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# Deciphering the role of the death receptor Fas/CD95 in T cell co-stimulation

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# THÈSE DE DOCTORAT

## Déterminer le rôle du récepteur de mort Fas/CD95 dans la co-stimulation des cellules T

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d'Université Côte d'Azur

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# Déterminer le rôle du récepteur de mort Fas/CD95 dans la co-stimulation des cellules T

## Deciphering the role of the death receptor Fas/CD95 in T cell co-stimulation

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## Résumé

Fas (CD95 / TNFRSF6), un récepteur transmembranaire de type I de la superfamille des récepteurs au TNF (TNFR), est un activateur de mort cellulaire bien connu. Cependant, il a également été impliqué dans des fonctions de non-mort cellulaires, telles que la survie, la différenciation et la migration. Alors que la cascade moléculaire qui initie l'apoptose lors de l'engagement de Fas avec son ligand FasL est particulièrement bien décrite, les informations concernant les mécanismes moléculaires sous-tendant les voies non apoptotiques médiées par Fas sont rares.

Comme indiqué par les manifestations d'auto-immunité et de lymphoprolifération chez les patients ALPS porteurs de mutations dans le récepteur ou dans son ligand, le système Fas / FasL joue un rôle majeur dans l'homéostasie des lymphocytes T et dans le contrôle de l'auto-immunité et du cancer. D'un côté, la mort médiée par Fas a été décrite comme critique pour (i) la suppression des lymphocytes autoréactifs, et donc dans le maintien de la tolérance périphérique; (ii) le contrôle du nombre de lymphocytes activés par des antigènes faibles lors d'infections par des pathogènes.

De l'autre côté, certaines fonctions de non mort de Fas ont été décrites dans les cellules T, parmi lesquelles le rôle de Fas comme récepteur co-régulateur de l'activation du TCR. Malgré l'importance potentielle de ce rôle dans les stratégies immunothérapeutiques, seules quelques études controversées liées à cette implication ont été réalisées. En effet, alors que plusieurs études ont décrit Fas comme un récepteur co-stimulateur du TCR, d'autres ont défini une inhibition de l'activation des lymphocytes T lors d'une stimulation concomitante de Fas et du TCR. Dans ce contexte, l'objectif de mon projet de thèse consistait à disséquer moléculairement la co-signalisation Fas-TCR.

En utilisant à la fois des cellules T primaires et des lignées cellulaires portant un TCR transgénique spécifique, nous avons pu définir Fas comme un récepteur co-stimulateur. En exploitant les approches biochimiques ainsi que la cytométrie en flux et la microscopie, nous avons déchiffré la co-stimulation Fas-TCR à la fois au niveau fonctionnel et moléculaire. Premièrement, nous avons montré que la co-stimulation Fas-TCR se produit à la fois dans les cellules T naïves et les cellules T mémoire ainsi que dans les sous-populations CD4 + et CD8 +. Moléculairement, nous avons décrit que Fas renforce la signalisation TCR dès les étapes précoces, puisque la phosphorylation des premières protéines impliquées dans l'activation du TCR est augmentée. En outre, les formes membranaires et solubles de FasL sont capables d'initier le signal co-stimulateur de Fas. Enfin, nous avons pu exclure l'implication de FADD et Caspase-8, premiers acteurs de la signalisation Fas, dans la co-activation, et, de manière importante, l'implication du domaine de mort de Fas, suggérant le rôle d'un autre domaine de Fas.

Décrire les mécanismes moléculaires et le contexte dans lequel la co-stimulation Fas-TCR se produit pourrait être d'une importance cruciale dans la compréhension de la physiopathologie de Fas dans les cellules T, mais également pour l'établissement de futures stratégies immunothérapeutiques.

**Mots clés :** Récepteur de mort Fas, activation des lymphocytes T, point de contrôle immunitaire du TCR, immunothérapie, co-stimulation du TCR, système immunitaire.

## Abstract

Fas (CD95/TNFRSF6), a type-I transmembrane receptor of the tumor necrosis factor receptor (TNFR) superfamily, is a well-known cell death activator. However, it has been also implicated in non-cell death processes including cell survival, differentiation, migration. Whereas the molecular cascade that initiates apoptosis upon Fas engagement with its ligand FasL is particularly well described, the informations concerning the molecular mechanisms underlying the Fas mediated non-apoptotic pathways are sparse.

As indicated by the induction of autoimmunity and lymphoproliferation in ALPS patients harboring mutations in either the receptor or its ligand, the Fas/FasL system plays a major role in T cell immune homeostasis and thus, in the control of autoimmunity and cancer. On one side, the Fas mediated death has been described critical for (i) the deletion of autoreactive lymphocytes, and thus in the maintenance of peripheral tolerance; (ii) the control of the number of lymphocytes activated by weak antigens during pathogen infections.

On the other side, and beyond cell death induction, some Fas non-death pathways have been described in T cells, among which the role of Fas as co-regulatory receptor for the TCR during its activation. Despite the potential importance of this role in immunotherapeutic strategies, only few and controversial studies related to this involvement were done. Indeed, whereas several studies have described Fas as a TCR co-stimulatory receptor, others defined an inhibition of T cell activation by Fas-TCR concomitant stimulation. In this context, the aim of my PhD project consisted into molecularly dissect the Fas-TCR co-signaling.

By using both primary T cells and cell lines bearing a specific transgenic TCR, we could define Fas as a co-stimulatory receptor. By exploiting biochemical approaches as well as flow cytometry and microscopy we could decipher the Fas-TCR crosstalk both at functional and molecular level. First, we show that Fas-TCR co-stimulation occurs in both naïve and in memory T cells as well as in both CD4+ and CD8+ T cell subpopulations. Molecularly, we could describe that Fas enhances the TCR signaling at membrane proximal level, since the phosphorylation of the first proteins involved in TCR activation is increased. Furthermore, both membrane-bound and soluble FasL are capable to initiate Fas co-stimulatory signal. Lastly, we could exclude the involvement of FADD and Caspase-8, first actors of Fas signaling, in the co-activation, and even more importantly, the involvement of the death domain of Fas cytoplasmic tail, unveiling the implication of another Fas receptor domain.

To describe the molecular mechanisms and the context where Fas-TCR co-stimulation occurs might be of an outstanding importance in the comprehension of Fas physiopathology in T cells and for future studies that might involve its potential for immunotherapeutic strategies.

**Keywords:** Death receptor Fas, T cell activation, TCR immune checkpoint, immunotherapy, TCR co-stimulation, immune system.

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## List of Abbreviations

ACT: Adoptive Cell transfer  
ADAM: A disintegrin and metalloprotease  
ADAP: Adhesion and degranulation promoting adapter protein  
Ag: Antigen  
AJ: Adherens junction  
ALPS: Autoimmune lymphoproliferative disease  
AP-1: Activator Protein 1  
Apaf-1: Apoptotic protease-activating factor 1  
APC: Antigen presenting cell  
Arp2/3: Actin related protein 2/3  
Bak: Bcl-2 homologous antagonist/killer  
Bax: Bcl-2 associated X protein  
Bcl-2/10/XL: B cell lymphoma 2/10/XL  
BCR: B cell receptor  
Bid/tBid: BH3 interacting-domain death agonist/ truncated  
BIM: Bcl-2-like protein 11  
BTK: Bruton tyrosine Kinase  
BTLA-4: B- and T-lymphocyte attenuator  
CAF: Cancer associated fibroblast  
CARMA-1: Caspase recruitment domain-containing membrane-associated guanylate kinase protein-1  
CAR-T: Chimeric antigen receptor T  
Cbl b/c: Casitas B-cell lymphoma b/c  
Cdc42: Cell division control protein 42 homolog  
cFLIPL/S/R: cellular FLICE-like inhibitory protein Long/Short/Raji  
c-IAP1/2: cellular Inhibitor of apoptosis protein-1/2  
CKI: Casein kinase I substrate  
CMV: Cytomegalovirus  
CRAC: Calcium release-activated channels  
CRD: Cysteine rich domain  
CSK: C-term src kinase  
CTL: Cytotoxic T lymphocyte

CTLA-4: Cytotoxic T-Lymphocyte Antigen 4  
DAG: Diacylglycerol  
DAXX: Death domain associated protein 6  
DC: Dendritic cell  
DcR: Decoy receptor  
DD: Death Domain  
DED: Death Effector Domain  
DISC: Death-inducing signaling *complex*  
Dlg: Disc large protein  
DNT: Double negative T cell  
DP: Double positive T cell  
DR: Death receptor  
DRM: Detergent resistant membrane  
EAE: Experimental autoimmune encephalomyelitis  
ECD: Extracellular domain  
EMT: Epidermal to mesenchymal transition  
ER: Endoplasmic reticulum  
ERK1/2: Extracellular regulated kinases 1/2  
ERM: Ezrin, radixin and moesin  
FADD: Fas-associated protein with death domain  
Fap-1: Fas associated phosphatase-1  
GADS: Grb2-related adaptor downstream of Shc  
GBM: Glycosphingolipid binding motif  
GCE: Genetic code expansion  
gld: Generalized lymphoproliferative disease  
Grb2: Growth factor receptor-bound *protein 2*  
GSK3 $\beta$ : *Glycogen synthase kinase 3 beta*  
GVHD: Graft versus host disease  
GVT: Graft versus tumor  
HLA: Human leukocyte antigen  
HVEM: Herpesvirus entry mediator  
ICAM: Intercellular Adhesion Molecule 1  
ICB: Immune checkpoint blockade

ICD: Intracellular domain  
ICOS: Inducible T-cell COStimulator  
IFN $\alpha/\beta/\gamma$ : Interferon  $\alpha/\beta/\gamma$   
IgG: Immunoglobulin G  
IgSF: Immunoglobulin superfamily  
I $\kappa$ B: Inhibitor of nuclear factor kappa B  
IKK: I $\kappa$ B kinase  
IL-1R1: Interleukin-1 receptor1  
IL-2/4/6/8/10: Interleukin-2/4/6/8/10  
IP3: Inositol triphosphate  
IP3R: Inositol triphosphate receptor  
IS: Immune synapse  
ITAM: Immunoreceptor Tyrosine-based Activation *Motif*  
ITIM: Immunoreceptor Tyrosine-based inhibition *Motif*  
Itk: IL-2-inducible T-cell kinase  
ITSM: Immunoreceptor Tyrosine-based Switch *Motif*  
JNK: c-Jun N-terminal kinase  
LAT: Linker for activation of T cells  
Lck: Lymphocyte-specific protein tyrosine kinase  
LFA-1: Lymphocyte function-associated antigen 1  
Lpr: Lymphoproliferation  
LRR: Lysine rich region  
LT $\alpha$ : Lymphotoxin  
LUBAC: Linear ubiquitination assembly *complex*  
MALT-1: Mucosa-associated lymphoid tissue lymphoma translocation protein 1  
MAPK: *Mitogen-activated protein kinase*  
MC: Microcluster  
mbFasL: membrane bound FasL  
MHC I / II / pMHC: Major histocompatibility complex I / II / peptide  
MISC: Migration-inducing signaling complex  
MMP: Metalloprotease  
MTOC: Microtubule-organizing center  
Nck: Non-catalytic region of tyrosine kinase adaptor *protein 1*

NFAT: Nuclear factor of activated T cells  
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells  
NK: Natural killer  
NMR: Nuclear magnetic resonance  
PARP: Poly (ADP-ribose) polymerase  
PBMC: Peripheral blood mononuclear cell  
PCD: Programmed cell death  
PD-1: Programmed cell death protein 1  
PI3K: Phosphoinositide 3-kinases  
PIP2: Phosphatidylinositol (4,5)-bisphosphate  
PIP3: Phosphatidylinositol (3,4,5)-trisphosphate  
PKC β/θ: *Protein Kinase C β/θ*  
PLAD: Pre-ligand binding assembly *domain*  
PLCγ: *Phospholipase C gamma*  
PP2A: Protein *phosphatase 2 A*  
PRD: Proline rich domain  
PRR: Proline rich region  
PTEN: Phosphatase and tensin homolog  
PTM: Post translational modification  
PTPN: *Protein tyrosine phosphatases, non-receptor type*  
Rac1: Ras-related C3 botulinum toxin substrate 1  
Rap1: Ras-related *protein 1*  
RICD: Restimulation induced cell death  
RIPK1-3: Receptor-interacting serine/threonine-protein kinase 1/3  
ROS: Reactive oxygen species  
sFasL: soluble FasL  
SH2/3: Src homology 2/3  
SHP-1/2: Src homology 2 domain-containing protein tyrosine phosphatase 1/2  
SKF: Src kinase family  
SLE: Systemic lupus erythematosus  
SLP-76: SH2 domain-containing leukocyte phosphoprotein of 76kDa  
SMAC c/p/d: Supramolecular activation cluster central/peripheral/distal  
SMase: Sphingomyelin phosphodiesterase

SP: Single positive

STAT-1/3/4: Signal transducer and activator of *transcription* 1/3/4

TAB2/3: TGF-Beta Activated Kinase 1 (MAP3K7) Binding protein 2/3

TAK1: Mitogen-activated protein kinase kinase kinase 7

TCM: Central memory T lymphocytes

TCR: T cell receptor

TEFF: Effector T lymphocyte

TEM: Effector memory T lymphocyte

Tfh: Follicular helper T lymphocyte

TGF $\beta$ : Transcription growth factor  $\beta$

Th: Helper T lymphocyte

THD: TNF homology domain

TIL: Tumor infiltrating lymphocyte

TIM: TRAF interacting motif

TKB: Tyrosine kinase binding domain

TM: Memory T lymphocyte

TM/TMD: Transmembrane/ Transmembrane domain

TN: Naive T lymphocyte

TNF(SF): Tumor necrosis factor (Superfamily)

TNFR(SF): Tumor necrosis factor receptor (Superfamily)

TRADD: Tumor necrosis factor receptor type 1-associated Death domain

TRAF: TNF receptor associated factors

Treg: Regulatory T lymphocyte

VCAM: Vascular cell adhesion *protein* 1

ZAP70: Zeta Chain of T Cell Receptor Associated Protein Kinase 70



# **INTRODUCTION**

## T cell mediated immune response

Vertebrates have evolved multiple strategies to counteract invasion and aggression coming from a large panel of insults, from attack by microorganisms such as bacteria and viruses, to chemical and physical injuries. Immune system consists of a large network of chemical (humoral) and cellular mediators all finely regulated. Each cell type plays a specific role to respond efficiently to external invasions or even to internal modifications not recognized as physiologic. Both cellular and molecular events are differently involved in the two main types of immunity: the innate and the adaptive immunity.

The first barrier against non-self-invasion is fulfilled by innate immunity, which recognizes intrinsic or pathogenic patterns, without any specificity for the type of agent. Conversely, the adaptive immunity requires specificity against a determined pathogen, in order to amplify the strength of the response and efficiently defeat the foreigner invaders. Adaptive immunity is based on cell-cell cooperation mechanisms where different cell populations participate to maintain homeostasis and assure constant surveillance of the organism. Innate and adaptive immunity, furthermore, collaborate with each other, where first recognition of an unknown agent by innate system can lead to activation of the adaptive one as will be simplistically described below.

One vital characteristic of lot of cells that are part of the innate immunity is the ability to ingest (phagocytosis) their target or part of them, processing it inside their cytoplasm and exposing on their surface pathogen-specific peptides (antigens). All these cells act indeed as **antigen presenting cells (APCs)**, able to induce and activate the adaptive immunity. The APCs expose the antigen to be presented to the adaptive cells through the Major Histocompatibility Complex (MHC). MHC can be divided in two main subclasses (I and II) and shows one of the highest genetic variability of our genome, in order to load on their extracellular part specific antigens that will be recognized by membrane receptors of the adaptive immune cells, the T and B lymphocytes and natural killer (NK) cells. In this manuscript we will focus on the role of **T lymphocytes**, and how their interaction with APCs is important to modulate the immune response of an organism.

T lymphocytes that have completed their maturation process, briefly described in the second chapter of this thesis, are called naïve or resting T cells ( $T_N$ ). Upon encounters and recognition of antigens presented by APCs by their T cell receptor (TCR), T lymphocytes get activated and they can clonally expand to efficiently respond to the infection becoming effector cells ( $T_{EF}$ ). Mature T lymphocytes can be mainly divided in two classes: the one expressing the CD4 receptor and the one expressing the CD8 receptor. When CD8+ cells get activated they differentiate in cytotoxic T lymphocytes (CTL) that together with the cells of the innate system and NK cells can act in destroying the non-self invaders, by inducing their death. On the other side, CD4+ cells, once activated, will differentiate in one of the subclasses of “helper” population. Multiple classes of effector cells exist and their differentiation into one or the other subtype depends on intrinsic and extrinsic

factors such as type and strength of initial TCR activation, cytokines present in the stroma and the presence of different TCR co-receptors (immune checkpoint receptors) which act in modulating T cell fate. The main effector cell categories known so far are T helper 1, 2, 9, 17 (respectively called Th1, Th2, Th9 and Th17), the T helper follicular cells (Tfh) and the regulatory T cells (Treg) of which this last presents suppressive functions against other T helpers in order to avoid an excessive reaction of the immune system to any type of injury.

Moreover, T lymphocytes can mutually downregulate themselves by induction of programmed cell death (PCD) at the end of the immune response, a mechanism known as T cell contraction. Furthermore, after the shutdown of the immune response, T lymphocytes can further differentiate into cells that keep the recognition pattern of the external agent that they were in contact with, to be able to respond rapidly to a second attack, the so called memory cells (further divided into effector and central memory cells T<sub>EM</sub> and T<sub>CM</sub>), that circulate in the bloodstream as prepared and equipped guardians. These cells are, however, just a small fraction of the pool.

One of the main roles of helper T cells is, therefore, to « help » other T cells and B lymphocytes, the other main lymphocyte subclass, to get properly activated and expand. Once they get activated, by their B cell receptor (BCR) in concert with other co-receptors, B lymphocytes can differentiate into plasma cells, a population that is able to create, expose and secrete antibodies, main actors of the humoral response. One important feature of the B lymphocytes is that, once activated, they can act as APCs, amplifying the propagation of the signal activating in a feedback loop other T lymphocyte.

Both deficiency or exacerbation of the immune response are cause of insurgence of severe diseases, ranging from immunodeficiency-related pathologies, when patients cannot properly activate the immune system upon infections, to autoimmune diseases when the immune response cannot be shut down.

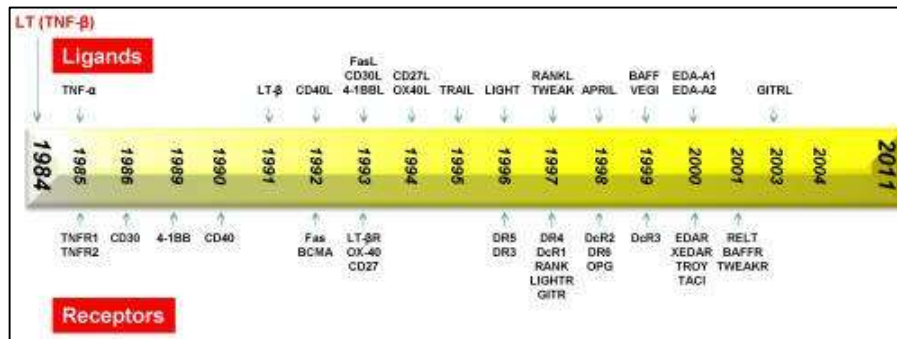
Fas and FasL proteins are components of the Tumor Necrosis Factor Receptor/Tumor Necrosis Factor Superfamilies (TNFRSF/TNFSF) respectively. The Fas/FasL couple plays a fundamental role in the homeostasis of the immune cells. Fas/FasL interaction is actually one of the main mediators of the peripheral tolerance in the elimination of autoreactive T cells, and their deficiency is the main cause of the appearance of autoimmune disorders such as autoimmune lymphoproliferative diseases (ALPS) and systemic lupus erythematosus (SLE), pathologies characterized by the presence of excessive number of lymphocytes and production of autoantibodies. This subject will be deeply faced in the section of the first chapter dedicated to the role of Fas in physiopathology. Fas/FasL interaction is, furthermore, mediator of multiple pro-survival pathways, which are strictly context and cell type dependent. As for other members of TNFR superfamily, a role for Fas in the modulation of the T lymphocyte activation has been established, but nor its importance and neither its features as well as the molecular mechanism have never been elucidated so far. It is thus, of an extremely interest to go deeper into this field and try to define how these players influence T cell fate.

## A. Fas/FasL system: structure, functions and signaling

### 1. TNF/TNFR superfamilies

#### 1.1 Generalities

More than thirty years ago some scientists started to suspect that specific proteins secreted by immune cells could have a critical role in some human diseases such as tumor development, inflammations, autoimmunity and pathogen infection (Berke et al 1972, Erard et al 1984, Decker et al 1987, Goeddel et al 1986). In a tumoral context it was for instance observed that factors expressed by activated macrophages and lymphocytes were able to induce tumor rejection by necrotic phenotype (Granger et al 1969, Carswell et al 1975). Their identification led to the cloning, in 1984, of two proteins respectively named the tumor necrosis factor (TNF) and the lymphotoxin (LT $\alpha$ ) (Pennica et al, Grey et al 1984). These two factors, that shared 50% of homology at the protein level, were the first two members of a superfamily that count nowadays 19 members (see list in Table 1) and that was named tumor necrosis factors superfamily (TNFSF)(Aggarwal 2003).



**Fig.1 Timeline of the discovery of various members of the TNF superfamily and their receptors.**

Aggarwall et al 2012.

One year later, the identification of the receptors that could bind to these two polypeptides was done by Aggarwal's team and gave rise to the identification of the tumor necrosis factor receptor (TNFR) superfamily (TNFRSF)(Aggarwal et al 1985) that comprises currently 29 members (Aggarwal 2003) (Table 1). The figure 1 represents the timeline of the discovery of several members of TNFSF and TNFRSF. The receptor/ligand couples possess unique structural attributes that induce diverse signaling outcomes ranging from death to cell proliferation, survival, and differentiation (Alderson et al 1993, Aggarwal et al 1995, Klebanoff et al 2016). It is therefore not surprising that they play a fundamental role in a plethora of physiologic and pathologic processes from development and organogenesis to tumorigenesis and chronic diseases (Desbarats and Newell 2000, Kleber et al 2008, Straus et al 1999, Fisher et al 1995, Rieux-Laucat et al 1995). The notion that both ligands and receptors can bind multiple partners renders this network quite complex and with a high level of regulation.

Cytokine	Cells	Receptor	Cells
LT $\alpha$	NK, T and B cells	TNFR1 TNFR2	Most normal and transformed cells Endothelial cells and immune cells
TNF	Macrophages, NK, T and B cells	TNFR1, TNFR2	See above
LT $\beta$	DCs, macrophages, T, B and NK cells	LT $\beta$ R	NK cells, CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells
CD95L	Activated splenocytes, thymocytes and non-lymphoid tissues, such as the eye and testis	CD95	Most normal and transformed cells
		DCR3	Lung and colon cells
TRAIL	NK cells, T cells and DCs	DR4, DR5 DCR1, DCR2, OPG	Most normal and transformed cells Most normal and transformed cells
TWEAK	Monocytes	FN14	Endothelial cells and fibroblasts
CD27L	NK, T and B cells	CD27	CD4 <sup>+</sup> and CD8 <sup>+</sup> T cells
CD30L	T cells and monocytes	CD30	Reed–Sternberg cells
CD40L	T and B cells	CD40	Reed–Sternberg cells
4-1BBL	B cells, DCs and macrophages	4-1BB	Activated T cells, monocytes and NK cells
OX40L	T and B cells	OX40	T cells
APRIL	Macrophages, lymphoid cells and tumour cells	BCMA TACI	B cells, PBLs, spleen, thymus, lymph nodes, liver and adrenals B cells, PBLs, activated T cells, spleen, thymus and small intestine
BAFF	T cells, DCs, monocytes and macrophages	TACI BCMA BAFFR	See above See above B cells, PBLs, resting T cells, spleen and lymph nodes
LIGHT*	T cells, granulocytes, monocytes and DCs	HVEM LT $\beta$ R	T cells Non-lymphoid haematopoietic cells and stromal cells
VEGI*	Endothelial cells	DR3, DCR3	Activated T cells
GITRL	N. D.	GITR	CD4 <sup>+</sup> CD25 <sup>+</sup> T cells
RANKL	Activated T cells and osteoblasts	RANK OPG	Osteoclasts, osteoblasts and activated T cells Osteoclast precursors, endothelial cells and others
EDA1	Skin	EDAR	Ectodermal derivative
EDA2	Skin	XEDAR	Ectodermal derivative
N. D.		DR6	Resting T cells
N. D.		RELT	Lymphoid tissues
N. D.		TROY	Embryo skin, epithelium, hair follicles and brain

**Table 1: Cellular expression of ligands and receptors of the tumor-necrosis factor superfamily. Aggarwall et al 2003.**

## 1.2 TNFs

Despite several differences in their conformation, in their roles and in their evolutionary conservation among the species, all members of the TNFSF share structural common features.

The TNFSF members are type II transmembrane proteins. They exist under different forms that were all described as active despite that they differ in their physiologic role: a membrane-bound form and soluble forms (Schneider et al 1998, Holler et al 2002, O'Reilly et al 2009). These latter can be secreted either as full-length proteins or as proteolytically shed extracellular fragments generated upon cleavage by metalloproteases (Gearing et al 1994).

Even if TNFSF proteins exist also as monomers their proactive forms require homo or hetero trimerization (Pennica et al 1984).

The most conserved region among the TNFSF members (25-30% of homology) resides in the extracellular part that is involved in the trimer assembly and that was therefore called TNF homology domain (THD) (Cha et al 1999, Jones et al 1989, Schneider et al 1998). Each monomer adopts a “jelly roll” conformation organized in beta sheets that form the THD. This structure allows a naturally occurring trimer formation (Bodmer et al

2000, Eck and Sprang 1989). In contrast, the domain which shows the lowest similarity in amino acid sequence is the C-terminus extracellular part (Reviewed in Locksley et al 2001).

Six members of TNFSF contain in their cytoplasmic region a casein kinase I substrate (CKI) domain that has been described to be involved in reverse signaling giving to these ligands a role of receptors that could transmit pro-survival signals involved in multiple cellular context, such as T lymphocyte activation and induction of gene expression (Smith et al 1994, Suzuki et al 1998, Watts et al 2005, Eissner et al 2004).

### **1.3 TNFRs**

On the other side, the TNFRs are type I transmembrane proteins. The extracellular portion is characterized by the presence of 6 or more cysteines which form disulfide bonds and define the cysteine-rich-domains (CRDs), a structural common feature among all the members of this superfamily. The number of CRDs varies among the different TNFRs (from 1 to 6) and plays a fundamental role for receptor-ligand interaction, as will be described later (Ashkenazi 2002).

The TNFRSF receptors cluster on the cell surface independently of the presence of their ligand. Their trimerization is mediated by their pre-ligand assembly domains (PLADs) formed by the N-terminal, the CRD1 and part of the CRD2 (Chan et al 2000, Clancy et al 2005, Siegel et al 2000). Conversely, the binding to the ligand was suggested to be mediated by a conformational change that occurs when receptors trimerize (Locksley et al 2001, Scott et al 2009).

These receptors can be furthermore divided in 3 categories according to the nature of the domain present in their cytoplasmic part: the death receptors, the non-death receptors and the decoy receptors. A schema comprising the main TNFRs/TNFs interaction and their cellular localization can be found in figure 2. The death receptors (DR) harbor a region called death domain (DD) (see the definition below). The ones that do not contain this domain are instead defined by the presence of a TRAF-interacting motif (TIM) (non-DR). In addition, the members that do not have any cytoplasmic domain cannot signal directly although they can compete with the signaling members and they have therefore been called decoy receptors (see below) (Ashkenazi 2002, Kwon et al 1998).

### **1.3.1 Death Receptors**

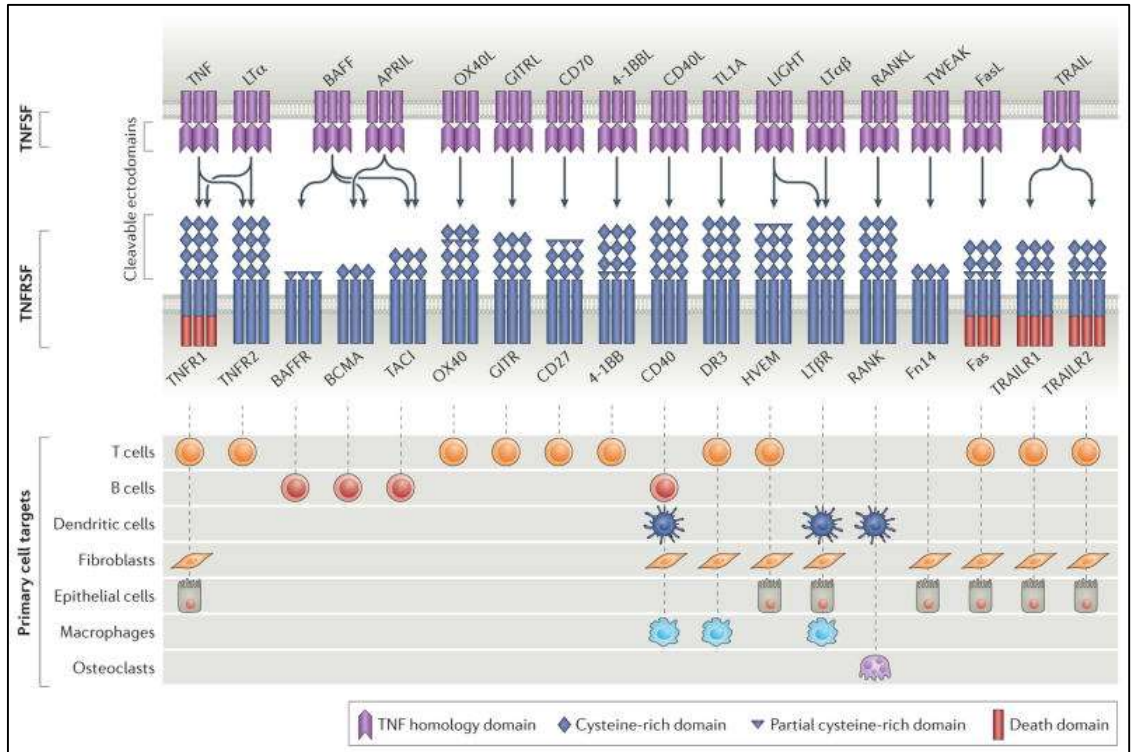
Members of this subclass of TNFR are TNFR1, Fas, TRAIL-R1, TRAIL-R2, DR6, NGFR and EDAR (Wajant 2003). The DD is a region of around 80 amino acids, composed of 6 alpha helix bundle structure (Park et al 2007, Kohl and Grutter 2004). This domain constitutes a scaffold for DD-containing adaptor proteins upon engagement of the ligand. This homotypic DD-DD interaction (described below at page 33) initiates different molecular pathways that, depending on partners, cellular context, membrane localization or receptor's post-translational modifications lead to cell death or survival signaling (Chakrabandhu et al 2016).

### **1.3.2 Non-Death Receptors**

Non-DRs are characterized by the presence of a motif which binds other classes of adaptor proteins belonging to the TNF receptor associated factors (TRAF) family. TRAFs, through adaptor and ubiquitin ligase activity (Wallach et al 1999), can initiate the activation of Nuclear factor kB (NF- $\kappa$ B), Jun N-terminal kinase (JNK), Extracellular signal-regulated kinase (ERK) and Phosphoinositide-3 kinase (PI3K) (Dempsey et al 2003). Even though, this concept has been reevaluated because again, depending of multiple factors, these TNFRs are able also to induce cell death, as, on the other side, the DRs can mediate non-death signals (Aktas et al 2006, Guicciardi et al 2009).

### **1.3.3 Decoy Receptors**

As mentioned above, these receptors do not contain any functional intracellular motif, but through the binding to the TNFSF ligands they can compete with the other TNFRs, acting as negative regulators of the signaling. Four members of this subcategory have been identified so far: DcR1 (TRID/TRAIL-R3), DcR2 (TRUNDD/TRAIL-R4), DcR3 and Osteoprogenin (OPG) (Ashkenazi et al 2002).



**Fig.2 Schema of some DR and non-DR of TNF/TNFR superfamilies and their tissue localization.**

**Croft and Siegel 2017.**

### 1.4 Tissue repartition

Concerning their tissue expression, TNFR members have quite ubiquitous localization, with higher level in thymus, liver, heart and kidney. Conversely, the members of the TNFSF are mostly restricted to immune cells such as activated lymphocytes, monocytes, dendritic cells and natural killer (NK) cells (Aggarwall et al 2003) (Fig.2). Additionally, they are expressed in tissues defined as immunoprivileged sites, such as eyes and testis, where their expression avoids unwanted immune response (Griffith et al 1996). Variation of the expression level of some TNF/TNFR members can be also found in a tumoral context, a feature that will be discussed later in this manuscript (page 26).



## 2. Fas/FasL proteins

Fas receptor was discovered by Yonehara and coworkers in 1989. They found that another receptor was able to bind an antibody originally directed against TNFR1 and to mediate cell apoptosis (Yonehara et al 1989).

By molecular and biochemical approaches, the team of Nagata and the one of Krammer respectively, were able to clone the Fas protein (Inazawa et al 1992, Lichter et al 1992). Its specific ligand, called Fas ligand (FasL), was cloned the year after by the team of Suda where they demonstrated its belonging to TNFSF (Suda et al 1993). This discovery initiated a new field, the one of cell death by “apoptosis” (see page 31). Since that moment hundreds of papers came out, by defining partners and way of action of this interaction.

In the following years, accumulation of molecular informations modified the paradigm of the role of Fas/FasL interaction, initially restricted to cell death induction, and comprising instead nowadays a large number of cell mechanisms in physiology and diseases, including non-apoptotic roles such as cell proliferation, differentiation and cell survival (Alderson et al 1993, Klebanoff et al 2016).

### 2.1 Fas protein structure

Human Fas receptor, also known as CD95/TNFRSF6/APO-1, is one DR of the TNFRSF. It is a type I transmembrane protein of 335 amino acids with a molecular weight that ranges from 42 to 56 kD, depending on its differential level of glycosylation. At the genomic level, the *FAS* gene is located at the 10th chromosome (10q23) and consists of 9 exons (Inazawa et al 1992, Yan et al 2005) that give rise to at least 6 different protein isoforms created by alternative splicing. These isoforms, being soluble truncated forms, might act as Fas full-length negative regulators or play unidentified roles (Papoff et al 1996).

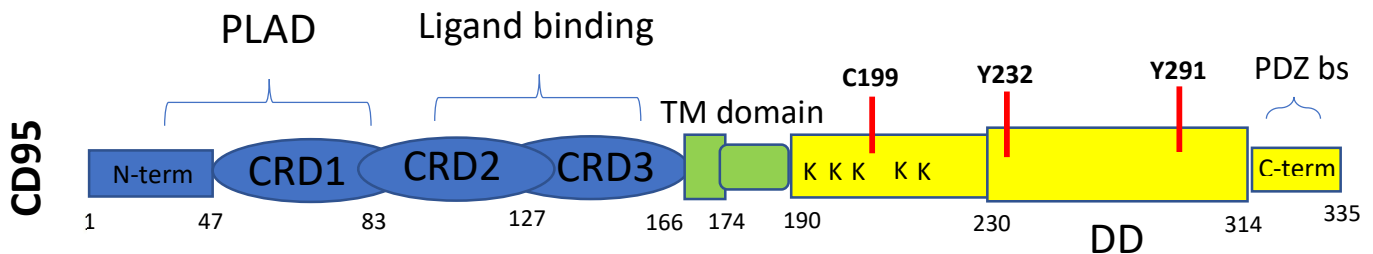


Fig.3 Human Fas structure.

High homology level with Fas protein was found for the soluble decoy receptor DcR3, which lacks the transmembrane domain and that can compete for the ligand binding with Fas protein (Pitti et al 1998).

Fas protein, schematized in Fig.3, is composed by:

- N-terminus extracellular part (1-173), containing:
  - A) the PLAD, by which the pre-associated homotrimer can form from homotypic interaction.
  - B) three CRDs critical for the interaction with the ligand (see page 29 for more detailed explanations).
  - C) a glycosphingolipid binding motif (GBM) that plays an important role in Fas membrane localization and clathrin-dependent internalization (Chakrabandhu et al 2008).
- One transmembrane domain (174-190) that can form also stable homotrimer in the lipid bilayer. At the position 183 a proline is present to allow sufficient flexibility for the accommodation of the hydrophobic core (Fu et al 2016).
- C-terminus cytoplasmic domain (191-335), which includes:
  - A) in its most membrane proximal part a lysine rich region (LRR) important for lipid raft localization, together with the cysteine present at position 199.
  - B) From position 202 starts a 68 amino acid DD, fundamental for the binding of the adaptor protein FADD and the initiation of the apoptotic signaling. Fas DD-FADD interaction models will be described in the section dedicated to the apoptotic signal. Interestingly the DD contains two different tyrosines at the position 232 and 291 subjected to phosphorylation by the Src kinase family members (Chakrabandhu et al 2016).
  - C) Finally, a PDZ binding site (SLV) at the extreme C-terminus end, important for protein stability and interaction with other partners, mainly PDZ-containing proteins (Gagnoux-Palacios et al 2018).

Beside the glycosylation, Fas protein is subjected to different kind of post translational modifications (PTMs), such as phosphorylation, palmitoylation, nitrosylation and glutathionylation which modulate its stability, cellular localization and functions (reviewed by Seyrek-Lavrik 2019). The role of Fas post translational modifications will be developed in the section concerning Fas signal regulation (pages 42 and 45).

As all members of TNFRSF, Fas exists as monomer but it has to trimerize in order to accomplish its function. We have already mentioned the presence of a PLAD in the extreme N-terminus of Fas (Papoff et al 1999) that is essential for its ability to self-associate independently of its ligand binding (Siegel et al 2000, Chan et al 2000). Recently, Fu *et al* defined also the importance of the TM domain for transduction of optimal intracellular signal, by nuclear magnetic resonance (NMR) technique, that resides in the presence of prolines which allow a proper steric conformation of the liganded trimers (Fu et al 2016).

At cellular level Fas is mostly localized at the plasma membrane, but it can internalize and so it can be found also in the membrane of endosomes or of other intracellular compartments (Reviewed by Tchikov et al 2011).

1-50	C-term	<b>MLGIWTLPL</b>	<b>VLTSVARLSS</b>	<b>KSVNAQVTDI</b>	<b>NSKGLELRKT</b>	<b>VTTVETQNL</b>
		MLWIWAVLPL	VLAG----SQ	LRVHTQGTNS	ISESLKLRRR	VRETDKNCSE
51-100		<b>GLHHDGQFCH</b>	<b>KPCPPGERKA</b>	<b>RDCTVNGDEP</b>	<b>DCVPCQEGKE</b>	<b>YTDKAHFSSK</b>
		GLYQGGPFCC	QPCQPGKKV	EDCKMNGGTP	TCAPCTEGKE	YMDKNHYADK
101-150		<b>CRRRCRLDEG</b>	<b>HGLEVEINCT</b>	<b>RTQNTKCRCK</b>	<b>PNFFCNSTVC</b>	<b>EHCDPCTKCE</b>
		CRRCTLCDEE	HGLEVEINCT	LTQNTKCRCK	PDFYCDSPGC	EHCVRCASCE
151-200		<b>HGIIECTLT</b>	<b>SNTKCKEEGS</b>	<b>RSNLGWLCLL</b>	<b>LLPIPLIVVW</b>	<b>KRKEVQKTCR</b>
		HGTLEPCTAT	SNTNCRKQSP	RNR LWLLTIL	VLLIPLVFIY	RKYRKRKCWK
201-250		<b>KHRKENQGSH</b>	<b>ESPTLNPETV</b>	<b>AINLSDVDLS</b>	<b>KYITTIAGVM</b>	<b>TLSQVKGfVR</b>
		RRQDDP----	ESRTSSRETI	PMNASNLSLS	KYIPRIAEDM	TIQEAKKFAR
251-300		<b>KNGVNEAKID</b>	<b>EIKNDNVQDT</b>	<b>AEQKVQLLRN</b>	<b>WHQLHGKKEA</b>	<b>YDTLIKDLKK</b>
		ENNIKEGKID	EIMHDSIQDT	AEQKVQLLLC	WYQSHGKSDA	YQDLIKGLKK
301-335		<b>ANLCTLAEKI</b>	<b>QTIILKDITS</b>	<b>DSSENSFRNE</b>	<b>IQSLV</b>	<b>N-term</b>
		AECRRTLDFE	QDMVQKDLGK	STPDTGNEENE	GQCLE	
<b>HUMAN</b>		<b>MOUSE</b>	<b>DOMAIN DIVISION</b>			

**Fig.4 Comparison of human/mouse aa sequence. In bold the common aa between the species.**

The molecular mechanisms underlying Fas internalization will be further described later in this manuscript (pag 43).

In mouse, the *FAS* gene is located at the distal part of the chromosome 19 and it contains 13 exons. Mouse genome does not contain a sequence for Dcr3, which is conserved instead just in primates. Mouse Fas is a protein of 327 amino acids. Despite the conservation of the function of apoptosis inducer of this protein among vertebrates, their amino acid sequences differ (Fig.4). Murine homologue of Fas protein is indeed quite divergent from the human one in term of primary sequence. These differences are mostly localized in the cytoplasmic region rather than in the extracellular one. Indeed, murine Fas is able to bind with human FasL. Therefore, data obtained with murine Fas have to take into account these differences before transposing the results on humans, considering that the signaling regulation can variate in a consistent way.

Despite the primary sequence divergence, most of the functional and structural informations given for the human Fas are also present in mouse Fas, from the post translational modifications (to be adapted at the species-specific amino acid sequence), the necessity of trimerization to be able to signalize, the ubiquitous expression in all the tissues of the organism, to the presence of the three regions divided in: N-term extracellular domain (1-169), one transmembrane domain (170-186) and a C-term cytoplasmic tail (187-327). Interestingly the PDZ-binding site at C-terminus expressed in human Fas is not found in mouse.

## 2.2 FasL protein structure

Fas ligand (FasL/ APO-1L/ CD95L/CD178/TNFSF6) is a cytokine of the TNFSF and the only known ligand for Fas. Conversely, FasL can bind also to DcR3. It is a type II transmembrane protein of 281 amino acids with a molecular weight that ranges from 38 to 42kD depending on its glycosylation status.

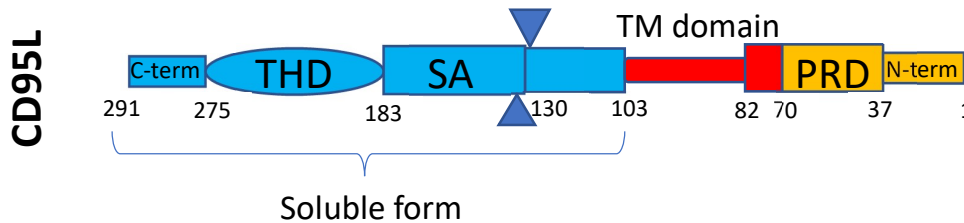


Fig.5 Human FasL structure.

FasL exists in 2 main forms: as a membrane-bound protein, and as a soluble protein. Furthermore, the membrane-bound form can be expressed at the plasma membrane or in membrane of exosomes that can be released by the cells in the stroma, a process that occurs in NK and CTL cells during their cytotoxic action (Wasem et al 2001, Bossi et al 1999, Blott et al 2001). The soluble form, on the other side, can be a full-length secreted protein or a cleaved one lacking its intracellular domain (Schulte et al 2007, Kirkin et al 2007).

As the other members of TNFSF, FasL is subjected to ectodomain shedding. This process is mediated by both matrix metalloproteases, such as MMP7, and disintegrins of the ADAM family. ADAM 10 was identified as the main FasL sheddase in T cells (Schulte et al 2007, Kirkin et al 2007) that releases in the stroma a 26-29kD form (Schulte et al 2007). The functional meaning of this cleavage was initially attributed to a down-modulation of the apoptotic signal since the soluble form of FasL resulted to be unable to induce apoptosis. Lines of evidence supporting functional role of the cleaved form of FasL are now several and attributed mostly to induction of non-apoptotic signals. Examples of these evidence will be found in this manuscript especially in the section dedicated to the role of Fas and FasL in pro-survival signaling (from page 36). The cleavage can be constitutive (Huovila et al 2005) or induced by several stimuli ranging from variation of Ca<sup>2+</sup> level (Endres et al 2003, Nagano et al 2004), activation of growth factors (Fisher et al 2003), and even binding of ligands to receptors (Janes et al 2005). ADAM10 cleavage was found to be coupled to intracellular shedding, mediated by the signal peptide peptidase-like 2a (SPPL2A), which generates a free intracellular FasL form that can translocate into the nucleus and modulate the expression of target genes (Kirkin et al 2007). Furthermore, this mechanism generates a reverse signaling that was found to be involved in the down-modulation of the immune response (Luckerath et al 2011).

At the genomic level, *FASLG* resides in chromosome 1 (1q24.3) and consists in 4 exons, and only another isoform of the protein, created by alternative splicing, has been identified.

FasL, as all the members of TNFSF, acts as a trimer in order to interact with its binding partner. FasL monomer, schematized in Fig.5, consists of:

- N-terminus intracellular domain (1-80), the longest among the TNFSF members (Takahashi et al 1994) containing:
  - A) a proline rich domain (PRD), a protein-protein interaction motif for SH3 or WW bearing proteins, important for secretory lysosome storage and trafficking to the plasma membrane (Blott et al 2001).
  - B) a CKI-S described to be necessary for FasL reverse signaling and regulation of gene expression (Luckerath et al 2011).
- One transmembrane domain (81-102) with SPPL2A cleavage site at position 81-82
- C-terminus extracellular domain (103-281) containing:
  - A) the THD, conserved among the members of TNFSF, necessary for binding to CRDs of Fas
  - B) a self-assembly (SA) motif, necessary for ligand trimerization together with the THD.

The site for ADAM10 cleavage for the production of the soluble form is located at the position 129-130.

Other PTMs identified for FasL are the phosphorylation on tyrosine (Zuccato et al 2007), palmitoylation and monoubiquitination, important for FasL localization at membrane level and for internalization (Cahuzac et al 2006 and Reviewed in Voss et al 2008).

At cellular level FasL is a membrane-bound protein, but its localization and level of expression are finely regulated. Because its constitutive expression would cause inappropriate cell death in Fas positive cells, it can be stored in secretory lysosome by immune cells, such as cytotoxic T lymphocytes and Natural killer cells, until they receive the signal that allows its membrane exposure or secretion (Wasem et al. 2001, Bossi and Griffith 1999, Blott et al 2001). FasL expression regulation in immune system will be described below at page 13 in this manuscript.

Differently from its receptor counterpart, FasL is extremely conserved compared to its mouse homologue, sharing more than the 77% of primary sequence (Nagata et al 1997), and consequently most of the structural and functional characteristics. Palmitoylation represents an exception, which is absent in murine FasL. The mouse protein consists of 279 amino acids. Human and mouse FasL have been considered as interchangeable polypeptides. Its gene locus resides also in mouse at the chromosome 1 and is divided in 4 exons.

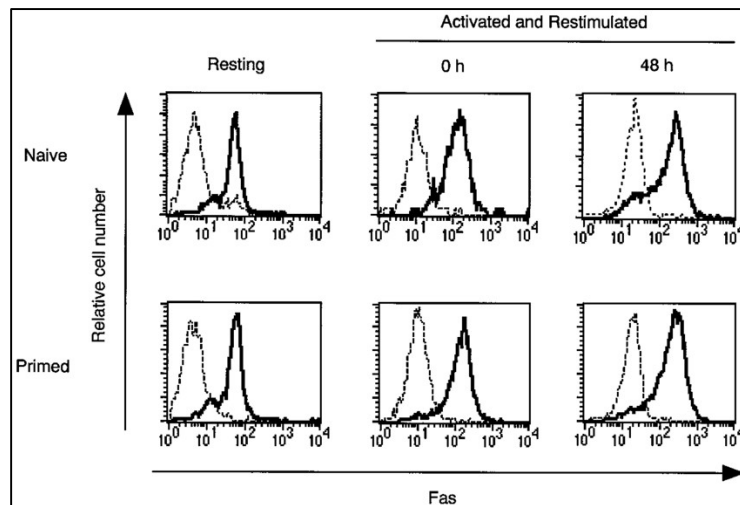
### 3. Fas/FasL in physiopathology

#### 3.1 Role of Fas and FasL in immune system

##### 3.1.1 Regulation of Fas/FasL expression in immune cells

Even if Fas expression among the tissues is mostly ubiquitous, it is well established that it plays a central role in the immune system, mainly by regulating lymphocytes homeostasis, via its expression on T cells, B cells and APCs in general.

It is expressed in all the immune cell types, even though its expression level is finely regulated among the different subpopulations to guarantee an efficient response to the FasL engagement at the precise timepoint.



**Fig.6 Fas expression in naïve and activated T cells. Inaba et al 1999.**

It has been described that human naïve T and B lymphocytes that have never been activated show extremely low level of Fas and are insensitive to FasL induced apoptosis (Fig.6). Interestingly, it is still controversial if these basal levels are anyway able to initiate a non-death signal, as the T cell co-stimulation, but this subject will be further discussed later. When T cells get activated upon the recognition of the antigens, they start to upregulate Fas (Trauth et al 1989), thus becoming sensitive to apoptosis, in order to shut down in a proper way the immune response and avoid an inappropriate response that can be detrimental for body homeostasis. Even though, the involvement of Fas in the elimination of the T lymphocytes is still controversial and will be faced in the section 3.1.5. Even the dendritic cells (DC) follow the same trend. Once they have been recognized by T cells, they start to upregulate Fas to become sensitive to apoptosis. This is a mechanism to avoid T cell over-activation by modulating the activated APCs circulation.

Expression of FasL is even more tightly regulated. We have already mentioned that FasL expression is restricted to few cell populations, such as the activated lymphocytes and neutrophils or other antigen

presenting cells. It is upregulated in T cells upon activation but its form and cellular localization are critical to regulate its activity. In cells bearing secretory lysosomes (NK and CTL) FasL is retained in these organelles until they receive a signal to release them at the plasma membrane, making them able to kill Fas bearing cells, in a mechanism known as « kiss of the death » (Bossi and Griffith 1999, Lowin et al 1996). The explanation of this process will be further discussed below in this chapter.

### 3.1.2 CTL and NK use FasL to kill

NK and CTL are immune cells that have an extremely important complementary role in the innate and adaptive immune system respectively. They have the capacity to recognize and eliminate virus infected cells and tumoral cells.

CTL and NK use the same systems to kill their target: the perforin (pore forming protein) /granzyme (serine proteases) and TNF/TNFR system where FasL mediated cell death is broadly exploited. These cells, as mentioned above, contain in their cytoplasm secretory lysosomes where FasL protein and perforins/granzymes are stored (Wasem et al 2001, Bossi and Griffith 1999, Blott et al 2001). Whether FasL and perforin/granzyme share the same granules and are released upon the same stimuli remains a matter of debate (review of Lettau 2015).

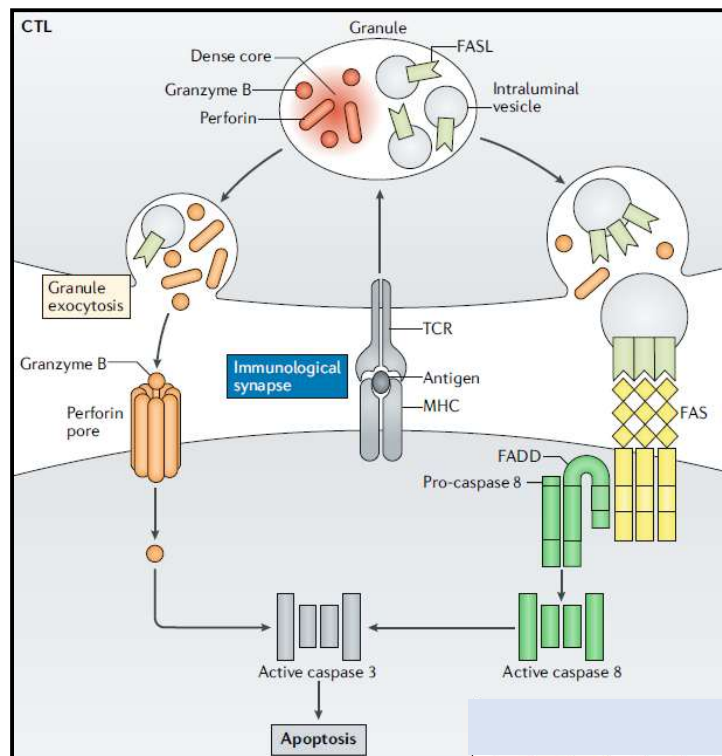


Fig.7 Mechanism of CTL mediated cytotoxicity. Adapted from Golstein and Griffiths 2018.

In response to non-self Ag recognition (for NK cells) or T cell restimulation (for CTL) (He et al 2007) the secretory lysosomes move from the cytoplasm to the cell membrane and release in the extracellular

environment the lytic agents by a process known as degranulation or expose at the plasma membrane the FasL (Fig.7). Interestingly, the FasL that is transported at the cell surface upon restimulation is mostly the preformed protein contained in the secretory lysosome more than the *de novo* synthesized, since the inhibition of new transcription by cyclosporin A after restimulation minimally affect the exposure of FasL at the membrane (Wasem et al 2001). Once exposed on the plasma membrane FasL can mediate its apoptotic function by localizing in the lipid raft (Nachbur et al 2006, Cahuzac et al 2006) or can be cleaved by MMPs (Mariani et al 1995) which enhances FasL mediated inflammation.

Another compartment of FasL storage in these type of cells are the multivesicular bodies (MVB) that can be directly targeted on the immune synapse upon T cell restimulation and allow the release of FasL-bearing exosomes, together with other proteins involved in T cell activation, directly at the contact zone with the target cell (Blanchard et al 2002).

### **3.1.3 *Lpr* and *gld* mice models in the discovery of Fas/FasL as mediators of T cell homeostasis**

The discovery by Andrews *et al* in 1978 of a naturally occurring mouse of the MLR strain developing lymphadenopathy and splenomegaly opened the gates in the description of Fas role in the immune system (Andrews et al 1978). This mouse was called *lpr* for lymphoproliferation and a second mutant with a similar phenotype *gld* for generalized lymphoproliferative disease was then discovered (Roths JB 1984). Indeed, mutation in the *FAS* or *FASLG* gene were identified as the origin of the systemic lupus erythematosus (SLE), phenotype observed in *lpr* and *gld* mice respectively (Ramsdell et al 1994b, Watanabe-Fukunaga et al 1992a). Hallmarks of these mutations are the accumulation of normal CD4+ and CD8+ lymphocytes and of the unusual double negative T lymphocyte population (CD4-/CD8-/B220+), that are causing the lymphadenopathy and splenomegaly. They also show autoimmune features, such as the presence of autoreactive T cells, amplification of B cell activation and proliferation, as well as the presence of a high content of autoantibodies of the families IgG and IgM. All these dysregulations lead to severe glomerulonephritis and arthritis symptoms that finally provoke death of the mice few months after their birth. It was later proved that the severity of the phenotype was strictly dependent on the mouse genetic background (Nagata et al 1995).

Adachi *et al* in 1993 identified that the cause of the extremely low level of Fas mRNA and protein (leaky mutation) was due to the insertion of a transposable element in the intron 2 of the *FAS* locus that was creating a truncated and loss of function protein (Adachi et al 1993). Another Fas mutation was identified in another strain called *lpr*<sup>CE</sup>. It consists of a single point mutation (T to A) inside *FAS* exon 9 (just after recognized as coding for the death domain) thus creating a full-length protein without its apoptotic capacity. The *gld* strain, on the other side, was caused by a point mutation, T to C, of the *FASLG* gene in the external region of the translated protein (C-term) making it unable to bind the Fas receptor.



It was therefore clear that the Fas/FasL system was playing a fundamental role in maintaining the homeostasis of immune cells and in controlling autoimmunity. Even though, *lpr* and *gld* mice did not show apparent defects in the thymocyte positive and negative selection (Singer and Abbas 1994, Sidman et al 1992). Instead, the role of Fas in preventing autoimmunity insurgence was addressed to the peripheral tolerance, in the clonal deletion of autoreactive T cells mediated by APCs (Vignaux et al 1994).

#### **3.1.4 Autoimmune lymphoproliferative disease: the human deficiency of Fas/FasL system**

The crucial role of the Fas/FasL couple in a human context was due to the discovery that patients lacking a functional Fas receptor developed a chronic non-malignant autoimmune lymphoproliferative syndrome (ALPS) (Rieux-Laucat et al 1995). It is also known as Canale and Smith syndrome referring to the names of the two scientists that initially described it in 1967 (Canale and Smith 1967). The most frequent symptoms that appear (usually before the age of 5) in this rare genetic disease are a benign lymphoproliferation through the accumulation of double negative TCR $\alpha\beta$  lymphocyte (DNT) in the lymphoid organs that lead to spleen and lymph nodes enlargement. These DNT lymphocytes have some defined features, such as the presence of the MHC II, high level of CD28 protein, the presence of the markers CD57, associated with senescent cells and, on the other side, of the naïve cell marker CD45RA (Rensing-Ehl et al 2014 and reviewed by Rieux-Laucat et al 2018). These features led to the hypothesis that this population might have arisen from abnormal differentiation and accumulation of CD4+ and CD8+ single positive cells in absence of Fas (Rensing-Ehl et al 2014, Volkl et al 2016). In two third of the patients, autoimmunity features are found with a high level in the serum of FasL, IL-10 and B123 vitamin and hyper immunoglobulinemia G and A. These manifestations could be the cause of c hepatitis, glomerulonephritis and dermatitis (Fisher et al 1995). In addition, ALPS patients are more prone in the onset of certain type of cancer such as Hodgkins and non Hodgkins lymphomas (Straus et al 2001).

The identification of mutations in other genes than in *FAS* in ALPS patients allow the classification of this disease in different subgroups:

- **The ALPS-0 and Ia subgroups** (ALPS-FAS) describe the ALPS presenting respectively recessive and dominant inherited mutations of the *FAS* gene. The ALPS Ia form found in 70% of the patients is the most common one (Straus et al 1999). The Fas translated protein, unable to trimerize, fails to induce the apoptotic cascade. In ALPS type 0 a form of recessive mutation of Fas is caused by a large deletion on the last exon (Rieux-Laucat et al 1995). This latest type of mutation is, furthermore, prone to become homozygous by loss of heterozygosity (LOH) and thus can cause the development of severe symptoms. The insurgence of symptoms in heterozygous siblings with healthy heterozygous parents enhanced the point that Fas mutations could be both dominant or recessive and that the incomplete penetrance of the mutation was interfering with the onset of the disease

(Bettinardi et al 1997). The discovery that ALPS insurgence could have arisen from somatic mutation in the hematopoietic progenitors, detectable in DNT lymphocytes, increased the level of complexity of this pathology (ALPS-sFAS Im). Indeed, even if in a small proportion compared to the wild type T cells, these apoptotic resistant mutants could have caused the manifestation of the symptoms for their selective advantages. Up to now, somatic ALPS represent the 15% of the cases (Dowdell et al 2010).

- **The ALPS-Ib group** (ALPS-FASLG) contains the mutations in the gene encoding Fas-ligand. Nowadays there are 8 cases of this rare form of the disease and only one is a dominant mutation, which however does not reflect totally the criteria of classical ALPS (low DNT accumulation and no splenomegaly) (Del-Rey et al 2006, Nabhani et al 2014).
- **The ALPS-II group** involves patients that bear mutations within Fas apoptotic cascade such as in Caspase-8 (-IIb) and 10 (-IIa) (Wang et al 1999, Salmena et al 2005). The latest, however, is often associated with Fas mutation in order to be symptomatic (Cerutti et al 2007, Martinez-Feito et al 2016). Moreover, Caspase-8 deficiency was not reflecting totally the ALPS features, since was leading to combined immune deficiency with defect in lymphocyte proliferation and recurrent bacterial and viral infections. Homozygous missense mutation of FADD, described to lead to an autosomal recessive inherited disorder, was identified to show biological features of ALPS with different clinical features (no lymphoproliferation and autoimmunity) such as predisposition to bacterial and viral infections (Bolze et al 2010).
- **The ALPS-III group** (ALPS-U) collect all the cases that show clinical phenotype close to ALPS but for which the genetic defects were not yet defined. It was suggested anyway that might be associated in some cases with reduced CTLA-4 expression at the cell surface of activated effector T cells and Tregs (Kuehn et al 2014, Schubert et al 2014).

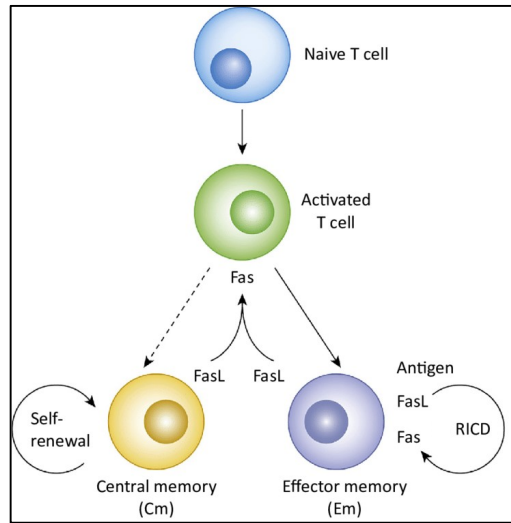
Even if in this manuscript I focused just on the Fas-related ALPS types, as we saw for the ALPS-U group, not all the forms of ALPS are strictly related with Fas pathway. Insurgence of ALPS-like disease was, indeed, described for mutations in other molecules such as KRAS or NRAS which give rise to the so called Ras-Autoimmune Leukoproliferative Disease (RALD) (ALPS IV)(Oliveira et al 2010, Takagi et al 2011, Calvo et al 2015).

### **3.1.5 RICD *in vitro* and *in vivo*: controversies**

Activated peripheral T cells are eliminated in order to silence the immune response at the end of an infection, a mechanism known as T cell contraction (Krueger et al 2003). Indeed, upon the first TCR stimulation T cells start to upregulate Fas and FasL expression, in a way that, after another contact with the same Ag presented by the MHC on the APCs they might be eliminated by apoptosis, in a process known as restimulation induced cell death (RICD) (Fig.8) (Zheng et al 2017). The observed apoptosis was first attributed

to the FasL/Fas system. In line, central memory T cells, which are known to be fundamental in activating a second immune response upon reinfection with the same antigen, are less sensitive to Fas induced cell death *in vitro*.

Nowadays, the role of Fas in RICD has been reevaluated. If Fas is able to induce apoptosis in restimulated cells *in vitro*, this is not necessary true for *in vivo* models. More recent studies showed that Fas-induced apoptosis is the main mechanism for cell death only after repetitive contacts with low affinity Ags for their TCR receptor (Fortner et al 2011), which occur for cells that have escaped to selection in the central tolerance setting (autoreactive T cells) or in response to a chronic infection (Varanasi et al 2014).



**Fig.8 RICD model. Adapted from Yi et al 2018.**

The self Ags which show a high affinity for TCR are mostly eliminated by negative selection in the thymus during cell development, in a process that involves the mitochondrial apoptotic pathway and the deprivation of cytokines in the microenvironment and is independent from Fas. In the same way, acute infections show a high Ag affinity for T cell receptor and upon this interaction, T cell death has been found to be more dependent from the proapoptotic protein BIM, known member in the induction of mitochondrial dependent cell death (Hildeman et al 2002, Pellegrini et al 2003, Strasser et al 2009), as well from the withdraw of survival cytokines, such as IL-2 and IL-7. Pellegrini and coworkers defined, indeed, that upon acute infection with Herpes simplex virus (HSV) Fas and TNFR1 were dispensable for the response and elimination (Pellegrini et al 2003). Furthermore, the viral response in a double knock-out of both Caspase-8 and RIPK3, fundamental proteins for induction of Fas mediated cell death, was not impaired and still worked at high strength (Feng et al 2018).

Conversely, in chronic response, there is a persistence of the pathogen in the host, and a « state of cohabitation » is achieved to keep a tolerated titer and avoid damageable response (Sprent and Tough 2001).

Hughes and his team in 2008 found that upon challenge with CMV (which causes a chronic infection) both Fas/FasL system and the apoptotic BIM protein are necessary for clonal T cell deletion (Hughes et al 2008).

However, the need of Fas in response to repetitive stimulations is also questioned. In the paper of Stranges of 2007 by using a mouse model where Fas was removed in total T cells or just in activated T cells (Th1 CD4+ or CD8+), they did not find any selective advantage in the survival of these cells compared to control mice (Fas sufficient mice) in response to primary or repetitive antigenic stimulations, doubting on the role for Fas in the elimination of T cells in both T cell contraction (primary response to stimulation) or upon restimulation (Stranges et al 2007). What was instead confirmed is the effect of Fas deficiency in the onset of autoimmune features, confirming a role for Fas in the deletion of autoreactive cells (Stranges et al 2007).

### 3.1.6 Fas in preventing and promoting autoimmunity

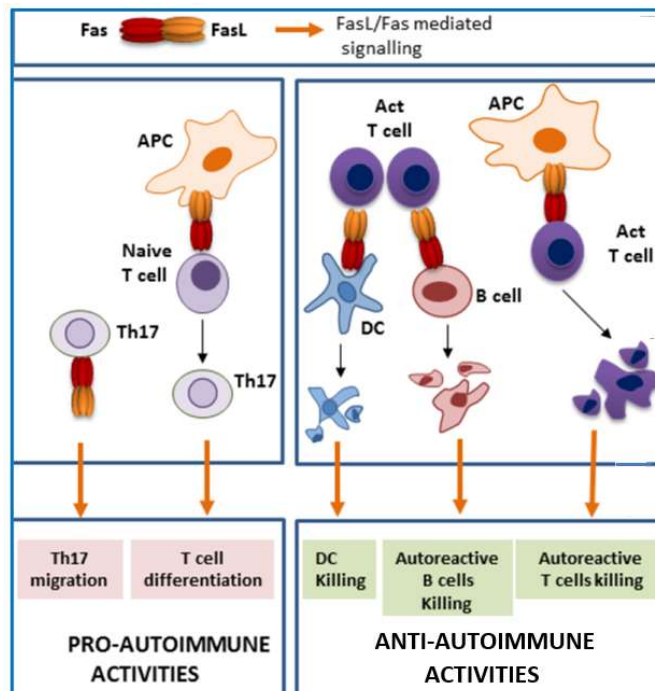


Fig.9 Pro and anti-autoimmunity Fas/FasL activities. Adapted from Rossin et al 2019.

At the state of the art it is well established that Fas/FasL proteins play a fundamental role in preventing the systemic autoimmunity insurgence and progression. However, Fas activation can also promote autoimmunity development in other specific cases rendering the situation quite complex (Fig.9).

As both *lpr* and *gld* mice models show evident autoimmune features, the role of Fas/FasL system was at first highly investigated in its property to prevent autoimmunity. Fas/FasL system has been shown to be determinant in the control of peripheral tolerance as its deficiency is causing spontaneous autoimmune

disorders. Its capacities have been elucidated in *lpr* and *gld* models, as well as in models lacking Fas expression just in specific immune cell subsets (T, B and APCs).

Fukuyama *et al* in 1998 could show in *lpr* mice that the reintroduction of Fas specifically in T cells prevents the lymphoaccumulation syndrome but not the SLE phenotype (Fukuyama *et al* 1998). This indicates that the presence of Fas in T cells is sufficient to control lymphocyte hyperproliferation but not to prevent autoimmunity. Indeed, it was demonstrated, using more sophisticated conditional knock-out mice in which Fas was specifically deleted in T, B or DC, that Fas expression in these 3 cell types was determinant for induction of peripheral tolerance.

Indeed, Stranges *et al* demonstrated that Fas expression in T cells is necessary for the deletion of autoreactive T cells (Stranges *et al* 2007). Even though, Fas plays also an important role also in the negative selection of autoreactive B cells and low affinity BCR cells in the germinal center (GC) (Hao *et al* 2008). GC is the zone of encounter between B cells and Th cells, where B cells bearing the best fit receptors are selected and antibody-secreting plasma cells are formed as well as the long living memory B cells. The reintroduction in a *lpr* model of Fas specifically in B cells could indeed prevent the SLE manifestation but not the lymphoaccumulation (Komano *et al* 1999). Furthermore, as explained by Stranges *et al* in 2007, the deletion of Fas in DCs is sufficient to induce systemic autoimmunity, caused by an increase of the Ag presentation to T cells (Stranges *et al* 2007). We can easily understand that, thus, elimination mechanism is coordinated by the presence of Fas in each of these populations. FasL upregulation in activated T cells contributes to the death of the APCs (which can be DC or B cells), which will in turn decrease the T cell activation. DC or B cells that lack Fas cannot be eliminated by T cells and in turn continue to act as APCs, enhancing T cells activation and the immune response (Hao *et al* 2008). Furthermore, autoreactive B cells start to produce autoantibodies, detrimental for the integrity of the target organs. Thus, the importance of Fas in maintaining the homeostasis in the immune system through the establishment of peripheral tolerance is crucial.

A contribution of the non-death functions of Fas has been proposed in the prevention of the autoimmunity. Inhibition of T cell proliferation, independently from its capacity to induce cell death, was suggested to be one of the mechanism that Fas would utilize to maintain the homeostasis, as a study revealed that overexpression of p21 (inhibitor of cell proliferation) in *lpr* mice could reduce the lymphoproliferative and autoimmune symptoms (Daszkiewicz *et al* 2015). Furthermore, it was shown that the introduction by pBAC transfection of the palmitoylation mutant of mouse Fas (C194V), which is known to fail to induce apoptosis for its inability to stabilize at the lipid raft, was able to rescue the lymphoproliferation and autoimmunity insurgence. This would suggest that Fas prevent autoimmunity onset not only by its apoptotic function but also by its pro-survival functions (Cruz *et al* 2016). It is anyway known that *lpr* is a leaky mutation, so the low reliability of the recipient which contains a low level of endogenous Fas might have affected the

results of this study. The generation of a knock in mice expressing such a mutant would unambiguously prove the role of non-death function of Fas in preventing lymphoaccumulation and autoimmunity.

On the other side, Fas functions were proved to contribute to the promotion of autoimmune diseases. Indeed it was shown by several studies that mice lacking Fas or FasL are protected from the development of experimentally induced autoimmune diseases such as experimental autoimmune encephalomyelitis (EAE), rheumatoid arthritis (RA) and type I diabetes (Waldner et al 1997, Sabelko et al 1997, Tu-Rapp et al 2004, Itoh et al 1997, Chervonsky et al 1997, Su et al 2000). It was proposed that this Fas role was promoted by its capacity to drive the differentiation of Th17 cells compared to a Th1 and Th2 phenotype (Yosef et al 2013, Meyer Zu Horste et al 2018). Th17 is a known subpopulation with high inflammatory properties and so would be mediator of the exacerbation of the autoimmune phenotype. Moreover, in the SLE model used by Poissonnier *et al* in 2016, the capacity of Fas expressing Th17 to transmigrate through the endothelial compartment is cause of aggravation of the symptoms (Poissonnier et al 2016). In a recent paper of 2019, a role for Fas in the promotion of the Th9 population has been identified (Shen et al 2019). Th9 population exacerbates autoimmune and allergic diseases (Jager et al 2009, Staudt et al 2010, Nalleweg et al 2015). In this paper, Fas-mediated Th9 differentiation causes the aggravation of inflammatory bowel disease (IBD). *lpr* mice, indeed, were producing less IL-9 cytokine and were enriched of the Th1 and Th2 populations at the expenses of the Th9 one. This was not caused by developmental defect of *lpr* mice because also the Fas knock-down was leading to a decrease of Th9 subpopulation (Shen et al 2019).

Fas/FasL system in autoimmunity, for its high versatility, context and cell dependent, results to be an extreme complex model that need to be further investigated (Reviewed by Rossin et al in 2019).

### **3.1.7 Fas/FasL in T cell activation**

Among the non-apoptotic roles of Fas/FasL proteins, modulation of cell proliferation was described for both immune and non-immune cells (Alderson et al 1993, Jelaska and Korn 1998, Desbarats and Newell 2000). Fas has been described in several studies in modulating the TCR activation in different T cell subpopulations. As this involvement is at the heart of my PhD project, an exhaustive dissection of this subject will be deeply faced in the chapter C of this manuscript.

### **3.1.8 Chemoattraction property of FasL**

The properties of FasL protein are not limited to cell death induction. It is known, indeed, that tissue expression of FasL recruit neutrophils and proinflammatory Th1 and CD8+ CTL, enhancing the inflammation on the site of recruitment. This is for instance, one of the main causes in the rejection of grafts. It was hypothesized that ectopic expression of FasL could have limited the damages caused by host immune system by depleting the infiltrated T cells in the graft organs. It was instead proved that the recruitment of proinflammatory agents by FasL was cause of faster destruction in transplanted beta island cells in the

pancreas and rejection of colon cancer cells (Kang et al 1997, Chen et al 1998). Even if the graft versus tumor (GVT) effect looks to be a promising strategy in the fight against tumor invasion, the other side of the story reveals an undesired effect that cannot be undervalued: GVHD (Graft versus host disease) is actually a counter effect of bone marrow transplantation in leukemia treatment. The transplanted T cells will indeed unleash the FasL mediated cytotoxicity against host organs which express Fas (Hartmann et al 2013).

### **3.1.9 FasL in immunoprivileged sites**

As briefly stated at the beginning of this manuscript, some tissues of our body have been shown to constitutively express FasL in order to inhibit immune mediated inflammation. Obviously, this mechanism acts by inducing cell death of the Fas positive activated lymphocytes that enter in the sites. These regions have been called immune privileged sites and include the eyes, the Sertoli cells in testis, encephalic barrier and the placenta of a pregnant woman. Together with FasL expression other mechanisms are exploited to avoid the occurrence of an inflammation process. Among them, the production of immunosuppressive cytokines, such as transforming growth factor  $\beta$  (TGF $\beta$ ), neuropeptides, complement protein regulation, as well as inhibitors of NK cells cytotoxic activity have been described (Reviewed by Ferguson et al 2006).

### 3.2 Fas/FasL roles in cancer immunoediting

The concept of dynamic interactions between tumor cells and immune system was defined as « Cancer immunoediting ». These interactions can, on one side, inhibit tumor progression by inducing the death of malignant cells and on the other side facilitate tumor development providing signals that enables tumor growth. It was soon discovered that the majority of tumors *in vivo*, after the first phase in which they are susceptible to apoptosis defined as « elimination phase », become able to overcome this initial loss selecting apoptosis-resistant clones. This adaptation occurs in a phase called « equilibrium », where cancer cells start to acquire phenotypic hallmarks that make them able to « escape » to cell death in order to survive and grow (Fig.10).

In this section we will focus on the role of Fas/FasL proteins in the cancer immunoediting concept, even though, obviously, also other members of TNF/TNFRSF and of other protein families play a vital role. Fas/FasL expression and activities are exploited by both immune and cancer microenvironment cells in the different phases of tumor progression, acting in both suppressing tumor and enhancing its survival.

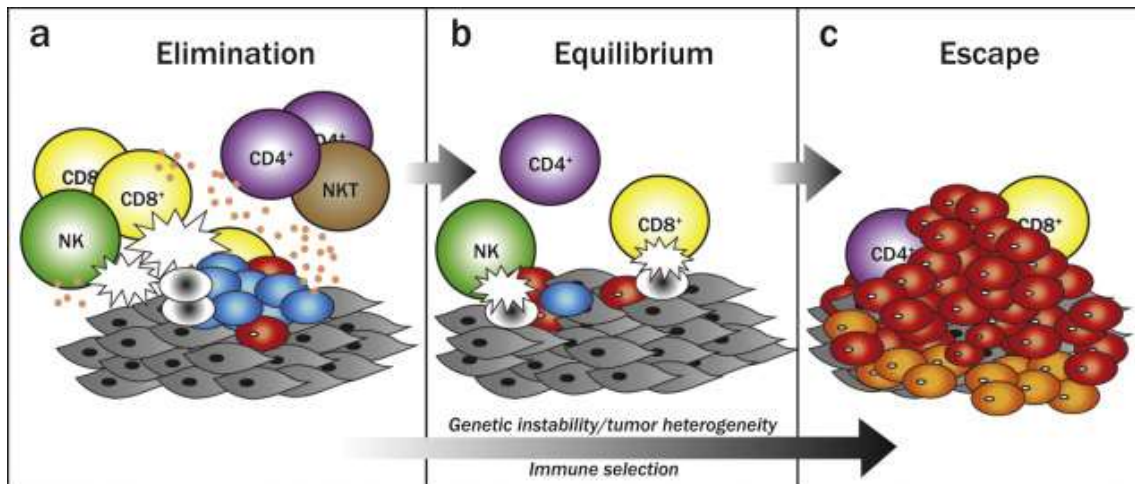


Fig.10 Cancer immunoediting phases. Pennell 2015.

#### 3.2.1. Fas/FasL in cancer suppression

Since the discovery of TNF/TNFR SF, their role in shaping cancer environment has been identified. The first clues came for instance, as mentioned at the beginning of this manuscript, by the capacity of the cytokines of the TNFSF to induce tumor rejection by necrotic phenotype (Granger et al 1969, Carswell et al 1975).

Several studies correlated the deficiency of Fas with an increase of tumor insurgence, and for that it was defined as tumor suppressor (Muschen et al 1998, Moers et al 1999, Keane et al 1996, Butler et al 1999).



A specific role of Fas in preventing tumor formation was stated by the high recurrence of Hodgkin and non-Hodgkin lymphomas in ALPS patients (Straus et al 2001). The absence of Fas in *E mu L-myc* transgenic mice was correlated to a more rapid lymphoma development (Zornig et al 1995). Conversely, the *lpr* and *gld* mice due their already altered phenotype are not optimal models to study the role of Fas in cancer and indeed there is no spontaneous occurrence of tumors in these models, with the exception for an increase of insurgence of malignant plasmacytoid lymphoma in aged *gld* mice (Davidson et al 1998).

Somatic mutations of Fas were described in hematologic and non-hematologic tumors, such as in bladder cancer, non-small cells lung cancer (Lee et al 1999), malignant melanoma and squamous cells carcinoma (Shin et al 1999). More than 180 mutations of the *FAS* gene have been identified and most of them clustered in the exons 8 and 9 (corresponding to the DD) provoking the loss of functionality of the protein, not anymore capable to mediate Fas-induced apoptosis (Grønbaek et al 1998, Lee et al 1999, Shen et al 2002). Despite that, these mutations are often present on just one allele, with a low rate of induction of loss of heterozygosity. Interestingly, some authors have demonstrated that whereas both alleles are necessary for the induction of an efficient apoptotic signal, only one is sufficient in the induction of the pro-survival ones (Peter et al 2007, Guicciardi and Gores 2009, Legembre et al 2004) suggesting that the non-death signaling might be conserved in a cancer context.

Elimination phase is mediated by Natural killer cells (NK) and cytotoxic T lymphocytes (CTL) by exploiting the perforin/granzyme system and/or the FasL targeting of the tumor cells (Seki et al 2002, Caldwell et al 2003, Morales-Kastresana et al 2013), as described above. The discriminant for the strategy to use in elimination of tumor cells has been uncovered by the study of Shanker and his coworkers. They have identified that low amount of Ag exposed by tumor cells would promote FasL-induced killing whereas high amount would promote the perforin/granzyme (Shanker et al 2009).

An interesting paper of Dellabona team of 2018, found that a specific class of NK cells, the CD1d-restricted iNKT, was responsible of blocking the advancement of prostate cancer. These cells were able to target and kill of M2-like macrophages, promoters of cancer progression, and instead enhance the M1-like macrophages, with a high proinflammatory activity against the tumor. This dual function is mediated by a bimodal FasL/CD40 signals (Cortesi et al 2018).

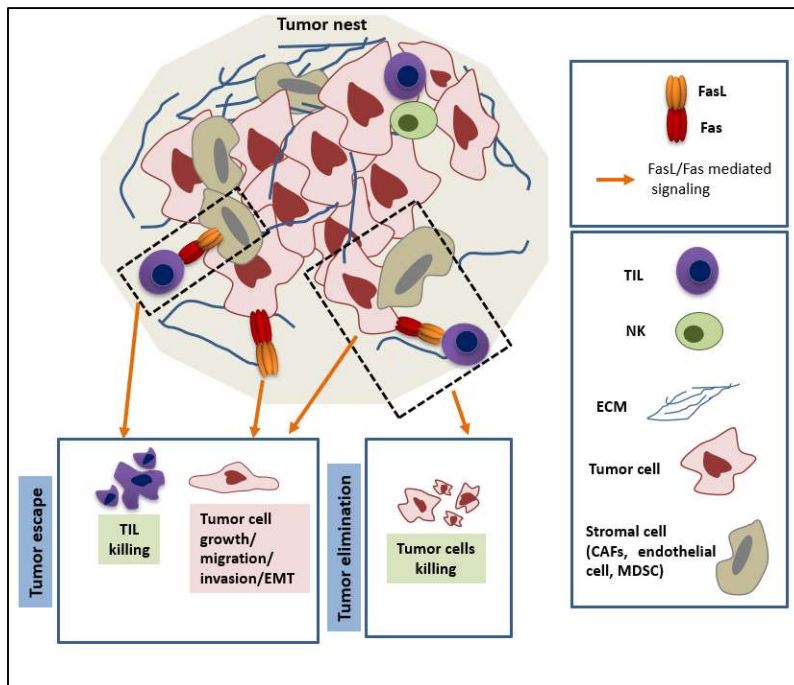


Fig.11 Fas/FasL roles in tumors. Adapted from Rossin et al 2019.

### 3.2.2 Pro-tumoral roles of Fas

Modulation of the expression of Fas and FasL by tumor cells can confer them advantages to support tumor progression. Pro-tumoral roles of Fas can be exerted by both its pro and antiapoptotic functions (Fig.11). In the progression of cancer development, multiple hallmarks contribute to the creation of a well-adapted environment that support the malignancy and cancer cells become able to “escape the immune system”.

- a) FasL expression and proapoptotic function can be exploited by tumors in order to escape from immune cells attack in a process known as “tumor counterattack”.
- b) Moreover, the modulation of the level of receptor and components of the apoptotic machinery by cancer cells is a hallmark strategy used by tumors to escape from cell death.
- c) Importantly, tumor cells exploit multiple Fas-induced signaling that are not leading to cell death. Fas/FasL can signal for multiple pro-survival processes (for molecular details see from pag 36), that are known to be used by tumor cells in order to support the malignant progression. These FasL-induced signaling can support migration, invasion and thus metastasis promotion, epithelial to mesenchymal transition (EMT), cancer cell stemness maintenance, as well as tumor growth.

### **a) Tumor counterattack**

The tumor can escape from immune system mediated killing by evolving different strategies. The first of these was initially defined as « tumor counterattack ».

Indeed, an abnormal level of FasL in the tumor microenvironment is often found and actively plays a role in the tumor escape by inducing the death of the tumor infiltrating lymphocytes (TIL) which express high level of Fas. However, the expression of FasL by tumor cells arose a long debate in the scientific community. As we explained above, it was at first claimed that the expression of FasL by cancer cells could have conferred them the status of the immuneprivileged site, permitting the killing of the TIL in tumor microenvironment. This concept was challenged in several contexts, such as in colorectal cancer cells (CRC) and xenograft transplantation of tumor in mice where it was proved that the expression of FasL by tumor cells, instead of killing the TILs, could promote neutrophils attraction, causing the tumor eradication (Arai et al 1997, Graf et al 2001, Reed et al 2006). However, FasL might be produced by tumoral cells and cleaved by metalloproteases or simply released by exosomes in the stroma. This latest form was shown to contribute to the death of TIL (Gearing et al 1994).

More recently, several studies identified that the FasL expression in tumor was not observed on the tumor cells themselves but on cells present in the tumoral microenvironment, such as endothelial cells, MDSC, and cancer-associated fibroblast (CAF) (Motz et al 2014, Lakins et al 2018, Zhu et al 2017).

### **b) Modulation of Fas and Fas-related proteins level**

Downregulation of Fas expression at the cell surface is another hallmark of tumoral cells that allow them to escape from apoptosis. This can be mediated by the CpG methylation of *FAS* gene or promoter (Petak et al 2003, Peli et al 1999), a process that can result from the oncogenic RAS signaling (Peli et al 1999).

Furthermore, overexpression of the decoy receptor DcR3, the Fas competitor for FasL binding, has been found in several type of cancers, such as lymphomas of viral origin (EBV or HTLV-1), colon, lung, pancreatic adenocarcinomas and gliomas (Toda et al 2013).

In addition, the regulation of mediators of the death signaling was found to be impaired in several kind of cancers, such as the overexpression of the antiapoptotic regulator cFLIP in colon cancer (Li et al 2009).

### **c) Tumor cell intrinsic Fas signaling driving tumor progression**

It is now well established that Fas-induced survival signaling does not only trigger resistance of the cancer cells to apoptosis but also provide them with tumor-promoting properties.

#### **Migration, invasion and metastasis promotion**

Fas/FasL can enhance tumor migration and invasion and thus trigger the metastatic processes in different cancer types such as colorectal cancers or glioblastomas (Ta et al 2018, Kleber et al 2008). Interestingly, it was demonstrated that oncogenic mutations such as KRAS or constitutively active PI3K can switch the Fas signaling from death to migration and invasion (Hoogwater et al 2010, Ehrenschwender et al 2010). Cell protrusion formation mediated by actin and cytoskeleton modulating proteins is a fundamental event in promoting invasion and metastasis. Fas activation was shown to drive the formation of such actin-mediated cell protrusion by modulating the cofilin pathway (Hoogwater et al 2010, Steller et al 2011). Fas can also activate amoeboid cell motility through activation of the ROCK pathway (Ehrenschwender et al 2010).

Moreover, the expression of FasL by endothelial cells, which is often found in tumoral context, can allow the binding of Fas positive myeloid cells and enhance their transmigration contributing to the roll and adhesion mechanism for extravasation (Gao et al 2013).

The properties of FasL in increasing the motility and invasiveness of cancer have been associated to its soluble form (sFasL) (Poissonnier et al 2016). In several kind of cancers, such as ovary carcinoma and melanoma, high level of FasL are detected in the serum of the patients. sFasL indeed promote the migration of brain cells by promoting the secretion of vascular endothelium growth factor (VEGF) (Zhang et al 2015). At the same time this mechanism leads also to angiogenesis and vascularization at the primary tumor site.

#### **EMT transition and cancer cell stemness maintenance**

EMT is a cellular process in which epithelial cells lose their characteristics to acquire mesenchymal features. EMT has been involved in malignant progression by promoting cancer cell migration, metastasis formation or tumor stemness maintenance (Jolly et al 2017, Shell et al 2007, Ye and Weinberg 2015). The tumor promoting functions of Fas rely also in its capacity to mediate EMT. It was shown in gastrointestinal cancer model that Fas inhibits the epithelial markers such as E-cadherin while promoting the mesenchymal ones (Li et al 2009). This induction is based on ERK1/2 activation and GSK-3 $\beta$  (Zheng et al 2013).

Furthermore, chronic Fas stimulation, that occurs in cancer progression, can promote type I interferons production that cause the activation of STAT1, one important mediator of the switching of cell phenotype to stemness (Qadir et al 2017). This is also in line with previous studies that defined a role for Fas in driving and maintaining cancer cell stemness (Ceppi et al 2014, Teodorczyk et al 2014).

### **Tumor growth by proliferation and cell survival**

FasL has been shown to promote cancer cell survival and proliferation. In the same way as it promotes liver regeneration, function that will be mentioned below, cancer cells use Fas induced JNK activation to promote cell proliferation. It was found that upon knock-down of Fas expression by shRNA approach the transcription factor Egr-1 and c-Fos, known to be promoter of growth and proliferation, were downregulated (Chen et al 2010). Furthermore, in a colon cancer model it is shown that a high percentage of Fas bearing cancer cells are apoptosis resistant because of the upregulation of antiapoptotic proteins such as c-FLIP. This leads to enhanced cell proliferation and expression of FasL that is able to induce cell death on the liver and endothelial cells surrounding it and create a niche for optimal expansion (Li et al 2009).

FasL recruitment of proinflammatory cells can indirectly lead to tumor promoting functions creating a microenvironment that stimulates tumor growth. It is known for instance, that the establishment of a chronic inflammation leads to cell proliferation and tumor development (Philip et al 2004).

It is thus hard to predict Fas/FasL signaling outcomes as they are multifaceted in a context dependent manner. All these considerations, that raise from its antitumoral to its pro-tumorigenic effects have to be taken into account in order to define if a Fas targeted therapeutic approach has to be used or not. A deeper discussion on how therapeutic approaches implying the enhancement or the blockade of Fas/FasL interaction are evolving will be one of the subjects of the discussion of this manuscript, after the integration of the results obtained during my PhD.

#### 4. Fas/FasL signaling has multiple outcomes

The outcomes of Fas/FasL interaction were originally restricted to the induction of cell death by apoptotic pathway. It is now well recognized that Fas/FasL is a pleiotropic system giving rise also to several non-apoptotic signaling that lead to cell survival, proliferation, inflammation, migration and differentiation. The non-apoptotic functions of Fas have been associated to both physiologic and pathologic conditions such as in tumorigenesis as described above (Alderson et al 1993, Klebanoff et al 2016, Li et al 2009). Before starting the description of these multiple outcomes it is worth to mention which are the features that allow Fas/FasL interaction and consequently the signals to occur.

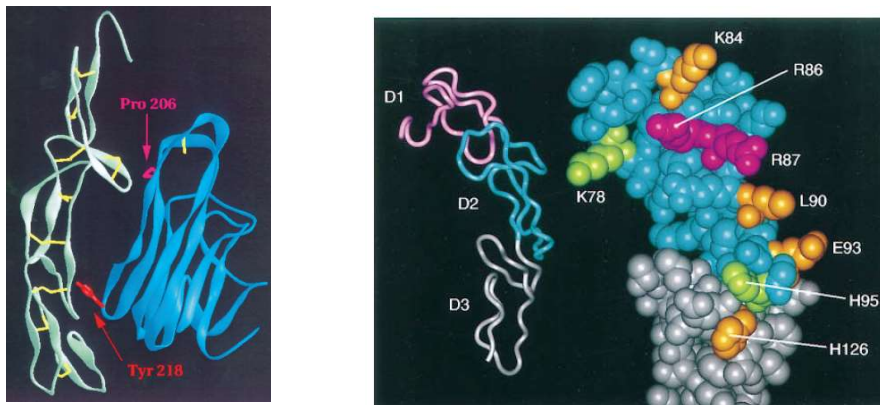
##### 4.1 Fas/FasL interaction

Fas/FasL interaction is a critical step for the initiation of the signal that activate the molecular cascade downstream the plasma membrane, independently from the type of response induced.

##### 4.1.1 Fas/FasL interaction site

Schneider in 1997, exploiting mutagenesis experiments, defined three amino acids on human FasL that were important for Fas binding: the Proline 206, the Phenylalanine 275 and the Tyrosine 238 (Fig.12). The last one is a conserved site among the members of TNF family for interaction of TNF $\alpha$  and LT $\alpha$  with TNFR1, hypothesizing that the negative charge of FasL was interacting with a basic amino acid on Fas as the Arginine 86 (Schneider et al 1997).

In the same year Starling and his team used the same approaches to identify the region of Fas involved in the interaction. They defined a critical region in CRD2 corresponding to the amino acids R86-R87, conserved in TNFR and CD40, and important but not necessary amino acids residing on the CRD3, which help the correct structure to be formed, identified as L90, E93 and H126 (Fig.12)(Starling et al 1997).



**Fig. 12 Fas/FasL residues for binding. On the left two amino acids on FasL monomers important for Fas binding. Schneider et al 1997. On the right amino acids on Fas involved in FasL binding. Starling et al 1997.**

Interestingly, some studies demonstrated a ligand-independent activation of Fas: in activated T cells, Baker and Moulian show that the presence of proinflammatory cytokines, such as TNF $\alpha$  and IFN $\gamma$ , lead to an increasing of Fas density at the cell surface which is promoting the apoptotic signals independently from FasL binding (Moulian et al 1997, 1998). Furthermore, it has been established from Boldin *et al* that trimerization of Fas DD is able to transduce an apoptotic signal *in vitro* (Boldin et al 1995). On the other hand, other groups demonstrated that even a high association of Fas on its own is not enough to induce strong Death Inducing Signaling Complex (DISC) formation (Siegel et al 2000) and consequently fails in cell death induction. The controversies existing in literature on this subject might derive on the utilization of different cell types and models.

#### **4.1.2 FasL forms that bind to Fas receptor**

Another theme of debate resides in the forms of FasL which are able to lead the initiation cascade upon Fas binding. All the FasL forms, membrane-bound and proteolytically cleaved (soluble usually present as a unique trimer), are able to bind to Fas receptor.

Initial studies identified the membrane-bound FasL as the only FasL form able to initiate an appropriate signal and that the soluble FasL was instead acting as a negative regulator of apoptosis (Schneider et al 1998, Hohlbaum et al 2000, Gregory et al 2011, O'Reilly et al 2009). Evidence coming from studies on inflammation and tumorigenesis in both human and *gld* mice models challenged this dogma defining also the soluble FasL as an active molecule that was able to induce apoptosis if used in high amounts (Bajou et al 2008, Vargo-Gogola et al 2002).

Furthermore *in vitro* studies confirmed the proactive role of the soluble ligand in inducing apoptosis, but only after hexameric crosslink (Holler et al 2002), that would mimic a soluble FasL caught by extracellular matrix proteins. A consequence of the hexameric minimal requirement for active ligand would imply the formation of dimers of trimers also for Fas receptor (receptor clustering) in order to initiate the death signal (Graves et al 2014). This will be further explained in the deciphering of the apoptotic cascade.

The soluble FasL has been described also for non-apoptotic signaling induction, thus enhancing inflammation, cell proliferation as well as cell motility and invasiveness (Ahn et al 2001, Niu et al 2012, Janssen et al 2003, Paulsen et al 2011, Tazuin et al 2011, Monet et al 2016).

Thus, the form of FasL binding the receptor combined with its aggregation state, might be discriminant factors for the different signaling that Fas can mediate, directing it through cell death or cell survival pathways.

## **4.2 The Fas/FasL death signaling**

### **4.2.1 The apoptotic pathway**

Several types of cell death have been described but Fas-induced cell death mainly relies on the induction of apoptotic pathway.

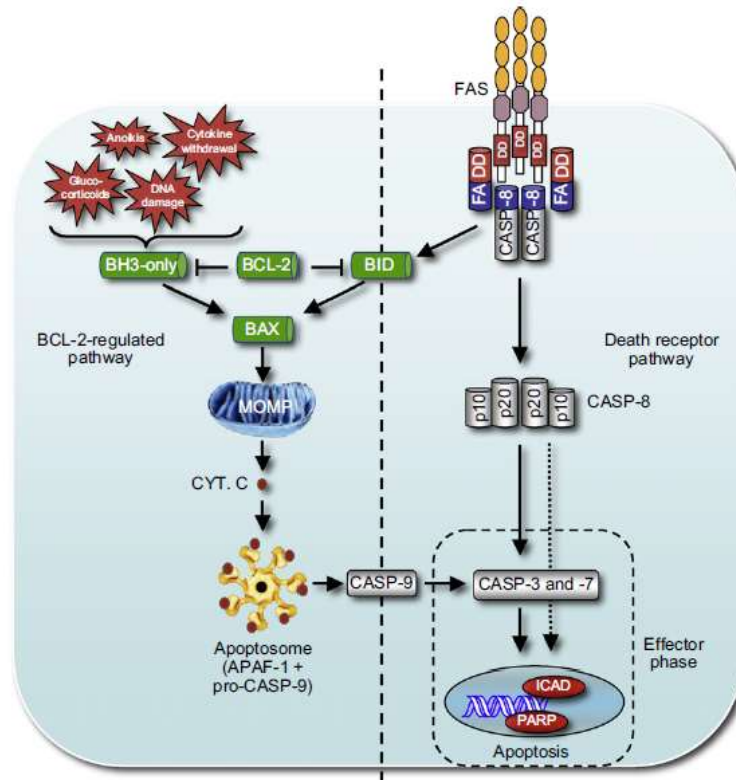
Apoptosis is a type of programmed cell death (PCD) that is triggered in response to external or internal stimuli of different nature. Morphological hallmarks of apoptosis are cell contraction and membrane blebbing, DNA laddering, chromatin condensation and DNA fragmentation (Kerr et al 1972, Gavrieli et al 1992, Lazebnik et al 1993). It is a mechanism defined as « cell suicide » which preserves the integrity of surrounding cells. Apoptosis occurs physiologically during development and organogenesis, notably for atrophy of regions of organs, but also in conditions of cellular stresses (Hengartner et al 2000).

Apoptotic process is strictly dependent and relies on the presence of the Caspases family (Janicke et al 1993). Caspases are aspartyl specific cysteine proteases that exist in the cells as inactive zymogens (Yuan et al 1993, Wang et al 1994). These proteins can be divided in two classes according to their structure and function: the initiator caspases (Caspases-8/10/2 and 9) and the effector caspases (Caspases-3/6 and 7). The first are apical sensors which, after homo or hetero dimerization facilitated by binding to adaptor proteins, can promote their self-cleavage in order to be activated (Muzio et al 1998, Degterev et al 2003, Walczak and Krammer 2000). Once activated, they can activate by proteolytic cleavage the effector Caspases, that will finally process their targets and lead to cell death. The substrates of effector caspases include essential structural proteins or enzymes such as the poly-ADP-ribose polymerase (PARP) protein, involved in DNA repairing, the nuclear protein Lamin and cytoskeleton proteins such as filamin, phosphomyosin and actin monomers, involved in the morphological features of apoptotic cells (Lazebnik et al 1993).

Two different pathways have been described for apoptosis induction: the extrinsic pathway and the intrinsic pathway.

The extrinsic apoptotic pathway occurs upon external events and involves the sensing of a specific signal, such as the binding of a death ligand to a death receptor, which initiates the cascade of events leading to cell death. FasL is one of the external stimuli that initiates the extrinsic apoptotic pathway (Walczak and Krammer 2000). The intrinsic apoptotic pathway, on the other hand, can be initiated by an internal stimulus, that can result from the accumulation of reactive oxygen species (ROS) in the intracellular environment, as well as from increasing DNA damages (Candé et al 2002; Saelens et al 2004). Extrinsic and intrinsic pathway can work cooperatively to amplify the signals that bring to cell death (Cory and Adams 2002) (see Fig.13).





**Fig.13 intrinsic and extrinsic apoptotic pathways. Strasser et al 2009.**

Fas has also been described as an inducer of necroptotic cell death. Necroptosis is a form of programmed cell death independent of Caspases and which presents the morphological features of necrosis (Majno et al 1995). Morphological hallmarks of necroptosis are cell and organelles swelling, rupture of plasma membrane and cell lysis. The molecular pathway that leads to necroptosis will be described in the section dedicated to the regulation of Fas/FasL signaling (page 48). Necroptosis is important for the activation of innate and adaptive immune system against infection and is pathologically linked to inflammatory diseases. Even if Fas can induce cell death by either apoptosis or necroptosis, the apoptotic pathway is the preferential mechanism when all the components necessary for its occurrence are functional.

#### 4.2.2 Molecular dissection of Fas-induced apoptosis

To avoid an uncontrolled cell death, apoptosis is a tightly controlled process that requires preliminary steps in order to occur.

##### a. Fas receptor clustering

Upon FasL engagement, Fas homotrimers assemble in a supramolecular protein complex formed by Fas and associated intracellular proteins with which will form the DISC. Fas homotrimers start aggregate in clusters at specific membrane nanodomains (lipid raft) to create high order units (Hueber et al 2001, Kischkel et al 1995, Kamitani et al 1997, Papoff et al 1999, Siegel et al 2004, Henkler et al 2005). These aggregates create amplification platforms necessary for the initiation and the propagation of the apoptotic signal (Fig.14) (Feig et al 2007). In T cells these big aggregates were shown to cause the so called “capping” of the receptor, where Fas re-localizes in one main pole of the membrane at the contact with the cells that exposed the FasL (Cremesti et al 2001). DISC is essentially composed by the adaptor protein FADD and the initiator procaspase-8.

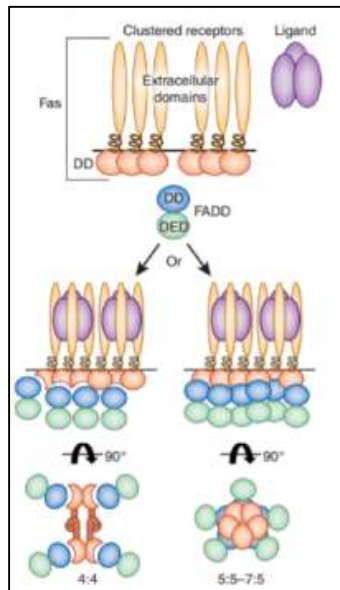
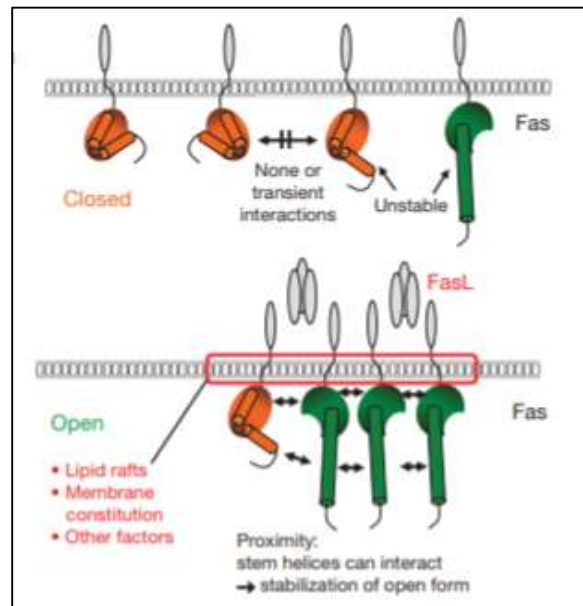


Fig.14 Receptor cluster and two models for Fas DD-FADD binding. Hymowitz and Dixit 2010.

##### b. Models for Fas DD-FADD interactions

The first protein that contributes to the formation of this complex is the adaptor Fas-associated protein with death domain (FADD), which contains a DD in its C-terminus able to interact with Fas via a homotypic interaction. The Fas-FADD interaction has not been completely solved and currently two models coexist: The 4:4 and 5:5. It is noteworthy to mention that the interaction models were created with the Fas death domain and FADD and not with the entire Fas protein. Indeed, we still lack a global 3D Fas structure in both resting and activated condition.



**Fig.15 Fas conformation in 4:4 model. Scott et al 2008.**

#### **4:4 model**

In the 4:4 model, tetrameric units of the complex exist (4 Fas DD and 4 FADD) and were observed in their crystal structure and corroborated by their maintenance in solubility conditions. Scott *et al* described that Fas DD exists in a closed or opened conformation. In absence of signals, Fas DD is balanced on the stable closed conformation. Upon FasL binding, Fas is recruited on membrane nanodomains with a different density (lipid raft) in proximity to other Fas DDs. This proximity in a different cell environment would shift the equilibrium to the open conformation that expose the hydrophobic residues and would allow the Fas tetramer formation and FADD binding and thus the initiation of the signaling (Fig.15) (Scott et al 2009). However, the low pH in which the crystal structures were obtained and the discrepancy of the measurements in solution lead to formulate other hypothesis for this cluster formation (Hymowitz and Dixit 2010).

#### **5:5 model**

Alternatively, two other teams used complementary techniques other than crystallography, such as nuclear magnetic resonance (NMR), multi angle light scattering microscopy, chromatography and electron microscopy, to define the stoichiometry of this complex. Both Wang *et al* and Esposito *et al* describe the primitive complex formed of 5-7 Fas DD recruiting 5 FADD proteins in neutral pH similar to the physiologic condition (Wang et al 2010, Esposito et al 2010).

This second model, however, does not explain how the FADD binding to Fas is selectively induced upon FasL engagement.

### c. FADD DED interacts with procaspase-8

FADD bears also the so called Death Effector Domain (DED) which is exposed after interaction with Fas in the 4:4 model (Scott et al 2009), that allows the homotypic binding of dimers of initiator procaspase to Fas/FADD complex, creating the DISC (Boldin et al 1996, Muzio et al 1996).

The pivotal initiator caspase identified was called MACH/FLICE and was later identified as procaspase-8. The binding to FADD allows the procaspase-8 to initiate a molecular cascade which sequentially will activate the effector caspases, whose targets disruption are leading to cell death. The close proximity of procaspases-8 allows their self-cleavage in the DISC and release in the cytoplasm the active form of the protein, consisting in a large tetrameric form containing 2 p18 subunits and 2 smaller p10.

A second initiator caspase was later identified in the DISC, the Caspase-10, absent in mice, with high sequence homology with Caspase-8. It consequently shows redundant function and can initiate on its own, or in dimer with Caspase-8, the apoptotic signaling (Kishkel et al 2001, Wang et al 1999). However, in literature, speculations of an alternative role of Caspase-10 in DISC association, such as the possibility to have different targets with non-apoptotic roles, have been exploited (Wang et al 2001). In a study, Sprick *et al* define that Caspase-10 has not a redundant role with Caspase-8 since it is unable to compensate the apoptotic pathway in Jurkat line deficient of Caspase-8 (Sprick et al 2002). Horn *et al* even found that Caspase-10 can be a negative regulator of Caspase-8 (Horn et al 2017).

Furthermore, the regulation of the DISC is mediated by other accessory proteins, first of all the FLICE inhibitory protein (FLIP) that exists in at least 3 different forms. This type of regulation will be described on this manuscript at page 43.

If a high amount of DISC complex is formed activated Caspase-8 or 10 can directly cleave the effector Caspases 3, 6 and 7 which in turn can cleave their target proteins, and finally lead to cell death. If the amount of DISC is not sufficient to initiate this pathway, mitochondrial dependent reactions are started giving rise to the intrinsic apoptotic pathway (Cande et al 2002; Saelens et al 2004). In this activation mechanism, the active Caspase-8 cleaves the BH3-only proapoptotic protein Bid, creating the truncated form tBid that can translocate into the mitochondria. This translocation inhibits the antiapoptotic proteins of the Bcl-2 family causing the aggregation of Bak/Bax proapoptotic proteins. This leads to the release of cytochrome c and of the activator of caspases SMAC/DIABLO (Saelens et al 2004). Association of procaspase-9 with cytochrome c and Apaf-1 forms a structure known as the apoptosome. Within the apoptosome, the Caspase-9 gets activated and can cleave the effector caspases downstream causing cell death as described above.

### 4.3 Fas pro-survival signal: an overview of outcomes and pathways involved

The concept of Fas/FasL as a death promoting interaction has been completely reevaluated in the past 20 years. It is now known that Fas can also induce several non-apoptotic pathways such as cell proliferation, differentiation, migration, production of inflammatory cytokines or chemoattraction.

The difficulty on the determination of the discriminants that mediates the shift of the response from death to non-death is based on the high number of parameters that can play a role. Examples of these parameters are cell type, threshold of signaling activation, resistance to Fas-mediated apoptosis, presence of cellular stresses and even the variation of the pool of pro and antiapoptotic proteins inside the cells including DISC interacting proteins.

Noteworthy, Fas-induced non-death pathway has been observed in physiologic conditions but also in a pathologic context. As we stated in the previous section of physiopathology, for instance, in a tumoral context, clones selected for their resistance to cell death have shown to induce non-death signals upon Fas engagement. Furthermore, in tumors, other parameters have been involved to collaborate in the promotion of Fas induced pro-survival signals, such as the switch of cell metabolism and the variation of gene expression by mutation in oncogene and tumor suppression genes.

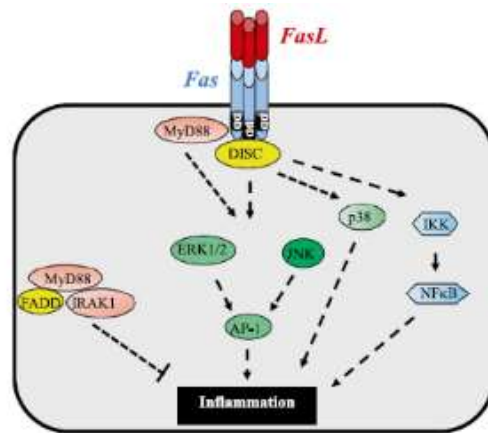
The first discovery that TNFRSF could promote pro-survival signaling by induction of NF- $\kappa$ B comes from about thirty years ago when Shalaby and Hollman in 1990 described the activation of this transcription factor upon ligation of TNF $\alpha$  and TNF $\beta$  to TNFR1 even at high doses of cytokines (Shalaby et al 1990). Then, Alderson in 1993, showed for the first time that Fas receptor could also stimulate a proliferation signal, acting as a co-stimulator of the T cell receptor and increasing the production and secretion of the proliferation marker IL-2 in purified T cells (Alderson et al 1993). Later, Fas and FasL have been described to induce cell proliferation in several tissues and organs, from the immune system to hepatocytes or fibroblasts (Alderson et al 1993, Aggarwal et al 1995, Desbarats et al 2000, Jelaska and Korn 1998).

Moreover, Fas is a promoter of cell differentiation, a mechanism that was evidenced in immune context and outside the immune system, by promoting neuronal stem cell differentiation and formation of new neurite branches (Desbarats et al 2003, Zuliani et al 2006). Furthermore, FasL is a known chemoattractant molecule, recruiting immune cells on the site of the body where it is expressed, so promoting inflammatory processes (Kang et al 1997, Chen et al 1998, Poissonnier et al 2016). In line with that, its interaction with Fas can favors the formation of cell protrusions, by acting with cytoskeleton proteins, and so promoting migration and invasiveness (Kleber et al 2008, Poissonnier et al 2016).

Importantly, if the molecular cascade of the apoptotic pathway has been extensively deciphered, pro-survival pathways were initially only defined by the activation of late signaling, downstream the early events that occur upon Fas engagement. This downstream activation of non-apoptotic molecules mediated by Fas

receptor were likely to reside, as for the other members of the superfamily, in the activation of the classical survival pathways such as NF- $\kappa$ B transcription factor, MAPK or PI3K/Akt. Thus, for long the molecular link between Fas and these downstream pathways remained unsolved but in the past years numbers of studies came out to dissect this point. I decide to only detail few of them below that I choose based on the amount of accumulated data.

#### 4.3.1. Production of proinflammatory cytokines



**Fig.16 Fas mediated cytokines production. Brint et al 2013.**

Fas/FasL interaction can play a role in enhancement of granulocytes and macrophages recruitment by the capacity to induce secretion of proinflammatory cytokines and chemokines, such as IL-6, IL-8 and MCP-1. This process has been observed in several cell types such as primary mouse hepatocytes, primary fibroblasts, primary mouse thymocytes as well as in cell lines such as HeLa cells and the colon adenocarcinoma HT-29 (Cullen et al 2013). Even if this process occurs in parallel with the induction of apoptosis, these pathways have been uncoupled.

DISC-interacting proteins, of which FADD and Caspase-8 are the first components, are of vital importance also for this kind of signalization. FADD or Caspase-8 knockdown, for instance, abrogates completely cytokines production in HeLa cells. However, the activity of Caspase-8 was proved to not be necessary, since the pancaspase activity inhibitor zVAD was able to block apoptosis but not cytokines production (Cullen et al 2013). Caspase-8 acts thus as scaffold to allow recruitment of proteins that will form an intracellular complex important for NF- $\kappa$ B and MAPKs activation and therefore for cytokine genes transcription (Fig.16) (Cullen et al 2013, Lafont et al 2017, Varfolomeev et al 2005).

Cullen and colleagues found that in HeLa cells FADD/procaspase-8 can form a complex with the kinase RIPK1. RIPK1 silencing by siRNAs strongly reduces cytokines production, as well as NF- $\kappa$ B activation (Cullen et al 2013, Kataoka and Tschopp 2004, Koenig et al 2014). Considering that regulation by polyubiquitination of

RIPK1 from c-IAPs was described for TNFR-mediated NF- $\kappa$ B activation (Hsu et al 1996), the role of IAPs was investigated in this context. Using first an inhibitor of their activity (BV6) and then knocking down c-IAP1 and c-IAP2, Cullen and co-workers have suggested a similar mechanism upon Fas stimulation in HUVEC cells. Beside this example, molecules involved in the regulation of this supramolecular complex important for gene transcription and cytokine production will be described at page 49.

Furthermore, chronic inflammation induced by cytokines production can be mediated by the IL-1R1-TLR4 pathway that promotes NF- $\kappa$ B activation. Fas signaling has been shown to indirectly enhance this pathway. Indeed, it was evidenced that FADD sequestering of the MyD88 protein via interaction of their respective DD, is shutting down this activation (Fig.16). However, upon Fas ligation, FADD is recruited to the DISC and the protein MyD88 is unleashed to solve its role of activator of the IL-1R1/TLR4 pathway (Ma et al 2004).

#### **4.3.2. T cell differentiation**

It is now well established that induction of T cell differentiation is one of the non-apoptotic function of Fas.

An example of contribution of Fas in cell differentiation comes from the study on Th17 cells, already mentioned in the description of Fas in promotion of autoimmunity in the physiopathology paragraph. A role for Fas has been described in its capacity to promote the differentiation and stability of Th17 cells, thus promoting EAE development, and at the same time negatively regulates the Th1 and Th2 phenotype (Meyer Zu Horste et al 2017). This process was uncoupled from apoptosis considering that inhibitors of Caspases were not able to interfere with the Th17 promoting program induced by Fas, identifying an apoptosis independent pathway involved in the process.

Meyer Zu Horste and colleagues performed RNA-seq to identify a potential mechanism for the occurrence of this process and STAT1 was identified to control the differentially expressed genes between WT and Fas<sup>-/-</sup> mice. STAT1 immunoprecipitated with Fas and the level of binding was enhanced upon FasL engagement. Th17 differentiation requires IL-6 mediated phosphorylation of STAT3 which is in turn inhibited by STAT1-induced heterodimerization. Thus, the model proposed is that Fas sequesters STAT1 at the membrane and therefore blocks STAT1-mediated inhibition of STAT3, which is free to translocate into the nucleus and promote Th17 differentiation. The confirmation of this hypothesis was identified by reverting defect of Fas deficiency by concomitant removal of STAT1 (Yosef et al 2013, Meyer Zu Horste et al 2018).

A recent paper of 2019 also revealed a role for Fas in induction Th9 differentiation. The mRNA encoding for IL-9 cytokine, responsible for Th9 differentiation, was extremely downregulated in Fas *lpr* mice. Considering that Fas on its own does not show enzymatic activity, Shen and colleagues started to decipher the molecular cascade that is involved in Th9 differentiation. The process was shown to be independent from Caspase activity, since the inhibitor zVAD did not alter Fas capacity to induce Th9 differentiation. Thus, they

hypothesized that ZAP70 binding to Fas, a process already described in T cells (Letellier et al 2010, Williams et al 1999), could have played a role in this process. Knock down of ZAP70 or mutation of tyrosines responsible for ZAP70 binding on Fas were strongly reducing Fas-mediated Th9 differentiation. The link from ZAP70 to gene activation was found by exploiting inhibitors of several pathways that could be in some way connected with the process. The mechanism was shown to rely on ZAP70 activation of PLC $\gamma$  that in turn enhance Ca<sup>2+</sup> influx, promote PKC $\beta$  and finally activation of NF- $\kappa$ B (Shen et al 2019).

#### 4.3.3 Cell migration

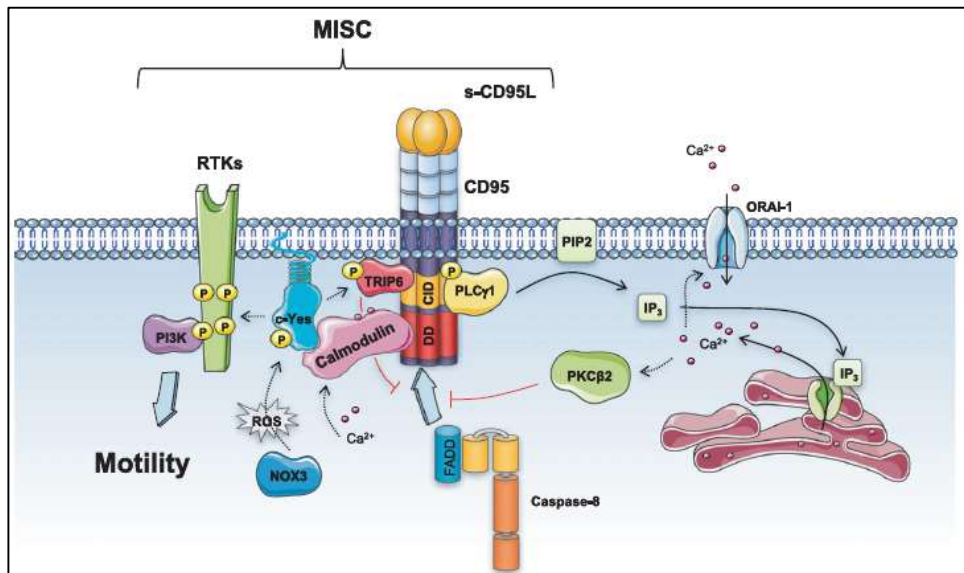


Fig. 17 Molecular deciphering of MISC complex. Guégan and Legembre 2018.

Fas-induced cell migration was described to occur in a DISC-independent manner in different cellular systems such as glioblastomas and Th17. The name of the complex formed by Fas upon FasL ligation which allows cell migration has been named “motility induced signaling complex” (MISC)(Fig.17) in reference to the DISC (Tauzin et al 2011, Monet et al 2016, Poissonnier et al 2016).

The formation of this complex was shown to be influenced by the form of FasL involved in Fas engagement. In the work of Kleber of 2008 the team shows that soluble FasL induced activation of src family kinases (SKFs) and subsequently the Akt pathway. They dissect molecularly, both *in vitro* and *in vivo*, the increased migration and invasion of the tumoral cells in response to FasL stimulation. They describe that Fas engagement recruits in its DD the SH2 domain of the Src kinase Yes, which in turn engages and activate the p85 fragment of PI3K. Activated PI3K is able to inhibit, by Akt mediated phosphorylation, the glycogen-synthase-kinase 3b (GSK3b) and to induce metalloproteases, such as MMP9. Finally, they suggest that cleaved FasL can act as a chemoattractive molecule increasing the migration and invasiveness of tumoral cells (Kleber et al 2008).



In a recent study led by Legembre team, the binding of PLC $\gamma$  protein to Fas was found in the proximal membrane part of Fas cytoplasmic domain, in a DD independent way. The interaction of PLC $\gamma$  to Fas allows the increase of intracellular Ca $^{2+}$  which in turn can mediate an increasing of cell motility and migration of endothelial Th17 cells in a SLE model (Poissonnier et al 2016).

Khadra *et al* found that PLC $\gamma$  dependent Ca $^{2+}$  releasing upon Fas ligation was indeed reducing DISC formation and protecting cells from cell death. They also show that an association in supramolecular clusters between the ER and membrane Ca $^{2+}$  protein channels (STIM1 and ORAI1 respectively) occurs upon Fas activation (Khadra et al 2011). Furthermore, the protein Calmodulin, an important Ca $^{2+}$  sensor, was found to bind the DD of Fas and that this binding was increasing upon the receptor activation. Interestingly, Calmodulin was described to mediate the interaction of Fas with the protein of Src family and to block the recruitment of FADD (Ahn et al 2004, Chen et al 2008, Yuan et al 2011).

Thus, cell migration partially relies on mechanisms that are DISC independent. DD of Fas can play a role in the formation of the MISC complex, but also DD-independent pathways exist, thus confirming the presence of alternative mechanisms for Fas induced migration process.

#### 4.4 Regulation of Fas/FasL signaling: events that modulate Fas signaling

Fas/FasL interaction and the outcome of the ligation is tightly regulated and can depend on several factors: (i) the cell type can play a role in the determination of Fas/FasL interaction. (ii) Fas localization in the cells can deeply affect the type of response. (iii) Fas PTMs play an important role in regulating the outcome of response.

##### 4.4.1 Fas signaling in polarized cells

Epithelial cells are characterized by a defined apico-basal polarity and cell-cell adherence. These features are maintained by the presence of protein complexes that play a role in forming cell-cell junction. Among them the adherens junctions (AJ) are formed in the baso-lateral domain of polarized cells. Dysregulation of the components involved in their formation, such as E-cadherin and the Scrib-Dlg1-Hugl1 complex, is cause of insurgence of diseases and promote tumorigenesis (Jeanes et al 2008, Stairs et al 2011).

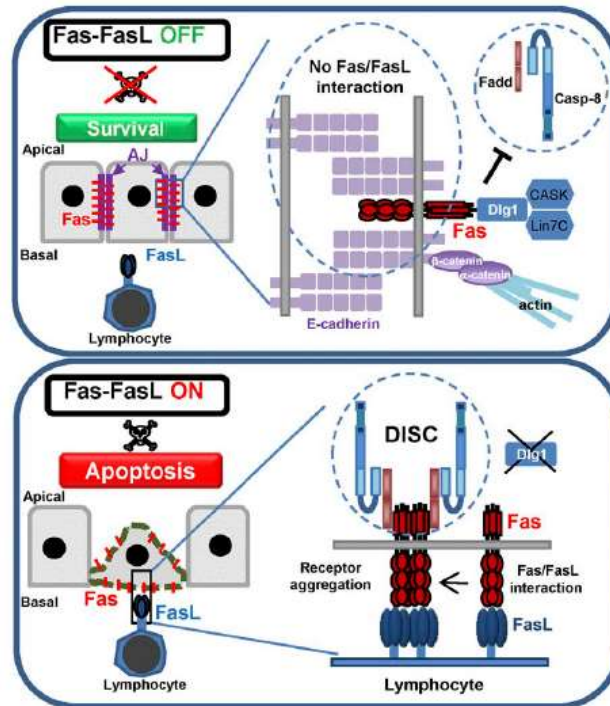


Fig.18 Fas in AJ. Gagnoux-Palacios et al 2018.

A study from our laboratory defined that Fas is localized to AJ in colorectal epithelial cells. The sequestration of Fas in these domains prevents FasL binding, DISC formation and apoptosis induction. The disruption of AJ therefore sensitize cell to Fas-mediated cell death (Fig.18). The protein scaffold Dlg1 was identified as one of the main interactors of Fas at the AJ through the PDZ-binding motif, present at the last amino acids at Fas C-terminus part. The binding of this protein to Fas contributes to the prevention of DISC formation and cell death (Gagnoux-Palacios et al 2018). Conversely, the presence of this complex could promote some non-apoptotic pathways.

#### 4.4.2 Lipid raft localization

Some fundamental events have been shown to be necessary for Fas-induced extrinsic apoptosis to occur. One of this is the clustering of Fas in specific membrane nanodomains, defined as lipid raft.

##### a. Fas in lipid raft: a step required for apoptosis induction

There are several lines of evidence describing Fas localization at the lipid raft. Some studies, including those from our laboratory, have identified a fraction of Fas constitutively located in the lipid raft, further corroborated by a high increasing of FADD and Caspase-8 at lipid raft upon FasL engagement (Hueber et al 2002, Eramo et al 2004, Henkler et al 2005, Chakrabandhu et al 2007). Other groups have found that Fas localization in the raft occurs only after ligand binding (Muppidi and Siegel 2004, Legembre et al 2006).

Importantly, the localization of Fas in these membrane domains is fundamental for a sufficient DISC formation in order to initiate the apoptotic signal. Treatment of the cells with detergent resistant membrane microdomain (DRM) disrupting agents was strongly inhibiting cell death. Moreover, point mutation that abrogates Fas palmitoylation, and therefore its accumulation at lipid raft, as explained below, is able to completely block Fas induced cell death (Chakrabandhu et al 2007). Fas aggregation is, furthermore, a prerequisite for appropriate cell death induction (Kischkel et al, 1995; Kamitani et al, 1997; Papoff et al, 1999; Siegel et al, 2004; Henkler et al, 2005).

Little is known about lipid raft role in promoting Fas death independent functions. However, Cruz *et al* demonstrate that expression of a Fas mutant that cannot localize in lipid raft, in Fas *lpr* mice model, can still properly prevent autoimmunity insurgence caused by *lpr* background and allow naïve T cell differentiation (Cruz et al 2016). This would imply that lipid raft localization is not an absolute prerequisite for Fas pro-survival functions. Further investigations are required in order to elucidate this area of research.

##### b. Prerequisites for Fas localization in lipid raft

###### Palmitoylation of Fas

Palmitoylation is a reversible post-translational modification that consists in the addition of a 16 carbon-palmitate moiety to a cysteine residue of a protein. This post-translational modification is found in transmembrane proteins or in intracellular proteins whose functions are close to plasma membrane, allowing their anchoring to the lipid bilayer, such as for Lck (pivotal member of Src kinase family in T lymphocyte) (Resh 1999, 2004). Palmitoylation of Fas on the Cysteine 199/194, in human and mouse respectively, is a necessary requisite for the localization of the protein in the lipid raft (Chakrabandhu et al 2007). It was indeed proved by our laboratory that a single point mutation on the palmitoylation site is able to block Fas aggregation, interaction with actin cytoskeleton and consequently internalization and cell death

(Chakrabandhu et al 2007). The palmitoylation of Fas was also shown to be important for its expression and stability at the plasma membrane (Rossin et al 2015).

#### **LRR motif**

Our team defined that the presence of lysines (K) at the beginning of the cytoplasmic domain (LRR motif) is an important feature for Fas lipid raft localization, since their mutations impairs its constitutive raft localization. This highlights the role of positive charged residues in stabilization of Fas in these structures (Rossin et al 2010).

#### **SMase activity**

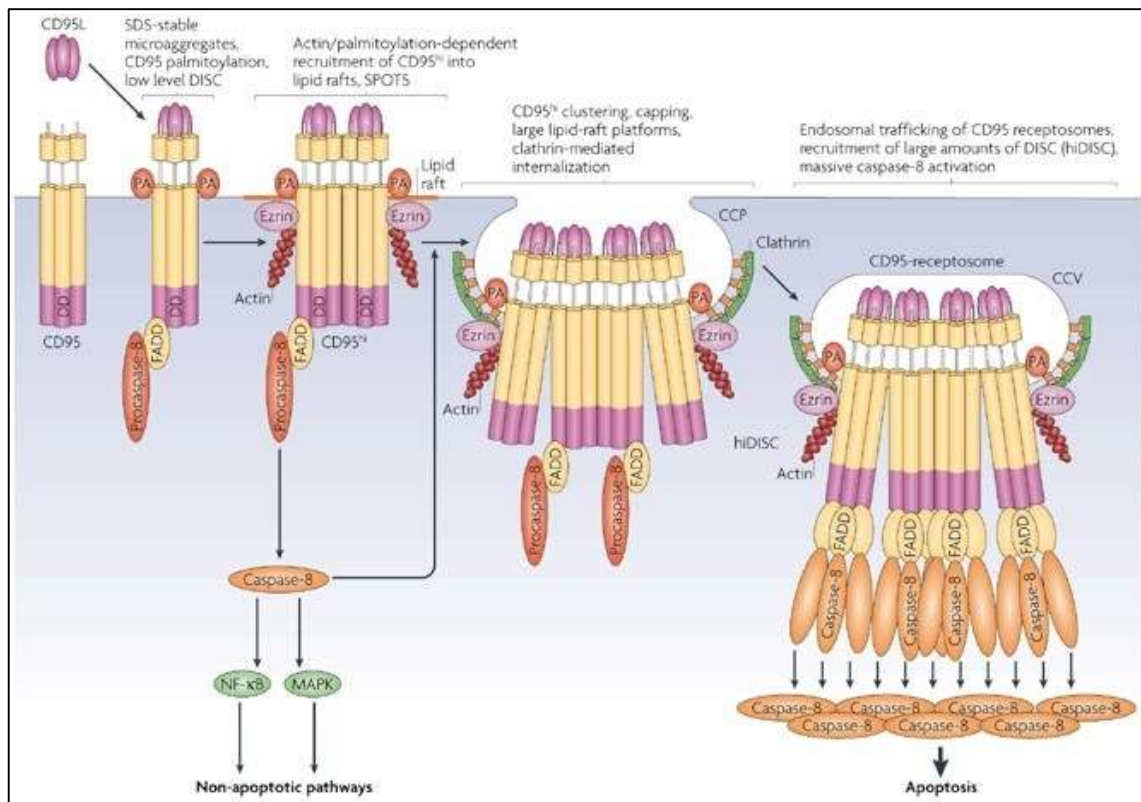
The role of ceramide (sphingolipid molecule consisting of a sphingosine and a fatty acid chain) in Fas aggregation and cluster formation at the lipid raft was evidenced since an increased level of sphingomyelinase (SMase), the enzyme that catalyzes its formation, was found in raft upon FasL engagement (Grassme et al 2001, Cremesti et al 2001). Deficiency in SMase indeed blocks Fas aggregation and cell death, a process that is restored by addition of exogenous ceramide (Grassme et al 2001).

#### **4.4.3 Fas internalization**

Receptor internalization by endocytosis is a major sorting step which allows regulating the receptor trafficking and signaling. Depending on its membrane localization, association with adaptor proteins and binding of a ligand, the receptor can follow different endocytic routes which affect the signaling outcome. It can be a constitutive process or induced by ligation of the cognate ligand. The functional meaning of receptor internalization can be multiple, such as the downmodulation of the surface expression, in order to limit a cellular response, or the amplification of a signal by the formation of an intracellular signaling platforms. Once internalized in endosomes, the receptors can be targeted to degradation to lysosomes or be recycled at the cell surface.

The role of actin cytoskeleton in receptor internalization in mammalian cells is well established. The actin monomers are directly involved in the later stage of clathrin-coated vesicles budding (Lamaze et al 1997). However, internalization route is not always associated to clathrin coating, but can be mediated by calveolae or even be a clathrin and calveolin independent process.

The group of Chan in 2006 described that clathrin-mediated Fas internalization is a fundamental step for a proper transduction of the apoptotic signals and that an efficient assembly of the DISC complex occurs after Fas internalization at endosomal level (Lee et al 2006). Disrupting filamentous actin indeed inhibits Fas induced apoptosis (Algeciras-Schimmich et al 2002, Peter et al 2003). Receptor internalization occurs upon FasL engagement through association of Fas with actin cytoskeleton via the ezrin protein (Fig.19) (Parlato et al 2000, Smith et al 2003, Chakrabandhu et al 2007).



**Fig.19 Model for Fas mediated apoptosis. Schutze et al 2008.**

Furthermore, the association of Fas to ezrin was proved to be dependent on Fas palmitoylation and therefore from the localization on the lipid raft (Chakrabandhu et al 2007, Rossin et al 2010). The model proposed is that palmitoylation stabilizes Fas to lipid raft and, upon FasL interaction, the receptor can be internalized thanks to its ezrin/actin association, allowing a sufficient amount of DISC formation at endosomal level. In this way the apoptotic cascade can be efficiently amplified and propagated (Chakrabandhu 2007).

Indeed, proteins that are involved in the rearrangement of actin cytoskeleton such as the Rho family protein Rac1-2, Rho and Cdc42 can directly have an effect on apoptosis susceptibility. Their involvement in remodeling cytoskeleton by interacting with the ERM (Ezrin/radixin/moesin) family sensitize T cells to Fas-induced apoptosis (Koncz et al 2008, Ramaswamy et al 2007). Furthermore, the phosphatase SHP-1 has a role in the regulation of actin cytoskeleton by dephosphorylating Vav1 protein, allowing its association with the actin filaments. Our group and others described an association of SHP-1 to Fas (Daigle et al 2002, Koncz

et al 2008). Upon Fas engagement, the SHP-1 mediated Vav1 dephosphorylation allows Fas/cytoskeleton association and the receptor internalization.

However, Fas internalization is not restricted to the induction of apoptosis but may be fundamental also to transduce pro-survival functions. The presence of a glycosphingolipid binding domain (GBM) between the CRD2 and CRD3 of Fas (Chakrabandhu et al 2008) play a role in the dynamic of multimolecular organization of Fas allowing internalization mediated by clathrin (CME). Mutation of this domain totally blocks Fas-induced cell death but allows the induction of non-death functions such as motility and proliferation involving a clathrin independent Fas internalization. Thus, Fas internalization route could be an important early event in the determination of Fas mediated cell fate. This hypothesis is also in line with an increase in phosphorylation of Akt and ERK upon FasL activation found for the LRR mutant which presents an altered cell death (Rossin et al 2010). Interestingly, the above cited LRR mutation impairs the lipid raft localization but not the internalization process demonstrating that non-death sustaining Fas internalization can occur outside the rafts.

#### **4.4.4 Fas phosphorylation**

As we described above, human Fas contains in its cytoplasmic domain two tyrosines that can be phosphorylated at the position 232 and 291. In the past 20 years, few contrasting reports were facing the role of Fas phosphorylation (Reinehr et al 2003, 2006, Daigle et al 2002). It was noticed that pY291 was increasing Fas-induced apoptosis in mouse neutrophils (Daigle et al 2002) and was associated with rat hepatocyte death. Even though, the rat Fas does not contain the equivalent of Y291 phosphorylation site (Reinher et al 2003). Several branches of research up to now have identified this post-translational modification as a switch mechanism to pass from the apoptotic to survival signals in tumoral context (Kleber et al 2008, Chakrabandhu et al 2016, Ta et al 2018, Gulculer-Balta et al 2019).

Our laboratory demonstrated that one phosphorylation (at position Y232 or Y291) is able to prevent DISC recruitment and that, on the other side, two dephosphorylations are needed for Fas mediated apoptosis to occur in CRC lines (Chakrabandhu et al 2016). We have also shown that pY291 promotes clathrin-mediated Fas internalization and cell migration, giving another clue to the hypothesis that Fas internalization is also involved in Fas pro-survival signaling. Furthermore, a recent report from our laboratory described that in CRC model the phosphorylation of Fas at 291 position bring to Fas/EGFR association. This event correlates with an increase of the EGFR signal by Yes-1/STAT-3 activation pathway and consequently cell proliferation and migration (Ta et al 2018).

Fas phosphorylation is mediated by the members of the SKFs (Chakrabadhu et al 2016). It was shown that the Fyn and Yes Src kinase proteins can interact with Fas (Atkinson et al 1996, Kleber et al 2008). What is the readout for Fas signaling from their interaction is still matter of debate, but it may vary depending on the cell

context (cell type and cellular condition). In the paper of 2016 from our laboratory it was proved that the inhibition of SHP members lead to an increased Fas-induced cell death and reduction of Fas mediated proliferation. The opposite results were obtained by the treatment of cells with an inhibitor of SHP-1 phosphatase establishing a role for this protein in dephosphorylating Fas (Chakrabadhu et al 2016). Other than the already described palmitoylation and phosphorylation, Fas is subjected to other PTMs that might affect stability, conformation and differential signaling pathway. This subject is efficiently reviewed by Seyrek and Lavrik in 2019.

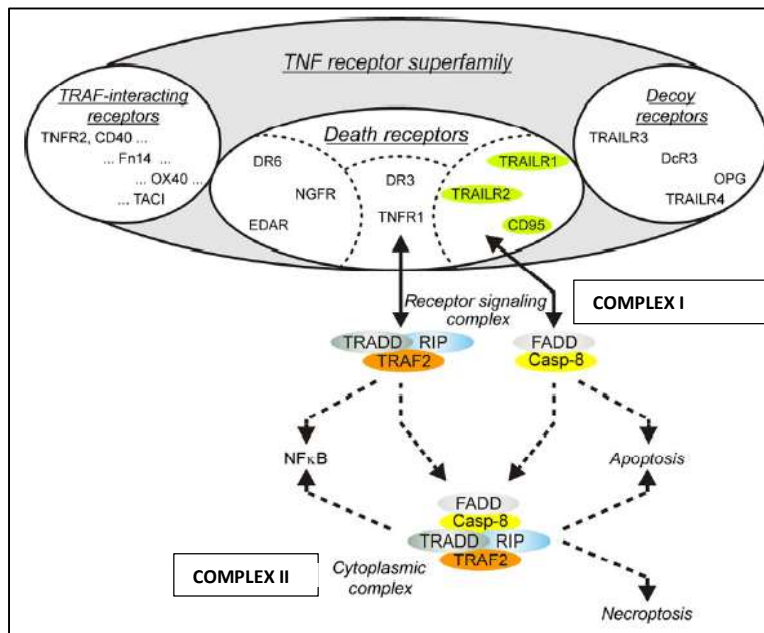
#### **4.5 Regulation of Fas/FasL signaling: proteins that modulate Fas signaling**

Multiple proteins are regulating Fas/FasL signaling, both at the level of the receptor and downstream, such as at the cytoplasm or at nuclear level, in order to modulate the kind of response.

##### **4.5.1 Role of DISC-interacting proteins**

Regulation of signaling complexes generated upon TNFSF/TNFRSF interaction is a finely controlled process that involves a high number of proteins, which regulates the switch of the death versus the non-death signaling. Association of proteins and regulation of their stability can influence the outcome of the signaling response (described below). Several ubiquitin ligases are part of these complexes and protein ubiquitination is a mechanism that the cells use to keep under control the threshold for activation of one pathway or the others (Huang Fu and Fuchs 2010).

Upon TNF ligation to TNFR1, two complexes can be formed: the complex I and the complex II. The first recruits proteins including TRADD and TRAF2 that activate the pro-survival signal NF- $\kappa$ B, and the second will turn this balance to apoptotic pathway by Caspase-8 recruitment (Micheau and Tshopp 2003). The formation of the two kind of complexes for TRAIL and Fas have also been established (Lafont et al 2017). However, most of the evidence in the characterization of these complexes are made for the TRAIL system and might be conserved also for Fas, even if few data have been reported so far. The main difference between complexes formed by TNF and TRAIL/FasL is that in case of Fas/TRAIL-R both complex I and II can mediate proapoptotic and pro-survival signals (Geserick et al 2009, Lavrik et al 2008). The complex I create a DISC complex at the membrane, the complex II instead is a cytosolic one deprived of the receptor (Fig.20) (Dondelinger et al 2016). This requires a further level of regulation that finally modulates the balance between the two responses.



**Fig.20 Schematization of signaling complex I and II in TNFR and TRAIL/Fas. Adapted from Siegmund et al 2017.**

**Death signals regulation by DISC-interacting proteins:** The first component of the chain is the adapter protein FADD by its homotypic interaction with the DD of Fas. FADD is subjected to degradative ubiquitination by Makorin ring finger protein 1 (MKRN1) (Lee et al 2016). Caspase-8 is instead regulated both positively and negatively by ubiquitination from Cullin-3 and LUBAC respectively (Jin et al 2009, Gonzalez et al 2012, Henry et al 2017). This is known anyway to be a late event compared to initial FADD/Casp-8 association, implying a role of these ubiquitin ligases in the termination of apoptotic signaling. Caspase-8 at the DISC can homodimerize or forming heterodimers with the protein FLIP (Kataota et al 2004, Riley et al 2015).

**The protein FLIP** was discovered by its sequence homology with Caspase-8 (Irmeler et al 1997, Goltsev et al 1997). Upon FasL binding to Fas the protein FLIP binds to FADD by its DED domain as the procaspase-8 (Scaffidi et al 1999, Krueger et al 2001). Up to now there is at least three different forms of FLIP protein described: FLIPL (Long 55kD), FLIPs and FLIPR (Short and Raji respectively 26-24kD) (Ewald et al 2011). The short forms of this protein (FLIPs and FLIPR) contain the DED domain, allowing the binding to the adaptor protein FADD, but lack the catalytic domain that is necessary to self-cleave and activation. For this reason these two forms have been defined as inhibitors of DISC competing with Caspase-8 binding to FADD and inhibiting the signal transduction cascade (Riley et al 2015).

The FLIPL form, instead, is structurally similar to Caspase-8, since it contains two death effector domains and a Caspase-like domain. However, this domain lacks residues that are important for its catalytic activity, most notably the cysteine within the active site (Scaffidi et al 1999). Its role therefore has been intensively investigated giving rise to controversial outputs concerning Fas mediated apoptosis (Ewald et al 2011, Safa



et al 2012). The ratio between FLIP and Caspase-8 at the DISC level was found extremely important in the regulation of the signal (Krueger et al 2001). In 2004, Boatright found that Caspase-8 has a higher affinity for FLIP<sub>L</sub> than for Caspase-8 itself, and so that FLIP<sub>L</sub> induces Caspase-8 recruitment at the DISC, explaining the increasing of this ratio at the DISC (Boatright et al 2004). Thus, this line of evidence indicates a role for FLIP<sub>L</sub> in enhancing the apoptotic process. However, other studies defined FLIP<sub>L</sub> as an inhibitor of the apoptotic process. FLIP<sub>L</sub> is able for instance to process itself and the procaspase-8 full length until the p43 form, so partially activating Caspase-8 but anchoring it at the DISC and so avoiding the releasing of the proactive forms in the cytoplasm (Tsuchiya et al 2015).

Furthermore, the E3 ubiquitin ligase Itch plays a role in decreasing the stability of both cFLIP<sub>L</sub> and cFLIP<sub>S</sub> enhancing the apoptotic signaling (Chang et al 2006, Yang et al 2010).

The enzyme Receptor-interacting serine/threonine-protein kinase 1 (**RIPK1**), known inducers of necroptosis, is present as a heavily ubiquitinated component and its ubiquitination is mediated by LUBAC (de Almagro et al 2015, 2017). RIPK1 contain a serine/threonine kinase domain, a DD and an intermediate domain. RIPK3 associate with RIPK1 and phosphorylates its downstream target MLKL, a substrate known to initiate the necroptotic pathway (Wang et al 2014, Holler et al 2000, Matsumura et al 2000). In cells bearing a normal Caspase activity the homodimer of Caspase-8 can cleave both RIPK1 and RIPK3, thereby limiting necroptosis occurrence (Tait et al 2014, Dondelinger et al 2016). Schematization of the regulation or pro-death signal mediated by DISC-interacting proteins is shown in the upper panels of Fig.21.

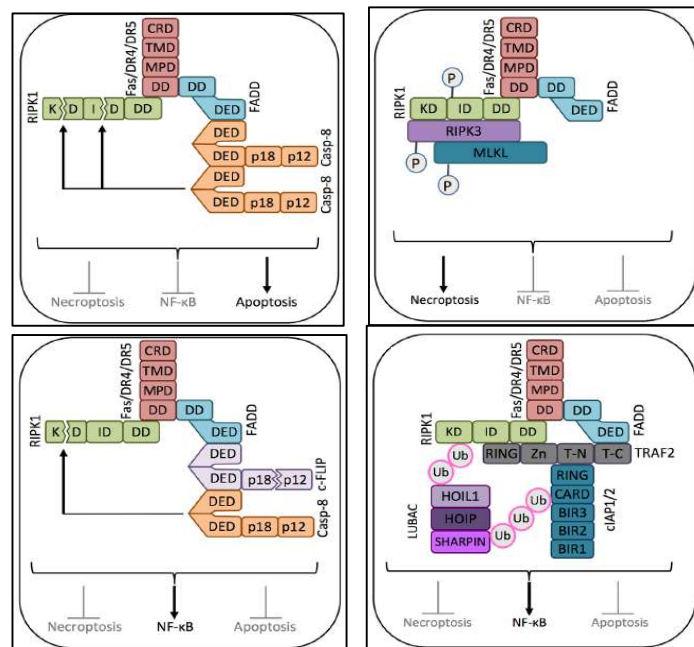


Fig.21 DISC-interacting proteins mediated regulation of Fas response. Adapted from O'Reilly et al 2016.

Additionally, recruitment of E3 ubiquitin ligase LUBAC is regulated by the presence of the **clAP1/2 protein**. These proteins of the IAP family have a well-established role of inhibitor of the apoptotic process (Labbé et al 2011). Indeed, the IAP family, of which XIAP, clAP-1 and clAP-2 are the main representants in inhibition of Fas induced apoptosis (Deveraux and Reed 1999, Holcik et al 2001) are potent Caspase inhibitors by interacting with them through their BIR motif (baculoviral IAP repeat) (Altieri et al 2010). They are inhibited by the activators of Caspases SMAC/ DIABLO at the apoptosome (Du et al 2000, Sun et al 2002). Their expression level in response of Fas stimulation may anyway variate in different cell types (Jost et al 2009).

**Pro-survival signals regulation by DISC interacting proteins:** As both complex I and complex II can mediate the two opposite signaling, it is suggested that the elements composing the pro-survival signaling platform are shared (Lafont et al 2017, Henry and Martin 2017, Jin et al 2006). However, for Fas the equilibrium of the main membrane bound complex I is shifted to the proapoptotic activity in contrast with the TNF/TNFR modulation which is, on the other side, promoting the activation of NF- $\kappa$ B (Reviewed in Guégan JP et al 2018).

FADD and Caspase-8 are indeed the first and crucial elements for the formation of the complex that will promote NF- $\kappa$ B induced gene activation and cytokines production as we already mentioned in the description of Fas pro-survival signals (Wajant et al 2000, Kreuz et al 2004, Cullen et al 2013, Lafont et al 2017, Varfolomeev et al 2005). Even though, the level at which these proteins contribute to the induction of gene activation is not known. The complex II acts, indeed, intracellularly, and therefore it might interfere with membrane proximal or more downstream events. Some groups evidenced a positive role of FLIP-Caspase-8 in enhancing FasL induction of NF- $\kappa$ B (Siegmond et al 2007, Imamura et al 2004).

Acting as antiapoptotic proteins, both TRAF2 and clAP1/2 promote FasL-mediated gene activation (Lafont et al 2017, Cullen et al 2013, Henry and Martin 2017, Trauzold et al 2005). clAP1/2 are necessary for LUBAC recruitment. LUBAC can promote the activation of the enzyme IKK, that mediate phosphorylation and degradation of I $\kappa$ B that sequestrate the active form of NF- $\kappa$ B in the cytoplasm. The bottom panels of Fig.21 show a schema of pro-survival regulation mediated by DISC-interacting proteins.

#### **4.5.2 Generic apoptosis inhibitor: anti apoptotic Bcl-2 family**

The generic apoptosis inhibitors of the Bcl-2 family participate in interfering with apoptotic signal. The main members are Bcl-2, Bcl-xl, Bcl-w, A1 and Mcl-1. They play a role in avoiding depolarization of mitochondria membrane, preventing apoptosome formation (Lacronique et al 1996). They act by sequestering the proapoptotic members Bak and Bax in a resting state. However, upon FasL engagement, BH3-only (pro-apoptotic) proteins bind Bak and Bax with higher affinity, inhibiting the role of the antiapoptotic Bcl-2 family proteins and starting protein release from mitochondria. Furthermore Bcl-2

proteins have been implicated also in other signalization ways, like the production of antiapoptotic proteins or activation of NF- $\kappa$ B (Mandal et al 1996).

#### 4.5.3 Other examples of Fas associated proteins in regulation of cell death and cell survival

**DAXX:** The adaptor protein DAXX was found to bind Fas receptor in the region of the DD, without possessing itself a DD. This association is mediated by the C-terminus region of the protein and does not necessary depend on FADD or Caspase-8.

The role of DAXX in Fas/FasL system has given multiple readouts in different contexts. It is involved in Fas-induced cell death by activating a non-canonical Fas signaling which includes the activation of the Ask1-JNK pathway (Chang et al 1998). Our laboratory could demonstrate that a recruitment of DAXX at the DISC level occurs, and that FADD and Caspase-8 are sensibly reduced in DISC in T cells in DAXX-Dominant negative transgenic mice (DN-DAXX Tg mice) (Leal-Sanchez et al 2007). An involvement of DAXX in the regulation of cell proliferation has been also suggested. Our laboratory demonstrated *in vivo* that the expression of a DN-DAXX form in Tg mice increased T cell proliferation. DAXX protein indeed, interfere with the normal signaling response after TCR activation, inhibiting the proteins involved in the activation-induced proliferation of the cells (Leal-Sanchez et al 2007). Other studies claimed an opposing effect on DAXX role in T cells, which would be dispensable for Fas induced cell death and on the contrary necessary for the stimulation of their proliferation. However, the results of this paper are giving a weak readout and are obtained in a not physiological setting, thus the process need to be further investigated (Li et al 2017).

**FAP1:** Not so long after the discovery of Fas protein structure, the extreme C-terminus region of Fas was found to have a role in the exacerbation of cell death. By looking at Fas C-terminal binding partners, the protein phosphatase FAP1 was found to bind to this part and to play a role in inhibiting cell death induction (Sato et al 1995, Li et al 2000). It is also interesting to note that the few amino acids involved to FAP-1 binding are not present in mouse Fas protein (Yanagisawa et al 1997). As described above in the description of Fas in AJ at page 41, also the protein Dlg1 bind human Fas in the same domain, underlying a species-specific regulation of Fas in the modulation of apoptosis (Gagnoux-Palacios et al 2018).

**BTK:** The non-receptor tyrosine kinase Btk was shown to have an inhibitory effect on Fas induced cell death in B cells through direct binding (Vassilev et al 1999, Tumang et al 2002). By looking at negative regulators of Fas-induced cell death in colorectal cancer model, our laboratory found that the binding of Btk to Fas works as a negative cell death regulator by recruiting PIP5K1 $\gamma$ . Thus, upon FasL activation, a PIP5K1 $\gamma$ -mediated production of PI(4,5)P2 has been detected, which might be involved in the generation of FasL-induced pro-survival signal cascade (Rossin et al 2017). Indeed, PI(4,5)P2 is second messenger involved in multiple signaling such as the increasing of cytosolic Ca<sup>2+</sup> and the activation of the PI3K/Akt pathway.

## **B. T cell receptor: structure, functions and signaling**

### **1. Generalities**

#### **1.1 TCR discovery and early studies**

First lines of evidence for the identification of the receptor able to activate T lymphocytes come from the early 1980s. In 1982, a study identified a tumoral antigen able to specifically activate a murine T lymphoma line (Allison et al 1982). The next year, in a study from Haskins *et al*, the researchers identified by antibody isolation the receptor present on the membrane of the T lymphocyte that was able to respond to the antigen exposed on the MHC of the APC (Haskins et al 1983). Because of its specificity for T lymphocytes it was called T cell receptor (TCR). By developing molecular approaches, two parallel teams were able to clone its cDNA in 1984 and defined the composing polypeptides as  $\alpha$  and  $\beta$  proteins (Yanagi et al, Hedrick et al 1984). Thus, it became soon clear that the  $\alpha\beta$  complex was responsible for the antigen binding, due to its homology with the immunoglobulins and high polymorphic nature. The fact that this identified receptor was responding, by activating the T cells, to OKT3 antibodies (directed against CD3 $\epsilon$  protein) led to the conclusion of the existence of a supramolecular complex including several subunits, all indispensable for the activation to occur properly (Borst et al 1984). Indeed, several cell mutants, coming from Jurkat cell line, were created, missing the  $\alpha\beta$  complex or other CD3 subunits and all failed to respond to antigen stimulation, implying a role for all these polypeptides in cell activation (Weiss and Stobo 1984, Abraham and Weiss 2004).

#### **1.2 Genetic localization and organization of TCR chains**

It is now known that the TCR can be constituted by  $\alpha\beta$  or alternative  $\gamma\delta$  polypeptides. The description of the composition of the TCR-CD3 complex will follow this short paragraph. At genomic level, the mentioned four chains of TCR are located in three distinct loci at the chromosomes 14 and 7 in humans. The  $\delta$  chain is actually residing in the the  $\alpha$  locus. All the loci contain different genes divided in segments: the Variable (V), the Joining (J) and the Constant (C). The  $\beta$  and the  $\delta$  contain also the Diversity (D) segment (Tonegawa 1987, Glusman et al 2001). The Constant region is constituted of four exons that encode four different domains: the extracellular IgG domain, the hinge, the transmembrane domain and the cytoplasmic tail. The organization of Variable, Joining and Diversity elements is a complex mechanism called V(D)J rearrangement since the genes composing the different V, D and J segments are casually rearranged and modified (Fig.22) (Matsuda et al 1998, Fugmann et al 2000, Lewis et al 1994, Gauss et al 1996). Casual recombination together with voluntary DNA deletions or insertion of palindromic or new sequences lead to the formation of extremely variable chains. This process, as it occurs for the formation of the immunoglobulins, allows the creation of a potential infinite number of different receptors, with consequently, an extreme selectivity and specificity for all the self and non-self antigens that they might encounter.

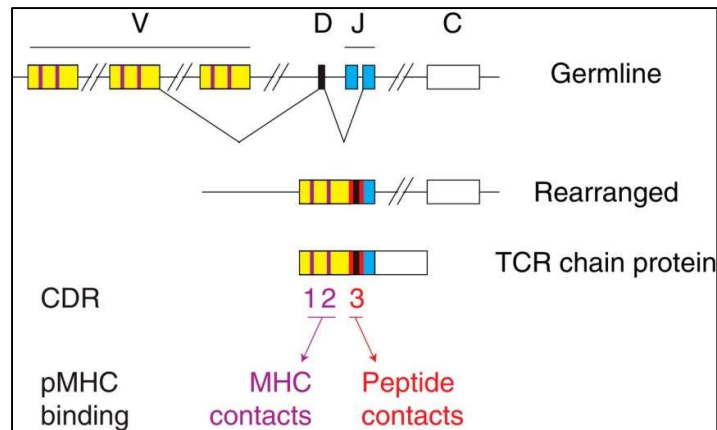


Fig.22 Schematic representation of TCR chain locus before and after rearrangement. Bosselut 2019.

## 2. Structure of TCR and partner proteins

### 2.1 TCR-CD3 complex

I will now describe the single elements composing the TCR-CD3 complex and how it is organized at the plasma membrane.

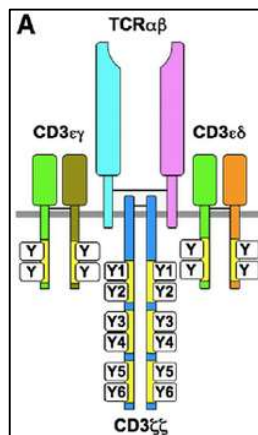


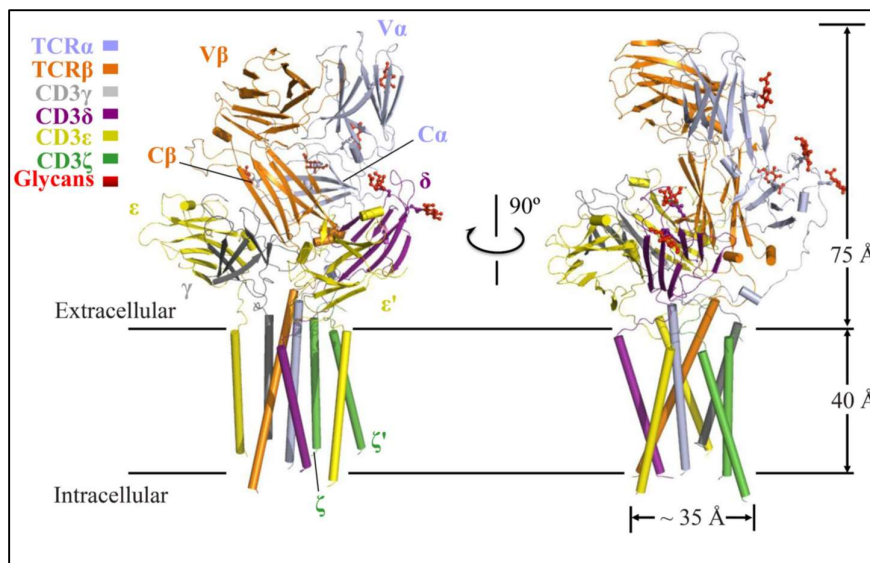
Fig.23 TCR/CD3 complex. Gelkop et al 2012.

The TCR-CD3 complex is constituted by clonally variable classical  $\alpha\beta$  or alternative  $\gamma\delta$  polypeptides covalently associated with different invariant CD3 polypeptides as shown in Fig.23 above. The  $\gamma\delta$  dimer is rare and is mainly expressed by a subclass of lymphocytes defined as intra-epithelial (from 1% to 5%). An important feature of this atypical TCR complex is that it does not require MHC presentation to be activated. Antigens activating the  $\gamma\delta$  heterodimer are largely unknown but might be lipid antigen or phospho-antigens (Holtmeier et al 2005, Jameson et al 2007). Phenomenon of antigen recognition and physiological role of TCR  $\gamma\delta$  are well reviewed in Deseke and Prinz work of 2020 (Deseke and Prinz 2020).

We will focus on the composition of the TCR  $\alpha\beta$ /CD3 complex. Four CD3 chains have been identified and respectively named  $\gamma, \delta, \epsilon$  and  $\zeta$  chains (Krissansen et al 1986, Gold et al 1987). Both  $\alpha$  and  $\beta$  TCR chains contain an extracellular domain (ECD) composed by a constant ( $C\alpha/C\beta$ ) domain, a variable ( $V\alpha/V\beta$ ) domain and by a membrane proximal connecting peptide (CP) region. In addition, the  $\alpha\beta$  chains contain a transmembrane domain (TMD) and very short cytoplasmic tail unable to transduce signal. The CD3 chains associate in dimers,  $CD3\gamma\epsilon/CD3\delta\epsilon/CD3\zeta\zeta$ . They are also composed of an ECD comprising an IgG domain and a short CP, a TMD and a cytoplasmic tail variable among the subunits. A common feature of the cytoplasmic tails of the CD3 subunits is the presence of one or more immunoreceptor tyrosine-based activating-motif (ITAM) responsible of the initiation of the activation signal upon phosphorylation. The conserved pattern of the motif is Tyr-X-X-Leu and they are separated from each other by 6 to 8 amino acids (Ivashkiv 2009). The  $\gamma, \delta$  and  $\epsilon$  subunits contain a single ITAM, whereas the  $\zeta$  chains have a longer cytoplasmic tail that includes three ITAMs. Mutations in ITAMs or variation of the spaces between two ITAMs abrogate TCR signal initiation defining a crucial role for these regions for the transduction of the activation signal (Irving and Weiss 1991, Romeo et al 1992, Wegener et al 1992).

### Structure of the TCR-CD3 complex

The TCR-CD3 complex is octameric and formed in a 1:1:1:1 stoichiometry composed by TCR  $\alpha/\beta$ :CD3 $\gamma/\epsilon$ :CD3 $\delta/\epsilon'$ :CD3 $\zeta/\zeta'$  (Call ME 2004 EMBO J). A recent paper from Dong *et al* solved the structure of the unligated complex by cryo-electron microscopy. The authors confirmed the cited stoichiometry and specified the assembly of the ECDs and TM domains of the complex (Fig.24) (Dong et al 2019).



**Fig.24 Reconstruction of human TCR/CD3 complex. Dong et al 2019.**

The structure of the TCR-CD3 complex was described as an ice cream cone, with the  $V\alpha V\beta$  of the TCR $\alpha\beta$  protruding in the most external part of the receptors and the  $C\alpha C\beta$  part of the heterodimers forming stable

contacts with the ECDs of the CD3  $\gamma/\epsilon$  and CD3 $\delta/\epsilon'$  subunits in a three-fold symmetry. Furthermore, the TMD of the complex contains 8 helices forming a helix bundle-like structure, where the TCR $\alpha\beta$  helices are located in the middle and surrounded by the TMDs of the CD3 subunits (Dong et al 2019). The intracellular regions of the CD3 subunits were poorly defined by this technique because of the high dynamic nature of these domains (described below at page 61).

## **2.2 Major histocompatibility complex and antigen presenting cells**

One vital prerequisite to allow antigen presentation to T lymphocytes is the expression, on plasma membrane of other immune cells, of the MHC, a surface protein heterodimer that can expose on its most distal extracellular part the processed peptide that will activate the matching TCR. In human, these molecules are also called Human Lymphocyte Antigen (HLA). Two classes of MHC molecules exist: the MHC class I (HLA-A, HLA-B, and HLA-C) and MHC class II (HLA-DP, HLA-DM, HLA-DOA, HLA-DOB, HLA-DQ, and HLA-DR). The two kind of complexes are composed by the non-covalent association of two polypeptide chains. In MHC class I, these chains are identified as  $\alpha$ , bearing the pocket responsible for the peptide binding, and  $\beta$ -microglobulin (Saper et al 1991).  $\alpha$  and  $\beta$  chains of around 30kD constitute the MHC II complex where, conversely, peptide binding site is shared by the two polypeptide chains (Wang et al 2011).

The extremely high variability of this complex is due to the pocket region in the extracellular part which contains polymorphic amino acids and is, therefore, the domain responsible for the binding of the processed antigen. The peptide binding on the MHC complex occur in the assembly phase and is responsible for the stability of the complex at the surface of the cells, which can have a long half-life in order to allow the encounter and recognition from T cell. MHC molecules are not able to discriminate between self and non-self antigens, a process that is mediated by T cells and acquired during their maturation phase in the thymus. Two Ig-like domains mediate their binding on the plasma membrane and the recognition site for the T cell by the co-receptors CD4 and CD8, in MHC II and MHC I respectively (Saper et al 1991, Gao et al 1997, Wang et al 2011) thus defining two distinct roles.

The MHC class I is expressed by most of the nucleate cells and platelets. MHC I molecules present the peptides derived from endogenous protein antigens formed by proteolysis of non-self cells (as tumoral or virally infected cells) to CD8+ lymphocytes. MHC class I expression is finely controlled by the external environment, such as the presence of cytokines like IFN- $\alpha/\beta$  or  $\gamma$ , which augment their expression in response to a viral infection.

On the other side the MHC class II molecules are involved in the phenomenon of cellular cooperation for the immune response and are expressed by the APC populations to activate CD4+ helper T lymphocytes. The peptide that is loaded on this type of MHC molecule indeed derives from extracellular environment by endocytosis or phagocytosis. Different types of myeloid cells can process and expose the self or foreign

antigens in order to allow T lymphocyte activation. The main myeloid subpopulation that plays a role in the antigen exposure is the dendritic cells but this role can be accomplished also by macrophages and B lymphocytes through a mechanism that involves phagocytosis and selection of short pathogen peptides processed and loaded on their surface (Hivroz et al 2012, Janeway et al 2001a, 2001b). As for MHC I, the level of MHC II molecule expression can be modulated by IFN $\gamma$  or by stimuli of specialized molecules that bind the antigen, such as the Toll-like receptor and the antibodies on B lymphocytes surface (Aderem and Underhill 1999, Stern et al 2016).

### **2.3 CD4 and CD8 co-receptors**

The CD4 and CD8 molecules are often called co-receptors as they stabilize the TCR-peptide-MHC (TCR-pMHC) interaction. Indeed, whereas they are not directly connected to the TCR-CD3 complex, they establish upon TCR engagement an association with the MHC complex on the APC. As we mentioned above, the CD4 receptor is specific for MHC class II binding and conversely the CD8 receptor binds the MHC Class I of APCs (Gay et al 1987, Gallagher et al 1989). For a detailed description of the structure and binding to the MHC complex by these co-receptors the readers can refer to the review of Li et al of 2013.

These two co-receptors are specific markers for the T cell subtypes. CD4 co-receptor is expressed by the T cell lineages which will give rise to T helper population (that is consequently often named CD4+ T cells). Conversely the CD8 co-receptor is expressed at the surface of the T cells that differentiate in the cytotoxic T lymphocytes (that are consequently often named CD8+ T cells).

The two co-receptors are known to bind the Src kinase protein Lck and to play a strong contribute to the initial stages of T cell activation (Veillette et al 1988, Barber et al 1989). This part will be further described below at page 63.



### 3. TCR activation in physiology

The TCR-pMHC interaction is involved in both the processes of T cell maturation/development and in the activation of the mature T cells.

#### 3.1 T cell development

The generation of mature T lymphocytes ensues two main steps. The production of immature T lymphocyte occurs, as for the other cells of the immune system, in the bone marrow. Once these progenitors have been produced, they translocate in the thymus (they are then called thymocytes) where the process of selection allows the production a mature T cell as shown in Fig.25.

Immature T cells have to pass through three different developmental stages: the double negative (DN, CD4- and CD8-), the double positive (DP, CD4+ and CD8+) and the single positive (SP, CD4+ or CD8+) stages. the DN T cells first reach the DP stage where they can initiate a maturation process through the interaction of their TCR with self-antigens presented by MHC on APC (Klein et al 2014). The affinity of the antigen for the TCR is the parameter that determines the fate of the thymocyte selection defining their specificity and selectivity.

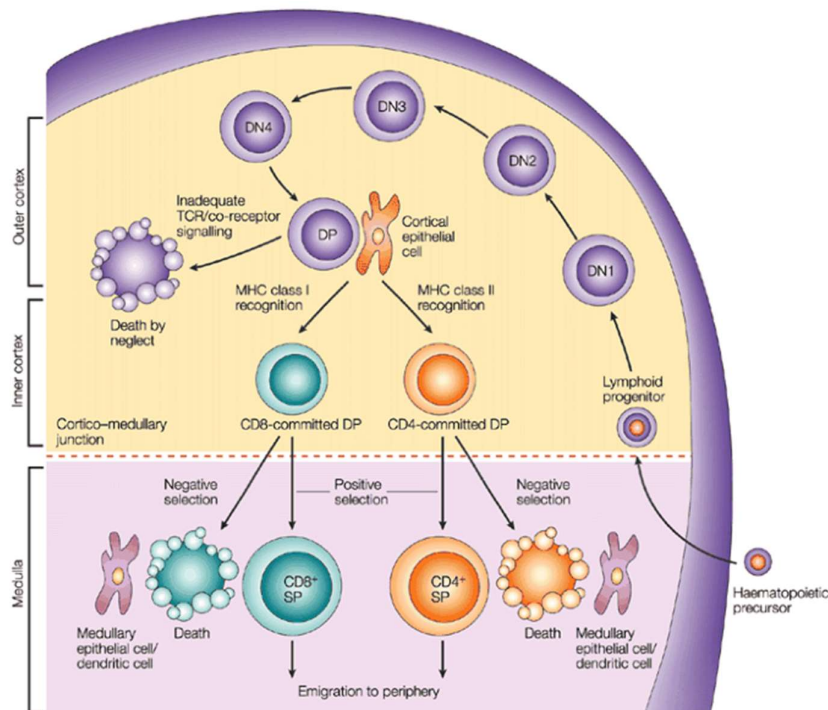


Fig.25 T cell development in the thymus. Germain 2002.

There are two main passages that assure the formation of a functional mature T cell. These are called positive and negative selection. The self pMHC-TCR association has a minimum and maximum threshold of affinity. If the threshold is higher than the minimum level it allows the transduction of the signal through the TCR and

thus the survival of the cells and their “positive selection”. If the level of affinity to the self-pMHC is lower than the minimum threshold or the T cells fail to engage the pMHC complex, they undergo programmed cell death (Klein et al 2014). This positive selection allows, therefore, the transition to the SP stage. Thymocytes then undergo a “negative selection”: if the affinity of the pMHC-TCR binding is too high and overcome the threshold level, the strong TCR activation induces their death by apoptosis.

Thus, cells that successfully pass these two selection phases, that are less than the 5% of the initial amount, can be delivered to the peripheral tissues and are considered as mature naïve T cells (T<sub>N</sub>). At this stage, the TCR has an adequate specificity, which means the ability to recognize the molecules of non-self, and selectivity, a condition that allow the discrimination from the self-antigen. This selection process allows the establishment of the self-tolerance and defects in the self-tolerance are the cause of the production of autoreactive T cells and autoimmune diseases.

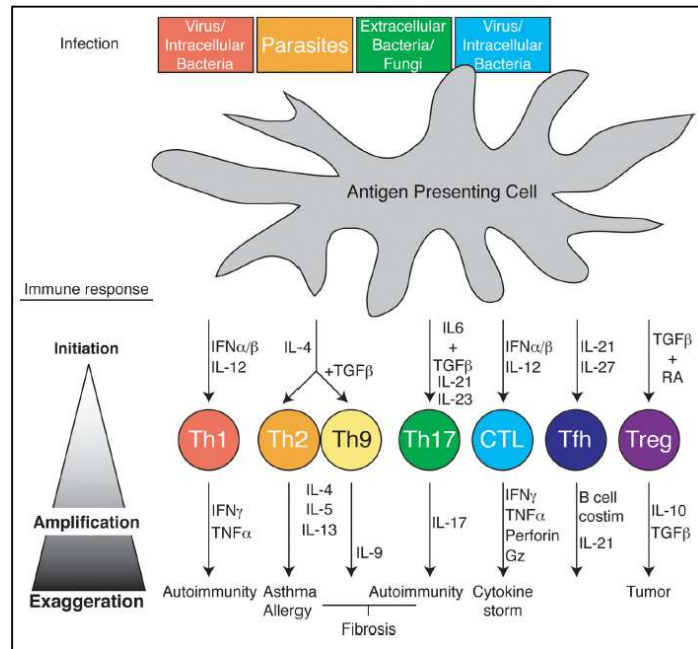
### **3.2 Mature T cell activation in the periphery**

When a mature naïve T cell encounters its specific antigen presented by MHC complex in the peripheral compartments, the processes of proliferation and differentiation are initiated. Optimal TCR activation culminates with the transcription of an entire set of genes. Thus, the upregulation of numerous proteins is achieved and among them several cytokines such as IL-2, TNF $\alpha$  and IFN $\gamma$  play an important role in sustaining cell survival, proliferation and selective differentiation programs (Openshaw et al 1995, Rothenberg et al 1996). This leads to a fast proliferation stage defined as “clonal expansion” (Cho et al 1999, Mosmann et al 1989). Some of these up-regulated proteins start to be expressed at the plasma membrane and are used as markers to define the activation state of the cells, such as CD69, CD44 and CD45RO, and co-signaling molecules such as the receptors ICOS and PD-1 as well as some of the members of TNF/TNFR SF, Fas and FasL included (Lopez-Cabrera et al 1993, Budd et al 1987, Picker et al 1993, Hutloff et al 1999, Trauth et al 1989).

Indeed, if the TCR activation efficiently promotes downstream signals that leads to activation of the program for gene transcription, this ends also into differentiation of the activated naïve cell in one of the subpopulations of effector or memory cells. As we mentioned at the beginning of the manuscript, once the infection has been defeated, the T cells that have expanded start their contraction phase. Most of the cells will be committed to programmed cell death (PCD) and a small subset will instead form the population of memory cells, which will efficiently and rapidly respond to a second encounter with the same antigen (Strasser et al 2009, Li et al 2006, Garcia et al 1999).

Thus, both naïve CD4<sup>+</sup> and CD8<sup>+</sup> cells differentiate in effectors cells and a small proportion in memory cells. CD8<sup>+</sup> effector cells are mainly the cytotoxic T lymphocyte population (CTL), which exert their function by killing the target cells, as we have described in the first chapter of this manuscript (page 14).

On the other side, the CD4+ effector cells are the helper T cells that comprise multiple categories which exert different and specific class-related roles. The main subclasses that have been identified so far include: the Th1, Th2, Th9, Th17, Tfh and Treg cells.



**Fig.26 Cytokines involved in T cell differentiation and produced by T cell subsets. In the bottom part are mentioned the main diseases occurring from exaggerate response of the effector cells. Pennock et al 2013.**

Memory cells can also be divided in two main categories: The effector memory ( $T_{EM}$ ), responsible for the first rapid response to a second stimulation but with low proliferative capacities and the central memory ( $T_{CM}$ ), which in response to the signals received from the effector memory cells start to proliferate at high rate in order to guarantee and efficient response to the known pathogen. The detailed description of the multiple differentiation programs of effector and memory cells is out of the scope of this manuscript but can be found in a review by Pennock et al 2013.

The differentiation program is influenced by several factors that determine in which population the cells will be oriented. Some of these factors have been determined and are, but not limited to: the cytokines present in the environment (that can depend on the pathogen that cause the infection and the populations of immune cells activated by it), other inflammatory and immunomodulatory products and tissue-specific factors (Openshaw et al 1995). Examples of cytokines that influence the differentiation in the Th subsets and their main cytokines secreted upon completing the differentiation program are summarized in the Fig.26.

Other important factors have been described to affect the differentiation program, such as the strength and dwell time of TCR activation, but also the affinity to self-peptide of the TCRs, intracellular proteins that

modulate the TCR early signaling and the presence of other surface receptors, that if ligated with their cognate ligand can influence the potential of differentiation (Snook et al 2018, Sood et al 2019, Aki et al 2018, Meyer zu Horste 2018, Shen et al 2019). These last ones, also defined as TCR co-signaling receptors or immune checkpoints, such as ICOS, CD137 and OX-40, gained in the last years increased importance, since it was proved that their modulation is one of the main determinants of the T cell fate. For example, ICOS receptor was associated to the promotion of T<sub>fh</sub> effector population and maintenance of memory T cells (Weber et al 2015, Choi et al 2011). Furthermore, as we already described in the first chapter, Fas can promote both the shift of differentiation in Th17 and Th9 populations compared to the Th1, and that can also cause the precocious differentiation of naïve cells in the memory subset (Klebanoff et al 2016, Meyer zu Horste et al 2018, Shen et al 2019). A deep analysis of TCR co-signaling receptors will be detailed in the next chapter.

#### **4. TCR signaling**

Briefly, the transduction of the signaling starting from the antigen recognition by the TCR begins with the phosphorylation of the ITAMs on the CD3 tails. This phosphorylation event is mediated by Lck, main member of the Src kinase family in T cells. Lck activation and ITAM phosphorylation lead to activation of ZAP70 and LAT proteins. Activated LAT can bring to the formation of a big signaling platform which enlarge the network of activated proteins. The creation of the signaling platform allows the activation of Vav1, PLC $\gamma$ , and other intermediates and subsequent promotion of the main activation pathways, such as MAPKs and NF- $\kappa$ B, that in turn culminate to gene transcription and functional events as described above.

However, if the main actors have been identified, the dissection of the molecular mechanism involved in the signal transduction revealed complex regulatory steps which are still not fully elucidated.

##### **4.1 Initiation of the signal**

The initiation of the TCR signaling is a critical point that has to be tightly regulated to avoid inappropriate T cell activation and immune response. The complex regulatory mechanism controlling the antigen discrimination by the TCR and the generation of an intracellular signal upon the pMHC-TCR interaction are still not completely understood and several models have been proposed.

##### **4.1.1 Ligand discrimination based on the sensing of TCR affinity**

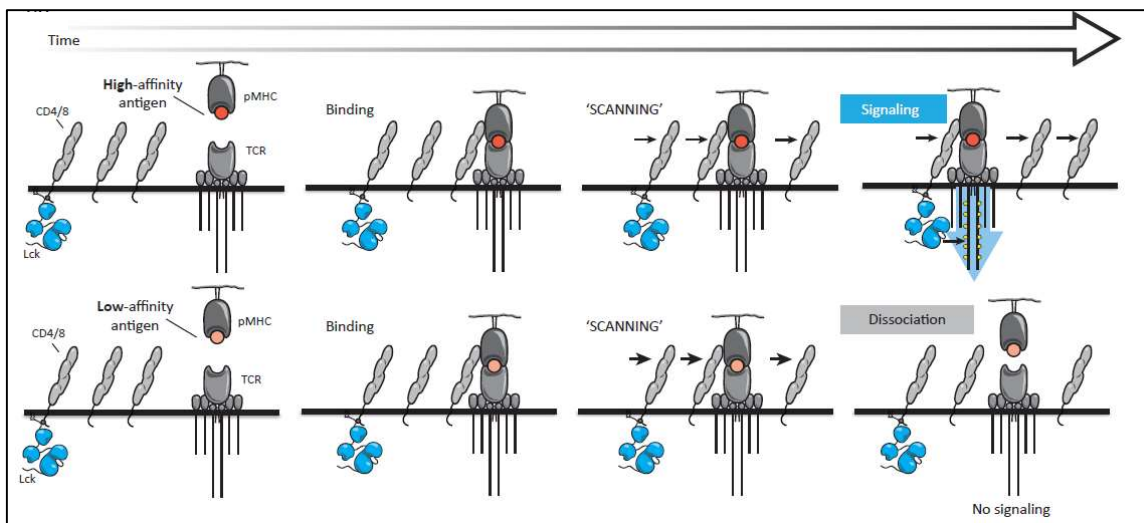
The strength of pMHC-TCR ligation has to reach a certain threshold to initiate a signal that will finally lead to cell activation (Love et al 2000, Stephen et al 2009, Klein et al 2014). It is indeed known that the foreign antigens can have a strong or a weak affinity for the TCR, and thus a way to guarantee a good level of activation have to overcome the weak initial contact between pMHC and TCR in some cases. Furthermore, on peripheral APCs are present also self-peptides with similar affinity and more abundant than foreigner

antigens (Birnbaum et al 2014). How can the T cells avoid activation from the high number of self-antigen and instead allow the one from the low number of foreign antigens?

We have to take into account the main features that distinguish the T cell receptor from a conventional membrane receptor: the high level of sensitivity, selectivity, versatility and structural diversity (Sykulev et al 1996, Irvine et al 2002, Ernst et al 1999, Viret, et al 1999, Schamel et al 2006, Garcia et al 1999, Marrack et al 2008).

We have already mentioned above about the meaning of sensitivity and selectivity so the capacity to recognize the few molecules of agonist peptides (foreign) and the one of discriminating them from the noise produced by the self-peptide respectively (Irvine et al 2002, Ernst et al 1999, Viret et al 1999). The versatility is the capacity of the TCR to give a different response depending on the affinity thresholds (Starr et al 2003). The structural diversity is based on the nature of the binding interface. Even if common features can be found in pMHC-TCR bindings, the diversity at the interface is at atomic level, which make any kind of binding unique (Marrack et al 2008, Rudolph et al 2006).

The proofreading kinetics and the serial engagement are models that describe the regulation of antigen discrimination. Noteworthy these models might coexist.



**Fig.27 Co-receptor scanning model. Courtney et al 2018.**

- Briefly the proofreading kinetics model comprises events that sense the threshold of affinity and promote or inhibit the start of activation. They are based on the co-receptor scanning model, where CD4/CD8 coreceptor scan several TCR-CD3 complexes and by their binding with Lck can allow the initial ITAMs phosphorylation (Fig.27). Another level of regulation is based on the strength of binding between Lck and one of its target ZAP70, which is the following molecule activated after CD3 phosphorylation (McKeithan et al 1995, Rabinowitz et al 1996). Other events, such as the

phosphorylation of specific LAT residues, are all involved in the control of the threshold affinity for activation, and the rapidity of the events before dissociation of the pMHC-TCR complex would promote the proper activation.

- The serial engagement model propose that a pMHC can bind several TCR-CD3 complexes in order to overcome the threshold limit for the activation, or on the other side, the pMHC can rebind to the same TCR-CD3 complex increasing the half-life to allow the reaching of the threshold (Valitutti et al 1995).

#### **4.1.2 Models for signal initiation**

One of the main questions that remain unsolved is how the contact between the antigen presented by the MHC complex and the TCR ECD can generate a signal that allows the molecular cascade to begin. Multiple models have been proposed and they do not mutually exclude each other, as their cooperation has been described in some specific circumstances. The three main models will be discussed here and are: the conformational change model, the aggregation model and the segregation model.

##### **a) The conformational change model**

The first event that has been reported upon TCR triggering involves the Lck-driven ITAM phosphorylation of CD3 cytoplasmic tails. The conformational change model has as main consequence the variation of the localization of these ITAMs that are “safely caught” in resting conditions and start to be exposed upon TCR engagement by a reorientation that renders their phosphorylation sites available.

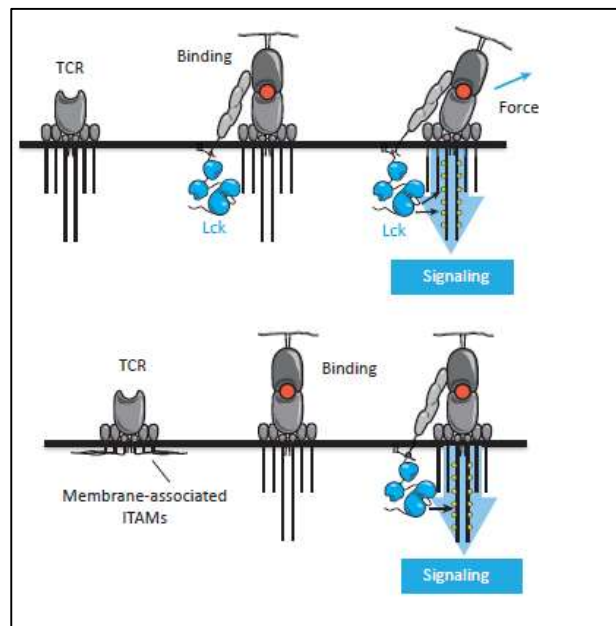
A conformational change of the cytoplasmic tail of the CD3 subunits has been observed for the CD3 $\epsilon$  and CD3 $\zeta$ . It was described that, in resting condition, the cytoplasmic tails of CD3 $\zeta$  and CD3 $\epsilon$  are retained in the lipid layer by the presence of basic amino acid residues (BRS) and that the TCR triggering would induce their release in the cytoplasm (Fig.28 bottom) (Aivazian et al 2000, Xu et al 2008, Deford-Watts et al 2009, Kuhns and Davis 2008).

However, the question of how the TCR triggering can cause the conformational change of the CD3 cytoplasmic tail remains unsolved. One of the explanations is based on the response of TCR to pulling mechanical forces. We will describe here two of them: the piston-like movement and the deformation of the receptor.

- External forces applied on the receptor upon T cell-APC contact could induce a piston-like movement that in turn induces alteration of the conformation of TCR-CD3 complex that allows the exposure of the CD3 ITAMs to the cytosolic environment and/or cause variation in the conformation of TCR-CD3 ectodomain allowing aggregation of several complexes. It has been shown that depending on the

nature of the Ag-TCR binding the external force applied can cause a “catch bond” effect, when high tension increases the strength of the bond, or on the other side a “slip bond” that cause instead a dissociation with the increasing of the strength of the force (Liu et al 2014).

- The force is caused by the movement of the T cells on the surface of the APC upon binding. This would cause a conformational change in the ectodomain and/or transmembrane domain of the TCR that would be responsible of the following variations. This model has been indeed called “receptor deformation model” (Fig.28 upper). The TCR triggering can occur only if the force for conformational change is less strong than the force that causes the dissociation of the complex (Ma et al 2008). Due to the lack of a resolved structure of the entire pMHC-TCR complex it is still elusive if a conformational change of the ECD or TMD of TCR-CD3 complex occurs upon antigen contact. (Garcia 1999, Reiser et al 2002, Feng et al 2017, Das et al 2015, Dong et al 2019).



**Fig.28 Receptor deformation (upper) and CD3 tail conformational change (bottom) models. Courtney et al 2018.**

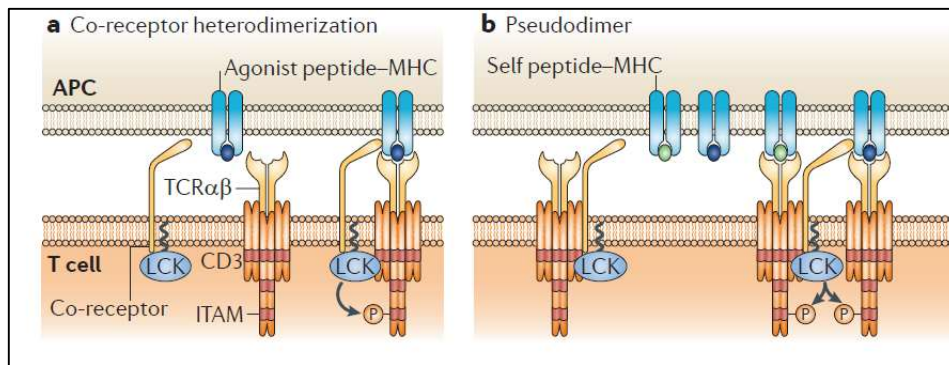
The bias of these models is that they do not explain the data that reported the activation event also by utilization of soluble antibodies that thus, would not apply these mechanical forces.

### **b) Aggregation model**

The TCR ectodomain conformational change has been also proved to be important for receptor clustering (Kuhns et al 2010), which would increase the strength of the Ag-TCR binding to reach the minimal threshold that activate the signal. In line with this, it was proved that high affinity binding induces receptor clustering (Pageon et al 2016), defining an important role of receptor aggregation for induction of the signal. It is indeed intuitive that aggregation of receptor would cause an increase of the signal, even just allowing

the transphosphorylation of the different ITAMs by associated Lck. It is also proved that the presence of multimeric pMHC enhances the signal initiation even in soluble form by forcing TCR clustering (Cooper et al 2008). What is instead less clear is how a single agonist pMHC-TCR binding with low affinity is able to initiate the signals. The co-receptor heterodimerization and the pseudo-dimerization have been proposed as main models (Fig.29).

- In the co-receptor heterodimerization model the co-receptors CD4 and CD8, in mature CD4+ and CD8+ T cells respectively, flank the TCR-CD3 without forming any contact. Upon antigen engagement, they establish a contact with the pMHC. The cytoplasmic tail of these co-receptors interacts directly with Lck. Thus, they actually recruit Lck to the TCR-CD3 complex, leading to ITAM phosphorylation and propagation of the signal. Nevertheless, TCR triggering was found to occur even in absence of co-receptors (Locksley et al 1993, Schilham et al 1993).



**Fig.29 Schematization of aggregation models. van der Merwe and Dushek 2011**

However, this model has been questioned. Casas *et al* could demonstrate in a mouse model and in transgenic mice T cells that TCR triggering occurs in two stages: there is a first pMHC-TCR interaction that leads to an initial CD3 phosphorylation mediated by free Lck (not bound to CD8) that allows CD8 association to TCR-CD3 complex in a MHC independent manner. This is followed by CD8 binding to pMHC I that stabilizes the complex (Casas et al 2014).

- The pseudo-dimer model, on the other side, imply the contribution of self-pMHC-TCR binding in helping to reach the threshold for signal activation of a low affinity agonist antigen (Irvine et al 2002, Krogsgaard et al 2005).

### c) Segregation model

In the kinetic segregation model, adaptor proteins and co-receptors with short extracellular domain (for example the co-receptor CD2) are involved in the formation of the first contacts between the APC and the T cells. There is a spatial reorganization of the surface proteins next to the interface. The TCRs that are engaged



by pMHC start to be in a close contact zone, defined as “diffusion trapping” structure, and the ones that are not engaged are free to diffuse in the plasma membrane. Co-receptors with long extracellular chain remain excluded from this aggregation zone (van der Merwe et al 2000). Among these segregated proteins, the CD45 phosphatase, that can have an inhibitory role in the initiation of the cascade, has been described (Imbert et al 1994, Secrét et al 1993).

Other than the kinetic segregation, the role of the variation of the lipid microenvironment around TCR has been evidenced by immobilizing and clustering the TCR-CD3 complex. Thus, TCR-CD3 complex upon engagement is redistributed in lipid raft nanodomains, a region enriched in proteins such as Lck, coherent with the increase of the initial signaling (Horejsi et al 2014, Gaus et al 2005, Zech et al 2009, Harder et al 2009). However, some discrepancies have been found in literature for this model and so it needs to be corroborated with further approaches (Munro et al 2003, Hashimoto-Tane et al 2010).

#### 4.2 Molecular dissection of T cell activation

The signaling cascade that starts from the membrane allows the sequential activation of Lck, CD3 chains, ZAP70 and LAT. Signalosome formed at LAT level create a platform and amplify the signal transduction to promote downstream events. A schema of the main pathways activated by TCR signaling is shown in Fig.30. Proteins are involved in both fulfilling their main function (catalytic or scaffold) and regulating in a feedback loop the previous and following interactions, thus sustaining and/or inhibiting the signaling.

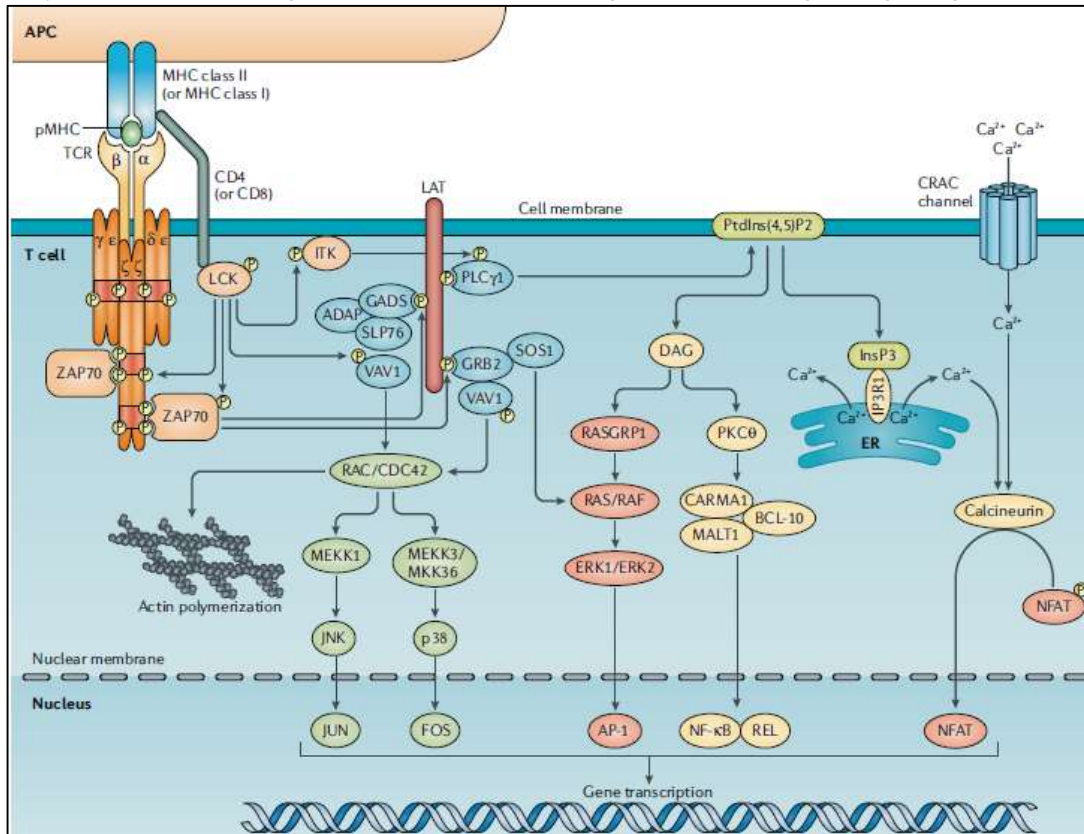


Fig.30 Major TCR signaling pathways. Gaud et al 2018.

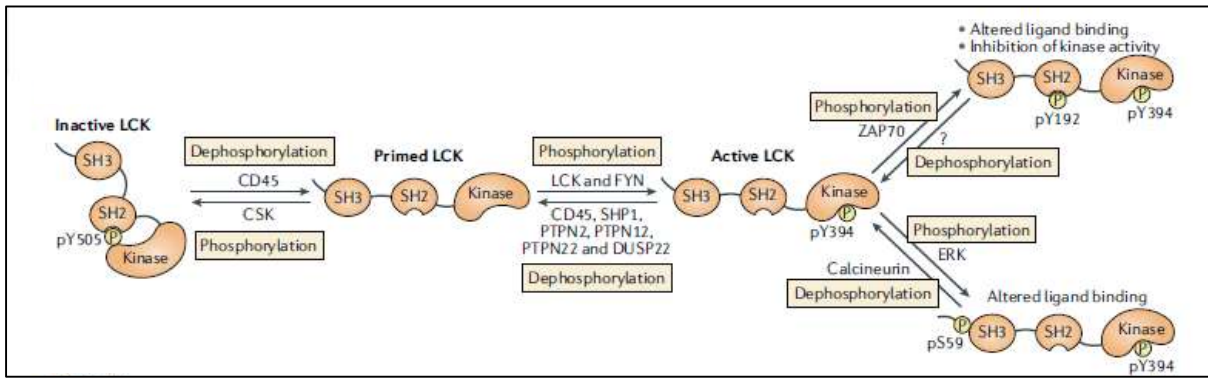
#### 4.2.1 Lck activation and regulation

Lymphocyte-specific protein tyrosine kinase of 56kD (p56Lck) is the main Src kinase protein expressed in T lymphocytes. The protein contains a SH3 domain, a SH2 domain and in the C-terminal part the tyrosine kinase domain. In addition, two post translational modifications of its N-terminal tail (myristoylation and palmitoylation) allow the tethering of the kinase to the plasma membrane. Lck was found to initiate the cascade activation upon TCR engagement through its catalytic activity by phosphorylating the ITAMs of the CD3 cytoplasmic tails. The activity of Lck is tightly controlled by the phosphorylation on different tyrosine present in its amino acid sequence.

The catalytically active Lck is phosphorylated on the Y394 which promote an open conformation. On the other side phosphorylation of Y505 inactivates it promoting a close conformation that masks Lck catalytic site (Stirnweiss et al 2013, Philipson et al 2017). Lck can auto-phosphorylate its Y394 to promote the open conformation.

Even if its structure is well defined, how its conformational change and post-translational modifications are affecting its kinase activity is still a matter of debate. It has been described that a pool of active Lck (around 40% of the total molecules) is already present in resting condition giving no real role of the TCR activation in the level of Lck phosphorylation. Both the localization of the protein with the co-receptors CD4/CD8 and the conformational changes on CD3 tails occurring upon TCR ligation might explain why this active pool is not phosphorylating the ITAMs in resting condition (Nika et al 2010). Conversely, other groups have found that the fraction of Lck protein phosphorylated in resting condition is much lower (around 2%) and that TCR engagement would allow the increase of phosphorylation of its active site and promotion of the open conformation responsible of the ITAMs phosphorylation (Simeoni 2017). Upon ITAM phosphorylation, Lck recruits ZAP70 ( $\zeta$ -associated protein of 70kD) by direct interaction and phosphorylate it promoting its binding to CD3 $\zeta$  ITAMs (Chan et al 1992). The role of ZAP70 will be briefly discussed after this paragraph.

Proteins that regulate the phosphorylation of the different tyrosine of Lck have been described. The kinase CSK is acting as a negative regulator by phosphorylating the Y505 and promoting the folding and inactive state of the protein. The phosphatases of the family PTPN, such as SHP-1 (page 75 for more details), have been also involved in negatively regulating Lck activity by dephosphorylating the Y394 (Pao et al 2007). Moreover, the phosphatase CD45 acts both as activator and repressor by dephosphorylating both tyrosines (Courtney et al 2019). Noteworthy, variation of CD45 expression modifies the pool of active Lck.



**Fig.31 Model of Lck activity regulation. Gaud et al 2018.**

Another Lck phosphorylation site, the Y192, have been shown to act in the modulation of Lck activity but it is still not yet completely uncovered. It has been shown that phosphorylation of Y192 mediated by active ZAP70 decreases the CD3 ITAM phosphorylation, acting as a negative feedback (Goodfellow et al 2015). It appears that phosphorylation of Y192 by ZAP70 affects Lck capacity to interact with CD45, promoting the close conformation and thus switching off the membrane proximal signal (Courtney et al 2017). However, other evidence found this phosphorylation site important for actin recruitment (a later event occurring in TCR activation), and this led to the hypothesis that it is a mechanism that mediates the switching of Lck substrates from early to later events (Granum et al 2014). The figure 31 summarizes the main proteins involved in Lck activity regulation.

#### **4.2.2 ZAP70 activation and regulation**

$\zeta$ -associated protein of 70kD (ZAP70) is a tyrosine kinase protein which was described to associate with the CD3  $\zeta$  chain (as suggested by its name) and to act as a second messenger to propagate the activation signal from the TCR-CD3 complex to the following interactors. As for Lck, ZAP70 exists in an inactive and active conformation depending on the phosphorylation status of tyrosine present in its SH2 domains. In resting condition, ZAP70 is found in the cytoplasm in an auto-inhibitory conformation. Its activity is strictly dependent on its activation through phosphorylation by Lck. Active Lck interacts with ZAP-70 by its SH2 domain and first phosphorylate the Y315 and Y319. These phosphorylations promote its active open conformation and recruitment to the CD3 $\zeta$  ITAMs where it is further stabilized by Lck mediated phosphorylation of its Y493.

ZAP70 cannot further phosphorylate ITAMs but is able to phosphorylate tyrosine flanked by acidic residues such as in adaptor protein LAT and SLP-76 (Shah et al 2016). However, LAT is not located in the same membrane islet than CD3 $\zeta$  ITAMs. Two models have been described to explain how ZAP70 mediates LAT phosphorylation and activation. In a recent paper, Lo and colleagues have shown that the binding of Lck to the proline rich region present on LAT protein through its SH3 domain would function as a bridge connecting

ZAP70 and LAT. Indeed, Lck binding to ZAP70 occurs through SH2 domain, so it would allow the simultaneous binding of the two proteins making them able to interact with each other (Lo et al 2018). In the model of “catch and release”, once activated and recruited to the ITAMs, ZAP70 can be released by a further phosphorylation at the Y126 and move through the cytoplasm to phosphorylate the LAT adaptor protein (Katz et al 2017).

ZAP70 is also regulated by a negative feedback loop that involves ubiquitination process. The non-degradative polyubiquitination mediated by the E3 ubiquitin ligase NRDP1 leads to the recruitment of tyrosine phosphatases, such as STS1 and STS2, that can act in downmodulation of phosphorylation of proteins involved in the TCR signaling (Yang et al 2015, Mikhailik et al 2007, San Luis et al 2011). This effect is counterbalanced by the deubiquitinase OTUD7B (Hu et al 2016).

#### **4.2.3 LAT signalosome**

The linker for activation of T cell (LAT) is a transmembrane protein that works as a scaffold once it gets phosphorylated by ZAP70. It contains 9 different phosphorylation sites that allow the docking of the adaptors and SH2 containing kinases that will follow in the transduction cascade. LAT aggregates in nanoclusters in resting conditions. It is found to reside in different islets of the plasma membrane compared to the TCR-CD3 complex (Balagopalan et al 2015). Adaptors and kinases that associate with LAT will form the LAT signalosome that contributes to the activation of multiple cell pathways that finally lead to gene transcription and T cell activation.

LAT protein can mediate the interaction with multiple partners, such as PLC $\gamma$ , the p85 subunit of PI3K, the adapter growth factor receptor bound 2 (Grb2), Grb2-related adapter downstream of Shc (GADS) and Son of Sevenless (SOS) (Houtman et al 2006, Sommers et al 2004). Interaction with GADS mediates the binding with SH2 domain containing leukocyte protein of 76kD (SLP-76) (Liu et al 1999). Furthermore, SLP-76 is able to interact with even a larger group of proteins such as the guanine nuclear exchange factor (GEF) Vav1, the adapter protein Nck and the IL-2-inducible T-cell kinase Itk (Qi et al 2007, Jordan et al 2008).

The interaction of LAT with Grb2 and SOS upon TCR activation promotes the enlargement of LAT signalosome that from nanoclusters form larger microclusters (Houtman et al 2006). The formation of these microclusters and LAT-SLP-76 association is fundamental for the activation of at least three different downstream pathways involved in T cell activation: Vav1-mediated JNK/p38 activation, IP3-mediated Ca<sup>2+</sup> flux and DAG-mediated RAS/PKC activation. All these processes will be further described below. All the components of LAT signalosome are important for the stabilization of this complex. Experiments where single proteins of the platform were deleted in cell lines or in knock-out mice models indeed resulted in partial abrogation or impairment of downstream signals (Miletic et al 2005, Jordan et al 2008).

Stability of LAT microclusters is found to be important for the propagation of an optimal T cell activation and contributes also to the kinetic proofreading. Indeed, stability of LAT signalosome was proved to depend on the affinity of pMHC-TCR binding. Furthermore, a recent paper evidenced that a delay of the phosphorylation of the Y132 residue of LAT contributes to the proofreading, since the acceleration of the phosphorylation of this site decreases the threshold for T cell activation (Lo et al 2019).

There are lines of evidence of non-canonical TCR signaling that include the participation of other molecules able to compensate the absence of the “official” proteins. One example of this diversification is given by the study of Roncagalli and colleagues. They could describe that activation of Vav1 can be induced by a signaling hub that is LAT independent. They find that the surface protein CD6 is able to bind SLP-76 on its own and promote Vav1 activation without LAT involvement (Roncagalli et al 2014).

#### **4.2.4 PLC $\gamma$ activation**

Among the proteins recruited to the LAT signalosome upon TCR activation there is the phospholipase C  $\gamma$ 1 enzyme (PLC $\gamma$ ) on the phosphorylated Y132 of LAT. It binds to LAT through its N-terminal SH2 domain and to SLP-76 via its SH3 domain (Gresset et al 2012). PLC $\gamma$  gets activated through the phosphorylation at positions 472, 771, 775, 783, and 1254 by receptor and non-receptor tyrosine kinases (Bae et al 2009). Lck-induced PI3K activity, Vav1 and SLP-76 contribute to the phosphorylation and activation of the protein Itk. Activated Itk can in turn phosphorylate PLC $\gamma$  (Reynolds et al 2002, Liu et al 1998, Berg et al 2005).

PLC $\gamma$  mediates the PI(4,5)P<sub>2</sub> hydrolysis which generates the second messengers diacylglycerol (DAG) and inositol triphosphate (IP<sub>3</sub>). Thus, PLC $\gamma$  activates three main pathways from its catalytic activity: The DAG dependent PKC $\theta$  and RAS activation pathways and the IP<sub>3</sub> induced Ca<sup>2+</sup> mobilization.

##### **a) DAG-mediated activation of PKC $\theta$ and RAS pathways**

Lck-mediated phosphorylation of PKC $\theta$  allows its conformational change and the recruitment of DAG. DAG binding in turn promotes PKC $\theta$  activation (Hayashi and Altman 2007, Melowic et al 2007). Activated PKC $\theta$  phosphorylates the protein CARMA1 allowing its trimolecular association with Bcl10 and MALT1. This complex leads the regulatory subunits of IKK (regulator of NF- $\kappa$ B transcription factor activation) to degradation (Sommer et al 2005). This event allows I $\kappa$ B phosphorylation, leading to the release of the p65 subunit of NF- $\kappa$ B, that become able to translocate into the nucleus and through association with transcription factors, such as REL, promoting the transcription of proinflammatory cytokines.

The guanine nucleotide binding protein RAS is a small GTPase protein activated by the guanine exchange factors (GEFs) Ras-GRP and SOS. These two GEFs are recruited to the LAT signalosome upon TCR activation and Ras-GRP is in turn activated by DAG mediated PKC $\theta$  activation (Ebinu et al 1998, Roose et al 2005, Finco et al 1998). RAS is thus recruited at the signalosome complex and through its catalytic activity induces the

activation of the serine-threonine kinase Raf-1. Raf-1 can further promote ERK1/2 activation and the nuclear activation of the transcription factor AP-1 (D'Ambrosio et al 1994).

#### **b) Ca<sup>2+</sup> intracellular influx**

Ca<sup>2+</sup> intracellular mobilization is a crucial step that promotes multiple pro-survival signaling pathways. PLC $\gamma$  production of IP<sub>3</sub> leads to the stimulation of the IP<sub>3</sub>R, a Ca<sup>2+</sup> permeable ion channel residing on the membrane of the endoplasmic reticulum (ER). This stimulation favors the release of Ca<sup>2+</sup> from the ER to the cytoplasm that in turn activates the CRAC channel at the plasma membrane initiating an Ca<sup>2+</sup> influx from the extracellular environment, in a process defined as store operated Ca<sup>2+</sup> entry (SOCE) (Oh-Hora et al 2008). CRAC channels are activated by the stimulation of the STIM1/2 proteins that are sensors of the Ca<sup>2+</sup> depletion from the ER (Roos et al 2005, Liou et al 2005).

Elevation of Ca<sup>2+</sup> intracellular level leads to activation of the phosphatase Calcineurin, together with the kinase CamK and the transcription factor dependent from calmodulin MEF2 (Savignac et al 2007). Calcineurin thus dephosphorylates NFAT that moves into the nucleus and associates with other transcription factor such as AP-1 (Wu et al 2006).

#### **4.2.5 Vav1 activation, rearrangement of cytoskeleton and cell polarization**

Vav1 is a guanine nucleotide exchanging factor (GEF) for the Rho family GTP proteins. In addition to its catalytic function, it has also an important scaffold function which is notably responsible for PLC $\gamma$  activation. Its main catalytic function, instead, relies on the recruitment and interaction with Rac1 and Cdc42 proteins which are the main mediators of the cytoskeleton rearrangement, fundamental process that together with cellular polarization allows the formation of the Immune Synapse (IS). The IS is a supramolecular structure that is formed at the interface between the T lymphocyte and the APC that will be described below at page 70.

Binding of SLP-76 to Vav1 protein induces its phosphorylation at the Y174, and thus its activation. Through the activation of Rac1 and Cdc42 target proteins, Vav1 activation leads to the transient dephosphorylation of the ezrin/radixin/moesin (ERM) proteins that in turn lose their ability to bind the plasma membrane and to connect the actin cytoskeleton to it (Faure et al 2004). This process causes an increase of membrane fluidity and decrease of cell motility that is the responsible for the morphological changes that result upon TCR-APC contact. The cells round up and start to accumulate F-actin at the borders of the site of cell-cell interaction. Accumulation of F-actin is caused by the activation of several proteins of which the best known are Arp2/3. Other important pathways activated by Vav1 mediated Rac1/Cdc42 stimulation are the JNK and p38 from MAPKs family, whose function is to promote the activity of transcription factors cJun and Fos for IL-2 production.

#### 4.2.6 Inside-out Integrin signal

T cell-APC conjugation is associated with the formation of adhesion contacts which promote the stabilization of pMHC-TCR interaction and sustain the signal for activation. This mechanism is based on the activation of the integrin proteins LFA-1 and VLA-4, which, upon TCR signals-induced conformational change, become able to interact with their ligands expressed on the APCs, ICAM and VCAM respectively. This process is known as “inside-out signaling” (Menasche et al 2007a).

Activation of LFA-1 and its adhesion to ICAM1 is mediated by the protein Rap1. Rap1 can be activated by LAT, SLP-76, PLC $\gamma$  and by the PKC $\theta$  target PDK1 as well as from other downstream proteins such as ADAP (Menasche et al 2007b, Medeiros et al 2005). Furthermore, the role of Rap1 is important for association of actin cytoskeleton components to the integrins. Through its association with RIAM, Riap1 can allow the binding of Talin protein to the integrins, further stabilizing the complex together with other actin cytoskeleton proteins involved in this association (Han et al 2006).

#### 4.2.7 TCR-Microclusters and IS formation

The interaction between pMHC and TCR leads to the formation of a well known structure called Immune synapse (IS). This structure consists in a platform where TCR membrane proximal events take place. IS is defined as a Supra Molecular Association Cluster. It can be divided in three main regions: the cSMAC (central), the pSMAC (peripheral), that together form the so called “bull-eyes” structure, and the dSMAC (distal) where excluded protein are retained (Monks et al 1998). These three regions have been associated with distinct protein pools, with small extracellular portion proteins accumulating at the cSMAC and long extracellular portion proteins are retained the pSMAC and dSMAC, point that gave rise to the theory of kinetic segregation mentioned above (Davis et al 2006). If TCR accumulation occurs in the cSMAC, protein associated with adhesion mechanism such as the LFA-1 resides in the pSMAC (Grakoui et al 1999).

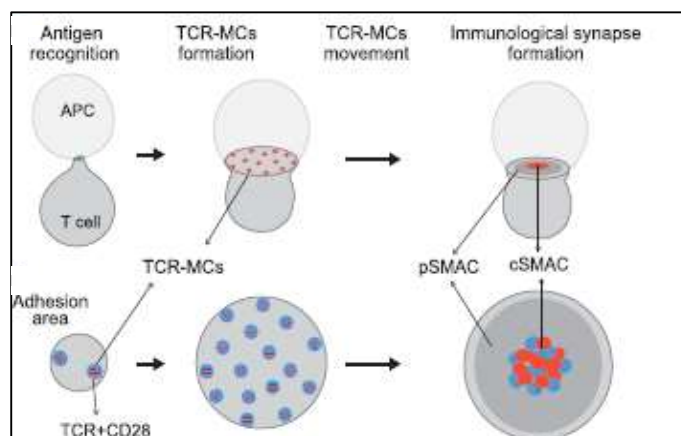


Fig.32 Schema of Immune synapse formation. Hashimoto-Tane and Saito 2016.

Despite that, the formation of the Immune synapse is a morphological feature that can be appreciated just after some minutes upon TCR-pMHC interaction. Therefore, the initiation of the signaling does not occur at the IS. TCR clustering start immediately after the contact with the antigen and form structures associated with ZAP70 and adapter proteins such as LAT and SLP-76 called microclusters (MC) (Campi et al 2005, Yokosuka et al 2005, Varma et al 2006). These are constituted by the association of around 100 TCR-CD3 complexes and together with the associated proteins can start the initial signaling after few seconds from the contact (Hashimoto-Tane et al 2016).

Immediately after TCR ligation, formed MCs reside at the T cell-APC contact region. The following rearrangement of cytoskeleton leads to a spreading of the interface area and the T cell will start to establish a larger contact with the APC. Upon TCR engagement, indeed, F-actin rearrangement plays a role at the periphery of the contact promoting the T cell spreading and the formation of a ring on the peripheral region of the interface (Barda-Saad et al 2005, Kumari et al 2015). Furthermore, in TCR-MCs an important role is played by the proteins involved in the integrin mediated adhesion (Huse et al 2006, Stinchcombe et al 2006). The peripheral regions of TCR-MCs are indeed surrounded by the LFA-1 protein that form a transient adhesion ring that promotes the binding with ICAMs (Springer et al 2012, Comrie et al 2015, Hashimoto-Tane et al 2016). As described, these interactions further stabilize the association between TCR and pMHC in order to prolongate the activation signal in weak Ag-TCR association and allow reaching the threshold needed to promote the proper T cell activation.

New TCR-MCs are thus formed and, upon sustained activation, they start moving to the center of the cell-cell contact together with the F-actin retrograde flow to form the cSMAC (Yokosuka et al 2005, Smoligovets et al 2012). Interestingly the TCR-CD3 complexes move rapidly to the center to form the cSMAC, and on the other side, the signaling proteins associated to it are less prone to reach the center of the interface and do not accumulate at the cSMAC (Yokosuka et al 2005, Varma et al 2006). The concomitant microtubule organizing center (MTOC) movement through the interface allows TCR-MCs microtubule association by the motor dynein and promote their movement at the center of the interaction interface (Hashimoto-Tane et al 2011).

The structure consisting of TCR-MC and the surrounding adhesion ring has been called microsynapse and disappears upon the TCR-MC movement through the center (Hashimoto-Tane et al 2016) to subsequently lead to the formation of the pSMAC region around the cSMAC. In Fig.32 the formation of the IS is schematized.

Noteworthy, not all the T cell-APC interactions lead to the formation of an IS. Some cell types such as thymocytes were shown to do not form this structure, suggesting that this process might occur only upon



strong activation. Weak activations indeed could be sufficiently sustained by TCR-MCs signaling (Hashimoto-Tane et al 2016). The explanation of this observation might reside in the role of the IS.

There are divergent lines of evidence about the positive or negative role of the IS in T cell activation. Early studies have described the IS as an amplifier of TCR signaling, undoubtedly describing it as an optimizer of T cell activation. Indeed, a positive role of the IS in sustaining the T cell activation is evidenced by the presence of the co-stimulatory receptors of TCR (described later in chapter C) at the level of the cSMAC (Yokosuka et al 2008, Yokosuka and Saito 2009). Despite that, some studies indicate that IS might negatively regulate the TCR signaling. IS was indeed identified as the region where both cytokine secretion and TCR down modulation by endocytosis, two final outcomes of T cell activation, occur, and for that it was suggested to be a structure related to activation shutdown (Singleton et al 2006).

Moreover, TCR-CD3 complexes that accumulate at the cSMAC can be classified as CD3<sup>high</sup> or CD3<sup>low</sup>. The ones that are actively involved into the activation signaling are the CD3<sup>low</sup>, suggesting that the CD3<sup>high</sup> have instead completed their function and are part of the termination of the signal consisting of too rigid structures to be able to signalize (Saito et al 2010, Yokosuka et al 2010).

#### **4.2.8 FADD and Caspase-8 in T cell activation**

Even if lot of TCR signaling events have been clarified, as we described above, lines of evidence highlight that much more proteins might participate in the activation process. Among them, the role of FADD and Caspase-8, main components of the DISC, has been investigated, pinpointing a potential connection between TCR and death receptor signaling.

Already in 1998, three studies from Walsh *et al*, Newton *et al* and Zornig *et al* described that transgenic mice bearing a dominant negative form of FADD (truncated protein FADD-DN containing the aa sequence 80-208) were defective in thymocyte development both *in vitro* and *in vivo* and in peripheral T cell activation and proliferation *in vitro* (Zornig et al 1998, Walsh et al 1998, Newton et al 1998, Hueber et al 2000). The same results were found in Rag1<sup>-/-</sup> mice defective of FADD protein *in vivo* in the study of Zhang of the same year (Zhang et al 1998). Other studies, however, described that FADD role is more implicated in the proliferation of mature cell, since the development of thymocytes was found to be normal in FADD:GFP knock-out mice (Zhang et al 2005). In 2003, Beisner described that the deficit observed in the population of mature T cells with truncated FADD was not related with TCR early signaling but more with a defect in cell cycle progression (Beisner et al 2003).

Caspase-8 role in T cell activation was also deeply investigated. The first findings for a role of Caspase-8 in T cell activation comes from studies in 1999 where Kennedy *et al* could demonstrate that treatment of T

lymphocytes with inhibitors of caspases activity upon TCR triggering caused a strong decrease in IL-2 production (Kennedy et al 1999). In 2002, Chun and colleagues could describe a defect in T cell proliferation in ALPS patients with Caspase-8 homozygous mutation that was leading to an enzymatically inactive protein. Exploiting PBMC from these patients, purified PBMC from healthy donors transfected with siRNA for Caspase-8, and Jurkat cell deficient for Caspase-8, they could describe a decrease of IL-2 secretion, activation markers CD69 and CD25 and impaired Ca<sup>2+</sup> flux, followed by a lower rate in proliferation compared to Caspase-8 sufficient cells (Chun et al 2002). These defects were furthermore present in B and NK cells, leading to recurrent bacterial and viral infections.

Furthermore, conditional knock-out of Caspase-8 in T cell was shown to lead to decreased IL-2 secretion and low proliferation at late time point (evident after 48h) without any apparent defect in TCR early signaling. This effect was clear in peripheral T lymphocytes without apparent defect in thymocyte development (Salmena et al 2003).

Caspase-8 association with MALT-1 might represent the link of Caspase-8 to cell activation. It is known that MALT1 also associates with Bcl-10 and CARMA1 to form the trimolecular complex upon PKC $\theta$  activation and that this complex is known to be important for NF- $\kappa$ B activation. Thus Caspase-8 association with MALT-1 would allow the strict contact with the abovementioned trimolecular complex. Moreover, through TRAF6, which bind the protein complex, the recruitment of TAB2/3 and TAK1 can occur with subsequently IKK activation and NF- $\kappa$ B nuclear translocation (Thome et al 2004).

Association of Caspase-8 with the ubiquitin ligase TRAF6 was shown to be furthermore responsible for Caspase-8 localization at the lipid raft upon TCR stimulation (Bidere et al 2006). It was suggested that the difference in the localization of Caspase-8 between apoptosis and TCR activation might be a mechanism for variation of Caspase-8 substrate that leads or not to cell death and that mediates the shift from death versus non death function of the protein (Koenig et al 2008). Indeed association of Caspase-8 to lipid raft and colocalization with CD3 molecules was observed by confocal microscopy upon TCR activation and both presence of active full-length protein and at less extent the p43 form have been described at this nanodomain just upon TCR activation (Koenig et al 2008).

Despite all these findings, the role of both FADD and Caspase-8 during T cell activation has been questioned and reevaluated. The finding that the defect in T cell accumulation in cells deficient of Caspase-8 was not due to an impairment of cell cycle progression but to differences in the rate of cell survival was described in the study of Ch'en *et al* of 2008. Indeed, it was described that T cell deficient for Caspase-8 were dying at higher rate compared to WT cells upon TCR activation by necroptotic cell death. Necroptosis is a mechanism that, as we described at page 48, relies on the activation of RIPK1 and RIPK3 and that is strongly induced in absence of Caspase-8. Caspase-8 cleavage of these proteins is, indeed, necessary to avoid

necroptosis to occur upon DRs as well as upon TCR ligation. Utilization of an inhibitor of necroptosis, necrostatin, was sufficient to rescue the survival defect present in Caspase-8 deficient cells *in vitro* (Ch'en et al 2008). In addition, a publication of 2011 evidenced that the FADD binding to RIPK1 could be responsible for the maintenance of proliferation in T cells. FADD<sup>-/-</sup> T cells showed a high level of RIPK1 that was inducing massive necroptosis of the cells. Elimination of RIPK1 indeed restored proliferation defect of FADD<sup>-/-</sup> T cells (Zhang et al 2011). *In vivo* studies confirmed this founding. In a publication of Lu of the same year, the defects of clonal expansion and proliferation showed by FADD<sup>dd</sup> (dominant interfering form of FADD) mice were completely rescued by crossing them with RIPK3<sup>-/-</sup> mice. Furthermore, they could demonstrate that the immune response to hepatitis infection regulated by CD8<sup>+</sup> cells, was normal in FADD<sup>dd</sup>-RIPK3<sup>-/-</sup> mice (Lu et al 2011). The results were confirmed by the following study of Ch'en conducted in the double KO mouse for Caspase-8 and RIPK3 (Ch'en et al 2011).

Interestingly the Caspase-8 form that was suggested to have a role in T cell survival during clonal expansion is a catalytically active full-length caspase, since the Caspase-8 D387A mutant (unable to be cleaved) was able to rescue Casp8-deficient T-cell proliferation (Leverrier et al 2011).

A recent study could describe that Caspase-8 represses the CD8<sup>+</sup> proliferation in response to viral challenge in mice in a death-independent manner. They could demonstrate that the double KO Casp-8-RIPK3 (DKO) highly accumulate CD8<sup>+</sup> cells (especially the terminally differentiated effector CD8<sup>+</sup> cell population) and have enhanced immune response compared to WT in the early phase of mouse cytomegalovirus infection (MCMV). These cells were proliferating more than the Caspase-8 sufficient counterpart, describing a suppressive role of Caspase-8 on proliferation of CD8<sup>+</sup> population in expansion phase upon antigen encounter (Feng et al 2019).

Thus, up to now, the role of FADD and Caspase-8 in TCR activation is still elusive and subject of debate, making of an extreme interest the continuation of the research on the subject.

### **4.3 Modulation of TCR signaling**

#### **4.3.1 Role of tuning molecules**

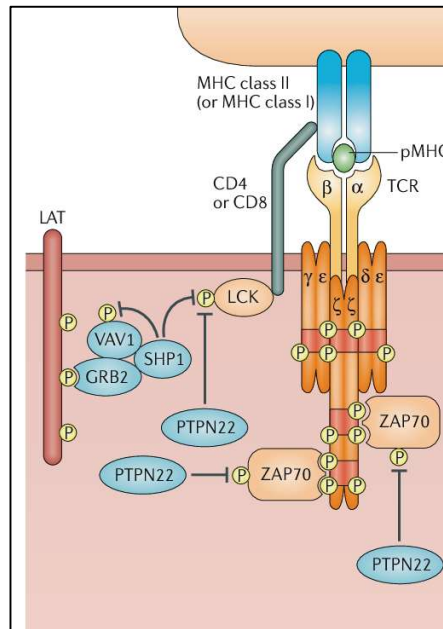
During T cell activation several proteins have been identified to negatively modulate the strength of the TCR signaling. Their constitutive expression in naïve as in effector T cells, (i) allows the shutdown the T cell activation in response to self-antigens, (ii) avoids activation induced by extremely weak antigens and (iii) blocks the overactivation of the cells in response to strong antigens. Being constitutive modulators of the signaling, proteins in this category have been defined as “tuning” molecules. Their mode of action differs from proteins that have a restricted pattern of expression depending on the state of the mature cell, such as co-inhibitory or co-stimulatory immune checkpoints. The main classes that are involved in this type of

modulation are phosphatases and E3 ubiquitin ligases. Furthermore, TCR activation increases the expression of the proteins responsible of the RICD that we described in the previous chapter, so the role of inhibitors of cell activation can promote cell survival (Paster et al 2014). Here I will describe few examples of these proteins.

**a) SHP-1/PTPN6 and its regulators**

Among the phosphatases of the protein tyrosine phosphatase non-receptor type (PTPN) family one of the best studied in the inhibition of TCR signaling is the phosphatase SHP-1. SHP-1 contains two SH2 domains for interaction with target proteins and a C-terminal catalytic domain with phosphatase activity. The relevance of this protein in the downmodulation of T cell activation has been revealed thanks to the analysis of the mice KO model *motheaten (me)*, which bears mutations that alter the splicing site of SHP-1 transcript and lead to a truncated non-functional protein (Tsui et al 1993). The KO mouse model shows severe systemic inflammation and autoimmunity features that lead to premature death (Shultz et al 1997). Autoimmunity features in *motheaten* mice partially rely on hyper-responsive T cells that lead to increased IL-2 production as well as activation of Lck (Lorenz et al 1996). These evidence were also confirmed in peripheral T cells deficient for SHP-1 (Carter et al 1999).

Its role in the dephosphorylation of TCR early signaling proteins is also well established (Pani et al 1996, Plas et al 1996, 1999, Jin et al 1998, Cuevas 1999). Noteworthy, lipid raft association of SHP-1 has been also described. A deletion of 68 amino acids in its C-term abrogates the recruitment of the protein to raft implicating the importance of this region for the raft association (Poole and Jones 2005).



**Fig. 33 SHP-1/PTPN phosphatases targets. Gaud et al 2018.**

The binding of SHP-1 to Lck was proved to occur upon TCR engagement. The role was described as a negative feedback loop, considering that activation of SHP-1 is mediated by its phosphorylation on Y564 which is provided by Lck and in turn, SHP-1 can bind and dephosphorylate Lck to limit its activity (Criado and Madrenas 2004). A publication of 2003 using mice model and *ex vivo* experiments evidenced that the negative SHP-1 activity and the positive activity of ERK proteins compete in the determination of T cell activation outcome. Indeed, weak stimulation facilitates SHP-1 binding to Lck, contributing to downmodulate the signaling cascade. In strong TCR activation the early activation of ERK proteins inhibit SHP-1 binding to Lck, positively enhancing the T cell activation (Stefanovà et al 2003). Main SHP-1 and other PTPN phosphatases targets are shown in Fig.33.

A role of SHP-1 in downmodulation of TCR signaling was also found to reside in the regulation of phosphorylation of the Cbl-b protein, a known E3 ubiquitin ligase also involved in the negative regulation of T cell activation. Lck-mediated phosphorylation of Cbl-b promotes its degradation and SHP-1-mediated dephosphorylation abrogates this process (Xiao et al 2015). Furthermore, SHP-1 itself is subjected to ubiquitination. The E3 ligases Itch and WWP2 promote SHP-1 ubiquitination limiting its Lck association (Aki et al 2018). Other SHP-1 interactors have also been proved to affect its role of TCR modulation. An example is offered by a recent paper that evidenced a role for the kinase Taok3, by interfering with SHP-1 binding to Lck protein upon TCR engagement. Taok3 deficiency, indeed, causes a rapid block in T cell signaling because of an increased SHP-1 Lck interaction (Ormonde et al 2018).

The role of SHP-1 in tumor progression has been deeply investigated. Decreased or abolished SHP-1 expression has been related to both malignant transformation and tumor cell invasiveness in several hematologic malignancies (Oka et al 2001). The cause has been addressed to an increased methylation of CpG island of the promoter region of SHP-1 locus (Koyama et al 2003). Moreover SHP-1 has been found to negatively regulate the growth pathway JAK/STAT by dephosphorylating STAT3 in diffuse large B cell lymphoma (Demosthenous et al 2015), and therefore, SHP-1 deficiency would lead to abnormal cell growth.

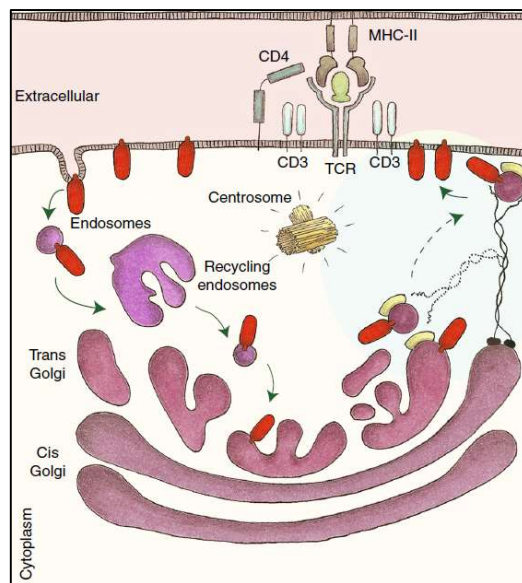
SHP-1 has also been implicated in the regulation of development of T cell subclasses, where it was shown to downmodulate Treg as well as Th-1 and Th-2 cells development (Carter et al 2005, Park et al 2005, Yu et al 2005). Moreover, absence of SHP-1 was related to increased activity in CD8+ cells, by increasing both the contact with APC and resistance to Treg mediated suppression (Sathish et al 2007, Stanford et al 2012, Mercadante et al 2017). For this last reason, targeting SHP-1 in cancer immunotherapy has been considered an attractive option. Up to now, however, the compounds that have been used to downmodulate SHP-1 activity in T cells did not go further than phase I clinical trial, since they did not show improvement in enhancing anti-tumor response. A brief mention to how SHP-1 might be integrated in the existing strategies of cancer immunotherapy can be found in the next chapter at pages 98-99.

## b) E3 ubiquitin ligases

Ubiquitination is also a factor that plays a role in the modulation of TCR signaling. Proteins of the Casitas B lineage lymphoma (Cbl) family have been identified to downmodulate T cell signal. Their members bear a tyrosine kinase binding domain (TKB) and a proline-rich region that specifically binds protein kinases for their degradation. Association of these proteins have been found for the members of Src family, CD3 $\zeta$ , ZAP70, as well as for other members of TCR signaling cascade such as Vav1 and PI3K (Meng et al 1999, Fang et al 2001, Magnan et al 2001, Andoniou et al 2000, Bachmaier et al 2000, De Sepulveda et al 2000). Several studies were conducted on Cbl deficient mice that showed hyperresponsiveness to T cell activation, confirming their negative role in regulation of T cell activation (Murphy et al 1998, Naramura et al 1998). Non redundant roles are evidenced for the c-Cbl protein and Cbl-b in mice models where the first was shown to play a wide negative regulation for PTKs and Cbl-b was mostly restricted to its role on Vav1 regulation (Thien et al 1999, Bachmaier et al 2000). Furthermore, this protein family has been recognized to ubiquitinate receptors that are subjected to tyrosine kinases modifications. Their monoubiquitination of Ag-liganded TCR have been shown to promote its surface downmodulation and targeting to lysosome (Wang et al 2001, Hicke et al 2001).

### 4.3.2 Role of receptor internalization

Receptors internalization has been described to modulate the signaling in several cellular processes, as we described for the TNFR members (see page 43). TCR-CD3 complex internalization occurs in resting state and upon antigen ligation, with no significant acceleration rate between the two conditions (Liu et al 2000). The functional meaning of the TCR-CD3 complex internalization, however, might be different in the two different contexts.



**Fig.34** Example of LAT protein (in red) endocytosis and movement through TCR/CD3 islands during TCR activation.

Zucchetti et al 2019.

In resting conditions, it has been shown that TCR has a long half-life even if it is subjected to constitutive internalization process. TCR expression is a balanced mechanism between *de novo* synthesis and recycling (Geisler et al 2004). Thus, most of internalized receptor is rapidly recycled and come back to the cell surface. Colocalization of TCR-CD3 complex with the endosome markers Rab5 (early endosomes), Rab4 (recycling endosomes) and Rab11 (late endosomes) has been extensively observed.

Moreover, the internalization guarantees the presence of a pool of intracellular receptor that is thus rapidly available to reach the IS in a moment where an amplification of the signal is required. This mechanism does not occur, indeed, just for TCR but also for proteins associated to the propagation and amplification of T cell signaling, such as LAT, Lck and other adapter proteins (Larghi et al 2013, Carpier et al 2018, Zucchetti et al 2019, Gorska et al 2009, Ehrlich et al 2002). Fig.34 shows as example LAT endocytosis and its movement to TCR-CD3 complex.

Upon TCR ligation, receptor internalization might give rise to opposite outcomes. First, even if the rate of internalization might not be clearly different, the downmodulation of the receptor at the surface is rapid and substantial. If the internalization is associated with downmodulation or to the amplification of the signal by recruitment of intracellular pool of proteins is still controversial.

On one side, down modulation has been associated to a feedback mechanism in order to keep under control the duration of cell activation and avoid an overactivation of the cells. It was found that TCR-CD3 complex upon activation is internalized and retained in intracellular compartments and can also be addressed to lysosome degradation. Indeed Cbl-b-dependent CD3 $\zeta$  ubiquitination has been documented. Interestingly, the recruitment of Cbl-b to the plasma membrane is dependent on Lck-mediated phosphorylation. The role of CD3 $\zeta$  was also shown to be important for the stability of the protein at the cell surface by a mechanism that is based on the length of its cytoplasmic tail more than on its primary structure. Mutation or abrogation of this, indeed, accelerate the rate of internalization of the complex and re-expression rescue the normal internalization rate (D'Oro et al 2002).

Conversely, recent lines of evidence described also that the intracellular TCR is able to signalize (Willinger et al 2015, Yudushkin et al 2010), as it occurs for other internalized receptors, thus opening a gate for the formation of another platform of signaling that might include also signaling proteins such as Lck and LAT. Moreover, TCR ligation was shown to induce also the internalization of non engaged receptor (San Jose et al 2000) that travel into the vesicles, by mechanisms that involve the microtubules and actin cytoskeleton, and can be re-exposed at the level of the cSMAC to allow further TCR-pMHC interaction. This is in line with the serial engagement model important for the threshold reaching and a proper T cell activation.

#### 4.4. Differential signals between naïve and memory T cells

Response of naïve and memory T cells to TCR stimulation has been shown to be quite diverse (Byrne et al 1988, Ehlers et al 1991, de Jong et al 1991). If memory cells show a rapid and robust activation with strong proliferation and cytokines production, the response of naïve cells is quite slower and weaker (Berard and Tough 2002, Garcia et al 1999, Veiga-Fernandes et al 2000).

The explanation to these differences has been searched at functional level. Indeed, memory cells have less stringent requirements to get activated, a lower antigen affinity level required to respond to the stimulation (Curtsinger et al 1998, Rogers et al 2000). Moreover, accumulation of mRNA and proteins prior the encounter confers selective advantages to the experienced population compared to the naïve (Stout et al 1992, Veiga-Fernandes et al 2000).

The description at molecular level remained for long time elusive. It is known that activation of MAPKs is an important step of cell activation, that on one side is important for AP-1, Jun/Fos and NFAT transcription factors activation, and on the other side, play an important role in the shutdown of the T cell response in naïve cells (Adachi and Davis 2010). It was indeed shown that ERK acts in a negative feedback loop mechanism, promoting the downmodulation of the signalosome and the intracellular Ca<sup>2+</sup> influx.

A study of Adachi and Davis of 2010 offers a molecular explanation for the difference of naïve and memory cells activation, which is based on the different ratio of ERK and p38 between the two cell populations. They found a higher ERK phosphorylation in naïve cells, that correlated with a stronger activation of SLP-76 compared to experienced memory cells. On the other side, memory T cells showed a higher phosphorylation of p38, activated by an alternative mechanism that relies on the binding to human Discs large (hDlg) (Round et al 2005). This alternative pathway was already shown to occur in several studies and is based on the formation of a complex between hDlg, Lck and ZAP70 upon TCR stimulation (Round et al 2007, Rebeaud et al 2007, Salvador et al 2005). The consequent activation of p38 would lead to NFAT activation and high production of IL-2 and IFN $\gamma$ .



## C. Regulation of the T cell response by the immune checkpoint receptors

### 1. Role of the immune checkpoints in TCR regulation

The discovery that, besides the TCR chains, other surface receptors were involved in the regulation of T cell activation comes from the observation that the solely TCR activation induces a state of cell unresponsiveness, called anergic state, giving rise to functionally inactivated T cells unable to initiate any productive response upon further stimuli (Jenkins and Chen 1990, Suzuki et al 1988). By utilization of directed monoclonal antibody, Harding *et al* identified CD28 as a co-stimulator of TCR activation allowing a full T cell activation (Harding et al 1992). This discovery led to the formulation of the “two signals model” required for T cell activation. The “signal 1” was initiated by the pMHC-TCR binding, the “signal 2” by the CD28 binding to its ligands, B7.1 or B7.2, expressed by activated APCs (June et al 1987, Mueller et al 1989). In the following years, an increasing number of molecules able to modulate, either positively or negatively, the TCR signaling were identified. The “two signals model” evolved then into “three” or even into “four signals model” clarifying the presence of an expanded network for the regulation of T cell activation. Receptors that, like CD28, were described to augment T cell signaling and allowing a full lymphocyte activation were defined as co-stimulatory receptors (or more recently stimulatory immune checkpoints). Their counterpart, co-receptors that negatively regulate the lymphocyte activation were named co-inhibitory receptors (or more recently inhibitory immune checkpoints) (Fig.35) (Reviewed by Ogawa and Abe 2019).

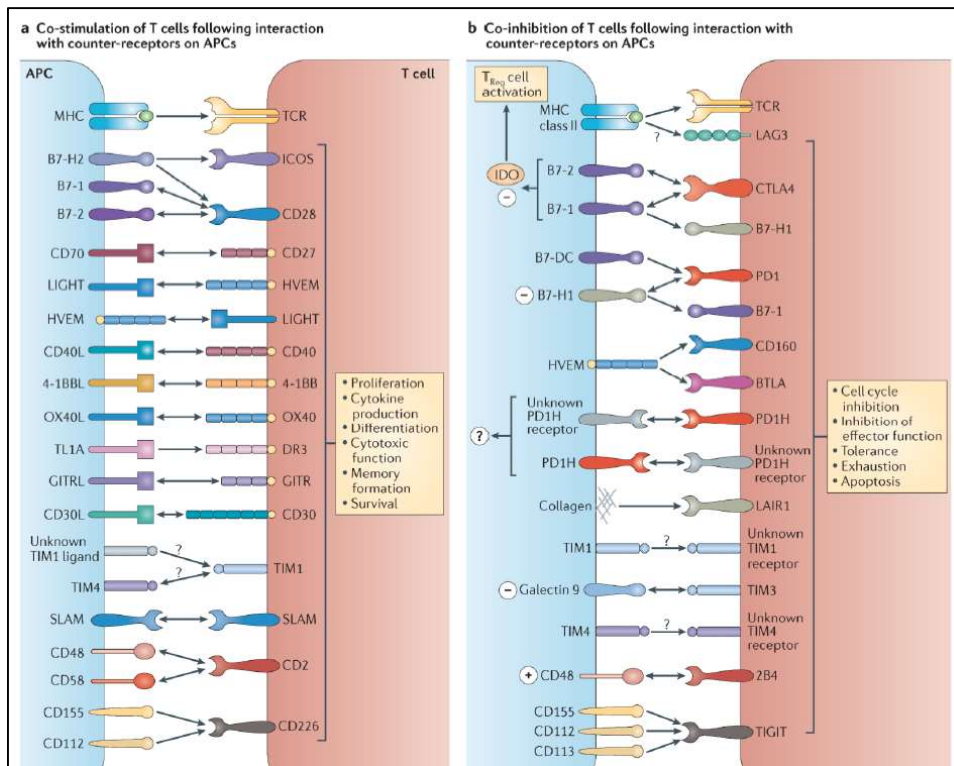


Fig.35 Schema of main TCR immune checkpoint. Adapted from Chen and Flies 2013.

Co-stimulatory and co-inhibitory receptors listed in the Table 2 can be grouped in two subfamilies: the immunoglobulin SF (IgSF) and the TNFRSF.

IgSF	TNFRSF
CD28	4-1BB (CD137)
ICOS (CD278)	OX40 (CD134)
CTLA4 (CD152)	CD27 (TNFRSF7)
PD1 (CD279)	GITR (CD357)
PD1H (VISTA)	CD30 (TNFRSF8)
BTLA (CD272)	TNFR1 (CD120a)
B71 (CD80)	TNFR2 (CD120b)
B7H1 (CD274,PDL1)	HVEM (CD270)
CD226 (DNAM1)	
CRTAM (CD355)	LT $\beta$ R (TNFRSF3)
TIGIT (VSI9,VSTM3)	DR3 (TNFRSF25)
CD96 (TACTILE)	DcR3 (TNFRSF6B)
TIM1 (HAVCR1,KIM1)	FAS (CD95)
TIM2 (TIMD2)	CD40 (TNFRSF5)
TIM3 (HAVCR2,KIM3)	RANK (CD265)
TIM4 (TIMD4)	OPG (TNFRSF11B)
CD2 (LFA2,OX34)	TRAILR1-4 (TNFRSF10A-D)
SLAM (CD150,SLAMF1)	TACI (CD267)
2B4 (CD244,SLAMF4)	BAFFR (CD268)
Ly108 (NTBA,CD352,SLAMF6)	BCMA (CD269)
CD84 (SLAMF5)	TWEAKR (CD266)
Ly9 (CD229,SLAMF3)	EDAR
CRACC (CD319,BLAME)	XEDAR (TNFRSF27)
BTN1 (BTN1A1)	RELT (TNFRSF19L)
BTN2 (BTN2A1-3)	DR6 (CD358)
BTN3 (BTN3A1-3)	TROY (TNFRSF19)
LAIR1	NGFR (CD271)
LAG3 (CD223)	
CD160 (BY55,NK28)	

**Table 2. Igs and TNFRs described as TCR immune checkpoints. In Green: stimulatory, in Red: inhibitory and in Yellow: categorization still unclear. Adapted from Chen and Flies 2013.**

The first class of the TCR co-receptor proteins belongs to **IgSF**. They are therefore constituted by an Immunoglobulin variable-like domain (IgV) bearing complementary determining region (CDR), site of antibody recognition of the antigen, and a short cytoplasmic tail (Zhang et al 2004). Their cytoplasmic tails bear tyrosines subjected to post-translational modifications able to transduce the ligand binding in signalization. I will focus on the most four described members that acquired in the last years a noteworthy importance for their therapeutic potential: the CD28 and ICOS as co-stimulatory receptors and the CTLA-4 and PD-1 as co-inhibitory receptors. The second class of the TCR co-receptors belong to the **TNFRSF**. They have been described mostly as co-stimulatory receptors so far. The most intensively studied are members bearing the TRAF-interacting motif (TIM) in their cytoplasmic domain such as OX-40, 4-1BB (CD137), CD30

and HVEM. However, members bearing the Death Domain (DD) have been also described as TCR co-receptors such as DR3 and Fas. As for the members of IgSF, I will focus on two members of this family which have been intensively studied for their therapeutic potential: 4-1BB and OX-40. The role of Fas as a TCR co-receptor, still a matter of debate, is at the heart of my PhD project and will be discussed in a separate section at the end of this chapter (Page 100).

The discovery of such a consistent number of co-receptors unraveled the notion of a spatio-temporal regulation at specific developmental or differentiation state. The co-signaling has, therefore, acquired an extreme importance, since regulation of co-receptors expression and signalization is crucial for determination of the fate of activated T lymphocytes. Furthermore, regulation of the expression of co-receptor ligands is also vital in maintaining immune system homeostasis in physiology and conversely to efficiently respond to a pathologic condition. At the state of the art we know that co-signaling receptors play an extremely important role not only in T cell activation, but also effector functions and memory T cell differentiation and maintenance (Linterman et al 2014, Tahiliani et al 2017, Weber et al 2015, Mousavi et al 2008).

In addition, exposure of T cells to chronic stimulation and in a proinflammatory environment for long time causes the upregulation of several co-inhibitory receptors. This overexpression makes the cells entering in a reversible stage defined as “exhaustion”. The exhausted T cells become less prone to proliferate and to respond to further stimuli, decreasing the potential of action of effector T cells as well as the differentiation to T memory phenotype (Takamura et al 2010, Angelosanto et al 2010). This unresponsiveness state can, however, be reverted by strong co-stimulatory signals, as well as from the artificial block of inhibitory receptors interaction (Wherrey et al 2011, Wang et al 2012).

Conversely, cell anergy is a process that occurs upon TCR stimulation with an extreme low affinity Ag or, in general, suboptimal stimulation. It can, for example, occur for naïve T cells without CD28 co-stimulation. The unresponsiveness state of the cells, always accompanied by upregulation of co-inhibitory receptors, looks to be irreversible, making the cells unable to respond to further following stimuli (Schwartz et al 2003).

## 2. Spatio-temporal expression and regulation

The spatial and temporal organization of a co-receptor defines its expression in term of regulation of its localization and timing, determining the network of interaction with other proteins, and thus affecting the readout of its action in T cells. The presence of an immune checkpoint at membrane level can determine an augmentation or inhibition of T cell response, that will contribute to shape its differentiation program. Moreover, the simultaneous presence of several co-receptors allows a possible synergic or competitive relation among them that could affect the outcome of the immune response. In order to define the immune checkpoints spatio-temporal organization, I will describe below their role for the establishment and maintenance of different T cell subsets, the competition that exists among TCR co-signaling receptors and ligands and their spatial localization at membrane level during T cell activation.

### 2.1 Immune checkpoints expression in different T cell subsets

Spatio-temporal organization of immune checkpoints is crucial in the determination of T cell response to T cell activation that occurs upon pathogen challenge. Presence as well as absence of a determined co-receptor can affect the promotion of a specific T cell differentiation program and thus the outcome of the immune response. The immune checkpoints functions in the different T cell subclassed are listed in the Table 3.

Receptor	T cell type	Priming	Cell growth	T <sub>H</sub> cell differentiation	Effector function	Survival	Memory
CD28	CD4 <sup>+</sup>	(+)	(+)	ND	ND	(+)	(+)
	CD8 <sup>+</sup>	(+)	(+)	ND	ND	(+)	(+)
ICOS	CD4 <sup>+</sup>	ND	(+)	(+)	(+)	(+)	(+)
	CD8 <sup>+</sup>	ND	ND	ND	ND	(+)	(+)
CTLA4	CD4 <sup>+</sup>	ND	(-)	ND	(-)	(-)	(-)
	CD8 <sup>+</sup>	ND	(-)	ND	(-)	(-)	(-)
PD1	CD4 <sup>+</sup>	ND	(-)	ND	(-)	(-)	
	CD8 <sup>+</sup>	ND	(-)	ND	(-)	(-)	(-)
4-1BB	CD4 <sup>+</sup>	ND	(+)	ND	(+)	(+)	(+)
	CD8 <sup>+</sup>	ND	(+)	ND	(+)	(+)	(+)
OX40	CD4 <sup>+</sup>	ND	(+)	(+)	(+)	(+)	(+)
	CD8 <sup>+</sup>	ND	(+)	ND	(+)	(+)	(+)
DR3	CD4 <sup>+</sup>	ND	(+)	(+)	(+)	ND	(+)

**Table 3. Examples of co-stimulatory and co-inhibitory receptor function in stages of T cell differentiation.**

**Adapted from Chen and Flies 2013.**

**CD28** has been shown to be the only co-receptor expressed at considerable high level in naïve T cells. It has been found that its presence is fundamental in accomplishing a proper naïve T cell activation (Acuto and Michel 2003, Boomer and Green 2010, Ogawa et al 2013, Xia et al 2018). Expression of CD28 on cell surface is generally constant but appears to show a transient down-regulation after stimulation with anti-CD28 antibodies or upon engagement of its ligands, the B7 proteins. Its expression starts to decrease after 12-24 hours of stimulation and returns to basal level after 48 hours. On the other hand, its role within the main effector T cell subpopulations is still elusive (Bour-Jordan et al 2009).

Up to now, increasing data have evidenced the role of CD28 in activated CD4+ T subpopulations. Indeed, analysis of *CD28<sup>flox/flox</sup> O<sub>x</sub>40<sup>cre/+</sup>* mice where CD28 deficiency is restricted to cells that have been already primed, demonstrated that CD28 plays a fundamental role in the differentiation and maintenance of T follicular helper, T helper 1 and T regulatory populations (Tfh, Th1 and Treg) upon viral and bacterial challenge (Linterman et al 2014). Other studies evidenced the role of CD28 on modulation of Treg development and activity. Indeed, CD28 was defined to be important in the development of Treg cells in the thymus, but also in their maintenance and effectiveness (Salomon et al 2000). Conditional deletion of CD28 in Treg cells caused an accumulation of activated T cells, severe autoimmunity, implying a role for CD28 in modulating their activity. Furthermore, the CD28 depleted Treg cells showed a dampened CTLA-4 and PD-1 expression (Zhang et al 2013).

Even though, several controversies exist concerning the CD28 role in promoting and modulating different T effector subpopulations, since differences existing between human and mouse protein affect the production of proinflammatory cytokines and thus the different T subpopulations homeostasis (reviewed by Porciello et al 2018). Ligands of CD28, B7.1 and B7.2, start to be expressed on APC that have been already challenged with the pathogen. This mechanism would further contribute to avoid T cell response upon self-antigen binding on MHC of naïve cells, promoting central and peripheral T cell tolerance.

The first TCR stimulation induces the upregulation and increased expression at membrane level of others known co-receptors of IgSF, such as the co-stimulatory receptor ICOS but also of some co-inhibitory receptors such as CTLA-4 and PD-1, which therefore acts mainly in activated T cells.

**ICOS** (CD278/ Inducible T-cell COStimulator) is upregulated upon first TCR activation thus directing the effector T cell differentiation towards specific T helper phenotypes such as Th1, Th2, Th17, Tfh and Treg. Indeed, ICOS co-stimulation has been recognized to induce and promote T helper cells development, as well as their survival and maintenance (Simpson et al 2010, Kroenke et al 2012, Choi et al 2011). The deficiency of ICOS receptor was associated in humans to “antibody deficiency syndrome” caused by impaired T cell dependent B cell responses, of which Tfh cells are important mediators. A defective formation of germinal centers, main site of encounter of T and B cells, was indeed described because of re-localization of Tfh cells

in the T zone upon ICOS signaling blockade (Warnatz et al 2006, Weber et al 2015). Moreover, ICOS was involved in maintenance and proliferation of memory T cells since its deficiency impairs T memory capacity to efficiently respond to the Ag stimulation (Simpson et al 2010).

**CTLA-4** (CD152/ Cytotoxic T-lymphocyte antigen 4) upregulation at the cell surface peaks 24-48 hours after activation and plays a crucial role in counteracting CD28 co-stimulation by competing for the same ligands. The mechanism of competition and its way of action is further described in the next paragraphs 2.2 and 3.2.3. CTLA-4 expression has been found at high level in Treg cells, T effector cells and T memory cells. It was proposed that it may act in the regulation of the interaction between T effector cells with Treg in a cell-extrinsic manner (Walker et al 2011). Signalization through CTLA-4 receptor would promote secretion of cytokines important for immune suppression properties, such as TGF $\beta$ , IL-10 and IL-35, that would act in suppressing the immune response of T helper cells maintaining T cell homeostasis (Chen et al 1998, Nakamura et al 2001). Human patients with insufficiency of CTLA-4 signaling display multiple features of lymphoproliferative disorders (Schubert et al 2014, Verma et al 2017) as well as hypogammaglobulinemia caused by impairment of B cell development upon overactivation of T cells (Schubert et al 2014). Upregulation of CTLA-4 in several type of cancers, such as acute myeloid leukemia (AML), was related to limitation of antitumor response by suppression of tumor infiltrating lymphocytes (TILs) functions (Dyck et al 2017, Ok et al 2017).

**PD1** (Programmed cell death-1/CD279) is another important co-inhibitory receptor of the IgSF which is upregulated around 24-48 hour upon T cell activation (Barber et al 2006, Day et al 2006). Its ligation with one of its two ligands, named PD-L1 (B7-H1) and PD-L2 (B7-DC), initiate a signaling that lead to inhibition of TCR activation. If antigen is acutely cleared, PD-1 levels decrease on responding T cells. However, in chronic infections and cancers, where the antigen persists, PD-1 expression remains sustained. It shares several functional features with the previously described CTLA-4 receptor, such as the T cell subsets where it operates (Tfh, Treg and memory T cells).

PD-1/PD-L1 interaction plays indeed an important role in maintaining T cell homeostasis, limiting the overactivation of immune response and the insurgence of features of autoimmune disorders. Its deficiency have been involved in several kinds of human autoimmune diseases, such as systemic lupus erythematosus, rheumatoid arthritis, type I diabetes and multiple sclerosis (Prokunina et al 2002, Nielsen et al 2003, James et al 2005). As for CTLA4, its role in modulation of Treg response was identified in suppression of effector T cells by cell extrinsic mechanisms as described above. Its cell intrinsic role in modulating Treg is instead still elusive, since there are studies that described PD-1 as a suppressor of Treg (Bour-Jordan et al 2009, Franceschini et al 2009, Francisco et al 2009) and other studies that have defined a role for PD-1 in promotion Treg differentiation phenotype (Amarnath et al 2011). PD-L1 expression is greatly

increased in cancer tissues undergoing immune attack. This contributes to the shutdown of the response of TILs in the tumor microenvironment, thus contributing to tumor escape.

Most of TNFR co-receptors expression is upregulated upon T cell activation, thus, as well as for the other main immune checkpoints, they act mostly in activated T cells. They act specifically in the promotion of the T effector subsets, Treg proliferative capacities and T memory survival and proliferation (Xiao et al 2012, So et al 2008, Hendriks et al 2005).

**OX-40 (CD134)** expression peaks 48-72 hours after TCR stimulation and it decreases approximately 5 days after (Baumann et al 2004). Its ligand, OX-40L, is expressed in activated professional APCs, as well as in non-professional APCs such as vascular endothelial cells and activated CD4+ and CD8+ T cells. Rather than promoting the differentiation of a specific subclass of T effector or memory cells, it is suggested that OX-40/OX40L interaction increases the threshold of the overall response to TCR activation (Jenkins et al 2007). Despite that, OX-40 presence is important for the response against viral infections, as well as for the other co-stimulatory receptors of TNFRSF, thus its role in enhancing CD8+ response was intensively investigated. It was shown that the extent of this response is strongly dependent on the nature of the pathogen, since OX-40 deficient background leads to no abnormalities in priming of CD8+ cells upon infection with CMV, influenza or *Listeria monocytogenes*, but impairs the CD8+ effector accumulation upon adenovirus and vaccinia virus (Humphreys et al 2007, Kopf et al 1999). Furthermore, OX-40 was demonstrated to be necessary for the generation and maintenance of CD8+ memory T cells (Hendriks et al 2005, Mousavi et al 2008).

**4-1BB (CD137)** is upregulated also 48-72 hours after TCR activation. Its expression is, however, more strictly cell type specific compared to other co-receptors. It was suggested to mostly play a role in NK lymphocytes and on the CD8+ population, both promoting effector CTL function and proliferation as well as survival of memory CD8+ T cells (Shuford et al 1997, Lin et al 2008). The level of its expression differs between CD8+ cells and other cell types expressing the receptor. Indeed, it is expressed marginally in CD4+ T cells and is strongly upregulated in CD8+ T cells, suggesting a correlation between its expression level and the ability of co-stimulate different class of lymphocytes.

4-1BB ligand is expressed on macrophages, activated B cells and dendritic cells and it is kept at high level in tumor associated cells which allows CTL effector activation (Pollok et al 1994, Kang et al 2017).

Regarding its abilities to respond efficiently to viral infection and tumors antigens, immunotherapeutic strategies exploiting the signaling through 41BB are broadly developed.

## 2.2 Competition among co-receptors and ligands

Competition for the same ligands is one example of spatio-temporal regulation and is commonly spread among receptors. We can briefly give here two examples of this type of regulation:

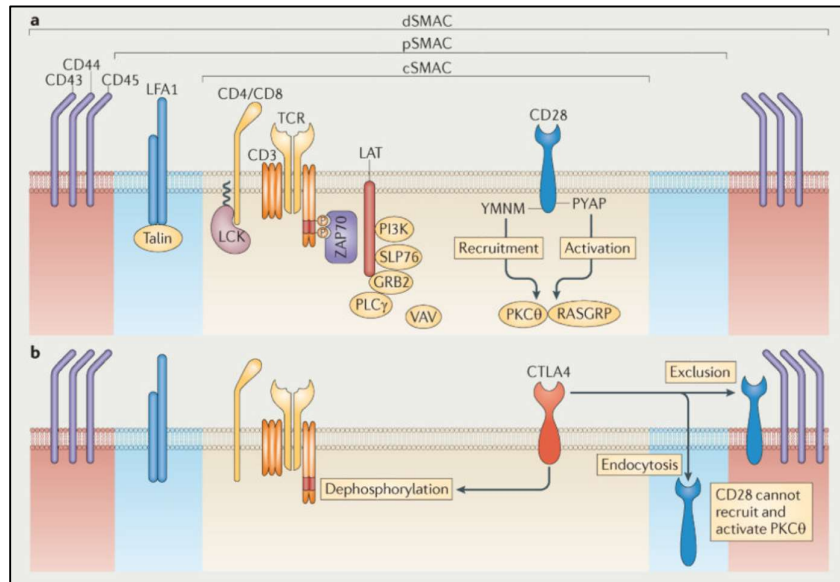
- CTLA-4 negatively regulate TCR-CD28 co-stimulation, by competing with CD28 for the binding to the ligands B7.1 and B7.2 for which it has higher affinity (Balzano et al 1992, van der Merwe et al 1997). This is, thus, one of the reasons why increased expression of CTLA-4 at the cell surface upon first TCR activation leads to the inhibition of T cell activation (Stamper et al 2001). Competition is further enhanced by the presence of the ligand B7.H2 for which CD28, CTLA-4 and ICOS are all receptors. Although they compete for its binding, ICOS is the one with higher affinity (Yao et al 2011).
- HVEM is a TIM-containing receptor of TNFRSF expressed on T lymphocyte surface. HVEM binding to one of its ligands LIGHT, expressed on activated DC or B cells, can induce a co-stimulatory effect on the T lymphocytes (Sedy et al 2005, Ware et al 2008). LIGHT, however, works also as ligand for LT $\beta$ R, and DcR3 (TNFRSF6B) and so these receptors can act as competitors of HVEM for LIGHT binding (Kroczek et al 2004, Shi et al 2002). The situation become even more complex when HVEM expressed by the activated APC acts as ligand for BTLA, a co-inhibitory receptor of the IgSF expressed on lymphocytes, leading to a co-inhibitory signal (Chemnitz et al 2006, Tamada et al 2000).

## 2.3 Co-localization with TCR upon activation

The co-localization of the immune checkpoint receptors and the TCR complex within the cSMAC of the IS, a structure described in the previous chapter at page 70, is another feature of the spatial regulation of the co-receptors. Interestingly, a minimal distance between TCR and the co-receptors CD28 and CTLA4 has been defined to accomplish the co-signaling (Fig.36) (Bashour et al 2014).

By biochemical and microscopy approaches, CD28 was found to colocalize with TCR-CD3 complex upon TCR and CD28 engagement both in microclusters (MC), described in last chapter at page 70, to promote early activation, and at the cSMAC of the IS (Yokosuka et al 2008 and 2009), sustaining T cell signaling after several minutes upon first contact.





**Fig. 36 Localization of CD28 and CTLA-4 in the cSMAC upon TCR activation. Chen and Flies 2013.**

Several molecular mechanisms are involved in the recruitment of CD28 to the MCs and IS. In both situations, the association of CD28 to TCR-CD3 complex relies on the presence of the Y188 present on the cytoplasmic tail of CD28, since a point mutation is able to decrease cSMAC association of more than the 70% (Sanchez-Lockhart et al 2008). Furthermore, it was shown that CD28 ligation with B7.1 or B7.2 is important to promote its recruitment to the IS (Bromley et al 2001). In addition, both TCR and CD28 signaling are necessary for sustained localization of CD28 to IS, since blocking either MHC class II or B7.1 results in a rapid displacement of CD28 from the IS (Sanchez-Lockhart et al 2008). The appearance of CTLA-4 at the cSMAC after 24-48h from the stimulation could explain its inhibitory role towards CD28 signaling. Localization of PD-1 at the IS has also been observed, but its localization at the cSMAC or at the more distal pSMAC is still controversial.

### **3. Molecular mechanisms of co-receptor signaling**

The way of action of the immune checkpoints is still far to be completely elucidated. If the deciphering of the molecular mechanisms by which they act through has been partially uncovered, it remains still elusive how they can be integrated in the TCR signaling pathways. Two models have been currently described to define this integration: the qualitative and the quantitative model.

#### **3.1 Qualitative and quantitative models**

The qualitative model assumes that the TCR and its co-receptors, activated by their ligands, initiate two independent signals and act in parallel to culminate in a response which is the summation of the signals activated by the two receptors on their own. Conversely, the quantitative model defines a synergistic or antagonist role for the co-receptors in modulating the TCR signal transduction.

CD28 and ICOS receptors have been shown to co-stimulate TCR signaling in both qualitative and quantitative way but the real interpretation of the data is still unclear. TCR independent activation of proteins upon co-receptor ligation was described, such as for Vav1 and PI3K for CD28 and PI3K for ICOS receptor (Raab et al 2001, Kovacs 2005, Wan et al 2020). This notion leads to the hypothesis that two pathways, initiated by TCR and CD28 or ICOS, could independently collaborate to enhance T cell activation. This model would define CD28 or ICOS stimulation as a qualitative signal to promote T cell activation. However, the activation of target proteins by these receptors is mediated by Lck induced phosphorylation of tyrosine present in their cytoplasmic domain. Thus, their response is strongly augmented by simultaneous TCR activation. Furthermore, it was claimed that the independent signaling coming from CD28 stimulation alone was obtained just in overexpressing conditions, or in activation induced by “superagonist” antibodies, which might not reflect their physiologic role.

The presence of IgSF members in the IS would contribute to sustain the quantitative model. For example CD28, colocalizing at the cSMAC together with TCR, is known to activate Vav1, PI3K and consequently PLC $\gamma$ , all components of TCR signaling cascade, leading to the idea that CD28 contributes to augment T cell activation acting synergistically to enhance TCR targets.

Co-receptors from the TNFRSF that bear TIM motif have been associated to the qualitative model, acting through the TRAF proteins to stimulate the MAPK and NF- $\kappa$ B pathways as will be briefly described in the paragraph 3.3. Even though, a movement to lipid raft nanodomains was also described for TNFRs upon engagement, in accordance with the quantitative model, that would potentially contribute to sustain or antagonize the TCR signal (Nam et al 2005, Starck et al 2005, Lee et al 2013).

## 3.2 IgSF co-receptors

### 3.2.1 CD28

CD28 was identified in early 80's by antibodies recognizing a 44 kDa transmembrane homodimeric glycoprotein at the surface of human T cells. It represents the founding member of the IgSF of costimulatory receptors (Hansen et al 1980, Gmünder and Lesslauer 1984). Whereas in humans CD28 is expressed on approximately 80% of CD4+ T cells and 50% of CD8+ T cells, all CD4+ and CD8+ murine T cells express CD28 (Gross et al 1990). Noteworthy, an important difference between amino acid sequence of human and mouse CD28 was recently described, identified as a single point mutation in the cytoplasmic tail of the protein, which affects the following signaling cascade induction in the two species (Porciello et al 2018).

The intracellular region of CD28 is quite short (41aa in humans) and do not contain any motifs with intrinsic enzymatic activity. Nevertheless, several sequences were described as critical for its localization in close proximity with TCR upon ligation and target binding:

- the membrane proximal YMMN, upon tyrosine phosphorylation via Src family kinases, allows the binding to the SH2 containing proteins p85, a subunit of PI3K, and Grb2 or GADS.
- The more distal proline-rich motif binds the SH3 containing proteins Itk, at the sequence PRRP.
- the sequence PYAP binds Grb2 (via its SH3 domain), filamin-A and Lck (Raab et al 1995).

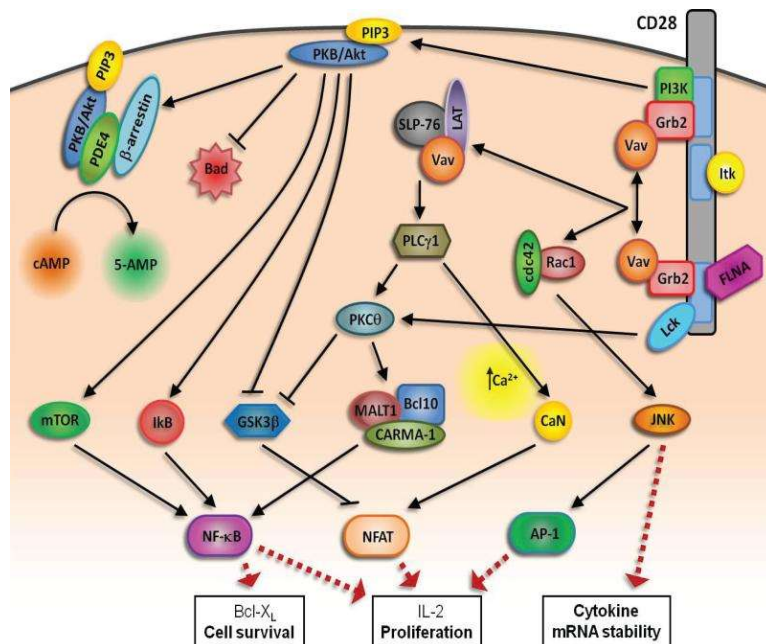
Notably, it was recently shown that CD28 intracellular domain is subjected to conformational change. This is due to the sequester of the cytoplasmic tail, containing polybasic residues, by the negatively charged lipid layer in resting condition, reverted upon ligand interaction (Dobbins et al 2016). Initiation of TCR signaling cascade, which causes an increase of intracellular Ca<sup>2+</sup>, would, furthermore, promote the release of the cytoplasmic tail in the cytoplasmic environment allowing the starting of CD28 signaling (Yang et al 2018).

CD28 interacting partners cited above allow the activation of several signaling pathways which promote the co-stimulatory functions of CD28. The interaction of the membrane proximal YMMN domain with the p85 subunit of PI3K leads to PIP<sub>2</sub>-PIP<sub>3</sub> conversion and activation of the PI3K/Akt pathway (Pages et al 1993), critical for the NF-κB activation. In addition to its role in IL-2 production, NF-κB activation has been also shown to upregulate some antiapoptotic proteins, such as Bcl-XL, leading to cell survival. Activation of PI3K together with PLCγ has, moreover, a fundamental role in the promotion of Ca<sup>2+</sup> influx. The publication of Xia *et al* describes that the dependency of naïve T cell from the co-stimulation is at least in part due to CD28 capacity to prolongate the time of decay of the Ca<sup>2+</sup> signal amplitudes (Xia et al 2018).

Interestingly, it was demonstrated that Lck-mediated phosphorylation of PYAP motif of CD28, and the following binding of SH2 domain of Lck to the phosphorylated site, is one important mediator of the interaction of CD28 with the following targets such as the PKCθ (Kong et al 2011). Dobbins and colleagues

could demonstrate that the basic residues contained in the intracellular region of CD28 are crucial for Lck-mediated phosphorylation. CD28 phosphorylation would permit Lck binding to phosphorylated Y207 (in PYAP) of CD28, further stabilizing the intracellular tail in the cytoplasm, and allowing the following PKC $\theta$  recruitment to the complex (Dobbing et al 2016). Furthermore, the recruitment of the GEF Ras-GRP, through association with the adapter Grb2, is important for the activation of RAS (Boomer et al 2010, Janardhan et al 2011). The results of these activations will lead to Akt, JNK and ERK pathways activation.

Of importance is also the CD28-mediated activation of Vav1 protein (Hehner et al 2000). As previously mentioned, Vav1 is a crucial mediator for the subsequent cytoskeleton rearrangement occurring for IS formation and sustained signal. Furthermore, Vav1-dependent activation of Rac1 and Cdc42 induces activation of JNK and p38 and thus, promotes activation of transcription factors, such as NFAT and AP-1, involved in IL-2 production. In addition, activated Vav1 and Itk proteins joins the complex at LAT signalosome through SLP-76 binding, increasing the membrane proximal TCR events. This CD28 feedback loop shows how, even if it can independently activate its own pathways, the contribution in enhancement of TCR signals is crucial for co-stimulation. In figure 37, CD28 co-stimulatory pathways are schematized.



**Fig.37 Scheme of CD28 molecular pathways induced upon concomitant TCR ligation. Bluestone et al 2011**

All the above-mentioned findings are in line with the finding that CD28 engagement at the IS was described to be responsible of Lck recruitment and stabilization to the T cell-APC contact zone. This process was shown to be mediated by CD28-induced Vav1 activation and subsequent cytoskeleton rearrangement (Tavano et al 2004). Thus, at late time point, when the IS has been formed, CD28 might work in sustaining Lck recruitment

in close proximity to the TCR-CD3 complex and to enhance protein kinase phosphorylation and signaling through the canonic TCR signaling.

Noteworthy, a publication of 2015 from Morin and colleagues questioned the dogma that CD28 signalization is strictly dependent on the domains cited above. They could show that the receptor lacking its cytoplasmic domain was still able to signalize and contribute to TCR activation. Even though, in this case the resulting co-stimulation is impaired, thus they do not exclude the importance of the intracellular regions of CD28 for the process to occur (Morin et al 2015).

### **3.2.2 ICOS**

ICOS shares with CD28 similar molecular mechanisms to promote sustained activation. ICOS harbors in its cytoplasmic domain a YMXM motif that allows the binding of p85 PI3K, thus enhancing the PI3K/Akt signal and Itk activation (Coyle et al 2000, Arimura et al 2002).

In addition, this domain can further bind the more active PI3K subunit p50 and this binding results in stronger Akt activation capacity of ICOS compared to CD28 (Simpson et al 2010). Despite that, the role for ICOS in IL-2 production has been proved to be extremely weak compared to CD28, mainly because of its failure in binding the adapter protein Grb2 and Lck due to the lack of the PXXP region in its cytoplasmic domain (Harada et al 2003). Even though, a recent paper challenged these findings establishing a potential Lck binding site on ICOS in its TMD, which would be responsible of increased Ca<sup>2+</sup> mobilization, uncoupled from PI3K activation (Wan et al 2020). This latest model, however, needs to be further investigated.

### **3.2.3 CTLA-4**

CTLA-4 inhibitory function has also been intensively deciphered. Other than competing with CD28 for ligand binding, as described above, it inhibits TCR and CD28 signaling pathway in a more direct way by its own signalization. By associating with phosphatases SHP-2 and PP2A, through the YVKM motif present in its intracellular domain, it promotes dephosphorylation of TCR proximal molecules such as CD3 $\zeta$ , ZAP70, LAT, and PKC $\theta$  as well as downstream signaling proteins (Chemnitz et al 2004, Schneider et al 2008). Presence of CTLA-4 during T cell activation causes the unbalancing of the downstream pathways such as ERK and JNK, which therefore cause a downmodulation of TCR-CD28 associated cell-cycle proteins and cytokines production (Brunner et al 1999, Kubsch et al 2003). Conversely, it promotes the expression of other modulatory proteins, such as the E3 ubiquitin ligase Cbl-b, whose role in the downmodulation of TCR and its components has already been described in the previous chapter (Page 77)(Li et al 2004). It is worth to mention that the suppressive role of CTLA-4 is also achieved by mechanisms that involve the process of trans-

endocytosis which consist in the uptake of B7 ligands from APCs surface by T cells expressing CTLA-4 and thus limiting T cell-APCs contact and the following T cell activation (Samson et al 2015).

### **3.2.4 PD-1**

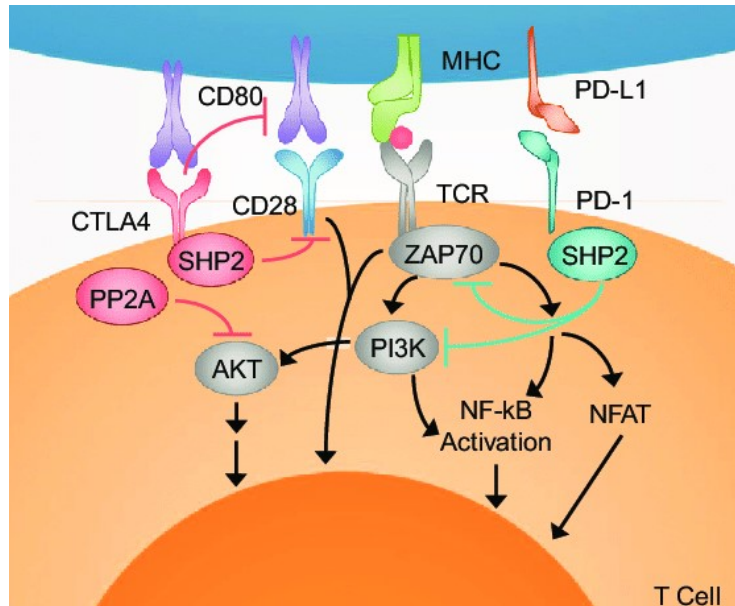
The cytoplasmic tail of PD-1 bears two motifs important for the binding of SH2 containing phosphatases: the ITIM (immunoreceptor tyrosine-based inhibitory motif) and the ITSM (immunoreceptor tyrosine-based switch motif) (Riley et al 2009). These two domains, once phosphorylated by Lck, can bind the proteins SHP-2 and SHP-1 and induce the dephosphorylation of some TCR-activated molecules such as ZAP70 and Vav1, and as a consequence reducing the downstream pathways involved in T cell activation (Sheppard et al 2004, Yokosuka et al 2012). Furthermore, a feedback loop has been identified by the mutual action of Lck and SHP-2 on PD-1. Lck allows SHP-2 binding, which in turn dephosphorylates the ITSM and causes the disassemble of the complex. Thus, constant Lck activity, coupled with PD-L1 ligation are fundamental to keep the activity of PD-1-SHP-2 complex (Hui et al 2017).

The main interactor for PD-1 has been shown to be SHP-2 which binds preferentially to the phosphorylated ITSM motif. Even though, the action of PD-1 has been also identified in absence of this phosphatase. A recent publication from the team of Malissen reports a proteomic profiling revealing that PD-1 binds SHP-2 with extremely higher affinity than SHP-1. Despite that, SHP-1 is able to compensate the absence of SHP-2 and to still perform efficiently the negative modulation of TCR signaling (Celis-Gutierrez et al 2019).

Acting mostly on common TCR and CD28 downstream targets, it was questioned if PD-1 was exerting its action during TCR activation or upon TCR-CD28 co-activation (Yokosuka et al 2012, Zikherman et al 2010, Zinselmeyer et al 2013, Parry et al 2005, Bennet et al 2003). It was claimed that CD28 was the primary target of PD-1/SHP-2 complex, describing a stronger dephosphorylation of CD28 and its targets compared to the TCR proximal signaling molecules (Hui et al 2017). Even though, tumor-related APCs rarely express B7.1 and B7.2, therefore CD28 inhibition cannot be the solely mechanism by which PD-1 exerts its suppression of cytotoxicity against tumoral cells (Juneja et al 2017). A recent paper of 2019, on the contrary, identifies the TCR signaling as the primary target of PD-1 inhibition by showing a strong dephosphorylation of the TCR membrane proximal molecules, such as CD3 $\zeta$ , ZAP70, Vav1 and PLC $\gamma$  and an abrogation of IL-2 production both in primary cells and in co-culture system of modified cell lines (Mizuno et al 2019).

Other than acting in dephosphorylation of the main TCR targets, the action of PD-1 can be exerted by indirect mechanisms. For example, cell cycle inhibition has been described to rely on the downmodulation of CK2 (cyclin) that activates the PTEN, important down modulator of PI3K/Akt pathway (Sheppard et al 2004). Furthermore, PD-1 also modulates CDKs expression by inhibiting them. This inhibition causes a cell cycle arrest and thus blockage of T cell proliferation (Patsoukis et al 2012). In addition, PD-1, as CTLA4, acts

in regulating the expression of ubiquitin ligases such as Cbl-b or c-Cbl, thus promoting the degradation of components of the signaling pathways, as well as TCR surface downmodulation. The figure 38 represents the main mechanism of action of both CTLA-4 and PD-1.



**Fig. 38 Schematization of CTLA-4 and PD-1 mechanism of action. Adapted from Borchering et al 2018.**

### 3.3 TNFRSF co-receptors: 4-1BB and OX-40

As the other co-stimulatory receptors, the activation of TNFRSF co-receptors including, 4-1BB and OX-40, lead to the activation of the classical signaling pathways such as PI3K/Akt, NF-κB, JNK, p38 which culminate in the activation of gene transcription (Watts et al 2005). Despite that, the TNFRSF co-receptors do not directly interact with the protein kinases to activate them, but they need the intracellular association with the adaptor proteins of the TRAF family (Chattopadhyay et al 2009). Tumor necrosis factor receptor-associated factors (TRAFs) are adaptor proteins initially identified for their interaction, occurring through the common C-terminal region conserved in all the components of the family, with tumor necrosis factor receptor-2 (TNFR2). Currently we know that TRAFs are able to serve as adaptor proteins for a wide variety of receptors that are involved in regulating cell death and survival. Up to now, 7 members of this family have been recognized (TRAF1-7) and some members, such as TRAF2 and TRAF3 act as E3 ubiquitin ligases enzymes. 4-1BB and OX-40 have been associated with different TRAFs proteins. 4-1BB was shown to interact with TRAF1,2 and 3. Deletion or TRAF1 or TRAF2 proteins was shown to strongly inhibit its co-stimulatory role

(Martinez-Forero et al 2013, Wang et al 2012, McPherson et al 2012). Conversely, OX40 can bind TRAF2, TRAF3 and TRAF5 where the latest was identified as a negative regulator of the NF- $\kappa$ B signaling.

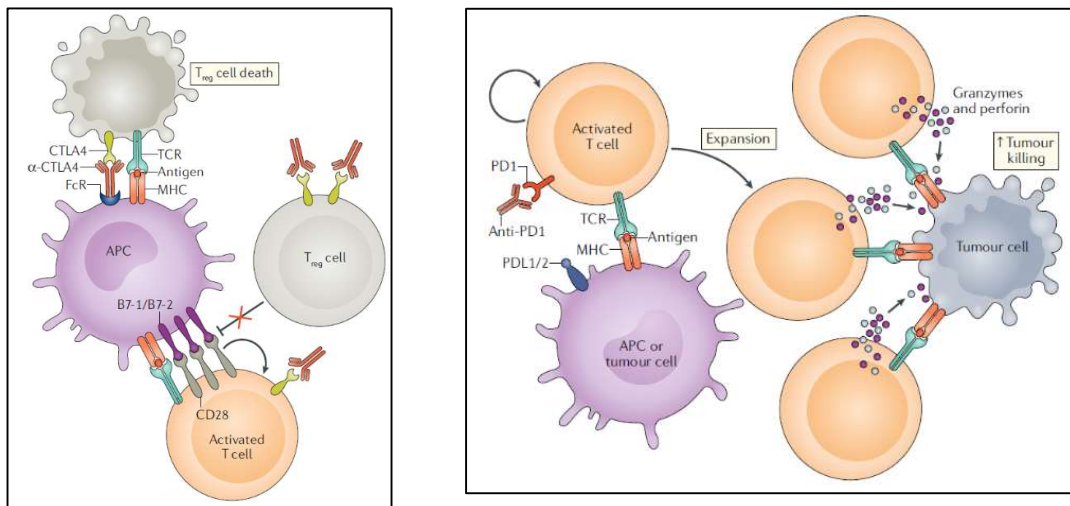
The intracellular region of 4-1BB contains two TRAFs potential binding sites, identified by mutational analysis: the regions <sup>234</sup>TTQEE<sup>238</sup> and <sup>246</sup>PEEEE<sup>250</sup>, sequences that find similarities with other TNFR members (Li et al 2018). The binding of 4-1BB to extracellular matrix proteins, such as fibronectin, laminin and galectin-9 allows the aggregation of the receptor by forming a net that enhance the TRAFs binding (Chalupny et al 1992, Madireddi et al 2014). The RING finger domain present in TRAF2 would be responsible of its E3 ubiquitin-ligase activity. Through the formation of polyubiquitin chains it would be able to bind cIAP1/2 proteins, other known ubiquitin ligases. The complex composed by receptors, TRAFs and cIAP1/2 would act as a signaling platform that recruits a complex formed by transforming growth factor beta activated kinase-1 (TAK-1) and TAK-1 binding proteins TAB-1/2/3. TAK-1 is responsible of the IKK- $\beta$  phosphorylation, that would in turn activate the canonical NF- $\kappa$ B transcription factor and ERK1/2 (Sabbagh et al 2008, Chen et al 2012). In addition, TAK-1 was shown to activate MAPKs leading to p38-mitogen activation (Cannons et al 2000). On the other side, also ubiquitin editing enzyme and DUBs have been found to associate to 4-1BB upon activation. These enzymes, such as A20 and CYLD, might contribute to the downregulation of co-stimulatory signals (Skaug et al 2011, Tokunaga et al 2012, Verhelst et al 2012, Kovalenko et al 2003).



#### 4. Immune checkpoints in cancer therapy

It has been soon evident that targeting the activity of either co-stimulatory or co-inhibitory checkpoints could have been exploited for the optimization of T cell capacities in therapeutic approaches. Main cancer immunotherapeutic strategies rely on the manipulation of co-receptor signaling, and they are in constant evolution due to their extremely strong potential. I will focus here on how targeting regulatory pathways in T cells can enhance antitumor immune response. To develop strategies acted to improve the efficiency of T cell mediated immunity is of vital importance, since the current therapies, such as chemotherapy and radiotherapy, have multiple limitations such as the elevated toxicity, elevated costs, low rate of efficiency and high relapse rate. Nowadays immunotherapeutic approaches that have been mostly described are based on two major strategies: immune checkpoint blockade (ICB) or Adoptive cell transfer (ACT) coupled with T cell engineering with chimeric antigen receptor (CAR).

**ICB** uses antibodies and chemical compounds to modulate the T cell performance in cancers (Zhao et al 2018, Arasanz et al 2017 and Fu et al 2019). Currently, blockade of co-inhibitory receptors, such as PD-1 and CTLA-4 (Fig.39), are diffusely used to treat several types of carcinomas, such advanced metastatic melanoma, non small cell lung cancer (NSCLC), renal cell carcinoma (RCC), urothelial bladder cancer, triple negative breast cancer, head and neck cancer, as well as leukemias and lymphomas, alone or in combination with stimulation of co-stimulatory receptors (Celis-Gutierrez et al 2019, Huang et al 2016, Yang et al 2012, Menk et al 2018, An et al 2019).



**Fig.39 Examples of ICB strategies of CTLA-4 and PD-1 (left and right respectively). Waldman et al 2020.**

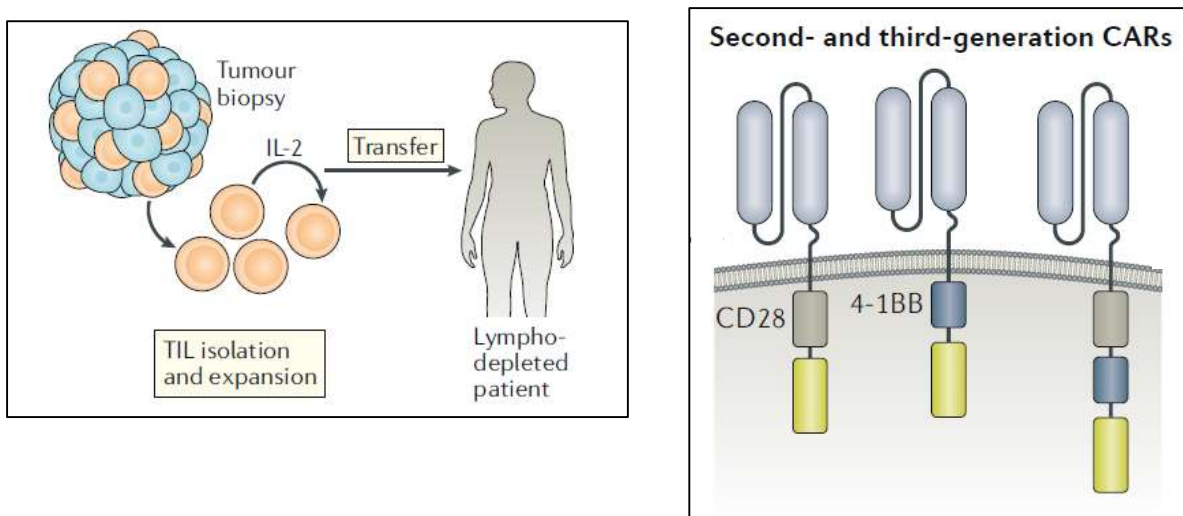
Treatments with ipilimumab, an antibody directed against CTLA-4, have been accepted from Food and Drug Administration (FDA) and successfully passed the phase III of clinical trial for advanced melanomas (Hodi et al 2010, Robert et al 2011). Benefits of this therapy have been considered relatively infrequent but highly

effective (Maio et al 2015). However, when tested in other type of cancers, is often not meeting its primary objective (van den Eertwegh et al 2012). Combination of the treatment with chemotherapy was shown to increase the overall survival in a phase II treatment of NSCLC (Lynch et al 2010). Other than the low rate of efficiency, several adverse effects have been encountered in the utilization of this approach, first of all the occurrence of “immune-related adverse events”, which consists in inflammation and immune activation in normal tissues (Fecher et al 2013). Clinical symptoms are often manageable but rare cases can be severe. Thus, the treatment can often be discontinuous and accompanied by immunosuppressive drugs (Horvat et al 2015). The utilization of PD-1/PD-L1 as target have been considered a better choice depending on the type of tumor, since the high expression of PD-L1 from solid tumor tissues have been discovered as marker for the effectiveness of the therapy. Nivolumab and pembrolizumab, two antibodies directed against PD-1, have been approved by FDA for the treatment of melanoma, NSCLC and RCC (Topalian et al 2014). Several compounds targeting both PD-1 or PD-L1 are passing through phase II and III of clinical trial. Another advantage of compounds based on PD-1/PD-L1 targeting is the lower rate and aggressiveness of the adverse effects compared to CTLA-4 based therapies (Naidoo et al 2016).

Combinatorial therapies have been proved to be more effective in some cases, as the utilization of low doses of both PD-1 and CTLA-4 antibodies (Hodi et al 2016, Callahan et al 2018, Overman et al 2018), or the coupling of the ICB with chemotherapy or radiation therapy (Arriola et al 2016, Tang et al 2017, Boyer et al 2016) as well as the combination of ICB and agonist of stimulatory immune checkpoints such as 4-1BB (CD137), OX-40, CD27, ICOS, GITR and others (Guo et al 2014, Linch et al 2014, Redmond et al 2014). CD137 is acquiring an increasing importance given its capability to strongly stimulate NK and CTL response against cancer (Menk et al 2018). Up to now it is passing the phase I of clinical treatment for several type of cancers (Morales-Kastresana et al 2013, Chester et al 2018). Furthermore, the utilization of compounds that target negative regulator of T cell activation, such as the phosphatase SHP-1, has been evaluated in order to improve the ICB strategies. A recent study on mice models have evidenced how the downmodulation of SHP-1 could lower the threshold of T cell activation, thus enhancing T cell mediated anti-tumor activities in combination with PD-1 targeting (Snook et al 2020).

However, even in case of combinatorial approaches, adverse effects have been shown to compromise the therapy, a limitation that will be overcome just with a deeper understanding of the molecular mechanisms surrounding the immune checkpoint stimulator and inhibitors and their behavior in the tumor landscape. This is one of the reasons why new compounds are continuously evaluated and the research of more effective new molecules with less disadvantages is one of the main goals of current research.

**ACT** is another approach which acts through the in vitro proliferation of autologous lymphocytes followed by reintroduction into the cancer affected patient. It was initially based on the enhancement of TILs expansion. However, the low persistence of these cells upon transfer and thus the low rate of complete response from the patients was one of the main adverse effect of this strategy (Rosenberg et al 1994). Even though, the ACT coupled with previous lymphodepletion from the patient has shown promising results in metastatic melanoma (Fig.40 left) (Rosenberg et al 2011). Moreover, it was shown that concomitant abrogation of catalytic activity of phosphatase SHP-1 during ex vivo proliferation of TILs might be useful in the enhancement of T cell anti-tumor activities (Stromnes et al 2012). In the recent years the approach has improved including the engineering of T cells by introduction of CAR-T molecules.



**Fig.40 ACT technique (left) and example of second and third generation of CARs (right). Adapted from Waldman et al 2020.**

Chimeric antigen receptors are composed by an extracellular antigen recognition site, which differently from canonic TCR does recognize pathogenic molecules even in absence of MHC on APCs or cancer cells, as for example the CD19 marker expressed by B cells in related malignancies. The TM and intracellular domains are instead composed of co-stimulatory domains which enhance the T cell activation, persistence in tumor microenvironment and boosting of antitumoral response. Up to now second and third generation CAR-T are under deep investigations and have been approved especially against B cell malignancies (Fig.40 right). Clinical trials with second-generation CAR T cells have identified efficacious response rates for B cell acute lymphoblastic leukemia (B-ALL), diffuse large B cell lymphoma (DLBCL), and other non-Hodgkin lymphoma (NHL) (Davila et al 2014, Maude et al 2015, Turtle et al 2016, Park et al 2018, Maude et al 2018). Co-receptor intracellular domains that have so far obtained the most promising results come from the utilization of CD28 and/or 4-1BB intracellular domain, but also other members of IgSF and TNFRSF are under investigation. CAR-T strategies are made up in order to promote T cell activation and resistance to cancer

counterattack. This optimization of T cell activation is usually acting through enhancement of NF- $\kappa$ B signaling, thus cytokines production and upregulation of antiapoptotic proteins.

However, several limitations are also present in the utilization of this strategy. The loss of antigen CD19, often occurring in B malignancies, is one of the causes of failure of this therapy. The detection of specific antigens present in the solid tumor limits the utilization of this approach to few types of cancer up to now (Beatty et al 2014, Park et al 2007). The research of other molecules able to specifically target tumor cells is currently under deep investigation (Majzner et al 2019, Koneru et al 2015, Yeku et al 2017) and found to be successful when CAR-T are engineered in multiple receptor specificity manner (Bielamowics et al 2018). Moreover, the utilization of CAR-T based strategies, as for ICB, has been found to cause organ toxicity caused by cytokine release syndrome (CRS). Furthermore, therapies based on this approach have to be designed in a patient-specific way and thus the costs of the utilization can be up to now extremely prohibitive (Hernandez et al 2018).

An exhaustive summary of the current investigation on cancer immunotherapy strategies is reviewed in Waldman et al 2020. To dissect the molecular pathways involved in these process is, therefore, fundamental, in order to improve the effect of the therapy, broad the spectrum of molecules that can be exploited and reduce the adverse effects that still limit the efficiency of these promising strategies.

## 5. Fas-TCR crosstalk

Conversely to the stimulatory immune checkpoints of TNFRSF cited above which have been extensively investigated for their physiologic and pathologic role, the role of Fas receptor in TCR activation, although mentioned very early after its discovery (Alderson et al 1993), was not deeply investigated and therefore remains still after 25 years a matter of debate. Even if several studies identified Fas as a TCR co-stimulatory receptor, few data report that under certain specific circumstances Fas could also play an inhibitory role. Since my PhD project aims to improve the understanding of the role of Fas as an immune checkpoint (see Thesis Aim), I will present now the data collected in the literature on Fas-TCR crosstalk in order to present these odds and discrepancies and try to understand their origins.

Fas is a crucial regulator of T cell homeostasis. For this reason, the literature describing Fas and TCR relation deeply explored the role of Fas as apoptosis inducer upon TCR stimulation and, on the other side, the role of TCR activation for Fas signaling. We have described in the first chapter of this manuscript the controversies behind the role of Fas in the death induction upon repetitive TCR stimulation (RICD), where it is suggested that Fas has only a marginal role in the elimination of the already activated cells, limited to the elimination of the autoreactive T cells (Stranges et al 2007). It was moreover evidenced how TCR activation, on the other side, might protect the cells from Fas induced cell death, in order to keep the T lymphocytes alive during the immune response. A publication by Karas *et al* describes that in the first hours upon TCR activation, upregulation of FasL is coupled with a decreasing of both caspase-8 and Fas expression, a phenomenon that would limit the initiation of a precocious death signaling (Karas et al 2001). It was furthermore hypothesized how, conversely, Fas signaling might depend on the presence of functional TCR in T cells. The study of Akimzhanov and colleagues suggested that TCR is essential for the calcium release occurring during Fas mediated apoptosis (Akimzhanov et al 2010).

### 5.1 Inhibitory or co-stimulatory Fas signaling?

The role of Fas as a TCR co-stimulatory receptor was first described in 1993, by Alderson and colleagues, that described that Fas/FasL interaction could positively regulate T cell activation and proliferation upon concomitant stimulation of the two receptors. The study was conducted on human peripheral blood mononuclear cells (PBMC) activated *ex vivo* by immobilized anti-CD3 and agonist anti-Fas antibodies (Alderson et al 1993). This description was the first one of a current long-lasting list of pro-survival functions of the receptor Fas.

The role as TCR co-stimulatory molecule for Fas has been further described by several studies, both in human and mice purified primary cells (Desbarats et al 1999, Kennedy et al 1999, Bae et al 2000, Maksimow et al 2003 and 2006, Paulsen et al 2011). However, this co-stimulation was only studied in *ex-vivo* models,

leaving a lack of information for what concern the physiologic role of Fas as a co-stimulatory receptor *in vivo*. *Lpr* and *gld* mice models, as well as human patients affected by ALPS, share the common feature of lymphoproliferation of an unusual double negative T (DNT) cell population. It is hard to find in literature findings which describe how the absence of Fas in CD4 and CD8 T cells might affect or impair their activation and proliferation, since the already altered phenotype of these mice might influence their response to pathogen challenge. The evidence that found that the insurgence in peripheral blood of ALPS patients of the DNT is caused by the loss of CD4 or CD8 co-receptors from single positive cells suggests that an impairment on their T cells activation and differentiation might occur in absence of Fas (Rensing-Ehl et al 2014, Volkl et al 2016), also corroborated by the accumulation of single positive CD4 and CD8 cells in *lpr* and *gld* mice. However, the debates on the origin of the DNT population as well as the point if the accumulation of single positive cells is caused by augmented cell proliferation in absence of Fas or from defects in the apoptotic pathway remain still unsolved.

The publication of Puliaeva of 2008 described the role of Fas in T cell activation *in vivo* but in antigen activated T cells using a pathologic model of mice subjected to T cell transfer leading to GVHD condition, thus not reflecting which might be the role of Fas in the physiology of T cell activation. In this study the authors could establish that Fas has a co-stimulatory role in both CD4+ and CD8+ populations and a helper role in promoting CD8+ cytotoxic lymphocytes effector cells. It is further demonstrated that Fas contribute also in the downmodulation of CTL response to maintain T cell homeostasis (Puliaeva et al 2008).

However, four teams challenged the co-stimulatory role by reporting also a co-inhibitory implication for Fas:

- Some studies confirmed, indeed, Fas-TCR co-stimulation just in specific conditions: the publication of Desbarats could describe a differential outcome to Fas-TCR co-engagement in naïve and memory T cells, where the first show an inhibition of T cell proliferation, reverted by addition of cytokines in the culture medium, and a co-stimulatory effect of Fas during TCR activation was shown for memory T cells (Desbarats et al 1999).

Furthermore, Maksimow study of 2003 described that naïve cells first pass through an apoptotic wave, which eliminate part of the population, but that the survivors were instead capable to be vigorously co-stimulated upon a second challenge because of the acquisition of apoptotic resistance features, such as increase of anti-apoptotic markers (Maksimow et al 2003).

Paulsen also describe a co-stimulation induced by Fas-TCR co-engagement, which is limited by the amount of FasL utilized for Fas ligation. Indeed, they found that low doses of FasL lead to co-stimulatory effect and conversely high doses are inhibiting T cells activation (Paulsen et al 2011).

- A publication of Strauss of 2007 completely disagrees on the co-stimulatory role of Fas in T cell activation: the studies are conducted on an alloantigen specific T cells and artificial APCs. They

describe a complete inhibition of T cell activation in response of Fas-TCR co-engagement (Strauss et al 2007). However, the setting conditions are altered by the utilization of not physiologic doses and form (not cleavable) of FasL expressed on the APCs. The same team conducted another research on the co-inhibitory role of Fas in T cell activation by utilization of naïve T cells as model. Even in this case the results might be affected by the non-physiologic doses of FasL used to ligate Fas receptor (Strauss et al 2009).

## 5.2 Molecular mechanisms

Molecular mechanisms underlying Fas-TCR co-engagement are largely unknown. It was never reported if the activation of the two receptors cause the activation of shared molecules (quantitative) or if they proceed by different pathways converging just at nuclear level (qualitative).

A particular importance was given to try to define caspases involvement, that was already described to participate in canonical T cell activation (Alam et al 1999, Kennedy et al 1999, Salmena et al 2003, Su et al 2005, Leverrier et al 2011). Maksimow *et al* described that activation of both Caspase-8 and Caspase-3 occurred upon co-stimulation but this activity was not leading to canonical Caspase-3 substrates cleavage, such as PARP protein. However, nor IFN $\gamma$  production or expression of activation marker were reduced upon pharmacological treatment with caspases inhibitors (Maksimow et al 2006). In line with that, Paulsen *et al* described cleavage of both Caspase-8 and Caspase-3 upon Fas-TCR co-stimulation but no cleavage of Caspase-3 substrates (PARP or PLC $\gamma$ ).

In addition, an upregulation of antiapoptotic proteins such as cFLIP<sub>R/S</sub>, Bcl-XL and plkB was detected and this was complemented by resistance to cell death and upregulation of cell cycle proteins such as cyclins, CDKs, p-Rb and PCNA (Paulsen et al 2011). Few other molecular events were dissected in TCR-Fas co-stimulation. Paulsen *et al* described an increase of ERK phosphorylation upon Fas-TCR co-triggering. The increasing of cytokines production observed was attributed to augmentation of the phosphorylation of the transcription factors T-bet, STAT1 and STAT4. Moreover, they describe an increase of TCR and Fas internalization upon low doses of FasL in co-stimulation. Interestingly, both TCR and Fas internalization, as we mentioned in the previous chapters, have been suggested to increase their signaling in some studies (Willinger et al 2015, Yudushkin et al 2010, Chakrabandhu et al 2007).

Conversely, the study of Strauss and Debatin of 2009 described that Fas engagement upon TCR triggering reduced caspases activation and its recruitment, together with the TCR-associated proteins, to lipid raft fraction at very late time point (up to 48 hours). Together with the lower recruitment of phospho-tyrosine proteins to lipid raft upon Fas-TCR co-stimulation, an inhibition of TCR membrane proximal signal, as well as late signal, was additionally described: a decrease of pPLC $\gamma$ , pLAT, pZAP70, and of MAPKs as well as less

activation of NF- $\kappa$ B, AP-1 and NFAT transcription factors upon co-triggering. Despite that, the high concentration of FasL used, which is a condition that diverges from the physiology, might explain such strong decrease of the TCR signaling. This latest consideration is also in line with the findings of a dose threshold of FasL which can shift the co-engagement from co-stimulatory (low doses of FasL) to co-inhibitory (high doses of FasL) (Paulsen et al 2011).

No data addressed the identification of the regions of Fas involved in the co-stimulation. In addition, the involvement of specific cytoplasmic domains, like the death domain critical for cell death signaling, was not afforded. In addition, the implication of the extracellular domains of Fas in allowing the Fas-TCR co-stimulation was never investigated.

The lack of notions concerning molecular mechanisms that underlie Fas-TCR co-engagement make of an extreme interest the investigation field on how this receptor works in the context of T cell activation. Given its vital importance in the physiopathology of the immune system, to decipher its way of action is crucial to unveil new important receptor features and for that reason the laboratory where I have carried out my PhD decided to face this subject.



## **THESIS AIM and RESULTS**

## 1. Thesis Aim

As we discussed in the introduction, both the nature (co-stimulatory or inhibitory effect) and the physiological (and pathological) importance of the Fas-TCR co-signaling need to be clarified. So far it appears that the apparent discrepancies on the data reported in the literature might be link to several factors, such as the cell type and status, the nature of Fas ligand used and type (nature and strength) of the TCR activation. The aim of my thesis was to progress on both the definition of the role of Fas as an (inhibitory or stimulatory) immune checkpoint and on the identification of the events involved at a molecular level.

In order to do that, we decided to answer to what we believe are still unresolved critical questions:

**a. Does concomitant Fas ligation contribute to TCR activation in a stimulatory or inhibitory manner? Does the quality of the response depend on cell developmental stage or T cell type? Could Fas TCR co-activation be described in a TCR specific manner and with native membrane ligand/receptors?**

We chose to approach this question by using both

- Total PBMCs, and not only purified naïve or memory T cells (as done in literature), as the response of the cells is strongly dependent on the cytokines present in the stroma. Therefore, the behavior of the subclasses of T cells can be different depending on the neighboring cells.
- A dedicated co-culture: a mouse hybridoma CD4<sup>+</sup> T cell line bearing a transgenic TCR $\alpha\beta$  recognizing a specific portion of a HEL (Henn eggs lysozyme) peptide (fraction 46-61) was co-incubated with modified HEK293 cells expressing the MHCII complex I-A<sup>k</sup> loaded or not with the peptide to simulate a physiologic activation, and expressing or not human FasL protein, to simulate a proper and simultaneous co-activation. A HEK293 cell line expressing also just the FasL protein was used to compare the effect of FasL stimulation to TCR and Fas/TCR stimulation, in order to understand if our outcomes were the readout of the co-engagement or if existed a contribution to the signal by the single FasL stimulation.

Moreover, it is still not elucidated if a difference exists between the two main T lymphocyte populations, CD4<sup>+</sup> and CD8<sup>+</sup>. The explanation also in this case might be that the observations have not been made in a physiologic setting, or even that the requirements or the sensitivity to Fas of the two populations might differ giving discrepancies in the results when cells are co-stimulated in the same way.

**b. Does Fas contribute to modulate T cell activation in a qualitative or quantitative manner? When and where the crosstalk between the receptor signaling occurs? And where Fas localize at the moment of the co-activation?**

There are no clues on how the TCR integrate the signal coming from Fas triggering. More precisely there are no evidence that shows if, when and where the crosstalk take place and so if the modulation of T cell activation occurs in a qualitative or quantitative manner. We decided therefore to activate and co-

activate primary cells and our co-culture 3A9-APCs model and to perform biochemical and microscopy approaches to address this point.

The qualitative or quantitative way of integration of the signal was also shown to be dependent from the localization of immune checkpoints during the co-activation (Yokosuka et al 2008, Sanchez-Lockhart et al 2008, Bashour et al 2014, Saito et al 2019). The localization of Fas during the Fas-TCR co-engagement has never been investigated. We thought that it would have been of an extreme importance to understand if upon co-triggering Fas localize at the Immune synapse or with TCR in membrane microclusters, of if the crosstalk occurs at cytoplasmic level by protein-protein interactions, since it was suggested that both TCR and other signaling proteins related to T cell activation signaling can operate at cytosol level. We afforded this point by performing microscopy techniques on 3A9 co-culture system and on PBMC activated by CD3 coated beads.

**c. Does the nature of Fas ligand and/or the type of Fas ligation modify the outcome of the crosstalk?**

Whereas the dose of the ligand was already studied in this context by Paulsen *et al* that reported that low doses of FasL promotes co-stimulation and high doses inhibit TCR signal, the nature of Fas ligand (membrane vs soluble) was never addressed. This point is of particular importance since it was reported that the nature of Fas ligand is a discriminant for the outcome of Fas signaling, defining the membrane bound form of FasL as apoptosis inducer and the soluble form of FasL the one capable to initiate pro-survival signals. We decided to address this question by using in different cell systems (primary cells and Jurkat cell line) soluble or cross-linked FasL recombinant protein for the co-stimulation. In addition, we used the 3A9 co-culture system to address the role of mbFasL for the co-engagement. Furthermore, we corroborated this technique by co-culture of primary cells coupled with a pharmacological treatment leading to the inhibition of the metalloprotease involved in FasL cleavage. Finally, we also used Fas agonist antibodies and compared the effect with FasL recombinant protein.

**d. Are the key molecules critical for the Fas induced cell death pathway also involved in Fas-TCR crosstalk?**

Role of DISC proteins Caspase-8 and FADD in Fas-TCR co-activation was still undefined. No lines of evidence in literature can be found for FADD role in Fas-TCR co-stimulation. The role of Caspase-8 has been instead mostly analyzed in term of its activity at late time point and late molecular events (activation markers, cytokines secretion). There is no description of a potential role of DISC in Fas-TCR co-activation. We afforded this point by looking at TCR proximal signal after pharmacological treatment with pan-caspases inhibitor on 3A9 co-culture system and PBMC, RNA interference on Jurkat cells and co-activating Jurkat clones deficient for FADD or Caspase-8.

**e. Which Fas domains are involved in TCR modulation?**

The DD of Fas is well known not only for being critical in its pro-apoptotic functions but also to achieve some pro-survival functions such as pro-inflammatory cytokines gene activation (Cullen et al 2013, Varfolomeev et al 2005, Ma et al 2004). However, DD-independent mechanisms have been described for Fas

non-apoptotic roles, such as for cell migration (Poissonnier et al 2016). Its importance in Fas TCR crosstalk was never described and we decided to clone a Fas mutant deprived of death domain and analyze: the behavior upon activation and co-activation by looking at TCR membrane proximal signal, by biochemical approaches, and Fas binding proteins in this mutant, using the genetic code expansion technology (described in Additional results at page 149).

**f. Does Fas TCR type of co-activation resemble the one of other co-stimulatory/inhibitory receptors? Or also, does it contribute to synergize or antagonize other co-receptors?**

Fas-TCR co-activation has not been extensively compared and correlated with other immune checkpoint receptors, such as CD28. We thought it would have been interesting to molecularly compare the similarities and differences between Fas TCR co-triggering and other co-receptors. Moreover, it would have been worth to describe if Fas ligation was able to stimulate or inhibits TCR-CD28 co-stimulation. We afforded this point by both comparing Fas-CD3 and CD28-CD3 co-activation and analyzing the cell behavior upon concomitant stimulation of the three above mentioned receptor. We used as model both PBMCs and a modified 3A9 co-culture system, where T cells were transfected with CD28 protein and the artificial APCs were transfected with the CD28 ligands, B7.1 or B7.2.

## 2. Results

**Fas enhances T cell activation by increasing the initial steps of the TCR signaling pathway independently of its death domain.**

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## **Abstract**

Immune responses are finely regulated by both co-stimulatory and inhibitory signaling pathways, driven by receptors and their cognate ligands, designated now as immune checkpoints and belonging to two important superfamilies: the immunoglobulin superfamily and the tumor necrosis factor receptors superfamily (TNFRSF). The ability to interfere in the balance of these two divergent types of signals is at the heart of cancer immunotherapy strategies. So far, all the immune checkpoints belonging to the TNFRSF appear to play a role in promoting T effector cell activity by supporting the proliferation, the survival and expansion of activated T cells, or the establishment of T cell memory.

The TNF receptor superfamily member Fas (CD95, TNFRSF6) is known as the prototypic death receptor inside and outside the immune system. The receptor Fas is well known for its abilities to trigger cell death and has been also described to induce non-death functions, including modulation of T cell activation. Despite the potential importance of the role of Fas as a co-regulatory receptor for the TCR during its activation in an immunotherapy context, only few controversial studies related to this involvement were addressed, and no associated molecular mechanism were described.

With the aim to better characterize the impact of Fas triggering on T cell activation, we demonstrate that Fas is a T cell co-stimulatory receptor acting to enhance TCR activation in both memory and naïve T cells from CD4+ and CD8+ human subsets. We show that Fas is also able to co-stimulate a physiologic TCR-specific response with native receptors and ligands. Moreover, this co-stimulatory function can be triggered both by soluble FasL and by the membrane-bound form of the ligand expressed on antigen presenting cells. At the molecular level, we demonstrate that Fas-induced signaling enhances the initial steps of the TCR-induced pathway to sustain T cell activation. Importantly, Fas co-stimulatory function does not rely on the recruitment of Fas main partners FADD and Caspase-8 to transduce its signal and neither requires the presence of its intracellular death domain. In summary, our results not only describe a new death domain independent non-death function of Fas but also a new way of action for a co-stimulatory protein of the TNSFR.

## Introduction

T cells are a major component of the adaptive immune system and play an essential role in elimination of pathogen-infected or tumor cells. Upon specific antigen encounter, T cells are activated and enter in a clonal expansion phase. Activation relies on the interaction between the T cell receptor (TCR) and the antigen, presented by antigen-presenting cells (APCs) via major histocompatibility complex (MHC) molecules. T cell response is finely tuned by co-stimulatory or co-inhibitory receptors belonging to the immunoglobulin (i.e. CD28, PD1) or the TNFR superfamilies (ie 4-1BB, OX40) engaged by their ligands present on the APC, and the integration of these co-signals respectively promote or inhibit T cell activation<sup>1, 2</sup>.

The TNFR superfamily member Fas and its ligand Fas ligand (FasL) play crucial roles in regulating T cell homeostasis and functions<sup>3, 4, 5</sup>. Indeed, mutations in the genes coding for Fas or FasL are responsible for the occurrence of the autoimmune lymphoproliferative (ALPS) syndrome, a genetic disease characterized by an uncontrolled lymphocyte proliferation accompanied by auto-immune manifestations<sup>6, 7, 8, 9</sup>. Even if FasL was first described as a cell death inducer<sup>10</sup> responsible upon binding to its receptor Fas of the elimination of activated lymphocytes recognizing low-affinity antigens<sup>11, 12, 13, 14</sup>, the Fas/FasL system was also reported to induce non-death pathways as cell differentiation through the Th17 or Th9 effector pathway, the Th17 migration and the pro-inflammatory cytokines production<sup>15, 16, 17</sup>.

At the molecular level, Fas belongs to the death receptor subfamily and contains in its intracellular part a death domain (DD) necessary for its apoptogenic capacities through the FasL-induced recruitment of the adaptor protein FADD, which allows the binding of the Caspase-8 thus generating the death inducing signaling complex (DISC)<sup>18, 19</sup>. Upon Caspase-8 oligomerization and auto-processing, active caspases are released and propagate the death signal by cleavage of downstream effector caspases 3, 6 and 7, which leads to substrate processing and ultimately to cell destruction<sup>19</sup>. Alternatively, the non-death signals triggered by Fas activation can be divided in those that require FADD and Caspases-8 to

propagate, through the recruitment of alternative proteins to the DISC such as cFLIP, TRAF-2 and RIP-1 and further activation of the MAPK and NF- $\kappa$ B pathways<sup>20, 21, 22</sup> and those which activate DISC-independent pathways<sup>15, 17, 23</sup>. Due to the high diversity of the non-death functions sustained by Fas activation, the molecular mechanisms involved, including the identification of the Fas domains and the interacting partners involved, require further investigations.

Among the non-death functions induced by Fas in T cells, several studies demonstrated its ability to modulate T cell activation<sup>24, 25, 26, 27, 28, 29, 30, 31, 32</sup>. Even if Fas has been mainly described to stimulate the T cell activation<sup>24, 25, 26, 27, 29, 30, 31</sup>, some studies also reported the opposite effect<sup>25, 28, 32</sup> and the exact nature of Fas as a TCR co-stimulatory or co-inhibitory receptor is still a matter of debate. Moreover, little knowledge is available on the specific T cell subsets affected by Fas co-signaling, or the molecular mechanism underlying this function, notably the putative involvement of the DISC proteins FADD and Caspase-8. We report here that Fas exerts TCR co-stimulatory functions in both human primary T cells and in a TCR-specific model and that all tested T cell subsets can respond to Fas co-stimulatory function. Moreover, we show that both membrane-bound and soluble FasL are able to trigger such signal. Finally, we shed light on the molecular mechanism underlying Fas co-stimulatory function and demonstrate that Fas enhances T cell activation by increasing the initial steps of the TCR signaling pathway, in a DISC and DD independent manner.

## Results

### **Fas is a T cell co-stimulatory receptor in human primary T cells and in a TCR specific murine cell line.**

Fas triggering, simultaneously with the TCR activation, has been reported to generate several types of outcome in T cells including cell death<sup>26, 27</sup>, co-stimulation<sup>24, 25, 26, 27, 30</sup> or inhibition of cell activation<sup>25, 28</sup>. In order to solve these discrepancies, we used two complementary models to assess the consequence of Fas triggering during TCR activation. First, we purified human peripheral blood



mononuclear cells (PBMC) from the blood of healthy donors. Upon cytometry phenotyping, we could quantify that between 68 and 89 % of the cells obtained were T cells depending on the donor (data not shown). Moreover, as expected, all T cells express Fas at their cell surface, with memory T cells expressing more than the naïve T cells (data not shown). CFSE-stained purified PBMC were activated for 5 days on anti-CD3 antibody coated plates to activate TCR signaling, which gave rise to around 20% of cell proliferation. Importantly, addition of FasL enhances T cell proliferation dependently on dose, as demonstrated by the increase of total dividing cells (Fig 1a left panel). More precisely, the analysis of the CFSE profile indicates that 48,1 % of cells co-stimulated with 100 ng/ml FasL replicated between 3 and 6 times compared to 20% in the anti-CD3 antibody treated condition (Fig 1a right panel). Note that the highest FasL dose gives similar results compared to the anti-CD28 antibody used as positive control in addition to TCR activation. In parallel, we detected an increase of interleukin-2 (IL-2) secretion upon FasL and anti-CD3 antibody treatment compared to anti-CD3 antibody alone (Fig 1b). Notably, we could not observe any increase in T cell death upon FasL exposure alone or in combination with anti-CD3 antibodies by propidium iodide incorporation. Furthermore, the biochemical analysis of caspases cleavage shows a slight appearance of the first Caspase-8 fragment upon exposure to anti-CD3 antibody alone, FasL alone, or a combination of both (p43), but not of the active p18 fragment neither of the downstream Caspase-8 target Caspase-3 (Fig 1c).

Knowing that our results obtained on human PBMC relied only on TCR engagement by anti-CD3 antibodies, we tested the Fas co-stimulatory function under a more physiologic TCR-specific context using the hybridoma cell line 3A9 expressing a transgenic TCR specific for the HEL peptide. 3A9 cells can be activated by co-culture with the HEK cells expressing the MHC class II protein I-A<sup>k</sup>, presenting the HEL peptide as APC (called HEK MHC), or HEK cells expressing both the I-A<sup>k</sup> protein and FasL (called HEK MHC FasL) (Fig 1d left panel). After validation of equivalent I-A<sup>k</sup> expression on HEK MHC and HEK MHC FasL and proper FasL expression in HEK MHC FasL (Fig 1d left panel), we observed that 3A9 cells co-cultured with HEK MHC, loaded with increasing amount of HEL peptide, show a dose-dependent IL-2 production and ERK phosphorylation as read-out of T cell activation (Fig.1d). Importantly, the co-

culture with HEK MHC FasL enhances both the IL-2 secretion and the ERK phosphorylation (Fig 1d). Thus, in two complementary models, we could identify a T cell co-stimulatory function for Fas.

#### **Fas T cell co-stimulatory function occurs in the main T cell subsets.**

In order to further identify which populations of T cells within the PBMC were responsive to T cell co-stimulatory function, we used a cytometry phenotyping strategy (using CD4, CD8 and CD45RO as T cell markers) and quantified the expression of three activation markers CD69, HLA-DR and Fas within the different gated populations. Importantly, we could detect an increase of the expression of all three selected activation markers when cells were co-stimulated with anti-CD3 antibody and FasL compared to anti-CD3 antibody alone in both naïve and memory T cells, thus demonstrating that Fas co-stimulatory function occurs in both populations (Fig 2a). Moreover, Fas-induced co-stimulation is detected in both CD4+ and CD8+ T cells (Fig 2b). Thus, Fas T cell co-stimulatory function is not restricted to one T cell sub-type but occurs in the main T cell subsets.

#### **Fas-induced co-stimulation occurs at TCR membrane proximal level.**

In order to determine whether FasL-induced co-stimulatory function enhances the TCR signaling or activates parallel TCR-independent pathways, we analyzed by immunoblot the early TCR signaling proteins of human PBMC activated with coated anti-CD3 antibodies and increasing doses of FasL. Importantly, a dose-dependent increase of the phosphorylation of the CD3 $\zeta$  chain, one of the earliest events in the TCR signaling, is observed after five minutes upon FasL co-stimulation (Fig 3a) and in the following steps of TCR activation, since pLAT, pPLC $\gamma$  and pERK signals are also significantly amplified (Fig 3a). The increase of LAT activation in T cells upon Fas co-stimulation condition was confirmed by confocal analysis, since the number of T cells presenting pLAT clusters on their plasma membrane doubled compared to activation conditions (Fig 3b). Interestingly, this experiment demonstrates that FasL co-stimulation allows the multiplication of the number of cells in which the TCR activation reaches a threshold and allows the propagation of the signal. In addition, when the incubation time reaches forty-five minutes, a specific polarization upon cytoskeletal rearrangement is observed in activated T

cells, which change their circular shape to a more elongated form. FasL co-stimulation significantly enhances this phenomenon as shown in figure 3c and quantified by cell circularity values (Fig 3c).

Moreover, we could confirm the increase of TCR early signaling activation upon FasL co-stimulation in the 3A9 co-culture system using a phosphotyrosine immunoprecipitation experiment. We could detect an increase of pCD3 $\zeta$ , pLAT, pZAP0 and even pLck which is the first activated protein in the TCR signaling cascade, which we did not manage to reveal in human PBMC for technical reasons (Fig 3d). In summary, our data collected in two complementary models demonstrate that Fas-induced signaling enhances the TCR pathway at early membrane proximal level.

More importantly, in the co-culture conditions described above, not only lck, LAT and PLC $\gamma$  are activated by phosphorylation, but they also co-immunoprecipitate with Fas upon Fas and TCR concomitant triggering (Fig 3e). This finding suggests that Fas brings these crucial players to TCR, thus increasing the activation of the signaling pathway, or that Fas is translocated to TCR signaling hub upon Fas and TCR activation. Both suggestions reinforce the idea that Fas and TCR signaling converge at TCR membrane proximal level.

#### **Different forms of Fas ligands can trigger Fas co-stimulatory functions.**

The nature of Fas activators, as well as the doses at which they were used, has been a matter of debate in the Fas-TCR co-stimulation studies<sup>25</sup>. In the human T cell line Jurkat, we demonstrated that both agonistic anti-Fas antibodies (Apo1.3) and recombinant FasL can induce Fas-TCR co-stimulation, as demonstrated by immunoblot analysis of TCR signaling molecules (Fig 4a). The recombinant FasL protein we used in this study contains a Flag tag in its C-terminal part, allowing crosslinking by an anti-Flag antibody (called M2), thus allowing FasL oligomerization. FasL crosslinking was demonstrated to amplify of a log the FasL killing capacity<sup>33, 34</sup>. In the same Jurkat model, we show that both soluble and cross-linked FasL generate TCR co-stimulatory signal and that cross-linking enhances FasL co-stimulatory function, as it is the case for its apoptogenic capacities (Fig 4b).

Most importantly, Fas activation by a membrane-bound FasL, obtained upon co-culture of the 3A9 hybridoma cells and the artificial APC stably expressing FasL, induces the enhancement of TCR early signaling activation as efficiently as the soluble form (Fig 4c). Moreover, we demonstrated in Figure 1d that such culture conditions not only generate TCR co-stimulation at signaling level but also at functional level since it generates interleukin-2 production. However, we could not quantitatively compare the efficiency of both forms since the concentration of membrane bound FasL is difficult to assess. We could confirm the capacity of membrane-bound FasL to trigger TCR co-stimulation on human primary T cells by co-incubating human PBMC with HEK cells stably expressing FasL and activated with anti-CD3 antibodies (Fig 4d). Membrane-bound FasL has been shown to be cleaved by the metalloprotease ADAM10, thus releasing soluble FasL<sup>35, 36</sup>. In order to assess which FasL form effectively activates Fas in our co-culture conditions to trigger TCR co-stimulation, we incubated FasL-expressing cells with the ADAM10 inhibitor GI254023X, at a dose allowing the increase of membrane-bound FasL expression (see flow cytometry analysis in Fig 4d). Importantly, the presence of ADAM10 inhibitor not only allows Fas co-stimulatory function but also increases it, thus firmly demonstrating that the membrane-bound FasL can trigger TCR co-stimulatory function.

#### **Fas co-stimulatory function is independent on the DISC proteins and Fas death domain.**

Most of the currently described Fas functions depend on the recruitment of the adaptor protein FADD and the subsequent Caspase-8 binding, which initiates the Fas signaling into both death and non-death pathways<sup>19,20</sup>. Whereas Caspase-8 self-oligomerization and auto-processing is necessary for apoptosis-induction, it is not automatically the case for non-death functions, for which both enzymatic and scaffold role have been described<sup>37</sup>. To understand whether caspase activity is necessary for Fas co-stimulatory function, we pre-incubated both human PBMC and 3A9 cells with the pan-caspase inhibitor Z-VAD prior Fas and TCR co-engagement with FasL and anti-CD3 antibodies or with the artificial APC HEK MHC FasL respectively. In both models, Z-VAD treatment did not inhibit Fas TCR co-stimulatory

functions, thus demonstrating that caspases activity is not necessary for Fas TCR co-stimulation to occur (Fig 5a and b). We could also detect an increase of TCR early signaling protein activation, which might suggest either that caspases have a negative role on that function or that by blocking the death signaling with ZVAD the pool of Fas molecules available for TCR-co-stimulation is enlarged. In addition, we demonstrated that Z-VAD did not interfere with later functional manifestation of co-stimulation, since the increase of Fas expression as an activation marker is not affected (supplementary Fig 1a).

We then took advantage of the Jurkat clones JA3 which are deficient for either FADD or Caspase-8 expression. We reported that Fas-induced TCR co-stimulation is still occurring in these cell lines, demonstrating that the Fas co-stimulatory function does not require the presence of FADD and Caspase-8 (Fig 5b). Note that the weaker stimulation and co-stimulation observed in Caspase-8 deficient cells can be attributed to the already high activation level of the TCR pathway basally observed in these cells. Moreover, FADD knock-down by specific siRNA in Jurkat cells, gave similar results and allowed the Fas-TCR co-stimulation to occur (supplementary Fig 1b). In conclusion, neither FADD nor Caspase-8 are necessary or contribute to Fas-TCR co-stimulatory function.

The domain of Fas that binds to FADD and allows the DISC formation is the DD localized intracellularly from position 230 to 314. By mutagenesis, we inserted a stop codon at position 232, thus creating a mutant that we called Fas  $\Delta$ DD that lacks the whole intracellular part from amino acid 232 (fig 5d top panel). We stably expressed through lentiviral infection Fas WT and Fas  $\Delta$ DD in the Jurkat Rapo clone which does not express endogenous Fas on its surface. Upon verification of equivalent expression of Fas and TCR on the surface of the infected cells (supplementary Fig 1c), we submitted them to activation (anti-CD3 antibody treatment) and FasL co-activation. Importantly, while Fas WT expression allows the Fas co-stimulatory function to occur compared to cells infected with empty vector lentivirus (EV) and not expressing Fas, the introduction of Fas  $\Delta$ DD also allows the generation of Fas co-stimulatory signal (Fig 5d). The same results were obtained with the reintroduction of these mutants in the 3A9 cells expressing the endogenous mouse Fas (supplementary Fig 1c). Thus, the Fas

intracellular domain located after position 232 including the DD and the extreme C-terminal domain is not necessary for Fas to generate TCR co-stimulation function in these cells.

## Discussion

Even if Fas was mainly studied for its apoptosis-inducing capacities, a first report in 1993 pointed its role as a T cell co-stimulatory receptor<sup>24</sup>, and was followed by several studies addressing this function<sup>25, 26, 27, 28, 29, 30, 31</sup>. The results obtained are various and sometimes contradictory, and the difficulties to compare them rely on the use of different models (mouse and human), different T cell subsets (total T cells, naïve, memory, CD4+,CD8+) and the type of experiments (ex vivo or in vivo). Furthermore, the way in which Fas is activated, by agonistic antibodies, recombinant FasL or membrane bound FasL and the way TCR is activated, by anti-CD3 antibodies or in a TCR-specific manner, can vary and might contribute to the observed discrepancies. Our primary goal was to precisely describe the impact of Fas triggering on T cell activation. We tested several models, cell subsets and activation conditions to finally conclude that, in all tested conditions, Fas activation had a co-stimulatory effect on T cell activation. Even if the majority of previous studies ended to the same conclusions,<sup>24, 29, 30, 31</sup> by observing an induction of T cell proliferation, interleukin secretion and activation marker expression upon Fas co-stimulation, two of them could detect some FasL-induced cell death in naïve CD4+ cells concomitantly with FasL-induced co-stimulation<sup>26, 27</sup>. In addition, Paulsen *et al* showed a FasL dose effect, demonstrating that low doses induces T-cell co-stimulation while higher doses switches the response to the inhibition of TCR-mediated T cell activation<sup>25</sup>. These findings explain the results obtained by Strauss *et al*, which concluded to an inhibition of TCR activation by Fas triggering using high FasL conditions<sup>28</sup>. With the doses we used (200 times lower), we were not able to detect any cell death nor inhibition of T cell activation. Importantly, with the aim of comparing the response of the different T cell subsets, we show that FasL treatment generally promotes TCR-mediated T cell activation in human PBMC and that this co-stimulatory effect can be observed in both naïve and memory subsets, as well as in both CD4+ and CD8+ T cells. This universal role of Fas as a co-

stimulatory receptor is of great interest, since it could maximize the effect on immune response by cumulating the response of all subsets. However, these results are obtained in *ex vivo* culture and should be confirmed *in vivo*. Indeed, the only *in vivo* study addressing the role of Fas in T cell co-stimulation, used the graft versus host disease model by transferring T cells from WT or from the *lpr* Fas mutated mice into a WT host and reached identical conclusions. They could reveal a co-stimulatory role for Fas in initial expansion in both CD4 and CD8 T cells, with a critical role of Fas expressed in CD4 cells to help CD8 cytotoxic effector cells<sup>29</sup>.

Importantly, we can also confirm the role of Fas as co-stimulatory T cell receptor in a more physiologic model, using native membrane-bound receptors and ligands in a TCR-specific system. We can demonstrate in this model that FasL is able to give a strong co-stimulatory signal to a specific TCR response generated by antigen presented by an artificial APC. Notably, the membrane-bound FasL expressed on the APC is also able to induce TCR co-stimulation as well as the soluble one. This critical aspect was definitely validated by obtaining a stronger T cell co-stimulation upon ADAM10 inhibitor treatment, which inhibits FasL cleavage into soluble FasL<sup>35, 36</sup>. This result is critical since it shows that membrane-bound FasL is not only able to generate cell death but also non-death signals. It was generally thought that membrane-bound FasL, by being associated in highly aggregated trimers, gave rise to death induction upon Fas activation while soluble or cleaved forms, by losing its oligomerization capacity, led to non-death functions<sup>38, 39, 40</sup>. In accordance with our results, the study of Klebanoff *et al* demonstrated that FasL expressed on memory T cells induces a precocious differentiation of the naïve T cells and identified the membrane-bound FasL form as the one responsible for that signal<sup>41</sup>. The exact source of membrane-bound FasL, expressed at plasma membrane or at the surface of exosomes has to be clearly established. In addition, we proved that the soluble recombinant FasL can generate Fas co-stimulatory signal independently of its oligomerization status. We still need to define whether the naturally occurring cleaved FasL displays the same capacities.

TCR and co-stimulatory receptors both generate signaling cascade that converge to allow full T cell activation<sup>1</sup>. However, an intriguing point regards the level of integration of their signaling. Even if Fas has been described as a T cell co-stimulatory receptor in several studies, the molecular mechanism underlying this function remains unsolved. Importantly, we demonstrate here for the first time that, opposite to the other TNFR which trigger TCR-independent signaling pathways to enhance T cell activation<sup>1,2</sup>, Fas co-stimulatory function occurs at TCR membrane proximal level, since Fas activation significantly enhances the first events of TCR-mediated cascade, i.e. Lck, CD3 $\zeta$ , ZAP70, LAT and PLC $\gamma$  phosphorylation. These results suggest that Fas acts quantitatively on TCR signaling even if we cannot exclude the additional involvement of TCR-independent pathways. For comparison, both CD28 and ICOS co-stimulatory capacities have been shown to rely partially on calcium mobilization, an early PLC $\gamma$ -dependent event that is essential for T cell activation, suggesting that the pathway they induce converge, at least in part, with the TCR signaling at that level<sup>42,43</sup>. In addition, Lck binding to ICOS was recently demonstrated, and ICOS was proposed to bring Lck to TCR complexes, thus explaining how ICOS could stimulate calcium mobilization triggered by TCR<sup>44</sup>. Remarkably, we also show that Lck, PLC $\gamma$  and LAT co-immunoprecipitate with Fas upon Fas-TCR co-stimulation, demonstrating that they are part of the same protein complex. This last observation might suggest that, upon co-stimulation, Fas recruits these proteins and bring them to TCR signaling complex, which would explain how Fas enhances TCR early signaling. However, these co-interactions might also argue in favor of a recruitment of Fas to the immunological synapse where TCR signaling is initiated, Lck, PLC $\gamma$  and LAT, thus found associated with Fas as a consequence of its localization. Both hypotheses imply a close proximity between Fas and TCR upon their concomitant activation that we need to further clarify. Such close localization at the immunological synapse of TCR and CD28 has been described to be necessary for its co-stimulatory functions<sup>45</sup>.

Several members of the TNFR superfamily display great potential in inducing T cell co-activation as GITR, 4-1BB, CD27, DR3 or OX40<sup>1,2</sup>. Importantly, all these TNFRs recruit adaptors of the TRAF (TNF receptor associated factors) family, which allows the activation of the NF- $\kappa$ B and MAPK survival



pathways supporting their co-stimulatory function<sup>46, 47, 48</sup>. Indeed, TNFR superfamily members are divided in three subgroups depending on their intracellular domain<sup>49</sup>: (i) those bearing a TRAF-interacting motif (TIM) which represents most of the T cell co-stimulatory cited above, (ii) those bearing a death domain (DD) recruiting TRAF through the adaptor TRADD such as DR3<sup>48</sup>, (iii) those also bearing a DD allowing the recruitment of the adaptor FADD and Caspase-8 to signal. Fas is so far the only member of the third group described to display T cell co-stimulatory function. Surprisingly, by using a Fas mutant devoid of its C-terminal part including the death domain, we showed that the DD of Fas is not necessary for its TCR co-stimulatory function. This is consistent with our additional results demonstrating that neither FADD, which is the main adaptor linking Fas DD to signaling, nor Caspase-8 are required for Fas to trigger T cell co-stimulation. In fact, non-death functions of Fas independent of the DD have already been reported<sup>17, 50</sup>. Poissonnier *et al* demonstrated that Th17 cell migration depends on the recruitment of PLC $\gamma$  to the membrane proximal domain of Fas without any contribution of the death domain. It is now crucial to identify which domain(s) of Fas, among the membrane proximal, the transmembrane domain or even extracellular domain would be necessary or contribute to its co-stimulatory function. The involvement of FADD and Caspase-8 in TCR activation has been described for long, but does not impact the TCR early signaling<sup>51, 52</sup>. It is now clear that it relies on the ability of Caspase-8 to inhibit RIPK-dependent signaling and necroptosis<sup>53, 54</sup>. The role of caspases in Fas-TCR co-stimulation has been also assessed and two studies concluded that the caspase inhibitor Z-VAD treatment impairs the Fas-TCR-co-stimulatory functions<sup>25, 55</sup>. However, their conclusions rely on the use of very high concentration of the caspases inhibitor. By using lowest but efficient Z-VAD concentration, we did not obtain any decrease of Fas-co-stimulatory function in human PBMC and 3A9 co-culture model in both early and later signaling. We strengthened this point by showing that Jurkat cells deficient for FADD or Caspase-8 still display Fas co-stimulatory signaling. Interestingly, recent publications soundly demonstrate that Fas is required for Th17 and Th9 differentiation. These works showed that caspase inhibition does not block the Fas-mediated role in T cell differentiation<sup>15, 16</sup>. Thus,

we identified a new Fas signaling pathway that is independent of its DD and independent of FADD and Caspase-8 recruitment.

Altogether, our results clearly established that Fas is a T cell co-stimulatory receptor which is able to trigger the co-stimulation of the different T cell subsets tested, upon activation by both membrane-bound and soluble FasL. Moreover, we demonstrate that Fas is an atypical TNFR co-stimulatory receptor since (i) Fas is the only DD-bearing member of its TNFR subgroup to trigger TCR co-stimulatory function (ii) Fas-induced co-stimulatory function occurs independently from the DD and the recruitment of its main partners FADD and Caspase-8 and (iii) Fas enhances TCR co-stimulation on the early steps of TCR signaling (Figure 6). The fact that receptors from the TNFR superfamily can boost the magnitude of T cell response and the generation of effector and memory T cells imposed them as part of the primary targets for immunotherapies. Further studies are necessary to unravel the precise molecular mechanism underlying the role of Fas as a co-stimulatory receptor and to consider the targeting of Fas as an alternative immunotherapeutic strategy.

## **Material and methods**

### **Antibodies and reagents**

Fas antibody used for immunoprecipitation (Apo1.3) is from Adipogen. For plate coating, anti-human CD3 (clone UCHT-1), anti-human CD28 (clone CD28.2) and anti-mouse CD3 (clone 2C-11) are from BD Biosciences and the control mouse IgG from Sigma. For FACS analysis, PE-anti-Fas (clone DX2) is from Miltenyi Biotech, biotin-anti Fas from Invitrogen, PE-anti-human TCR $\alpha\beta$  from Biolegend, anti-FasL (clone Nok-1) from TEBU, FITC-anti-CD8, PE-Cy7-anti-CD4, BV421-anti-CD69, BV421-anti-HLA-DR, APC-anti-CD45RO, PE-CF594-anti-CD3, FITC-anti-mouse TCR $\beta$  (clone H57) and PE-anti-mouse I-A<sup>k</sup> from BD Biosciences. For immunoblotting, anti-pY174 Vav is from Abcam, anti-ZAP-70, anti-PLC $\gamma$  and anti-caspase-3 from Transduction Laboratories, anti-Ezrin from Invitrogen, anti-FADD from MBL, anti-pY142 CD3 $\zeta$  and anti-Lck from BD biosciences, anti-Akt, anti-pY783 PLC $\gamma$ , anti-pY191 LAT, anti-caspase-8 and

anti-pT202 pY204 ERK1/2 from Cell Signaling Technology. HRP-coupled secondary antibodies are from Jackson Immunosearch.

Flag-recombinant human FasL (rhFasL) from Enzo Life Science is crosslinked with anti-Flag M2 from Sigma. Human recombinant Interleukin-2 is from Roche. Z-VAD is from Enzo Life Science and GI254023X from Sigma. CFSE, DAPI, and fluorescent-coupled secondary antibodies from Molecular Probe.

### **Constructs**

The pLenti6-hFas 232TAG was obtained by site-directed mutagenesis using the pLenti6-hFasWT as template, CTTGAGTAAATAGATCACCACTATTGC and GCAATAGTGGTGATCTATTTACTCAAG as forward and reverse primers respectively.

### **Cell culture, treatment and transfection**

Human PBMC were purified from blood samples of healthy donors (Etablissement français du sang, Marseille) by density gradient centrifugation over a Ficoll solution (GE Healthcare) and cultured in RPMI supplemented with 10% fetal calf serum and recombinant Interleukin-2. Naïve T cells were purified using negative selection kits (Milteniy Biotech). Jurkat cells subclones (JA3, JA3 FADD<sup>-/-</sup>, JA3 caspase-8<sup>-/-</sup> and Jurkat Rapo) were grown in RPMI supplemented with 10% fetal calf serum, 3A9 in RPMI supplemented with 5% fetal calf serum and HEK293 in DMEM supplemented with 10% fetal calf serum.

3A9 hybridoma cells displays a TCR specific for the hen egg lysozyme (HEL) bound to MHC class II molecule I-A<sup>k</sup> and were transfected to express the CD4 co-receptor. Jurkat Rapo EV, Fas WT and Fas  $\Delta$ DD and 3A9 EV, Fas WT and Fas  $\Delta$ DD were obtained by transduction of the corresponding lentiviral construct in Jurkat Rapo cells and 3A9 cells respectively followed by blasticidin (Invitrogen) selection. To do so, lentivirus stock were produced by co-transfection of HEK293 cells with the pseudotyping vector pMD2.G, the packaging vector p8.91 and the lentiviral cloning vector pLenti6 encoding the

protein of interest. HEK293 MHC and HEK293 MHC FasL were obtained by stable transfection of the I-A<sup>k</sup> encoding vector into HEK293 and HEK293 FasL respectively.

For caspase inhibition, cells were pre-incubated for 1 hour with Z-VAD-FMK (Enzo Life Science) which was kept during the activation time. In order to inhibit the FasL cleavage, the ADAM10 inhibitor GI254023X was added to the culture medium 5 minutes before the activation.

Transient transfection of siRNA was obtained by using the nucleofection technology (Amaxa). siRNA control (CGUACGCGAAUACUUCGA) and siRNA FADD (GCAAUUCUACAGUUUCUACU) were obtained from Eurofins.

### **T cell activation**

Human PBMC were activated by immobilized anti-human CD3 antibodies (0.5µg/ml) alone or in combination with anti-human CD28 antibodies (5 µg/ml) used as positive control. Isotypic control IgG was used as negative control. 96, 48 or 24 well plates were incubated overnight at 4°C with 50, 200 and 400µl respectively with the indicated concentration of the antibody solution in PBS, washed and further incubated for 20 min at RT with 0.1% BSA. Cells were plated and indicated doses of recombinant human FLAG-FasL (crosslinked or not with 1µg/ml anti-Flag antibody M2) or anti-Fas antibodies (Apo1.3) was added in solution. Jurkat cells were activated as the PBMC but with 2µg/ml anti-CD3 antibodies.

3A9 cells were activated by co-culture in a 3:1 ratio with HEK MHC or HEK MHC FasL previously incubated overnight with the indicated concentration of the HEL peptide 46-61.

### **Western blotting**

Western blot analysis of total proteins was performed according to standard protocols. Briefly, cells were lysed with Laemmli buffer pre-heated at 95°C. After sonication, quantification, denaturing and reduction (dithiothreitol 1 mM, 95°C, 5 min) the solubilised proteins were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), followed by transfer to a PVDF membrane (Immobilon-P; Millipore). Immunoblotting was performed using indicated primary antibody and HRP-coupled secondary antibody (Jackson ImmunoResearch). Quantitative densitometry was performed using Image J.

### **Immunoprecipitation**

Cells were lysed with buffer A (25 mM Hepes, 150 mM NaCl, 1 mM EGTA, 10 mM NaF, 10 mM Na-pyrophosphate, 5 mM sodium orthovanadate, 25 mM  $\beta$ -glycerophosphate, protease inhibitors cocktail) containing 1 % NP-40 and 10 % glycerol (lysis buffer) at 4 °C. The post-nuclear supernatants obtained after sonication and centrifugation at 10000g for 10 minutes at 4°C were incubated with the anti-Fas antibody Apo1.3 (Alexis) or anti-phosphotyrosine antibody (4G10) coupled to protein G-sepharose beads (Zymed) for 2 hours. After 4 washes with lysis buffer, beads were eluted with Laemmli buffer at 95°C for 5 min and subjected to western blot.

### **Cell proliferation analysis**

Human PBMC (10 million) were stained with 0.3 $\mu$ M CFSE in PBS for 5 minutes at RT and immediately washed twice in PBS 10 % FCS. Stained cells were plated on coated 96 wells plates and treated with the indicated amount of recombinant human FLAG-FasL for 5 days. At the end of the incubation, cells were detached by pipetting, stained with 1  $\mu$ g/ml propidium iodide to allow dead cells exclusion and analysed on the BD LSRFortessa SORP (Becton Dickinson).

### **Cell death analysis**

Human PBMC (1 million) were activated as indicated in presence or not of recombinant human FLAG-FasL (100 ng/ml). After 24 hours, cells were detached by pipetting, incubated with 1 µg/ml propidium iodide which specifically stains dead cells and analysed on the BD LSRFortessa SORP (Becton Dickinson).

### **Interleukin-2 production**

Human naïve T cells were activated for 24 hours as indicated without Il-2 supplementation. Cell medium was collected, centrifuged twice at 800g and 10000g to get rid of dead cells and debris respectively and analysed by ELISA to quantify Il2 (MSD U-PLEX). The same protocol was used for 3A9 cells co-cultured with HEK MHC or HEK MHC FasL in a 3:1 ratio for 24 hours (eBiosciences).

### **Analysis of protein surface expression by FACS**

Human PBMC (1 million) activated for 24 hours as indicated were harvested and simultaneously stained at 4°C for 30 minutes with APC-anti-CD45RO antibody, FITC-anti-CD8 antibody, Cy7-PE-anti-CD4 antibody, biotin-anti-Fas and either with BV421-anti-CD69 antibody or anti BV421-anti-HLA-DR. Samples were washed and stained at 4°C for 20 minutes with streptavidin-PE. Cells were finally analyzed on a BD LSRFortessa SORP (Becton Dickinson) equipped with four lasers and the BD FACSDiva software. For CD8-FITC, fluorescence was excited with the 488 nm laser and measured with a 530/30 bandpass filter. For Fas-PE, fluorescence was excited with the 561 nm laser and measured with a 586/15 bandpass filter. For CD45RO-APC, fluorescence was excited with the 633 nm laser and measured with a 670/14 bandpass filter. For CD69-BV421 or HLA-DR-BV421, fluorescence was excited with the 405 nm laser and measured with a 450/40 bandpass filter. T cells were gated for CD4 or CD8 cells positivity. Among them, CD45RO<sup>+</sup> and Fas<sup>High</sup> cells were defined as memory cells and CD45RO<sup>-</sup> and Fas<sup>Low</sup> as naïve. The analysis of CD69, HLA-DR and Fas expression was performed for each gated cell population and the median of fluorescence intensity determined for each marker.

Fas and TCR surface expression on Jurkat Rapo and 3A9 cells was also assessed by FACS analysis using anti-Fas DX2 antibody, anti-human TCR $\alpha\beta$  antibody and anti-mouse TCR $\alpha\beta$  antibody. MHC class II protein I-A<sup>k</sup> and FasL expression was analyzed on HEK MHC and HEK MHC FasL using the PE-anti-mouse I-A<sup>k</sup> and anti-FasL Nok1 antibody. Briefly, 500000 cells were stained on ice for 20 min with the indicated antibody before analysis on a BD LSRFortessa SORP (Becton Dickinson).

### **Immunofluorescence**

For pY191 LAT staining experiments, human PBMC were plated in the presence of recombinant human FLAG-FasL (100 ng/ml) on anti-CD3 antibody (2  $\mu$ g/ml) or control IgG-coated coverslips previously treated with poly-L-lysine. Upon 20 min activation, cells were fixed with 4% paraformaldehyde for 15 min at RT and permeabilized in 0.005% saponin buffer for 10 min. After 1 hour incubation with anti-pY191 LAT and AF594-anti-CD3 antibodies for 1 hour at RT, cells were washed and stained for 45 min with goat anti-rabbit-AF488 secondary antibody and DAPI. Alternatively, in order to assess the activation-induced morphological changes cells were incubated for 45 min before fixation and stained with AF594-anti-CD3 antibody and DAPI for 45 min at RT. In both conditions, samples were analyzed by confocal microscopy using a Zeiss LSM 880 on an inverted Axio Observer.Z1 stand (Carl Zeiss Microscopy GmbH, Jena, Germany), using objective Plan Apo 63X oil 1.4 NA. The LASERs used were LASER diode 405 nm, Argon LASER (458, 488, 514 nm) and DPSS 561 nm.

For pLAT staining experiments, the number of cells presenting pLAT clusters was determined by using the Image J software. For the experiments assessing the activation-induced morphological changes, the Image J software was used to determine cell circularity values, 1 defining round cells while lower values were attributed to polarized cells.

## Statistical analysis

Most statistical analyses were performed using a non-parametric two-tailed unpaired Mann–Whitney test for comparison between two groups with 0.1% range of confidence ( $p < 0.001$ ). For the evaluation of the activation-induced morphological changes, data were analyzed using a two-tailed t test.

## Figure Legends

**Figure 1: Fas is a T cell co-stimulatory receptor in human primary T cells and in a TCR-specific murine cell line.**

**a:** Human PBMC were stained with CFSE and cultured for 5 days in the presence of control IgG, anti-CD3 antibodies alone or in combination with FasL (10 or 100 ng/ml). A co-treatment with anti-CD3 and anti-CD28 antibodies was used as positive control. A fold increase of dividing cells compared to the TCR activation condition (anti-CD3 antibody) for 3 experiments is shown on the left panel and an immunoblot showing pERK activation as a biochemical marker of T activation is included. Given the high variability of the CFSE profiles obtained from different healthy donors, a representative analysis of the number of division is shown on the right panel, P0 corresponding to non-dividing cells and P6 to cells obtained after 6 cycles **b.** Human naïve T cells were exposed for 24 hours to control IgG, anti-CD3 antibodies alone or in combination with FasL (10 or 100 ng/ml). IL-2 secreted in the medium was quantified by ELISA and a fold increase of IL-2 secreted compared to anti-CD3 antibodies condition is shown ( $n=3$ ). **c.** Human PBMC were treated for 24 hours with anti-CD3 antibodies, FasL (100 ng/ml) or both and analyzed for cell death by propidium iodide (PI) incorporation (left panel). The numbers included in the histograms represents the percentage of PI positive cells. Alternatively, the cells were lysed for immunoblot analysis of Caspase-3 and Caspase-8 profiles (right panel). FL represents the full-length pro-form of the caspases, cl the cleaved forms. A representative experiment out of 3 is shown. **d.** Schematic representation of the co-culture model between the TCR-specific 3A9 murine T cells and



the artificial APC system. FACS profiles on the left panel show the surface expression of FasL and the MHC class II protein I-A<sup>k</sup> on the HEK MHC and HEK MHC FasL cells used as APC. 3A9 cells were co-cultured for 24 hours in a 3:1 ratio with HEK MHC or HEK MHC FasL cells loaded with increasing concentration of HEL peptide. IL-2 secreted in the medium was quantified by ELISA and the fold induction compared to 3A9 cultured alone is represented (n=2). Alternatively, cells were lysed for immunoblot analysis of ERK activation. In all graphs, error bars represent mean $\pm$  SD. \*\*\* represents p <0.001 in a Mann-Whitney statistic test.

**Figure 2: Fas T cell co-stimulatory function occurs in the main T cell subsets.**

Human PBMC were cultured for 24 hours in the presence of control IgG, anti-CD3 antibodies, FasL (100 ng/ml) or a combination of both. Upon staining with phenotyping antibodies and gating for defined population as indicated in material and methods, the expression of the activation markers CD69, Fas and HLA-DR were assessed by FACS analysis. Histograms from one representative experiment out of three are shown for both naïve and memory cells (a). The numbers included inside the FACS histograms represent the median of the studied activation markers. Graphs comparing the expression of CD69, Fas and HLA-DR in CD4<sup>+</sup> or CD8<sup>+</sup> cells are shown (b). They represent the fold induction of the median for each sample compared to the value obtained by the naïve CD4 cells treated with anti-CD3 antibodies. The error bars represent the SD (n=3 for CD69 and Fas, n=2 for HLA-DR). \*\*\* represents p <0.001 in a Mann-Whitney statistic test.

**Figure 3: Fas-induced co-stimulation occurs at TCR membrane proximal level.**

a. Human PBMC were activated for 5 minutes with control IgG, FasL (100 ng/ml), anti-CD3 antibodies alone or in combination with increasing doses FasL (1, 10 or 100 ng/ml) before lysis and immunoblot analysis with the indicated antibodies. One representative immunoblot of phosphorylated proteins from the TCR signaling pathway is shown in the left panel. A quantification by densitometry is shown on the right panel (n=3) and represents the fold induction compared to anti-CD3 antibody activation alone for each studied protein. b. Human PBMC were activated for 20 minutes with control IgG, anti-

CD3 antibodies alone or in combination with FasL (100 ng/ml) and stained for confocal analysis. Anti-CD3 antibody coupled to AF-594 was used to identify T cells. pLAT staining is shown and the percentage included represents the percentage of T cells presenting pLAT clusters quantified by Image J. **c.** Human PBMC were activated for 45 minutes as in (b) and stained for confocal analysis. Activation-induced morphological changes and polarization was evaluated by cell circularity values using the image J software, 1 defining round cells while lower values were attributed to polarized cells. A graph representing the median of these circularity values depending on the cell treatment is shown. \*\*\*\* represents  $p < 0.001$  in a Student t test. **d-e.** 3A9 cells were co-cultured for 5 minutes with the indicated APC (MHC and MHC FasL) previously loaded with 1  $\mu$ M HEL before lysis. Anti phosphotyrosine immunoprecipitation (**d**) or anti Fas immunoprecipitation (**e**) was performed and followed by immunoblot analysis of the indicated proteins. The included IP control represents beads and antibody condition. One representative experiment is shown on the top panel and a quantification by densitometry is shown below ( $n=3$ ) and represented by the fold induction compared to condition of co-culture with HEL-loaded MHC cells for each studied protein. For graphs presented in (a), (d) and (e), error bars show the mean  $\pm$  SD. \*\*\* represents  $p < 0.001$  in a Mann-Whitney statistic test.

**Figure 4: Different forms of Fas ligands can trigger Fas co-stimulatory functions.**

**a-b.** The Jurkat cells (clone JA3) were treated for 5 minutes with anti-CD3 antibodies alone or in combination with Fas activating molecules before lysis and immunoblot analysis. Anti-Fas antibodies Apo1.3 (1 $\mu$ g/ml) or FasL (10 ng/ml) were tested in (**a**) while indicated doses of FasL (in ng/ml) crosslinked or not with M2 antibody (XL) were tested in (**b**). **c.** 3A9 cells were co-cultured with the MHC or MHC FasL in a 3:1 ratio for 5 minutes before lysis and immunoblot resolution. Increasing doses of FasL (ng/ml) were included for co-culture conditions with MHC cells. **d.** Human PBMC were activated with anti-CD3 antibodies and co-cultured in a 5:1 ratio with HEK cells or HEK FasL cells stably expressing FasL in the presence of the ADAM10 inhibitor GI254023X (10  $\mu$ M) before lysis and immunoblot resolution. A FACS histogram shows the FasL expression on cell surface upon incubation with ADAM10

inhibitor (GI) and numbers represent the FasL median for both conditions. For all experiments (a-d), a representative immunoblot is shown on the top panel and quantification by densitometry is shown below (n=3). The represented fold induction is calculated relatively to TCR activation alone (anti-CD3 antibodies for a and b, co-culture with HEL-loaded MHC cells for c and co-culture with HEK cells treated with anti-CD3 antibodies for d) for each studied protein. Error bars show the mean +/- SD. \*\*\* represents  $p < 0.001$  in a Mann-Whitney statistic test.

**Figure 5: Fas co-stimulatory function is independent on the DISC proteins and Fas DD.**

**a.** Human primary T cells pre-incubated for 1 hour with the pan caspase inhibitor Z-VAD (10  $\mu$ M) were activated by control IgG, anti-CD3 antibody alone or in combination with indicated doses of FasL for 5 minutes before lysis and immunoblot analysis. **b.** 3A9 cells were pre-incubated for 1 hour with Z-VAD (10  $\mu$ M) and co-cultured for 5 minutes with the indicated APC before lysis and immunoblot analysis. **c.** The Jurkat cell clones JA3 wild type (JA3), JA3 deficient for FADD (JA3 FADD  $-/-$ ) and JA3 deficient for Caspase-8 (JA3 Casp8 $-/-$ ) were activated for 5 minutes with control IgG, anti-CD3 antibodies alone or in combination with FasL (10 ng/ml) before lysis and immunoblot resolution. **d.** The Jurkat Rapo cells deficient for Fas expression and stably transduced with empty vector (EV), Fas WT or Fas  $\Delta$ DD lentivirus were activated for 5 minutes with control IgG, anti-CD3 antibodies alone or in combination with FasL (10 ng/ml) before lysis and immunoblot resolution. A simplified scheme describing the Fas WT and Fas  $\Delta$ DD mutant is shown above. For all experiments (a-d), a representative immunoblot and quantification by densitometry (n=3) is shown. The represented fold induction is calculated relatively to TCR activation alone (anti-CD3 antibodies for a, c and d, co-culture with HEL-loaded MHC cells for b) for each studied protein. Error bars show the mean +/- SD. \*\*\* represents  $p < 0.001$  in a Mann-Whitney statistic test.

**Figure 6: Fas-induced T cell co-stimulatory function occurs at early stage of TCR signaling independently of the Fas DD and DISC proteins.**

This figure shows our conclusion model. In the left panel, Fas-induced cell death pathway is schematically represented. Membrane-bound FasL binding to Fas induces the recruitment of the DISC proteins FADD and Caspase-8 on the Fas DD which lead to caspase cascade and cell death. Our current main results are depicted on the right panel: membrane-bound FasL activates a DD-independent pathway which enhances the early steps of the TCR signaling without the contribution of DISC proteins, thus resulting in increased T cell activation.

**Supplementary figure 1:**

**a.** Human primary T cells pre-incubated with Z-VAD (10  $\mu$ M) for 30 minutes were activated for 24 hours with control IgG, anti-CD3 antibodies alone or in combination with FasL (100 ng/ml) in the presence of Z-VAD. The expression of Fas as activation marker was assessed by FACS analysis on T cells and Fas median represented on a graph (n=3). **b.** JA3 T cells were transfected with siRNA control or siRNA targeting FADD prior activation of 5 minutes with control IgG, anti-CD3 antibodies alone or in combination with FasL (10 ng/ml) before lysis and immunoblot resolution. **c.** FACS histograms showing the surface expression of human Fas and TCR on lentivirus transduced Jurkat Rapo cells (left panel) or 3A9 cells (right panel). The 3A9 cells stably transduced with empty vector (EV), Fas WT or Fas  $\Delta$ DD lentivirus were activated for 5 minutes with control IgG, anti-CD3 antibodies alone or in combination with FasL (10 ng/ml) before lysis and immunoblot resolution. For b and c, a representative immunoblot and quantification by densitometry (n=3) is shown. The represented fold induction is calculated relatively to TCR activation alone (anti-CD3 antibodies). For all graphs, error bars show the mean  $\pm$  SD.

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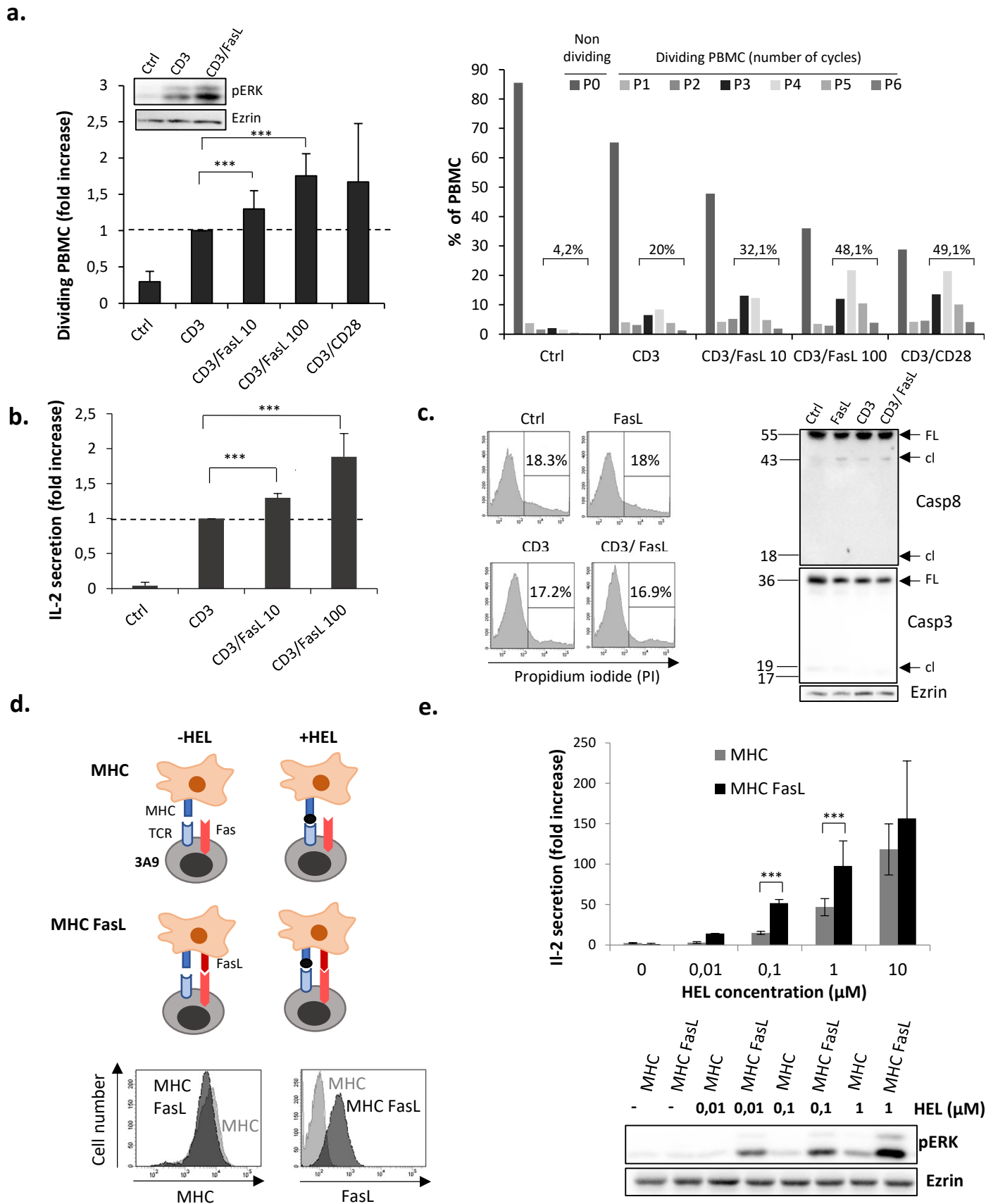
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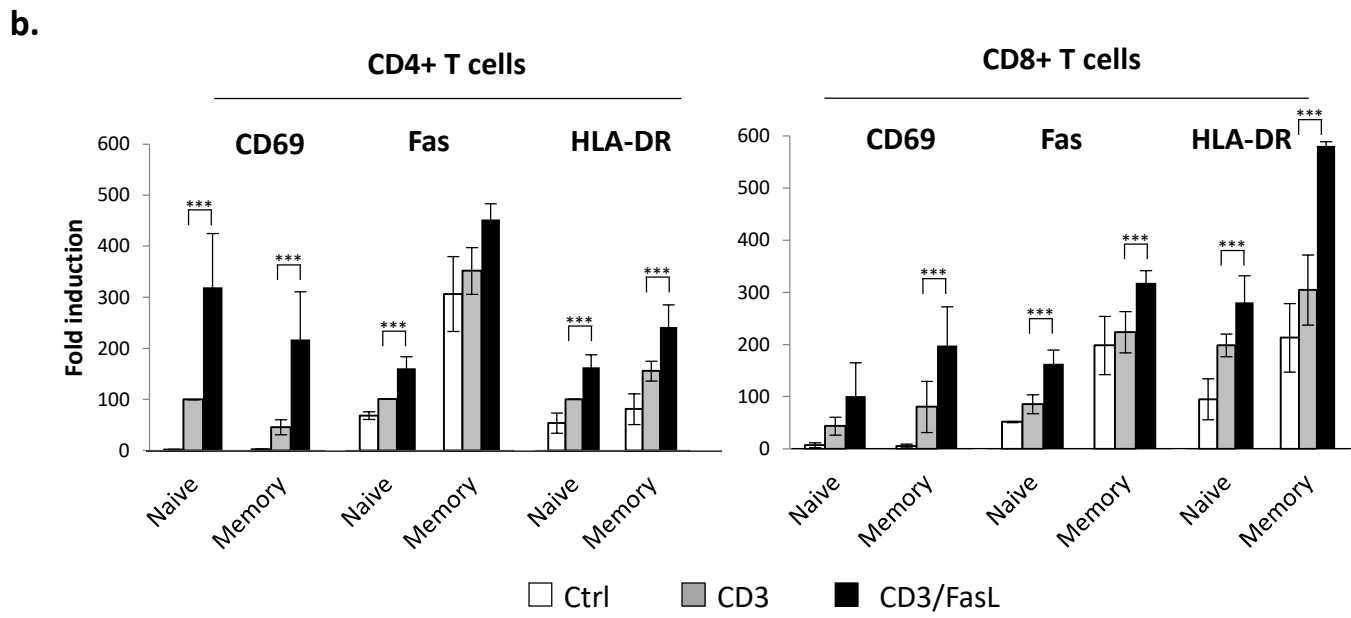
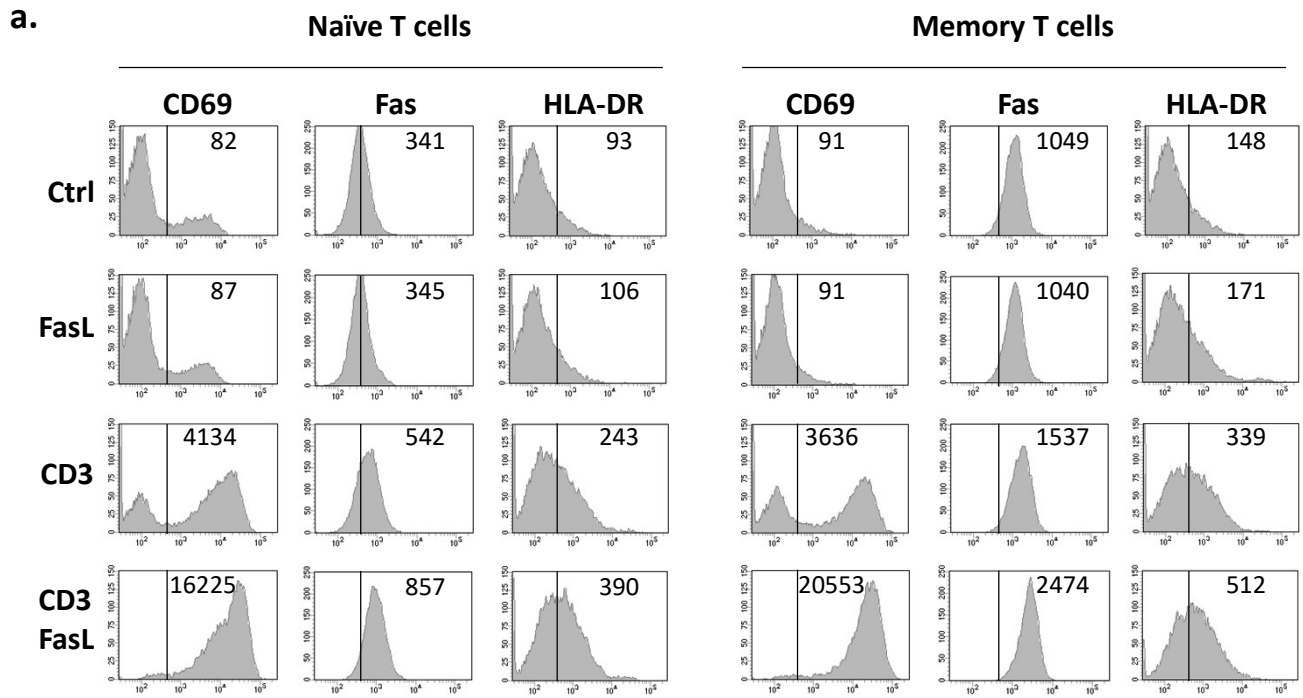
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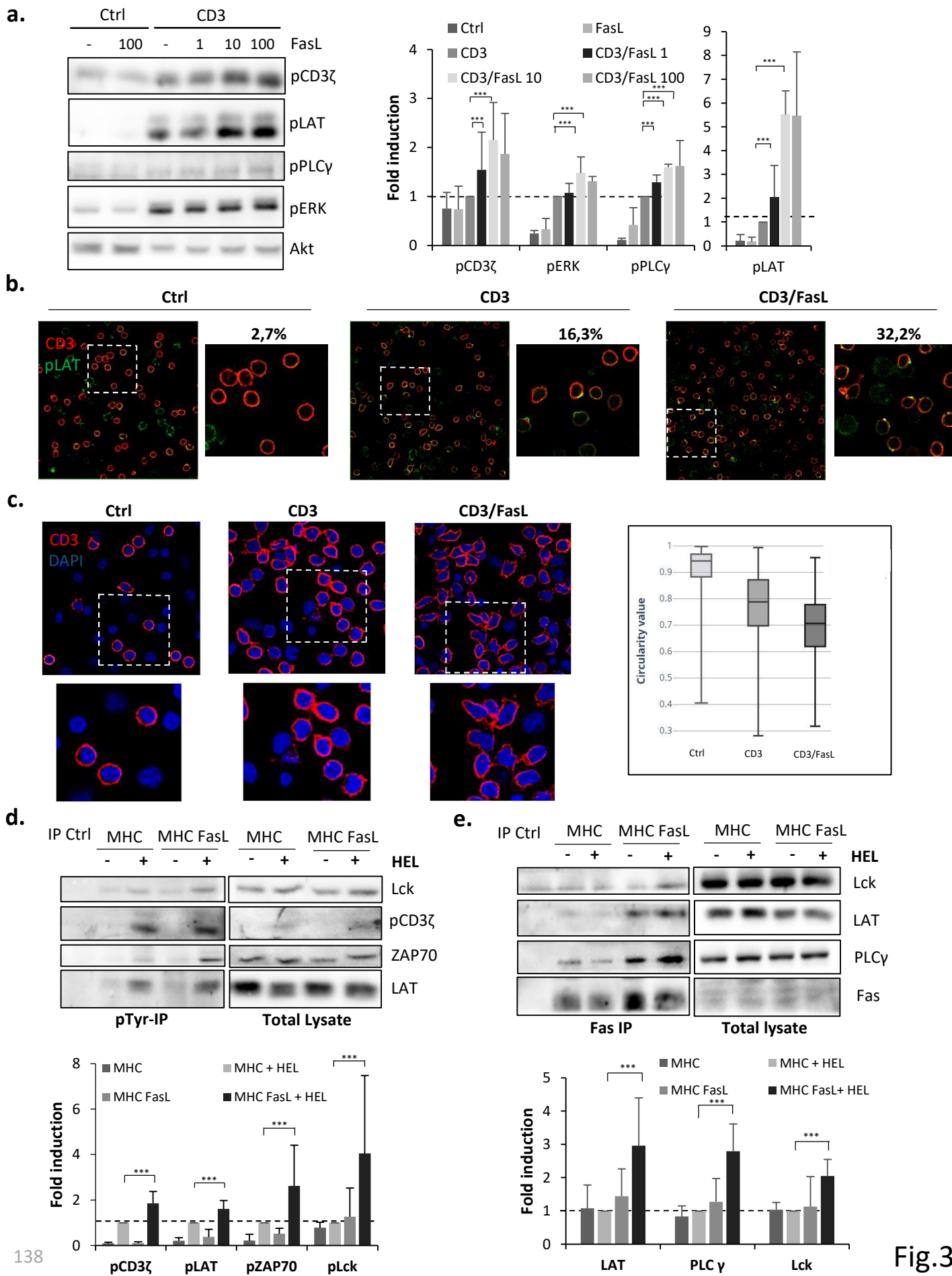
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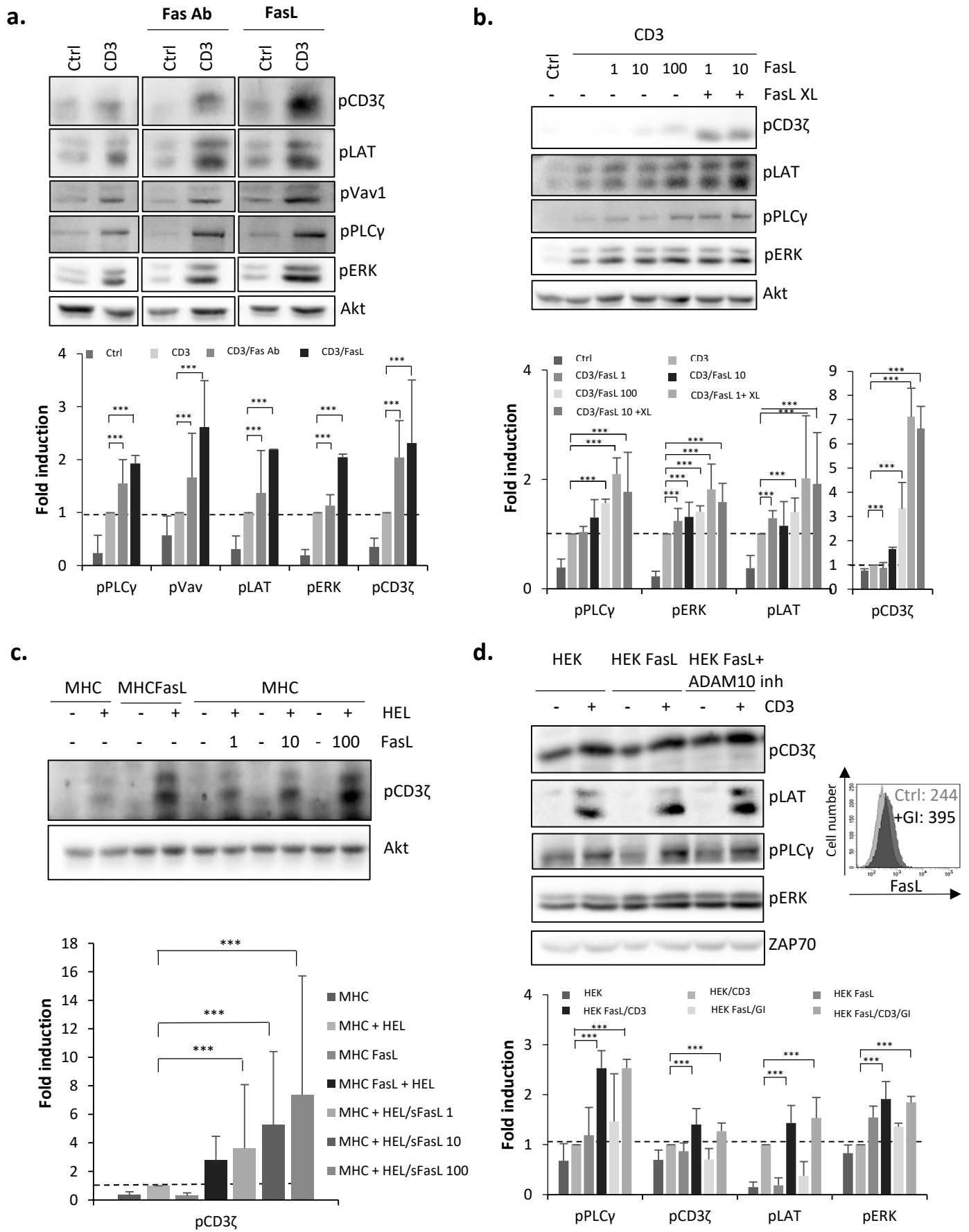


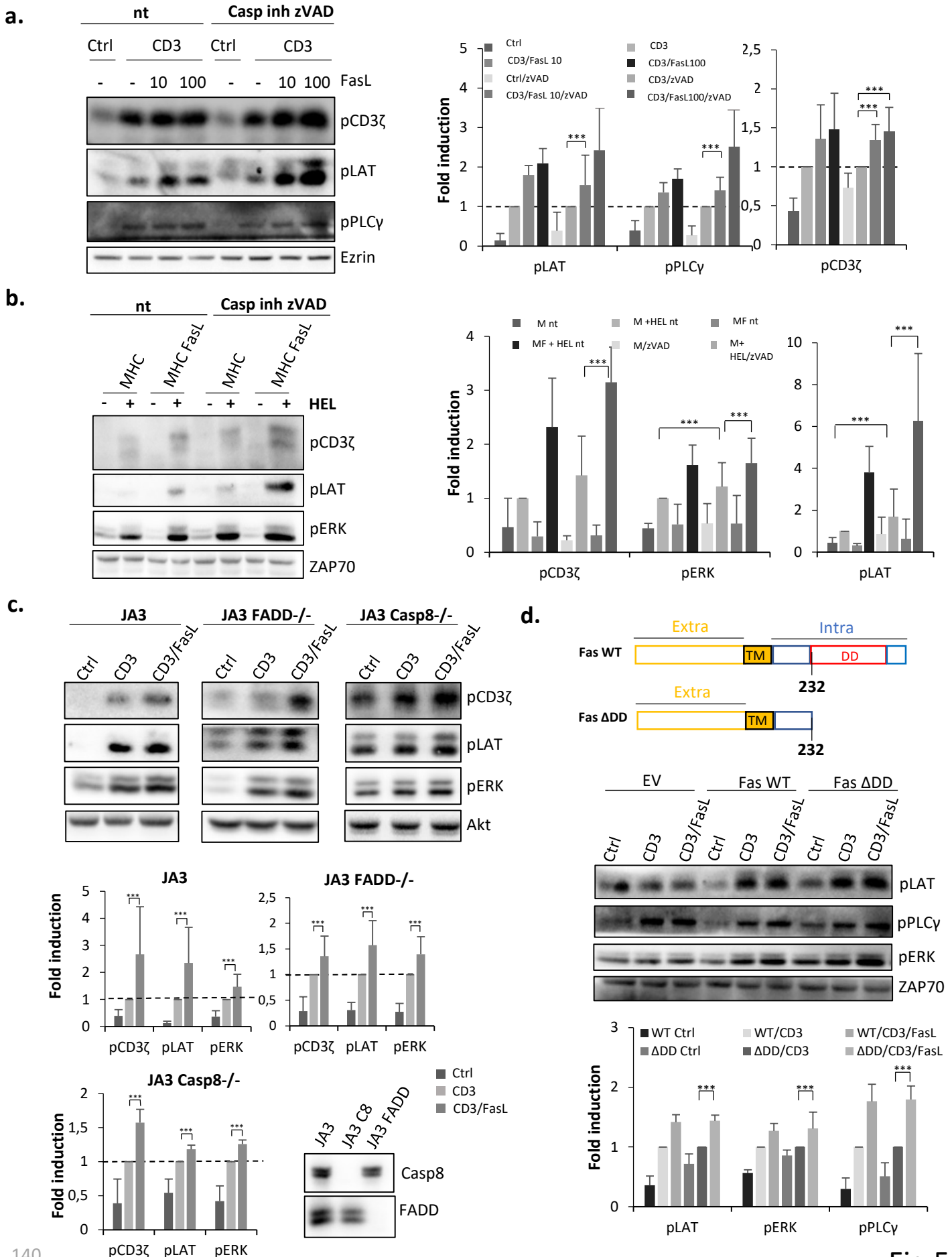
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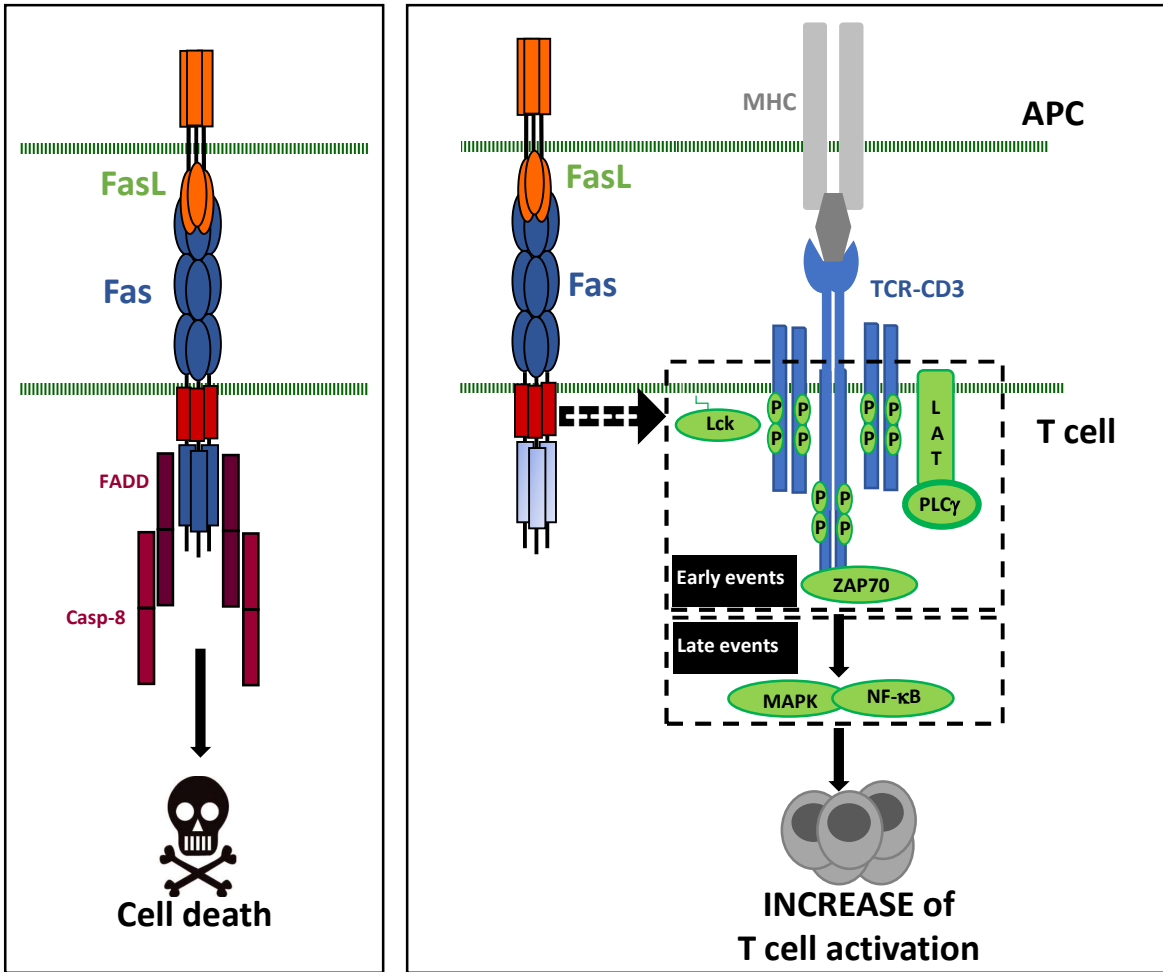


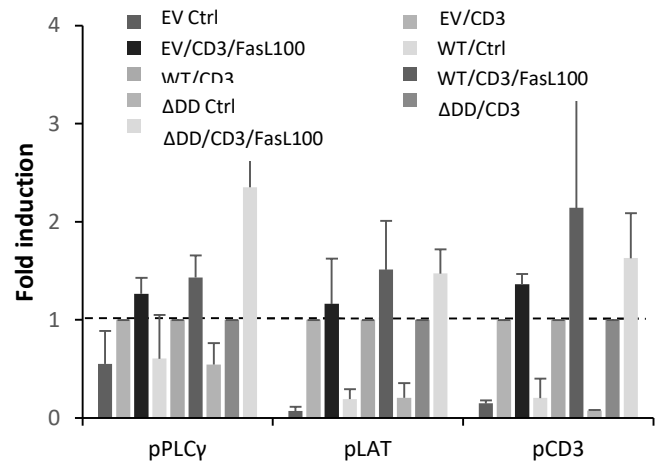
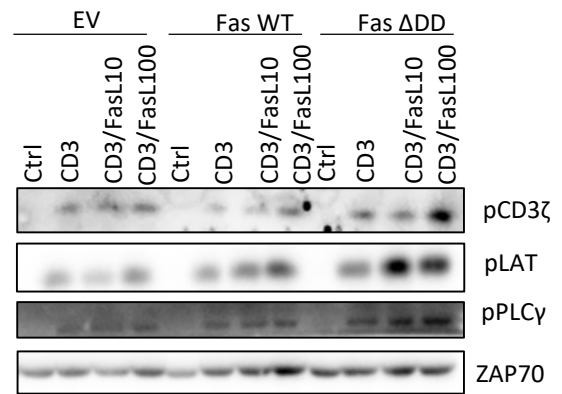
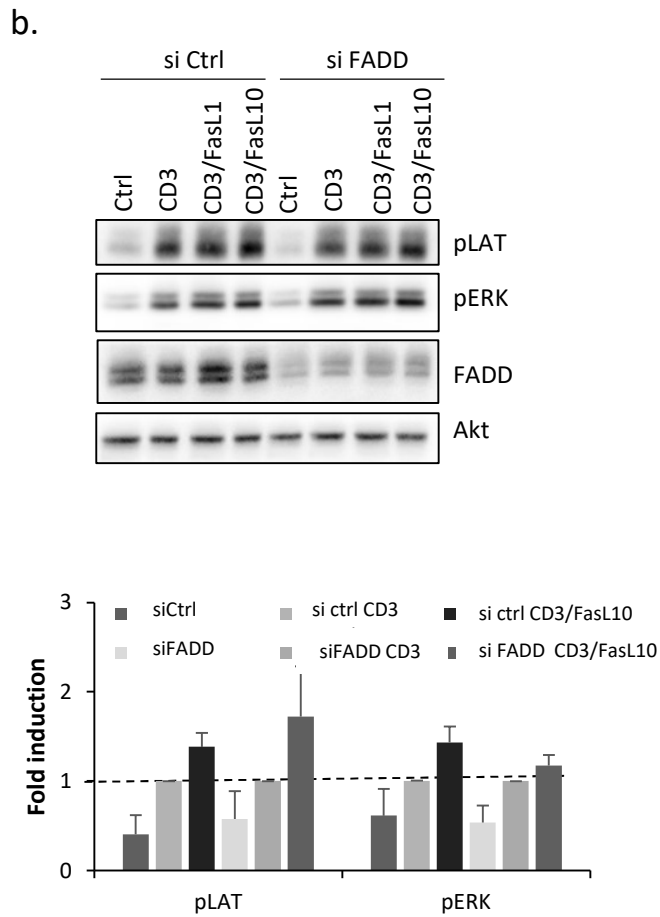
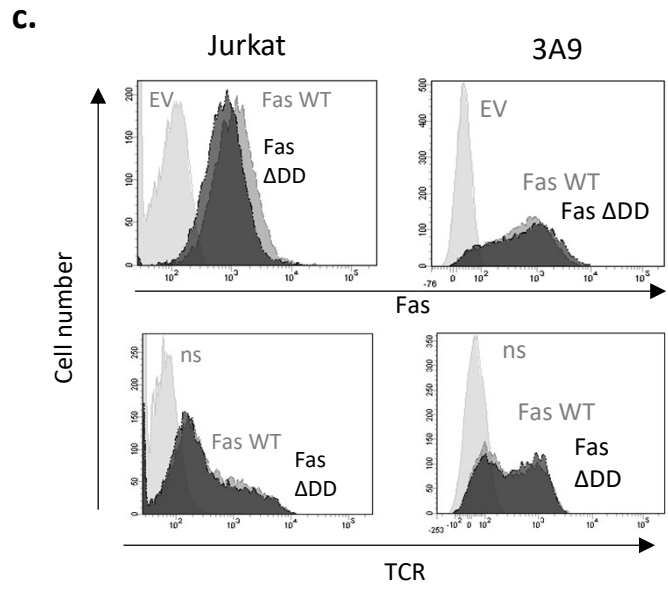
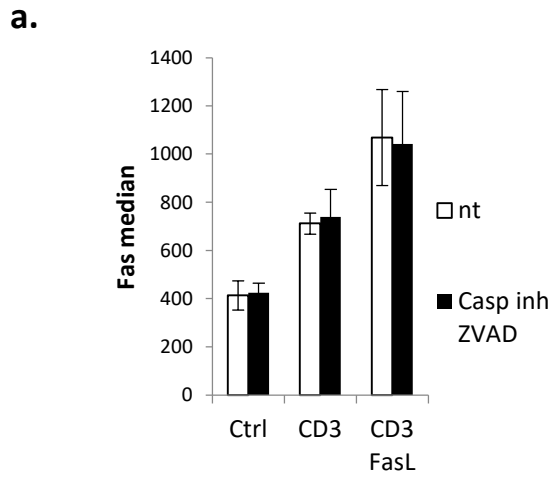














### **3. Additional results**

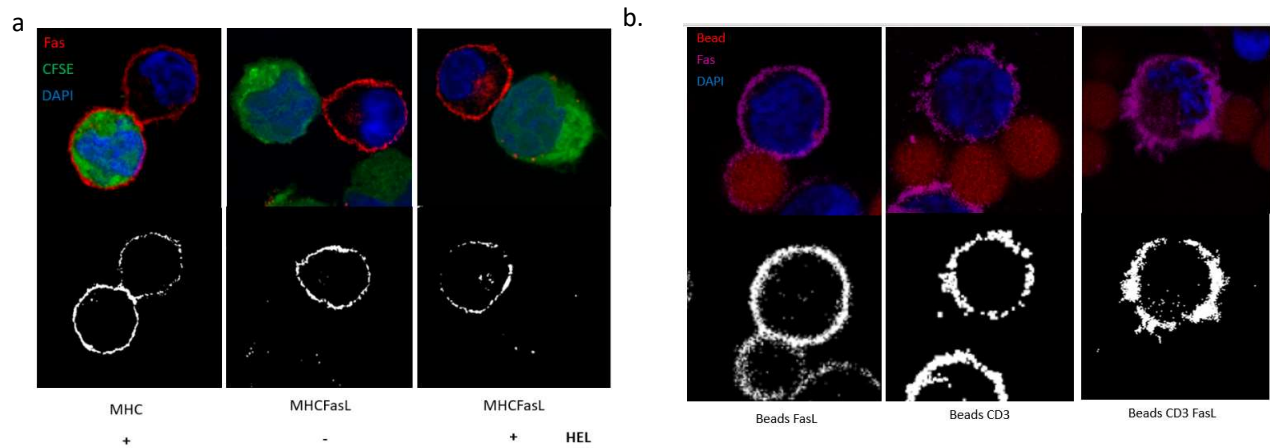
#### **3.1 Qualitative or quantitative signal?**

The data obtained during my PhD showing an early integration of Fas induced signaling to TCR membrane proximal cascade suggest that Fas-TCR co-stimulation occurs in a quantitative manner, allowing an augmentation of TCR activated phospho-proteins five minutes after the Fas-TCR co-engagement.

##### **3.1.1 Investigation on Fas localization at membrane level during co-stimulation**

The co-localization of TCR and other co-stimulatory molecules, such as CD28 receptor, at the level of TCR microclusters and at the immune synapse has been described and would support the quantitative model. Thus, we investigated on the localization of Fas during co-stimulation. Indeed, if Fas-TCR co-localization in same membrane regions is a necessary event for the co-stimulation to occur and, on the other side, the increased distance between the two receptors would block the co-stimulatory effect, this would support the hypothesis that the integration of Fas-TCR signal occurs in a quantitative way. This is consistent with our results on 3A9 Fas Co-IP that identified a binding to Fas of Lck, PLC $\gamma$  and LAT upon Fas-TCR co-engagement (Results Fig. 3d).

We have therefore performed Immunofluorescence experiments to identify Fas localization at the membrane on two complementary models. We have used the mouse hybridoma/APC co-culture system (Fig.1a) and primary T cells activated with CD3-coupled beads with or without FasL (Fig.1b). From co-culture experiments we can conclude that both single (TCR or Fas) and concomitant (TCR+Fas) stimulation induce cell-cell or cell-bead interaction. Fas distribution on the membrane is not modified by FasL stimulation alone as well as from CD3 stimulation on the experiment on the primary cells (1b, middle panel). The localization of Fas for the 3A9 upon TCR activation is less clear (1a, left panel) and the confusion might be caused by the presence of Fas on the plasma membrane of the HEK293-MHCII. Co-stimulation conditions in both experiments (right panel of 1a and 1b) support the hypothesis that there is an accumulation of Fas at cell-cell/cell-beads interaction upon concomitant Fas-TCR stimulation. These preliminary results suggest that Fas might accumulate at the site of TCR stimulation, supporting the hypothesis of a quantitative integration of Fas to TCR signaling. However, the experiment still needs to be optimally set in order to consider exhaustive our conclusions and repeated a statistical relevant number of times to be validated.



**Fig.1 Fas localization upon TCR and/or Fas stimulation. a.** IF of co-culture system 3A9-APC (HEK293-MHCII and HEK293-MHCII-FasL). The APCs were stained with the green dye CFSE to distinguish them from the T cells. Upon co-incubation of 5 minutes on the coverslip, cells were fixed in 4% paraformaldehyde, permeabilized with Saponin 0,01% and probed with Fas antibody for 1h. DAPI was added together with the secondary antibody for 45 minutes. Images were analyzed and the threshold of intensity of Fas probing was regulated to discriminate the regions where the accumulation of the staining occurred. **b.** IF of PBMC activated with beads coated with anti-CD3 antibody alone or in presence of soluble FasL. The control on the left panel was done by coating the beads with an IgG antibody in presence of FasL. Upon 5 minutes activation on coverslip, cells were treated as described for figure 1a.

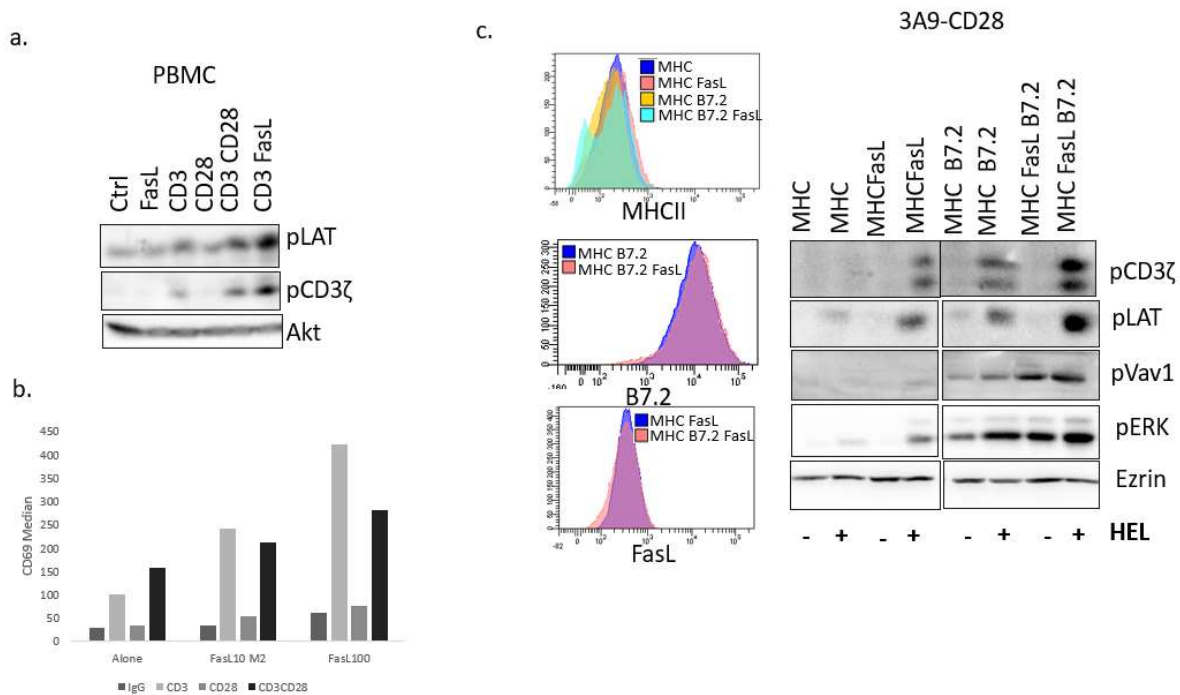
### 3.1.2 CD28 comparison

I also investigated another important question concerning the molecular mechanism underlying the Fas-TCR co-stimulation: can we compare Fas to other co-stimulatory receptors, such as CD28? Do they exploit similar molecular mechanism? Do they reciprocally affect each other signals?

CD28 co-stimulation, as described in the chapter C of this manuscript, was partially molecularly deciphered and some shared molecule between TCR and CD28 have been identified, such as Vav1, PLC $\gamma$  and PI3K. Even though, no membrane proximal events of TCR signaling have been so far involved in CD28 co-stimulation et early time points.

We co-stimulated PBMCs with anti-CD28 Ab and/or FasL (Fig.2a). Whereas we noticed an increase of the phosphorylation of both the CD3 $\zeta$ chain and the LAT protein upon CD3/Fas stimulation compared to CD3 antibody alone, in line with the literature, we were not able to see an increase of early TCR signaling upon CD3/CD28 co-stimulation, which indicate a different way of action of the two co-stimulatory receptors. To further investigate the difference between Fas and CD28 co-stimulation, we evaluated in the same model the effect of CD3/CD28 co-stimulation with or without the presence of FasL by quantifying the expression of the activation marker CD69 after 24 hours of incubation (Fig. 2b). We observed that both FasL and CD28 induced TCR co-stimulation (as indicated by the increase of CD69 expression), and that the concomitant CD3-CD28-FasL stimulation enhanced the CD69 median compared to CD3/CD28 stimulation.

To confirm the additive effect of Fas ligation on CD3/CD28 co-stimulation observed for PBMC, we used the mouse 3A9 cell line overexpressing CD28 protein and co-incubated with HEK293MHCII cells expressing one of the ligand of CD28 B7.2 and/or expressing FasL. Both 3A9-CD28 and HEK293 expressing B7.2 and/or FasL were kindly provided from the team of Dr Hai-Tao He. We observe an augmentation of pCD3 $\zeta$ , pVav1, pLAT and pERK upon simultaneous stimulation of the three receptors compared to Fas-TCR and CD28-TCR stimulations (Fig.2c). However, the high and not physiologic level of expression of both CD28 on 3A9 and of B7.2 ligand on the APC used for the co-culture experiment provoke a membrane proximal CD3-CD28 co-stimulation, data that have not been observed by previous literature and in our experiments performed in primary cells. We can conclude that, in line with the literature, TCR-CD28 co-stimulation does not act at membrane proximal level, implying a different mechanism of action between Fas and CD28 co-receptors. Furthermore, it is worth to suggest that there is an additive or synergic role of Fas on CD3/CD28 during T cell activation.



**Fig.2 Fas-CD28 comparison.** **a.** PBMC activation for 5 minutes on wells coated with anti-CD3 antibody, anti-CD28 antibody and FasL, alone or in combination between anti-CD3 and anti-CD28 antibodies or anti-CD3 antibody and FasL. Cells were lysed and loaded on SDS-PAGE and pCD3ζ and pLAT were probed as marker of TCR membrane proximal signals. **b.** FACS median of CD69 activation marker upon 24h incubation of PBMC with anti-CD3 antibody and/or anti-CD28 antibody coated on plate in presence of absence of FasL. **c.** The comparison of the surface expression of MHCII, B7.2 and FasL on the APCs is shown on the left panel. On the right panel cell were co-incubated for 5 minutes followed by cell lysis and immunoblot analysis. pCD3ζ, pLAT, pVav1 and pERK have been used as marker of short-term activation.

### **3.2 Fas partners during co-stimulation**

As we described that the Fas co-stimulatory effect could occur in absence of the death domain of Fas and of the main DISC components, FADD and Caspase-8, we hypothesize that there might be a negative regulator binding to the death domain that, in resting physiologic condition, would limit Fas pro-survival signal to occur. I will summarize the data that we obtained by focusing on one potential negative regulator, the phosphatase SHP-1. I will afterwards summarize data collected on the identification of the domains of Fas involved in the co-stimulation and which are the specific partners that are crucial for this process to occur.

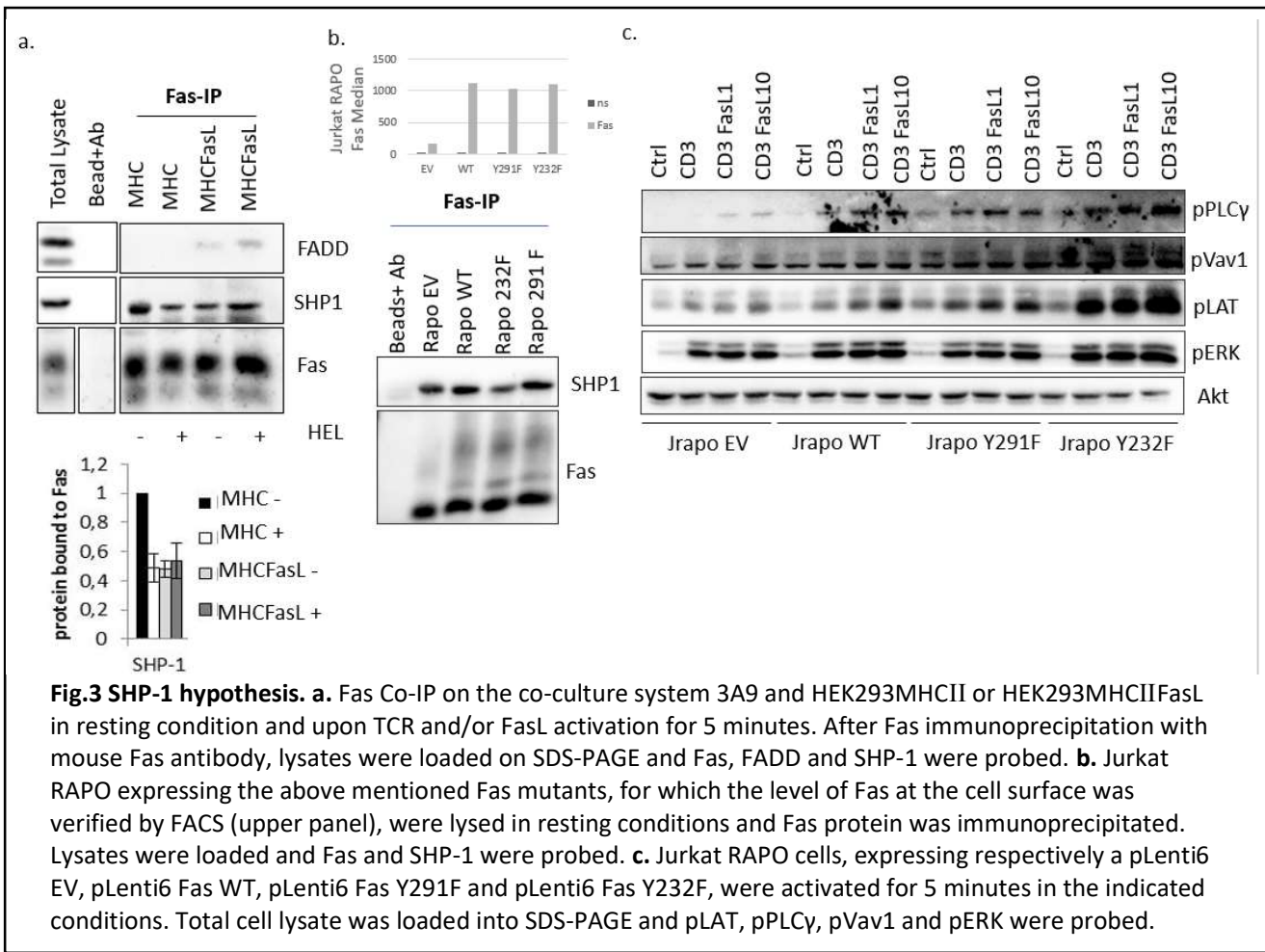
#### **3.2.1 SHP-1 hypothesis**

Data coming from our laboratory and others showed that SHP-1 phosphatase binds to Fas receptor (Koncz et al 2008, Daigle et al 2002). Moreover, SHP-1 is a well-known negative regulator of TCR activation. We have, therefore, started to investigate on the potential role of SHP-1 in Fas-TCR co-stimulation.

To verify SHP-1 binding to Fas in our model and to determine the dynamic of SHP-1 binding to Fas upon Fas-TCR co-activation we have performed Fas Co-IP on 3A9/APCs co-culture system in resting condition and upon activation and co-activation (Fig.3a). We can observe that SHP-1 binds to Fas in resting condition and that the binding diminishes upon both Fas and/or TCR activation. We can conclude that SHP-1 might act as negative regulator of Fas-TCR co-activation by binding to Fas in resting conditions and dissociating from the receptor upon Fas and/or TCR engagement.

SHP1 has been described to bind to several receptors, including immune checkpoint receptors on the phosphorylated tyrosine of ITIM motifs. Fas presents 2 tyrosines within its death domain, Y232 and Y291, even though only Y291 is part of a real ITIM motif. We have exploited Fas mutant constructs Y232F and Y291F which abrogate the phosphorylation site to define potential binding sites of SHP-1 to Fas. We have stably expressed them on a Jurkat line clone which does not express endogenous Fas at the cell surface (Jurkat RAPO) and verified their equivalent surface expression (Fig 3b). We performed a Fas Co-IP (Fig. 3b bottom): The Jurkat RAPO Fas Y232F mutant showed a strong decrease of SHP-1 binding to Fas in resting condition compared to Fas WT, thus identifying the tyrosine at the position 232 of cytoplasmic Fas as a putative SHP-1 binding site. Importantly, activation/coactivation of cells bearing Fas Y232F mutant is highly augmented compared to Jurkat RAPO Fas WT supporting the notion of a negative role of SHP-1 on Fas during Fas-TCR co-stimulation and identifying a putative necessary site on Fas for SHP-1 binding (Fig.3c). We can conclude that

the position Y232 on cytoplasmic tail of Fas might be a necessary region to allow SHP-1 binding and therefore that Fas DD might contribute to negatively regulate Fas TCR co-stimulation.

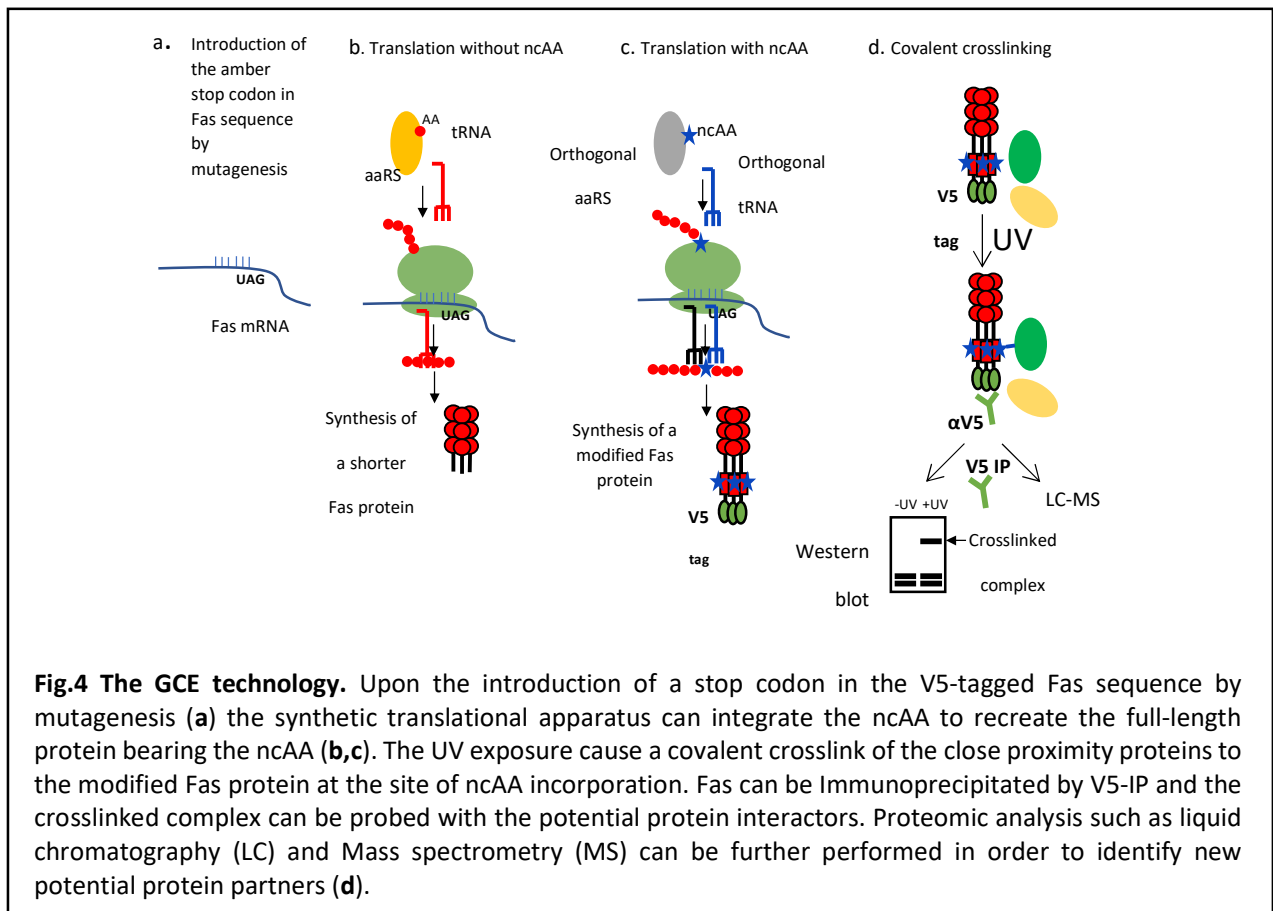


**Fig.3 SHP-1 hypothesis.** **a.** Fas Co-IP on the co-culture system 3A9 and HEK293MHCII or HEK293MHCII FasL in resting condition and upon TCR and/or FasL activation for 5 minutes. After Fas immunoprecipitation with mouse Fas antibody, lysates were loaded on SDS-PAGE and Fas, FADD and SHP-1 were probed. **b.** Jurkat RAPO expressing the above mentioned Fas mutants, for which the level of Fas at the cell surface was verified by FACS (upper panel), were lysed in resting conditions and Fas protein was immunoprecipitated. Lysates were loaded and Fas and SHP-1 were probed. **c.** Jurkat RAPO cells, expressing respectively a pLenti6 EV, pLenti6 Fas WT, pLenti6 Fas Y291F and pLenti6 Fas Y232F, were activated for 5 minutes in the indicated conditions. Total cell lysate was loaded into SDS-PAGE and pLAT, pPLCy, pVav1 and pERK were probed.

### 3.2.2 Defining Fas binding protein in Fas-TCR co-stimulation using the Genetic Code Expansion technology (GCE)

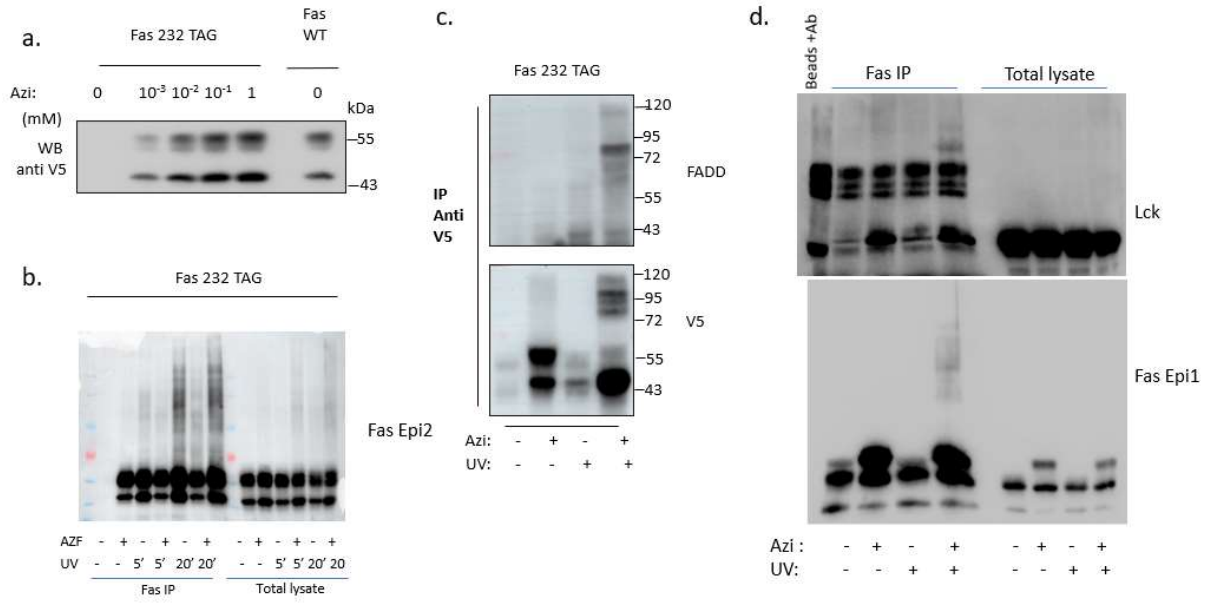
Due to the limitation of biochemical techniques in defining transient protein-protein interactions, especially because of the utilization of strong detergents that compromise the ternary structure of the proteins, the laboratory decided to exploit the power of the GCE technology thanks to the presence of a new facility in our institute managed by Dr. Krittalak Chakrabandhu.

GCE allows the covalent capture of proteins through the site-specific integration of non-canonical amino acids (ncAA) bearing chemical groups allowing the crosslinking of proteins in the close proximity. GCE uses an orthogonal UAA-specific tRNA/aminoacyl-tRNA synthetase pair to direct the incorporation of a ncAA in response to an unassigned codon, such as an amber stop codon, in a gene of interest. These ncAAs can have fluorescent side chain, the capacity of photocrosslink, or reactive handles for facile conjugation with small probes (Nguyen et al 2018, Young et al 2018). The schema in the figure 4 briefly explain how GCE is performed on Fas protein and potential crosslinking applications.



We generated the Fas mutant with a stop codon (amber TAG) at the position 232. We could efficiently express the Fas full-length containing the unnatural Azi amino acid upon transfection of Fas mutant together with the synthetic translational machinery and Azi addition to culture medium. The creation of the full-length Fas after addition of Azi, was followed by UV crosslink to identify Fas interactors in 293 cells. We have performed preliminary experiments, testing the optimal Azi dose that allow the translation of a proper amount of Fas full-length for the following crosslink and IP experiments (Fig.5a). We decided to use the 2 $\mu$ M Azi to perform the following experiments. To confirm the efficiency of the UV crosslink we have performed control experiments by immunoprecipitating the V5tag of Fas construct and we have identified the formation of different Fas complexes, that can correspond both to Fas aggregates or Fas interaction with other proteins (Fig.5b). To further corroborate the efficiency of the technique we have transfected 293 cells with FADD protein, which is a positive control for Fas binding, together with the Fas-232TAG-V5 and the translational machinery-containing plasmid (Fig. 5c). We could identify the Fas-FADD complex by V5-IP by the formation of the band at 80kDa (size of Fas+FADD) common for both probing with FADD (upper panel) and V5 (bottom panel) antibodies. Furthermore, we have transfected Lck protein in 293 cells to confirm the protein binding to Fas that we have already visualized by canonical Fas Co-IP in the 3A9 model (Fig.5d). Also in this case, upon Fas IP we could identify the formation of the protein complex by the visualization of the band at 110kDa (size of Fas+Lck) present upon Azi addition and UV treatment after probing with both Lck and Fas antibodies. From the figure 5 we can conclude that the integration of the Azi amino acid occurs in a dose dependent manner and that the technique has been correctly set in order to identify transient Fas interactors present in the close proximity of the protein. We will use this Fas mutant or others, such as Fas 210TAG and Fas 220TAG and Fas196TAG, to describe other potential Fas binding partners during Fas-TCR co-stimulation.





**Fig.5. Preliminary results of GCE technique.** **a.** Different amounts of Azi amino acid were added to the culture medium of 293 cells transfected with pLenti6-Fas232TAG-V5 plasmid and the nCAA-specific tRNA/aminoacyl-tRNA synthetase encoding pBAC vector. V5 antibody has been used to reveal Fas full-length protein formation. **b.** Fas Co-IP on transfected 293 cells. Fas antibody that recognize a cytoplasmic portion of Fas (Epi2) was used for the probing. **c.** V5 Co-IP on 293 transfected with the above-mentioned vectors and FADD encoding plasmid, Azi addition and UV treatment. Both FADD antibody and V5 were used to probe the membranes. **d.** Fas Co-IP on 293 transfected with the above-mentioned vectors and Lck encoding plasmid, Azi addition and UV treatment. Both Lck and a Fas antibody recognizing an extracellular portion of the protein (Epi1) were used to probe the membranes.

## **DISCUSSION and PERSPECTIVES**

## Discussion and Perspectives

This discussion part will focus on three main critical issues regarding the co-stimulatory function of the Fas receptor that I have faced during my PhD project: the subtype of T immune cells affected by the co-stimulatory role of Fas; the nature of the ligand able to induce this co-signaling; the regions of Fas (and the signaling proteins) underlying this function. Finally, future scientific directions putting our results in perspective in a context of immuno-oncology will be proposed and discussed.

### 1. Fas as an immune checkpoint

#### 1.1 Co-stimulatory or co-inhibitory receptor?

Even if nowadays it is clear that immune checkpoints of TNFRSF mostly act as co-stimulatory receptors, the investigation have mainly focused on the members of the family containing the TIM motif. Few evidence described the DD-bearing TNFRs capable to initiate co-stimulatory events. An example of these last is the DR3 receptor, even though, it was suggested to perform its function by binding of TRADD which subsequently might lead to TRAFs binding. The role of the protein Fas as a co-stimulatory or co-inhibitory receptor remained a controversial subject for more than 25 years. Using both primary PBMCs and a physiologic co-culture T-APC system we identified a co-stimulatory role of Fas on T cell activation confirming the results obtained by the majority of the previous reported studies (Alderson et al 1993, Kennedy et al 1999, Chung et al 2000, Maksimow et al 2006, Puliaeva et al 2008, Paulsen et al 2011). The use of the co-culture system was of particular importance: indeed, in contrast to the *in vitro* activation that bypass the TCR chains by directly acting on the CD3 $\epsilon$  chain, we were able to observe a co-activation initiated by a specific pMHC-TCR interaction. The presence of a transgenic TCR on the hybridoma 3A9 cells allows, indeed, the direct activation through the contact with the peptide expressed on the APC HEK293 cells expressing specific MHCII (I-A<sup>k</sup>).

The co-stimulatory effect of Fas on T cell activation was evaluated by different readouts: the T cell proliferation rate, the IL-2 secretion and the expression of multiple activation markers at the T cell surface (Results Fig.1 and 2).

- One of the first question we addressed is the capacity Fas ligation and of Fas and TCR concomitant engagement to induce cell death in the models used in our study. It was indeed of a crucial importance to understand if these cells were capable to initiate Fas mediated apoptosis in order to better define the behavior of our models and discriminate potential apoptosis sensitive T cells from the resistant ones, to further determine proliferation rate of the surviving cells. We did not observe Fas mediated cell death upon FasL and/or TCR-Fas engagement neither in PBMCs nor in the co-culture system (Results Fig.1 and data not shown). This result is in contrast with what was reported

in naïve T cells by Maskimow *et al* 2003 and in the T cell co-culture system (HLA-A1 specific T cells with OVA-pulsed HLA-A1 expressing APCs with or without mbCD95L) used by Strauss *et al* (Maksimow *et al* 2003, Strauss *et al* 2007 and 2009). The origin of this discrepancy could rely on the difference in expression of Fas on the T cell populations or on the strength of Fas stimulation as well as from the absence of external stimuli in the naïve pool used by Maksimow, separated from the rest of myeloid cells.

The co-stimulatory effect that we describe are in contrast with two studies (Paulsen *et al* 2011; Strauss *et al* 2007,2009) that reported that Fas could induce a co-inhibition of the TCR signal. We suggest that the main reason that could explain these discrepancies is that the amount of Fas ligand used in their studies to engage the Fas receptor might interfere with a physiological relevant role and consecutively give a co-inhibitory effect. This possibility is particularly well reflected in the manuscript by Paulsen of 2011 where a co-stimulatory effect is observed in CD4+ T cells upon treatment with low doses of FasL whereas a co-inhibition is found with high doses of FasL. It is important to notice that the “low doses” used in this study reach 25 times more the maximal dose of FasL that we have used in our study. The high doses raising to the co-inhibition effect are extremely prohibitive (up to 20µg/ml for the recombinant FasL, 200 times more than the concentration we routinely used).

This problem is still present in the a co-culture system used by Strauss *et al* since level of FasL expressed by the OVA-pulsed APCs is far from the physiologic FasL present on the cell surface of natural APCs (Strauss *et al* 2007,2009).

Although our data confirmed the function of Fas a T cell co-stimulator, we believe that more important informations still need to be collected to firmly confirm this role as a broader conclusion.

- We cannot totally exclude that the outcome of co-activation (co-stimulation versus co-inhibition) is not dependent on the Fas level at the T cell surface. Even if in our study the TCR-Fas co-engagement lead to a stimulatory effect in both naïve (which show basal low level of Fas) and memory cells (which express a higher basal level of Fas) (Results Fig.2), it might be important to understand if there is a threshold level of Fas at the cell surface above which we block the co-stimulatory event.
- Noteworthy, another parameter that can affect cell activation is the strength of TCR binding (i.e. the affinity of the antigen presented by the APC for TCR). It would be interesting to verify if the co-stimulation can depend or variate depending on the TCR affinity for the antigen, maybe testing on the transgenic TCR present in 3A9 cells low and high affinity peptides together with Fas stimulation.
- Importantly, none of these studies (including ours) concerning the importance of the Fas-TCR co-stimulation was addressed in an *in vivo* context. Future work might propose to study the behavior of T cell activation in *lpr* or *gld* mice models for the different T cell populations in response to pathogen

challenge. However, the immune system of these mice, already severely compromised, might affect the conclusion of such type of experiment and thus it might be delicate to really attribute to Fas the effect on T cell activation. One can keep in mind the data obtained by Puliaeva and colleagues (Puliaeva et al 2008) on the role of Fas *in vivo*: transferring T cells from *lpr* mice into Fas sufficient recipient mice they could observe an impaired T cell activation on Fas deficient T cells, confirming a role for Fas as co-stimulator of TCR. Even though, the T cell transfer caused in the recipient mice the insurgence of GVHD, thus not reflecting which might be the role of Fas in T cell activation physiologically.

### 1.2 Fas role on different T cell subsets

In order to assess if Fas has a co-stimulatory function in the different T cells subsets, we gated the different populations of T cells in PBMC using different T cells markers: the co-receptors CD4 and CD8 and the memory cell markers CD45RO using flow cytometry. As shown in our results (Results Fig. 2) all the populations analyzed, the CD4+ and CD8+ T cells and inside each group naïve and memory cells, present a co-stimulatory effect of FasL based on the upregulation of the three tested activation markers, CD69, HLA-DR and Fas itself, at the T cell surface.

Noteworthy the description of the phenotype of the populations in purified T cell subclasses was only addressed twice: (i) one study reporting that blockage of Fas was leading to inhibition of T cell activation did not observe any particular difference between CD4+ and CD8+ cells (Paulsen et al 2009) (ii) in the *in vivo* model studied by Puliaeva, that as we mentioned, simulate a pathologic GVHD condition, an impaired T cell activation and proliferation was observed in both CD4+ and CD8+ cells lacking Fas and as consequence this resulted in aberrant CTL effector development due to a lack of “help” coming from the CD4+. Anyway, as we mentioned, these observations have been made in a pathologic landscape, thus far from the physiologic context and therefore the information need to be further investigated.

In contrast to our results, Desbarats *et al* noticed a marked difference towards the importance of Fas in the T cell activation between naïve and memory T cell compartments (Desbarats et al 1999). Indeed, they could describe a co-stimulatory effect of Fas in TCR stimulation in case of memory cells but not in naïve cells where even cell apoptosis was identified. Two differences with our study are obvious: the human versus murine model and the use of total PBMCs (preserving the pool of cytokines) versus the purified T populations of their study.

- In human T lymphocytes the level of Fas consistently differs between naïve and memory cells. The level of the receptor in naïve cells is extremely lower than the one of memory cells. It is indeed known that human memory cells are mainly apoptosis resistant even with high level of Fas, mainly for the presence of high level of antiapoptotic proteins (Reviewed by Zheng et al 2017).

Murine T cells used in the study of Desbarats *et al* do not show this difference and naïve cells used for this study show high level of Fas expression, maybe implying a different way of Fas regulation in a murine context. A similar Fas level on human naïve cells, which do not express high level of antiapoptotic proteins, is potentially leading to Fas induced cell death upon Fas stimulation.

- Moreover, the apoptotic effect on naïve cells observed by Desbarats could have been affected by the isolation of naïve population that was thus deprived of certain specific stimuli that could be necessary for the occurring of the co-stimulation for this specific population. Indeed, they reported that the addition of IL-4 and IL-12, cytokines produced by Th2 and Th1 respectively, was able to revert the effect from cell death to co-stimulation on naïve cells.

Importantly, Fas involvement has been reported in T cell differentiation. Klebanoff *et al* described that Fas expressed at the surface of human memory T cells was shown to promote the precocious differentiation of naïve T cells (Klebanoff *et al* 2016). Other studies have instead demonstrated the role of Fas in the differentiation into Th9 and Th17 populations (Meyer zu Horste *et al* 2018, Shen *et al* 2019). Furthermore, Fas deficiency in ALPS patients was shown to impair differentiation of CD4+ and CD8+ T cells causing the accumulation of the abnormal DNT cells (Rensing-Ehl *et al* 2014).

It might be interesting to try to understand if the co-activation and differentiation are coupled events. We plan to analyze whether Fas-TCR co-stimulation could lead to specific cell differentiation phenotypes and how the different effector subpopulations respond to Fas-TCR co-stimulation once differentiated. Additional progress could be done by dissecting the molecular pathways of both Fas mediated co-activation and differentiation and analyze whether they share some similitudes or not.

## **2. Impact of Fas agonists on Fas-TCR co-stimulation**

Researchers that have investigated Fas-TCR co-stimulation have used three main methods to achieve Fas engagement: agonist anti-Fas antibodies, FasL recombinant protein or membrane-bound FasL expressed on “artificial” APCs:

\*Fas agonist antibodies were mainly coated on plate together with anti-CD3 antibody (Alderson *et al* 1993, Chung *et al* 2000, Desbarats *et al* 1999).

\*The FasL recombinant proteins mostly used contain a FLAG-tag allowing the crosslinking of the protein with anti-FLAG antibody M2 and, therefore, its aggregation. Recombinant FasL was considered effective in TCR-co-stimulation only upon crosslinking with FLAG-M2 protein, thus in aggregated form and not as soluble protein (Kennedy *et al* 1999, Maksimow *et al* 2003, Paulsen *et al* 2011).

\*The only study using a membrane-bound FasL form concluded to an inhibitory role of Fas on TCR signaling (Strauss et al 2007, 2009). However, this membrane-bound form was expressed at very high level and was an uncleavable form of the protein, not found in physiologic conditions.

Data obtained from our experiments could instead:

\* Confirm the capability of Fas agonist antibodies (Apo1-3, Jo-2) to induce a co-stimulatory effect in human and murine cells respectively either by coating them on the plate and by adding them soluble in the medium at the moment of the activation (Results Fig.4a).

\*Show that the recombinant soluble form of Fas ligand acts as an inducer of co-stimulation even in a uncross-linked form at doses varying from 1ng/ml to 100 ng/ml depending on the model used (primary or Jurkat cells). Upon cross-linking, the co-stimulation is detected even at lower doses and reach the saturation plateau earlier (Results Fig. 4b).

\*Demonstrate that in the 3A9 and human primary models the membrane bound form is effective in inducing the T cell co-stimulatory process. As membrane-bound FasL has been shown to be cleaved by the ADAM 10 metalloprotease raising a soluble form, we chose to study the co-stimulation effect in presence of specific ADAM10 inhibitor (GI254023X). We still observe co-stimulation in absence of ADAM10 cleavage firmly demonstrating that the membrane-bound form could induce this signaling (Results 4d). We could not anyway compare the efficiency of co-activation between soluble and membrane-bound FasL since we could not precisely quantify the number of FasL molecules that actually ligate Fas receptor, both for the co-culture system (mb-form) and for the recombinant Fas ligand (crosslinked or not crosslinked).

As described in the first chapter of this manuscript, the forms of FasL (membrane bound or soluble) have been described to generate different specific outcomes of the Fas signaling. It was proposed that the membrane-bound forms (both on the cell surface and in exosomes membrane) of FasL were responsible of the apoptotic signal whereas the soluble form could rather act as negative regulator of apoptosis and as an inducer of pro-survival signals (O'Reilly et al 2009, Kleber et al 2008, Tauzin et al 2011, Alonso et al 2011, Poissonnier et al 2016). Nevertheless, this concept is far away to be accepted by all. There are several lines of evidence that, could identify, both *in vitro* and *in vivo* the soluble form of FasL able to induce cell death by apoptosis in certain circumstances, for example when other cleavage of FasL are induced by MMP-7 or Plasmin, or when FasL in the extracellular environment is able to aggregate by association with matrix proteins (Holler et al 2002, Vargo-Gogola et al 2002, Bajou et al 2008). Before our study, only one study reported the ability of membrane-bound FasL to induce a non-death signaling through Fas: the study by Klebanoff *et al*, which implicated the membrane-bound FasL in the Fas induced differentiation (Klebanoff et al 2016). We believe that this result is crucial since it emphasizes the versatility of the Fas signaling in the

context of Fas-TCR co-activation, which does not depend on the form of FasL with which the receptor interacts.

Several experiments are already planned in the lab to further consolidate this result.

- We will test in our system the ability to induce co-stimulation of a non-cleavable form of FasL, the D4 mutant described by Nagata (Nagata et al 1998). This mutant has a deletion of the extracellular FasL region from the amino acid 111 to the 133, which totally abrogates the ADAM 10 cleavage site (position 129-130). The surface level of FasL in this mutant was just slightly decreasing upon treatment with a generic metalloprotease inhibitor BB2116, strongly suggesting that this form of FasL can be considered a not cleavable form by all metalloproteases.
- We will also test the ability of another membrane-bound FasL, the one found at the membrane exosomes. Indeed, FasL bearing exosomes will be isolated by a sorter present in the flow cytometry platform in our institute or by ultracentrifugation and will be co-cultured with T cell; the response to Fas-TCR co-stimulation will be analyzed.
- We will also perform FasL dose dependency assays, to identify the threshold level of FasL (both minimum and maximum) outside of which we cannot obtain anymore the co-stimulation.

### **3. Molecular insight of Fas-TCR co-stimulation**

#### **3.1 Fas co-stimulates TCR signaling in a quantitative way**

Even more than for other TCR co-receptors, the way in which Fas co-stimulatory contribution can be integrated in TCR activation is still far to be elucidated. Our results demonstrate that Fas-TCR co-stimulation occurs at TCR membrane proximal level, by augmentation of the phosphorylation of Lck and of the CD3zeta chain, the first proteins of the TCR signaling, already five minutes after co-stimulation, followed by the augmentation of the main TCR membrane proximal targets, such as pLAT, pPLC $\gamma$ , pVav1 and the downstream pERK molecule. No data reporting co-stimulation of membrane proximal TCR protein has been described so far at so early timepoint for other co-receptors, such as CD28. Coupling this finding with Lck, PLC $\gamma$  and LAT association to Fas upon co-ligation, it is worth to conclude that Fas might act in a quantitative way in the co-stimulation of TCR signaling (Results Fig.3).

Noteworthy, this way of co-stimulation would indicate that Fas acts in a different way compared to all the other co-stimulatory receptors analyzed so far. The qualitative model was reported for the TNFRs, which bind to the TRAFs proteins and activate the NF- $\kappa$ B pathway. Even if a potential integration in a quantitative manner for the co-receptors of this family has been suggested, since their co-localization with TCR has been described in several studies, it is still controversial and not definitively clarified (Nam et al 2005, Starck et al 2005, Lee et al 2013).



The quantitative model that we observe for Fas-TCR crosstalk is instead more in line with the co-signaling receptors of the IgSF. Despite that, important differences have been described between Fas-TCR crosstalk and the co-stimulation of the members of IgSF, such as by CD28. Indeed, there are no lines of evidence that described an increasing of the first membrane proximal proteins of TCR signaling upon TCR-CD28 co-activation. It is commonly accepted that CD28 co-stimulation can induce an increase of the PI3K, Vav1 and PLC $\gamma$  phosphorylation but no earlier TCR membrane proximal signal seems to be affected. We have already started to investigate on comparison between Fas and CD28 co-stimulation and so far we could confirm the data obtained by the previous literature (Additional results Fig.2a and 2b). Furthermore, we decided to investigate the effect of concomitant stimulation of the three receptors. We could observe a cumulative effect of the co-activation of the three receptors compared to the TCR activation alone and to TCR-CD28 co-stimulation (Additional results Fig.2b and 2c).

We can suggest that the recruitment of Lck, PLC $\gamma$  and LAT to Fas that we have described upon co-stimulation condition can give some clues to the molecular mechanism involved. Noteworthy, two publications coming from the same team describe the binding of Lck and PLC $\gamma$  to Fas, but they relate it to Fas induced apoptosis (Akimzhanov et al 2010, 2015). They describe that presence of TCR signaling as well as Lck, ZAP70 and PLC $\gamma$  are essential for Fas-induced Ca $^{2+}$  increase and apoptosis in T cells. However, we can question this hypothesis since the only model used in these publications are Jurkat cell lines, known to be sensitive to Fas induced cell death and therefore for which the apoptotic signaling might mask the pro-survival one. For this reason our studies were indeed mostly conducted on cell types that are closer to physiologic models, limiting the utilization of the Jurkat model to confirm our results and for further applications that are more difficult to perform in the primary cells, such as RNA interference and viral infection. Indeed, we could immediately exclude that the above-mentioned protein binding to Fas was leading to apoptosis by looking at long term readout of Fas-TCR co-stimulation that, we now know that is instead leading to cell activation and proliferation.

For immune checkpoints of IgSF, such as CD28 and ICOS, an interaction with Lck has been described upon co-stimulation (Raab et al 1995, Wan et al 2018, Hui et al 2017). This would suggest that these immune checkpoints are in the same membrane region where TCR is at the moment of co-stimulation. The identification of the binding of Lck, PLC $\gamma$  and LAT to Fas during co-stimulation, that might be direct or indirect, would not only describe a potential co-localization of the two receptors, but also indicate the hypothesis that Fas might be responsible of re-localization of Lck, PLC $\gamma$  and LAT in close proximity of TCR, allowing the co-stimulatory event.

Moreover, our laboratory will continue the investigation on this subject by starting from the preliminary results that we were able to obtain during these three years. The visualization of Fas-TCR co-localization at the T cell-APC contact interface by confocal microscopy can be addressed with both the co-culture 3A9 model

and the PBMC activated with coated beads (Additional results Fig.1). Furthermore, by utilization of this latest model the laboratory can investigate on the minimal/maximal distance required between the two receptors for the co-activation to occur.

The quantitative model that we started to decipher here does not exclude the presence of an additional Fas signal that can proceed in parallel until the nucleus without interfering with TCR cascade. Thus, up to now, we cannot exclude the involvement of a qualitative signal in Fas-TCR co-stimulation. This last point can be addressed by evaluating variations of the transcriptome upon TCR and Fas-TCR co-stimulation.

### **3.2 Fas-TCR co-stimulation is a FADD and Caspase-8 independent mechanism**

The molecular deciphering of Fas-TCR co-stimulation in the preexisting literature left completely unsolved the potential involvement of the DISC components FADD and Caspase-8. We could demonstrate that neither FADD nor Caspase-8 or even caspases activity are indispensable for the initiation of the co-stimulatory signaling (Results Fig.5). Furthermore, in our hands, treatment of cells with the pan-caspase inhibitor zVAD does not block neither the upregulation of Fas in both TCR and Fas-TCR co-stimulation (Results, Supplementary figure a).

The literature behind the implication of these proteins in normal TCR activation has supported their role for the occurrence of a normal T cell proliferation and homeostasis (See Chapter B paragraph 4.2.8). Even though, their role concerning the T cell activation remains still controversial and need to be further corroborated. Interestingly, a publication of Salmena of 2003, by exploiting an conditional T cell KO for Caspase-8 demonstrate that T cell Casp8<sup>-/-</sup> do not show defect neither in TCR early signaling nor in the activation marker of the cell surface, and that the defects can be associated to altered IL-2 secretion, a feature more related to cell proliferation (Salmena et al 2003). These conclusions are in line with our observations.

Most of the studies deciphering the role of Caspase-8 in Fas-TCR co-stimulation, report caspases activity and partial Caspase-8 and Caspase-3 cleavage at late time points, after a minimum of 24 hours, until 6 days after induction of cell activation, much later than the occurring of the Fas-TCR crosstalk (Paulsen et al 2011, Maksimow et al 2006). They all conclude that Caspase-8 activity was crucial for the occurring of Fas-TCR induced proliferation and that the caspase activity observed was not leading to cell death. Caspase-3 cleavage observed by Paulsen was, indeed, resulting in a band detected at 20kD (different from the classical 19 and 17kD bands), thus they hypothesized that this form of Caspase-3 would have acted on other substrates (Paulsen et al 2011). The reason why the caspases activity described by Maksimow in his publication of 2006 was not leading to cell death was, instead, addressed to the elevated activation of NF- $\kappa$ B that would have worked as survival signal for co-stimulated cells with high levels of active caspases (Maksimow et al 2006). However, the high doses of Fas agonists or FasL protein used to stimulate Fas receptor might have an effect on the caspase cleavage, since Caspase-8 activation is strictly dependent on Fas activation strength.

Moreover, the role of caspases has been investigated by utilization of caspases activity inhibitors, which might indirectly affect other pathways in which caspases are involved, and often at high doses, which already block the canonical TCR activation, not excluding possible non-specific side effect of the cell treatment (Kennedy et al 1999, Maksimow et al 2006, Paulsen et al 2011).

Interestingly our results show that pan-caspase activity inhibitor zVAD not only allows the Fas co-stimulatory signaling to occur but that it even enhances co-stimulation and even the simple TCR stimulation in 3A9 model (Results Fig. 5b). This might suggest that, in contrast on what was described, caspases could act as negative regulators of Fas-TCR activation or even broadly of TCR activation itself. We can hypothesize that upon Fas ligation, both pro-death and pro-survival signals are initiated and that if a fraction of Fas is involved in the DISC formation it would limit the amount of Fas that is free for the co-stimulation.

The role of DISC and DISC-interacting proteins will to be further investigated by utilization of specific Caspase-8 activity inhibitor and siRNA directed against Caspase-8 in primary cells. We might also investigate on the role of Caspase-10 in co-activation, since its role is still not elucidated. Indeed, if several studies identified Caspase-10 redundant to Caspase-8 for the initiation of apoptotic signals, other studies suggested an alternative pro-survival role for this protein or even that it might negatively regulate Caspase-8 (Wang et al 2001, Horn et al 2017). Furthermore, the long-term effect of absence of FADD, Caspase-8 has to be analyzed to define their potential function at later time points. Moreover, the role of TRAF family members will be evaluated by siRNA strategy in order to unveil an alternative way of activation upon Fas ligation such as the one that exists for the others TNFRSF members.

### **3.3 Role of Fas domains and Fas post-translational modifications in Fas-mediated TCR co-stimulation.**

Noteworthy, we demonstrated also that the entire C terminal part of Fas, including the death domain, is not necessary for the occurrence of Fas-TCR co-stimulation. Indeed, we generated a Fas mutant by the introduction of a stop codon at position 232 (Fas 232TAG), which is the start of the death domain, and we could still allow the initiation of a co-stimulatory signal. We stably expressed human Fas WT and Fas 232TAG in a Jurkat clone which lacks endogenous Fas surface expression and in the 3A9 model, which expresses the murine Fas. In both models, the reintroduction of human Fas WT and Fas 232TAG enables the induction of TCR co-stimulation (Results Fig 5d and Supplementary Fig.c). This result further confirms that co-stimulation is a DISC-independent mechanism since the binding of FADD occurs through the DD of cytoplasmic tail of Fas. We can hypothesize that the co-stimulation involves other regions of Fas protein. We will, therefore, investigate on the potential role of: membrane proximal region, TMD and extracellular domain of Fas, as well as on the role of PTMs of the protein, that might be crucial for the occurrence of the co-stimulatory event.

\*We can hypothesize that the membrane proximal region or the transmembrane domain of Fas could be necessary for the occurrence of the co-activation, since we can describe the binding of Lck, PLC $\gamma$  and LAT to Fas. This concept would be in line with the finding of Poissonnier publication that identified that Fas activation by cleaved FasL was inducing transmigration of Th17 through a mechanism that involve PLC $\gamma$  binding to Fas in the membrane proximal region of cytoplasmic Fas, excluding the involvement of the DD for the binding (Poissonnier et al 2016). Moreover, this is also in accordance with the way of action of other co-receptors. A recent publication observed that ICOS co-stimulatory receptor can bind to Lck through its TMD and initiate the co-stimulation (Wan et al 2020).

We have, for this purpose, created a Fas mutant with almost complete deletion of the intracellular domain (Fas 196TAG), to investigate the effect of the absence of the membrane proximal region on the co-stimulation. However, we could not test the co-activation on our mutant, since the Fas 196TAG was not stably expressed at the cell surface. Thus, creation of new mutants, such as Fas 210TAG and Fas 220TAG, will be further performed in order to identify the intracellular residues of Fas important for the co-stimulation.

\*The importance of critical post translational modifications of Fas will be tested:

- The membrane proximal region contains the palmitoylation site at position C199, or lysine rich region (LRR region), both of them important for Fas localization in the lipid raft, for which mutants, already present in the lab (C199V, LRR and double mutation C199V/LRR) will be fundamental to understand if Fas localization is a determinant for the co-stimulatory event.
- We need to study the involvement of Fas phosphorylation in the co-stimulation. Mutants of tyrosine of cytoplasmic Fas generated by our team that either block or simulate a constant phosphorylation (Y $\rightarrow$ F and Y $\rightarrow$ D respectively) will be tested. So far we show that preventing phosphorylation of the Y232 (Y232F mutant) is enhancing the Fas-TCR co-stimulation, as will be briefly explained below.

\*We can also hypothesize that the extracellular regions of Fas might participate or even be necessary for the co-stimulatory event. Indeed, a paper from 2015 shows that CD28 lacking most of cytoplasmic tail is still able to signalize, thus that the TMD and/or the ECD are involved in the co-stimulation (Morin et al 2015). Moreover, Madireddi and colleagues demonstrate that the co-receptor 4-1BB interacts with galectin-9 in the extracellular matrix, which promote the aggregation of the receptor and allow the co-stimulatory event (Madireddi et al 2014). We will test a mutant already present in our laboratory, the GBM, cited in the first chapter of the manuscript at page 9 and 45, which already demonstrated to have an important role in the in Fas membrane localization and clathrin-dependent internalization (Chakrabandhu et al 2008). We will, moreover, test the co-stimulation by creation of Fas mutant that can interfere with its capacity to self-aggregate in its TMD or in the extracellular domain to test the potential involvement of these regions of Fas

in the co-stimulation. The creation of these mutants, however, do not have to compromise the site of FasL binding, to avoid artefacts of outcome upon co-stimulation.

Furthermore, our preliminary data could also demonstrate that not only the DD of Fas is not necessary, but that this domain binds to negative regulators of the co-stimulation. Starting from the data obtained from our laboratory and others we confirmed that SHP-1 phosphatase binds to Fas in resting condition in our models and that co-stimulation was leading to dissociation of SHP-1 from Fas (Additional results Fig.3a). We could identify the putative site of SHP-1 binding, since the point mutation in one tyrosine of cytoplasmic Fas that would block its phosphorylation (Y232F) was sufficient to strongly decrease SHP-1 binding and to increase the co-activation (Additional results Fig. 3b and 3c). However, we need to perform other experiments to confirm this theory.

In order to confirm SHP-1 role in co-stimulation and to identify new regulators of Fas-TCR co-stimulation we will use the GCE technology, described in the additional results at page 149. Indeed, the covalent crosslink on the region of Fas where will incorporate the nAA will be exploited to perform the proteomic analysis and identify the new regulators.

### **Future perspective: Fas as immunotherapeutic target**

Two current Fas targeted therapies in cancer exist so far:

- The first one aims to target the receptor by crosslinking it in order to activate the Fas death signal. APO010 is Fas agonist that mimic an hexameric form of FasL that is able to bind to Fas expressed on tumor cell and induce their death. This treatment is currently under investigation for glioblastoma and multiple myeloma tumors (Eisele et al 2011, Jandu et al 2020).
- The second one aims to rather sequester the Fas ligand, preventing it from binding to Fas and therefore inhibiting both death and survival signals. APG101 consists of the extracellular domain of Fas fused to Fc domain of IgG that block FasL interaction with Fas. It is currently in phase II of clinical trial for glioblastoma (Wick et al 2014, Blaes et al 2018).

Whether to block or to enhance Fas/FasL signaling remains thus a controversial subject. As we have described in the first chapter of this manuscript, Fas has pro-tumorigenic properties especially in inflammatory condition where the binding to FasL enhances tumor progression and metastasis. Thus, blocking Fas/FasL interaction would offer numerous benefits to contrast tumor progression and enhance the potential of current cancer immunotherapeutic strategies. Moreover, Fas/FasL interaction it is partially responsible of the RICD of immune cells and of the apoptosis of TILs. Thus, the blockage of Fas/FasL interaction would increase the persistence of T cells in the tumor environment and would contrast the immunotherapeutic resistance often found for several patients. Furthermore, the study of Klebanoff described that Fas is responsible of precocious differentiation of naïve cells, a mechanism that compromise the adoptive cell transfer (ACT) based strategies (Klebanoff et al 2016, Waldman et al 2020).

However, enhancement of Fas/FasL interaction would increase the cytotoxic properties of CTL and NK lymphocytes against the tumor cells, a fundamental strategy that our organism exploits to fight tumors.

Unfortunately, these therapies face a major challenge. Indeed it is impossible so far to choose which strategy is the right one since we still cannot predict when Fas will go on death or on survival mode and furthermore, the landscape of tumor microenvironment is extremely heterogeneous and composed by cells that allows the tumor progression and cells that have anti-tumorigenic properties. Indeed, none of the two mentioned strategy is selective since in both cases all the signaling for Fas will be either promoted either inhibited.

Despite that, our data support the co-stimulatory properties of Fas/FasL interaction in T cell activation, that would optimize the proliferation of immune cells in a tumor environment. Currently there are strategies exploiting the co-stimulatory potential of TNFRSF TCR co-receptors such as the CART strategies (see Chapter C pages 98-99). The discovery of the regions of Fas that are involved in the co-stimulation, coupled with the

finding that Fas-TCR co-stimulation is a DD independent mechanism, might allow the creation of CARs that utilize the co-stimulatory region of Fas without involving its pro-death signaling to promote persistence of TILs in tumor microenvironment. In addition, defining the parameters and balance between the pro-death and co-stimulatory functions of Fas might be useful in the utilization for Fas agonist antibody in combinatorial therapy with the ICB based approaches to increase cell activation preserving its cytotoxic properties. In this context the main goals of our team is to deeply understand the molecular basis underlying the Fas signaling versatility in order maybe one day to be able to develop new Fas targeted strategies with a selective mode of action. It is therefore of extreme interest the investigation on the mechanisms underlying Fas-TCR co-stimulation in order to have a complete overview of the advantages that Fas targeting might have in cancer immunotherapeutic treatments.

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**ANNEXES**

Review

# TRAIL and FasL Functions in Cancer and Autoimmune Diseases: Towards an Increasing Complexity

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**Abstract:** Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL/TNFSF10) and Fas Ligand (FasL/TNFSF6), two major cytokines of the TNF (Tumor Necrosis Factor) superfamily, exert their main functions from the immune system compartment. Mice model studies revealed that TRAIL and FasL-mediated signalling both control the homeostasis of the immune cells, mainly from the lymphoid lineage, and function on cytotoxic cells as effector proteins to eliminate the compromised cells. The first clues in the physiological functions of TRAIL arose from the analysis of TRAIL deficient mice, which, even though they are viable and fertile, are prone to cancer and autoimmune diseases development, revealing TRAIL as an important safeguard against autoimmunity and cancer. The naturally occurring *gld* (generalized lymphoproliferative disease) and *lpr* (lymphoproliferation) mutant mice develop lymphadenopathy and lupus-like autoimmune disease. The discovery that they are mutated in the *fasl* and the *fas* receptor gene, respectively, demonstrates the critical role of the FasL/Fas system in lymphocyte homeostasis and autoimmunity. This review summarizes the state of current knowledge regarding the key death and non-death immune functions that TRAIL and FasL play in the initiation and progression of cancer and autoimmune diseases.

**Keywords:** death receptors; autoimmunity; cancer; immune system; cell death

## 1. Introduction

Human Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL), which was cloned in 1995 as the third death inducing ligand of the Tumor Necrosis Factor (TNF) superfamily (TNFSF), shares 28% and 23% homology with Fas Ligand (FasL) and Tumor Necrosis Factor  $\alpha$  (TNF $\alpha$ ), respectively [1]. TRAIL and FasL are type II membrane proteins that exert optimal biologic activity in a trimeric form [2]. Both can be further cleaved by endopeptidases (metalloproteases for FasL or cathepsin E and cysteine protease for TRAIL) producing soluble trimer versions of the ligands [3–5]. Membrane bound forms are more potent in death induction [6–8], but soluble proteins are also biologically active, and soluble FasL was reported to exert alternative non-death functions [9]. The human TRAIL receptor system is the most complex of the two: TRAIL binds to five different receptors: the two membrane receptors TRAIL-R1 (DR4/TNFRSF10A) and TRAIL-R2 (DR5/TNFRSF10B) contain a full length cytoplasmic domain that harbors an intracellular death domain (DD), whereas the receptors TRAIL-R3 (TNFRSF10C) and TRAIL-R4 (TNFRSF10D) show a truncated intracellular part and, in the absence of death domain, might function as decoy receptors [10]. Lastly, osteoprotegerin (OPG/TNFRSF11B), which was initially reported as ligand for RANK (receptor activator of nuclear factor  $\kappa$ ) (TNFRSF11A), was described as a TRAIL soluble receptor [10]. For its part, human FasL binds to one DD-containing membrane receptor, Fas (CD95/TNFRSF6), and to one soluble decoy receptor (DcR3) [10]. On their side, all Fas and TRAIL receptors exist as ligand-independent homotrimers rather than as monomer receptors,

thanks to the presence of a preligand assembly domain in their extracellular region [2]. Interestingly, ligand independent heteromers between some TNFRS members (such as TRAIL-R, Fas, and CD40) were also reported, suggesting that hetero interaction might be a way to modulate the initial signalling steps [11]. The TRAIL and the FasL signalling regulation can differ appreciably between species, which emphasizes that a particular attention should be paid before transposing data obtained in mice into human contexts, as rodents only possess one DD-containing TRAIL receptor and no FasL decoy receptor, and that the primary sequence of the human and mice protein of each receptor is quite divergent.

The expression of FasL and TRAIL is tightly controlled and restricted in physiological conditions to innate and adaptive immune system cells as well as to immune privilege sites, such as the eyes, the placenta, or the testis [12–14]. Both ligands are expressed at the surface of the two main immune effector cells, i.e., activated T cells and natural killer (NK) cells, but also on macrophages, neutrophils, and dendritic cells [15–24]. Their expression can be induced in response to TCR (T cell receptor) activation, but also upon cytokine stimulation particularly interferons (INF) through transcriptional regulation [16,18]. In contrast to their specific ligands, the TRAIL receptors and Fas are ubiquitously expressed inside and outside the immune system.

As TRAIL and FasL were initially described as apoptosis inducers, their death-inducing capacities and the associated molecular mechanisms were extensively studied [10,25]. Briefly, their binding to their respective cognate DD-containing receptors triggers the recruitment of several adaptor proteins that form a death-inducing signalling complex (DISC), which initiates the caspase activation and ultimately leads to the death of the sensitive target cell. This death-inducing function is mainly used by cytotoxic T cells and NK cells to eliminate the unwanted cells, such as cancer cells and virus-infected cells, but also autoreactive lymphocytes and activated lymphocytes during the contraction phase of an infection. In the latter, the involvement of the FasL/Fas system is particularly reported: the TCR restimulation through the upregulation of FasL expression leads to the death of Fas bearing clones in an antigen (ag) specific manner, a mechanism that is known as restimulation-induced cell death (RICD) [26].

It is now well established that, besides these well-known killing functions, FasL and TRAIL also generate other signalling pathways, leading to non-death functions or cell survival. In immune cells, they were reported affecting differentiation, migration processes, as well as cytokines production [27–31]. The molecular mechanisms underlying these non-death signals, although less well described than the apoptotic ones, start to be molecularly deciphered. Their description is beyond the scope of this review, but it can be found in recent literature [32–35].

## 2. TRAIL and FasL Functions in the Control of Autoimmunity

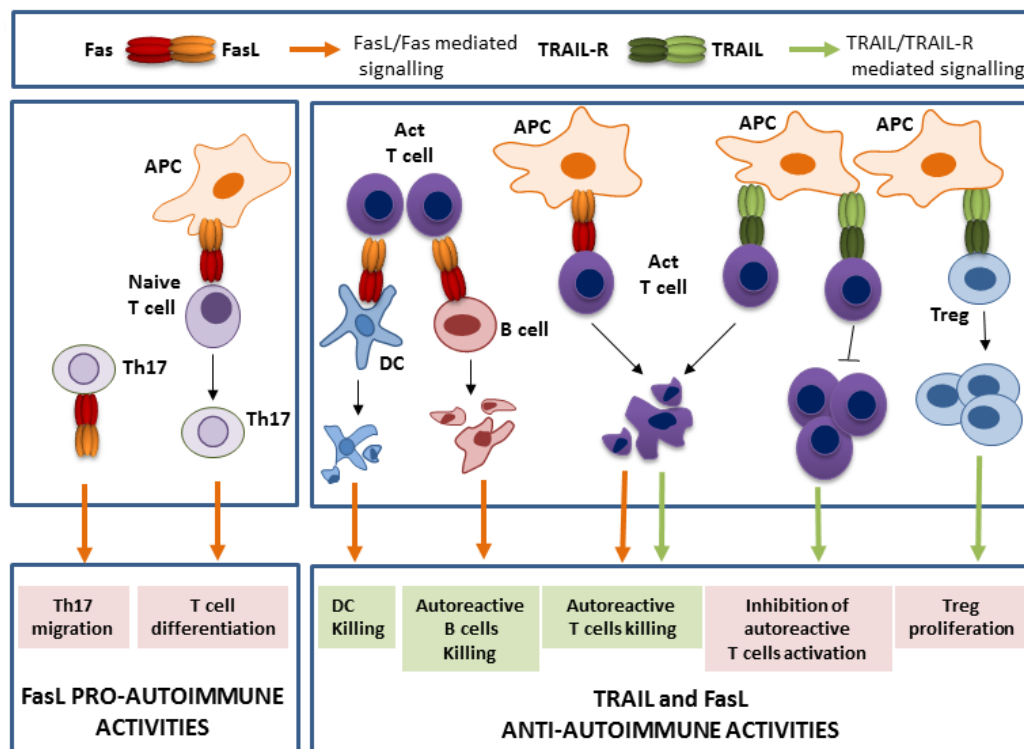
The TRAIL and TRAIL-R knock out mice are viable and fertile [36,37]. In contrast to the FasL/Fas deficient mice, they do not spontaneously develop autoimmune diseases, but they rather present an increased severity of experimentally-induced autoimmune diseases (see Table 1). Autoimmune diseases can be induced by immunizing mice with specific ags, as it is the case for experimental autoimmune encephalomyelitis (EAE) (a mouse model of human multiple sclerosis), experimental induced thyroiditis (EAT), and collagen-induced rheumatoid arthritis (RA) or by the chemical treatment of mice in streptozotocin-induced type I diabetes. In these models, the activation of autoreactive T cells, mainly from CD4+ Th1 and Th17 subtypes, are the principal actors of the immune-mediated defects. An increase of the symptoms observed when inducing EAE [38–40], RA [41], or type I diabetes [41] in TRAIL or TRAIL-R deficient mice (demonstrated mainly by using the C57BL/6 TRAIL<sup>-/-</sup> mice, C57BL/6 TRAIL-R<sup>-/-</sup>, but also the BALB/c TRAIL<sup>-/-</sup> mice) when compared to their wild type counterpart clearly demonstrated the protective role of the TRAIL/TRAIL-R systems (Table 1). Moreover, intraperitoneal TRAIL injection decreases inflammation and autoimmune damages in mice, in which EAE or EAT was previously induced [38,42,43]. Conversely, TRAIL neutralization by the injection of either antibodies or soluble TRAIL receptor was shown to enhance the development of EAE [38,44], collagen-induced

RA [45], as well as insulinitis in non-obese diabetic (NOD) mice [46]. All together, these results identified TRAIL as a guardian against autoimmunity in several autoimmune diseases models.

**Table 1.** The role of Tumor Necrosis Factor-Related Apoptosis Inducing Ligand (TRAIL)/TRAIL-R and Fas Ligand (FasL)/Fas systems in experimentally-induced autoimmune disease mice models.

Autoimmune Mouse Model	Ligand /Receptor Status	Outcome	References
Experimental autoimmune encephalomyelitis (EAE)	TRAIL neutralization (sDR5)	Exacerbation of symptoms	[44]
	TRAIL neutralization (TRAIL Abs)		[38]
Experimental autoimmune thyroiditis (EAT)	TRAIL $^{-/-}$ mice	Attenuation of symptoms	[38,39]
	TRAIL-R $^{-/-}$ mice		[40]
	TRAIL injection		[38]
	TRAIL expressing DC		[47,48]
Collagen-induced rheumatoid arthritis (RA)	TRAIL $^{-/-}$ mice	Attenuation of symptoms	[49–51]
	TRAIL expressing DC		[49,50]
Type 1 diabetes	TRAIL $^{-/-}$ mice	Attenuation of symptoms	[42,43]
	TRAIL neutralization (sDR5)		[45]
Type 1 diabetes	TRAIL $^{-/-}$ mice	Attenuation of symptoms	[41]
	NOD lpr mice		[52]
Type 1 diabetes	NOD gld mice	Attenuation of symptoms	[53]
	NOD gld mice		[54–56]
Type 1 diabetes	NOD gld mice	Attenuation of symptoms	[57]
	NOD gld mice		[57]

The mechanism underlying these effects of TRAIL relies on its critical role in the maintenance of peripheral tolerance. Peripheral tolerance, a mechanism that is controlled by dendritic cells (DC), deals with the control of autoreactive immune cells at the periphery, in opposition to central tolerance, which consists in the deletion of developing autoreactive T cells or B cells in the thymus and bone marrow, respectively. Actually, Hirata et al. could prevent the development of EAE in mice by the transfer of DC presenting the auto ag and genetically modified to express TRAIL [47,48]. The same strategy was used to prevent the development of collagen II-induced arthritis [52]. TRAIL was described to control peripheral tolerance through the regulation of T cell compartment homeostasis by at least three different actions, leading to the neutralization of CD4+ autoreactive T cells (Figure 1). The main explanation of the protective role of TRAIL was initially attributed to an increased death of autoreactive T cells [47,52]. Later, it was also reported that TRAIL is able to stimulate the immunosuppressive regulatory T cells (Treg) proliferation by a so-far unknown mechanism, a process that leads to the inhibition of autoreactive T cell proliferation [43,48]. Indeed, Hirata et al. elegantly demonstrated that the adoptive transfer of Treg cells from mice transferred with TRAIL-expressing DC is sufficient to protect recipient mice from EAE induction [39,48]. Lastly, TRAIL was reported to directly inhibit the activation and proliferation of pathogenic Th1 or Th17 cells [38–40,47,58], notably by interfering with TCR proximal signal activation [40,58]. Nevertheless, the generation of cell specific TRAIL knockout mice would allow strictly identifying the precise role of each immune cell type in the anti-autoimmune function of TRAIL. However, it is important to remember that neither mutations nor modulation of the TRAIL/TRAIL-R expression have been reported so far in the etiology of autoimmune disease in humans.



**Figure 1.** TRAIL and FasL activities in the control of autoimmunity. Whereas, TRAIL acts as a guardian against autoimmunity, FasL exerts both a role of guardian, but also promoter of autoimmunity. The main respective roles of their death (highlighted in green) and non-death (highlighted in pink) functions are represented. APC is for antigen presenting cells, DC for dendritic cells, and Act T cell for activated T cells.

In contrast, a human genetic disease, called autoimmune lymphoproliferative syndrome (ALPS), arises from mutation in the *fas* gene, in the *fasl* gene, or in genes encoding for effector proteins that are involved in Fas-induced cell death signalling, such as FADD (Fas-Associated protein with Death Domain) and caspases-8 or-10 [59]. ALPS patients present an uncontrolled lymphocyte proliferation that is accompanied by autoimmune manifestations, such as cytopenia or organ-specific autoimmunity, including hepatitis, glomerulonephritis, or dermatitis [60]. Notably, the naturally occurring *lpr* or *gld* mice, which bear mutations in the *fas* and *fasl*-encoding gene, respectively, are phenotypically similar to ALPS patients and they represent a model for autoimmune disease [61,62]. These mice develop progressive lymphadenopathy, splenomegaly, and an excess of immunoglobulins, including auto antibodies and systemic lupus erythematosus (SLE)-like autoimmune symptoms. However, the severity of the phenotype depends on the genetic background [63]. The lymphocytes that accumulate in the secondary lymphoid organs predominantly present the pathognomonic TCR $\alpha/\beta$  CD4 $^-$  CD8 $^-$  double negative phenotype, even though the conventional CD4 $^+$ , CD8 $^+$  T lymphocytes and B lymphocytes also show a several fold increase [63]. Altogether, these observations demonstrate a critical role of the FasL/Fas system in the prevention of autoimmunity and the control of lymphocytes homeostasis. Interestingly, TRAIL deficiency in a *gld* background significantly exacerbates both lymphoproliferation and autoimmune disease when compared to the *gld* mice, suggesting some redundancy between FasL and TRAIL and a role for these two receptors in the control of T cell homeostasis and autoimmunity [64].

The attribution of autoimmunity regulation to one specific cell type has been a debate for several years. However, it is now recognized that Fas expression on T cells, B cells, and DC is determinant for induction of peripheral tolerance. The first experiments conducted in *lpr* mice showed that the transgenic expression of Fas in their T cells is sufficient to prevent the lymphocytes accumulation (including the double negative T cells), but not the SLE development [65]. In contrast, the re-introduction



of Fas in the *lpr* mice B cells avoids the SLE autoimmune manifestation, but do not prevent the T cell lymphoproliferation [66]. Additional critical informations came from tissue-specific deletion of Fas. Indeed, Stranges et al. reported that: (i) loss of Fas expression in T cells, which is critical for autoreactive T cell death recognizing low affinity self-ags, lead to autoimmune symptoms development and (ii) the conditional deletion of Fas in DC is sufficient to cause systemic autoimmunity, which is mainly due to DC accumulation and increased ag presentation [67] (Figure 1). They revealed a negative regulatory loop, in which T cells that recognize ags on the DC eliminate them by Fas-induced cell death to limit the immune response. On its side, the specific deletion of Fas in B cells lead to autoimmune disease, since Fas expression on B cells is involved in the negative selection of autoreactive B cells [67–69] (Figure 1). Interestingly, as B cells have also a role in ag presentation and thus T cell activation, the increased B cell number that is obtained due to lack of Fas-induced apoptosis in turn activates a large number of T cells, resulting in both B and T lymphoproliferation and exacerbating autoimmunity through the survival of autoimmune T cells [67,69].

More recently, some authors proposed that the control of the T cell compartment by Fas could be independent of its death function [70–73]. Fas palmitoylation is required for Fas localization in lipid raft, receptor aggregation and stability, and finally efficient cell death induction [74–76]. Palmitoylation-deficient mutant is defective in inducing death in primary mouse T cells, B cells, and DC, while retaining the capacity to trigger naïve T cell differentiation [72]. Interestingly, the palmitoylation-deficient receptor reintroduced in *lpr* mice reverses the lymphoproliferation and autoimmunity, suggesting that Fas does not protect from autoimmunity through its death-inducing capacity, but rather through its survival functions [72]. Additionally, Daszkiewicz et al. have suggested that Fas controls T cell homeostasis by inhibiting their proliferation following their observation of a reduction of effector and memory T cell accumulation and autoimmune associated symptoms upon the expression of p21 in T cells of *lpr* mice [73]. Indeed, FasL has been shown to induce p21 expression, and thus block the cell cycle progression of activated T cell, leading to the inhibition of their proliferation [71].

Paradoxically, while Fas has a suppressive effect on spontaneous systemic immunity, as seen with the development of autoimmunity in *lpr* or *gld* mice, Fas also plays a promoting role in the development of experimentally-induced autoimmune disease, since *lpr* or *gld* mice are resistant to EAE, RA, and type I diabetes induction [49,50,53–55,57] (Table 1). The promotion of EAE development by Fas was elegantly recently solved by Meyer Zu Horste et al., who revealed that Fas, through a death independent function, drives Th17 differentiation, which are the critical promoters of autoimmune tissue inflammation [51] (Figure 1). By transcriptional profiling analysis, Fas was shown to control the Th1/Th17 balance by a STAT-1 mediated mechanism [51,77]. Indeed, as both RA and type I diabetes development also involve Th17-mediated inflammation, the same molecular mechanism could be transposed and explain the role of Fas in supporting their progression. An additional autoimmune-promoting role of Fas involving Th17 has been recently described in SLE patients. The authors demonstrated that cleaved FasL in inflamed tissue chemoattracts Fas expressing Th17 cells and favors their transmigration across the endothelial barrier [78]. Despite intense research, controversies regarding the precise cellular etiology of the milder development of insulinitis in NOD/*lpr* mice remain. Some of the authors suggest that the decreased  $\beta$  cells destruction that was observed was due to their own inability to signal through Fas upon binding to FasL from effector T cells [54]. However, others demonstrated that Fas deficiency on the T cells impaired their reactivity and thus initiates the phenotype [56].

In contrast to the role of TRAIL, which, to our current knowledge, seems limited in preventing autoimmunity through its action on different T cells subtypes, the contribution of the FasL/Fas system in autoimmune disease development reaches a high level of complexity, depending on the multiplicity of the cellular subsets involved and the multiplicity of functions activated by Fas triggering (Figure 1). While it can prevent autoimmune development by targeting T cells, B cells and DC, it can also

promote it, notably through the differentiation of the pathogenic Th17 cells. The integration of all these informations is necessary to complete the general picture, which is far from being solved.

### 3. TRAIL and FasL Functions in Cancer Immunoediting

The TRAIL/TRAIL-R and FasL/Fas systems are critical players in the complex and reciprocal relationship that exists between cancer cells and the immune system, which is recapitulated within the cancer immunoediting concept. This dynamic process integrates the coexistence of promoting and constraining tumoral functions that are operated by different immune cells subtypes within the tumor environment through three consecutive key phases: elimination, equilibrium, and escape. The elimination phase relies on the removal of the malignant cells by both innate and adaptive immune cells, notably through perforin/granzyme and FasL/TRAIL-mediated killing. An immune selective pressure is maintained on the tumor cells that had survived this first surveillance stage, during which the highly resistant clones are selected. Therefore, they could escape from the immune system thanks to their abilities to survive in an immunosuppressive environment, proliferate, and disseminate in other organs, which can be mediated in part by TRAIL and FasL-induced signalling. The demonstration of the critical implication of the immune system in the tumor development has allowed great advances in the design of therapies, namely immunotherapies, in which TRAIL and FasL signalling could be targeted.

#### 3.1. Role of TRAIL and FasL in the Elimination Phase

The evidence for the involvement of the endogenous TRAIL/TRAIL-R system in tumor elimination came from animal studies using TRAIL<sup>-/-</sup> [36,79,80] or TRAIL-R<sup>-/-</sup> mice [81–83] or mice that were injected with TRAIL neutralizing antibodies [84–86] and mostly conclude to a protective role of the TRAIL/TRAIL-R system against cancer development (Table 2).

**Table 2.** The role of TRAIL/TRAIL-R and FasL/Fas systems in cancer development in mice models.

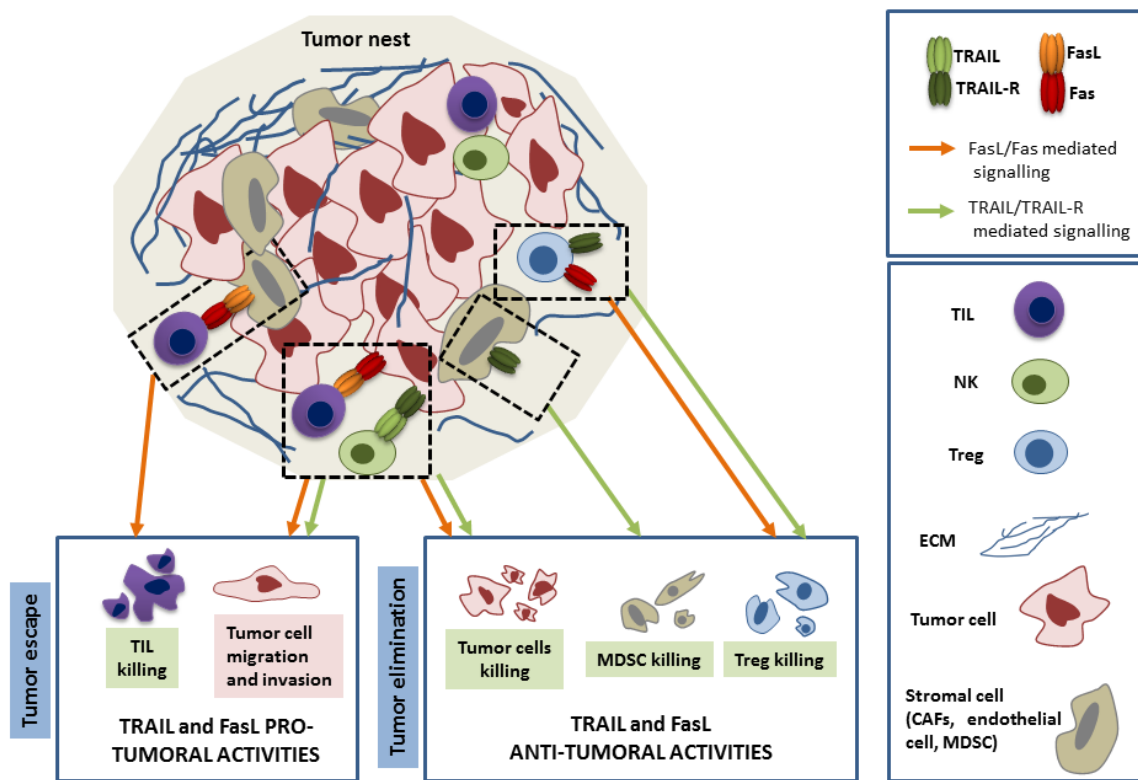
Ligand/Receptor Status	Cancer Induction	Outcome	References
TRAIL <sup>-/-</sup> mice	Spontaneous	Late-age lymphoma	[79]
	p53 <sup>+/-</sup> mice	Sarcoma, lymphoma	[79]
	Her2/neu mice	No symptoms	[79]
	A20 cell line transfer	Lymphoma	[36]
	Renca cell line transfer	Liver metastasis	[80]
	4T1 cell line transfer	Mammary carcinoma Lung and liver metastasis	[80]
	MCA induction	Fibrosarcoma	[80]
TRAIL neutralization by Abs	p53 <sup>+/-</sup> mice	Sarcoma, lymphoma	[84]
	L929 cell line transfer	Liver metastasis	[85]
	Renca cell line transfer	Liver metastasis	[86]
	MCA induction	Fibrosarcoma	[84]
TRAIL-R <sup>-/-</sup> mice	Eu-myc mice	Lymphoma	[81]
	p53 <sup>-/-</sup> mice	No symptoms	[82]
	APC <sup>min/+</sup> mice	No symptoms	[82]
	DMBA/TPA	Lymph node metastasis	[83]
	DEN treatment	Hepato carcinoma	[81]
lpr mice	T cell deficient	Lymphoma	[87]
	Eu-myc mice	Lymphoma	[88]
gld mice	Spontaneous	Lymphoma	[89]
FasL <sup>-/-</sup> T cells (CD8+)	Lymphoma cells transfer in rag1 <sup>-/-</sup> mice	Lymphoma	[90]

In TRAIL<sup>-/-</sup> mice, an increased susceptibility to both tumor initiation and metastasis development was demonstrated by several experimental methods, including transplantation of syngenic cancer cell lines, the spontaneous occurrence of late age lymphoma or the development of chemical carcinogen methylcholanthrene-induced fibrosarcomas [36,79,80,84]. The TRAIL-R invalidation leads to more contrasting results: on one hand, the TRAIL-R deficiency was found to recapitulate the TRAIL deficient phenotype, since the loss of TRAIL-R in the lymphoma-prone E $\mu$ myc genetic background significantly reduced the lymphoma-free survival and enhanced diethylnitrosamine (DEN)-induced hepatocarcinogenesis [81]. On the other hand, Yue et al. identified no tumor promotion in TRAIL-R<sup>-/-</sup> mice, even when the deficient mice were crossed with p53<sup>-/-</sup> mice or APC<sup>min/+</sup> mice [82]. The discrepancies that were observed between this work and others backcrossing with p53<sup>+/-</sup> mice could be due to the complete loss of p53, which might affect the TRAIL/TRAIL-R system expression and function. A study performed by Grosse-Wilde et al. brings interesting clues: the authors reported that, whereas an increased lymph node metastasis is detected in TRAIL-R deficient mice that were subjected to the DMBA/TPA-induced model of squamous cell carcinomas, no incidence is found in the primary tumor. Interestingly, while the adherent primary tumor cells were resistant to TRAIL-mediated cell death, their detachment, which is a mandatory step in metastasis formation, sensitize them to TRAIL-induced apoptosis and provide an explanation for the role of TRAIL as a metastasis suppressor [83]. This finding can be read in light with a recent work from our laboratory demonstrating that inhibition of cell-cell adhesion of epithelial cells sensitizes them to Fas-induced cell death [91]. A sequestration of Fas by cadherins in adherens junctions, as well as the association of Fas and the polarity molecule Dlg1, which inhibits DISC formation, molecularly account for the inhibition of Fas-induced cell death in polarized cells. Thus, it is tempting to speculate that similar molecular control of TRAIL-R localization and association could account for their sensitization to TRAIL upon the loss of adherence.

The role of the FasL/Fas system in preventing cancer development was highlighted in ALPS patients who present a higher incidence of non-Hodgkin and Hodgkin lymphoma [92]. It is more difficult to assess in mice models due to the altered immune system that results from FasL/Fas deficient signalling. Nevertheless, some studies demonstrated that aging gld mice develop spontaneously malignant plasmacytoid lymphomas, even though the late development of the tumors strongly suggests that additional genetic mutations are necessary for malignant transformation to occur [89] (Table 2). Actually, no spontaneous occurrence of cancer development was reported in lpr mice, but the loss of Fas was shown to accelerate the lymphoma development in E $\mu$  L-myc transgenic mice [88]. In addition, T cell deficient lpr mice develop intraperitoneal B cell lymphoma and the authors propose that the loss of Fas promotes a pool of premalignant B cells that progress to malignancy in the absence of T cells [87].

Cytotoxic T lymphocytes (CTL) and NK cells are the two main players in the immune-mediated elimination of cancer cells. Even though they are activated by different ways, they use the same mechanisms to kill their target: (i) the granule exocytosis pathway using the pore-forming protein perforin and the serine proteases granzymes and (ii) the expression of the death ligands FasL and TRAIL. The origin of the protective role of TRAIL in cancer development has been mainly attributed to its cytotoxic function on hepatic NK cells (Figure 2). Indeed, while neutralizing TRAIL with anti-TRAIL antibodies significantly enhance metastasis formation in mice, previous NK cells depletion abolished this effect, indicating that the cytotoxic activity of TRAIL expressed on NK cells is responsible for its anti-metastatic action [85]. Moreover, the ex vivo mediated NK cells cytotoxicity was dramatically reduced in TRAIL<sup>-/-</sup> mice when compared to their wild type counterparts, leading to the conclusion that TRAIL, as well as FasL and perforin, contribute to the anti-metastatic effect of the NK cells [80]. Importantly, TRAIL contributes to the INF $\gamma$ -mediated anti-metastatic effect of NK cells, probably through the upregulation of TRAIL expression upon INF $\gamma$  treatment [86]. Nevertheless, the contribution of other cell types to TRAIL-mediated anti-cancer effect has been demonstrated and TRAIL has been reported as a mediator of tumor-specific CD4<sup>+</sup> cytotoxic lymphocytes-mediated cell death in lung cancer [93]. Importantly, not only the cancer cell killing, but also the targeting of tumor supportive

cells that are present within the tumor nest, such as myeloid-derived suppressor cells (MDSC) and Treg, might account for some anti-cancer role of TRAIL [94,95] (Figure 2).



**Figure 2.** TRAIL and FasL pro- and anti-tumoral activities within the tumor nest. The main respective roles of their death (highlighted in green) and non-death (highlighted in pink) functions are represented. Briefly, TRAIL and FasL, mainly from tumor-infiltrating lymphocytes (TIL) and natural killer (NK) cells origin target the different cells of the tumor nest. They trigger the death of immunosuppressive cells such as regulatory T cells (Treg) and myeloid-derived suppressor cells (MDSC). The tumor cells can respond either by death but also non death pro tumoral functions which lead to tumor escape. Moreover, the tumor can escape from the immune system by specific killing of the TIL through abnormal expression of FasL on stromal cells.

If the perforin-granzyme system has been shown to play a major role in tumor cell elimination, as demonstrated by the development of spontaneous B cell lymphomas in the perforin-deficient mice, FasL expressed on CTL is a second way used by lymphocytes to kill cancer cells (Figure 2). Indeed, while in *perf<sup>-/-</sup>* mice an anti-tumor response is still carried out by the CTL, this cytotoxic effect cannot be seen in *ex vivo* experiments where FasL has been neutralized [96]. Therefore, both systems appear to be critical and complementary to optimally achieve tumor rejection [96–98]. However, the exact contribution of these two cytotoxic pathways used by CTL to kill the cancer cells is still unsolved. Shanker et al. propose that the ag concentration presented by tumor cells might determinate the type of system used: the FasL system might be prominently used when a limited amount of ag is presented by the tumor cells, while the perforin system might become more present when this amount increases [99]. This hypothesis is coherent with previous studies that were done in a cancer-independent context, describing that a weak TCR signalling is able to activate a FasL, but not a perforin-mediated CTL death [100]. Interestingly, a recent study questioned the role of the immune system in controlling the development of B lymphoma. Afshar-Sterle et al. not only show that the lymphoma development was inhibited by different CD8+T cell clones presenting a large TCR range, but also identified FasL expression on their surface as a way of limiting the lymphoma development [90]. Based on these observations, the authors develop an interesting hypothesis that could explain the unique lymphoma

nature of cancers that developed in a deficient FasL/Fas context: since B lymphoma cells could behave as APC, T cells bearing a low affinity TCR for self-ags eliminate through a FasL/Fas system the developing B lymphoma cells, an elimination that cannot be taken over in *lpr* and *gld* mice or in ALPS patients.

### 3.2. Role of TRAIL and FasL in Immune Escape

The cancer cells use different strategies to escape from the NK cells and CTL-mediated killing. Amongst them, the apoptosis of tumor-infiltrating lymphocytes (TILs) represents an effective way to prevent anti-tumor immunity and it can be reached by the death ligand expression within the tumor microenvironment and the death receptor expression on the TIL. This old idea of tumor counterattack gave rise to intense debates that were initiated 20 years ago. Some clarifications recently came from several studies that showed that an abnormal FasL expression within the tumor is playing an active role in the tumor development by allowing the tumor to escape. They identified the cellular origins of the FasL expression within the tumor microenvironment: in contrast to what was initially thought, FasL was not expressed at the surface of the tumor cells, but by endothelium cells, cancer-associated fibroblast (CAFs), and myeloid-derived suppressor cells (MDSC) [101–103]. Indeed, FasL neutralization in each of these three cell populations lead to the Fas expressing TILs inhibition of apoptosis and increased tumor rejection. Such a clear demonstration has not been reported so far for TRAIL. Nevertheless, the observation relating a correlation between the rate of apoptotic TILs and their TRAIL-R1 expression suggest that TRAIL-R1-mediated TIL apoptosis could also participate to tumor immune escape, a hypothesis that is coherent with the expression of TRAIL by the colorectal tumor cells [104].

The tumor cells themselves could adapt to counteract the death signal that is induced by TRAIL-R or Fas activation by down regulating their surface expression. A decreased expression of TRAIL-R1 upon TGF $\beta$  (Transforming growth factor beta) treatment has been detected in pancreatic cancer cell lines and correlates with a decrease in TRAIL-induced cell death [105]. Afshar-Sterle et al. demonstrate that B-lymphoma which develops in a T cell sufficient mouse model has a weaker Fas expression when compared to those arisen from T cell deficient mice and are more resistant to FasL-induced apoptosis [90]. In the case of TRAIL, the overexpression of the decoy receptors TRAIL-R3 and TRAIL-R4 have been reported in some tumor types (including acute myeloid leukemia), where they might sequester TRAIL, but also form heteromers with TRAIL-R1 and TRAIL-R2, thereby preventing TRAIL-R1 and TRAIL-R2 activation and proper DISC formation [25,106]. The overexpression of DcR3 has also been reported in tumors, such as colon and lung [25]. However, the consequence on Fas signalling is more difficult to assess, since it also acts as decoy receptor for TL1A and LIGHT and presents independent non-decoy functions. Additionally, some tumor cells also modulate their expression of essential mediators of cell death signalling, as it is the case for the anti-apoptotic regulator c-FLIP, which is found to be frequently overexpressed in tumors of different origins [107].

Cancer cells not only develop resistance to FasL and TRAIL-mediated immune cell death, but they also hijack the TRAIL and FasL non-death signalling pathway in order to support migration and invasion. This is mainly true in the presence of an oncogenic mutation. Both FasL and TRAIL were reported to stimulate the invasion of colorectal tumor cells and liver metastasis in mice through a K-Ras-dependent way [108]. The authors demonstrated that mutated K-Ras and its effector Raf-1 can switch the death receptor signalling from death to invasion through the inhibition of the Rho/ROCK/LIMK pathway. More recently, Von Karstedt et al. demonstrated, via the cancer cell restricted genetic ablation of TRAIL-R, that spontaneous metastasis in a K-Ras driven pancreatic ductal adenocarcinoma requires the expression of TRAIL-R [109]. Working in human cell lines, they only identified TRAIL-R2, but not TRAIL-R1, as the driver of this cancer progression, but also reported the critical importance of the membrane proximal domain of TRAIL-R2 to induce a Rac1 mediated pro-migratory signalling pathway. Interestingly, a kinome analysis of the non-death pathway induced by TRAIL in non-small-cell lung carcinoma identified a RIPK1/Src/STAT3 pathway that was only activated by the TRAIL-R2 receptor and lead to cell migration and invasion [110]. Thus, the two TRAIL receptors might have different abilities in activating cancer-promoting functions. The recruitment of the Src kinase Yes and the p85

PI3K subunit to Fas upon FasL activation was demonstrated in glioblastoma cells and it could give rise to an invasion signal through the activation of the Akt/GSK3 $\beta$  axis, which leads to subsequent matrix metalloproteases expression [111]. Neutralizing the FasL pathway in mice dramatically reduces the number of invading cells [111]. A recent report from our laboratory demonstrated that Src kinase-mediated phosphorylation of Fas within its death domain is a key event in switching Fas signalling from death to non-death: the dephosphorylation of both tyrosines in the death domain of Fas by the SHP-1 phosphatase turns on the apoptotic signal, whereas the tyrosine phosphorylation turns off the pro-apoptotic signal and turns on the prosurvival. Furthermore, we provide evidence that Fas tyrosine phosphorylation status may vary among different cancer types and influence the response to anti-cancer treatments [112].

#### 4. TRAIL and FasL in Clinical Interventions

Huge expectations in the use of TRAIL in cancer treatment were generated, since systemic administration of TRAIL in mice was shown to be not only effective in killing human breast or colon xenografted tumor cells, but also in causing less toxicity than FasL or TNF administration [113]. Based on these initial observations, TRAIL was considered as the most promising tumor selective ligand, and intense world-wide research efforts were made on TRAIL-R agonists as potential novel cancer therapeutics.

Nevertheless, this initial optimism to target TRAIL for anticancer therapy was stunted by the disappointing results that were obtained with the TRAIL-R agonists clinical trials that, although confirming the good tolerance of the treatment, did not show any robust benefits for patients [106,114]. Two classes of molecules have been tested so far in clinic, recombinant form of the ligand (i.e., Dulanermin) or agonistic antibodies targeting the receptors (i.e., TAS266, AMG-655) [106]. The lack of success for TRAIL targeting molecules was mainly attributed to an insufficient agonistic activity, a short half-life of the molecule in situ, and the resistance of the majority of cancer cells to TRAIL-induced cell death. Several attempts have been made to overcome these difficulties. Recently, a combination of recombinant TRAIL and agonistic TRAIL-R2 was shown to synergize in the killing of cancer cells via enhanced multimerization of TRAIL-R2, which brings new hope for their use in therapy [115]. New TRAIL formulations with increased activity have been designed and evaluated in preclinical studies [116]. They aim at increasing the stability of the molecules, but also the specific targeting to cancer cells [116]. If TRAIL system targeting drugs were not yet tested in auto-immune disease contexts, the progress that has been made in cancer therapy could benefit to auto-immune affected patients and the activation of the TRAIL pathway could be validated as a therapeutic option.

The idea to use the FasL system as a potential therapeutic intervention was abandoned when it was described that activating Fas antibody injection in mice induces hepatocyte apoptosis, liver failure, and death. It is now known that the main cause of such toxicity is attributed to the Fc part of the antibody [117,118]. Later, recombinant FasL or Fas molecules have been designed. One of them, called megaFasL or APO010, is a synthetic hexameric Fas agonist that is generated by the fusion of two trimeric FasL, is currently in clinical trials evaluation after successful assays in animal models [119]. A second molecule, APG101, which consists of the extracellular domain of Fas fused to the Fc domain of IgG binds FasL, thereby acting as an antagonist, is in a phase II clinical trial for glioblastoma treatment [120,121].

Importantly, some critical questions regarding the death receptor targeting in cancer treatment remain. First, as TRAIL and FasL are both involved in the death of primary cells, such as hepatocytes, immune cells, or neurons, the administration mode and the molecule design has to be carefully thought. Secondly, the question of blocking or activating Fas or TRAIL-R signalling is still opened. The TRAIL-R agonists, by inducing apoptosis of cancer cells, regulatory T cells, and MDSC, could represent a real improvement for cancer therapy. In the other hand, in some other tumors, such as those that bear the mutated K-Ras, neutralizing the TRAIL/TRAIL-R axis might exert a beneficial effect on tumor development. The same question arises for Fas. On the contrary to what has been initially anticipated,

several independent studies came to the conclusion that FasL/Fas axis neutralization would extend the benefits of cancer immunotherapy: (i) one major role of FasL/Fas in the immune cells is to promote the RICD of activated T lymphocyte at the end of the immune response. Blocking this function would improve T cell persistence at the tumor site; (ii) the expression of FasL by some stromal cells has been shown to trigger TILs apoptosis therefore participating to the immunotherapy resistance [101–103]; (iii) Fas expressed on memory T cells induces the precocious differentiation of naïve Fas-expressing T cells that limits their anti-tumor action through an Akt-dependent pathway [122]. Thus, neutralizing the FasL/Fas axis could synergize with several immunotherapy strategies by improving T cell activity. However, maintaining or even enhancing the FasL/Fas activity might also be of high interest by potentiating the T cell-mediated cytotoxicity, an important process that achieves tumor rejection. In conclusion, major precautions need to be taken before applying a FasL/Fas blockage strategy that could definitively alter the efficient T cell activation, and thus the anti-cancer response.

Important lessons that arise from the disappointing translation of death ligands mouse model studies into human therapeutic must be learnt in order to improve the design of future drugs. First of all, mouse and human death receptor/ligand systems display major differences in terms of sequence, number and nature of receptors, and post-translational modifications, which make the comparison of the systems and adaptation of results obtained from mouse in humans difficult. Moreover, despite the proper expression of TRAIL-R or Fas on tumor cells, the response to the receptors activation may vary extremely from death-induction, no response, to tumor promotion. Consequently, the identification of valid biomarkers that are able to predict the response of each patient to TRAIL-Rs or Fas stimulation is crucial to determine which patient will benefit from such treatment. In conclusion, a better understanding of the molecular mechanisms underlying TRAIL or FasL-mediated signalling pathways in human is necessary to bring death-receptor-based therapy to success.

## 5. Conclusions

As described in this review, whereas TRAIL is only known for its anti-autoimmune activities through targeting several T cell subsets, the situation is more complicated for FasL, which presents both pro and anti-autoimmune functions. In the context of cancer, both FasL and TRAIL are involved in killing cancer cells, but whereas the role of FasL mainly seems attributed to its expression on cytotoxic T cells, the role of TRAIL seems to rely on its expression on NK cells. In this way, FasL and TRAIL may exert complementary functions that involve both adaptive and innate immune cells in order to more efficiently limit the cancer cells expansion. On the other hand, the role of both molecules in tumor escape is now well documented, notably by their abilities to induce tumor cell migration.

In conclusion, despite a similar cellular expression pattern and comparable molecular signalling mechanisms used by their receptors, the individual participation of TRAIL and FasL in autoimmunity and cancer development are mainly not redundant, but rather complementary. A deep analysis of their precise role revealed a huge complexity that arises from the different cell subsets that are involved either as effector or targets. A second level of complexity is added by the different outcomes that can be triggered by TRAIL and FasL signalling, since, in the two pathological contexts, both the death and the non-death activities of the ligands are involved. This general complex picture is important to keep in mind, especially when considering the targeting of the TRAIL/TRAIL-R and FasL/Fas systems in therapeutic strategies.

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