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Dorota Klysz

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Dorota Klysz. Impact des conditionnements lymphopéniques et de l'environnement métabolique sur le devenir des cellules T greffées. Immunité adaptative. Université Montpellier II - Sciences et Techniques du Languedoc, 2014. Français. NNT : 2014MON20184 . tel-01898676

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THÈSE

Pour obtenir le grade de Docteur

Délivré par UNIVERSITÉ MONTPELLIER 2
Sciences et Techniques

Préparée au sein de l'école doctorale
Sciences Chimiques et Biologiques pour la Santé

Et de l'unité de recherche IGMM-CNRS UMR5535

Spécialité: Immunology

Présentée par Dorota Klysz

Impact of lymphopenia-inducing regimens and energetic resources on the fate of adoptively transferred T cells

Soutenue le 08.07.2014 devant le jury composé de

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Acknowledgements

First, I would like to thank my Ph.D. committee, Valérie Zimmermann, Emmanuel Donnadieu, Hans Stauss, Reno Debets and David Klatzmann for the time devoted to reading my manuscript, all their helpful suggestions and comments and for accepting the invitation to come to Montpellier as members of my jury.

I would like to acknowledge all members of the ATTRACT ITN network. My sincere gratitude goes to all the PIs and in particular to Dave Gilham, Hans Stauss, Reno Debets and Anna Mondino, for their guidance and advice regarding my project as well as on many other levels. I would also like to acknowledge Helena Kondryn and Nikki Price for their amazing patience and help with the organization of meetings, network, reports, etc etc…. To all ATTRACT fellows. I will always be grateful to Naomi and Valérie for giving me the chance to meet you all. Thank you guys for all the fun that we had together.

I would like to thank all the collaborators I had the chance to work with during my thesis. A huge thank you to Al Singer and Xuguang Tai; it was an unforgettable experience to work with you. I am extremely grateful to Emmanuel Donnadieu, Alain Trautmann and Nadège Bercovici, for their advice and great discussions on our microscopy analyses. Charles D. Surh and Juhee Kim, thank you very much for giving us the opportunity to extend our study to germ-free mice, perform OTI/OTII experiments and think about some of our questions differently. Julien Marie, for so kindly providing us with Foxp3-reporter mice, whenever it was possible, as well as for all your tips and suggestions. Finally, last but not least, I would like to acknowledge Marc Sitbon and his team for their daily help, support and suggestions.

I would like to express my gratitude to Naomi Taylor. Thank you for giving me the chance to do this PhD and accepting me in your lab. I am extremely thankful for your constant guidance, passion, scientific lead, and criticisms and, most importantly, for showing me that work can also be fun. In the end, I can say that it was a great "marriage".

To Valérie Dardalhon. I have so many things to thank you for, that I would have to make a separate chapter just for this. That is why I will just simply say THANK YOU not only for being an amazing supervisor who taught me to "try not to make my life more complicated than it is" but also for becoming my friend.

I would like to thank all my colleagues from the lab, present and former.
Sandrina, Céd(e)ric and Patrice for trying to make me speak French. Sorry guys, I am just a lost case!
Myriam, for all the time that you spent at the sort and for saving my experiments when I messed things up. It took me three years but finally I always remember about single colors.
Leal, Peggy, Valérie Z., Sarah, Vanessa, Isabelle, Gaspard, and Mathieu thank you very much for all your help and support and care.
Marco, all beginnings are difficult ;) Thank you for making me laugh (btw, I still think

that Chablis is the best wine ever).
Philou! Thank you for being a really good friend even from abroad. I think you are the only one person who always agrees with all my crazy ideas.
I am really grateful also to Stephanie and Marine for helping me to settle down in Montpellier.
Thank you all. Meeting you and working with you was a real pleasure!

Je voudrais remercier tous mes collègues, ici, à l'IGMM, et tout particulièrement Sarah, Françoise, Lysiane et Anne. Je remercie tout spécialement les services communs tels que la laverie (Stephan, Ludyvine, …), le magasin (Emile, Laurent,…), l'atelier (Gilles, Greg, Lionel, Simon, Bruno,…), MRI (Myriam, Cédric) et l'animalerie (Sarah, Marc, Thomas, Eric, AnaBella, Melodie, Sandie,…). Cette thèse n'aurait pas pu voir le jour sans votre aide!

To the best-flatmate-ever, Caroline, for all your optimism. To Basia and Jakub for making sure I would not lose too much weight and trying to teach me how to cook.
To Ewa and Mathieu, it is mainly because of you I ended up in Montpellier. Thank you for keeping your doors always open for me. Ewa, you are close to me like family.
Finally, I would like to thank Dominik for his patience, longanimity, support and love.

Indeed, these were amazing four years.

“We must not see any person as an abstraction. Instead, we must see in every person a universe with its own secrets, with its own treasures, with its own sources of anguish, and with some measure of triumph.”

- Elie Wiesel

For Z.,
You made me who I am today.

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Summary

La persistance et l'efficacité des lymphocytes T transférés chez des patients atteints de cancer sont fortement accrues après chimiothérapie et/ou radiothérapie. Toutefois, l'impact de ces traitements sur le devenir de ces lymphocytes n'est pas connu. Le but de mon projet était d'identifier les facteurs permettant de moduler le devenir des lymphocytes greffés. Par le biais de modèles murins, nous avons pu démontrer que le devenir des cellules T diffère après leur transfert dans des souris irradiées ou traitées par chimiothérapie (Bu/Cy). Ainsi, le transfert après irradiation s'accompagne d'une prolifération préférentielle des cellules T $CD8^+$, dépendante de l'IL-7; tandis qu'après chimiothérapie le transfert est associé à une prolifération rapide et indépendante de l'IL-7, des cellules T $CD4^+$. De plus, ces comportements sont associés à d'importantes modifications de l'environnement généré chez l'hôte, notamment des cellules dendritiques qui régulent la prolifération rapide des cellules T CD4 chez les souris traitées Bu/Cy. D'autre part, la disponibilité en nutriments peut moduler les fonctions des lymphocytes T. Ainsi, nous avons montré qu'une carence en glutamine retarde l'activation de mTORC1 médiée par le TCR et favorise la différenciation de cellules $CD4^+$ T naïves en cellules régulatrices $Foxp3^+$, y compris en conditions de polarisation. Ce phénomène est dépendant de TGF-β et les cellules CD4 T $Foxp3^+$ ainsi générées présentent *in vivo* une prolifération accrue et des fonctions suppressives efficaces. L'ensemble de mes travaux de thèse a mis en évidence de nouveaux paramètres capables de modifier la survie et la réactivité des cellules T greffées.

Summary

It is known that the persistence and reactivity of adoptively transferred T cells in tumor-bearing patients is highly increased after chemotherapy and/or irradiation. However, the direct impact of these treatments on the fate of the injected lymphocytes has not been investigated. My research focused on the identification of microenvironmental factors that modulate the fate of adoptively transferred T cells. We found that the proliferation of T cells injected into mice rendered lymphopenic by irradiation as compared to a Busulfan/Cyclophosphamide (Bu/Cy) chemotherapy regimen is strikingly different. We observed a skewed IL-7-dependent proliferation of donor $CD8^+$ T cells in irradiated mice, whereas Bu/Cy conditioning led to an increased IL-7-independent and rapid $CD4^+$ T cell proliferation. These alterations were associated with distinct changes in the host microenvironment, and particularly dendritic cells, which regulated the rapid $CD4^+$ division in Bu/Cy-treated mice. We also determined that nutrient availability regulates $CD4^+$ effector differentiation. Specifically, deprivation of glutamine, the most abundant amino acid in blood, delayed mTORC1 activation following TCR stimulation and resulted in the conversion of naïve $CD4^+$ T cells into $Foxp3^+$ regulatory cells. This differentiation also occurred under polarizing conditions and was TGF-β-dependent. Importantly, the converted $CD4^+$ lymphocytes exhibited augmented *in vivo* proliferation and potent suppressor function. Altogether, the data generated during my PhD provide new insights into the identification of parameters that can potentially alter the survival and reactivity of adoptively-transferred T cells.

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ABBREVIATIONS

4E-BP1 - initiating factor 4E binding protein
A2AR - A2A adenosine receptor
Ac-CoA - acetyl-CoA
ACT - adoptive T cell therapy
ADP - adenosine diphosphate
AML- acute myeloid leukemi
AMP - adenosine monosphosphate
AMPK - AMP-activated protein kinase
APC - antigen presenting cell
ATP - adenosine triphosphate
BAC - bacterial artificial chromosome
BCR - B cell receptor
c-Kit - stem cell factor receptor (CD117)
CA9 - carbonic anhydrase IX
CAR - chimeric antigen receptor
CCR5 - chemokine receptor 5
CCR7 - chemokine receptor 7
CCR8 - chemokine receptor 8
cDC- classical dendritic cell
CEA - carcinoembryonic antigen
Cit - citrate
CNS2- T_{reg}-specific demethylated region, TSDR
cTEC - cortical thymic epithelial cell
CTL - cytotoxic T lymphocyte
CTLA-4 - cytotoxic T cell antigen 4, CD152
CXCR3 - C-X-C chemokine receptor 3
CXCR5 - C-X-C chemokine receptor 5
Cys - cysteine
DC - dendritic cell
DN - double negative
DP – double positive
EAA - essential amino acid
EAE - experimental autoimmune encephalomyelitis
EAE - experimental autoimmune encephalomyelitis
ECM - extracellular matrix
EGP2 - epithelial glycoprotein 2
ERBB2 - folate-binding protein, HER2/NEU
ERK - extracellular-signal-regulated kinase
ETP - early thymic progenitor
FAO - fatty acid oxidation
FOXO - forkhead box O family transcription factor
Foxp3 - forkhead box P3 transcription factor
GC- ganglioside
GD2 - diasialoganglioside
GF - germ-free
GIRT - glucocorticoid-induced TNFR family-related protein
Glc - glucose
GlcNAc - *N*-Acetylglucosamine
Gln - glutamine
GLS - glutaminase
Glu - glutamate
GLUT - glucose transporter family, SLC2A family
GM-CSF - granulocyte macrophage–colony-stimulating factor
GSH - glutathione
GVHD - graft versus host disease
HEV - high endothelial venules
HIF - hypoxia-inducible factor
HLA - human leukocyte antigen
IFNγ - interferon gamma
IL-12 - interleukin 12
IL-15 - interleukin 15
IL-17 - interleukin 17
IL-17A - interleukin 17A
IL-17F - interleukin 17F
IL-1β - interleukin 1β
IL-21 - interleukin 21
IL-22 - interleukin 22
IL-2Rβ - interleukin 2 receptor β
IL-3R - IL-3 receptor
IL-4 - interleukin 4
IL-7 - interleukin 7
ILT2 - immunoglobulin-like transcript 2
ILT3 - immunoglobulin-like transcript 3
ILT4 - immunoglobulin-like transcript 4
IRES - internal ribosomal entry site

IRF4 - interferon regulatory factor 4
ITAM - immunoreceptor tyrosine-based activation motif
KD - ketogenic diet
KDR - kinase insert domain receptor
KLF2 - Krüppel-like factor 2
LAT - linker for activation of T cells
LC - Langerhans cell
LIP – lymphopenia-induced proliferation
Mal - malate
MCT - medium chain triglyceride
ME - malic enzyme
MHC - major histocompatibility complex
mTEC - medullary thymic epithelial cell
mTOR - mammalian target of rapamycin
NEAA - non-essential amino acids
NF-κB - nuclear factor-κB
NK - natural killer
NLRs - NOD-like receptors
Nrp1 - neuropilin-1
OAA - oxaloacetate
OXPHOS - oxidative phosphorylation
pDC- plasmacytoid dendritic cell
PDK1 - phosphoinositol-dependent protein kinase 1
PH - pleckstrin homology domain
PI3K - phosphoinositide 3-kinase
PKC - protein kinase C
PPP- pentose phosphate pathway
PSMA - prostate specific membrane antigen
PtdIns(3,4,5)P3 - phosphatidylinositol-3,4,5-trisphosphate
Pyr - pyruvate
Rag2 - recombinase activating gene 2
Rheb - RAS homologue enriched in brain
$S1P_1$ - sphingosine-1-phosphate
S6K - ribosomal S6 kinase, p70S6K1
ScFv - single chain variable fragment
SGK1 - serum- and glucocorticoid-regulated kinase 1
SLC1A5 - solute carrier family 1, member 5
SLC7A5 - solute carrier family 7, member 5
SPF mice - specific-pathogen free mice
SRC - spare respiratory capacity
STAT1 - signal transducer and activator of transcription 1
STAT4 - signal transducer and activator of transcription 4
STAT6 - signal transducer and activator of transcription 6
Syk - spleen tyrosine kinase
TAA - tumor-associated antigens
TAG72 - tumor-associated glycoprotein 72
TCA - tricarboxylic acid
TCR - T cell receptor
TEC - thymic epithelial cell
T_{eff} - effector T cell
TGF-β - transforming growth factor β
TIL- tumor infiltrating lymphocyte
T_m - memory T cell
T_n - naïve T cell
TNFα - tumor necrosis factor α
T_{reg} - regulatory T cell
TSC1/TSC2 - tuberous sclerosis 1/2
TSLP - thymic stromal lymphopoetin
TSP - thymic settling progenitor
WT - wild type
ZAP-70 - zeta associated protein
α-KG - α-ketoglutarate
β_2m - β_2-microglobulin

INTRODUCTION

INTRODUCTION

1. The immune system

Humans are continually exposed to millions of potential pathogens but only rarely get sick. This resistance to disease, called immunity, is the result of cooperation between different tissues, cells and molecules that together establish the immune system. Two main defense mechanisms of immunity have been described: innate and adaptive immunity [Figure 1].

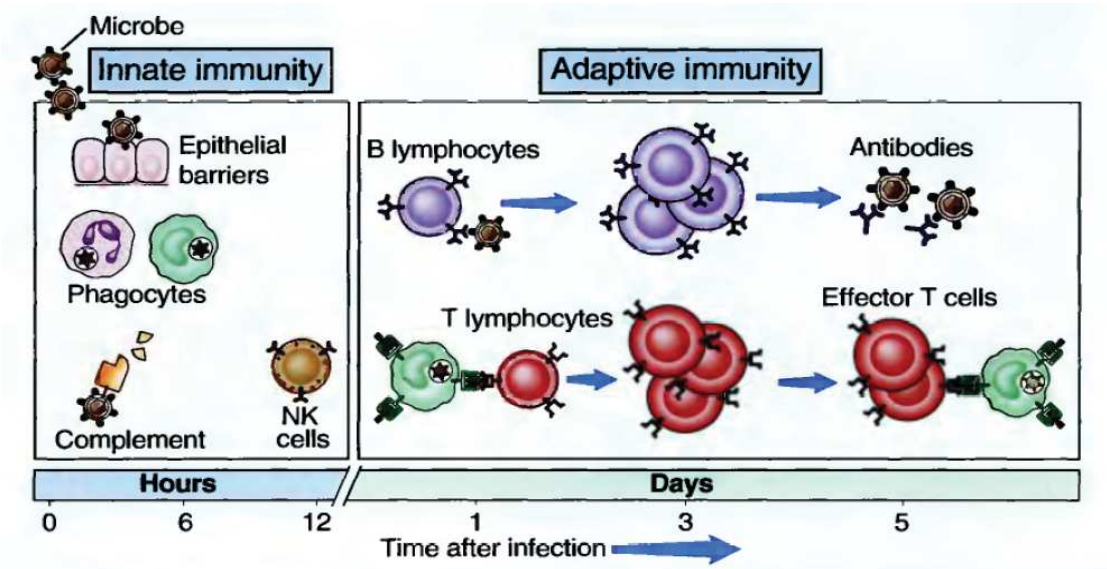


Figure 1. Innate and adaptive immunity. Innate immunity provides the initial defense against infections. It consists of elements that prevent pathogens from penetrating an organism (e.g., epithelium) and elements that rapidly eliminate pathogens (e.g., phagocytes, NK cells, complement system). Adaptive immunity develops after the innate response and is mediated by lymphocytes (B and T cells) and their products such as antibodies or cytokines. Copied from (Abbas and Lichtman, 2004).

1.1 Innate immunity

Innate immunity, also called natural or native immunity, is already active in infants and its components act at the front line of the battle against pathogens. The innate response is genetically fixed and thus invariant. It relies on a defined set of receptors called pattern recognition receptors (PRRs), which are non-specific as they target whole classes of microbes. These receptors can be expressed by different kinds of cells (dendritic cells (DCs), monocytes, macrophages) which are classified together into the one family of antigen-presenting cells (APCs) (refer to section 1.1.2) (Akira, 2001).

1.1.1 The PPRs

PPRs can distinguish host components from pathogens by recognizing small molecular motifs that are conserved within pathogens and known as pathogen-associated molecular patterns (PAMPs) or damage-activated molecular patterns (DAMPs).

At the present time, several classes of PRRs have been distinguished on the basis of their localization and function. The first class of PPRs, consisting of mannose-binding lectin (MBL), is involved in activation of complement system and opsonization. The second class comprises receptors, such as the scavenger receptor or mannose-binding receptor (MR) that induce phagocytosis on DCs and macrophages. The third class induces the production of antimicrobial peptides, proinflammatory cytokines and chemokines and can be subdivided into: cytoplasmic PRRs detecting intracellular infections (RIG-I-like receptors, NOD-like receptors (NLRs), DNA sensors) while membrane-bound PRRs monitor the extracellular milieu (TLRs and some C-type lectins) (Schenten and Medzhitov, 2011). Toll-like receptors (TLRs) were the first identified PRR and they have been extensively studied. All of the TLRs are type I transmembrane proteins that are composed of an amino-terminal leucine-rich repeat-containing ectodomain responsible for PAMP recognition, a transmembrane domain and a cytoplasmic carboxy-terminal Toll-interleukin-1 receptor (IL-1R) homology (TIR) domain that activates downstream signal transduction (Akira and Takeda, 2004; Bowie, 2000). The 13 members of the TLR familly have been characterized in mammals and they are activated by a diverse range of PAMPs such as zymosan, bacterial lipoproteins, LPS and flagellin (Alexopoulou, 2001; Hemmi, 2000; Poltorak, 1998; Qureshi, 1999; Takeuchi, 1999). Stimulation of a Toll-like receptor pathway can activate adoptive response; once recognized by the APCs, PAMPs or DAMPs are captured by endocytosis, processed and presented in a complex with Major Histocompatibility Complex (MHC) molecules to T lymphocytes (cells of the adoptive immunity, refer to section 1.2). These events initiate signaling cascades resulting in the expression of antimicrobial products, inflammatory cytokines and chemokines (reviewed in (Kawai and Akira, 2008, 2011; Medzhitov, 2002) [Figure 2].

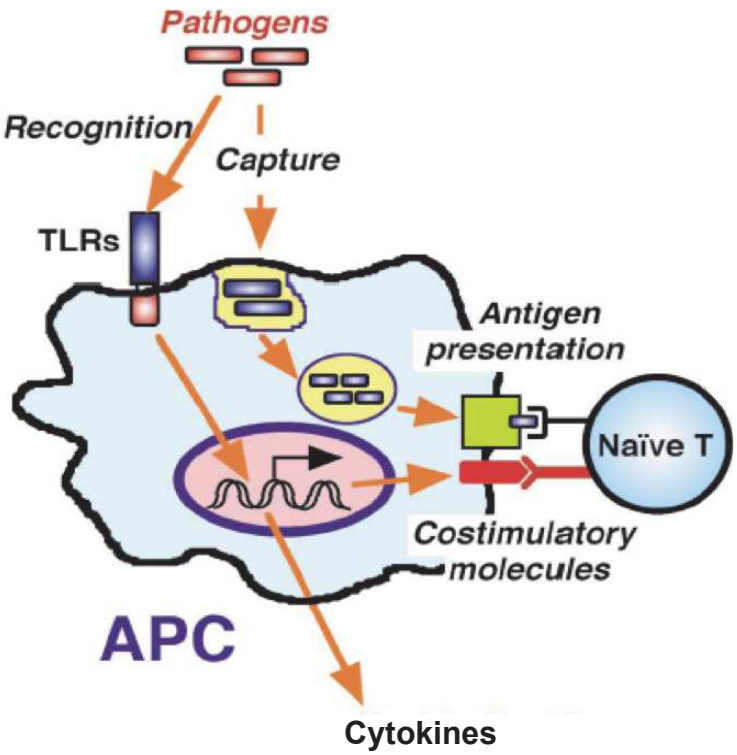


Figure 2. T cell activation by an APC. Upon TLR activation, pathogens are captured by phagocytosis, endocytosis or *via* TLRs themselves. They are processed and presented by the MHC complex to naïve T cells. In turn, TLR stimulation can induce cytokine production, which then drives T cell differentiation towards different effector phenotypes. Adapted from (Akira, 2001).

1.1.2 Antigen presenting cells

Stimulation of TCRαβ$^+$ T cells requires the presentation of antigens in the form of fragmented peptides, displayed by major histocompatibility complexes (MHC) expressed on the surface of specialized antigen-presenting cells (APCs) such as dendritic cells or macrophages. In addition to MHC-antigen presentation, a full activation of T cell effector functions (clonal expansion, cytokine production) required antigen-independent co-stimulatory signals. In the absence of these co-stimulatory signals, T cells enter into a state called anergy (also named peripheral tolerance), where they do not proliferate even upon further TCR stimulation (Jenkins, 1988; Schwartz, 1989).

Major Histocompatibility Complex

The MHC was initially discovered in humans in the 1950s on the short arm of chromosome 6 and has been called human leukocyte antigens (HLA). Later on, MHC

was also identified on the mouse chromosome 17 (Bjorkman, 1987). MHC is a large genetic complex with multiple *loci* that encodes two classes of membrane-bound glycoproteins: MHC I and II. MHC class I complex (MHC-I) is a heterodimer which consists of a polymorphic heavy and light chain called β_2-microglobulin (β_2m) (Hughes, 1997; Koopmann, 2000). Class II MHC (MHC-II) composed of two chains, α and β, which both contain two domains and the peptide-binding groove formed by the heterodimer of α1 and β1 (reviewed in (Adams and Luoma, 2013).

In general MHC-I is responsible for presenting intracellular peptides to $CD8^+$ T cells, whereas MHC-II presents exogenous peptides to $CD4^+$ T cells (reviewed in (Vyas, 2008). MHC-I molecules are expressed on the vast majority of cells and present antigens to $CD8^+$ T cells. In contrast, the constitutive expression of MHC-II molecules is confined to those cells, which are termed "professional" antigen presenting cells. Notably though, MHC-II expression can be induced on non-professional APCs, such as fibroblasts, epithelial cells, keratinocytes in inflammatory environment (Bal, 1990; Frasca, 1998; Gaspari, 1988). Furthermore, TCR stimulation results in the induction of MHC-II on the surface of T cells from many species, with the exception of mice (Holling, 2004).

Some antigen-presenting cells, especially dendritic cells, are able to load peptides that are derived from exogenous antigens onto MHC class I molecules. This atypical property is termed cross-presentation and is particularly important in the case of viruses that do not infect antigen-presenting cells. One of the mechanisms involved in cross-presentation is the transfer of antigen to a neighboring cell through gap junctions (Neijssen, 2005). *Saccheri et al.* have shown that cross-presentation through bacteria-induced gap junctions favors the generation of strong anti-tumor responses (Saccheri, 2010).

Dendritic cells

Dendritic cells, the major professional APC, are a heterogenous population that can be divided in distinct sub-population. Mouse DCs can be divided into different subsets based on the surface expression of markers such as the integrin-α chain /CD11c, co-stimulatory molecules such as CD80 and CD86, CD40 which was

previously described as a CD4- and CD8- T cell marker, CD11b (integrin αM chain of Mac-1) and CD205 (the multilectin domain molecule DEC205, originally known as NLDC-145). These subsets include classical DCs (cDCs), plasmacytoid DCs (pDCs), Langerhans cells (LCs) and monocyte-derived DCs (reviewed in (Satpathy, 2012). Classical DCs are highly phagocytic with a very short half-life (approximately 3-5 days) and undergo continuous replacement from bone marrow precursors (Liu, 2007; Waskow, 2008). Under steady state conditions, cDCs are located mainly in non-lymphoid tissues but can also be found in the spleen. They produce IL-12 and IFNγ upon activation. Plasmacytoid DCs, on the other hand, are a small population of dendritic cells that can be found mainly in the blood and lymphoid organs. They express low levels of MHC-II, co-stimulatory molecules and CD11c. Upon recognition of foreign antigens they secrete type I IFN. LCs are located in the epidermal layer of the skin. As compared to the previously described dendritic cells, Langerhans cells express low level of MHC-II, intermediate CD11c and high levels of CD207 (C-type lectin langerin). Interestingly, at steady-state, LCs are able to self-renewal *in situ* independently of the bone marrow (Merad, 2002), but upon inflammation, BM-derived cells can be mobilized to replenish the LC network (Ghigo, 2013). The *in vitro* activation of $CD14^+$ monocytes with granulocyte macrophage–colony-stimulating factor (GM-CSF)/interleukin 4 (IL-4) results in the generation of monocyte-derived DCs (Sallusto, 1994; Zhou, 1996). This differentiation of monocytes into DCs can also occur *in vivo* (Bogunovic, 2009). At least in mice, during colitis, transferred monocytes differentiate into inflammatory DCs in the colon (Rivollier, 2012). These cells are similar to the true cDCs taking under consideration their function and phenotype, but it is not know if they are dependent on GM-CSF and their gene-expression patterns has not been reported (Chomarat, 2003; Lapteva, 2001; Le Naour, 2001; Pereira, 2005; Rivollier, 2012; Tamoutounour, 2013). Monocyte-derived DCs can be characterized by the absence of expression of CD8 and a low CD11c expression (Randolph, 1999). The phenotype of murine dendritic cell subsets is summarized in Table 4.

Phenotypical marker	pDC	Lymphoid tissue cDC		Nonlymphoid tissue cDC			Langerhans cell
		$CD8^+$ cDC	$CD11b^+$ cDC	$CD103^+$ $CD11b^-$ cDC	$CD103^+$ $CD11b^+$ intestinal cDC	$CD103^-$ $CD11b^+$ cDC	
CD45	+	+	+	+	+	+	+
CD11c	+	+++	+++	++	++	++	++
MHC class II	+	++	++	++	++	++	++
CD8	subset	+	–	–	–	–	–
CD4	+	–	+/–	–	–	–	ND
CD11b	–	–	+	–	+	+	+
CD103	–	subset	–	++	++	–	–
Langerin	–	subset	–	+	–	–	++
EpCAM	–	–	–	–	–	–	++
B220	+	–	–	–	–	–	–
CD24	ND	++	+	++	++	+/–	++
Btla	+	++	+	++	+	+/–	ND
c-kit	–	+	+	+	+	+/–	ND
CD26	+	+	+	+	+	+/–	ND
Xcr1	–	+	–	+	–	–	–
CD36	–	+	–	+	ND	–	–
Cystatin C	+	++	+	ND	ND	ND	ND
Clec9a (DNGR1)	+	++	–	++	–	–	–
Cadm1 (Necl2)	–	+	–	ND	ND	ND	ND
CD205	–	++	+	++	ND	ND	++
CX_3CR1	–	subset	–	–	–	++	+
CD209 (dc-sign)	++	–	+	–	+	+/–	–
F4/80	–	–	+	–	–	+	+
CD172a (Sirpα)	+	–	++	–	–	++	+
CD64 (Fcγr1)	–	–	–	–	–	++	ND
Ly6C	++	–	–	–	–	+/–	–

Table 4. Phenotype of murine lymphoid and non-lymphoid tissue DCs. ND- not determined. Copied from (Merad, 2013).

A full T cell activation requires the interaction of an antigen:MHC complex with the TCR together with a second, antigen-independent co-stimulatory signal. TCR stimulation alone can produce long-lived, functionally anergic T cells (Schwartz, 2003). The main co-stimulatory signals are provided by the B7 protein family (CD80/B7-1 and CD86/B7-1). Co-stimulatory signals initiated by the interaction between CD28 on the T cell surface and B7 proteins expressed by APCs strengthen and sustain a T-cell response, preventing anergy (reviwed in (McAdam, 1998). Cells expressing MHCs and providing co-stimulatory signals simultaneously are called "professional" antigen-presenting cells.

1.2 Adaptive immunity

In all vertebrates, beside innate immunity, exists a second line of defense, called the adaptive immune response. Adaptive immunity is mediated by two types of cells: B

lymphocyte (humoral immunity) and T lymphocyte (cell-mediated immunity) (reviewed in (Litman, 2010). It takes at least several days for it be fully effective as it requires 1) the specific recognition of an antigen by few selected T or B lymphocytes and 2) the clonal expansion of those cells and 3) their differentiation into effector cells.

B cells

B cells are crucial actors of humoral immunity due to their ability to secrete specific antibodies. On their surface, B cell receptors (BCRs) are generated through the recombination of variable (V), diversity (D) and joining (J) gene segments in a process called V(D)J recombination and are expressed in thousands of identical copies (Tonegawa, 1983). Every BCR has a unique binding site, which binds to a part of its specific antigen called an antigenic determinant or epitope. Upon encountering its specific antigen, the cells proliferate and terminal differentiation gives rise to immunoglobulin-secreting plasma cells. Immunoglobulins (antibodies) promote the neutralization of toxins, activation of complement, phagocytosis and elimination of extracellular microbes.

T cells

Cell-mediated responses involve the activation of T cells. T cells express a specific T-cell receptor (TCR) on their surface, comprised of a heterodimer of αβ or γδ chains. αβ heterodimers show high similarity in structure to B cell receptors. A TCR consists of a N-terminal variable (V) region that is responsible for direct binding of an antigen and a constant C-terminal cytoplasmic (C) region (Akamatsu, 2003). T-cell receptors are responsible for binding to the antigen-MHC complex presented by APCs leading to T cell activation. Due to the role played by T cells in anti-cancer immune responses, they represent the main focus of my thesis.

2. T cell differentiation and function

Based on the expression of two cell-surface glycoproteins, TCR-αβ T cells can be divided into two distinct lineages: $CD4^+$ and $CD8^+$ T cells. $CD4^+$ is a transmembrane monomeric glycoprotein that consists of hydrophobic transmembrane residues and four extracellular domains (Garrett, 1993; Harrison, 1993). Two *CD8* genes in mammals encode for the CD8α and CD8β chains and although most peripheral $CD8^+$ T cells express CD8αβ heterodimers, there is also a small population of T cells expressing CD8αα homodimers on their surface (found in the skin and gut epithelium) (Jarry, 1990; Patrnes, 1989; Zhu, 2013), reviewed in (Hayday, 2001). $CD4^+$ and $CD8^+$ molecules stabilize TCR signaling by interacting with MHC class I and II, respectively, during T cell activation.

Both, $CD4^+$ and $CD8^+$ T cells develop in a thymus from progenitor cells coming from the bone marrow in a process called thymopoiesis. They leave the thymus as a mature but not activated, naïve T cells (T_n). Activation of a T cell occurs upon TCR stimulation, after which $CD4^+$ T cells differentiate into helper (Th) or regulatory (T_{reg}) cells. These different subsets can be divided based on their functions, cytokine secretion and upregulation of specific transcription factors. $CD8^+$ T cells acquire cytotoxic properties and are able to directly kill cells presenting antigens (Jenkins and Griffiths, 2010) (refer to section 2.2). These processes will be described in detail in sections 2.1 and 2.2.

2.1 T cell development

2.1.1 Thymus

The immune system consists of primary (thymus, bone marrow) and secondary (lymph nodes, tonsils, spleen, appendix, Peyer's patches in the gut) lymphoid organs. T lymphocyte development occurs in the thymus, which is a two-lobed organ constituted of thymocytes, thymic epithelial cells (TECs), dendritic cells (DCs), macrophages, endothelial cells and mesenchymal cells (Miller, 1961). The structure of the thymus can be divided into distinct regions containing different cell types and specifically contributing to different stages of T cell development.

The cortex, the sub-capsular zone surrounding the thymus, consists of cortical mesenchymatous (cMES) and thymic epithelial cells (cTEC) (Muller, 2005). The latter are involved in the recruitment of thymic settling precursors (TSP) *via* expression of adhesion molecules (Rossi, 2005) and chemokines such as CCL21 or CXCL12 (Calderon and Boehm, 2011; Liu, 2005). The central region of the thymus, called the medulla, is composed of medullary thymic epithelial cells (mTECs) characterized by the expression of co-stimulatory molecules CD80, MHC-I and MHC-II (Gray, 2006). Finally, between the cortex and the medulla is a blood vessel-rich region called the cortico-medullary junction, which is a site of entry for T lymphoid progenitors into the thymus and exit site for already generated T cells (Lind, 2001). Furthermore, TECs present at the cortico-medullary junction have been described as the cells producing the highest level of IL-7, a cytokine critical for T cell development (Alves, 2009; Hara, 2012).

2.1.2 Thymopoiesis

It takes around one week for a T cell precursor to become fully mature in the process of thympoesis. Every day around 5×10^7 new cells are generated in the thymus but only around 10^6 to 2×10^6 (roughly 2–4%) of these will entry the blood stream each day as mature T cells (Janeway, 2001). Thymopoiesis is characterized by changes in the expression of several surface markers including CD117 (c-Kit), the CD44 adhesion molecule, CD25 (α chain of the IL-2 receptor), and CD4 and CD8 co-receptors. Additionally, changes in the expression of surface molecules correspond with migration through spatially distinct areas of the adult thymus (Lind, 2001; Plotkin, 2003; Prockop and Petrie, 2000) [Figure 3].

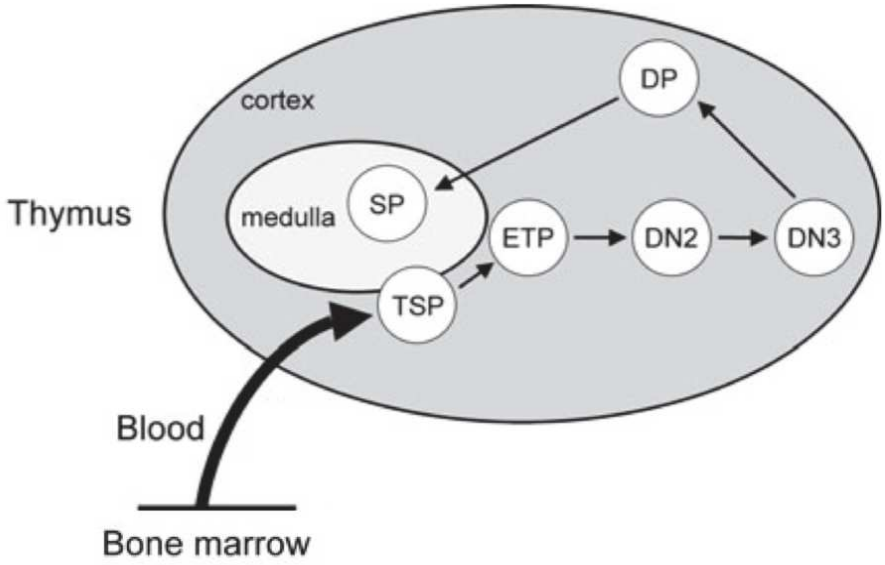


Figure 3. Overview of T cell development. T cell progenitors migrate from the bone marrow to the thymus *via* blood vessels and cross the thymic epithelium around the cortico-medullary junction as thymic settling precursors (TSPs). Once in the thymus, TSPs differentiate into early thymic progenitors (ETP). Interaction of ETPs with the thymic stroma result in the generation of CD4/CD8 double-negative 2 and 3 cells (DN2 and DN3, respectively). Following TCR rearrangement in DN thymocytes, CD4 and CD8 expression is initiated, resulting in the development of double positive (DP) thymocytes. DP cells that undergo positive selection differentiate into $CD4^+$ or $CD8^+$ single-positive (SP) cells. Maturation of SP cells occurs following their migration from the cortex to the medulla and thymocytes with non-autoreactive TCRs survive negative selection and can migrate into the periphery. Copied from (Zlotoff and Bhandoola, 2011).

The first step of T cell development requires migration of thymic settling precursors through the thymic epithelium and their differentiation into early thymic progenitors (ETP). ETPs are also called "double-negative 1" (DN1) thymocytes because they do not express CD4 or CD8 co-receptors. The DN1 thymocytes differentiate further into three populations: DN2, DN3 and DN4 cells, respectively (Hoffman, 1996; Porritt, 2004; Taghon, 2006). Whereas ETPs express very low levels of recombination activating genes 1 and 2 (*Rag 1* and *Rag 2*), these genes are upregulated at the DN2 stages. They are important for the initiation of T cell receptor (TCR) gene *loci* rearrangements of TCRδ, TCRγ and TCRβ gene (Capone, 1998; Livák, 1999). Further on, thymocytes give rise to the DN3 (Wu, 1996; Bhandoola, 2007). Successful rearrangement of the βTCR or γδTCR results in the transmission of signals that rescue the cells from death. Whereas most of the γδT lineage cells remain DN, cells with functional β chain differentiate further towards the $CD4^+CD8^+$ double positive (DP) stage (Kruisbeek, 2000; Michie and Zúñiga-Pflücker, 2002) and rearrange the

αTCR chain (Labrecque, 2010). DP cells represent the majority of thymocytes in the adult thymus (around 80%) (Huesmann, 1991) and are eliminated through a process called "death by neglect", unless they are capable of interacting with self-antigens presented within the thymic cortex. The expression of these tissue-restricted antigens has been shown to be regulated by the autoimmune regulator transcription factor AIRE (Anderson, 2002; Blechschmidt, 1999; Liston, 2003). This checkpoint stage is called positive selection (Huesmann, 1991; Suzuki, 1995), reviewed in (Starr, 2003).

To avoid the selection of potentially auto-reactive T cells, thymocytes undergo through negative selection. DP thymocytes harboring a TCR with a high avidity for self-peptide-MHC complexes are eliminated by apoptosis (Siggs, 2006; Starr, 2003). After being positively selected, cells in the medulla are subjected towards $CD4^+$ or $CD8^+$ single positive fate (SP4 or SP8 respectively) *via* a process known as CD4/CD8-lineage choice, which depends on TCR and IL-7 signaling (reviewed in (Starr, 2003) and in (Singer, 2008). In general, DP thymocytes receiving signals through MHC II-restricted TCRs differentiate into $CD4^+$ T cells, whereas DP thymocytes that receive signals through MHC class I-restricted TCRs become $CD8^+$ T cells (reviewed in (Singer, 2008).

However, there are reports describing the involvement of other transcriptional factors. T-helper inducing POZ/Kruppel-like factor (ThPOK) zinc finger protein, its transcription regulator – Gata3 (He, 2005; Hernández-Hoyos, 2003; Pai, 2003; Sun, 2005; Wang, 2008) and TOX (Thymocyte selection-associated high-mobility group box transcription factor) (Aliahmad and Kaye, 2008) have been shown to be crucial for the $CD4^+$ SP differentiation. In contrast, $CD8^+$ lineage commitment is promoted by runt-related transcription factor 3 (RUNX 3) (Taniuchi, 2002) that induces *Cd8* transcription in $CD4^+CD8^{lo}$ thymocytes (Collins, 2009; Naito, 2011) and inhibits ThPOK expression (reviewed in (Xiong and Bosselut, 2012). Other factors, such as Mazr or AP4 also contribute to the $CD8^+$ T cell generation either by repression of ThPOK (Sakaguchi, 2010) or by *Cd4* gene expression (Egawa and Littman, 2011).

The process of migration and differentiation of a thymocyte in the thymus has been presented in the Figure 4.

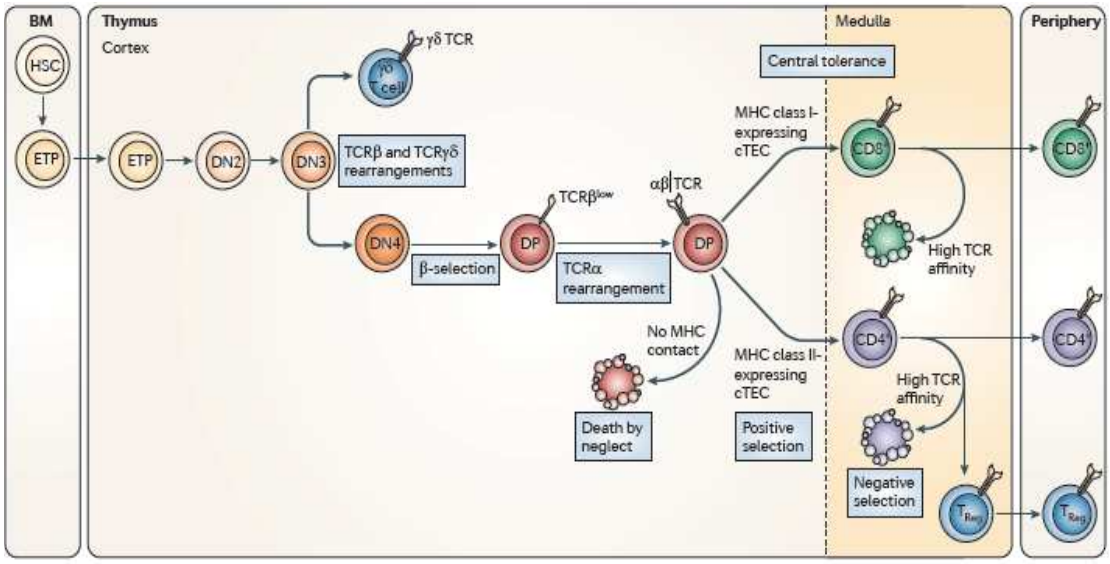


Figure 4. Major events in thymocyte differentiation. HSCs from the bone marrow give rise to ETPs. ETPs lose the potential to differentiate into B cells and DCs within the thymus. DN cells, thus named because they do not express the TCR or the co-receptors CD4 and CD8, begin to undergo crucial rearrangements of TCR genes. TCRβ gene selection leads to the generation of DP cells, which express a properly rearranged TCR β-chain (TCRβ^{low}) and both the CD4 and CD8 co-receptors. This is followed by the rearrangement of the TCR α-chain locus and expression of an αβ TCR. Failure of a DP thymocyte to be activated via its TCR results in death by neglect. Cells with TCRs that bind to MHC class I-Ag complexes upregulate CD8 and lose CD4, whereas cells that bind to MHC class II-Ag complexes maintain CD4 and lose CD8; this process is called positive selection. Selected thymocytes migrate to the medulla but If the binding avidity to a specific MHC–peptide ligand exceeds a certain threshold, they are deleted by a process termed negative selection. Undeleted SP cells migrate to the periphery from the medulla. Copied from (Miller, 2011).

2.1.3 Development of natural occurring regulatory T cells

The negative selection of thymocytes with high reactivity to self-antigens limits autoimmunity but other thymus-mediated mechanisms also regulate self-tolerance. The thymus is the source of CD4^{+} natural-occurring regulatory T cells (nT$_{reg}$), which are involved in suppressing immune responses (the properties and function of these cells will be discussed in the section 2.2.6) (reviewed in (Apostolou, 2002; Dujardin, 2004; Klein, 2003; Shevach, 2000), reviewed in (Spence and Green, 2008) and in (Stritesky, 2012). While the concept of immunological tolerance, involving a specialized population of regulatory T cells, has been described for more than 30 years (Gershon, 1971), the precise mechanisms regulating T$_{reg}$ development are still

not completely understood, although TCR specificity to self-antigens seems to be a crucial determinant for T_{reg} cell linage commitment.

Early studies using TCR transgenic mice have suggested that certain TCR specificities could facilitate thymic T_{reg} cell development (Itoh, 1999; Olivares-Villagomez, 2000). For example, hemagglutinin (HA)-specific TCR transgenic cells were selected to become T_{reg} cells only when HA was expressed in the thymus (Jordan, 2001). Furthermore, experiments performed on mice with limited diversity of TCR have shown that the TCR repertoires of T_{reg} cells and conventional $CD4^+$ T cells are different, with only a small overlap (Hsieh, 2004; Pacholczyk, 2006; Wong, 2007). From these data arose a concept that nT_{reg} cell development occurs when the TCR avidity for self-antigens lies between the TCR avidities that drive positive selection and negative selection (Maloy, 2001). This is in agreement with the observation that T cells expressing TCRs isolated from T_{reg} cells were more likely to proliferate in a lymphopenic environment, where the access to self-peptides is increased (Hsieh, 2004).

Although it has been shown that the percentage of T_{reg} cells increases in the thymus after stimulation by their cognate antigen, the absolute numbers of T_{reg} is only slightly changed (van Santen, 2004). This finding together with the observation that regulatory cells are more resistant to TCR-induced apoptosis (Papiernik, 1998) suggest that their development is not only dependent on the TCR binding avidity to the MHC complexes.

Interestingly, it has been reported that frequency of nT_{reg} was inversely correlated with the clonal frequency of TCR transgenic cells. Moreover, the number of T_{reg} cells reached plateau at high clonal frequency and was much smaller than the total number of $CD4^+CD8^-$ (SP4) cells that could be generated by positive selection. Based on these data, rose up the hypothesis that thymocytes have to compete with each other for a small niche with limited microenvironmental factors important for thymic T_{reg} cell development. Therefore, antigens involved in T_{reg} selection are more likely rare and tissue specific. Moreover the size of the niche is not equal for all TCR, suggesting that some T_{reg} TCR ligands are more abundant than others (Killebrew, 2011).

Recently, it has been apparent that interactions with the thymic stromal cells play an

additional role in promoting negative selection of self-reactive T cells and T_{reg} production. Cells in the thymus can express a selected set of molecules called tissue-restricted antigens (TRAs) such as insulin (reviewed in (Fan, 2009; Su and Anderson, 2004), which are normally limited to specific tissues within the body (Anderson, 2002; Derbinski, 2001; Pugliese, 1997; Smith, 1997; Sospedra, 1998). TRAs are mainly expressed by medullary thymic epithelial cells (Anderson, 2002; Derbinski, 2001; Klein, 1998, 2001; Smith, 1997; Sospedra, 1998), reviewed in (Kyewski, 2002) and in (Derbinski and Kyewski, 2005), to a smaller degree by dendritic cells (Garcia, 2005; Throsby, 1998) and in peripheral lymphoid tissues (Fletcher, 2010a; Lee, 2006; Magnusson, 2008; Nichols, 2007; Zheng, 2004). Expression of the TRAs on mTEC is regulated by the transcription factor AIRE (Anderson, 2002; Blechschmidt, 1999; Liston, 2003). Mutations in *AIRE* gene in humans result in the autoimmune disease 'APECED' (autoimmune polyendocrinopathy candidiasis ectodermal dystrophy) (Aaltonen, 1999; Kuroda, 2005) or 'APS-1' (polyglandular syndrome type 1) (Ahonen, 1990). However, AIRE-deficient mice develop autoimmune disorders that are similar to the one occurring in T_{reg}-depleted mice, although the knockout mice have normal number of T_{reg} cells (Su and Anderson, 2004). This suggests that TRAs expression by mTEC is crucial to maintain tolerance and T_{reg} functions but not their generation.

Other studies indicate that besides mTEC, dendritic cells are also involved in negative selection (Aschenbrenner, 2007; Gallegos, 2004; Watanabe, 2005). *Gallegos et al.* have proposed that thymic DCs presenting self-antigens negatively select both $CD4^+$ and $CD8^+$ T cells, whereas mTECs efficiently delete $CD8^+$, but not $CD4^+$ T cells (Gallegos, 2004).

Moreover, co-stimulatory signals expressed on thymic APCs have been involved in T_{reg} cell generation. The first identified signal required for T_{reg} homeostasis was the co-stimulation by CD28 (Salomon, 2000). Data from *Tai et al.* suggested that CD28 was involved in providing a cell-intrinsic signal, rather than increasing the production of cytokines (Tai, 2005). Furthermore, an approximately 80% decrease in the frequency of thymic T_{reg} cells has been observed in the CD28-deficient mice (Lohr, 2004; Tai, 2005). Taken together, these data demonstrate the important implication of CD28 in regulatory T cell selection.

Although TCR signaling for thymic T_{reg} cell differentiation is not fully understood yet,

it has been shown that sufficient interaction of SP4 thymocytes with self-antigen and co-stimulatory molecules leads to the activation of several pathways, such as NF-κB. Activation of c-REL, a member of the NF-κB family, results in formation of c-REL homodimers and their migration to the locus of forkhead box P3 (*Foxp3*) gene. This *locus*, encodes Foxp3 protein (the master regulator for T_{reg} cells) (Zheng, 2010). Deletion of c-REL severely impaired Foxp3$^+$ T_{reg} generation (Ruan, 2009; Zheng, 2010). Possibly, c-REL after TCR- and CD28- induced activation opened the *Foxp3 locus* in a comparable way as it has been shown for the correct chromatin remodeling at the IL-2 promoter site (Malek, 2002; Rao, 2003; Tai, 2005; Zheng, 2010). Further studies have suggested that TGF-β cytokine may contribute to the thymic T_{reg} differentiation (Liu, 2008; Tone, 2007). Although, it is still not clear whether TGF-β role relates more to survival or selection of the Foxp3$^+$ cells (Liu, 2008; Ouyang, 2010). In contrast, recently published data have demonstrated that TGF-β limits the proliferation of the mTECs, including the AIRE$^+$ subpopulation and the generation of the regulatory T cells (Hauri-Hohl, 2014).

Recent studies point out the importance of the CD27-CD70 pathway in the thymic development of regulatory T cells. CD70 is exclusively found in the thymic medulla and the interactions between the CD27 expressed on thymocytes and CD70 has been recently shown to support the generation of T_{reg} cells (Coquet, 2013; Cowan, 2013). Thus, T_{reg} generation requires high TCR avidity and the developmental niche provided by the thymic medulla.

2.2 Peripheral T cell subsets

After final maturation in the medulla, T cells leave the thymus as naïve lymphocytes. They migrate to the periphery towards the lymph nodes *via* specialized high endothelial venules (HEV) (Gowans, 1964). Once in the lymph nodes, T cells stay there for approximately 6-21 hours and scan antigen-presenting cells for cognate antigens. If they do not receive a signal activating their TCRs, they return to the circulation (Grigorova, 2010; Mandl, 2012; Rowley, 1972; Tomura, 2008; Westermann, 1988). Upon encountering its cognate antigen, T cells get activated,

undergo extensive proliferation (clonal expansion) and differentiate into effector T cells (T_{eff}). After clearance of the foreign antigen, generally representing an infectious agent, up to 90–95% effector cells die. Besides T_{eff} we can distinguish memory T cells (T_m) that are long-lived and have antigen-specific memory. Naïve, effector and memory T cells express different adhesion molecules and chemokine receptors, associated with different migratory patterns. Murine naïve and memory T cells express high levels of CD62L (L-lectin), whose ligands (e.g., CD34, GlyCAM-1, MAdCAM-1) (Berg, 1991; Hemmerich, 1994; Malý, 1996; Michie, 1993) are expressed on HEV in the peripheral lymph nodes and therefore facilitate rolling upon endothelium at inflammation sites (Bradley, 1992; Ley, 1995; Tedder, 1995). CD44 (H-CAM) is an adhesion molecule whose expression is upregulated on activated and memory T cells (Budd, 1987; Butterfield, 1989; MacDonald, 1990). Additionally, upon TCR-stimulation, lymphocytes upregulate inflammatory chemokine receptors such as CCR3, CCR5 or CXCR3 and these receptors are downregulated following transition to a memory phenotype (Sallusto, 1999). There are also other molecules, expressed upon activation, such as the IL-2R α-chain (CD25) that allow also allow to distinguish different T cell phenotypes. It has been shown that the massive rate of proliferation in response to foreign antigens is driven by interleukin-2 (IL-2). In accord with these observations, conventional naïve T cells do not express CD25, whereas effector and memory T cells exhibit high levels of CD25 (Cho, 2007; Ramsey, 2008). Natural-occuring regulatory T cells are an exception and their survival is dependent on IL-2 (Setoguchi, 2005).

2.2.1 T cell antigen receptor signal transduction

Upon TCR stimulation by an antigen, a T cell is activated by engagement of the three dimeric signaling modules made up of the CD3γ, CD3δ, CD3ε and TCRζ chains (CD3γ/ε, CD3δ/ε and TCRζ/ζ) [Figure 5]. All of these chains contain similar signaling motifs called ITAMs. The tyrosine residues in the ITAMs motifs are phosphorylated by the Src-family Lck/Fyn tyrosine kinases upon antigen-TCR recognition (Chan, 1994; Iwashima, 1994; Wange, 1992). This initiates conformational changes in the

ZAP-70 kinase, its recruitment to the ITAMs (Deindl, 2007; Ottinger, 1998) and phosphorylation by Src kinases (Steinberg, 2004; Wange, 1992). In turn, activation of ZAP-70 leads to the phosphorylation of multiple intermediate proteins such as the key adaptor molecule linker for activation of T cells (LAT). Activated LAT recruits further downstream adaptors and signaling molecules, which activate several major signaling branches involved in cell proliferation, apoptosis and effector differentiation (reviewed in (Lineberry and Fathman, 2006) and in (Brownlie, 2013).

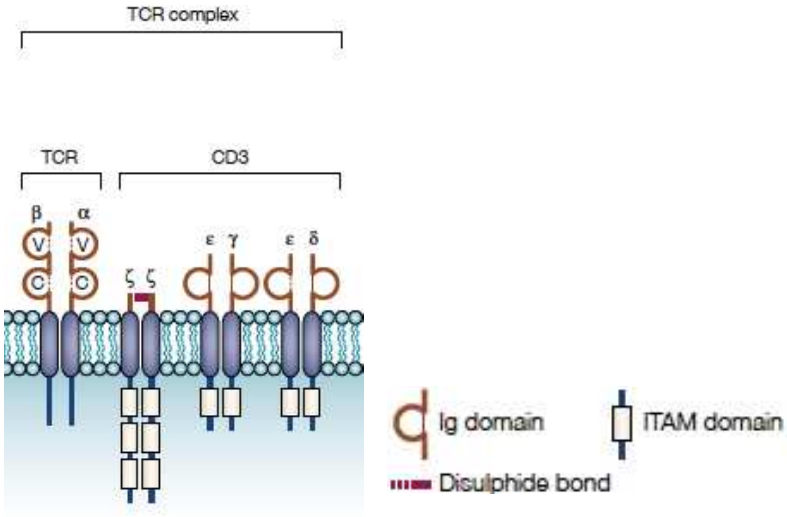


Figure 5. Structure of a TCR complex. TCR consists of α and β chains that contain variable (V) region responsible for antigen recognition and a constant (C) cytoplasmic region. CD3 complex contains γ/ ε, δ/ ε, heterodimers, a homidimer of ζ chains and tyrosine-based activation motifs (ITAM). Upon TCR stimulation by antigen, CD3 complex is phosphorylated at the ITAM sites, which leads to initiation of activation signal transduction. Copied from (Sadelain, 2003).

Among the LAT targets are phosphoinositide-3-kinases (PI3Ks), enzymes regulating different key functions in cells including cell growth, differentiation, proliferation and metabolism (Zhang, 1998), reviewed in (Vanhaesebroeck, 2001). PI3Ks are phosphorylated within seconds of TCR stimulation and remain active for nine or more hours (Costello, 2002; Ward, 1993). Activation of PI3K results in a cascade of events: increases levels of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3), binding of 3-phosphoinositol-dependent protein kinase 1 (PDK1) to

the PtdIns(3,4,5)P3, transport of the AKT kinase to the plasma membrane and its phosphorylation on Thr308 by PDK1. Finally, to fully activate its kinase activity, AKT has to be phosphorylated by the mammalian target of rapamycin (mTOR) complex 2 on Ser473 (Sarbassov, 2005) [Figure 6].

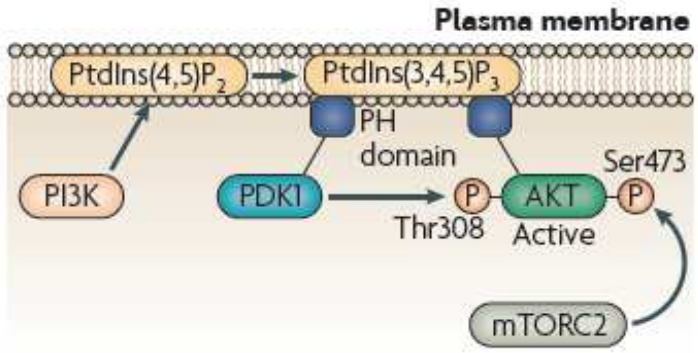


Figure 6. Mechanisms of AKT activation. Following phosphoinositide 3-kinase (PI3K) phosphorylation, the levels of phosphatidylinositol-3,4,5-trisphosphate (PtdIns(3,4,5)P3) increases in the plasma membrane. AKT binds PtdIns(3,4,5)P3 at its pleckstrin homology (PH) domain, which induces conformational changes in AKT and allows its phosphorylation by PDK1 at Thr308. AKT is also phosphorylated on its carboxy-terminal Ser473 by mammalian target of rapamycin (mTOR) complex 2 which triggers its full kinase activity. Copied from (Finlay and Cantrell, 2011).

AKT is involved in many processes including T cell migration (Bai, 2007; Brunet, 1999; Carlson, 2006; Fabre, 2008, 2005; Guertin, 2006; Kerdiles, 2009). AKT activates the forkhead box O (FOXO) family transcription factors such as FOXO1 and FOXO3A (Brunet, 1999; Fabre, 2005; Guertin, 2006), reviewed in (Coffer and Burgering, 2004), thereby controlling the expression of the transcription factor Krüppel-like factor 2 (KLF2), which in turn regulates the expression of the *Cd62l* gene (Bai, 2007; Carlson, 2006; Fabre, 2008, 2005; Kerdiles, 2009). Interestingly, the KLF2 transcription factor is rapidly downregulated following T cell activation (Kuo, 1997; Schober, 1999), resulting in the downregulation of CD62L at the T cell surface and its migration to peripheral tissues [Figure 7]. FOXO1 is in itself sensitive to growth-factor availability and survival signals such as those delivered by IL-7 (Kerdiles, 2009). Furthermore, FOXO1 has been shown to modulate metabolic functions in insulin-sensitive tissues, a process mediated by AKT phosphorylation (reviewed in (Accili, 2004) and in (Gross, 2008). These effects together with the AKT-mediated activation of the mTOR

complex 1, a serine-threonine kinase integrating environmental cues, point to the important involvement of this kinase in regulating T cell activation and metabolism (Delgoffe, 2011; Nicklin, 2009).

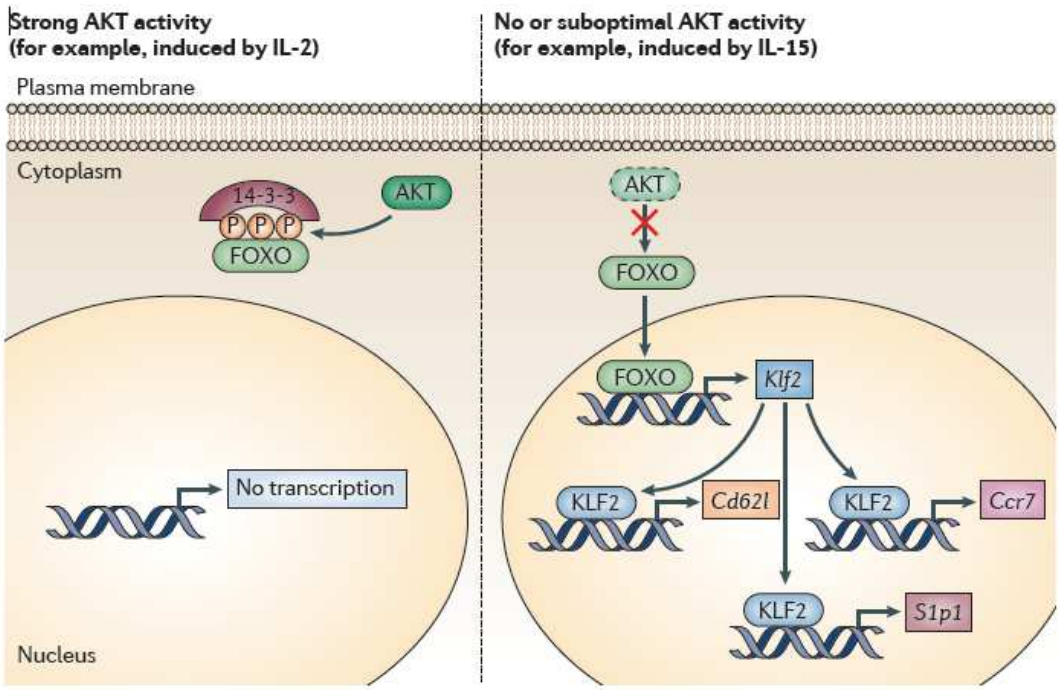


Figure 7. AKT activation status controls adhesion molecules and chemokine receptor expression. Strong AKT activation is followed by translocation of the forkhead box O (FOXO) transcription factors from the nucleus to the cytoplasm, where they bind to 14-3-3 proteins. When AKT is inactive or its activation is not optimal, FOXO transcription factors drive expression of the Krüppel-like factor 2 (KLF2) transcription factor. KLF2 induces the expression of CD62L (L-selectin), chemokine CC-chemokine receptor 7 (CCR7) and sphingosine-1-phosphate receptor ($S1P_1$) all involved in T cell migration. Copied from (Finlay and Cantrell, 2011).

2.2.2 T cell subsets

Upon encountering their cognate antigen, T cells start to differentiate and acquire effector functions. Notably, $CD8^+$ T cell differentiation results in the generation of effector cytotoxic T lymphocytes (CTLs) with the ability to directly kill infected cells through a targeted secretion of perforin and granzymes from lytic granules (Jenkins and Griffiths, 2010) and cytokines such as IFNγ (Ghanekar, 2001; Kuwano, 1993). TCR stimulation of $CD4^+$ T cells initiates their differentiation towards functionally distinct helper subsets, an outcome that is dependent on the cytokine microenvironment surrounding the T cell. T helper subsets are discriminated on the basis of their cytokine profiles and more recently, they are also classified according to their

"master regulator" transcription factors. Furthermore, years of studies have shown that it is possible to mimic T cell differentiation *in vitro* and prime a naïve $CD4^+$ T cell towards a specific pathway by modulating the cytokine environment in a process called polarization [Figure 8].

Some of the Th subsets identified so far are described below and summarized in Table 1.

Subset	T_H1	T_H1	T_H2	T_H1/T_H2	T_{REG}	T_H17
Cytokine	IFNγ	IL-12	IL-4	IL-2	IL-2	IL-6, IL-21, IL-23
Stat member	Stat1	Stat4	Stat6	Stat5	Stat5	Stat3
TF	Tbet	Tbet	Gata3		Foxp3	Rorγt (Rorα)

Table 1. Expression of T lineage-specific cytokines, and transcription factors in different Th effector subsets. TF- transcription factor. Copied from (Zygmunt and Veldhoen, 2011).

2.2.3 Effector T cell subsets

Th1/Th2 cells

In 1986, Mosmann and Coffman described two subsets of T helper cells- Th1 and Th2, based on cytokine production profiles (Mosmann, 2005). Th1 cells were associated mainly with IFNγ production but they also produced IL-2 and TNF-α. In contrast, Th2 cells produced IL-4, IL-5 and IL-13. Since that time, a huge effort has been invested to identify the critical signaling pathways and transcription factors driving the differentiation of each helper T cell subset. As a result, it has been demonstrated that the Th1 gene-expression program requires the activation of a number of transcriptional regulatory proteins, such as the signal transducer and activator of transcription 1 (STAT1) and 4 (STAT4) and the T-box transcription factor T-bet as the "master regulator" (Szabo, 2000). Th2 differentiation involves the expression of the STAT6 and Gata3 transcriptional factors (Chang and Aune, 2007; Kanhere, 2012; Scheinman and Avni, 2008; Zheng, 1997). These transcriptional factors are expressed in similar manner during human Th1/Th2 differentiation, with some differences. *Cousin and colleagues* have demonstrated that Gata3 is detected in resting naïve $CD4^+$ T cells and its expression is retained in Th1 cells, however to a

lower extend as compare to the Th2. On the other hand, although T-bet is expressed in resting Th1 cells but not in Th2, its expression is upregulated upon Th2 cells activation (Cousins, 2002). To generate Th1 or Th2 cells *in vitro,* naïve T cells need to be activated in the presence of IFNγ or IL-12/ IL-4, respectively (Hsieh, 1993; Kopf, 1993; Manetti, 1993). Th1 cells are primarily involved in the immune response against intracellular microorganisms (reviewed in (Zhu, 2008) whereas Th2 cells are involved in killing extracellular parasites such as helminths (reviewed in (Paul and Zhu, 2010) and in (Anthony, 2007). Various cell-mediated autoimmune diseases such as multiple sclerosis appear to be characterized by a bias towards a Th1 type of response (reviewed in (Fletcher, 2010b). Th2 cells, on the other hand, play a predominant role in allergic inflammation and elicit a high-level humoral immunity, with the secretion of IL-5, IL-4, and IL-13 playing roles in B cell maturation, clonal expansion, and class switching (Rafiq, 2002), reviewed in (Maggi, 1998).

***Th17* cells**

Th17 cells have been characterized by the expression of the pro-inflammatory cytokines interleukin-17A (IL-17A), IL-17F and IL-22. *In vitro* polarization of naïve $CD4^+$ T cells to a Th17 fate requires the simultaneous presence of TGF-β and IL-6 (Bettelli, 2006; Mangan, 2006; Veldhoen, 2006). Two other cytokines, IL-21 and IL-1β, have also been found to be important factors affecting Th17 differentiation (Nurieva, 2007; Zhou, 2007b). It has also been shown that also IL-23 is essential for the maintenance of murine Th17 cells (Stritesky, 2008). However, T_n cells do not differentiate *in vitro* into Th17 phenotype in only presence of IL-23 and this is largely due to a lack of expression of IL-23 receptor (IL-23R) on naive murine T cells (Zhou, 2007b). Interestingly, in human, IL-23R may be constitutively expressed on $CD4^+$ cells; therefore *in vitro* IL-17 secretion can be induced by IL-23 (Manel, 2008). Moreover, some studies have shown that human Th17 can differentiate from naïve $CD4^+$ cells in the presence of IL-1 β and IL-6 (Acosta-Rodriguez, 2007), whereas TGF-β is not critical (Wilson, 2007), reviewed in (Chen and O'Shea, 2008) or even may act as an inhibitor (Acosta-Rodriguez, 2007). The Th17 cell subset is characterized by a high expression of several transcription factors including RORγt (Ivanov, 2006),

STAT3 (Yang, 2007), IRF4 (Brüstle, 2007) and RORα (Yang, 2008). One of the main physiological roles of Th17 cells is to promote host defense against infectious agents, including certain bacteria, fungi, viruses and protozoa. Thus, this subset is involved in immune response at mucosal surfaces (i.e. guts or lungs) but also the skin (Dubin, 2008).

***Th9* cells**

Th9 is a subset that was first identified *in vitro* under conditions where murine naïve $CD4^+$ T cells were activated with a combination of TGF-β and IL-4 (Dardalhon, 2008). T cells cultured under these conditions were primed for IL-9 secretion and required transcription factors that include STAT6, PU.1, IRF4 (interferon response factor 4) (Chang, 2010; Staudt, 2010), and GATA3 (Dardalhon, 2008; Veldhoen, 2006). Similarly to mice, human Th9 differentiation is driven in naïve $CD4^+$ T cells by the combination of TGF-β and IL-4 (Beriou, 2010) and it has been shown that Thymic Stromal Lymphopoetin (TSLP) can enhance IL-9 production (Yao, 2013). Given the pleiotropic functions of IL-9, Th9 cells have been associated with pathogen immunity and immune-mediated disease. It has been demonstrated that the adoptive transfer of Th9 cells is crucial in the protection against helminth infections such as *Nippostrongylus brasiliensis* (Licona-Limón, 2013; Veldhoen, 2006). In this manner, human Th9 cells secrete TNF-α and granzyme B and can display skin-tropism where they primarily function to protect against pathogens but may also contribute to inflammatory skin diseases (Schlapbach, 2014). Th9 cells ($CD4^+IL\text{-}9^+IL\text{-}13^-IFN\gamma^-$) were also found in the peripheral blood of allergic patients (Jones, 2012). Consistent with a role for Th9 cells in allergy, increased IL-9 secretion has been observed in peripheral blood mononuclear cell cultures of adult asthmatics in response to pollen and mite allergen (Jones, 2012; Umezu-Goto, 2007) as also during allergic contact dermatitis (Liu, 2014).

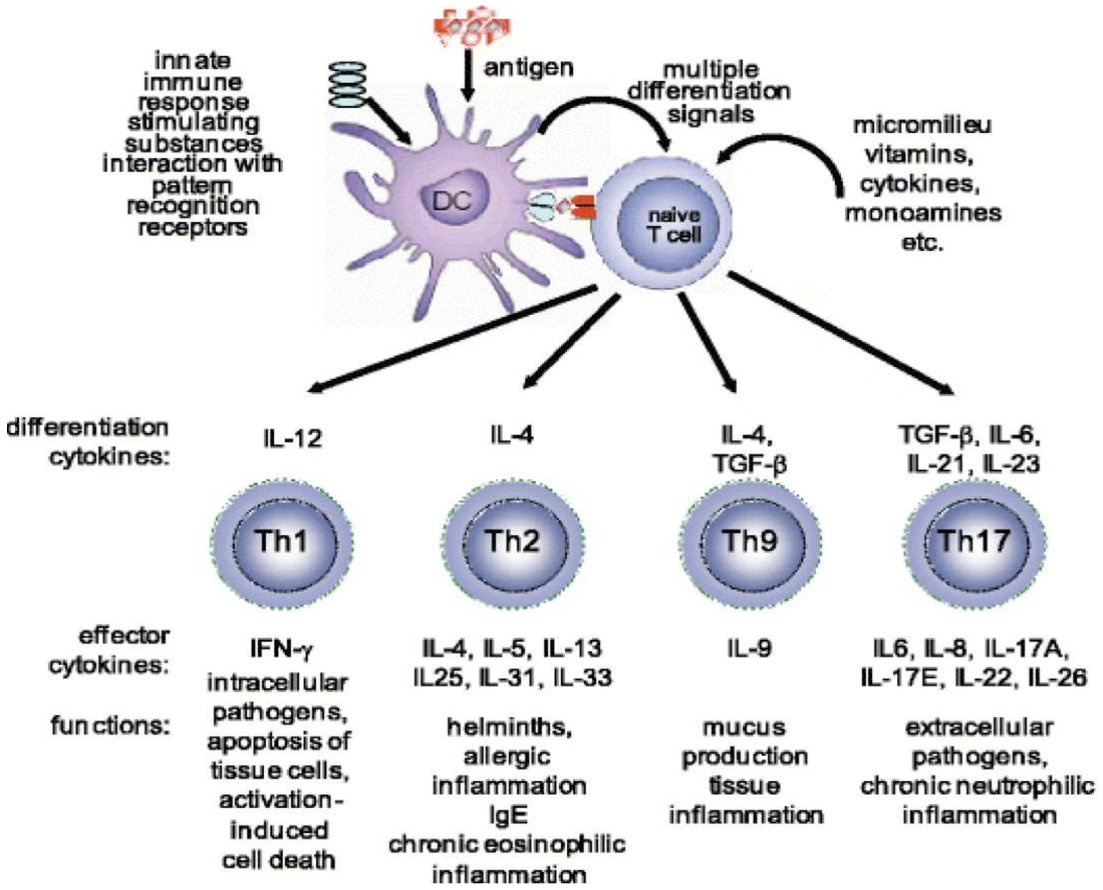


Figure 8. Differentiation of naïve T cells. Upon antigen encounter naïve $CD4^+$T cells can differentiate into T-helper subset such as Th1, Th2, Th9 and Th17 lymphocytes depending on the activation signals and the cytokines in the microenvironment. Based on their cytokine profiles, responses to chemokines, and interactions with other cells, these T-cell subsets can promote different types of inflammatory responses. IFNγ- interferon, TGFβ- transforming growth factor, TNFα- tumor necrosis factor α. Copied from (Jutel and Akdis, 2011).

2.2.5 Helper T cell plasticity

The concept of distinct T helper subsets represents a useful simplification but it remains important to remember that, although some cytokines are selectively produced by specific subsets, many are broadly expressed. Furthermore, the expression of the transcription factors regulating Th differentiation appears to be more flexible than originally described (Nakayamada, 2012; O'Shea and Paul, 2010) [Figure 9]. For example, it has been shown that Th17 cells primarily associated with the production of IL-17 can become IFNγ producers (Th1 signature cytokine) (Lee, 2009). Other evidence shows that Th2 cells can be reprogrammed into Th1 effectors, expressing T-bet and IFNγ (Hegazy, 2010). Furthermore, some $Foxp3^+$ T cells residing in the gut and skin can also express the Th2-related transcription factor Gata3,

crucial for their accumulation at inflammatory sites (Wohlfert, 2011). While Th phenotype plasticity can be advantageous in terms of host defense, it can also generate unpredictable risks for the development of anti-cancer immune therapies and vaccine development.

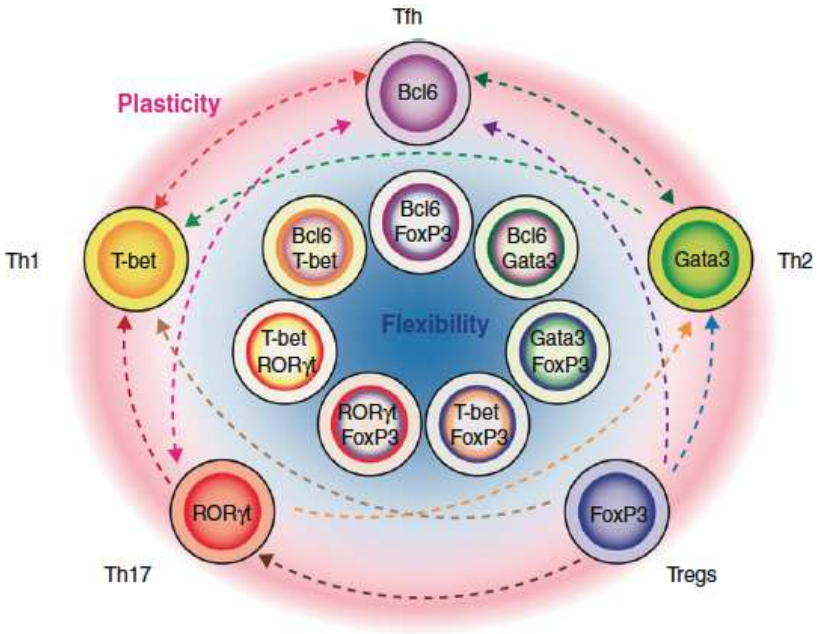


Figure 9. Plasticity and flexibility of helper T cells. However**,** naïve T cells differentiate in response to TCR-stimulation, they exhibit high levels of flexibility and plasticity. Depending on the micro-environmental signals such as cytokines, even a fully committed cell can switch its phenotype. Furthermore, T helper cells can express more than one master regulator. Copied from (Nakayamada, 2012).

Role of T helper cells in mediating an anti-tumor response

Many studies suggest that CTL are important mediators of anti-tumor responses. CTL recognize tumor antigen peptide fragments presented by MHC molecules. Experiments performed in murine models have demonstrated that the adoptive transfer of CTLs into tumor-bearing animals can result in an anti-tumor effect (Chakraborty, 2003; Dennis, 1984; Stevens, 2003). However, CTLs are unable to effectively mediate a long-term response in the absence of T helper activity (Dudley, 2001; Yee, 2002). *Fallarino et al.* have shown that the infusion of Th1 cells into tumor-bearing animals directly activate a CTL-mediated anti-tumor response (Fallarino, 2000). Indeed, Th1 cells promoted the activation and persistence of CTLs

and the generation of opsonizing antibodies. This occurs *via* several mechanisms: (1) IFNγ produced by Th1 cells can result in the upregulation of MHC-I on APCs, leading to an increased antigen presentation to CTLs (Früh, 1999); (2) Th1 cell-induced upregulation of Fas expression on tumor cells can trigger a Fas/FasL-mediated cell death. This has been shown to occur in Burkitt's lymphoma cells wherein the ligation of the CD40 TNF receptor family member facilitates the coupling of Fas to caspase-mediated apoptosis (An, 1997; Eliopoulos, 2000; Schattner, 1996); (3) Th1-induced death of T lymphoma cells by a granzymes/perforin-dependent pathway (Echchakir, 2000; Khanolkar, 2001). Although, Th2 cells does not seem to posses potent anti-tumoral function, it has been reported that they may promote anti-tumor immune responses and necrosis (Fallarino, 2000; Nishimura, 1999). Further, recently published data has shown that after chemotherapy, engagement of one of the co-stimulatory molecule belonging to the TNFR family called OX40, promoted transferred $CD4^+$ T cell differentiation towards an unique lymphocyte subset. This subset expressed both Th1 and Th2 cytokines, and it was associated with the regression of very large tumors (Hirschhorn-Cymerman, 2012).

The role of $IL17^+$ cells in anti-tumor immunity is still unclear due to contradictory observations (reviewed in (Wilke, 2011). Overexpression of IL-17 has been associated with enhanced tumor growth and tumor vascularization In immune-deficient nude mice and severe combined immunodeficient (SCID) mice (Numasaki, 2002; Numasaki, 2005). Further, endogenous IL-17 promotes tumor growth in transplanted murine tumor models such as B16 melanoma and bladder carcinoma MC49 (Wang, 2009), suggesting a pro-tumoral activity. In contrast, several studies or observations highlighted the anti-tumoral functions for Th17 cells. Th17 cells were described to promote the activation of tumor-specific $CD8^+$ T cells against the lung melanoma, by increasing DCs recruitment into the tumor site and the number of $CD8\alpha^+$ dendritic cells containing tumor antigens in draining lymph nodes (Martin-Orozco, 2009). In humans, tumor-associated Th17 level positively correlate with the level of intra-tumoral Th1 cells, cytotoxic $CD8^+$ T cells and natural killer cells and are inversely correlated with the number of T_{reg} (Curiel, 2004; Kryczek, 2009). Furthermore, Th17 cells have been found within tumor-infiltrating lymphocyte populations (Su, 2009). Moreover, in ovarian cancer patients, Th17-derived IL-17 in

ascites fluid was decreased in more advanced stage of disease and was positively associated with patient survival (Kryczek, 2009).

More recently, Th9 cells have been shown to be involved in anti-tumor immunity (Purwar, 2012). In the absence of Th17 cells, IL-9-secreting $CD4^+$ T cells inhibited growth of a B16 melanoma. Furthermore, transfer of Th9 cells or administration of recombinant IL-9 inhibited melanoma and lung cancer growth, a phenomenon requiring the presence of mast cells but not T cells (Purwar, 2012). Moreover, the transfer of OVA-specific Th9 cells in several models of OVA-expressing melanoma strongly promoted $CD8^+$ CTL activity and protected mice from tumor development (Lu, 2012).

2.2.6 Regulatory T cells

Regulatory T cells suppress immune responses and thereby, function to prevent autoimmunity, uncontrolled responses to pathogens or allergens and maintain a balance with the obligate microbial flora. These cells were initially identified in mice based on their high surface expression of the IL-2 α chain receptor (CD25) as compared to "conventional" naïve $CD4^+$ T cells. *Sakaguchi et al.* demonstrated that the autoimmunity induced by the injection of $CD25^-CD4^+$ T cells in athymic mice could be prevented by the co-injection of $CD25^+CD4^+$ cells (Sakaguchi, 1995). However, CD25 is not exclusively expressed by regulatory lymphocytes, it is also upregulated on conventional T cells following TCR stimulation. The characterization of these cells was significantly improved by the identification of the Foxp3 factor (also known as scurfin) as a critical regulator of suppressor function. Indeed, mutations in the *Foxp3* gene are responsible for a syndrome termed Immune dysregulation, Polyendocrinopathy, Enteropathy, X-linked (IPEX) in humans and multi-organ autoimmunity in mice (*scurfy* mice) (Bennett, 2001; Fontenot, 2005; Hori, 2003; Khattri, 2003; Wan and Flavell, 2005). Most $Foxp3^+$ T cells are $CD4^+$, however, a relatively small population of peripheral $CD8^+$ $Foxp3^+$ cells have also been described (Chaput, 2009; Fontenot, 2005), reviewed in (Pomié, 2008).

While Foxp3 has been described as the "master regulator" of the T_{reg} cell lineage, several reports have demonstrated the existence of $Foxp3^-$ regulatory T cells such as

the type 1 regulatory T cells (Tr1) and Th3 cells. Tr1 were first described by *Groux et al.* (Groux, 1997) and can be characterized by the secretion of large amounts of IL-10 and TGF-β, intermediate amount of IL-5 low levels of IL-2 and IFNγ but no IL-4 (IL-10^{high}, TGF-β^{high}, IL-5^{int}, IL-2^{low}, IFNγ^{low}, IL-4^{-}) (Battaglia, 2006). Tr1 upregulated Foxp3 expression upon activation but to a much lower degree as compare to the $Foxp3^{+}$ regulatory T cells (Vieira, 2004). Interestingly, in patients suffering from X-linked (IPEX) syndrome, $Foxp3^{-}$ T cells with regulatory functions have been detected. These $CD4^{+}CD25^{+}CD127^{low}Foxp3^{-}$ cells were able to suppress conventional T cells proliferation, although in much lower degree as compare to the Foxp3-expressing regulatory T cells (Otsubo, 2011). However, even though IL-10 was described, both in human (Groux, 1997) and in mice (Battaglia, 2006), to play a crucial role in Tr1 cells differentiation, it is not sufficient for *in vitro* human Tr1 cells generation and it also requires the presence of APCs (Battaglia, 2006), reviewed in (Roncarolo, 2006). It has been demonstrated that stimulation of human naïve T cells with immature DCs drive the generation of Tr1 *in vitro* through an IL-10-dependent mechanism, which is IL-10-dependent (Jonuleit, 2000; Levings, 2004). Recently, *Gregori et al.* have characterized in human peripheral blood and in secondary lymphoid tissues a dendritic cell subset called DC-10 that could be also generated *in vitro* from peripheral blood monocytes in the presence of IL-10 (Gregori, 2010). DC-10 express significantly high levels of the tolerogenic molecules such as immunoglobulin-like transcript ILT2, ILT3, ILT4, and HLA-G and secrete IL-10 and IL-6. These dendritic cells induce Tr1 differentiation *in vitro via* the ILT4/HLA-G pathway that is dependent on IL-10. Autocrine secretion of the IL-10 by DC-10 sustains ILT4 expression on DCs and HLA-G1 on T cells (Gregori, 2010).

Another regulatory subset- Th3, produce preferentially TGF-β (Chen, 1994; Fukaura, 1996). While some studies have reported expression of Foxp3 in Th3 cells, others have not detected its expression (Okamura, 2012; Ramsdell, 2003). Both, Tr1 and Th3 have been found to be involved in intestinal immunity and oral tolerance (Battaglia, 2004).

These studies showed a role for regulatory $Foxp3^{-}$ subsets in controlling immune responses. Nevertheless, the $Foxp3^{+}CD4^{+}$ population represents the major regulatory subset and is presented in more detail in the section below.

Natural and induced Foxp3+ CD4+ regulatory T cells

In vivo, at least two types of Foxp3$^+$ regulatory T cells have been distinguished; natural-occurring T_{reg} (nT_{reg}) and induced T_{reg} (iT_{reg}). Natural T_{reg} refers to regulatory T cells that differentiate in the thymus (detailed in paragraph 2.1.2) whereas induced T_{reg} characterizes conventional CD4$^+$ T cells wherein Foxp3 expression is upregulated in the periphery. The latter cells are generated following TCR stimulation under specific environmental and cytokine conditions, especially those associated with inflammation (Walker, 2003), reviewed in (La Cava, 2008). Although it has been shown that the *in vitro* conversion of conventional CD4$^+$ T cells towards a regulatory phenotype is relatively simple and requires the presence of TGF-β (Chen, 2003), the question of how these cells are induced and maintained in the periphery remains unclear. Recently, the role of the microbiota has started to be appreciated as an important factor shaping the phenotype of lymphocytes residing within the gut (Guarner, 2003), reviewed in (Round and Mazmanian, 2009) and in (Round, 2010). Some studies performed with germ-free (GF) mice have shown that the microbiota can modulate T_{reg} suppressive functions and affect oral tolerance (Min, 2007; Moreau, 1996; Östman, 2006; Rask, 2005). Further experiments including colonizing GF mice with a single species of bacteria or treatment of specific pathogen free (SPF) mice with antibiotics have shown that some microbiota, such as *Bacteroides fragilis* or *Clostridium*, can specifically induce regulatory T cells, (Atarashi, 2010; Round, 2010) and are critical for maintaining intestinal homeostasis (Atarashi, 2013; Atarashi, 2010). The exact mechanisms linking T_{reg} to the commensal microbiota are still not fully understood, but recent studies demonstrate an important role for bacterial metabolite- butyrate in T_{reg} generation (Arpaia, 2013; Furusawa, 2013).

Markers distinguishing natural and induced T_{reg} cells

In humans, but not mice, the Foxp3 gene is upregulated upon activation by conventional T cells (Gavin, 2006; Wang, 2007; Ziegler, 2006). As such, there has been an extensive search for other T_{reg}-specific markers. Additional markers including CD25, glucocorticoid-induced TNFR family-related protein (GITR) and CTL-associated molecule-4 (CTLA-4) have been proposed as being T_{reg}-specific (Kavanagh, 2008; Kolar, 2009; Ronchetti, 2002). While none of these proteins is expressed

exclusively on T_{reg}, they have been used to identify T_{reg} subsets presenting different suppressive functions.

To date, no single marker has allowed nT_{reg} to be distinguished from iT_{reg}. However, studies using microarray analyses have shown that *Ikzf2* (Helios) and *Nrp1* (Neuropilin-1) are upregulated in nT_{reg} as compare to iT_{reg} (Sugimoto, 2006). Helios is a transcription factor belonging to the Ikaros family whose role is still not fully understood. *Thornton et al.* proposed Helios as a nT_{reg}-specific marker as it was found to be expressed in $Foxp3^+$ T cells isolated from healthy mice but not in iT_{reg} (Thornton, 2010). However, this conclusion was challenged by *Akimova et al.*, who found that Helios was transiently induced in activated and intensively proliferating conventional T cells (Akimova, 2011). Furthermore, more recently, Helios was found to be a hallmark of strongly autoreactive T cells (Daley, 2013).

Neuropilin-1 (Nrp-1) is a surface glycoprotein mediating prolonged interactions between T_{reg} and dendritic cells (Sarris, 2008). It has been recently proposed as a surface molecule specific for nT_{reg} (Hansen, 2012; Yadav, 2012). However, these results have been challenged by other studies. Conditional knockout of Nrp-1 on T cells do not affect T_{reg} generation (Corbel, 2007). Further, it has been shown that Nrp-1 expression is positively regulated by TGF-β and can also be expressed on iT_{reg} at very high level under inflammatory conditions such as in the spinal cord of mice developing experimental autoimmune encephalomyelitis (EAE) (Weiss, 2012). Moreover, exclusive deletion of this gene in $CD4^+$ T cells provokes the expansion and cytokine production of Th17 both *in vitro* and *in vivo* (Solomon, 2011). Therefore, it is difficult to accept the notion that Nrp-1 distinguishes nT_{reg} from iT_{reg}. Nonetheless, *Weiss et al.* observed that low Nrp-1 levels were associated with the expression of Dapl1 (death-associated protein-like 1), which is absent in natural T_{reg} cells (Weiss, 2012). Thus, the profile of expression of both Nrp-1 and Dapl1 in T_{reg} may provide an useful tool to distinguish between these 2 regulatory T cell subsets.

A summary of some of the differences between natural-occuring and induced regulatory T cells are presented in Table 2.

Characteristic	Natural Treg cell	Induced Treg cell
Site of induction	Thymus	Secondary lymphoid organs/inflamed tissues
Costimulation requirement	CD28	CTLA-4
Cytokine requirement	IL-2, TGF-β?	TGF-β, IL-2, RA (potentiation)
Specificity	Self-antigens	Self- and foreign antigens
Common markers	Foxp3, CD25, GITR, CTLA-4	
Specific markers	Helios, Nrp1, PD-1, Swap70	Dapl1, Igfbp4
Mechanism of suppression	Cell contact–dependent	Cytokine-dependent?

Table 2. Comparison between natural and induced regulatory T cells. Copied from (Bilate and Lafaille, 2012).

Foxp3 gene

The critical role of Foxp3 in T_{reg} function has been demonstrated, at least in part, by the finding that the ectopic expression of Foxp3 in conventional T cells results in a suppressive phenotype (Fontenot, 2003). Interestingly, the suppressive functions appear to depend on the quantity of Foxp3 protein (Wan and Flavell, 2007) but importantly, introduction of Foxp3 into conventional T cells from IPEX patients converts them into stable and functional regulatory T cells (Passerini, 2013). Genome-wide analyses of Foxp3 target genes suggest that Foxp3 acts both as a transcriptional activator and inhibitor (Zheng, 2007). Studies employing epigenetic methods provided new insights into the molecular regulation of Foxp3 expression and stability. Analysis of proximal conserved non-coding sequences (CNS) revealed that several intronic elements play important roles in controlling Foxp3 expression. CNS1 (TGF-β-sensitive enhancer element), the first Foxp3 regulatory element, contains NFAT and Smad3 binding sites. Although CNS1 deletion resulted in severely impaired Foxp3 induction in the periphery, it did not affect natural-occurring T cell development (Zheng, 2007). This observation suggests that CNS1 role is redundant in nT_{reg} but not in iT_{reg}, consistent with the role of TGF-β/Smad pathway (Schlenner, 2012) in the generation of peripheral iT_{reg}.

Another intronic element termed CNS2 (also called T_{reg}-specific demethylated region-TSDR) contains a CpG islands whose methylation correlates tightly with Foxp3 expression and stability (Floess, 2007; Kim and Leonard, 2007). CNS2 appears to be completely demethylated in nT_{reg} but only partially in iT_{reg} (Baron, 2007; Floess, 2007). Consistent with these data, the TGF-β-dependent induction of Foxp3 is

associated with only a partial demethylation of CNS2, and can be reversed upon restimulation (Kim and Leonard, 2007). The finding that CNS2-deficient mice exhibit a reduced number of regulatory T cells only after 6 months of age suggests that CNS2 is not critical for initial Foxp3 expression but rather, for its stable maintenance (Zheng, 2010).

CNS3 (a pioneer element) contains a DNase I hypersensitive site (Zheng, 2010). It is bound by c-Rel in cooperation with other transcriptional factors, e.g., NFAT, Smad3, CREB and facilitates the formation of a c-Rel-containing enhanceosome at the Foxp3 promoter (Ruan, 2009). In addition to c-Rel, it has been demonstrated that Foxo1/3 and Stat5 can facilitate Foxp3 transcription by binding to its promoter and other regulatory elements (Harada, 2010; Ouyang, 2010; Yao, 2007).

The complexity of the conserved non-coding sequences in the Foxp3 promoter suggests that expression of this gene is highly regulated and indeed, a genetic tagging system allowing Foxp3 induction and downregulation to be concurrently followed *in vivo* demonstrated that Foxp3 expression can be extremely unstable (Yurchenko, 2012; Zhou, 2009). T_{reg} that downregulated Foxp3 (called here 'Ex-Foxp3') T cells display an activated-memory T cell phenotype and secrete IFNγ and low levels of IL-17 (Zhou, 2009). These data are in agreement with the observation of *Duarte et al.* demonstrating that under lymphopenic conditions, part of the T_{reg} cells can spontaneously lose Foxp3 expression and convert to helper phenotype due to the competition for IL-2. These pathogenic ex-Foxp3 cells can secrete IL-2, but do not exhibit suppressive functions and promote tissue infiltration (Duarte, 2009). Interestingly, expression of Foxp3 is not sufficient to maintain a suppressive T_{reg} cell phenotype. Interaction of Foxp3 with the Ikaros family member Eos is required for inhibition of Foxp3 target genes and in its absence, $Foxp3^+$ lymphocytes acquire partial effector function with IL-2 and IFNγ expression (Pan, 2009). Furthermore, in the presence of IL-6, the downregulation of Eos in $Foxp3^+$ T_{reg} has been shown to drive their rapid reprogramming to an effector phenotype (Sharma, 2013).

It has been demonstrated that stability of the Foxp3 expression is regulated by the level of methylation (Sauer, 2008) and that regulatory cells exhibit characteristic hypometylation that is required for the full suppressive activity of these cells (Morikawa, 2014; Ohkura, 2012). The establishment of T_{reg}-specific DNA methylation

pattern is associated with expression of other T_{reg}-associated genes such as the Helios, Eos or CTLA-4 (Morikawa, 2014; Ohkura, 2012).

The studies discussed here show the complexity of transcriptional and phenotypic regulation in T_{reg} cells, involving multiple factors. The stability of T_{reg} cells needs to be considered when devising therapeutic strategies based on the adoptive transfer of these lymphocytes.

T_{reg} suppression mechanisms

Suppressive functions of regulatory cells involve many different mechanisms still not fully understood that vary between humans and mice [Table 3]. In particular, immune tolerance can be mediated by the cytokines produced by regulatory cells, such as TGF-β and IL-10. These cytokines can suppress conventional T cell proliferation (Battaglia, 2004; Bettelli, 1998; Miyara and Sakaguchi, 2007) but can also drive naïve T cell conversion to an iT_{reg} phenotype (Chen, 2003). Moreover, some T_{reg} are able to produce granzyme B, thereby directly killing NK cells and CTLs (Cao, 2007). CTLA-4, a Foxp3-dependent gene, is a T_{reg}-associated molecule that plays an important role in T_{reg} suppressive functions (Hori, 2003). *Ctla4*$^{-/-}$ mice develop a severe lethal autoimmunity and die by 3-4 weeks of age (Chambers, 1997; Jain, 2010; Tivol, 1995). Furthermore, the specific deletion of *Ctla4* in Foxp3^{+} T cells also results in the development of autoimmunity, although the life span of these mice is significantly increased in comparison to constitutional CTLA-4 deletion (Wing, 2008). CTLA-4 competes with the co-stimulatory molecule CD28 for the binding to CD80 and CD86 on the surface of APCs (Tai, 2012; Thompson, 1997). Another proposed mechanism of suppression involves the ectoenzymes CD39 and CD73, which together hydrolyze extracellular ATP into ADP and/or AMP into adenosine. Extracellular adenosine inhibits effector T cell function (proliferation and IL-2 production) through the activation of the adenosine receptor 2a (A2aR) (reviewed in (Vignali, 2008).

Key molecule(s)	Function	Refs	
		Mouse	Human
Mechanisms of contact-dependent suppression			
CTLA4	Downregulation of APC co-stimulatory function	76	–
	Interaction with CD80 and CD86 on conventional T cells	101	–
CD73–CD39	Hydrolysis of inflammatory extracellular ATP	102	–
LAG3	Induction of inhibitory signalling through MHC class II molecules	103	–
Granzyme B (mouse) and granzyme A (human)	Lysis of conventional T cells	104,105	106
CD95–CD95 ligand	Induction of apoptosis in conventional T cells	–	107
Mechanisms of cytokine-mediated suppression			
TGFβ and LAP	Induction of FOXP3 in conventional T cells	108,109	–
IL-10	Attentuation of DC function	–	20
	Conversion of conventional T cells to T_R1 cells	110,111	111
Galectin 1	Cell cycle arrest and apoptosis in conventional T cells	112	–
CD25	Adsorption of IL-2	113	–
IL-35	Induction of conventional T cell expression of IL-35 by T_{Reg} cells enhances suppression (IL-35 is not expressed by human T_{Reg} cells)	114	115

Table 3. Different mechanisms of suppression. APC- antigen presenting cell, CTLA-4- cytotoxic T lymphocyte antigen 4, DC- dendritic cell, Foxp3- forkhead box P3, IL- interleukin, LAG3- lymphocyte activation gene 3, LAP- latency-associated peptide, TGF-β- transforming growth factor β, T_{reg}- regulatory T cell. Copied from (Sakaguchi, 2010).

Regulatory Foxp3$^+$ T cells in tumor immunity

Due to their suppressive function, Foxp3$^+$ T_{reg} can facilitate tumor escape from the immune response (Onizuka, 1999; Shimizu, 1999). It has been long known that the tumor environment can be highly enriched in Foxp3$^+$ regulatory T cells (Nishikawa, 2010; Wilke, 2010). Although the origin of tumor-infiltrating T_{reg} remains controversial, it is probably the result of two non-exclusive phenomena; the *in situ* generation of iT_{reg} (Valzasina, 2006) and the enhanced migration and/or proliferation of nT_{reg} (Curiel, 2004; Zhou, 2007a) [Figure 10]. This is particularly important as an increased ratio of T_{reg}:T_{eff} in tumor tissues has been associated with a poor prognosis for patients with ovarian, breast and gastric cancers (Sato, 2005), reviewed in (Nishikawa, 2010). Moreover, it has been reported that CD25$^+$CD4$^+$ T cells suppress the expansion of antigen-specific effector cells (Darrasse-Jèze, 2009; Sugiyama, 2013; Touil, 2012). Nevertheless, high infiltration of Foxp3$^+$ T_{reg} has also been associated with improved survival of patients with colon, head/neck cancers, Hodgkin lymphoma, diabetes, (Alvaro, 2005; Badoual, 2006; Salama, 2008; Xu, 2013;

Yoon, 2012). As this observation appears to be counterintuitive, it will be important to elucidate the mechanisms *via* which T_{reg} result in an improved survival in these patients.

Generally though, one strategy to increase an anti-tumoral response is based on decreasing the number of regulatory cells, and more specifically intra-tumoral T_{reg}. As T_{reg} constitutively express CD25 (the IL-2 high affinity α-receptor), this molecule has been proposed as a suitable target for T_{reg} cell depletion (Sakaguchi, 1995). Indeed, in mouse models, administration of a cell-depleting anti-CD25 mAb before tumor inoculation resulted in its eradication (Shimizu, 1999). While some clinical trials employing the *in vivo* administration of an anti-CD25 mAb have shown the potential of this strategy to augment anti-tumor immune responses, other studies have failed to obtain clinically relevant outcomes (Attia, 2005; Jacobs, 2010; Rech, 2012). The contradictory data may, at least in part, be explained by the fact that activated effector T cells also express CD25 and their IL-2 production is required for the expansion of $CD8^+$ CTLs (Williams, 2006). As such, a CD25-based mAb therapy resulting in the depletion of activated effector T cells can also negatively impact an anti-tumor immune response. Thus, the combination of a strategy reducing T_{reg} numbers or attenuating their suppressive activity in tumor tissues together with the activation of tumor-specific effector T cells (e.g., by cancer vaccine) should enhance the efficiency of current cancer immunotherapies.

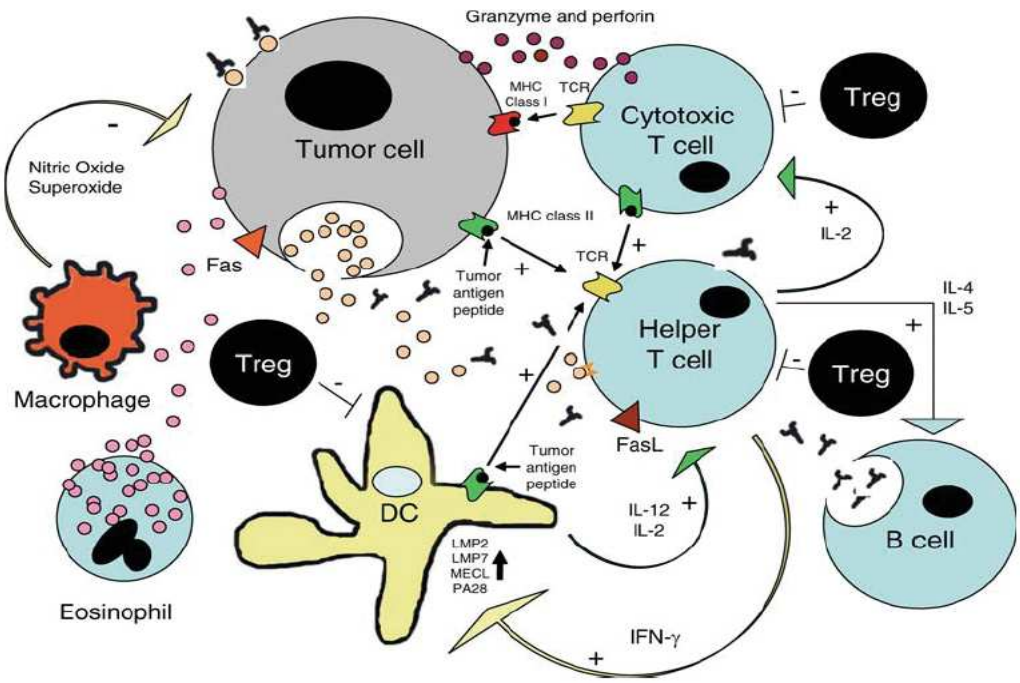


Figure 10. The role of T helper cells in the tumor micro-environment. Helper cells can interact with a tumor cell *via* direct and indirect mechanisms and also induce anti-tumor responses by activation of other immune cells. "+": positive interactions; "-": detrimental effect. Copied from (Nakayamada, 2012).

3. T cell metabolism

Upon TCR stimulation, naïve T cells increase in size, initiate proliferation and acquire effector functions (refer to section 2.2). These processes are energetically demanding and T cells undergo metabolic reprogramming to meet the various biochemical and biophysical requirements (reviewed in (Pearce, 2013) [Figure 11]. The changes in lymphocyte metabolism are dynamic and tightly correlate with the activation state of the cell.

Naïve T cells have low rates of glucose uptake, glycolysis and translation. Glucose oxidation *via* OXPHOS and β-oxidation of fatty acids (FAO) in the mitochondria are the primary sources of ATP. Upon antigen encounter, the acquisition of effector function is associated with a suppression of the β-oxidation pathway and induction of a metabolic reprogramming to glycolytic, pentose-phosphate and glutaminolytic pathways (Wang, 2011), reviewed in (Gerriets, 2012) and in (Wang and Green,

2012). The switch from OXPHOS to aerobic glycolysis, even when the oxygen supply is not limited, is called the Warburg effect. While O. Warburg first described this metabolic signature in cancer cells (Warburg, 1956, 1958), it has more recently been shown to be a characteristic of actively proliferating T lymphocytes (Vander Heiden, 2009). The Warburg effect is intriguing, as it appears to be counter-productive, providing cells with fewer ATP molecules than OXPHOS (2 vs 36 molecules, respectively). Nevertheless, it may present several advantages. Indeed, the speed of ATP generation in aerobic glycolysis is quicker than in OXPHOS, which is suited to the energy demands of rapid proliferation. Moreover, it generates intermediates that fuel biosynthetic pathways which are critical for cell growth and proliferation. It is also important to point out that even though the glycolytic pathway is dominant in proliferating T lymphocytes, OXPHOS remains active (Wang, 2011).

Memory T cells, on the other hand, exhibit a metabolic profile similar to naïve T cells. After infection clearance, $CD8^+$ T cells decrease nutrient uptake and exhibit lower biosynthesis, a state associated with a switch back to the OXPHOS and FAO (Araki, 2009; Pearce, 2009). However, they display a higher mitochondrial biomass than naïve or effector cells, suggesting a potentially higher mitochondrial spare respiratory capacity (SRC). SRC represents the additional capacity of a cell to produce ATP in response to stress, such as infection, that correlates with cellular survival. Recently *Windt and colleagues* have shown that high mitochondrial mass allows a rapid ATP synthesis in memory cells upon TCR stimulation, giving the cells a bioenergetic advantage upon re-challenge (van der Windt, 2012).

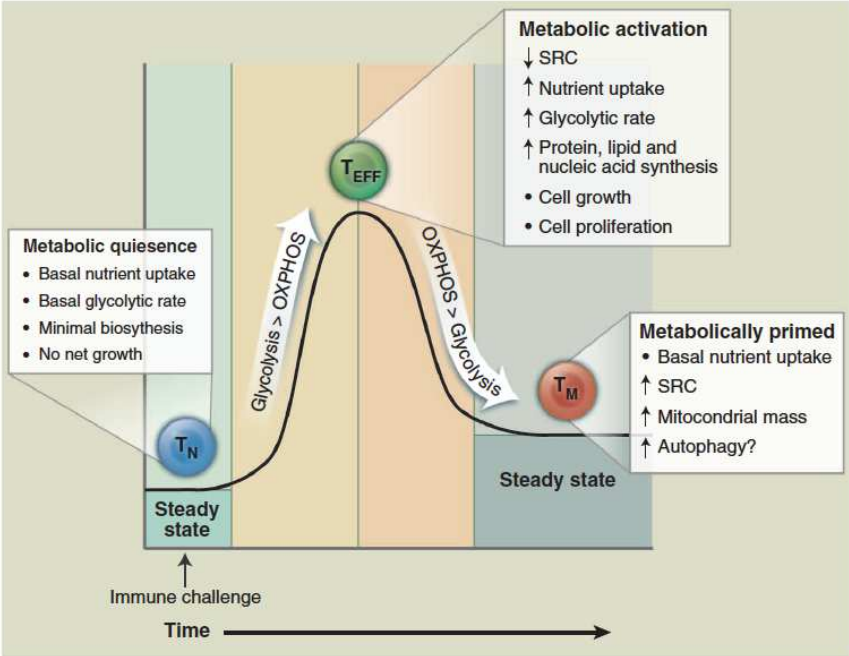


Figure 11. Metabolic shifts over the course of an immune response. T cells depending on their activation state display distinct metabolic profiles. Naïve T cells (T_N) are metabolically quiescent, and exhibit basal levels of nutrient uptake and use OXPHOS as their primary source of energy. Following TCR stimuation, T_{EFF} cells shift to a state of metabolic activation characterized by increased nutrient uptake, elevated glycolytic and glutaminolytic metabolism, biomass accumulation and reduced mitochondrial SRC. Effector T cells mainly use glycolysis for ATP production. Memory T cells (T_M) shift back to the quiescent metabolism, but with increased reliance on FAO to fuel OXPHOS. Mitochondrial mass and SRC are increased in T_M, suggesting that these cells are metabolically primed to respond upon reinfection. Copied from (Pearce, 2013).

In order to fulfill all their bioenergetic and biosynthetic needs, lymphocytes, like all other cells, depend on cellular energy supplies. In the following paragraphs, I will focus on two crucial sources of ATP, glucose and amino acids (refer to section 3.1 to 3.3)

3.1 Role of glucose

The primary source of energy and carbon for T lymphocytes is glucose (Rathmell, 2000), reviewed in (Pearce, 2010). Upon mitogen-induced activation of lymphocytes, glucose uptake and consumption are highly increased (Kinet, 2007; Manel, 2002). Additionally, in the absence of glucose, proliferating T cells undergo apoptosis (Frauwirth, 2002; Jacobs, 2008; Rathmell, 2000).

Glucose transporters

In most mammalian cells, glucose is transported by several members of the glucose transporter family called Gluts. The Glut family comprises 14 isoforms in humans and 13 in mice (Uldry, 2004; Zhao, 2007). The structure of all isoforms is highly conserved and they all contain 12 transmembrane helices. All Gluts are highly glycosylated proteins harboring a N-linked glycosylation site and depending on their localization can be divided into three classes. Class I and II family members, have an N-linked glycosylation positioned in the first exofacial loop between transmembrane helices 1 and 2. On the other hand, the class III family members contain a shorter extracellular loop 1 and harbor a glycosylation site within the larger loop 9 (Augustin, 2010). Schematic structures of the class I, II and III Gluts are presented in [Figure 12].

T cell activation is associated with the upregulation at the cell-surface of several nutrient transporters, including Glut1 (MacIver, 2008; Manel, 2002, 2005; Rathmell, 2000). Glut1 surface expression can be detected as early as 4 hours after TCR stimulation and requires *de novo* protein synthesis (Manel, 2002). Although the precise mechanisms by which T cells control Glut1 expression and trafficking have not yet been fully characterized, PI3K signaling through AKT is recognized to be essential for this process (MacIver, 2008). Interestingly, over-expression of Glut1 does not alter T cell development or homeostasis but has been associated with increased cell size, glucose uptake, a higher proliferation rate and an increased IL-2 and IFNγ secretion (Jacobs, 2008; Michalek, 2011).

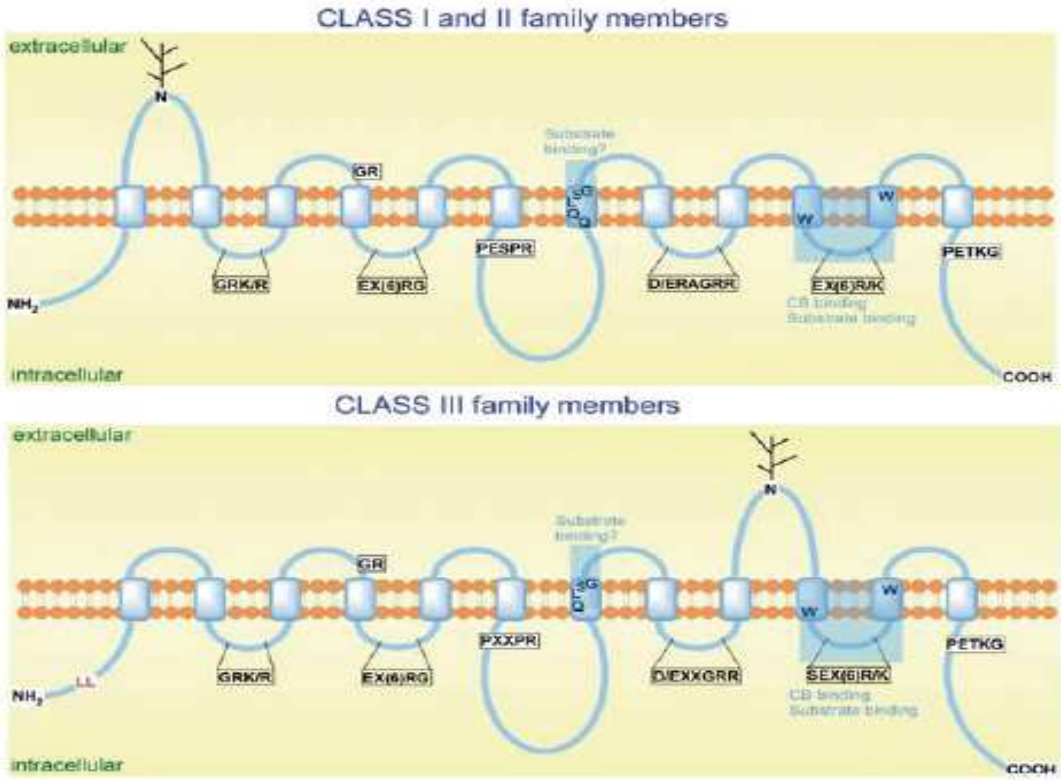


Figure 12. Structure of GLUT family members. Schematic of class I/II (upper panel) and class III (lower panel) GLUT family members are presented. Copied from (Augustin, 2010).

3.2 Importance of amino acids in T cell activation

T lymphocytes not only use glucose for energy but also amino acids [Figure 13]. Amino acids serve as the basic substrate for protein synthesis and many metabolic processes. One of the most important sources of carbon and a major carrier of nitrogen between organs is glutamine. Glutamine is a non-essential amino acid that accounts for approximately 50-60% of the intracellular free amino acid pool in skeletal muscle and more than 20% in plasma (Newsholme, 1990; Roth, 1990). Cells can produce glutamine in sufficient quantities under stable conditions, but it becomes a limiting substrate during T cell proliferation (Hörig, 1993).

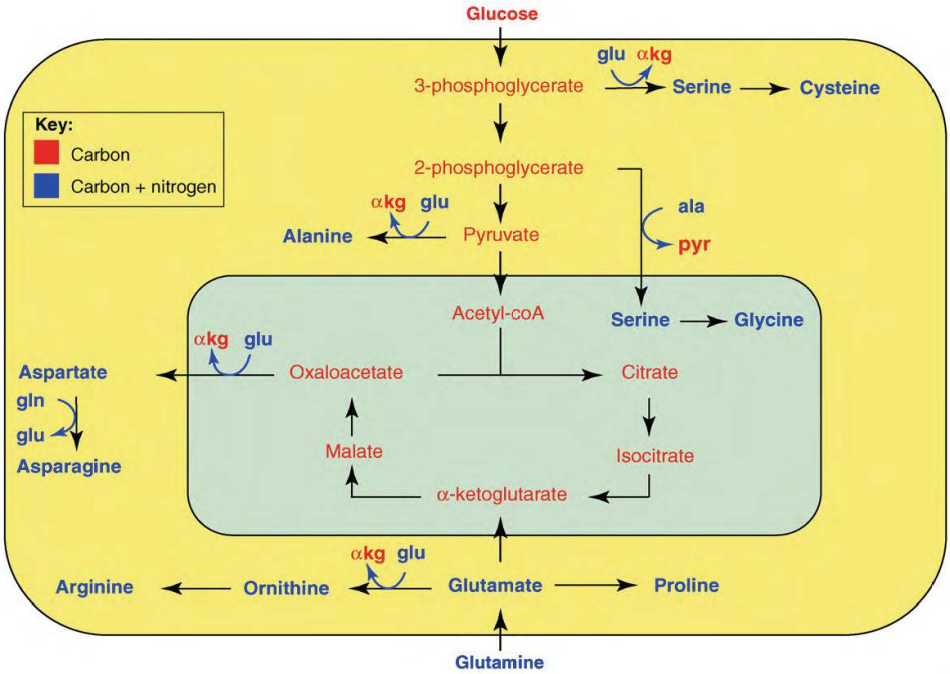


Figure 13. Utilisation of glucose and glutamine in a T cell. Glucose (glu) and glutamine (gln) serve as a source of carbon and nitrogen for the biosynthesis of nonessential amino acids. Substrates containing carbon, but not nitrogen (in red) and containing carbon and nitrogen (in blue) are shown. Carbon precursors derived from glycolysis (3-phosphoglycerate, 2- phosphoglycerate, pyruvate) and glutaminolysis (OAA, GA g-semialdehyde) serve as substrates for amino acid biosynthesis. Glutamine-derived GA (glu) donates its amine group to these carbon substrates to produce nonessential amino acids (serine, alanine, aspartate, ornithine) and α-ketoglutarate (αkg). Alanine (ala) serves as substrate for production of serine and pyruvate (pyr) in the mitochondria for the synthesis of glycine. Glutamine provides the carbon and nitrogen for the synthesis of proline, ornithine and arginine. Glutamine can also serve as a direct nitrogen donor in the synthesis of asparagine from aspartic acid. Copied from (Wise, 2010).

As indicated above, glutamine is involved in many cellular processes. It serves as a source of nitrogen for *de novo* nucleotide biosynthesis (both purines and pyrimidines). The conversion to glutamate by glutaminase (GLS), a mitochondrial phosphate-dependent enzyme, is used by a proliferating cell to generate non-essential amino acids such as arginine or proline.

Until recently ROS (reactive oxygen species), by-products of oxygen metabolism were associated with damaging effects on DNA (reviewed in (Cooke, 2003), proteins (Mallis, 2001) or lipids (Morel, 1983). However, it has been shown that ROS are also important mediators and take part in the regulation of different biological processes, such as signal transduction or control of protein synthesis (Gill, 2013; O'Loghlen, 2003), reviewed in (Gough and Cotter, 2011). Glutamine is involved in production of

the glutathione (GSH), a major endogenous antioxidant, protecting cells against ROS damage (Klimberg, 1996; Xu, 1997). Glutathione consists of three peptides (glutamate, cysteine and glycine) and its production is highly dependent on glutamine.

Glutamine's carbon backbone can also be oxidized in the mitochondria to produce ATP (reviewed in (Calder, 1994) and in (Calder, 1995; Fan, 2014; Newsholme, 1996a). This process requires conversion of glutamine through glutamate into α-ketoglutarate, by transaminases or glutamate dehydrogenase. α-ketoglutarate is then introduced into the TCA cycle. Mitochondrial oxidation of α-ketoglutarate generates malate, which can then be converted by $NADP^+$-dependent malic enzyme to pyruvate and generates NADPH. NADPH is required for biosynthetic reactions such as fatty acid synthesis, the production of free radicals, and the activity of the gluthatione reductase. The process of fueling the TCA cycle by glutamine is called anaplerosis. The diverse pathways in which glutamine is used are depicted in [Figure 14].

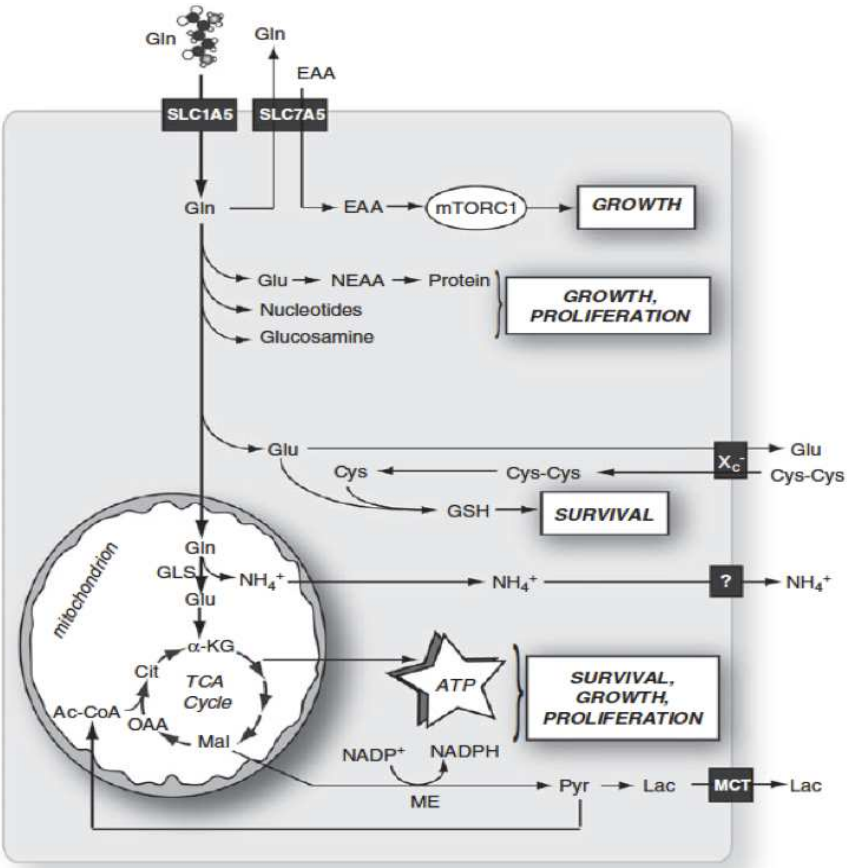


Figure 14. Glutamine supports cell survival, growth and proliferation through various mechanisms. Glutamine is transported via transporters such as SLC1A5. It can then either exit the cells in exchange for other essential amino acids (EAA) or be utilized in protein/nucleotide synthesis, signaling via glutaminate, or the TCA cycle. These pathways support the survival and proliferation of different cell types, Ac-CoA, acetyl-CoA; Cit, citrate; Cys, cysteine; Cys-Cys, cystine; GLS, glutaminase; Glu, glutamate; GSH, glutathione; Lac, lactate; Mal, malate; ME, malic enzyme; mTORC1, mammalian target of rapamycin complex 1; NEAA, nonessential amino acids; OAA, oxaloacetate; Pyr, pyruvate; SLC1A5, solute carrier family 1 (neutral amino acid transporter), member 5; SLC7A5, solute carrier family 7 (cationic amino acid transporter, y+ system), member 5; TCA, tricarboxylic acid; α-KG, α-ketoglutarate. Copied from (DeBerardinis 2010).

Glutamine transporters

Transfer of glutamine across the plasma membrane of mammalian cells occurs through multiple transporters that can be divided into two categories: Na^+-dependent and Na^+-independent. The Na^+-dependent transporters can be further divided into alanine–serine–cysteine transporters (ASCT) and the sodium-dependent neutral amino acid transporter (SNAT or SLC38) family (reviewed in (Bode, 2001).

Among ASCT transporters, the most important is ASCT2 (SLC1A5), which was in fact the first mammalian glutamine transporter isolated in 1996 (Utsunomiya-Tate,

1996). ASCT2 is specific for small, neutral amino acids (Newsholme, 1996b; Shafqat, 1993) and can transport them only in exchange with the same or other neutral amino acids (Bröer, 2000). Therefore, it can mediate either glutamine uptake or release (Bröer, 1999).

The SNAT family can be subdivided according to their properties into two groups: System A or System N. System A is an ubiquitous Na^+-dependent amino acid transport system that carriages small, neutral amino acids with short, unbranched side chains including alanine, asparagine, cysteine, glutamine, glycine, methionine, and serine (Christensen, 1965; Johnson and Smith, 1988; Oxender, 1963; Shotwell, 1981). This system composed of three different members of the *SLC38* gene family (*Slc38a1*, *Slc38a2*, and *Slc38a4*), gave rise to the three subtypes: SNAT1, SNAT2, and SNAT4 (reviewed in (Mackenzie and Erickson, 2004). System N on the other hand, consists of SNAT3 and SNAT5 and is characterized by its specific substrate affinity towards glutamine, histidine and asparagine (Chaudhry, 1999; Fei, 2000; Nakanishi, 2001a, b). Interstingly, System N can be driven not only by the Na^+ but also by Li^+ ions (Fei, 2000).

Further, Na^+-independent category of transporters includes LAT1 and LAT2, which function as amino acid exchangers (Kanai, 1998; Pineda, 1999; Segawa, 1999). Both isoforms are heterodimeric and consist of 4F2hc (CD98) as the heavy chain and either LAT1 (SLC7A5) or LAT2 (SLC7A8) as the light chain (Fei, 1995; Pfeiffer, 1999; Rajan, 2000; Torrents, 1998). Moreover, it is the light chain that determines the extent of the resulting transport activity (Campbell, 2000).

Although, the mechanisms of regulation of amino acid transporters have been described for many tissues, not much is known regarding the glutamine transport in lymphocytes. It has been shown that resting T cells express no or very low levels of neutral amino acid transporters such as ASCT2 (SLC1A5) and SLC7A5. However, upon TCR stimulation, a strong upregulation of these transporters (Carr, 2010; Edinger, 2007; Gottesdiener, 1988; Levring, 2012; Sinclair, 2013) and other glutamine transporters (SLC3A2, SLC38A1) has been observed (Wang, 2011). Furthermore, there are growing evidences suggesting the importance of glutamine transport for optimal mTORC1 pathway activation (Nakaya, 2014; Nicklin, 2009) and its link with T cell differentiation (Nakaya, 2014).

3.3 Energy sensing pathways

It is becoming increasingly clear that T cell functions are linked to metabolism. The mechanisms underlying the "choice" of the specific metabolic programs adopted by T cells and the impact of these pathways on immunological outcome remain unclear. However, several metabolic pathways, including those regulated by mTOR, LKB1 and c-Myc, have been demonstrated to be intricately involved in T cell function.

3.3.1 mTOR in T cell activation

The mTOR (mammalian target of rapamycin) pathway is one of the central sensors of nutrients and extracellular signals transducer coordinating T cell metabolism, protein synthesis, energy balance, proliferation and survival (reviewed in (Hay, 2004) and in (Laplante and Sabatini, 2009) and in (Sengupta, 2010).

The kinase mTOR is an evolutionarily conserved member of the phosphatidylinositol-3-OH kinase (PI(3)K)-related kinase family that exists in two multiprotein complexes in metazoans. The mTOR complex 1 (mTORC1) contains a regulatory associated protein of mTOR (RAPTOR), as well as the mLST8, PRAS40 and Deptor subunits (Laplante and Sabatini, 2009). The mTORC1 pathway can be activated by many signals such as the small GTPase RHEB (RAS homologue enriched in brain). The tuberous sclerosis 2 (TSC2) gene product and its obligate partner TSC1 inhibit mTORC1 indirectly *via* inhibition of RHEB. Moreover, loss of either TSC1 or TSC2 was associated with hyperactivation of mTORC1 (reviewed in (Huang and Manning, 2008).

The PI3K-AKT pathway, further upstream, can inactivate the TSC1/TSC2 complex whereas the AMP-activated protein kinase (AMPK) enhances its activity (refer to section 3.3.2). The activity of the mTORC1 complex can be canonically measured by the phosphorylation of its substrates: ribosomal S6 kinase (p70S6K1) and eukaryotic Initiating Factor 4E Binding Protein (4E-BP1) (Beugnet, 2003), reviewed in (Harris, 2003) and in (Jastrzebski, 2007). S6K has many targets that play a role in the translational machinery such as S6 small ribosomal subunit, eIF4B and programmed cell death 4 protein (PDCD4) (Beugnet, 2003; Schmid, 2008). The phosphorylation of 4E-BP1 releases the translation-initiation factor eIF-4e, which in turn leads to

increased protein synthesis. mTORC1 is nutrient-sensitive and acutely inhibited by the immunosuppressant rapamycin (Choo, 2008; Hunter, 2009).

The mTORC2 complex is composed of a distinct scaffolding protein, the rapamycin-insensitive companion of mTOR (RICTOR), and its activity can be measured by phosphorylation of AKT at Ser473. Interestingly, AKT can both induce mTORC1 activity as well as act as a downstream target of mTORC2. Moreover, in T cells, the AKT-dependent induction of mTORC1 activity does not require mTORC2 (Delgoffe, 2011; Guertin, 2006; Lee, 2010b). mTORC2 is involved in regulating metabolism, apoptosis and cytoskeletal organization (reviewed in (Laplante and Sabatini, 2012). While mTORC2 was considered to be insensitive to rapamycin, it has become clear that prolonged treatment with rapamycin can lead to the inhibition of mTORC2 signaling in some tissues and cell types (Sarbassov, 2006) mTORC regulation and signaling pathways are presented in [Figure 15].

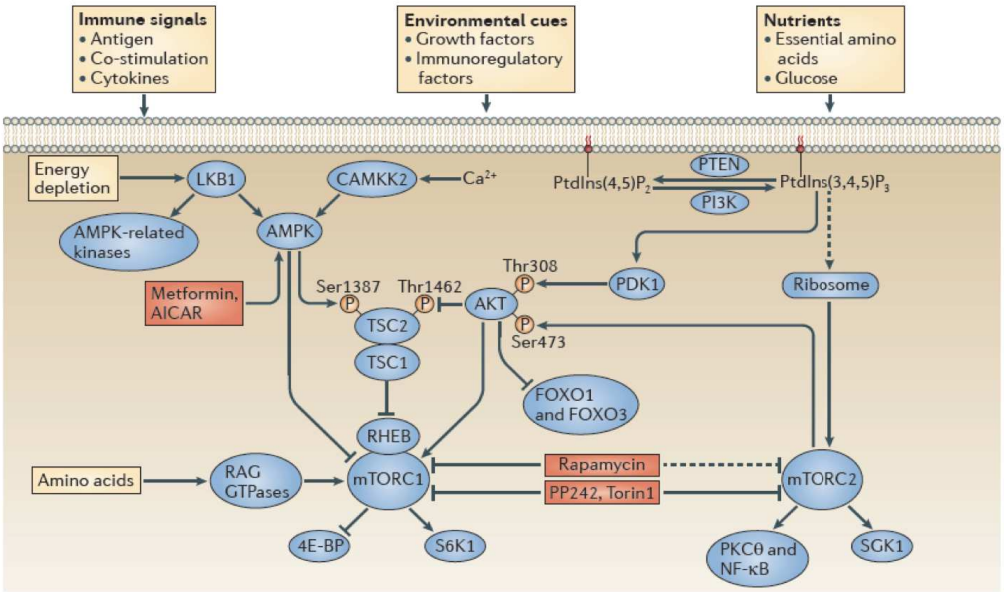


Figure 15. mTOR signaling pathways in T cells. mTOR pathways can be activated by many signals such as antigenic stimulation, co-stimulation and cytokines; growth factors and immunomodulatory factors such as leptin, sphingosine-1-phosphate (S1P) or nutrients. The signals from the phosphoinositide 3-kinase (PI3K)–AKT and liver kinase B1 (LKB1)–AMP-activated protein kinase (AMPK) pathways are integrated by the tuberous sclerosis 1 (TSC1)–TSC2 complex. TSC2 can be activated through AKT-dependent phosphorylation of threonine 1462 (Thr1462) and AMPK-dependent phosphorylation of serine 1387 (Ser1387). Upon TCR stimulation the TSC complex is inactivated which allows the activation of mTOR complex 1 (mTORC1). In contrast, TSC function is maintained in naive T cells, whereas mTOR activation is low. Moreover, AKT and AMPK can directly modulate mTORC1 functions independently of TSC and the small GTPase RHEB. Also amino acids can activate mTORC1 *via* the RAG family of small GTPases. mTORC1 activation results in promoting translation initiation and protein synthesis by directly phosphorylating ribosomal protein S6 kinases (S6Ks) and eIF4E-binding proteins

(4E-BPs). Further, mTORC1 is involved in cell signaling, metabolism and autophagy. mTORC2 is crucial for the optimal activation of AKT through the phosphorylation of Ser473. Additionally, mTORC2 phosphorylates various protein kinase C (PKC) isoforms, such as PKCθ, which activates nuclear factor-κB (NF-κB) as well as serum- and glucocorticoid-regulated kinase 1 SGK1). Copied from (Chi, 2012).

mTOR in T cell differentiation

While the mTOR pathway is a central regulator of cell growth and metabolism, there are a growing number of studies suggesting that mTOR signaling is tightly linked to the activation state of T lymphocytes (reviewed in (Powell, 2012). Following TCR stimulation, the level of mTOR activation depends on the antigen dose and the duration of the T cell/APC interaction (Katzman, 2010; Turner, 2011). Moreover, co-stimulatory signaling through CD28 is necessary for the optimal activation of mTORC1 (Zheng, 2009). In contrast, ligation of inhibitory receptors such as CTLA-4 and PD-1, results in diminished mTOR activity and is associated with induction of T_{reg} (Francisco, 2009; Karman, 2012). Another example of the interplay between mTOR and TCR signaling is the fact that both, Lck and Fyn phosphorylation, directly downstream of the TCR (see section 2.2), are required for the TCR-induced mTOR activation (Salmond, 2009). This is in agreement with the observation that the rapamycin-dependent block of mTOR impairs Lck and ZAP-70 activation (Procaccini, 2012). Moreover, PI(3)-K/mTOR activation is required to prevent atrophy and loss of glucose metabolism in response to IL-7 (Rathmell, 2001).

mTOR complexes are involved not only in T cell activation but also in T cell differentiation in response to diverse cytokines. $CD8^+$ effector T cell differentiation is facilitated by the IL-12-mediated activation of mTOR (Rao, 2010). The mTOR pathway is also a crucial mechanism that governs the lineage choice of $CD4^+$ helper T cells. Notably, *Delgoffe and colleagues* have shown that mTOR activation is necessary for the up-regulation of Th1 and Th2 lineage-specific transcription factors (T-bet/STAT4 and Gata-3/STAT6, respectively) (Delgoffe, 2009). Specifically, the selective inhibition of mTORC1 impairs the differentiation of naïve T cells towards Th1 and Th17 subsets, whereas the generation of Th2 cells is dependent on active mTORC2 but not on mTORC1 (Delgoffe, 2011). Furthermore, in the absence of mTOR signaling, $CD4^+$ T cells fail to differentiate into effector cells and undergo a *de novo*

expression of Foxp3 (Sauer, 2008), (Delgoffe, 2009; Powell, 2012). Regulatory T cells have been associated with lower levels of mTOR activity (Haxhinasto, 2008; Kang, 2008; Sauer, 2008; Zeiser, 2008). Nevertheless, there is clearly some controversy that remains to be resolved, as *Zeng and colleagues* have demonstrated that mTORC1 signaling is required for T_{reg} function, activating cholesterol and lipid metabolism. They found that the mevalonate pathway is significant for orchestrating T_{reg} proliferation and expression of suppressive molecules such as ICOS or CTLA-4 (Zeng, 2013). In contrast, activation of mTOR, *via* deletion of the TSC1 negative regulator, was found to inhibit Foxp3 expression and T_{reg} function, while increasing Th1 and Th17 effector function (Park, 2013). Similarly, activation of mTOR *via* sphingosine 1-phosphate (S1P) receptor signaling has been shown to suppress T_{reg} generation and promote the development of Th1 cells (Liu, 2010). Moreover, activated mTORC2 promotes the transcription of the Foxp3 gene in iT_{reg} through Foxo transcription factors (Harada, 2010; Kerdiles, 2009; Ouyang, 2010). The proposed roles of different mTOR complexes during T cell differentiation are depicted in [Figure 16].

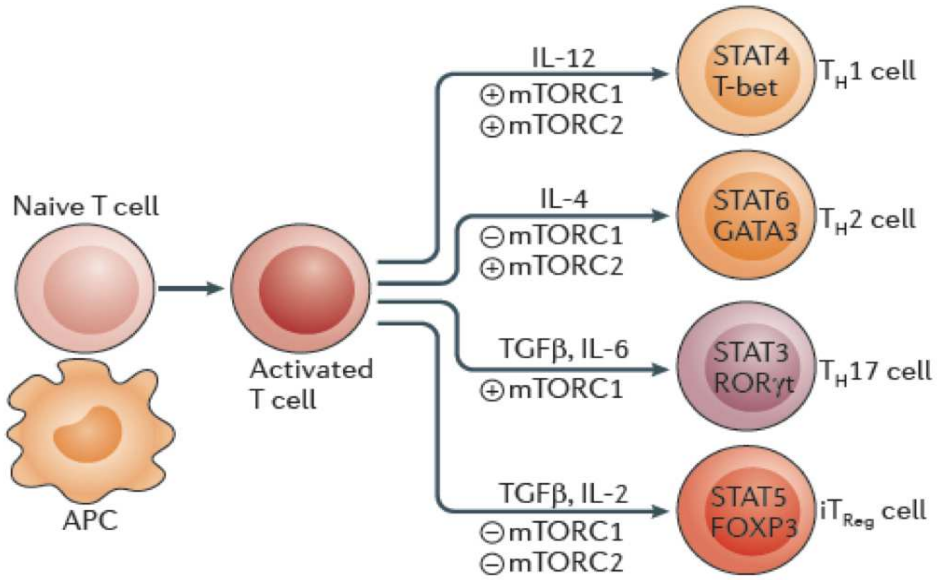


Figure 16. mTORCs play a critical role in T effector cells differentiation. Upon T cell stimulation by antigen presenting cells (APC), mTOR signaling promotes the differentiation of naïve T cells towards effector phenotype, such as Th1, Th2 or Th17, and inhibits the generation of induced regulatory T cells (iT_{reg}). Copied from (Chi, 2012).

3.3.2 The AMPK/LKB1 pathway

AMP-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase constituted of a catalytic α subunit and two regulatory subunits (β and γ). AMPK plays an important role in the control of proliferation, cell growth and autophagy by antagonizing mRNA translation through the negative regulation of mTORC1 (reviewed in (Shackelford and Shaw, 2009) and in (Hardie, 2011). ATP, ADP and AMP compete for nucleotide-binding sites of the γ regulatory subunit of AMPK, with AMP or ADP to promote, and ATP antagonize AMPK activation. Following an increase in the cellular AMP/ATP ratio (low energy level), AMP binding causes an allosteric change in AMPKγ conformation, thus exposing the AMPKα Thr172 residue for phosphorylation by LKB1, which markedly activates AMPK (Xiao, 2011). LKB1 was first characterized as the suppressor of Peutz-Jeghers syndrome; a disorder associated with intestinal polyps (hamartomas), mucocutaneous lesions, and a high risk of spontaneous epithelial carcinomas (Hemminki, 1998; Jenne, 1998). AMPK can also be activated by $Ca2^{+}$ (*via* CamKKb) (Hawley, 2005; Hurley, 2005; Woods, 2005) and TGF-β (*via* TAK1). Together, AMPK and LKB1 function as sensors of cellular energy homeostasis (Hardie, 2011; Oakhill, 2011; Xiao, 2011). Activation of AMPK acts to maintain a cellular energy balance by promoting catabolic ATP-generating pathways such as glucose and fatty acid oxidation, while simultaneously inhibiting anabolic ATP-utilizing pathways such as proteins and fatty acid synthesis. A schematic representation of the AMPK/LKB1 pathway is presented in [Figure 17].

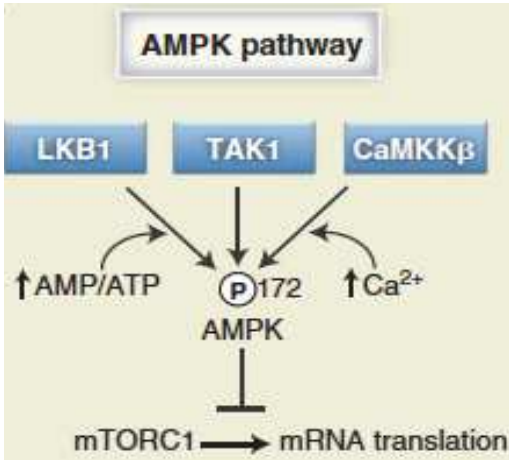


Figure 17. The AMPK pathway. AMPKa is activated by phosphorylation on Thr172 of its activation loop by the kinases LKB1, TAK1, or CaMKKb. LKB1 promotes enhanced AMPK phosphorylation under a high AMP:ATP ratio. One biological outcome of AMPK activity is the inhibition of mRNA translation under low-energy conditions through inhibition of mTORC1 activity. Copied from (Pearce, 2013).

It has been demonstrated that in conditions of bioenergetic stress, LKB1 is crucial for AMPK activation (Hawley, 2003; Shaw, 2004; Woods, 2003). Moreover, LKB1 is important for thymocyte development (Cao, 2009) and AMPK has been linked to stress resistance in lymphocytes (Mayer, 2008). Mouse and human T cells express the AMPKα1 catalytic domain but no detectable levels of AMPKα2 (Tamas, 2006). Additionally, TCR stimulation promotes LKB1-dependent AMPK activation in lymphocytes (MacIver, 2011; Tamas, 2006). *MacIver et al.* have shown that LKB1-deficient T cells exhibit defects in proliferation and viability in conditions of metabolic stress. Moreover, loss of LKB1 in the T cell compartment leads to an accumulation of IFNγ– producing $CD8^+$ and $CD4^+$ T cells in peripheral lymphoid organs. AMPKα1- and LKB1-deficient T cells exhibit elevated activation of mTORC1 and production of IFNγ (MacIver, 2011). Additionally, control of lipid metabolism by AMPK seems to regulate T cell fate. Stimulation of AMPK activity by its agonist metformin in activated T cells was shown to enhance $CD8^+$ memory T cells generation through the promotion of FAO (Pearce, 2009). Furthermore, it was recently shown that AMPK-deficient T cells are defective in their ability to generate $CD8^+$ memory T cells during infection (Rolf, 2013). These data suggest that the LKB1-AMK pathway functions as a critical regulator of T cell metabolism and function. The function of LKB1 is summarized in [Figure 18].

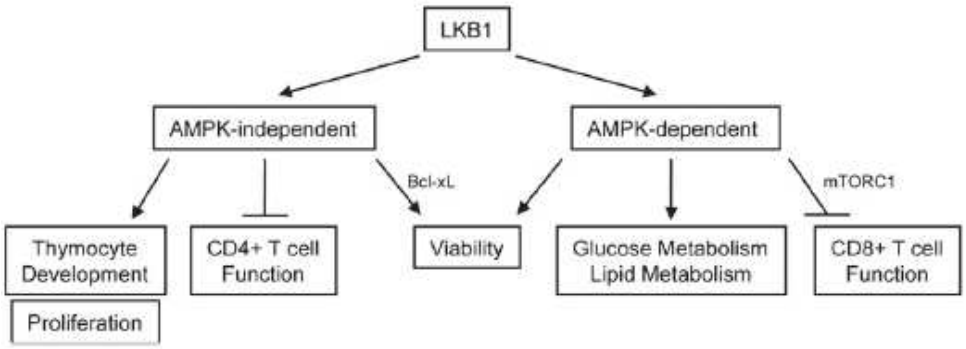


Figure 18. Model of the LKB1 role in T cell development and function. Copied from (MacIver, 2011).

3.3.3 cMYC

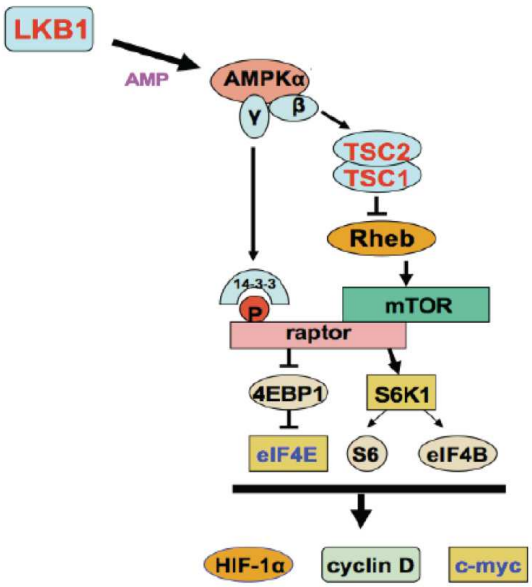
The basic region/helix-loop-helix/leucine zipper (bHLHZip) transcription factor cMyc is essential for embryonic development and disruption of the *c-myc* gene leads to death at an early embryonic stage (Davis, 1993; Trumpp, 2001) whereas overexpression may lead to development various types cancers, such as T-ALL (Felsher, 1999; Girard, 1996; Langenau, 2003). cMyc has been described to play a role in many cellular processes such as lymphocyte development (Dose, 2006; Huang, 2008), cell cycle entry (Mateyak, 1999; Walker, 2005), transcriptional activation, and transcriptional suppression, amongst others (Grandori, 2005; Nilsson and Cleveland, 2003). Expression of cMyc increases rapidly in response to growth factors such as cytokines (Lindsten, 1988). Furthermore, cMyc has been proposed by *Bianchi et al*. to be involved in IL-15-dependent homeostatic proliferation of $CD8^+$ memory T cells (Bianchi, 2006; Itsumi, 2009). TCR ligation can also lead to cMyc activation (Lindsten, 1988) and more recent data have been demonstrated that cMyc activation can be regulated by the strength of TCR signaling. Its expression has been increased upon high dose peptide stimulation as compared to low dose stimulation (Mehlhop-Williams and Bevan, 2014).

cMyc expression has been associated with lymphocyte development. Loss of cMyc gene in thymocytes resulted in blocking maturation of the DN stage (Douglas, 2001). *Iritani et al.* have proposed that a balance between cMyc and its antagonist Mad1 controls thymocytes development and expansion (Iritani, 2002). Moreover, cMyc has been also proposed as an enhancer of positive selection (Broussard-Diehl, 1996; Rudolph, 2000).

Importantly, cMyc target genes are also implicated in cell metabolism. In the studies of *Wang and colleagues* cMyc was found to play a critical role in promoting the metabolic programs necessary for shifting from fatty acid β-oxidation and pyruvate oxidation *via* the TCA cycle to aerobic glycolysis, pentose phosphate pathway (PPP), and glutaminolysis, thereby sustaining T cell activation (Wang, 2011). Deletion of cMyc significantly impaired activation-induced glutamine oxidation, which was associated with transcriptional downregulation of nutrient transporters such as SLC1A5, SLC3A2, and the glutaminase 2 enzyme (GLS2) that catalyzes the hydrolysis of glutamine to glutamate. The cMyc-dependent induction of glutaminolysis leads to

production of α-ketoglutarate and nucleotide synthesis. In addition, cMyc regulates polyamine biosynthesis to meet the significantly increased polyamine demands upon cells proliferation (Wang, 2011). Furthermore, *Wang et al.* have proposed that cMyc is coupled to the mTOR pathway. mTORC1 activity is required for sustaining the upregulation of cMyc activity following TCR stimulation. Acute deletion of cMyc impairs the activation of downstream targets of the mTOR pathway such as 4E-BP and S6. Indeed, mTOR activation requires glutamine entry *via* the SLC3A2 (CD98)/SLC7A5 glutamine transporter (Nicklin, 2009), and the transcription of both of these subunits is impaired in cMyc-deficient T cells upon *in vitro* and *in vivo* activation (Wang, 2011). These results demonstrate the existence of a cross-talk between the mTOR and cMyc-dependent pathways.

Taking together, these data provide valuable insights into the mechanisms integrating metabolic pathways and lymphocyte function and survival. Moreover, they show that there is more than one mechanism orchestrating the metabolic and functional changes in T cells. The immune response provided by a given lymphocyte occurs as a result of the collaboration between these different pathways. The interplay between LKB1, mTOR and c-Myc is summarized in [Figure 19].



F

igure 19. The LKB1/AMPK/mTORC1-dependent regulation of protein translation. LKB1 regulates at least 14 substrates, including AMPK. After AMP binding to the γ-subunit, the AMPKα catalytic site is activated *via* LKB1-mediated T172 phosphorylation. AMPK activation results in TSC2 and raptor phosphorylation and mTORC1 inhibition. mTORC1 controls the translation of a number of cell growth regulators. Copied from (Han 2013).

3.3.4 Role of the metabolic environment in modulating T cell function

Despite the advances in understanding how metabolic pathways influence T cell homeostasis, multiple questions remain to be answered. T cells have to adapt their metabolism to changes in nutrient availability *in vivo*. This adaptation of T cells to the environment may be regulated at different levels: 1) availability of extracellular and intracellular metabolites 2) expression and activity of metabolites transporters 3) activity of enzymes regulating cellular metabolic pathways. One area of much recent interest is whether this metabolic plasticity shapes T cell fate. The converse, i.e. that different T cell fates are associated with distinct metabolic phenotypes, has been shown. Specifically, *Michalek and colleagues* have shown that effector helper cells such as Th1, Th2 and Th17 are highly glycolytic and exhibit high surface expression of Glut1, whereas iT_{reg} express low levels of Glut1 and displayed a mixed metabolism involving glycolysis, lipid oxidation and OXPHOS (Michalek, 2011). In addition, the differentiation of naïve T cell towards a Th17 phenotype is induced by hypoxic conditions which result in the upregulation of genes important for glycolysis including the hypoxia inducible factor-1α (HIF-1α) and its downstream target Glut1 (Dang, 2011; Shi, 2011). In contrast, blocking glycolysis inhibits Th17 differentiation and skews T cells towards an iT_{reg} fate (Shi, 2011; Wang, 2011). Therefore, the direct targeting of cellular metabolic pathways may provide a new strategy to modulate immune responses.

The intracellular availability of metabolites such as glucose, glutamine and leucine has also recently been shown to affect T effector function. Glucose deprivation strongly inhibits IFNγ production by $CD8^+$ effector cells (Cham and Gajewski, 2005; Jacobs, 2008), whereas, low levels of glutamine have been correlated with low IL-2 and IFNγ production (Rohde, 1996).

Moreover, interfering with amino acid uptake by a T cell *via* a leucine/glutamine transporter such as SLC7A5 abrogates the differentiation of naïve $CD4^+$ T cells into Th1 or Th17 effectors as well as the generation of CTL from naive $CD8^+$ T cells (Sinclair, 2013). Interestingly, the ability of $CD4^+$ T cells to differentiate into regulatory T cells was not altered. These effects are likely due, at least in part, to a loss in mTORC1 activity as a result of decreased leucine intracellular transport in exchange for glutamine (Sinclair, 2013). Interestingly, deletion of another glutamine

transporter SLC1A5 (ASCT2) has also been associated with decreased phosphorylation of mTORC1 substrates, S6 and 4EBP-1 (Nakaya, 2014). Moreover, SLC1A5$^{-/-}$ T cells have demonstrated impaired ability to differentiate *in vitro* towards Th1 and Th17. In addition, knockout of the CD98 heavy chain (SLC3A2) resulted in increased differentiation of regulatory cells (Bhuyan, 2014; Liu, 2012b). Thus, T cell fate can be modulated by extracellular nutrient-deprivation as well as by inhibiting the transport of metabolites into a cell. In addition, depletion of methionine and cysteine has been shown to abrogate Th17 differentiation but not T_{reg} (Sundrud, 2009).

While not directly related to T cell metabolism, recent research indicates that modulation of amino acid utilization can play a critical role in cell transformation and other pathological processes. Studies of acute myeloid leukemia (AML) growth in a mouse xenograft model suggest that blocking the transport of amino acids may represents an attractive new strategy for treating cancer. Specifically, downregulation of the SLC1A5 glutamine transporter inhibited the growth of an AML cell line. This phenomenon correlated with decreased mTORC1 activity, protein synthesis suppression and induction of the apoptosis (Willems, 2013).

Recently *Son et al*. have demonstrated that pancreatic tumor cells highly depend on glutamine availability (Lyssiotis, 2013; Son, 2013). Although, it is known that cancer cells are highly “addicted” to glutamine (reviewed in (Wise, 2010), it was however surprising that human pancreatic ductal adenocarcinoma (PDAC) cells do not utilize glutamine to produce ATP but rather to generate NADPH and maintain the proper ROS balance. In these cells, glutamine-derived α-ketoglutarate is transformed into aspartate and converted into oxaloacetate by cytoplasmic transaminase GOT1. Oxaloacetate is then transformed into malate and further into pyruvate through series of reactions that are associated with production of reducing potential in the form of NADPH. Interestingly, only cells with the Kras mutation displayed dependence on this glutamine metabolic pathway. Inhibition of transaminases or GOT1 knockdown in non-transduced human pancreatic ductal cells (HPDE) or human diploid fibroblasts (IMR90) cells had only a modest influence on cells proliferation. Taken together, inhibition of glutamine metabolism in pancreatic Kras-mutated tumors may be beneficial applied together with chemotherapy or irradiation, which

are known to increase ROS.

During the course of their life, lymphocytes move from lymphoid organs, which are presumably nutrient-rich, to sites of infection or cancer, where oxygen, nutrients, growth factors and other signals are limiting. In the latter environments, T cells must compete with multiple other highly proliferating cells for nutrients such us glucose and amino acids. Understanding how cellular metabolism influences the T cell-mediated immune response is an exciting question and elucidating the mechanisms involved may create new prospects for improving immunotherapy strategies. Therefore, part of my thesis project focused on this specific question (refer to section "Results: Part II").

4 Anti-cancer immunotherapies

Despites many advances in anti-tumor treatments, chemotherapy and radiotherapy have limited efficacy in the treatment of metastatic cancers such as melanoma or breast cancer (American Cancer Society 2013, (Stefanovic, 2013). As such, during the past few decades a strong effort has been invested in improving current treatment strategies. Amongst them, one of the most attractive is immunotherapy, designed to increase the immune responses of the patient against the tumor. Although, the concept of immunotherapy has been developed for more than 30 years, the potential of this promising strategy has only recently been realized. Due to these recent successes, immunotherapy has been highlighted by *Science* magazine as "*Breakthrough of the Year for 2013*" (Couzin-Frankel, 2013). Testimony to the success of this approach is shown by the FDA approval of several immunotherapy agents including sipuleucel-T (for the treatment of advanced prostate cancer, (Kantoff, 2010), ipilimumab (for advanced melanoma) and recombinant IFNα2 (adjuvant therapy for melanoma). Currently, more than 800 clinical trials are ongoing (http://www.fightcancerwithimmunotherapy.com/ImmunotherapyAndCancer/StateOfCancerImmunotherapy.aspx; reviewed in (Kirkwood, 2012)). An overview of the immunotherapies undergoing phase III clinical trials is presented in [Figure 20] (from

<http://www.cancerresearch.org/cancer-immunotherapy>).

The first immunotherapy approved by FDA in 1998 was high-dose of IL-2 administration to treat patients with metastatic melanoma. However, this treatment demonstrates significant adverse side effects such as fever, chills cardiac arythmias and delirium (Atkins, 1999), reviewed in (Petrella, 2007). One of the strategies involves administration of monoclonal antibodies that block T cell inhibitory molecules such as CTLA-4 (ipilimumab) or PD-1 (nivolumab). The expression of both CTLA-4 and PD-1 is upregulated on activated T cells inhibiting their activation, cytokine secretion and proliferation of T cells (Parry, 2005). Moreover, it is also important to note that CTLA and PD-1 are highly expressed on regulatory T cells, wherein they may directly promote T_{reg} cell–mediated suppression of effector immune responses (Francisco, 2009; Peggs, 2009; Read, 2000; Wing, 2008). Notably, MDX-1106 (nivolumab, from Bristol-Meyers Squib) against PD-1 has shown very interesting and potentially clinically relevant preliminary effects and this drug as well as MK3475 (Merck) is now under consideration for FDA approval (Topalian, 2012). Ipilimumab (blocking CTLA-4), which has been tested in clinical trials since 2000, demonstrated a statistically significant survival benefit for patients with metastatic melanoma in a randomized clinical trial (Hodi, 2010) and the FDA approved this drug in 2011 for the treatment of metastatic melanoma. Trials assessing its efficacy in other types of cancers are ongoing (Lipson, 2012), reviewed in (Pardoll, 2012). Importantly, results of a recent clinical trial involving a combined administration of both nivolumab and ipilimumab to patients with advanced melanoma cancer have been remarkable, demonstrating that it is not only a safe and feasible therapy but also a highly efficient treatment. Within this group of patients who were non-responsive to chemotherapy, evidence of clinical activity was observed in 65% of patients (Wolchok, 2013).

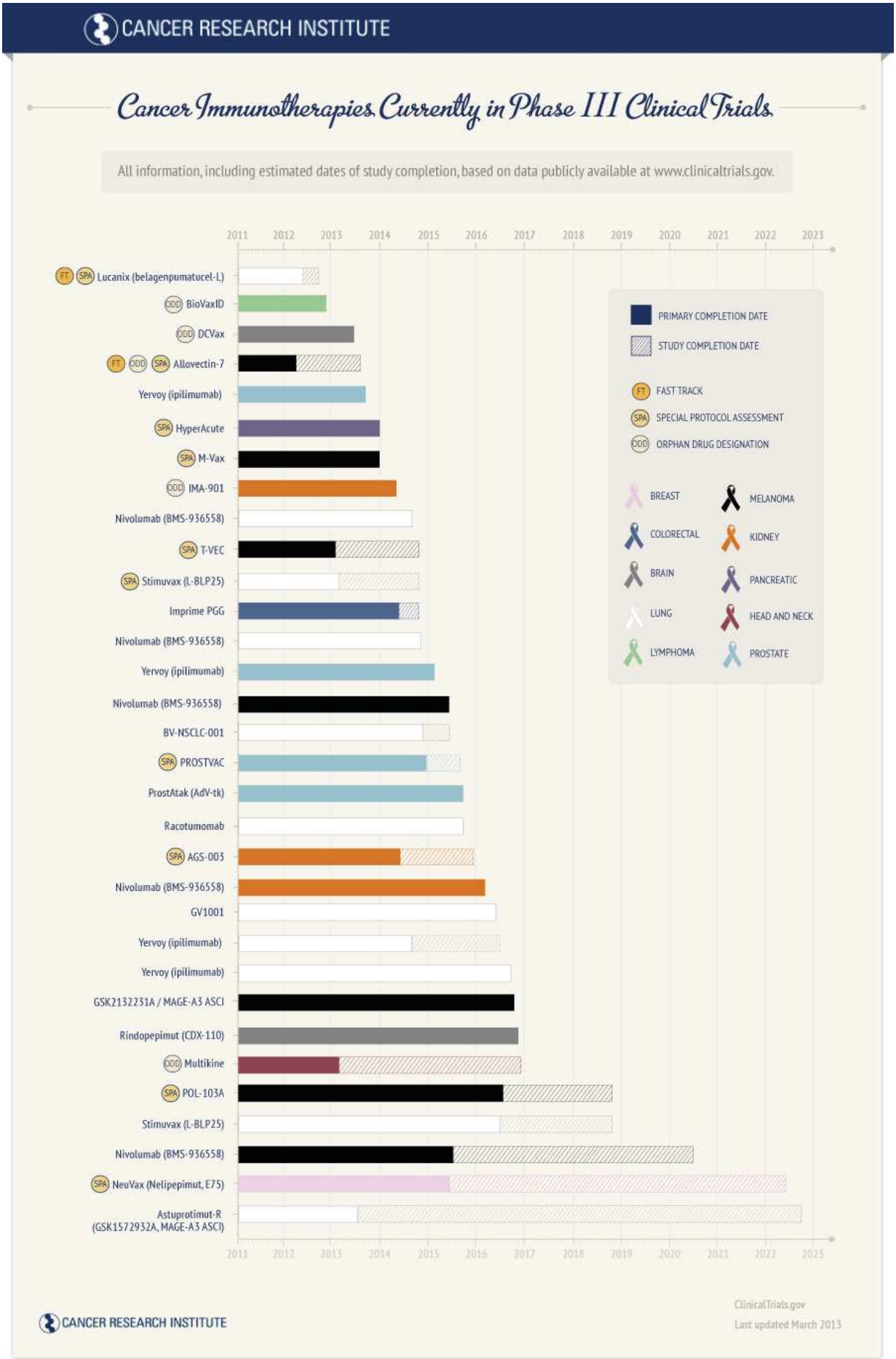


Figure 20. Phase III of clinical trials of the various immunotherapies. Copied from http://www.cancerresearch.org/cancer-immunotherapy).

4.1 Adoptive T cell therapy (ACT)

Adoptive T cell therapy (ACT) is a highly promising anti-cancer immunotherapy. It is based on the re-infusion of autologous tumor-antigen-specific T cells that have been expanded *ex vivo* into pre-conditioned patients. Conditioning (also called preparative lymphodepletion) is a transient ablation of the immune cells of a patient. It can be performed using chemotherapy alone or in combination with total-body irradiation and has been associated with enhanced persistence of the transferred T cells (Dudley, 2005; Dudley, 2008). ACT has been performed using two different approaches: tumor infiltrating T cells (or TILs) and redirected-T cells (TCR-engineered T cells and CARs), wherein antigen specificity has been redirected to a tumoral antigen [Figure 21].

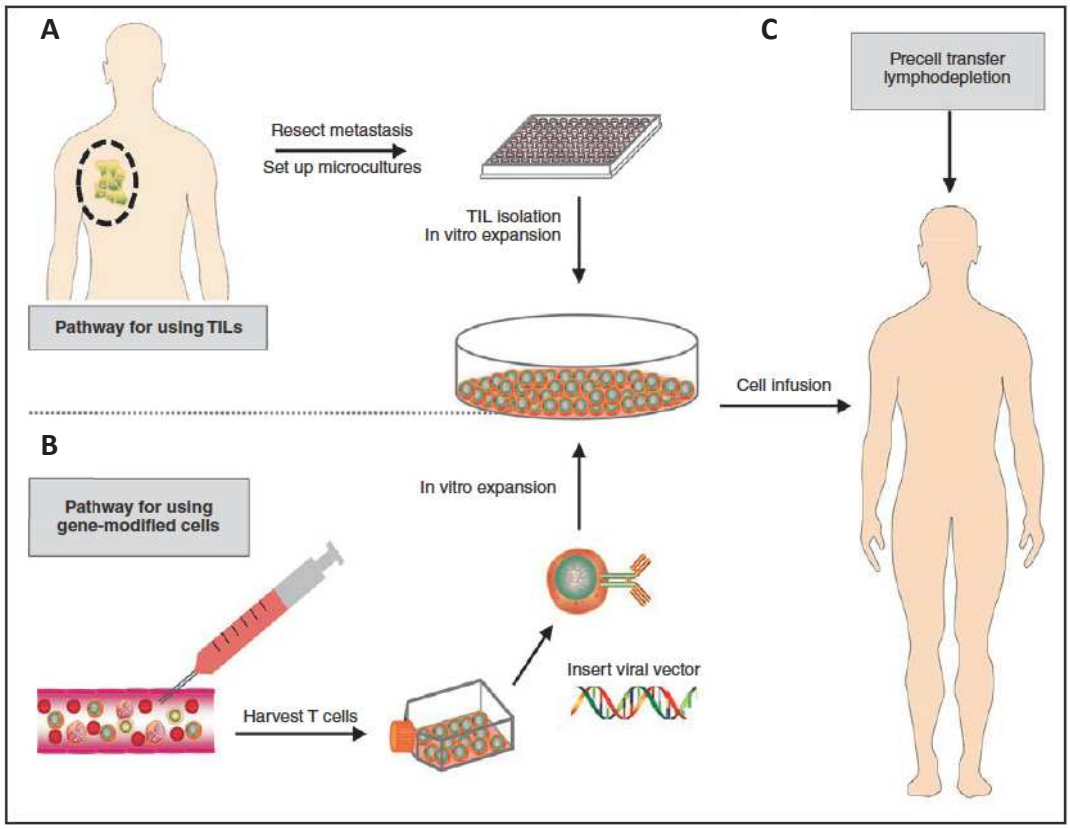


Figure 21. Two strategies used in the context of anti-tumoral adoptive T Cell therapy. Adoptive cell transfer therapy using: **(A)** autologous tumor-infiltrating lymphocytes (TILs) isolated from tumor fragments or **(B)** autologous peripheral blood lymphocytes genetically modified *ex vivo* to express engineered T-cell receptors or chimeric antigen receptors. **(C)** T cell injection into pre-conditioned lymphopenic patients. Copied from (Phan, 2013).

4.1.1 TILs-Tumor Infiltrating Lymphocytes

Some tumor types demonstrate high T lymphocyte infiltration such as small cell lung carcinoma (Eerola, 2000), melanoma (Ellebaek, 2012), breast cancer (Liu, 2012a; Melichar, 2014) and colorectal cancer (Koch, 2006). It has been demonstrated that these so-called tumor-infiltrating T cells (TILs) can be activated *ex vivo* (free from a potentially suppressive tumor environment) and are capable of *in vitro* tumor killing (Dudley, 2013; Muul, 1987; Rosenberg, 1986). The *ex vivo* expansion of TILs comprises several steps. The first step consists of a resection of a patient biopsy (1-2 cm in diameter) and the *in vitro* establishment of numerous micro-cultures, which can originate form single-cell suspensions or 1 to 2 mm^3 tumor fragments. These cells are grown in media containing IL-2 to stimulate T cells. Tumor-specific T cells should get activated and kill autologous tumor cells present within the cultures and reach a number sufficient for clinical use (usually 10^8 or more are required per injection) during 21 to 36 days. T cells are then screened for their reactivity against tumor antigen, viability, phenotype and then infused into a lymphodepleted patient (Goff 2010; Nguyen, 2010), reviewed in (Dudley and Rosenberg, 2003). Adoptive cell therapy (ACT) with tumor infiltrating lymphocytes was pioneered by *Rosenberg and co-workers* and has demonstrated very encouraging results in term of response rates and long-term survival (Rosenberg, 1985). Moreover, the combination of ACT together with the administration of IL-2 has been shown to lead to prolonged tumor eradication in patients who have exhausted other treatment options. This treatment has led to beneficial outcomes in patients with metastatic melanoma as well as in patients with other tumor histologies such as cervical cancer (Rosenberg, 2011) (Hinrichs, 2013; Piersma, 2007). Nevertheless, the widespread application of ACT has been limited by the complex and labor-intense procedure for the generation of clinical-grade TILs. Thus, in order to improve and simplify TIL generation, modified methods of TILs preparation such as the 'Young' TILs protocol, are being explored with promising results (Dudley, 2010; Dudley, 2013; Itzhaki, 2011; Tran, 2008). This protocol is based on $CD8^+$ T cell enrichment combined with regulatory T cell depletion, a shorter time of culture (10 to 18 days) and don't require tumor antigen specificity testing (Dudley, 2005). In this context, IL-2 is injected to support T cell proliferation and persistence of the adoptively-transferred T cells but it is not clear

whether high doses of IL-2 are necessary for the induction of the clinical response. Given the toxicity associated with high-dose IL-2 administration (Schwartz, 2002), different TIL clinical trials were performed using varying doses of administered IL-2 (Dudley, 2002; Yang, 2003) but the optimal levels of IL-2 administration have not yet been established.

In addition, preclinical and clinical studies have identified multiple factors associated with successful ACT, including: 1) host-related factors (e.g. magnitude of the lymphodepletion) (Dudley, 2008) 2) phenotype and functionality of the transferred cells (number of $CD8^+$ T cells) (Itzhaki, 2011) 3) *in vivo* persistence (Robbins 2004) 4) duration of the *ex vivo* culture (Tran, 2008) and 5) the differentiation state of the infused TILs (Huang, 2006; Shen, 2007). The effects of altering these parameters need to be further investigated to improve clinical trials.

4.1.2 T cell receptor gene therapy

In cancer patients where TILs cannot be generated (e.g., no resectable and non-solid tumors or an inability to expand TILs to adequate numbers for adoptive transfer), two promising alternatives have been developed: adoptive transfer of T cells expressing an engineered TCR or a chimeric antigen receptor (CAR) [Figure 22].

The engineered-TCR approach is based on the transduction of a tumor-specific TCR into autologous T cells followed by their *ex vivo* expansion and re-injection into patient. These TCRs are isolated from T cells showing high avidity to tumor-associated antigens (TAAs) and introduced in lymphocytes using a retroviral/lentiviral vectors based gene transfer. This procedure has been applied to several tumor-associated antigens such as: minor histocompatibility antigen (Bleakley and Riddell, 2011), CEA (Parkhurst, 2009), gp100 (Morgan, 2003; Schaft, 2003), MART-1 (Morgan, 2006), p53 (Theoret, 2008) and WT1 (Xue, 2009). The first trial performed in 2006 clearly demonstrated the feasibility and anti-tumor activity of the TCR-engineered T cells (Morgan, 2006). Unfortunately, despite its promise, this strategy is associated with an important risk of cross-pairing between the transduced TCR chains and the endogenous TCR chains, potentially resulting in the formation of hybrid TCRs with unexpected autoimmune reactivity (Bendle, 2010). To

date, several strategies have been developed to prevent TCR missed-pairing. One of these is based on the generation of TCRs containing a human variable region and a murine constant region (Cohen, 2006; Goff, 2010). Further, it has been demonstrated that the introduction of cysteines at structurally favorable positions allows formation of an additional disulfide bond between the α-and β-chains of the ectopic TCRs, increasing their total surface expression and reducing cross pairing with endogenous TCR chains (Boulter, 2003; Kuball, 2007). Other strategies involve the optimization of vectors (Lamers, 2008; Perro, 2010), and transgene cassettes to increase transduction efficacy (Leisegang, 2008). There is growing evidence that introduction of both TCRα and TCRβ into one vector downstream of a single promoter separated by an internal ribosomal entry site (IRES) or a 2A peptide sequence results in improved efficiency of TCR expression. Nevertheless, in the context of IRES, there is a problem of expression of the downstream gene. Notably, the genes linked by IRES are expressed as a single RNA molecule under the control of the LTR. The initiation of the translation of the first gene is cap-dependent while the translation of the second one is mediated by the IRES element (Morgan, 2006). Often, this results in a lower expression of the gene inserted downstream of the IRES (Herbreteau, 2005; Mizuguchi, 2000). In contrast, the use of a 2A peptide linker does not hamper expression of the 3' gene (de Felipe, 1999; Klump, 2001). In this configuration, a single TCRα- and β-chain-encoding mRNA is also generated and upon translation process, a ribosomal "skip" mechanism at the sequence of 2A peptide results in translation of two separate proteins by the same ribosome from the same mRNA. While the major part of the 2A peptide stays attached to the C-terminus of the upstream TCR chain, only one glycine residue remains at the N-terminus of the downstream-encoded protein (de Felipe, 1999; Klump, 2001). Importantly, 2A-mediated cleavage is close to 100% efficient and leads to a near stoichiometric ratio of the encoded gene products (Szymczak, 2004).

Other genetic strategies improving the expression and function of TCR transgenes include: the introduction of mutations in the transduced TCR structure for codon optimization, affinity maturation and incorporation of stimulatory T cell molecules (Chames, 2002; Scholten, 2006), usage of antigen-specific TCR vectors encoding siRNA for endogenous TCR genes (Ochi, 2011) or permanent knockout of the

endogenous TCR genes by zinc-finger nucleases (ZFNs) (Provasi, 2012). Incorporation of stimulatory molecules like CD3ζ into transduced TCRs also results in highly preferred pairing between TCRα:CD3ζ and TCRβ:CD3ζ and prevents TCR cross pairing with TCR chains: CD3ζ and endogenous TCR chains (Roszik, 2011; Sebestyen, 2008),reviewed in (Nicholson, 2012). Furthermore, incorporation of CD28 domain into TCRαβ:CD3ζ constructs has been shown to increase TCR-mediated IFNγ production by the engineered T cells (Schaft, 2006).

The biggest limitation of TCR gene therapy is the interaction between αβ-TCRs and peptide–MHC complexes. Since the cognate peptide recognized by the receptor will be presented on the major histocompatibility complex (MHC) these therapies are restricted to a single MHC subtype. In general, TCRs recognizing HLA-A2 restricted epitopes are chosen because this HLA is the most frequent within several ethnic groups. Nevertheless, this is a subject of high importance to develop T cell strategies that are not limited by HLA expression. One possibility is the use of TCRs that recognize a tumor antigen independently of MHC. While this is very rare, such a TCR, recognizing a renal carcinoma antigen was identified by *Chen and colleagues* (Chen, 2008). Another strategy has been developed based on the use of chimeric antigen receptors.

4.1.3 Chimeric Antigen Receptors (CARs)

CARs, or T-bodies, are generated by joining the light and heavy chain variable regions of a monoclonal antibody as a single chain Fc (scFv) molecule that consists of a hinge domain, transmembrane domain, and cytoplasmic signaling domains derived from the CD3ζ chain or Fc receptor γ chains (reviewed in (Sadelain, 2009). This structure represented the first generation of CARs. Second generation CARs contain the CD3ζ chain in the intracellular tail as well as one extra domain derived from a co-stimulatory molecule such as CD28 or 4-1BB (Kowolik, 2006; Maher, 2002). The most recent, the third generation of CARs, contains at least two signaling domains and other co-stimulatory molecules to enhance the therapeutic potential of engineered T cells (reviewed in (Maus, 2014). The different types of CARs used in the clinical trials are summarized in Table 3 (Cieri, 2014).

CAR-transduced cells have the specificity of an antibody coupled with the cytotoxic effector mechanisms of a T cell, therefore CARs recognize MHC-non-restricted structures. In contrast to a TCR, CARs cannot target an intracellular antigen, including melanoma-associated proteins such as gp100 and MART-1. This clearly represents an important disadvantage of CAR-transduced T cells as compared to TCR-transduced cells, which can recognize intracellular antigens that have been processed and presented as peptide/MHC complexes (Sadelain, 2009).

The first study to show clinical responses using CAR-transduced lymphocytes was performed in patients with neuroblastoma. In this clinical trial, the CAR was directed against the GD2 (diasialoganglioside) antigen expressed by most neuroblastoma cells (Pule, 2008). More recently, extremely promising results have been obtained using CARs targeting CD19, a surface marker constitutively expressed on most acute and chronic B-cell malignancies. Trials led by the group of Carl June have used a CD19-specific CAR containing a 4-1BB co-stimulatory domain (Grupp, 2013; Kalos, 2011) while the group of M Sadelain/M Brentjens have used CARs with a CD28 domain (Brentjens, 2013; Davila, 2014b). These CAR-modified T cells have been shown to expand and clear CD19-positive leukemia cells and one pediatric patient is now cancer-free for two years since T cell treatment (Kochenderfer, 2013). A trial is presently underway to compare the efficacy of CARs containing a CD28 as compared to a 4-1BB domain (clinicaltrials.gov NCT01044069).

Tumor	Antigen	CAR – Vector	Activation	Pts	Results	Comments	Ref.
Ovarian cancer	FBP	1G – RV	OKT3	14	No responses	Poor persistence	(131)
Renal cell cancer	CAIX	1G – RV	OKT3	3	No responses	On-target, off-tumor liver toxicity	(142)
Neuroblastoma	L1-CAM	1G – EP	OKT3	6	1 PR	Poor persistence	(123)
NHL/MCL	CD20	1G – EP	OKT3	7	1 PR	Better persistence with IL-2	(125)
Neuroblastoma	GD2	1G T versus CTL – RV	OKT3	11	1 CR, 5 PR	Better persistence with EBV CTLs	(134)
Colon cancer	HER2	3G – RV	OKT3	1	Death	On-target, off-tumor lung toxicity	(143)
CLL/ALL	CD19	2G – RV	Beads	10	1 PR, 2 SD, 1 death	Cytokine-release syndrome	(141)
NHL	CD19	1G versus 2G – RV	OKT3	6	2 SD	Persistence better with 2G	(133)
NHL/CLL	CD19	2G – RV	OKT3	8	1 CR, 5 PR	B-cell aplasia Cytokine-release syndrome	(140)
CLL	CD19	2G – LV	Beads	3	2 CR, 1 PR	Long persistence (high levels) B-cell aplasia Cytokine-release syndrome	(136, 137)
NHL/MCL	CD20	3G – EP	OKT3	3	1 PR, 2 SD	Long persistence (low levels)	(126)
ALL	CD19	2G – LV	Beads	2	2 CR	B-cell aplasia Cytokine-release syndrome CAR T cells in spinal fluid CD19-less relapse in one case	(138)
ALL	CD19	2G – RV	Beads	5	5 CR	Cytokine release syndrome	(139)

Table 5. Overview of clinical results with using CAR-T cells. NHL- non-Hodgkin lymphoma; MCL mantle lymphoma; CCL- chronic lymphocytic leukemia; AAL- acute lymphocytic leukemia; IG- first generation of CARs; 2G- second generation of CARs; 3G- third generation of CARs, RV- retroviral

vector; EP- electroporation; LV- lentiviral vector; PR- partial response, CR- complete response; SD- stable disease; Beads- cell-sized beads conjugated with antibodies specific for CD3 and CD28; EBV- Epstein Barr Virus; CTL- cytotoxic T lymphocyte. Copied from (Cieri, 2014).

4.1.4 Therapy-related toxicities

It is important to note that this type of treatment is not without risk (reviewed in (Kunert, 2013). Ideally, a tumor antigen chosen as a target for adoptive cell therapy would be tumor specific only and not expressed on any other tissues. Unfortunately, there have been cases of "on-target" toxicity, due to antigen expression on normal cells. Thus, clinical trials with gene redirected-T cells against MART1 and gp100 have demonstrated that beside partial tumor rejection, these T cells also caused destruction of normal melanocytes in the skin, eye, and ear in some patients, leading to severe skin rashes, uveitis, or hearing loss (Johnson, 2009). *Lamers et al.* have reported that adoptive transfer of autologous T-lymphocytes recognizing an epitope on carboxy-anhydrase-IX (CAIX), frequently overexpressed on renal cell carcinoma, may cause a liver toxicity associated with discrete expression of the $CAIX^+$ by the bile duct epithelial cells (Lamers, 2006; Lamers, 2013). Moreover, unexpected deaths have been reported in 2 trials using respectively anti-CD19 CAR cells (one patient) (Brentjens, 2010) and anti-HER2/NEU CAR cells (one patient) (Morgan, 2010). Soon after cell infusion, the 2 patients developed profound hypotension, respiratory distress, and multi-organ failure associated with markedly elevated levels of inflammatory and homeostatic cytokines such as IL-2, IL-7, IL-15 and IL-12. This lethal "cytokine storm" may have been due to the overwhelming activation and proliferation of the transferred anti-CD19 CAR cells upon target recognition (Brentjens, 2010). Several patients in anti-CD19 CAR trials displayed reversible obtundation, seizures, aphasia and mental status changes, which could have been associated with increased the blood brain barrier crossing by cytokines such as IL-6 or CARs (reviewed in (Davila, 2014a) and in (Maus, 2014). The cytokine storm can also cause a life-threating condition named tumor lysis syndrome (TLS). TLS is a group of metabolic abnormalities leading to a massive and abrupt release of cellular components into the blood after rapid lysis of tumor cells (Cairo and Bishop, 2004; Davidson, 2004; Yim, 2003). Therefore, to minimalize the potential severity of the

TLS, it is crucial to identify and characterize different risk factors such as cell proliferation, size and stage of the tumor (Cairo, 2010; Canet, 2014).

Moreover, due to the CD19 expression by normal B cells, one of the expected results of anti-CD19 CAR treatment is B cell aplasia and increased risk of infections. Fortunately, patients may be injected with gamma globulins as a replacement therapy.

Therefore, TILs, TCR-engineered T cells and CARs are extremely encouraging strategies allowing a broadly applicable therapeutic treatment of cancer. Nethertheless, the progress in this field is heavily dependent on the identification of new tumor-specific antigens (TSAs). This would allow a specific targeting of a malignancies and the development of new strategies to reduce toxic side effects (Stauss, 2013). One approach used to prevent "on-target" reactivity towards healthy tissues is to direct T cell responses against specific tumor antigen epitopes. In this context, *Straetemans et al.* characterized two MAGE antigen epitopes belonging to the group of cancer testis antigens (CTAs), MAGE-C2/HLA-A2 and MAGE-A3/HLA-DP4, that may safely be used as a target for TCR-engineered T cells (Straetemans, 2012). Similarly, a cancer exome-guided analysis of T cell reactivity, has been proposed as a promising method to characterize a range of mutations that may be specific only for cancer cells and thereby, potentially give rise to neo-antigen proteins that would be recognized by T cells (Tran, 2014) (Rajasagi, 2014; Robbins, 2013; van Rooij, 2013). This approach involves comparative whole exome sequencing of cancer and healthy cells in order to identify cancer-specific somatic mutations. Selected mutated sequences are then further analyzed *in silico* in order to determine peptide-MHC binding epitopes and perform screening for TIL recognition (Robbins, 2013). Using this type of approach, *Van Rooij and colleagues* have identified two neo-antigens specifically expressed in melanoma, which they identified as mutant epitopes of ataxia telangiectasia and Rad3 related (ATR) gene products. Further analyses have revealed the presence of neo-antigen-specific T cells amongst melanoma tumor infiltrating lymphocytes (van Rooij, 2013). Moreover, this approach has also been successfully applied to enrich for neo-antigen-specific T cells injected into a patient with metastatic cholangiocarcinoma, which resulted in a rapid tumor regression, which was maintained for at least 6 months (Tran, 2014). Finally,

cancer exome sequencing may also be applicable for the generation of personalized tumor vaccines (reviewed in (Hacohen, 2013)).

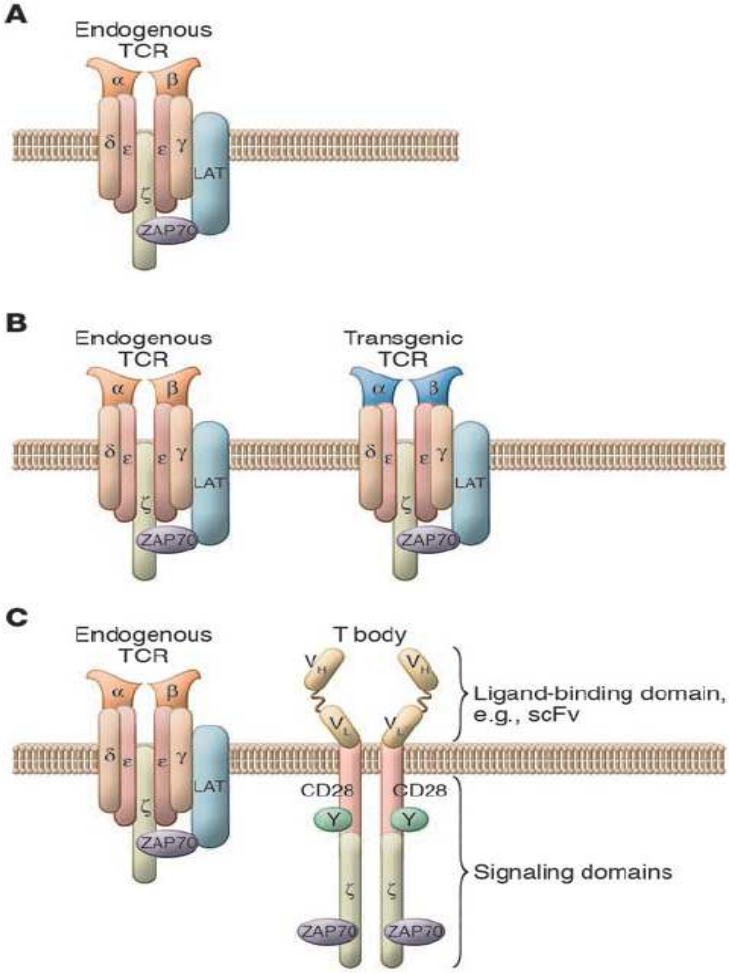


Figure 22. T cell redirecting strategies. (A) Endogenous T cells express a single heterodimeric TCR. **(B)** A transgenic TCR recognizing an antigen expressed by target tumor cells. **(C)** Chimeric tumor antigen–specific receptors (T bodies) target surface antigens in an MHC-independent fashion. T bodies express an extracellular ligand generally derived from an antibody and intracellular signaling modules derived from T cell–signaling proteins. LAT, Linker for Activation of T cells; ScFv, Single Chain Variable Fragment; ZAP70, ζ -chain associated protein kinase 70 kDa. Copied from (June, 2007).

4.1.5 Factors affecting the persistence of adoptively transferred T cells

To best exploit adoptive T cell therapies, it is necessary to improve the short peripheral persistence of adoptively-transferred cells)(Lamers, 2010). Characterization of factors modulating the fate of these T cells is therefore crucial for the success of ACT.

Role of conditioning

More than 30 years ago, *Greenberg* et *al.* demonstrated that leukemia-specific T cells, adoptively-transferred into leukemic mice, are effective only if they are administered following chemotherapy (Greenberg, 1991a) reviewed in (Greenberg, 1991b). Indeed, in patients, the ability of adoptively transferred T cells to mount an effective anti-tumor response is significantly improved by first rendering patients lymphopenic (objective response rates increased from 40% to 72%) (Rosenberg, 2011). Therefore, in recent years conditioning has been studied not only for its direct role in tumor death but also its ability to contribute to the immune response (Apetoh, 2007a; Apetoh, 2007b; Ding, 2010; Nowak, 2003; van der Most, 2009).

Chemotherapy is an immunomodulatory factor that may promote anti-tumor immunity through many ways, such as induction of tumor cells apoptosis, enhancement of tumor antigen cross-priming (Casares, 2005; Nowak, 2003) or increase expression of MHC class I molecules (Adair and Hogan, 2008; Fonsatti, 2007). The therapeutic agent, Cyclophosphamide has been shown to promote maturation of dendritic cells (Wada, 2009) and induce interferon I production which was associated with increased number of $CD44^{hi}$ T cells (Schiavoni, 2000). Furthermore, low doses of cyclophosphamide decrease the number of regulatory T cells by downregulating the Foxp3 and GITR expression. Further, this low number of T_{reg} was associated with enhanced $CD8^{+}$ T cell anti-tumoral functions (Ghiringhelli, 2004) 2004, (Ding, 2010; Ghiringhelli, 2007; Lutsiak, 2005). Moreover, cyclophosphamide has been demonstrated to promote Th17 helper cells (Moschella, 2011; Viaud, 2010). Also other types of chemotherapy, such as fludarabine or paclitaxel have been shown to decrease T_{reg} in cancer patients (Beyer, 2005; Zhang, 2008a), reviewed in (Zhang, 2008b).

Irradiation affects cells and tissues in ways that may stimulate an immune response.

Low doses of irradiation has been shown to increase expression of MHC molecules, tumor-associated antigens, such as carcinoembryonic antigen and mucin 1 and CD95 (FAS) by tumor cells, as well as adhesion molecule such as I-CAM (Gaugler, 1997) by endothelial cells and therefore increase CTL activity (Garnett, 2004).

Moreover, it has been reported that total body irradiation could cause mucosal barrier increased permeability (Nejdfors, 2000) and allows translocation of the commensal microbial antigen into blood vessels. Once in the blood, microbial antigens may induce an immune response, increase T cell proliferation and tumor regression (Paulos, 2007).

Furthermore, conditioning-induced lymphopenia may enhance the anti-tumor efficacy of transferred $CD8^+$ T cells by decreasing the competition for the access to antigens presented on APCs (Cho, 2010; Kedl, 2000).

Lymphopenia is associated with improved anti-tumor responses of transferred T cells but has also been associated with autoimmunity in physiological human conditions (systemic lupus erythematosus, rheumatoid arthritis, and Crohn and Sjogren syndromes) (reviewed in (Khoruts and Fraser, 2005) as well as in animal models. The mechanism(s) driving autoimmunity in these situations remain unclear. In some cases it has been attributed to a loss of regulatory cells. However, even though the treatment of lymphopenic hosts with $CD4^+CD25^+$ T_{reg} can abrogate autoimmunity induced by co-transferred $CD25^-$ cells, the depletion of T_{reg} from T-replete animals rarely evokes autoimmunity (McHugh and Shevach, 2002). Moreover, in clinical trials, Fludarabine- or Cyclophosphamide-induced lymphodepletion has been shown to promote the *in vivo* expansion of transferred T cells by increasing the availability of cytokines such as IL-7 and IL-15, therefore promoting the homeostatic proliferation of the existing T cell compartment, and decreasing the number of regulatory T cells (Ao, 2009; Wrzesinski, 2010), reviewed in (Muranski, 2006).

Many studies shown that the “open space” created by lymphopenia leads to a radically altered T cell homeostasis, resulting in a proliferative response and a conversion of T cells from a naive to a memory-like phenotype (reviewed in (Almeida, 2005; Prlic, 2002; Surh and Sprent, 2008). This response has been called lymphopenia-induced proliferation (LIP), lymphopenia-driven proliferation or homeostatic proliferation. TCR signaling from interaction with self-peptide–MHC

complexes (Goldrath, 1999; Kieper, 1999), IL-7 and to a lesser degree IL-15 are involved in this process (Ramsey, 2008; Seddon, 2003; Tan, 2001; Vella, 1997). Due to the fact that LIP is driven by interaction with self-peptides and leads to decreased activation requirements and accumulation of T cells with memory-like phenotype, it has been proposed that this phenomenon causes in the autoimmune diseases detected in individuals with lymphopenia (Asano, 1996; Davey, 2005; King, 2004), reviewed in (Khoruts and Fraser, 2005) and in (Surh and Sprent, 2008) and (Datta and Sarvetnick, 2009).

T cell homeostasis in lymphopenic environment- role of cytokines

IL-7 has been identified as a major cytokine supporting the survival of various populations of immune cells; αβ T cells as well as γδT, NKT, and innate lymphoid cells (reviewed in (Rochman, 2009). IL-7 is a member of the common γc cytokine family that comprises IL-2, IL-4, IL-7, IL-15 and IL-21. These cytokines share the γc chain (also known as IL-2Rγ and CD132). IL-7 is produced by stromal and epithelial cells in the bone marrow and thymus and by fibroblastic reticular cells in the T cell zones of secondary lymphoid organs (Miller, 2013; Onder, 2012; Zamisch, 2004; Zubkova, 2005). Recently, lymphatic epithelial cells (LECs) in lymph nodes have been shown to be an important source of IL-7 *in vivo* (Iolyeva, 2013; Miller, 2013; Onder, 2012; Watanabe, 1995). These data suggest that circulating lymphocytes are likely to be exposed to IL-7 whenever they traffic through the extensive lymphatic network. Furthermore, LEC-derived IL-7 can act in an autocrine manner. LEC tube formation, migration of immune cells under normal as well as lymphopenic conditions is regulated by IL-7-secreting LEC (Miller, 2013). Moreover, the availability of IL-7 is regulated by both its production and its consumption by T cells (reviewed in (Surh and Sprent, 2008) and in (Fry and Mackall, 2005). IL-7Rα is expressed by naïve and memory T cells but its expression is downregulated after TCR stimulation. These data indicate that IL-7 regulates the fate of naïve and memory T cells to a significantly greater extent than effector cells (Alves, 2008; Schluns, 2000).

IL-15 is particularly important for the homeostasis of memory $CD8^+$ T cells that express high levels of IL-2Rβ (also known as CD122) (Judge, 2002). Although IL-15

does not play an essential role in the homeostatic proliferation of memory $CD4^+$ T cells, upon depletion of $CD8^+$ T cells and NK cells, the main consumers of IL-15, there is a greater availability of IL-15 and the homeostatic proliferation of memory $CD4^+$ T cells is increased (Purton, 2007).

T cells expansion and activation under homeostatic condition have been shown to enhance anti-tumor responses detected after adoptive transfer into lymphodepleted hosts (Dummer, 2001; Dummer, 2002). These results are in accord with recent studies demonstrating that T-cell homeostatic proliferation can lead to autoimmunity in humans (Jones, 2013). Surprisingly, *Gattinoni et al.* have shown that increased anti-tumor efficacy of ACT regimen after lymphodepletion is not the result of increased numbers of tumor-reactive T cells but rather the enhanced effector functions of the transferred cells. Thus, the ensemble of these data suggests that increased availability of cytokines and self-peptides are not the only mechanisms accounting for the enhanced anti-tumor immunity observed after lymphodepletion (Gattinoni, 2005).

Taken together, these data demonstrate the importance of lymphodepletion before adoptive transfer of tumor-specific lymphocytes. Conditioning enhances anti-tumor immune response *via* many molecular and cellular effects such as the removal of T_{reg}, an increased availability of cytokines or the activation of self/tumor antigen-specific T cells, and other mechanisms that still have to be determined.

Tumor microenvironment

The tumor microenvironment (TME) has gained attention over the past few years (reviewed in (Hanahan, 2011). The interactions between tumors and their microenvironment have become increasingly significant (reviewed in (Peranzoni, 2013), and clinical trials using agents that specifically target pathways between neoplastic and stromal cells has risen (Barker, 2012; Eisinger-Mathason, 2013). The tumor extracellular matrix is constantly changing as a result of tissue remodeling, metabolic modifications and alterations, all in the presence of non-tumor cells, including endothelial cells, fibroblasts as well as diverse immune cells. Such remodeling creates a tumor-promoting inflammatory environment similar to a wound environment, characterized by an influx of Th2, macrophages, abundant

fibrillar collagen, and increased cyclooxygenases (COX)-2 activity (Lyons, 2011).

One of the features of the TME is its low oxygen tension. It has been demonstrated that hypoxia can also influence immune cell in the TME, depending on the type of hypoxia-inducible factor (HIF) involved (reviewed in (Keith, 2011). HIF-1α and HIF-2α possess distinct and occasionally overlapping roles. However, both have been suggested to actively promote the progression of a variety of cancers, including cell renal carcinoma, neuroblastoma, hepatocellular carcinoma and colorectal cancer (Skuli, 2012) Keith *2012,* reviewed in (Mucaj, 2012). Recently, *Eisinger-Mathason et al.* have demonstrated that the loss of HIF-1α or PLOD2 (the endoplasmic reticulum–associated enzyme pro-collagen-lysine, 2-oxoglutarate 5-dioxygenase) expression disrupts collagen modification, cell migration, and pulmonary metastasis but not primary tumor growth (Eisinger-Mathason, 2013).

Another important characteristic of the tumor microenvironment is the mechanical properties of the extracellular matrix (ECM), which can affect neoplastic cell differentiation, invasiveness and drug delivery (reviewed in (Salmon, 2012a). In humans an increased stiffness of stroma in breast tissue is a risk factor of breast cancer and it has been demonstrated that inhibition of collagen cross-linking by lysyl oxidase (LOX) in murine models delays and decreases tumor invasion (Barker, 2012; Dufort, 2012). Furthermore, extracellular matrix organization can highly influence adoptive T cell therapy outcome. Cancer-specific effector T cells must enter into the tumor after leaving the blood vessels and the presence of T cells in cancer stroma has been associated with favorable prognosis in human lung cancers (Wakabayashi, 2003). However, the entry of lymphocytes into the tumor does not always occur easily, mostly due to abnormal vessel formation and reduced expression of adhesion molecules (Fisher, 2006). Indeed, immune cells are not randomly distributed within tumors and a dense collagen network surrounding the tumor can limit T cell trafficking into tumor islets (Salmon, 2012b). Taken together these data suggest that targeting tumoral ECM can become an extremely attractive strategy to improve the anti-tumor surveillance of T cells.

Influence of nutrients on anti-cancer therapies

As mentioned in previous chapters, there is a close functional and molecular integration of the immune and metabolic systems. Thus, it seems obvious that the proper balance between the nutrients available in the blood circulation and the metabolic need of a cell represents a crucial regulatory mechanism, the dysfunction of which underlies many chronic metabolic diseases. The initiation and maintenance of immunity is a metabolically costly task and cannot operate efficiently under conditions of energy deficit (Demas, 1997). Moreover, starvation and malnutrition can impair immune function. A total reduction in body fat has been shown to result in a decrease of energy for immune responses in rodents (Demas, 2003). Furthermore, diet is known to be a risk factor for disorders ranging from diabetes and inflammatory bowel disorders (Spooren, 2013) to metabolic diseases (reviewed in (Veldhoen and Brucklacher-Waldert, 2012). Recent studies have shown that obesity affects primary immune tissues. *Trottier et al.* found that hematopoietic and lymphopoietic functions in the bone marrow (BM) and the thymus are significantly increased in mice treated with a high-fat diet. Specifically, the total number of lymphocytic lineage cells in the BM is increased and the thymus is greatly enlarged, associated with higher numbers of $CD4^{+}CD8^{+}$ thymocytes (Trottier, 2012).

The role of diet has also been appreciated in cancer treatment. The reprogramming of energy metabolism called Warburg effect is one of the “hallmarks” of cancer (reviewed in (Hanahan, 2011). Moreover, with progressive tumorigenesis, cancer cells become more and more “addicted” to aerobic glycolysis (reviewed in (Koppenol, 2011) and vulnerable to glucose deprivation. Indeed, several studies have shown that malignant cells quickly lose ATP *in vitro* and undergo apoptosis when starved of glucose (Demetrakopoulos, 1978; Priebe, 2011; Shim, 1998). Therefore, it become of high interest to investigate whether changes in the glucose concentration in the bloodstream would have comparable effects on tumor growth *in vivo*. Caloric restriction in a number of animal models and in epidemiologic studies suggests that it may prolong life span (Hursting, 2009), reviewed in (Longo, 2010). Further, experiments performed on yeast and mice suggested that starvation could result in protection of normal but not cancer cells from various chemotherapy drugs, a phenomenon called Differential Stress Resistance (DSR) (Lee, 2010a; Raffaghello,

2008). These data are in agreement with the observations that patients reduced side effects associated with the treatment, such as vomiting, diarrhea, fatigue and weakness by being protected from oxidative stress (Raffaghello, 2010; Safdie, 2009).

Most malignant cells are not able to metabolize significant amounts of fatty acids or ketone bodies due to mitochondrial dysfunction and cannot use them to replace glucose as an energy source. This finding led to the possibility of providing a high-fat, ketogenic diet (KD) as a supportive strategy to patients with cancer, who often suffer from severe weight loss (called cachectic syndrome) (Seyfried 2010, (Tisdale, 2007; Tisdale, 2010; Zhou, 2010). However, there are not so many clinical studies studying the effects of high-fat diets. *Fearon et al.* applied a 70% medium chain triglyceride diet containing β-hydroxybutyrate to five patients in a late-stage of cachexia. After a week on this diet, they observed an increase in body weight by 2 kg, which was correlated with an improvement in their physical condition (Fearon, 1988). Also, in prostate cancer mouse model, a ketogenic diet has been suggested to support tumor volume decrease and improve survival time, when compared to mice fed with the standard "Western diet" (Freedland, 2008). Moreover, a pilot trial administering a ketogenic diet, consisting mainly of medium-chain triglycerides, to patients with advanced metastatic tumors of different origin (such as ovarian, breast cancer, parotis carcinoma among others) was demonstrated potentially beneficial. Notably, severe side effects were not observed and almost all standard blood parameters such as cholesterol, triglycerides, glucose and leukocyte count as were quality of life were improved (Schmidt, 2011).

Another important nutrient for the immune system, and mentioned above, is glutamine. Increased glutamine in the diet has been associated with improved functional and structural integrity of the gut (Garrel, 2004; van der Hulst, 1993) and protection against endotoxic shock, hyperthermic stress, and other potentially fatal side effects of chemotherapy (Anderson, 1998). *Xue et al.* have shown that oral glutamine supplementation limits the development of severe diarrhea in rats treated with CPT-11, a regimen used for the treatment of patients with colorectal cancer (Xue, 2007). Although these observations open new avenues and indicate a potential combination of dietary changes, modulation of immune response and cancer treatment, further investigations are required.

Objectives

Objectives

Adoptive T cell immunotherapy has resulted in impressive remissions in some patients with chemotherapy-refractory tumors (Brentjens, 2013; Davila, 2014b; Dudley, 2002; Dudley, 2008; Kochenderfer, 2013). However, expanding the use of this strategy will require improvements in therapeutic efficacy, prevention of toxicity and enhanced persistence of the adoptively transferred T cells. During my PhD, I carried out two projects, both of which were aimed at understanding how different pathophysiological conditions alter the survival and reactivity of adoptively transferred T cells. The first project focused on the role of the host lymphopenic environment while the objective of the second project was to elucidate how cell metabolism impacts on the effector function of adoptively transferred T cells. The data emerging from these studies are presented as two articles in the ensuing results section and the objectives are summarized below.

One important aspect impacting on T cell proliferation and function is the host environment. Under conditions of lymphopenia, there is an increased homeostatic proliferation of T lymphocytes that results in the expansion of conventional T cells, in the apparent absence of antigenic stimulation. Notably, this non-specific proliferation has been shown to enhance the reactivity of T cells and this effect has been exploited to augment the responsiveness of T lymphocytes in cancer patients (Dudley, 2002; Gattinoni, 2005; Rosenberg, 2011). Indeed, the ability of adoptively-transferred autologous tumor infiltrating lymphocytes to mount an effective response in tumor-bearing patients is significantly enhanced by first rendering the patients lymphopenic (Dudley, 2005; Lim, 2013; Rosenberg, 2011). Nevertheless, the precise mechanisms regulating this phenomenon are not yet clear.

The vast majority of conclusions concerning the influence of the lymphopenic environment on the enhanced responsiveness of adoptively transferred $CD4^+$ and $CD8^+$ T cells have been drawn from mice rendered lymphopenic either by genetic means (i.e. $Rag^{-/-}$ background) or by total body irradiation (TBI) ((Wrzesinski, 2010), reviewed in (Surh and Sprent, 2008) and in (Sprent and Surh, 2011)). While these experiments have provided significant information about the role of homeostatic

proliferation in modulating anti-tumor T cell reactivity, they may not accurately reflect the situation occurring in cancer patients who are pre-conditioned with varied chemotherapy regimens including fludarabine, melphalan, busulphan and cyclophosphamide. Therefore, one goal of my PhD research was to assess whether the fate of adoptively-transferred T cells is modulated by the type of conditioning that is used to induce lymphopenia. In an attempt to respond to this question, we rendered mice lymphopenic by either sublethal irradiation or by a busulphan/cyclophosphamide chemotherapy regimen. The data that I obtained are included in the first part of the result section and are presented as a manuscript entitled "***Lymphopenia-inducing regimens differentially condition the fate of adoptively transferred T cells***". In the context of this research, we addressed the following issues:

- The consequences of host conditioning on T cell reconstitution:
    - Impact on host and adoptively-transferred T cell subsets
    - Effects on the proliferation of adoptively-transferred $CD4^+$ and $CD8^+$ T cell subsets
    - Effects on the responsiveness of adoptively-transferred T cells to the IL-7 and IL-6 cytokines
    - Alterations in the balance between conventional and regulatory T cells

- The consequences of host conditioning on the microenvironment:
    - Changes in lymphoid organ structure and reconstitution
    - Changes in host DC subsets
    - Role of host DC subsets in mediating the proliferation of adoptively-transferred T cells

In the second project, I focused on the role of metabolism in T cell effector function. Activation of naïve $CD4^+$ T lymphocytes results in their effector differentiation, defined by a specific cytokine secretion profile. Th1 and Th17 effectors, characterized by IFN-γ and IL-17 secretion respectively, appear to be the most

potent players in anti-tumor responses ((Nishimura, 1999; Sadanaga, 1999) reviewed in (Kennedy, 2008; Zou, 2010)) while regulatory T cells inhibit anti-tumor immunity ((Arendt, 2006; Onizuka, 1999; Shimizu, 1999) reviewed in (Beyer, 2006)). Multiple studies have shown the importance of the T_{eff}:T_{reg} balance in mediating anti-tumor responses, focusing on the critical roles of cytokines, the migration of T cells into the tumor as well as the hypoxic microenvironment of the tumor itself (Billiard, 2006; Chaput, 2009; Salmon, 2012b; Sato, 2005; Skuli, 2012). Nevertheless, it is intriguing that there is a paucity of studies, at least to date, on how the metabolic environment of a tumor may impact on T cell responses. Indeed, the activation of a T cell in response to a tumor antigen, or any antigen, requires a considerable expenditure of energy and cellular resources. Activated T cells undergo a metabolic shift, fueling the increased energetic and biosynthetic demands of the cell (reviewed in (Pearce, 2013)). Recent studies have demonstrated that T lymphocyte growth and proliferation depend on glucose as well as on other nutrients such as fatty acids and amino acids (Michalek, 2011; Sundrud, 2009; Wang, 2011). Furthermore, changes in lymphocyte metabolism condition the differentiation potential of a naïve T cell (Michalek, 2011; Pearce, 2009; Sundrud, 2009; Wang, 2011). Therefore, targeting T cell metabolism may be a new strategy for directing effector function (Lyssiotis, 2013; Michalek, 2011; Son, 2013). We were interested in studying the role of glutamine in $CD4^+$ T cell effector function as it is the most abundant amino acid in the plasma and certain tumor cells are "addicted" to glutamine (reviewed in (Wise, 2010)), thereby potentially changing the availability of glutamine within a tumor microenvironment. Furthermore, after starting this research, 2 groups reported that inactivation of either the SLC7A5 or SCL1A5 amino acid transporter negatively impacts T cell function, abrogating $CD8^+$ T cell cytolytic activity and Th17 differentiation potential, respectively (Nakaya, 2014; Sinclair, 2013).

During my PhD, I studied the effects of glutamine availability and glutaminolysis on the effector function of $CD4^+$ T cells. The data obtained are presented in the second part of the results section in an article entitled ***"Glutamine deprivation during TCR stimulation results in the conversion of naive $CD4^+$ T cells to a regulatory T cell fate"***. In this article, I addressed the following questions:

- What is the impact of glutamine deprivation on the TCR-mediated activation of the mTOR pathway and ATP production?
- Does glutamine metabolism alter the effector function of *ex vivo*-stimulated $CD4^+$ T cells?
- Under conditions of limiting glutaminolysis, what are the mechanisms regulating the *ex vivo* conversion of TCR-engaged $CD4^+$ T cells to a regulatory T cell fate?
- Does glutamine deprivation affect the *in vivo* persistence and suppressor function of adoptively-transferred T cells?

Paper I

The fate of adoptively-transferred T cells is conditioned by the lymphopenia-inducing regimen

The fate of adoptively-transferred T cells is conditioned by the lymphopenia-inducing regimen

Running title: **Regulation of T cell transfer by host conditioning**

Abstract

Adoptive T cell immunotherapy has demonstrated promising potential but enhancing T cell persistence remains a challenge. One approach involves a pre-transfer lymphodepletion to improve the engraftment and reactivity of injected T lymphocytes. Indeed, different lymphopenia-inducing regimens are used in clinical trials but it is not known whether they are equivalent in their capacity to promote expansion and effector function. Here, we show that the fate of donor T cells is strikingly different in host mice rendered lymphopenic by sub-lethal irradiation versus a busulphan/cyclophosphamide (Bu/Cy) chemotherapy regimen. Donor CD8 T cells underwent homeostatic proliferation in irradiated but not Bu/Cy-conditioned mice due to low IL-7 bioavailability. In contrast, Bu/Cy treatment induced a massive antigen-driven proliferation of donor CD4 T cells. This CD4 proliferation was associated with changes in lymphoid organ architecture and was dependent on $CD11c^+$ dendritic cells (DCs). Notably, the Bu/Cy-modulated environment resulted in a 2-3-fold increase in donor CD4+Foxp3+ regulatory T cells, due to their extensive proliferation. Thus, lymphopenia-inducing regimens differentially regulate the fate of adoptively-transferred T lymphocytes.

Introduction

Despite many advances in cancer therapies, metastatic disease remain difficult to treat. As immune cells are theoretically capable of responding against tumor antigens, new immunotherapy approaches, aimed at modifying the balance between tumor-specific immune cells and cancer cells, are being developed. This type of therapy is based on the infusion of tumor-specific immune cells that have been manipulated and expanded *ex vivo* (Gill and Kalos, 2013; Hawkins et al., 2010; Kalos and June, 2013; Kershaw et al., 2013b). Most of the clinical work in adoptive T cell therapy has been applied to metastatic melanoma because of the relatively frequent existence of tumor-specific T cells (Dudley et al., 2002; Rosenberg and Dudley, 2004). However, recent promising results have also been reported in refractory B leukemias/lymphomas treated with T cells engineered to express a CD19-expressing chimeric antigen receptor (Brentjens et al., 2013; Davila et al., 2013; Davila et al., 2014; Kalos et al., 2011; Kochenderfer et al., 2013; Kochenderfer et al., 2012; Kochenderfer et al., 2010; Koehler et al., 2012). Nevertheless, finding optimal conditions that support long-term persistence and function of adoptively transferred T cells remains a significant challenge.

One important aspect impacting on the proliferation and effector function of adoptively transferred T cells is the host environment. Under conditions of lymphopenia, there is an increased homeostatic proliferation of T lymphocytes which results in the expansion of conventional T cells, in the apparent absence of antigenic stimulation (reviewed in (Jameson, 2002; Prlic and Jameson, 2002; Takada and Jameson, 2009)). Notably, this non-specific proliferation has been shown to enhance the functionality of T cells and this effect has been exploited to augment the responsiveness of T lymphocytes in cancer patients (Dudley et al., 2002 ; Kershaw et al., 2013a). However, many

conclusions on the mechanisms regulating the link between lymphopenia and T cell responsiveness have been drawn from mice that are rendered lymphopenic by either genetic mutations or by total body irradiation (Do and Min, 2009; Goldrath et al., 2002; Hamilton and Jameson, 2008; Tan et al., 2001; Voehringer et al., 2008). While these experiments have provided significant information, they may not accurately reflect the situation in cancer patients who are often rendered lymphopenic by chemotherapy regimens (Kershaw et al., 2013a). Moreover, it is important to note that in ongoing T cell immunotherapy trials, patients are being pre-conditioned with a wide array of chemotherapies, including cyclophosphamide, fludarabine, bendamustine, pentostain/ cyclophosphamide, etoposide/ cyclophosphamide, and fludarabine/ cyclophosphamide as well as combined irradiation/chemotherapy protocols ((Maus et al., 2014). While these conditioning protocols all induce severe lymphopenia, it is important to consider the possibility that they may alter lymphocyte behavior via differential effects on the microenvironment.

Here, we assessed whether donor T cell fate is dependent upon the conditioning regimen used to achieve lymphopenia. We induced lymphopenia in C57Bl/6 mice by either sub-lethal irradiation or a chemotherapy regimen consisting of busulfan and cyclophosphamide (Bu/Cy) and analyzed the proliferation and fate of adoptively-transferred T lymphocytes. We found that lymphopenic conditions are not equivalent in their potential to foster the regeneration of the T cell pool. The extensive homeostatic proliferation of donor CD8 T cells was observed in irradiated, but not Bu/Cy-conditioned, hosts, due to the increased bioavailability of endogenous IL-7 in the former group. In the latter group, CD8 proliferation could be rescued by exogenous IL-7 administration. Furthermore, a rapid antigen-driven proliferation of CD4 T cells was observed in Bu/Cy-conditioned, but not irradiated hosts, associated with changes in lymphoid organ architecture and the presence of distinct

CD11c$^+$ DC subsets. Indeed, we found CD11c$^+$ DC to be required for this rapid antigen-driven proliferation. Moreover, this DC-mediated proliferation was particularly pronounced for CD4$^+$Foxp3$^+$ regulatory T cells, resulting in a significant increase in the Treg: conventional T cell ratio within the CD4$^+$ compartment. Thus, the design of future immunotherapy trials should carefully consider the complex interplay between cytokines and dendritic cells in the lymphopenic conditions created by various conditioning regimens, as these factors differentially regulate the proliferation and function of adoptively-transferred T cells.

Results and Discussion

Lymphopenia-inducing conditioning regimens alter the proliferation potential and phenotype of adoptively transferred T cells

It has previously been shown that in mice with either genetically-induced or radiation-induced lymphopenia, transferred T cells undergo a homeostatic lymphopenia-induced proliferation (LIP), filling the available "space" (Almeida et al., 2005; Goldrath et al., 2000; Rocha et al., 1989). To assess whether different lymphodepletion regimens modulate the fate of transferred T cells, C57Bl/6 mice were treated with either sub-lethal irradiation (600 rads), or a busulphan (16.5 mg/kg X4)/cyclophosphamide (200 mg/kg IP) chemotherapy regimen followed by T cell transfer. It is important to note that while both regimens resulted in an initial massive depletion of endogenous immune cells (data not shown), the relative recovery of host CD4 and CD8 T cells differed with significantly fewer endogenous CD8 T cells recovered in irradiated mice as compared to Bu/Cy-treated mice (**Supplemental Figure 1**). Thus, even prior to assessing the effects of conditioning on the fate of adoptively-transferred T cells, these data reveal the conditioning regimen to be an important factor in the relative recovery of endogenous CD4 and CD8 T cell subsets.

The lymphopenia induced by both conditioning regimens significantly increased the percentage of donor T cells recovered after 7 days (<1% in normal hosts as compared to 8-30% after conditioning, **Figure 1A** and data not shown). Moreover, with the doses of chemotherapy used here, the relative engraftment of donor T cells was comparable with that obtained following radiation. Interestingly though, the relative proportion of donor CD4 and CD8 cells was strongly modulated by the type of conditioning; In irradiated mice, there was a significantly higher percentage of donor CD8 T

cells whereas in Bu/Cy treated mice, the proportion of CD4 T cells was markedly higher. As previously shown in irradiated mice (Do and Min, 2009; Goldrath et al., 2002; Hamilton and Jameson, 2008; Schluns et al., 2000; Tan et al., 2001; Voehringer et al., 2008), CD8 T cells underwent a faster homeostatic than CD4 T cells, resulting in a CD4:CD8 ratio of <1. In contrast, in Bu/Cy-conditioned hosts, CD4 T cells underwent a massive proliferation of more than 6 divisions while CD8 T cells exhibited only a minimal proliferation (**Figure 1A**). This resulted in a significantly altered CD4:CD8 profile in these mice as compared to irradiated mice, with a ratio of >5:1 (**Figure 1A**).

The homeostatic proliferation of adoptively-transferred naïve T cells into T cell-deficient mice as well as in mice rendered lymphopenic by irradiation requires low affinity interactions with self-peptide/MHC complexes (Ernst et al., 1999; Goldrath and Bevan, 1999; Kieper and Jameson, 1999; Viret et al., 1999). In contrast, memory cells have significantly less stringent requirements for homeostatic proliferation (Surh and Sprent, 2008). As such, it was important to determine whether the massive increase in CD4 T cells detected in Bu/Cy-conditioned mice reflected the proliferation of a small percentage of memory cells or rather, a more global proliferation of naïve lymphocytes. We therefore compared the proliferation profiles of adoptively-transferred naïve T cells as compared to total T cells. Importantly, the distinct proliferation profiles of donor CD4 and CD8 T cells in irradiated vs. Bu/Cy-conditioned mice were also observed in mice receiving FACS-sorted naïve CD4 and CD8 T cells ($CD62L^{+}CD44^{-}CD4^{+}$ and $CD62L^{+}CD44^{-}CD8^{+}$, respectively; **Figure 1B**). These data strongly suggest that the massive division of donor CD4 T cells in Bu/Cy-conditioned recipients reflects a strong proliferation of memory as well as naive CD4 T cells.

Under lymphopenic conditions, the homeostatic proliferation of adoptively transferred naïve T lymphocytes results in their differentiation, with an acquisition of a memory-like phenotype (Cho et al., 2007; Goldrath et al., 2000; Kieper and Jameson, 1999; Murali-Krishna and Ahmed, 2000; Takada and Jameson, 2009). This phenotype change has been detected in irradiated mice and genetic T cell-deficient models (**Figure 1C)** but has not been analyzed in Bu/Cy-conditioned hosts. Notably, donor CD4 T cells in Bu/Cy-conditioned mice acquired either a memory or effector phenotype while the non-dividing CD8 T cells remained, in large majority, naïve (**Figure 1C**). Indeed, in both conditioning environments, cell proliferation was linked to changes in phenotype; non-dividing donor CD4 T cells in irradiated mice also retained their naïve phenotype (**Figure 1C**). Thus, the phenotype of the adoptively transferred T cells correlates strongly with their proliferation profile.

IL-7 is not required for the rapid proliferation of CD4 T cells in Bu/Cy-conditioned mice

The homeostatic proliferation occurring in lymphopenic hosts is mediated by environmental cues, amongst which the IL-7 cytokine plays a critical role ((Bosco et al., 2005; Kimura et al., 2013; Schluns et al., 2000; Tan et al., 2001) also reviewed in (Sprent and Surh, 2011; Surh and Sprent, 2008)). Moreover, under conditions of lymphopenia, there is an increased availability of IL-7, due, at least in part, to a reduced utilization by the few lymphocytes that remain (Fry et al., 2001; Fry and Mackall, 2001; Gattinoni et al., 2005). It was therefore important to determine whether the lack of CD8 T cell proliferation in Bu/Cy-conditioned mice was related to IL-7 availability. Indeed, rIL-7 administration dramatically increased donor CD8 proliferation in Bu/Cy-treated hosts (27% to 79%; **Figure 2A**). Furthermore, as in irradiated hosts, the IL-7-induced proliferation of CD8 T cells in Bu/Cy mice resulted in

their increased differentiation; more than 50% of CD8 cells exhibited a memory or effector phenotype (**Supplemental Figure 2**). These data strongly suggest that there is a significantly lower level of biologically-active endogenous IL-7 in Bu/Cy-conditioned mice than following irradiation. Furthermore, the finding that exogenous IL-7 promotes a homeostatic CD8 T cell expansion indicates that there is no intrinsic deficit preventing CD8 proliferation in Bu/Cy-conditioned mice.

We next assessed whether the fast proliferation of CD4 T cells was sensitive to IL-7 signaling. As indicated above, homeostatic proliferation is cytokine-sensitive (Sprent and Surh, 2011; Surh and Sprent, 2008) whereas the rapid proliferation of adoptively transferred T cells is generally induced by antigens derived from self or microbial antigens (Kieper et al., 2005; Min et al., 2005; Paulos et al., 2007) or is fostered by the translocation of commensal bacteria (Viaud et al., 2013) . Indeed, blocking IL-7 signaling inhibited LIP in irradiated hosts as well as the minor level of homeostatic CD8 T cell proliferation detected in Bu/Cy-conditioned hosts (**Figure 2B**). Notably though, blocking IL-7 signaling did not affect the striking CD4 T cell proliferation in Bu/Cy hosts, indicating that this division occurs in an IL-7-independent manner.

To further investigate the rapid proliferation of CD4 T cells in Bu/Cy-conditioned mice, we first focused on IL-2 as this pro-inflammatory cytokine has been shown to be required for the effector function of naïve CD4 T cells in lymphopenic hosts (Atreya et al., 2000; Noguchi et al., 2007; Tajima et al., 2008). Furthermore, while IL-6-dependent proliferation is generally antigen-specific (Feng et al., 2010), it can also augment CD4 T cell proliferation in a TCR-independent manner (Gagnon et al., 2008). To assess the specific role of IL-6 in Bu/Cy-conditioned hosts, the proliferation of adoptively-transferred T cells in WT and IL-6$^{-/-}$ mice were compared. As expected, the homeostatic

proliferation of donor CD8 T cells in irradiated mice was not altered in the absence of IL-6 (**Figure 3**). Interestingly though, the rapid CD4 proliferation in Bu/Cy-conditioned hosts was also not affected in the absence of IL-6 and the CD4:CD8 ratio of host T cells was not modified (data not shown). The ensemble of these data indicates that endogenous IL-6 production is not driving this rapid proliferation of adoptively-transferred CD4 T cells.

These data raised the issue of whether the striking CD4 T cell proliferation detected in Bu/Cy-conditioned mice was antigen-dependent. To respond to this question, we assessed whether OTII CD4 T cells, expressing an MHCII-restricted TCR specific for ovalbumin (OVA), would proliferate following their adoptive transfer. Importantly, OTII T cells did not proliferate in Bu/Cy-conditioned mice in the absence of the OVA antigen (**Figure 4**) These data strongly suggest that the rapid, or spontaneous, CD4 T cell proliferation occurring in Bu/Cy-conditioned mice is dependent on antigen signaling. As expected from the absence of polyclonal CD4 T cell proliferation in irradiated hosts, OTII transgenic CD4 T cell cells also did not proliferate in this setting. In contrast, OTI transgenic CD8 T cells, expressing an MHCI-restricted OVA-specific TCR, underwent homeostatic proliferation in irradiated hosts. These data are consistent with an IL-7-dependent, antigen-independent, behavior (Sprent and Surh, 2011; Surh and Sprent, 2008). Furthermore, the lower level of homeostatic OTI T cell proliferation in Bu/Cy as compared to irradiated hosts (**Figure 4**) points to lower IL-7 bioavailability in the former environment. Altogether, these data, demonstrates that the behavior of polyclonal as well as monoclonal CD4 and CD8 T cells is significantly modulated by the conditioning environment.

Conditioning regimens modulate the host microenvironment and dendritic cell populations

To elucidate why the rapid antigen-driven proliferation of adoptively transferred CD4 T cells occurs only in Bu/Cy-conditioned mice, we next focused on the host microenvironment. While several studies have assessed the effects of cylophosphamide conditioning on myeloid and APC subsets (Nakahara et al., 2010; Salem et al., 2009; Salem et al., 2007), there is a severe paucity of research on how such conditioning affects the structure of lymphoid organs. This is even more surprising given the importance of tissue anatomy and positioning of immune cells in immune responsiveness (Ansel et al., 1999; Garside et al., 1998; Reif et al., 2002). In control LN and splenic histological sections, B follicles were clearly defined with other areas showing a wide distribution of $CD11c^+$ dendritic cells (DCs). However, following irradiation, the architecture was highly perturbed. By 7 days post treatment, B cell follicles were non-distinct but a significantly higher frequency of $CD11c^+$ DCs was detected throughout. Interestingly, lymphoid organs from Bu/Cy-conditioned hosts revealed a more “intermediate” conformation; sections showed distinct B cell follicles, albeit smaller and fewer in number, and $CD11c^+$ DCs were less frequent than in irradiated tissues (**Figures 5A and 5B**). Thus, conditioning regimens result in distinct changes to lymphoid organ architecture (**Figures 5A and 5B**). Furthermore, it is important to note that the restitution of the architecture in secondary lymphoid organs is not immediately re-established and remains perturbed for at least one week following adoptive transfer.

Given the critical input of DC in antigen presentation and therefore in T cell proliferation (Do and Min, 2009; Gruber and Brocker, 2005; Sapoznikov et al., 2007; Zaft et al., 2005), it was of interest to evaluate the kinetics of DC perturbation following conditioning. At two days post either irradiation or Bu/Cy treatment, the percentages of DCs in lymphoid organs was significantly decreased as were $CD8^+CD11c^+$ cross-presenting DCs ($p<0.005$; **Figures**

5B, and data not shown). However, by day 7 post treatment, the relative percentages of CD11c$^+$DCs in Bu/Cy-treated mice reached levels equivalent to those in control mice. Moreover, CD11c$^+$ DCs accounted for a significantly higher percentage of immune cells in the lymphoid organs of irradiated mice ($p<0.005$). Furthermore, the sub-populations of DCs also differed; CD8$^+$CD11c$^+$ DCs were present at more than 2-fold higher levels in irradiated mice than in Bu/Cy-conditioned mice (means of 30% vs 63%, $p<0.005$; **Figure 5B**). Thus, the host microenvironment, and specifically DCs, are markedly affected by the type of conditioning which is used to achieve a lymphopenic state in immune-competent recipients.

Interplay between CD11c$^+$DC and IL-7 signals in the proliferation of adoptively-transferred T cells

Based on the important differences in DC subsets in irradiated as compare to Bu/Cy-conditioned mice, we assessed whether the T cell proliferation in these mice was dependent on DCs. Previous research has shown that the rapid antigen-dependent proliferation of adoptively transferred T cells requires DCs (Do and Min, 2009; Gruber and Brocker, 2005; Sapoznikov et al., 2007; Zaft et al., 2005) but the role of DCs in distinct lymphopenia-induced environments is not known. To address this question, we made use of BAC transgenic CD11c.DOG mice in which expression of the human DTR is under the control of the CD11c promoter (Hochweller et al., 2008). Unlike CD11c-DTR mice with an integrated copy of the gene, the CD11c.DOG mice can be repeatedly injected with diphteria toxin in order to maintain DC depletion without causing lethality (**Figure 6A and 6B**). Importantly, the massive CD4 proliferation detected in Bu/Cy-conditioned mice was markedly decreased in the absence of CD11c$^+$ DCs and this effect was also observed for the proliferating CD8 T cell population (**Figure 6C and 6D**). Together, these results demonstrate that

the rapid CD4 T cell proliferation in Bu/Cy-conditioned hosts is independent of the IL-6 and IL-7 cytokines but requires an interaction with CD11c+ DCs.

It was somewhat surprising to find that the homeostatic proliferation of both CD4 and CD8 T cells in irradiated mice was dependent on the presence of CD11c$^+$DC ($p<0.00005$; **Figure 6C and 6D**) because this division requires IL-7 signaling (Bosco et al., 2005; Kimura et al., 2013; Schluns et al., 2000; Tan et al., 2001) and **Figure 2).** There has been some controversy on the link between DCs and IL-7 signaling in homeostatic T cell proliferation (Guimond et al., 2009; Martin et al., 2010) and here, we attempted to dissect the relative role(s) of DC and IL-7 signaling in different lymphopenic environments. T cell transfers were performed under conditions where rIL-7 was administered, in the presence or absence of host CD11c$^+$DCs. In DC-depleted conditions, exogenous IL-7 markedly increased the slow homeostatic proliferation of CD8 as well as CD4 T cells in Bu/Cy-conditioned hosts ($p<0.00005$ and $p<0.005$, respectively; **Figure 7**). However, in the absence of DCs, exogenous IL-7 was not sufficient to promote the rapid antigen-driven proliferation of adoptively-transferred CD4 or CD8 T cell subsets (**Figure 7**). Thus, DCs promote homeostatic T cell proliferation, likely by increasing self-pMHC ligands, but their presence is required for the rapid proliferation of CD4 and CD8 T cell subsets.

While CD8α$^+$ DCs have been shown to play a critical role in antigen presentation and T cell proliferation (den Haan et al., 2000), it is interesting to note that in certain settings, CD4 T cell proliferation is preferentially induced by CD8α$^-$CD11b$^+$ DCs (Chung et al., 2007; Pooley et al., 2001). Furthermore, the LN environment itself is likely to modulate the ability of adoptively transferred T cells to come into contact with antigen presenting cells. Indeed, in the context of chronic inflammation where there is a reduction in the T cell

zone, there is an impaired T cell response to tumor antigens (Soudja et al., 2011). Thus, it is interesting to speculate that the loss of T cell zones in irradiated mice reduces the ability of transferred CD4 T cells to encounter antigen and proliferate while in Bu/Cy-treated hosts, the presence of these zones together with high percentages of $CD8\alpha^{-}CD11b^{+}$ DCs promotes their extensive proliferation.

Regulatory T cells proliferate extensively in Bu/Cy-conditioned hosts. The CD4 T cell pool that was adoptively-transferred into conditioned hosts harbored a small percentage of $CD4^{+}CD25^{+}$ regulatory T cells (Tregs). We therefore assessed whether the relative percentage of Tregs was altered following adoptive transfer. While the percentage of Tregs was not significantly different in control and irradiated mice, the percentage in Bu/Cy-conditioned mice was markedly increased (9% to 24%; **Figure 8A**). The augmentation of donor $Foxp3^{+}$ T cells in Bu/Cy-conditioned hosts represents an expansion, rather than a *de novo* conversion, as only low levels of $Foxp3^{+}$ T cells were recovered following adoptive transfer of Treg-depleted T cells ($CD25^{-}CD3^{+}$; **Figure 8B**). Nevertheless, it is important to note that even following Treg depletion, the vast majority of conventional CD4 T cells underwent a rapid proliferation following their transfer into Bu/Cy hosts (data not shown). Thus, both conventional as well as $Foxp3^{+}$ Tregs proliferate rapidly in these mice but under conditions where Tregs are present, it is their division that is favored. As expected, the accumulation of Tregs was also dependent on endogenous IL-2; blocking IL-2 signaling significant decreased the percentage of regulatory T cells (**Figure 8C**). Furthermore, as expected from our data showing that the rapid proliferation of CD4 T cells is dependent on the presence of $CD11c^{+}DC$ (**Figure 7**), the elimination of this subset also decreased the relative abundance of $Foxp3^{+}$ T cells within the CD4 T cell population (mean of 22% to 9%; $p<0.05$; Figure 7D). Indeed, the absence of

DCs dramatically affected the rapid proliferation of both conventional CD4 T cells and Tregs ($p<0.0005$). High Treg proliferation was still detected because of the extremely high baseline levels of proliferation (90%; **Figure 8D**).

The ensemble of these data reveal the conditioning regimen to be a critical factor in the proliferation of adoptively-transferred T cells as well as in the expansion of specific donor T cell subsets. Furthermore, the host microenvironment as well as the relative abundance of available cytokines such as IL-7, a master regulator of T cell homeostasis, are significantly affected by conditioning, thereby modulating the fate of donor as well as host T cells. It will be of utmost important to take these factors into consideration upon treatment of patients with adoptively-transferred T lymphocytes. In this regard, it is important to note that while the adoptive transfer of genetically modified T cells bearing a chimeric antigen receptor against the CD19 receptor has resulted in spectacular outcomes in some patients with chemotherapy-resistant $CD19^+$ leukemias, the conditioning regimens that the patients have undergone prior to T cell injection have differed (Brentjens et al., 2013; Brentjens et al., 2007; Davila et al., 2013; Davila et al., 2014; Kochenderfer et al., 2013; Kochenderfer et al., 2012; Kochenderfer et al., 2010). Thus, it will be important to assess how these regimens impact on the fate of the adopted cells. The data presented here clearly indicate that host conditioning cannot just be treated as a simple lymphopenia-inducing regimen. Each regimen has specific effects on the lymphoid, APC and stromal environments, shaping the destiny of transferred CD4 and CD8 T lymphocytes.

Methods and Materials

Mice. C57Bl/6-Thy1.2, C57Bl/6-Thy1.1 and C57Bl/6-CD45.1 mice were purchased from Charles River laboratories and CD11c-DOG as well as IL-6 KO mice have been previously described (Hochweller et al., 2008; Kopf et al., 1994). Mice were housed in conventional pathogen-free facilities at the Institut de Génétique Moléculaire de Montpellier and NCI-Frederick. OTI and OTII TCR-transgenic mice, on a Rag-1 null background, were housed in a pathogen-free facility at the POSTECH Biotech Center. Animal care and experiments were performed in accordance with National Institutes of Health (NIH), French national guidelines and South Korean national guidelines.

Cell isolations and proliferation assays. Donor cells were isolated from the lymph nodes of Thy1.1^{+}C57BL/6 or CD45.1^{+}C57BL/6 mice. CD3^{+} T cells were purified using Dynal isolation kits (Dynabeads Untouched Mouse T cells, #11413D, Invitrogen) according to the manufacturer's instructions. For experiments with naïve or CD25-depleted CD3^{+} T cells, enriched CD3^{+} T cells were sorted on a FACSaria flow cytometer (BD Biosciences) on the basis of a CD3^{+}CD62L^{hi}CD44^{-} and CD4^{+}CD62L^{hi}CD44^{-}CD25^{-} expression profile respectively. To monitor proliferation, cells were labeled with CFSE (Life Technologies; 2.5 uM) or CTV (Life Technologies; 5 μM) for 8-10 mins at 37°C. Reactions were stopped with ice-cold PBS.

Adoptive T cell transfer into irradiated and Bu/Cy-conditioned hosts. Thy1.2^{+}C57Bl/6 mice were sublethally irradiated (6 Gy) or conditioned with busulphan (16.5 mg/kg *bid* for 2 days; Inresa Pharma, Bartenheim, FR) followed by cyclophosphamide (200 mg/kg for 1 day; Sigma). Twenty four to thirty six hours post conditioning, mice received IV injections of CFSE-labeled

Thy1.1$^+$ lymph node T cells (5 x 10^6 cells/mouse). Followin g transfer, mice were either left untreated, treated with αIL7 (M25)/αIL7R (A7R34) antibodies (500 μg/mouse qd for 7 days), treated with rIL-7 (5 μg/mouse bid for 7 days) or IL-7 immunocomplexes (rIL-7 (3 μg) premixed with the M25 anti-IL-7 mAb (15 μg)) every other day for 7 days, or treated with an αIL2 (S4B6) mAb (800 μg/mouse followed by 500 μg/mouse *qod* for 7 days). For dendritic cell depletion experiments, CD11c-DOG mice were injected IP with DT (32 ng/gbw) from *Corynebacterium diphtheriae* (every other day, starting 2 days before conditioning). Animals were sacrificed at the indicated time points and lymph nodes and spleens were harvested for analysis.

Immunophenotyping and flow cytometry analyses. Cells were immunophenotyped with the following fluorochrome-conjugated antibodies to the following markers: CD4 (RM4-5), CD8 (53-6.7), Thy1.1 (OX7), CD45.1 (A20), CD62L (MEL-14), CD44 (IM7), CD11c (HL3), CD11b (M1/70) and Foxp3; all obtained from BD Biosciences and EBiosciences. Intracellular staining for Foxp3 protein was performed using the eBioscience Fixation/Permeabilization kit. Cells were analyzed on a FACScanto (BD Biosciences) or sorted on a BD FACSaria flow cytometer. Data analyses were performed using FlowJo Mac v.8.8.7 software (Tree Star) and FCAP Array Software (CBA analysis).

Tissue digestion for DC phenotying. For DC phenotyping, lymph nodes and spleens were gently dissected into small pieces and incubated in RPMI medium 1640 medium containing 1mg/mL collagenase D (Roche) at 37°C for 45 min. The digested tissues were then mashed and filtered through a cell strainer to obtain a single cell suspension.

Immunohistochemistry. Freshly collected organs were incubated overnight at 4°C in fixing solution (4% paraformaldehyde/10% sucrose in PBS) and further immersed in a sucrose solution for 3 hours (30% sucrose in PBS). After fixation, tissues were 'snap-frozen' in OCT (CellPath) with a slurry of isopentane and liquid nitrogen. Tissue sections (8 μm in thickness) were prepared, air-dried, and stored at -80°C. Cryosections were blocked for 30 min with Fc-blocking antibody (2.4G2), washed in PBS with 2% FCS, and incubated for 1-12h at 4°C with anti-B220 (RA3-6B2) and anti-CD11c (HL3) antibodies (BD Biosciences). Sections were then washed with PBS/ 2% FCS and stained with DAPI. All slides were mounted with Prolong Antifade Reagent (Invitrogen) and images were captured with a Leica DC500 camera on a Confocal Leica TCS SP5 microscope and analyzed with Leica LAS AF Lite software.

Statistical analyses. Statistical significance was determined using a paired Student's t-test with a 2-tailed distribution unless otherwise indicated (Graph Pad Software, La Jolla, CA). Data were considered to be statistically different for $P \leq 0.05$ (*$P<0.05$, **$P<0.005$, ***$P<0.0005$, ****$P<0.00005$). All data are presented as means ± SD.

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Figure Legends

Figure 1. **Lymphopenia-inducing regimens alter the proliferation and phenotype of adoptively transferred T cells.**

(**A**) C57Bl/6-Thy1.2 mice, preconditioned by irradiation (6 Gy) or busulphan (16.5 mg/kg x 4doses)/cyclophosphamide (200 mg/kg), were injected with CFSE-labelled Thy1.1$^+$ CD4 and CD8 T cells (5x10^6). Mice were sacrificed 7 days post adoptive transfer and the recovery of donor T cells was monitored by gating on Thy1.1$^+$ positive cells (left plots). Dot plots showing the relative repartition of donor CD4 and CD8 T cells are presented and division of gated CD4 and CD8 donor T cells was monitored as a function of CFSE fluorescence (right histograms). (**B**) C57Bl/6 mice, conditioned as above, were injected with either total CFSE-labelled T cells or purified naïve CFSE-labelled T cells (CD3$^+$CD62L$^+$CD44$^-$). CFSE profiles of the adoptively transferred cells were assessed at day 7 and representative histograms are presented. (**C**) The phenotype of adoptively-transferred T cells was monitored as a function of CD62L and CD44 expression and the percentages of naïve (N), central memory (CM) and effector memory (EM) cells are shown. Results are representative of data obtained in at least 8 independent experiments.

Figure 2. **IL-7 signalling differentially regulates the proliferation of adoptively-transferred T cells in irradiated and Bu/Cy-conditioned mice.**

(**A**) Irradiated and Bu/Cy-conditioned C57Bl/6-Thy1.2 mice were injected with Thy1.1$^+$ CFSE-labelled T cells in the absence or presence of recombinant IL-7. Mice were sacrificed at day 7 and the percentages of Thy1.1$^+$ donor cells were monitored. The percentages of donor CD4 and CD8 T cells are indicated for each group and CFSE fluorescence was monitored in gated subsets as indicated. (**B**) CFSE-labelled T cells were injected into conditioned mice in the absence or presence of αIL7/αIL7R antibodies (500 μg/mouse per day). The

relative engraftment, CD4/CD8 repartition and proliferation of donor CD4 and CD8 Thy1.1^{+} T cells were monitored and representative plots are presented.

Figure 3. **IL-6 is not required for the rapid proliferation of CD4 T cells in Bu/Cy-conditioned mice.**

The role of IL-6 in regulating donor T cell proliferation in irradiated- and Bu/Cy-induced lymphopenic environments was assessed by comparing the fate of T cells transferred into WT as compared to IL-6-deficient C57Bl/6 hosts. Mice were sacrificed 6 days following adoptive transfer and representative plots showing the percentage recovery of donor T cells in each condition and the proliferation of gated CD4 and CD8 donor T cells (by CFSE dilution) are presented (left panels). Quantification of CD4 and CD8 T cell proliferation, as a function of the number of divisions, is shown as mean levels ± SD (n= 4 per group; *P<0.05).

Figure 4. **Donor CD4 T cell proliferation in Bu/Cy conditioned hosts is antigen-dependent.**

C57Bl/6-Thy1.2 mice, preconditioned by irradiation (6 Gy) or busulphan (16.5 mg/kg x 4doses)/cyclophosphamide (200 mg/kg), were injected with CFSE-labelled Thy1.1^{+} OTII/CD4 or OTI/CD8 T cells from a Rag-1 null background. Mice were sacrificed 7 days post adoptive transfer and the recovery of donor OTII and OT1 T cells was monitored by gating on Thy1.1^{+}CD4^{+} (left panels) or Thy1.1^{+}CD8^{+} (right panels) cells, respectively (left plots). Representative histograms showing division of gated OTII and OTI donor T cells are presented as a function of CFSE or CTV fluorescence (right panels).

Figure 5. **Distinct effects of conditioning regimens on the host microenvironment and recovery of dendritic cell subsets.**

(**A**) The localization of B cells and dendritic cells was monitored on 8 mm thick cryosections of spleen sections from control mice as well as 7 days post irradiation or busulfan/cyclophosphamide treatment. Sections were stained with DAPI (nucleus), anti-B220 (B cells) and anti-CD11c (dendritic cells) antibodies and assessed by confocal microscopy. Representative sections are shown (40 x magnification) with white dashed lines delineating B cell follicles and white arrows indicating disrupted B cell follicles. (**B**) The impact of conditioning on the fate of host dendritic cells (DC) was monitored in spleens of control, irradiated and busulfan/cyclophosphamide-conditioned mice at days 3 and 7 post treatment. DCs were analysed as a function of CD11c expression and representative plots showing the percentages of $CD11c^+$ are presented. The repartition of $CD11c^+$ cells subsets was monitored as a function of CD8 and CD11b expression and percentages are indicated in each plot. The percentages of total $CD11c^+$ and $CD11c^+CD8^+$ cells at each time point were quantified and mean levels ± SD are indicated (n=2-6 mice per group; **P<0.005; ***P<0.0005).

Figure 6. **Depletion of dendritic cells decreases the proliferation of adoptively transferred T cells in both irradiated and Bu/Cy-conditioned mice.**

(**A**) The role of DCs in regulating donor T cell proliferation in irradiated- and Bu/Cy-induced lymphopenic environments was assessed by comparing the fate of T cells transferred into WT as compared to DC-depleted C57Bl/6 hosts. DCs were eliminated in CD11c-DOG mice by injection of diphtheria toxin (DT; 32 ng/gbw every 2 days) and the level of depletion at day 7 was monitored as a function of CD11c expression. Representative dot plots of splenic $CD11c^+$ DC are shown. (**B**) The percentages of splenic $CD11c^+$ DC in untreated and DT-treated mice were quantified. Levels in untreated mice were fixed at 100% and percent depletion ± SD is presented (n=12 mice per group;

***P<0.0005, ****P<0.00005). (**C**) Untreated and DT-treated CD11c-DOG mice were injected with CFSE-labelled Thy1.1^{+} T cells and proliferation of gated CD4 and CD8 donor T cells was monitored by dilution of CFSE fluorescence. Representative histograms are shown and the percentages of non-dividing, homeostatically proliferating, and rapidly proliferating T cells are indicated. (**D**) Quantification of CD4 and CD8 T cell proliferation, separated as 1-6 divisions or >6 divisions, are shown for each condition and are presented as mean levels ± SD (n=12 mice per group; **P<0.005, ****P<0.00005).

Figure 7. **IL-7 increases the homeostatic but not rapid proliferation of adoptively transferred T cells in DC-depleted hosts following Bu/Cy conditioning.**

(**A**) To assess the respective roles of DCs and IL-7 in driving donor T cell proliferation in Bu/Cy-conditioned mice, CD11c^{+}DC were depleted from CD11c-DOG mice by DT treatment in the absence or presence of IL-7 immunocomplexes. The presence of Thy1.1$_{+}$ donor T cells was analyzed 7 days post adoptive transfer (left plots) and the proliferation of CD4 and CD8 donor T cells was monitored by dilution of CFSE fluorescence (right histograms). The percentages of non-dividing, homeostatic dividing (1-6 divisions), and rapidly dividing (>6 divisions) are indicated in each histogram. (**B**) Quantification of CD4 and CD8 T cell proliferation, separated as 1-6 divisions or >6 divisions, are shown as mean levels ± SD for each condition (n=3 mice per group; *P<0.05, **P<0.005, ***P<0.0005, ****P<0.00005).

Figure 8. **Bu/Cy-generated lymphopenia results in the expansion of adoptively-transferred Foxp3^{+} regulatory T cells.**

(**A**) Following transfer of T cells into irradiated and Bu/Cy-conditioned hosts, the relative repartition of conventional (Foxp3^{-}) and regulatory (Foxp3^{+}) T cells

within gated donor Th1.1^{+}CD4^{+} T cells was monitored. Representative plots showing the percentages of Foxp3^{+} T cells 7 days post transfer are shown. (**B**) To assess whether the significant donor regulatory T cell subset in Bu/Cy-conditioned mice resulted from a conversion or an expansion, mice were injected with either total T cells or CD25^{hi}-depleted T cells (CD62L^{+}CD44^{-} CD25^{-}CD3^{+}). The repartition of CD4/CD8 donor T cells are shown (left plots). The presence of Foxp3^{+} cells within the gated CD4 donor T cell subset was monitored and percentages are indicated in each plot (right plots). (**C**) The role of IL-2 in the expansion of donor Foxp3^{+} cells in Bu/Cy-conditioned mice was evaluated by injection of an αIL-2 antibody (0.8 mg every other injection). The repartition of CD4/CD8 donor T cells and the presence of Foxp3^{+} cells within the gated CD4 donor T cell subset were monitored as above and representative plots at day 6 post adoptive transfer are shown. (**D**) Total T cells were adoptively transferred into Bu/Cy-conditioned CD11c-DOG mice. Injected mice were either maintained or further treated with DT to deplete CD11c^{+}DC. The percentages of Foxp3^{+} T cells, within the CD4 donor gate, were monitored in the two conditions and representative dot plots (left upper plots) as well as a quantification (right histogram; n=6 mice per group) are presented. Changes in the proliferation of donor CD4^{+}Foxp3^{-} and CD4^{+}Foxp3^{+} T cells as a function of DT treatment were monitored and representative histograms (bottom left) and a quantification of the percentages of dividing cells (bottom right; means ± SD (*P<0.05, ***P<0.0005) are presented.

Supplemental Figure Legends

Supplemental Figure 1. **The phenotype of host T cells is modulated by irradiation and Bu/Cy conditioning.**

C57Bl/6-Thy1.2 mice were irradiated (6 Gy) or conditioned with busulphan (16.5 mg/kg x 4 doses)/cyclophosphamide (200 mg/kg). Mice were sacrified 7 days later and representative dot plots showing the relative repartitions of host CD4 and CD8 T cells within the CD3+ LN gate are presented (left plots). The percentages of naïve (N), central memory (CM) and effector memory (EM) CD4 and CD8 host T cells were monitored by CD44/CD62L staining as presented.

Supplemental Figure 2. **Changes in IL-7 availability alters the phenotype of donor T cells in irradiated and Bu/Cy-conditioned mice.**

Irrradiated and Bu/Cy-conditioned C57Bl/6-Thy1.2 mice were injected with Thy1.1+ donor T cells in the absence or presence of recombinant IL-7 or IL-7 blocking antibodies (αIL-7/αIL-7R) as described in figure 2. The phenotype of host CD4 and CD8 T cells was monitored at the time of sacrifice, 7 days post adoptive transfer. Phenotypes are presented as a function of CD44/CD62L expression and the percentages of naïve (N), central memory (CM) and effector memory (EM) T cells are indicated.

Figure 1: Lymphopenia-inducing regimens alter the proliferation and phenotype of adoptively transferred T cells

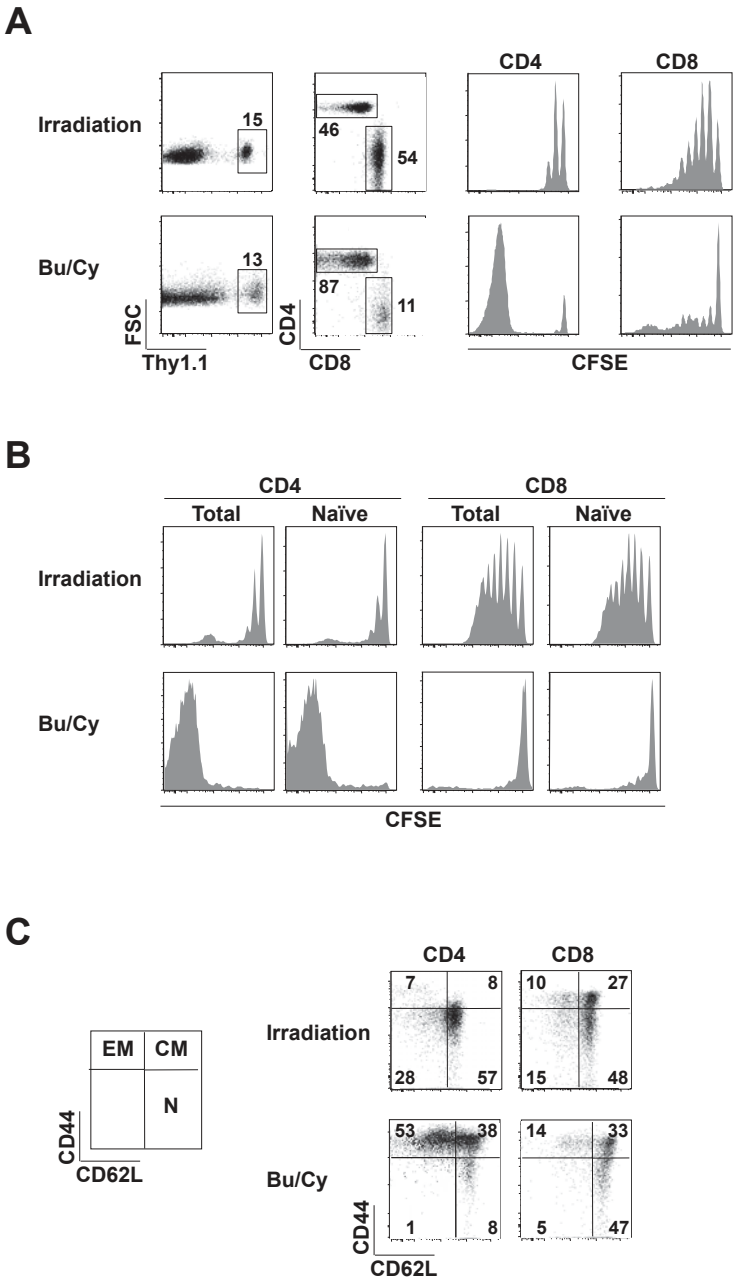


Figure 2: IL-7 signalling differentially regulates the proliferation of adoptively-transferred T cells in irradiated and Bu/Cy-conditioned mice

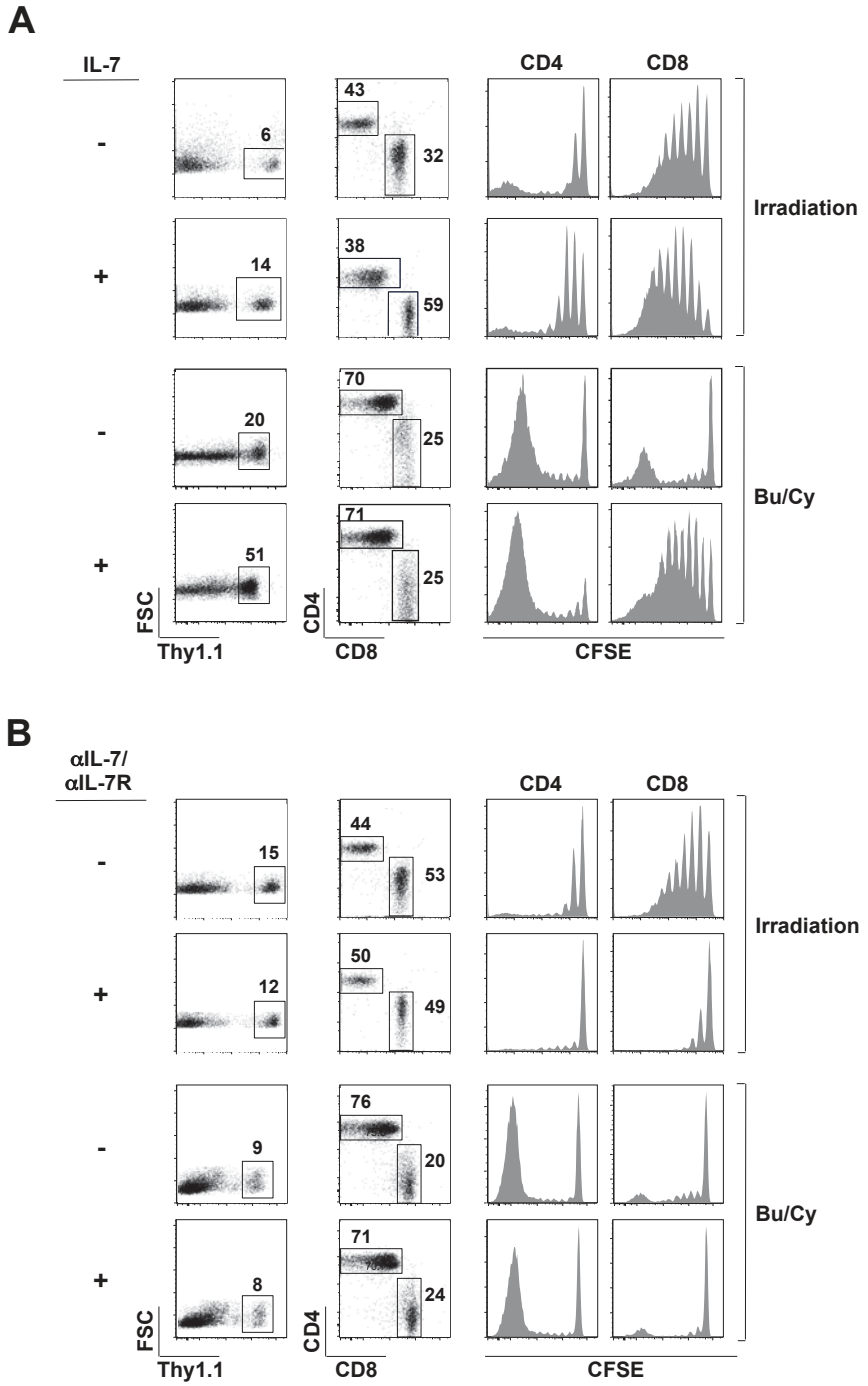


Figure 3: IL-6 is not required for the rapid proliferation of CD4 T cells in Bu/Cy-conditioned mice

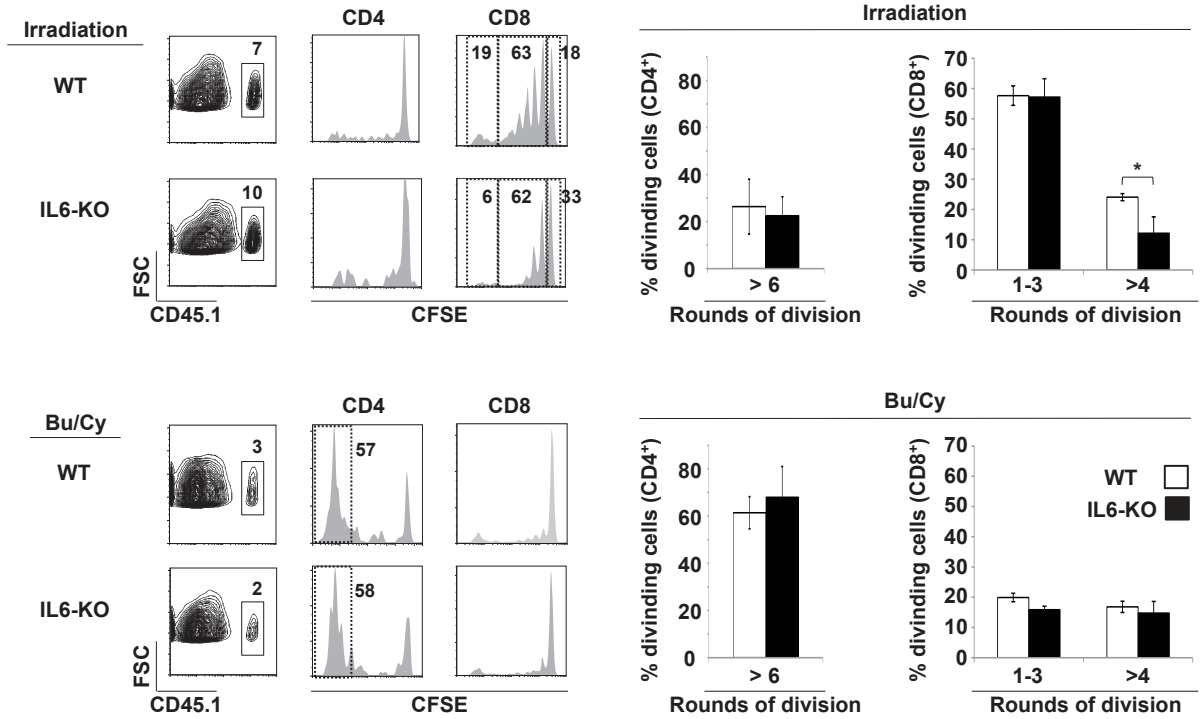


Figure 4: Donor CD4 T cell proliferation in Bu/Cy-conditioned hosts is antigen-dependent

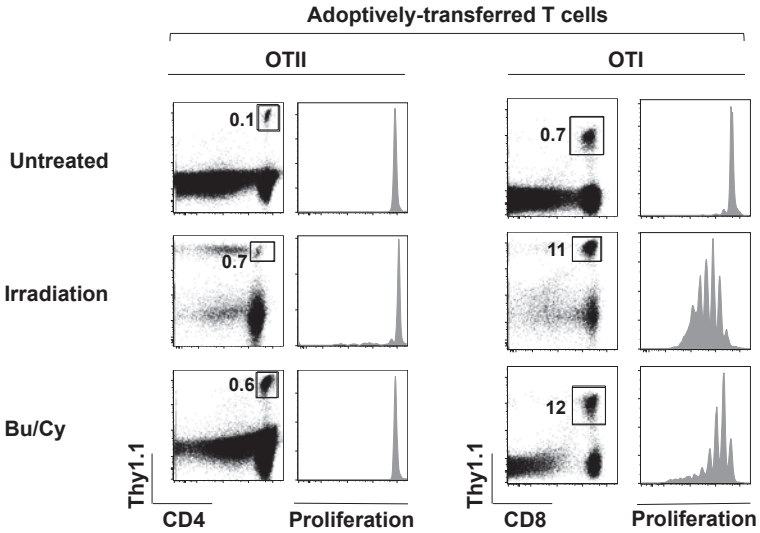


Figure 5: Distinct effects of conditioning regimens on the host microenvironment and recovery of dendritic cell subsets

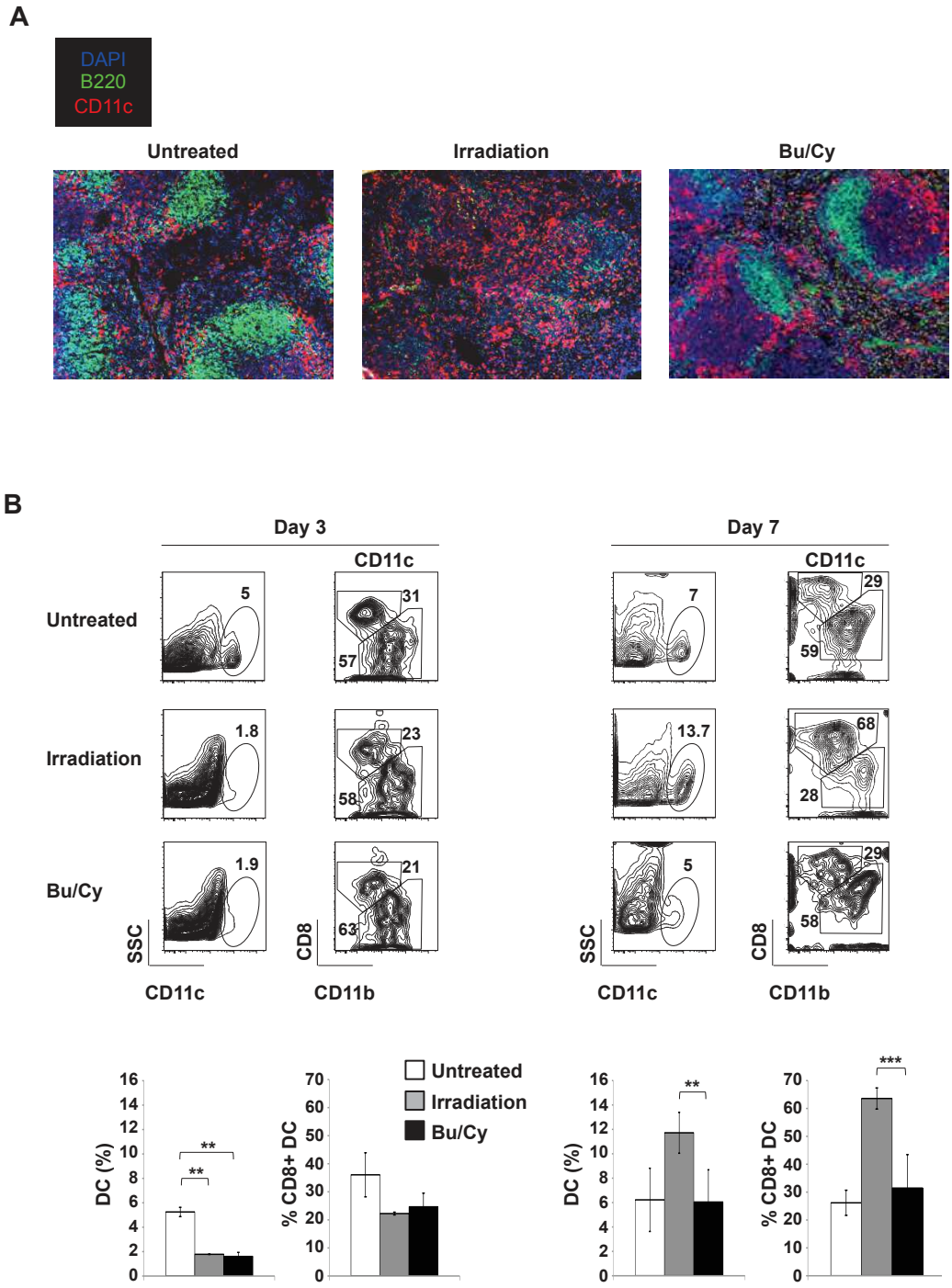


Figure 6: Depletion of dendritic cells decreases the proliferation of adoptively transferred T cells in both irradiated and Bu/Cy-conditioned mice

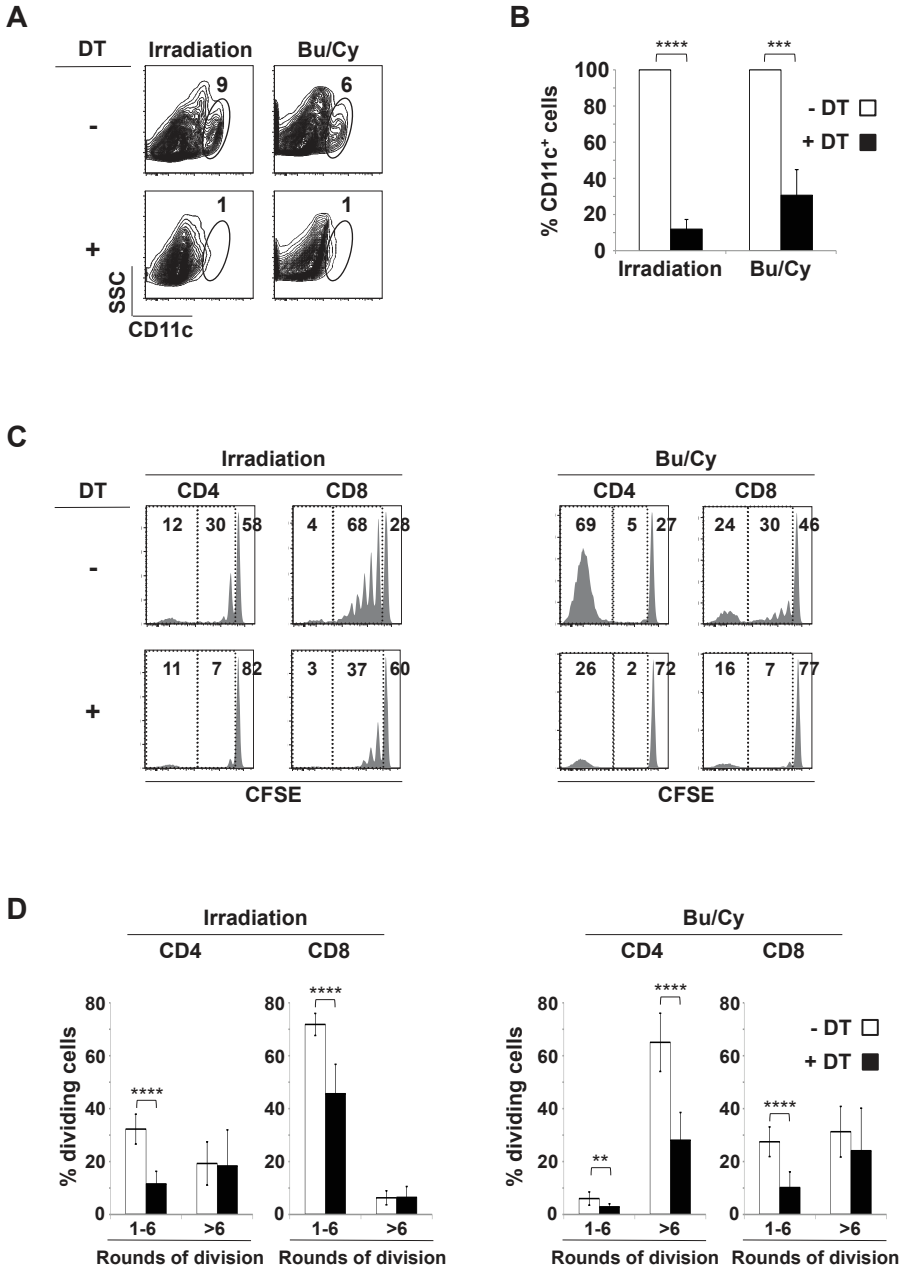


Figure 7: IL-7 increases the homeostatic but not rapid proliferation of adoptively transferred T cells in DC-depleted hosts following Bu/Cy conditioning

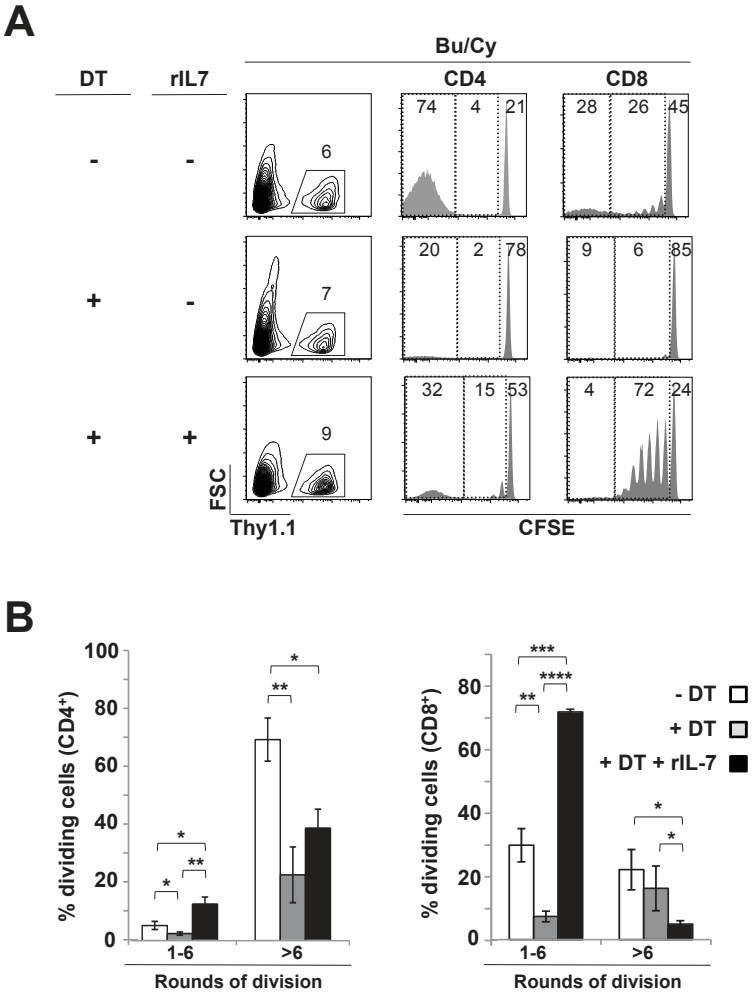
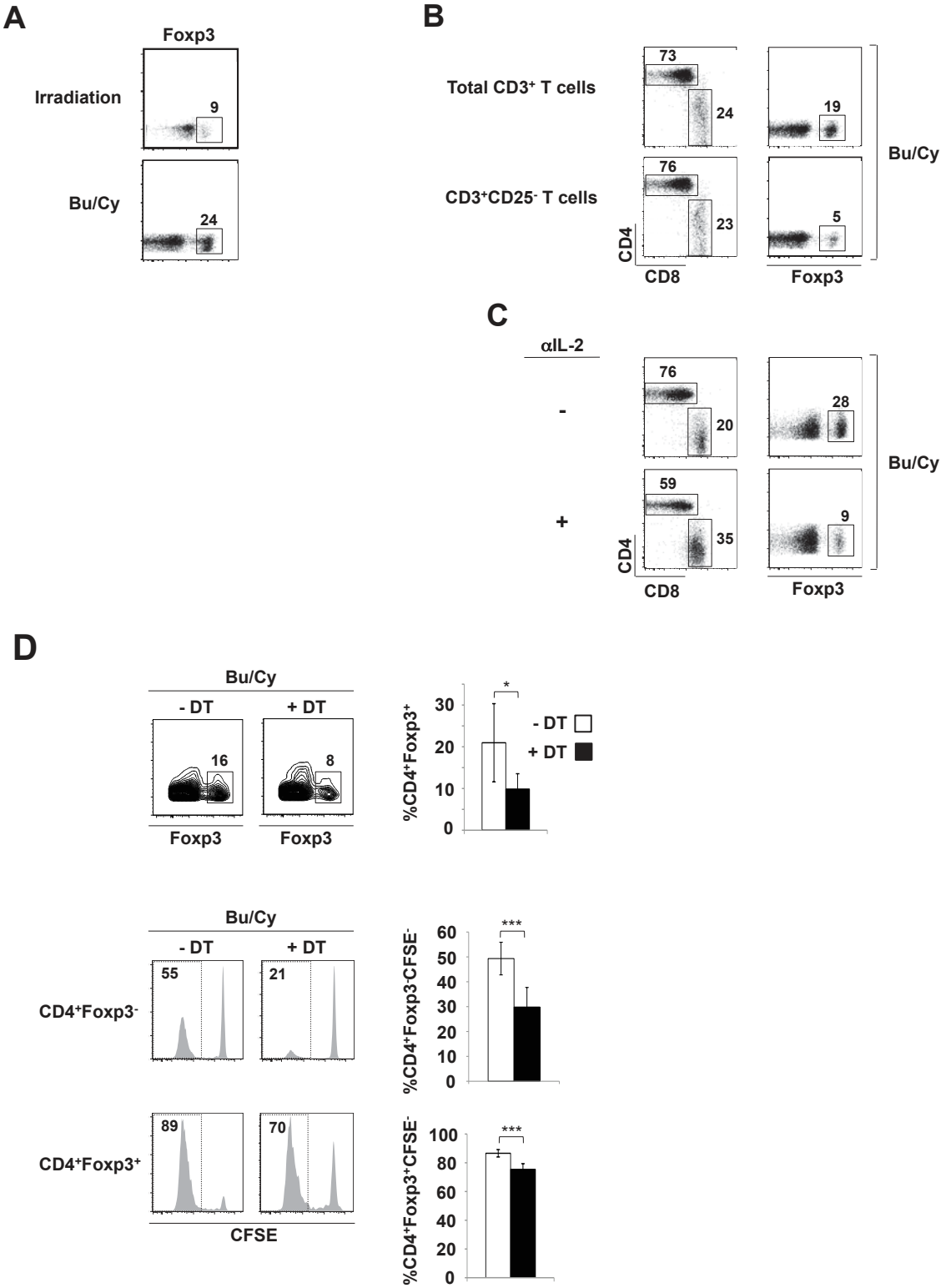
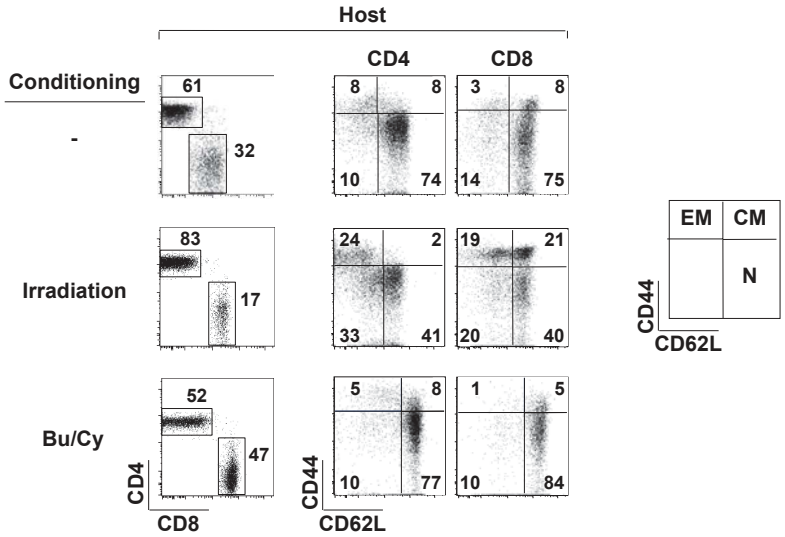


Figure 8: Bu/Cy-generated lymphopenia results in the expansion of adoptively-transferred Foxp3^{+} regulatory T cells

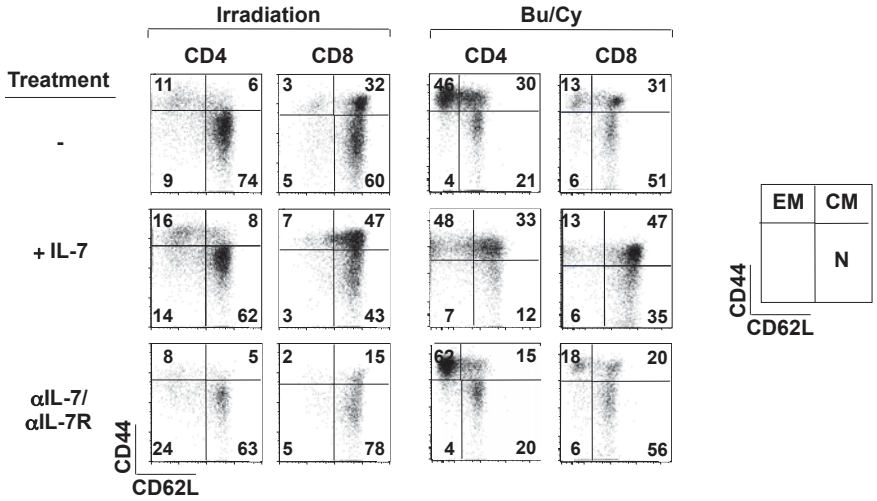


Supplementary figures

Supplementary Figure 1: The phenotype of host T cells is modulated by irradiation and Bu/Cy conditioning



Supplementary Figure 2: Changes in IL-7 availability alters the phenotype of donor T cells in irradiated and Bu/Cy-conditioned mice



Paper II

Glutamine deprivation during TCR stimulation results in the conversion of naive CD4 T cells to a regulatory T cell fate

Glutamine deprivation during TCR stimulation results in the conversion of naive CD4 T cells to a regulatory T cell fate

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Condensed title: **Glutamine control of Treg differentiation**

Abstract

The mammalian target of rapamycin (mTOR) integrates environmental cues, regulating T cell activation and function. While genetic manipulations have provided insights into the effects of mTOR signaling on T cell functions, mTOR signaling is physiologically regulated by changes in amino acid transport and availability. Here we show that glutamine deprivation of TCR-stimulated naïve $CD4^+$ T cells delays mTORC1 activation, decreasing the early energetic resources of the cell and resulting in the conversion of naïve $CD4^+$ T cells into $Foxp3^+$ regulatory T cells (Tregs). This *de novo* Treg differentiation even occurs even under Th1-polarizing conditions and is strictly dependent on TGFβ. $Foxp3^+CD4^+$ lymphocytes generated by glutamine deprivation exhibit augmented *in vivo* proliferation, upregulated Helios expression and potent suppressor function. Thus, the differential utilization of nutrient resources, conditioned by fluctuating microenvironments, converts TCR-signaled $CD4^+$ T cells into potent regulatory T cells. This may represent a mechanism via which an organism avoids mounting an energetically costly immune reaction under conditions of limiting fuel resources.

Introduction

The mammalian target of rapamycin (mTOR) integrates environmental cues, regulating T cell activation and function. Glutamine uptake, via cell surface amino acid transporters, has been shown to be a rate-limiting step in the activation of the mTOR pathway in transformed cell lines (Fuchs et al., 2007; Nicklin et al., 2009). The mTOR serine threonine kinase forms two functionally distinct complexes termed mTORC1 and mTORC2. In T cells, activation of mTORC1 regulates Th1 and Th17 differentiation and is critical for the cytolytic activity of $CD8^+$ memory T cells while induction of mTORC2 promotes Th2 differentiation (Delgoffe et al., 2011; Finlay et al.; Lee et al.; Li et al., 2011; Rao et al.). Moreover, inhibition of mTOR activity has been shown to block the generation of Th effector cells, promoting the generation and function of Foxp3-expressing Tregs (Delgoffe et al., 2009; Powell et al., 2012; Sauer et al., 2008) and enhancing memory T cell generation (Araki et al., 2009; He et al.; Rao et al.). The finding that recruitment of distinct mTOR signaling cascades promotes disparate fates for T lymphocytes suggests that different T cell subsets have distinct metabolic requirements (Wang and Green).

Presently, it is not known whether metabolite-induced changes in mTOR activity alter T effector differentiation or function. Notably, amino acid levels themselves can regulate T cell differentiation, with low levels of cysteine and methionine abrogating Th17 development (Sundrud et al., 2009). It is interesting to note that multiple amino acid transporters are highly upregulated within the first hours of TCR stimulation and knock down of one of these transporters, SLC7A5, completely blocks the capacity of CD8 T cells to undergo clonal expansion and effector differentiation (Sinclair et al.). These results are in accord with several recent studies showing the critical role of metabolic reprogramming in T cell activation, from fatty acid oxidation to

glycolytic and glutaminolytic pathways ((Wang et al.) and reviewed in (Wang and Green)).

We now report that glutamine deprivation delays the activation of the mTOR pathway in T cells, with a corresponding lower energetic state of the cell that is associated with an enhanced conversion of naïve $CD4^+$ T cells to a $Foxp3^+$ Treg cell fate. This phenomenon also occurred under Th1-polarizing conditions and abrogated Th1 but not Th2 differentiation. We found that TGFβ-signaling controlled this Treg conversion. Importantly, $Foxp3^+$ T cells induced by glutamine deprivation showed enhanced *in vivo* persistence and were highly suppressive, completely protecting Rag-deficient mice from the development of autoimmune colitis. Thus, energetic resources metabolically modulate peripheral T lymphocyte differentiation to distinct cell fates.

Results

The kinetics and magnitude of TCR-induced mTOR activity and energy production are altered by glutamine availability and catabolism

mTOR promotes cellular growth and proliferation by activating the ribosomal protein S6 kinase (S6K) which in turn phosphorylates the S6 ribosomal protein, enhancing translation of multiple mRNA transcripts (Meyuhas and Dreazen, 2009). TCR engagement of $CD4^+$ T cells, under optimal nutrient conditions, resulted in a rapid upregulation of S6 phosphorylation (pS6) that peaked at 48h of activation and then declined (**Fig. 1**). Notably, under conditions of glutamine depletion (Nutr-GLN), pS6 upregulation was significantly delayed and reduced, with 4-fold lower levels of pS6 at 48h post activation. To assure that this alteration in pS6 induction was due to changes in glutamine catabolism *per se*, we also blocked glutamine utilization with 6-diazo-5-oxo-L-norleucine (DON), a mitochondrial glutaminase inhibitor that functions only when the enzyme is active. Importantly, S6 phosphorylation was equivalently delayed and reduced, confirming the critical role of glutamine in mTOR activation in TCR-signaled T cells (**Fig. 1**).

The delayed activation of the mTOR pathway in response to glutamine deprivation was also detected at the level of mitochondrial function. mTOR promotes anabolic processes by increasing mitochondrial biogenesis and oxidative function (Powell et al., 2012; Xu et al., 2012), and in T cells, it has been specifically shown that fueling the generation of mitochondrial reactive oxygen species (ROS) creates a positive feedback for TCR signaling and antigen-specific T cell expansion (Devadas et al., 2002; Sena et al.). Indeed, similar to the phosphorylation of S6, TCR-induced increases in ROS and mitochondrial transmembrane potential ($\Delta\Psi m$) were delayed in glutamine-deprived conditions. Notably though, ROS and $\Delta\Psi m$ reached equivalent

levels to those detected in nutrient replete conditions by 96h post activation (**Fig. 1**).

ATP is required to meet the energetic needs of an activated T cell and ATP-generating pathways are regulated by mTOR (D'Souza et al., 2007; Schieke et al., 2006). It was therefore important to determine whether glutamine deprivation affected mTOR activity alters the energetic resources of a TCR-engaged lymphocyte. Importantly, intracellular ATP levels did not increase above baseline during the first 48h post TCR stimulation but were comparable to those detected in nutrient-replete T cells by 96h (**Fig. 1**). These data strongly suggest that the initial activation of mTOR in TCR-engaged CD4 T lymphocytes is strongly dependent on glutamine but that at later time points, other nutrient sources can meet the cells' metabolic requirements.

Reduced glutamine uptake results in a decreased TCR-induced proliferation

As the activation of mTOR in transformed cells requires glutamine transport (Duran et al.; Fuchs et al., 2007; Nicklin et al., 2009), we assessed whether the potential of a TCR-engaged T cell to transport glutamine was affected by glutamine deprivation. Notably, while T cell stimulation in nutrient-replete conditions resulted in a 20-fold increase in glutamine uptake, the induction in glutamine-depleted conditions was significantly lower ($p<0.05$; **Supplementary Fig. 1A**). Concomitant with this decrease, glucose uptake was also attenuated. As a control, we monitored uptake in T cells activated in glucose-depleted conditions and unexpectedly, found that glucose uptake was induced to equivalent levels as that detected in nutrient-replete cells (**Supplementary Fig. 1A**).

Based on these data, it was of interest to assess how changes in metabolite entry affect T lymphocyte proliferation. Interestingly, the significant decrease in glucose and glutamine uptake associated with glutamine deprivation resulted in a lower level of proliferation for up to 72h post activation. Nevertheless, consistent with the delay in mTOR activation, proliferation increased at 96h (**Supplementary Fig. 1B**). In contrast, the positive feedback loop detected in glucose-deprived T cells, resulting in a higher glucose uptake, is likely responsible for the significantly higher proliferation detected in this condition (**Supplementary Fig. 1B**). Thus, decreases in the extracellular glutamine available to a TCR-engaged $CD4^+$ T cells limits the potential of the cell to transport glutamine, affecting mTOR activation and resulting in a reduced proliferation.

Glutamine availability modulates the TCR-mediated induction of effector-specific transcription factors

The modification of genes encoding proteins directly involved in mTOR signaling has revealed a critical role for this pathway in naïve T cell differentiation to specific effector cell fates. Activation of mTORC1 promotes Th1 and Th17 differentiation (Delgoffe et al., 2011; Finlay et al.; Lee et al.; Park et al.; Rao et al.) while inhibition of mTOR activity blocks the production of Th effector cells and promote the generation of $Foxp3^+$ Tregs (Delgoffe and Powell, 2009; Powell et al., 2012; Sauer et al., 2008). Consequently, we wondered whether glutamine deprivation, resulting in delayed mTORC1 activation, would alter the effector fate of activated CD4 T cells.

While the TCR-induced upregulation of IFNγ expression in total naïve $CD4^+$ T cells was not altered by glutamine availability, a non-polarizing TCR activation in glutamine-deprived conditions resulted in a massive upregulation of Foxp3-expressing cells, from 5% to 39% (**Fig. 2A**). To assure that the augmented

Foxp3 expression was due to changes in glutamine catabolism, we also blocked glutamine utilization with DON. In this context as well, the percentage of Foxp3-expressing cells was increased by approximately 10-fold, from 5% to 46% (**Fig. 2A**). Furthermore, the induction of Foxp3 was also detected at the mRNA level with a 50-100 fold increase in Foxp3 transcripts in glutamine-deprived or DON-supplemented conditions (**Fig. 2B**). Early decreases in mTORC1 activity therefore appear to be associated with an altered differentiation potential of CD4 T cells.

Given the striking increase in Foxp3$^+$ T cells following TCR activation in conditions of glutamine deprivation, it was important to determine the kinetics of this augmentation. In control nutrient-replete conditions, the percentage of Foxp3$^+$ T cells actually decreased during the 96h activation period (9% to 2%). In marked contrast, in glutamine-deplete or DON-treated cultures, the percentages of Foxp3$^+$ T cells increased significantly, but only at 96h post stimulation (**Fig. 2C**, middle panels). To determine whether the massive increase in Foxp3$^+$ T cells was a response to nutrient-deprivation *per se* or alternatively was a specific consequence of glutamine starvation, we assessed the fate of CD4 T cells stimulated in the absence of glucose. In the absence of glucose, neither the percentages of Foxp3$^+$ cells nor the level of Foxp3 mRNA transcripts was increased (**Fig. 2B and 2C)**. Furthermore, we found that the increased percentage of Foxp3$^+$ cells detected in glutamine-deprived conditions was not due to their preferential proliferation; the global proliferation of all CD4 T cells was decreased in the absence of glutamine or presence of DON but the relative proliferation profiles of Foxp3$^-$ and Foxp3$^+$ lymphocytes were similar in all conditions (**Fig. 2C**, right panels). Moreover, this phenomenon was rapidly reversed by the addition of low amounts of ectopic glutamine; While the concentration of glutamine in the cell culture media is 2mM, the percentage of Foxp3$^+$ cells dropped from 44% to 3% upon

addition of 0.8 mM glutamine and Foxp3 transcripts decreased to negligible levels (**Supplementary Fig. 2**). Thus, Foxp3$^+$ cells are preferentially generated under conditions of decreased glutamine, but not glucose utilization.

These data suggested that either the survival of Foxp3$^+$ cells relative to Foxp3$^-$ cells was modulated when glutamine catabolism was inhibited and/or that under these conditions, Foxp3$^-$ cells were converted to a Foxp3$^+$ fate. As neuropilin-1 is expressed on *in vivo*-generated natural Treg (nTreg) but not on induced Treg (Yadav et al., 2012), the absence of detectable neuropilin-1 under conditions of inhibited glutamine catabolism suggested that these Tregs resulted from conversion, supporting the second hypothesis (**Fig. 2D**). To further test the second hypothesis, FACS-purified CD4$^+$CD62L$^+$CD44$^-$CD25$^-$ conventional T cells, wherein nTregs were depleted, were activated in glutamine-deprived conditions. Following depletion, Foxp3-expressing T cells represented 0.1% of the total CD4 naïve cell population. Notably though, by 96h post TCR activation, the percentages of Foxp3$^+$ T cells increased to 23% and 24% in glutamine-depleted or DON-treated cultures, respectively **(Fig. 2E**). These data indicate that under neutral TCR stimulation conditions, glutamine deprivation favors the conversion of Foxp3$^-$ cells to a Foxp3$^+$ fate.

The preferential glutamine-deprived conversion of naïve CD4$^+$ T cells to a Foxp3$^+$ T cell fate occurs under neutral as well as Th1-polarizing conditions

As the experiments described above were performed in neutral conditions, we wondered whether glutamine deprivation would also impact Foxp3 induction under iTreg, Th1 or Th2 polarizing conditions. In iTreg conditions, wherein ectopic TGFβ was present, the vast majority of naïve T cells upregulated Foxp3, irrespective of the presence of glutamine (75% and 79%, respectively,

Fig. 3A). Notably though, Foxp3 was also highly upregulated in CD4 T cells activated in Th1-polarizing conditions in the absence of glutamine. The level of Foxp3$^+$ cells was equivalent to that detected following a neutral Th0 activation (37-39%, **Fig. 3A**, right panels) and this change was also detected at the mRNA level (**Fig. 3B**). Interestingly, under Th1 conditions, this induction of Foxp3 was associated with an inability of these cells to upregulate Tbet, the master regulator of Th1 differentiation, and as expected from these data, the cells did not secrete IFNγ (3% as compared to 48%, **Fig. 3B** and **3C**). The situation in Th2 conditions was markedly different; Foxp3 expression was not augmented whereas the Th2 transcriptional regulator GATA3 as well as IL-4 production were induced to levels comparable to that detected in control conditions (**Fig. 3B and 3C**). The ensemble of these experiments shows that in the absence of glutamine, TCR stimulation results in a delayed activation of the mTORC1 pathway, promoting a Foxp3$^+$ T cell differentiation. Intriguingly, this phenomenon occurs in neutral and Th1 conditions, but not in Th2-skewing conditions. Therefore, the nutrient milieu regulates the differentiation potential of a naïve T cell, even when the cytokine environment would normally be expected to bias the lymphocyte to a distinct effector fate.

Foxp3$^+$ T cell conversion in glutamine-deprived conditions results from TGFβ-dependent signaling

Given the central role of TGFβ in the conversion of naïve T cells to a Foxp3$^+$ regulatory T cell fate, we evaluated its potential implication in the enhanced differentiation detected in glutamine-deprived conditions. In the experiments above, exogenous TGFβ had not been added to the cultures but it was possible that the cells themselves were producing sufficient levels of this cytokine to promote a conversion. Interestingly, analysis of TGFβ transcript levels did not reveal any significant increases as compared to control conditions (data not shown). We therefore assessed whether the low amount

of TGFβ present in the cultures was sufficient to drive differentiation to a Foxp3$^+$ cell fate in glutamine-deprived conditions. CD4 T cells were TCR-stimulated in glutamine-deplete or -replete conditions, in the presence or absence of an anti-TGFβ neutralizing antibody. While neutralizing anti-TGFβ treatment did not alter the low percentage of Foxp3$^+$ T cells detected in glutamine-replete conditions (1%), there was a massive reduction in the generation of Foxp3$^+$ T cells in glutamine-deprived conditions (33% to 6%; **Fig. 4A**). This was not due to a change in the relative proliferation of Foxp3$^-$ as compared to Foxp3$^+$ cells; glutamine deprivation itself decreased proliferation but this was not further altered by anti-TGFβ treatment (**Fig. 4A**, right panels). We further assessed the specific role of TGFβ signaling by treating cells with the SB431542 kinase inhibitor, a molecule that specifically inhibits the TGFβR1 receptor transducing pathway (Inman et al., 2002). While this inhibitor did not alter Foxp3 expression in control cultures, it significantly decreased the appearance of Foxp3$^+$ cells under conditions where glutamine was limiting (**Fig. 4B**). Together, these data indicate that endogenously-produced TGFβ promotes Treg conversion under the stress conditions of glutamine deprivation.

Based on these data, it was of interest to determine whether endogenous TGFβ was also responsible for the emergence of Foxp3$^+$ T cells detected in Th1 polarizing conditions (**Fig. 4C**). Indeed, anti-TGFβ antibodies inhibited Foxp3 conversion under Th1 conditions (**Fig. 4C**). It is though important to note that while endogenous TGFβ was required for induction of Foxp3 expression, neutralizing this activity was not sufficient to promote Th1 polarization under glutamine-deprived conditions. The cytokine-polarizing induction of Tbet, abrogated under conditions of glutamine depletion, was not restored by anti-TGFβ treatment (**Fig. 4D**). Consistent with these data, IFNγ was not produced (**Fig. 4E**) and as expected, the induction of GATA3

expression in Th2-polarizing conditions was not altered (**Fig. 4D**). Altogether, these results indicate that glutamine deprivation promotes a TGFβ-dependent conversion of Foxp3$^-$ cells to a Foxp3$^+$ fate under Th0 as well as Th1 polarizing conditions. Nevertheless, under conditions of glutamine deprivation, inhibiting TGFβ signals was not sufficient to allow Th1 differentiation, consistent with previous studies showing that this phenomenon is dependent on increased mTORC1 activity (Delgoffe et al., 2009; Delgoffe et al., 2011; Liu et al.).

***In vivo* proliferation of Foxp3$^+$ T cells generated in the absence of glutamine is markedly enhanced**

The experiments presented above demonstrate that glutamine depletion promotes the *in vitro* differentiation of Foxp3$^+$ T cells but it was not clear whether this phenotype would be stable and maintained *in vivo*. Importantly, it has recently been shown that the stability of a Treg cell fate is dependent not only on Foxp3 expression but on the epigenetic programming of the cell (Ohkura et al.). To assess whether glutamine deprivation during TCR activation promotes the *in vivo* persistence of Foxp3$^+$ T cells, naive CD4$^+$ T lymphocytes from C57Bl/6-Thy1.1 mice were activated for 5 days in nutrient-replete or glutamine-deprived conditions or in the presence of DON (Nutr+, Nutr-GLN and Nutr+DON; **Fig. 5A**). These lymphocytes were then CFSE-labeled and adoptively-transferred into sublethally irradiated C57BL/6-Thy1.2 recipients. Notably, the ratio between donor Foxp3$^+$ and Foxp3$^-$ T cells was significantly higher in both the glutamine-depleted and DON groups than in the control group (up to 48%, **Fig. 5B**). This high percentage of Foxp3$^+$ T cells was associated with a significantly higher *in vivo* proliferation of donor Foxp3$^+$ as compared to donor Foxp3$^-$ T cells (**Fig. 5B**, lower histograms). Thus, the phenotype of the Foxp3$^+$ cells was stable following a 7 day *in vivo* transfer, even under conditions of significant proliferation.

Helios, an Ikaros family transcription factor, has been found to be upregulated in Foxp3^{+} T cells. While the origin of Helios^{+} regulatory T cells is not clear, some studies have suggested that expression of this transcription factor may be associated with Treg proliferation and suppressive function (Akimova et al., 2011; Baine et al., 2013; Getnet et al., 2010). Indeed, the adoptive transfer of T cells pre-activated in glutamine-depleted or DON conditions resulted in the recovery of a significantly higher percentage of Helios^{hi}Foxp3^{+} T cells, with means of 46 and 50% as compared to 14%, respectively (**Fig. 5C**, top panels). Furthermore, irrespective of the pre-activation conditions, the vast majority of fast-proliferating Foxp3^{+} T cells expressed high levels of Helios (**Fig. 5C**, bottom histograms). Thus, Foxp3^{+} T cells generated by glutamine deprivation upregulate Helios expression *in vivo* and this is associated with a significantly enhanced *in vivo* proliferation.

Treg generated in glutamine-deficient conditions show *in vivo* suppressive activity protecting Rag-deficient mice from the development of autoimmune colitis

Based on the high *in vivo* proliferation of Foxp3^{+} cells generated under conditions of limiting glutamine metabolism, it was important to assess their *in vivo* suppressive potential. A well-adapted model in which this question can be addressed is based on the potential of Treg to prevent inflammatory bowel disease (IBD) induced by the transfer of naïve CD4 effector cells into Rag2$^{-/-}$ mice. Briefly, CD4^{+} T cells isolated from Foxp3-GFP reporter mice were TCR-stimulated in glutamine-deprived (Treg$^{Nutr\text{-}GLN}$) conditions and after 5 days, they were sorted on the basis of GFP expression (**Supplementary Fig. 3**). As expected, mice injected with CD4^{+}CD45RB^{hi} effector T cells alone lost body weight because they developed IBD, a diagnosis confirmed by histopathology (**Fig. 6A and 6B**). Co-injection of *in vivo*-generated Foxp3^{+}CD4^{+} lymphocytes

(nTreg) protected Rag2$^{-/-}$ from IBD during the entire 9 week follow up period, a result previously reported by numerous groups (Denning et al., 2005; Mottet et al., 2003; Murai et al., 2009; Pan et al., 2009; Uhlig et al., 2006). Notably, co-injection with glutamine-deprived *in vitro*-generated Foxp3^{+}CD4^{+} lymphocytes (Treg$^{Nutr\text{-}GLN}$) also fully protected mice from IBD during the entire follow up period (**Fig. 6A and 6B**).

Disease evolution correlated with the expression of CD44, a marker upregulated in memory and effector T cells: Expression was significantly higher in the mice developing IBD as compared to the disease-free groups which received either *in vivo*-generated nTreg or glutamine-deprived *in vitro*-generated Treg activated in glutamine-deficient conditions (Treg^{Nutr+}; **Fig. 6C**). We also assessed the relative persistence of the Treg populations. In accord with the increased short term *in vivo* proliferation of iTreg activated in glutamine-deprived conditions (**Fig. 5**), the long-term persistence of injected CD45.2^{+} Tregs was significantly higher than that of nTregs, with means of 53% and 30%, respectively (**Fig. 6D**). Thus, the deprivation of glutamine during TCR activation leads to the preferential persistence of this T cell subset and a strong *in vivo* suppressive activity.

Discussion

In the present study, we demonstrate the critical impact of glutamine availability on the fate of naïve $CD4^+$ T cells. Low glutamine levels promoted the generation of $Foxp3^+$ lymphocytes and inhibited Th1 differentiation. Notably, the striking increase in $Foxp3^+$ T cells was due to a conversion of conventional $CD4^+$ T cells to a $Foxp3^+$ fate and this process was regulated by endogenous TGFβ signaling. The skewing of naive $CD4^+$ T cells to a $Foxp3^+$ fate occurred even under Th1-polarizing conditions, blocking Tbet upregulation. Furthermore, these converted $Foxp3^+$ T cells had robust suppressor activity, controlling colitis in a T cell adoptive transfer model. Our data reveal the external metabolic environment to be a key regulator of a CD4 T lymphocyte's differentiation potential, modulating the immune responsiveness of the organism under conditions of amino acid starvation.

Conversion of naive $CD4^+$ T cells to a $Foxp3^+$ T cell fate was associated with diminished mTORC1 activity and a decreased energetic state of the cell, as shown by lower mitochondrial function and intracellular ATP levels. mTOR is a critical factor in the metabolic reprogramming of TCR-stimulated T cells and serves to integrate environmental cues such as nutrients, growth factors and stress signals into an "optimal" cellular response (Laplante and Sabatini, 2009; Sengupta et al.). Indeed, inhibition of mTOR activity has been found to foster the generation and function of Foxp3-expressing Treg (Delgoffe et al., 2009; Powell et al., 2012; Sauer et al., 2008) while mTORC2-mediated activation of Foxo1 and Foxo3a abrogates Foxp3 expression (Harada et al.; Kerdiles et al.; Ouyang et al.). Similarly, activation of mTORC1, via deletion of the upstream TSC1 negative regulator, results in a loss of Foxp3 expression and a gain of effector function (Park et al.). Nevertheless, the relationship between the mTOR pathway and regulatory T cells is not yet completely

understood because disruption of mTORC1 was recently shown to reduce Treg activity (Zeng et al.). The results presented here show that conversion of TCR-stimulated CD4 T cells to a Foxp3$^+$ T cell fate simply requires delayed induction of mTORC1 activity.

iTreg can easily be generated *ex vivo* by TCR stimulation of naive CD4+ T cells in the presence of exogenous TGF-β. We found this conversion to be independent of glutamine levels; a high level differentiation of regulatory T cells was obtained irrespective of the glutamine concentration. Importantly, in glutamine-deficient conditions, endogenous TGF-β was sufficient to promote the extensive Treg differentiation that we detected following either Th0- or Th1-type stimulations. Indeed, this conversion was abrogated by either an anti-TGF-β neutralizing antibody or an inhibitor of TGF-β signaling. These data are in accord with previous elegant work demonstrating that endogenous low level TGF-β is sufficient to generate inducible regulatory T cells in an mTOR-deficient background (Delgoffe et al., 2009). Interestingly though, and in marked contrast with the outcome of Th0- and Th1-type stimulations in the absence of glutamine, Th2 polarization was not dependent on extracellular glutamine concentrations. The Th2 transcription regulator GATA3 was highly upregulated and IL-4 levels were even higher than those detected under control conditions. Thus, we show that there are striking differences in the amino acid requirements of Th effector subsets; Th1, but not Th2, differentiation is strictly dependent on extracellular glutamine.

There is a large debate concerning the stability of Foxp3$^+$ Treg, especially *in vivo*. Some groups have reported that Tregs are stable under inflammatory conditions (Rubtsov et al.) while others have shown that they can lose Foxp3 expression and convert into pathogenic Th cells (Duarte et al., 2009; Yurchenko et al.; Zhou et al., 2009). Interestingly, treatment with an mTOR

inhibitor was shown to attenuate the *in vivo* reprogramming of Foxp3$^+$ Tregs to effector lymphocytes (Yurchenko et al.). We found that an early *ex vivo* inhibition of mTOR in Foxp3$^+$ T cells, prior to their adoptive transfer into mice, was sufficient to promote *in vivo* maintenance. Moreover, the relative recovery of adoptively-transferred Tregs to T effectors was significantly higher for Tregs converted in glutamine-deplete conditions as compared to nTregs (50% vs 30%, $p<0.005$; **Fig. 6d**). While the precise mechanisms accounting for this difference have not yet been fully elucidated, it is interesting to note that the Treg converted under our conditions of glutamine deprivation demonstrated an *in vivo* upregulation of the Helios transcription factor. The expression of this factor has been shown to be dependent on the establishment of a hypomethylation signature and it is noteworthy that a Treg-type hypomethylation is required for full suppressive activity (Ohkura et al.). Moreover, we found that Foxp3$^+$Helios$^+$ cells exhibited a significantly enhanced level of *in vivo* proliferation as compared to the Foxp$^+$Helios$^-$ subset. Therefore, the initial *ex vivo* activation of naïve T cells in limiting glutamine conditions, resulting in delayed mTORC1 activation, strongly promotes the generation of Foxp3-expressing regulatory T cells with *in vivo* suppressive activity.

In transformed cell lines, glutamine transport and utilization are thought to be a rate-limiting step in the activation of mTOR (Duran et al.; Fuchs et al., 2007; Nicklin et al., 2009; Sundrud et al., 2009). Prior to this study, it was not known whether glutamine availability regulates mTOR activity in primary T lymphocytes, but elegant research from several labs has shown that TCR stimulation results in shifts in oxidative phosphorylation, aerobic glycolysis, lipid metabolism, glycogen metabolism, nucleotide synthesis, fatty acid/ cholesterol synthesis, pH regulation, and glutaminolysis (reviews; (Powell et al., 2012; Wang and Green)). Our experiments reveal a critical sensitivity of T

lymphocytes to extracellular glutamine levels, with replenishment of even 20% of extracellular levels inhibiting the enhanced conversion of conventional T cells to a Foxp3$^+$ fate (**Supplementary Fig. 2**). In this context, it is interesting to note that the deletion of a single amino acid transporter (SLC7A5) completely blocks the clonal expansion and effector differentiation of CD8 T cells (Sinclair et al.). Furthermore, in accord with the data that we present here, knockout of the CD98 heavy chain (SLC3A2), regulating integrin signaling as well as amino acid transport, has been found to increase the generation of regulatory T cells (Bhuyan et al.; Liu et al.). Finally, depletion of two other amino acids, methionine and cysteine, abrogates Th17, but not Treg, differentiation (Sundrud et al., 2009). Together, these data unveil the importance of intracellular amino acid availability in T cell activation but also point to specificities of different amino acids in directing T cell differentiation and effector function.

Cytokines play a central role in controlling T cell activation but the ensemble of these results demonstrate a critical position for the external nutrient environment in modulating effector function. Intriguingly, the activation and differentiation profiles of CD4 T lymphocytes was only mildly perturbed in glucose-deprived conditions but was significantly modulated in the absence of glutamine. This likely reflects the wide-ranging nature of the amino acid starvation response (AAR), resulting in the activation of a network of signaling pathways in response to changes in even a single essential amino acid (reviewed in (Kilberg et al.)). This has significant implications in tumor and inflammatory environments, wherein the milieu in which a T cell is localized can fluctuate. Our results highlight a previously unknown relationship between glutamine availability and the establishment of a tolerogenic environment. Specifically, a TCR-stimulated CD4 T cell responds to limiting glutamine availability with an early decrease in mTORC1 activity, the induction of Foxp3

expression and subsequent suppressor function. Manipulating the metabolic state of the T lymphocyte can therefore modulate the balance between effector and suppressor functions, opening new avenues for the development of immunotherapies. From an evolutionary perspective, it is tempting to speculate that it is not in the interest of the organism to mount an energetically costly immune response under conditions of amino acid starvation.

Materials and Methods

Mice.

C57BL/6 mice and C57BL/6-Thy1.1 were purchased from Charles River laboratories and Foxp3-GFP mice have been previously described (Bettelli et al., 2006). Mice were housed in conventional pathogen-free facilities at the Institut de Génétique Moléculaire de Montpellier and NCI-Frederick. Animal care and experiments were performed in accordance with National Institutes of Health (NIH) and French national guidelines.

Cell isolations and activations.

$CD4^+$ T cells were purified using MACS $CD4^+$ T cell isolation kits (Miltenyi Biotec) according to the manufacturer's instructions. For experiments with CD25-depleted $CD4^+$ T cells, enriched $CD4^+$ T cells were sorted on the basis of a $CD4^+CD62L^{hi}CD44^-CD25^-$ expression profile on a FACSaria flow cytometer (BD Biosciences). For Th1, Th2 and iTreg polarizing conditions, IL-12 (10 ng/ml) + anti-IL-4 (5 µg/ml), IL-4 (10 ng/ml) + anti-IFNγ (10 µg/ml), and hTGFβ (3 ng/ml), respectively, were added to the cultures. TGFβ or its signaling pathway were neutralized by addition of the anti-TGFβ mAb (clone 1D11, 10 µg/ml) or the inhibitor SB431542 (Sigma, 5µM), respectively.

Cell activations were performed using plate-bound anti-CD3 (clone17A2 or clone 2C11; 1 µg/ml) and anti-CD28 (clone PV-1 or clone 37.5; 1 µg/ml) mAbs in RPMI 1640 media (Life Technologies) supplemented with 10% FCS in the presence or absence of glutamine (2 mM) or glucose (11 mM). In some experiments, glutamine was added at the indicated concentrations. Exogenous IL-2 (100 U/ml) was added every other day starting at day 2 post-activation. To block glutaminolysis, $CD4^+$ T cells were activated in the presence of L-DON (6-Diazo-5-oxo-L-norleucine, Sigma-Aldrich; 3 µM). To

monitor proliferation, cells were labeled with CFSE (Life Technologies; 2.5 uM) or CTV (Life Technologies; 5 μM) for 3 min at RT or 8-10 mins at 37°C for *in vitro* and *in vivo* manipulations, respectively. Reactions were stopped with 2% FCS/PBS or ice-cold PBS alone for *in vitro* and *in vivo* experiments respectively.

Adoptive T cell transfer and induction of colitis.

Thy1.1^{+} lymph node T cells were activated with plate-bound anti-CD3 and anti-CD28 mAbs in nutrient-replete RPMI, in the absence of glutamine or in the presence of DON (3 μM) for 5 days. To assess the *in vivo* persistence of these cells, they were then labeled with CFSE and transferred intravenously (6 x 10^{6} cells/mouse) into sublethally irradiated (5.5 Gy) Thy1.2^{+}C57Bl/6 recipient mice. Seven days post adoptive T cell transfer, animals were sacrificed, and lymph nodes and spleens were harvested for analyses.

For induction of colitis, C57BL/6 RAG-2$^{-/-}$ mice were injected with sorted CD45.1^{+}CD4^{+}CD45RB^{hi} T cells (2.5 x 10^{5}) either alone or in combination with sorted CD4^{+}Foxp3/GFP^{+} T cells (2.5 x 10^{5}) from CD45.2^{+}Foxp3-GFP mice. Mice were injected with either natural Treg or Foxp3/GFP^{+} T cells obtained following a 5 day TCR activation in glutamine-deplete conditions. Following T cell transfer, mice were weighed weekly for 9 weeks or were sacrificed if their weight loss exceeded 20%. Lymphoid tissues were harvested and colons were fixed in 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin.

Gene expression analyses.

RNA was isolated from purified CD4^{+} T cells using the RNeasy Micro Kit (Qiagen) and reverse transcribed into cDNA by oligonucleotide priming with the QuantiTect Reverse Transription Kit (Qiagen). Quantitative RT-PCR was

performed using the LightCycler 480 SYBR Green I Master kit (Roche) with specific primers to Tbet (S: 5'-TCCCCCAAGCAGTTGACAGT, AS: 5'-CAACAACCCCTTTGCCAAAG), GATA3 (S: 5'-AGTTCGCGCAGGATGTCC, AS: 5'-AGAACCGGCCCCTTATCAA), and Foxp3 (S: 5-CCCAGGAAAGACAGCAACCTT, AS: 5”-TTCTCACAACCAGGCCACTTG). Gene expression values were normalized to HPRT (S:5’- CTGGTGAAAAGGACCTCTCG, AS: 5’-TGAAGTACTCATTATAGTCAAGGGCA) and are presented as means +/- SD.

Immunophenotyping and flow cytometry analyses. Immunophenotyping of cells was performed with fluorochrome-conjugated antibodies to the following markers: GATA3 (L50-823), Foxp3 (FJK-16s), Helios (22F6), IFNγ (XMG1.2), Thy1.1 (OX-7), and Thy1.2 (53-2.1; all obtained from BD Biosciences) and CD4 (RM4-5) from EBiosciences and Neuropilin-1 (Q9QWJ9) from R&D Systems. Phosphorylation of S6 was assessed by staining with an anti-pS6 antibody (clone 91B2; Cell Signaling) and revealed with a secondary anti-rabbit IgG antibody coupled to AlexaFluor647 (Life Technologies). Mitochondrial membrane potential and ROS production were analyzed following incubation with Tetramethylrhodamine (TMRE, Abcam) and 1,2,3-Rhodamine (Sigma-Aldricht), respectively, at 37° C for 30 mins. IL-4 production was assessed at day 6 of polarization in glutamine deprived and replete conditions using a Cytometric Bead Array (CBA) Kit (BD Biosciences) according to the manufacturer’s instructions.

Prior to intracellular cytokine stainings, cells were activated with PMA (Sigma-Aldrich; 100 ng/ml)/ Ionomycin (Sigma-Aldrich; 1 µg/ml) in the presence of brefeldin A (Sigma-Aldrich; 10ug/ml) for 3.5-4h at 37°C. Cell surface staining was performed, and cells were then fixed and permeabilized using a fixation/permeabilization kit (BD Biosciences or eBioscience) prior to intracellular cytokine staining. Intracellular staining for Foxp3, Tbet, GATA3

and the phosphorylated form of S6 ribosomal protein was performed without any prior stimulation, using the eBioscience Fixation/Permeabilization kit. Cells were analyzed on a FACScanto (BD Biosciences) or sorted on a BD FACSaria flow cytometer. Data analyses were performed using FlowJo Mac v.8.8.7 software (Tree Star) and FCAP Array Software (CBA analysis).

Glucose/Glutamine uptake assays.

Prior to transport analyses, $CD4^+$ T cells (0.5×10^6) were starved in glucose or glutamine-free RPMI for 30 min at 37°C. Glucose and glutamine uptake assays were initiated by addition of 2-deoxy-D[1-^{3}H]glucose (2 µCi; Perkin Elmer) or L-2,3,4-[^{3}H]glutamine (0,5 µCi); Perkin Elmer) for 5 min at room temperature, respectively. Cells were then washed and solubilized in 0.1% SDS (500 µl) and radioactivity was measured by liquid scintillation. Uptake for each cell subset is expressed as mean counts per minute (CPM) for triplicate samples and error bars indicate standard deviation (SD).

ATP detection.

Intracellular ATP production was quantified using the ATPlite- Luminescence ATP Detection Assay System (Perkin Elmer) according to the manufacturer's instructions.

Statistical analyses.

Statistical significance was determined using an unpaired Student's t-test with a 2-tailed distribution unless otherwise indicated (Graph Pad Software, La Jolla, CA). Data were considered to be statistically different for $P \leq 0.05$ (*$P<0.05$, **$P<0.01$, ***$P<0.001$). All data are presented as means ± SD.

Acknowledgments

We thank all members of our laboratories for discussions, scientific critique and continual support. We are grateful to Montpellier Rio Imaging for support in cytometry experiments and the RAM animal facility of the IGMM. D.K. has been supported by an EU Marie Curie fellowship (ATTACK), P.R. by a Research Ministry fellowship administered by the University of Montpellier II, L.O. and G.C. by fellowships from the Ligue Contre le Cancer and L.O. is presently supported by ARC, and M.C. by a fellowship from the Portuguese Foundation for Science and Technology. X.T. and A.S. are supported by the NIH intramural program, C.M., S.K. and V.D. by the CNRS and J.M. and N.T. by INSERM. This work was supported by generous funding from the European Community (contracts LSHC-CT-2005-018914 "ATTACK" and PIRG5-GA-2009-249227 “T cell homeostasis”), Association de la Recherche contre le Cancer (ARC), French national (ANR) research grants (PolarATTACK and GlutStem), INCa and the French laboratory consortiums (Labex) EpiGenMed and GrEX.

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Figure Legends

Figure 1: **The kinetics and magnitude of TCR-induced mTOR activity and energy production are altered by glutamine availability and catabolism.**
$CD4^+$ T cells were activated by immobilized αCD3/αCD28 mAbs in complete medium, designated here as "nutrient+" (Nutr+), glutamine-free media (Nutr-GLN) or in the presence of the 6-diazo-5-oxo-L-norleucine (DON) glutamine analogue. Phosphorylation of the mTOR substrate, S6 ribosomal protein, ROS levels and mitochondrial membrane potential were all monitored by flow cytometry, as a function of mean fluorescence intensity (MFI) and intracellular ATP levels were measured biochemically. Quantification at the indicated time points is presented. Results are representative of data obtained in 3 independent experiments.

Figure 2: **Glutamine deprivation promotes the conversion of naive $CD4^+$ T cells into $Foxp3^+$ Tregs.**
(**A**) IFNγ production and Foxp3 expression were assessed 96h post TCR stimulation in the presence or absence of glutamine or in the presence of DON. The percentages of cytokine-secreting and Foxp3-expressing cells within the $CD4^+$ T cell populations are indicated in the representative dot plots. (**B**) The level of Foxp3 transcripts was monitored 96h following stimulation in the indicated conditions by qRT-PCR. mRNA levels ±SD relative to HPRT are presented (*$p<0.05$). (**C**) Changes in the relative percentages of Foxp3-expressing $CD4^+$ T cells were monitored as a function of time and nutrient composition. Representative dot plots showing the percentages of $Foxp3^+$ cells within freshly isolated $CD4^+$ lymph node T cells as well as 24 and 96h post TCR stimulation are presented (left panels). The proliferation profiles of $Foxp3^-$ (dashed line) relative to $Foxp3^+$ (solid line) $CD4^+$ T cells were monitored by dilution of CTV fluorescence and representative histograms at

96h are presented. (**D**) The expression of neuropilin-1 (solid line) was monitored in *in vivo*-generated nTregs and $Foxp3^{+}CD4^{+}$ cells generated *ex vivo* by glutamine deprivation or DON treatment. Histograms relative to IgG isotype staining (dashed line) are shown. (**E**) Naïve conventional $CD4^{+}CD62L^{+}CD44^{-}CD25^{-}$ T cells were FACS-sorted and purity, as a function of $Foxp3^{+}$ cell elimination, was monitored (left panel; day 0). Induction of Foxp3 expression in these purified $Foxp3^{-}CD4^{+}$ naïve T cells was assessed following activation in glutamine-deprived or DON-supplemented conditions. Representative plots are shown at 96 hours and the percentages of $Foxp3^{+}$ cells are indicated. Results are representative of data obtained in 4 independent experiments.

Figure 3: **The preferential glutamine-deprived conversion of naïve $CD4^{+}$ T cells to a $Foxp3^{+}$ T cell fate occurs under neutral as well as Th1-polarizing conditions.**

(**A**) Naïve $CD4^{+}CD62L^{+}CD44^{-}CD25^{-}$ T cells were FACS-sorted and TCR-activated under iTreg-, Th0-, Th1- and Th2-polarizing conditions in nutrient-replete or glutamine-deprived conditions. Expression of Foxp3 was assessed at day 6 post-activation and representative plots are presented. (**B**) Foxp3, Tbet and GATA3 mRNAs were quantified in cells activated under Th0-, Th1- and Th2-polarizing conditions. Transcripts were monitored by qRT-PCR and mean levels relative to HPRT are presented. (**C**) The percentages of $CD4^{+}$ T cells expressing IFNγ following polarization in nutrient-replete as compared to glutamine-deprived conditions were detected by intracellular staining at day 6 and representative plots are shown (left plots). IL-4 production was assessed at the same time point by cytometric bead array and mean levels are presented (right graph). Results are representative of data obtained in 4 representative experiments.

Figure 4: **Glutamine deprivation promotes a TGF-β-dependent conversion of naïve $CD4^+$ T cells to a $Foxp3^+$ iTreg fate.**

(**A**) The impact of TGFβ neutralization on the conversion of naïve $CD4^+$ T cells to a $Foxp3^+$ T cell fate in glutamine-deprived conditions was assessed by addition of an anti-TGFβ neutralizing antibody. The percentages of $Foxp3^+$ cells were monitored at day 4 of activation and representative dot plots are presented (left panels). The relative proliferation profiles of $Foxp3^-CD4^+$ and $Foxp3^+CD4^+$ T cells in each condition were monitored as a function of CTV fluorescence and histogram overlays at day 4 of activation are presented (right panels). (**B**) The percentages of $Foxp3^+$ T cells was assessed following 5 days of activation in nutrient-replete and glutamine-deprived conditions, in the presence or absence of SB431542, an inhibitor of TGFβ receptor signaling (Inman et al., 2002) (**C**) Naïve T cells were TCR-activated under Th0-, Th1- or Th2-polarizing conditions in nutrient-replete and glutamine-deprived conditions, in the presence or absence of an anti-TGFβ neutralizing antibody. Expression of Foxp3 was assessed at 96 hours post activation and representative plots are shown. (**D**) Expression of Tbet and GATA3 were assessed in Th1- and Th2-polarizing conditions, respectively, in the conditions described above. Representative histograms showing intracellular levels, relative to Th0 conditions, are presented. (**E**) IFNγ production was monitored following activation in Th1-polarizing conditions in the presence or absence of glutamine, with or without TGFβ neutralization. Data are representative of three independent experiments.

Figure 5: **Enhanced *in vivo* proliferation of $Foxp3^+$ T cells generated in the absence of glutamine.**

(**A**) $Thy1.1^+$ $CD4^+$ T cells were TCR-stimulated in complete medium (Nutr+), in the absence of glutamine (Nutr-GLN), or in the presence of DON for 5 days. Cells were then CTV-labelled and transferred into congenic $Thy1.2^+$ C57Bl/6

mice rendered lymphopenic by irradiation (5.5 Gy). Mice were sacrificed 7 days later and the status of adoptively-transferred T cells was assessed. (**B**) The engraftment of transferred T cells was monitored as a function of Thy1.1 expression (upper plots) and expression of Foxp3 within the donor $CD4^+$ T cell populations was assessed (middle plots). The percentages of positively stained cells are indicated in each plot. Quantification of the percentages of $Foxp3^+$ donor T cells is shown with each point representing data from a single mouse and mean levels indicated by a horizontal line (right graph; *P<0.05; **P<0.01; ***P<0.001). Proliferation of $Foxp3^-CD4^+$ relative to $Foxp3^+CD4^+$ T cells was monitored within individual mice as a function of CTV fluorescence and representative histograms for the indicated conditions are shown (lower histograms). Note that the recovery of $Foxp3^+$ cells in the Nutr+ condition was insufficient for evaluation of proliferation. (**C**) Expression of the Helios transcription factor in $CD4^+Foxp3^+$ donor T cells was assessed and representative dot plots are shown. The percentages of $Helios^{hi}$ cells are indicated (upper panels). Quantification of $Helios^{hi}Foxp3^+$ cells is shown with each point representing data from a single mouse and mean levels indicated with a line (right graph; ***P<0.001). Proliferation profiles of $Helios^{lo}Foxp3^+$ and $Helios^{hi}Foxp3^+$ cells in the different conditions are shown. Data are representative of three independent experiments and each experiment was performed with 3-4 mice per group.

Figure 6: Tregs generated in glutamine-deficient conditions efficiently inhibit effector T cell-mediated autoimmune colitis.

(**A**) Rag2-deficient mice were injected with sorted $CD4^+CD45RB^{hi}$ ($CD45.1^+$) T effector cells, alone or together with FACS-purified *in vivo*-generated nTreg or glutamine-deprived *in vitro*-generated $Foxp3^+CD4^+$ lymphocytes ($Treg^{Nutr\text{-}GLN}$). Injected Rag2-deficient mice were examined for weight loss on a weekly basis and the mean weights in each group are presented as a function of the initial

weight (mean±SD; n=5 per group; upper graph). The survival rate in each group is presented as a function of time post T cell transfer (lower graph). (**B**) Colons were collected, fixed and H/E stained. Representative sections from each of the groups of mice are shown. The magnification in the upper and lower panels is 5x and 10x respectively. (**C**) Surface levels of the CD44 marker were assessed on $CD4^+$ effector cells ($CD45.1^+$) isolated from the different groups of Rag2-deficient-injected mice. Representative histograms are shown. The vertical dotted line delineates the MFI of CD44 staining on $CD45.1^+$ T cells isolated from Rag2-deficient mice injected with effectors alone. The mean MFI of CD44 expression in each group is presented (right graph). (**D**) The relative recovery of injected effector ($CD45.1^+$) and regulatory ($CD45.2^+$) T cells in the different groups of mice was assessed at week 9. Representative dot plots discriminating between $CD45.1^+$ effectors and $CD45.2^+$ Tregs in mLN are presented for each condition. The proportion of $CD45.2^+$ Tregs recovered in the mLN of individual Rag2-deficient-injected mice from each group is quantified with each point representing data from one mouse. Mean levels are indicated with a line (*P<0.05; ***P<0.001). Data from 5 mice per group are presented.

Figure 1: The kinetics and magnitude of TCR-induced mTOR activity and energy production are altered by glutamine availability and catabolism

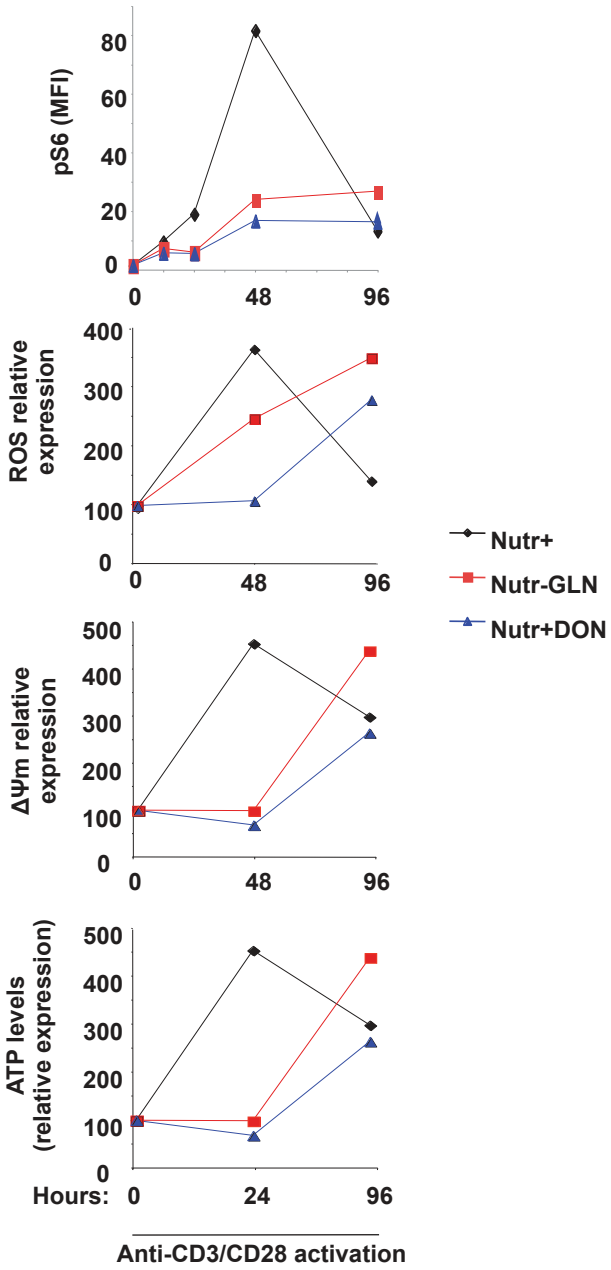


Figure 2: Glutamine deprivation promotes the conversion of naive CD4$^+$ T cells into Foxp3$^+$ Tregs

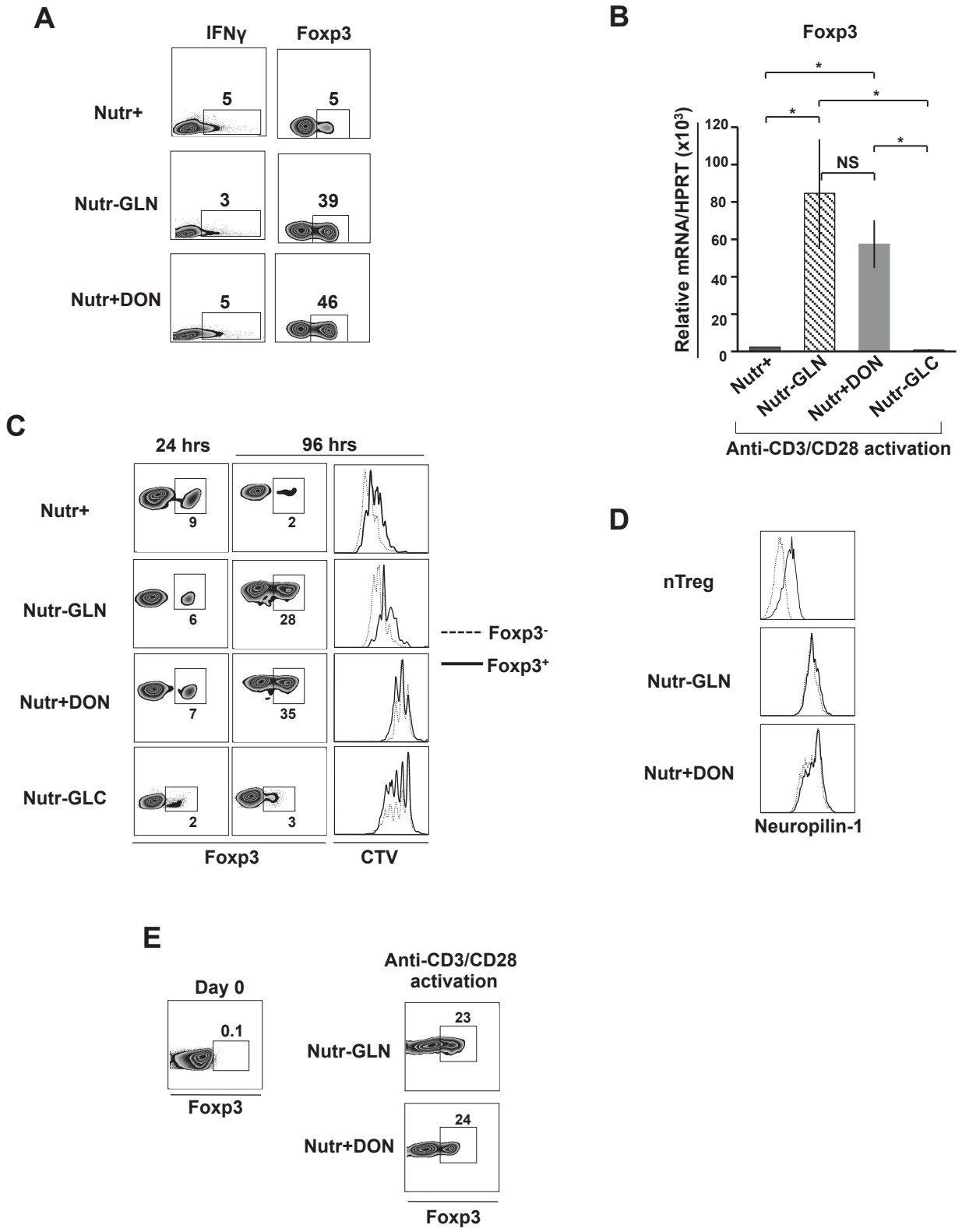


Figure 3: The preferential glutamine-deprived conversion of naïve CD4$^+$ T cells to a Foxp3$^+$ T cell fate occurs under neutral as well as Th1-polarizing conditions

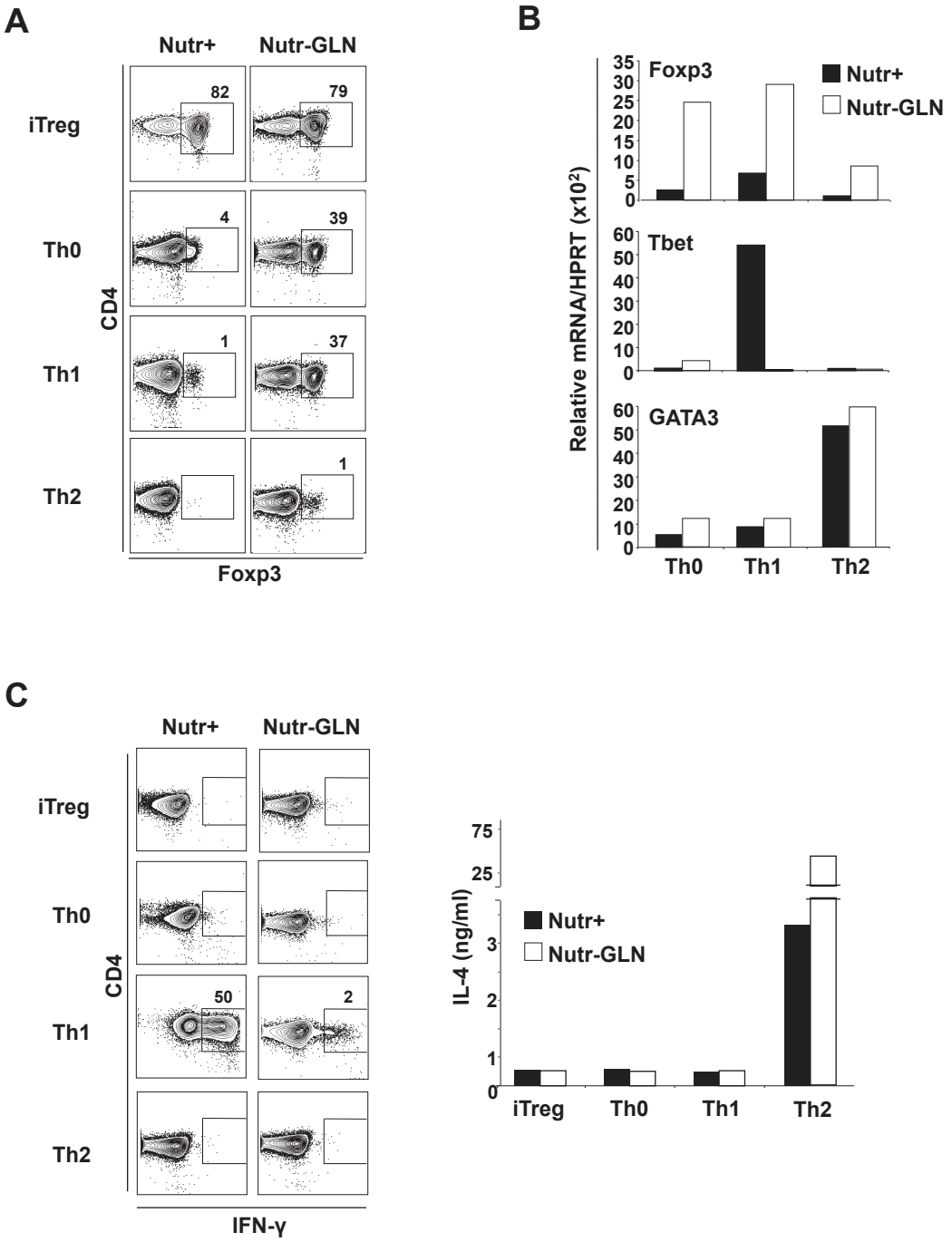


Figure 4: Glutamine deprivation promotes a TGF-β-dependent conversion of naïve CD4⁺ T cells to a Foxp3⁺ iTreg fate

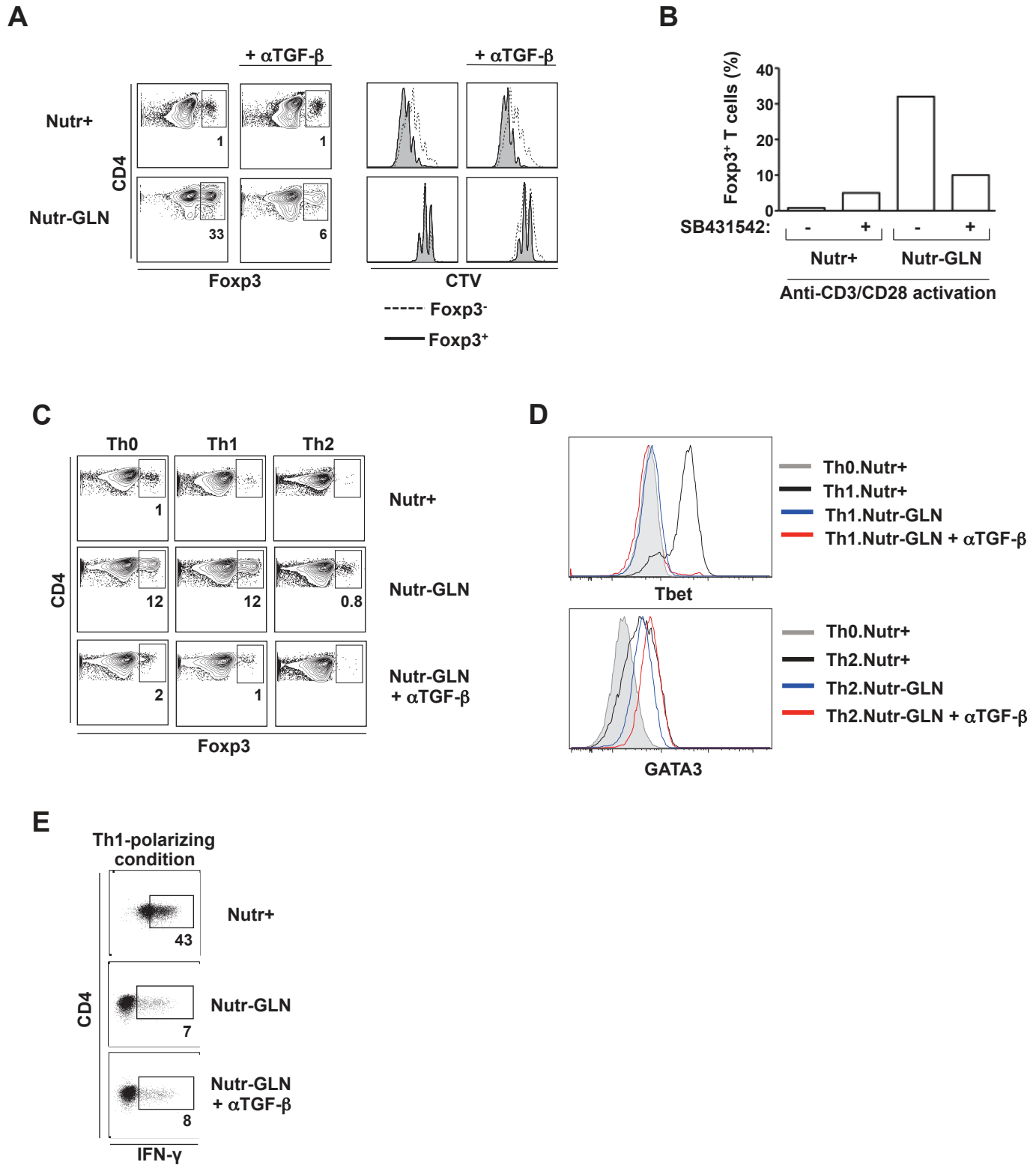


Figure 5: Enhanced *in vivo* proliferation of Foxp3^{+} T cells generated in the absence of glutamine

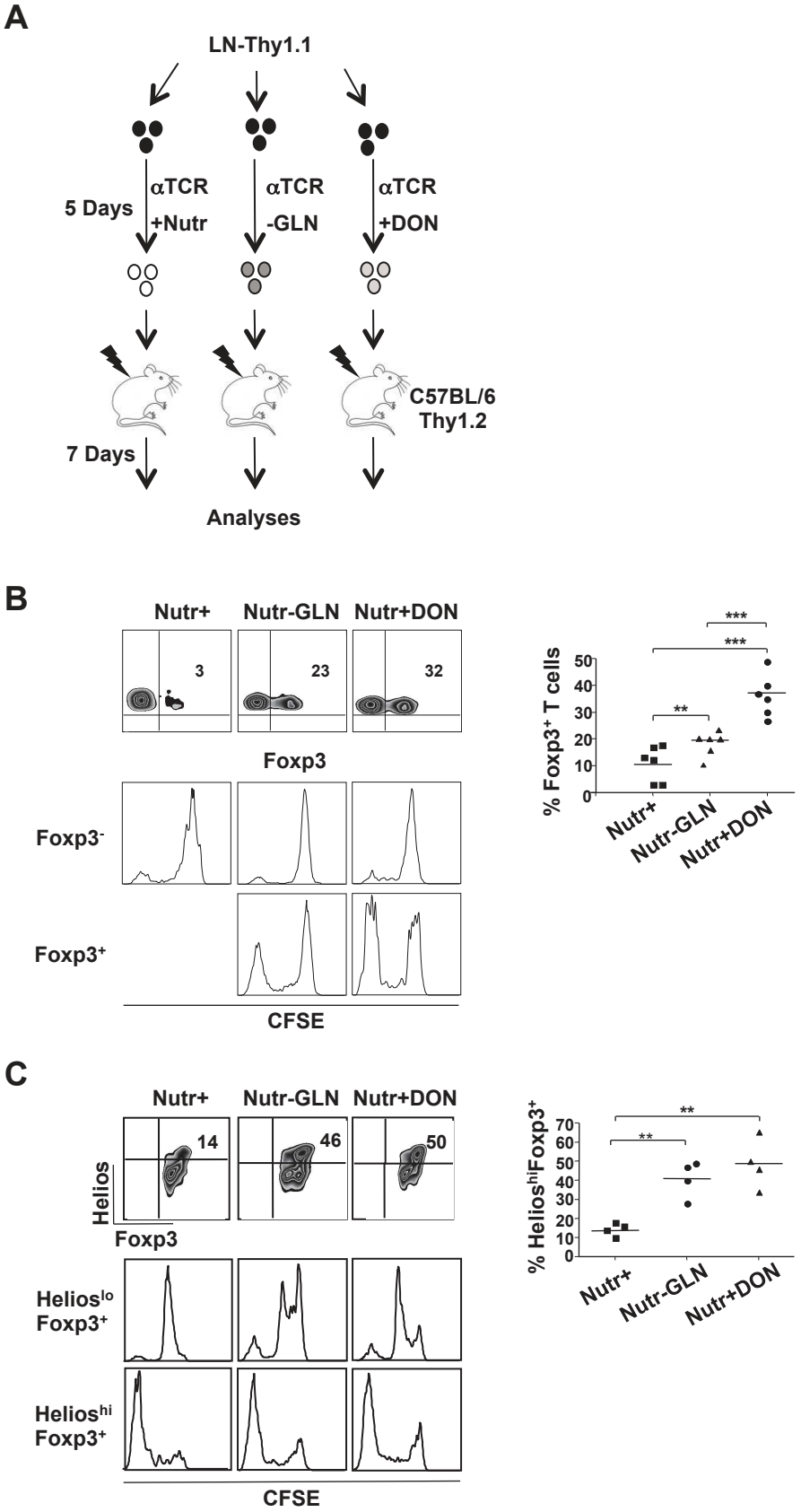
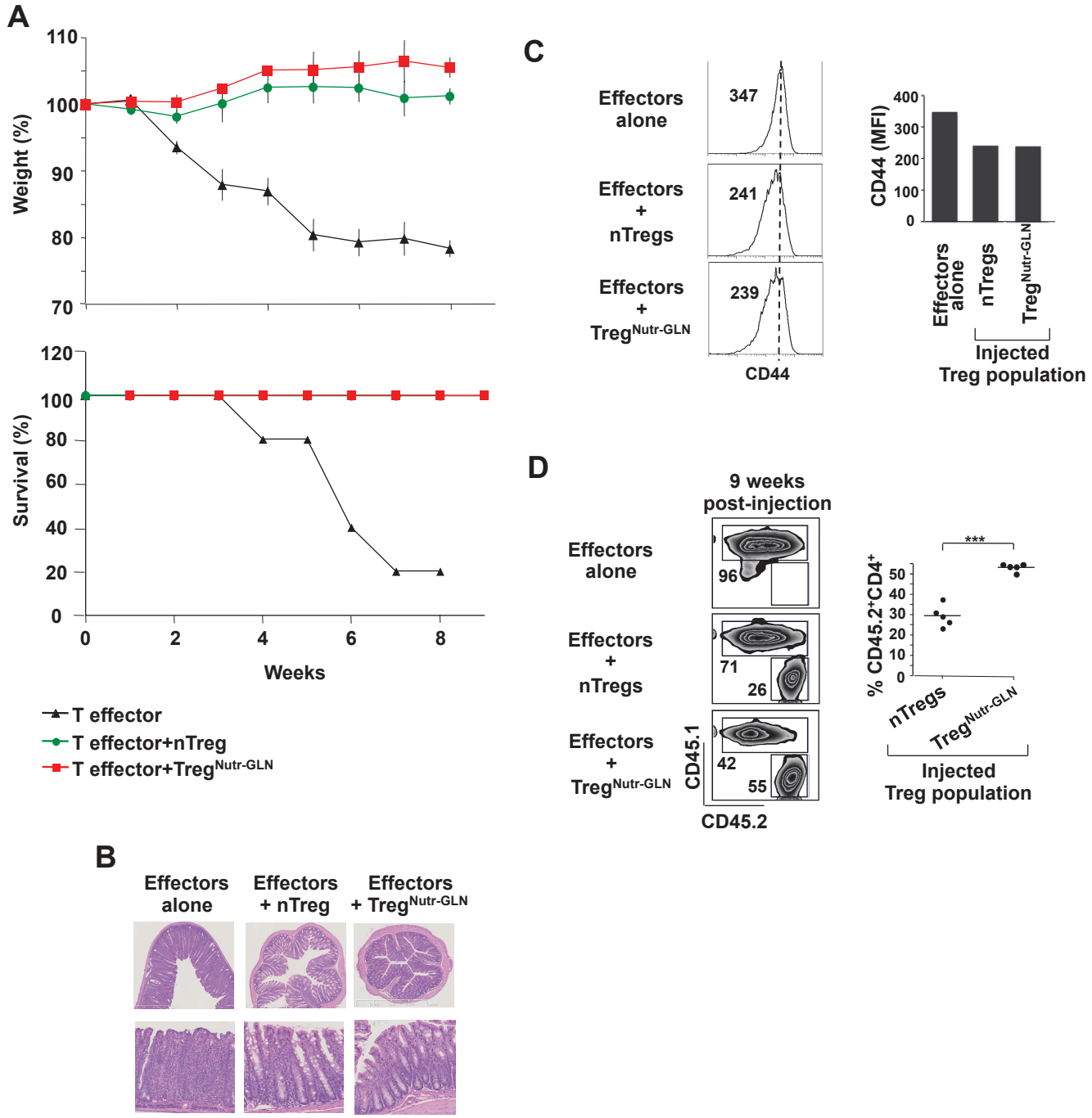
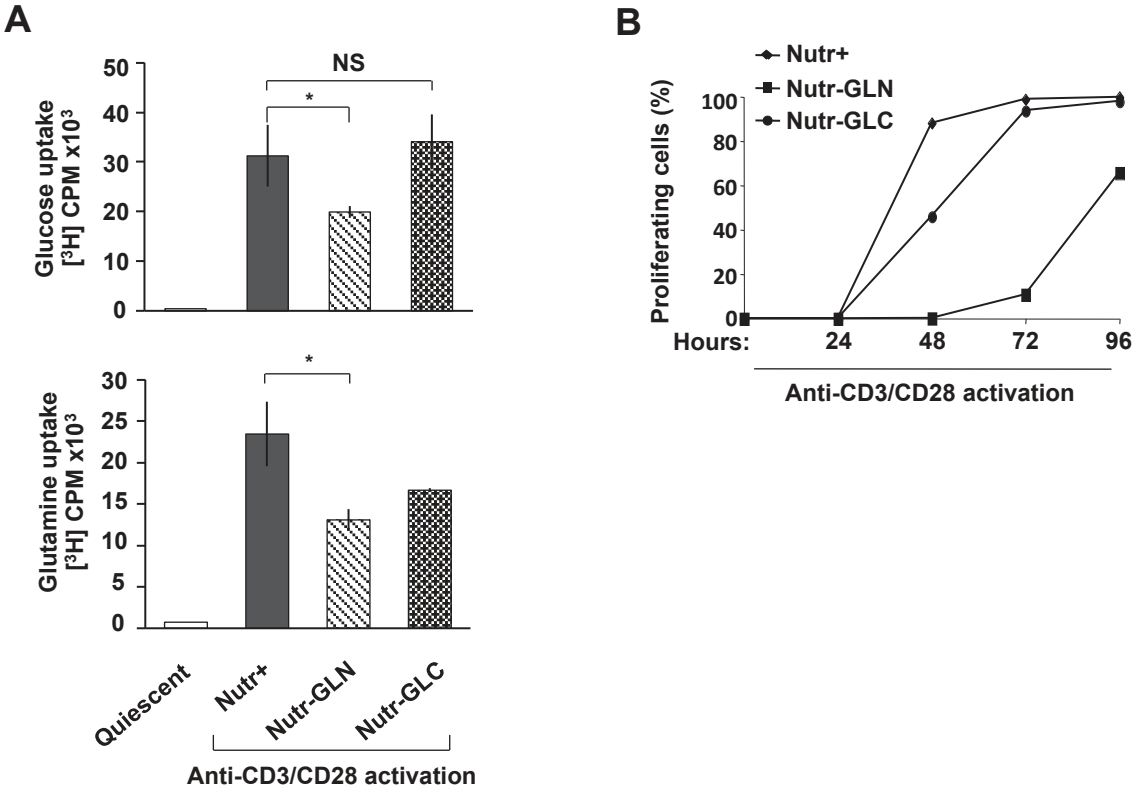


Figure 6: Tregs generated in glutamine-deficient conditions efficiently inhibit effector T cell-mediated autoimmune colitis

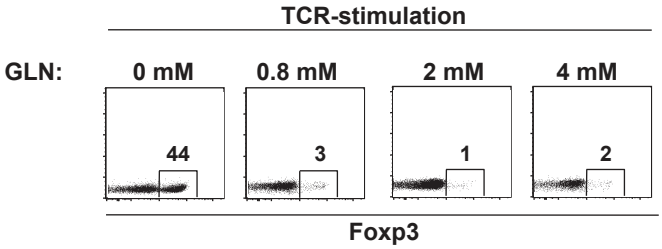


Supplementary Figure 1: Reduced glucose and glutamine uptake is associated with decreased TCR-induced proliferation in glutamine-deprived conditions.



Supplementary Fig. 2: Low levels of ectopic glutamine inhibit the conversion of naïve CD4$^+$ T cells to a Foxp3$^+$ T cell fate

A



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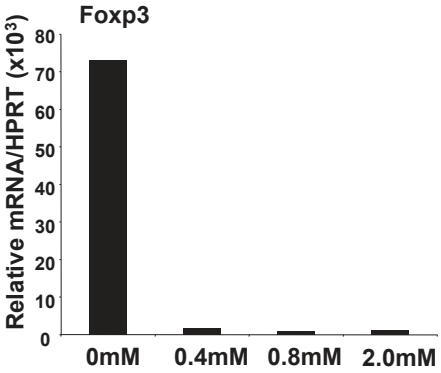
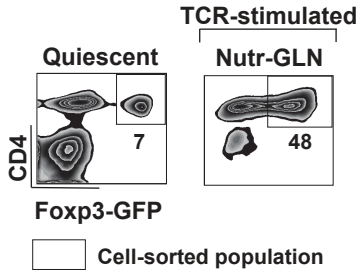


Supplementary Fig. 3: Strategy for FACS purification of Foxp3^{+}CD4^{+} T cells



Supplementary Figure Legends

Supplementary Figure 1: **Reduced glucose and glutamine uptake is associated with decreased TCR-induced proliferation in glutamine-deprived conditions.**

(**A**) $CD4^+$ T cells were activated in Nutr+, Nutr-GLN and Nutr GLC conditions and glucose (upper panel) and glutamine (lower panel) uptake was assayed by incubating quiescent or activated $CD4^+$ T cells in the indicated conditions ($5x10^5$) with L-[3,4-^{3}H (N)]glutamine (0.5 才Ci) or 2-deoxy-D[1-^{3}H]glucose (2 才Ci), respectively, for 5 min at RT. Uptake for each condition, at day 3 of stimulation, is expressed as mean counts per minute (CPM) for triplicate samples; error bars indicate SD. (**B**) $CD4^+$ T cells were activated as indicated and proliferation was monitored by dilution of CTV fluorescence. Quantification of T cell proliferation, as a function of the percentage of proliferating cells, is shown at 24, 48, 72 and 96 hours post stimulation. Experiments were performed in triplicate and results are representative of two independent experiments.

Supplementary Figure 2: **Low levels of ectopic glutamine inhibit the conversion of naïve $CD4^+$ T cells to a $Foxp3^+$ T cell fate. Changes in the relative percentages of Foxp3-expressing $CD4^+$ T cells were monitored as a function of glutamine concentration.**

(**A**) The percentages of $Foxp3^+$ cells within the $CD4^+$ T cell population was monitored 6 days post TCR stimulation as a function of the exogenous glutamine concentration, as indicated (0, 0.8, 2 and 4 mM). Representative dot plots are presented. (**B**) The level of Foxp3 transcripts was quantified in the cells activated in the indicated conditions by qRT-PCR and mean levels

relative to HPRT are presented. Data are representative of three representative experiments.

Supplementary Figure 3: **Strategy for FACS purification of Foxp3$^+$CD4$^+$ T cells.**

For injection of Rag2-deficient mice with natural Treg (left dot plot) or Treg (right dot plot) converted following stimulation with immobilized anti-CD3/anti-CD28 mAbs in the absence of glutamine (Nutr-GLN), CD45.2$^+$Foxp3$^+$CD4$^+$ cells in the indicated gates were FACS-purified. The percentages of Foxp3$^+$ cells present prior to purification are indicated.

Discussion and Perspectives

Discussion and Perspectives

Adoptive T cell therapy represents a very promising therapeutic strategy for treating patients with chemotherapy-resistant tumors. Conditioning of cancer-bearing patients prior to adoptive T cell transfer has been shown to significantly increase anti-tumor responses (Greenberg, 1991a; Rosenberg, 1986; Rosenberg, 2011). The work performed during my thesis demonstrates the importance of the type of conditioning used to achieve a lymphopenic state, and not just the lymphopenic state itself, in the fate of adoptively-transferred T cell fate. Specifically, the transfer of T cells into irradiated hosts was associated with a preferential IL-7-dependent homeostatic proliferation of donor $CD8^+$ T cell whereas in mice preconditioned with busulphan/cyclophosphamide (Bu/Cy), donor $CD4^+$ T cells proliferated extensively in an IL-7-independent manner. The phenotype of adoptively-transferred T cells was also differentially affected by the conditioning protocol. In irradiated hosts, $CD8^+$ T cells acquired a memory-like phenotype and $CD4^+$ T cells remained in vast majority naïve, whereas in Bu/Cy-treated mice, $CD4^+$ T cells acquired an effector/memory phenotype while $CD8^+$ T cells remained naïve. Furthermore, in Bu/Cy-conditioned hosts, $CD4^+$ T cell proliferation resulted in a relative expansion of $Foxp3^+$ regulatory T cells.

The distinct fates of these adoptively transferred T cells were associated with significant changes in the environment generated by these conditioning regimens. Notably, we found a different localization and repartition of dendritic cell subsets in lymphoid organs following irradiation and Bu/Cy conditioning. Moreover, we showed that the presence of dendritic cells was required for the antigen-dependent proliferation of $CD4^+$ T cells transferred into Bu/Cy-conditioned mice but also played a crucial role in homeostatic T cell proliferation. Thus, our results provide new insights into the identification of parameters that can potentially alter the survival and reactivity of adoptively-transferred T cells in lymphopenic hosts.

The role of pre-conditioning in improving the efficacy of T cell-mediated anti-tumor responses in mice is associated with an expansion of the adoptively-transferred T cells. This phenomenon has been shown to be due, at least in part, to the “open

space" created by lymphopenic regimens, resulting in an increased availability of homeostatic cytokines such as IL-7 and IL-15 (Ramsey, 2008; Seddon, 2003; Tan, 2001; Vella, 1997). However, it is important to note that these conclusions were drawn from murine studies performed in lymphopenic environments created by either genetic means or irradiation (reviewed in (Lim, 2013; Sprent and Surh, 2011; Surh and Sprent, 2008)). Our knowledge about the lymphopenic environments generated by different chemotherapeutic agents, even in mice, is much more sparse (Cheadle, 2014; Nakahara, 2010; Salem, 2012, 2010).

As chemotherapeutic agents are commonly used to induce lymphopenia in patients, it was of much interest to investigate how they impact on the fate of adoptively-transferred T cells. During my PhD, we specifically focused on a busulphan/cyclophosphamide (Bu/Cy) conditioning protocol and established a dose allowing us to achieve a level of lymphopenia similar to that obtained by exposure of mice to 600 rads of gamma radiation (from a ^{60}Cobolt source). Importantly, the destiny of adoptively-transferred T cells was distinctly modulated by these 2 lymphopenia-inducing treatments, strongly suggesting that they affect not only the available "space" but also the "quality" of the host environment.

Indeed, it is well known that the environment, comprising stromal cells and APCs as well as soluble factors such as cytokines/chemokines, plays a crucial role in T cell maintenance and homeostasis. The IL-7 cytokine is required for optimal lymphocyte generation and survival (Tan, 2001; von Freeden-Jeffry, 1995) and also plays a major role in the homeostatic T cell proliferation occurring in lymphopenic conditions (Khoruts and Fraser, 2005; Kieper, 2005; Min, 2005; Schluns, 2000; Surh and Sprent, 2008; Tan, 2001). In accord with these previously published studies (Tan, 2002), we detected an IL-7-dependent homeostatic CD8$^+$ T cell proliferation in irradiated mice. However, we were intrigued by the finding that even though Bu/Cy conditioning eliminated host CD8$^+$ T cells as efficiently as irradiation, a homeostatic proliferation of transferred CD8$^+$ T cells was not detected. This did not reflect an inability of CD8$^+$ T cells to respond to IL-7 in the environment of a Bu/Cy-conditioned mouse because administration of exogenous IL-7 promoted a homeostatic proliferation of donor

$CD8^+$ T cells. As these data indicate that the transferred $CD8^+$ T cells are sensitive to IL-7, our data suggest that the bio-availability of IL-7 in Bu/Cy-conditioned mice may be decreased as compared to irradiated mice.

Within lymphoid tissues, several stromal cell types produce IL-7 (Iolyeva, 2013; Miller, 2013; Onder, 2012; Schluns, 2000; Watanabe, 1995), including lymphatic endothelial cells (LEV) and fibroblastic reticular cells (FRC) but not lymphatic endothelial cells (BEC), (Hara et al., 2012; Link, 2007; Malhotra and Brown, 2012; Onder, 2012). We were therefore interested in assessing whether these subsets were differentially affected by irradiation and Bu/Cy conditioning. Intriguingly, preliminary data from the group shows that there is a significantly greater reduction in LEVs following Bu/Cy treatment as compared to irradiation and moreover, the overall organization of stromal cells is distinctly affected by these two conditioning regimens (M. Epardaud, unpublished observations). Thus, these data may explain the significantly lower level of homeostatic T cell proliferation in Bu/Cy-treated as compared to irradiated mice. From a clinical perspective, our data suggest that the utilization of adjuvant cytokine therapies (such as rIL-7) should be adapted as a function of the pre-conditioning regimen. Patients receiving tumor-specific T cells after a Bu/Cy conditioning may benefit more from rIL-7 than patients who are rendered lymphopenic by irradiation.

Another important difference between irradiated and Bu/Cy-treated mice was the proliferation profile of adoptively-transferred $CD4^+$ T cells. In the latter, we detected a rapid $CD4^+$ T cell proliferation. This type of “rapid” (also termed “spontaneous” and “endogenous”) proliferation of T lymphocytes has previously been described to be antigen-driven (Kieper, 2005; Min, 2005). Indeed, our experiments modulating the IL-7 pathway upon Bu/Cy conditioning demonstrated that the rapid division of donor $CD4^+$ T cells was not dependent on IL-7. To determine whether the division was antigen-dependent, we used T cells harboring a single transgenic TCR against an antigen not present in C57Bl/6 mice. OTII $CD4^+$ T cells, harboring a transgenic TCR against ovalbumin (backcrossed onto a RAG-KO background) were adoptively-transferred into Bu/Cy-conditioned mice. Importantly, these OTII T cells did not proliferate in this context (Juhee Kim and Charlie Surh, unpublished observations),

demonstrating that the rapid $CD4^+$ T cell proliferation observed in Bu/Cy conditioned mice is antigen-dependent.

Dendritic cells are critical for antigen presentation, thereby driving T cell proliferation (Do, 2009; Sapoznikov, 2007; Zaft, 2005). Considering the antigen-dependent nature of the rapid $CD4^+$ T cell proliferation observed in Bu/Cy-treated host, it was important to determine how the conditioning protocols that we used affected peripheral dendritic cells. Interestingly, cross-sections of lymphoid organs from irradiated mice revealed a significantly disrupted tissue organization and an increased staining for $CD11c^+$DCs as compare to control mice. In contrast, the architecture of lymphoid tissues in Bu/Cy-conditioned hosts was partially retained, with clearly distinct B cell follicles, albeit smaller and fewer in number, and aggregated $CD11c^+$ DCs. Nevertheless, we detected a significantly higher percentage of $CD11c^+CD8^+$ cross-presenting dendritic cells in irradiated as compared to Bu/Cy-conditioned or untreated hosts (reviewed in (Joffre, 2012)). Given the role of DC in antigen-driven T cell proliferation (reviewed in (Kieper, 2005; Min, 2005)), these data were actually the opposite of what we were expecting. It is though important to note that $CD8^+$ DC can also have immune-inhibitory or tolerogenic properties (Bonifaz, 2002; Hawiger, 2001; Kronin, 2001). Indeed, Nakahara and colleagues found that $CD8^+$ DC can inhibit the function of CD8-negative DC in cyclophosphamide-treated mice. Moreover, CD8-negative DC isolated from cyclophosphamide-treated mice secrete increased levels of IL-12 and are more potent in stimulating T cell proliferation and IFNγ secretion than DC from non-treated mice (Nakahara, 2010). Thus, these data may, at least partially, explain the rapid antigen-specific $CD4^+$ T cell proliferation following Bu/Cy conditioning.

To specifically assess whether the DC subsets reconstituting Bu/Cy-conditioned mice are important for the rapid proliferation of donor $CD4^+$ T cells, we attempted to eliminate DCs following conditioning. Using the CD11c/DOG-DTR mouse model (Hochweller, 2008), we demonstrated that DC depletion in Bu/Cy-treated mice was associated with a significant reduction in the rapid proliferation of donor $CD4^+$ T cells, clearly demonstrating the importance of dendritic cells in this process. In light

of the study by Nakahara et al. (Nakahara, 2010), it will be of interest to determine the relative impact of $CD8^-$ as compared to $CD8^+$ DCs in this T cell proliferation. Based on their data (Nakahara, 2010), we would predict an important role for the $CD8^-$ DC subset.

There are several mechanisms *via* which DCs stimulate T cell proliferation (Mildner and Jung, 2014), including the secretion of soluble factors such as IL-6. IL-6 is a pro-inflammatory cytokine (Suzuki, 2009) produced by different cell types including dendritic cells (Pasare, 2003) and it plays a pivotal role in T cell differentiation (Bettelli, 2006; Korn, 2008; Smith and Maizels, 2014; Zhou, 2007b) as well as proliferation (Feng, 2010; Yoshida, 2010a, b). Furthermore, it is interesting to note that IL-6 is required for the antigen-dependent proliferation of $CD4^+$ T cells in the lymphopenic environment of Rag KO mice. In our studies, we detected 60-fold higher levels of IL-6 in the serum of Bu/Cy-conditioned as compared to irradiated mice (M. Epardaud and D.Klysz, unpublished observations). We therefore evaluated the possibility that the DC-dependent proliferation of $CD4^+$ T cells in Bu/Cy-conditioned mice was mediated by IL-6. However, in our system, donor $CD4^+$ T cells rapidly proliferated following transfer into Bu/Cy-conditioned IL-6 KO mice. Thus, the donor $CD4^+$ T cell proliferation in Bu/Cy-treated mice requires DCs but is independent of IL-6. It will be interesting to determine whether other soluble factors such as the inflammatory IL-12 cytokine, which regulates T cell responses ((Henry, 2008; King and Segal, 2005) reviewed in (Vignali and Kuchroo, 2012)) and is induced in DCs by cyclophosphamide treatment (Nakahara, 2010), are involved in this process.

Microbial-derived antigens also play an important role in T cell proliferation (Feng, 2010) and it is known that chemotherapy and irradiation damage mucosal barriers, leading to their increased permeability (Ferreira, 2014; Nejdfors, 2000). Total body irradiation and cyclophosphamide both result in the translocation of commensal microbial antigens into blood vessels resulting in the induction of immune responses (Feng, 2010; Paulos, 2007). Furthermore, in the case of cyclophosphamide treatment, microbial antigens have been shown to drive differentiation of naive

$CD4^+$ lymphocytes (Viaud, 2013). Indeed, in the studies that we performed here, we found that gut integrity, as monitored by the translocation of FITC-dextran, was negatively affected by both irradiation and Bu/Cy-conditioning treatments (M. Epardaud, unpublished data).

Based on these data, we were interested in determining whether microbial-derived antigens drive the fast proliferation observed in Bu/Cy-conditioned mice. In an attempt to respond to this question, we performed two different types of experiments. First, we treated mice with a cocktail of antibiotics (ampicillin, neomycin and polymixin B) beginning 14 days prior to Bu/Cy conditioning and continuing throughout the 7day period of adoptive T cell transfer. Notably, a rapid proliferation of donor $CD4^+$ T cells was still observed (V. Dardalhon, unpublished observations). However, as this treatment does not eliminate many commensal bacteria, we next assessed whether the rapid donor CD4 T cell proliferation would be observed in Bu/Cy-conditioned germ-free mice (collaboration J-H. Kim and C. Surh, POSTECH, Korea). Surprisingly, in these mice as well, donor $CD4^+$ T cells continued to proliferate extensively. There is some disparity between these data and a recently published study showing that the generation of Th1 and Th17 cells in cyclophosphamide-treated mice is significantly reduced in germ-free mice (Viaud, 2013). The differences between these studies may be due to the fact that Viaud et al were assessing effects on endogenous CD4 T cells while we assessed the fate of adoptively-transferred T cells. Alternatively, it is possible that different types of commensal bacteria, differing between germ-free animal facilities, may account for these discrepancies.

In our experimental system, the conditioning regimens also modulated the effector functions of the adoptively-transferred T cells. Our preliminary data suggests that the percentage of donor T cells secreting IFNγ, a cytokine playing a crucial role in anti-tumor responses (Lim, 2013; Schroder, 2003; Starbeck-Miller, 2013) is higher in irradiated than Bu/Cy-conditioned mice (26% vs 7%, respectively as compare to about 5% in non-treated mice) whereas the proportion of IL-17-producing donor T cells is higher in Bu/Cy-treated mice (34%). Of note, we observed a significantly

higher expansion of donor regulatory Foxp3$^+$ cells in Bu/Cy-conditioned (27%) as compared to irradiated hosts (9%). As T_{reg} can impair anti-tumoral responses by suppressing tumor-specific T cells and thereby facilitating tumor escape (Onizuka, 1999; Shimizu, 1999), their presence during anti-tumor immunotherapy is undesirable. That being said, we found that the *in vivo* expansion of these T_{reg} could be significantly reduced by depleting CD25^{hi} T cells prior to adoptive transfer. Furthermore, even though cyclophosphamide treatment has previously been shown to be associated with an expansion of regulatory T cells, *Nakahara et al.* reported that these cells have impaired suppressive functions as compared to T_{reg} isolated from control mice (Nakahara, 2010). Therefore, it will be crucial to determine the relative suppressor activity of these expanded donor T_{reg} cells. Moreover, the role of IL-17 in anti-tumor responses is not completely clear, with both positive as well as negative effects reported (reviewed in (Wilke, 2011)). As such, it will be important to assess whether Bu/Cy-conditioning alters IL-17 production by endogenous as well as adoptively-transferred T cells and monitor how this may impact on anti-tumor responsiveness. Irrespective of the data, it is probably optimal to deplete T_{reg} before engineering conventional T cells to express a tumor-specific CAR or TCR. Taken together, our data demonstrate that conditioning protocols modify T cell fate by shaping the host microenvironment and as such, modifications that may be induced by other clinically relevant chemotherapy protocols need to be taken into account. It is though important to note that the Bu/Cy conditioning regimen efficiently promotes an anti-melanoma response in mice transplanted with T cells engineered to express a melanoma-specific TCR (Straetemans/Debets, manuscript in revision). In light of our results, it will be of interest to compare the efficacy and persistence of these cells following transfer into lymphopenic environments generated by irradiation as compared to Bu/Cy conditioning.

As discussed above, it is well known that cytokines and DCs modulate T cell activation and differentiation but it is important to reiterate that the metabolic environment of a T cell, and specifically the nutrient environment, can also play a critical role in T cell fate. During my PhD, we demonstrated that nutrient availability regulates CD4$^+$ effector differentiation. Indeed, this field has taken off since I began my PhD and there is now significant data showing that different T cell effector

subsets can be distinguished based on their metabolic needs (reviewed in (Pearce, 2010; Wang and Green, 2012) and differential levels of lipid oxidation, glycolysis and oxidative phosphorylation (Chang, 2013; Dang, 2011; Michalek, 2011; Pearce, 2009; Shi, 2011; Zeng, 2013).

mTOR is a critical sensor in the metabolic reprogramming of TCR-stimulated T cells, integrating environmental signals such as nutrients, growth factors and stress signals and translating them into an "optimal" cellular response (reviewed in (Hay, 2004; Laplante and Sabatini, 2009; Sengupta et al., 2010)). Our experiments demonstrated that the activation of $CD4^+$ T lymphocytes in glutamine-deprived conditions resulted in slower and decreased activation of mTORC1, measured as a function of S6 phosphorylation, and impaired proliferation. These data are in agreement with previous work performed in the HeLA cell line showing that the mTORC1 pathway is regulated by the intracellular transport of essential amino acids (such as leucine) in exchange for glutamine (Nicklin, 2009). Importantly, under these conditions of decreased mTORC1 activation, we detected an increased conversion of naïve $CD4^+$ T cells to Foxp3-expressing regulatory T cells. Furthermore, Th1, but not Th2 differentiation, was inhibited in glutamine-deprived conditions. This finding is consistent with previous work from *Delgolffe et al.* showing that the generation of Th2 cells is dependent on mTORC2, but not mTORC1, signaling (Delgoffe, 2011). It is nonetheless important to note that other metabolic pathways may also play a role in this conversion. Metabolic sensors such as cMYC regulate glutaminolysis (Gao, 2009; Le, 2012; Wang, 2011), and intersect with the mTOR pathway as well as with the AMPK/LKB1 pathway, upstream of mTOR (Hardie, 2011; Shackelford and Shaw, 2009) play also a role. The complex interplay *via* which these pathways regulate T cell differentiation has not yet been completely elucidated.

In our system, we found that the conversion of naïve $CD4^+$ T cells to regulatory $Foxp3^+$ T cells was dependent on TGF-β. Blocking this signaling pathway inhibited the generation of $Foxp3^+$ T cells under Th0 as well as Th1 conditions. Earlier research by *Delgoffe et al.* demonstrated that mTOR-deficient T cells are hypersensitive to TGF-β and even low levels of this cytokine were sufficient to generate inducible T_{reg}

(Delgoffe, 2009). Indeed, we did not detect significant increases in TGF-β production, either at the mRNA or protein level, in cells converting to a Foxp3$^+$ T cell fate under glutamine-deficient conditions (our unpublished observations). Thus, it is likely that in the absence of glutaminolysis, TCR-activated T cells exhibit increased responsiveness to TGF-β signaling. It will be of interest to assess whether this signaling cascade is mediated by receptor-associated Smad2 and Smad3.

While abrogating TGF-β signaling inhibited T_{reg} conversion under conditions of limiting glutaminolysis, it was not sufficient to promote the induction of T-bet, even under optimal Th1-polarizing conditions. Thus, our data strongly suggest that the impairment of Th1 differentiation in glutamine-deprived condition occurs through a TGF-β-independent mechanism, potentially associated with lower activation of the mTORC1 pathway. This conclusion is supported by a very recent study showing that the absence of the ASCT2 glutamine transporter results in decreased mTORC1 activation and impaired Th1 as well as Th17 differentiation (Nakaya, 2014).

The Foxp3$^+$ T cells generated under conditions of glutamine-deprivation were fully protecting mice from the development of inflammatory bowel disease. Furthermore, the percent recovery of T_{reg} activated in glutamine-depleted conditions was significantly higher than the recovery of nT_{reg}. This is of interest as it is known that regulatory T cells can lose Foxp3 expression in lymphopenic environments (Duarte, 2009; Komatsu, 2009; Murai, 2009; Tsuji, 2009; Yurchenko, 2012; Zhou, 2009) even when co-transfered with effector cells (Komatsu, 2009; Yurchenko, 2012; Zhou, 2009). These data suggest that the viability and/or persistence of T_{reg} generated under glutamine-deprivation is increased because of their initial metabolic state. Interestingly, as compared with other T effector subsets, $T_{reg,}$ have a greater dependence on lipid metabolism (Michalek, 2011; Zeng, 2013). As such, conversion to a lipid metabolism may be promoted by limiting glutaminę metabolism. Another possibility is that Foxp3 expression and function, regulated by hypomethylation at the Foxp3 locus (Morikawa, 2014; Ohkura, 2012; Sauer, 2008), may be more stable under specific nutrient conditions. It will therefore be of interest to compare the methylation levels of the Foxp3 locus in T_{reg} generated in glutamine-deprived

conditions as compared to nT_{reg}. It will also be important to characterize the mechanisms *via* which these glutamine-deprived T_{reg} exert suppressive functions, assessing the expression levels of supressive molecules such as CTLA-4 (Tai, 2012; Wing, 2008) and CD39 (Vignali et al., 2008), and monitoring production of the immunosuppressive enzyme indoleamine 2,3-dioxygenase (IDO) (Grohmann, 2002) and anti-inflammatory cytokines such as IL-10 (Boks, 2012). Finally, in the context of the significant lipid oxidation occuring in regulatory T cells, it will be of interest to assess the implication of the melanovate pathway in the upregulation of CTLA4 (Zeng, 2013).

In conclusion, our data together with other recent elegant studies (Lyssiotis, 2013; Michalek, 2011; Shi, 2011; Son, 2013; Wang, 2011; Willems, 2013) suggest that the targeting of metabolic pathways may present an attractive new strategy for modulating immune responses. This is especially interesting in the context of the tumor microenvironment where fluctuations in nutrient availability may force T cells to adapt their metabolism, resulting in modifications of their effector functions. Furthermore, in the context of anti-tumor therapies, chemotherapeutic agents can negatively impact on patients' nutritional state (Raffaghello, 2010; Safdie, 2009; Shelton, 2010; Tisdale, 2007; Zhou, 2010) and tumor growth can contribute to cachexia. It will be important to evaluate how the inter-relationships between the metabolic requirements of tumors for nutrients, including glucose (Demetrakopoulos, 1978; Koppenol, 2011; Priebe, 2011; Shim, 1998; Warburg, 1956, 1958) and glutamine (Ahluwalia, 1990; Lyssiotis, 2013; Qin, 2010; Shelton, 2010; Son, 2013; Wise, 2010; Wu, 1978; Yuneva, 2007), impact on the metabolism of endogenous as well as adoptively-transferred T cells. It is interesting to speculate that the increased percentage of donor regulatory T cells in Bu/Cy-conditioned as compared to irradiated mice may be related to lower glutamine concentrations in specific microenvironments of the former group (Yoshida, 1998). Within the context of tumor-bearing mice, it will therefore be of interest to compare nutrient concentrations in the plasma, as well as LN and tumor microenvironments in the absence or presence of chemotherapy/irradiation. These data may also impact on the possible role of a nutrient-supplemented diet in anti-tumor responses.

Furthermore, it is interesting to note that oral glutamine supplementation has been shown to reduce some of the side effects of chemotherapy (Anderson, 1998; Xue et al., 2007) and to promote anti-tumor treatments (Cardaci, 2012). Our data strongly suggest that glutamine deprivation during T cell stimulation may result in the conversion of an effector T cell into a suppressive regulatory T cell. While this is clearly not optimal for an anti-tumor therapy, it can be beneficial for the treatment of autoimmune disorders (reviewed in (Wright, 2011), (Kong, 2012; Pilat, 2014)). Therefore, this type of approach may be useful as an adjuvant for promoting regulatory T cell differentiation (Keever-Taylor, 2007; Prevel and Klatzmann, 2013; Shin, 2011; Stauss, 2013; Steiner, 2006; Sugiyama, 2014). Taken together, our results highlight a previously unknown relationship between glutamine availability and the establishment of a tolerogenic environment.

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