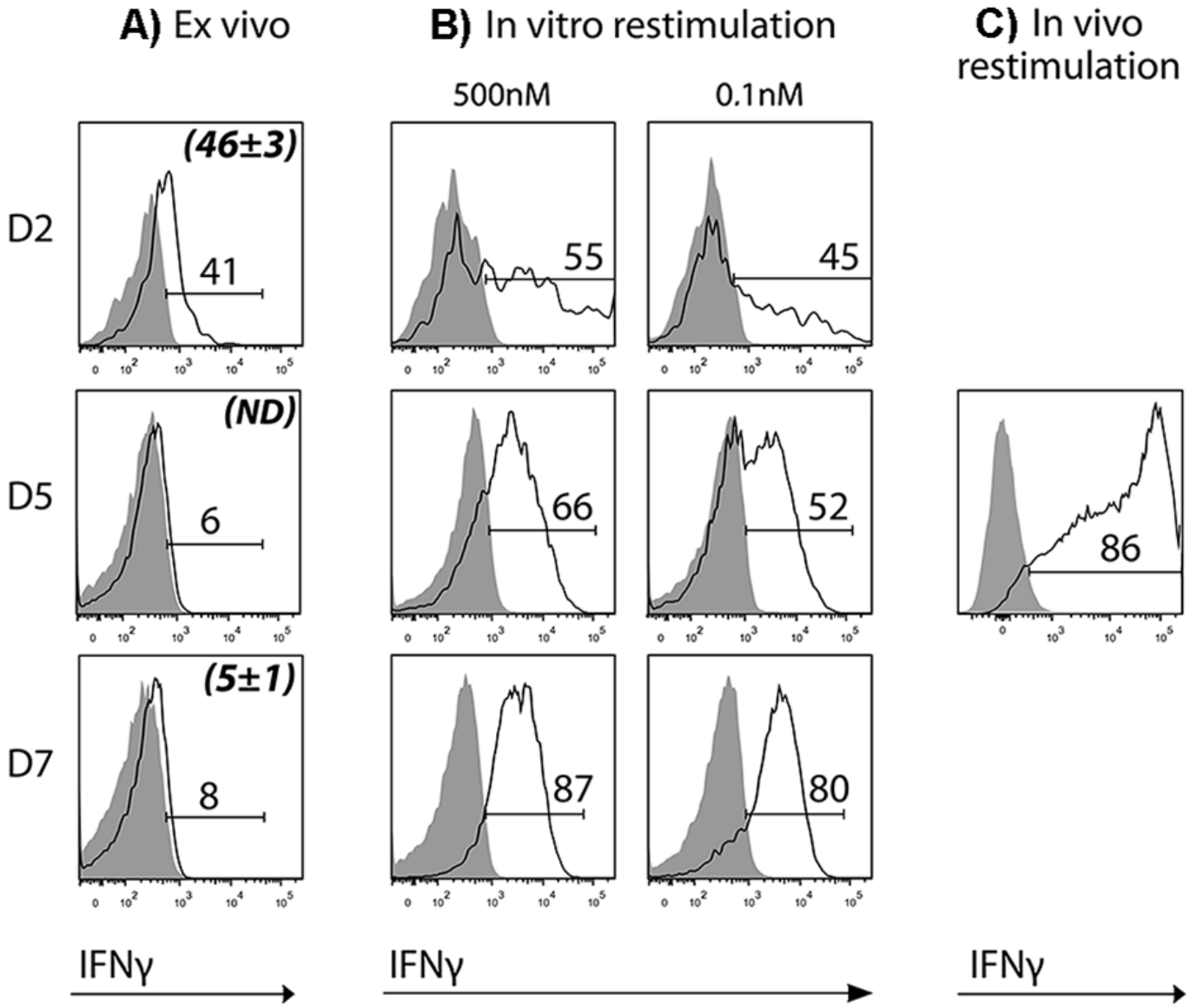
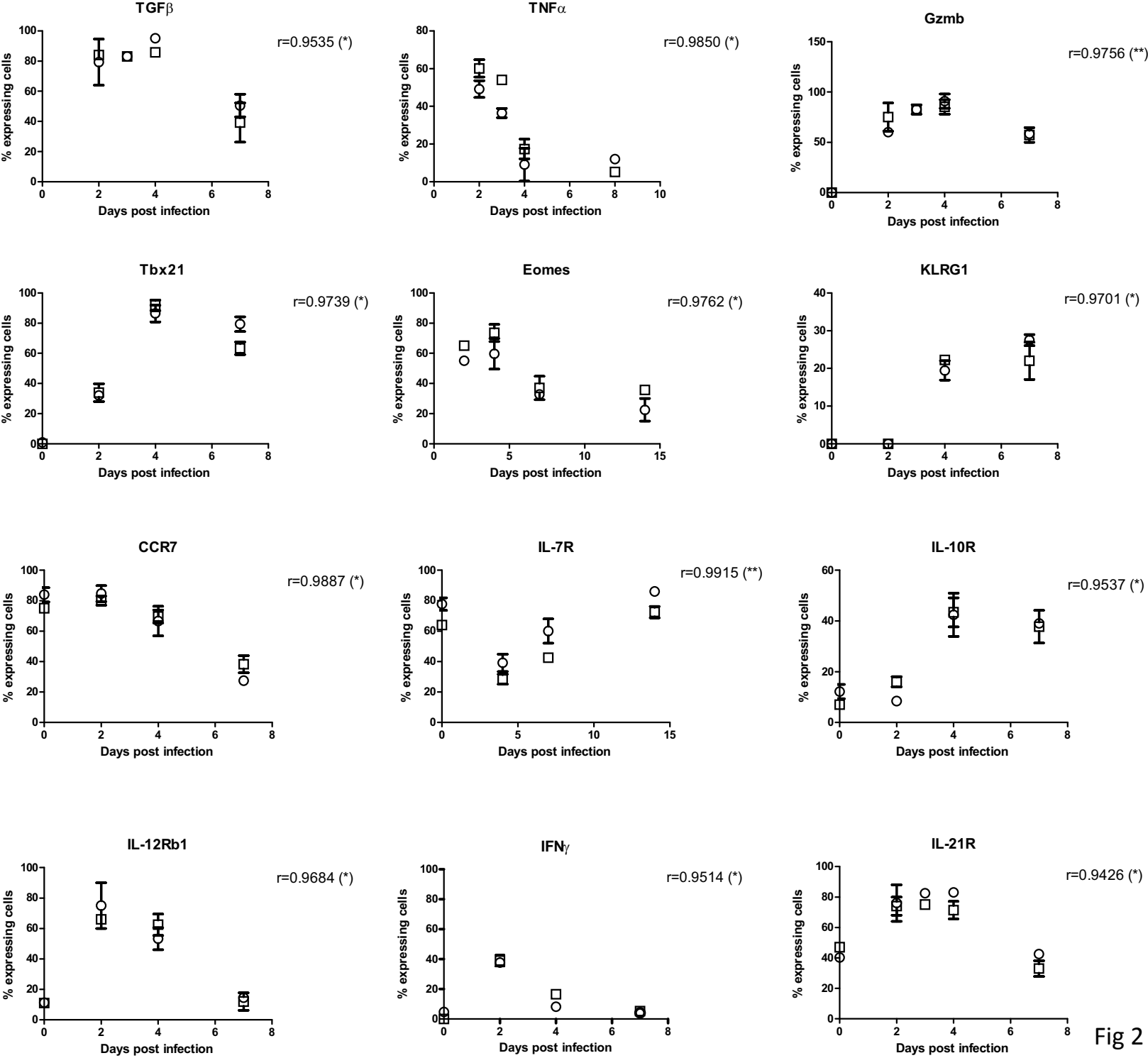

A) Ex vivo
B) In vitro restimulation
C) In vivo restimulation
500nM
0.1nM
D2
D5
D7
(46±3)
41
55
45
(ND)
6
66
52
86
(5±1)
8
87
80
IFNγ
IFNγ
IFNγ

Fig 1


TGFβ
r=0.9535 (*)
TNFα
r=0.9850 (*)
Gzmb
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Eomes
r=0.9762 (*)
KLRG1
r=0.9701 (*)
CCR7
r=0.9887 (*)
IL-7R
r=0.9915 (**)
IL-10R
r=0.9537 (*)
IL-12Rb1
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IFNγ
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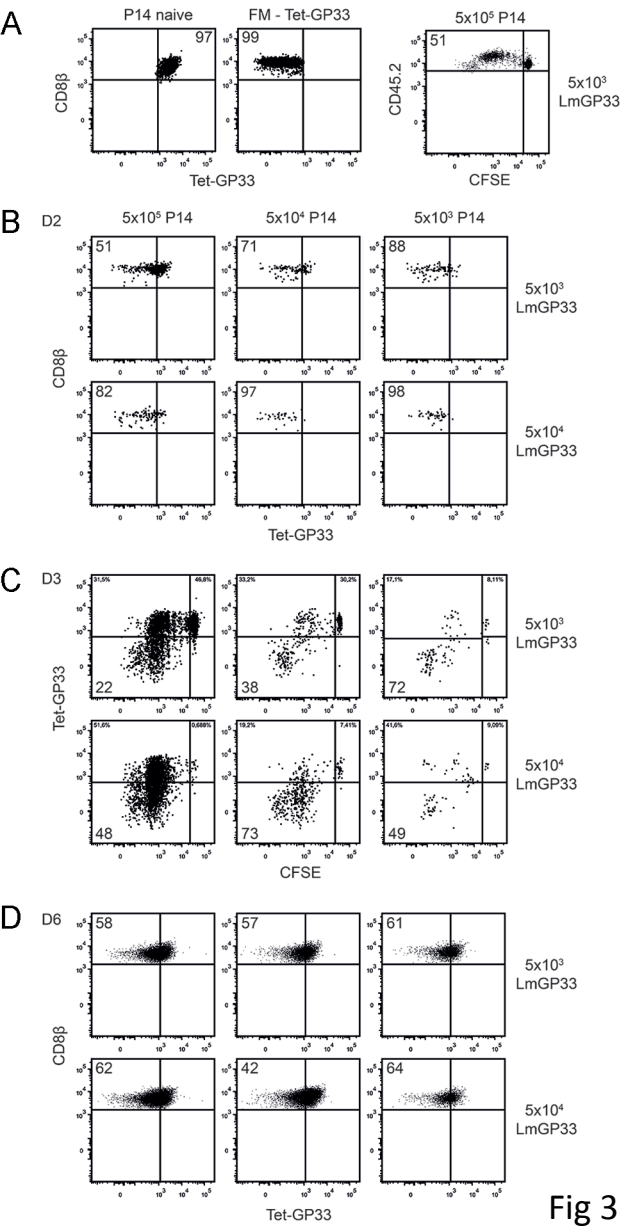
IL-21R
r=0.9426 (*)
% expressing cells
Days post infection

Fig 2


A
P14 naive
FM - Tet-GP33
5x10⁵ P14
97
99
51
CD8β
Tet-GP33
CD45.2
CFSE
5x10³ LmGP33
B
D2
5x10⁵ P14
5x10⁴ P14
5x10³ P14
51
71
88
82
97
98
5x10³ LmGP33
5x10⁴ LmGP33
CD8β
Tet-GP33
C
D3
22
38
72
48
73
49
Tet-GP33
CFSE
5x10³ LmGP33
5x10⁴ LmGP33
D
D6
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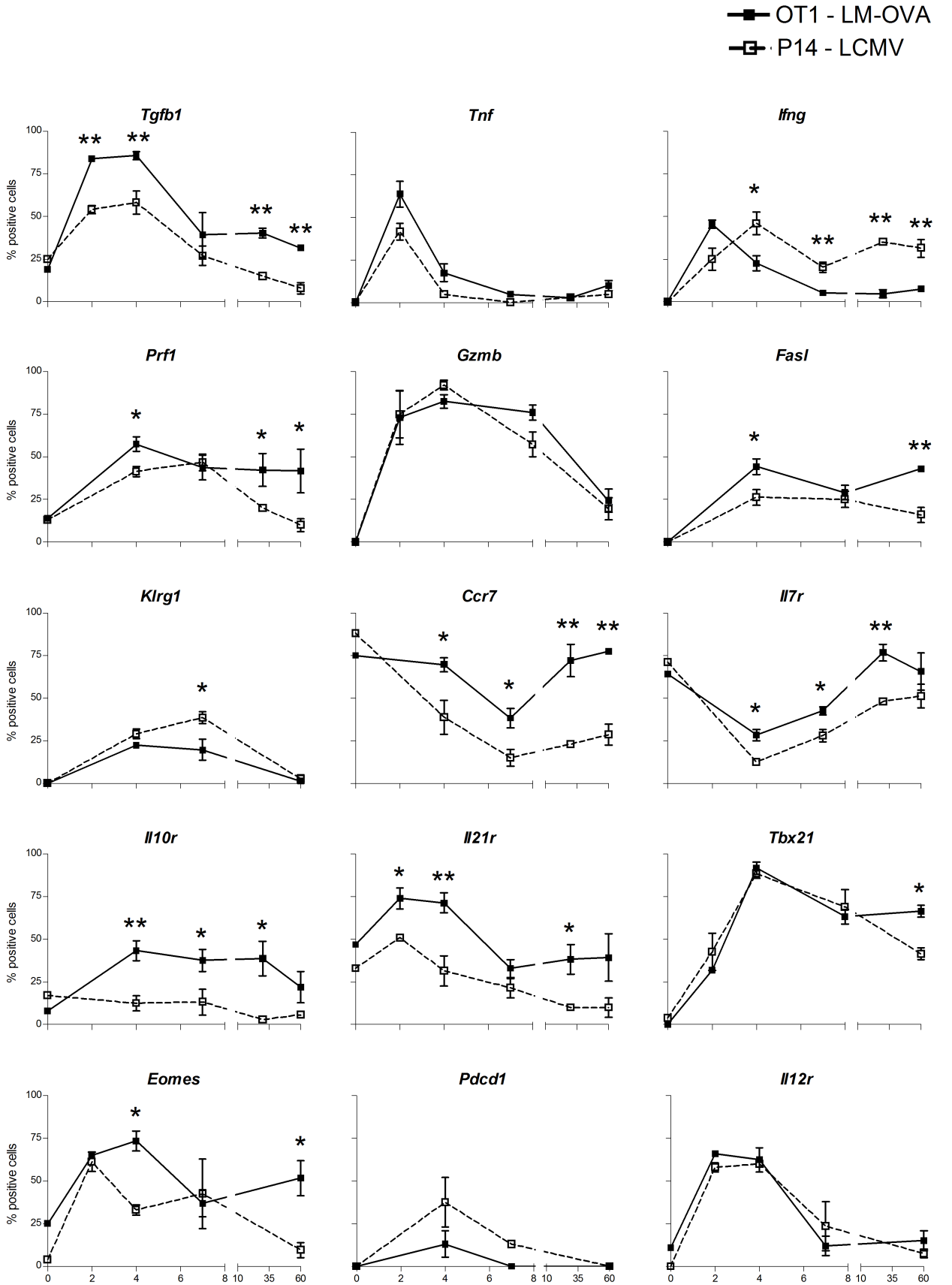
CD8β
Tet-GP33

Fig 3


OT1 - LM-OVA
P14 - LCMV
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Tnf
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Gzmb
Fasl
Klrg1
Ccr7
Il7r
Il10r
Il21r
Tbx21
Eomes
Pdcd1
Il12r
% positive cells
0
25
50
75
100
0
2
4
6
8
10
35
60

Fig 4

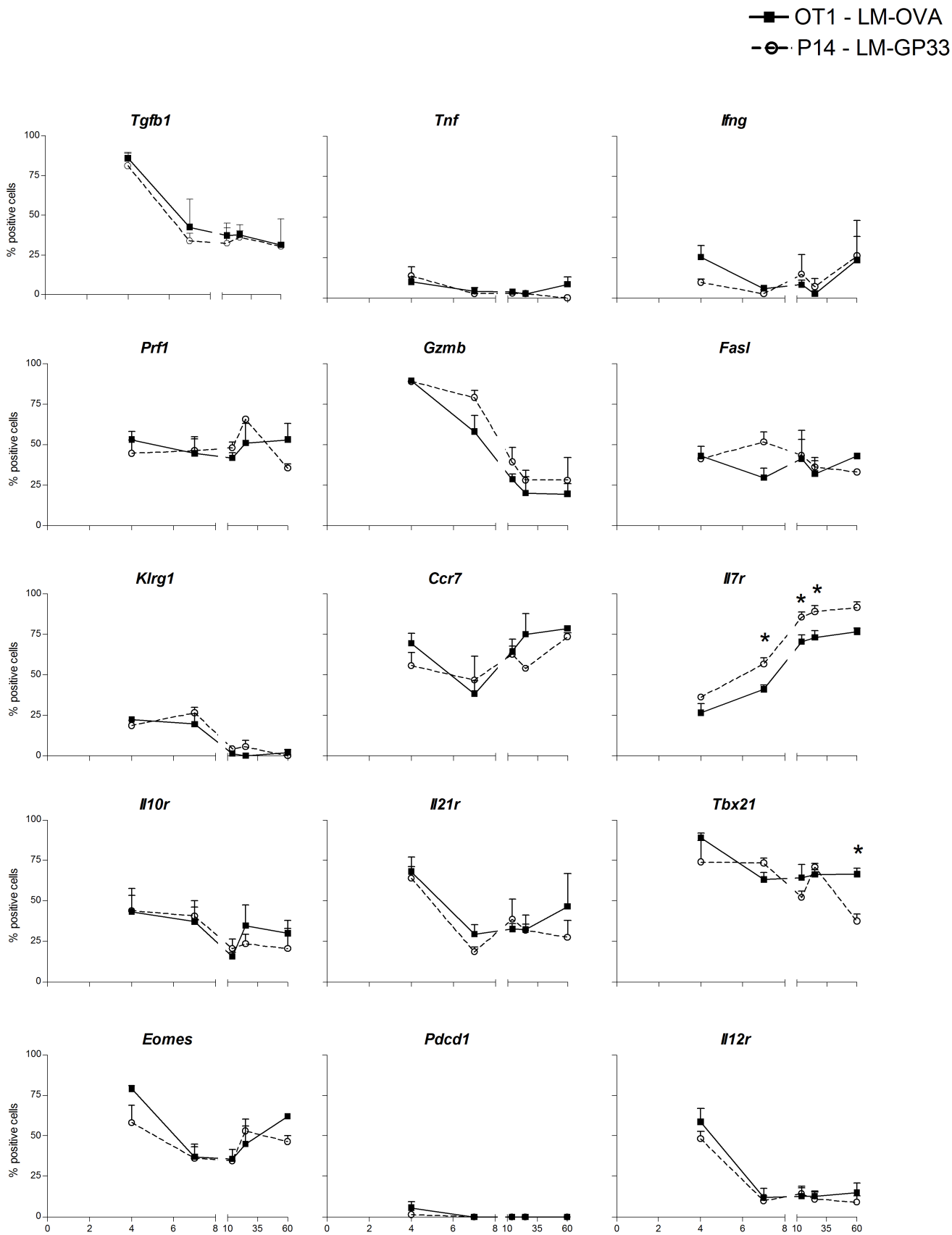
OT1 - LM-OVA
P14 - LM-GP33
Tgfb1
Tnf
Ifng
Prf1
Gzmb
Fasl
Klrg1
Ccr7
Il7r
Il10r
Il21r
Tbx21
Eomes
Pdcd1
Il12r
% positive cells
100
75
50
25
0
0
2
4
6
8
10
35
60
*

Fig 5

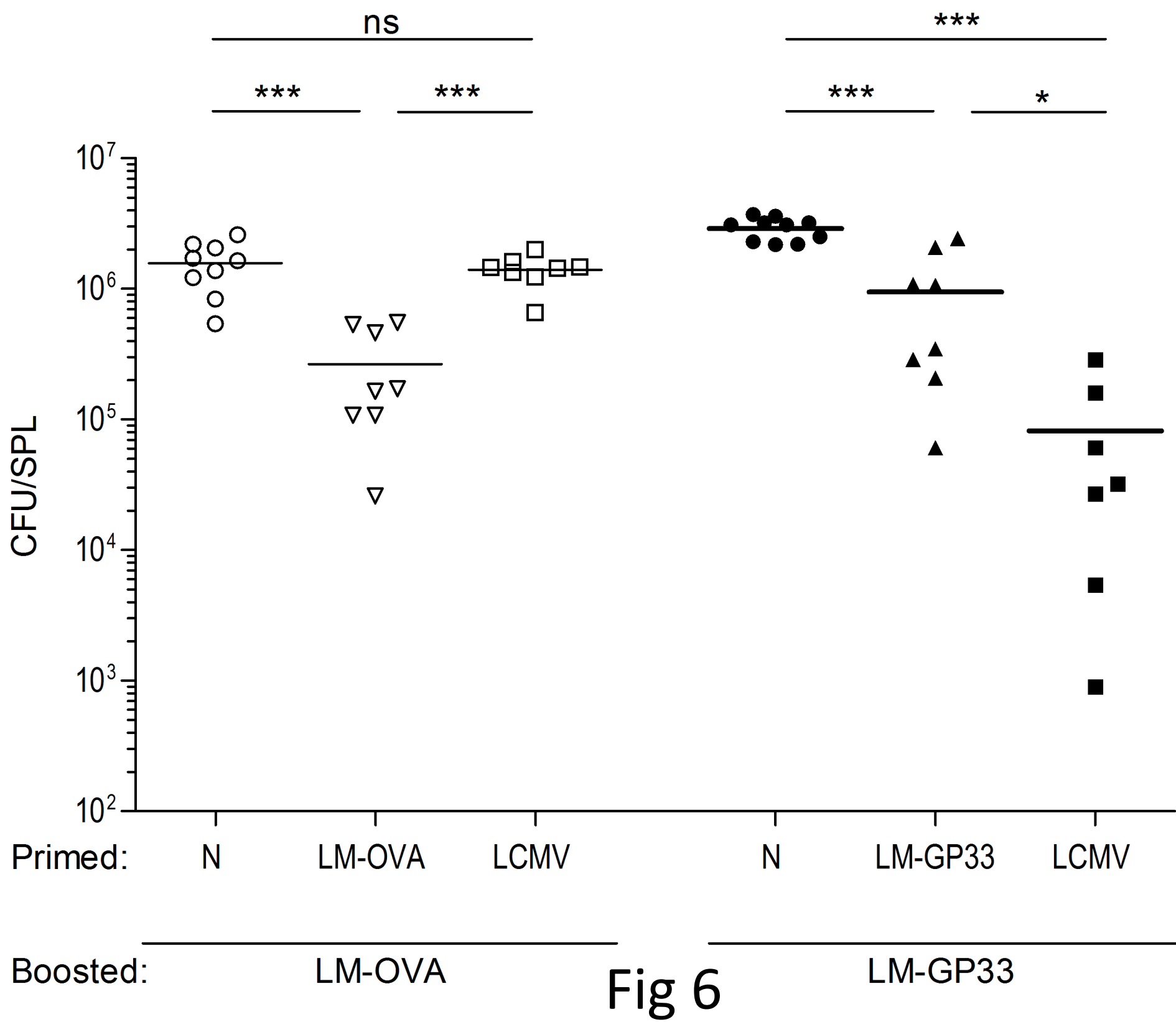
ns
***
***
***
***
*
CFU/SPL
10^7
10^6
10^5
10^4
10^3
10^2
Primed:
N
LM-OVA
LCMV
N
LM-GP33
LCMV
Boosted:
LM-OVA
LM-GP33

Fig 6

Table S1: List of primer sequences for single cell multiplex RT-PCR. Three specific primers were designed for each gene. Reverse transcription reactions were carried out using B primers. For the first and second PCR reactions A B and B C primer pairs were used respectively. A and C are sense primers, B are anti-sense primers.

Gene	Primer	Sequence 5' --> 3'
Tgfb1	A	ACCATCCATGACATGAACCG
	B	CAATCATGTTGGACAACTGC
	C	GCTACCATGCCAACTTCTGT
Tnf	A	AGCACAGAAAGCATGATCCG
	B	AACCTGGGAGTAGACAAGGT
	C	CCTCCCTCTCATCAGTTCTA
Ifng	A	GCTCTGAGACAATGAACGCT
	B	AAAGAGATAATCTGGCTCTGC
	C	TGTTTCTGGCTGTTACTGCC
Prf1	A	TCACACTGCCAGCGTAATGT
	B	CTGTGGTAAGCATGCTCTGT
	C	CACAGTAGAGTGTCGCATGT
Gzmb	A	GTCAATGTGAAGCCAGGAGA
	B	AGGATCCATGTTGCTTCTGT
	C	GGGAGTGTGAGTCCTACTTT
Fasl	A	TTCATGGTTCTGGTGGCTCT
	B	GAGCGGTTCCATATGTGTCT
	C	TGTATCAGCTCTTCCACCTG
Klrg1	A	AGGGATTTGGTAGAGATGGC
	B	GAGCCTTTGTCTGCACAGAA
	C	ATTCAACACTAGAGCTGCCG
Ccr7	A	AGGGAAACCCAGGAAAAACG
	B	TATCCGTCATGGTCTTGAGC
	C	TACGAGTCGGTGTGCTTCAA
Il7r	A	GAGTCCAAGTTCTACCTTCG
	B	CGGTTTGCACTGTGTACAGC
	C	AACCTGTCGTATGGCCTAGT
Il10r	A	AACAGTCAGTACTCCAACT
	B	CTGCTCCGTCGTGATAAGTA
	C	CGGCATCATCTATGGGACAA
Il21r	A	CATTGTCAATGTGACGGACC
	B	CTGCAGCTGGTAGCTAGAAT
	C	CAGGACGCTATGATATCTCC
Tbx21	A	AACCACCTGTTGTGGTCCAA
	B	CCCTTGTTGTTGGTGAGCTT
	C	AACCAGCACCAGACAGAGAT
Eomes	A	CCCCTATGGCTCAAATTCCA
	B	GGAGCCAGTGTTAGGAGATT
	C	GGCACCAAACTGAGATGATC
Pdcd1	A	AGGAAGAGGAGACTGCTACT
	B	ACGGGTTGGCTCAAACCATT
	C	AGGTACCCTGGTCATTCACT
Il12rb	A	ATGAGTGCTCCTGGCAGTAT
	B	TTCCAGTCCATTCGCAACTG
	C	TGGCCCTGAGGACAATGTTT
CD3e	A	ACCAGTGTAGAGTTGACGTG
	B	TATGGCTACTGCTGTCAGGT
	C	GCTACTACGTCTGCTACACA

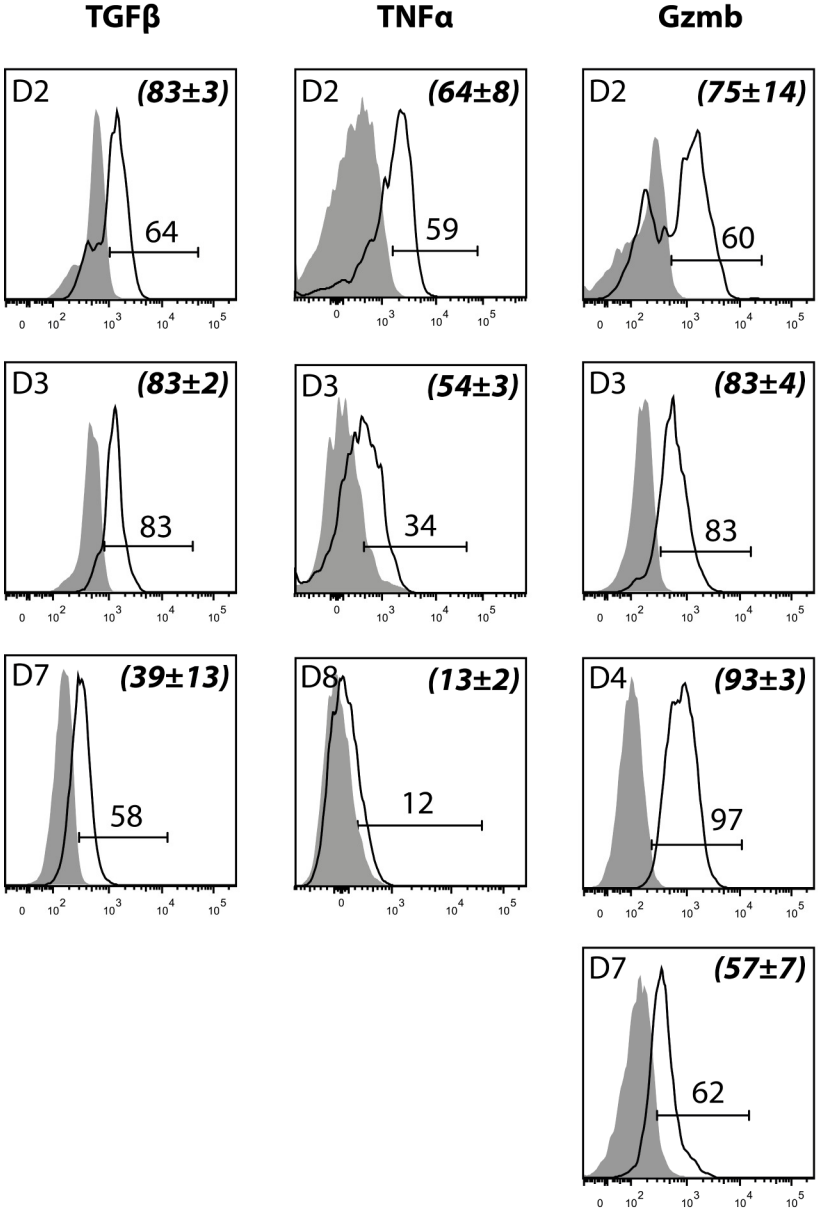
TGFβ
TNFα
Gzmb
D2
(83±3)
64
D2
(64±8)
59
D2
(75±14)
60
D3
(83±2)
83
D3
(54±3)
34
D3
(83±4)
83
D7
(39±13)
58
D8
(13±2)
12
D4
(93±3)
97
D7
(57±7)
62
0
10^2
10^3
10^4
10^5

Fig S1

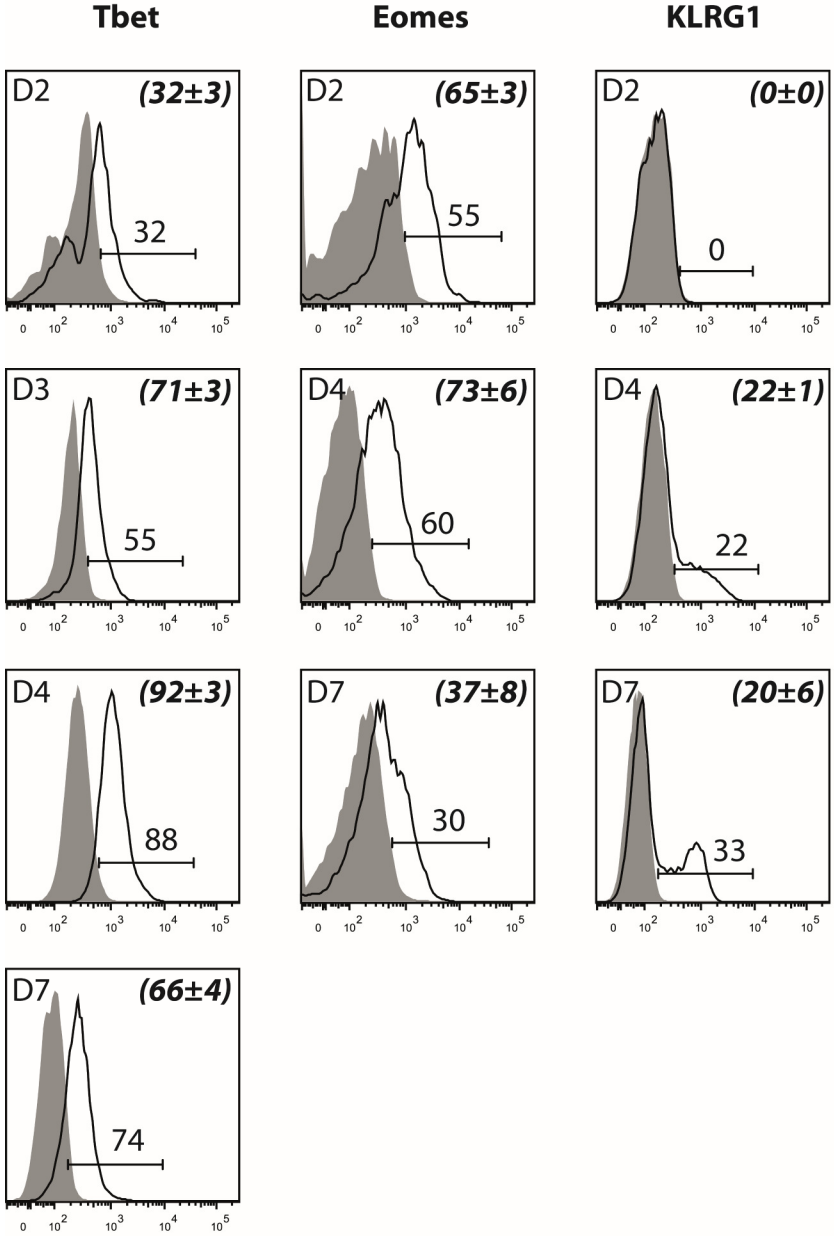
Tbet
Eomes
KLRG1
D2
(32±3)
32
D2
(65±3)
55
D2
(0±0)
0
D3
(71±3)
55
D4
(73±6)
60
D4
(22±1)
22
D4
(92±3)
88
D7
(37±8)
30
D7
(20±6)
33
D7
(66±4)
74

Fig S2

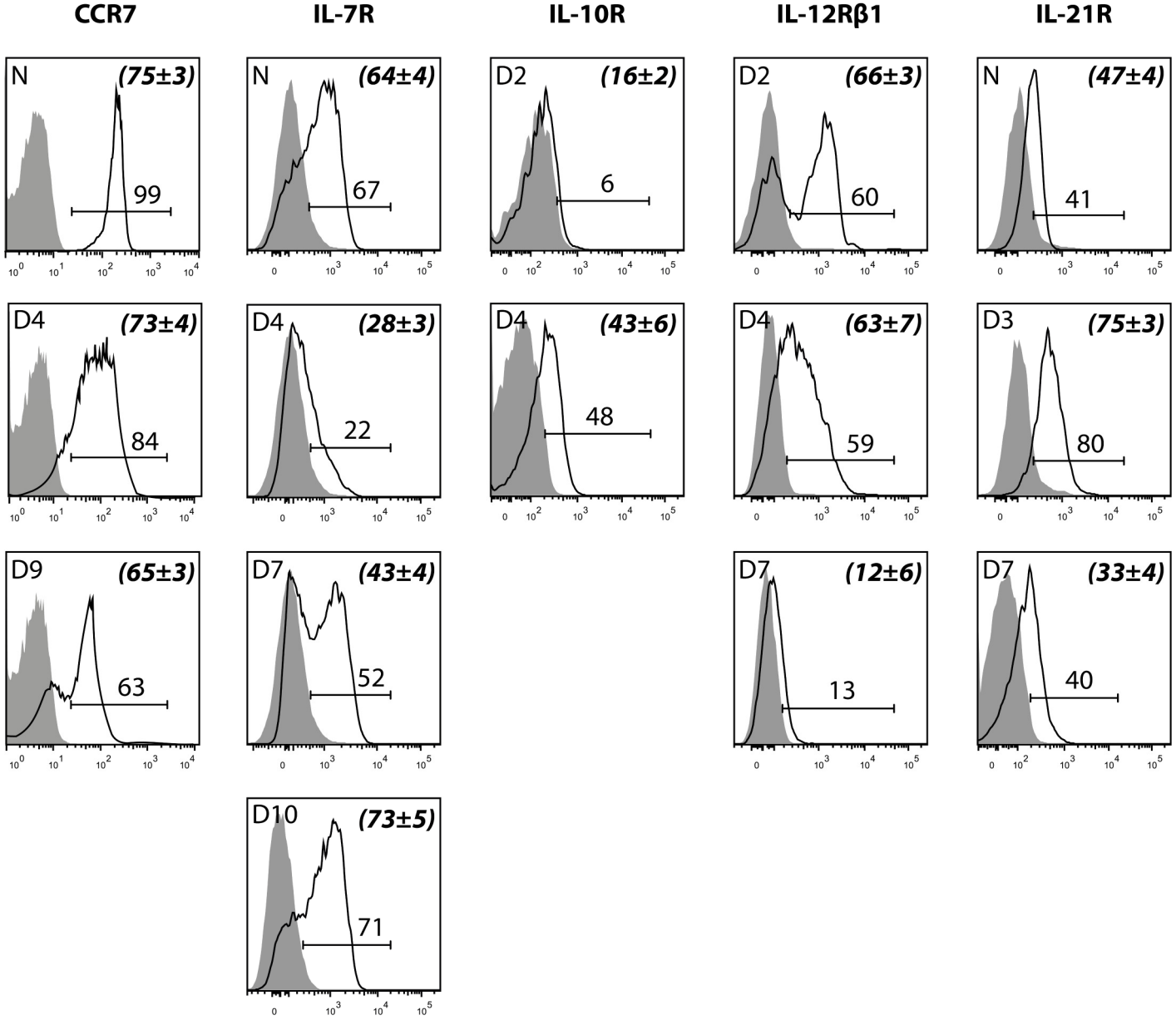
CCR7
IL-7R
IL-10R
IL-12Rβ1
IL-21R
N (75±3) 99
N (64±4) 67
D2 (16±2) 6
D2 (66±3) 60
N (47±4) 41
D4 (73±4) 84
D4 (28±3) 22
D4 (43±6) 48
D4 (63±7) 59
D3 (75±3) 80
D9 (65±3) 63
D7 (43±4) 52
D7 (12±6) 13
D7 (33±4) 40
D10 (73±5) 71

Fig S3

Article III

Cognate antigen stimulation generates potent CD8$^+$ inflammatory effector T cells

Sung H-C*, **Lemos S***, Ribeiro-Santos P, Kozyrytska K, Vasseur F, Legrand A, Charbit A, Rocha B and Evaristo C

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Cognate antigen stimulation generates potent CD8$^+$ inflammatory effector T cells

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Inflammatory reactions are believed to be triggered by innate signals and have a major protective role by recruiting innate immunity cells, favoring lymphocyte activation and differentiation, and thus contributing to the sequestration and elimination of the injurious stimuli. Although certain lymphocyte types such as TH17 cells co-participate in inflammatory reactions, their generation from the naïve pool requires the pre-existence of an inflammatory milieu. In this context, inflammation is always regarded as beginning with an innate response that may be eventually perpetuated and amplified by certain lymphocyte types. In contrast, we here show that even in sterile immunizations or in MyD88-deficient mice, CD8 T cells produce a burst of pro-inflammatory cytokines and chemokines. These functions follow opposite rules to the classic CD8 effector functions since they are generated prior to cell expansion and decline before antigen elimination. As few as 56 CD8$^+$ inflammatory effector cells in a lymph node can mobilize 10^7 cells in 24 h, including lymphocytes, natural killer cells, and several accessory cell types involved in inflammatory reactions. Thus, although inflammation modulates cognate responses, CD8 cognate responses also initiate local inflammatory reactions.

Keywords: CD8 T cells, immune responses, effector functions, lymphocyte trapping, lymph-node shut-down-phase

INTRODUCTION

The main CD8 effector functions are believed to be the production of IFN-γ and cytotoxic activity (CTL), which are induced after extensive division. However, while studying CD8 T cell responses at day 4 after priming we found that these cells had other properties. We named them "inflammatory effectors" because they expressed abundant *Tgfb*, but none co-expressed *TgfbR1* and *R2*, indicating that TGF-β could only act in *trans*, where it is pro-inflammatory [reviewed in Ref. (1)]. While testing their CTL activity (by co-injecting them with antigen-loaded and non-loaded targets directly into the spleen), these effectors did not kill loaded targets, but rather induced the local retention of both antigen-loaded and non-loaded targets (2), mimicking the events described in non-specific phase of lymphocyte trapping (3–5). Indeed, during the first 2–4 days of an immune response all antigen-specific-cells dispersed throughout the body are retained in the restricted site where the antigen is first presented (a phenomenon named lymphocyte trapping). Recent studies suggested that this local retention was due to the formation of stable interactions between antigen-specific T cells and the antigen-presenting cells (APCs). These stable interactions would lead to T cell activation and the subsequent down-regulation of the sphingosine-1-phosphate (S1P) receptor $S1P_1$ at the T cell surface, preventing antigen-specific T cells to egress the lymphoid organ (6). It was also shown that CD69 expression induced the down-regulation of $S1P_1$ since cells from CD69$^-$ mice failed to be retained (7, 8). However, these events only explain why antigen-specific-cells remain in contact with the APCs presenting the antigen. They do not explain how all lymphocytes dispersed throughout the body are "screened" for such binding capacity during a very short time-period after immunization. This is particularly problematic since it was shown that immediately after infection the number of APCs is very low: using the dose L50 of influenza virus in aerosols only four infectious particles were transmitted (9). In these circumstances, some circulating antigen-specific-cells may fail to contact these rare APCs, unless their transit time through the draining lymph node (DLNs) is considerably modified.

Indeed, early studies on lymphocyte trapping revealed that local recruitment of antigen-specific-cells was always preceded by profound modifications of migration affecting all lymphocytes. Elegant experiments in the sheep (3, 4) where the entry from the blood and the traffic in afferent and efferent lymphatic vessels were directly evaluated, as well as experiments in the mouse (5) showed that shortly after antigen administration, the influx of both antigen-specific and non-specific T cells into the DLN was much increased (3, 5) whereas egress was totally blocked for 1–3 days (3–5). This early reaction named "antigen non-specific trapping" or "lymph-node shut-down-phase" was considered fundamental

to allow every lymphocyte enough time to move among resident cells until meeting the rare APCs first presenting the Ag. Early studies proposed that such accumulation was due to an increase in local blood flow (10), but other studies indicated that perfusion rates were not modified: the apparent increase in blood flow was only due to an increase in the size of the organ (11). Moreover, an increase in the blood flow may explain the increase in lymphocyte input, but does not explain concomitant "shut-down-phase." Other studies reported that several inflammatory mediators could modify cell egress. Interferons (12) and TNF were reported to have these effects, but a detailed study of the effects of TNF injection showed they did not mimic those induced by antigen (13).

We here describe that CD8 T cells express a burst of pro-inflammatory cytokines immediately after cognate antigen stimulation. These inflammatory effectors follow opposite rules to classical CD8 functions, and are very potent effectors. They recruit non-resident cells, 56 cells injected into a LN recruiting up to 10^7 cells in 24 h, including lymphocytes and multiple accessory cell types involved in inflammatory reactions. They are generated even in sterile immunizations. These results describe the characteristics of a new CD8 effector differentiation phase. They show that although local inflammation modulates T cell responses, CD8 cognate responses also initiate local inflammatory reactions.

MATERIALS AND METHODS

MICE AND IMMUNIZATION PROTOCOLS

C57BL/6 mice expressing the CD90.1 allotype marker (Ba mice) and monoclonal (Mo) Rag2$^{-/-}$ mice expressing TCRαβ Tgs specific for the GP33 peptide of the LCMV (P14) or for the HY antigen (HY) expressing different CD45 allotypes were obtained from our breeding colonies at the Center for the Development of Advanced Experimental Techniques, Orleans, France. CD3ε$^{-/-}$CD45.1$^+$ mice, and CD90.2$^+$ Mo Rag2$^{-/-}$ mice expressing MoTCRαβ Tg receptor specific for OVA peptides (OT1 or OT2) and MyD88-deficient mice were gifts respectively from Antonio Freitas, and from Mathew Albert and were bred at the Pasteur Institute' animal facilities. *Listeria monocytogenesis* (LM) (expressing both the OT1 and the OT2 OVA peptides: LM-OVA) or LM-GP33 were kind gifts from L. Lefrançois – University of Connecticut Healthcare Center, Farmington, CT. For immunization with LM, sex-matched 6–8 weeks old CD90.1$^+$ B6 mice were adoptively transferred with 10^6 lymph-node cells derived from either MoP14 Tg mice or MoOT-1 Tg mice. One day later, LM were recovered during the exponential growth phase, and mice were injected i.v. with 5000 CFU LM. When specified in the text, naïve MoTg cells were labeled with 5 μM CFSE (Molecular Probes, Eugene, OR, USA) prior to injection. GP33-specific endogenous cells were obtained from wild type or MyD88-deficient mice immunized with the 5,000 CFU LM-GP33. Under both these infection conditions, bacterial loads (determined as CFU per liver or spleen) peaked at post-infection days 2–3, and the response peak was by day 8–10 after infection (not shown). For the generation of CD8 HY-specific effector cells, 6–8 weeks Rag2$^{-/-}$ female mice were injected i.v. with a mixture of 10^6 female and 10^5 male bone marrow cells from CD3ε deficient mice (14). Two days later these mice were injected i.v. with 0.5×10^5 CD4$^+$ (Marilyn) and CD8$^+$ Mo TCR-Tg cells specific for the male antigen.

ANTIBODIES USED FOR FLOW CYTOMETRY ANALYSIS AND CELL SORTING

The following monoclonal antibodies (MoAbs) used for flow cytometry and cell sorting were obtained from BD Pharmingen (San Diego, CA, USA): anti-CD3, anti-CD4, anti-CD8 (53-6.7), anti-CD8b (H35-172), anti-CD11b/Mac-1 (M1/70), anti-CD11c, anti-CD19, anti-CD44 (1M781), anti-CD45.2 (104-2.1), anti-CD69, anti-CD90.2/Thy1.2 (53-2-1), anti-DX5, anti-NK1.1 (PK136), anti-Ly6G/Gr1 (RB6-8C5), anti-Ly6c, anti-PDCA-1. All the above-mentioned mAbs were directly coupled to FITC, PE, PerCP, PECy7, allophycocyanin or Pacific Blue, or conjugated with biotin. Biotinylated mAbs were revealed with streptavidin-allophycocyanin (BD Pharmingen, San Diego, USA), or streptavidin-Pacific Orange (Molecular Probes, Eugene, USA). Innate cell populations present in brachial lymph node (BRLN) after the injection of naïve or effector cells were defined as following: NKs: DX5$^+$ NK1.1$^+$; cDCs: CD11c^{high}PDCA-1$^-$; pDC: CD11c^{low}PDCA-1$^+$; monocytes: CD11b^{high} LyC6^{high}; granulocytes (PMNs): CD11b^{high}Ly6C^{low}. For the *ex vivo* detection of cytokines and chemokines, mice were injected with 0.25 mg of Brefeldin A (Sigma-Aldrich, St. Louis, USA) and intracellular staining performed 6 h later (15), with the following Abs: rat anti-mouse CCL3 (clone IC450A, R&D Systems, Minneapolis, MN, USA); rat anti-mouse TNF-α (clone 557644, BD Pharmingen, San Diego, CA, USA), rat anti-mouse CCL4 (clone MAB451, R&D systems). Antibodies for phosphorylated signal transduction molecules and the respective isotype controls were purchased from Cell Signaling Technology (Danvers, MA, USA): Akt (Ser473, clone D9E)-PE, NF-kB p65 (Ser536, clone 93H1)-Alexa Fluor 488, p44/42 MAPK (Thr202/Tyr204, clone E10)-Alexa Fluor 488, p38 MAPK (Thr180/Tyr182, clone 28B10)-Alexa Fluor 647 and SAPK/JNK (Thr183/Tyr185, clone G9)–PE. Cells were analyzed on a FACSCanto system and sorted on a FACS Aria system (Becton Dickinson, Franklin Lakes, NJ, USA).

QUANTIFICATION OF ANTIGEN-SPECIFIC ENDOGENOUS CELLS

All the individual steps of this method are required to achieve optimal recovery and quantification of naïve cells. Organs were totally cleaned of fat and other adjoining tissues and distributed in 24-well plates in RPMI medium supplemented with 2% fetal calf serum and HEPES buffer. Cell suspensions were obtained by mechanical disruption with forceps followed by digestion with 0.5 mg/ml collagenase type IV (Worthington Biochemical Corporation, Lakewood, NJ, USA) and 5 μg/ml deoxyribonuclease I (Sigma-Aldrich, St. Louis, MN, USA) for 30 min at 37°C in 5% CO2 with agitation. We found that this digestion step was critical, since cell yields were much higher and the resulting cell suspensions cleaner when compared with those obtained by mechanical disruption alone.

For counting GP33-specific naïve cells, a known number of LN Mo P14 Tg cells expressing different allotypes were added directly to these suspensions prior to any further manipulation. The cells were then washed and depleted of non-CD8 T cells with a cocktail of MoAbs (TER119, CD19, Mac-1, GR1, CD4, B220) and Dynabeads (Dynal AS, Oslo, Norway). All these Abs were previously titrated to determine the binding efficiency and the absence of non-specific binding/depletion. We found that

this enrichment step was required to optimize the labeling and discrimination of endogenous antigen-specific-cells. Cells were labeled with PE- and APC-labeled multimers of MHC class I loaded with GP33 peptide (Dextramers®, Immudex, Copenhagen, Denmark) previously titrated on P14 Tg cells, and antigen-specific-cells recovered by pull-down (16). In contrast to MHC tetramers, these multimers associate a higher number of fluorochrome and peptide-loaded MHC class I molecules, enabling a better discrimination between antigen-specific and non-specific endogenous cells. For counting antigen-specific-cells, the labeled populations were diluted in 0.5–1 ml of FACS flow buffer and acquired using the low-speed mode in a FACS-Canto. The use of low-speed mode was also found important, since it reduced both the cell loss during acquisition and the background non-specific labeling.

CYTOKINE EXPRESSION. CORRELATIONS BETWEEN CELL DIFFERENTIATION AND DIVISION "*IN VIVO*"

Assessment of the correlations between the expression of several pro-inflammatory mediators and molecules involved in cytotoxicity with division *in vivo* was hindered by (i) the very low number of cells present at each division at early time points of the response and (ii) the small number of parameters than can be reliably used with CFSE. In order to overcome these difficulties and study the expression of several effector molecules simultaneously, we used a single-cell multiplex RT-PCR technique previously developed in our laboratory (17). This technique allows the simultaneous detection of the expression of *Tgfb1*, *Tnf*, and cytotoxic genes in each individual cell and we created compatible primers for the simultaneously detection of chemokines (Figures S1 and S2 in Supplementary Material). Briefly, individual cells were sorted and lysed and the mRNA reverse-transcribed by using specific 3′ primers for all genes. The 5′ primers are then added, and a first 15-cycle amplification step is initiated. The PCR products are then split into different wells and a second, nested PCR is performed for each gene separately. Given that the primers amplifying all the mRNAs are present in the first PCR, the arrays have two major requirements. Firstly, all PCRs should have the same efficiency, to prevent preferential amplification. Secondly, neither the primers nor the amplicons should compete during the first PCR reaction. For new primers, validation of these two requirements are shown in Figures S1 and S2 in Supplementary Material. Validation of the other primers has been described previously (17).

The following primers were selected:

Xcl1: forward 5′-GAC-TTC-TCC-TCC-TGA-CTT-TC-3′, nested forward 5′-GGA-CTG-AAG-TCC-TAG-AAG-AG-3′, and reverse 5′-TGC-CAT-CCA-CAG-TCT-TGA-TC-3′
Ccl3: forward 5′-AAG-GAT-ACA-AGC-AGC-AGC-GA-3′, nested forward 5′-CCA-GTC-CCT-TTT-CTG-TTC-TG-3′, and reverse 5′-GAT-CTG-CCG-GTT-TCT-CTT-AG-3′
Ccl4: forward 5′-CCA-GCT-CTG-TGC-AAA-CCT-AA-3′, nested forward 5′-GAG-CAA-CAC-CAT-GAA-GCT-CT-3′, and reverse 5′-GCT-CAG-TTC-AAC-TCC-AAG-TC-3′
28S: forward 5′-TAC-CGG-ACC-CTG-AAC-AGA-AT-3′, and reverse 5′-GAT-GAT-CCT-CCG-GCA-TGT-TT-3′ (28S amplification was used to test the plating efficiency).

We used two independent approaches to show that this method can detect as few as two mRNA molecules per cell (17). *NB*: the expression of all these effector molecules requires cognate antigen stimulation. Naïve cells do not express these mRNAs. The infection milieu does not induce the expression of these mediators: when mice are infected with LM that does not express OVA, OT-1 cells do not express these mediators.

Detection of cytokine proteins *ex vivo* was performed after injection of Brefeldin A, as described (15).

THE INFLAMMATORY CAPACITY OF CD8 T CELLS *IN VIVO*

Sorted naïve or effector CD90.2 Tg T cells obtained from the spleen 2.5 days after immunization. In some experiments they were mixed at 4°C with 50% "High Concentration" Growth factor-reduced Matrigel (BD Biosciences, San Jose, USA), and injected subcutaneously in the ear of CD90.1 recipient mice. In other experiments, the BRLN was accessed by a small vertical skin incision parallel to medial border of the scapula in anesthetized mice. The same cells were injected directly in the BRLN in a 10 μl volume using an insulin syringe, and the skin incision was closed with one wound clip. We aimed to inject 60 or 600 cells/mouse but variations in cell numbers were to be expected after such high dilutions and injection. To determine the actual number of cells present in the BRLN after injection, we counted them in the final cell suspension and in the BRLN of control mice shortly after injection.

To determine the best time point for studying the effect of these effectors, we measured the weight of LNs at different times after injection of effector T cells. For evaluation of S1P, LNs were studied 16 h after the injection of effectors, since their weight had not yet increased at that time point. For the detection of cell accumulation, LNs were studied between 24 and 30 h post-injection, when they had already increased in size. For the detection of S1P, we used the S1P bioassay described previously (18) with WEHI231 cells expressing Flag-S1P1 (a kind gift of J. Cyster). This method was slightly modified to increase sensitivity. To concentrate the S1P amounts recovered from the LN, we extracted S1P in a 50 μl volume, and reduced the reaction volumes to 40 μl and the number of WEHI231 cells to 2×10^4/well. To reduce background labeling (variations of Flag-S1P1 expression in WEHI231 cells incubated with medium alone) these cells were previously sorted to obtain homogeneous populations expressing the same level of Flag-S1P1.

RESULTS

CD8$^+$ INFLAMMATORY EFFECTOR T CELLS DO NOT FOLLOW THE RULES THOUGHT TO GOVERN CD8$^+$ DIFFERENTIATION INTO EFFECTOR CELLS

Since in the first 1–4 days after antigen administration cell input to the DLN is much increased, we studied if CD8 T cells would express mediators justifying this increase. When Mo TCR-Tg cells were stimulated with LM expressing the OT1 and the OT2 epitopes, CD8 OT1, but not the CD4 OT2 cells abundantly expressed a panoply of chemokines – *Xcl1*, *Ccl3*, and *Ccl4* by day 2 after immunization, while this expression was lost before the immune response peak (**Figure 1A**). OT1 cells also expressed *Tnf* (**Figure 1A**), reported to increase HEV permeability and reduce LN egress (19). *Ex vivo* detection of protein in Brefeldin A injected mice confirmed that secretion of pro-inflammatory mediators was restricted to the early response (**Figure 1B**). Production of

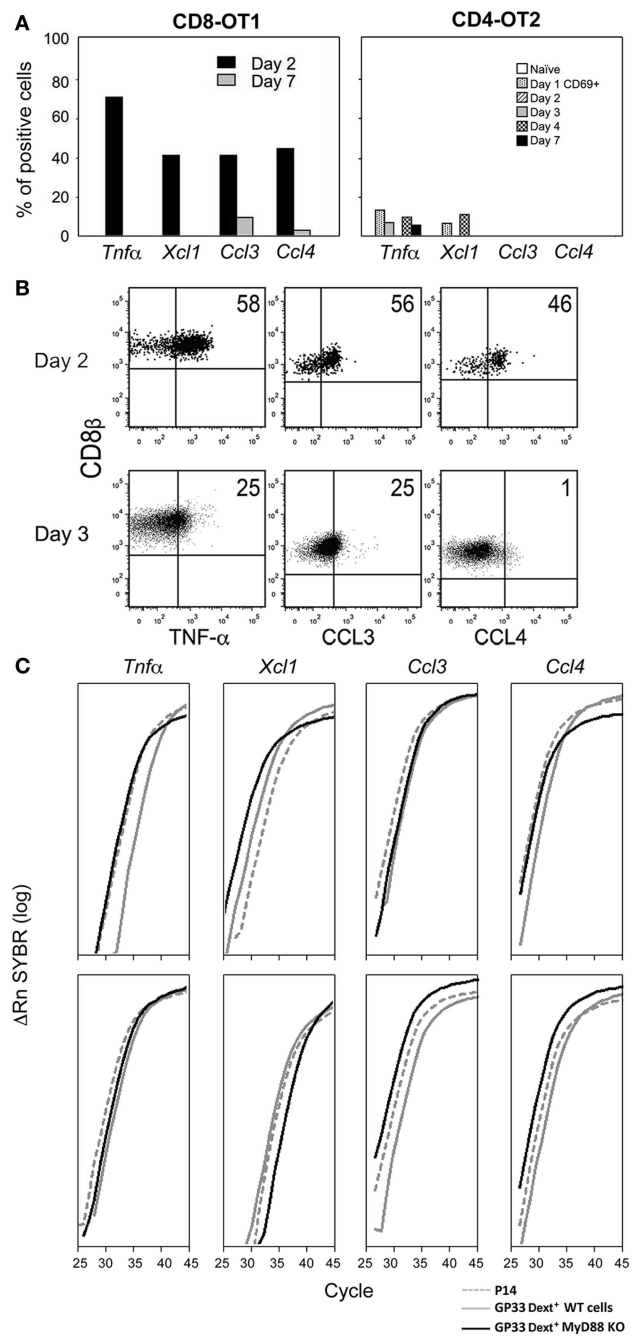


FIGURE 1 | Expression of inflammatory mediators by CD8 T cells. **(A,B)** Mo CD90.2 TCR-Tg cells were transferred to CD90.1 B6 mice, and immunized with LM-OVA expressing the OT1 and OT2 epitopes. At different time points after infection we determined: **(A)** The proportion of OT1 and OT2 Tg cells expressing these mRNAs evaluated in 72 individual cells by single-cell RT-PCRs. **(B)** The *ex vivo* intracellular expression of pro-inflammatory proteins in Brefeldin A injected mice. Barriers to identify positive cells were based on the labeling of the same cell suspension with isotype control antibodies. The lack of suitable Abs prevented us from evaluating XCL1 expression. **(C)** Mo CD90.2 P14 TCR-Tg cells were transferred to CD90.1 B6 mice. 24 h later, these transferred mice, as well as My88^{+} and MyD88^{-} mice were immunized simultaneously with LM-GP33. *(Continued)*

FIGURE 1 | Continued
CD8^{+} CD69^{+} GP33-specific-cells were sorted from these three mice types at days 2 and 3 after priming. For that purpose, spleen cell suspensions were depleted of non-CD8 T cells. The P14 Tg cells were identified by their co-expression of CD90.2 and CD8β. Endogenous cells from MyD88^{+} and MyD88 B6^{-} mice were identified by triple co-expression of CD8β, and APC, and PE labeled GP33-Dext. Results show the levels of these mRNAs in CD69^{+} GP33-specific-cells 2 (upper graphs) and 3 days (lower graphs) after immunization, evaluated by qRT-PCR.

these mediators was dependent on the recognition of the Ag by antigen-specific-cells. When these mice were injected with LM not expressing OVA, OT-1 cells did not express any effector function. Mo CD8 anti-HY TCR-Tg cells but not Mo CD4 anti-HY Marilyn TCR-Tg immunized with male cells also produced a similar cytokine/chemokine burst, although *Ccl3* and *Ccl4* levels were lower than found in OT1 CD8 cells (not shown).

Since the above results were obtained with TCR-Tg cells we wished to evaluate if endogenous cells shared the same properties. Moreover, since immunization with male cells does not involve obvious innate signals we wished to study the impact of such signaling in inflammatory effector functions. We compared by qRT-PCR the expression of the mRNAs coding for these mediators by different types of CD69^{+} GP33-specific CD8 T cells, immunized with LM-GP33. We found equivalent expression in TCR-Tg cells from P14 mice and in endogenous cells from MyD88^{+} or MyD88^{-} B6 mice (**Figure 1C**). These results show that endogenous and TCR-Tg cells share the same properties. Moreover, such properties are not affected by the abrogation of the MyD88 pathway.

The kinetics of expression of these mediators was surprising, since it is generally believed that all CD8 effector functions increase with cell division, and decline when antigen is eliminated (20, 21). Correlation of cell division with differentiation (**Figure 2A**) confirmed that the expression of cytolyic effector molecules does follow these rules: they increase with division (lower panel), and we previous confirmed that they decline only after antigen elimination (2). In contrast, inflammatory mRNAs were expressed in CD69^{+} cells before any division, declining with division. While effector functions are believed to decline only when antigen is eliminated, inflammatory effectors also contradicted this rule, as they were lost by day 4 after immunization (**Figure 2A**), when we previously showed that antigen concentrations were still (2).

We next wished to determine the cause for this early decline. Given that the expression of these mediators requires MAPK activation, we looked at whether such activation could be interrupted during the early phases of the CD8 response. Indeed, both OT-1 TCR-Tg cells and endogenous cells down-regulated their TCR (**Figures 2B,C**) and CD3ε (not shown) losing the ability to bind peptide-loaded dextramers (Dext: MHC multimers with a higher number peptide – MHC complexes than tetramers). The MAPK activation declined progressively and was fully abrogated before the immune response peak (**Figure 3**), restricting CD8s' inflammatory profile to the first days after priming.

Overall, these results demonstrate that the classic description of the sequential steps of CD8 responses (activation, expansion, differentiation into effector functions, antigen elimination, effector function loss) must be revised. CD8 T cells actually undergo two

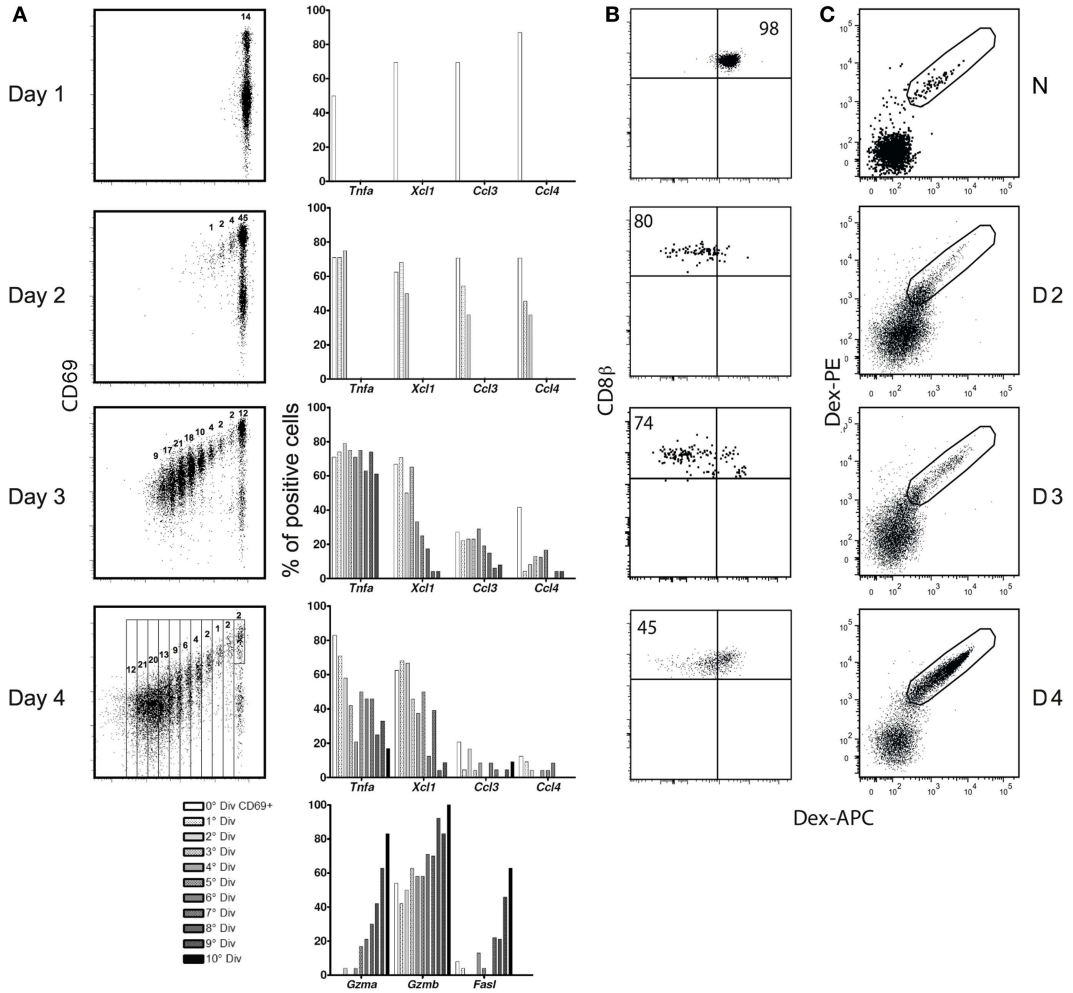


FIGURE 2 | (A) Correlation between CD8 division and differentiation. **(A)** CFSE-labeled Mo CD90.2 OT-1 Tg cells were transferred to CD90.1 B6 mice and studied at different time points after infection with LM-OVA. Left: CFSE dilution and the gates used to sort individual cells at each division. Right: expression frequency, as evaluated in RT-PCRs in 96 individual cells. **(B,C)** TCR expression after immunization with LM-GP33. **(B)** TCR-Tg cells. 5,000 Mo CD90.2$^+$ P14 TCR-Tg cells were injected into CD90.1$^+$ B6 mice prior to infection. Results show APC-Dext-GP33 binding in CD90.2$^+$ CD8$^+$ Tg T cells in naïve (upper graph) and infected mice, at different days (D) after immunization. **(C)** Endogenous cells. CD90.1 B6 mice were immunized with LM-GP33. Results show Dext-GP33 PE and APC double-labeling in gated CD8 spleen cells in naïve mice (upper graph) and primed mice at different days (D) after infection.

independent differentiation phases, governed by opposite rules. Immediately after activation they differentiate into inflammatory effectors, but then lose their pro-inflammatory properties as they divide and gradually differentiate into CTLs.

THE PHYSIOLOGIC ROLE OF CD8$^+$ INFLAMMATORY EFFECTOR T CELLS: I. QUANTIFICATION OF THEIR NUMBER

Since the generation of inflammatory effectors is restricted to the first three-four divisions, their number should never exceed that of naïve cells by more than a factor of eight. It is difficult to envisage that such low effector number has any major role in the immune response, since the number of naïve cells for a single GP33 epitope was described to average 200 cells/mouse (16). However, this number is known to be an underestimate (16). Indeed, from the 50 LN reported to be present in the mouse, only nine were included in this calculation. The identification of the naïve cells after tetramer pull-down was not controlled for the efficiency of tetramer binding nor for losses during purification.

We revised these estimates by treating individual organs with collagenase/DNase (increasing substantially total cell recovery), and adding a known number of antigen-specific "reference populations" (RP) to these cell suspensions. These RP bind the same tetramers as endogenous cells and therefore can be used as controls for the efficiency of tetramer binding. Since they undergo the same purification steps, their recovery rate can be used to control non-specific cell losses during purification. We also used the more efficient Dext instead of tetramers for the purification of antigen-specific-cells. Using these combined approaches, we evaluated the total number of naïve cells for the LCMV response. This evaluation can only be determined for this response, since this calculation

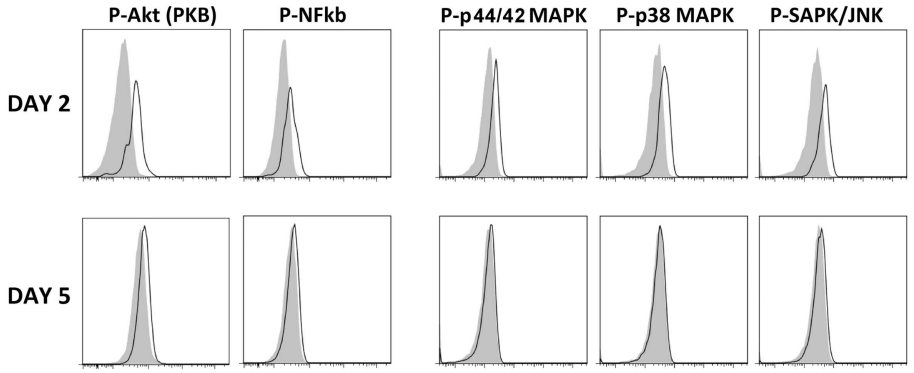


FIGURE 3 | TCR signaling transduction at different time points after immunization. Mo CD90.2$^+$CD45.1$^+$ OT-1 TCR-Tg cells were transferred into CD90.1$^+$ CD45.2$^+$ B6 hosts and infected with LM-OVA. The figure shows the expression of phosphorylated signal transduction molecules in CD90.2$^+$ CD45.1$^+$ CD8$^+$ Tg cells at different time points after priming. The labeling with isotype controls is shown in gray.

requires the identification of all antigen epitopes and their relative representation in the response. These data are only available for the LCMV response, where it is known that the GP33 specificity corresponds to 10% of the LCMV specific-cells (22).

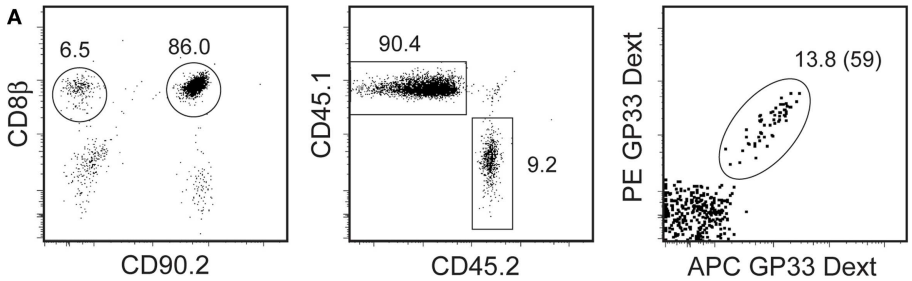
When two RP of monoclonal P14 TCR-Tg LN T cells (10^4 CD90.2 CD45.1 and 10^3 CD90.2 CD45.2 P14 cells) were added to BRLN of CD90.1 CD45.2 mice, endogenous CD90.1 CD8 T cells could be easily differentiated from CD90.2 RP populations (**Figure 4A**: left). Both RP (CD45.1 and CD45.2) were fully labeled with GP33-Dext, demonstrating that Dext binding of the suspension was efficient (not shown). Moreover, CD45.2 and CD45.1 RP populations maintained the same relative representation after purification (10:1) demonstrating that less abundant cells were not preferentially lost (**Figure 4A**, middle). Within CD90.1$^+$ endogenous CD8 T cells, Dext$^+$ cells were clearly visualized (**Figure 4A**, right), the number of GP33-specific-cells in the BRLN averaging 60. Similar results were obtained for the number of epitope HY-specific or SIINFEKL – OVA specific naïve CD8$^+$ cells.

To calculate the number of naïve cells/mouse identical studies were performed in the spleen and in 39 other LN. In the three independent experiments we performed we found about 3,800 naïve GP33-specific-cells (**Figure 4B**). Since the GP33 specificity corresponds to 10% of the LCMV response (22), total number of naïve cells responding to LCMV should be about 3.8×10^4, and the total number of inflammatory effectors could reach 3.04×10^5. It must be noted that this number is still an underestimate, since we only managed to recover 39 LN of the 50 LN described, and naïve cells present in the blood and bone marrow were not counted. However, it is likely that the number of naïve cells would not be much higher since the LN we did not harvest were described as relatively small, and the BM harbors mostly antigen-experienced cells.

THE PHYSIOLOGIC ROLE OF CD8$^+$ INFLAMMATORY EFFECTOR T CELLS: II. CHEMOKINES CAN ATTRACT CELLS AT DISTANCE

A role of the chemokine burst produced by CD8 T cells in the increase in cell input during antigen-non-specific trapping would imply that these chemokines should create a gradient able to recruit cells located out-side the DNL. However, it is yet unclear if chemokines only modify the migration of resident cells (23). To test if CD8 inflammatory effectors attract cells at distance, they should be isolated from the *in vivo* inflammatory milieu, transferred to a normal mouse and retained in a particular site where non-resident cells would not migrate, and their effect studied at distance. We found that this was possible when sorted effectors were immobilized subcutaneously in the ear with the collagen-laminin matrix Matrigel. Matrigel is liquid at 4°C but solidifies after injection, and is frequently used to promote viability of tumor cell lines injected subcutaneously. When injected with Matrigel, effectors remained viable and at the injection site and could be recovered and from the Matrigel plug, but were absent in the auricular LN (ALN) (**Figure 5A**) and other organs (not shown) 24 h later. When 5×10^4 naïve OT-1 were immobilized in the ear, naïve cells did not modified the number or cellular composition of the ALN as compared to non-injected mice (not shown). In contrast, when similar numbers of OT1 inflammatory effectors were retained, the ALN increased in size, accumulating both T and B lymphocytes (**Figure 5B**). We also observed a preferential accumulation of NK cells, CD11b$^+$Ly6C^{hi} inflammatory monocytes, cDCs, and CD11b$^+$Ly6C^{low} PMNs (**Figures 5B,C**) reflecting the production of MIP-1 and XCL1 chemokines by these CD8 T cells. Similar effects were observed when the effector number was reduced 20-fold. These results show that CD8 inflammatory effectors are able to recruit non-resident cells.

When immobilized in the ear, HY naïve cells had no effect, while HY-specific inflammatory effectors induced a major accumulation of XCR1 expressing cells (24, 25): T and B lymphocytes, NK cells, and CD11b$^+$Ly6C^{hi} inflammatory monocytes. Indeed, upon injection of HY effectors the ALN could triple in size, i.e., LN hypertrophy was more marked than that found after injection of OT-1 effectors. Accordingly to their lower expression of MIP-1 chemokines, DCs and PMNs that are recruited by MIP-1 chemokines (26, 27), were less affected (**Figure 6**). Thus, CD8



B

Minimal Number of G933-specific naive cells

Exp #	LNs (39)	Spleen	Total
1	2,802	981	3,783
2	1,972	1,200	3,172
3	3,320	1,169	4, 489
AVERAGE			**3, 815**
N° Naive/mouse: 38,000			

FIGURE 4 | Quantification of the naïve T cell pool. **(A)** Quantification of antigen-specific naïve cells in the BRLN. Reference populations (RPs: 10^4 CD90.2 CD45.1 cells and 10^3 CD90.2 CD45.2 P14 cells) were added to collagenase/DNase digests of the BRLN from CD90.1 B6 mice. Cell suspensions were depleted of non-CD8 T cells, and antigen-specific-cells purified by Dext pull-down. Left: the gates used to identify CD90.2$^+$ RP and CD90.1 endogenous cells. Middle: CD45 allotypes distribution in RPs. Right: GP33-Dext labeling in CD90.1 endogenous CD8 T cells. **(B)** Estimation of the size of the naïve GP33-specific T cell pool. Results show the number of Dext-GP33-specific-cells recovered/mouse in three independent experiments.

inflammatory effectors generated after a sterile immunization are also able to recruit non-resident cells at distance.

THE PHYSIOLOGIC ROLE OF CD8$^+$ INFLAMMATORY EFFECTOR T CELLS: III-LOCAL MODIFICATIONS AFTER INTRA-NODAL INJECTION OF PHYSIOLOGIC NUMBERS

Since at the beginning of a natural infection the inflammatory effectors should only be the rare resident cells already present in a lymphoid organ, we aimed to study the role of such effector numbers after intra-nodal injection. We first selected the target LN used for injection, which should be relative large (facilitating calculation of the number of naïve resident cells) and giving easy access to injection. We studied the positions and sizes of several superficial LNs and the best procedure to access them and found the BRLN suitable in both respects. After collagen and DNase digestion it harbors 5–8 × 10^6 cells. It can be reproducibly accessed through a very small skin incision near the scapula. Since in this node the number of naïve cells specific for the GP33 epitope averages 60 cells (**Figure 4A**), the number of naïve LCMV specific-cells should be 600, since the GP33 specificity corresponds to 10% of the LCMV response (22). Therefore the total number of resident effectors should be lower than 4,800. Remarkably, even a much lower number of OT-1 (**Figure 7A**) or anti-HY inflammatory effectors (**Figure 7B**) injected directly in the BRLN induced a major local recruitment. Again, the type of recruited cells reflected the chemokine profile expressed by each effector type.

We were surprised that as few as 56 effectors could attract up to 10^7 cells to a LN in 24 h (**Figure 7B**), and wondered whether other mediators could be involved. Given that (i) LN egress is totally blocked during early trapping (3, 4) and (ii) S1P gradients control this egress (18), we hypothesized that inflammatory effectors could increase local S1P concentrations and thus contribute to the local retention. Indeed, since inflammatory mediators induce the release of S1P by certain resident cell lines *in vitro* (28, 29), it was hypothesized that local inflammation could induce S1P release by tissue-resident cells and thus contribute to cell accumulation at the inflammatory site (30). This attractive hypothesis could not be confirmed experimentally since previous methods did not allow S1P measurements in small tissue fragments. We modified these methods to increase sensitivity and found that intra-nodal injection of inflammatory effectors induced the up-regulation of S1P in tissue extracts from that BRLN 16 h later (**Figure 7C**). By comparison with a known concentration of standard, synthetic S1P, the amounts of S1P recovered from BRLNs injected with effectors ranged from 1422 ng/g to about 2800 ng/g, i.e., 2.4- to 5-fold higher than the value reported after direct extraction of S1P from a large LN pool (18). This value is likely an underestimate, since the S1P levels in BRLNs injected with 56 effectors were 10-fold higher than

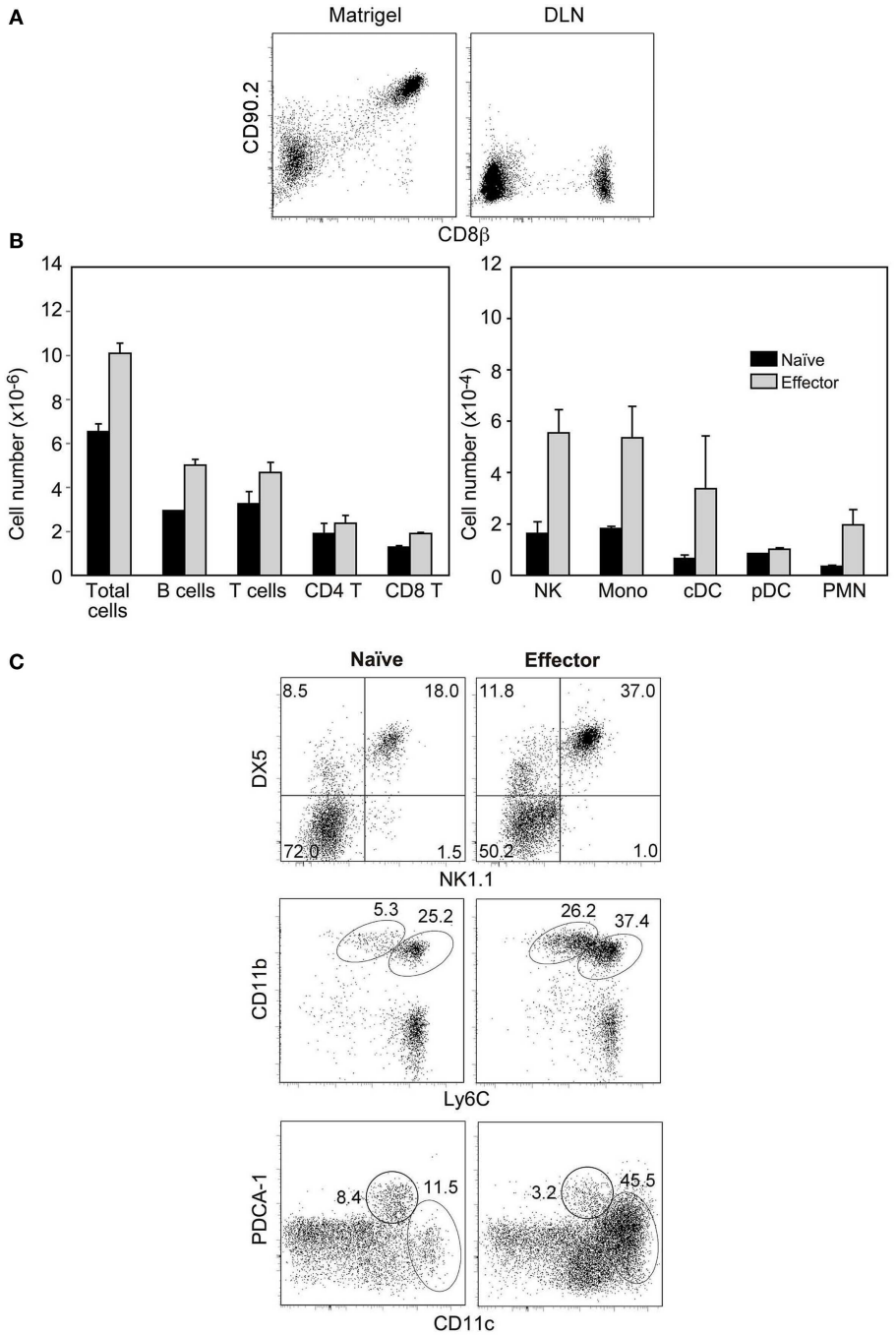


FIGURE 5 | Inflammatory effector CD8 T cells recruit non-resident cells. Sorted Mo CD90.2$^+$ OT-1 naïve cells or inflammatory effectors (recovered 2.5 days after LM-OVA infection) were injected with matrigel subcutaneously in the ear of CD90.1$^+$ mice. **(A)** Recovery of injected cells from the matrigel plug (left) and the auricular LN (ALN) (right) 24 h after injection. **(B,C)** Cell populations recovered in the ALN of injected mice, 24 h after injection. **(B)** Results show cell numbers and are mean±SEM of three independent experiments. **(C)** Results show the gates used to identify different cell types and are from one representative experiment out of the three we performed.

in BRLNs injected with a 600 naïve cells, studied simultaneously (**Figure 7C**). Since a twofold increase in the S1P concentrations is enough to induce major changes in lymphocyte egress (6) the S1P amounts detected here justify the blockage of LN egress.

DISCUSSION

Our present results characterize an important function of CD8 T cells, which modifies the actual paradigms on: (i) when CD8 cells acquire and lose effector functions; (ii) the roles that CD8

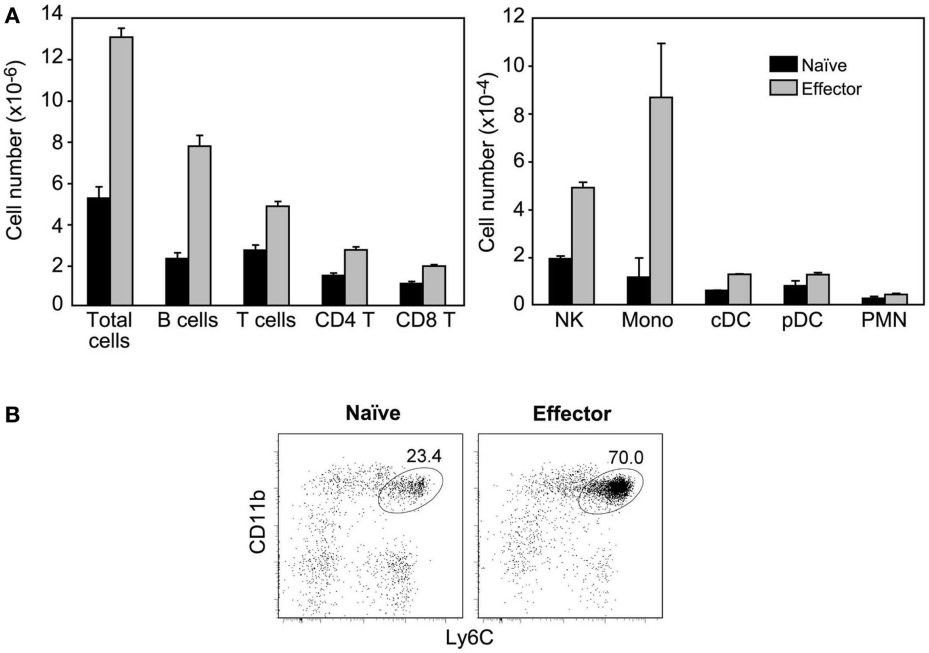


FIGURE 6 | CD8$^+$ inflammatory effector T cells generated in sterile immunizations also recruit non-resident cells. Sorted Mo CD90.2$^+$ HY-specific CD8 naïve cells or inflammatory effectors (recovered at 2.5 days after immunization with male cells) were injected with matrigel subcutaneously in the ear of CD90.1$^+$ mice. **(A)** Results show the number of different cell types recovered in the ALN of injected mice, 24 h after injection and are the mean±SEM of three independent experiments. Cell populations were identified as described in **Figure 5**. **(B)** Major accumulation of inflammatory monocytes in the ALN of mice injected with inflammatory effectors. Results are from one representative experiment, *out of the three performed*.

cells have in immune responses; and (iii) how inflammation and cognate immune responses interact.

It is generally believed that after antigen stimulation CD8 T cells effector functions increase with division and decline once the antigen is eliminated. Our results allow comparing the differentiation into killer or inflammatory functions by the same CD8 population throughout divisions/throughout time. While cytotoxicity follows the "classic" rules of CD8 differentiation, opposite rules govern CD8 inflammatory functions. These are induced before division, decrease while cells divide and decline at the time point of the response when we previously showed antigen concentrations to be quite high (2). The shortness of this inflammatory burst is likely due to extensive TCR down-regulation, which abrogates MAPK activation preventing the expression of these mediators.

The loss of TCR surface expression after *in vitro* T cell activation is well documented, but it was shown to reverse by 24 h (31). Down-regulation of the TCR was also reported after *in vivo* immunization (32, 33). However, we were now able to quantify this phenomenon. By visualizing Mo TCR-Tg cells recognizable by a different allotype, present in frequencies equivalent to those of endogenous antigen-specific-cells, we found that 80% lost their TCR expression by day two after priming. Moreover, this down-regulation persisted for several days: by day 4 after immunization 50% of the responding cells yet fail to express the TCR. Comparison of Dext labeling in naïve and primed endogenous cells also shows considerable down-regulation during these time points. These results identify a major limitation to the study of endogenous CD8 responses. They show that the majority of endogenous antigen-specific-cells cannot be identified during early responses.

While it is generally believed that the major CD8 effector functions are γ-IFN production and cytotoxicity, we now describe in detail a new CD8 effector phase: the secretion of a major burst of pro-inflammatory mediators shortly after activation which directly or indirectly promote the recruitment of lymphocytes and accessory cells types to the location where antigen is present. These cells are likely involved in the "antigen non-specific/shut-down-phase" of lymphocyte trapping-a process proposed to have a fundamental role in allowing rare APCs at a restricted location to screen the total lymphocyte pool in 24–48 h, in order that all antigen-specific-cells dispersed throughout the body may be selected for APC binding. Elegant experiments performed 4–5 decades ago identified a major increase in cell input shortly after antigen stimulation, and a total block in cell egress, which retained both antigen-specific and non-specific T cells at the location where antigen is first presented (3–5). It was proposed that these modifications were induced by an increase in blood flow (10), but our previous measures of blood flow during trapping indicated that the reported increases were due to an increase in organ size. Indeed, when the blood flow was related to the organ weight, perfusion rates were not modified (11). In contrast, our

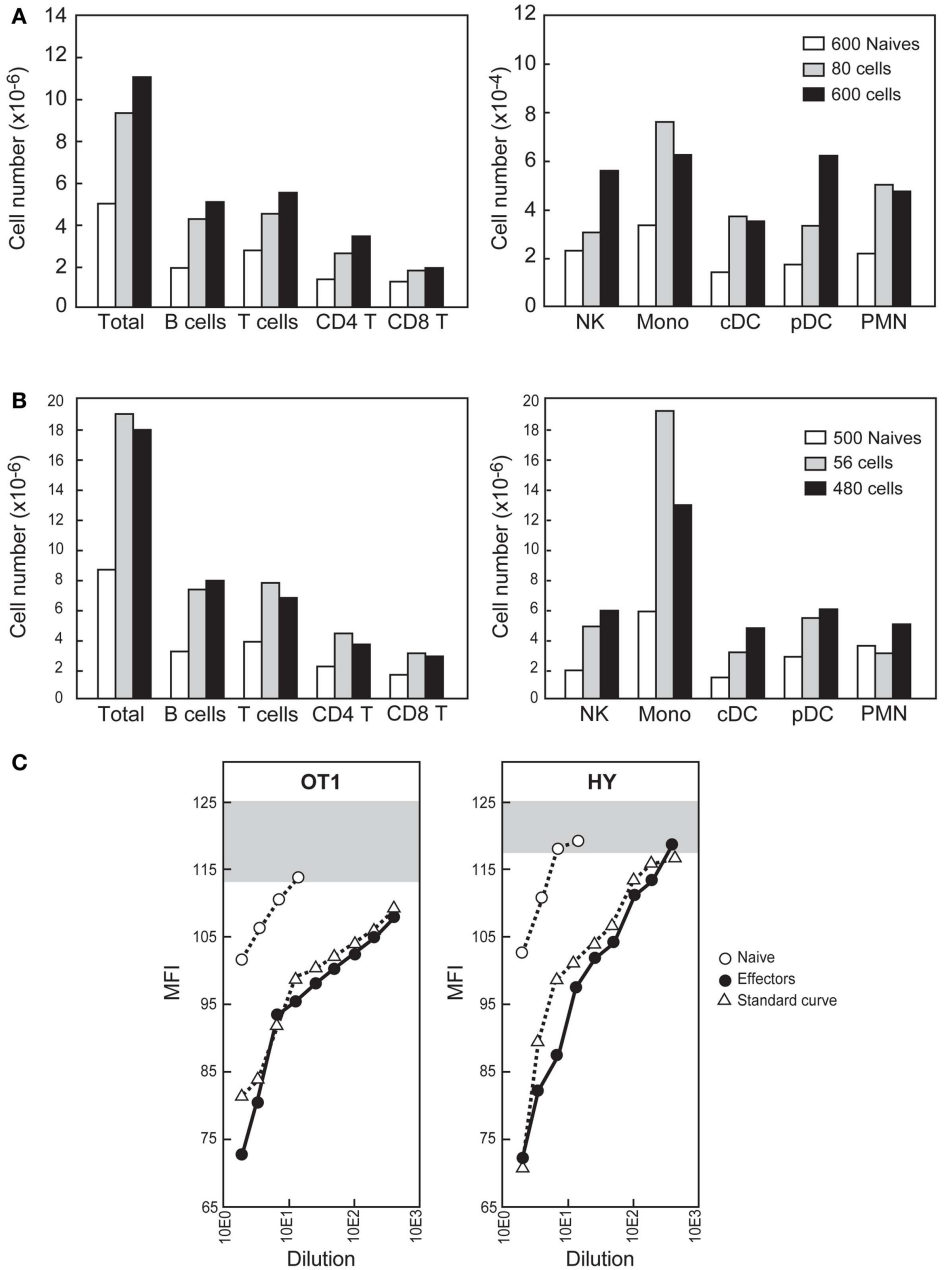


FIGURE 7 | Effects of intra-nodal injection of physiologic numbers of CD8⁺ inflammatory effector T cells. **(A,B)** The indicated number of sorted CD90.2⁺ naïve or inflammatory effectors (recovered 2.5 days after priming) were injected into the BRLN of CD90.1⁺ B6 mice. The BRLNs were removed 24 h later and the different cell types identified as shown in **Figure 5**. Results compare cell recovery in mice injected with naïve CD8+ T cells or inflammatory effector CD8⁺ T cells **(A)** Mice were injected with OT-1 Tg cells. **(B)** Mice injected with HY Tg cells. Similar results were obtained after injection of P14 Tg cells. **(C)** The BRLNs were injected with 500 naïve cells, 80 OT-1 effectors or 56 HY effectors. Results show of S1P amounts in LN lysates 16 h later, compared with known amounts of a synthetic S1P. Gray bars represent the Flag-S1P1 expression of WEHI231 incubated with medium alone. Results are representative of the seven experiments we performed.

results implicate the chemokine burst produced by inflammatory effectors in this recruitment. This burst is able to mobilize non-resident cells. Importantly, the cell types recruited either at distance or after the intra-nodal injection of inflammatory effectors correlate with the chemokine profile of effector cells: HY effectors which express mainly XCL1 recruiting predominantly inflammatory monocytes and lymphocytes, while OT-1 effectors secreting CCL2 and CCL3 also promoting DC and granulocyte recruitment. Inflammatory effectors also secrete TNF, known to participate in DC and T cell recruitment besides having other functions in DC stimulation, T cell co-stimulation and survival [review in Ref. (19)].

When we first detected these early effector functions we were unconvinced that they would have any relevant role in immune responses, since the number of these effectors should be very low. Surprising, they are very potent effectors since 56 present in a LN harboring 5–8 × 10^6 cells are able to recruit up to 10^7 cells in 24 h. It must be noted that the different cell types recruited to the DLN may co-participate in this recruitment, since they are also able to secrete these mediators. In particular, injection of antigen-loaded DCs was shown to increase LN cellularity, which was potentiated by co-injection of TNF (34). However, it was not established if this effect was direct, or mediated by the recruited antigen-specific T cells. In spite of the possible contribution of other cell types, the time course of cell recruitment and LN "shut-down" phase overlaps that of the CD8 inflammatory burst, indicating that the latter has a fundamental role in the overall regulation of these phenomena.

Besides secreting inflammatory mediators, the intra-nodal injection of very low effector numbers also induced a 10-fold increase in S1P recovery from the injected LN, as compared to LN injected with eightfold more naïve cells. S1P up-regulation precedes LN hyperplasia, since it is already detected at 16 h after effector injection, and thus may also contribute to the local cell accumulation by immobilizing recruited cells. The kinetics of S1P up-regulation also mimics the CD8 inflammatory burst, since it is stable at 24 h and declines by days 2–3 after effector injection (data not shown). It must be noted that we studied both intracellular and secreted S1P, since it is not possible to discriminate soluble S1P from tissue samples (J. Pereira, personal communication). However, it is likely that part of this S1P is secreted, being involved in the LN "shut-down" phase of lymphocyte trapping, since (i) a twofold increase in secreted S1P was found sufficient to modify lymphocyte migration (6); and (ii) the injection of FTY720 (which is rapidly phosphorylated *in vivo* generating an SIP analog) mimics the DLN shut-down-phase (23). Part of the S1P may also be intracellular, where it was shown to have important functions, by co-participating in TNF signaling, activating NF-Kb, and contributing to the anti-apoptotic role of TNF (35).

Concerning the production of S1P, we confirmed that T cells do not secrete it (23). We failed to detect this molecule in supernatants of activated T cells *in vitro*. Quantitative evaluation of *Sphk1* and *Sphk2* showed equivalent *ex vivo* expression levels in naïve and inflammatory effectors. The S1P recovery was similar in LN injected with 56 or 1,000 effectors (not shown). Therefore, as described in all the systems studied so far, tissue-resident cells likely produced this mediator (23). In contrast, our data does not support the general believe that the TNF/SIP pathway is always responsible for S1P production and cell recruitment during inflammation. This notion issued from the demonstration that TNF induces the up-regulation of *Sphk1* expression in certain cell lines *in vitro*. We could not confirm the role of this pathway in lymphocyte trapping, since blockage of TNF activity by Abs did not modify S1P recovery in LN injected with inflammatory effectors (not shown). A detailed comparison between the effects of TNF versus antigen injection on lymphocyte migration also reported that TNF does not recapitulate the effects of antigen (13). The complexity of sphingolipids role and the regulatory mechanisms governing their metabolism is yet being revealed, but these studies are yet seriously handicapped by the low sensitivity of the methods for sphingolipids detection.

We were surprised that CD4 T cells reportedly producing MIP-1 chemokines to recruit CD8 T cells to provide help (36) had no equivalent functions. It is possible that the relative roles of CD4 and CD8 T cells in recruitment depend of the immunization context. MIP-1^+ CD4 T cells were detected after immunization with peptide and complete Freund's adjuvant, whereas we here studied CD8-dependent responses. Moreover, it should be noted that MIP-1 chemokines only attract previously activated T cells (37) and thus are unlikely to be involved in the recruitment of naïve cells during trapping.

Our results also modify perspectives on the putative relationships between inflammation and immune responses. Inflammation is generally regarded as starting with an innate immune response that is mediated by tissue-resident cells (38, 39) and may eventually be perpetuated and amplified by certain T cell types (such as TH17 cells). However, Th17 cells cannot initiate inflammatory reactions since their generation requires a previously inflammatory milieu (40). We did not fully block innate signaling in LM infection, since this signaling is complex and involves multiple mediators [reviewed in Ref. (41)]. However, the elimination of the MyD88 pathway reported to be the most important in LM infection (42) did not modify the pro-inflammatory burst. The strong inflammatory bursts of CD8 T cells after sterile immunizations with male, sheep red blood or allogeneic cells indicate that CD8 cognate interactions are also able to initiate inflammatory reactions. This feature may have an important role in a number of "sterile" responses to tissue antigens (such as in transplantation) or in responses to self-antigens or tumor antigens. Thus, while inflammation modulates cognate responses, CD8 cognate responses may also initiate local inflammatory reactions.

AUTHOR CONTRIBUTIONS

Hsueh-Cheng Sung and Sara Lemos performed most experiments; Patricia Ribeiro-Santos and Florence Vasseur did the cell sorting; Kateryna Kozyrytska, the CD4 studies, Agnès Legrand helped with the single-cell PCRs; César Evaristo trained Hsueh-Cheng Sung and contributed to the experiment shown in **Figure 2A**. Alain Charbit contributed to LM preparation, Benedita Rocha devised and supervised the study, and wrote the MS.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/journal/10.3389/fimmu.2013.00452/abstract

REFERENCES

1. Luethviksson BR, Gunnlaugsdottir B. Transforming growth factor-beta as a regulator of site-specific T-cell inflammatory response. *Scand J Immunol* (2003) **58**:129–38. doi:10.1046/j.1365-3083.2003.01297.x
2. Peixoto A, Evaristo C, Munitic I, Monteiro M, Charbit A, Rocha B, et al. CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response. *J Exp Med* (2007) **204**:1193–205. doi:10.1084/jem.20062349
3. Cahill RN, Frost H, Trnka Z. The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes. *J Exp Med* (1976) **143**:870–88. doi:10.1084/jem.143.4.870
4. Hall JG, Morris B. The immediate effect of antigens on the cell output of a lymph node. *Br J Exp Pathol* (1965) **46**:450–4.
5. Zatz MM, Lance EM. The distribution of 51Cr-labeled lymphocytes into antigen-stimulated mice. Lymphocyte trapping. *J Exp Med* (1971) **134**:224–41. doi:10.1084/jem.134.1.224
6. Pham TH, Okada T, Matloubian M, Lo CG, Cyster JG. S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress. *Immunity* (2008) **28**:122–33. doi:10.1016/j.immuni.2007.11.017
7. Grigorova IL, Panteleev M, Cyster JG. Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and antigen exposure. *Proc Natl Acad Sci U S A* (2010) **107**:20447–52. doi:10.1073/pnas.1009968107
8. Shiow LR, Rosen DB, Brdickova N, Xu Y, An J, Lanier LL, et al. CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs. *Nature* (2006) **440**:540–4. doi:10.1038/nature04606
9. MacInnes H, Zhou Y, Gouveia K, Cromwell J, Lowery K, Layton RC, et al. Transmission of aerosolized seasonal H1N1 influenza A to ferrets. *PLoS One* (2011) **6**:e24448. doi:10.1371/journal.pone.0024448
10. Hay JB, Hobbs BB. The flow of blood to lymph nodes and its relation to lymphocyte traffic and the immune response. *J Exp Med* (1977) **145**:31–44. doi:10.1084/jem.145.1.31
11. Freitas AA, Rocha B, Bognacki J. Effects of intravenous administration of antigen on lymphocyte localization and blood flow into the mouse spleen. *Acta Med Port* (1983) **4**:147–50.
12. Mann EA, Markovic SN, Murasko DM. Inhibition of lymphocyte recirculation by murine interferon: effects of various interferon preparations and timing of administration. *J Interferon Res* (1989) **9**:35–51. doi:10.1089/jir.1989.9.35
13. Young AJ, Seabrook TJ, Marston WL, Dudler L, Hay JB. A role for lymphatic endothelium in the sequestration of recirculating gamma delta T cells in TNF-alpha-stimulated lymph nodes. *Eur J Immunol* (2000) **30**:327–34. doi:10.1002/1521-4141(200001)30:1<327::AID-IMMU327>3.3.CO;2-K
14. Tanchot C, Guillaume S, Delon J, Bourgeois C, Franzke A, Sarukhan A, et al. Modifications of CD8+ T cell function during in vivo memory or tolerance induction. *Immunity* (1998) **8**:581–90. doi:10.1016/S1074-7613(00)80563-4
15. Liu F, Whitton JL. Cutting edge: re-evaluating the in vivo cytokine responses of CD8+ T cells during primary and secondary viral infections. *J Immunol* (2005) **174**:5936–40.
16. Obar JJ, Khanna KM, Lefrancois L. Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection. *Immunity* (2008) **28**:859–69. doi:10.1016/j.immuni.2008.04.010
17. Peixoto A, Monteiro M, Rocha B, Veiga-Fernandes H. Quantification of multiple gene expression in individual cells. *Genome Res* (2004) **14**:1938–47. doi:10.1101/gr.2890204
18. Pappu R, Schwab SR, Cornelissen I, Pereira JP, Regard JB, Xu Y, et al. Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate. *Science* (2007) **316**:295–8. doi:10.1126/science.1139221
19. Watts TH. TNF/TNFR family members in costimulation of T cell responses. *Annu Rev Immunol* (2005) **23**:23–68. doi:10.1146/annurev.immunol.23.021704.115839
20. Iezzi G, Karjalainen K, Lanzavecchia A. The duration of antigenic stimulation determines the fate of naive and effector T cells. *Immunity* (1998) **8**:89–95. doi:10.1016/S1074-7613(00)80461-6
21. Kaech SM, Wherry EJ, Ahmed R. Effector and memory T-cell differentiation: implications for vaccine development. *Nat Rev Immunol* (2002) **2**:251–62. doi:10.1038/nri778
22. Kotturi MF, Peters B, Buendia-Laysa F, Sidney J Jr, Oseroff C, Botten J, et al. The CD8+ T-cell response to lymphocytic choriomeningitis virus involves the L antigen: uncovering new tricks for an old virus. *J Virol* (2007) **81**:4928–40. doi:10.1128/JVI.02632-06
23. Cyster JG. Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs. *Annu Rev Immunol* (2005) **23**:127–59. doi:10.1146/annurev.immunol.23.021704.115628
24. Dong C, Chua A, Ganguly B, Krensky AM, Clayberger C. Glycosylated recombinant human XCL1/lymphotactin exhibits enhanced biologic activity. *J Immunol Methods* (2005) **302**:136–44. doi:10.1016/j.jim.2005.05.008
25. Dorner BG, Scheffold A, Rolph MS, Huser MB, Kaufmann SH, Radbruch A, et al. MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines. *Proc Natl Acad Sci U S A* (2002) **99**:6181–6. doi:10.1073/pnas.092141999
26. Lee SC, Brummet ME, Shahabuddin S, Woodworth TG, Georas SN, Leiferman KM, et al. Cutaneous injection of human subjects with macrophage inflammatory protein-1 alpha induces significant recruitment of neutrophils and monocytes. *J Immunol* (2000) **164**:3392–401.
27. Rot A, von Andrian UH. Chemokines in innate and adaptive host defense: basic chemokinese grammar for immune cells. *Annu Rev Immunol* (2004) **22**:891–928. doi:10.1146/annurev.immunol.22.012703.104543
28. Pettus BJ, Bielawski J, Porcelli AM, Reames DL, Johnson KR, Morrow J, et al. The sphingosine kinase 1/sphingosine-1-phosphate pathway mediates COX-2 induction and PGE2 production in response to TNF-alpha. *FASEB J* (2003) **17**:1411–21. doi:10.1096/fj.02-1038com
29. Zhi L, Leung BP, Melendez AJ. Sphingosine kinase 1 regulates pro-inflammatory responses triggered by TNFalpha in primary human monocytes. *J Cell Physiol* (2006) **208**:109–15. doi:10.1002/jcp.20646
30. Olivera A, Mizugishi K, Tikhonova A, Ciaccia L, Odom S, Proia RL, et al. The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis. *Immunity* (2007) **26**:287–97. doi:10.1016/j.immuni.2007.02.008
31. Valitutti S, Muller S, Cella M, Padovan E, Lanzavecchia A. Serial triggering of many T-cell receptors by a few peptide-MHC complexes. *Nature* (1995) **375**:148–51. doi:10.1038/375148a0
32. Drake DR III, Ream RM, Lawrence CW, Braciale TJ. Transient loss of MHC class I tetramer binding after CD8+ T cell activation reflects altered T cell effector function. *J Immunol* (2005) **175**:1507–15.
33. Xiao Z, Mescher MF, Jameson SC. Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation. *J Exp Med* (2007) **204**:2667–77. doi:10.1084/jem.20062376
34. Martin-Fontecha A, Sebastiani S, Hopken UE, Uguccioni M, Lipp M, Lanzavecchia A, et al. Regulation of dendritic cell migration to the draining lymph node: impact on T lymphocyte traffic and priming. *J Exp Med* (2003) **198**:615–21. doi:10.1084/jem.20030448
35. Spiegel S, Milstien S. The outs and the ins of sphingosine-1-phosphate in immunity. *Nat Rev Immunol* (2011) **11**:403–15. doi:10.1038/nri2974
36. Castellino F, Huang AY, Altan-Bonnet G, Stoll S, Scheinecker C, Germain RN. Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction. *Nature* (2006) **440**:890–5. doi:10.1038/nature04651
37. Sallusto F, Mackay CR, Lanzavecchia A. The role of chemokine receptors in primary, effector, and memory immune responses. *Annu Rev Immunol* (2000) **18**:593–620. doi:10.1146/annurev.immunol.18.1.593
38. Medzhitov R. Origin and physiological roles of inflammation. *Nature* (2008) **454**:428–35. doi:10.1038/nature07201
39. Nathan C. Points of control in inflammation. *Nature* (2002) **420**:846–52. doi:10.1038/nature01320
40. Stockinger B, Veldhoen M, Martin B. Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* (2007) **19**:353–61. doi:10.1016/j.smim.2007.10.008
41. Schuppler M, Loessner MJ. The opportunistic pathogen *Listeria monocytogenes*: pathogenicity and interaction with the mucosal immune system. *Int J Inflam.* (2010) **2010**:704321. doi:10.4061/2010/704321
42. Shen Y, Kawamura I, Nomura T, Tsuchiya K, Hara H, Dewamitta SR, et al. Toll-like receptor 2- and MyD88-dependent phosphatidylinositol 3-kinase and

Rac1 activation facilitates the phagocytosis of *Listeria monocytogenes* by murine macrophages. *Infect Immun* (2010) **78**:2857–67. doi:10.1128/IAI.01138-09

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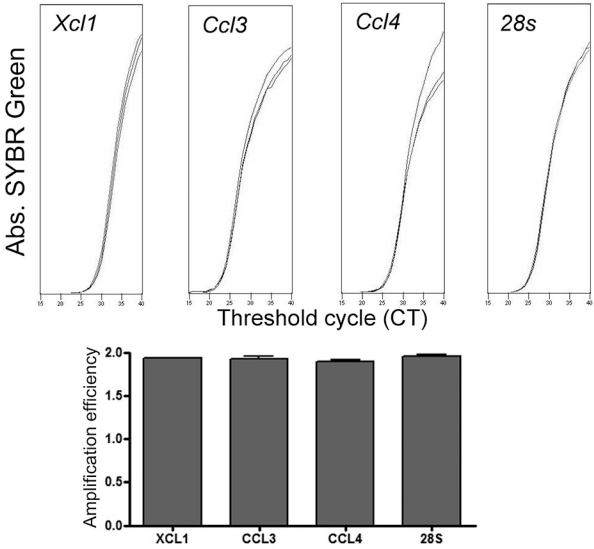
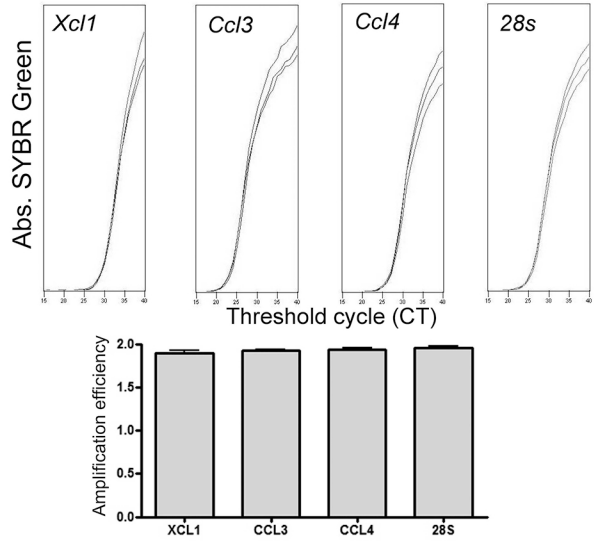
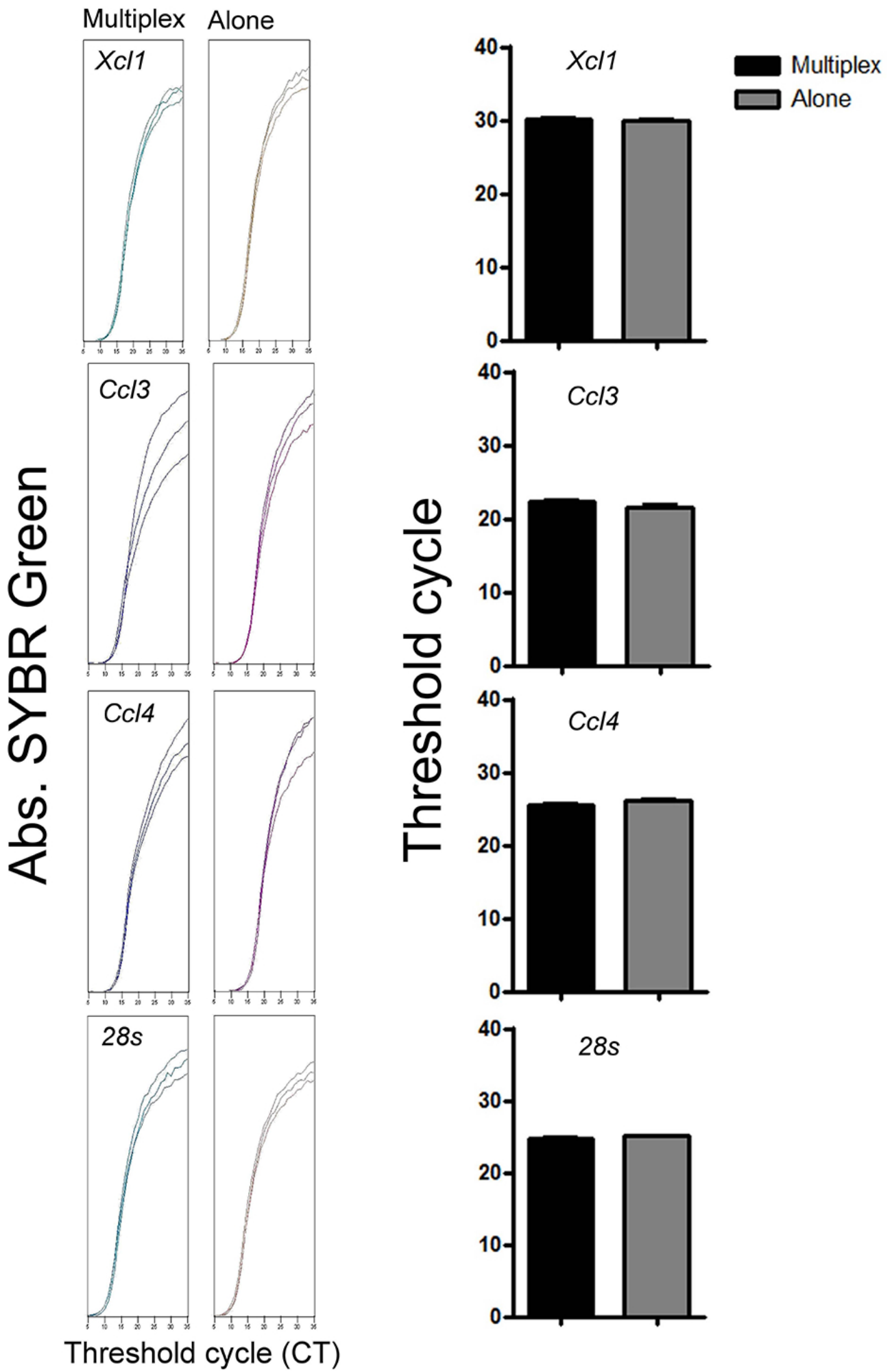
A 1st PCR



B 2nd PCR




Multiplex
Alone
Xcl1
Ccl3
Ccl4
28s
Abs. SYBR Green
Threshold cycle (CT)
Threshold cycle
Multiplex
Alone
0
10
20
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40

Discussion

During immune responses, naïve CD8 T cells face the great challenge of developing multiple activities required to control antigen load and to generate memory cells. All these functional activities are initiated by TCR triggering occurring within a very limited time frame, but several other signals (like co-stimulatory or cytokine signals) do also drive effector cell proliferation and cell differentiation fates. Addressing how these differentiation programs (modifications of multiple genes expression, which define new cell properties) are established is of great interest to understand the establishment of successful immunity and vaccination protocols.

Thus, in this work we attempted to characterize the diversity of CD8 T cell behavior (differentiation programs) during immune responses. We hope that this could lead to a better understanding on: (a) the generation of effector CD8 T cells responsible for an efficient and quick elimination of the pathogen in primary responses, where rare specific-naïve CD8 T cells should quickly meet APCs presenting the cognate antigen; (b) the generation of long lived and competent memory T cells to counter pathogens in future encounters and to engender efficient immunization protocols . Unraveling the complexity of the adaptive immune response requires the study of T cells *in vivo,* and thus in this thesis work we also attempted to revise the strengths and pitfalls of some of these strategies to accurately monitor the diversity of CD8 T cell immune responses (c).

I. Experimental strategies to monitor the diversity of CD8 T cell immune responses: Single cell multiplex RT-PCR technique, *in vitro* reactivation method, and adoptive transfer of TCR-Tg cells system

The study of *in vivo* CD8 T cell differentiation requires the use of reliable methods, not only to induce adequate immune responses mimicking pathogen infections or diseases, but also to monitor and/or predict the outcome of diseases or immunizing protocols.

In vitro reactivation tests, currently used to test T cell function, have been shown to not correlate with the *in vivo* behavior of T cells for both CD4 (Panus *et al.* 2000) and CD8 (Veiga-Fernandes *et al.* 2000) cells in mice. However, this method is still frequently used to access T cell function and it has been argued that *in vitro* readouts reflect how T cells react once they encounter the antigen *in vivo*. On the other hand, it has been shown that the three-dimensional structure of organs significantly modifies CD8 responses (Surh *et al.* 2006) and that cytokine secretion greatly depends on the strength of stimulation (Itoh and Germain 1997; Slifka and Whitton 2001). Therefore, the *in vitro* environment may fail to reproduce the *in vivo* cell interactions, the peculiar inflammatory environment induced by infections, and CD8 T cells may never meet the peptide or

cytokine concentrations used to differentiate them *in vitro*. Indeed, using human T cell clones it was shown that the number and the type of effector function induced after *in vitro* reactivation depends on both TCR avidity and the peptide concentration used. Low antigen concentration was optimal to reveal effector functions in high affinity clones, whereas higher antigen concentration was needed for low affinity clones (Almeida *et al.* 2009).

To overcome these *in vitro* reactivation limitations, we monitored *in vivo* CD8 T cell differentiation by *ex vivo* evaluation of mRNA expression, using the single-cell multiplex RT-PCR technique. Compared to other methods to assess gene expression, which most rely on population analysis, this technique allows us to evaluate the frequency of expressing cells (evaluation of cell heterogeneity inside a population), and the gene co-expression (indicative of the cell function potential). To correlate mRNA expression with protein expression levels, we evaluated protein expression either by *ex vivo* analysis of surface proteins or intracellular protein secretion in Brefeldin A injected mice (secretion blocked); and further, we evaluated protein expression after *in vitro* reactivation.

Comparing these three approaches to monitor the diversity of CD8 T cell immune responses, it was clear that when studying the same population of cells (recovered from the same immunized mice), further *in vitro* restimulation with two peptide concentration ranging 5000x never recapitulated the *ex vivo* protein readouts. Indeed, neither the percentage of expressing cells, nor the intensity of the expression (MFI) obtained after *in vitro* restimulation correlated with *ex vivo* mRNA or protein analysis. Thus, *ex vivo* methods to access *in vivo* diversity of CD8 properties should definitely be preferred to *in vitro* restimulation procedures.

We additionally performed a detailed correlation between *ex vivo* mRNA and *ex vivo* protein detection for different molecules associated with CD8 T cell function and differentiation. The results were clear, a maximal correlation and a significant identity was observed between these two approaches. Although the toxicity of Brefeldin A prevents its use in humans, due to the maximal correlation between *ex vivo* protein and mRNA expression, the quantification of mRNA expression by single-cell multiplex RT-PCR constitutes a powerful method to access the diversity of T cell properties occurring *in vivo*. The use of *ex vivo* mRNA detection also has additional advantages: due to their sensitivity, it allows a better discrimination between poor expressing and non expressing cells; it is particularly useful when antibodies are not available or do not have enough quality; and it is extremely valuable to use when studying rare populations like infrequent antigen-specific cells present at early time points of the immune response.

In conclusion, these results reinforced the powerful of using single-cell *ex vivo* methods, over *in vitro* reactivation ones, to access the diversity of CD8 T cell differentiation occurring after *in vivo* activation. Moreover, single-cell multiplex RT-PCR is the sole approach allowing to assess CD8 T cell properties at the very beginning of an immune response, when antigen-specific CD8 T cells are infrequent. Pointing out the strengths of these two *ex vivo* techniques (single-cell multiplex RT-PCR, and Brefeldin A injected mice) was of major importance to answer to the other two main question of this thesis, where both prediction of heterogeneous CD8 T cell behaviors and analysis of very rare CD8 population were required.

After having chosen an accurate method to access the diversity of *in vivo* CD8 T cell properties, it was also imperative to chose an appropriate system to track antigen-specific cells during CD8 T cell immune responses.

A major goal in studying CD8 T cell differentiation is to understand the developmental progression of antigen-specific T cells from naïve precursors to activated effectors and long-lived memory cells. The achievement of this goal requires tracking distinct populations of T cells through the course of an immune response. This tracking can be accomplished by pMHC multimers labeling, which allows the identification of antigen-specific cells T cells by their TCRs, or by using allelic variation of surface receptors (congenic markers) to discriminate donor T cells from recipient's own cells.

During CD8 immune responses, the endogenous repertoire of antigen-specific cells is polyclonal. As potential heterogeneity of the response could be attributed to TCR diversity of cells specific for a given antigen, $Rag^{-/-}$ TCR-Tg cells are frequently used to guarantee the study of the same clone. Thus, the adoptive transfer of TCR-Tg cells has been an experimental system frequently used not only to: i) study specific T cell responses at early time points when endogenous specific cells are difficult to detect (by transferring high frequency of antigen-specific precursors); but also ii) to study putative heterogeneity that might arrive during immune responses independently of TCR affinity differences (by studying the same clone in different contexts). Thus, the use of TCR-Tg cells to study heterogeneity/diversity on CD8 T cell differentiation during immune responses was crucial to achieve this thesis' aims.

However, several studies have criticized the use of TCR-Tg cells as experimental approach to study T cell differentiation during immune responses. It has been argued that TCR-Tg cells may not mimic the behavior of endogenous cells due to a possible bias in T cell differentiation of responding cells generated from artificial-high numbers of naïve precursors (Marzo *et al.* 2005; van Faassen *et al.* 2005; Badovinac *et al.* 2007). On the other hand, the study of endogenous CD8 immune responses may also be affected by TCR down-regulation occurring after antigen stimulation

(Valitutti *et al.* 1995), which prevents the use of pMHC multimers to detect antigen-specific cells by their TCRs.

TCR-down regulation has been mainly studied in TCR-Tg cells and after *in vitro* activation, and has only rarely been studied after *in vivo* infections (Drake *et al.* 2005; Xiao *et al.* 2007). Drake *et al.* despite of showing TCR downregulation *in vivo*, did not study TCR downregulation in endogenous cells, and Xiao *et al.* reported TCR downregulation on endogenous cells only on later days after infection (day 5 and 7 after VV-OVAp infection). In both studies, the correlation of TCR downregulation with CD8 T cell responsiveness was assessed after *in vitro* restimulation of TCR-Tg cells and never assessed in unmanipulated endogenous CD8 cells. In addition, correlation of TCR downregulation with antigen dose was also analyzed after *in vitro* stimulations. Thus, we further evaluated the impact of TCR downregulation in the assessment of CD8 T cell immune responses. Taking advantage of the congenic markers to track antigen-specific TCR-Tg cells, we showed a marked TCR downregulation occurring at the very early time points of the immune responses, and that it occurs after cell division. In a $5x10^5$ P14 adoptively transferred mouse (high frequency of naïve precursors transferred), at day 2 after LM-GP33 infection, we showed that 51% of Tg cells were unable to bind pMHC multimer. At day 3 after LCMV infection, 40-60% of Tg cells were also unable to be identified by pMHC multimers. Importantly, we also showed that *in vivo* TCR downregulation is inversely proportional to the number of adoptively transferred cells and directly proportional to the antigen doses, suggesting that it is conditioned by the strength of antigen stimulation. This is particularly relevant to endogenous-specific cells present in the first days of infection, where they are present at low numbers and where pathogen levels are high. Applying a modified tetramer pull down technique, we could also observe a strong TCR downregulation on specific-endogenous CD8 T cells in the early days after infection. Moreover, when accessing *ex vivo* granzyme B expression of multimer^{high} and multimer^{low} labeled endogenous cells, we observed that multimer^{low} cells expressed higher levels of granzyme B, suggesting that cells that received the highest level of stimulation, and are highly responsive, are in fact the ones that render “invisible” by TCR-multimer identification.

In an attempt to quantify the extension of TCR downregulation occurring in specific-endogenous cells (that are rendered indistinguishable from other specificities upon TCR downregulation), we analyzed TCR downregulation in TCR-Tg cells present at a frequency equivalent to those of endogenous antigen-specific cells. In this condition, at day 2 post infection, 80% of TCR-Tg cells have lost their capacity to be identified by pMHC-dextramers. Moreover, we also observed that lost of TCR surface expression persists for several days after infection in contrast to faster TCR re-expression occurring after *in vitro* studies.

In conclusion our observations have particular importance for multimer-based techniques currently used either to monitor T cell-mediated immune responses through direct detection and quantification of endogenous-specific T cells, or to isolate/purify antigen-specific cells for further manipulation and adoptive T cell therapies. Thus, conclusions based on procedures using pMHC-multimers should be taken with caution as this approach is not appropriated for an accurate analysis of the entire (early) immune response, not only because it prevents the accurate identification and enumeration of responding cells, but also because it selects non-representative cell subsets, which bias endogenous T cell studies. Therefore, we reinforced the merit of using TCR-Tg cells versus endogenous cells to study *in vivo* CD8 T cell differentiation.

II. Impact of naïve-precursor frequency on the diversity of CD8 T cell immune responses

It has been suggested that artificially induced immunodominance leads to an intra-clonal competition of high precursor cells resulting in their suboptimal cell activation, and also, that the presence of high frequency naïve precursors out-competed with the polyclonal endogenous response (Marzo *et al.* 2005; van Faassen *et al.* 2005; Badovinac *et al.* 2007). These experiments were mostly performed with adoptive transfer of different numbers of naïve TCR-Tg CD8 T cells into naïve host mice, and their behavior were studied after immunization. These studies showed that the fold expansion of T cells present at high frequency was lower than what was observed in low frequency clones, and also, that high frequency clones reached the peak of the expansion earlier. Despite these observations suggesting differences in the kinetic of the response, the phenotype of effector cells differentiating from high and low frequency precursor were directly compared in distinct mice, for the same time point. This comparison also revealed differences in the expression of IL-7R and CD62-L and, the kinetics of re-expression of these receptors started earlier when a high number of precursors were transferred than when fewer cells were transfer.

Additional reasons prompted us to reexamine the impact of the naive-precursor frequency on the diversity of CD8 T cell properties. Competition for antigen also regularly occurs in natural infections, where dominant populations are reported to suppress the expansion of subdominant ones (Yewdell and Bennink 1999). Moreover, adoptive transfers of more than 10^5 precursors have been reported to interfere with: the kinetics of pathogen clearance; the magnitude of the inflammatory responses; and the peak of CD8 T cell expansion (Ehl *et al.* 1998; Zimmermann *et al.* 1999; Sarkar *et al.* 2007; Wirth *et al.* 2009). Thus, we investigated if immunodominance can interfere with CD8 T cell differentiation programs by studying both dominant and subdominant

populations in the same mice. This procedure ensured comparison of cells with different initial precursor frequencies in the same infectious context/antigen load.

Our results showed no differences between the gene expression profiles of two dominant (GP33 and NP396) vs one subdominant (GP276) antigen-specific endogenous population, which are naturally generated in mouse responding to an acute LCMV infection. We screened the expression of cytokine and cytotoxic effector molecules, as well the expression of molecules associated with CD8 T cell effector and memory differentiation. As a result, we observed a fully overlapping behavior between these populations at early time points of the immune response, at the peak of the response, and at the memory phase. We further forced a pronounced immunodominance hierarchy between two populations responding to the same epitope. We adoptively transferred high numbers of P14 cells into a host mouse and compared both P14 and endogenous GP33-specific cells in the same mouse. We observed that both effector and memory cells generated from either abundant or rare precursors (P14 or endogenous $GP33^{+}$ cells, respectively) had strikingly identical differentiation patterns. Moreover, we also found that the presence of high frequencies of naïve P14 cells did not affect the frequency nor the functional properties of endogenous CD8 T cells recognizing other LCMV epitopes.

In conclusion, these results showed that both high and low frequencies of naïve CD8 T cells follow the same rules and differentiate in equivalent effector and memory CD8 T cells. Thus clonal abundance does not affect CD8 T differentiation, nor is immunodominace responsible for diversity in CD8 T cell responses. These results also indicated that high frequency of TCR-Tg transfers do not have an impact on CD8 T cell properties, and thus they further support the merit of TCR-Tg cells to study *in vivo* CD8 T cell differentiation.

III. Distinct pathogens and diversity of CD8 differentiation programs

The previously reported cell-to-cell heterogeneity and the stochastic association of the different effector genes raised the possibility for the existence of multiple programs of differentiation giving rise to CD8 T cells with different fates. This putative plasticity would then allow CD8 T cells to adapt to different challenges. Thus, we wondered if different pathogens, by providing different infectious contexts, could induce such heterogeneity in CD8 T cell properties. We therefore compared OT1 cells responding to LM-OVA with P14 cells responding to LCMV. Specifically, we analyzed the expression of: effector molecules involved in inflammation and

cytotoxicity (TGFβ, TNFα, IFNγ, Prf1, Gzmb, FasL); transcription factors involved in CD8 T cell function and memory differentiation (Tbet and Eomes); receptors of molecules involved in the responsiveness to environmental cues: IL-10R, IL-12Rb1 and IL-21R; inhibitory molecules such as KLRG1 and PD1 (associated with terminal differentiation and exhaustion, respectively); the chemokine receptor CCR7, involved in secondary lymphoid organs trafficking and in the characterization of T_{CM} subtype of memory population; and the cytokine receptor IL-7R involved in cell survival and in the classification of memory precursor effector cells (MPECs). We found that in the response of P14 to LCMV infection, the frequencies of cells expressing TGFβ, IL-10R and IL-21R throughout the response were significantly lower than in the response of OT1 cells to LM-OVA infection. We also found that P14 responding to LCMV reverted to a $CCR7^-$ T_{EM} phenotype at the memory stage in contrast to a $CCR7^+$ T_{CM} phenotype found on OT1 cells responding to LM-OVA. Accordingly, the frequency of IL-7R expression, defining memory precursor effector cells, was higher in OT1 cells. In addition, although at the memory phase of the OT1/LM-OVA response half of CD8 T cells expressed Prf1 and FasL, these cells almost scored negative for IFNγ. In contrast, P14 cells responding to LCMV showed ~50% of cell expressing IFNγ at the memory stage. However, the above observed differences between the 2 systems (OT1/LM-OVA vs P14/LCMV) could be due either to infectious context (LM-OVA vs LCMV) or to clone-specific TCRs (OT1 vs P14). To clarify this, we then compared the gene expression profile of OT1 and P14 cells throughout the response to LM-OVA and LM-GP33 in the same animal. This approach ensured that both populations were primed and differentiated in exactly the same environment and bacterial load. In this context, the gene expression profiles of OT1 and P14 cells did not showed statistically significant differences, showing that under the same circumstances, OT1 and P14 cells behave similarly. Thus, the previous differences in these two clones in response to Listeria and LCMV can be attributed to the infectious context in which these cells were stimulated and not to TCR-specificity and avidity. In sum, these results showed diversity in the differentiation patterns *in vivo*, according to the environment of the infection.

One of the striking differences between the innate response to Listeria and LCMV is the significant IL-12 production in the Listeria responses (virtually absent in LCMV infections) (Orange *et al.* 1994; Cousens *et al.* 1997; Way *et al.* 2007). Surprisingly, we did not find differences in the IL-12R expression in these two infections and the presence of IL-12 may only influence early LM responses, as cells lost IL-12 receptor by the peak of the response. However it must be noted that we only detected IL-12Rβ1, which is a component of two distinct heterodimeric receptors: it associates with IL-12Rβ2 to bind IL-12, and can also associate with IL-23R to bind IL-23. Thus, it would be highly informative to analyze IL-12Rβ2 too, as IL-12 responsiveness in CD4 T cells is made via IL-12Rβ2 modulation (Szabo *et al.* 1997).

In contrast, we found that most of the differences between Listeria vs LCMV responses can be attributed to IL-21. The receptor for this cytokine was more frequently expressed in OT1 than in P14 cells. In the memory phase, 40% of OT1 cells expressed IL-21R against only 8% of P14 cells.

It has been shown that IL-21 favors the response to IL-10, a regulatory cytokine that restricts T cell responses. IL-21 increases IL-10 expression in TCR-stimulated naïve CD4 and CD8 T cells and TCR priming in the presence of IL-21 results in accumulation of CD8 T cells with immunosuppressive activity that is dependent on IL-10 signaling (Spolski *et al.* 2009). This could explain the synchronized differences found between OT1 and P14 cells for both IL-21R and IL-10R.

IL-21 has also been implicated in abrogation of IFNγ production: OT1 cells stimulated with artificial APCs displaying Ag and B7-1 on their surface in the presence of IL-21 were unable to produce IFNγ (Casey and Mescher 2007). This is in agreement with our results that showed no IFNγ expression on memory OT1/LM-OVA responding cells, which actually also had a high percentage of cells expressing IL-21R. Accordingly, P14 in response to LCMV exhibited high precentage of cells expressing IFNγ and low percentage of IL-21R expressing cells.

Moreover, *ex vivo* treatment of purified CD8 T cells from HIV-infected donors with IL-21 increases perforin expression (Parmigiani *et al.* 2011). We also observed that cells expressing IL-21R (OT1/LM-OVA) also expressed perforin, in contrast with P14/LCMV responses, which do not expressed either of these molecules at the memory phase. However, as we did not observe a difference in granzyme b expression between OT1 and P14 cells, it cannot be argued that differences in perforin would lead to differences in the cytotoxic function, as both molecules are needed to achieve the target cell killing.

Importantly, it has been described that IL-21 favors the generation of T_{CM} memory cells. Over-expression of IL-21 in mice results in an increase of the memory CD8 T cell population, implicating this cytokine in the development and/or maintenance of memory cells (Allard *et al.* 2007). IL-21 signaling during the priming phase limits the effector phase and leads the majority of responding T cells to express a T_{CM} phenotype (Kaka *et al.* 2009). Accordingly, we also associated a T_{CM} phenotype with cell expressing IL-21R (OT1/LM-OVA response).

Another characteristic of the LM response was the high percentage of cells expressing TGFβ. In the appropriate context (namely in the presence of IL-6), TGFβ contributes to Th17 differentiation, which are a major source of IL-21 cytokine (Wei *et al.* 2007).

Currently, not only it remains a challenge to understand how different cytokines (such as type I IFNs, IFNγ, IL-2, IL-12 and IL-21) regulate the gene expression in effector CD8 T cells, but also how distinct innate signals are induced by different pathogens. In the case of LM and LCMV infections it must be noted differences concerning: the route of infection, the type of infected cells, the localization/organ of pathogen replication, the type of PRRs receptors activated, the amount and

the diversity of host damage sensed by innate cells, and the maturation state and nature of costimulatory signals provided by APCs.

Concerning the route of infection, intravenous for LM and intraperitoneal for LCMV, it determines the type of cells firstly sensing the pathogen (with phagocytic capacity or not) and consequently it determines the first line of defense available to counter the pathogen before dissemination. Besides being able to infect a broad variety of mammalian cell types, Listeria quickly disseminates to splenocytes and hepatocytes, whereas LCMV uses a ubiquitous receptor to infect cells. In systemic LM administration, DCs transport bacteria to the white-pulp areas of the spleen where they form clusters with NK cells and monocytes. DCs secrete IL-12 and IL-18 that activate recruited NK cells to produce IFNγ, which in turns induces monocyte activation leading to MHC II and iNOS upregulation, and to monocyte differentiation into TNFα and NO secreting cells (TipDCs: $CD11b^{+}Ly6C^{+}CD11c^{+}$) (reviewed in Serbina and Pamer 2008). In contrast, LCMV infection is associated with viral replication in the red pulp of the spleen, and with minimal replication in $CD11c^{+}$ and DEC-205^{+} splenic DCs (reviewed in Humphreys et al. 2008). Concerning the type PRRs activated by LM and LCMV, LM elicits a strong innate response by triggering TLR2 and TLR5, whereas viral infection induces large amounts of type I IFN secretion through TLR7 and TLR9 activation on pDCs (Segura et al. 2007; Merad et al. 2013). TLR3 is also used to recognize dsRNA produced during replication of ssRNA like LCMV. Additionally to the type of TLR used to sense LM and LCMV, the type of adaptor molecules used in TLR signaling also affects the outcome of innate responses. TLR3 and TLR4 signaling (through TRIF-dependent pathway) generate both type I IFN and inflammatory cytokine expression, whereas TLR1-TLR2, TLR2-TLR6 and TLR5 signaling (through MyD88-dependent pathway) induce mainly inflammatory cytokines (reviewed in Kawai and Akira 2010; Kawai and Akira 2011). It has also been suggested that type I IFN response to TLR2 ligands differs according on the cell type involved (Barbalat et al. 2009). Since PRRs can also sense damage-associated molecular patterns (DAMPs) [in addition to PAMPs], the amount of host damage sensed by innate cells may also modulate the innate context generated in LM or LCMV infection. Indeed, LCMV is a non cytophatic virus, whereas LM infection is characterized by a massive activation of macrophages and TipDCs responsible for the production of reactive oxygen species, which are mediators of necrosis. It would also be worthwhile to directly investigate the maturation state and the co-stimulatory ligands expressed on APCs in both LM and LCMV infection, as the co-receptors OX40 and 4-1BB are also involved in memory T cell formation (Watts 2005).

As we found that different pathogens modulate CD8 T cell differentiation programs, creating a diversity in CD8 properties, we wondered if those distinct differentiation patterns between Listeria and LCMV infections (as described above) could also be responsible for a diversity in the protection capacity of memory cells. Clarifying this issue is important for optimal T cell vaccine design, as several hypotheses can be put forward for the generation of an efficient T cell memory pool. Should

vaccines use attenuated pathogens in order to recapitulate the behavior of the pathogen that it aims to control? Should vaccines guarantee the generation of as many memory cells as possible and do different pathogens induce different memory cell numbers with identical memory cell types? Should vaccines guarantee the generation of a peculiar memory type and do different pathogens originate distinct memory subtypes?

To answer these questions, we immunized one group of mice with LM-GP33 and other with LCMV, and two months later both groups were challenged with a lethal dose of LM-GP33. We found that memory cells induced after LCMV priming could faster eliminate the pathogen in a secondary response, than those generated after LM-GP33 priming. Although in LM-GP33 primed mice we were inducing a secondary response to other LM epitopes too (when rechallenging them again with LM-GP33), this hypothetical favorable condition did not favor a faster protection in these mice compared to the LCMV primed mice. In addition, a slightly higher number of memory cells present in LM-GP33 primed mice did not confer a faster protection too.

Thus, reproduction of the infectious context is not a pre-requisite for efficient memory generation and not all immunizations have an identical capacity of protection.

In conclusion, these results demonstrated that different pathogens modulate CD8 T cells differentiation programs, ensuring a considerable heterogeneity/plasticity of CD8 T cell responses that could then result in memory cells with different capacities to confer protection. In particular, LCMV infectious context promoted memory differentiation into a T_{EM} subtype, which was associated with a higher protection capacity upon rechallenging. In addition, these cells did not respond to IL-10 and IL-21 and ~50% of them were able to secrete IFNγ.

IV. Diversity of CD8 T cell effector functions

Inflammation is a key component of innate responses and has a major role in protecting the host against infectious agents by recruiting additional innate cells to the injury site. These recruited cells contribute not only for the first line of pathogens elimination, but also for lymphocyte activation and differentiation. By recognizing pathogen invasion or cell damage through pathogen-recognition receptors (PRRs), several innate cells such as mast cells, monocytes, macrophages, NK cells, neutrophils and DCs, as well endothelial cells are regarded as initiators of inflammation that will then result in antigen presentation augmentation to trigger T and B cell responses. In this perspective T cells are not generally believed to be initiators of a pro-inflammatory response. Although Th17 cells do co-participate in inflammatory reactions, their differentiation into effectors

cell producing pro-inflammatory mediators does require the presence of a pre-existent inflammatory milieu (TGF-β, IL-6, IL-21 and IL-23). Thus, in contrast with this view, where inflammation is initiated by innate signals that are recognized by innate cells, we showed for the first time that: i) shortly after antigen recognition, CD8 T cells can initiate an inflammatory response by secreting pro-inflammatory cytokines and chemokines (contrasting with the classical cytotoxic function of CD8 T cells) ; ii) that these pro-inflammatory molecules are also secreted in apparent absence of pathogen innate signals (in "sterile" immunizations with male cells, or in MyD88$^{-/-}$ mice).

We had previously noted a potential pro-inflammatory role by CD8 T effector cells present at early time points of immune responses and have named these early effectors cells as inflammatory effectors. This designation was based in two observations. First, CD8 T cells earlier after priming highly expressed TGF-β but did not co-express the TGF-β receptor subunits necessary for signaling. Secondly, these early effectors were able to promote local retention of both specific and non-specific target cells, instead of showing the classical killer function. Thus, in this thesis, we characterized the diversity of CD8 T cell effector functions and investigate their role during immune responses.

Using two experimental systems: the response of OT-1 TCR-Tg cells to LM-OVA and the response of anti-HY TCR-Tg cells to male cells, we found that immediately after activation CD8 T cells expressed other pro-inflammatory mediators such as XCL1, CCL3 and CCL4, in addition to TNFα and TGF-β. Moreover, at the peak of the response, CD8 T cells were no longer producing these pro-inflammatory mediators and instead expressed cytotoxic molecules. Although it was shown that OT-2 CD4 T cells may produce CCL3 and CCL4 after s.c. immunization with admixture of pOVA and CpGs in alum or with peptide pulsed DCs administration (Castellino *et al.* 2006), we did not detect CCL3 neither CCL4 expression on OT-2 cells when activated *in vivo* with i.v. LM-OVA expressing both OT-1 and OT-2 peptides. Differences in the context of immunization as well differences in the route of administration may explain the different outcomes. Additionally, we also confirmed absence of CCL3 and CCL4 expression on Maryrin cells (CD4 male-specific cells).

Similarly to the expression of these pro-inflammatory mediators in the absence of pathogen innate signals (male immunization) we also detected their expression in OVA-specific endogenous cells of MyD88$^{-/-}$ mice after LM-OVA administration.

Although LM infection elicits a strong innate immune response, and MyD88$^{-/-}$ mice do not completely abrogate all innate signals that could be sensed by other PAMPs or DAMPs (namely by TLR4 through TRIF adaptor, or by NOD1 and NOD2 receptors), several studies report MyD88 adaptor as essential for the innate immune defense against LM (Pamer 2004). It was also shown that in LM infection, DCs undergo MyD88-dependent activation and secrete IL-12 and IL-18 to

recruit NK cells to produce IFNγ. NK-derived IFNγ induces monocyte activation and MHC II and iNOS upregulation and subsequent differentiation of monocytes into TipDCs. TipDCs also sense infection in a MyD88-dependent manner, and are responsible for TNFα and NO secretion leading to bacterial replication restriction (Kang *et al.* 2008; Serbina and Pamer 2008). Our results using MyD88$^{-/-}$ mice showed that even in the absence of the major pathway sensing LM innate signals, CD8 endogenous do also express these pro-inflammatory mediators. Thus, together with anti-HY results, these results support the conclusion that CD8 cognate interactions are sufficient to induce CD8 T cell differentiation into inflammatory effectors. In addition, when MyD88$^{-/-}$ and WT cells are directly compared for the expression of pro-inflammatory mediators, it seems that MyD88$^{-/-}$ cells express higher levels than WT cells. This could be due the fact that MyD88$^{-/-}$ mice are more susceptible to LM infection, and hence have higher antigen levels, which further supports the notion that cognate stimulation are sufficient to induce a burst of pro-inflammatory mediators.

When comparing the rules leading to differentiation of CD8 T cells into inflammatory effectors or cytotoxic effectors, we observed that expression of inflammatory effector genes is induced even before cell division, decreases while the cell divides, and declines when Ag concentration is still high. These features are completely the opposite of what occurs in the classical acquisition of cytotoxic effector functions: after Ag stimulation cytotoxic functions increase with division and decline once Ag is eliminated.

As expression of pro-inflammatory genes is restricted to early days of the immune response, when antigen loads are still high (such as on days 2 and 3), and as the expression of pro-inflammatory genes is dependent on MAPkinase pathways, we correlated the restriction of pro-inflammatory mediators expression with the TCR downregulation phenomena massively occurring in early days after priming. We further supported this correlation by showing that several TCR-signaling pathways were completely abrogated from day 2 to day 5 post infection. We detected a decrease in the expression of MAPkinases proteins when dual phosphorylated (active form that enters the nucleus and regulates gene transcription). The expression of phosphorylated NF-kB and phosphorylated Akt (CD28 co-stimulation pathway) also decreased from day 2 to day 5.

Related to the rules that govern the diversity of CD8 effector functions (inflammatory vs cytotoxic) is also the question of why pro-inflammatory genes are immediately expressed after activation while cytotoxic genes are not. Unfortunately, nowadays not much can be argued beyond broad differences in: transcription factors availability, transcription factor networks and chromatin accessibility. Indeed, it is known that association of different transcription factors leads to different patterns of gene expression: NFAT/AP1 induce IL-2 expression (Macian *et al.* 2002); NFAT/Foxp3 antagonize IL-2 expression and results in Treg functional gene expression (Wu *et al.* 2006);

NFAT/STATs induce Th1 or Th2 gene expression (Savignac *et al.* 2007). In addition, it is also known that different modes of activation can occur on p38 MAP kinase: in response to a variety of growth factors, cytokines and stress signals p38 kinase is dual activated through a MAPK cascade, while in T cells TCR ligation lead to direct phosphorylation of p38 with further autophosphorylation in a MAPK cascade independent mechanism (Salvador *et al.* 2005). p38 MAPK signaling pathways through TCR-triggering can also selectively regulate functions in CD8 and CD4 cells: p38 MAP kinase signaling pathway controls IFNγ production in both CD4 and CD8 T cells, but only regulates apoptosis selectively in CD8 T cells and not in CD4 T cells (Merritt *et al.* 2000). Directly concerning MAPKinase and inflammatory chemokines expression, different cell types can use different families of MAPkinases to express the same chemokine (reviewed in the chemokines section of introduction).

Concerning the question related to the physiologic role of diverse CD8 effector properties and, in particular, the role of pro-inflammatory CD8 effectors in immune responses, we tested if these effectors were able to induce "in vivo" cell recruitment, which is a characteristic of inflammatory reactions. Thus, we isolated pro-inflammatory effectors at day 2,5 of the response (when the percentage of cell expressing pro-inflammatory mediators is higher) and immobilized them subcutaneously either in the ear or in a braquial lymph node (BRLN) of a host immunocompetent mouse. In both cases, there was a massive leukocyte recruitment, either into the auricular draining lymph node or into the injected BRLN, 24h upon effector's immobilization. Likewise, we also observed a LN hypertrophy associated with this massive cell recruitment. Using both OT1 and anti-HY effectors, we observed that 80 or 56 pro-inflammatory effectors were able to recruit up to 10^7 cells in 24h. When compared with immobilization of naïve CD8 T cells, intra-nodal injection of physiologic numbers of CD8 inflammatory effectors revealed higher numbers of B, T, NK, cDCs, pDCs and PMN cells presented in the injected LN. Thus, the new CD8 effector phase secreting a burst of pro-inflammatory mediators shortly after activation promote the recruitment of lymphocytes and accessory innate cells to the local where antigen is present. This massive cell recruitment into LNs occurring at early time points of the response may thus provide a rapid screen for the maximum number of naïve-specific circulating throughout the body, and thus assure a proper encounter of T cells with cognate antigens.

It must be noted that the different cell types recruited to the draining LNs may also co-participate and amplify to this massive recruitment, since macrophages, neurophils and DCs are also able to secrete these mediators. It has also been shown that after the initial inflammatory challenge LNs undergo substantial remodeling, including the expression of the primary feed arterioles and HEV networks, and that the flow of afferent lymphatic vessels also increases (von Andrian and Mempel 2003; Drayton *et al.* 2006; Bajenoff *et al.* 2007; Lammermann *et al.* 2008). So, even that other cells may also participate on the recruitment and also be responsible for LN remodeling, it must be noted that naïve cells immobilized in the LN do not induce LN hypertrophy

and that OT1 cells responding to wild type LM do not express these inflammatory mediators. Thus, these features are dependent (directly or indirectly) on CD8 cognate antigen interaction.

As we were surprised that as few as 80 or 56 pro-inflammatory effectors could attract up to 10^7 cells in 24h, we wondered if other mediators could be involved in the accumulation of such number of cells in a LN. Given that LN egress is totally blocked during early trapping (for 1-3 days) and that S1P gradients control LN egress, we tested if pro-inflammatory effectors could increase local S1P concentrations. We observed that 16h after intra-nodal injection of CD8 effectors, S1P levels increased by 10x on LN extracts. Since a twofold increase in the S1P concentration is reported to induce major changes in lymphocyte egress (Pham *et al.* 2008), the S1P levels detected in the presence of inflammatory effectors justify the contribution of these effectors to the blockage of LN egress and to the massive retention of cells on LNs at early time points of immune responses.

Thus, both the increase in cell numbers and the blockage of cell egress contributed to a rapid accumulation of recirculating cells at the inflamed LN to ensure that a large and rare repertoire of antigen-specific lymphocytes will have enough time to move among resident cells until they meet the rare APCs first presenting cognate antigens.

In conclusion, the diversity of CD8 T cell effector functions during immune responses contributes:

i) Initially: for a quick and proper encounter of naïve CD8 T cells dispersed throughout the body with their cognate antigens, in order to activate a maximum repertoire of naïve CD8 T cells;
ii) Later on: for a potent elimination of the pathogen by killing infected cells.

These features might be relevant for “sterile” responses to tissue antigens, such as in transplantation, in response to self-antigens, as well as in response to tumor antigens. Promoting or dampening the differentiation of CD8 inflammatory effectors could then bring new perspectives on tumor combat or in anti-inflammatory therapies and transplant rejection.

Overall, this thesis work shows that the <u>frequency of naïve-precursor cells</u> do not have an impact in the **diversity of CD8 T cell responses**, that <u>different pathogens</u> are able to modulate CD8 T cell properties and thus generate diversity on CD8 T cell behaviors, and that during CD8 immune responses there are <u>two distinct effector phases governed by distinct rules</u>: inflammatory (at the early days of the response) and cytotoxic (on later days). Moreover, <u>cognate interactions seem to be sufficient for CD8 T cell differentiation into inflammatory effectors</u>.

In future work it would be interesting to study if different infectious contexts (shaped by different pathogens innate signals) also impact the CD8 T cell differentiation into pro-inflammatory effectors. In particular, assessing the expression of pro-inflammatory mediators using the viral infection model LCMV, and then comparing it with the LM model. P14 TCR-Tg CD8 T cells responding to LCMV (expressing GP33) could be used to analyze the pro-inflammatory profiles in viral context.

As during secondary responses, memory CD8 T cells have a more rapid exertion of effector functions, it will be interesting to investigate if memory CD8 T cells could also generate pro-inflammatory effectors faster and more extensively.

Since pro-inflammatory CD8 T cell effectors produce a large amount of TGFβ and do not express the corresponding receptor subunits, it would be relevant to address the question if CD8 T cell derived TGFβ could be a source of CD4 differentiation into Treg or Th17 cells. By injecting pro-inflammatory CD8 T cells subcutaneously in the ear, the percentage of $CD4^{+}CD25^{+}$ T cells and IL-17 producing cells could be analyzed in the draining lymph node.

As IL-21 signaling seems to have a relevant role in the diversity of CD8 T cell differentiation in efficient memory cells, it would be interesting to directly assess the impact of IL-21 on CD8 T cell differentiation modulation. Thus, by adoptive transferring IL-21R$^{-/-}$ P14 TCR-Tg ($CD45.2^{+}$) and WT P14 TCR-Tg ($CD45.1^{+}$) cells into a $CD45.2^{+}$ $CD45.1^{+}$ B6 host mouse and by studying the response of both clones to LM-GP33 infection, we could investigate if in the absence of IL-21 signaling, CD8 T cells are able to adopt a T_{EM} phenotype (characteristic of the LCMV infection).

Bibliography

Acuto, O. and F. Michel (2003). "CD28-mediated co-stimulation: a quantitative support for TCR signalling." Nat Rev Immunol 3(12): 939-951.

Aichele, P., H. Unsoeld, M. Koschella, O. Schweier, U. Kalinke and S. Vucikuja (2006). "CD8 T cells specific for lymphocytic choriomeningitis virus require type I IFN receptor for clonal expansion." J Immunol 176(8): 4525-4529.

Akira, S. and K. Takeda (2004). "Toll-like receptor signalling." Nat Rev Immunol 4(7): 499-511.

Akira, S., S. Uematsu and O. Takeuchi (2006). "Pathogen recognition and innate immunity." Cell 124(4): 783-801.

Alam, R., S. Stafford, P. Forsythe, R. Harrison, D. Faubion, M. A. Lett-Brown and J. A. Grant (1993). "RANTES is a chemotactic and activating factor for human eosinophils." J Immunol 150(8 Pt 1): 3442-3448.

Allan, R. S., J. Waithman, S. Bedoui, C. M. Jones, J. A. Villadangos, Y. Zhan, A. M. Lew, K. Shortman, W. R. Heath and F. R. Carbone (2006). "Migratory dendritic cells transfer antigen to a lymph node-resident dendritic cell population for efficient CTL priming." Immunity 25(1): 153-162.

Allard, E. L., M. P. Hardy, J. Leignadier, M. Marquis, J. Rooney, D. Lehoux and N. Labrecque (2007). "Overexpression of IL-21 promotes massive CD8+ memory T cell accumulation." Eur J Immunol 37(11): 3069-3077.

Allman, D., A. Sambandam, S. Kim, J. P. Miller, A. Pagan, D. Well, A. Meraz and A. Bhandoola (2003). "Thymopoiesis independent of common lymphoid progenitors." Nat Immunol 4(2): 168-174.

Almeida, J. R., D. Sauce, D. A. Price, L. Papagno, S. Y. Shin, A. Moris, M. Larsen, G. Pancino, D. C. Douek, B. Autran, A. Saez-Cirion and V. Appay (2009). "Antigen sensitivity is a major determinant of CD8+ T-cell polyfunctionality and HIV-suppressive activity." Blood 113(25): 6351-6360.

Altman, J. D., P. A. Moss, P. J. Goulder, D. H. Barouch, M. G. McHeyzer-Williams, J. I. Bell, A. J. McMichael and M. M. Davis (1996). "Phenotypic analysis of antigen-specific T lymphocytes." Science 274(5284): 94-96.

Alves, N. L., N. D. Huntington, H. R. Rodewald and J. P. Di Santo (2009a). "Thymic epithelial cells: the multi-tasking framework of the T cell "cradle"." Trends Immunol 30(10): 468-474.

Alves, N. L., O. Richard-Le Goff, N. D. Huntington, A. P. Sousa, V. S. Ribeiro, A. Bordack, F. L. Vives, L. Peduto, A. Chidgey, A. Cumano, R. Boyd, G. Eberl and J. P. Di Santo (2009b). "Characterization of the thymic IL-7 niche in vivo." Proc Natl Acad Sci U S A 106(5): 1512-1517.

Amulic, B., C. Cazalet, G. L. Hayes, K. D. Metzler and A. Zychlinsky (2012). "Neutrophil function: from mechanisms to disease." Annu Rev Immunol 30: 459-489.

Apte, S. H., A. Baz, P. Groves, A. Kelso and N. Kienzle (2008). "Interferon-gamma and interleukin-4 reciprocally regulate CD8 expression in CD8+ T cells." Proc Natl Acad Sci U S A 105(45): 17475-17480.

Araki, K., A. P. Turner, V. O. Shaffer, S. Gangappa, S. A. Keller, M. F. Bachmann, C. P. Larsen and R. Ahmed (2009). "mTOR regulates memory CD8 T-cell differentiation." Nature 460(7251): 108-112.

Arbour, N., D. Naniche, D. Homann, R. J. Davis, R. A. Flavell and M. B. Oldstone (2002). "c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 signaling pathways have divergent roles in CD8(+) T cell-mediated antiviral immunity." J Exp Med 195(7): 801-810.

Arnon, T. I., Y. Xu, C. Lo, T. Pham, J. An, S. Coughlin, G. W. Dorn and J. G. Cyster (2011). "GRK2-dependent S1PR1 desensitization is required for lymphocytes to overcome their attraction to blood." Science 333(6051): 1898-1903.

Auffray, C., D. Fogg, M. Garfa, G. Elain, O. Join-Lambert, S. Kayal, S. Sarnacki, A. Cumano, G. Lauvau and F. Geissmann (2007). "Monitoring of blood vessels and tissues by a population of monocytes with patrolling behavior." Science 317(5838): 666-670.

Bachmann, M. F., P. Wolint, K. Schwarz, P. Jager and A. Oxenius (2005). "Functional properties and lineage relationship of CD8+ T cell subsets identified by expression of IL-7 receptor alpha and CD62L." J Immunol 175(7): 4686-4696.

Bacon, K. B., B. A. Premack, P. Gardner and T. J. Schall (1995). "Activation of dual T cell signaling pathways by the chemokine RANTES." Science 269(5231): 1727-1730.

Badovinac, V. P., J. S. Haring and J. T. Harty (2007). "Initial T cell receptor transgenic cell precursor frequency dictates critical aspects of the CD8(+) T cell response to infection." Immunity 26(6): 827-841.

Badovinac, V. P. and J. T. Harty (2007). "Manipulating the rate of memory CD8+ T cell generation after acute infection." J Immunol 179(1): 53-63.

Bai, A., H. Hu, M. Yeung and J. Chen (2007). "Kruppel-like factor 2 controls T cell trafficking by activating L-selectin (CD62L) and sphingosine-1-phosphate receptor 1 transcription." J Immunol 178(12): 7632-7639.

Bajenoff, M., J. G. Egen, H. Qi, A. Y. Huang, F. Castellino and R. N. Germain (2007). "Highways, byways and breadcrumbs: directing lymphocyte traffic in the lymph node." Trends Immunol 28(8): 346-352.

Bakker, A. H. and T. N. Schumacher (2005). "MHC multimer technology: current status and future prospects." Curr Opin Immunol 17(4): 428-433.

Balagopalan, L., V. A. Barr, C. L. Sommers, M. Barda-Saad, A. Goyal, M. S. Isakowitz and L. E. Samelson (2007). "c-Cbl-mediated regulation of LAT-nucleated signaling complexes." Mol Cell Biol 27(24): 8622-8636.

Balciunaite, G., R. Ceredig and A. G. Rolink (2005). "The earliest subpopulation of mouse thymocytes contains potent T, significant macrophage, and natural killer cell but no B-lymphocyte potential." Blood 105(5): 1930-1936.

Banchereau, J. and R. M. Steinman (1998). "Dendritic cells and the control of immunity." Nature 392(6673): 245-252.

Banerjee, A., S. M. Gordon, A. M. Intlekofer, M. A. Paley, E. C. Mooney, T. Lindsten, E. J. Wherry and S. L. Reiner (2010). "Cutting edge: The transcription factor eomesodermin enables CD8+ T cells to compete for the memory cell niche." J Immunol 185(9): 4988-4992.

Barbalat, R., L. Lau, R. M. Locksley and G. M. Barton (2009). "Toll-like receptor 2 on inflammatory monocytes induces type I interferon in response to viral but not bacterial ligands." Nat Immunol 10(11): 1200-1207.

Barber, D. L., E. J. Wherry, D. Masopust, B. Zhu, J. P. Allison, A. H. Sharpe, G. J. Freeman and R. Ahmed (2006). "Restoring function in exhausted CD8 T cells during chronic viral infection." Nature 439(7077): 682-687.

Barnich, N., J. E. Aguirre, H. C. Reinecker, R. Xavier and D. K. Podolsky (2005). "Membrane recruitment of NOD2 in intestinal epithelial cells is essential for nuclear factor-{kappa}B activation in muramyl dipeptide recognition." J Cell Biol 170(1): 21-26.

Batard, P., D. A. Peterson, E. Devevre, P. Guillaume, J. C. Cerottini, D. Rimoldi, D. E. Speiser, L. Winther and P. Romero (2006). "Dextramers: new generation of fluorescent MHC class I/peptide multimers for visualization of antigen-specific CD8+ T cells." J Immunol Methods 310(1-2): 136-148.

Beals, C. R., C. M. Sheridan, C. W. Turck, P. Gardner and G. R. Crabtree (1997). "Nuclear export of NF-ATc enhanced by glycogen synthase kinase-3." Science 275(5308): 1930-1934.

Bell, J. K., J. Askins, P. R. Hall, D. R. Davies and D. M. Segal (2006). "The dsRNA binding site of human Toll-like receptor 3." Proc Natl Acad Sci U S A 103(23): 8792-8797.

Belz, G. T. and A. Kallies (2010). "Effector and memory CD8+ T cell differentiation: toward a molecular understanding of fate determination." Curr Opin Immunol 22(3): 279-285.

Belz, G. T., K. Shortman, M. J. Bevan and W. R. Heath (2005). "CD8alpha+ dendritic cells selectively present MHC class I-restricted noncytolytic viral and intracellular bacterial antigens in vivo." J Immunol 175(1): 196-200.

Benz, C., V. C. Martins, F. Radtke and C. C. Bleul (2008). "The stream of precursors that colonizes the thymus proceeds selectively through the early T lineage precursor stage of T cell development." J Exp Med 205(5): 1187-1199.

Berche, P., J. L. Gaillard, C. Geoffroy and J. E. Alouf (1987). "T cell recognition of listeriolysin O is induced during infection with Listeria monocytogenes." J Immunol 139(11): 3813-3821.

Bernatoniene, J., Q. Zhang, S. Dogan, T. J. Mitchell, J. C. Paton and A. Finn (2008). "Induction of CC and CXC chemokines in human antigen-presenting dendritic cells by the pneumococcal proteins pneumolysin and CbpA, and the role played by toll-like receptor 4, NF-kappaB, and mitogen-activated protein kinases." J Infect Dis 198(12): 1823-1833.

Berzins, S. P., R. L. Boyd and J. F. Miller (1998). "The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool." J Exp Med 187(11): 1839-1848.

Best, J. A., D. A. Blair, J. Knell, E. Yang, V. Mayya, A. Doedens, M. L. Dustin and A. W. Goldrath (2013). "Transcriptional insights into the CD8(+) T cell response to infection and memory T cell formation." Nat Immunol 14(4): 404-412.

Bettelli, E., Y. Carrier, W. Gao, T. Korn, T. B. Strom, M. Oukka, H. L. Weiner and V. K. Kuchroo (2006). "Reciprocal developmental pathways for the generation of pathogenic effector TH17 and regulatory T cells." Nature 441(7090): 235-238.

Bhandoola, A., H. von Boehmer, H. T. Petrie and J. C. Zuniga-Pflucker (2007). "Commitment and developmental potential of extrathymic and intrathymic T cell precursors: plenty to choose from." Immunity 26(6): 678-689.

Bielecki, J., P. Youngman, P. Connelly and D. A. Portnoy (1990). "Bacillus subtilis expressing a haemolysin gene from Listeria monocytogenes can grow in mammalian cells." Nature 345(6271): 175-176.

Billadeau, D. D., J. C. Nolz and T. S. Gomez (2007). "Regulation of T-cell activation by the cytoskeleton." Nat Rev Immunol 7(2): 131-143.

Blackburn, S. D., H. Shin, W. N. Haining, T. Zou, C. J. Workman, A. Polley, M. R. Betts, G. J. Freeman, D. A. Vignali and E. J. Wherry (2009). "Coregulation of CD8+ T cell exhaustion by multiple inhibitory receptors during chronic viral infection." Nat Immunol 10(1): 29-37.

Blattman, J. N., R. Antia, D. J. Sourdive, X. Wang, S. M. Kaech, K. Murali-Krishna, J. D. Altman and R. Ahmed (2002). "Estimating the precursor frequency of naive antigen-specific CD8 T cells." J Exp Med 195(5): 657-664.

Bluthmann, H., P. Kisielow, Y. Uematsu, M. Malissen, P. Krimpenfort, A. Berns, H. von Boehmer and M. Steinmetz (1988). "T-cell-specific deletion of T-cell receptor transgenes allows functional rearrangement of endogenous alpha- and beta-genes." Nature 334(6178): 156-159.

Boehm, U., T. Klamp, M. Groot and J. C. Howard (1997). "Cellular responses to interferon-gamma." Annu Rev Immunol 15: 749-795.

Borregaard, N. (2010). "Neutrophils, from marrow to microbes." Immunity 33(5): 657-670.

Boscacci, R. T., F. Pfeiffer, K. Gollmer, A. I. Sevilla, A. M. Martin, S. F. Soriano, D. Natale, S. Henrickson, U. H. von Andrian, Y. Fukui, M. Mellado, U. Deutsch, B. Engelhardt and J. V. Stein (2010). "Comprehensive analysis of lymph node stroma-expressed Ig superfamily members reveals redundant and nonredundant roles for ICAM-1, ICAM-2, and VCAM-1 in lymphocyte homing." Blood 116(6): 915-925.

Bots, M., I. G. Kolfschoten, S. A. Bres, M. T. Rademaker, G. M. de Roo, M. Kruse, K. L. Franken, M. Hahne, C. J. Froelich, C. J. Melief, R. Offringa and J. P. Medema (2005). "SPI-CI and SPI-6 cooperate in the protection from effector cell-mediated cytotoxicity." Blood 105(3): 1153-1161.

Bourgeois, C., B. Rocha and C. Tanchot (2002a). "A role for CD40 expression on CD8+ T cells in the generation of CD8+ T cell memory." Science 297(5589): 2060-2063.

Bourgeois, C., H. Veiga-Fernandes, A. M. Joret, B. Rocha and C. Tanchot (2002b). "CD8 lethargy in the absence of CD4 help." Eur J Immunol 32(8): 2199-2207.

Bousso, P. and E. Robey (2003). "Dynamics of CD8+ T cell priming by dendritic cells in intact lymph nodes." Nat Immunol 4(6): 579-585.

Brandt, K., S. Bulfone-Paus, D. C. Foster and R. Ruckert (2003). "Interleukin-21 inhibits dendritic cell activation and maturation." Blood 102(12): 4090-4098.

Brinkmann, V., M. D. Davis, C. E. Heise, R. Albert, S. Cottens, R. Hof, C. Bruns, E. Prieschl, T. Baumruker, P. Hiestand, C. A. Foster, M. Zollinger and K. R. Lynch (2002). "The immune modulator FTY720 targets sphingosine 1-phosphate receptors." J Biol Chem 277(24): 21453-21457.

Brinkmann, V. and K. R. Lynch (2002). "FTY720: targeting G-protein-coupled receptors for sphingosine 1-phosphate in transplantation and autoimmunity." Curr Opin Immunol 14(5): 569-575.

Brinkmann, V., U. Reichard, C. Goosmann, B. Fauler, Y. Uhlemann, D. S. Weiss, Y. Weinrauch and A. Zychlinsky (2004). "Neutrophil extracellular traps kill bacteria." Science 303(5663): 1532-1535.

Brown, K. E., G. J. Freeman, E. J. Wherry and A. H. Sharpe (2010). "Role of PD-1 in regulating acute infections." Curr Opin Immunol 22(3): 397-401.

Busch, D. H., K. Kerksiek and E. G. Pamer (1999). "Processing of Listeria monocytogenes antigens and the in vivo T-cell response to bacterial infection." Immunol Rev 172: 163-169.

Busch, D. H., I. M. Pilip, S. Vijh and E. G. Pamer (1998). "Coordinate regulation of complex T cell populations responding to bacterial infection." Immunity 8(3): 353-362.

Cahill, R. N., H. Frost and Z. Trnka (1976). "The effects of antigen on the migration of recirculating lymphocytes through single lymph nodes." J Exp Med 143(4): 870-888.

Calame, K. (2006). "Transcription factors that regulate memory in humoral responses." Immunol Rev 211: 269-279.

Cao, X., E. W. Shores, J. Hu-Li, M. R. Anver, B. L. Kelsall, S. M. Russell, J. Drago, M. Noguchi, A. Grinberg, E. T. Bloom and et al. (1995). "Defective lymphoid development in mice lacking expression of the common cytokine receptor gamma chain." Immunity 2(3): 223-238.

Carlson, C. M., B. T. Endrizzi, J. Wu, X. Ding, M. A. Weinreich, E. R. Walsh, M. A. Wani, J. B. Lingrel, K. A. Hogquist and S. C. Jameson (2006). "Kruppel-like factor 2 regulates thymocyte and T-cell migration." Nature 442(7100): 299-302.

Carpenter, A. C. and R. Bosselut (2010). "Decision checkpoints in the thymus." Nat Immunol 11(8): 666-673.

Casalegno-Garduno, R., A. Schmitt, J. Yao, X. Wang, X. Xu, M. Freund and M. Schmitt (2010). "Multimer technologies for detection and adoptive transfer of antigen-specific T cells." Cancer Immunol Immunother 59(2): 195-202.

Casey, K. A. and M. F. Mescher (2007). "IL-21 promotes differentiation of naive CD8 T cells to a unique effector phenotype." Journal of immunology 178(12): 7640-7648.

Castellino, F. and R. N. Germain (2007). "Chemokine-guided CD4+ T cell help enhances generation of IL-6RalphahighIL-7Ralpha high prememory CD8+ T cells." J Immunol 178(2): 778-787.

Castellino, F., A. Y. Huang, G. Altan-Bonnet, S. Stoll, C. Scheinecker and R. N. Germain (2006). "Chemokines enhance immunity by guiding naive CD8+ T cells to sites of CD4+ T cell-dendritic cell interaction." Nature 440(7086): 890-895.

Chaffin, K. E. and R. M. Perlmutter (1991). "A pertussis toxin-sensitive process controls thymocyte emigration." Eur J Immunol 21(10): 2565-2573.

Chalfant, C. E. and S. Spiegel (2005). "Sphingosine 1-phosphate and ceramide 1-phosphate: expanding roles in cell signaling." J Cell Sci 118(Pt 20): 4605-4612.

Chaplin, D. D. (2010). "Overview of the immune response." J Allergy Clin Immunol 125(2 Suppl 2): S3-23.

Charlton, J. J., I. Chatzidakis, D. Tsoukatou, D. T. Boumpas, G. A. Garinis and C. Mamalaki (2013). "Programmed death-1 shapes memory phenotype CD8 T cell subsets in a cell-intrinsic manner." J Immunol 190(12): 6104-6114.

Charmoy, M., S. Brunner-Agten, D. Aebischer, F. Auderset, P. Launois, G. Milon, A. E. Proudfoot and F. Tacchini-Cottier (2010). "Neutrophil-derived CCL3 is essential for the rapid recruitment of dendritic cells to the site of Leishmania major inoculation in resistant mice." PLoS Pathog 6(2): e1000755.

Charo, I. F. and R. M. Ransohoff (2006). "The many roles of chemokines and chemokine receptors in inflammation." N Engl J Med 354(6): 610-621.

Chemnitz, J. M., R. V. Parry, K. E. Nichols, C. H. June and J. L. Riley (2004). "SHP-1 and SHP-2 associate with immunoreceptor tyrosine-based switch motif of programmed death 1 upon primary human T cell stimulation, but only receptor ligation prevents T cell activation." J Immunol 173(2): 945-954.

Chi, H. (2012). "Regulation and function of mTOR signalling in T cell fate decisions." Nat Rev Immunol 12(5): 325-338.

Choe, J., M. S. Kelker and I. A. Wilson (2005). "Crystal structure of human toll-like receptor 3 (TLR3) ectodomain." Science 309(5734): 581-585.

Ciofani, M. and J. C. Zuniga-Pflucker (2005). "Notch promotes survival of pre-T cells at the beta-selection checkpoint by regulating cellular metabolism." Nat Immunol 6(9): 881-888.

Ciofani, M. and J. C. Zuniga-Pflucker (2007). "The thymus as an inductive site for T lymphopoiesis." Annu Rev Cell Dev Biol 23: 463-493.

Coban, C., Y. Igari, M. Yagi, T. Reimer, S. Koyama, T. Aoshi, K. Ohata, T. Tsukui, F. Takeshita, K. Sakurai, T. Ikegami, A. Nakagawa, T. Horii, G. Nunez, K. J. Ishii and S. Akira (2010). "Immunogenicity of whole-parasite vaccines against Plasmodium falciparum involves malarial hemozoin and host TLR9." Cell Host Microbe 7(1): 50-61.

Colonna, M. (2006). "Cytolytic responses: cadherins put out the fire." J Exp Med 203(2): 261-264.

Conlan, J. W., P. L. Dunn and R. J. North (1993). "Leukocyte-mediated lysis of infected hepatocytes during listeriosis occurs in mice depleted of NK cells or CD4+ CD8+ Thy1.2+ T cells." Infect Immun 61(6): 2703-2707.

Conze, D., T. Krahl, N. Kennedy, L. Weiss, J. Lumsden, P. Hess, R. A. Flavell, G. Le Gros, R. J. Davis and M. Rincon (2002). "c-Jun NH(2)-terminal kinase (JNK)1 and JNK2 have distinct roles in CD8(+) T cell activation." J Exp Med 195(7): 811-823.

Cousens, L. P., J. S. Orange, H. C. Su and C. A. Biron (1997). "Interferon-alpha/beta inhibition of interleukin 12 and interferon-gamma production in vitro and endogenously during viral infection." Proc Natl Acad Sci U S A 94(2): 634-639.

Cox, M. A., L. E. Harrington and A. J. Zajac (2011). "Cytokines and the inception of CD8 T cell responses." Trends Immunol 32(4): 180-186.

Crotty, S., R. J. Johnston and S. P. Schoenberger (2010). "Effectors and memories: Bcl-6 and Blimp-1 in T and B lymphocyte differentiation." Nat Immunol 11(2): 114-120.

Crozat, K., R. Guiton, V. Contreras, V. Feuillet, C. A. Dutertre, E. Ventre, T. P. Vu Manh, T. Baranek, A. K. Storset, J. Marvel, P. Boudinot, A. Hosmalin, I. Schwartz-Cornil and M. Dalod (2010). "The XC chemokine receptor 1 is a conserved selective marker of mammalian cells homologous to mouse CD8alpha+ dendritic cells." J Exp Med 207(6): 1283-1292.

Cruz-Guilloty, F., M. E. Pipkin, I. M. Djuretic, D. Levanon, J. Lotem, M. G. Lichtenheld, Y. Groner and A. Rao (2009). "Runx3 and T-box proteins cooperate to establish the transcriptional program of effector CTLs." J Exp Med 206(1): 51-59.

Cui, W., Y. Liu, J. S. Weinstein, J. Craft and S. M. Kaech (2011). "An interleukin-21-interleukin-10-STAT3 pathway is critical for functional maturation of memory CD8+ T cells." Immunity 35(5): 792-805.

Curtsinger, J. M., C. M. Johnson and M. F. Mescher (2003). "CD8 T cell clonal expansion and development of effector function require prolonged exposure to antigen, costimulation, and signal 3 cytokine." J Immunol 171(10): 5165-5171.

Curtsinger, J. M., C. S. Schmidt, A. Mondino, D. C. Lins, R. M. Kedl, M. K. Jenkins and M. F. Mescher (1999). "Inflammatory cytokines provide a third signal for activation of naive CD4+ and CD8+ T cells." J Immunol 162(6): 3256-3262.

Curtsinger, J. M., J. O. Valenzuela, P. Agarwal, D. Lins and M. F. Mescher (2005). "Type I IFNs provide a third signal to CD8 T cells to stimulate clonal expansion and differentiation." J Immunol 174(8): 4465-4469.

Cyster, J. G. (2005). "Chemokines, sphingosine-1-phosphate, and cell migration in secondary lymphoid organs." Annu Rev Immunol 23: 127-159.

Cyster, J. G. and S. R. Schwab (2012). "Sphingosine-1-phosphate and lymphocyte egress from lymphoid organs." Annu Rev Immunol 30: 69-94.

D'Oro, U., M. S. Vacchio, A. M. Weissman and J. D. Ashwell (1997). "Activation of the Lck tyrosine kinase targets cell surface T cell antigen receptors for lysosomal degradation." Immunity 7(5): 619-628.

Damjanovic, D., M. Divangahi, K. Kugathasan, C. L. Small, A. Zganiacz, E. G. Brown, C. M. Hogaboam, J. Gauldie and Z. Xing (2011). "Negative regulation of lung inflammation and immunopathology by TNF-alpha during acute influenza infection." Am J Pathol 179(6): 2963-2976.

Davey, G. M., M. Wojtasiak, A. I. Proietto, F. R. Carbone, W. R. Heath and S. Bedoui (2010). "Cutting edge: priming of CD8 T cell immunity to herpes simplex virus type 1 requires cognate TLR3 expression in vivo." J Immunol 184(5): 2243-2246.

Davis, M. M., J. D. Altman and E. W. Newell (2011). "Interrogating the repertoire: broadening the scope of peptide-MHC multimer analysis." Nat Rev Immunol 11(8): 551-558.

Davis, S. J. and P. A. van der Merwe (2006). "The kinetic-segregation model: TCR triggering and beyond." Nat Immunol 7(8): 803-809.

Degli-Esposti, M. A. and M. J. Smyth (2005). "Close encounters of different kinds: dendritic cells and NK cells take centre stage." Nat Rev Immunol 5(2): 112-124.

DeGrendele, H. C., P. Estess and M. H. Siegelman (1997a). "Requirement for CD44 in activated T cell extravasation into an inflammatory site." Science 278(5338): 672-675.

DeGrendele, H. C., M. Kosfiszer, P. Estess and M. H. Siegelman (1997b). "CD44 activation and associated primary adhesion is inducible via T cell receptor stimulation." J Immunol 159(6): 2549-2553.

DeJarnette, J. B., C. L. Sommers, K. Huang, K. J. Woodside, R. Emmons, K. Katz, E. W. Shores and P. E. Love (1998). "Specific requirement for CD3epsilon in T cell development." Proc Natl Acad Sci U S A 95(25): 14909-14914.

Del Corno, M., Q. H. Liu, D. Schols, E. de Clercq, S. Gessani, B. D. Freedman and R. G. Collman (2001). "HIV-1 gp120 and chemokine activation of Pyk2 and mitogen-activated protein kinases in primary macrophages mediated by calcium-dependent, pertussis toxin-insensitive chemokine receptor signaling." Blood 98(10): 2909-2916.

Delgado, P. and B. Alarcon (2005). "An orderly inactivation of intracellular retention signals controls surface expression of the T cell antigen receptor." J Exp Med 201(4): 555-566.

Denkers, E. Y., G. Yap, T. Scharton-Kersten, H. Charest, B. A. Butcher, P. Caspar, S. Heiny and A. Sher (1997). "Perforin-mediated cytolysis plays a limited role in host resistance to Toxoplasma gondii." J Immunol 159(4): 1903-1908.

Dietrich, J., J. Kastrup, J. P. Lauritsen, C. Menne, F. von Bulow and C. Geisler (1999). "TCRzeta is transported to and retained in the Golgi apparatus independently of other TCR chains: implications for TCR assembly." Eur J Immunol 29(5): 1719-1728.

Dietrich, J., J. Kastrup, B. L. Nielsen, N. Odum and C. Geisler (1997). "Regulation and function of the CD3gamma DxxxLL motif: a binding site for adaptor protein-1 and adaptor protein-2 in vitro." J Cell Biol 138(2): 271-281.

Doherty, P. C. and R. M. Zinkernagel (1975). "H-2 compatibility is required for T-cell-mediated lysis of target cells infected with lymphocytic choriomeningitis virus." J Exp Med 141(2): 502-507.

Domann, E., J. Wehland, M. Rohde, S. Pistor, M. Hartl, W. Goebel, M. Leimeister-Wachter, M. Wuenscher and T. Chakraborty (1992). "A novel bacterial virulence gene in Listeria monocytogenes required for host cell microfilament interaction with homology to the proline-rich region of vinculin." EMBO J 11(5): 1981-1990.

Dorner, B., S. Muller, F. Entschladen, J. M. Schroder, P. Franke, R. Kraft, P. Friedl, I. Clark-Lewis and R. A. Kroczek (1997). "Purification, structural analysis, and function of natural ATAC, a cytokine secreted by CD8(+) T cells." J Biol Chem 272(13): 8817-8823.

Dorner, B. G., M. B. Dorner, X. Zhou, C. Opitz, A. Mora, S. Guttler, A. Hutloff, H. W. Mages, K. Ranke, M. Schaefer, R. S. Jack, V. Henn and R. A. Kroczek (2009). "Selective expression of the chemokine receptor XCR1 on cross-presenting dendritic cells determines cooperation with CD8+ T cells." Immunity 31(5): 823-833.

Dorner, B. G., A. Scheffold, M. S. Rolph, M. B. Huser, S. H. Kaufmann, A. Radbruch, I. E. Flesch and R. A. Kroczek (2002). "MIP-1alpha, MIP-1beta, RANTES, and ATAC/lymphotactin function together with IFN-gamma as type 1 cytokines." Proc Natl Acad Sci U S A 99(9): 6181-6186.

Drake, D. R., 3rd, R. M. Ream, C. W. Lawrence and T. J. Braciale (2005). "Transient loss of MHC class I tetramer binding after CD8+ T cell activation reflects altered T cell effector function." J Immunol 175(3): 1507-1515.

Drayton, D. L., S. Liao, R. H. Mounzer and N. H. Ruddle (2006). "Lymphoid organ development: from ontogeny to neogenesis." Nat Immunol 7(4): 344-353.

Duffy, D., H. Perrin, V. Abadie, N. Benhabiles, A. Boissonnas, C. Liard, B. Descours, D. Reboulleau, O. Bonduelle, B. Verrier, N. Van Rooijen, C. Combadiere and B. Combadiere (2012). "Neutrophils transport antigen from the dermis to the bone marrow, initiating a source of memory CD8+ T cells." Immunity 37(5): 917-929.

Dutko, F. J. and M. B. Oldstone (1983). "Genomic and biological variation among commonly used lymphocytic choriomeningitis virus strains." J Gen Virol 64 (Pt 8): 1689-1698.

Edelson, B. T. and E. R. Unanue (2002). "MyD88-dependent but Toll-like receptor 2-independent innate immunity to Listeria: no role for either in macrophage listericidal activity." J Immunol 169(7): 3869-3875.

Ehl, S., P. Klenerman, R. M. Zinkernagel and G. Bocharov (1998). "The impact of variation in the number of CD8(+) T-cell precursors on the outcome of virus infection." Cell Immunol 189(1): 67-73.

Elsaesser, H., K. Sauer and D. G. Brooks (2009). "IL-21 is required to control chronic viral infection." Science 324(5934): 1569-1572.

Finlay, D. and D. Cantrell (2010). "Phosphoinositide 3-kinase and the mammalian target of rapamycin pathways control T cell migration." Ann N Y Acad Sci 1183: 149-157.

Franchini, G. (2009). "Choosing the right memory T cell for HIV." Nat Med 15(3): 244-246.

Frauwirth, K. A., J. L. Riley, M. H. Harris, R. V. Parry, J. C. Rathmell, D. R. Plas, R. L. Elstrom, C. H. June and C. B. Thompson (2002). "The CD28 signaling pathway regulates glucose metabolism." Immunity 16(6): 769-777.

Freitas, A. A. and B. Rocha (2000). "Population biology of lymphocytes: the flight for survival." Annu Rev Immunol 18: 83-111.

Frey, J. R., B. Ernst, C. D. Surh and J. Sprent (1992). "Thymus-grafted SCID mice show transient thymopoiesis and limited depletion of V beta 11+ T cells." J Exp Med 175(4): 1067-1071.

Frohlich, A., J. Kisielow, I. Schmitz, S. Freigang, A. T. Shamshiev, J. Weber, B. J. Marsland, A. Oxenius and M. Kopf (2009). "IL-21R on T cells is critical for sustained functionality and control of chronic viral infection." Science 324(5934): 1576-1580.

Frucht, D. M., T. Fukao, C. Bogdan, H. Schindler, J. J. O'Shea and S. Koyasu (2001). "IFN-gamma production by antigen-presenting cells: mechanisms emerge." Trends Immunol 22(10): 556-560.

Fruh, K. and Y. Yang (1999). "Antigen presentation by MHC class I and its regulation by interferon gamma." Curr Opin Immunol 11(1): 76-81.

Fukuda, T., T. Yoshida, S. Okada, M. Hatano, T. Miki, K. Ishibashi, S. Okabe, H. Koseki, S. Hirosawa, M. Taniguchi, N. Miyasaka and T. Tokuhisa (1997). "Disruption of the Bcl6 gene results in an impaired germinal center formation." J Exp Med 186(3): 439-448.

Gaillard, J. L., P. Berche, C. Frehel, E. Gouin and P. Cossart (1991). "Entry of L. monocytogenes into cells is mediated by internalin, a repeat protein reminiscent of surface antigens from gram-positive cocci." Cell 65(7): 1127-1141.

Gao, Y., J. Tao, M. O. Li, D. Zhang, H. Chi, O. Henegariu, S. M. Kaech, R. J. Davis, R. A. Flavell and Z. Yin (2005). "JNK1 is essential for CD8+ T cell-mediated tumor immune surveillance." J Immunol 175(9): 5783-5789.

Gebhardt, T., L. M. Wakim, L. Eidsmo, P. C. Reading, W. R. Heath and F. R. Carbone (2009). "Memory T cells in nonlymphoid tissue that provide enhanced local immunity during infection with herpes simplex virus." Nat Immunol 10(5): 524-530.

Geisler, C. (2004). "TCR trafficking in resting and stimulated T cells." Crit Rev Immunol 24(1): 67-86.

Geissmann, F., S. Jung and D. R. Littman (2003). "Blood monocytes consist of two principal subsets with distinct migratory properties." Immunity 19(1): 71-82.

Girard, J. P., C. Moussion and R. Forster (2012). "HEVs, lymphatics and homeostatic immune cell trafficking in lymph nodes." Nat Rev Immunol 12(11): 762-773.

Godfrey, D. I., J. Kennedy, T. Suda and A. Zlotnik (1993). "A developmental pathway involving four phenotypically and functionally distinct subsets of CD3-CD4-CD8- triple-negative adult mouse thymocytes defined by CD44 and CD25 expression." J Immunol 150(10): 4244-4252.

Goossens, P. L. and G. Milon (1992). "Induction of protective CD8+ T lymphocytes by an attenuated Listeria monocytogenes actA mutant." Int Immunol 4(12): 1413-1418.

Gordon, S. (2007). "The macrophage: past, present and future." Eur J Immunol 37 Suppl 1: S9-17.

Grigorova, I. L., M. Panteleev and J. G. Cyster (2010). "Lymph node cortical sinus organization and relationship to lymphocyte egress dynamics and antigen exposure." Proc Natl Acad Sci U S A 107(47): 20447-20452.

Grigorova, I. L., S. R. Schwab, T. G. Phan, T. H. Pham, T. Okada and J. G. Cyster (2009). "Cortical sinus probing, S1P1-dependent entry and flow-based capture of egressing T cells." Nat Immunol 10(1): 58-65.

Gu, L., S. Tseng, R. M. Horner, C. Tam, M. Loda and B. J. Rollins (2000). "Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1." Nature 404(6776): 407-411.

Guarda, G., M. Hons, S. F. Soriano, A. Y. Huang, R. Polley, A. Martin-Fontecha, J. V. Stein, R. N. Germain, A. Lanzavecchia and F. Sallusto (2007). "L-selectin-negative CCR7- effector and memory CD8+ T cells enter reactive lymph nodes and kill dendritic cells." Nat Immunol 8(7): 743-752.

Guidotti, L. G., P. Borrow, A. Brown, H. McClary, R. Koch and F. V. Chisari (1999). "Noncytopathic clearance of lymphocytic choriomeningitis virus from the hepatocyte." J Exp Med 189(10): 1555-1564.

Guidotti, L. G., T. Ishikawa, M. V. Hobbs, B. Matzke, R. Schreiber and F. V. Chisari (1996). "Intracellular inactivation of the hepatitis B virus by cytotoxic T lymphocytes." Immunity 4(1): 25-36.

Guy, C. S., K. M. Vignali, J. Temirov, M. L. Bettini, A. E. Overacre, M. Smeltzer, H. Zhang, J. B. Huppa, Y. H. Tsai, C. Lobry, J. Xie, P. J. Dempsey, H. C. Crawford, I. Aifantis, M. M. Davis and D. A. Vignali (2013). "Distinct TCR signaling pathways drive proliferation and cytokine production in T cells." Nat Immunol 14(3): 262-270.

Hadrup, S. R., A. H. Bakker, C. J. Shu, R. S. Andersen, J. van Veluw, P. Hombrink, E. Castermans, P. Thor Straten, C. Blank, J. B. Haanen, M. H. Heemskerk and T. N. Schumacher (2009). "Parallel detection of antigen-specific T-cell responses by multidimensional encoding of MHC multimers." Nat Methods 6(7): 520-526.

Hall, J. G. and B. Morris (1965). "The immediate effect of antigens on the cell output of a lymph node." Br J Exp Pathol 46(4): 450-454.

Hammad, S. M., H. G. Crellin, B. X. Wu, J. Melton, V. Anelli and L. M. Obeid (2008). "Dual and distinct roles for sphingosine kinase 1 and sphingosine 1 phosphate in the response to inflammatory stimuli in RAW macrophages." Prostaglandins Other Lipid Mediat 85(3-4): 107-114.

Hand, T. W., M. Morre and S. M. Kaech (2007). "Expression of IL-7 receptor alpha is necessary but not sufficient for the formation of memory CD8 T cells during viral infection." Proc Natl Acad Sci U S A 104(28): 11730-11735.

Hansen, S. G., M. Piatak, Jr., A. B. Ventura, C. M. Hughes, R. M. Gilbride, J. C. Ford, K. Oswald, R. Shoemaker, Y. Li, M. S. Lewis, A. N. Gilliam, G. Xu, N. Whizin, B. J. Burwitz, S. L. Planer, J. M. Turner, A. W. Legasse, M. K. Axthelm, J. A. Nelson, K. Fruh, J. B. Sacha, J. D. Estes, B. F. Keele, P. T. Edlefsen, J. D. Lifson and L. J. Picker (2013). "Immune clearance of highly pathogenic SIV infection." Nature 502(7469): 100-104.

Hansen, S. G., C. Vieville, N. Whizin, L. Coyne-Johnson, D. C. Siess, D. D. Drummond, A. W. Legasse, M. K. Axthelm, K. Oswald, C. M. Trubey, M. Piatak, Jr., J. D. Lifson, J. A. Nelson, M. A. Jarvis and L. J. Picker (2009). "Effector memory T cell responses are associated with protection of rhesus monkeys from mucosal simian immunodeficiency virus challenge." Nat Med 15(3): 293-299.

Hao, Y., N. Legrand and A. A. Freitas (2006). "The clone size of peripheral CD8 T cells is regulated by TCR promiscuity." J Exp Med 203(7): 1643-1649.

Haring, J. S., V. P. Badovinac and J. T. Harty (2006). "Inflaming the CD8+ T cell response." Immunity 25(1): 19-29.

Harty, J. T., A. R. Tvinnereim and D. W. White (2000). "CD8+ T cell effector mechanisms in resistance to infection." Annu Rev Immunol 18: 275-308.

Hashimoto, S., K. Matsumoto, Y. Gon, S. Maruoka, K. Kujime, S. Hayashi, I. Takeshita and T. Horie (2000). "p38 MAP kinase regulates TNF alpha-, IL-1 alpha- and PAF-induced RANTES and GM-CSF production by human bronchial epithelial cells." Clin Exp Allergy 30(1): 48-55.

Hayashi, F., K. D. Smith, A. Ozinsky, T. R. Hawn, E. C. Yi, D. R. Goodlett, J. K. Eng, S. Akira, D. M. Underhill and A. Aderem (2001). "The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5." Nature 410(6832): 1099-1103.

Hedrick, J. A., V. Saylor, D. Figueroa, L. Mizoue, Y. Xu, S. Menon, J. Abrams, T. Handel and A. Zlotnik (1997). "Lymphotactin is produced by NK cells and attracts both NK cells and T cells in vivo." J Immunol 158(4): 1533-1540.

Hemmerich, S., A. Bistrup, M. S. Singer, A. van Zante, J. K. Lee, D. Tsay, M. Peters, J. L. Carminati, T. J. Brennan, K. Carver-Moore, M. Leviten, M. E. Fuentes, N. H. Ruddle and S. D. Rosen (2001). "Sulfation of L-selectin ligands by an HEV-restricted sulfotransferase regulates lymphocyte homing to lymph nodes." Immunity 15(2): 237-247.

Henson, S. M. and A. N. Akbar (2009). "KLRG1--more than a marker for T cell senescence." Age (Dordr) 31(4): 285-291.

Henson, S. M., O. Franzese, R. Macaulay, V. Libri, R. I. Azevedo, S. Kiani-Alikhan, F. J. Plunkett, J. E. Masters, S. Jackson, S. J. Griffiths, H. P. Pircher, M. V. Soares and A. N. Akbar (2009). "KLRG1 signaling induces defective Akt (ser473) phosphorylation and proliferative dysfunction of highly differentiated CD8+ T cells." Blood 113(26): 6619-6628.

Heyeck, S. D., H. M. Wilcox, S. C. Bunnell and L. J. Berg (1997). "Lck phosphorylates the activation loop tyrosine of the Itk kinase domain and activates Itk kinase activity." J Biol Chem 272(40): 25401-25408.

Hikono, H., J. E. Kohlmeier, S. Takamura, S. T. Wittmer, A. D. Roberts and D. L. Woodland (2007). "Activation phenotype, rather than central- or effector-memory phenotype, predicts the recall efficacy of memory CD8+ T cells." J Exp Med 204(7): 1625-1636.

Hirotani, T., M. Yamamoto, Y. Kumagai, S. Uematsu, I. Kawase, O. Takeuchi and S. Akira (2005). "Regulation of lipopolysaccharide-inducible genes by MyD88 and Toll/IL-1 domain containing adaptor inducing IFN-beta." Biochem Biophys Res Commun 328(2): 383-392.

Hoebe, K., E. M. Janssen, S. O. Kim, L. Alexopoulou, R. A. Flavell, J. Han and B. Beutler (2003). "Upregulation of costimulatory molecules induced by lipopolysaccharide and double-stranded RNA occurs by Trif-dependent and Trif-independent pathways." Nat Immunol 4(12): 1223-1229.

Hornung, V., M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres and G. Hartmann (2005). "Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7." Nat Med 11(3): 263-270.

Huang, A. Y., P. Golumbek, M. Ahmadzadeh, E. Jaffee, D. Pardoll and H. Levitsky (1994). "Role of bone marrow-derived cells in presenting MHC class I-restricted tumor antigens." Science 264(5161): 961-965.

Humphreys, T. D., A. Khanolkar, V. P. Badovinac and J. T. Harty (2008). "Generation and maintenance of Listeria-specific CD8+ T cell responses in perforin-deficient mice chronically infected with LCMV." Virology 370(2): 310-322.

Huster, K. M., V. Busch, M. Schiemann, K. Linkemann, K. M. Kerksiek, H. Wagner and D. H. Busch (2004). "Selective expression of IL-7 receptor on memory T cells identifies early CD40L-dependent generation of distinct CD8+ memory T cell subsets." Proc Natl Acad Sci U S A 101(15): 5610-5615.

Huster, K. M., M. Koffler, C. Stemberger, M. Schiemann, H. Wagner and D. H. Busch (2006). "Unidirectional development of CD8+ central memory T cells into protective Listeria-specific effector memory T cells." Eur J Immunol 36(6): 1453-1464.

Ichii, H., A. Sakamoto, M. Hatano, S. Okada, H. Toyama, S. Taki, M. Arima, Y. Kuroda and T. Tokuhisa (2002). "Role for Bcl-6 in the generation and maintenance of memory CD8+ T cells." Nat Immunol 3(6): 558-563.

Ichii, H., A. Sakamoto, Y. Kuroda and T. Tokuhisa (2004). "Bcl6 acts as an amplifier for the generation and proliferative capacity of central memory CD8+ T cells." J Immunol 173(2): 883-891.

Imai, Y., K. Kuba, G. G. Neely, R. Yaghubian-Malhami, T. Perkmann, G. van Loo, M. Ermolaeva, R. Veldhuizen, Y. H. Leung, H. Wang, H. Liu, Y. Sun, M. Pasparakis, M. Kopf, C. Mech, S. Bavari, J. S. Peiris, A. S. Slutsky, S. Akira, M. Hultqvist, R. Holmdahl, J. Nicholls, C. Jiang, C. J. Binder and J. M. Penninger (2008). "Identification of oxidative stress and Toll-like receptor 4 signaling as a key pathway of acute lung injury." Cell 133(2): 235-249.

Intlekofer, A. M., A. Banerjee, N. Takemoto, S. M. Gordon, C. S. Dejong, H. Shin, C. A. Hunter, E. J. Wherry, T. Lindsten and S. L. Reiner (2008). "Anomalous type 17 response to viral infection by CD8+ T cells lacking T-bet and eomesodermin." Science 321(5887): 408-411.

Intlekofer, A. M., N. Takemoto, C. Kao, A. Banerjee, F. Schambach, J. K. Northrop, H. Shen, E. J. Wherry and S. L. Reiner (2007). "Requirement for T-bet in the aberrant differentiation of unhelped memory CD8+ T cells." J Exp Med 204(9): 2015-2021.

Intlekofer, A. M., N. Takemoto, E. J. Wherry, S. A. Longworth, J. T. Northrup, V. R. Palanivel, A. C. Mullen, C. R. Gasink, S. M. Kaech, J. D. Miller, L. Gapin, K. Ryan, A. P. Russ, T. Lindsten, J. S. Orange, A. W. Goldrath, R. Ahmed and S. L. Reiner (2005). "Effector and memory CD8+ T cell fate coupled by T-bet and eomesodermin." Nat Immunol 6(12): 1236-1244.

Ito, K., Y. Anada, M. Tani, M. Ikeda, T. Sano, A. Kihara and Y. Igarashi (2007). "Lack of sphingosine 1-phosphate-degrading enzymes in erythrocytes." Biochem Biophys Res Commun 357(1): 212-217.

Itoh, Y. and R. N. Germain (1997). "Single cell analysis reveals regulated hierarchical T cell antigen receptor signaling thresholds and intraclonal heterogeneity for individual cytokine responses of CD4+ T cells." J Exp Med 186(5): 757-766.

Jacobs, S. R., C. E. Herman, N. J. Maciver, J. A. Wofford, H. L. Wieman, J. J. Hammen and J. C. Rathmell (2008). "Glucose uptake is limiting in T cell activation and requires CD28-mediated Akt-dependent and independent pathways." J Immunol 180(7): 4476-4486.

Jalonen, T. O., K. Pulkkinen, M. Ukkonen, M. Saarela and I. Elovaara (2002). "Differential intracellular expression of CCR5 and chemokines in multiple sclerosis subtypes." J Neurol 249(5): 576-583.

Jameson, S. C. and D. Masopust (2009). "Diversity in T cell memory: an embarrassment of riches." Immunity 31(6): 859-871.

Jang, E., S. H. Cho, H. Park, D. J. Paik, J. M. Kim and J. Youn (2009). "A positive feedback loop of IL-21 signaling provoked by homeostatic CD4+CD25- T cell expansion is essential for the development of arthritis in autoimmune K/BxN mice." J Immunol 182(8): 4649-4656.

Janssen, E. M., E. E. Lemmens, T. Wolfe, U. Christen, M. G. von Herrath and S. P. Schoenberger (2003). "CD4+ T cells are required for secondary expansion and memory in CD8+ T lymphocytes." Nature 421(6925): 852-856.

Jeannet, G., C. Boudousquie, N. Gardiol, J. Kang, J. Huelsken and W. Held (2010). "Essential role of the Wnt pathway effector Tcf-1 for the establishment of functional CD8 T cell memory." Proc Natl Acad Sci U S A 107(21): 9777-9782.

Jia, T., I. Leiner, G. Dorothee, K. Brandl and E. G. Pamer (2009). "MyD88 and Type I interferon receptor-mediated chemokine induction and monocyte recruitment during Listeria monocytogenes infection." J Immunol 183(2): 1271-1278.

Jia, T., N. V. Serbina, K. Brandl, M. X. Zhong, I. M. Leiner, I. F. Charo and E. G. Pamer (2008). "Additive roles for MCP-1 and MCP-3 in CCR2-mediated recruitment of inflammatory monocytes during Listeria monocytogenes infection." J Immunol 180(10): 6846-6853.

Johnston, R. J., A. C. Poholek, D. DiToro, I. Yusuf, D. Eto, B. Barnett, A. L. Dent, J. Craft and S. Crotty (2009). "Bcl6 and Blimp-1 are reciprocal and antagonistic regulators of T follicular helper cell differentiation." Science 325(5943): 1006-1010.

Joshi, N. S., W. Cui, A. Chandele, H. K. Lee, D. R. Urso, J. Hagman, L. Gapin and S. M. Kaech (2007). "Inflammation directs memory precursor and short-lived effector CD8(+) T cell fates via the graded expression of T-bet transcription factor." Immunity 27(2): 281-295.

Joshi, N. S., W. Cui, C. X. Dominguez, J. H. Chen, T. W. Hand and S. M. Kaech (2011). "Increased numbers of preexisting memory CD8 T cells and decreased T-bet expression can restrain terminal differentiation of secondary effector and memory CD8 T cells." J Immunol 187(8): 4068-4076.

Jung, I. D., J. S. Lee, Y. J. Kim, Y. I. Jeong, C. M. Lee, T. Baumruker, A. Billlich, Y. Banno, M. G. Lee, S. C. Ahn, W. S. Park, J. Han and Y. M. Park (2007). "Sphingosine kinase inhibitor suppresses a Th1 polarization via the inhibition of immunostimulatory activity in murine bone marrow-derived dendritic cells." Int Immunol 19(4): 411-426.

Jung, S., D. Unutmaz, P. Wong, G. Sano, K. De los Santos, T. Sparwasser, S. Wu, S. Vuthoori, K. Ko, F. Zavala, E. G. Pamer, D. R. Littman and R. A. Lang (2002). "In vivo depletion of CD11c+ dendritic cells abrogates priming of CD8+ T cells by exogenous cell-associated antigens." Immunity 17(2): 211-220.

Kaech, S. M. and R. Ahmed (2001). "Memory CD8+ T cell differentiation: initial antigen encounter triggers a developmental program in naive cells." Nat Immunol 2(5): 415-422.

Kaech, S. M. and W. Cui (2012). "Transcriptional control of effector and memory CD8+ T cell differentiation." Nat Rev Immunol 12(11): 749-761.

Kaech, S. M., J. T. Tan, E. J. Wherry, B. T. Konieczny, C. D. Surh and R. Ahmed (2003). "Selective expression of the interleukin 7 receptor identifies effector CD8 T cells that give rise to long-lived memory cells." Nat Immunol 4(12): 1191-1198.

Kagi, D., B. Ledermann, K. Burki, H. Hengartner and R. M. Zinkernagel (1994a). "CD8+ T cell-mediated protection against an intracellular bacterium by perforin-dependent cytotoxicity." Eur J Immunol 24(12): 3068-3072.

Kagi, D., B. Ledermann, K. Burki, P. Seiler, B. Odermatt, K. J. Olsen, E. R. Podack, R. M. Zinkernagel and H. Hengartner (1994b). "Cytotoxicity mediated by T cells and natural killer cells is greatly impaired in perforin-deficient mice." Nature 369(6475): 31-37.

Kagi, D., F. Vignaux, B. Ledermann, K. Burki, V. Depraetere, S. Nagata, H. Hengartner and P. Golstein (1994c). "Fas and perforin pathways as major mechanisms of T cell-mediated cytotoxicity." Science 265(5171): 528-530.

Kaka, A. S., D. R. Shaffer, R. Hartmaier, A. M. Leen, A. Lu, A. Bear, C. M. Rooney and A. E. Foster (2009). "Genetic modification of T cells with IL-21 enhances antigen presentation and generation of central memory tumor-specific cytotoxic T-lymphocytes." J Immunother 32(7): 726-736.

Kalia, V., S. Sarkar, S. Subramaniam, W. N. Haining, K. A. Smith and R. Ahmed (2010). "Prolonged interleukin-2Ralpha expression on virus-specific CD8+ T cells favors terminal-effector differentiation in vivo." Immunity 32(1): 91-103.

Kallies, A., J. Hasbold, D. M. Tarlinton, W. Dietrich, L. M. Corcoran, P. D. Hodgkin and S. L. Nutt (2004). "Plasma cell ontogeny defined by quantitative changes in blimp-1 expression." J Exp Med 200(8): 967-977.

Kallies, A., A. Xin, G. T. Belz and S. L. Nutt (2009). "Blimp-1 transcription factor is required for the differentiation of effector CD8(+) T cells and memory responses." Immunity 31(2): 283-295.

Kang, S. J., H. E. Liang, B. Reizis and R. M. Locksley (2008). "Regulation of hierarchical clustering and activation of innate immune cells by dendritic cells." Immunity 29(5): 819-833.

Kanneganti, T. D., M. Lamkanfi and G. Nunez (2007). "Intracellular NOD-like receptors in host defense and disease." Immunity 27(4): 549-559.

Kao, C., K. J. Oestreich, M. A. Paley, A. Crawford, J. M. Angelosanto, M. A. Ali, A. M. Intlekofer, J. M. Boss, S. L. Reiner, A. S. Weinmann and E. J. Wherry (2011). "Transcription factor T-bet represses expression of the inhibitory receptor PD-1 and sustains virus-specific CD8+ T cell responses during chronic infection." Nat Immunol 12(7): 663-671.

Karwacz, K., C. Bricogne, D. MacDonald, F. Arce, C. L. Bennett, M. Collins and D. Escors (2011). "PD-L1 co-stimulation contributes to ligand-induced T cell receptor down-modulation on CD8+ T cells." EMBO Mol Med 3(10): 581-592.

Kassiotis, G., S. Garcia, E. Simpson and B. Stockinger (2002). "Impairment of immunological memory in the absence of MHC despite survival of memory T cells." Nat Immunol 3(3): 244-250.

Kassiotis, G., R. Zamoyska and B. Stockinger (2003). "Involvement of avidity for major histocompatibility complex in homeostasis of naive and memory T cells." J Exp Med 197(8): 1007-1016.

Kawai, T. and S. Akira (2006). "Innate immune recognition of viral infection." Nat Immunol 7(2): 131-137.

Kawai, T. and S. Akira (2010). "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors." Nat Immunol 11(5): 373-384.

Kawai, T. and S. Akira (2011). "Toll-like receptors and their crosstalk with other innate receptors in infection and immunity." Immunity 34(5): 637-650.

Kawai, T., O. Takeuchi, T. Fujita, J. Inoue, P. F. Muhlradt, S. Sato, K. Hoshino and S. Akira (2001). "Lipopolysaccharide stimulates the MyD88-independent pathway and results in activation of IFN-regulatory factor 3 and the expression of a subset of lipopolysaccharide-inducible genes." J Immunol 167(10): 5887-5894.

Kayal, S. and A. Charbit (2006). "Listeriolysin O: a key protein of Listeria monocytogenes with multiple functions." FEMS Microbiol Rev 30(4): 514-529.

Kedzierska, K., J. Stambas, M. R. Jenkins, R. Keating, S. J. Turner and P. C. Doherty (2007). "Location rather than CD62L phenotype is critical in the early establishment of influenza-specific CD8+ T cell memory." Proc Natl Acad Sci U S A 104(23): 9782-9787.

Kelner, G. S., J. Kennedy, K. B. Bacon, S. Kleyensteuber, D. A. Largaespada, N. A. Jenkins, N. G. Copeland, J. F. Bazan, K. W. Moore, T. J. Schall and et al. (1994). "Lymphotactin: a cytokine that represents a new class of chemokine." Science 266(5189): 1395-1399.

Keppler, S. J., K. Rosenits, T. Koegl, S. Vucikuja and P. Aichele (2012). "Signal 3 cytokines as modulators of primary immune responses during infections: the interplay of type I IFN and IL-12 in CD8 T cell responses." PLoS One 7(7): e40865.

Keppler, S. J., K. Theil, S. Vucikuja and P. Aichele (2009). "Effector T-cell differentiation during viral and bacterial infections: Role of direct IL-12 signals for cell fate decision of CD8(+) T cells." Eur J Immunol 39(7): 1774-1783.

Kim, E. Y., J. J. Priatel, S. J. Teh and H. S. Teh (2006). "TNF receptor type 2 (p75) functions as a costimulator for antigen-driven T cell responses in vivo." J Immunol 176(2): 1026-1035.

Kim, K., C. K. Lee, T. J. Sayers, K. Muegge and S. K. Durum (1998). "The trophic action of IL-7 on pro-T cells: inhibition of apoptosis of pro-T1, -T2, and -T3 cells correlates with Bcl-2 and Bax levels and is independent of Fas and p53 pathways." J Immunol 160(12): 5735-5741.

Kim, S., K. Iizuka, H. S. Kang, A. Dokun, A. R. French, S. Greco and W. M. Yokoyama (2002). "In vivo developmental stages in murine natural killer cell maturation." Nat Immunol 3(6): 523-528.

Kisielow, P., H. Bluthmann, U. D. Staerz, M. Steinmetz and H. von Boehmer (1988). "Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature CD4+8+ thymocytes." Nature 333(6175): 742-746.

Klein, U. and R. Dalla-Favera (2008). "Germinal centres: role in B-cell physiology and malignancy." Nat Rev Immunol 8(1): 22-33.

Kocks, C., E. Gouin, M. Tabouret, P. Berche, H. Ohayon and P. Cossart (1992). "L. monocytogenes-induced actin assembly requires the actA gene product, a surface protein." Cell 68(3): 521-531.

Kolumam, G. A., S. Thomas, L. J. Thompson, J. Sprent and K. Murali-Krishna (2005). "Type I interferons act directly on CD8 T cells to allow clonal expansion and memory formation in response to viral infection." J Exp Med 202(5): 637-650.

Korn, T., E. Bettelli, W. Gao, A. Awasthi, A. Jager, T. B. Strom, M. Oukka and V. K. Kuchroo (2007). "IL-21 initiates an alternative pathway to induce proinflammatory T(H)17 cells." Nature 448(7152): 484-487.

Krangel, M. S. (1987). "Endocytosis and recycling of the T3-T cell receptor complex. The role of T3 phosphorylation." J Exp Med 165(4): 1141-1159.

Kroczek, R. A. and V. Henn (2012). "The Role of XCR1 and its Ligand XCL1 in Antigen Cross-Presentation by Murine and Human Dendritic Cells." Front Immunol 3: 14.

Kufer, T. A., E. Kremmer, A. C. Adam, D. J. Philpott and P. J. Sansonetti (2008). "The pattern-recognition molecule Nod1 is localized at the plasma membrane at sites of bacterial interaction." Cell Microbiol 10(2): 477-486.

Kumar, H., T. Kawai and S. Akira (2011). "Pathogen recognition by the innate immune system." Int Rev Immunol 30(1): 16-34.

Kumar, S. N. and J. M. Boss (2000). "Site A of the MCP-1 distal regulatory region functions as a transcriptional modulator through the transcription factor NF1." Mol Immunol 37(11): 623-632.

Kuna, P., S. R. Reddigari, T. J. Schall, D. Rucinski, M. Y. Viksman and A. P. Kaplan (1992). "RANTES, a monocyte and T lymphocyte chemotactic cytokine releases histamine from human basophils." J Immunol 149(2): 636-642.

Lacombe, M. H., M. P. Hardy, J. Rooney and N. Labrecque (2005). "IL-7 receptor expression levels do not identify CD8+ memory T lymphocyte precursors following peptide immunization." J Immunol 175(7): 4400-4407.

Ladel, C. H., I. E. Flesch, J. Arnoldi and S. H. Kaufmann (1994). "Studies with MHC-deficient knock-out mice reveal impact of both MHC I- and MHC II-dependent T cell responses on Listeria monocytogenes infection." J Immunol 153(7): 3116-3122.

Lammermann, T., B. L. Bader, S. J. Monkley, T. Worbs, R. Wedlich-Soldner, K. Hirsch, M. Keller, R. Forster, D. R. Critchley, R. Fassler and M. Sixt (2008). "Rapid leukocyte migration by integrin-independent flowing and squeezing." Nature 453(7191): 51-55.

Latchman, Y., C. R. Wood, T. Chernova, D. Chaudhary, M. Borde, I. Chernova, Y. Iwai, A. J. Long, J. A. Brown, R. Nunes, E. A. Greenfield, K. Bourque, V. A. Boussiotis, L. L. Carter, B. M. Carreno, N. Malenkovich, H. Nishimura, T. Okazaki, T. Honjo, A. H. Sharpe and G. J. Freeman (2001). "PD-L2 is a second ligand for PD-1 and inhibits T cell activation." Nat Immunol 2(3): 261-268.

Lauritsen, J. P., M. D. Christensen, J. Dietrich, J. Kastrup, N. Odum and C. Geisler (1998). "Two distinct pathways exist for down-regulation of the TCR." J Immunol 161(1): 260-267.

Lazarevic, V., L. H. Glimcher and G. M. Lord (2013). "T-bet: a bridge between innate and adaptive immunity." Nat Rev Immunol 13(11): 777-789.

Lecuit, M., S. Vandormael-Pournin, J. Lefort, M. Huerre, P. Gounon, C. Dupuy, C. Babinet and P. Cossart (2001). "A transgenic model for listeriosis: role of internalin in crossing the intestinal barrier." Science 292(5522): 1722-1725.

Lee, H. K., J. M. Lund, B. Ramanathan, N. Mizushima and A. Iwasaki (2007). "Autophagy-dependent viral recognition by plasmacytoid dendritic cells." Science 315(5817): 1398-1401.

Lei, Y. and Y. Takahama (2012). "XCL1 and XCR1 in the immune system." Microbes Infect 14(3): 262-267.

Lenz, L. L., S. Mohammadi, A. Geissler and D. A. Portnoy (2003). "SecA2-dependent secretion of autolytic enzymes promotes Listeria monocytogenes pathogenesis." Proc Natl Acad Sci U S A 100(21): 12432-12437.

Levy, J. A. (2009). "The unexpected pleiotropic activities of RANTES." J Immunol 182(7): 3945-3946.

Ley, K., C. Laudanna, M. I. Cybulsky and S. Nourshargh (2007). "Getting to the site of inflammation: the leukocyte adhesion cascade updated." Nat Rev Immunol 7(9): 678-689.

Lighvani, A. A., D. M. Frucht, D. Jankovic, H. Yamane, J. Aliberti, B. D. Hissong, B. V. Nguyen, M. Gadina, A. Sher, W. E. Paul and J. J. O'Shea (2001). "T-bet is rapidly induced by interferon-gamma in lymphoid and myeloid cells." Proc Natl Acad Sci U S A 98(26): 15137-15142.

Lim, S. P. and A. Garzino-Demo (2000). "The human immunodeficiency virus type 1 Tat protein up-regulates the promoter activity of the beta-chemokine monocyte chemoattractant protein 1 in the human astrocytoma cell line U-87 MG: role of SP-1, AP-1, and NF-kappaB consensus sites." J Virol 74(4): 1632-1640.

Lo, C. G., Y. Xu, R. L. Proia and J. G. Cyster (2005). "Cyclical modulation of sphingosine-1-phosphate receptor 1 surface expression during lymphocyte recirculation and relationship to lymphoid organ transit." J Exp Med 201(2): 291-301.

Lowsky, R., T. Takahashi, Y. P. Liu, S. Dejbakhsh-Jones, F. C. Grumet, J. A. Shizuru, G. G. Laport, K. E. Stockerl-Goldstein, L. J. Johnston, R. T. Hoppe, D. A. Bloch, K. G. Blume, R. S. Negrin and S. Strober (2005). "Protective conditioning for acute graft-versus-host disease." N Engl J Med 353(13): 1321-1331.

Ludviksson, B. R. and B. Gunnlaugsdottir (2003). "Transforming growth factor-beta as a regulator of site-specific T-cell inflammatory response." Scand J Immunol 58(2): 129-138.

Luther, S. A. and J. G. Cyster (2001). "Chemokines as regulators of T cell differentiation." Nat Immunol 2(2): 102-107.

Macian, F., F. Garcia-Cozar, S. H. Im, H. F. Horton, M. C. Byrne and A. Rao (2002). "Transcriptional mechanisms underlying lymphocyte tolerance." Cell 109(6): 719-731.

MacInnes, H., Y. Zhou, K. Gouveia, J. Cromwell, K. Lowery, R. C. Layton, M. Zubelewicz, R. Sampath, S. Hofstadler, Y. Liu, Y. S. Cheng and F. Koster (2011). "Transmission of aerosolized seasonal H1N1 influenza A to ferrets." PLoS One 6(9): e24448.

Madakamutil, L. T., U. Christen, C. J. Lena, Y. Wang-Zhu, A. Attinger, M. Sundarrajan, W. Ellmeier, M. G. von Herrath, P. Jensen, D. R. Littman and H. Cheroutre (2004). "CD8alphaalpha-mediated survival and differentiation of CD8 memory T cell precursors." Science 304(5670): 590-593.

Mancuso, G., M. Gambuzza, A. Midiri, C. Biondo, S. Papasergi, S. Akira, G. Teti and C. Beninati (2009). "Bacterial recognition by TLR7 in the lysosomes of conventional dendritic cells." Nat Immunol 10(6): 587-594.

Mandala, S., R. Hajdu, J. Bergstrom, E. Quackenbush, J. Xie, J. Milligan, R. Thornton, G. J. Shei, D. Card, C. Keohane, M. Rosenbach, J. Hale, C. L. Lynch, K. Rupprecht, W. Parsons and H. Rosen (2002). "Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists." Science 296(5566): 346-349.

Marcais, A., M. Tomkowiak, T. Walzer, C. A. Coupet, A. Ravel-Chapuis and J. Marvel (2006). "Maintenance of CCL5 mRNA stores by post-effector and memory CD8 T cells is dependent on transcription and is coupled to increased mRNA stability." Eur J Immunol 36(10): 2745-2754.

Markert, M. L., B. H. Devlin, M. J. Alexieff, J. Li, E. A. McCarthy, S. E. Gupton, I. K. Chinn, L. P. Hale, T. B. Kepler, M. He, M. Sarzotti, M. A. Skinner, H. E. Rice and J. C. Hoehner (2007). "Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: outcome of 44 consecutive transplants." Blood 109(10): 4539-4547.

Markert, M. L., J. G. Marques, B. Neven, B. H. Devlin, E. A. McCarthy, I. K. Chinn, A. S. Albuquerque, S. L. Silva, C. Pignata, G. de Saint Basile, R. M. Victorino, C. Picard, M. Debre, N. Mahlaoui, A. Fischer and A. E. Sousa (2011). "First use of thymus transplantation therapy for FOXN1 deficiency (nude/SCID): a report of 2 cases." Blood 117(2): 688-696.

Marzo, A. L., K. D. Klonowski, A. Le Bon, P. Borrow, D. F. Tough and L. Lefrancois (2005). "Initial T cell frequency dictates memory CD8+ T cell lineage commitment." Nat Immunol 6(8): 793-799.

Masopust, D., D. Choo, V. Vezys, E. J. Wherry, J. Duraiswamy, R. Akondy, J. Wang, K. A. Casey, D. L. Barber, K. S. Kawamura, K. A. Fraser, R. J. Webby, V. Brinkmann, E. C. Butcher, K. A. Newell and R. Ahmed (2010). "Dynamic T cell migration program provides resident memory within intestinal epithelium." J Exp Med 207(3): 553-564.

Masopust, D., V. Vezys, A. L. Marzo and L. Lefrancois (2001). "Preferential localization of effector memory cells in nonlymphoid tissue." Science 291(5512): 2413-2417.

Matloubian, M., C. G. Lo, G. Cinamon, M. J. Lesneski, Y. Xu, V. Brinkmann, M. L. Allende, R. L. Proia and J. G. Cyster (2004). "Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1." Nature 427(6972): 355-360.

Maurer, M. and E. von Stebut (2004). "Macrophage inflammatory protein-1." Int J Biochem Cell Biol 36(10): 1882-1886.

Mauri, C. and A. Bosma (2012). "Immune regulatory function of B cells." Annu Rev Immunol 30: 221-241.

Mbitikon-Kobo, F. M., M. Vocanson, M. C. Michallet, M. Tomkowiak, A. Cottalorda, G. S. Angelov, C. A. Coupet, S. Djebali, A. Marcais, B. Dubois, N. Bonnefoy-Berard, J. F. Nicolas, C. Arpin and J. Marvel (2009). "Characterization of a CD44/CD122int memory CD8 T cell subset generated under sterile inflammatory conditions." J Immunol 182(6): 3846-3854.

McGregor, D. D., F. T. Koster and G. B. Mackaness (1970). "The short lived small lymphocyte as a mediator of cellular immunity." Nature 228(5274): 855-856.

Medzhitov, R. (2008). "Origin and physiological roles of inflammation." Nature 454(7203): 428-435.

Medzhitov, R. (2010). "Inflammation 2010: new adventures of an old flame." Cell 140(6): 771-776.

Melgarejo, E., M. A. Medina, F. Sanchez-Jimenez and J. L. Urdiales (2009). "Monocyte chemoattractant protein-1: a key mediator in inflammatory processes." Int J Biochem Cell Biol 41(5): 998-1001.

Merad, M., P. Sathe, J. Helft, J. Miller and A. Mortha (2013). "The dendritic cell lineage: ontogeny and function of dendritic cells and their subsets in the steady state and the inflamed setting." Annu Rev Immunol 31: 563-604.

Merritt, C., H. Enslen, N. Diehl, D. Conze, R. J. Davis and M. Rincon (2000). "Activation of p38 mitogen-activated protein kinase in vivo selectively induces apoptosis of CD8(+) but not CD4(+) T cells." Mol Cell Biol 20(3): 936-946.

Messingham, K. A., V. P. Badovinac, A. Jabbari and J. T. Harty (2007). "A role for IFN-gamma from antigen-specific CD8+ T cells in protective immunity to Listeria monocytogenes." J Immunol 179(4): 2457-2466.

Mestas, J. and C. C. Hughes (2004). "Of mice and not men: differences between mouse and human immunology." J Immunol 172(5): 2731-2738.

Mielke, M. E., S. Ehlers and H. Hahn (1988). "T-cell subsets in delayed-type hypersensitivity, protection, and granuloma formation in primary and secondary Listeria infection in mice: superior role of Lyt-2+ cells in acquired immunity." Infect Immun 56(8): 1920-1925.

Miller, J. F. (1961). "Immunological function of the thymus." Lancet 2(7205): 748-749.

Miller, M. J., A. S. Hejazi, S. H. Wei, M. D. Cahalan and I. Parker (2004). "T cell repertoire scanning is promoted by dynamic dendritic cell behavior and random T cell motility in the lymph node." Proc Natl Acad Sci U S A 101(4): 998-1003.

Minami, Y., L. E. Samelson and R. D. Klausner (1987a). "Internalization and cycling of the T cell antigen receptor. Role of protein kinase C." J Biol Chem 262(27): 13342-13347.

Minami, Y., A. M. Weissman, L. E. Samelson and R. D. Klausner (1987b). "Building a multichain receptor: synthesis, degradation, and assembly of the T-cell antigen receptor." Proc Natl Acad Sci U S A 84(9): 2688-2692.

Mionnet, C., S. L. Sanos, I. Mondor, A. Jorquera, J. P. Laugier, R. N. Germain and M. Bajenoff (2011). "High endothelial venules as traffic control points maintaining lymphocyte population homeostasis in lymph nodes." Blood 118(23): 6115-6122.

Mittrucker, H. W., T. Matsuyama, A. Grossman, T. M. Kundig, J. Potter, A. Shahinian, A. Wakeham, B. Patterson, P. S. Ohashi and T. W. Mak (1997). "Requirement for the transcription factor LSIRF/IRF4 for mature B and T lymphocyte function." Science 275(5299): 540-543.

Miyasaka, M. and T. Tanaka (2004). "Lymphocyte trafficking across high endothelial venules: dogmas and enigmas." Nat Rev Immunol 4(5): 360-370.

Mogensen, T. H. (2009). "Pathogen recognition and inflammatory signaling in innate immune defenses." Clin Microbiol Rev 22(2): 240-273, Table of Contents.

Monteiro, M., C. Evaristo, A. Legrand, A. Nicoletti and B. Rocha (2007). "Cartography of gene expression in CD8 single cells: novel CCR7- subsets suggest differentiation independent of CD45RA expression." Blood 109(7): 2863-2870.

Monteleone, G., F. Pallone and T. T. Macdonald (2009). "Interleukin-21 as a new therapeutic target for immune-mediated diseases." Trends Pharmacol Sci 30(8): 441-447.

Moore, T. A., U. von Freeden-Jeffry, R. Murray and A. Zlotnik (1996). "Inhibition of gamma delta T cell development and early thymocyte maturation in IL-7 -/- mice." J Immunol 157(6): 2366-2373.

Mori, N., N. Mukaida, D. W. Ballard, K. Matsushima and N. Yamamoto (1998). "Human T-cell leukemia virus type I Tax transactivates human interleukin 8 gene through acting concurrently on AP-1 and nuclear factor-kappaB-like sites." Cancer Res 58(17): 3993-4000.

Moser, B. and P. Loetscher (2001). "Lymphocyte traffic control by chemokines." Nat Immunol 2(2): 123-128.

Moskophidis, D., M. Battegay, M. A. Bruendler, E. Laine, I. Gresser and R. M. Zinkernagel (1994). "Resistance of lymphocytic choriomeningitis virus to alpha/beta interferon and to gamma interferon." J Virol 68(3): 1951-1955.

Mount, A. M., C. M. Smith, F. Kupresanin, K. Stoermer, W. R. Heath and G. T. Belz (2008). "Multiple dendritic cell populations activate CD4+ T cells after viral stimulation." PLoS One 3(2): e1691.

Mullbacher, A., R. T. Hla, C. Museteanu and M. M. Simon (1999). "Perforin is essential for control of ectromelia virus but not related poxviruses in mice." J Virol 73(2): 1665-1667.

Munitic, I., H. Decaluwe, C. Evaristo, S. Lemos, M. Wlodarczyk, A. Worth, A. Le Bon, L. K. Selin, Y. Riviere, J. P. Di Santo, P. Borrow and B. Rocha (2009). "Epitope specificity and relative clonal abundance do not affect CD8 differentiation patterns during lymphocytic choriomeningitis virus infection." J Virol 83(22): 11795-11807.

Muraille, E., R. Giannino, P. Guirnalda, I. Leiner, S. Jung, E. G. Pamer and G. Lauvau (2005). "Distinct in vivo dendritic cell activation by live versus killed Listeria monocytogenes." Eur J Immunol 35(5): 1463-1471.

Muraille, E., E. Narni-Mancinelli, P. Gounon, D. Bassand, N. Glaichenhaus, L. L. Lenz and G. Lauvau (2007). "Cytosolic expression of SecA2 is a prerequisite for long-term protective immunity." Cell Microbiol 9(6): 1445-1454.

Murali-Krishna, K., J. D. Altman, M. Suresh, D. J. Sourdive, A. J. Zajac, J. D. Miller, J. Slansky and R. Ahmed (1998). "Counting antigen-specific CD8 T cells: a reevaluation of bystander activation during viral infection." Immunity 8(2): 177-187.

Murphy, M. A., R. G. Schnall, D. J. Venter, L. Barnett, I. Bertoncello, C. B. Thien, W. Y. Langdon and D. D. Bowtell (1998). "Tissue hyperplasia and enhanced T-cell signalling via ZAP-70 in c-Cbl-deficient mice." Mol Cell Biol 18(8): 4872-4882.

Murray, P. J. and T. A. Wynn (2011). "Protective and pathogenic functions of macrophage subsets." Nat Rev Immunol 11(11): 723-737.

Nansen, A., T. Jensen, J. P. Christensen, S. O. Andreasen, C. Ropke, O. Marker and A. R. Thomsen (1999). "Compromised virus control and augmented perforin-mediated immunopathology in IFN-gamma-deficient mice infected with lymphocytic choriomeningitis virus." J Immunol 163(11): 6114-6122.

Naramura, M., I. K. Jang, H. Kole, F. Huang, D. Haines and H. Gu (2002). "c-Cbl and Cbl-b regulate T cell responsiveness by promoting ligand-induced TCR down-modulation." Nat Immunol 3(12): 1192-1199.

Narayan, P., B. Holt, R. Tosti and L. P. Kane (2006). "CARMA1 is required for Akt-mediated NF-kappaB activation in T cells." Mol Cell Biol 26(6): 2327-2336.

Narni-Mancinelli, E., L. Campisi, D. Bassand, J. Cazareth, P. Gounon, N. Glaichenhaus and G. Lauvau (2007). "Memory CD8+ T cells mediate antibacterial immunity via CCL3 activation of TNF/ROI+ phagocytes." J Exp Med 204(9): 2075-2087.

Nathan, C. (2002). "Points of control in inflammation." Nature 420(6917): 846-852.

Nembrini, C., B. Abel, M. Kopf and B. J. Marsland (2006). "Strong TCR signaling, TLR ligands, and cytokine redundancies ensure robust development of type 1 effector T cells." J Immunol 176(12): 7180-7188.

Newton, K. and V. M. Dixit (2012). "Signaling in innate immunity and inflammation." Cold Spring Harb Perspect Biol 4(3).

Nganou-Makamdop, K. and R. W. Sauerwein (2013). "Liver or blood-stage arrest during malaria sporozoite immunization: the later the better?" Trends Parasitol 29(6): 304-310.

Nolz, J. C. and J. T. Harty (2011). "Protective capacity of memory CD8+ T cells is dictated by antigen exposure history and nature of the infection." Immunity 34(5): 781-793.

Nolz, J. C., G. R. Starbeck-Miller and J. T. Harty (2011). "Naive, effector and memory CD8 T-cell trafficking: parallels and distinctions." Immunotherapy 3(10): 1223-1233.

Nurieva, R., X. O. Yang, G. Martinez, Y. Zhang, A. D. Panopoulos, L. Ma, K. Schluns, Q. Tian, S. S. Watowich, A. M. Jetten and C. Dong (2007). "Essential autocrine regulation by IL-21 in the generation of inflammatory T cells." Nature 448(7152): 480-483.

Nurieva, R. I., Y. Chung, G. J. Martinez, X. O. Yang, S. Tanaka, T. D. Matskevitch, Y. H. Wang and C. Dong (2009). "Bcl6 mediates the development of T follicular helper cells." Science 325(5943): 1001-1005.

Nutt, S. L., K. A. Fairfax and A. Kallies (2007). "BLIMP1 guides the fate of effector B and T cells." Nat Rev Immunol 7(12): 923-927.

O'Hayre, M., C. L. Salanga, T. M. Handel and S. J. Allen (2008). "Chemokines and cancer: migration, intracellular signalling and intercellular communication in the microenvironment." Biochem J 409(3): 635-649.

Obar, J. J., K. M. Khanna and L. Lefrancois (2008). "Endogenous naive CD8+ T cell precursor frequency regulates primary and memory responses to infection." Immunity 28(6): 859-869.

Olivera, A., K. Mizugishi, A. Tikhonova, L. Ciaccia, S. Odom, R. L. Proia and J. Rivera (2007). "The sphingosine kinase-sphingosine-1-phosphate axis is a determinant of mast cell function and anaphylaxis." Immunity 26(3): 287-297.

Orange, J. S. and C. A. Biron (1996). "An absolute and restricted requirement for IL-12 in natural killer cell IFN-gamma production and antiviral defense. Studies of natural killer and T cell responses in contrasting viral infections." J Immunol 156(3): 1138-1142.

Ouyang, W., J. K. Kolls and Y. Zheng (2008). "The biological functions of T helper 17 cell effector cytokines in inflammation." Immunity 28(4): 454-467.

Pamer, E. G. (2004). "Immune responses to Listeria monocytogenes." Nat Rev Immunol 4(10): 812-823.
Panus, J. F., L. J. McHeyzer-Williams and M. G. McHeyzer-Williams (2000). "Antigen-specific T helper cell function: differential cytokine expression in primary and memory responses." J Exp Med 192(9): 1301-1316.
Pappu, R., S. R. Schwab, I. Cornelissen, J. P. Pereira, J. B. Regard, Y. Xu, E. Camerer, Y. W. Zheng, Y. Huang, J. G. Cyster and S. R. Coughlin (2007). "Promotion of lymphocyte egress into blood and lymph by distinct sources of sphingosine-1-phosphate." Science 316(5822): 295-298.
Park, B. S., D. H. Song, H. M. Kim, B. S. Choi, H. Lee and J. O. Lee (2009). "The structural basis of lipopolysaccharide recognition by the TLR4-MD-2 complex." Nature 458(7242): 1191-1195.
Park, J. H., Y. G. Kim, C. McDonald, T. D. Kanneganti, M. Hasegawa, M. Body-Malapel, N. Inohara and G. Nunez (2007). "RICK/RIP2 mediates innate immune responses induced through Nod1 and Nod2 but not TLRs." J Immunol 178(4): 2380-2386.
Parmigiani, A., M. F. Pallin, H. Schmidtmayerova, M. G. Lichtenheld and S. Pahwa (2011). "Interleukin-21 and cellular activation concurrently induce potent cytotoxic function and promote antiviral activity in human CD8 T cells." Hum Immunol 72(2): 115-123.
Pearce, E. L., A. C. Mullen, G. A. Martins, C. M. Krawczyk, A. S. Hutchins, V. P. Zediak, M. Banica, C. B. DiCioccio, D. A. Gross, C. A. Mao, H. Shen, N. Cereb, S. Y. Yang, T. Lindsten, J. Rossant, C. A. Hunter and S. L. Reiner (2003). "Control of effector CD8+ T cell function by the transcription factor Eomesodermin." Science 302(5647): 1041-1043.
Pearce, E. L., M. C. Walsh, P. J. Cejas, G. M. Harms, H. Shen, L. S. Wang, R. G. Jones and Y. Choi (2009). "Enhancing CD8 T-cell memory by modulating fatty acid metabolism." Nature 460(7251): 103-107.
Peaudecerf, L., P. R. dos Santos, A. Boudil, S. Ezine, N. Pardigon and B. Rocha (2011). "The role of the gut as a primary lymphoid organ: CD8alphaalpha intraepithelial T lymphocytes in euthymic mice derive from very immature CD44+ thymocyte precursors." Mucosal Immunol 4(1): 93-101.
Peixoto, A., C. Evaristo, I. Munitic, M. Monteiro, A. Charbit, B. Rocha and H. Veiga-Fernandes (2007). "CD8 single-cell gene coexpression reveals three different effector types present at distinct phases of the immune response." J Exp Med 204(5): 1193-1205.
Peixoto, A., M. Monteiro, B. Rocha and H. Veiga-Fernandes (2004). "Quantification of multiple gene expression in individual cells." Genome Res 14(10A): 1938-1947.
Peschon, J. J., P. J. Morrissey, K. H. Grabstein, F. J. Ramsdell, E. Maraskovsky, B. C. Gliniak, L. S. Park, S. F. Ziegler, D. E. Williams, C. B. Ware, J. D. Meyer and B. L. Davison (1994). "Early lymphocyte expansion is severely impaired in interleukin 7 receptor-deficient mice." J Exp Med 180(5): 1955-1960.
Peters, N. C., J. G. Egen, N. Secundino, A. Debrabant, N. Kimblin, S. Kamhawi, P. Lawyer, M. P. Fay, R. N. Germain and D. Sacks (2008). "In vivo imaging reveals an essential role for neutrophils in leishmaniasis transmitted by sand flies." Science 321(5891): 970-974.
Pham, T. H., P. Baluk, Y. Xu, I. Grigorova, A. J. Bankovich, R. Pappu, S. R. Coughlin, D. M. McDonald, S. R. Schwab and J. G. Cyster (2010). "Lymphatic endothelial cell sphingosine kinase activity is required for lymphocyte egress and lymphatic patterning." J Exp Med 207(1): 17-27.
Pham, T. H., T. Okada, M. Matloubian, C. G. Lo and J. G. Cyster (2008). "S1P1 receptor signaling overrides retention mediated by G alpha i-coupled receptors to promote T cell egress." Immunity 28(1): 122-133.
Pipkin, M. E., J. A. Sacks, F. Cruz-Guilloty, M. G. Lichtenheld, M. J. Bevan and A. Rao (2010). "Interleukin-2 and inflammation induce distinct transcriptional programs that promote the differentiation of effector cytolytic T cells." Immunity 32(1): 79-90.
Pircher, H., K. Burki, R. Lang, H. Hengartner and R. M. Zinkernagel (1989). "Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen." Nature 342(6249): 559-561.
Porritt, H. E., L. L. Rumfelt, S. Tabrizifard, T. M. Schmitt, J. C. Zuniga-Pflucker and H. T. Petrie (2004). "Heterogeneity among DN1 prothymocytes reveals multiple progenitors with different capacities to generate T cell and non-T cell lineages." Immunity 20(6): 735-745.
Portnoy, D. A., R. D. Schreiber, P. Connelly and L. G. Tilney (1989). "Gamma interferon limits access of Listeria monocytogenes to the macrophage cytoplasm." J Exp Med 170(6): 2141-2146.
Powell, J. D., K. N. Pollizzi, E. B. Heikamp and M. R. Horton (2012). "Regulation of immune responses by mTOR." Annu Rev Immunol 30: 39-68.
Preece, G., G. Murphy and A. Ager (1996). "Metalloproteinase-mediated regulation of L-selectin levels on leucocytes." J Biol Chem 271(20): 11634-11640.
Puga, I., M. Cols, C. M. Barra, B. He, L. Cassis, M. Gentile, L. Comerma, A. Chorny, M. Shan, W. Xu, G. Magri, D. M. Knowles, W. Tam, A. Chiu, J. B. Bussel, S. Serrano, J. A. Lorente, B. Bellosillo, J. Lloreta, N. Juanpere, F. Alameda, T. Baro, C. D. de Heredia, N. Toran, A. Catala, M. Torrebadell, C. Fortuny, V. Cusi, C. Carreras, G. A. Diaz, J. M. Blander, C. M. Farber, G. Silvestri, C. Cunningham-Rundles, M. Calvillo, C. Dufour, L. D. Notarangelo, V. Lougaris, A. Plebani, J. L. Casanova, S. C. Ganal, A. Diefenbach, J. I. Arostegui, M. Juan, J. Yague, N. Mahlaoui, J. Donadieu, K. Chen and A. Cerutti (2012). "B cell-helper neutrophils stimulate the

diversification and production of immunoglobulin in the marginal zone of the spleen." Nat Immunol 13(2): 170-180.
Pulendran, B., K. Palucka and J. Banchereau (2001). "Sensing pathogens and tuning immune responses." Science 293(5528): 253-256.
Puneet, P., C. T. Yap, L. Wong, Y. Lam, D. R. Koh, S. Moochhala, J. Pfeilschifter, A. Huwiler and A. J. Melendez (2010). "SphK1 regulates proinflammatory responses associated with endotoxin and polymicrobial sepsis." Science 328(5983): 1290-1294.
Rahman, A. H., W. Cui, D. F. Larosa, D. K. Taylor, J. Zhang, D. R. Goldstein, E. J. Wherry, S. M. Kaech and L. A. Turka (2008). "MyD88 plays a critical T cell-intrinsic role in supporting CD8 T cell expansion during acute lymphocytic choriomeningitis virus infection." J Immunol 181(6): 3804-3810.
Rao, R. R., Q. Li, M. R. Gubbels Bupp and P. A. Shrikant (2012). "Transcription factor Foxo1 represses T-bet-mediated effector functions and promotes memory CD8(+) T cell differentiation." Immunity 36(3): 374-387.
Rao, R. R., Q. Li, K. Odunsi and P. A. Shrikant (2010). "The mTOR kinase determines effector versus memory CD8+ T cell fate by regulating the expression of transcription factors T-bet and Eomesodermin." Immunity 32(1): 67-78.
Rapetti, L., S. Meunier, C. Pontoux and C. Tanchot (2008). "CD4 help regulates expression of crucial genes involved in CD8 T cell memory and sensitivity to regulatory elements." J Immunol 181(1): 299-308.
Ribeiro-dos-Santos, P., E. L. Turnbull, M. Monteiro, A. Legrand, K. Conrod, J. Baalwa, P. Pellegrino, G. M. Shaw, I. Williams, P. Borrow and B. Rocha (2012). "Chronic HIV infection affects the expression of the 2 transcription factors required for CD8 T-cell differentiation into cytolytic effectors." Blood 119(21): 4928-4938.
Rincon, M. and R. J. Davis (2009). "Regulation of the immune response by stress-activated protein kinases." Immunol Rev 228(1): 212-224.
Rivera, J., R. L. Proia and A. Olivera (2008). "The alliance of sphingosine-1-phosphate and its receptors in immunity." Nat Rev Immunol 8(10): 753-763.
Robbins, S. H., S. C. Terrizzi, B. C. Sydora, T. Mikayama and L. Brossay (2003). "Differential regulation of killer cell lectin-like receptor G1 expression on T cells." J Immunol 170(12): 5876-5885.
Rocha, B. and C. Tanchot (2004a). "CD8 T cell memory." Semin Immunol 16(5): 305-314.
Rocha, B. and C. Tanchot (2004b). "Towards a cellular definition of CD8+ T-cell memory: the role of CD4+ T-cell help in CD8+ T-cell responses." Curr Opin Immunol 16(3): 259-263.
Rocha, B., C. Tanchot and H. Von Boehmer (1993). "Clonal anergy blocks in vivo growth of mature T cells and can be reversed in the absence of antigen." J Exp Med 177(5): 1517-1521.
Rocha, B. and H. von Boehmer (1991). "Peripheral selection of the T cell repertoire." Science 251(4998): 1225-1228.
Roost, H., S. Charan, R. Gobet, E. Ruedi, H. Hengartner, A. Althage and R. M. Zinkernagel (1988). "An acquired immune suppression in mice caused by infection with lymphocytic choriomeningitis virus." Eur J Immunol 18(4): 511-518.
Rudd, C. E. (2005). "MAPK p38: alternative and nonstressful in T cells." Nat Immunol 6(4): 368-370.
Russell, J. H. and T. J. Ley (2002). "Lymphocyte-mediated cytotoxicity." Annu Rev Immunol 20: 323-370.
Rutishauser, R. L., G. A. Martins, S. Kalachikov, A. Chandele, I. A. Parish, E. Meffre, J. Jacob, K. Calame and S. M. Kaech (2009). "Transcriptional repressor Blimp-1 promotes CD8(+) T cell terminal differentiation and represses the acquisition of central memory T cell properties." Immunity 31(2): 296-308.
Sabbah, A., T. H. Chang, R. Harnack, V. Frohlich, K. Tominaga, P. H. Dube, Y. Xiang and S. Bose (2009). "Activation of innate immune antiviral responses by Nod2." Nat Immunol 10(10): 1073-1080.
Sabroe, I., S. K. Dower and M. K. Whyte (2005). "The role of Toll-like receptors in the regulation of neutrophil migration, activation, and apoptosis." Clin Infect Dis 41 Suppl 7: S421-426.
Sallusto, F. and A. Lanzavecchia (2009). "Heterogeneity of CD4+ memory T cells: functional modules for tailored immunity." Eur J Immunol 39(8): 2076-2082.
Sallusto, F., D. Lenig, R. Forster, M. Lipp and A. Lanzavecchia (1999). "Two subsets of memory T lymphocytes with distinct homing potentials and effector functions." Nature 401(6754): 708-712.
Salvador, J. M., P. R. Mittelstadt, T. Guszczynski, T. D. Copeland, H. Yamaguchi, E. Appella, A. J. Fornace, Jr. and J. D. Ashwell (2005). "Alternative p38 activation pathway mediated by T cell receptor-proximal tyrosine kinases." Nat Immunol 6(4): 390-395.
San Jose, E., A. Borroto, F. Niedergang, A. Alcover and B. Alarcon (2000). "Triggering the TCR complex causes the downregulation of nonengaged receptors by a signal transduction-dependent mechanism." Immunity 12(2): 161-170.
Sanchez, T. and T. Hla (2004). "Structural and functional characteristics of S1P receptors." J Cell Biochem 92(5): 913-922.
Sancho, D., O. P. Joffre, A. M. Keller, N. C. Rogers, D. Martinez, P. Hernanz-Falcon, I. Rosewell and C. Reis e Sousa (2009). "Identification of a dendritic cell receptor that couples sensing of necrosis to immunity." Nature 458(7240): 899-903.

Sarkar, S., V. Kalia, W. N. Haining, B. T. Konieczny, S. Subramaniam and R. Ahmed (2008). "Functional and genomic profiling of effector CD8 T cell subsets with distinct memory fates." J Exp Med 205(3): 625-640.

Sarkar, S., V. Teichgraber, V. Kalia, A. Polley, D. Masopust, L. E. Harrington, R. Ahmed and E. J. Wherry (2007). "Strength of stimulus and clonal competition impact the rate of memory CD8 T cell differentiation." J Immunol 179(10): 6704-6714.

Savignac, M., B. Mellstrom and J. R. Naranjo (2007). "Calcium-dependent transcription of cytokine genes in T lymphocytes." Pflugers Arch 454(4): 523-533.

Scapini, P., C. Laudanna, C. Pinardi, P. Allavena, A. Mantovani, S. Sozzani and M. A. Cassatella (2001). "Neutrophils produce biologically active macrophage inflammatory protein-3alpha (MIP-3alpha)/CCL20 and MIP-3beta/CCL19." Eur J Immunol 31(7): 1981-1988.

Schluns, K. S., W. C. Kieper, S. C. Jameson and L. Lefrancois (2000). "Interleukin-7 mediates the homeostasis of naive and memory CD8 T cells in vivo." Nat Immunol 1(5): 426-432.

Schoenberger, S. P. (2003). "BLT for speed." Nat Immunol 4(10): 937-939.

Schuppler, M. and M. J. Loessner (2010). "The Opportunistic Pathogen Listeria monocytogenes: Pathogenicity and Interaction with the Mucosal Immune System." Int J Inflam 2010: 704321.

Schwab, S. R. and J. G. Cyster (2007). "Finding a way out: lymphocyte egress from lymphoid organs." Nat Immunol 8(12): 1295-1301.

Schwab, S. R., J. P. Pereira, M. Matloubian, Y. Xu, Y. Huang and J. G. Cyster (2005). "Lymphocyte sequestration through S1P lyase inhibition and disruption of S1P gradients." Science 309(5741): 1735-1739.

Segura, E., C. Guerin, N. Hogg, S. Amigorena and C. Thery (2007). "CD8+ dendritic cells use LFA-1 to capture MHC-peptide complexes from exosomes in vivo." J Immunol 179(3): 1489-1496.

Seki, E., H. Tsutsui, N. M. Tsuji, N. Hayashi, K. Adachi, H. Nakano, S. Futatsugi-Yumikura, O. Takeuchi, K. Hoshino, S. Akira, J. Fujimoto and K. Nakanishi (2002). "Critical roles of myeloid differentiation factor 88-dependent proinflammatory cytokine release in early phase clearance of Listeria monocytogenes in mice." J Immunol 169(7): 3863-3868.

Serbina, N. V., T. Jia, T. M. Hohl and E. G. Pamer (2008). "Monocyte-mediated defense against microbial pathogens." Annu Rev Immunol 26: 421-452.

Serbina, N. V. and E. G. Pamer (2008). "Coordinating innate immune cells to optimize microbial killing." Immunity 29(5): 672-674.

Serbina, N. V., T. P. Salazar-Mather, C. A. Biron, W. A. Kuziel and E. G. Pamer (2003). "TNF/iNOS-producing dendritic cells mediate innate immune defense against bacterial infection." Immunity 19(1): 59-70.

Sevilla, N., S. Kunz, A. Holz, H. Lewicki, D. Homann, H. Yamada, K. P. Campbell, J. C. de La Torre and M. B. Oldstone (2000). "Immunosuppression and resultant viral persistence by specific viral targeting of dendritic cells." J Exp Med 192(9): 1249-1260.

Shapiro-Shelef, M., K. I. Lin, L. J. McHeyzer-Williams, J. Liao, M. G. McHeyzer-Williams and K. Calame (2003). "Blimp-1 is required for the formation of immunoglobulin secreting plasma cells and pre-plasma memory B cells." Immunity 19(4): 607-620.

Sharpe, A. H. and G. J. Freeman (2002). "The B7-CD28 superfamily." Nat Rev Immunol 2(2): 116-126.

Shaw, M. H., T. Reimer, C. Sanchez-Valdepenas, N. Warner, Y. G. Kim, M. Fresno and G. Nunez (2009). "T cell-intrinsic role of Nod2 in promoting type 1 immunity to Toxoplasma gondii." Nat Immunol 10(12): 1267-1274.

Shedlock, D. J. and H. Shen (2003). "Requirement for CD4 T cell help in generating functional CD8 T cell memory." Science 300(5617): 337-339.

Shen, H., J. K. Whitmire, X. Fan, D. J. Shedlock, S. M. Kaech and R. Ahmed (2003). "A specific role for B cells in the generation of CD8 T cell memory by recombinant Listeria monocytogenes." J Immunol 170(3): 1443-1451.

Shen, Y., M. Naujokas, M. Park and K. Ireton (2000). "InIB-dependent internalization of Listeria is mediated by the Met receptor tyrosine kinase." Cell 103(3): 501-510.

Sheppard, K. A., L. J. Fitz, J. M. Lee, C. Benander, J. A. George, J. Wooters, Y. Qiu, J. M. Jussif, L. L. Carter, C. R. Wood and D. Chaudhary (2004). "PD-1 inhibits T-cell receptor induced phosphorylation of the ZAP70/CD3zeta signalosome and downstream signaling to PKCtheta." FEBS Lett 574(1-3): 37-41.

Sheridan, B. S. and L. Lefrancois (2011). "Regional and mucosal memory T cells." Nat Immunol 12(6): 485-491.

Shi, C., T. Jia, S. Mendez-Ferrer, T. M. Hohl, N. V. Serbina, L. Lipuma, I. Leiner, M. O. Li, P. S. Frenette and E. G. Pamer (2011). "Bone marrow mesenchymal stem and progenitor cells induce monocyte emigration in response to circulating toll-like receptor ligands." Immunity 34(4): 590-601.

Shi, C. and E. G. Pamer (2011). "Monocyte recruitment during infection and inflammation." Nat Rev Immunol 11(11): 762-774.

Shinkai, Y., S. Koyasu, K. Nakayama, K. M. Murphy, D. Y. Loh, E. L. Reinherz and F. W. Alt (1993). "Restoration of T cell development in RAG-2-deficient mice by functional TCR transgenes." Science 259(5096): 822-825.

Shinkai, Y., G. Rathbun, K. P. Lam, E. M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A. M. Stall and et al. (1992). "RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement." Cell 68(5): 855-867.

Shiow, L. R., D. B. Rosen, N. Brdickova, Y. Xu, J. An, L. L. Lanier, J. G. Cyster and M. Matloubian (2006). "CD69 acts downstream of interferon-alpha/beta to inhibit S1P1 and lymphocyte egress from lymphoid organs." Nature 440(7083): 540-544.

Shortman, K. and S. H. Naik (2007). "Steady-state and inflammatory dendritic-cell development." Nat Rev Immunol 7(1): 19-30.

Slifka, M. K. and J. L. Whitton (2001). "Functional avidity maturation of CD8(+) T cells without selection of higher affinity TCR." Nat Immunol 2(8): 711-717.

Smith-Garvin, J. E., G. A. Koretzky and M. S. Jordan (2009). "T cell activation." Annu Rev Immunol 27: 591-619.

Soehnlein, O. and L. Lindbom (2010). "Phagocyte partnership during the onset and resolution of inflammation." Nat Rev Immunol 10(6): 427-439.

Sommers, C. L., L. E. Samelson and P. E. Love (2004). "LAT: a T lymphocyte adapter protein that couples the antigen receptor to downstream signaling pathways." Bioessays 26(1): 61-67.

Sondergaard, H., J. M. Coquet, A. P. Uldrich, N. McLaughlin, D. I. Godfrey, P. V. Sivakumar, K. Skak and M. J. Smyth (2009). "Endogenous IL-21 restricts CD8+ T cell expansion and is not required for tumor immunity." J Immunol 183(11): 7326-7336.

Spiegel, S. and S. Milstien (2003). "Sphingosine-1-phosphate: an enigmatic signalling lipid." Nat Rev Mol Cell Biol 4(5): 397-407.

Spiegel, S. and S. Milstien (2011). "The outs and the ins of sphingosine-1-phosphate in immunity." Nat Rev Immunol 11(6): 403-415.

Spolski, R., H. P. Kim, W. Zhu, D. E. Levy and W. J. Leonard (2009). "IL-21 mediates suppressive effects via its induction of IL-10." J Immunol 182(5): 2859-2867.

Sponaas, A. M., E. T. Cadman, C. Voisine, V. Harrison, A. Boonstra, A. O'Garra and J. Langhorne (2006). "Malaria infection changes the ability of splenic dendritic cell populations to stimulate antigen-specific T cells." J Exp Med 203(6): 1427-1433.

Sporri, R., N. Joller, H. Hilbi and A. Oxenius (2008). "A novel role for neutrophils as critical activators of NK cells." J Immunol 181(10): 7121-7130.

Sprent, J. and J. F. Miller (1976). "Effect of recent antigen priming on adoptive immune responses. III. Antigen-induced selective recruitment of subsets of recirculating lymphocytes reactive to H-2 determinants." J Exp Med 143(3): 585-600.

Sprent, J., J. F. Miller and G. F. Mitchell (1971). "Antigen-induced selective recruitment of circulating lymphocytes." Cell Immunol 2(2): 171-181.

Sprent, J. and C. D. Surh (2002). "T cell memory." Annu Rev Immunol 20: 551-579.

Spring, M., J. Murphy, R. Nielsen, M. Dowler, J. W. Bennett, S. Zarling, J. Williams, P. de la Vega, L. Ware, J. Komisar, M. Polhemus, T. L. Richie, J. Epstein, C. Tamminga, I. Chuang, N. Richie, M. O'Neil, D. G. Heppner, J. Healer, M. O'Neill, H. Smithers, O. C. Finney, S. A. Mikolajczak, R. Wang, A. Cowman, C. Ockenhouse, U. Krzych and S. H. Kappe (2013). "First-in-human evaluation of genetically attenuated Plasmodium falciparum sporozoites administered by bite of Anopheles mosquitoes to adult volunteers." Vaccine 31(43): 4975-4983.

Stark, M. A., Y. Huo, T. L. Burcin, M. A. Morris, T. S. Olson and K. Ley (2005). "Phagocytosis of apoptotic neutrophils regulates granulopoiesis via IL-23 and IL-17." Immunity 22(3): 285-294.

Starnbach, M. N., M. J. Bevan and M. F. Lampe (1994). "Protective cytotoxic T lymphocytes are induced during murine infection with Chlamydia trachomatis." J Immunol 153(11): 5183-5189.

Stefanova, I., B. Hemmer, M. Vergelli, R. Martin, W. E. Biddison and R. N. Germain (2003). "TCR ligand discrimination is enforced by competing ERK positive and SHP-1 negative feedback pathways." Nat Immunol 4(3): 248-254.

Sun, J. C. and M. J. Bevan (2003). "Defective CD8 T cell memory following acute infection without CD4 T cell help." Science 300(5617): 339-342.

Sun, J. C. and L. L. Lanier (2011). "NK cell development, homeostasis and function: parallels with CD8(+) T cells." Nat Rev Immunol 11(10): 645-657.

Suresh, M., A. Singh and C. Fischer (2005). "Role of tumor necrosis factor receptors in regulating CD8 T-cell responses during acute lymphocytic choriomeningitis virus infection." J Virol 79(1): 202-213.

Surh, C. D., O. Boyman, J. F. Purton and J. Sprent (2006). "Homeostasis of memory T cells." Immunol Rev 211: 154-163.

Suzuki, Y. and J. S. Remington (1990). "The effect of anti-IFN-gamma antibody on the protective effect of Lyt-2+ immune T cells against toxoplasmosis in mice." J Immunol 144(5): 1954-1956.

Szymczak, A. L. and D. A. Vignali (2005). "Plasticity and rigidity in adaptor protein-2-mediated internalization of the TCR:CD3 complex." J Immunol 174(7): 4153-4160.

Tabeta, K., P. Georgel, E. Janssen, X. Du, K. Hoebe, K. Crozat, S. Mudd, L. Shamel, S. Sovath, J. Goode, L. Alexopoulou, R. A. Flavell and B. Beutler (2004). "Toll-like receptors 9 and 3 as essential components of innate immune defense against mouse cytomegalovirus infection." Proc Natl Acad Sci U S A 101(10): 3516-3521.

Taghon, T. N., E. S. David, J. C. Zuniga-Pflucker and E. V. Rothenberg (2005). "Delayed, asynchronous, and reversible T-lineage specification induced by Notch/Delta signaling." Genes Dev 19(8): 965-978.

Takahama, Y. (2006). "Journey through the thymus: stromal guides for T-cell development and selection." Nat Rev Immunol 6(2): 127-135.

Takeda, S., H. R. Rodewald, H. Arakawa, H. Bluethmann and T. Shimizu (1996). "MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span." Immunity 5(3): 217-228.

Takemoto, N., A. M. Intlekofer, J. T. Northrup, E. J. Wherry and S. L. Reiner (2006). "Cutting Edge: IL-12 inversely regulates T-bet and eomesodermin expression during pathogen-induced CD8+ T cell differentiation." J Immunol 177(11): 7515-7519.

Takeuchi, O. and S. Akira (2010). "Pattern recognition receptors and inflammation." Cell 140(6): 805-820.

Tanchot, C., S. Guillaume, J. Delon, C. Bourgeois, A. Franzke, A. Sarukhan, A. Trautmann and B. Rocha (1998). "Modifications of CD8+ T cell function during in vivo memory or tolerance induction." Immunity 8(5): 581-590.

Tanchot, C., F. A. Lemonnier, B. Perarnau, A. A. Freitas and B. Rocha (1997). "Differential requirements for survival and proliferation of CD8 naive or memory T cells." Science 276(5321): 2057-2062.

Tanchot, C. and B. Rocha (2003). "CD8 and B cell memory: same strategy, same signals." Nat Immunol 4(5): 431-432.

Teirlinck, A. C., M. Roestenberg, M. van de Vegte-Bolmer, A. Scholzen, M. J. Heinrichs, R. Siebelink-Stoter, W. Graumans, G. J. van Gemert, K. Teelen, M. W. Vos, K. Nganou-Makamdop, S. Borrmann, Y. P. Rozier, M. A. Erkens, A. J. Luty, C. C. Hermsen, B. K. Sim, L. van Lieshout, S. L. Hoffman, L. G. Visser and R. W. Sauerwein (2013). "NF135.C10: a new Plasmodium falciparum clone for controlled human malaria infections." J Infect Dis 207(4): 656-660.

Testi, R., J. H. Phillips and L. L. Lanier (1989). "Leu 23 induction as an early marker of functional CD3/T cell antigen receptor triggering. Requirement for receptor cross-linking, prolonged elevation of intracellular [Ca++] and stimulation of protein kinase C." J Immunol 142(6): 1854-1860.

Thimme, R., V. Appay, M. Koschella, E. Panther, E. Roth, A. D. Hislop, A. B. Rickinson, S. L. Rowland-Jones, H. E. Blum and H. Pircher (2005). "Increased expression of the NK cell receptor KLRG1 by virus-specific CD8 T cells during persistent antigen stimulation." J Virol 79(18): 12112-12116.

Thompson, C. B. and J. P. Allison (1997). "The emerging role of CTLA-4 as an immune attenuator." Immunity 7(4): 445-450.

Thompson, L. J., G. A. Kolumam, S. Thomas and K. Murali-Krishna (2006). "Innate inflammatory signals induced by various pathogens differentially dictate the IFN-I dependence of CD8 T cells for clonal expansion and memory formation." J Immunol 177(3): 1746-1754.

Tillack, K., P. Breiden, R. Martin and M. Sospedra (2012). "T lymphocyte priming by neutrophil extracellular traps links innate and adaptive immune responses." J Immunol 188(7): 3150-3159.

Toebes, M., M. Coccoris, A. Bins, B. Rodenko, R. Gomez, N. J. Nieuwkoop, W. van de Kasteele, G. F. Rimmelzwaan, J. B. Haanen, H. Ovaa and T. N. Schumacher (2006). "Design and use of conditional MHC class I ligands." Nat Med 12(2): 246-251.

Travassos, L. H., L. A. Carneiro, M. Ramjeet, S. Hussey, Y. G. Kim, J. G. Magalhaes, L. Yuan, F. Soares, E. Chea, L. Le Bourhis, I. G. Boneca, A. Allaoui, N. L. Jones, G. Nunez, S. E. Girardin and D. J. Philpott (2010). "Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane at the site of bacterial entry." Nat Immunol 11(1): 55-62.

Traynor, T. R., W. A. Kuziel, G. B. Toews and G. B. Huffnagle (2000). "CCR2 expression determines T1 versus T2 polarization during pulmonary Cryptococcus neoformans infection." J Immunol 164(4): 2021-2027.

Turner, S. J., N. L. La Gruta, J. Stambas, G. Diaz and P. C. Doherty (2004). "Differential tumor necrosis factor receptor 2-mediated editing of virus-specific CD8+ effector T cells." Proc Natl Acad Sci U S A 101(10): 3545-3550.

Uematsu, S., K. Fujimoto, M. H. Jang, B. G. Yang, Y. J. Jung, M. Nishiyama, S. Sato, T. Tsujimura, M. Yamamoto, Y. Yokota, H. Kiyono, M. Miyasaka, K. J. Ishii and S. Akira (2008). "Regulation of humoral and cellular gut immunity by lamina propria dendritic cells expressing Toll-like receptor 5." Nat Immunol 9(7): 769-776.

Valenzuela, J., C. Schmidt and M. Mescher (2002). "The roles of IL-12 in providing a third signal for clonal expansion of naive CD8 T cells." J Immunol 169(12): 6842-6849.

Valitutti, S., S. Muller, M. Cella, E. Padovan and A. Lanzavecchia (1995). "Serial triggering of many T-cell receptors by a few peptide-MHC complexes." Nature 375(6527): 148-151.

Valitutti, S., S. Muller, M. Salio and A. Lanzavecchia (1997). "Degradation of T cell receptor (TCR)-CD3-zeta complexes after antigenic stimulation." J Exp Med 185(10): 1859-1864.

van der Merwe, P. A., D. L. Bodian, S. Daenke, P. Linsley and S. J. Davis (1997). "CD80 (B7-1) binds both CD28 and CTLA-4 with a low affinity and very fast kinetics." J Exp Med 185(3): 393-403.

van der Merwe, P. A. and O. Dushek (2011). "Mechanisms for T cell receptor triggering." Nat Rev Immunol 11(1): 47-55.

van Faassen, H., M. Saldanha, D. Gilbertson, R. Dudani, L. Krishnan and S. Sad (2005). "Reducing the stimulation of CD8+ T cells during infection with intracellular bacteria promotes differentiation primarily into a central (CD62LhighCD44high) subset." J Immunol 174(9): 5341-5350.

van Gisbergen, K. P., M. Sanchez-Hernandez, T. B. Geijtenbeek and Y. van Kooyk (2005). "Neutrophils mediate immune modulation of dendritic cells through glycosylation-dependent interactions between Mac-1 and DC-SIGN." J Exp Med 201(8): 1281-1292.

Vang, T., H. Abrahamsen, S. Myklebust, J. Enserink, H. Prydz, T. Mustelin, M. Amarzguioui and K. Tasken (2004). "Knockdown of C-terminal Src kinase by siRNA-mediated RNA interference augments T cell receptor signaling in mature T cells." Eur J Immunol 34(8): 2191-2199.

Veiga-Fernandes, H. and B. Rocha (2004). "High expression of active CDK6 in the cytoplasm of CD8 memory cells favors rapid division." Nat Immunol 5(1): 31-37.

Veiga-Fernandes, H., U. Walter, C. Bourgeois, A. McLean and B. Rocha (2000). "Response of naive and memory CD8+ T cells to antigen stimulation in vivo." Nat Immunol 1(1): 47-53.

Veldhoen, M., R. J. Hocking, C. J. Atkins, R. M. Locksley and B. Stockinger (2006). "TGFbeta in the context of an inflammatory cytokine milieu supports de novo differentiation of IL-17-producing T cells." Immunity 24(2): 179-189.

Venkataraman, K., Y. M. Lee, J. Michaud, S. Thangada, Y. Ai, H. L. Bonkovsky, N. S. Parikh, C. Habrukowich and T. Hla (2008). "Vascular endothelium as a contributor of plasma sphingosine 1-phosphate." Circ Res 102(6): 669-676.

Villadangos, J. A. and P. Schnorrer (2007). "Intrinsic and cooperative antigen-presenting functions of dendritic-cell subsets in vivo." Nat Rev Immunol 7(7): 543-555.

Vinuesa, C. G. and P. P. Chang (2013). "Innate B cell helpers reveal novel types of antibody responses." Nat Immunol 14(2): 119-126.

Viola, A. and A. Lanzavecchia (1996). "T cell activation determined by T cell receptor number and tunable thresholds." Science 273(5271): 104-106.

Virgin, H. W., E. J. Wherry and R. Ahmed (2009). "Redefining chronic viral infection." Cell 138(1): 30-50.

von Andrian, U. H. and C. R. Mackay (2000). "T-cell function and migration. Two sides of the same coin." N Engl J Med 343(14): 1020-1034.

von Andrian, U. H. and T. R. Mempel (2003). "Homing and cellular traffic in lymph nodes." Nat Rev Immunol 3(11): 867-878.

von Boehmer, H., I. Aifantis, O. Azogui, J. Feinberg, C. Saint-Ruf, C. Zober, C. Garcia and J. Buer (1998). "Crucial function of the pre-T-cell receptor (TCR) in TCR beta selection, TCR beta allelic exclusion and alpha beta versus gamma delta lineage commitment." Immunol Rev 165: 111-119.

von Essen, M., C. M. Bonefeld, V. Siersma, A. B. Rasmussen, J. P. Lauritsen, B. L. Nielsen and C. Geisler (2004). "Constitutive and ligand-induced TCR degradation." J Immunol 173(1): 384-393.

Walsh, C. M., M. Matloubian, C. C. Liu, R. Ueda, C. G. Kurahara, J. L. Christensen, M. T. Huang, J. D. Young, R. Ahmed and W. R. Clark (1994). "Immune function in mice lacking the perforin gene." Proc Natl Acad Sci U S A 91(23): 10854-10858.

Wang, J., R. Alvarez, G. Roderiquez, E. Guan, Q. Caldwell, M. Phelan and M. A. Norcross (2005). "CpG-independent synergistic induction of beta-chemokines and a dendritic cell phenotype by orthophosphorothioate oligodeoxynucleotides and granulocyte-macrophage colony-stimulating factor in elutriated human primary monocytes." J Immunol 174(10): 6113-6121.

Wang, J. H. and E. L. Reinherz (2012). "The structural basis of alphabeta T-lineage immune recognition: TCR docking topologies, mechanotransduction, and co-receptor function." Immunol Rev 250(1): 102-119.

Wang, T., H. Dai, N. Wan, Y. Moore and Z. Dai (2008). "The role for monocyte chemoattractant protein-1 in the generation and function of memory CD8+ T cells." J Immunol 180(5): 2886-2893.

Watts, T. H. (2005). "TNF/TNFR family members in costimulation of T cell responses." Annu Rev Immunol 23: 23-68.

Wegener, A. M., F. Letourneur, A. Hoeveler, T. Brocker, F. Luton and B. Malissen (1992). "The T cell receptor/CD3 complex is composed of at least two autonomous transduction modules." Cell 68(1): 83-95.

Wei, L., A. Laurence, K. M. Elias and J. J. O'Shea (2007). "IL-21 is produced by Th17 cells and drives IL-17 production in a STAT3-dependent manner." J Biol Chem 282(48): 34605-34610.

Weinreich, M. A. and K. A. Hogquist (2008). "Thymic emigration: when and how T cells leave home." J Immunol 181(4): 2265-2270.

Weiss, A. and D. R. Littman (1994). "Signal transduction by lymphocyte antigen receptors." Cell 76(2): 263-274.

Weiss, A. and J. D. Stobo (1984). "Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line." J Exp Med 160(5): 1284-1299.

Weiss, W. R., J. A. Berzofsky, R. A. Houghten, M. Sedegah, M. Hollindale and S. L. Hoffman (1992). "A T cell clone directed at the circumsporozoite protein which protects mice against both Plasmodium yoelii and Plasmodium berghei." J Immunol 149(6): 2103-2109.

Weissman, A. M., M. Baniyash, D. Hou, L. E. Samelson, W. H. Burgess and R. D. Klausner (1988). "Molecular cloning of the zeta chain of the T cell antigen receptor." Science 239(4843): 1018-1021.

Weissman, I. L. and J. A. Shizuru (2008). "The origins of the identification and isolation of hematopoietic stem cells, and their capability to induce donor-specific transplantation tolerance and treat autoimmune diseases." Blood 112(9): 3543-3553.

West, A. P., I. E. Brodsky, C. Rahner, D. K. Woo, H. Erdjument-Bromage, P. Tempst, M. C. Walsh, Y. Choi, G. S. Shadel and S. Ghosh (2011). "TLR signalling augments macrophage bactericidal activity through mitochondrial ROS." Nature 472(7344): 476-480.

Wherry, E. J., S. J. Ha, S. M. Kaech, W. N. Haining, S. Sarkar, V. Kalia, S. Subramaniam, J. N. Blattman, D. L. Barber and R. Ahmed (2007). "Molecular signature of CD8+ T cell exhaustion during chronic viral infection." Immunity 27(4): 670-684.

Wherry, E. J., V. Teichgraber, T. C. Becker, D. Masopust, S. M. Kaech, R. Antia, U. H. von Andrian and R. Ahmed (2003). "Lineage relationship and protective immunity of memory CD8 T cell subsets." Nat Immunol 4(3): 225-234.

White, D. W. and J. T. Harty (1998). "Perforin-deficient CD8+ T cells provide immunity to Listeria monocytogenes by a mechanism that is independent of CD95 and IFN-gamma but requires TNF-alpha." J Immunol 160(2): 898-905.

White, D. W., A. MacNeil, D. H. Busch, I. M. Pilip, E. G. Pamer and J. T. Harty (1999). "Perforin-deficient CD8+ T cells: in vivo priming and antigen-specific immunity against Listeria monocytogenes." J Immunol 162(2): 980-988.

Wiesel, M., J. Crouse, G. Bedenikovic, A. Sutherland, N. Joller and A. Oxenius (2012). "Type-I IFN drives the differentiation of short-lived effector CD8+ T cells in vivo." Eur J Immunol 42(2): 320-329.

Williams, M. A. and M. J. Bevan (2007). "Effector and memory CTL differentiation." Annu Rev Immunol 25: 171-192.

Wirth, T. C., N. L. Pham, J. T. Harty and V. P. Badovinac (2009). "High initial frequency of TCR-transgenic CD8 T cells alters inflammation and pathogen clearance without affecting memory T cell function." Mol Immunol 47(1): 71-78.

Witte, C. E., K. A. Archer, C. S. Rae, J. D. Sauer, J. J. Woodward and D. A. Portnoy (2012). "Innate immune pathways triggered by Listeria monocytogenes and their role in the induction of cell-mediated immunity." Adv Immunol 113: 135-156.

Wong, P. and E. G. Pamer (2003). "CD8 T cell responses to infectious pathogens." Annu Rev Immunol 21: 29-70.

Wooldridge, L., A. Lissina, D. K. Cole, H. A. van den Berg, D. A. Price and A. K. Sewell (2009). "Tricks with tetramers: how to get the most from multimeric peptide-MHC." Immunology 126(2): 147-164.

Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A. D. Lapan, J. C. Stroud, D. L. Bates, L. Guo, A. Han, S. F. Ziegler, D. Mathis, C. Benoist, L. Chen and A. Rao (2006). "FOXP3 controls regulatory T cell function through cooperation with NFAT." Cell 126(2): 375-387.

Xiao, Z., M. F. Mescher and S. C. Jameson (2007). "Detuning CD8 T cells: down-regulation of CD8 expression, tetramer binding, and response during CTL activation." J Exp Med 204(11): 2667-2677.

Xu, C., E. Gagnon, M. E. Call, J. R. Schnell, C. D. Schwieters, C. V. Carman, J. J. Chou and K. W. Wucherpfennig (2008). "Regulation of T cell receptor activation by dynamic membrane binding of the CD3epsilon cytoplasmic tyrosine-based motif." Cell 135(4): 702-713.

Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda and S. Akira (2003). "Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway." Science 301(5633): 640-643.

Yamane, H. and W. E. Paul (2013). "Early signaling events that underlie fate decisions of naive CD4(+) T cells toward distinct T-helper cell subsets." Immunol Rev 252(1): 12-23.

Yarovinsky, F., H. Kanzler, S. Hieny, R. L. Coffman and A. Sher (2006). "Toll-like receptor recognition regulates immunodominance in an antimicrobial CD4+ T cell response." Immunity 25(4): 655-664.

Yarovinsky, F., D. Zhang, J. F. Andersen, G. L. Bannenberg, C. N. Serhan, M. S. Hayden, S. Hieny, F. S. Sutterwala, R. A. Flavell, S. Ghosh and A. Sher (2005). "TLR11 activation of dendritic cells by a protozoan profilin-like protein." Science 308(5728): 1626-1629.

Yatomi, Y., Y. Ozaki, T. Ohmori and Y. Igarashi (2001). "Sphingosine 1-phosphate: synthesis and release." Prostaglandins 64(1-4): 107-122.

Yewdell, J. W. and J. R. Bennink (1999). "Immunodominance in major histocompatibility complex class I-restricted T lymphocyte responses." Annu Rev Immunol 17: 51-88.

Yi, J. S., M. A. Cox and A. J. Zajac (2010a). "Interleukin-21: a multifunctional regulator of immunity to infections." Microbes Infect 12(14-15): 1111-1119.

Yi, J. S., M. A. Cox and A. J. Zajac (2010b). "T-cell exhaustion: characteristics, causes and conversion." Immunology 129(4): 474-481.

Yi, J. S., M. Du and A. J. Zajac (2009). "A vital role for interleukin-21 in the control of a chronic viral infection." Science 324(5934): 1572-1576.

Yi, J. S., J. T. Ingram and A. J. Zajac (2010c). "IL-21 deficiency influences CD8 T cell quality and recall responses following an acute viral infection." J Immunol 185(8): 4835-4845.

Yin, J. and T. A. Ferguson (2009). "Identification of an IFN-gamma-producing neutrophil early in the response to Listeria monocytogenes." J Immunol 182(11): 7069-7073.

Yoshida, K., A. Sakamoto, K. Yamashita, E. Arguni, S. Horigome, M. Arima, M. Hatano, N. Seki, T. Ichikawa and T. Tokuhisa (2006). "Bcl6 controls granzyme B expression in effector CD8+ T cells." Eur J Immunol 36(12): 3146-3156.

Yu, D., S. Rao, L. M. Tsai, S. K. Lee, Y. He, E. L. Sutcliffe, M. Srivastava, M. Linterman, L. Zheng, N. Simpson, J. I. Ellyard, I. A. Parish, C. S. Ma, Q. J. Li, C. R. Parish, C. R. Mackay and C. G. Vinuesa (2009). "The transcriptional repressor Bcl-6 directs T follicular helper cell lineage commitment." Immunity 31(3): 457-468.

Yuseff, M. I., P. Pierobon, A. Reversat and A. M. Lennon-Dumenil (2013). "How B cells capture, process and present antigens: a crucial role for cell polarity." Nat Rev Immunol 13(7): 475-486.

Zatz, M. M. and E. M. Lance (1971). "The distribution of 51Cr-labeled lymphocytes into antigen-stimulated mice. Lymphocyte trapping." J Exp Med 134(1): 224-241.

Zehn, D., C. King, M. J. Bevan and E. Palmer (2012). "TCR signaling requirements for activating T cells and for generating memory." Cell Mol Life Sci 69(10): 1565-1575.

Zeng, R., R. Spolski, S. E. Finkelstein, S. Oh, P. E. Kovanen, C. S. Hinrichs, C. A. Pise-Masison, M. F. Radonovich, J. N. Brady, N. P. Restifo, J. A. Berzofsky and W. J. Leonard (2005). "Synergy of IL-21 and IL-15 in regulating CD8+ T cell expansion and function." J Exp Med 201(1): 139-148.

Zeremski, M., L. M. Petrovic and A. H. Talal (2007). "The role of chemokines as inflammatory mediators in chronic hepatitis C virus infection." J Viral Hepat 14(10): 675-687.

Zhang, D., G. Zhang, M. S. Hayden, M. B. Greenblatt, C. Bussey, R. A. Flavell and S. Ghosh (2004). "A toll-like receptor that prevents infection by uropathogenic bacteria." Science 303(5663): 1522-1526.

Zhang, Q., M. Raoof, Y. Chen, Y. Sumi, T. Sursal, W. Junger, K. Brohi, K. Itagaki and C. J. Hauser (2010). "Circulating mitochondrial DAMPs cause inflammatory responses to injury." Nature 464(7285): 104-107.

Zhang, S. Y., E. Jouanguy, S. Ugolini, A. Smahi, G. Elain, P. Romero, D. Segal, V. Sancho-Shimizu, L. Lorenzo, A. Puel, C. Picard, A. Chapgier, S. Plancoulaine, M. Titeux, C. Cognet, H. von Bernuth, C. L. Ku, A. Casrouge, X. X. Zhang, L. Barreiro, J. Leonard, C. Hamilton, P. Lebon, B. Heron, L. Vallee, L. Quintana-Murci, A. Hovnanian, F. Rozenberg, E. Vivier, F. Geissmann, M. Tardieu, L. Abel and J. L. Casanova (2007). "TLR3 deficiency in patients with herpes simplex encephalitis." Science 317(5844): 1522-1527.

Zhou, X., S. Yu, D. M. Zhao, J. T. Harty, V. P. Badovinac and H. H. Xue (2010). "Differentiation and persistence of memory CD8(+) T cells depend on T cell factor 1." Immunity 33(2): 229-240.

Ziegler, S. F., F. Ramsdell and M. R. Alderson (1994). "The activation antigen CD69." Stem Cells 12(5): 456-465.

Zimmermann, C., A. Prevost-Blondel, C. Blaser and H. Pircher (1999). "Kinetics of the response of naive and memory CD8 T cells to antigen: similarities and differences." Eur J Immunol 29(1): 284-290.

Annexes

Article IV

Thymocytes may persist and differentiate without any input from bone-marrow progenitors

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Thymocytes may persist and differentiate without any input from bone marrow progenitors

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Thymus transplants can correct deficiencies of the thymus epithelium caused by the complete DiGeorge syndrome or FOXN1 mutations. However, thymus transplants were never used to correct T cell–intrinsic deficiencies because it is generally believed that thymocytes have short intrinsic lifespans. This notion is based on thymus transplantation experiments where it was shown that thymus-resident cells were rapidly replaced by progenitors originating in the bone marrow. In contrast, here we show that neonatal thymi transplanted into interleukin 7 receptor–deficient hosts harbor populations with extensive capacity to self-renew, and maintain continuous thymocyte generation and export. These thymus transplants reconstitute the full diversity of peripheral T cell repertoires one month after surgery, which is the earliest time point studied. Moreover, transplantation experiments performed across major histocompatibility barriers show that allogeneic transplanted thymi are not rejected, and allogeneic cells do not induce graft-versus-host disease; transplants induced partial or total protection to infection. These results challenge the current dogma that thymocytes cannot self-renew, and indicate a potential use of neonatal thymus transplants to correct T cell–intrinsic deficiencies. Finally, as found with mature T cells, they show that thymocyte survival is determined by the competition between incoming progenitors and resident cells.

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Abbreviations used: DP, $CD4^+CD8\alpha\beta^+$ double-positive thymocytes; GVH, graft-versus-host; LM, *Listeria monocytogenes*; LSK, $Lineage^-$ $Sca\text{-}1^+$ $c\text{-}Kit^+$ BM precursors; TN, $Lineage^-$ $CD4^-CD8^-CD3\varepsilon^-$ triple-negative thymocyte.

T lymphocytes are fundamental for the control of infection. In rare cases, T cell deficiencies are congenital, caused by mutations preventing the expression of any gene required for T cell generation. However, in most cases they are induced in the adult either by infections such as AIDS, by aggressive anticancer therapies, or by aging. In the current clinical practice, these situations have become frequent, rendering the reconstitution of the peripheral T cell pool an important clinical goal. In children who do not yet have competent thymus epithelia, T cell reconstitution may be achieved by the transplantation of a competent BM. However, because BM precursors must transit through the thymus to generate T cells, the peripheral T cell reconstitution is delayed by many months, during which time patients are very susceptible to infections (Parkman and Weinberg, 1997; Holländer, 2008; Cavazzana-Calvo et al., 2009; Reimann et al., 2010). In addition, BM transplantation cannot correct T cell deficiencies once the thymus atrophies in adults.

Thymus transplants could constitute an advantageous alternative or complementary therapy; these grafts could be a source of both a functional thymus epithelia and functional T cells, and thus might correct T cell deficiencies in both children and adults. They would not necessarily require the conditioning of the patient and should export mature T cells immediately, overcoming the long lag-time required for thymus T cell generation after BM transplantation. However, although thymus grafts were successful in correcting deficiencies of the thymus epithelium, as found in the complete DiGeorge syndrome (Markert et al., 2007) or

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in FOXN1 mutations (Markert et al., 2011), they were never used to correct intrinsic T cell deficiencies, as it is generally accepted that the thymus does not harbor precursors with self-renewal capacities. Indeed, in thymus transplants, resident thymocytes generate a single wave of mature T cells because precursors originating from the BM rapidly replace resident cells (Berzins et al., 1998). Moreover, this occurs even when the host cannot generate mature T cells. When WT thymi are transplanted into SCID or Rag2-deficient hosts, the competent thymocyte populations from the graft are rapidly replaced by the incompetent precursors from the host BM (Frey et al., 1992; Takeda et al., 1996), and mature T cell export fails by 3 wk after surgery. Based on these data, the current dogma postulates that all thymocyte subpopulations are short-lived, with their maintenance being strictly dependent on the continuous input of BM-derived progenitors.

In contrast, we describe that the neonatal thymus harbors populations with self-renewal capacity that maintain the thymocyte populations independently of any input from the BM, rapidly reconstitute the peripheral T cell pools, and the capacity to clear infections in T cell–deficient mice. Moreover, these transplants function across histocompatibility barriers, recalling early studies on the susceptibility of neonatal cells to become tolerant to alloantigens (Billingham et al., 1953). These results highlight a possible application of thymus transplants in correcting intrinsic T cell deficiencies.

RESULTS AND DISCUSSION

The fate of thymus transplants in IL-7R–deficient hosts

When searching for thymocyte populations able to generate gut intraepithelial lymphocytes (Lambolez et al., 2006; Peaudecerf et al., 2011) we found that thymus transplants behaved differently when transplanted into Ragγ_c^- hosts. Indeed, as described previously (Berzins et al., 1998), when a single neonatal CD45.1$^+$ thymus lobe is grafted into WT CD45.2$^+$ hosts, the transplant is rapidly invaded by precursor cells derived from the host BM; 1 mo later, only rare mature T cells from the graft remain (Fig. 1 A). This substitution occurs even when the host cannot generate mature T cells (Frey et al., 1992; Takeda et al., 1996); when CD45.1$^+$ thymi are transplanted into CD45.2$^+$ Rag2- or CD3ε-deficient mice (not depicted) thymocytes of graft origin are also substituted by incompetent CD45.2$^+$ precursors from the host BM (Fig. 1 A). In contrast, we were surprised to observe that this substitution did not occur when the hosts could not respond to IL-7; in CD45.2$^+$ Rag2 IL-7R$^-$ or Rag2γ_c^- hosts, the vast majority of thymocytes residing in the graft were CD45.1$^+$ cells of graft origin (Fig. 1 A). These resident cells persisted and maintained a normal CD4/CD8$\alpha\beta$ profile up to 7 mo after transplant, the latest time point studied (Fig. 1 B). However, from 4 mo after transplantation onwards, thymus cellularity usually declined, with 25% of the grafts undergoing full atrophy by 4 mo, and 50% by 7 mo after surgery. This decline may be caused by age-related hormonal influences because the host mice were 6–9 mo old. Alternatively, resident thymocytes might have exhausted self-renewal capacity.

Mechanisms involved in autonomous thymocyte renewal

Several possibilities could explain the persistence of resident thymocytes in the thymus transplants grafted into IL-7R–deficient hosts. We excluded contamination by circulating hematopoietic stem cells, thereby ensuring continuous graft colonization. The host BM did not contain CD45.1$^+$ precursors derived from the graft; the only precursors present were CD45.2$^+$; LSK, Lineage$^-$ Sca-1$^+$c-Kit$^+$ BM precursors (LSKs) from the host, as it is characteristic for Rag IL-7R$^-$ or Ragγ_c^- mice (Fig. 1 C). Thymocyte persistence was also not caused by any mechanism preventing the colonization of the graft by the host BM: all grafts were colonized by host BM-derived progenitors, which progressed through differentiation as characteristic of each set of host mice: WT BM generating all Lineage$^-$ CD4$^-$CD8$^-$CD3ε^- triple-negative thymocytes (TN) precursor types, Rag2$^-$ BM-derived cells arresting their differentiation at the TN3, and IL-7R$^-$ or Ragγ_c^- BM at the TN2 differentiation stage (Fig. 1 D; Egerton et al., 1990; Mombaerts et al., 1992; Di Santo et al., 1999). Finally, Ly5.1$^+$ thymocytes of graft origin could have stopped dividing, but this was not the case (Fig. 2): we concluded that the persistence of a conserved CD4$^+$/CD8$\alpha\beta^+$ profile in the transplanted thymi indicated the presence of one or several populations with self-renewal capacity.

We first considered that CD4$^+$CD8$\alpha\beta^+$ double-positive thymocytes(DP) cells might continuously renew in the absence of any intake from more immature precursors, although their BrdU incorporation was similar to that of DP cells from a normal thymus (Fig. 2 A). To address this possibility, we investigated DP cells' TCRA repertoires, as the continuous division of the same cohort of DP cells should reduce diversity and/or switch TCRA repertoires to the preferential usage of 5′ Vα and 3′ Jα genes (Guo et al., 2002; Pasqual et al., 2002; Krangel et al., 2004). However, the high-throughput analysis of $\sim 10^7$ TCRA chains expressed by normal DP thymocytes and 8×10^6 TCRA chains expressed by DP cells from transplanted thymi showed no modifications of TCRA repertoires (Fig. S1). Diversity was maintained because samples had equivalent number of unique in-frame CDR3s (Fig. S1 A), and VA and JA usage were also comparable (Fig. S1 B). Therefore, DP differentiation was not modified, indicating that maintenance of DP cells in these grafts should be ensured by more immature progenitors.

Analysis of CD45.1$^+$ TN cells revealed the persistence of T cell progenitors of graft origin. Resident ETPs and TN2s declined rapidly (Fig. 2 B), likely substituted by the hosts' TN precursors (Fig. 1 D). CD45.1$^+$ TNs were progressively enriched in CD44$^+$CD25^{low} TN1–TN2 transition cells, with a CD44$^+$ Sca-1$^+$ c-kit$^+$ IL-7R^{low}, Fl3L$^{+/-}$ phenotype (Fig. 2, B and C). We previously studied this population in detail in the WT thymus and showed that it is more abundant in neonatal than in adult thymi (Peaudecerf et al., 2011) and is mostly T cell but not $\alpha\beta/\gamma\delta$ lineage committed, yet may generate few NK cells. Moreover, these cells are capable of considerable expansion, generating all thymocyte sets as well as the gut nonconventional TCR$\alpha\beta/\gamma\delta$ gut intraepithelial

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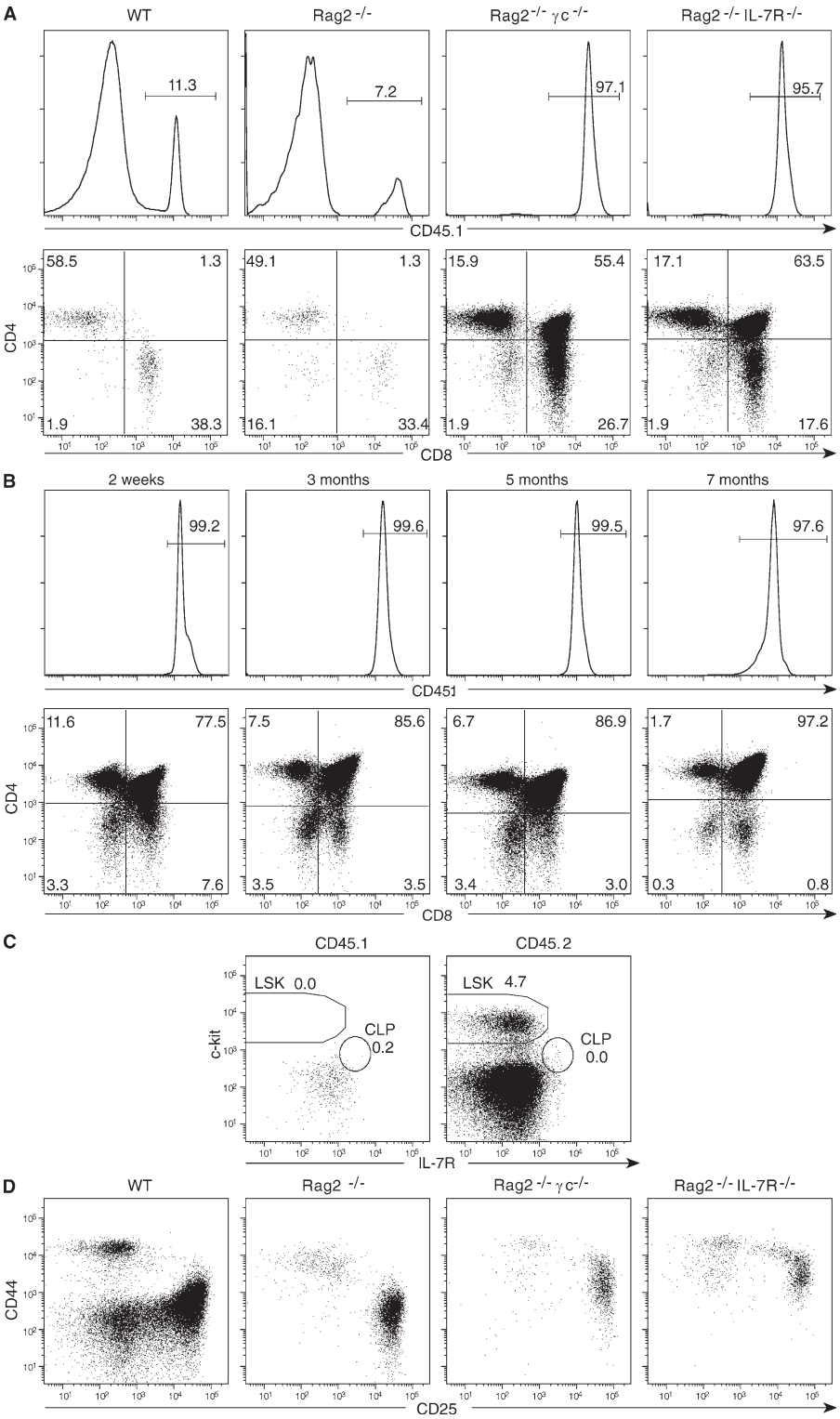

Figure 1. CD45.2⁺ B6 mice were transplanted with a single thymus lobe from 1-d-old CD45.1⁺ B6 mice. (A and B). Frequencies (histograms) and CD4/CD8β phenotypes (dot plots) of CD45.1⁺ donor thymocytes persisting in the thymus graft. (A). Hosts were WT, Rag2, Rag2γ_c, or Rag2 IL-7R–deficient, studied 1 mo after transplantation. (B) Hosts were Rag2γ_c-deficient, studied at different time points after transplantation. Similar results were obtained when hosts were Rag2 IL-7R–deficient. Results are from 1 experiment representative of 8 WT, 12 RagIL-7R–deficient, and 45 Rag2γ_c-deficient grafted mice, studied from 2 wk to 7 mo after grafting. (C) Stem cell precursors in the BM of host Rag2γ_c^- transplanted mice, 3 mo after grafting. Results show Ter119⁻GR1⁻Mac1⁻ cells of graft (CD45.1⁺) and the host (CD45.2⁺), and are representative of 5 individual mice studied in two experiments. (D) Repopulation of the transplanted thymi in different host mice. The CD44/CD25 phenotypes of CD45.2⁺ TN thymocytes derived from the host BM, 1 mo after grafting. They are representative of three experiments.

lymphocytes (Lambolez et al., 2006; Peaudecerf et al., 2011). Similar to neonatal TN1–TN2 cells, resident progenitors expressed all the molecular markers of T cell–committed progenitors (*Notch1*, *Gata3*, *Bcl11b*, and *Rag1*) and a constant small fraction divided; however, differentiation potential was restricted to T cell lineages because we could not visualize other hematopoietic populations of thymus graft origin in transplanted mice (unpublished data). Up to 5 mo after transplantation, TN3 and TN4 populations were also present, with TN3 division rates increasing by 3 mo after surgery. Coinciding with transplant atrophy, the TN3 and TN4 populations eventually disappeared (Fig. 2 B). The characteristics of such precursors explain the overall aspects of our data. They ensure that TCR rearrangements will follow relatively normal kinetics, justifying the transplants' DP unbiased TCRA repertoires (Fig. S1), and the unbiased TCRB and TCRA repertoires found in the periphery (Fig. S2). They also explain why thymocytes from the transplanted thymi are only able to persist in hosts that cannot respond to IL-7. In other hosts, the BM will have the capacity to generate such cells, and thus substitute resident TN1–TN2 populations. In contrast, IL-7R– and γc-deficient hosts cannot generate these cells, explaining why TN1–TN2 transitional cells from the transplanted thymi persist in these hosts. Overall, these results showed that T cell production in the thymus is not necessarily dependent of a

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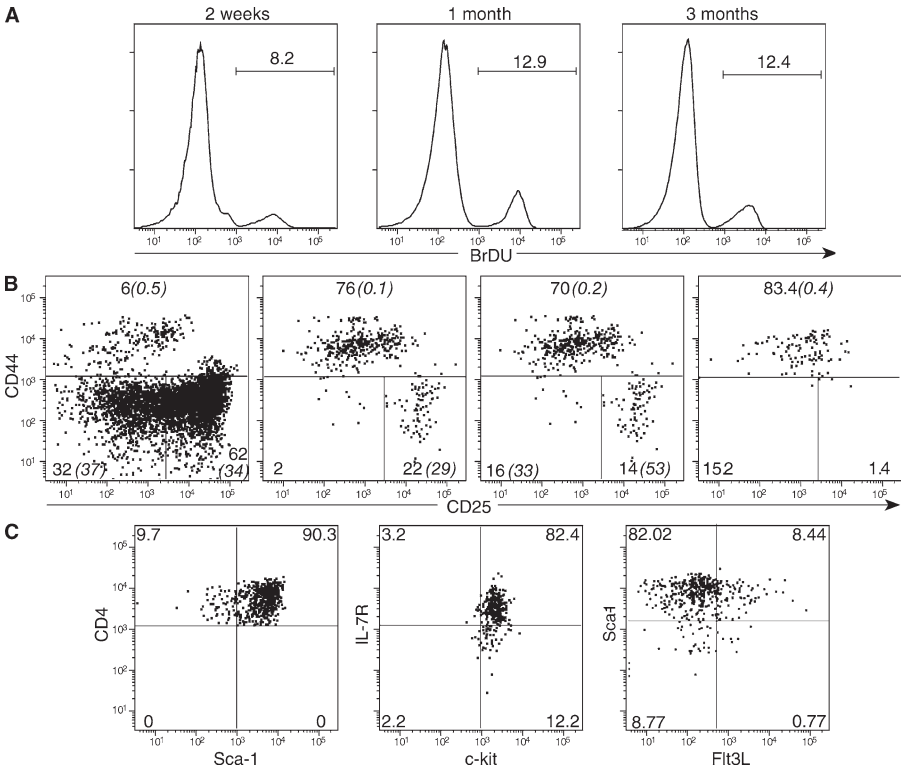

Figure 2. The phenotype and division rates of the grafts' thymocytes. Grafts were performed as in Fig. 1 B. At different time points, mice were injected with BrdU 1 h before sacrifice. (A) BrdU incorporation in DP cells in 1 out of 8 experiments with similar results. (B) The phenotype of CD45.1+ TN donor thymocytes at different time points after grafting. The percentage of cells incorporating BrdU in is italicized and in brackets. Results are from one out of 15 equivalent experiments (C) The phenotype of CD45.1+ TN1–TN2 thymocytes. Results are from one of five identical experiments.

continuous input from the BM. In certain conditions, the thymus may be self-sufficient, continuously generating mature T cells.

The capacity of thymus transplants to reconstitute the peripheral T cell pools

After BM transplantation in the mouse, mature T cells were reported to start leaving the thymus by 4 wk, and peripheral pools were found to be fully reconstituted only 2.5–3 mo later (Almeida et al., 2001). To directly compare the kinetics of peripheral reconstitution after BM or thymus transplants, sublethally irradiated CD45.2$^+$ Ragγ_c^- mice were injected simultaneously with 2×10^4 CD45.1$^+$ LSKs and transplanted with one CD45.1$^+$x CD45.2$^+$ neonatal thymus lobe. 2 wk later, the progeny of the injected LSKs had barely reached the graft, most having the CD44$^+$CD25$^-$ TN1 phenotype, whereas resident thymocytes maintained a normal CD4$^+$CD8$\alpha\beta^+$ profile (Fig. 3 A). The spleen of these mice, however, already harbored a substantial pool of naive T cells of graft origin, although CD44$^+$s were enriched (Fig. 3 B). In contrast, 1 mo later, LSK-derived cells had replaced most resident thymocytes in the graft (Fig. 3 C), but had not yet exported mature T cells. Virtually all T lymphocytes present in the spleen were still of grafted thymus origin (Fig. 3 D). Compared to the CD4 and CD8 naive cells found in normal mice, the frequency of the naive CD4$^+$ pool was equivalent and the frequency of CD8$^+$ naive pool was slightly lower in mice receiving thymus transplants (Fig. 4, A and B), supporting the observation that peripheral reconstitution is caused by the export of naive T cells (see Martins et al. in this issue). These results formally demonstrate that thymus transplants reconstitute the peripheral T cell pools much earlier than BM-derived precursors. Moreover, they also show that the kinetics of BM reconstitution is not modified by the presence of a thymus graft, and that when competent BM precursors are present, resident thymocytes are substituted even in IL-7R–deficient hosts.

To further evaluate the quality of the mature T cell repertoires generated by thymus transplantation, we analyzed the spleen T lymphocytes of Ragγ_c^--transplanted mice. High-throughput sequencing of $\sim 10^7$ TCRB and TCRA chains showed the same number of unique CDR3s as found in normal mice (Fig. S2 A). VB/JB and VA/JA usage was also very similar (Fig. S2 B). In contrast to Martins et al. (2012), we did not find clonal expansions. In the rare cases when some V genes were used at slightly higher frequency (for example TCRB V12-2 or TCRA V12D-3 in the grafted spleen; Fig. S2 C) the analysis of these apparent "expansion peaks" showed that each corresponded to multiple T cells using the same V gene but otherwise differing in both TCRBJ usage and CDR3 composition. We conclude that thymus transplants already reconstitute the diversity of the peripheral T cell repertoires 1 mo after grafting.

Peripheral pools generated by thymus transplants in different conditions

IL-7R–deficient hosts are but a small minority of potential candidates for thymus transplantation, and fully histocompatible fetal thymi would not likely always be available for transplantation. For a wider therapeutic use, thymus grafts should also be beneficial in other conditions, i.e., partial histocompatibility match between the host and the donor thymi, and in hosts able to respond to IL-7, where thymus substitution by BM precursors occurs. We found a substantial reconstitution of the peripheral pools in Ragγ_c^- B6 mice transplanted with (BALB/c x B6) F1 thymi, although the frequency of CD8$^+$ naive T cells but not of naive CD4$^+$ cells was slightly

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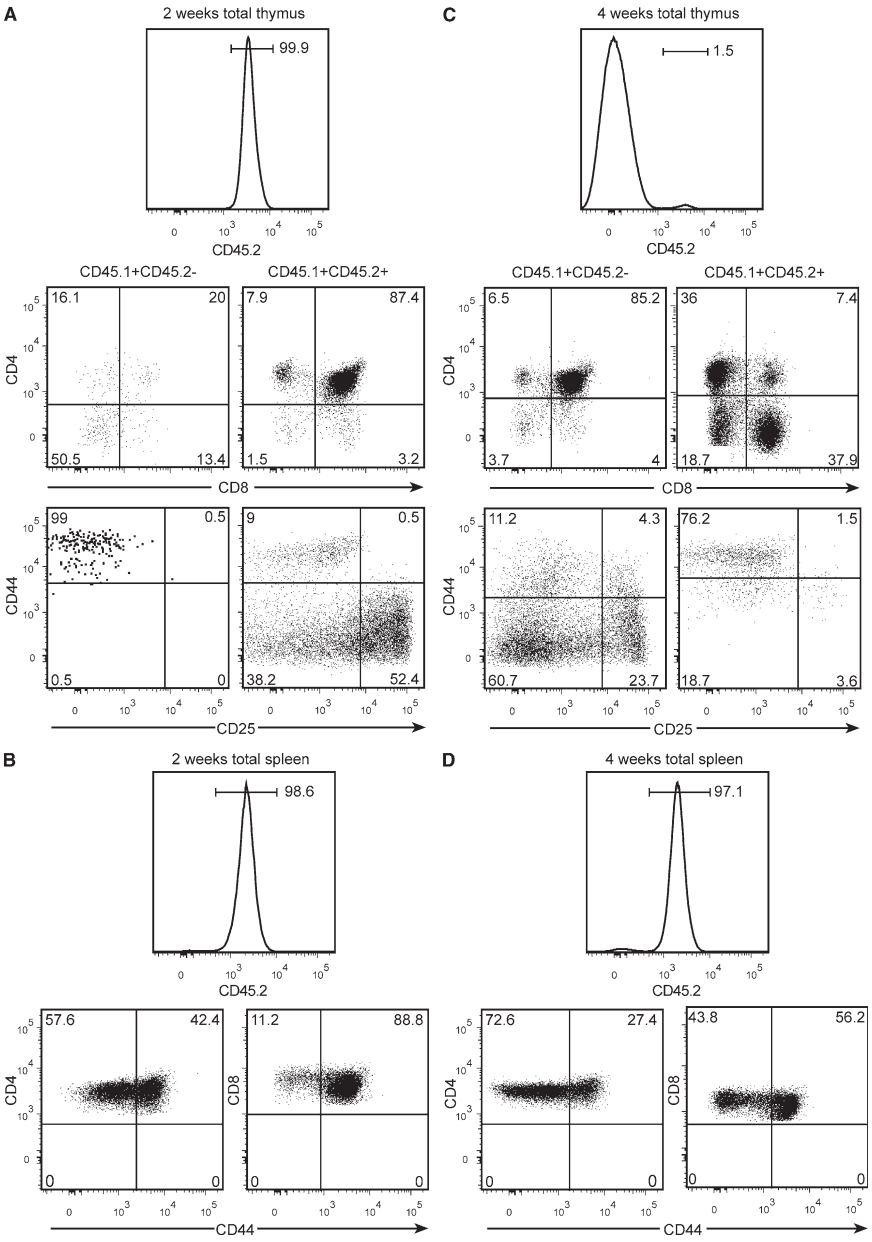

Figure 3. The kinetics of peripheral reconstitution after BM or thymus grafts. CD45.2+ B6 Rag2γ_c^- mice were sublethally irradiated (600 rads) and grafted simultaneously with 2×10^4 WT CD45.1+ LSKs, and a single thymus lobe from CD45.2+xCD45.1+ WT B6 neonatal mice and studied for 2 wk (A and B) and 1 mo later (C and D). (A and C) Percentages (histograms) and the phenotypes (dot plots) of thymus graft-derived (right) and BM-derived (left) cells in the grafted thymus. Top dot plots showing the CD4/CD8β profile, and bottom dot plots show TN cells. (B and D) Percentages (histograms) and CD44 expression (dot plots) of CD45.2+ T cells of thymus graft origin in the spleen. Results are from one mouse at each time point representative for the four mice studied.

reduced when compared with syngeneic transplants (Fig. 4, B and C). When a single thymus lobe was transplanted into IL-7-competent, CD3ε-deficient mice a substantial peripheral T cell pool was generated and naive T cells were present, although, as expected, CD44+ cells were enriched by homeostatic proliferation (Fig. 4 D). These results recall early studies reporting that 10% of a normal thymus export is sufficient to reconstitute the peripheral T cell pools (Almeida et al., 2001).

Thymus transplants in children lacking thymus epithelium were reported to ensure peripheral reconstitution across full histocompatibility barriers, T cells selected in these thymi neither inducing graft-versus-host (GVH) reactions nor rejecting the transplanted tissue, and conferring protection to infections (Markert et al., 2007, 2011). We investigated if the same phenomena would occur when Ragγ_c^- B6 hosts were transplanted with a fully allogeneic BALB/c neonatal thymus. As expected, these grafts were less efficient in reconstituting the periphery: naive cells, which require the recognition of the same MHC that induced their positive selection in the thymus to survive (Tanchot et al., 1997), were absent in these mice (Fig. 4 E). However, these mice were healthy, not showing any signs of GVH, indicating that the neonatal T cells exported by these thymi became tolerant to the host MHC. These results recall early studies (Billingham et al., 1953) reporting that neonatal T cells were easily tolerized. This was attributed to the neonatal environment, peculiar circulatory characteristics of neonatal mice promoting the extensive migration of T cells throughout nonlymphoid tissues (Arnold et al., 2005), or/and a putative immaturity of neonatal dendritic cells (Ridge et al., 1996) favoring tolerance induction. Because we transplanted adult mice, both of these mechanisms can be excluded, indicating that the properties of neonatal cells (do Canto et al., 2008) rather than their environment are responsible for tolerance induction. Besides the induction of T cell tolerance, early studies also described tolerance to fetal thymus tissue. When nude mice were transplanted into each kidney capsule with fetal thymi expressing different MHCs, neither thymus was rejected (Zamoyska et al., 1989). To investigate if a neonatal thymus epithelium would be tolerated, sublethally irradiated immunocompetent B6 mice were grafted with neonatal (B6xBALB/c) thymus and followed up for 2 mo after surgery. These transplants were invaded

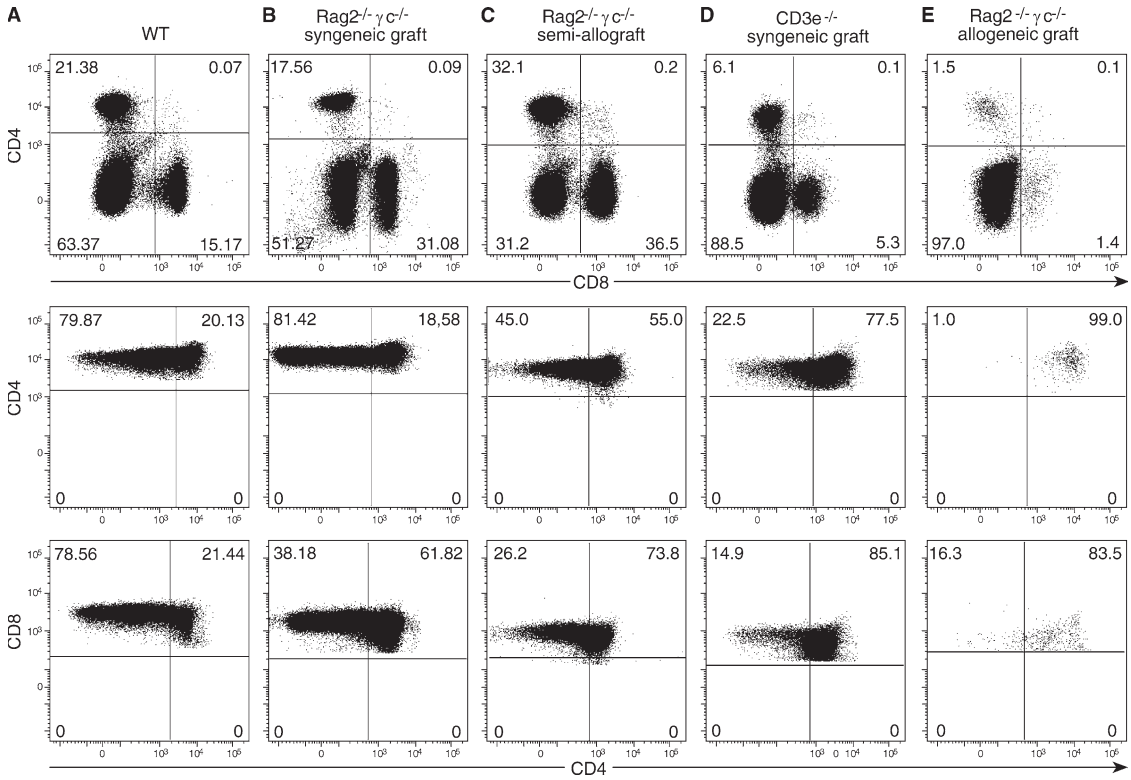

Figure 4. Peripheral reconstitution after thymus transplantation in different conditions. Different host mice were transplanted with neonatal thymi and studied 1 mo later. Top dot plots show the distribution of CD4/CD8 T cells, and bottom dot blots show the ratio of naive and CD44+ activated cells in the spleen, in one out of five equivalent experiments.

by BM-derived precursors from the host, but the thymus tissue was not rejected (unpublished data). The capacity of the thymus tissue to be tolerated, and the relative "indifference" of neonatal T lymphocytes issuing from transplanted thymi to MHC mismatches contrasts with the frequent GVH reactions induced by mismatched BM transplantation. It is tempting to speculate that this behavior may have evolved to prevent the possibly ill effects of MHC mismatches during pregnancy/delivery and that the use of fetal hematopoietic precursors for BM transplantation may minimize/prevent GVH reactions.

The capacity of thymus transplants to confer protection

To determine the capacity of different types of transplanted mice to deal with infection, we studied the T cell–dependent response to *Listeria monocytogenes* (LM). We first determined the kinetics of LM elimination in WT mice in the conditions we used. We found that bacterial loads reached the highest levels by 2 d after infection. Bacteria elimination varied in individual mice, but by day 5, LM was still detectable in all mice (Fig. 5), whereas by day 6 several infected mice had already fully eliminated LM (not depicted). We thus selected day 5 after infection as the best time point to compare bacterial clearance between different transplanted mice. We found that transplants conferred protection to infection in all mice: LM was cleared with similar kinetics in normal mice, Ragγ_c^- mice transplanted with syngeneic or semiallogeneic thymi, or even in IL-7R–competent CD3ε^- mice transplanted with semiallogeneic thymi, where besides a partial MHC mismatch the thymus transplants originated a single wave of T cell export. These results indicate that thymus transplants may have wide applications as they are capable of precocious T cell generation and confer the capacity to clear infections even in IL-7R$^+$ adult mice. Bacterial clearance was less efficient in mice transplanted with fully allogeneic BALB/c thymi, but even these mice were able to reduce bacterial loads (Fig. 5).

To summarize, the present data and also those from Martins et al. (2012) show that in contrast to the current dogma, maintenance of thymocyte populations does not depend on the continuous input of BM-derived progenitors. The thymus harbors populations with self-renewal capacities that are capable of maintaining apparently normal CD4/CD8$\alpha\beta$ profiles for several months. However, our combined data show that autonomous thymocyte renewal may be achieved by different mechanisms. In our experiments, the unbiased TCRA repertoires of DP cells indicate a major contribution of early TN thymocyte progenitors, which are indeed present and persist for long time periods. In contrast, Martins et al. (2012) did not detect these progenitors, with the TCRA repertoires generated having reduced diversity and showing several

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clonal expansions even at early points after transplantation, indicating that autonomous thymocyte renewal in their conditions is instead ensured by the continuous division of the same cohort of DP cells. Importantly, the outcomes of these two types of autonomous renewal are also very different. While our mice survive, are protected from infection, and transplanted thymi eventually atrophy, the continuous division of the same DP cells likely favors genetic instability because a large fraction of them develop T cell lymphomas later in life. The reasons behind these differences are yet unclear, but our combined data provide an unbiased and complete perspective on the advantages and possible dangers of thymus transplantation. Thymus transplants may pose a risk when used alone in the therapy of congenital IL-7R deficiencies. However, thymus transplants rapidly export T cells, function across histocompatibility barriers, and confer rapid protection to infection even in IL-7R–competent hosts where the thymocytes from the graft are substituted by precursors originated in the host BM. Therefore, thymus transplantation may be of major therapeutic value for the rapid correction of other T cell deficiencies, either when used alone or in combination with simultaneous BM transplantation.

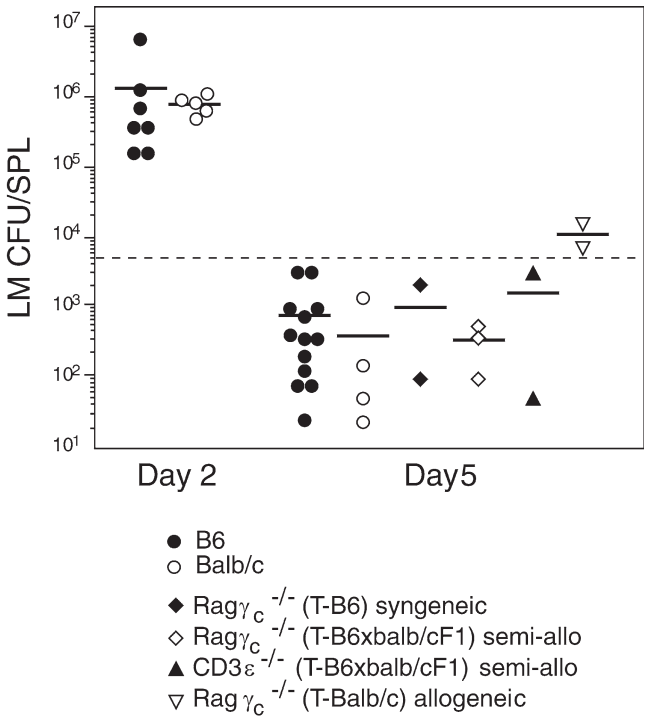

Figure 5. The capacity of thymus transplants to protect from infection. WT B6 and BALB/c controls and the transplanted mice described in Fig. 4 were injected i.v with live LM. The dotted line shows the number of injected LM. Results show bacteria loads evaluated as LM CFU/spleen at day 2 and 5 after infection, each point showing an individual mouse. T denotes the genotype of donor thymus.

MATERIALS AND METHODS

Mice, transplantation procedures, and infection with LM. BALB/c mice were purchased from Charles River, and all other mice were obtained from our breeding colonies. Host mice were 6–8-wk-old CD45.2$^+$ and were transplanted under the kidney capsule with a single thymus lobe from 1-d-old mice. CD45.1$^+$ B6, Rag2$^-$, Rag2$^-$ IL7R$^-$, or CD3ε^- mice received a lobe from CD45.1$^+$ or CD45.1xCD45.2 donor mice. For allogeneic transfers, CD45.1$^+$ Rag2γ_c^- mice were transplanted with a lobe from CD45.2$^+$ Balb/c donors. Fully allogeneic transplants were performed in Rag2γ_c^- CD45.1$^+$ hosts, grafted with one thymus lobe from 1-d-old CD45.2$^+$ BALB/c mice. When mentioned mice were injected with 5×10^3 live LM. Bacterial loads were evaluated at different time points after infection as CFU per spleen. Experiments were approved by Comitié d'ethique pour l'experimentation animale, licence # CEEA34.BR.020.12.

Cytofluorometry analysis. For surface staining, the following mAbs obtained from BD were used: anti-CD45.1/Ly5.1 (A20-1.7), anti-CD45.2/Ly5.2 (104–2.1), anti-CD3 (145-2C11), anti-CD11b/Mac1 (M1/70), anti-CD25 (PC-61), anti-CD117/c-kit (2B8), anti-TCRb (H57-597), anti-GR1 (8C5), anti-erythroid cells (TER-119), and anti–Sca-1 (E13-161.7). Anti-CD4 (GK1.5), anti-CD8β (H35-172), and anti-CD127/IL7-Rα (A7R34, a gift from Dr S.-I. Nishikawa, Kyoto University, and Institute of Physical and Chemical Research Center, Kyoto, Japan) were purified and conjugated in our laboratory. Anti-CD44 (1M781) was obtained from eBioscience. All of the aforementioned mAbs were directly coupled to FITC, PE, PerCP-Cy5.5, PECy7, APC, APC-Alexa Fluor 750, and Pacific blue or conjugated with biotin. Biotinylated mAbs were revealed with PECy7-streptavidin (BD), PE-Alexa Fluor 750-streptavidin (Invitrogen), Pacific blue–streptavidin (Invitrogen), or Pacific orange–streptavidin (Invitrogen). Cells were analyzed in a FACSCanto and sorted in a FACSAria (BD). For the determination of cell division, each mouse received a 1-h pulse of 1 mg of BrDU i.p., and BrDU incorporation was determined using a BrdU Flow kit (BD).

High-throughput sequence analysis. The populations analyzed were sorted from the thymus or the spleen of transplanted mice or age-matched controls. Deep sequence analysis of the TCRA and TCRB repertoires was performed as described previously (Wang et al., 2010).

Analysis of gene expression in the progenitors persisting in the thymus graft. B6 CD45.2 Ragγ_c-deficient mice were transplanted with a single neonatal thymus lobe from CD45.1 syngeneic WT mice. 1 mo after surgery, TN1–TN2 populations were sorted as 10 or single cells into individual wells and tested for the expression of *Gata3*, *Notch1*, *Bcl11b*, *Rag1*, and a housekeeping gene by single-cell seminested RT-PCR, using the approach we described and validated previously (Peixoto et al., 2004). In total, we studied 23 wells recovered from three different transplanted mice, with similar results. The primers pairs used are listed in Table S1.

Online supplemental material. Fig. S1 shows the CD4$^+$CD8$\alpha\beta^+$ (DP) populations persisting in the grafts. Figure S2 displays the repertoire of the peripheral T cell pools 1 mo after grafting. The primer sequences used for single-cell genetic profiling are listed in Table S1. Online supplemental material is available at http://www.jem.org/cgi/content/full/jem.20120845/DC1.

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The authors declare no conflicts of interests.

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REFERENCES

Almeida, A.R., J.A. Borghans, and A.A. Freitas. 2001. T cell homeostasis: thymus regeneration and peripheral T cell restoration in mice with a reduced fraction of competent precursors. *J. Exp. Med.* 194:591–599. http://dx.doi.org/10.1084/jem.194.5.591

Arnold, B., T. Schüler, and G.J. Hämmerling. 2005. Control of peripheral T-lymphocyte tolerance in neonates and adults. *Trends Immunol.* 26:406–411. http://dx.doi.org/10.1016/j.it.2005.06.002

Berzins, S.P., R.L. Boyd, and J.F. Miller. 1998. The role of the thymus and recent thymic migrants in the maintenance of the adult peripheral lymphocyte pool. *J. Exp. Med.* 187:1839–1848. http://dx.doi.org/10.1084/jem.187.11.1839

Billingham, R.E., L. Brent, and P.B. Medawar. 1953. Actively acquired tolerance of foreign cells. *Nature*. 172:603–606. http://dx.doi.org/10.1038/172603a0

Cavazzana-Calvo, M., I. André-Schmutz, L. Dal Cortivo, B. Neven, S. Hacein-Bey-Abina, and A. Fischer. 2009. Immune reconstitution after haematopoietic stem cell transplantation: obstacles and anticipated progress. *Curr. Opin. Immunol.* 21:544–548. http://dx.doi.org/10.1016/j.coi.2009.08.001

Di Santo, J.P., I. Aifantis, E. Rosmaraki, C. Garcia, J. Feinberg, H.J. Fehling, A. Fischer, H. von Boehmer, and B. Rocha. 1999. The common cytokine receptor gamma chain and the pre-T cell receptor provide independent but critically overlapping signals in early alpha/beta T cell development. *J. Exp. Med.* 189:563–574. http://dx.doi.org/10.1084/jem.189.3.563

do Canto, F.B., C. Lima Junior, I.A. Teixeira, M. Bellio, A. Nóbrega, and R. Fucs. 2008. Susceptibility of neonatal T cells and adult thymocytes to peripheral tolerance to allogeneic stimuli. *Immunology*. 125:387–396. http://dx.doi.org/10.1111/j.1365-2567.2008.02855.x

Egerton, M., K. Shortman, and R. Scollay. 1990. The kinetics of immature murine thymocyte development in vivo. *Int. Immunol.* 2:501–507. http://dx.doi.org/10.1093/intimm/2.6.501

Frey, J.R., B. Ernst, C.D. Surh, and J. Sprent. 1992. Thymus-grafted SCID mice show transient thymopoiesis and limited depletion of V beta 11+ T cells. *J. Exp. Med.* 175:1067–1071. http://dx.doi.org/10.1084/jem.175.4.1067

Guo, J., A. Hawwari, H. Li, Z. Sun, S.K. Mahanta, D.R. Littman, M.S. Krangel, and Y.W. He. 2002. Regulation of the TCRalpha repertoire by the survival window of CD4(+)CD8(+) thymocytes. *Nat. Immunol.* 3:469–476. http://dx.doi.org/10.1038/ni791

Holländer, G.A. 2008. Lymphoid reconstitution following hematopoietic stem cell transplantation. Of mice and men: progress made in HSCT immunobiology. *Semin. Immunopathol.* 30:369–370. http://dx.doi.org/10.1007/s00281-008-0139-y

Krangel, M.S., J. Carabana, I. Abbarategui, R. Schlimgen, and A. Hawwari. 2004. Enforcing order within a complex locus: current perspectives on the control of V(D)J recombination at the murine T-cell receptor alpha/delta locus. *Immunol. Rev.* 200:224–232. http://dx.doi.org/10.1111/j.0105-2896.2004.00155.x

Lambolez, F., M.L. Arcangeli, A.M. Joret, V. Pasqualetto, C. Cordier, J.P. Di Santo, B. Rocha, and S. Ezine. 2006. The thymus exports long-lived fully committed T cell precursors that can colonize primary lymphoid organs. *Nat. Immunol.* 7:76–82. http://dx.doi.org/10.1038/ni1293

Markert, M.L., B.H. Devlin, M.J. Alexieff, J. Li, E.A. McCarthy, S.E. Gupton, I.K. Chinn, L.P. Hale, T.B. Kepler, M. He, et al. 2007. Review of 54 patients with complete DiGeorge anomaly enrolled in protocols for thymus transplantation: outcome of 44 consecutive transplants. *Blood*. 109:4539–4547. http://dx.doi.org/10.1182/blood-2006-10-048652

Markert, M.L., J.G. Marques, B. Neven, B.H. Devlin, E.A. McCarthy, I.K. Chinn, A.S. Albuquerque, S.L. Silva, C. Pignata, G. de Saint Basile, et al. 2011. First use of thymus transplantation therapy for FOXN1 deficiency (nude/SCID): a report of 2 cases. *Blood*. 117:688–696. http://dx.doi.org/10.1182/blood-2010-06-292490

Martins, V.C., E. Ruggiero, S.M. Schlenner, V. Madan, M. Schmidt, P.J. Fink, C. von Kalle, and H.-R. Rodewald. 2012. Thymus-autonomous T cell development in the absence of progenitor import. *J. Exp. Med.* 209:1409–1417.

Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 68:869–877. http://dx.doi.org/10.1016/0092-8674(92)90030-G

Parkman, R., and K.I. Weinberg. 1997. Immunological reconstitution following bone marrow transplantation. *Immunol. Rev.* 157:73–78. http://dx.doi.org/10.1111/j.1600-065X.1997.tb00975.x

Pasqual, N., M. Gallagher, C. Aude-Garcia, M. Loiodice, F. Thuderoz, J. Demongeot, R. Ceredig, P.N. Marche, and E. Jouvin-Marche. 2002. Quantitative and qualitative changes in V-J α rearrangements during mouse thymocytes differentiation: implication for a limited T cell receptor alpha chain repertoire. *J. Exp. Med.* 196:1163–1173. http://dx.doi.org/10.1084/jem.20021074

Peaudecerf, L., P.R. dos Santos, A. Boudil, S. Ezine, N. Pardigon, and B. Rocha. 2011. The role of the gut as a primary lymphoid organ: CD8αα intraepithelial T lymphocytes in euthymic mice derive from very immature CD44+ thymocyte precursors. *Mucosal Immunol.* 4:93–101. http://dx.doi.org/10.1038/mi.2010.47

Peixoto, A., M. Monteiro, B. Rocha, and H. Veiga-Fernandes. 2004. Quantification of multiple gene expression in individual cells. *Genome Res.* 14:1938–1947. http://dx.doi.org/10.1101/gr.2890204

Reimann, C., L. Dal Cortivo, S. Hacein-Bey-Abina, A. Fischer, I. André-Schmutz, and M. Cavazzana-Calvo. 2010. Advances in adoptive immunotherapy to accelerate T-cellular immune reconstitution after HLA-incompatible hematopoietic stem cell transplantation. *Immunotherapy*. 2:481–496. http://dx.doi.org/10.2217/imt.10.36

Ridge, J.P., E.J. Fuchs, and P. Matzinger. 1996. Neonatal tolerance revisited: turning on newborn T cells with dendritic cells. *Science*. 271:1723–1726. http://dx.doi.org/10.1126/science.271.5256.1723

Takeda, S., H.R. Rodewald, H. Arakawa, H. Bluethmann, and T. Shimizu. 1996. MHC class II molecules are not required for survival of newly generated CD4+ T cells, but affect their long-term life span. *Immunity*. 5:217–228. http://dx.doi.org/10.1016/S1074-7613(00)80317-9

Tanchot, C., F.A. Lemonnier, B. Pérarnau, A.A. Freitas, and B. Rocha. 1997. Differential requirements for survival and proliferation of CD8 naïve or memory T cells. *Science*. 276:2057–2062. http://dx.doi.org/10.1126/science.276.5321.2057

Wang, C., C.M. Sanders, Q. Yang, H.W. Schroeder Jr., E. Wang, F. Babrzadeh, B. Gharizadeh, R.M. Myers, J.R. Hudson Jr., R.W. Davis, and J. Han. 2010. High throughput sequencing reveals a complex pattern of dynamic interrelationships among human T cell subsets. *Proc. Natl. Acad. Sci. USA*. 107:1518–1523. http://dx.doi.org/10.1073/pnas.0913939107

Zamoyska, R., H. Waldmann, and P. Matzinger. 1989. Peripheral tolerance mechanisms prevent the development of autoreactive T cells in chimeras grafted with two minor incompatible thymuses. *Eur. J. Immunol.* 19:111–117. http://dx.doi.org/10.1002/eji.1830190118

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SUPPLEMENTAL MATERIAL

Peaudecerf et al., http://www.jem.org/cgi/content/full/jem.20120845/DC1

A

TCRA diversity of DP thymocytes

	# of cells sorted	# of unique CDR3s
Control	200,000	96,449
Control	200,000	95,144
Graft	70,000	58,843

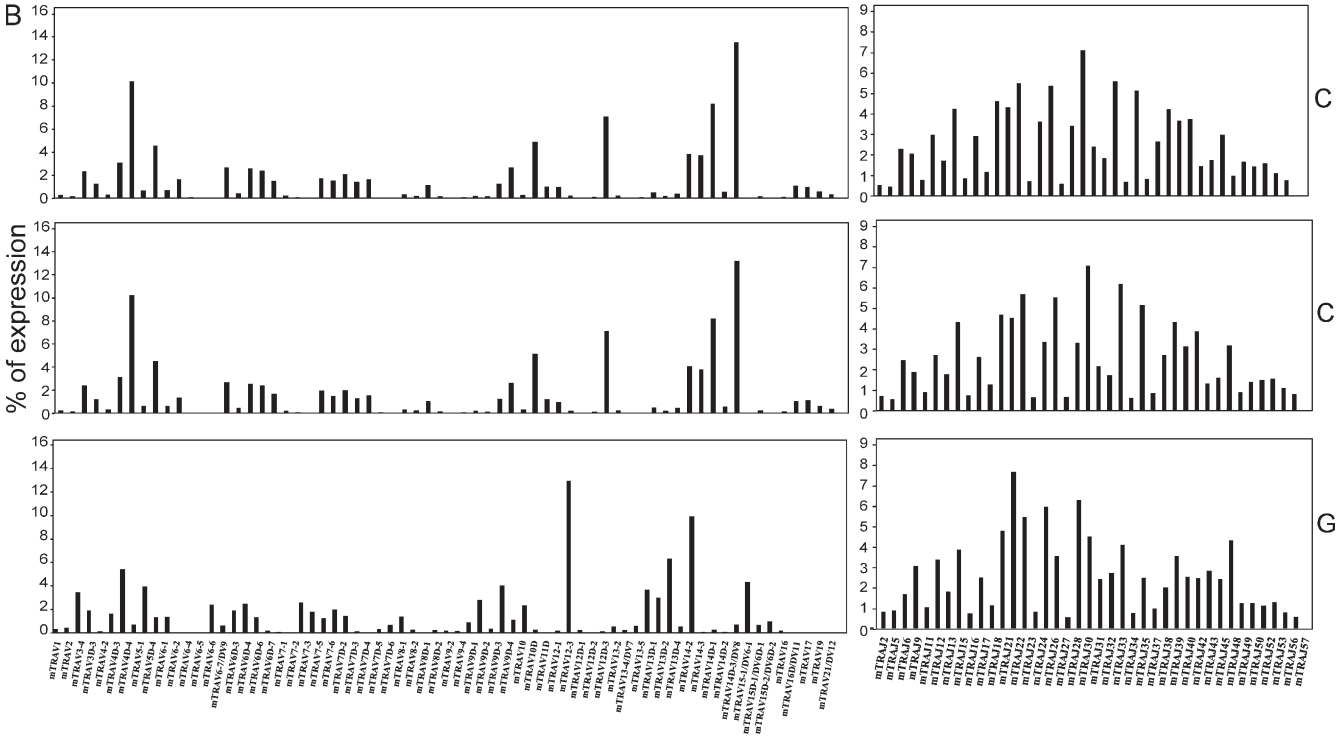

Figure S1. The CD4⁺CD8αβ⁺ (DP) populations persisting in the grafts. 6–8-wk-old CD45.2⁺ B6 Rag2γ_c^- mice were grafted with a single thymus lobe from CD45.1⁺ B6 WT neonatal mice. 2 mo later, DP cells from the graft or from age-matched controls were sorted and the TCRA repertoires were analyzed. (A) Numbers of cells sorted and of unique CDR3 regions identified. (B) TCRAV and TCRAJ usage in two controls (C, top graphs) and one transplanted thymus (G, bottom graph) from the four transplanted mice studied.

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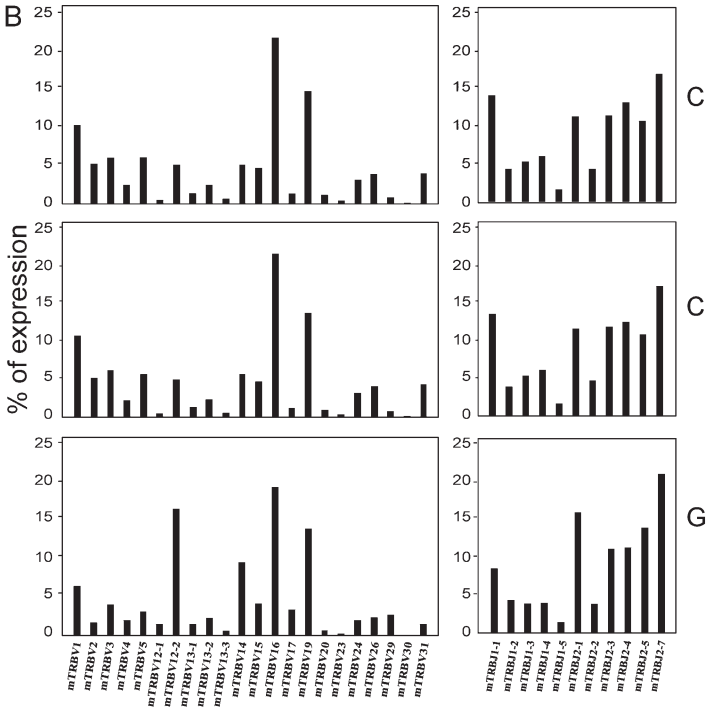
A TCR diversity of CD8 Tcells in the spleen

	# of cells sorted	# of unique CDR3s TCRB	TCRA
Control	200,000	113,032	140,070
Control	200,000	106,590	136,362
Graft (4w)	200,000	104,284	142,064
Graft (4w)	200,000	129,998	124,641
Graft (6w)	200,000	109,090	167,908
Graft (8w)	200,000	ND	185,346



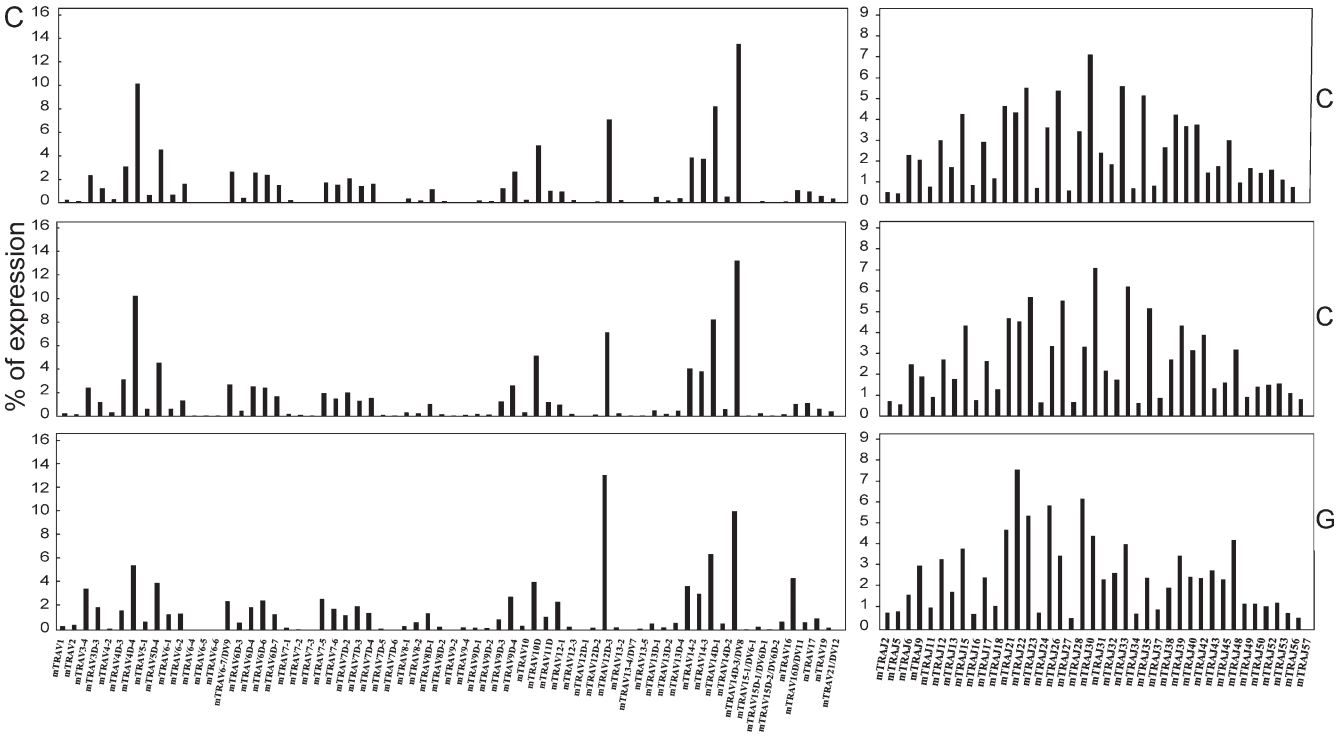


Figure S2. The repertoire of the peripheral T cell pools 1 mo after grafting. 6–8-wk-old CD45.2+ B6 Rag2γ_c^- mice were grafted with a single thymus lobe from CD45.1+ B6 WT neonatal mice. At different time points after surgery, 2 × 10^4 CD8 T cells were sorted from their spleen or from the spleen of age-matched controls and TCRB and TCRA repertoires were analyzed. (A) Numbers of unique CDR3 regions identified in the spleen. (B) TCRVB and TCRBJ usage (C) TCRVA and TCRJA usage in two WT mice (C, top graphs) and one transplanted mouse (G, bottom graph) at 1 mo after grafting. 4 mice were studied at different time points with the equivalent results.

Table S1. Primer sequences used for single-cell genetic profiling

Genes	Primer sequences
Bcl11b	A: GGCGATGCCAGAATAGATGC
	B: TTGTCCAGGACCTTGTCGTA
	C: TTGTCCCAGAGGGAACTCAT
Gata3	A: TCGGCCATTCGTACATGGAA
	B: TGGATGGACGTCTTGGAGAA
	C: ATCGATGGTCAAGGCAACCA
Notch1	A: GCTACGAATGTGCCTGTGAA
	B: CATACGTAGCCACTGGTCAT
	C: CAACGAGTGCAACAGTAACC
Rag1	A: CAACCAAGCTGCAGACATTC
	B: CTACTGGAGACTGTTCTAGG
	C: GCAGACATTCTAGCACTCTG

All primer sequences are in 5′→ 3′ direction. B primers are anti-sense, and were used for gene-specific reverse transcription. This was followed by a first RT-PCR associating primers A and B. A second, seminested PCR was performed by adding primers B and C. Primers for the house keeping gene were as described (Peixoto et al., 2004).

REFERENCE

Peixoto, A., M. Monteiro, B. Rocha, and H. Veiga-Fernandes. 2004. Quantification of multiple gene expression in individual cells. *Genome Res.* 14:1938–1947. http://dx.doi.org/10.1101/gr.2890204

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equivalents in human, a sub-group of DC that possesses specialised properties including a unique propensity to phagocytose dead cell debris and to crosspresent exogenous antigens to $CD8^+$ T cells. DNGR-1 signalling via Syk in DC regulates the retrieval and crosspresentation of dead cell-associated antigens and impacts CTL responses to infection. The study of DNGR-1 helps build a picture of the receptors and signalling pathways that regulate DC responses to self alterations and has applications in immunotherapy of cancer and infectious diseases.

S066
Control of B cell function by the Kinasel Phosphatase Equillibrium

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The early steps of B cell activation involve the dissociation of an autoinhibitory oligomeric B cell antigen receptor (BCR) complex and the exposure of the cytosolic tails of the Ig-a/Ig-b BCR signaling subunit to kinases that initiated several intracellular signaling pathways. The activation of kinases and the time of their association with the opened active BCR is tightly controlled by counteracting phosphatases. We have generated mice with a B cell specific deletion of phosphatases such as SHP-1 and PTP-1B and show these deletions result in aberrant extended B cell activation. Using substrate-trapping mutants of these phosphatases, we have identified several intracellular targets and identified critical signaling pathways which are regulated by these phosphatases. Interestingly, the B cell specific deletion of these phosphatases results in the generation of autoimmune antibodies. This finding lead us to the study of the phosphatase expression in human B cells where we found a down-regulation of phosphatases and hyperactive kinase activity in B cells of autoimmune disease patients. The molecular details of this regulation are currently under study.

We also generated mice with a B cell specific and inducible deletion of the spleen tyrosine kinase Syk. The analysis of these mice showed that Syk is required for B cell development but not for the survival of B2 B cells in the periphery. On contrast, B1 B cells are critical dependent on Syk expression.

S067
Cancer Therapy by targeted polymeric drugs evokes activation of the host's immune system

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Purpose: The possibility was tested to induce potent and long-lasting tumor-specific immunity during the treatment of experimental cancer with targeted water-soluble polymer based on *N*-(2-hydroxypropyl)methacrylamide (HPMA) containing doxorubicin. The drug (doxorubicin; DOX) was bound to its polymeric carrier through low pH-sensitive hydrazone bond from which drug is released only intracellularly in endosomes and lysosomes.
Material and methods: Three models of experimental cancer were used: BCL1-mouse B cell leukemia, EL4 T – mouse T cell lymphoma and 38C13 – mouse B cell lymphoma. The mice that developed palpable tumors reaching 8–9 mm^3 in diameter within 8–9 days after the implantation were i.v. treated with different doses (5–15 μg DOX eq./kg) of DOX^{HYD}-HPMA. Those surviving at least 60 days without any signs of a disease were considered as long-term survivors (LTS), and they were re-transplanted with a lethal dose of the same cancer cell line and left without treatment to determine the therapy-induced tumor resistance (TITR).
Results: It was seen that the treatment with DOX^{HYD}-HPMA regularly triggers a systemic anticancer response that protects mice from a second cancer attack. Such treatment – inducible 'autovaccination' is dose and time dependent; more aggressive treatment which facilitates very rapid elimination of tumor cells induces low or even undetectable tumor resistance whereas a slower eradication of tumor mass induces tumor resistance that is strong, long-lasting and could protect up to 100% of cancer-bearing animals. As proven by neutralization Winn's test, the chief mediators of the observed tumor resistance are CTL $CD8^+$. The immunogenic cancer cell death is probably involved in described phenomenon as translocation of CRT together with ERp57 and release of HMGB-1 alarmin was seen after cancer cell exposition of DOX^{HYD}-HPMA.
Conclusion: Targeted conjugates based on HPMA represent a new generation of polymeric anticancer drugs with improved therapeutic potential, considerably decreased nonspecific side effects and the ability to stimulate therapy-dependent tumor resistance.

S068
CD8 'inflammatory' effectors: a new population of effector cells with a fundamental role in early immune responses

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Inflammatory reactions are complex biological responses believed to be triggered by pathogens or tissue injure 'danger signals' that have a major protective role by recruiting innate immunity cells, favouring lymphocyte activation and differentiation, and thus contributing to the sequestration and eventually eliminating the injurious stimuli. Although certain T lymphocyte types such as TH17 cells may co-participate in inflammatory reactions, the generation of such cells from a naïve T cell pool requires previous differentiation steps within a pre-existing inflammatory milieu. In this context inflammation is regarded as beginning with an innate immune response mediated by tissue resident cells that may eventually be perpetuated and amplified by the recruitment and/or differentiation of certain T cell types. We show that CD8 T cells but not CD4 T cells, initiate a local inflammatory process shortly after antigen stimulation. Even in 'sterile immunizations' and in the apparent absence of danger signals, they secrete a burst of pro-inflammatory cytokines and chemokines. Inflammatory effectors have a most powerful effect *in vivo*: very low numbers present in a single LN are able to recruit not only lymphocytes, but also NK cells and multiple accessory cell types known to have a fundamental role in local defence. Moreover, they also induce an increase in the local concentration of sphingosine1-phosphate (S1P) what should hinder lymphocyte egress and immobilize circulating lymphocytes at the site of the immune reaction. These results demonstrate that CD8 differentiation into effector functions is not necessarily preceded by an expansion phase. They show that CD8 cognate interactions have a major role in inducing the early recruitment of multiple cell types to the place where the antigen is present. They demonstrate for the first time that local inflammatory reactions can modify S1P concentrations in tissues, thus modulating local S1P gradients. Conceptually, they also show that although inflammation does modulates cognate responses, CD8 T cells cognate responses also have a fundamental role in initiating and modulating inflammatory reactions.

List of courses, conferences and congresses during the thesis

➢ Modules de l' ECOLE DOCTORAL Gc2iD:

Modules scientifiques:

- **D.U. Thérapeutiques Immunologiques**: 2012/2013
  (Anticorps, Cytokines, Transplantation et Thérapie Cellulaire)
- **Biologie et signalisation cellulaire in vivo**: 22-26th Octobre 2012
- **Dynamique membranaire**: 15-19 th Octobre 2012

Module transdiciplinaires:

- **D.U. Formation spéciale à l'expérimentation animale – Niveau 1**: 7-18th Janvier 2013
- **English Scientific Writing**: 4-8th Mars 2013

Module d'insertion professionnelle :

- **Formation connaître et comprendre l'Entreprise**: 31th Janvier-1st Février 2011
- **Formation comment établir un CV**: 22th Mars 2011
- **Forum annuel de l'Ecole Doctorale Gc2iD**: 15-16th Mars 2012 **(poster presentation)**

➢ Formations INSERM:

- **Atelier n°201: Les vecteurs lentiviraux- outils pour la recherche fondamentale et thérapeutique**: 3-5th Mars 2010
- **Créer et exploiter les bibliographies à l'aide de logiciels de références bibliographiques-** EndNote and Zotero: 4-5th Avril 2011
- **Formation "Sensibilisation à la prévention des risques professionnels en laboratoire de recherche"**: 4-5th Novembre 2009
- **Modules de Français/French lessons** (30h): 2009/2010

➢ Communications scientifiques (CONFERENCES/CONGRES):

2010 - **5th ENII-EFIS/EJI Immunology Summer School** , May 9th-16th , Capo Caccia, Sardinia - Italy. **(poster presented)** *Epitope specificity and relative clonal abundance do not affect CD8 differentiation patterns during Lymphocytic Choriomeningitis Virus infection.* Munitic I, Decaluwe H, Evaristo C, **Lemos S**, Wlodarczyk M, Worth A, Le Bon A, Selin LK, Rivière Y, Di Santo JP, Borrow P, Rocha B

2012 - **1st EATI and 3rd ERI-ICP Conferences" entitled "Death, Danger, Inflammation and Immunity"** May 31th-June1st, Institut Pasteur, Paris - France. **(attendance)**

2012 - **3rd European Congress of Immunology**, September 5th-8th, Glasgow, Scotland - UK. *CD8 "inflammatory" effector: a new population of effector cells with a fundamental role in early immune responses.* B.Rocha, H. Sung, **S. Lemos**, P. Ribeiro-Santos, A. Charbit, C. Evaristo (**PhD work presented by the supervisor** B. Rocha as symposium invited speaker). **Immunology**. **2012** Sep; Vol 137. doi: 10.1111/imm.12001 (abstract S068 published pag.803) http://onlinelibrary.wiley.com/doi/10.1111/imm.12003_2/pdf

2013 - **3rd EFIS-EJI Summer School in Clinical Immunology**, June 1st-3rd, Paris - France **(attendance)**

Résumé

Les lymphocytes T CD8 ont un rôle essentiel dans la protection contre les agents pathogènes intracellulaires et la progression tumorale. Ainsi, la compréhension de la diversité des mécanismes de différenciation des lymphocytes T CD8 naïfs en cellules effectrices, ainsi qu'en cellules mémoires compétentes, est fondamentale pour le développement efficace de vaccins à cellules T.
Dans ce travail de thèse, nous avons abordé deux questions centrales:
(1)Très tôt après l'activation des cellules T CD8, quels sont les mécanismes par lesquels les cellules T effectrices agissent comme effecteurs pro-inflammatoires en recrutant d'autres cellules? Et quel est leur rôle dans la réponse immunitaire?
(2) Quel est le rôle du contexte infectieux dans le programme de différenciation des lymphocytes T CD8 ? Est-il responsable de l'hétérogénéité des cellules répondeuses et a-t-il un rôle dans les différents effets protecteurs des cellules mémoires?
Afin de répondre à ces questions, nous avons choisit d'utiliser des cellules T CD8 exprimant un récepteur pour l'antigène transgéniques (TCR-Tg) pour suivre la différentiation in vivo des lymphocytes T CD8. De plus, la méthode de RT-PCR sur des séries de cellules uniques, nous a permis d'analyser la co-expression des ARNm dans ces cellules.

Comme l'utilisation à haute fréquence de cellules TCR-Tg a été fortement critiquée, nous avons comparé la différenciation de ces cellules avec celle des cellules endogènes (non transgéniques et rares). Dans ce premier manuscrit nous avons observé un comportement similaire, ce qui a renforcé l'avantage d'utiliser des cellules TCR Tg pour étudier les réponses immunitaires des lymphocytes T CD8. De plus, nous avons conclu que la diversité des réponses immunitaires des lymphocytes T CD8 n'est pas conditionnée par la fréquence de cellules naïves.

Dans un deuxième manuscrit, nous avons comparé la réponse des cellules OT1 TCR-Tg (spécifiques de l'antigène OVA) à l'infection bactérienne LM-OVA (*Listeria Monocytogènes* exprimant OVA) avec la réponse des cellules P14 TCR-Tg (spécifiques de l'épitope GP33) à l'infection par le virus LCMV. Nous avons montré que les cellules OT1, stimulées par l'OVA dans un contexte bactérien (LM-OVA), présentent un profil d'expression génique distinct de celui des cellules P14 stimulées par le GP33 dans un contexte viral (LCMV). Nous avons également co-stimulé les cellules P14 et OT1 dans une même souris suivant le même contexte bactérien avec LM-GP33 et LM-OVA. Dans ce cas, nous n'avons pas observé de différence dans le profil d'expression génique. L'ensemble des résultats démontrent que les stimulations spécifiques des cellules T CD8 par différents agents pathogènes génèrent des cellules T CD8 présentant des caractéristiques différentes qui ne sont pas déterminées par la spécificité du TCR mais plutôt par le contexte infectieux. De plus, nous avons montré que les cellules mémoires endogènes résultant de la stimulation des CD8 en présence de LCMV ont été plus efficaces après une deuxième réponse immunitaire que des cellules mémoires générées après stimulation avec LM-GP33 (bactérie). Nous avons également observé que la protection plus efficace dans le contexte viral est associée à des cellules T CD8 qui présentent un phénotype de cellules T mémoires effectrices (T_{EM}) tandis que les cellules T CD8 générées dans un contexte bactérien ont plutôt un phénotype associé aux cellules T mémoires centrales (T_{CM}). Ces résultats démontrent que différents pathogènes induisent différents profils de différentiation des cellules T CD8 et que malgré l'élimination efficace des différents pathogènes dans une réponse primaire, la qualité des cellules mémoires générées au cours de cette réponse peut être différente.

Dans un troisième manuscrit, nous avons étudié les mécanismes de recrutement d'autres cellules par les lymphocytes T CD8 activés à un temps précoce de la réponse immunitaire. Nous avons analysé les réponses de cellules OT1 TCR-Tg à l'infection par LM-OVA, et la réponse des cellules anti- HY spécifiques des cellules mâles (contexte « stérile », non infectieux). Les résultats ont montré, qu'immédiatement après l'activation, les cellules T CD8 expriment des niveaux élevés de cytokines et de chimiokines pro-inflammatoires (TNFa, XCL1, CCL3 et CCL4). Nous avons aussi confirmé l'expression de ces médiateurs pro-inflammatoires dans des cellules endogènes activées. De plus, l'injection locale de ces effecteurs pro-inflammatoires dans l'oreille de souris induit l'hypertrophie du ganglion drainant, le recrutement de nombreux leucocytes (B, T, NK, monocytes, PMNs et DCs), et leur rétention dans le ganglion par l'augmentation de S1P. Ce potentiel inflammatoire a également été détecté après l'injection intra nodale de cellules T CD8. En contraste avec les fonctions classiques des lymphocytes T CD8 cytotoxiques, l'expression de médiateurs pro-inflammatoires diminue avec les divisions cellulaires alors que l'antigène est encore présent de manière abondante. La perte rapide de fonctions effectrices CD8 inflammatoires a été corrélée à la très forte diminution de l'expression du TCR à la surface des cellules activées et à la régulation des voies de signalisation du TCR (MAPkinases). Ces résultats démontrent, pour la première fois, que les réponses CD8 comportent deux phases effectrices distinctes qui suivent des règles opposées (inflammatoires et cytotoxiques), et que la stimulation spécifique par l'antigène est suffisante pour induire la phase effectrice inflammatoire des lymphocytes T CD8, ce qui est nécessaire pour faciliter la rencontre avec des cellules rares présentatrices de l'antigène.

En conclusion, l'ensemble de ces études ont démontré la diversité des fonctions des cellules T CD8 au cour de une réponse immunitaire (phase inflammatoire et phase cytotoxique) et que différents pathogènes induisent différents profils de différentiation des cellules T CD8, ce qui est crucial pour prédire la réponse T CD8 et envisager le développement de vaccins efficaces.

(Summary pag.11)