



Normal and pathological functions of the T cell signaling protein, THEMIS, in CD4⁺ T cells

Cui Yang

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Cui YANG

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Cui YANG**

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Abbreviations

- A -

ADAP	Adhesion and degranulation adaptor protein
APC	Antigen presenting cells
AhR	Aryl hydrocarbon receptor
AP-1	Activator protein 1

- B -

BN rats	Brown Norway rats
Bcl6	Bcl6 Transcription Repressor
BD	Behcet's disease

- C -

CABIT	Cysteine containing all beta in <i>themis</i>
c-Cbl	casistas B-lineage lymphoma
CAR	Chimeric antigen receptor
CCR	C-C chemokine receptor
CIA	Collagen-induced arthritis
cTECs	Cortical thymic epithelial cells
CNS	Central nervous system
CWD	Chronic wasting disease

- D -

DP	Double positive
DN	Double negative
DAG	Diacylglycerol
DSS	Dextran Sulfate Sodium

- E -

ENU	N-ethyl-N-nitrosourea
ERK	Ras-dependent extracellular signal-regulated kinase
EGF	Epidermal growth factor
ECM	Experimental cerebral malaria
EAE	Experimental autoimmune encephalomyelitis

- F -

FEZF2 FEZ family zinc finger protein 2

- G -

GAREM Grb2-associated regulator of ERK/MAPK1

Grb2 Growth factor receptor-bound protein 2

Grap GRB2 related adaptor protein

Gads GRB2-related adapter protein 2

GWASs Genome-wide association studies

GvHD Graft versus host disease

GM-CSF Granulocyte-macrophage colony-stimulating factor

- H -

HSC Hematopoietic stem cell

HIV Human Immunodeficiency Virus

HVEM Herpesvirus entry mediator

- I -

IL Interleukin

IP3 Inositol 1,4,5-trisphosphate

IKK IκB kinase

ITIM Immunoreceptor tyrosine-based inhibitory motif

ITAM Immunoreceptor tyrosines-based activation motifs

ITSM immunoreceptor tyrosine-based switch motif

ITK IL-2 inducible T cell kinase

iTreg induced T regulatory cells

IC Immune checkpoints

IBD Inflammatory bowel disease

ICOS Inducible T-cell COS stimulator

- J -

JNK c-Jun N-terminal kinases

- L -

Lck	Lymphocyte-specific protein tyrosine kinase
LAT	Linker for activation of T cell
LIP	T cell proliferation under lymphogenic conditions
LAIR1	Leukocyte-associated Ig-like receptor 1
LM – OVA	Listeria monocytogenes expressing OVA
- M -	
MAPK	Mitogen-activated protein kinases
MS	Multiple sclerosis
MOG ₃₅₋₅₅	Myelin oligodendrocyte glycoprotein epitope 35-55
mTECs	Medullary thymic epithelial cells
mTOR	mechanistic target of rapamycin
- N -	
NFAT	Nuclear factor of activated T cells
NF-κB	Nuclear factor-kappa B
NK	Natural killer cells
NKT	Natural killer T cells
NLS	Nuclear localization signal
NF-M	Neurofilament medium polypeptide
- P -	
PH domain	Pleckstrin homology domain
PIP2	Phosphatidylinositol 4,5-bisphosphate
PI3K	Phosphatidylinositol 3-kinase
PKCθ	Protein kinase C
PLC-γ1	Phospholipase C gamma
PTP	Protein tyrosine phosphatase
PRR	Proline-rich region
PDK1	Phosphoinositide-dependent kinase 1
pTrge	Peripheral T regulatory cells
PBMCs	Peripheral blood mononuclear cells

- R -

Rac1	Ras-related C3 botulinum toxin substrate 1
RAG1/2	Recombination activating protein
RhoA	Ras homolog gene family, member A
ROS	Reactive oxygen species
RUNX3	Runt-related transcription factor 3
RA	Rheumatoid arthritis

- S -

SP	Single positive
SH2	Src homology domain 2
SH3	Src homology domain 3
SLP-76	SH2 domain containing Leucocytes Phosphoprotein of 76Kda
SOCS	Suppressor of cytokine signaling protein
SOCS3	Cytokine-inducible SH2 protein-3
Sos1	Guanine nucleotide exchange factor

- T -

THEMIS	THymocyte Expressed Molecule Involved in Selection
Tespa1	Thymocytes-expressed positive selection-associated 1
tTreg	Thymic T regulatory cells
Tconv	Conventional T cells
TGF- β	Transforming growth factor- β
Th-POK	T-helper-inducing POZ/Krüppel-like factor
TILs	Tumor-infiltrating lymphocytes

- U -

USP9X	Ubiquitin Specific Peptidase 9 X-Linked
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- V -

VAV1	Vav Guanine Nucleotide Exchange Factor 1
------	--

- Z -

Zap-70	Zeta-chain-associated protein kinase 70
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Summary

Summary in English

CD4⁺ T cells or T helper (Th) cells play a pivotal role in triggering and shaping adaptive immune responses. Naïve CD4⁺ T cells may differentiate into multiple effector subsets with distinct lineages and specific immune functions which help to orchestrate host defense against various classes of pathogens. The mechanism that controls cell fate decision depends on the integration of multiple signals triggered by the T cell antigen receptor (TCR), which recognize Self or foreign peptide associated with the molecules of the Major Histocompatibility Complex (MHC), and by an array of cytokines receptors which are stimulated according to the cytokines present in the extracellular environment. In addition to those cell fate decision signals, additional signals are important to control the magnitude of T cell effector responses and set the balance between effective and pathologic immune responses.

THEMIS is a T cell lineage protein that plays an essential role during the positive selection of T cells in the thymus. THEMIS has been characterized as a TCR signaling protein, but whether it operates as an inhibitor or enhancer of TCR signals has been controversial. Its function in peripheral T cells, especially in peripheral CD4⁺ T cells, remains poorly characterized. The objective of this study was to investigate the role of THEMIS in normal and pathological CD4⁺ T cell responses.

By using a mouse model which is selectively deficient for THEMIS after the stage of positive selection (lateCD2-Cre *themis*^{flox/flox} mice; named *themis*-T^{KO}), we observed that THEMIS deficiency has opposite consequences on CD4⁺ T cell responses upon TCR stimulation *in vitro* and following immunization with foreign- and self-antigens *in vivo*. *In vitro*, THEMIS deficiency leads to a selective increase of interferon-gamma production in Th1 polarized CD4⁺ T cells. The absence of THEMIS did not affect pro-Th1 signals mediated by IL-12 and IFN γ receptors but results in a specific increase of the phosphorylation of ERK1/2 MAP kinases following TCR stimulation which is responsible for the increased production of Th1 cytokines observed in THEMIS deficient CD4⁺ T cells. *In vivo*, immunization of *themis*-T^{KO} with a foreign antigen leads to decreased production of IFN γ by antigen-specific CD4⁺ T cells associated with a lower expression level of the transcription factor T-bet. Also, susceptibility to the development of Experimental Autoimmune Encephalomyelitis (EAE), the murine model of Multiple Sclerosis, is decreased in *themis*-T^{KO} mice. This is associated with an attenuated encephalitogenic potential of *themis*-T^{KO} CD4⁺ T cells, which express lower levels of the pro-Th1 transcription factor T-bet and of the chemokine receptor CXCR3 and produce reduced amounts of

interferon-gamma. Altogether, these results suggest that THEMIS exerts inhibitory effects on TCR-mediated signaling and CD4⁺ T cell responses *in vitro*, which are overcome *in vivo* by TCR-independent signals that promote normal and neuro-inflammatory Th1 responses.

In conclusion, this thesis work presents insights that may explain the previous discrepancy associated with the function of THEMIS on TCR signaling and identifies a deleterious effect of THEMIS on the regulation of Central Nervous System autoimmunity.

Keywords: THEMIS, T cell antigen receptor, CD4⁺ T cells, TCR signaling, Experimental autoimmune encephalomyelitis

Summary in French

Les lymphocytes T (LT) CD4⁺ (aussi appelés LT helper ou LTh) jouent un rôle central dans le déclenchement et le contrôle des réponses immunitaires adaptatives. Les LT CD4⁺ dits naïfs peuvent se différencier en différentes sous-populations de LT effecteurs ayant des fonctions immunitaires spécifiques, qui contribuent à orchestrer la défense de l'hôte contre diverses classes d'agents pathogènes. Ce processus de différenciation dépend de l'intégration de plusieurs signaux déclenchés par le récepteur d'antigène des LT (TCR), qui reconnaît des peptides du Soi ou étrangers associés aux molécules du complexe majeur d'histocompatibilité (CMH), et par un ensemble de récepteurs aux cytokines plus ou moins stimulés selon les cytokines présentes dans le milieu extracellulaire. En plus de signaux contrôlant la différenciation des LT effecteurs, des signaux supplémentaires sont importants pour contrôler l'amplitude des réponses effectrices des LT et établir un équilibre optimal entre une bonne efficacité des réponses immunitaires et la prévention de réponses potentiellement pathologiques.

THEMIS est une protéine de la lignée des LT qui joue un rôle essentiel lors de leur sélection positive dans le thymus. THEMIS a été identifiée comme une protéine de signalisation du TCR mais sa fonction dans ce contexte est encore controversée. Son rôle dans les LT périphériques, en particulier dans les LT CD4⁺, reste mal caractérisé. L'objectif de cette étude a été d'étudier le rôle de THEMIS dans les réponses normales et pathologiques des lymphocytes T CD4⁺.

En utilisant un modèle murin sélectivement déficient pour THEMIS après l'étape de sélection positive dans le thymus (souris lateCD2-Cre *themis*^{flox/flox}; nommées *themis*-T^{KO}), nous avons observé que le déficit en THEMIS a des conséquences opposées sur les réponses des LT CD4⁺ lors de la stimulation du TCR *in vitro* et après immunisation avec des antigènes étrangers et/ou du Soi *in vivo*. *In vitro*, le déficit en THEMIS entraîne une augmentation sélective de la production d'IFN γ dans les LTh1 due à une augmentation spécifique de la phosphorylation des MAP kinases ERK1/2 suite à la stimulation du TCR. En revanche, l'absence de THEMIS n'a pas d'effet sur les signaux pro-Th1 déclenchés par les récepteurs à l'IL-12 et à l'IFN γ . *In vivo*, la déficience en THEMIS entraîne une diminution de la production d'IFN γ par les LT CD4⁺ spécifiques d'antigènes exogènes associée à des niveaux d'expression plus faible du facteur de transcription pro-Th1 T-bet. En outre, la susceptibilité au développement de l'encéphalomyélite auto-immune expérimentale (EAE), le modèle murin classique de sclérose en plaques, est diminuée chez les souris *themis*-T^{KO}. Les LT CD4⁺ déficients en THEMIS sont

caractérisés par un potentiel encéphalitogénique atténué, de faibles niveaux d'expression du facteur de transcription T-bet et du récepteur CXCR3 ainsi qu'une moindre capacité à produire de l'IFN γ . Dans l'ensemble, ces résultats suggèrent que THEMIS exerce des effets inhibiteurs sur la signalisation du TCR et les réponses des lymphocytes T CD4⁺ *in vitro* ; effets contrebalancés *in vivo* par des signaux indépendants du TCR qui amplifient les réponses Th1 normales et neuro-inflammatoires.

En conclusion, ce travail de thèse apporte un nouvel éclairage sur les fonctions de THEMIS sur la signalisation du TCR et identifie des effets potentiellement pathologiques de THEMIS dans les maladies auto-immunes affectant le système nerveux central.

Mots-clés : THEMIS, Récepteur d'antigène des lymphocytes T, Lymphocytes T CD4⁺, Signalisation du TCR, Encéphalomyélite Auto-immune Expérimentale.

Introduction

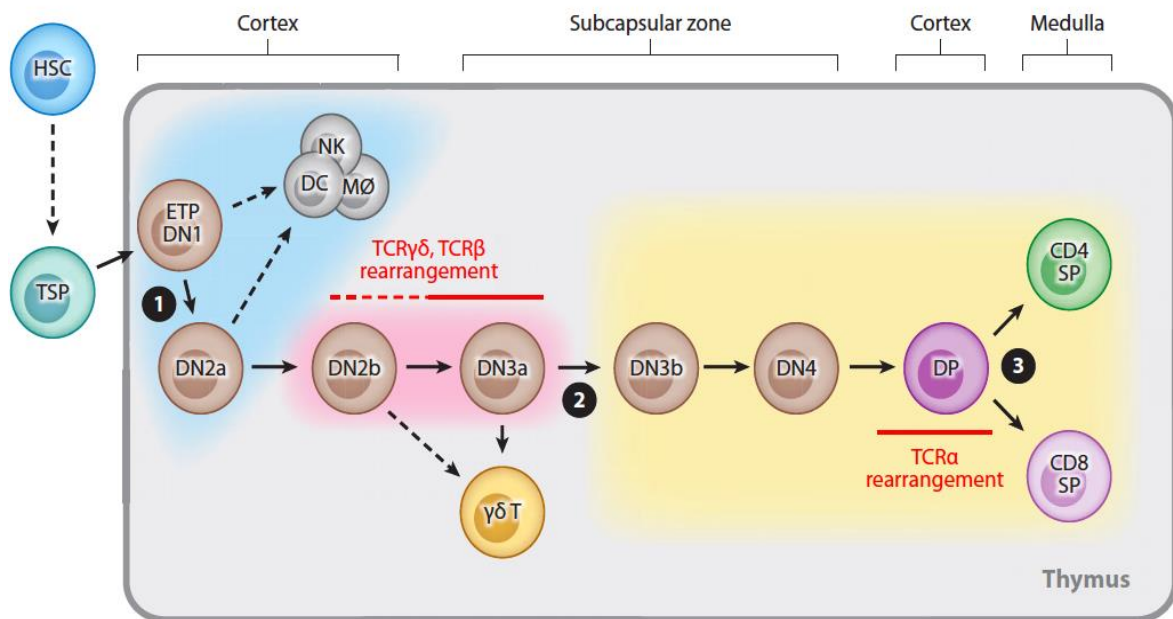


Figure 1. Overview of thymocytes development

The surface expression of CD4 and CD8 distinguishes four main thymocytes subsets which correspond to cell populations at distinct stages of T cell development: the CD4[−]CD8[−] double negative (DN) cells, the CD4⁺CD8⁺ double positive (DP) cells and CD4⁺CD8[−] and CD4⁺CD8⁺ single-positive (SP) cells. Expression of CD44 and CD25 characterizes four major DN cell sub-populations: CD44⁺CD25[−] (DN1), CD44⁺CD25⁺ (DN2), CD44[−]CD25⁺ (DN3), and CD44[−]CD25[−] (DN4). Critical checkpoints during early thymocytes development are indicated by circled numbers in Figure 1. Their key steps are framed in different colors: 1- DN1 checkpoint (blue shading), 2 - the β -selection checkpoint (pink shading), 3 - the positive and negative selection checkpoint (yellow shading). (Koch & Radtke, 2011)

Chapter I: T cell development in the thymus and TCR signaling

T cells play an essential role in the immune system, especially in the adaptive immune responses. Their precursors originate from hematopoietic stem cells (HSCs) in the bone marrow and develop in the thymus, maturing into distinct types of T cells. Some of these T cells stay in the thymus, while the majority migrate to peripheral lymphoid organs, becoming mature immunocompetent T cells which contribute to the immune response or recirculate back into the thymus (Hale & Fink, 2009; Takahama, 2006). T cell development in the thymus is tightly associated with signaling events triggered by the T cell antigen receptor (TCR). In this chapter, we will describe the different stages of T cell development, the main signaling events associated to TCR signaling and the role of the TCR signaling protein THEMIS in T cell development.

1. Stages of T cell development

As shown in **Figure 1**, T-cell progenitors originated from HSCs can migrate through the blood circulation into the thymus *via* the cortical-medullary junctions, acquiring the expression of CD117 and becoming early thymic progenitors (ETPs). Those ETPs are able to develop into natural killer (NK) cells, macrophages, dendritic cells (DC) and T cells in the cortex. The development of T cells in the thymus is characterized by 3 major stages depending on the expression of CD4 and CD8 co-receptors: double negative (DN, CD4-CD8-), double positive (DP, CD4+CD8+) and single positive (SP, CD4+CD8- or CD4-CD8+) cells. DN cells are subdivided into four distinct populations according to the expression level of CD44 and CD25 co-receptors on their surface: DN1 cells (CD44+ CD25-), DN2 cells (CD44+ CD25+), DN3 cells (CD44- CD25+) and DN4 cells (CD44- CD25-) (Godfrey *et al.*, 1993). At the early stage of T cell development, Notch1 receptor signaling allows ETP/DN1 cells to evolve into DN2a, preventing them from differentiating into other cells, which is also called **DN1 checkpoint**. The decreased expression of CD117 on the surface of DN2a cells is associated to the DN2b stage, in which the expression of RAG1 recombinase and pre-TCR α gradually increase along with the cell differentiation into the DN3a cell stage in the subscapular thymic zone (SCZ).

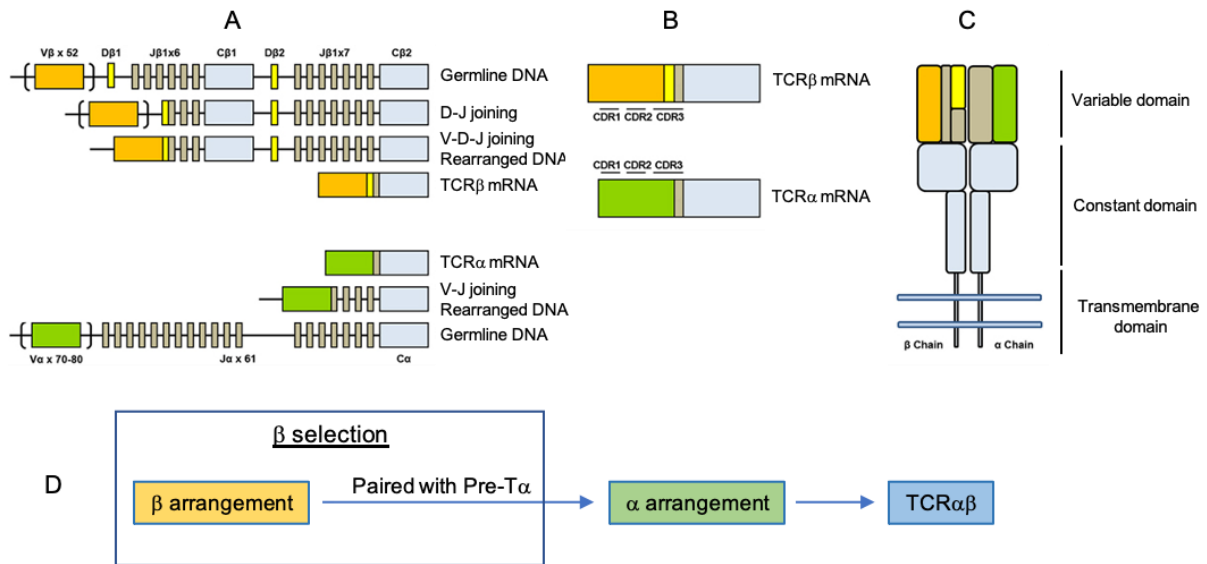


Figure 2. Formation of TCRαβ.

(A) Genomic organization and somatic rearrangement of *TCRβ* and *TCRα* loci. (B) Final α and β TCR subunits. (C) TCR organization. TCR α and TCR β organize in a constant region and a variable region responsible for antigen recognition. (D) Kinetics of TCRαβ rearrangement. (Adapted from Simone D, *et al.*, *Frontiers in immunology*. 2018)

1.1. Rearrangement of T cell receptor (TCR)

T cell responses to antigens are mediated by the T cell antigen receptor (TCR), a surface protein that can specifically recognize antigenic peptides presented by the major histocompatibility complex (MHC) at the surface of antigen-presenting cells. The TCR is a heterodimeric molecule composed of either α and β or γ and δ chains. Each chain comprises a constant and a variable domain, the latter being genetically encoded by a series of fragments on the same genetic locus. The cutting and joining of these gene fragments by RAG recombinases produce a rich variety of unique TCRs, which recognize different peptides and their multiplicity is referred to as the T cell repertoire. The heterodimeric association of the four transmembrane chains α , β , γ and δ enables the formation of TCR $\alpha\beta$ and TCR $\gamma\delta$.

TCR $\alpha\beta$ -expressing T cells represent the vast majority of T cells. They are restricted to the recognition of MHCII molecules for CD4⁺ T cells and of MHCI molecules for CD8⁺ T cells, and migrate toward the secondary lymphoid tissues after their development. By contrast, TCR $\gamma\delta$ -expressing T cells is a small subset of T cells whose ability to recognize antigens is not restricted by MHC molecules and which are mostly detected in intraepithelial tissues. The molecular events leading to the lineage commitment to the two types of T cells have not been fully resolved yet. High expression of the IL-7 receptor (IL-7R) on DN2 cells leads to the development of $\gamma\delta$ thymocytes, while low expression results in further development toward the $\alpha\beta$ cell lineage (Kang *et al.*, 2001). At the stage of DN3, Notch signaling is required for the recombination of the β chain that is able to pair with the α chain later. By contrast, the rearrangement of $\gamma\delta$ chains seems Notch-independent and can be promoted by the ID3 transcription factor (Lauritsen *et al.*, 2009; Wolfer *et al.*, 2002). So far, antigenic peptides recognized by $\gamma\delta$ T cells are mostly unknown and their response to pathogens is not fully understood. The rest of this manuscript will focus on TCR $\alpha\beta$ -expressing T cells.

As mentioned earlier, the variability in TCR specificity is generated through the rearrangement of gene fragments at the α and β loci, which is initiated from the DN2a stage using RAG 1/2 recombinases. Indeed, the diversity of α and β chains mainly depends on the semi-stochastic rearrangement of the V, D and J genes for the β chain and of the V and J genes for the chain α (**Figure 2**). Regarding TCR $\alpha\beta$ rearrangement, the β chain is first rearranged by semi-random gene recombination controlled by RAG1

and RAG2 nucleases which cleave DNA at the level of recombination signal sequence (RSS) on both sides of the D β and J β genes (Schatz *et al.*, 1992). The D β J β fragment then rearranges with the V β fragment at the DN3a stage. The enzyme Terminal deoxynucleotidyl Transferase (TdT) randomly adds nucleotides in the junction between the segments, adding “junctional diversity” to the TCR repertoire (Cabaniols *et al.*, 2001). Meanwhile, T cells begin to express the invariant α -chain, named pre-T α . The successful pairing of the rearranged β chain with the pre-T α results in the expression of a pre-TCR at the end of the DN3a phase. If this pre-TCR is functional, it triggers intracellular signals which lead to a decrease in the expression of RAG recombinases and lead to the allelic exclusion of TCR β to inhibit the formation of other β chains (von Boehmer, 2005). To eliminate the production of non-functional TCR, only cells that have successfully rearranged the β chain and express a functional pre-TCR are allowed to evolve towards the cell stage DN3b. The “failed” cells die through apoptosis. This process is called **β selection**. The engagement of pre-TCR with MHC molecules associated with Self-peptides (Self-pMHC) – on the surface of thymic epithelial cells – allows thymocytes to survive, proliferate and differentiate into DN4 cells. Notch signaling is essential for this process (Ciofani *et al.*, 2004). The DN4 thymocytes then leave the SCZ and acquire the expression of co-receptors CD4 and CD8 in the thymic cortex where they differentiate into DP cells (Porritt *et al.*, 2003). At this stage, the activity of RAG1 and RAG2 nucleases increases again to allow rearrangement of the V α and J α segments to form the TCR α chain, also known as α rearrangement. The DP cells continue to develop with a low expression of TCR $\alpha\beta$ (TCR $\alpha\beta^{\text{low}}$).

1.2. Positive and negative selections

The high variability of TCRs allows T cells to trigger immune responses which are adapted and specific to a broad spectrum of antigens. However, not all the TCRs which are generated in a semi-random manner are able to effectively recognize Self-pMHC. On the other hand, the generated TCRs can recognize Self-pMHC complexes, sometimes with an “excessive” affinity, which can lead to the generation of self-reactive T cells that may induce the development of autoimmune diseases. Therefore, in order to eliminate thymocytes which express ineffective or potentially self-reactive TCRs, positive and negative selection processes are set in the thymic cortex and medulla. **Positive selection (Figure 3)** takes place in the thymic cortex and involves cortical thymic epithelial cells (cTECs) which express

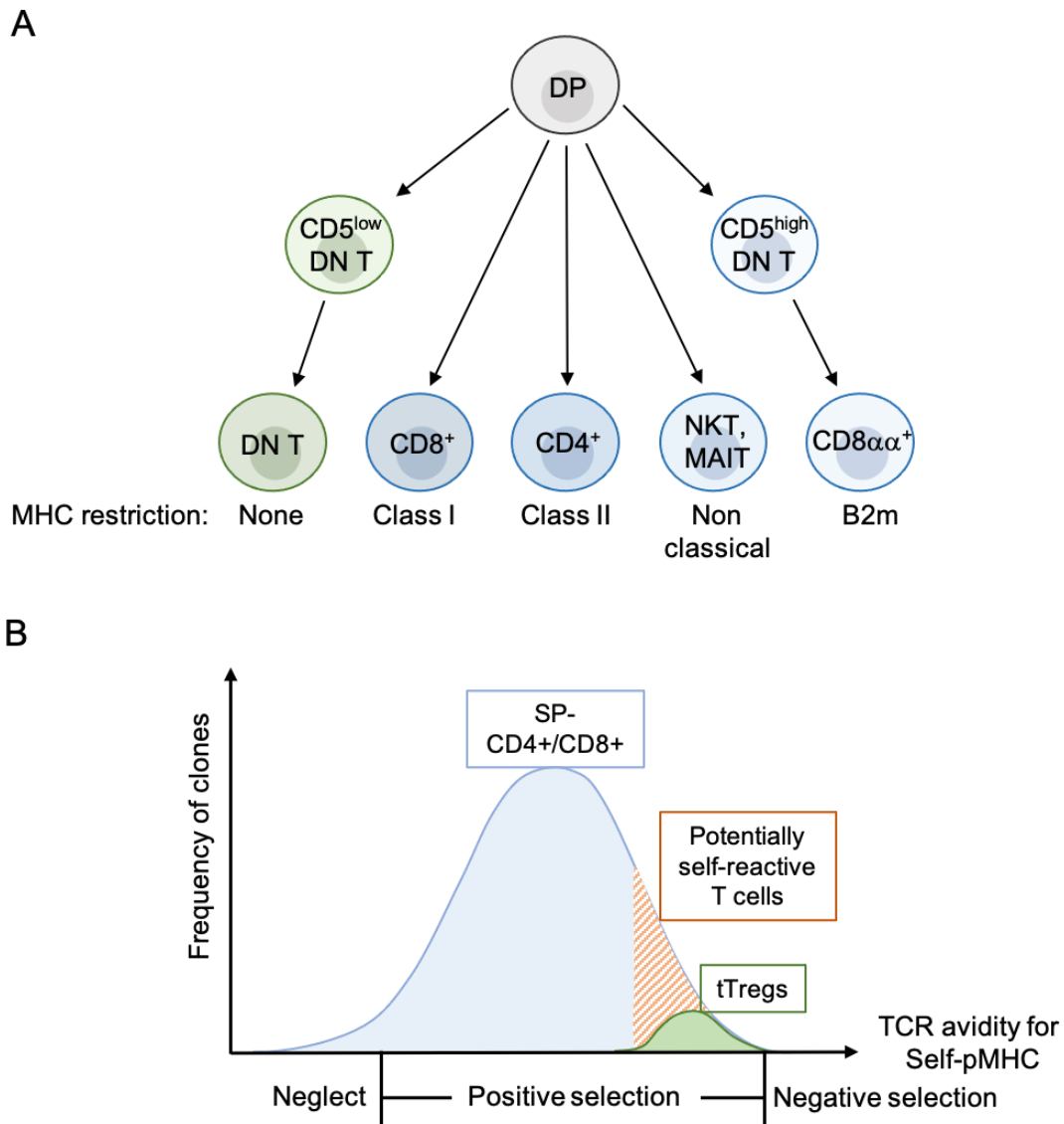


Figure 3. Cell lineage decisions and TCR affinity

(A) T cell lineage commitment. CD4⁺ and CD8⁺ T cells are conventional phenotypes, the rest are unconventional T cells, including MAIT (mucosal-associated invariant T cells); NKT (natural killer T cells); CD8αα⁺ (TCRαβ⁺ CD8αα⁺ intraepithelial lymphocyte). DP: CD4⁺CD8⁺ T cells; DN T: CD4[−]CD8[−] T cells.

(B) Schematic shows the relationship between TCR-pMHC affinity and thymic selection outcomes. High TCR avidity for self-pMHC reflects high TCR signal strength (X axis). Frequency of different T cells after the selection are framed by the indicated curves (Y axis). Intermediate TCR signal strength causes positive selection, while too low leads to neglect, too high results in negative selection. (Adapted from Espinosa E, *et al.*, *Immunologie*, 2006 and Collin R, *et al.*, *The journal of immunology*, 2020)

Self-pMHC that are presented to the TCRs of DP thymocytes. Successful positive selection results in the generation of SP-CD4⁺ cells (recognizing MHC class II) and SP-CD8⁺ cells (recognizing MHC I). Lysosomal cathepsin L and thymic-specific serine protease (TSSP) specifically expressed by cTECs are necessary to generate the spectrum of self-peptides involved in the selection of SP-CD4⁺ thymocytes (Gommeaux *et al.*, 2009; Viret *et al.*, 2011). Regarding SP-CD8⁺ cells, the thymoproteasome subunit $\beta 5t$ is required to generate TCRs recognizing efficiently MHC class I (Murata *et al.*, 2007). As shown in **Figure 3**, the positive selection is associated with TCRs which have a sufficient affinity for self-pMHC complexes (Merkenschlager *et al.*, 1997; Starr *et al.*, 2003). Thymocytes whose TCRs have relatively high affinity for Self-pMHC undergo negative selection, which restricts the susceptibility to develop autoimmune responses.

Negative selection occurs after positive selection. It requires the migration of the cells from the thymic cortex to the medulla (Nitta *et al.*, 2009). The expression of two transcription factors, AIRE (Autoimmune Regulator) and FEZF-2 (Family Zinc Finger 2), expressed by medullar thymic epithelial cells (mTEC) is necessary for the recognition of tissue restricted antigens by thymocytes, hence for negative selection. AIRE or FEZF-2 deficiency in mice leads to the development of severe autoimmunity (Anderson *et al.*, 2002; Takaba *et al.*, 2015). The process of negative selection involves co-stimulatory signals on thymocytes. Indeed, perinatal blockade of CD80/CD86 molecules which are the ligands of the co-stimulatory molecule CD28 expressed by thymocytes leads to a decrease in negative selection (Gao *et al.*, 2002). In addition to positive selection and negative selection, thymocytes whose TCRs cannot respond to self-peptide-MHC complexes are neglected and die by apoptosis.

1.3. CD4 *versus* CD8 lineage commitment

At the end of positive selection, SP-CD4⁺ and SP-CD8⁺ thymocytes express high level of TCR $\alpha\beta$ (TCR $\alpha\beta^{hi}$) and develop into mature SP T cells. It has been reported that the TCR signaling initiated by the recognition of TCR and Self-pMHC plays a major role in T cell commitment. Both strength and duration of the signal are critical for the lineage choice (Gascoigne *et al.*, 2016; Yasutomo *et al.*, 2000). The generation of SP-CD4⁺ T cells is associated with sustained and/or strong TCR signaling (although lower than the negative selection threshold), whereas transient and/or weak TCR signaling (although

higher than the neglect threshold) preferentially leads to the development of SP-CD8⁺ T cells. In addition, ThPOK and Runx3 are two key transcription factors implicated in the full commitment of SP T cells. ThPOK promotes CD4⁺ lineage commitment and blocks the expression of genes associated to the CD8⁺ lineage, whereas Runx3 contributes to the generation SP-CD8⁺ cells (He *et al.*, 2008; Liu *et al.*, 2005; Wang *et al.*, 2008); they repress the expression of each other (Egawa & Littman, 2008). Not all Self-pMHC specific T cells are eliminated in the thymic medulla by negative selection; some of them differentiate into thymic regulatory T cells (tTregs). These cells represent a population enriched in autoreactive thymocytes, which specifically express the transcription factor FoxP3 and are involved in the control of autoimmune responses (Brunkow *et al.*, 2001; Romagnoli *et al.*, 2002). The generation of tTregs requires a strong TCR signaling and is regulated by the stimulation of thymocytes with specific cytokines, including IL-2 and TGFβ (Liu *et al.*, 2008; Moran *et al.*, 2011; Soper *et al.*, 2007). In addition, some DP thymocytes can differentiate into invariant Natural Killer T cells (iNKTs). These cells do not express the co-receptor CD8, some express CD4. They have an invariant TCR with a single type of chain α (Vα14 Jα18) which can be associated with three types of β chain (Vβ8.2, Vβ7, Vβ2) and interacts with a CD1d “MHC I-like” lipid molecule (Arase *et al.*, 1992; Egawa *et al.*, 2005). With the development of further research, some new thymic-derived T cells were found in the thymus and peripheral tissues. TCRαβ+CD8αα+ intraepithelial lymphocytes (IELs) and CD4–CD8–TCRαβ+ thymocytes are two newly characterized unconventional T cells which develop from DN T cells with high or low expression of CD5, transiting through DP stage (Collin *et al.*, 2020; Ruscher *et al.*, 2017). The generation of CD4–CD8–TCRαβ+ T cells is MHC-independent (McDonald *et al.*, 2018). TCRαβ+CD8αα+ IELs can be selected by both classic and non-classic MHC I, as they recognize β2 macroglobulin (β2m), an essential component of these molecules (McDonald *et al.*, 2018; Ruscher *et al.*, 2017).

2. TCR signaling: signal transduction pathways

As mentioned above, the positive selection of thymocytes is dependent on the affinity between the TCR and Self-pMHC which determines the strength and the duration of TCR signaling. The second part of chapter I will describe the main steps of TCR signaling and their implication in thymocytes development.

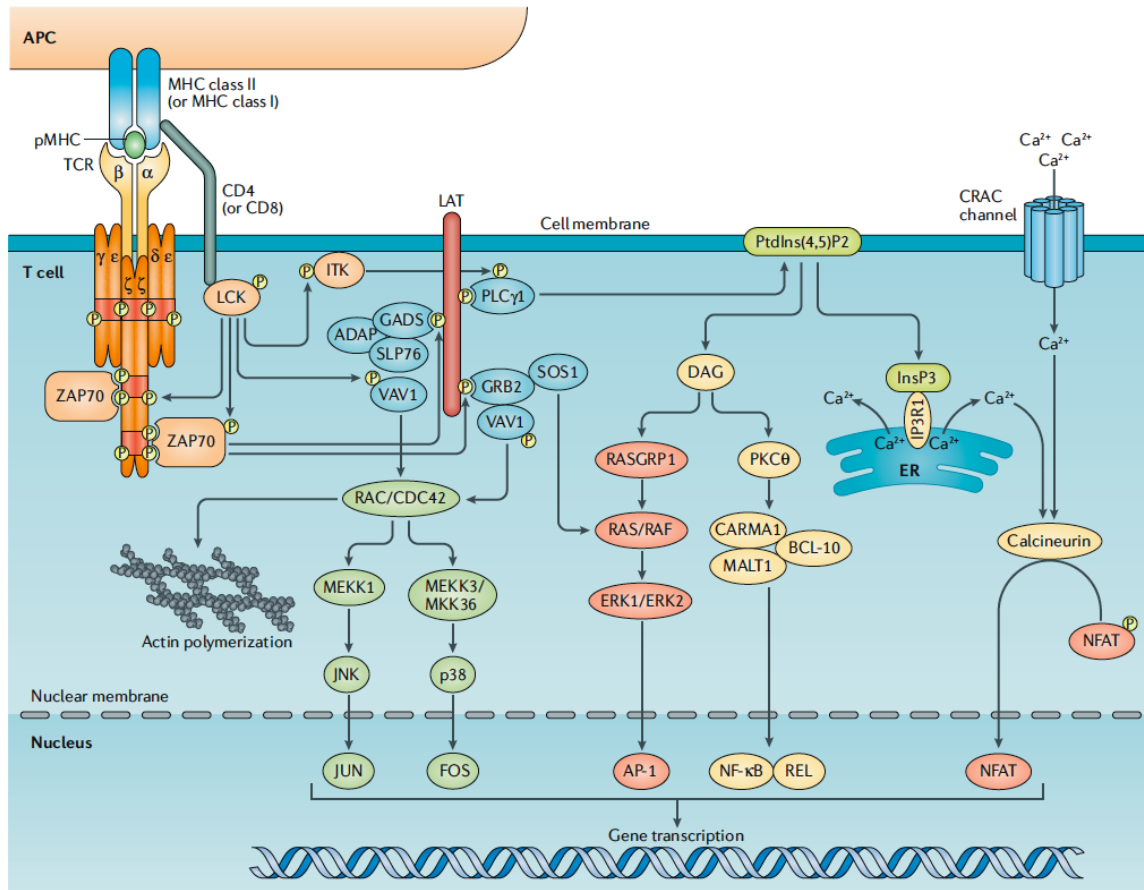


Figure 4. Overview of the major TCR signaling pathways

pMHC recognition by TCR drives the phosphorylation of TCR's ITAM through the kinase Lck, which then recruits and activates the kinase Zap-70. Zap-70 then phosphorylates the transmembrane linker LAT at several tyrosine sites. This leads to different molecules being recruited to LAT either directly or through adaptor proteins, which triggers different signaling pathways to propagate signals in T lymphocytes. (Gaud *et al.*, 2018)

2.1. TCR/CD3 complex

TCR signaling is initiated upon the stimulation of TCR/CD3 complex by peptides presented by MHC molecules (pMHC) and depends on a series of tyrosine kinases (**Figure 4**), adaptor proteins and additional enzymes which operate together to trigger more distal signaling events. In mammals, TCR/CD3 complex, also known as the TCR complex, is composed of TCR α and β chains, CD3 heterodimers $\delta\epsilon$ and $\gamma\epsilon$ and a TCR $\zeta\zeta$ homodimer. To express a functional TCR, the TCR $\alpha\beta$ associates with the CD3 $\delta\epsilon$ and $\gamma\epsilon$ heterodimers, forming a TCR $\alpha\beta$ / CD3 $\delta\epsilon$ / CD3 $\gamma\epsilon$ complex, then combines with TCR $\zeta\zeta$ before being expressed on the surface of T cells (San José *et al.*, 1998). The intracellular tail of each ϵ , γ and δ CD3 chains contains one immunoreceptor tyrosine-based activation motif (ITAM), which consists of two tyrosine residues separated by 6-8 amino acids (YxxLx6-8-YxxL), whereas each TCR ζ chain has three ITAMs (Samelson *et al.*, 1985).

2.2. Formation of proximal TCR signaling

TCR engagement by specific pMHC leads to the phosphorylation of ITAMs by the Src family kinases Lck or Fyn, two important proteins initiating the intracellular signaling events (Samelson *et al.*, 1990; Straus & Weiss, 1992). *In vitro* TCR stimulation of Lck-deficient T cells with an anti-CD3 antibody results in a remarkable reduction in the phosphorylation of many signaling events, including PLC γ 1, PKC, ERK kinases and mobilization of calcium flux (Lovatt *et al.*, 2006). The catalytic activity of Lck is regulated by a phosphorylation balance between the tyrosine Y394, an activating autophosphorylation site, and the tyrosine Y505, an inhibiting phosphorylation site which can be phosphorylated by the tyrosine kinase Csk, leading to the formation of a closed conformation of Lck, and dephosphorylated by phosphatase CD45 (Zikherman *et al.*, 2010). Regarding Fyn, upon the stimulation of anti-CD3 antibody *in vitro*, Fyn-deficient T cells with an anti-CD3 antibody leads to a reduction in the phosphorylation of ERK, an essential tyrosine kinase of distal TCR signaling (Sugie *et al.*, 2004).

After ITAMs phosphorylation by Lck or Fyn, these motifs serve as docking sites for other proteins containing SH2 domains, such as the Syk family enzymes Zap70 and Syk. Recruitment of Zap70 to phosphorylated ITAMs modifies its conformation and enables its phosphorylation on its tyrosine Y493 by Src kinases which enhances its catalytic activity and enables consequently the phosphorylation of the

transmembrane linker for activation of T cells LAT (Chan *et al.*, 1995). In addition, ZAP-70 contains three other phosphorylation sites Y292, Y315 and Y319 that recruit SH2-containing proteins which exert negative and positive regulatory effects on TCR signaling. For example, the recruitment of ubiquitin ligase c-Cbl to p-Y292 can inhibit TCR signaling by degrading signaling proteins (Wang *et al.*, 2001), whereas the recruitment of VAV1 to p-Y315 and Lck to p-Y319 enhances TCR signal (Pelosi *et al.*, 1999). LAT has four tyrosine sites, Y132, Y171, Y191 and Y226, all of which can be phosphorylated by Zap-70, thereby recruiting a variety of molecules to LAT, such as Phospholipase C gamma 1 (PLC γ 1), Growth factor receptor-bound protein 2 (Grb2) and Grb2-related adapter protein 2 (Gads), all of which are important for relaying early TCR-mediated signaling events to downstream pathways (Kuhné *et al.*, 2003). This suggests a central role for LAT in the signaling pathways triggered upon TCR engagement. PLC γ 1 plays a key role in the mobilization of calcium flux in T cells. The recruitment of PLC γ 1 to LAT leads to the hydrolysis of phosphatidylinositol (4,5)-biphosphate PI (4,5) P₂ to inositol 1, 4, 5-triphosphate (IP₃) and diacylglycerol (DAG), thereby activating the downstream signaling components. Gads constitutively interacts with the adapter protein Slp76, allowing its recruitment to LAT and forming a LAT-Gads-Slp76 complex (Liu *et al.*, 2001). This complex creates a platform which further recruits multiple signaling molecules to LAT, such as VAV1, Nck, c-Cbl, Interleukin 2 tyrosine kinase (Itk) and ADAP, transmitting TCR signaling (Wu & Koretzky, 2004).

2.3. Major distal signaling pathways mediated by TCR activation

Calcium flux:

The binding of IP₃ to its receptor (IP₃R) located on the membrane of the endoplasmic reticulum (ER) leads to the mobilization of calcium flux (Nagaleekar *et al.*, 2008). Indeed, following IP₃ binding, ER calcium is released into the cytoplasm and enables the activation of the Ca²⁺ Release-activated Ca²⁺ (CRAC) Channel allowing further entry of Ca²⁺ into the cell. The mobilization of calcium flux leads to the activation of calmodulin kinases and calcineurin. Calcineurin then dephosphorylates multiple phosphoserine residues on the transcription factor NFAT – a key regulator of gene expression in T cell development and function – leading to its activation and nuclear translocation (Macian, 2005). During this process, the LAT-Gads-Slp76 complex stabilizes the interaction of PLC γ 1 on LAT (Zhang *et al.*, 2000). The proteins Itk, RIAM (Rap1 GTP interacting adaptor molecule) and Tespa1 (Thymocyte-expressed positive selection-associated 1) all positively regulate the activation of PLC γ 1 and calcium

pathway (Patsoukis *et al.*, 2009; Schaeffer *et al.*, 2000). DAG is another product of PIP₂ hydrolysis, which functions as a lipid second messenger. It activates Ras guanyl nucleotide-releasing protein (RasGRP) and protein kinase C θ (PKC - θ), two initiators of the Ras/MAPK/ERK cascade and of the NF- κ B pathway, respectively (Ebinu *et al.*, 1998; Lin *et al.*, 2000).

MAP Kinases pathway:

The kinases ERK (extracellular signal-regulated protein kinase) belong to the class of mitogen-activated protein kinases (MAPK). They are associated with cytokines production, cell proliferation and T cell development through activation of several transcription factors such as AP1, which is highly expressed during cell division and can form a complex with NFAT to regulate the expression of various cytokines including IL-2, IL-4 and IFN γ (Angel & Karin, 1991; Macián *et al.*, 2001). The activation of an optimum ERK cascade is dependent mostly on the protein RasGRP (RAS guanyl nucleotide-releasing protein) and to a lesser extent on the Grb2-SOS complex which is recruited to LAT after the engagement of TCR by pMHC (Roose *et al.*, 2007). p38 kinases and c-Jun N terminal kinase (JNK) are two other MAPK. They can both be activated by the LAT-recruited protein VAV1 through regulating Ras-related GTP-binding proteins, such as Rac, Rho and Cdc42 (Kaminuma *et al.*, 2001; Salojin *et al.*, 1999). Phosphorylation of p38 and JNK leads to the activation of many transcription factors such as c-Jun, p53 and Fos, regulating the cell growth, differentiation, survival and apoptosis. Besides, VAV1-mediated activation of Ras-related GTP-binding proteins is also associated with the assembly of the actin cytoskeleton (Tapon & Hall, 1997).

PKC/NF κ B pathway:

Upon its activation by DAG, PKC θ is recruited near the plasma membrane where it phosphorylates the adapter protein CARD11 (CARMA1), which then forms a complex with the proteins Bcl10 and MALT1. This complex recruits TRAF2 and TRAF6, which then activates I κ B kinases (IKK), leading to the degradation of I κ B α and releasing the transcription factor NF κ B (Rosebeck *et al.*, 2011; Schulze-Luehrmann & Ghosh, 2006). In addition, the engagement of TNFR superfamily receptors, such as 4-1BB and OX40, stabilizes the NF κ B-inducing kinase (NIK), which can phosphorylate the inhibitor of NF- κ B kinase α (IKK α) and NF κ B2 precursor protein p100, thereby mediating activation of the

p52/RelB NFκB complex (Sun, 2012). As a central mediator of cytokines expression, NFκB can enter the nucleus and induce the expression of genes harboring specific NFκB DNA-binding sites.

PI3K/AKT/mTOR pathway:

The PI3K/AKT/mTOR pathway is important for the regulation of many T cells responses. It's activated by the recruitment to the plasma membrane of phosphoinositide 3-kinases (PI3K), a family of enzymes expressed by T cells, which allows the phosphorylation of membrane Pi (4,5) P2 to Pi (3,4,5) P3. The natural inhibitor of this process is PTEN (Machama & Dixon, 1998). mTOR is a member of PI3K family, which functions as the core component of mTORC1 and mTORC2 complexes, phosphorylating serines, threonines and tyrosines of many signaling proteins (Hay & Sonenberg, 2004).). PIP3 can activate and bind to proteins that contain pleckstrin homology domains (PHD), such as phosphoinositide-dependent kinase 1 (PDK1). Binding of PDK1 to PIP3, leading to the phosphorylation of AKT at the threonine 308 residue (T308). This triggers the phosphorylation of AKT at serine 473 (S473) by mTORC2 complex (Currie *et al.*, 1999; Franke *et al.*, 1995), which upregulates AKT serine/threonine kinase activity, enabling Akt to phosphorylate many downstream substrates in order to regulate cellular processes, such as survival and metabolism (Alessi *et al.*, 1996; Sarbassov *et al.*, 2005). Akt can further serves as an activator of many downstream molecules, regulating different cell processes. For example, it upregulates cell survival by phosphorylating FOXO1 – a positive regulator of apoptosis – which inhibit its translocation to the nucleus (Zhang *et al.*, 2011). It also promotes the expression of the cAMP response element (CREB), an important transcription factor related to proliferation (Kops *et al.*, 1999; Peltier *et al.*, 2007).

In addition to the major signaling proteins described above, TCR signaling is regulated by many other factors, such as phosphatases, co-receptors and microRNA. Previous studies have shown that the participation of tyrosine phosphatases SHP-1 can dephosphorylate several proximal TCR signaling components, including Zap-70 and Lck, leading to the down-regulation of TCR signal (Kosugi *et al.*, 2001). The engagement of stimulatory co-receptor CD28 and inhibitory co-receptor CTLA-4 enhances and limits the strength of TCR signaling, respectively (Holt *et al.*, 2017). The miR-181a dynamically regulates TCR signal strength, as its expression in T cells leads to reduced activity of multiple

phosphatases (Grewers & Krueger, 2020). Amongst these many proteins involved in TCR signaling, THEMIS (Thymocytes-Expressed Molecule Involved in Selection) was identified in 2009 as necessary for the development of T cells in the thymus. Being the main subject of my research work, the third part of this chapter will focus on how THEMIS regulates thymocytes development through TCR signaling.

3. Function and molecular roles of THEMIS in T cells development

3.1. Discovery and classification of THEMIS

THEMIS, also known as THEMIS1, is a protein initially found in murine thymus in 2009 (GenBank:E430004N04Rik). It was named “Thymocyte-Expressed Molecule Involved in Selection” following independent studies of five research teams headed by Paul Love (Lesourne *et al.*, 2009), Richard Cornall and Ron Schwartz (Johnson *et al.*, 2009), Nicholas Gascoigne (Fu *et al.*, 2009), Hiroshi Kawamoto and Hisahiro Yoshida (Kakugawa *et al.*, 2009) and Harumi Suzukia (Patrick *et al.*, 2009).

Orthologous *themis* genes are found in mammals (*Mus musculus*, *Homo sapiens*), birds (*Gallus gallus*) and bony fish (*Danio rerio*) (Fu *et al.*, 2009). THEMIS family gathers structurally related proteins, containing a specific and conserved globular domain named CABIT, either singular or in tandem copies (Johnson *et al.*, 2009). In mammals, THEMIS family counts five members. The first two, GAREM1 and GAREM2, ubiquitous and brain specific respectively, bear a single CABIT-module and have been characterized as adaptor proteins involved in EGFR signaling (Taniguchi *et al.*, 2013; Tashiro *et al.*, 2009). The other three homologs are THEMIS1, expressed in T cells, NKT cells and mast cells, THEMIS2 which is expressed in B cells, dendritic cells and macrophages and THEMIS3, which is expressed in the intestine (Fu *et al.*, 2009; Johnson *et al.*, 2009; Lesourne *et al.*, 2009).

As shown in **Figure 5**, THEMIS1, THEMIS2 and THEMIS3 contain two tandem copies of CABIT domain, a proline-rich region (PRS) and a bipartite nuclear localization sequence (NLS), which are all required for the function of THEMIS in thymocytes (Johnson *et al.*, 2009; Kakugawa *et al.*, 2009; Okada *et al.*, 2014). THEMIS lacks a consensus catalytic domain but there is a conserved cysteine residue in each CABIT domain, which suggests that THEMIS may possess some catalytic activity (Johnson *et al.*, 2009). In thymocytes, THEMIS is mainly localized in the cytoplasm but is still detected in the nucleus, which suggests its function in both cellular compartments (Lesourne *et al.*, 2009).

3.2. Critical role of THEMIS in T cells development

Murine THEMIS is highly expressed in thymus and less in lymph nodes and spleen, but it is not detected in any other organs or tissues. In thymocytes, THEMIS has the highest expression level in CD4⁺CD8⁺DP thymocytes, then is downregulated as thymocytes transition to the CD4⁺ or CD8⁺SP stage (Fu *et al.*, 2009; Lesourne *et al.*, 2009). Studies on *themis* knockout mouse models (*themis*^{-/-}) expressing different types of TCR transgene (MHC class II-restricted AND TCR transgene, MHC class I-restricted H-Y TCR transgene, MHC class I-restricted OT-I) showed that THEMIS is essential for efficient positive and negative selections during T cell development (Fu *et al.*, 2009; Lesourne *et al.*, 2009). In the absence of THEMIS, thymocytes development is blocked at the DP stage, which leads to a drastic reduction in CD4⁺ and CD8⁺ SP thymocytes and in peripheral T cells (Fu *et al.*, 2009; Johnson *et al.*, 2009; Lesourne *et al.*, 2009). Since thymocytes selection is dependent on TCR signals (Hogquist & Jameson, 2014), those results suggest that THEMIS is involved in TCR signaling. Moreover, in *themis*^{-/-} mice, CD4⁺ SP thymocytes development is more severely impacted than CD8⁺ SP thymocytes development (Fu *et al.*, 2009; Johnson *et al.*, 2009; Kakugawa *et al.*, 2009; Lesourne *et al.*, 2009; Patrick *et al.*, 2009). Data from the literature show that the development of CD4⁺ SP depends on persistent TCR signal transduction while the development of CD8⁺ SP depends on transient TCR signals combined with IL-7 mediated survival signals (Singer *et al.*, 2008).

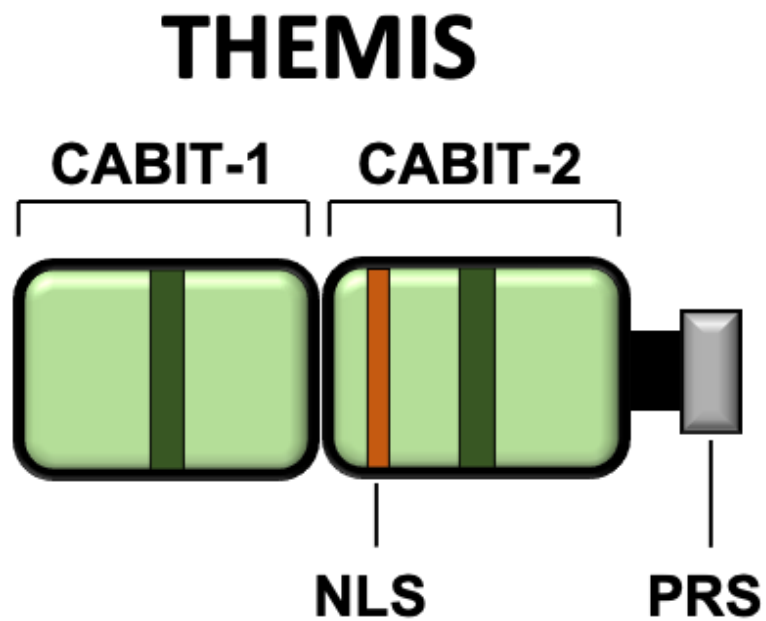


Figure 5. Domain architectures of THEMIS

THEMIS contains two globular CABIT domains (CABIT-1 and CABIT-2; Light green), a proline rich region (PRS, Gray), a nuclear localization sequence (NLS, Orange) and two conserved cysteine motifs (Dark green bars). (Adapted from Andy L Johnson, *et al.*, *Nature immunology*, 2009)

Analysis of T cell development in MHC class I-deficient $\beta 2m^{-/-}$ mice shows that part of the MHC class II restricted thymocytes are ‘redirected’ from the CD4 lineage to the CD8 lineage in the absence of THEMIS (Lesourne *et al.*, 2009), suggesting that THEMIS could be important to promote or sustain TCR signals following TCR engagement. However, initial studies on THEMIS failed to detect significant defect in TCR signaling in *themis*-deficient thymocytes following TCR stimulation *in vitro* (Johnson *et al.*, 2009; Patrick *et al.*, 2009). Impaired TCR signaling was however suspected given the reduced expression of the signaling sensor CD5 at the surface of CD4⁺CD8^{int} thymocytes (Fu *et al.*, 2009), raising questions about the molecular mechanisms by which THEMIS operates during thymocytes development.

3.3. THEMIS is involved in TCR signaling cascade

THEMIS binds to the ubiquitous cytosolic adaptor Grb2 by its PRS (Johnson *et al.*, 2009; Lesourne *et al.*, 2009; Lesourne *et al.*, 2012; Paster *et al.*, 2013; Patrick *et al.*, 2009). After TCR stimulation, THEMIS is rapidly phosphorylated by Lck and ZAP-70 (Brockmeyer *et al.*, 2011; Fu *et al.*, 2009), and recruited to LAT by Grb2 (Brockmeyer *et al.*, 2011; Lesourne *et al.*, 2012; Paster *et al.*, 2013). Grb2 expression is necessary to maintain the stability of THEMIS (Garreau *et al.*, 2017). Previous results obtained in our team indicate that THEMIS expression is reduced in Grb2^{+/-} thymocytes compared to wild type cells (Garreau *et al.*, 2017). This study also shows that the N-terminal CABIT domain of THEMIS directly binds to the ubiquitin-specific protease USP9X, which de-ubiquitinates THEMIS ubiquitin K48 chains after TCR engagement (Garreau *et al.*, 2017). Grb2 allows the recruitment of THEMIS/USP9X complexes to LAT, sustaining THEMIS expression in the process of positive selection, which indicates that THEMIS-Grb2 interaction is required for the function of THEMIS *in vivo* (Garreau *et al.*, 2017; Okada *et al.*, 2014; Paster *et al.*, 2013; Zvezdova *et al.*, 2014). THEMIS-Grb2 complex also bridges THEMIS to the phosphatase SHP-1, an inhibitory tyrosine phosphatase that dephosphorylates and inactivates several key molecules in the TCR signaling pathway including the tyrosine kinases Lck and ZAP-70 and the guanine nucleotide exchange factor VAV1 (Lorenz, 2009; Pao *et al.*, 2007; Stebbins *et al.*, 2003). THEMIS and SHP-1 separately interact with N-terminal and C-terminal SH3 domains of Grb2 (Lesourne *et al.*, 2012; Okada *et al.*, 2014; Paster *et al.*, 2013; Paster *et al.*, 2015), which suggests a tripartite complex involving THEMIS, Grb2 and SHP-1 (Paster *et al.*, 2015).

However, a recent publication shows that THEMIS directly interacts with the phosphatase SHP-1 through binding to SHP-1 PTP domain by its CABIT modules. This interaction is enhanced by Grb2 but is not Grb2 dependent (Choi, Warzecha, *et al.*, 2017). THEMIS can be co-immunoprecipitated with SHP-1 in Grb2^{-/-} thymocytes and this co-immunoprecipitation also occurs in the absence of the THEMIS PRS region (Choi, Warzecha, *et al.*, 2017). In *themis*^{-/-} thymocytes, tyrosine phosphorylation of SHP-1 is markedly diminished with or without TCR stimulation (Fu *et al.*, 2013; Zvezdova *et al.*, 2016), which suggests an important role for the THEMIS-SHP-1 interaction in thymocytes development.

Other studies show that THEMIS also co-immunoprecipitates with the phosphatase SHP-2 in Jurkat T cells and in transfected HEK293 cells, suggesting an interaction between THEMIS and this regulator – positive and negative – of TCR signaling (L. Chen & D. B. Flies, 2013; Fu *et al.*, 2013; Lorenz, 2009; Pao *et al.*, 2007; Paster *et al.*, 2015; Stanford *et al.*, 2012).

3.4. Two models of THEMIS function in T cells development

3.4.1. Model I: THEMIS suppresses TCR signaling in thymocytes to prevent crossing the threshold for negative selection

While initially studying THEMIS function in thymocytes development, Paul Love and collaborators found that the expression of CD5, IL-7 R and CD69 proteins, which are correlated with the intensity of TCR signals at the transition from DP to SP, was reduced in the CD4⁺CD8^{int} *themis*^{-/-} mice compared to *themis*^{+/+} mice (Lesourne *et al.*, 2009). In parallel, Nicholas Gascoigne's research group found that calcium flux and ERK activation were mildly decreased in *themis*^{-/-} thymocytes after TCR stimulation (Fu *et al.*, 2009). They later reported that there is a reduction of ERK phosphorylation, IL-2 production and NFAT/AP1 activation in response to TCR stimulation in *themis*-knockdown Jurkat T cells (Brockmeyer *et al.*, 2011; Gascoigne & Palmer, 2011). Altogether, these initial studies lead to identify THEMIS as a positive regulator of TCR signaling (Brockmeyer *et al.*, 2011; Gascoigne & Palmer, 2011; Lesourne *et al.*, 2009).

However, a new study by Nicholas Gascoigne's research team brought this group to new and nearly opposite conclusions about the function of THEMIS on TCR signaling. In a *themis*^{-/-} OT-I TCR transgenic mouse model, the authors engaged OT-I DP thymocytes with pMHC ligands of various affinity and found that THEMIS negatively regulate TCR signals when relatively low affinity ligands were used (Fu *et al.*, 2013). Low affinity tetramer/peptide ligands induced stronger phosphorylation of Lck, PLC- γ 1, LAT and ERK as well as calcium flux in *themis*^{-/-} thymocytes or in *themis*-knockdown Jurkat T cells than in wild-type controls (Fu *et al.*, 2013; Paster *et al.*, 2015). Moreover, in their studies, the absence of THEMIS increased activated caspase-3⁺ cells, a readout for apoptosis (Salvesen, 2002), and markedly decreased SHP-1 phosphorylation (Fu *et al.*, 2013; Paster *et al.*, 2015). When they crossed *themis*^{-/-} mice with mice knocked out for pro-apoptotic factor Bim (*Bim*^{-/-}), the proportions of CD4-SP and CD8-SP thymocytes were restored in the double knockout mice compared to *Bim*^{+/+}*themis*^{-/-} mice (Fu *et al.*, 2013). However, the proportions of SP cells in *Bim*^{-/-}*themis*^{-/-} mice remained decreased in comparison to that in control *Bim*^{-/-}*themis*^{+/+} mice, suggesting that the block in T cell development persists even in the absence of Bim. In addition, authors did not show whether BIM deficiency could restore normal numbers of peripheral CD4⁺ and CD8⁺ T cells. SHP-1 is a negative regulator of several signaling proteins, such as Lck, Zap70 and VAV1 (Lorenz, 2009; Stebbins *et al.*, 2003; Stefanová *et al.*, 2003). They hence proposed a model in which THEMIS attenuates TCR signaling by promoting SHP-1 recruitment to LAT or enhancing SHP-1 activity in response to low affinity peptide ligands stimulation (Fu *et al.*, 2013; Paster *et al.*, 2015), enhancing positive selection by reducing TCR signals below the threshold of negative selection (Fu *et al.*, 2013; Gascoigne & Acuto, 2015). Although the effect of THEMIS on SHP-1 activity and recruitment to LAT was not directly address in this study, this hypothesis is referred to as **model I** hereafter (**Figure 6**).

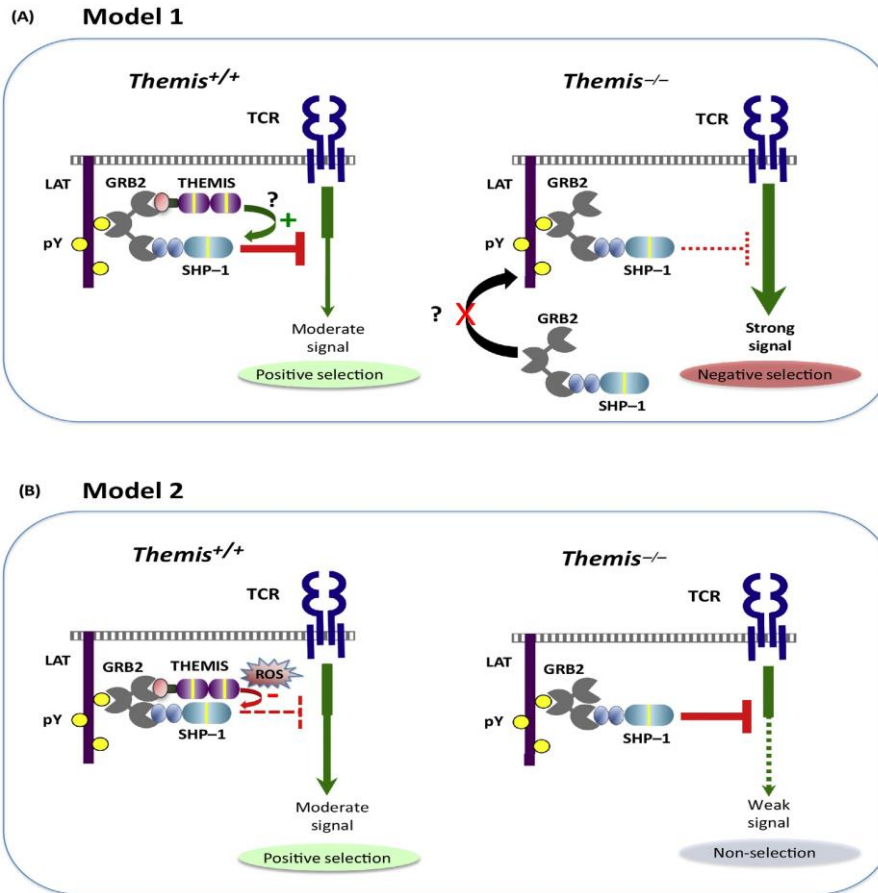


Figure 6. Controversial models: how THEMIS promotes the positive selection of thymocytes through the regulation of TCR signaling

(A) Model I. Left: In *themis*^{+/+} thymocytes, THEMIS enhances the recruitment of SHP-1 to LAT and/or positively regulates phosphatase activity of SHP-1 to attenuate TCR signal strength that are initiated by low-affinity TCR-self p-MHC interactions to be lower than the threshold for negative selection. Right: In *themis*^{-/-} thymocytes, SHP-1 activity or the recruitment of SHP-1 to LAT is reduced, leading to increased TCR signals and resulting in negative selection. (B) Model II. Left: In *themis*^{+/+} thymocytes, THEMIS promotes or stabilizes oxidation of SHP-1 catalytic cysteine in presence of ROS, thereby inhibiting SHP-1 phosphatase activity after TCR stimulation with low-affinity self-pMHC and generating signals sufficient for positive selection. Right: In *themis*^{-/-} thymocytes: SHP-1 impairs the signal strength to be lower than the required for positive selection, resulting in cell death by neglect.(Choi, Cornall, *et al.*, 2017)

3.4.2. Model II: THEMIS facilitates T cell development by enhancing TCR signaling above the threshold required for positive selection.

Model II is consistent with the initial conclusion that THEMIS positively regulates TCR signaling in thymocytes (Brockmeyer *et al.*, 2011; Fu *et al.*, 2009; Gascoigne & Palmer, 2011; Lesourne *et al.*, 2009). It is based on further results obtained by Renaud Lesourne and Paul Love's research groups. In 2017, they first crossed *themis*^{-/-} mice expressing the AND TCR with Nur77–green fluorescent protein (GFP) transgenic mouse model in which GFP abundance correlates with the intensity of TCR signals transmitted during positive or negative selection (Zvezdova *et al.*, 2016). They found that GFP expression was reduced in activated *themis*^{-/-} DP thymocytes, suggesting a positive function for THEMIS on TCR signaling. They also showed in this study that BIM deficiency does not restore normal numbers of peripheral T cells in *themis*^{-/-} mice, suggesting that the block of T cell development in *themis*^{-/-} mice is not the consequence of apoptosis due to stronger TCR signals (Zvezdova *et al.*, 2016). Analysis of THEMIS interactome in thymocytes by mass spectrometry confirmed that THEMIS interacts with SHP-1, SHP-2, Grb2 and LAT but also indicates that THEMIS strongly interacts with the Rac-GTP exchange factor VAV1 following thymocytes stimulation with pervanadate (**Figure 7**). Analysis of TCR signaling upon weak or strong stimulations with anti-CD3 antibodies showed that the phosphorylation of ERK is increased in the absence of THEMIS upon weak anti-CD3 stimulation, confirming the results from Nicholas's Gascoigne group in a different experimental setting. However, the absence of THEMIS led to a strong decrease of VAV1 phosphorylation and of the production of Rac-1 GTP independently of the strength of the TCR stimuli (Zvezdova *et al.*, 2016). Reduced phosphorylation of VAV1 was also observed *ex-vivo* in THEMIS deficient DP thymocytes expressing the AND TCR. Altogether, those results suggested that THEMIS could exert both positive and negative effects on TCR signals according to the type of signaling pathways. However, the positive effects of THEMIS seem to overcome its negative effects in T cells undergoing development.

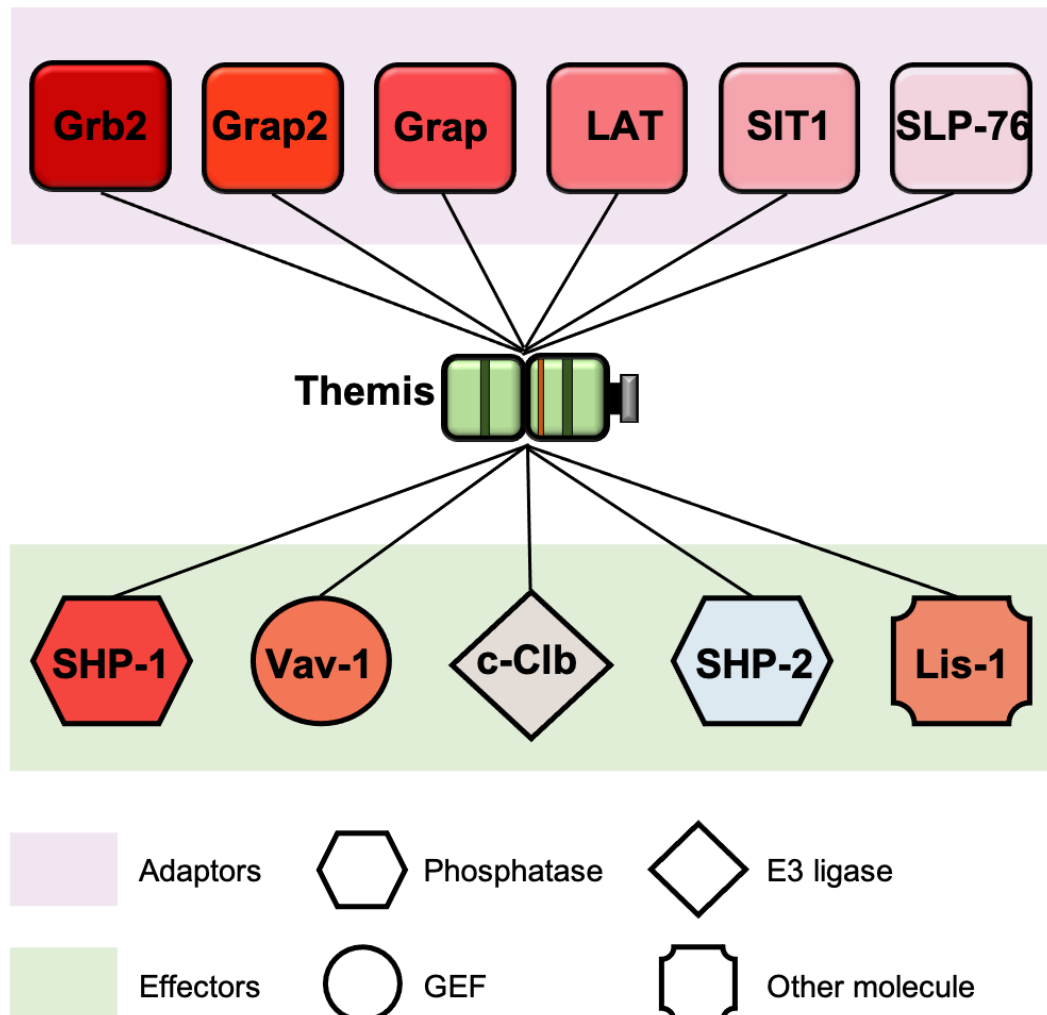


Figure 7. Schematic of 11 proteins that preferentially interact with THEMIS in pervanadate-stimulated thymocytes

Purple layout: adaptors, Green layout: effectors (different shapes stand for different protein functions). Shades of colour indicate the frequency of interaction with THEMIS (from blue = scarce, to red = abundant). Grb2 is the protein which interacts most extensively with THEMIS, while c-Cbl and SHP-2 have comparable lower interaction with THEMIS. (Adapted from Zvezdova E, *et al.*, *Science signaling*, 2016)

A later key study by the group of Paul Love demonstrated that THEMIS directly binds to the phosphatase domain of SHP-1 through its CABIT domains and inhibits SHP-1 PTP activity both *in vitro* and *in vivo* by promoting or sustaining the oxidization of SHP-1 catalytic cysteine (Choi, Warzecha, *et al.*, 2017). The phosphorylation of some of SHP-1 targets, such as LCK and ZAP-70, was decreased in *themis*^{-/-} thymocytes following TCR stimulation in the presence of ROS (Choi, Warzecha, *et al.*, 2017). Moreover, the tyrosine phosphorylation of both SHP-1 and SYK, one of its substrates, were increased in the cell lines co-transfected with these two molecules and THEMIS, suggesting that THEMIS inhibits the dephosphorylate function of SHP-1 on pSYK, and reflecting that the phosphorylation level of SHP-1 is not equal to its PTP activity (18). THEMIS also constitutively interacts with SHP-2 and represses its activity (Fu *et al.*, 2013; Paster *et al.*, 2015), although to a lesser extent than as for SHP-1. The role of SHP-2 on TCR signaling is complex since it has been ascribed with both stimulatory and inhibitory function according to the context of stimulation. Studies using cell lines expressing dominant-negative forms of SHP-2 or SHP-2 deficient mice show that SHP-2 is required to promote ERK activation as well as IL-2 production and CD25 and CD69 expression upon TCR stimulation (Lo *et al.*, 2012). Taken together, these findings raise the possibility that THEMIS could have distinct effect on TCR signaling according to the type of phosphatases with which it interacts, enhancing the activity of signaling proteins such as VAV1, Lck and ZAP-70 by blocking the inhibitory activity of SHP-1, and repressing the activity of ERK by blocking the stimulatory action of SHP-2 (Choi, Warzecha, *et al.*, 2017). It remains unclear whether this dual effect is biologically relevant in the context of T cell development or whether the enhancing role of THEMIS through SHP-1 is physiologically predominant *in vivo*. THEMIS is highly expressed in DP cells together with molecules which have been characterized as positive regulators of TCR signaling (Tesp1, mir181a and Scn4b), supporting the idea that THEMIS operates mainly as positive regulator TCR signaling at this stage of T cell development and functions in coordination with other regulators which enable thymocytes to reach the threshold for positive selection (Li *et al.*, 2007; Lo *et al.*, 2012; Mehta *et al.*, 2018; Nguyen *et al.*, 2006).

In addition, it has been shown that THEMIS2 positively regulates BCR signaling in response to low-avidity antigens during B-cell selection, which thereby enhances the positive selection of B1 cells and germinal center B cells (Cheng *et al.*, 2017). THEMIS and THEMIS2 are functionally exchangeable, since they have highly conserved molecular features (Lesourne *et al.*, 2012). Indeed, it has been shown that overexpression of THEMIS2 restored the thymocytes development in *themis*^{-/-} mice and that THEMIS2 could be phosphorylated and recruited to LAT following TCR engagement (Lesourne *et al.*, 2012). Altogether, a new model about how THEMIS regulates T cell development was proposed, referred here as Model II (**Figure 6**). In Model II, THEMIS enhances TCR signaling by blocking SHP-1 PTP activity, thereby promoting the signal strength beyond the threshold for positive selection (Choi, Warzecha, *et al.*, 2017).

To conclude on THEMIS function in T cell development, it is clear that THEMIS is essential for T cell development and the fact that THEMIS operates by blocking the catalytic function of SHP phosphatases seems also well established. Although the phenotype of *themis*^{-/-} mice clearly indicates positive function of THEMIS on TCR signaling, the relevance of its inhibitory effect on TCR-mediated activation events in thymocytes activation remains to be defined.

Chapter II Influence of TCR signaling on T cell responses in peripheral lymphoid organs

After leaving the thymus, T cells circulate through the lymphatic system in a resting state until they encounter antigens for which they are specific in secondary lymphoid organs. In order to be fully activated, T cells require three type of signals: TCR signals triggered by pMHC, co-stimulatory signals triggered by the interaction of co-receptors with their ligands and cytokine-driven signals (**Figure 8**).

The TCR of CD4⁺ and CD8⁺ T cells recognize their specific antigen by binding to the peptide-MHC (pMHC) complex displayed by APC, triggering the initial activation of T cells (signal 1). T cells full activation also requires the engagement of their stimulatory co-receptors, such as CD28 which binds to the costimulatory molecules B7-1 (CD80) and B7-2 (CD86) expressed on activated APCs (signal 2). The intracellular signaling pathways induced by the engagement of stimulatory co-receptors function together with signals triggered by antigen recognition to initiate T cells proliferation. These signals induce characteristic changes in the expression of various surface molecules in T cells, such as increased expression of CD69 (directs T cells to secondary lymphoid organs), CD25 (high affinity IL-2 receptor), CD44 (retains effector T cells at the sites of infection and inflammation), CTLA-4 (a negative regulator of T cell response). Activated T cells also increase expression of molecules that induce their migration to the inflammation sites, such as LFA-1 and VLA-4, and reduce the expression of molecules that retain them into lymphoid organs, such as CD62L and CCR7. In addition, the stimulation of T cells by cytokines, such as IL-2, stimulates the growth, survival and proliferation of activated T cells (signal 3). After several cycles of proliferation, under the control of different cytokines, activated T cells differentiate into different subpopulations, generating effector and memory immune response.

Four major subsets of CD4 effector T cells, also named helper T cells (Th), can be distinguished: Th1, Th2, Th17 and Tfh. They take part in host defense against different types of infections and diseases. Regulatory T cells (Tregs) constitute a distinct CD4⁺ T cell population which can inhibit responses to self- and foreign- antigens mediated by effector T cells.

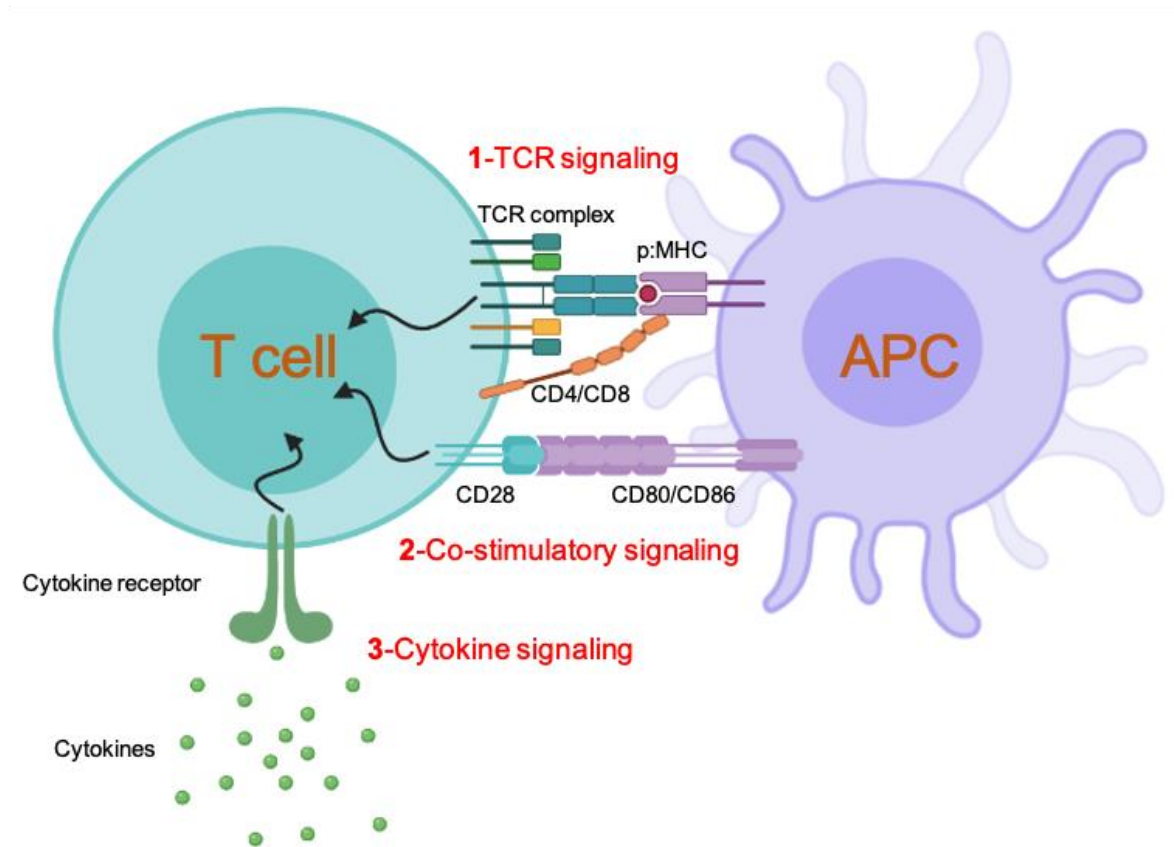


Figure 8. Schematic T cell activation

In order to eliminate pathogens, activated CD4⁺ and CD8⁺ T cells migrate to the site of inflammation caused by infection or autoimmune disorders, express surface molecules and secrete cytokines that activate other immune cells, such as B lymphocytes, macrophages and dendritic cells. Meanwhile, the release of cytokines, chemokines and other inflammatory molecules by activated T cells and by other immune cells induce further activation and proliferation of the T cells.

TCR signaling mediated by TCR-pMHC specific recognition as well as signals triggered by co-receptors and cytokine receptors influence the outcomes of T cell responses. The following part of this manuscript will focus on describing the mechanism by which TCR signals influence the activation and the differentiation of T cells into effector cells in the context of normal and autoimmune responses.

1. Role of the different T cell subsets during immune responses

1.1. CD4⁺ T cells subsets

According to the strength of TCR stimulation and the presence of specific cytokines secreted by the APC in the extracellular milieu, the activated CD4⁺ T cells may differentiate into distinct effector subtypes which are characterized by their ability to secrete a specific set of cytokines (**Figure 9**).

1.1.1. T helper 1 (Th1) cells

Th1 cells predominantly produce IFN γ in response to intracellular pathogens such as viruses, some bacteria, some parasites and protein antigens administrated with strong adjuvants (Cher & Mosmann, 1987). T-bet, a member of the T-box family, is essential for the polarization and maintenance of Th1 cells. T-bet^{-/-} CD4⁺ T cells stimulated with anti-CD3/anti-CD28 antibodies and naïve CD4⁺ T cells cultured under Th1-polarizing conditions produce low amount of IFN γ , suggesting a defective Th1 cell differentiation *in vitro* (Szabo *et al.*, 2002). Specific defect in Th1 differentiation was also shown *in vivo*, following immunization of T-bet^{-/-} mice with 2,4,6-trinitrophenol–keyhole limpet hemocyanin TNP-KLH emulsified in complete CFA (to induce a mixed Th1/Th2 response). Moreover, T-bet^{-/-} mice exhibited increased Th2 cells in response to *Leishmania major* injection, showing higher production of IL-4 and IL-5 (Szabo *et al.*, 2002).

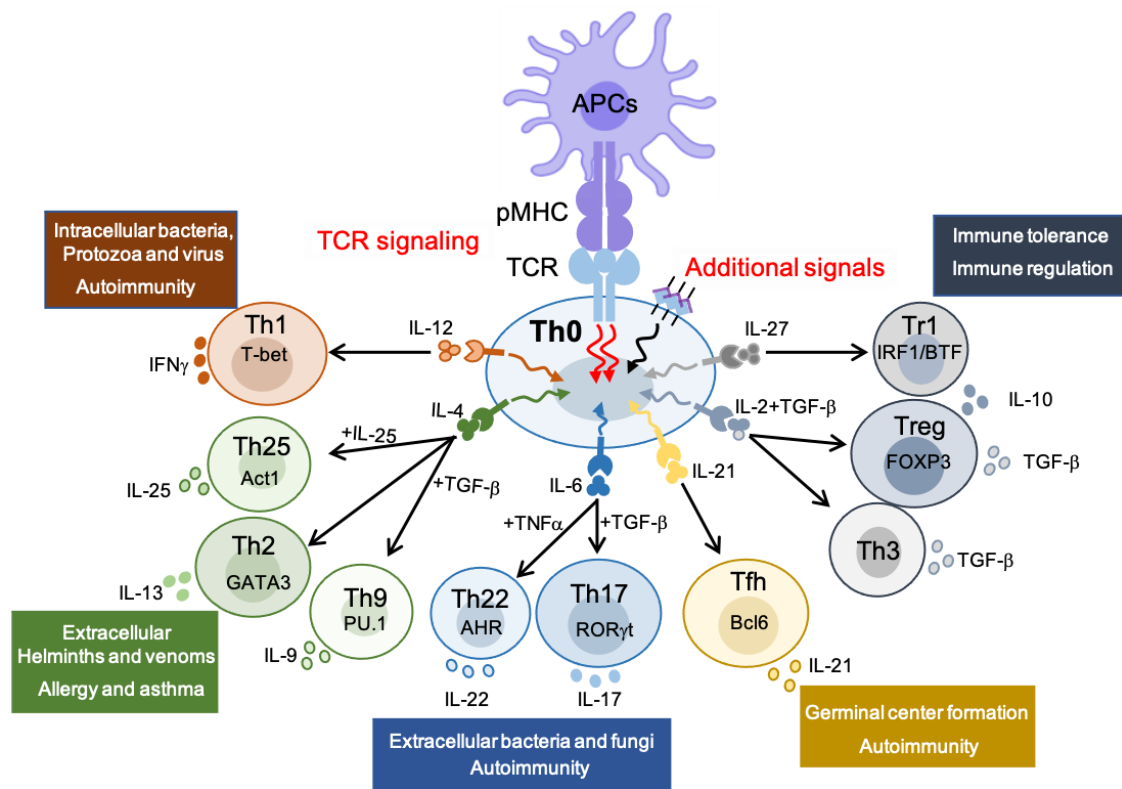


Figure 9. The differentiation of naïve CD4⁺ T cell into distinct effector subpopulations

Antigen-presenting cells present antigens through peptide-MHC complexes to naïve CD4⁺ T cells expressing the TCR and trigger TCR signals leading to the differentiation of naïve CD4⁺ T cells into distinct effector subsets, according to the cytokine micro-environment. The differentiation into each subtype is controlled by specific transcription factors. The participation of additional signals, such as co-inhibitory receptors, also has the ability to influence the outcome of differentiation. Each T helper subset possesses specific biological functions to achieve adaptive immune response. The functionally close subpopulations are marked with similar colors.

This study demonstrated the pivotal role of T-bet in the differentiation of naïve CD4⁺ T cells into Th1 cells and preventing the development of Th2 cells. T-bet is also a key suppressing factor in the generation of Th17 cells *in vitro* and *in vivo* (**Figure 10**). *In vitro*, CD4⁺ T cells with a deletion of both T-bet and STAT6 (a major actor of IL-4-mediated signaling) produce higher amount of IL-17 in Th17 polarizing condition, and generate a higher percentage of IL-17-producing cells in mixed Th1/Th17 polarizing conditions, compared with BALB/c mice (Y. Yang *et al.*, 2008). *In vivo*, Stat6/T-bet^{-/-} naïve CD4⁺ T cells develop more severe colitis and produce higher amount of IL-17 than control cells following transfer into Rag^{-/-} mice cells (Y. Yang *et al.*, 2008). Eomes, another member of the T-box family important in Th1 polarization, promotes the production of IFN γ and suppress the generation of Treg cells by limiting Foxp3 induction (Lupar *et al.*, 2015). T-bet^{-/-} CD4⁺ T cells transfected with Eomes-GFP retrovirus can partially rescue the production of IFN γ . However, Eomes is not able to fully compensate the defective function of Th1 cells due to the loss of T-bet, as Eomes expression in T-bet^{-/-} CD4⁺ T cells is not sufficient to block the inhibitory effect of pro-Th17 cytokines on Th1 polarization (Y. Yang *et al.*, 2008). In diseases models, mice transferred with T-bet^{-/-} naïve CD4⁺ T cells do not develop colitis, by contrast to Eomes^{-/-} naïve CD4⁺ T cells which induce severe disease (Lupar *et al.*, 2015; Neurath *et al.*, 2002; Y. Yang *et al.*, 2008). Previous analysis of CD4⁺ T cell responses against Influenza A Virus (IAV) showed that Eomes^{-/-} CD4⁺ T cells exhibited similar Th1 responses and protection against IAV, by contrast, T-bet^{-/-} CD4⁺ T cells provided partially inefficient protective responses whereas Th1 responses is almost completely impaired in T-bet^{-/-} Eomes^{-/-} CD4⁺ T cells (Dhume *et al.*, 2020). These findings indicate that Eomes enhances Th1 cell responses, but that T-bet is required for the function of Th1 cells.

IL-12 and IFN γ are two key cytokines for the generation of Th1 cells (Hsieh *et al.*, 1993; Szabo *et al.*, 2002). By binding to their receptor on CD4⁺ T cells, they induce the production of IFN γ by Jak2/STAT4 and Jak1/STAT1 pathways, respectively. Binding of IL-12 to its receptor causes the phosphorylation of STAT4, which leads to the transactivation of IFN γ gene and to the expression of T-bet and IL-12R β 2 subunit, amplifying Th1 response (Hsieh *et al.*, 1993; Thieu *et al.*, 2008). Activation of STAT1 by IFN γ stimulates the expression of T-bet, which upregulates IFN γ production by directly transactivating *Ifn γ* gene and by inducing chromatin remodeling of the IFN γ promoter region, forming a positive feedback loop (Lighvani *et al.*, 2001) (**Figure 10**).

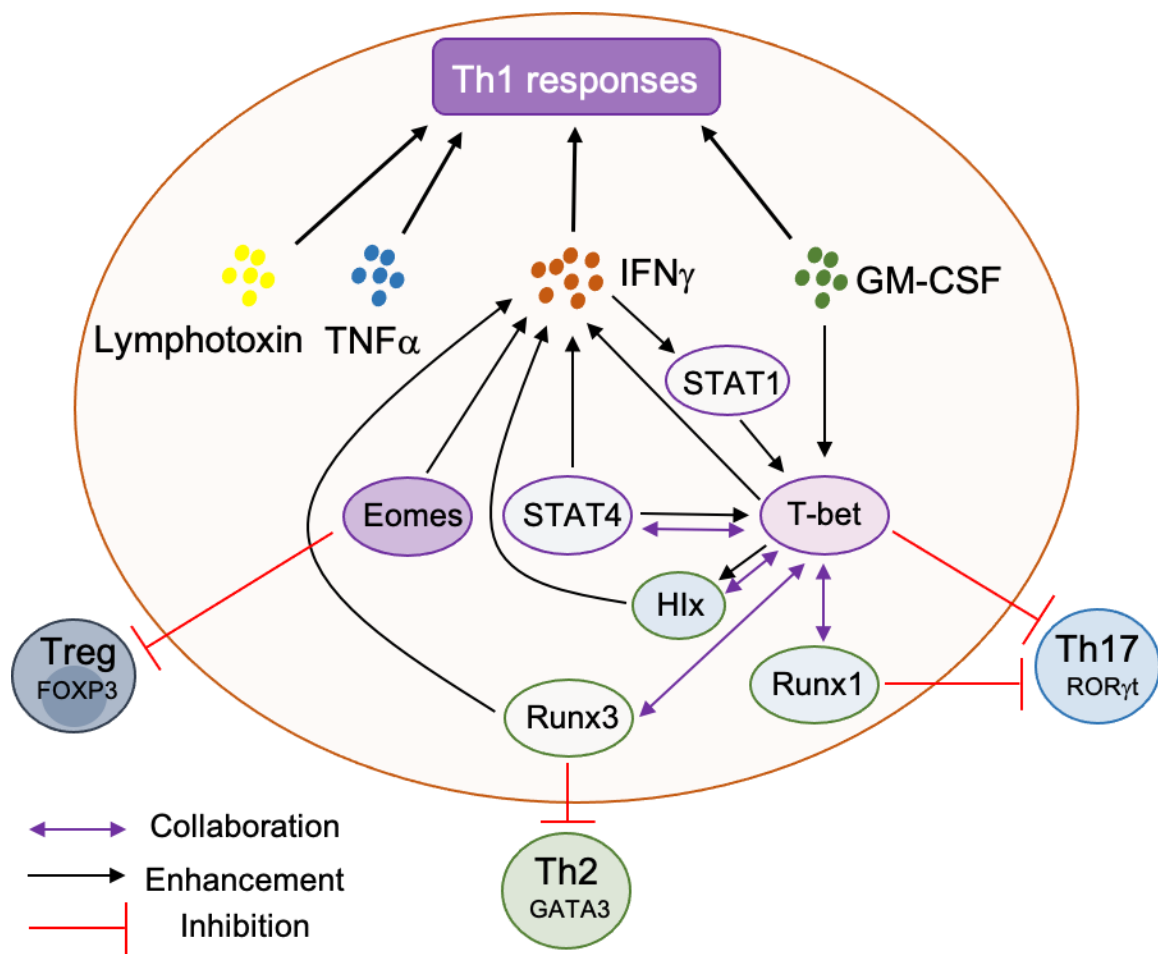


Figure 10. Networks of cytokines and transcription factors in Th1 cells

For optimal Th1 responses, collaboration between cytokines and transcription factors is required. On one hand, they promote the differentiation of Th1 cells, on the other hand, they negatively regulate the development of other T helper cells through repressing the expression of their key transcription factors. Similarly, IL-27, member of IL-12 family, contributes to promote Th1 differentiation by activating the phosphorylation of STAT1 (Amsen, Spilianakis, *et al.*, 2009). In addition, Runx1, Runx3 and H1x are three other transcription factors involved in the process of Th1 differentiation. Runx1, on one hand promotes the expression of T-bet, on the other hand coordinate with T-bet, interfering with the activation of ROR γ t to inhibit the development of Th17 cells (Lazarevic *et al.*, 2011). Runx3 cooperates with T-bet, promoting IFN γ production by binding to promoter regions controlling the expression of *IFNGR1* and *IFNGR2* and by silencing the gene encoding for IL-4, which inhibits Th1 development. Meanwhile, it inhibits Th2 differentiation by interacting with GATA3 (Djuretic *et al.*, 2007; Kohu *et al.*, 2009). H1x is induced after T-bet activation and also cooperates with T-bet to enhance IFN γ production by Th1 cells (244). In addition to the above-mentioned cytokines and transcription factors, Th1 cells also produce GM-CSF, TNF α and lymphotoxin. GM-CSF enforces the maintenance of Th1 cells by up-regulating T-bet and IFN γ *in vitro* and *in vivo* (Rasouli *et al.*, 2020). TNF α and lymphotoxin contribute to the recruitment of leukocytes and to the amplification of inflammation (Fiorentino *et al.*, 1989; Mosmann *et al.*, 1986).

Th1 and Th2 cells have been the first two CD4⁺ T cell subsets to be discovered. They were particularly well studied in seminal studies in the context of autoimmune disease such as Multiple Sclerosis (MS), Collagen-Induced Arthritis (CIA), Inflammatory Bowel Diseases (IBD) and Crohn's disease. Th1 cells were earlier described as the major pathogenic subset in the frame of autoimmune diseases while Th2 cells were shown to exert inhibitory effects on those pathologies (Liblau *et al.*, 1995; Nicholson & Kuchroo, 1996). Indeed, increased numbers of Th1 cells, especially IFN γ -producing cells are present in the central nervous system (CNS) inflammatory lesions at the peak of Experimental autoimmune encephalomyelitis (EAE), the mouse model of MS. On the contrary, the proportion of this phenotypic cell decreases during remission, accompanied by an increase in the production of Th2 cytokines (Imam *et al.*, 2007; Nicholson & Kuchroo, 1996; Renno *et al.*, 1995). In addition, the adoptive transfer of Th1 cells that express TCR recognizing MOG₃₅₋₅₅ into Rag^{-/-} mice develop severe EAE (Domingues *et al.*,

2010). Adoptive transfer of C57BL/6 mice with T-bet^{-/-} CD4⁺ T cells pre-activated with MOG₃₅₋₅₅ *in vivo* fails to induce EAE and shows low infiltration of the CNS by CD4⁺ T cells (O'Connor *et al.*, 2013). Adoptive transfer of T-bet^{-/-} CD4⁺ T cells also leads to reduced Th1-mediated colitis and Crohn's disease in mouse models (Neurath *et al.*, 2002). STAT4⁻ or IL-12p40⁻ (a subunit of IL-12) deficient mice are resistant to the development of EAE (Chitnis, Najafian, Benou, *et al.*, 2001; Gran *et al.*, 2002). Mice deficient for Foxo3, a transcription factor which enhances Th1 generation by activating *Eomes*, develop reduced EAE and have decreased numbers of GM-CSF- and IFN γ - producing CD4⁺ T cells (Stienne *et al.*, 2016). Besides, the treatment of mice with IL-12 during the development of CIA aggravates disease severity (Germann *et al.*, 1996). Taken together, these data suggest a pathogenic role of Th1 cells in autoimmune diseases. However, the Th1-driven nature of these autoimmune diseases was later challenged by studies on mice lacking IFN γ or other molecules important for Th1 differentiation. Mice deficient for IL-12p35 (the other subunit of IL-12), IL-12R β 2, IFN γ or IFN γ R are not protected from EAE but instead develop more severe disease, pointing-out a complex function of Th1 cells in autoimmune disorders (Ferber *et al.*, 1996; Gran *et al.*, 2002; Willenborg *et al.*, 1996; Zhang *et al.*, 2003). GM-CSF and TNF α are not Th1 specific cytokines, as they are produced by most T helper subsets. However, GM-CSF maintains the phenotype of Th1 cells by up-regulating T-bet and IFN γ in the CNS of EAE-developing mice, promoting neuro-inflammation (Rasouli *et al.*, 2020), hence representing a putative therapeutic target in MS (McQualter *et al.*, 2001). TNF α can promote Th1 and Th17 responses in peripheral lymphoid organs. Mice treated with anti-TNF α mAbs have reduced incidence and delayed onset of EAE, but show no difference in disease severity compared to untreated mice (Batoulis *et al.*, 2014).

1.1.2. T helper 2 (Th2) cells

CD4⁺ T cells differentiates into Th2 subtype when facing extracellular pathogens, helminths and allergens in the presence of IL-4. Together with TCR signals, binding of IL-4 on its receptor activates the transcription factor STAT6 which then induce GATA-3 expression (Amsen, Antov, *et al.*, 2009). As an important transcription factor in Th2 differentiation, GATA-3 enhances the production of IL-4, IL-5 and IL-13 by directly binding to the enhancer regions of *IL-4* gene and by upregulating the transcription of IL-5 and IL-13 (Agarwal *et al.*, 2000; Kishikawa *et al.*, 2001; Siegel *et al.*, 1995), generating a

positive feedback loop between GATA-3 and these Th2 cytokines. Furthermore, GATA-3 blocks Th1 differentiation by inhibiting T-bet expression and IFN γ synthesis (Zhu *et al.*, 2006). Likewise, the IL-10 produced by Th2 cells also blocks Th1 cells (Fiorentino *et al.*, 1989). Another essential transcription factor for Th2 subset is STAT5. STAT5-deficient mice show reduced generation of Th2 cells. In the presence of GATA-3, the activation of IL-2-mediated activation of STAT5 promotes Th2 differentiation by driving the early production of IL-4 (Zhu *et al.*, 2006). In addition, the transcription factor IRF4 promotes IL-4 secretion in Th2 cells by increasing GATA-3 expression and by co-operating with NLRP3 (Bruchard *et al.*, 2015; Lohoff *et al.*, 2002), whereas C-MAF specifically promotes IL-4 production by cooperating with KLF-13 (Kwon *et al.*, 2014). To protect the organism against helminths and allergic inflammation, Th2 cells promotes type II immune response by several mechanisms. They release IL-4 to promote IgE antibody responses, and produce IL-5 that activates eosinophils and IL-13 that stimulates mucus production by airway epithelial cells. Moreover, these cytokines work together to induce macrophage activation, which further stimulates B cells and recruits leukocytes (Jabeen *et al.*, 2013; Staudt *et al.*, 2010). Exacerbated type II responses may cause chronic and inflammatory diseases, such as allergies, asthma, rhinitis and dermatitis (Lloyd & Snelgrove, 2018; Walker & McKenzie, 2018).

Th9 and Th25 cells are two subsets that are closely related to Th2 cells and are the major T cell subpopulations involved in mucosal immunity (Locksley, 2009; Swaidani *et al.*, 2011). Th9 cells provide protection against helminthic infections and tumors by producing IL-9 and IL-21 (Kaplan *et al.*, 2015; Veldhoen *et al.*, 2008; Végran *et al.*, 2014). By contrast, these IL-9 producing cells promote the development of colitis and EAE in mouse and contribute to a group of human autoimmune diseases, such as asthma, MS, IBD and Rheumatoid Arthritis (RA) (Deng *et al.*, 2017; Gerlach *et al.*, 2014; Jäger *et al.*, 2009; Kaplan *et al.*, 2015). As for Th2 cells, the differentiation from activated T cells into Th9 subset needs IL-4. The activation of IL-4/Jak1/STAT-6 signaling induced by GATA3 cooperates with TGF- β to induce the maturation of Th9 cells (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008). It has been shown that the transcription factors PU.1, Gfi.1, BATF, IRF4 and Foxo1 are all important for the induction of IL-9 (Buttrick *et al.*, 2018; Chang *et al.*, 2005; Spooner *et al.*, 2009).

Regarding Th25 cells, they induce non-lymphoid cells to secrete cytokines against extracellular pathogens by producing IL-25 and facilitating the differentiation of Th2 cells *via* STAT5 activation (Fallon *et al.*, 2006; Wu *et al.*, 2015). The production of IL-25 cells still needs IL-4, but the main transcription factor is Act1 (Swaidani *et al.*, 2011).

1.1.3. T helper 17 (Th17) cells

Th17 cells are defined by their ability to produce IL-17. The differentiation from activated CD4⁺ T cells into Th17 cells requires IL-6 in combination with IL-23 or TGF β and is inhibited by IFN γ and IL-4 (Brucklacher-Waldert *et al.*, 2009; Korn *et al.*, 2009). The transcription factor ROR γ t is a master regulator for the development of Th17 cells; indeed ROR γ t^{-/-} mice exhibit diminished number and percentage of Th17 cells (Nurieva *et al.*, 2008). STAT-3 is another important transcription factor for Th17 differentiation, its activation by IL-6, IL-21 and IL-23 can induce ROR α and ROR γ t, leading to the production of Th17 cytokines IL-17, IL-17F and IL-22 (Romano *et al.*, 1997; X. O. Yang *et al.*, 2008). IL-23 is composed of two subunits, p19 and p40. The p40 subunit is shared with IL-12 which is an essential cytokine for the development of Th1 cell. But IL-12 and IL-23 have different receptors and effects, which leads to the generation of Th1 and Th17 subsets, respectively (Cauli *et al.*, 2015; Oppmann *et al.*, 2000). In addition, the transcription factors BATF and Runx1 can also promote the polarization of naïve CD4⁺ T cells into Th17 cells by inducing ROR γ t production (Schraml *et al.*, 2009; Zhang *et al.*, 2008). TGF β enhances the generation of Th17 subset through several ways, such as suppressing the expression of SOCS3, a major negative feedback regulator of Th17 cells development, promoting IL-22 production and cooperating with IL-6 for the induction of ROR γ t (Bettelli *et al.*, 2006; Perez *et al.*, 2020; Qin *et al.*, 2009; Veldhoen *et al.*, 2006).

Th17 cells provide protection against infections by extracellular bacteria, fungi at mucosal surfaces and against some microbes that are not targeted by Th1 or Th2 cells. They operate by recruiting leukocytes and neutrophils to infection sites *via* cytokines such as IL-17, IL-21 and IL-22 (Weaver *et al.*, 2006). The dysregulation of Th17 cells is responsible for a variety of autoimmune diseases and chronic inflammations, such as MS, RA and psoriasis (Zambrano-Zaragoza *et al.*, 2014).

IL-17 and other Th17 cytokines such as IL-21 and IL-22 strongly contribute to the pathogenicity of this lineage (Korn *et al.*, 2007; Zheng *et al.*, 2007). IL-23 plays a critical role in generating pathogenic Th17 cells during autoimmune inflammation (Langrish *et al.*, 2005). Mice deficient for IL-23p19, a subunit of IL-23, develop less severe autoimmune diseases, including EAE, CIA and IBD (Cua *et al.*, 2003; Murphy *et al.*, 2003). Adoptive transfer of MOG-specific Th17 cells differentiated *in vitro* by IL-6 and TGF β 1 fails to induce EAE while polarization of MOG-specific Th17 cells in the presence of IL-23 induces severe EAE in recipient mice (El-Behi *et al.*, 2011; Ghoreschi *et al.*, 2010; Langrish *et al.*, 2005). While IL-6 and TGF- β 1 induce non-pathogenic Th17 cells producing IL-10 and expressing high amount of PROCR, a negative regulator of pro-inflammatory genes (Ghoreschi *et al.*, 2010; Kishi *et al.*, 2016; Lee *et al.*, 2012), further investigations showed that IL-23 and ROR γ t, alone or in cooperation, contribute to induce GM-CSF expression in Th17 cells, a pro-inflammatory cytokine which is required for encephalitogenic potential of Th17 cells (Codarri *et al.*, 2011; El-Behi *et al.*, 2011).

Th22 cells are phenotypically and functionally related to Th17 cells (Eyerich *et al.*, 2009). Th22 subset contributes to mucosal immunity by preventing translocation of microbes across epithelia to limit infection (Kim *et al.*, 2012). Like Th17 cells, Th22 cells produce IL-22, but this production needs an environment poor in IFN γ and IL-17 (Trifari *et al.*, 2009). Both subsets express chemokine receptors CCR4 and CCR6 that are associated with cutaneous T cell homing, but CCR10 is specifically expressed on Th22 cells (Trifari *et al.*, 2009). In addition, Th22 cells produce IL-13, TNF α , IL-10, and the chemokines CCL15 and CCL17. The production of IL-22 needs the expression of aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor that is activated by small molecules (Monteleone *et al.*, 2011). STAT-3 is another important transcription factor for the generation of Th22 cells. It can be activated by the cytokines IL-6 and TNF α (Pickert *et al.*, 2009). *In vitro*, Th22 cells can be induced in the presence of IL-6, IL-23, IL-1 β , FICZ and TGF- β R inhibitor (Plank *et al.*, 2017).

1.1.4. T follicular helper (Tfh) cells

Tfh cells are CD4⁺ T cells that specifically express the chemokine receptor CXCR5 and markedly express ICOS, PD-1 and CD40L, which regulate B lymphocyte activity (Crotty, 2019; Nurieva *et al.*, 2008). Tfh cells migrate into the follicle in lymph nodes and allow B cells to initiate extra follicular and

germinal center responses, playing an important role in antibody class switching, affinity maturation of antibodies and more generally in the development of efficient humoral memory (Vinuesa *et al.*, 2016). However, exacerbated Tfh response may also lead to the development of autoimmune diseases, such as lupus and arthritis (N. Simpson *et al.*, 2010; H. Ueno *et al.*, 2015). Induction of Tfh cells requires strong TCR stimulation and depends on Bcl6, a nuclear transcription factor that is essential for the expression of CXCR5 (Johnston *et al.*, 2009; King, 2009; Nurieva *et al.*, 2009; Yu *et al.*, 2009). Bcl6 also inhibits the development of other CD4⁺ T cell subsets by suppressing the expression of the transcription factors T-bet, GATA-3 and ROR γ t and negatively regulates the expression of surface molecules, such as CCR6, PSGL1, CCR7, and S1PR1, that prevent the localization of effector CD4⁺ T cells into the follicle (Vinuesa *et al.*, 2016). In addition, ICOS is essential for the development of Tfh cells. Its activation promotes the phosphorylation of VAV1 and the activation of the Pi3K/Akt pathway, allowing the expression of the transcription factor c-Maf which then transactivates IL-21, a signature cytokine in Tfh cells that is necessary for the formation of the germinal center (Bauquet *et al.*, 2009; Gigoux *et al.*, 2009; King, 2009; Vogelzang *et al.*, 2008). ICOS also inhibits the expression of FoxP1 and Foxo1 which downregulate the formation of Tfh cells (Stone *et al.*, 2015; Wang *et al.*, 2014). Similar to Th17 cells, IL-6 and STAT3 are required for Tfh development but in an environment without Th17 cytokines IL-17, IL-17F, or TGF β (Nurieva *et al.*, 2008). Finally, upon TCR ligation, the costimulatory signal induced by OX40/OX40L interaction can promote the generation of Tfh cells by enhancing the nuclear accumulation of NFAT1 and NFAT2, two transcription factor downstream of Ca²⁺ signaling essential for effective Tfh development during virus infection (Croft, 2010; Martinez *et al.*, 2016). The signal mediated by engagement of this costimulatory also upregulates the expression of several Tfh-associated molecules in T cells, including Bcl6, CXCR5 and IL-21, and promotes the accumulation of Tfh cells in the B cell follicles in autoimmune conditions, like lupus (Jacquemin *et al.*, 2015).

1.1.5. Regulatory T cells (Tregs)

Tregs are CD4 T cells that maintain tolerance to self-antigens and suppress immune responses of other effector T cells responding to self- and foreign- antigens (Josefowicz & Rudensky, 2009). Dysfunction of Tregs can cause severe chronic pathologies, such as autoimmune disease, cancers, viral infections and allergies (Noval Rivas & Chatila, 2016; Sakaguchi *et al.*, 1995; Takeuchi & Nishikawa, 2016).

Tregs are identified as expressing high level of CD25 (IL-2Ra), and by the expression of Foxp3, a transcription factor which is required for the generation of Tregs and for their suppressive function (Hori *et al.*, 2003; Sakaguchi *et al.*, 1995; Zhou *et al.*, 2008). There are two major types of Tregs in the mammalian immune system: thymic-derived natural Tregs (nTreg or tTreg) and adaptively induced Tregs (pTreg or iTreg) differentiated from conventional CD4⁺ T cells in peripheral lymphoid organs. tTregs migrate out of the thymus and work together with pTregs to dampen immune response. tTregs derive from DP thymocytes harboring a TCR with a strong affinity for self-antigen p-MHC complex; the TCR repertoire of tTregs responding to foreign antigens is limited. pTregs derived from naïve CD4⁺ T cells mainly respond to foreign antigens present in tolerogenic microenvironment (Relland *et al.*, 2012). The co-stimulatory signal provided by CD28 enhances the generation of tTregs, by contrast to pTregs which are less dependent on this stimulation (Apostolou *et al.*, 2002; Kretschmer *et al.*, 2005). The cytokine signals from TGF β and IL-2 stimulate the generation of both tTregs in the thymus and pTregs *in vitro* and *in vivo* (Davidson *et al.*, 2007; Liu *et al.*, 2008; WanJun *et al.*, 2003). The expression of Foxp3 is promoted by STAT5 signaling activated by cytokines including IL-2 and IL-15 (Passerini *et al.*, 2008). Intestine is a privileged site for the generation of pTregs, as the high concentration of retinoic acid, a metabolite of vitamin A, and the anti-inflammatory cytokines secreted by tolerogenic APC presenting antigens from the normal microbiota, favor the differentiation of naïve CD4⁺ T cells into pTregs (Atarashi *et al.*, 2013; Coombes *et al.*, 2007).

Tregs can dampen immune responses of other effector T cells by absorbing extracellular IL-2, through IL-2R α which is highly expressed on their cell surface (Thornton & Shevach, 1998). Furthermore, Tregs suppress immune responses by cell-contact-dependent and -independent ways (Wan & Flavell, 2008). It has been demonstrated that tTregs perform their suppressive effects by cell-contact-dependent mechanisms through membrane-bound molecules while pTregs tend to suppress immune responses by secreting inhibitory cytokines including IL-10, TGF- β and IL-35 (Josefowicz *et al.*, 2012). There is no consistent and perfect marker that would allow to distinguish tTreg from pTregs *in vivo*, although Nr1, a receptor of TGF- β 1, has been suggested to specifically identify tTregs in some experimental models. It has been reported that pTreg only express Nr1 in certain inflammatory conditions, but it is still difficult to separate them from tTregs, as tTregs constitutively express this receptor (Szurek *et al.*, 2015).

Nrp1 is important and required for the function of Tregs. Indeed, one recent publication shows that Nrp1KO Tregs have defective suppressive function, producing reduced level of IL-10+ and increased level of IFN γ (Campos-Mora *et al.*, 2019).

Interestingly, some pTregs exert suppressive functions independently of Foxp3, including **Tr1 cells** and **Th3 cells** (Mills, 2004). Tr1 cells are characterized as producing high amount of IL-10 (Groux *et al.*, 1997). They also exhibit high production of TGF- β , intermediate level of IL-5 and low level of IFN γ and IL-4 (Battaglia *et al.*, 2006). Tr1 cells suppress the immune response *via* IL-10 secretion through several ways, such as downregulating costimulatory molecules, preventing the secretion of inflammatory mediators and inhibiting of the secretion of T cell cytokines (Pestka *et al.*, 2004). Indeed, the defect of Tr1 cells is associated with several T cell-mediated diseases, such as Graft-versus-host disease (GvHD), autoimmunity and chronic inflammatory diseases (Bluestone *et al.*, 2007). Previous studies on the influence of Tr1 cells on colitis and MS mouse models showed that mice with IBD or EAE were cured after receiving ovalbumin-specific Tr1 cells and administration of ovalbumin (Barrat *et al.*, 2002; Groux *et al.*, 1997). Clinical trials of Tr1 cell to treat GvHD and Crohn's disease are currently ongoing (Roncarolo & Battaglia, 2007; Weston *et al.*, 2006). The function and generation of Tr1 cells do not need Foxp3, but their activation leads to increased Foxp3 expression. Foxp3 can prevent Tr1 cells from converting to a Th2 phenotype and from losing their suppressive function (Roncarolo & Gregori, 2008; Veldman *et al.*, 2006; Vieira *et al.*, 2004). *In vitro*, Tr1 cells can be induced by continuous TCR stimulation in presence of IL-27, a heterodimeric cytokine of the IL-12 family, alone or working together with TGF- β (Chihara *et al.*, 2016; Stumhofer *et al.*, 2007). Compared with Tr1 cells, Th3 cells produce higher level of TGF β and lower amount of IL-10 in response to the stimulation of oral foreign antigens (Chen *et al.*, 1994; Wan & Flavell, 2008). They are involved in mucosal immunity and protecting mucosal surfaces in the gut from pathogens. TGF β enhances IgA secretion and suppress the effective responses of Th1 and Th2 cells (Fukaura *et al.*, 1996).

1.2. Plasticity of T helper cells

CD4⁺ T cells differentiate into distinct subpopulations in response to specific pathogens. Differentiated CD4⁺ T cells produce effector cytokines which in turn provide positive feedback to increase further the differentiation of naïve CD4⁺ T cells into this subset and inhibit the development of antagonist subpopulations (Rao & Avni, 2000). However, their fate isn't set for good, as T helper cells can re-differentiate into other subpopulation or adopt an intermediate state between two subsets in response to changing circumstances, showing their phenotype flexibility (O'Shea & Paul, 2010; Zhou *et al.*, 2009) (**Figure 11**). For example, naïve CD4⁺ T cells can produce both IFN γ and IL-4 at early stages after activation, although their polarization towards Th1 or Th2 subpopulation implies blocking the expression of either one or the other cytokine (Reiner, 2001). Under Th1 or Th2 differentiation conditions *in vitro*, Th22 cells can repolarize into IFN γ -producing Th1 or IL-13-producing Th2 cells (Plank *et al.*, 2017). IL-10 is the characteristic cytokine of Tr1 subset, but Th1, Th2, Th17 and Tregs are also able to produce IL-10 under certain circumstances. In addition, Th1/Th17 cells, also called ex-Th17 cells, have been identified as a distinct population that can produce IFN γ , GM-CSF and IL-17 (Bending *et al.*, 2009). Studies on Th17 cells during EAE development in mice show that adoptive transfer of Th17 cells induces more severe disease than Th1 cells (Langrish *et al.*, 2005). Some data also show that Th17 cells can co-express IL17 and IFN γ at the early stage of Th17 differentiation *in vitro* and increased number of IFN γ +IL-17⁺ cells are found in patients with autoimmune diseases, including MS, IBD and RA (Bazzazi *et al.*, 2018; Fletcher *et al.*, 2010; A. Ueno *et al.*, 2015). One recent publication shows that the behavior of IFN γ +IL-17⁺ cells in the CNS of EAE mice resembles more Th1 cells than Th17 cells, indicating strong plasticity of Th17 cells and strong pathogenicity of these double positive cells (Loos *et al.*, 2020).

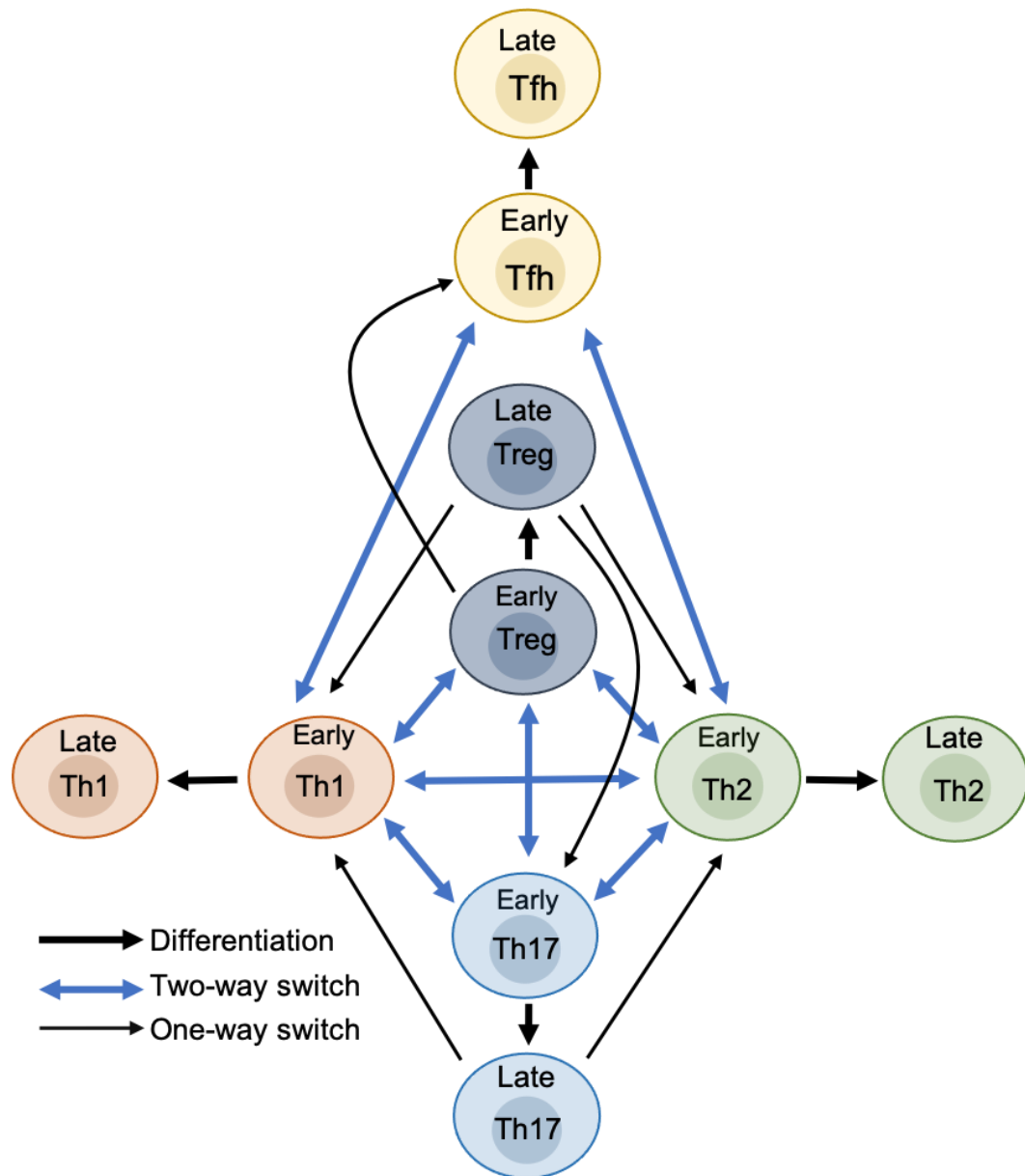


Figure 11. Plasticity of major T helper cells

At early stages of CD4⁺ T cell differentiation, each subpopulation can redirect to other effector T cell subsets. Most Th1 and Th2 cells have persistent phenotype at the last stage, while Th17 and pTregs conserve the ability of re-differentiation. (Adapted from Zhu & Paul, *Cell research*, 2010)

1.2.1. Need of specific cytokines

The plasticity of CD4⁺ T cells depends on different factors. The presence of specific cytokines in the environment is an essential factor. During the differentiation of CD4⁺ T cells into Th1 cells *in vitro*, enhanced IL-15 signaling from trans-presented IL15/IL-15R α increase and maintain the expression of Th1-associated genes including IFN γ and T-bet by promoting the expression of STAT5. However, when expose to IL-15 alone, those Th1 cells show increased expression of Bcl6, resulting in a re-differentiation from Th1 to Tfh cells (Cooley *et al.*, 2015). Similarly, IL-18, a member of the IL-1 family, was originally discovered as an enhancer of IFN γ production by anti-CD3 stimulation in Th1 cells (Nakanishi *et al.*, 2001; Okamura *et al.*, 1995). Later studies showed that IL-18 cooperates with IL-12 to promote Th1 responses *in vitro and in vivo* by enhancing IFN γ production in Th1 cells and non-polarized T cells. However, without IL-12, IL-18 drives the cells to produce IL-4 and IL-13, inducing Th2 polarization (Hoshino *et al.*, 1999; Nakanishi *et al.*, 2001; Yoshimoto *et al.*, 2000).

In addition, at early stage of CD4⁺T cell differentiation *in vitro*, Th1 cells can lose IFN γ expression and convert into Th2 cells by adding IL-4 to the culture medium. Likewise, Th2 cells can be easily redirected to Th1 cells by adding IL-12 (Perez *et al.*, 1995). Th17 cells can be re-differentiated into Th1 cells *in vitro* by adding IL-12 to the culture medium, in the absence of IL-6 (Zhou *et al.*, 2009). Interestingly, both Th1 and Th17 cells can be repolarized to Th2 cells *in vivo*. A study shows that injection of the gastrointestinal helminth *Nippostrongylus brasiliensis* into mice that were transferred with Th1 or Th17 cells caused these cells to repolarize into Th2 cells, producing IL-4 and losing the ability to express IFN γ or IL-17 (Panzer *et al.*, 2012). In addition, during the early development of Th17 cells, CD4⁺ T cells express both Foxp3 and ROR γ t, exhibiting an intermediate state between pTregs and Th17 cells (Huehn *et al.*, 2009). Adding IL-6, IL-21, IL-23 and low level of TGF β in the culture medium drives these intermediate cells to become Th17 cells, while the presence of high level of TGF β and IL-2 supports the development of pTregs (Huehn *et al.*, 2009; Yun Kyung Lee *et al.*, 2009; Weaver *et al.*, 2006). In the presence of IL-21, TGF β -induced Tregs are able to mature into Tfh cells by upregulating CXCR5 expression in aim to relocate into follicles (Nurieva *et al.*, 2008). Finally, in the presence of IL-4, Th2 cells also can become Tfh cells by increasing CXCR5 expression, the re-differentiated Tfh cells still produce IL-4 but do not produce other Th2 cytokines (Dardalhon *et al.*, 2008; Veldhoen *et al.*, 2008).

Since IL-4 is required for the development of Th2, Th9 and Tfh subpopulations, Th2 cells can become Th9 cells when IL-4 and TGF β are present in the surrounding environment (Cantor & Shinohara, 2009).

1.2.2. Need of the required Transcription factors

Transcription factors play an essential role for the redifferentiation of T helper cells. They are not just necessary for the polarization of the relevant CD4⁺ T cell subsets but also for suppressing the development of other populations. Overexpression of T-bet in Th2 cells results in a Th1 phenotype while overexpression of GATA3 in Th1 cells lead these cells to re-differentiate into Th2 cells (Cantor & Shinohara, 2009). In parallel, the absence of the key transcription factors in a defined lineage unlocks the expression of other transcription factors or polarizing mediators, leading to the differentiation of alternative CD4⁺ T cell subpopulations. For example, under strong Th1 polarizing conditions, the absence of T-bet results in upregulation of GATA3, IL-4 and IL-17 (E. L. Pearce *et al.*, 2003; Yagi *et al.*, 2010). The increased production of IL-17 can be further enhanced by knocking out another T-box family member, Eomes (Dhume *et al.*, 2020). Besides, some transcription factors are co-expressed in intermediate CD4⁺ T cells. For example, both T-bet and ROR γ t are expressed in IFN γ +IL-17+-producing cells, allowing them to maintain either a Th1/Th17 phenotype or to further develop into either Th1 or Th17 cells (Wilson *et al.*, 2009). In differentiated Th17 cells that are induced by myelin basic protein (MBP-Ac-11) *in vitro*, IFN γ +IL-17+ cells have higher expression of T-bet than IFN γ + cells (Yang *et al.*, 2009). Further studies demonstrated that T-bet is required for the switch from IL-17 to IFN γ . During the development of EAE, compared with WT mice, T-bet^{-/-} mice display a reduced production of IFN γ but increased expression of IL-17 and no IFN γ +IL-17+ CD4 T cells (O'Connor *et al.*, 2013).

The metabolic activity also plays a considerable role in CD4⁺ T cell plasticity. For example, Acetyl-CoA Carboxylase 1 (ACC1) enhances the generation of Th17 cells but decreases the development of Tregs in mouse models and human cells. Blocking ACC1 with Soraphen A inhibits *de novo* fatty acid synthesis and glycolytic-lipogenic metabolic pathway, leading to increased generation of FoxP3-producing cells and to decreased development of IL17-producing cells *in vitro* and *in vivo* (Berod *et al.*, 2014). Besides, other factors including cellular variations, genetic, epigenetic and TCR signal strength are involved in the regulation of the CD4⁺ T cell plasticity.

1.3. CD8+ T cells and other T cell subsets

1.3.1. CD8+ T cells

CD8+ T cells, also known as cytotoxic T lymphocytes (CTLs) when they are activated, constitute a T cell population that possess protective function towards intracellular pathogens, including intracellular bacteria, viruses and which can also contribute to the elimination of tumor cells. CTLs kill antigen-bearing cells by three major mechanisms. The first is the secretion of cytokines and chemokines with antimicrobial function, such as IFN γ , TNF α , MIP-1 α/β and RANTES (Guidotti *et al.*, 1994; Yang *et al.*, 1997). The second way is to release lytic granules containing perforin and granzymes which can induce caspase-dependent apoptosis of the target cell (Obar & Lefrançois, 2010). The third mechanism is *via* Fas/FasL interaction. Binding of Fas, expressed on the surface of the target cell, with FasL, expressed by activated CD8+ T cells, also causes the activation of caspases cascade and results in the apoptosis of the target cells (Bakshi *et al.*, 2014). In addition, evidence from studies in RA, MS and EAE have shown that CD8+ T cells play a complex role in autoimmune diseases (Blanco *et al.*, 2005; Friese & Fugger, 2009; Junker *et al.*, 2007). Effective CD8+ T cell responses have been associated with the development of acute MS plaques and oligoclonal expansion of CD8+ T cells in CNS of MS patients (Blanco *et al.*, 2005; Junker *et al.*, 2007). By contrast, in autoimmune diseases such as RA, IBD and MS and in murine model of MS, some CD8+ T cells develop regulatory function by targeting autoreactive CD4+ T cells through the recognition of the MHC class Ib molecule Qa-1 in mouse and the HLA-E in human (Baughman *et al.*, 2011; Brimnes *et al.*, 2005; Carneiro *et al.*, 2013; Jiang *et al.*, 1992; Lu & Cantor, 2008). During EAE development, the activation of those CD8+ regulatory T cells suppress the disease by inhibiting CD4+ T cells responses through releasing IFN β and perforin (Sinha *et al.*, 2014).

As for CD4+ T cells, the activation of CD8+ T cells is mediated by TCR-pMHC I engagement and needs additional signals from co-receptors, such as CD28, and immunomodulation by cytokines such as IL-2, IL-7, IL-15 and IL-18 (Au-Yeung *et al.*, 2017; Carrette & Surh, 2012; Obar & Lefrançois, 2010; Walsh *et al.*, 2014). Effector CD4+ T cells promote the activation of CD8+ T cells by secreting cytokines, such as IL-2, IL-12 and IL-21, and enhancing stimulatory signal from APCs to CD8+ T cells through CD40/CD40L interaction (Bennett *et al.*, 1998; Castellino & Germain, 2006). T-bet and especially Eomes are key transcription factors for the development and function of CTLs.

Eomes is responsible for T-bet-independent IFN γ production in CD8 $^{+}$ T cells (Erika L Pearce *et al.*, 2003). The absence of Eomes in CD8 $^{+}$ T cells leads to reduced production of perforin, IFN γ and granzyme B *in vitro* and *in vivo* (Erika L Pearce *et al.*, 2003). Mice that have T-bet/Eomes double-deficiency result in the development of chronic wasting disease (CWD) in response to LCMV infection (Intlekofer *et al.*, 2008).

1.3.2. $\gamma\delta$ T cells and NKT cells

In addition to CD4 $^{+}$ and CD8 $^{+}$ T cells, some populations of T cells have distinct features and functions in host defense, including $\gamma\delta$ T cells and NKT cells. The TCRs expressed by these T cells have limited diversity, but are able to recognize various antigens without being presented by MHC I or MHC II. According to reports, $\gamma\delta$ T cells predominantly recognize lipid antigens, and some can recognize MHC class Ib molecules, while it's still rarely known about the antigenic molecules that activate this phenotypical cells (Bonneville *et al.*, 2010). Likewise, many of NKT cells recognize lipid antigens presented by CD1d, a non-polymorphic major histocompatibility complex class I-like antigen presenting molecule (Godfrey & Kronenberg, 2004). Both cell types are abundant in epithelial tissues, being involved in and promoting innate and adaptive immunity by secreting cytokines and chemokines.

$\gamma\delta$ T cells have been associated with many autoimmune diseases. On one hand, they play a protective role at the inflammation site by secreting IL-22, a cytokine that is responsible for cell-mediated production of antimicrobial peptides and tissue repair (Holtmeier & Kabelitz, 2005; Paul & Lal, 2015). On the other hand, they promote Th17 response by releasing IL-17. High numbers of IL-17 producing $\gamma\delta$ T cells are detected during the development of IBD, RA, MS and Psoriasis (Cruz *et al.*, 2018; Paul & Lal, 2015; Shiromizu & Jancic, 2018).

NKT cells, also known as invariant NKT cells (iNKT), share some features with NK cells, such as Granzyme production. They also secrete large quantities of cytokines, such as IFN γ and IL-2 (Vivier & Anfossi, 2004). The interaction of iNKT and B cells can promote the prolonged antibody response and long-term memory of B cells (Bai *et al.*, 2013). It has been reported that the absence of iNKT cells can induce the development of autoimmune diseases, such as diabetes or atherosclerosis (Wu & Kaer, 2009).

This type of cells is also associated with allergic asthma, as iNKT cells can produce Th-2 type cytokines IL-4 and IL-13 (Iwamura & Nakayama, 2010). In addition, NK1.1^{neg} iNKT cells is an additional iNKT cells that lack the NK1.1 marker (NK1.1^{neg}) and secrete high amounts of IL-17 (Michel, 2007). This population is more found in the lung and enhances the recruitment of airway neutrophilia through the secreted IL-17, contributing to the natural immunity against inhaled antigens (Michel, 2007).

The generation of each T cell subset and their effector functions listed above requires TCR-induced signals. As described at the beginning of this chapter, the signal triggered by the engagement of TCR with pMHC induces T cell activation and early cytokine production, thereby maintaining cell survival and proliferation, and cooperating with other signals to guide differentiation and the production of characteristic cytokines. Previous findings suggest that the strength of TCR signaling regulates the differentiation of T cells. In general, strong TCR signaling induced by stimulation with highly concentrated antibodies or high affinity peptides favors the generation of Th1 cells *in vitro* and *in vivo*, whereas weak T cell signaling favors Th2 cell development (Constant & Bottomly, 1997; Pfeiffer *et al.*, 1995; Tao *et al.*, 1997). In addition, under Th17 polarizing conditions, IL-17 production can be promoted by increased concentration of antigen and persistent TCR stimulation (Iezzi *et al.*, 2009; Kastirr *et al.*, 2015). The mechanism by which TCR signaling regulates CD4⁺ T cell responses will be described in the next section.

2. Influence of TCR signal transduction on CD4 T cell response

As shown in the **Figure 12**, TCR signaling initiates the activation of the transcription factors AP-1, NFAT and NF- κ B. These transcription factors translocate into the nucleus and regulate the expression of cytokines, cytokine receptors and master transcription factors for differentiation, and cooperate with STAT transcription factors to drive the expression of specific cytokines.

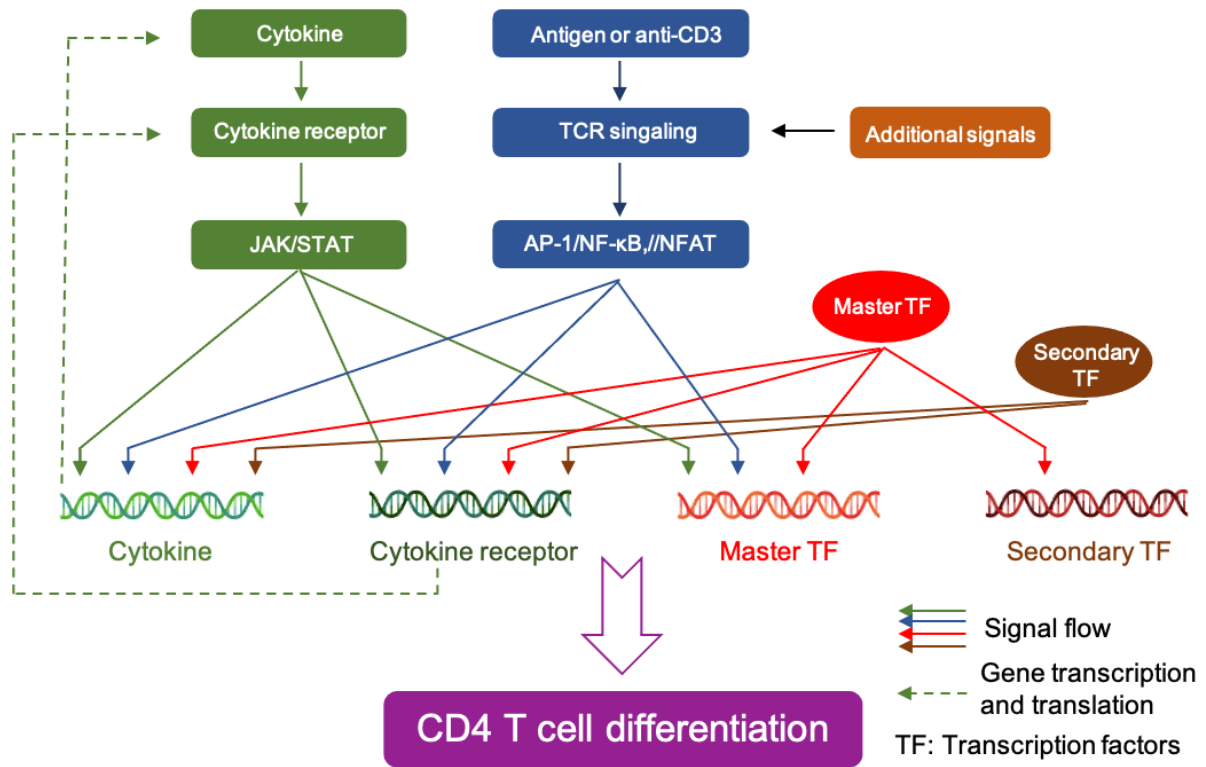


Figure 12. TCR signaling regulates CD4+ T cell differentiation

By cooperating with cytokines-mediated signals, TCR signaling induces the production of early cytokine and the expression of cytokine receptor and master transcription factors, thereby enhancing the expression of secondary transcription factors and cooperating with them to upregulate the expression of cytokine and cytokine receptor genes. Additional signals are able to affect the outcome of differentiation by modulating and cooperating with TCR signals. (Adapted from Zhu J, *et al.*, *Annual review of immunology*, 2010).

Meanwhile, master transcriptional regulators enhance the expression of secondary transcription factors, such as the enhancement of STAT-4 and Runx3 by T-bet in Th1 cells and ROR γ t - upregulated STAT-3 in Th17 cells (Zhu *et al.*, 2010). In addition, they directly upregulate cytokines and cytokine receptors, which in turn enhance initial cytokine/Jak/STAT pathways. In addition, other signals, such as co-inhibitory receptors and growth factors, are able to affect the outcomes of CD4⁺ T cell responses by modulating TCR-induced signals. Thus, signaling and transcriptional events associated to the TCR, the cytokine receptors and the transcription factors form an interconnected network which control the lineage fate of effector CD4⁺ T cells. All these pathways cooperate with each other to direct the differentiation of T cells as well as their functional characteristics and their response to diseases.

Notably, each transcription factor in the AP-1 family, NFAT and NF- κ B is activated by specific TCR signaling distal pathways, corresponding to their distinct effect on the different biological responses of CD4⁺ T cells. Proximal TCR signaling events occur early after TCR ligation and are important to translate early signals into cues which may modulate more ubiquitous downstream pathways. Defects in the proximal signaling components (such as CD3 ITAMs and LAT) often result in impaired T cell responses, leading to poor protection against pathogens. Whereas, excessive activation of proximal signaling or each downstream pathway may lead to autoimmunity. Their particular effects on CD4⁺ T cell responses and functions will be described in the following section.

2.1. Proximal TCR signaling

As described in Chapter I (1.2), the engagement of the TCR with pMHC induces the phosphorylation of CD3 ITAMs by Lck or Fyn. Lck is a member of Src family of tyrosine kinases, its expression is required for cell proliferation and IL-2 production (Seddon & Zamoyska, 2002). Furthermore, the absence of Lck results in impaired development of Th2 cells with decreased expression of GATA-3, whereas it does not affect the differentiation of Th1 subset (Kemp *et al.*, 2010). Fyn also belongs to Src family, functions similarly to Lck in T cells. It contributes to the activation of the ERK pathway, while its expression is not necessary for T cell activation, and it negatively regulates Th2 polarization *via* repressing IL-4R signaling (Lovatt *et al.*, 2006; Mamchak *et al.*, 2008; Tamura *et al.*, 2001). ITAMs phosphorylation by Lck promotes the recruitment and activation of Zap70. Zap70 activity is required

for T cell activation, survival and suppressive activity of Treg (Au-Yeung *et al.*, 2010; Schim van der Loeff *et al.*, 2014). At the molecular level, Zap70 functions by recruiting SH2-containing proteins like Cbl, VAV1 and Lck to the tyrosine sites Y292, Y315 and Y319, respectively (Kong *et al.*, 1996). It has been shown that the residues Y315 and Y319 enhances TCR signaling while the recruitment of Cbl to Y292 exerts a negative role on T cell activation and on the production of IL-2 and IFN γ (Magnan *et al.*, 2001).

Zap70 then phosphorylates LAT, an adaptor protein able to recruit many other actors of TCR signaling, such as PLC γ 1, SLP-76 and Grb2, forming a LAT signalosome and leading to the activation of distal signaling pathways (Wange, 2000). LAT being the link between proximal and distal TCR signaling pathways, the deletion or mutation of LAT in peripheral CD4⁺ T cells lead to severe defect of TCR signaling and T cell response, including reduced suppressive function of Tregs and lymphoproliferation of conventional CD4⁺ T cells in post-thymic LAT deficient mice (Chuck *et al.*, 2010; Mingueneau *et al.*, 2009; Shen *et al.*, 2010). Over-expression of PLC- γ 1 has been reported to occur in a variety of malignancies including Luminal-A and -B breast cancer patients (Lattanzio *et al.*, 2019). Both SLP-76 and Grb2 are essential for the transduction of TCR signals. Mice with conditional deficiencies for either SLP-76 or Grb2 are more resistant to EAE compared to wild type mice, and exhibit impaired T cells proliferation (Radtke *et al.*, 2016; Wu *et al.*, 2011). However, in terms of T cell differentiation, Grb2-deficient CD4⁺ T cells exhibit increased ability to differentiate into Th1 and Th17 cells (Radtke *et al.*, 2016).

The LAT signalosome incorporates several proteins involved in the regulation of T-cell biological response. One of them, Itk, belonging to the Tec family kinase, possesses the highest expression in T cells compared to the other family members (Fowell *et al.*, 1999). Once recruited, Itk activates PLC- γ 1, leading to the activation of Ca²⁺-calcineurin/NFAT pathway. Itk ^{-/-} CD4⁺ T cells have defective Ca²⁺/NFAT pathway, correlated with impaired Th2 differentiation *in vitro* and increased Th1 phenotype cells (Fowell *et al.*, 1999; Miller *et al.*, 2004). Loss of Itk specifically decreases IL-17A production in Th17 cells (Andreotti *et al.*, 2010; Gomez-Rodriguez *et al.*, 2009). In mouse models of asthma, IBD and MS, loss or inhibition of Itk expression in T cells alleviates the disease severity (Lechner *et al.*, 2020).

2.2. Distal signaling pathways

2.2.1. MAPK/ERK pathway

The MAPK/ERK pathway also known as Ras-Raf-MEK-ERK pathway is reported to regulate several cellular processes, such as proliferation, differentiation and cytokines/mediators secretion (Roux & Blenis, 2004). By using knockout mouse model, ERK1 has proven to be important for Th2 differentiation, whereas ERK2 is required for Th1 differentiation (N. Goplen *et al.*, 2012). ERK1^{-/-} mice have decreased Th2 cytokines, including IL-4 and IL-5, leading to the failure of asthma induction in these mice (N. Goplen *et al.*, 2012). ERK2 deficiency dramatically dampens IFN γ production in Th1 cells, but does not affect the differentiation of Th2 and Th17. Knocking out ERK2 also increases pTregs polarization *in vitro*, which indicates that ERK2 can combine positive and negative effect on Th1 and pTreg differentiation, respectively. More specifically, ERK2 suppresses GATA3 and inhibits TGF β signaling *via* suppressing FOXP3 and Smad2/3 (Chang *et al.*, 2012). Interestingly, cytokine environment also affects the expression of distinct ERK isoforms. A study conducted by Rafeul Alam and his collaborators shows that the expression of ERK1 was decreased by adding IL-12 and was increased by IL-4, while those cytokines have opposite effect on ERK2 production (Nicholas Goplen *et al.*, 2012).

ERK cascade is involved in innate immune system and in autoimmune diseases (Arthur & Ley, 2013). Studies showed that the MAPK activation is important for the initiation of EAE and hyperactive peripheral T cells with augmented ERK activity are detected in RA patients as well as in the relevant murine model (Shin *et al.*, 2003; Singh *et al.*, 2009). In EAE mouse model, the treatment of inhibitors targeting the kinases upstream of ERK can alleviate disease severity (Brereton *et al.*, 2009). ERK1^{-/-} mice have enhanced Th1 type immune response, developing precocious and more severe EAE as well as displaying higher IFN γ production than wild type mice (Agrawal *et al.*, 2006). In Th17 subset, MAPK/ERK signaling has a more complex role. Inhibition of kinases upstream of ERK in DCs leads to a decreased production of IL-23 and IL-1 β by those cells, resulting in reduced ability to induce IL-17 production in Th17 cells (Brereton *et al.*, 2009). Directly inhibiting ERK nuclear translocation with Glu-Pro-Glu (EPE) peptide impairs secretion of GM-CSF by Th17 cells, but does not affect their proliferation and IL-17 production *in vitro*. *In vivo*, this inhibition displays very minor impact on EAE development (Birkner *et al.*, 2017).

In addition to ERK1/2, p38 and JNK are two other typical MAP Kinases. Earlier studies proved that, in an EAE mouse model, treatment with pharmacologic inhibitors of p38 MAP kinases did not affect the production of IFN γ by CD4⁺ T cells but decreased the production of IL-17 by those cells (Lu *et al.*, 2010; Noubade *et al.*, 2011). Moreover, lack of T cell-specific alternative p38 activation pathway resulted in diminished Th17 response and reduced EAE severity, stressing out the important role of MAPK/p38 in Th17 cells (Jirmanova *et al.*, 2011). Further *in vitro* studies on the four p38 isoforms (p38 α , p38 β , p38 γ and p38 δ) show that p38 α /p38 β double-deficient CD4⁺ T cells are not affected in their differentiation into Th1, Th2 and Th17, but show an increase development of pTregs cells (Hayakawa *et al.*, 2017). The absence of p38 γ and p38 δ causes reduced production of IFN γ and IL-17 in Th1 cells and in Th17 cells, respectively. *In vivo*, mice lacking p38 γ and p38 δ display reduced disease severity during the development of collagen-induced arthritis (CIA) (Criado *et al.*, 2014).

Regarding JNK, in general, the apoptosis mediated by JNK activation is necessary to avoid exacerbated immune response, preventing the development of autoimmune pathologies (Nishina *et al.*, 1998). However, increased activity of JNKs has been detected in PBMCs of relapsing MS patients, in the CNS of EAE-developing mice and in intestine of IBD patients and relevant mouse model (Cui *et al.*, 2007; Ferrandi *et al.*, 2011; Roy *et al.*, 2008). In mammals, JNK has ten isoforms derived from three genes, JNK1 (four isoforms), JNK2 (four isoforms) and JNK3 (two isoforms). JNK1 and JNK2 are expressed in all cells, JNK3 is mainly found in the brain (Bode & Dong, 2007). By using a double-deficient mouse model for JNK1 and JNK2, it has been demonstrated that they both promote the differentiation of naïve CD4⁺ T cells into Th2 phenotype but have no influence on IL-2 production and cell proliferation (Dong *et al.*, 2000). JNK2, but not JNK1, increases the suppressive functions of Tregs and the production of regulatory cytokines, such as TGF β and IL-10 (Joetham *et al.*, 2014). In EAE model, knocking out JNK2 does not affect disease severity, and inactivation of JNK1 moderately decreases EAE development (Nicolson *et al.*, 2002; Tran *et al.*, 2006). One recent publication in *Cell* shows that pharmacological inhibition of all JNK isoforms with SP600125, a reversible adenosine triphosphate competitive pan-JNK inhibitor, can alleviate EAE severity and decreases the infiltration of T cells and macrophages in the spinal cord, suggesting a potential treatment strategy for MS (Bagnoud *et al.*, 2020).

Interestingly, this treatment does not affect the production of IFN γ , TNF α and IL-17 in brain and spleen, but increases IFN γ and TNF α mRNA levels in spinal cord, stressing out the complex role of those cytokines in this disease (Bagnoud *et al.*, 2020).

2.2.2. PKC- θ /NF- κ B pathway

Proteins from the NF- κ B family are activated and translocated into the cell nucleus to regulate gene expression (Hayden *et al.*, 2006). The PKC- θ /NF- κ B pathway plays a central role in cell activation, proliferation and cytokine production (Schulze-Luehrmann & Ghosh, 2006), and also regulates the differentiation of CD4⁺ T cell. Indeed, it upregulates Th1 cells by promoting the production of IL-2 and IFN γ , and enhances Th17 responses by promoting the expression of Th17 specific transcription factors, such as ROR γ t and ROR α (Corn *et al.*, 2003; Ruan *et al.*, 2011). PKC- θ /NF- κ B pathway is also important for Tregs. It has been reported that the activation of c-Rel and p65 (RelA), two family members of NF- κ B, is required for the development and function of tTregs and pTregs, respectively (Oh *et al.*, 2017). Deregulation of this pathway is associated with numerous autoimmune diseases, which include RA, MS and IBD (Pai & Thomas, 2008). Mice deficient for CARMA1, an essential adaptor protein which operates up-stream of the NF- κ B, have CD4⁺ T cells which produce less IL-17 and are resistant to EAE (Molinero *et al.*, 2012). In an allergy mouse model, the absence of CARMA1 leads to decreased pulmonary eosinophilia and reduced production of IL4, IL5 and IL13 in bronchoalveolar lavages (Medoff *et al.*, 2006). In addition, mice in which c-Rel and p65 are knocked out specifically in Tregs develop severe inflammatory diseases, such as lethal autoimmune syndrome and Scurfy-like autoimmune syndrome (Oh *et al.*, 2017).

2.2.3. Ca²⁺-calcineurin-NFAT pathway

Like NF- κ B, NFAT can also translocate into the nucleus. Ca²⁺-calcineurin signaling pathway activated by TCR stimulation leads to the dephosphorylation of NFAT, which then forms a complex with AP-1 and bind to cognate DNA response elements, inducing the expression of genes related to T cell activation, such as IL-2 (Serfling *et al.*, 1989). NFAT is found in almost each type of T cells. In effector T cells, depending on the cytokine environment, the NFAT/AP-1 complex activates the key transcription factors in the different T cell subsets, such as T-bet in Th1 polarizing condition, GATA-3 in Th2, ROR γ t in Th17

and BATF in Tfh (Hermann-Kleiter & Baier, 2010; Macián *et al.*, 2001). However, in Tregs, NFAT binds to SMAD3 instead of AP-1, leading to the activation of FOXP3 transcription (Tone *et al.*, 2008). Due to the essential role of Ca^{+2} -calcineurin-NFAT pathway in T cell response, the calcineurin inhibitors CsA and FK506, which target signaling events upstream of NFAT, have been well used in the treatment of autoimmune disease including atopic dermatitis and lupus nephritis and organ transplants, while their usage often leads to serious side effects, such as neurotoxicity and nephrotoxicity. Some other NFAT inhibitors with higher specificity or targeting its binding protein are under investigation (Lee *et al.*, 2018; Park *et al.*, 2020).

2.2.4. AKT/mTOR pathway

The AKT/mTOR pathway is a central regulator of cell growth and metabolism. It's involved in these cell activities by integrating extracellular signals and stimulations, associated to energetic metabolites, stress signals and cytokines. mTOR functions as the core component of mTOR complex1 (mTORC1) and mTOR complex2 (mTORC2). These two complexes differentially regulate T cells differentiation (Chi, 2012). mTORC1 promotes Th17 polarization. Mice deficient for Rheb (activator of mTORC1) or Raptor (part of mTORC1 complex) in T cells have defective Th17 differentiation (Delgoffe *et al.*, 2011). Regarding mTORC2, the absence of Rictor, component of mTORC2, leads to impaired differentiation of naïve CD4^{+} T cells into Th1 and Th2 cells (Delgoffe *et al.*, 2011; Lee *et al.*, 2010). Meanwhile, pharmacological inhibition of these two mTOR complexes results in increased Tregs differentiation *in vitro* (Sauer *et al.*, 2008). By using EAE mouse model, it has been shown that the treatment with rapamycin, an mTOR inhibitor, can significantly inhibit EAE development by suppressing the production of $\text{IFN}\gamma$ and IL-17 *via* mediating mTOR-STAT3 and TAM-TLRs-SOCS signaling pathways (Hou *et al.*, 2017; Li *et al.*, 2020). Akt is a kinase upstream of mTOR, its several isoforms Akt1, Akt2 and Akt3 have distinct function on T cell functions. In EAE mouse model, Akt1^{-/-} mice show decreased EAE, whereas Akt2^{-/-} mice exhibit an increase in disease severity (Li *et al.*, 2020). Zhangjiang and collaborators found that Akt1 inhibits the proliferation of tTregs, which promotes antigen-specific Th1 and Th17 responses. By contrast, Akt2 facilitates tTregs development *in vitro* and *in vivo* (Ouyang *et al.*, 2019). Regarding Akt3, it is highly expressed in CNS and is important for brain development (Ouyang *et al.*, 2019). During EAE development, it protects the mice from the disease (Tsiperson *et al.*, 2013).

Those four TCR signaling pathways exert mostly positive effect on cell survival, growth and effector functions. To avoid the occurrence of autoimmunity, TCR signaling is regulated by negative regulators which reduce T cell responses. Although many different types of inhibitory processes can be engaged, we will focus here on tyrosine phosphatases.

3. Tyrosine phosphatases as regulators of TCR signaling

Protein tyrosine phosphatases (PTPs) regulate T cell responses by dephosphorylating key proteins involved in proximal TCR and cytokine receptors signaling (**Figure 13**). So far, 37 PTPs have been detected in murine mature T cells (Howden *et al.*, 2019). Many of them contribute to adjust T cell responses through the regulation of TCR signaling. Deregulation of their activity is involved in autoimmunity. The regulation of TCR signaling by PTPs and their potential application to immunotherapy have been reviewed in a recent publication (Castro-Sanchez *et al.*, 2020). SHP-1 and SHP-2 are two PTPs with SH2 domains which have been described above(c.f. Chapter I §2). In short, SHP-1 negatively regulates positive selection in the thymus (Choi, Warzecha, *et al.*, 2017), whereas SHP-2 has been described as a positive regulator of the β selection at early stages of T cell development (Qu *et al.*, 2001). Besides, they both constitutively binds to THEMIS in the thymus. In peripheral T cells, their deficiency also affects either positively or negatively the effector function of T cells development (Qu *et al.*, 2001). Besides, they both constitutively binds to THEMIS in the thymus. In peripheral T cells, their deficiency also affects either positively or negatively the effector function of T cells.

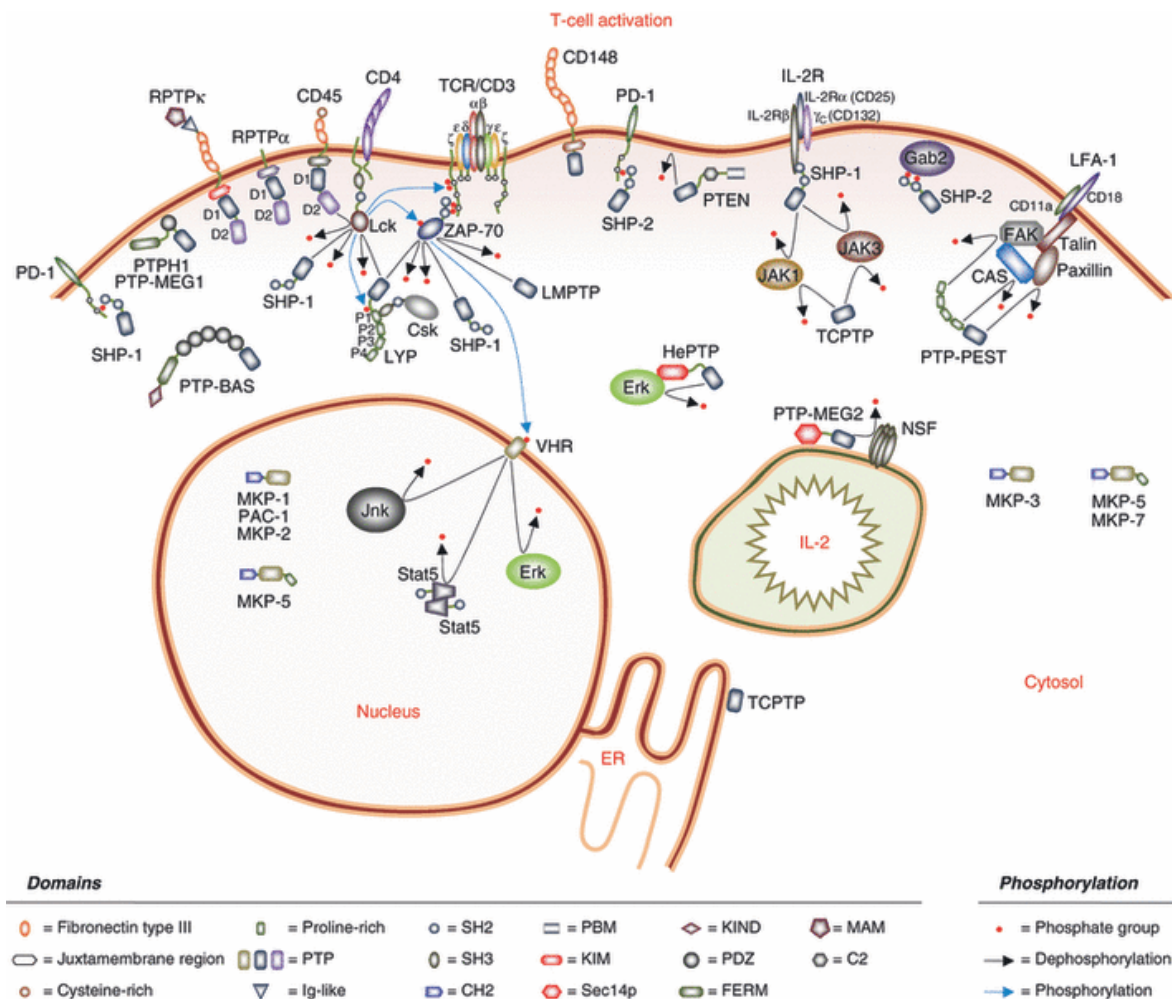


Figure 13. Several tyrosine phosphatases are involved in T lymphocytes responses

The TCR-pMHC engagement leads to tyrosine phosphorylation of many signaling proteins in T lymphocytes. Protein tyrosine phosphatase (PTP) plays an important role in establishing T cell activation and differentiation thresholds. Many types of PTP are involved in these processes, such as transmembrane, cytoplasmic and nuclear PTPs. Near the plasma membrane, these PTPs negatively regulate the phosphorylation levels of Src, TCR ITAM, and Zap-70 proteins, and weaken the TCR signal strength. PTP also de-phosphorylate some molecules that are involved in TCR-mediated distal signaling pathways and cytokine signaling pathways, such as MAP-K or STAT proteins, respectively. In some cases, PTPs can positively regulate T cell responses, such as SHP-2 which can enhance the activity of ERK1/2 when it is not recruited to co-inhibitory receptors.(Stanford *et al.*, 2012)

3.1. Effect of the Tyrosine phosphatase SHP-1 on T cell responses

SHP-1 is characterized as a broad negative regulator of TCR signaling. Mice deficient for SHP-1 exhibit increased frequencies of memory T cells characterized by highly expression of CD44 (Johnson *et al.*, 2013). Although conventional CD4⁺ T cells lacking SHP-1 respond normally to TCR stimulation *in vitro*, they are more resistant to Treg suppressive responses *in vitro* and *in vivo* (Johnson *et al.*, 2013; Mercadante & Lorenz, 2017). SHP-1 also regulates the suppressive function of Tregs. Tregs with absent or repressed expression of SHP-1 have a stronger ability to inhibit the response of conventional T cells *in vitro* and *in vivo*, and to repress inflammation *in vivo* (Iype *et al.*, 2010). Furthermore, SHP-1 represses signaling events associated to cytokine receptors. The absence of SHP-1 in CD4⁺ T cells leads to increased Th2 polarization *in vitro* by promoting the phosphorylation of STAT6 (Johnson *et al.*, 2013). In an OVA-induced allergic airway inflammation model, naturel SHP-1 mutant mice derived from viable motheaten (me^V) developed more severe allergic inflammation (Kamata *et al.*, 2003). In addition, loss of SHP-1 in CD4⁺ T cells causes increased phosphorylation of STAT3 and cell polarization towards Th17 phenotype (Mauldin *et al.*, 2012). Early studies on motheaten (me) and viable motheaten (me^V) found that undetectable and reduced SHP-1 expression can induce severe autoimmunity in the mice 2 to 3 weeks following their birth (Shultz *et al.*, 1993; Tsui *et al.*, 1993). During the development of EAE, SHP-1-deficient viable motheaten mice (me^{v+/-}) showed increased disease severity compared to wild type mice (Deng *et al.*, 2002). In the PBMCs of MS patients, higher level of pSTAT6 and low expression of SHP-1 were detected. Both SHP-1 siRNA and transduction of PBMCs with a lentiviral vector expressing SHP-1 decreased pSTAT6 (Christophi *et al.*, 2008).

3.2. Effect of the Tyrosine phosphatase SHP-2 on T cell responses

Unlike SHP-1, SHP-2 exerts both positive and negative roles on T cell responses according to its substrates and to the context of its recruitment to the membrane. Upon TCR stimulation alone, SHP-2 enhances effector T cell responses by positively regulating the activity of ERK, a positive actor of IFN γ production. Publications report that SHP-2^{-/-} T cells exhibit decreased proliferation and ERK activation upon anti-CD3 stimulation *in vitro* (Nguyen *et al.*, 2006). By dephosphorylating CBP, a Csk binding protein, SHP-2 inhibits the recruitment of Csk, a negative regulator of ERK activity, to ERK (Zhang *et al.*, 2004). SHP-2 also promotes ERK signaling upstream of the MAPK cascade by dephosphorylating

Ras-GAP, an inhibitor of Ras (Agazie & Hayman, 2003). SHP-2 has been reported to increase the severity of systemic lupus erythematosus (SLE) (Wang *et al.*, 2016). Treatment of lupus-prone MRL/lpr mice with SHP-2 inhibitor can ameliorate disease severity, leading to decreased cell proliferation, ERK/MAPK signaling and the production of IFN γ and IL-17A/F (Wang *et al.*, 2016), suggesting that SHP-2 also enhances ERK signaling in T cells during lupus erythematosus *in vivo*. One study on EAE found that treatment of C57BL/6 mice with SHP-2 inhibitor, NSC-87877, can almost completely protect the mice from EAE development (Luo *et al.*, 2014). This study demonstrates that NSC-87877 prevents EAE development by blocking the infiltration of T cells into the CNS. However, in the same study, mice with SHP-2 conditional knockout in peripheral T cells develop EAE comparable to that in wild-type C57BL/6 mice. The authors propose that NSC-87877 may also inhibit SHP-2 activity in other cells than T cells (Luo *et al.*, 2014). It may also be due to the off-target effects of NSC-87877.

It has been shown that SHP-2 negatively regulates cytokine induced Jak/STAT pathway in T cells. One previous study showed that over-activation of SHP-2 in Th1 cells decreases the production of Th1 cytokines by preventing the recruitment of STAT1 to IFN γ R through directly binding to STAT1 and sequestering it in cytoplasm (Wu *et al.*, 2012). Upon transfer with these cells, C57BL/6 mice present a significantly reduced 2, 4, 6-trinitrobenzene sulfonic acid-induced colitis. Furthermore, the decreased Th1 cytokine production and disease severity can be restored and even worsen by using SHP-2 inhibitor NSC-87877 or transferring conditional SHP-2 knockout T cells (Wu *et al.*, 2012). In the mouse model of Dextran sulfate sodium (DSS) -induced acute colitis, mice with T cell- specific conditional knockout of SHP-2 also exhibit more severe disease. Compared to the control mice, increased number of Th1 and Th17 cells are detected in their spleen and lamina propria, and higher amount of Th1 and Th17 cytokines are found in the mucosa (Liu *et al.*, 2017). Surprisingly, SHP-2 deficiency increases inflammation which can prevent the development of colitis associated cancers, such as carcinoma (Liu *et al.*, 2017). This protective phenomenon, however, is dependent on the location and stage of the tumor. In a mouse model of melanoma, the absence of SHP-2 in CD4⁺ T cells leads to increased melanoma metastasis in mice and inflammatory cytokines, especially IL-6 (Zhang *et al.*, 2013), suggesting that, in melanoma model, SHP-2 prevents the tumor progression and metastasis by repressing IL-6/STAT3 signaling in CD4⁺ T cells.

Taken together, SHP-2 can enhance T cell responses by positively regulating TCR signal-induced ERK activity, while SHP-1 operates more broadly as a negative regulator of TCR signaling. In the context of cytokine receptors signaling, both SHP-1 and SHP-2 dephosphorylate Jak/STAT and impair T cell differentiation and cytokine production. In the context of inhibitory co-receptors, they both dephosphorylate proteins in different signaling pathways, negatively regulating T cell responses. This will be described in detail in the following section (c.f. Chapter II §4).

4. Impact of Co-receptors on TCR signaling

In addition to specific antigen-induced signals, signals mediated by the engagement of co-stimulatory and co-inhibitory receptors on T cell surface are required for optimal T cell responses. Co-stimulatory signals contribute to full activation of T cells, while co-inhibitory signals function to repress T cell signaling, limiting T cell activation and preventing autoimmunity.

4.1. Immunoglobulin superfamily (IgSF)

IgSF is a large protein superfamily of cell surface and soluble proteins that share structural homology with immunoglobulins and are involved in cell recognition, binding and adhesion. CD4 and CD8 are two IgSF members that are required for T cell activation. They cooperate with TCR during the recognition of pMHC, causing the initiation of TCR signaling. Another IgSF subgroup that tightly regulate T cell activation is CD28 family, which is constituted of a large number of costimulatory receptors including CD28 and ICOS, and inhibitory receptors, such as CTLA-4, PD-1 and BTLA (Lieping Chen & Dallas B Flies, 2013) (**Figure 14**).

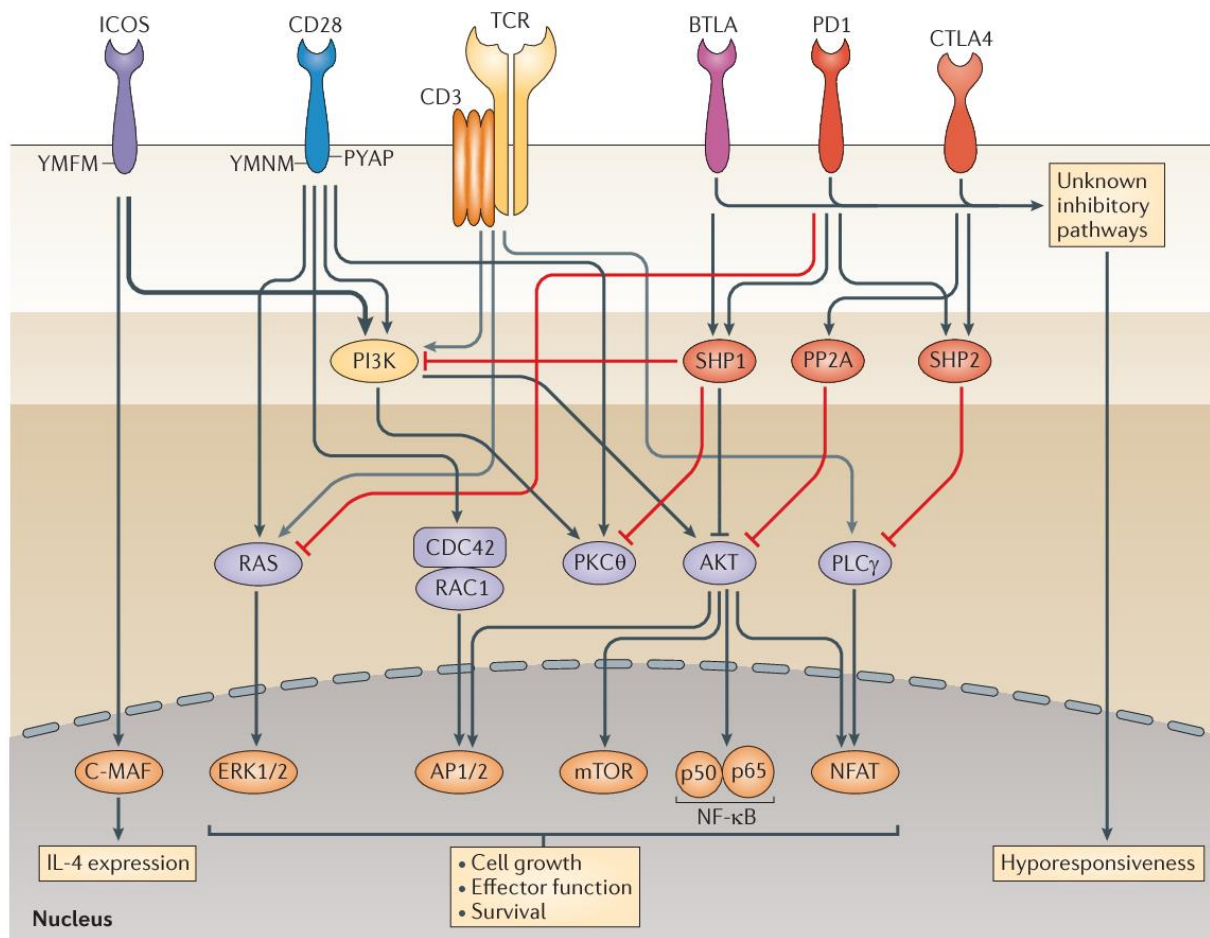


Figure 14. Co stimulatory and co inhibitory signaling pathways downstream of CD28 family receptors in T lymphocytes

Upon the activation of TCR complex, the signaling pathways initiated by the engagement of ICOS, CD28, BTLA, PD1 and CTLA-4 overlap, which allows fine regulation of the signals propagated by the TCR. The YMNM and PYAP motifs of CD28 recruit molecules involved in proximal TCR signaling to propagate positive signals in T lymphocytes. Recruitment of the Pi3K subunit p50a by ICOS leads to enhanced AKT signaling. Activated ICOS also induces IL-4 production through the c-Maf pathway. BTLA, PD1 and CTLA-4 can recruit different tyrosine phosphatases, including SHP-1, SHP-2 and PP2A, to dephosphorylate major signaling proteins that are important for T cell response, thereby leading to the down-regulation of T cell activation and function. In addition, these co-inhibitory receptors may also transduce positive signals in some context through additional pathways. (Lieping Chen & Dallas B Flies, 2013)

4.1.1. The Stimulatory co-receptor CD28

Among those co-receptors, only CD28 is expressed on naïve murine T cells, the others being induced only after T cell activation. CD28 has an intercellular domain containing a proximal YMNM motif and a distal PYAP motif, which enables CD28 to recruit several kinases and adaptors with SH2 and/or SH3 domains (Boomer & Green, 2010). The recruitment of SH2-containing proteins including PI3K, Grb2 and Gads to its YMNM motif as well as Src family kinases, such as Lck and Itk, to PYAP have been described as the major mechanisms to regulate TCR signaling (King *et al.*, 1997; Prasad *et al.*, 1994; Raab *et al.*, 1995). Upon TCR activation, their recruitment following the engagement of CD28 with its ligands B7-1 and B7-2 positively regulate the production of IL-2, pro-survival factor Bcl-XL, IFN γ and IL-4 by enhancing distal TCR signaling pathways (Bricker *et al.*, 2014; Kane *et al.*, 2001; Kubo *et al.*, 1999; Okkenhaug *et al.*, 2001; Shahinian *et al.*, 1993). T cells of CD28^{-/-} mice show normal activation but cannot induce IL-2 in response to lectins. CD28^{-/-} mice produce lower concentration of Immunoglobulins (Ig), and their CD8 T cells show delayed-type hypersensitivity respond to viruses *in vivo* (Shahinian *et al.*, 1993). CD28^{-/-} mice are resistant to EAE development, which corresponds to reduced MOG-specific IFN γ -producing cells and significantly less production of chemokine MIP-1 α (macrophage inflammatory protein-1 α) by T cells (Chitnis, Najafian, Abdallah, *et al.*, 2001; Karpus *et al.*, 1995; Perrin *et al.*, 1999). In CD4⁺ T cells, continuous CD28 signaling is required for the polarization and expansion of T cells in response to viral or bacterial infection (Linterman *et al.*, 2014). This signaling is also important for the generation of Treg *in vivo*. CD28^{-/-} and B7-1/B7-2^{-/-} mice present a dramatically decreased Treg count and develop spontaneous diabetes (Salomon *et al.*, 2000). However, in humans, increased CD4⁺CD28⁻ T cells, also called cytotoxic CD4⁺ T cells, are associated with aging and chronic diseases, such as RA (Martens *et al.*, 1997; Weyand *et al.*, 1998). Compared to normal CD4⁺ T cells, they express NK-cell receptors, produce more IFN γ and TNF α and downregulate Tregs activity (Appay *et al.*, 2002; Fasth *et al.*, 2010; Thewissen *et al.*, 2007; Téó *et al.*, 2013).

4.1.2. Stimulatory co-receptor ICOS

The signaling of ICOS, another co-stimulatory receptor that belongs to the CD28 family, is triggered by binding to ICOS-L. Besides its positive regulation of T cell activation, ICOS signaling has been reported to promote the development of Th2 cells (Dong *et al.*, 2001; Rudd & Schneider, 2003).

Indeed, ICOS $-/-$ CD4 $^{+}$ T cells produce less IL-4 but more IFN γ *in vitro* (Dong *et al.*, 2001). ICOS has also been reported to promote Th17 and Tfh responses (Bauquet *et al.*, 2009; Paulos *et al.*, 2010). One possible mechanism could be that it recruits SH2-domain containing protein PI3K, which upregulates downstream pathways, leading to the increased production of IL-4 and IL-21 (Gigoux *et al.*, 2009; T. R. Simpson *et al.*, 2010). Meanwhile, ICOS has been shown to promote the expression of IL-21 expression-related transcription factor c-Maf and the Tfh transcription factor Klf2, which therefore enhances the development of Th17 and Tfh (Bauquet *et al.*, 2009; Weber *et al.*, 2015). *In vivo*, ICOS has a complex role in different diseases model. In CIA, ICOS-deficient mice are completely resistant to disease induction (Dong & Nurieva, 2003). By contrast, these same mice exhibit extremely high susceptibility to EAE, presumably due to increased Th1 and Th17 cytokines (Dong & Nurieva, 2003; Galicia *et al.*, 2009). Moreover, in EAE model, selective blockade of ICOS during the priming stage (before day 10 after immunization) exacerbates disease development, whereas blocking ICOS later on (day 10 to 20) alleviates the disease (Sporici *et al.*, 2001).

4.1.3. Inhibitory co-receptor CTLA-4, PD-1 and BTLA

CTLA-4, PD-1 and BTLA act as “immune checkpoints” to prevent the over-activation of T cells in different immunological context. Among them, CTLA-4 was the first co-inhibitory receptor to be identified (Brunet *et al.*, 1987; Ishida *et al.*, 1992; Watanabe *et al.*, 2003). CTLA-4 is homologous to CD28 and they share the same ligands, B7-1(CD80) and B7-2(CD86), however their affinity for CTLA-4 is higher than for CD28 (Greene *et al.*, 1996). It has been shown that CTLA-4 negatively regulates IL-2 production as well as the duration of interaction between APC and T cells, thereby enhancing the inhibition of T cell activation and proliferation (Krummel & Allison, 1996; Schneider, Smith, *et al.*, 2008). CTLA-4 intracellular domain is similar to CD28, enabling CTLA-4 to recruit SH2- and SH3-domain containing proteins, such as the PI3K and tyrosine phosphatase SHP-2 and PP2A (Chuang *et al.*, 2000). The recruitment of PI3K by CTLA-4 leads to the activation of Rap1, a positive key regulator of T cell and APC interactions as well as of the polarization and motility of T cells (Wei *et al.*, 2007). In allergy, the recruitment of PI3K and AKT to CTLA-4 promotes the expression of anti-apoptotic factors Bcl2 and Bcl-XL to prevent cell death (Schneider, Valk, *et al.*, 2008). However, the recruitment of SHP-2 and PP2A to CTLA-4 dephosphorylates CD3, ZAP-70 and AKT (Parry *et al.*, 2005; Rudd *et al.*, 2009),

indicating that CTLA-4 has both positive and negative effects on T cell responses, depending on the molecules it recruits. In addition, CTLA-4 exhibits a dual influence on MAPK pathways since it inhibits the ERK activity but promotes JNK activation, driving the polarization towards Th2 lineage (Schneider *et al.*, 2002). Besides, although the expression of CTLA-4 is undetectable on naïve T cells, it is highly expressed on tTregs and activated pTregs (Perkins *et al.*, 1996; Walunas *et al.*, 1994). The conditional CTLA-4 knockout in Tregs leads to spontaneous development of systemic lymphoproliferation and autoimmune diseases, which demonstrates its crucial role in the suppressive function of Tregs (Wing *et al.*, 2008).

Compared to CTLA-4, **PD-1 and BTLA** both possess a cytoplasmic tail containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM), which binds and activates the tyrosine phosphatases SHP-1 and SHP-2, inhibiting tyrosine kinases-dependent signaling pathways (Chemnitz *et al.*, 2006; Chemnitz *et al.*, 2004). In a similar way to CD28, ICOS and CTLA-4, the ligands of PD-1, PD-L1 and PL-L2, belong to B7 family while the ligand of BTLA, herpesvirus entry mediator (HVEM), is part of the TNF-R family (Freeman *et al.*, 2000; Sedy *et al.*, 2005).

Following phosphorylation, PD-1 recruits SHP-2 rather than SHP-1 (Xu *et al.*, 2020). PD-1-associated SHP-2 dephosphorylates TCR proximal signaling molecules CD28, CD226 and CD3 (Hui *et al.*, 2017). PD-1 deficiency in mice results in the development of lupus-like autoimmune diseases (Nishimura *et al.*, 1999). *In vitro*, the engagement of PD-1 with PD-L1 leads to a decrease in the proliferation of anti-CD3-stimulated T cells, which is related to the reduction in IL-2 and IFN γ production (Carter *et al.*, 2002; Freeman *et al.*, 2000). PD-1/PD-L1 interaction leads to different outcomes on the responses of different CD4 subpopulations. A previous study on asthma showed that blocking PD-1/PD-L1 interaction in mice leads to enhanced airway hyper reactivity (McAlees *et al.*, 2015), which is associated with increased development of Th17 cells but no obvious change in Th2 response. *In vitro* analysis of this study showed that PD-1 ligation leads to reduced differentiation of naïve CD4⁺ T cells into Th1 and Th17 cells but to a slight increase of Th2 polarization.

PD-1^{-/-} CD4⁺ T cells produce higher amount of IFN γ and IL-17 but decrease amount of IL-13 in response to ConA or anti-CD3 antibody stimulation *ex vivo*, indicating that PD-1/PD-L1 interaction inhibits Th17 response but slightly enhances Th2 response, and that the increased Th17 cell development can lead to worse asthma. In addition, a study showed that the transfer of naïve CD4⁺ T cells to PD-L1(-/-) PD-L2(-/-) RAG(-/-) mice resulted in a significant reduction in the number of pTregs. This study demonstrated that PD-1 signaling promotes pTregs development by inhibiting the phosphorylation of AKT, mTOR and ERK (Francisco *et al.*, 2009).

Regarding BTLA, the HVEM/BTLA interaction allows the recruitment of both SHP-1 and SHP-2 phosphatases to BTLA (Celis-Gutierrez *et al.*, 2019; Gavrieli *et al.*, 2003). However, some studies have found that BTLA recruits preferentially SHP-1 over SHP-2 in activated CD4 T cells (Chemnitz *et al.*, 2006; Mintz *et al.*, 2019). BTLA-associated SHP-1 recruitment potentially dephosphorylates CD28 and CD3 (Sedy *et al.*, 2005). The binding of BTLA on T cells by HVEM leads to decreased proliferation and IL-2 production *in vitro* (Sedy *et al.*, 2005). *In vivo*, reduced level of BTLA was detected in PBMC and CD4⁺ T cells from patients with Behcet's disease (BD), a chronic inflammatory disease associated with hyperactive Th1 and Th17 responses (Ye *et al.*, 2016). In addition, transfer of CD4⁺CD45RB^{hi} BTLA^{-/-} T cells to RAG2^{-/-} mice can induce more severe IBD in the recipients than transfer with BTLA-expressing T cells (Steinberg *et al.*, 2008). However, in the same study, BTLA^{-/-} mice did not develop spontaneous autoimmune diseases such as PD-1^{-/-} mice. This observation could be explained by the dual regulatory influence of BTLA in T cells. Indeed, compared with PD-1, in addition to ITIM and ITSM motifs, BTLA possesses two additional tyrosine residues (Y226 and Y243) N-terminal to the ITIM, which may enable direct binding of Grb2 protein and indirect recruitment of PI3K through Grb2, resulting in the stimulation of the PI3K pathway and thereby promoting T cell proliferation and survival (Gavrieli & Murphy, 2006). Yet, SHP-1 and SHP-2 recruitment is not the only mechanisms used by PD-1 and BTLA to exert their inhibitory function. It has been shown that, in activated T cells in absence of both SHP-1 and SHP-2, proliferation and cytokines production can still be suppressed by PD-1 and BTLA (Xu *et al.*, 2020). In addition, PD-1 negatively regulates the activation of ERK, independently of SHP-1 and SHP-2, to block the G1-S transition of the cell cycle and proliferation of T cells (Patsoukis *et al.*, 2012).

Interestingly, suppressing T cells responses by activating immune checkpoints is an effective immune evasion strategy employed by tumor cells. Therefore, co-inhibitory signals involved in the development of various cancers have been extensively studied. Thanks to Nobel prize winners Dr. James P Allison and Dr. Tasuku Honjo's contribution on the discovery of cancer therapy by inhibition of immune checkpoints, many therapeutic antibodies based on CTLA-4 and PD-1 blockade are currently approved by the U.S. Food and Drug Administration for cancer treatment (FDA), or are evaluated in clinical trials (Ansell *et al.*, 2015; Topalian *et al.*, 2012). For example, ipilimumab (anti-CTLA4) has been used for the treatment of metastatic melanoma, nivolumab (anti-PD-1) is used for treating many types of tumor including melanoma and non-small cell lung cancer, and a combination therapy with ipilimumab plus nivolumab has been approved for the treatment of Renal cell carcinoma (Iwai *et al.*, 2017; Wei *et al.*, 2018). In addition to CTLA-4, PD-1 and BTLA, a lot of other inhibitory co-receptors in IgSF, such as LAG-3, VISTA and TIGIT have become new targets for research related to immune tolerance, autoimmune diseases and antitumoral immunotherapy (Attanasio & Wherry, 2016; Śledzińska *et al.*, 2015).

4.2. Engagement of CD5

During my PhD, I have contributed to a study on the signaling function of CD5 (Blaize *et al.*, 2020), a type 1 transmembrane cell surface glycoprotein that belongs to Scavenger Receptor Cysteine-Rich super family (SRCR-SF). Only two members of this family are expressed by T cells, CD5 being one of them. As for other co-receptors, several ligands of CD5 have been reported, but its biological activity doesn't require their interaction (Davies *et al.*, 1992). After TCR stimulation, the intracytoplasmic domain of CD5 is phosphorylated by Lck (Davies *et al.*, 1992; Dennehy *et al.*, 2001). Previous studies showed that increased expression of CD5 on T cells are correlated with the affinity of the TCR for self-pMHC in T cells, suggesting that CD5 could be important for the maintenance of tolerance (Azzam *et al.*, 1998; Mandl *et al.*, 2013). Based on TCR engagement, the loss of CD5 in T cells leads to increased mobilization of calcium flux and phosphorylation of several signaling proteins, such as TCR, Zap-70 and VAV1, and the engagement of CD5 by antibodies decreases the phosphorylation of those proteins, which indicates a negative role for CD5 on TCR signaling (Bhandoola *et al.*, 2002; Perez-Villar *et al.*, 1999; Tarakhovsky *et al.*, 1995). However, opposite role of CD5 were shown in other studies.

The co-engagement of CD3 and CD5 by antibodies leads to an increase phosphorylation of PI3K, ERK and AKT in both human T lymphocytes and murine CD4⁺CD8⁺ thymocytes *in vitro* (Gringhuis *et al.*, 1998; Zhou *et al.*, 2000). *In vivo*, CD5^{-/-} mice show decreased susceptibility to the development of EAE and IBD (Axtell *et al.*, 2004; Dasu *et al.*, 2008). Later findings show that the decreased T cell responses caused by the absence of CD5 is associated with increased number and activity of Tregs. Compared to CD5^{+/+} mice, CD5^{-/-} mice show alleviated IBD severity and increased Tregs count in their colon (Dasu *et al.*, 2008). The differentiation of CD5-deficient CD4⁺ T cells/thymocytes into Tregs can be inhibited by very low amount of IL-4, IL-6 and IFN γ in response to self and tolerizing antigens (Henderson *et al.*, 2015). In addition, CD5^{-/-} Balb/c mice show an increase in the number of Tregs in the thymus and in peripheral lymphoid organs, confirming the role of CD5 in the generation of Tregs (Ordoñez-Rueda *et al.*, 2009). This study also found that CD5 increases the phosphorylation of AKT in naïve CD4⁺ T cells but decreases the level of p-ERK in Tregs, suggesting a complex regulation mechanism of CD5 on TCR signaling. Together with other studies, a recent work conducted in our team to which I have contributed has identified several proteins interacting with CD5, such as PI3K, c-Cbl, CIN85, UBASH3A and SHIP, including thus both positive and negative regulators of TCR signaling (Blaize *et al.*, 2020; Dennehy *et al.*, 1998; Dennehy *et al.*, 1997; Gringhuis *et al.*, 1998). Our study found that, upon TCR activation, tyrosine 429 of CD5 is selectively phosphorylated and recruit a signalosome composed of c-Cbl, CIN85 and CRKL. This signalosome, especially c-Cbl, helps to connect CD5 with the positive (PI3K) and negative (UBASH3A, SHIP1) regulators of TCR signaling, coordinately repressing the activity of ERK and promoting AKT activity to inhibit FOXP3 expression (Blaize *et al.*, 2020).

Chapter III Role of THEMIS in peripheral T cells

THEMIS is expressed in peripheral T-cells although less than in thymocytes (Fu *et al.*, 2009; Lesourne *et al.*, 2009). It's well known that THEMIS is crucial for thymocyte development (Fu *et al.*, 2009; Johnson *et al.*, 2009; Kakugawa *et al.*, 2009; Lesourne *et al.*, 2009; Patrick *et al.*, 2009) and the mechanisms of THEMIS function in the thymus have been thoroughly studied (c.f. Chapter I §3). However, the role of THEMIS in peripheral T cells still remains elusive, as *themis*^{-/-} mice present a severe T cell lymphopenia. In thymocytes, THEMIS is involved in TCR signaling since it is indirectly recruited to LAT by interacting with Grb2 after TCR stimulation (Brockmeyer *et al.*, 2011; Lesourne *et al.*, 2012; Paster *et al.*, 2013). In peripheral CD4⁺ T cells, THEMIS and Grb2 show similar kinetics of interaction with LAT, with maximum binding observed at 2min and maintained for up to 5min after anti-CD3 and anti-CD4 stimulation (Roncagalli *et al.*, 2014), suggesting that THEMIS is involved in TCR signaling in peripheral T cells and may play a crucial role within these cells.

1. THEMIS is important for the suppressive function of regulatory T cells

An early study on THEMIS in Brown Norway (BN) rats showed that *themis*-deficient BN rats (BN^{*themis*-/-}) present a defect in the suppressive activity of CD4⁺ CD25^{bright} T cells, resulting in spontaneous development of IBD (Chabod *et al.*, 2012). These BN^{*themis*-/-} rats also exhibit a sharp and specific decrease in the number of effector CD4⁺ T cells and tTregs. In addition, they display an increase of activated CD4⁺ T cells producing elevated levels of Th2/Th17 cytokines, but which show reduced IFN γ production as compared to that in BN control rats (Chabod *et al.*, 2012). This study initially suggested that THEMIS might positively regulate the suppressive function of Tregs, preventing the pathogenesis of IBD. Indeed, further research on Tregs subset through proteomic analysis revealed the importance of THEMIS in controlling the inhibitory function of the cells (Duguet *et al.*, 2017). The authors found that the expression of THEMIS in Tregs was strongly down-regulated compared to conventional T cells. In addition, Tregs purified from *themis*_{tg} mice – over-expressing *themis* – showed better suppressive activity on the proliferation of conventional T cells *in vitro* compared to Tregs of *themis*_{wt} mice. Finally, co-injection of CD4⁺CD25⁻CD45RB^{high} colitogenic T cells with Tregs purified from *themis*_{tg} mice exhibited better protection against the development of IBD than coinjection with Tregs from *themis*_{wt} mice (Duguet *et al.*, 2017).

However, the positive regulation of THEMIS on the inhibitory function of Tregs does not only depend on THEMIS. In *themis*-deficient LEWIS (LEW) rats, the same research team found that these rats exhibited the same lymphopenia as BN^m rats, but had no impairment in Tregs suppressive function and no spontaneous development of IBD or other diseases (Pedros *et al.*, 2015). Compared with LEW background, BN rats contain a 117-kb interval region harboring a nonsynonymous polymorphism affecting the expression and functions of VAV1 (Colacios *et al.*, 2011). The authors hence generated BN^m rats deficient for THEMIS and in which the 117-kb interval from LEW rats was introduced. They found that the introduction of the 117-kb interval region from LEW rats restored Tregs function in BN^{themis-/-} rats and protected them from spontaneous IBD development, highlighting the importance of THEMIS/VAV1 interaction on Treg suppressive function and susceptibility to IBD development (Colacios *et al.*, 2011).

2. THEMIS regulates the metabolism reprogramming of CD4 T cells

Little is known about the function of THEMIS in peripheral T cells because of lymphopenia in *themis*^{-/-} mice. An initial study conducted by Paul Love and collaborators found that, compared with *themis*^{+/+} mice, *themis*^{-/-} peripheral CD4⁺ T cells had a comparable proliferation profile in response to anti-CD3 stimulation (Lesourne *et al.*, 2009). However, several publications from Nicholas R. J. Gascoigne's team show that *themis*^{-/-} peripheral CD4⁺ T cells are poorly responsive to anti-CD3 stimulation and show reduced proliferative responses and reduced cytokine production under stimulation with anti-CD3/anti-CD28 antibodies *in vitro* (Fu *et al.*, 2009; Prasad *et al.*, 2020). *In vivo*, *themis*^{-/-} CD4⁺ T cells have reduced ability to develop IBD, showing weaker inflammatory potential compared with *themis*^{+/+} peripheral CD4 T cells (Prasad *et al.*, 2020). In addition, their studies showed that *themis*^{-/-} CD4⁺ T cells have reduced glucose uptake, mitochondrial dysfunction and mTOR signaling responses, showing defective metabolomic reprogramming (Prasad *et al.*, 2020). Under the same conditions of TCR stimulation, the absence of THEMIS in CD4⁺ T cells leads to reduced expression of insulin receptor (IR), a protein that initializes insulin signaling pathway and, like THEMIS, binds to Grb2 and shares common downstream signaling pathways with TCR, such as ERK and mTOR activation (Brockmeyer *et al.*, 2011; Saltiel & Kahn, 2001). *themis*^{-/-} CD4⁺ T cells also exhibit defective nutrient uptake, mitochondrial function and mTOR upregulation under TCR stimulation, which indicates a connection

to the reduced TCR response (Prasad *et al.*, 2020). Indeed, the authors found that *themis*^{-/-} CD4⁺ T cells have decreased nuclear translocation of NFAT, a transcription factor which is regulated by TCR signaling and leads to transcription programs for T cell activation and effector functions, such as IL-2 (Macián *et al.*, 2001). As Chip-seq and RNA-seq demonstrated, that the nuclear translocation of NFAT controls IR gene expression, which suggests that the defective TCR signal in *themis*^{-/-} CD4⁺ T cells causes reduced NFAT nuclear translocation which in turn results in decreased IR expression, defective metabolic reprogramming and mTOR upregulation, culminating in impaired effector functions, including proliferation, cytokines production and inflammatory potential to cause colitis (Prasad *et al.*, 2020). Altogether, these data lead the authors to conclude that THEMIS regulates metabolic signaling and effector functions in CD4⁺ T cells by controlling NFAT nuclear translocation (Prasad *et al.*, 2020).

However, the defective metabolism and effector function of *themis*^{-/-} peripheral CD4 T cells may not be caused by the intrinsic deficiency of THEMIS in peripheral CD4⁺ T cells and may result from the abnormal maturation of CD4-SP caused by the impaired thymic development. This indirect defect due to lymphopenia could also result in CD4⁺ T cell functional anomalies. At this stage, in order to better understand the role of THEMIS in peripheral CD4⁺ T cells, an appropriate conditionally knocked model seems necessary.

3. THEMIS is required for the maintenance of peripheral CD8 T cells

One recent study published by Nicholas R. J. Gascoigne and collaborators shows THEMIS is required for the maintenance of peripheral CD8⁺ T cells and enhances homeostatic signals resulting from the integration of signals induce by weak TCR stimulation and by cytokines receptors such as IL-7R (Brzostek *et al.*, 2020). To investigate the role of THEMIS in peripheral T cells, the authors generated *themis* conditional knockout (cKO) mice by crossing *themis*^{flox/flox} mice with dlLck-Cre⁺ in the OT-I TCR transgenic background. In this way, their OT-I *themis*-cKO mice express H-2Kb-restricted TCR that can recognize a well-defined series of agonist peptides with different affinities (Hogquist *et al.*, 1994). In this study, OT-I *themis*-cKO mice exhibit normal thymocytes development and normal CD4⁺ T cells number but present a 50% reduction in peripheral CD8⁺ T cells count (Brzostek *et al.*, 2020).

OT-I *themis*-cKO mice and acute THEMIS deletion in peripheral T cells using tamoxifen-inducible ERT2-Cre both lead to reduced CD5 expression on CD8 SP thymocytes and on peripheral CD8⁺ T cells separately, leading to an increase in CD8 surface expression, which suggests a role of THEMIS in the regulation of signaling from Self-pMHC (Brzostek *et al.*, 2020). Results of lymphopenia induced proliferation (LIP) show that the transfer of OT-I *themis*-cKO CD8 T cells into RAG1^{-/-} mice induces an important loss in proliferation capacity, expression of CD44 and CD122 as well as IFN γ production, compared to OT-I *themis*-wt CD8 T cells, indicating that THEMIS is required for the activation and effector functions of CD8 T cells *in vivo* (Brzostek *et al.*, 2020). However, CD8⁺ T cells from OT-I *themis*-cKO and from its wild type control show a similar activation, proliferation, TCR signaling pathway response and cytokine production in response to different doses of OVA-based peptide ligands *in vitro*. Moreover, no difference is detected between these two types of CD8⁺ T cells concerning IL-7 signaling and response, a necessary component besides self-pMHC for CD8⁺ T cell maintenance and for LIP, suggesting that THEMIS deficiency does not alter agonistic pMHC ligand discrimination and IL-7 signaling responses in CD8⁺ T cells (Brzostek *et al.*, 2020). Other *in vitro* results of this study show that *themis*-cKO OT-I CD8⁺ T cells exhibit reduced proliferation, Akt signaling and c-Myc upregulation in response to stimulation with low-affinity pMHC in presence of IL-2 alone, IL-7 and IL-18 or IL-7 and IL-12. To assess the role of THEMIS in signal integration between TCR and cytokine receptors in CD8⁺ T cells, the authors investigated whether the absence of THEMIS affects CD8⁺ T cells response to *Listeria monocytogenes* expressing OVA (LM-OVA). The result showed that *themis*-cKO CD8⁺ T cells have reduced response to *Listeria monocytogenes* infection *in vivo*, exhibiting a decrease in the proliferation, cytokines production and IL-2/IL-12 signaling responses, which indicates that THEMIS promotes anti-bacterial CD8⁺ T cell responses *in vivo* by modulating signal integration from low-affinity TCR and cytokine receptor (Brzostek *et al.*, 2020). Furthermore, the conditional knockout of SHP-1 can rescue the reduced cell numbers of CD8 T cells in OT-I *themis*-cKO mice and the impaired activation and effector functions of these CD8 T cells in response to low-affinity TCR and cytokine signal integration, which suggests the negative role of SHP-1 in CD8 homeostasis and indicates that THEMIS function in peripheral CD8 T cells is mediated through SHP-1 phosphatase (Brzostek *et al.*, 2020). This study also checked the effect of conditional THEMIS deficiency in peripheral CD4⁺ T cells. Although peripheral CD4⁺ T cells from *themis*-cKO have reduced CD5 expression, they proliferate

normally in response to TCR ligation alone or in combination with IL-7 or IL-15 stimulation, suggesting a specific effect of THEMIS on signal integration in CD8⁺ T cells (Brzostek *et al.*, 2020). However, this study did not analyze the effect of THEMIS on the polarization of CD4⁺ T cells *in vitro* and *in vivo*.

Taken together, these data suggest that THEMIS enhances the maintenance and effector function of peripheral CD8⁺ T cells in response to low-affinity pMHC and cytokine-mediated signals by inhibiting the phosphatase SHP-1. However, the role of THEMIS in peripheral CD4⁺ T cells is still poorly explored particularly because the conditional knockout model based on the transgenic Cre expression driven by the distal Lck promoter doesn't allow complete gene deletion in peripheral CD4⁺ T cells (D'Souza *et al.*, 2008)

4. THEMIS is involved in CAR-T cell-induced tumor regression by recruiting SHP-1 to the CAR synapse

Chimeric Antigen Receptors (CARs) are synthetic molecules composed of a single chain variable fragment (scFv), co-stimulatory moieties (either CD28 or 4-1BB) and a CD3 ζ signaling domain. CARs expression in CD4⁺ and CD8⁺ T cells triggers their lytic machinery and co-stimulation after antigen engagement (Dotti *et al.*, 2014; Zhao *et al.*, 2015). CAR-T cell co-stimulation mediated by CD28 and 4-1BB plays an essential role in CAR-T cell-induced tumor regression and has been associated with distinct antitumor kinetics, as CD28-mediated CAR-T cell co-stimulation leads to faster antitumoral activity showing pronounced glycolytic metabolism and higher susceptibility to exhaustion while the 4-1BB-mediated co-stimulation results in predominantly oxidative metabolism and lower susceptibility to exhaustion (Kawalekar *et al.*, 2016; Long *et al.*, 2015; Zhao *et al.*, 2015). One recent publication in *Cancer Cell* shows that the antitumoral kinetics of CAR-T cells is correlated with the recruitment of Lck and THEMIS-SHP-1 complex to the CAR-T cell / APC synapse (Sun *et al.*, 2020). In this study, the recruitment of Lck to the synapse of CD28-encoding CAR-T cells causes antigen-independent CAR-CD3 ζ phosphorylation and increased antigen-dependent T cell activation. By contrast, the synapse formed by 4-1BB-engaged CAR-T cells recruits the THEMIS-SHP1 complex leading to reduced CAR-CD3 ζ phosphorylation, which can be overcome by overexpression of Lck (Sun *et al.*, 2020).

Co-immunoprecipitation experiments show that FLAG-tagged human THEMIS can pull down 4-1BB-encoding CAR in T cells and Jurkat cells. While this interaction is abolished when the gene encoding for 4-1BB is mutated, which corresponds to increased CAR-CD3 ζ phosphorylation, Ca²⁺ influx, CD69 expression and IFN γ production, indicating that THEMIS is able to directly interact with 4-1BB-encoded CAR synapse (Sun *et al.*, 2020). However, since THEMIS does not have direct phosphatase activity, the dephosphorylation function of THEMIS-SHP-1 complex must come from SHP-1. Indeed, this same study shows that modified SHP-1, able to be directly recruited to the CAR synapse without interacting with THEMIS, can attenuate the activation of CD28 encoding CAR-T cells *in vitro* and *in vivo* (Sun *et al.*, 2020). Taken together, these results demonstrate that the indirect recruitment of SHP-1 to 4-1BB through THEMIS tunes Lck-mediated CAR-induced T cells priming and that the balance between the recruitment of THEMIS-SHP-1 complex and Lck to CAR synapse regulates the antitumoral kinetics of CAR-induced T cells (Sun *et al.*, 2020).

5. Association between THEMIS genetic variants and autoimmune disorders

Genome-wide association studies (GWAS) have identified *themis* as a potential susceptibility gene for the development of autoimmune diseases such as MS (Davies *et al.*, 2016; Sawcer *et al.*, 2011) and Celiac disease (Bondar *et al.*, 2014) and inflammatory diseases including IBD (Jostins *et al.*, 2012; Liu *et al.*, 2015) and psoriasis (Kim *et al.*, 2015). Further investigation is needed to clarify the role of THEMIS in these pathologies. In the disease model of experimental cerebral malaria (ECM), ENU-induced *themis* mutant mice (*themis*^{I23N}) have reduced disease severity after infection by *Plasmodium berghei* ANKA compared to wild type mice (Torre *et al.*, 2015). This is associated with reduced cerebral cellular infiltration, retention of blood-brain barrier integrity and decreased production of pro-inflammatory cytokines including IFN γ and TNF α by *themis*^{I23N} CD4⁺ and CD8⁺ T cells, suggesting that THEMIS may enhance the pro-inflammatory function of peripheral T cells in response to parasitic infection *in vivo*. To confirm the effect of *themis* mutation on T cell responses *in vivo*, both control and *themis*^{I23N} mice were infected with *Mycobacterium tuberculosis*, leading to a more severe infection in *themis*^{I23N} mice. This result indicates that fully functional THEMIS is necessary for effector T cell response against mycobacterial infections (Torre *et al.*, 2015).

Overall, this study shows that THEMIS is required for the neuroinflammation of cerebral malaria and protection against *M. tuberculosis*, which may correspond to its enhancement on the pro-inflammatory responses of peripheral T cells. However, further study is necessary, as *themis*^{123N} mice display peripheral lymphopenia (Torre *et al.*, 2015).

In summary, human THEMIS is involved in the antitumoral kinetics of CAR T cells by recruiting SHP-1 to the synapse. In mouse, the studies in *themis*^{-/-} peripheral T cells demonstrate that THEMIS is important for the suppressive function of Tregs and for the metabolism of peripheral CD4⁺ T cells. It is also required for pathogenesis of Experimental Cerebral Malaria and protection against Pulmonary Tuberculosis. In addition, THEMIS sustains the maintenance of peripheral CD8⁺ T cells by regulating T cell receptor and cytokine signal integration. However, the various mouse models used for all these studies exhibit major drawbacks. Firstly, the impact of lymphopenia of *themis*^{-/-} mice cannot be ignored and secondly, it must be kept in mind that *themis* deletion in peripheral CD4 T cells is incomplete in the conditional knockout dILck-Cre *themis* mouse model.

Objectives

THEMIS is expressed in peripheral T cells, but its role in these cells, especially on CD4⁺ T cell responses, is still poorly understood. In humans, *themis* has been identified as a susceptibility gene in early GWAS on patients suffering from multiple sclerosis, a demyelinating disease which is tightly related to the inflammation process mediated by T cells. The functions of THEMIS in the frame of this disease needs to be explored using the relevant mouse model of EAE.

Therefore, this project aims to study the function of protein THEMIS in normal and pathological peripheral CD4⁺ T cell responses.

Studies on CD4⁺ T cells were based so far on mouse models with germline THEMIS deficiency, which may lead to indirect effects on those cells due to the block on T cell development and conditional models that do not always induce efficient deletion of candidate gene in this population. In order to avoid defects related to lymphopenia in peripheral T cells, we generated a conditional *themis* knockout mouse line CD2-Cre-*themis*^{fllox/fllox} (named as *themis*-T^{KO}), in which *themis* is invalidated by the Cre recombinase controlled by human CD2 promoter which is active after positive selection in the thymus. To focus on the effect of THEMIS on CD4⁺ T cells, we also generated CD4-Cre^{ERT2}-*themis*^{fllox/fllox}, in which *themis* is conditionally deleted in peripheral CD4⁺ T cells by CD4-Cre^{ERT2} following tamoxifen treatment.

The result section investigates the function of THEMIS on peripheral CD4⁺ T cell responses from the following three main aspects:

1. Phenotype of thymocytes and peripheral T cells in *themis*-T^{KO} mice.
2. Effect of THEMIS deficiency on the activation and differentiation of CD4⁺ T cells upon TCR triggering *in vitro* and foreign antigen recognition *in vivo*.
3. Effect of THEMIS deficiency on CD4⁺ T cells in the context of EAE development.

Results

**THEMIS enhances the magnitude of normal and neuroinflammatory Th1
responses by promoting TCR-independent signals**

(Under review)

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Abstract

Signals that determine the differentiation of naïve CD4⁺ helper T cells (Th) into specific effector subsets are primarily triggered by cytokines but additional signals are required to adjust the magnitude of Th responses and set the balance between effective immunity and immunological tolerance. By inducing a post-thymic deletion of the T-cell lineage signaling protein THEMIS, we show that THEMIS promotes the development of optimal Th1 responses to foreign antigens but triggers signals that in return favour encephalitogenic responses to self neuroantigens. THEMIS exerts positive effects on T-BET expression and on IFN γ production and enhances the ability of encephalitogenic CD4⁺ T cells to migrate into the central nervous system. Although the phenotype observed *in vivo* is indicative of positive function of THEMIS on TCR-mediated responses, we show that THEMIS exerts opposite effects *in vitro* by inhibiting TCR-mediated signals leading to Th1 responses, indicating that THEMIS exerts TCR independent functions which increase the magnitude of normal and pathogenic Th1-mediated responses.

Introduction

One essential feature of the immune system is its ability to engage distinct defensive strategies adapted to the variety of invading pathogens. This is partially dependent on CD4⁺ helper T cells (Th) which may differentiate from a so-called naïve state into distinct effector subsets (Th1, Th2, Th17, T-follicular helper cells [Tfh], induced-regulatory T cells [iTreg], Type-1 regulatory T cells [Tr1]) with specialized immune functions. The lineage choice decision of Th cells results from the complex integration of signaling events triggered by the T cell antigen receptor (TCR), which recognize self- or foreign-peptide ligands bound to major histocompatibility complex (pMHC) at the surface of antigen presenting cells, and by a combination of cytokine receptors which are stimulated specifically according to the cytokine milieu at the site of CD4⁺ T cell activation (1). In addition to those cell-fate decision signals, additional signaling events triggered by the TCR and by co-stimulatory and co-inhibitory receptors (2) contribute to regulate the magnitude of specific effector responses, promoting thereby effective immunity and preventing the development of potentially pathogenic inflammatory responses.

THEMIS is a T cell lineage-specific protein that has important role during T cell development (3-5). The phenotype of THEMIS deficient mice shows that THEMIS is essential for the positive selection of CD4⁺CD8⁺ (double-positive [DP]) thymocytes (3-5) and for their commitment to the CD4⁺ lineage (5). THEMIS contains a proline-rich C-terminal sequence (PRS), which binds to the adaptor protein GRB2 (5, 6) that is required for the recruitment of THEMIS to the transmembrane adaptor LAT following TCR engagement (5, 6), and two globular cysteine-containing, all- β in THEMIS (CABIT) domains (4), which bind to the phosphatase domains of the tyrosine phosphatases SHP-1 and SHP-2 (7).

Although THEMIS has been characterized as an important regulator of T cell development more than a decade ago, the mechanism by which it operates in this context has remained the subject to nearly opposite interpretations (8). A first model posits that THEMIS dampens TCR signaling initiated by low-affinity self-peptide-bound MHC (pMHC) interactions, preventing

the transmission of a strong signaling response to these stimuli that would trigger negative selection (9). THEMIS is speculated here to function by promoting SHP-1 and SHP-2 phosphatase domain activation and or by facilitating their recruitment to LAT where they are brought into contact with their primary targets (9, 10). This model was mostly deduced from *in vitro* studies in which preselected “naïve” DP thymocytes were stimulated with low-affinity pMHCs tetramers (9). More recently, a second model has been proposed in which THEMIS operates as an enhancer of TCR signaling, which enables thymocytes to reach the threshold for positive selection, avoiding death by neglect (7, 11). THEMIS operates here by blocking the catalytic function of the tyrosine phosphatase SHP-1, which has inhibitory effect on TCR signaling (7). This action is mediated by THEMIS’s CABIT modules, which bind to the phosphatase domain of SHP-1 and promote or stabilize the oxidation of SHP-1’s catalytic cysteine residue, inhibiting the tyrosine-phosphatase activity of SHP-1 (7). In this second model, the enhancing effects of THEMIS on TCR signaling were mainly deduced from *in vivo* observations using TCR-signal sensing mouse models (11) and complementation studies with SHP-1 deficient mice (7). Although the second model is more likely to explain the block of T cell development observed in THEMIS deficient mice and although the repressing effect of THEMIS on SHP-1 and SHP-2 phosphatase activity is based on solid molecular evidences (7), the reasons for the apparently contradictory findings remain unexplained.

The role of THEMIS during thymic selection has been well characterized, yet less is known about its function in peripheral T cells. Conditional disruption of the gene encoding THEMIS at late stage of T cell development using CRE recombinase transgenes driven by the distal *lck* promoter identified a positive role for THEMIS in controlling peripheral CD8⁺ T cell maintenance (12). The impaired homeostasis of peripheral CD8⁺ T in THEMIS-deficient mice was strongly suggestive of a positive function for THEMIS on tonic TCR signaling and could be rescue by deleting the gene encoding for SHP-1 (12). Interestingly, this effect was restricted to CD8⁺ T cells, since no effect of THEMIS on CD4⁺ T cell function could be detected in that study (12).

101 In the present study, we identified a positive function for THEMIS in CD4⁺ T cell responses
102 to self and foreign antigens. Post-thymic disruption of the *Themis* gene, using Cre recombinase
103 transgenes driven by the *hCD2* promoter, shows that THEMIS promotes Th1 responses *in vivo*
104 and enhances encephalitogenic responses of CD4⁺ T cells to a neuroantigen in a mouse model
105 of central nervous system (CNS) auto-immunity. Although THEMIS exerts a positive function
106 in Th1 responses following antigen immunization, we show that THEMIS deficiency leads to
107 opposite outcomes following TCR stimulation, suggesting that the inhibitory effects of
108 THEMIS on TCR signaling are overcome during immune responses by additional THEMIS-
109 mediated signals which promote rather than repress T-cell effector function.

Materials and Methods

Mice

For the generation of *Themis*- T^{KO} mice, ES cells from the EUCOMM consortium (clone HEPD0712-6-H05) were used to generate *Themis*^{lox/lox} mice in which the 4th exon of *themis* was surrounded by two *loxP* sites. These mice were bred with *hCD2-cre* transgenic mice (<https://www.jax.org/strain/027406>) in which the human *cd2* promoter directs the expression of the CRE recombinase at late stages of T cell development in mature CD4⁺ and CD8⁺ single-positive thymocytes. *Themis*^{lox/lox} mice were also bred with *CD4-Cre*^{ERT2} transgenic mice which contain tamoxifen inducible CRE under the control of the *cd4* promoter (<https://www.jax.org/strain/022356>). *Themis*- T^{KO} were crossed with transgenic mice expressing the 2D2 TCR (13). All the experiments were conducted with sex and age-matched mice between 8 and 12 weeks old housed under specific pathogen-free conditions at the INSERM animal facility (US-006; accreditation number A-31 55508 delivered by the French Ministry of Agriculture to perform experiments on live mice). All experimental protocols were approved by a Ministry-approved ethics committee (CEEA-122) and follow the French and European regulations on care and protection of the Laboratory Animals (EC Directive 2010/63).

Antibodies

For CD4⁺ T cell purifications: monoclonal antibodies anti-B220 (RA3-6-B2), anti-CD11b (M1/70), anti-MHC class II (m5-114), anti-FcγRs (2.4G2), NK1.1 (83PK136), anti-CD4 (GK1.5), anti-CD8 (SN-H59) and anti-CD25 (PC61) were generated in our Lab. *For stimulation and cell culture:* anti-CD3ε (145-2C11) and anti-CD28 (37.51) antibodies were from Biolegend. Recombinant murine IL-2, IL-12p70, IL-6, TGF-β and IL-27 are from PeproTech. Neutralizing anti-IL-4 (BVD4-1D11) and anti-IFNγ (XMG1.2) antibodies are from BD Bioscience. The ERK inhibitor U0126 is from Cell Signaling. *For ELISA analysis:* capture anti-IFNγ (AN-18), anti-IL-2 (JES6-1A12), anti-GM-CSF (MP1-22E9) and anti-IL-17 (TC11-18H10) antibodies and detection biotin anti-IFNγ (XMG 1.2), anti-IL-2 (JES6-SH4), anti-GM-CSF (MP1-31G6) and anti-IL-17 (TC11-8H4) antibodies are from BD Bioscience. Capture anti-IL-13 (eBio13A) and detection biotin anti-IL-13 (eBio1316H) antibodies were

from eBioscience. *For Immunoprecipitation and Western blot:* anti-THEMIS (06-1328) and anti-Rac (23A8) antibodies are from EMD Millipore. Anti-pERK (Thr202/Tyr204), anti-pSHP-1 (Tyr564), anti-pSHP-2 (Tyr580) and anti-pP38 MAPK (Thr180/Tyr182) antibodies were purchased from Cell Signaling. HRP-conjugated goat anti-rabbit and goat anti-mouse antibodies were from Santa Cruz Biotechnology. *For cell sorting and flow cytometry analysis:* anti-CD4 (RM 4-5), anti-GM-CSF (MP1-22E9), anti-IL-10 (JES5-16E3 and anti-BTLA (6A6) antibodies were from Biolegend; anti-CD8 α (53-6.7), anti-TCR β (H57-597), anti-CD25 (PC61.5), anti-CD44 (IM7), anti-CD69 (H1.2F3), anti-EOMES (Dan11mag), anti-T-bet (4B10), anti-IL-13 (eBio13A), anti-ROR γ t (B2D), anti-FOXP3 (FJK-16s), anti-CXCR3 (CXCR3-173), anti-PC-1 (J43), anti-IFN γ R (2E2), anti-pSTAT4 (4LURPIE) and anti-Granzyme B (NGZB) antibodies were from eBioscience. Anti-CD62L (MEL-14), anti-IFN γ (XMG1.2), anti-TNF α (MP6-XT22), anti-IL-17 (TC11-18H10), anti-Ki-67 (B56) antibodies were from BD Bioscience. Anti-CD45.1 (A20), anti-TCR V α 3.2 (REA359) and anti-TCR V β 11(REA657) were from Miltenyi Biotec.

Flow cytometry and cell sorting

For flow cytometry analysis, single-cell suspensions from thymus, spleen or lymph nodes were incubated with diluted eBioscience™ Fixable Viability Dye eFluor™ 780 (ThermoFisher) in phosphate-buffered saline (PBS), 1% Fetal Calf Serum (FCS) and 2 mM ethylenediaminetetraacetic acid disodium salt solution (EDTA) prior staining with fluorochrome-conjugated antibodies. Intracellular staining was performed using the FOXP3 staining buffer kit (e-Biosciences) according to the manufacturer's instructions before data acquisition on a BD LSRII flow cytometer and analysis with the FlowJo software. Purification of naïve CD62L^{hi}CD25⁻CD4⁺ and CD8⁺ T cells was performed by cell sorting using a FACS Aria-SORP (BD Biosciences).

Cell cultures

For proliferation assays, naïve CD62L^{hi}CD25⁻CD4⁺ T cells were labeled with 2 μ M Cell Trace Violet (CTV; Life Technologies) for 15 min at 37 °C and cultured in 96-well plates coated with variable doses of anti-CD3 antibodies at a density of 10⁶ per ml. The incorporation of CTV

was measured by flow cytometry after 3 days of culture. For polarization assays, naïve CD4⁺ T cells were stimulated for three (Th1, iTreg), four (Tr1) and five days (Th2, Th17) with the indicated doses of anti-CD3 antibodies and with 1 µg/ml of soluble anti-CD28 antibodies in presence of different cytokines and neutralizing antibodies cocktails, such as: Th1 : IL-12 (10ng/ml) and anti-IL-4 (10 µg/mL); Th2: IL-4 (20ng/ml) and anti-IFN γ (6 µg/ml); Th17: IL-6 (20ng/ml), TGF-beta (2ng/ml), anti-IFN γ (6 µg/ml) and anti-IL-4 (10 µg/ml); Tr1: IL-27 (50ng/mL); iTreg: TGF β (0.25ng/ml) and IL-2 (1ng/mL). For the analysis of cytokine production by flow cytometry, cells were re-stimulated for 4 hours with 500 ng/ml Phorbol 12-myristate 13-acetate (PMA), 1 µg/mL of ionomycin and 1 µg/ml of Golgi-plug (BD Bioscience). To analyze STAT4 phosphorylation, naïve CD4⁺ T cells were stimulated for two days with anti-CD3 (2 µg/ml) and anti-CD28 (1 µg/ml) antibodies. Cells were rested in RPMI at 37°C overnight and stimulated with the indicated doses of IL-12 at 37°C for 40 min. For *in vitro* stimulation of 2D2⁺ CD4⁺ T cells, naïve CD62L^{hi}CD25⁻ CD4⁺T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice expressing the 2D2 TCR were labeled with CTV and cultured in Th1 or Th17 polarizing conditions with MOG₃₅₋₅₅ or NF-M₁₅₋₃₅ at 10 or 50 µg/mL in presence of 1x10⁵ cells/well of irradiated splenocytes from C57BL/6J mice.

ELISA

Enzyme immunoassays were used to measure cytokines in culture supernatants. Briefly, 96 well plates were coated for 2h at 37°C with anti-IFN- γ , anti-IL13, anti-IL-17 or anti-GM-CSF in carbonate buffer 0.05 M pH 9.6. Culture supernatants or standards were incubated 2h at 37°C. The plates were then incubated 1h30min with a secondary biotinylated antibody specific for each cytokine, followed by 20 min of incubation with streptavidin-phosphatase alkaline at 37°C. Finally, plates were revealed by phosphatase alkaline substrate and absorbance was measured at 450/540 nm.

Immunization with 1W1K peptide

Mice were immunized subcutaneously on each side at the base of the tail with 4 µg of peptide 1W1K (EAWGALANKAVDKA from Genecust) in complete Freund's adjuvant (CFA, Sigma). CD4⁺ T cell populations from the draining lymph nodes were analyzed 9 days after

immunization. For staining of antigen-specific cells, 1W1K-IAb tetramers were obtained from the NIH Tetramer core facility. Overall, cells at 10^8 per ml were first stained at room temperature for two hours with an optimal concentration of tetramer. Then, cell staining for additional markers was performed as described previously. To measure cytokines production, 6×10^6 cells from the draining lymph nodes were stimulated with the indicated dose of 1W1K peptide overnight and incubated with Golgi-plug for 1h30min.

Experimental autoimmune encephalomyelitis

For active EAE, *Themis-T^{WT}* and *Themis-T^{KO}* mice were immunized with 50 μ g of MOG₃₅₋₅₅ peptide (Covalab) emulsified with CFA containing 1 mg/ml of Mycobacterium tuberculosis (Difco). 200 ng of pertussis toxin (List Biological Lab) was given through intravenous (i.v.) injection at day 0 and day 2 after immunization. *CD4-Cre^{ERT2}-Themis^{flox/flox}* mice were treated by oral gavage with 5mg of tamoxifen for 3 days and rested for two weeks prior immunization with 200 μ g of MOG₃₅₋₅₅ peptide emulsified with CFA containing 2 mg/ml of Mycobacterium tuberculosis. 200 ng of pertussis toxin was given i.v. at day 0 and day 2 after immunization. Analysis of CD4⁺ and CD8⁺T cells in the lymph nodes, the brain and the spinal cord was performed at day 28 after immunization. Clinical scores were evaluated on a five-stage scale from 0 to 5. For active EAE following adoptive transfer, naïve CD62L^{hi}CD25⁻CD45.1⁺CD4⁺ T cells were purified from lymph nodes and splenocytes from *Themis-T^{WT}* and *Themis-T^{KO}* mice expressing the 2D2 TCR. 2×10^5 cells in PBS were injected i.v. into C57BL/6J mice (CD45.2⁺) one day before immunization with 100 μ g of MOG₃₅₋₅₅ peptide emulsified with CFA containing 1 mg/ml of Mycobacterium tuberculosis. Analysis of CD45.1⁺CD4⁺ T cells in the brain and the spinal cord was performed at day 14 after immunization. For *in vivo* analysis of proliferation, 2D2⁺CD4⁺ T cells were stained with CTV and injected i.v. into C57BL/6J mice one day before immunization with MOG₃₅₋₅₅ peptide. The dilution of CTV was evaluated on day 3 after immunization. The phenotype of the CD45.1⁺CD4⁺ T cells from the draining lymph nodes was analyzed by flow cytometry on day 6 after immunization. For the analysis of cytokines production, 1×10^6 cells isolated from the draining lymph nodes were cultured in the presence of MOG₃₅₋₅₅ or NF-M₁₅₋₃₅ peptides (10 and 50 μ g/ml) for 2 days and then incubated with 1 μ g/mL of Golgi-plug for 4 hours.

Western blot analysis

To analyze TCR signaling, CD4⁺ T cells were resuspended at a density of 10^7 cells per 50 μ l and incubated at 37°C for 10 min before being stimulated. Antibody complexes were prepared at a 2 \times concentration before being used to stimulate cells at 37°C for 10 min with biotin-conjugated anti-CD3 and anti-CD4 antibodies mixed with equal concentrations of streptavidin. Cells were stimulated with 50 μ l of antibody complexes (60 μ g/ml) for the times indicated in the figure legends. Cell lysates were prepared by resuspending cells in 1ml of ice-cold lysis buffer (10 mM tris-HCl pH 7.4, 150 mM NaCl, 1% Triton, 2 mM Na₃VO₄, 5 mM NaF, 1 mM EDTA, and protease inhibitor tablets (Roche)) for 20min at 4°C. Lysates were cleared by centrifugation at 18,000g for 15 min at 4°C. For Western blot analysis, proteins were resolved by SDS-PAGE gel and transferred to PVDF membranes according standard protocols. Membranes were blocked with 5% milk in tris-buffered saline containing Tween at 0.05% for 1 hour at room temperature before being incubated with primary antibodies at 4°C overnight. After washing, membranes were incubated with secondary antibodies for 1 hour at room temperature. Subsequently, membranes were incubated with enhanced chemiluminescence solution (Sigma) for 5 minutes in the dark, and luminescence was captured with a Bio-Rad XRS+ imager. Band intensities were quantified with Bio-Rad ImageLab software. Values from quantitated bands were normalized for loading differences with Rac1 as a control. The intensity values at each time point were normalized to the highest intensity value obtained for wild-type controls in each individual experiment.

Statistical analysis

Data are presented as mean \pm SEM. Statistical analysis were performed using unpaired t test. Mann-Whitney test was used for nonparametric tests. For clinical score and incidence in EAE, two-ways ANOVA method was used for calculating the statistical difference. P values < 0.05 were considered statistically significant (*p<0,05 **p<0,01 ***p<0,001, ****p<0,001).

Results

THEMIS negatively regulates IFN γ production in polarized Th1 cells by repressing ERK1/2-mediated signaling

To evaluate the post-thymic functions of THEMIS in CD4⁺ T cells, we conditionally disrupted *Themis* gene using two different Cre recombinase transgenes driven by the human *Cd2* and the distal *lck* promoters. Contrary to other *Cd2*- and *lck*-driven transgenes, these constructs are not active in most DP thymocytes and CD4 single positive (CD4SP) thymocytes, but are upregulated mainly after thymic development in peripheral T cells (14, 15). THEMIS expression is comparable in thymocytes from *Themis*^{flx/flx} (thereafter referred as to *Themis*-*T*^{WT}) and *Cd2-Cre Themis*^{flx/flx} (thereafter called *Themis*-*T*^{KO}) mice but becomes undetectable in peripheral *Themis*-*T*^{KO} CD4⁺ and CD8⁺ T cells (Fig. S1A). Since a significant amount of THEMIS was detected in peripheral CD4⁺ T cells from *dLck-Cre Themis*^{flx/flx} (Fig. S1B), we decided to mainly focus our analysis on the *Cd2*-driven knockout model. Proportions and numbers of DP and SP thymocytes and of peripheral CD4⁺ T cells observed in *Themis*-*T*^{KO} mice were comparable to those observed in *Themis*-*T*^{WT} mice (Fig. S1C). The proportions and numbers of lymph nodes and splenic CD8⁺ T cells were slightly reduced in *Themis*-*T*^{KO} mice (Fig. S1D), confirming previously published findings obtained in the *dLck-Cre Themis*^{flx/flx} model indicating a role for THEMIS in peripheral CD8⁺ T cell maintenance (12). The absence of THEMIS in peripheral T cells did not affect the proportions and numbers of FOXP3⁺ regulatory T cells (Fig. S1E) and did not significantly modify the percentages of CD62L^{lo}CD44^{hi} memory-like CD4⁺ T cells as compared to that in the *Themis*-*T*^{WT} group (Fig. S1F).

Since THEMIS has been characterized as a regulator of TCR signaling, we first examined the effect of THEMIS deficiency on CD4⁺ T cell responses induced *in vitro* following TCR triggering with anti-CD3 and anti-CD28 antibodies. The viability and the ability of CD4⁺ T cells to proliferate in response to TCR stimulation were similar in *Themis*-*T*^{KO} and *Themis*-*T*^{WT} mice (Fig. 1A). TCR stimulation also resulted in similar expression of the activation markers CD25 and CD69 on CD4⁺ T cells in the two groups of mice but induced a higher expression

of CD44 at the surface of *Themis*- T^{KO} mice (Fig. 1B), suggesting that THEMIS may have an inhibitory function on a restricted set of TCR-mediated responses in CD4⁺ T cells. THEMIS deficiency had no impact on the production of IL-2, IFN γ and GM-CSF in a similar stimulating context (Fig. 1C and S2A), indicating that THEMIS does not regulate the secretion of cytokines associated to primary CD4⁺ T cell activation. The absence of THEMIS had minimal impact on *in vitro* CD8⁺ T cell responses following TCR stimulation (Fig. S2B, S2C and S2D), confirming previous findings obtained in the *dLck-Cre Themis^{lox/lox}* mouse model (12).

We next investigated whether THEMIS deficiency would impact the production of cytokines in CD4⁺ T cells stimulated in Th1, Th2, Th17, iTreg and Tr1 polarizing conditions. We observed that THEMIS deficient CD4⁺ T cells produced significantly higher amount of IFN γ in Th1 polarizing conditions as compared to that in *Themis*- T^{WT} CD4⁺ T cells (Fig. 2A). This difference was mostly detected when high doses of anti-CD3 antibodies were used, suggesting that THEMIS could be part of a negative feedback loop that may prevent excessive and potentially detrimental effects of IFN γ . This effect was specifically observed in Th1 polarized cells, since the production of IFN γ was not affected by THEMIS deficiency in Th2 and Th17 polarized cells (Fig. S3A). The production of IL-13, IL-17 and IL-10 by CD4⁺ T cells polarized respectively under Th2, Th17 and Tr1 conditions and the expression of FOXP3 in iTreg-polarized CD4⁺ T cells were also comparable in *Themis*- T^{KO} and *Themis*- T^{WT} mice (Fig. 2A, S3B, S3C), indicating that THEMIS selectively represses the production of cytokines in CD4⁺ T cells polarized in the Th1 subset. Accordingly, the absence of THEMIS also led to a moderate increase of TNF α production in Th1 polarized cells (Fig. S3D), although no significant effects were observed on IL-2 secretion in similar experimental settings (Fig. S3E). The transcription factors T-BET and EOMES were expressed at similar level in *Themis*- T^{KO} and *Themis*- T^{WT} CD4⁺ T cells polarized in Th1 conditions (Fig. 2B), suggesting that THEMIS represses Th1 responses rather than the differentiation program leading to the acquisition of the Th1 lineage.

We next investigated the mechanism by which THEMIS negatively regulates the secretion of cytokines in Th1 cells. The expression level of the IL-12 receptor (IL-12R) and the phosphorylation of STAT4 induced by IL-12 stimulation (16) were comparable in *Themis*- T^{KO}

and *Themis-T^{WT}* CD4⁺ T cells (Fig. S4A and S4B), indicating that the increased secretion of Th1 cytokines in *Themis-T^{KO}* CD4⁺ T cells was not the consequences of exacerbated IL-12R signaling. Since the binding of IFN γ to its receptor (IFN γ R) triggers a positive feedback loop in Th1 cells (17), we analyzed whether the inhibitory effects of THEMIS on IFN γ secretion were dependent on IFN γ R-mediated signaling. The expression levels of IFN γ R were similar in *Themis-T^{KO}* and *Themis-T^{WT}* CD4⁺ T cells (Fig. S4C). The production of IFN γ remained increased in *Themis-T^{KO}* CD4⁺ T as compared to that in control cells, when the IFN γ R was blocked using antagonist antibodies (Fig. S4D), suggesting that THEMIS operates independently of IFN γ R-mediated signaling.

THEMIS deficiency leads to a selective increase of the MAP kinases ERK1/2 phosphorylation following TCR stimulation in thymocytes (9, 11). Since ERK2 enhances selectively the production of Th1 cytokines without affecting T-BET expression (18), we speculated that THEMIS might negatively regulate Th1 responses by repressing ERK2 activity. Confirming previous observations in thymocytes (9, 11), the phosphorylation of ERK1/2 was increased in *Themis-T^{KO}* CD4⁺ T cells as compared to that in control cells (Fig. 3A and 3B). In comparison, the presence of THEMIS did not affect the phosphorylation of P38, indicative of specific effect of THEMIS on TCR signaling pathways (Fig. 3A and 3B). The phosphorylation of SHP-1 and SHP-2 were decreased in *Themis-T^{KO}* CD4⁺ T cells as compared to that in *Themis-T^{WT}* CD4⁺ T cells (Fig. 3A), suggesting that THEMIS negatively regulates ERK by regulating those phosphatases. To address whether exacerbated ERK activity could be responsible for the increased production of Th1 cytokines in *Themis-T^{KO}* CD4⁺ T cells, we next compared the responsiveness of *Themis-T^{KO}* and *Themis-T^{WT}* CD4⁺ T cells to ERK inhibitor treatments following TCR stimulation of CD4⁺ T cells in Th1 polarized conditions. The production of IFN γ by CD4⁺ T cells was progressively decreased as the doses of the ERK inhibitor U0126 increased, confirming the enhancing effect of ERK1/2-mediated signaling on IFN γ production in Th1 cells (Fig. 3C). By contrast the production of TNF α was not affected by those treatments, indicating that ERK1/2-dependent signals regulate a restricted set of Th1 cytokines (Fig. S4E). Higher doses of inhibitor were required to inhibit by 50% the production of IFN γ in *Themis-*

T^{KO} Th1 cells as compared to that in control cells (Fig. 3C), confirming that the increased activity of ERK1/2 enhances the production of IFN γ in *Themis-T^{KO}* CD4⁺ T cells.

THEMIS promotes the expression of T-BET and enhances the secretion of IFN γ by antigen specific CD4⁺ T cells

To examine the *in vivo* relevance of those observations, we next analyzed CD4⁺ T cell responses following immunization of *Themis-T^{WT}* and *Themis-T^{KO}* mice with a peptide variant (EAWGALANKAVDKA, called 1W1K peptide hereafter) of the I-E alpha chain immunodominant peptide 52-68 in the presence of the Th1/Th17 polarizing Complete Freund's Adjuvant (CFA). To follow antigen-specific CD4⁺ T cells, we stained cells from the draining lymph nodes with 1W1K-pMHCII tetramer and first analyzed the expression of CD44, T-BET and ROR γ t. The percentages of CD4⁺ T cells expressing high level of CD44 and the percentages of CD44^{hi}CD4⁺ T cells positive for tetramer staining were similar in *Themis-T^{WT}* and *Themis-T^{KO}* mice, indicating that THEMIS does not significantly impact the activation and the clonal expansion of antigen-specific CD4⁺ T cells in this experimental setting (Fig. 4A). The percentages of tetramer+CD4⁺ T cells expressing T-BET were decreased in *Themis-T^{KO}* mice as compared to those in *Themis-T^{WT}* mice (Fig. 4B). In comparison, the expression of ROR γ t in tetramer+CD4⁺ T cells was similar between *Themis-T^{WT}* and *Themis-T^{KO}* mice (Fig. 4C). The proportions of antigen-specific CD4⁺ T cells producing IFN γ were decreased in *Themis-T^{KO}* mice in comparison to that in *Themis-T^{WT}* mice, whereas the absence of THEMIS had no significant impact on the percentages of cells producing IL-17 and TNF α in the same experimental conditions (Fig. 4D). Thus, contrasting with results indicating inhibitory effects of THEMIS on CD4⁺ responses in Th1 polarized cells, immunization with a foreign antigen reveals positive effect of THEMIS on the development of type-1 responses.

THEMIS enhances the susceptibility to the development of Central nervous system autoimmunity

Initial studies on THEMIS suggest that it attenuates TCR signal strength in thymocytes in response to low- but not high-affinity TCR engagement (9), suggesting that THEMIS might have a different influence on CD4⁺ T cells responses when cells are stimulated by self rather

than foreign antigens. To address this possibility, we examined the effect of THEMIS expression on the development of Experimental Auto-immune Encephalomyelitis (EAE), a well-characterized mouse model of multiple sclerosis. The disease was induced by immunizing *Themis*-*T*^{KO} and *Themis*-*T*^{WT} mice with a peptide from the myelin oligodendrocyte glycoprotein (MOG₃₅₋₅₅) emulsified in CFA. The incidence and the clinical scores of EAE were decreased in the group of *Themis*-*T*^{KO} mice in comparison to that in the wild-type group (Fig. 5A). Although the *Cd2*-driven Cre transgene begins to be expressed at relatively late stage of T cell development (14), we could not definitively exclude that the effect of THEMIS on EAE development could be the consequence of an altered selection of the TCR repertoire in the thymus. To evaluate this possibility, we conditionally disrupted *Themis* gene in peripheral CD4⁺ T cells by administering tamoxifen to *Themis*^{flx/flx} mice expressing the *CD4-Cre*^{ERT2}-driven transgene (21) and analyzed the development of EAE in this new model. The incidence and clinical scores of EAE were decreased in THEMIS deficient mice similarly to what was observed in the *Cd2-Cre* driven transgenic model (Fig. 5B). Numbers of CD4⁺ and CD8⁺ T cells that have migrated into the brain were decreased in THEMIS deficient mice in comparison to that in littermate controls (Fig. 5C and 5D). This was associated with increased numbers of CD4⁺ and CD8⁺ T cells in the lymph nodes, whereas the numbers of cells recovered in the spinal cord were comparable between the two groups of mice (Fig. 5C and 5D).

THEMIS enhances the expression of T-BET and the production of IFN γ and GM-CSF in encephalitogenic CD4⁺ T cells

To investigate further the mechanism by which THEMIS modulates the development of EAE and to avoid potential bias due to the perturbation of the TCR repertoire or to tamoxifen treatment, we adoptively transferred *Themis*-*T*^{KO} and *Themis*-*T*^{WT} CD62L^{high}CD25⁻CD4⁺ T cells expressing the MOG₃₅₋₅₅ specific TCR 2D2 and the CD45.1 allotypic marker into C57BL/6 mice and analyzed the development of EAE in recipient mice following immunization with MOG₃₅₋₅₅ in the presence of CFA. The incidence and the clinical scores of EAE were decreased in recipient mice that have received *Themis*-*T*^{KO} 2D2+CD4⁺ T cells in comparison to that in the control group (Fig. 6A). The cumulative scores and the maximum scores were also lower in recipient mice injected with *Themis*-*T*^{KO} 2D2+CD4⁺ T cells (Fig.

6A), suggesting that THEMIS enhances the encephalitogenic potential of CD4⁺ T cells in this model. The numbers of CD45.1⁺CD4⁺ T cells infiltrating the brain and the spinal cord were decreased in recipient mice injected with *Themis*-T^{KO} CD4⁺ T cells fourteen days after immunization (Fig. 6B). However, similar percentages of 2D2⁺ T cells out of total CD4⁺ T cells were recovered in those organs, suggesting that THEMIS promotes the migration of encephalitogenic 2D2⁺CD4⁺ T cells and may also facilitate the migration of endogenous CD4⁺ T cells through bystander effects (Fig. 6B).

To analyze whether THEMIS regulates the activation and the differentiation of 2D2⁺ T cells, we performed a phenotypic analysis of lymph nodes CD45.1⁺CD4⁺ T cells prior the onset of the disease. The percentages and numbers of *Themis*-T^{KO} CD45.1⁺CD4⁺ T cells recovered in the draining lymph nodes were decreased in comparison to *Themis*-T^{WT} control cells, suggesting that THEMIS might be important for the maintenance or the clonal expansion of 2D2⁺ T cells (Fig. 6C). To examine whether THEMIS regulates the ability of CD4⁺ T cells to proliferate in response to MOG₃₅₋₅₅ peptide, we injected *Themis*-T^{KO} and *Themis*-T^{WT} 2D2⁺CD45.1⁺ CD4⁺ T cells stained with Cell-Trace-Violet (CTV) into C57BL/6 mice and analyzed CTV staining in CD45.1⁺CD4⁺ T cells three days after immunization. The absence of THEMIS had no detectable impact on the percentages of CTV^{low} and CTV^{very-low} CD45.1⁺CD4⁺ T cells, indicating that THEMIS is not required for the proliferation of antigen specific T cells (Fig. 6D). Confirming this observation, the percentages of 2D2⁺ T cells expressing the cell division marker Ki67 were not affected by THEMIS deficiency (Fig. 6E). The expression of the TCR signaling sensor CD5 (22) was decreased in *Themis*-T^{KO} CD45.1⁺CD4⁺ T cells as compared to that in wild-type controls, indicating positive function of THEMIS on TCR signaling during CD4⁺ T cell activation (Fig. 6F). Although the percentages of CD45.1⁺CD4⁺ T cells expressing the IL-2 receptor alpha chain, CD25, were lower in the absence of THEMIS, the activation markers CD44 and PD-1 were expressed at similar levels on *Themis*-T^{KO} and *Themis*-T^{WT} CD45.1⁺CD4⁺ T cells, indicating that THEMIS deficiency does not lead to an overall defect of CD4⁺ T cell priming in this model (Fig. 6F). We next analyzed the expression of T-BET and RORγt in CD45.1⁺CD4⁺ T cells to determine the influence of THEMIS on the generation of Th1 and Th17 effector subsets. The percentages

of CD45.1+CD4+ T cells expressing T-BET were decreased in the absence of THEMIS in comparison to those in control cells, whereas the expression of ROR γ t in CD45.1+CD4+ T cells was not affected by THEMIS expression (Fig. 6G). The chemokine receptor CXCR3, whose expression is positively regulated by T-BET (23), was expressed at lower level on *Themis*-T^{KO} CD45.1+CD4+ T cells as compared to that in control cells (Fig. 6H).

We next examined the production of inflammatory cytokines by THEMIS deficient peripheral 2D2+CD4+ T cells in this model. To evaluate whether the affinity of TCR engagement could influence the effect mediated by THEMIS on CD4+ T cell responses, 2D2+ T cells were harvested from the lymph nodes six days after immunization and re-stimulated *in vitro* with MOG₃₅₋₅₅ or with a peptide corresponding to the Neurofilament medium polypeptide epitope (NF-M₁₅₋₃₅), which exhibits a higher affinity than the MOG₃₅₋₅₅ peptide for the 2D2 TCR (24). Contrasting again with previous results obtained following TCR stimulation, the percentages of 2D2+CD44^{hi} cells producing IFN γ were decreased in the absence of THEMIS in responses to MOG₃₅₋₅₅ and NF-M₁₅₋₃₅ stimulation (Fig. 7A). The production of GM-CSF was also decreased in *Themis*-T^{KO} 2D2+CD44^{hi} T cells but only when cells were stimulated with low doses of MOG₃₅₋₅₅ (Fig. 7B). In comparison, the absence of THEMIS had no detectable effect on the production of IL-17 by 2D2+CD44^{hi} cells (Fig. 7C).

One discrepancy between the stimulation of naïve CD4+ T cells *in vitro* and the re-stimulation of effector CD4+ T cells *ex-vivo* relies on the nature of the TCR stimulations, which are based on anti-CD3 antibodies *in vitro* and on peptide presented by antigen presenting cells *ex-vivo*. To evaluate whether this could explain the difference between our *in vitro* and *in vivo* data, we purified naïve CD4+ T cells expressing the 2D2 TCR from *Themis*-T^{KO} and *Themis*-T^{WT} mice and stimulated them with irradiated splenic cells in presence of MOG₃₅₋₅₅ or NF-M₁₅₋₃₅ peptides in Th1 polarized conditions. The ability of 2D2+ T cells to proliferate in response to MOG₃₅₋₅₅ or NF-M₁₅₋₃₅ stimulation was not affected by THEMIS deficiency (Fig. S5A). Confirming previous data obtained with antibody-based stimulations, the percentages of 2D2+ T cells producing IFN γ and TNF α were enhanced in the absence of THEMIS as compared to those in control cells (Fig. 7D and 7E). The inhibitory effect of THEMIS on IFN γ production was

mostly observed at high doses of peptides and seemed more pronounced with NF-M₁₅₋₃₅ than with MOG₃₅₋₅₅ stimulations, supporting the idea that THEMIS might be part of a negative feedback loop engaged upon strong TCR stimulation. The expression of T-BET was comparable in *Themis*-T^{KO} and *Themis*-T^{WT} CD4⁺ T cells (Fig. S5B). Finally, the absence of THEMIS did not significantly affect the percentages of 2D2⁺ cells producing IL-17 when cells were polarized in Th17 conditions (Fig. 7F). Altogether those results indicate that THEMIS exerts dual inhibitory and enhancing effects on CD4⁺ T cell responses and that enhancing effects predominates in the physiological context of immune responses against self- and foreign antigens.

Discussion

Nearly opposite functions have been assigned to THEMIS to explain its role on T cell development (8). Whereas *in vitro* studies on DP thymocytes suggest that THEMIS could prevent “misplaced” negative selection events by negatively regulating TCR signals elicited by weak TCR stimulation (9), *in vivo* studies are indicative of an enhancing role on TCR signaling which enables thymocytes to reach the threshold for positive selection and prevent death by neglect (7, 11). One potential interpretation to explain this discrepancy is that THEMIS may promote different outcomes according to the context of stimulation, operating as a negative regulator of T cell responses when TCR signals emanate exclusively from the TCR, and as an enhancer of those responses when TCR signals integrate multiple regulatory inputs triggered by additional environmental stimuli. Biochemically speaking, THEMIS would operate similarly in both contexts *i.e* by repressing the phosphatase activity of SHP-1 and SHP-2, but the consequence of this effect might be different according to how those phosphatases are respectively engaged by the TCR and by additional receptors such as co-inhibitory and cytokine receptors.

Accordingly, we show here that THEMIS deficiency has nearly opposite consequences on CD4⁺ T cell responses according to the context of stimulation. THEMIS negatively regulates the production of IFN γ and TNF α in CD4⁺ T cells polarized under Th1 conditions. The inhibitory effect of THEMIS is restricted to Th1-mediated responses since the absence of THEMIS did not affect naïve CD4⁺ T cell priming and differentiation into Th2 and Th17 subsets. T-BET and EOMES were induced normally in THEMIS deficient CD4⁺ T cells stimulated in those polarizing conditions, suggesting that in those stimulatory conditions, THEMIS regulates Th1 responsiveness to TCR stimulation rather than the ability of naïve CD4⁺ T cells to differentiate into this subset. Paralleling this relatively narrow modulatory

effect of THEMIS on Th1 responses, we showed that THEMIS inhibits preferentially the phosphorylation of ERK1/2 but has no detectable effect on the phosphorylation of p38 upon TCR stimulation or on the phosphorylation STAT4 upon IL-12R stimulation. The effects of *erk2* conditional disruption in CD4⁺ T are relatively symmetrical to those observed in THEMIS deficient CD4⁺ T cells, leading to a specific decrease of IFN γ production in polarized Th1 cells associated with the absence of detectable effect on T-BET and on Th2 or Th17-mediated responses (18), suggesting that the selective effect of THEMIS on Th1 cytokine responses may be the consequence of a targeted effect of THEMIS on ERK2 activity. Supporting this possibility, ERK1/2 inhibitors repressed less efficiently IFN γ production in THEMIS-deficient CD4⁺ T cells as compared to that in control CD4⁺ T cells. Of note, the inhibition of ERK1/2 activity had no detectable effect on the production TNF α in Th1 polarized cells, suggesting that the regulatory effect of THEMIS on this cytokine might be dependent on alternative signaling pathways.

Previous analysis of TCR signaling in *Themis*^{-/-} thymocytes showed that THEMIS exerts uncoupled and dual outcomes on TCR-mediated signaling events by repressing on one hand the phosphorylation of ERK1/2 induced by weak TCR stimulation (9, 11) and by enhancing on the other hand the activity of Vav1 independently of the strength of the TCR stimulation (11). The molecular explanation of this combined effect is not yet resolved but may rely on the type of SHP phosphatases that interact with THEMIS. THEMIS binds and directly inhibits SHP-1 and SHP-2, with the inhibitory effect on SHP-2 being less pronounced as that on SHP-1 (7). SHP-1 appears to have an exclusively inhibitory role in T-cell signaling by dephosphorylating key positive regulators of TCR signaling among which Vav1 (25-27). By contrast, SHP-2 has been ascribed both inhibitory and activating roles depending on the substrate and the context of its recruitment to the membrane. Whereas SHP-2 is described as a negative regulator of TCR- and CD28-mediated signaling events following its recruitment to

the inhibitory receptor PD-1 (28-30), SHP-2 is associated to positive function on TCR signaling when TCR signals do not integrate additional stimulatory inputs (19, 31). Inactivation of SHP-2 catalytic function in Jurkat cells (31) and conditional disruption of *Ptpn11*, the gene encoding SHP-2, in thymocytes (19) result in impaired TCR-induced ERK activation, suggesting that the repressing effect of THEMIS on ERK1/2 activity may be the consequence of the inhibitory effect of THEMIS on SHP-2 catalytic function and that, by contrast, the activating effect of THEMIS on Vav1 may results from the inhibition of SHP-1.

THEMIS-mediated inhibition of SHP-1's phosphatase activity is associated with an enhanced phosphorylation of SHP-1 in THEMIS-deficient thymocytes, presumably due to reduced auto- or trans-dephosphorylation by SHP-1 (7, 9). We observed that THEMIS positively regulates the phosphorylation of SHP-2 in CD4⁺ T cells, supporting the possibility that THEMIS represses the phosphatase activity of SHP-2 in those cells and inhibit thereby Th1 cytokine responses. Although, we also detected a decreased phosphorylation of SHP-1 in THEMIS deficient CD4⁺ T cells, this was not associated with a significant decrease of VAV1 phosphorylation (data not shown) and, more generally, with any detectable decrease of CD4⁺ T cell responses. This suggests that the functional outcomes resulting from THEMIS-mediated SHP-2 inhibition could be predominant over those resulting from SHP-1 inhibition in these settings. Although several reports identified inhibitory function for SHP-1 on CD4⁺ T cell responses *in vivo* (32-35), the conditional deletion of *ptpn6*, the gene encoding for SHP-1, has minimal effect on CD4⁺ T cell responses *in vitro* when TCR signals emanate exclusively from TCR cross-linking (33, 35, 36), suggesting that SHP-1 might operate more effectively when recruited to inhibitory receptors (30, 37, 38), which might enrich SHP-1 in proximity of TCR signaling complexes, or in the context of cytokine receptor signaling (33, 39, 40), rather than though negative feedback loops triggered by the TCR itself. This might explain why THEMIS-

mediated positive effects on TCR signaling are not revealed in *in vitro* experiments where only the TCR is stimulated.

The effect of THEMIS on CD4⁺ T cell responses was nearly the opposite *in vivo* when mice were immunized with self or foreign antigens. THEMIS exacerbates auto-immunity following the immunization with a neuroantigen and enhanced the encephalitogenic potential of 2D2⁺ CD4⁺ T cells. This was associated with a positive effect of THEMIS on the secretion of IFN γ and to a lesser extent on the secretion of GM-CSF by 2D2⁺ CD4⁺ T cells. Similar to our *in vitro* findings, THEMIS expression did not significantly affect molecular and cellular events associated to naïve CD4⁺ T cell priming and to the development of efficient Th17 responses. The presence of THEMIS was associated with an enhanced expression of T-BET in MOG- and 1W1K-specific CD4⁺ T cells, suggesting that THEMIS might be important *in vivo* for the differentiation of naïve CD4⁺ T cells into type 1 effector cells by contrast to its selective effect observed *in vitro* on Th1 cell responsiveness. T-BET is required to promote full pathogenic responses during the development of EAE (41, 42) and may operate, at least partly, by inducing the secretion of IFN γ which promotes pathogenesis at the early phase of the disease (43, 44). The reduced susceptibility of THEMIS deficient mice to develop EAE might thus result from the inefficient up-regulation of T-BET which may consequently affect the production level of IFN γ by encephalitogenic CD4⁺ T cells. The absence of THEMIS is also associated with decreased numbers of encephalitogenic CD4⁺ T cells in the brain, suggesting that THEMIS might favor the migration of auto-reactive CD4⁺ T cells in the CNS. T-BET is required for optimal migration of CD4⁺ T cells to inflammatory sites by controlling the attachment of CD4⁺ T cells to P-selectin and by enhancing the expression of chemokine receptors such as CXCR3 (23). Accordingly, the surface expression of CXCR3 was reduced in THEMIS deficient CD4⁺ T cells, suggesting that THEMIS that may enhances CD4⁺ T cell migration into the CNS through a T-BET-mediated process.

581

582 As THEMIS is expressed in naïve CD4⁺ T cells prior their differentiation into T-helper subsets,
583 it is intriguing that it promotes more specifically the development of Th1 responses over other
584 type of effector cells. The differentiation of naïve CD4⁺ T cells into distinct Th subsets is
585 dependent on cytokine co-stimulation but also on the magnitude of TCR signaling events which
586 is contingent on antigen loads, on the affinity of the TCR for pMHC complex and on additional
587 input signals which emanate from co-stimulatory receptors. Several evidences suggest that
588 stronger TCR signals are required for the differentiation of naïve CD4⁺ T cells into the Th1
589 lineage than for the Th2 lineage (45, 46), suggesting that THEMIS might operate in CD4⁺ T
590 cells similarly as in thymocytes, by enabling reaching a TCR signaling threshold required for
591 the Th1 lineage commitment. Accordingly, the expression level of the TCR signaling sensor
592 CD5 was reduced in antigen specific CD4⁺ T cells following immunization with MOG₃₅₋₅₅.
593 This suggests that THEMIS could enhance TCR signals independently of the TCR itself by
594 regulating stimulatory and inhibitory signals triggered by additional receptors express at the
595 surface of T cells. Another possibility could be that THEMIS operates downstream of
596 receptors involved more selectively in the regulation of Th1 responses. Immune checkpoints
597 such as PD-1 and BTLA are characterized as effective repressors of Th1 responses (37, 47, 48)
598 and operate by recruiting directly SHP-1 and SHP-2 through inhibitory motifs located in their
599 cytoplasmic tail (28, 30, 37, 49, 50). THEMIS may therefore enhance Th1 responses by
600 blocking SHP-1 and SHP-2-mediated inhibitory effects associated to those receptors on Th1
601 responses. This possibility may explain why *in vitro* studies, which exclusively focused on
602 isolated TCR stimulation may have failed to identify THEMIS as a positive regulator of T cell
603 responses.

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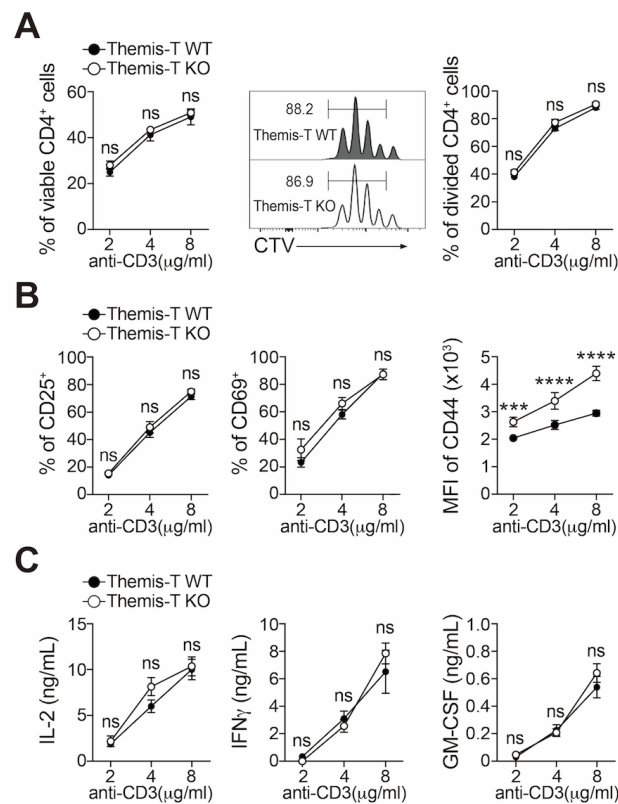
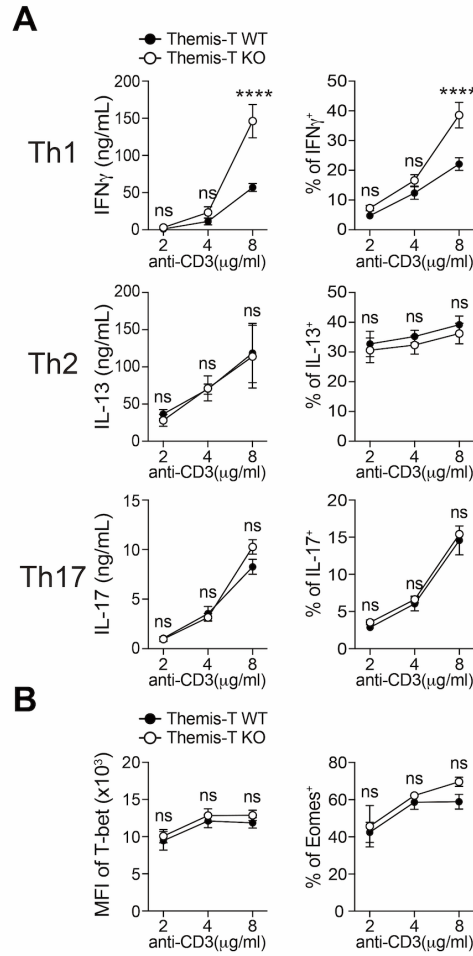


Figure 1. THEMIS deficiency has minimal effect on naïve CD4⁺ T cell responses following TCR stimulation.

(A) Naïve CD62L^{high}CD44⁺CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stained with CTV and stimulated with the indicated dose of anti-CD3 antibodies. Curve graphs represent the survival rate (left) and the proliferation (right) of cells determined by flow cytometry at day 3 after stimulation; the histogram graph (middle) shows CTV dilution on viable CD4⁺ T cells. (B and C) Naïve CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with the indicated dose of anti-CD3 antibodies. (B) Curve graphs represent the percentages of CD4⁺ T cells expressing CD25 (left) and CD69 (middle) as well as the MFI of CD44 staining on CD4⁺ T cells (right). (C) Curve graphs represent the secretion of IL-2 (left), IFN γ (middle) and GM-CSF (right) measured by ELISA. Data are mean values \pm S.E.M and are representative of at least three independent experiments each including n=3-4 mice per group. Unpaired two-tailed t test. ***P < 0.001. ****P < 0.0001.



810 **Figure 2. THEMIS negatively regulates the production of IFN γ in Th1 polarized cells.** Naïve
 811 CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with various doses of anti-CD3
 812 antibodies in presence of anti-CD28 antibodies in Th1, Th2 and Th17 polarizing conditions. (A) Curve graphs represent the
 813 production of IFN γ (upper panels), IL-13 (middle panels) and IL-17 (lower panels) in Th1, Th2 and Th17 polarizing conditions,
 814 respectively, analyzed by ELISA (left panels) and by flow cytometry after intracytoplasmic staining (right panels). (B) Naïve
 815 CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with various doses of anti-CD3
 816 antibodies in presence of anti-CD28 antibodies in Th1 polarizing conditions. Curve graphs represent the MFI of T-BET in
 817 CD4⁺ T cells (left) and the percentages of CD4⁺ T cells expressing EOMES (right) analyzed by flow cytometry after
 818 intracytoplasmic staining. Data are mean values \pm S.E.M. and represent of at least 3 independent experiments including n=3-
 819 4 mice per group. Unpaired two-tailed t test. ****P < 0.0001.

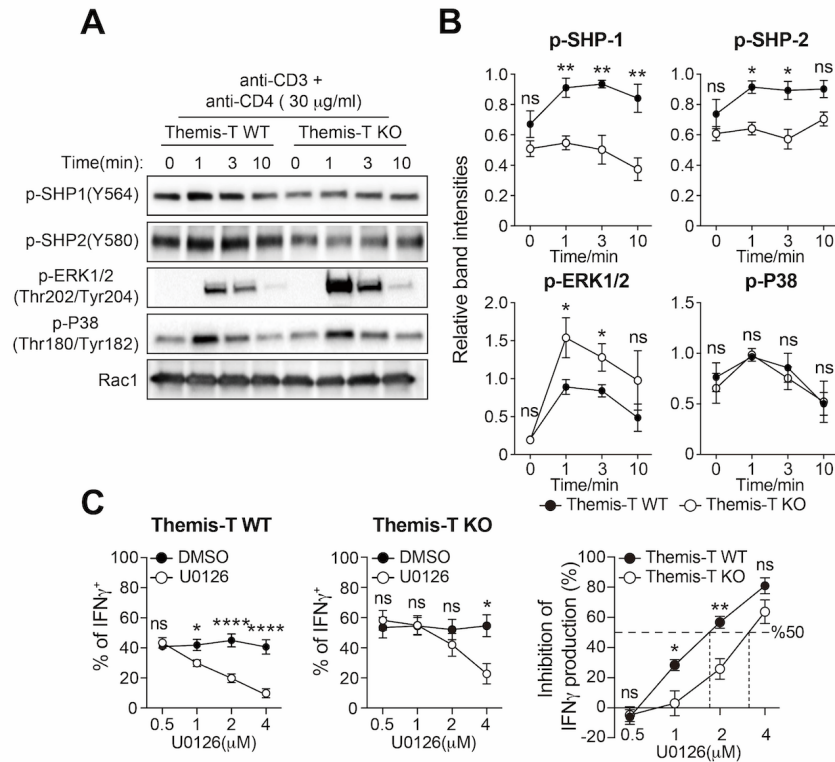


Figure 3. THEMIS represses the production of IFN_γ in Th1 cells by negatively regulating ERK-mediated signaling. (A) Total CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated anti-CD3 and anti-CD4 antibodies for the indicated times. Total cytoplasmic extracts of the cells were then analyzed by Western blotting with antibodies against phosphorylated forms of SHP-1 (Y564), SHP-2 (Y580), ERK (T202/Y204) and p38 (T180/Y182). (B) Curve graphs show the relative abundances of the indicated phosphorylated proteins as determined by calculating the ratios of the intensities of the bands corresponding to the phosphorylated proteins to those corresponding to Rac1, the loading control. The y axes represent means ± S.E.M. of the relative values calculated after normalization to the highest value in the *Themis-T^{WT}* cells. Data are representative of three to five independent experiments, each including one mouse of the indicated genotype. (C) Naïve CD62L^{high}CD25⁻CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with anti-CD3 antibodies in Th1 polarizing conditions in presence of the indicated doses of ERK inhibitor (U0126) or DMSO. Left and middle curve graphs represent the production of IFN_γ by CD4⁺ T cells analyzed by flow cytometry after intracytoplasmic staining. Right curve graph represents the percentages of inhibition of IFN_γ produced by CD4⁺ T cells following treatment with the indicated doses of U0126. Data are mean ± S.E.M. and are representative of one out of three independent experiments each including n=3 mice per group. Unpaired two-tailed t test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

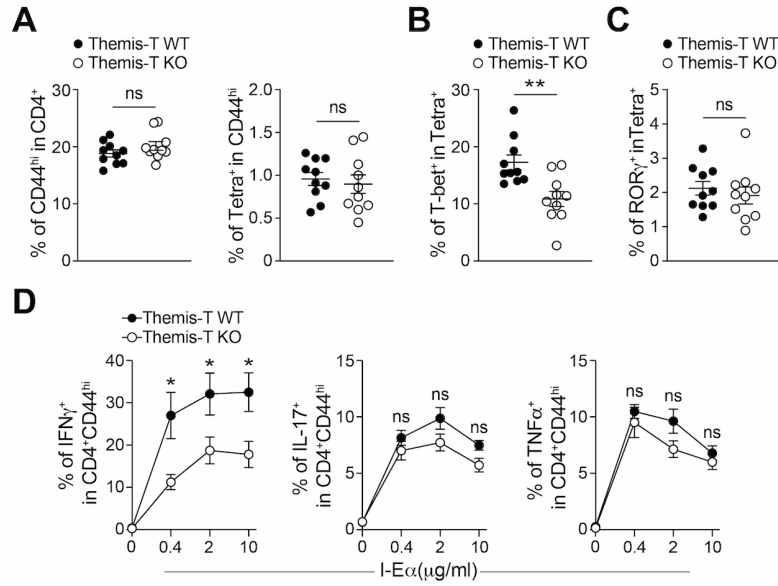


Figure 4. THEMIS enhances Th1 responses to foreign antigens. *Themis-T^{WT}* and *Themis-T^{KO}* mice were immunized with 1W1K peptide in presence of CFA. (A - C) Draining lymph nodes were collected after 9 days and analyzed by flow cytometry after staining of the cells with 1W1K-conjugated MHC tetramers. Graphs represent the percentages of CD44^{hi} among CD4⁺ T cells (A - left), the percentages of tetramer⁺ cells among CD44^{hi}CD4⁺ T cells (A - right), the percentages of T-BET⁺ (B) and RORγt⁺ (C) cells among tetramer⁺ cells. (D) At day 9 post-immunization, cells from the draining lymph nodes were re-stimulated *in vitro* with the indicated doses of the 1W1K peptide. Curves graphs represent the production of IFNγ, IL-17 and TNFα in CD44^{hi}CD4⁺ T cells analyzed by flow cytometry after intracytoplasmic staining. Data are means ± S.E.M. and are representative of one out of three independent experiments including n=5 mice per group. Unpaired two-tailed t test (A - C) and multiple t-test (D). *P < 0.05; **P < 0.01.

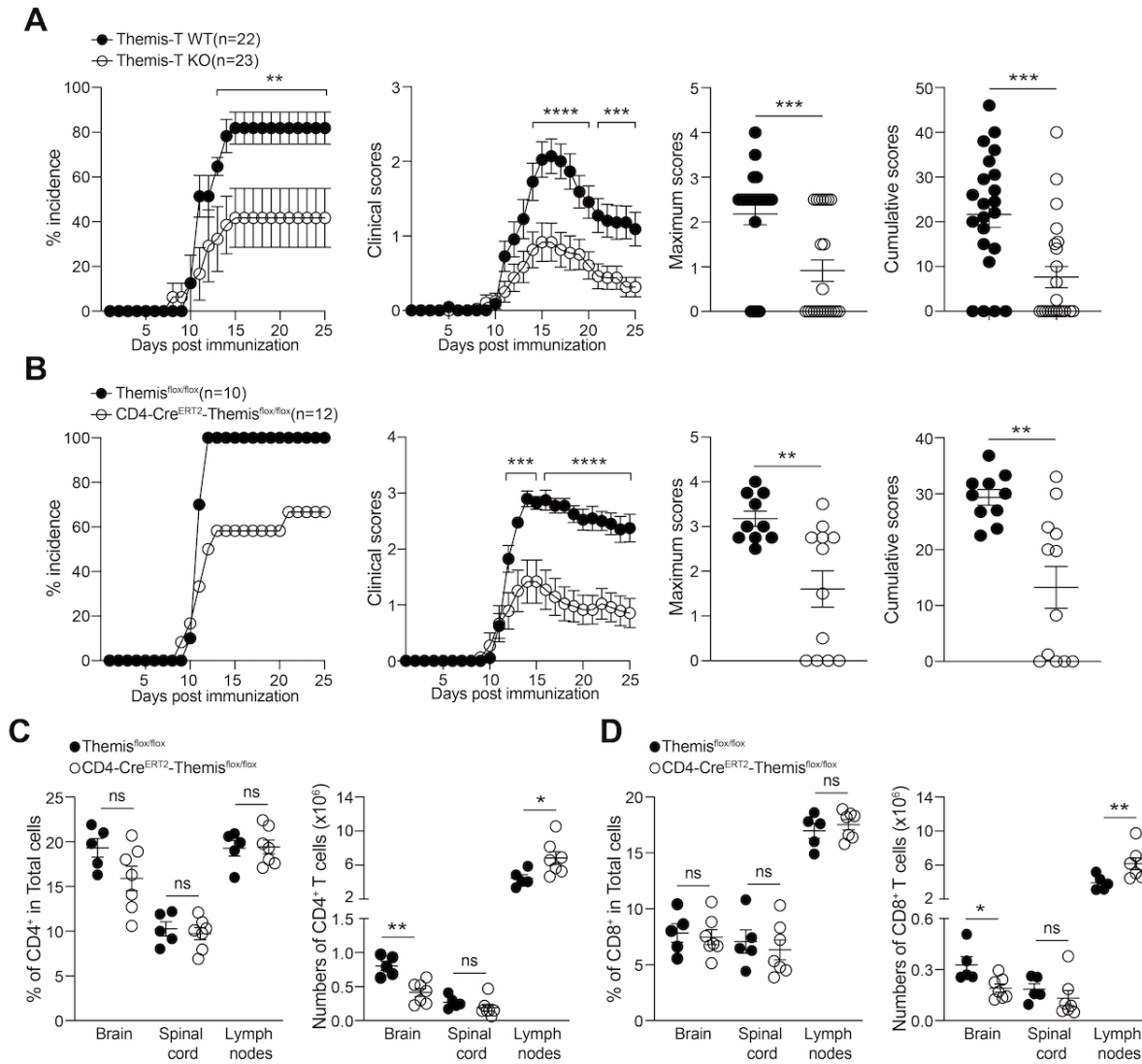


Figure 5. THEMIS expression in CD4⁺ T cells is associated with an enhanced susceptibility to the development of EAE. (A) *Themis*-T^{WT} (black circles) and *Themis*-T^{KO} (open circles) mice were immunized with 50 µg of peptide MOG₃₅₋₅₅ emulsified in CFA at day 0 and 200 ng of pertussis toxin was injected i.v. on day 0 and day 2. Incidence, clinical scores, maximum scores and cumulative clinical scores were calculated. Data represent four independent experiments including a total of n=22-23 mice per group. (B) *Themis*^{flox/flox} (black circles) and *CD4-Cre*^{ERT2}-*Themis*^{flox/flox} (open circles) mice were immunized with 200 µg of peptide MOG₃₅₋₅₅ emulsified in CFA at day 0 and 200 ng of pertussis toxin was injected i.v. on day 0 and day 2. Incidence, clinical scores, maximum scores and cumulative clinical scores were calculated. Data represent two independent experiments including a total of n=10-12 mice per group. (C, D) Dot graphs represent the percentages and numbers of CD4⁺ (C) and CD8⁺ (D) T cells in the lymph nodes, the brain and the spinal cord of *Themis*^{flox/flox} and *CD4 CreER(T2)-Themis*^{flox/flox} mice immunized with MOG₃₅₋₅₅. Data are representative of one experiment out of two independent experiments each including n=5-7 mice per group. Clinical scores and incidence were calculated using a two-way ANOVA. Unpaired two-tailed t test for other analysis. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

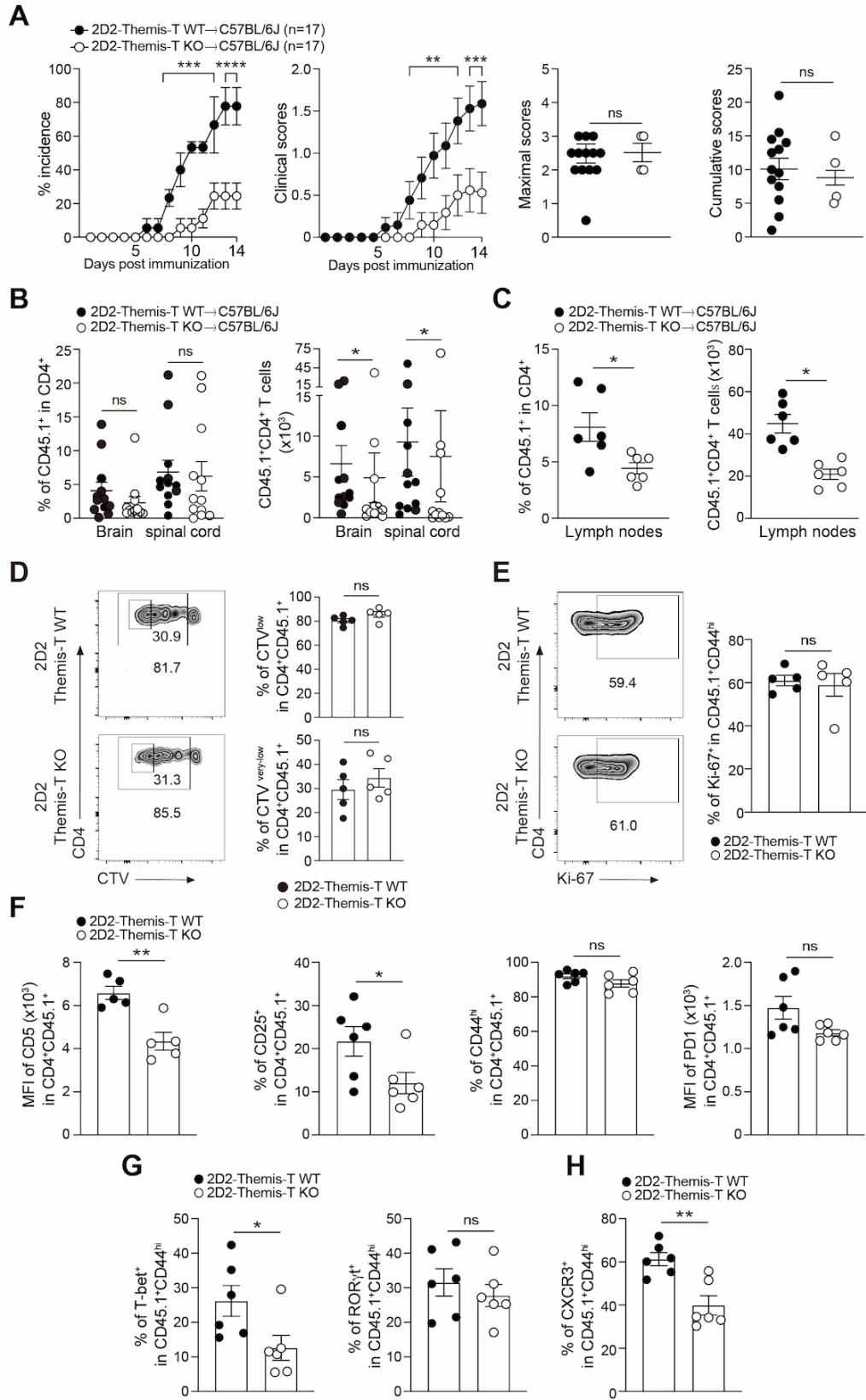


Figure 6. THEMIS enhances the encephalitogenic potential of CD4⁺ T cells expressing the MOG₃₅₋₅₅ specific 2D2 TCR. C57BL/6j mice (CD45.2⁺) were injected i.v. with 2×10^5 naïve CD45.1+CD4⁺ T cells from 2D2-*Themis*-*T*^{WT} and 2D2-*Themis*-*T*^{KO} mice. Mice were then immunized with 100 µg of peptide MOG₃₅₋₅₅ emulsified in CFA at day 0 and injected i.v. with 200 ng of pertussis toxin at day 0 and 2. (A) Incidence, clinical scores, maximum scores and cumulative clinical scores were calculated. Data represent three independent experiments including a total of n=17 mice per group. (B and C) Dot graph represent the percentages and numbers of CD45.1⁺ cells among CD4⁺ T cells in the brain and the spinal cord (B) and in the lymph nodes (C) at day 14 and 6 after immunization, respectively. Data in (B) represent two independent experiments including a total of n=12 mice per group. Data in C is representative of one experiment out of four independent experiments including n=6 mice per group (D) C57BL/6j mice (CD45.2⁺) were injected i.v. with 2×10^5 CTV stained naïve CD45.1+CD4⁺ T cells from 2D2-*Themis*-*T*^{WT} and 2D2-*Themis*-*T*^{KO} mice. Mice were then immunized with 100 µg of peptide MOG₃₅₋₅₅ emulsified in CFA at day 0 and injected i.v. with 200 ng of pertussis toxin at day 0 and 2. Proliferation was analyzed at day three after immunization. Contour plots represent CD4 vs CTV staining on CD45.1⁺ cells. Histogram bars represent the percentages of divided and highly divided cells based on the gating strategy indicated in the contour plots. (E) Contour plots represent CD4 vs Ki-67 staining on CD45.1⁺ cells. Histogram bars represent the percentage of Ki-67⁺ cells in CD45.1+CD44^{hi} cells. (F) Histogram bars represent the percentages of CD44^{hi} and CD25⁺ cells among CD45.1+CD4⁺ T cells as well as the MFI of PD-1 on CD45.1+CD4⁺ T cells. (G) Histogram bars represent the percentages of CD45.1+CD4⁺ T cells expressing T-BET and RORγt cells. (H) Histogram bars represent the percentages of CD45.1+CD4⁺ T cells expressing CXCR3. For D, E, F, G and H, data are representative of one experiment out of three to five independent experiments each including n=5-7 mice per group. Clinical scores and incidence were calculated using a two-way ANOVA. Unpaired two-tailed t test for the other analysis. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.

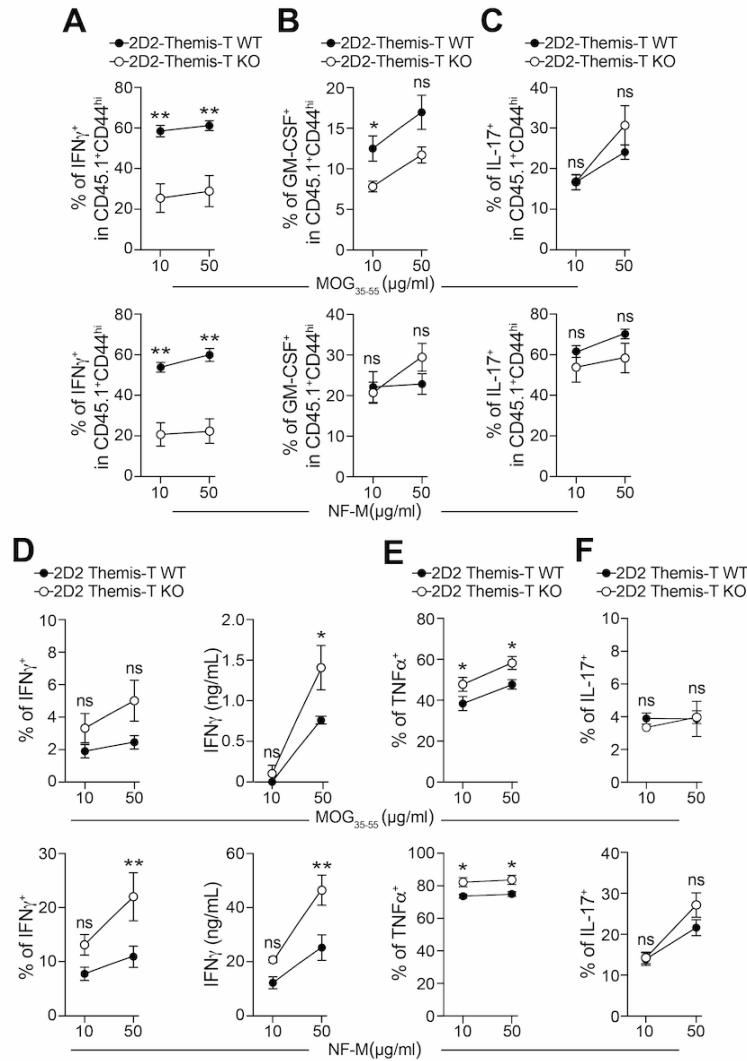
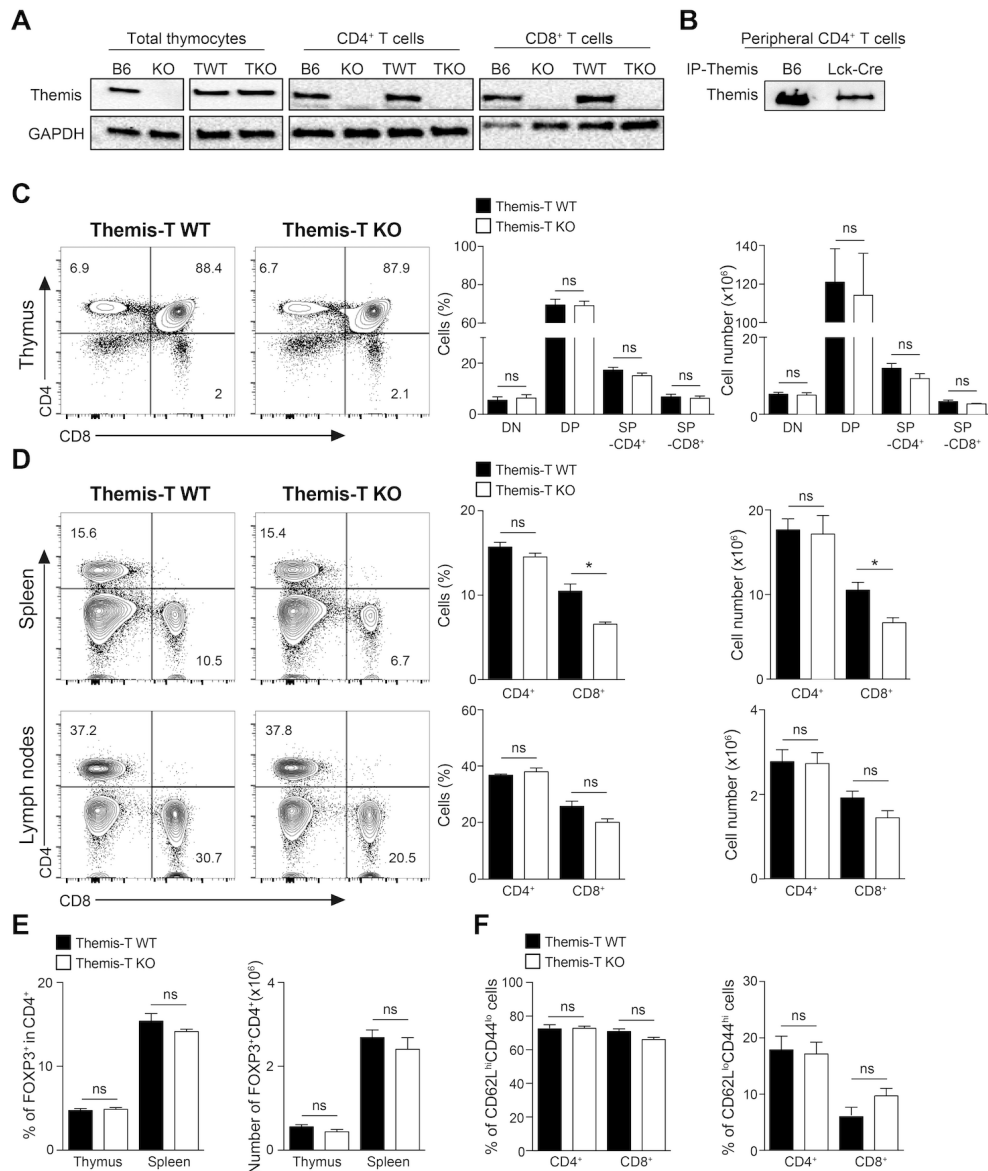
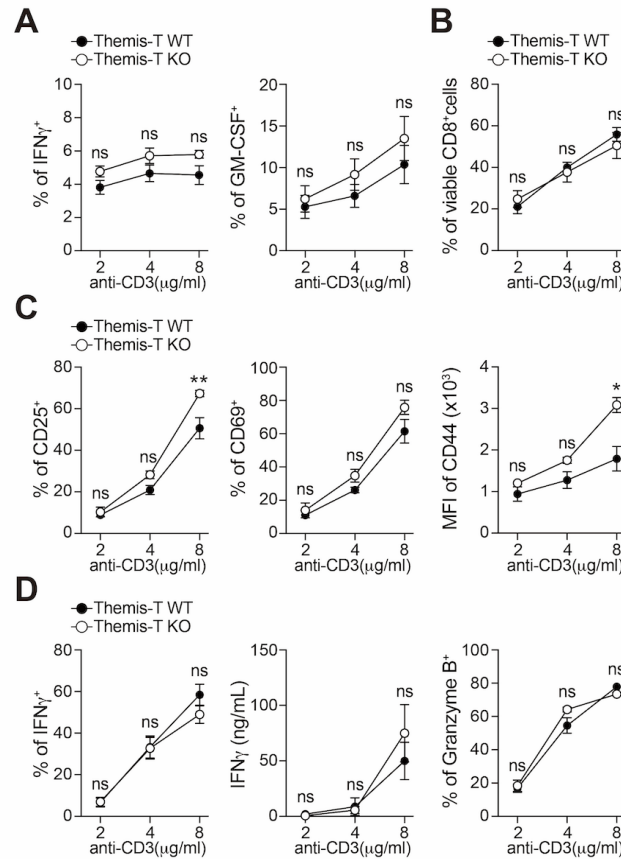


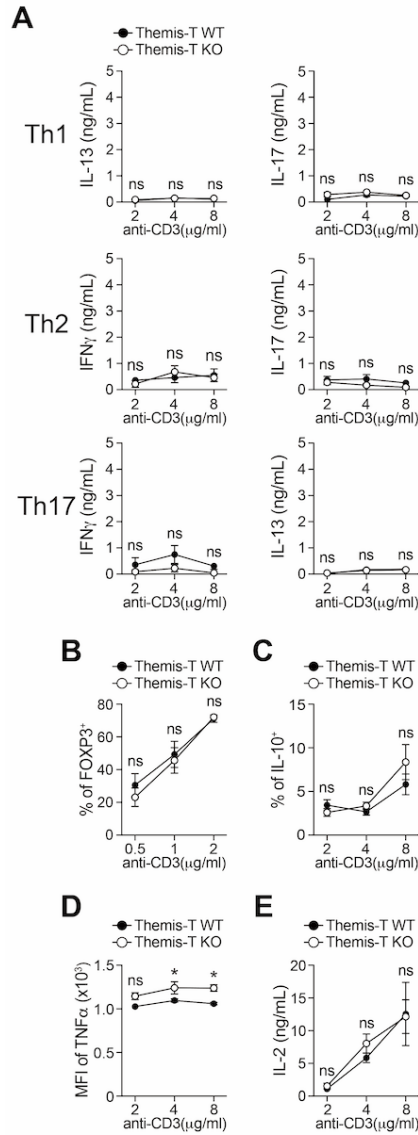
Figure 7. THEMIS exerts opposite effect on IFN γ production in effector CD4⁺ T cells and in Th1-polarized cells stimulated with neuroantigens. (A, B and C) C57BL/6j mice (CD45.2⁺) were injected i.v. with 2×10^5 naïve CD45.1⁺CD4⁺ T cells from 2D2-Themis-T^{WT} and 2D2-Themis-T^{KO} mice. Mice were then immunized with 100 μ g of peptide MOG₃₅₋₅₅ emulsified in CFA. Cells were collected from the draining lymph nodes at day six after immunization and stimulated with the indicated dose of MOG₃₅₋₅₅ (upper panel) and NF-M₁₅₋₃₅ (lower panel). The curve graphs represent the percentages of CD45.1⁺CD44^{hi} CD4⁺ T cells expressing IFN γ (A), GM-CSF (B) and IL-17 (C). Data are means \pm S.E.M. and are representative of one experiment out of five independent experiments each including n=5 mice per group. (D, E and F) Naïve CD62L^{hi}CD25⁻CD4⁺ T cells from 2D2-Themis-T^{WT} and 2D2-Themis-T^{KO} mice were stimulated with MOG₃₅₋₅₅ and NF-M₁₅₋₃₅ in presence of irradiated splenocytes in Th1 (D and E) and Th17 (D) polarizing conditions. Curve graphs represent the production of IFN γ (D), TNF α (E) and IL-17 (F) analyzed by flow cytometry after intracytoplasmic staining and by ELISA. Data are means \pm S.E.M. and are representative of one experiment out of two independent experiments each including n=3-4 mice per group. Unpaired two-tailed t test for the other analysis. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001.



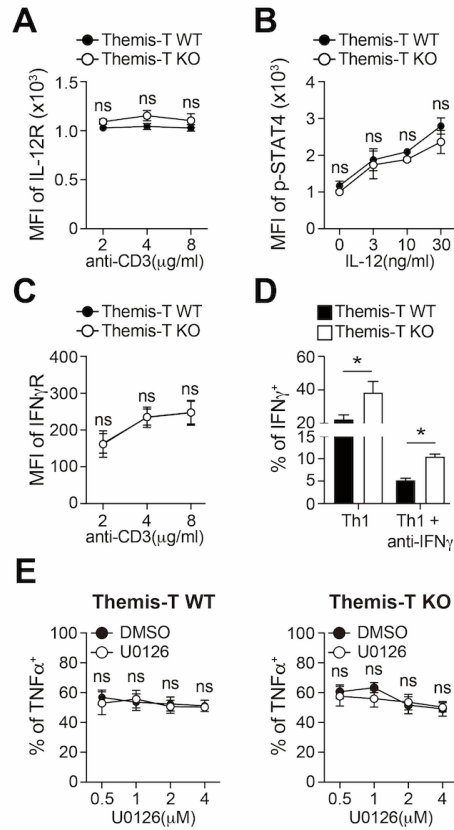
Supplementary Figure 1. Phenotypic analyses of thymocytes and peripheral T cells from *Themis*^{WT} and *Themis*^{KO} mice. (A) Total cytoplasmic extracts of thymocytes and peripheral T cells from *Themis*^{WT} (TWT) and *Themis*^{KO} (TKO) mice were analyzed by Western blot with antibodies against THEMIS and GAPDH. (B) THEMIS was immunoprecipitated from cellular extracts of peripheral CD4⁺ T cells from C57BL/6 and *dLck-Cre Themis*^{flox/flox} mice. (C and D) Dot plots show CD4 versus CD8 surface staining on thymocytes (C), splenocytes (D, middle panel) and lymph nodes (D, lower panel) from *Themis*^{WT} (TWT) and *Themis*^{KO} mice. Histogram bars represent the percentages of thymocytes and peripheral T cell subsets in mice from the indicated genotype. Data are mean ± S.E.M. and represent two to three independent experiments each including n=3-4 mice per group. (E) Histogram bars represent the percentages and numbers of CD4⁺ T cells expressing FOXP3 in mice from the indicated genotype. Data are mean ± S.E.M. and represent two independent experiments each including n=3 mice per group. (F) Histogram bars represent the percentages of CD62L^{hi}CD44^{lo} and CD62L^{lo}CD44^{hi} cells among CD4⁺ and CD8⁺ T cells in mice from the indicated genotype. Data are mean ± S.E.M. and represent three independent experiments each including n=3 mice per group. Unpaired two-tailed t test for the other analysis. *P < 0.05.



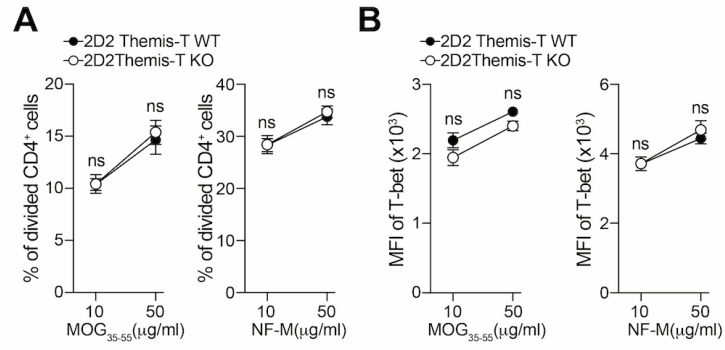
Supplementary Figure 2. Effect of THEMIS deficiency on CD4⁺ and CD8⁺ T cell responses induced after TCR stimulation. (A) Naïve CD62L^{hi}CD25⁻CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with the indicated dose of anti-CD3 antibodies in presence of anti-CD28 antibodies. Curve graphs represent the percentages of CD4⁺ producing IFN γ and GM-CSF analyzed by flow cytometry following intracytoplasmic staining. (B, C and D) Naïve CD62L^{hi}CD25⁻CD8⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with the indicated dose of anti-CD3 antibodies in presence of anti-CD28 antibodies for one (C) or 3 days (B and D). Curve graphs represent the percentages viable cells among CD8⁺ T cells (B), the percentages of CD4⁺ T cells expressing CD25 and CD69 as well as the MFI of CD44 staining on CD4⁺ T cells (C), the percentages of CD8⁺ T cells producing IFN γ and Granzyme B (D). Data are mean values \pm S.E.M and are representative of at least three independent experiments each including n=3-4 mice per group. Unpaired two-tailed t test for the other analysis. *P < 0.05; **P < 0.01.



Supplementary Figure 3. Effect of THEMIS deficiency on CD4 $^{+}$ T cell polarization. (A) Naïve CD62L $^{\text{high}}$ CD25 $^{-}$ CD4 $^{+}$ T cells from *Themis*-T $^{\text{WT}}$ vs *Themis*-T $^{\text{KO}}$ mice were stimulated with various doses of anti-CD3 antibodies in presence of anti-CD28 antibodies in Th1, Th2 and Th17 polarizing conditions. Curve graphs represent the production of IFN γ , IL-13, and IL-17 in Th1, Th2 and Th17 polarizing conditions analyzed by ELISA. (B) Naïve CD62L $^{\text{high}}$ CD25 $^{-}$ CD4 $^{+}$ T cells from *Themis*-T $^{\text{WT}}$ and *Themis*-T $^{\text{KO}}$ mice were stimulated with various doses of anti-CD3 antibodies in presence of anti-CD28 antibodies in iTreg polarizing conditions. Curve graphs represent the percentages of cells expressing FOXP3 among CD4 $^{+}$ T cells. (C) Naïve CD62L $^{\text{high}}$ CD25 $^{-}$ CD4 $^{+}$ T cells from *Themis*-T $^{\text{WT}}$ vs *Themis*-T $^{\text{KO}}$ mice were stimulated with various doses of anti-CD3 antibodies in presence of anti-CD28 antibodies in Tr1 polarizing conditions. Curve graphs represent the percentages of cells expressing IL-10 among CD4 $^{+}$ T cells. (D, E and F) Naïve CD62L $^{\text{high}}$ CD25 $^{-}$ CD4 $^{+}$ T cells from *Themis*-T $^{\text{WT}}$ and *Themis*-T $^{\text{KO}}$ mice were stimulated with various doses of anti-CD3 antibodies in presence of anti-CD28 antibodies in Th1 polarizing conditions. Curve graphs represent the MFI of TNF α staining gated on CD4 $^{+}$ TNF α $^{+}$ cells (D), the percentages of divided CD4 $^{+}$ T cells (CTV $^{\text{low}}$) (E), the secretion of IL-2 (F). Data are mean values \pm S.E.M and are representative of at least three independent experiments each including n=3-4 mice per group. Unpaired two-tailed t test for the other analysis. *P < 0.05.



Supplementary Figure 4. Effect of THEMIS deficiency on IL-12R-, IFN γ R- and TCR-mediated signaling in CD4⁺ T cells. (A and C) Naïve CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* vs *Themis-T^{KO}* mice were stimulated with various doses of anti-CD3 antibodies in presence of anti-CD28 antibodies in Th1 polarizing conditions. Curve graphs represent the MFI of IL-12R (A) and IFN γ R (C) staining on CD4⁺ T cells analyzed flow cytometry. B) Naïve CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* vs *Themis-T^{KO}* mice were stimulated with 8 $\mu\text{g/ml}$ of anti-CD3 antibodies in presence of anti-CD28 antibodies for two days. Cells were then stimulated following overnight resting with the indicated dose of IL-12 for 40 minutes. The curve graph represents the MFI of phosphorylated STAT4 in CD4⁺ T cells. (D) Naïve CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* vs *Themis-T^{KO}* mice were stimulated with anti-CD3 antibodies and anti-CD28 antibodies in Th1 polarizing conditions in the absence or in the presence of IFN γ R neutralizing antibody. Bar graph represents the percentages of CD4⁺ T cells producing IFN γ analyzed by flow cytometry after intracytoplasmic staining. (E) Naïve CD62L^{high}CD25⁻ CD4⁺ T cells from *Themis-T^{WT}* and *Themis-T^{KO}* mice were stimulated with anti-CD3 and anti-CD28 antibodies in Th1 polarizing conditions in presence of the indicated doses of ERK inhibitor (U0126) or DMSO. Curve graphs represent the percentages of CD4⁺ T producing TNF α analyzed by flow cytometry after intracytoplasmic staining. Data are mean values \pm S.E.M and are representative of two to three independent experiments each including n=3-4 mice per group. Unpaired two-tailed t test for the other analysis. *P < 0.05.



Supplementary Figure 5. Effect of THEMIS deficiency on Th1 cell responses following stimulation with MOG₃₅₋₅₅ and NF-M₁₅₋₃₅. (A and B) CTV stained naïve CD62L^{hi}CD25⁻CD4⁺ T cells from 2D2-*Themis-T^{WT}* and 2D2-*Themis-T^{KO}* mice were stimulated with MOG₃₅₋₅₅ and NF-M₁₅₋₃₅ in presence of irradiated splenocytes in Th1 polarizing condition for 3 days. Curve graphs represent the percentages of divided CD4⁺ T cells (CTV^{low}) (A) and the MFI of T-BET staining (B) in CD4⁺ T cells analyzed by flow cytometry following intracytoplasmic staining. Data are mean values ± S.E.M and are representative of two independent experiments each including n=3-4 mice per group. Unpaired two-tailed t test for the other analysis. *P < 0.05.

Discussion

Peripheral CD4⁺ T cells play a central role in the adaptive immune response. The activation of CD4⁺ T cells leads to their proliferation and to their differentiation into distinct Th subpopulations which migrate toward the inflammation sites. Each Th subset secretes a specific panel of cytokines and chemokines which contribute to activate other immune cells and promote their recruitment to infection sites. The exacerbated activation and differentiation of CD4⁺ T cells into specific subsets may lead to the development of organ-specific autoimmune diseases such as RA, MS or may enhance the susceptibility to develop inflammatory diseases such as IBD. The magnitude of CD4⁺ T cell responses is modulated by proteins which regulate signals triggered by the TCR, cytokine receptors and additional receptors. Identifying new signalling proteins involved in the regulation of CD4⁺ T cell responses may help to better understand the mechanism by which autoimmune and inflammatory diseases occur.

THEMIS is a T cell-lineage protein, which promotes the positive selection of thymocytes by regulating TCR signalling (Choi, Warzecha, *et al.*, 2017; Fu *et al.*, 2009; Lesourne *et al.*, 2009). The germline deletion of *themis* (*themis*^{-/-}) results in a strong reduction of CD4⁺ and CD8⁺ T cells in the thymus and in peripheral lymphoid organs. *themis*^{-/-} mice do not constitute a good model for exploring the function of THEMIS in peripheral T cells since the lymphopenia status of those mice may alter the profile of T cell responses independently of THEMIS expression. To avoid this, we created a model, in which the gene encoding for THEMIS is invalidated at late stages of T cell development in the thymus. By using this mouse model, my PhD work revealed that:

- THEMIS deficiency leads to opposite outcomes on peripheral CD4⁺ T cell responses following TCR triggering *in vitro* and antigen recognition *in vivo*.
- THEMIS enhances the susceptibility to EAE development by enhancing encephalitogenic Th1 responses in the early stage of the disease.

1. Opposite outcomes of THEMIS deficiency on peripheral CD4⁺ T cell responses *in vitro* and *in vivo*

Our results show that THEMIS deficiency leads to exacerbated production of Th1 cytokines when naïve CD4⁺ T cells were polarized into Th1 cells. THEMIS inhibits IFN γ production in those conditions by repressing ERK-dependent Th1 responses but has no effect on T-bet, the key transcription factor for differentiation into Th1 cells. Similar effects were observed when cells were stimulated with anti-CD3 antibodies and with the encephalitogenic peptides MOG₃₅₋₅₅ and NF-M₁₅₋₃₅, suggesting that this outcome is not due to the methodology of stimulation. The repressive effect of THEMIS on Th1 responses observed *in vitro* appeared stronger when high concentration of anti-CD3 antibodies and NF-M₁₅₋₃₅ peptide were used, suggesting that THEMIS may operate in a negative feedback loop triggered by strong TCR stimulation. By contrast to these *in vitro* data, THEMIS deficiency leads to decreased T-bet expression and reduced IFN γ production in effector CD4⁺ T cells re-stimulated with MOG₃₅₋₅₅ and NF-M₁₅₋₃₅ after immunization with MOG₃₅₋₅₅. Thus, the absence of THEMIS leads to nearly opposite outcomes upon the polarization of naïve CD4⁺ T cells into Th1 cells and the re-stimulation of effector CD4⁺ T cells with the same set of antigenic peptides. The stimulation environment *in vitro* presumably does not reflect the environment *in vivo*, where CD4⁺ T cells receive multiple stimuli that may affect the ability of cells to respond to antigen challenge. Analysis of Th1 responses *in vitro* were performed partly by using irradiated splenic cells as APC. Those CD4⁺ T cells may not express similar co-stimulatory and co-inhibitory molecules as the CD4⁺ T cells which have migrated into lymph nodes after immunization. The irradiation of cells may affect the expression of co-receptor ligands on APCs and the production of cytokines which may promote additional signals controlling Th1 responses. Although we could not formally explain the reasons of those opposite effects, we speculate that the phosphatases SHP-1 and SHP-2, which are the main target of THEMIS, may not be engaged similarly by the TCR and additional cell surface receptors in naïve CD4⁺ T cells polarized into Th1 cells and in effector T cells. Since SHP-1 and SHP-2 can have opposite outcomes on CD4⁺ T cell responses, we believe that THEMIS may exert distinct effect according to which phosphatases is functionally preponderant in a given stimulatory context.

A. What is the mechanism by which THEMIS regulates Th1 responsiveness *in vitro*?

THEMIS seems to repress Th1 responses independently of SHP-1

SHP-1 is mainly characterized as an inhibitory regulator of TCR signaling, which can repress the activity of several key TCR signaling molecules, such as the TCR ζ chain, the adaptor LAT and the Src-family kinases and the Rac-GTP exchange factor VAV1 (Carter *et al.*, 1999; Fawcett & Lorenz, 2005; Kosugi *et al.*, 2001; Stebbins *et al.*, 2003; Su *et al.*, 2001). Whereas SHP-2 has been ascribed with both inhibitory and enhancing roles on TCR signaling, depending on its substrate and on the context of its recruitment to the membrane (Arnaud *et al.*, 2004; Dong *et al.*, 2015; Frearson & Alexander, 1998; Nguyen *et al.*, 2006; Niogret *et al.*, 2019; Salmond *et al.*, 2005; Yokosuka *et al.*, 2012). In thymocytes, THEMIS constitutively binds with SHP-1 and to a lesser extent with SHP-2 (Choi, Warzecha, *et al.*, 2017). The role of THEMIS on SHP-1-mediated activity has been the subject of controversy and has led to nearly two opposite models to explain the mechanism by which THEMIS regulates T cell development. In the first model, THEMIS promotes positive selection by recruiting SHP-1 to TCR signaling complexes and/or by promoting its phosphatase activity, reducing thereby the strength of TCR signals below the threshold of negative selection (Fu *et al.*, 2013). In the second model, THEMIS enhances positive selection by blocking SHP-1 activity, promoting or stabilizing the oxidation of its catalytic cysteine, and enhancing thereby TCR signal strength beyond the TCR signalling threshold required for positive selection (Choi, Warzecha, *et al.*, 2017). Although THEMIS inhibits SHP-2 to a lesser extent than SHP-1 (Choi, Warzecha, *et al.*, 2017), the consequence of this inhibition on T cell development and T cell responses has not been clearly evaluated.

One can wonder therefore which of these two models would explain the best our results. Early studies using the motheaten mouse model identified SHP-1 as a negative regulator of the generation of Th1 subpopulation (Kamata *et al.*, 2003; Yu *et al.*, 2005). SHP-1 deficiency leads to increased proliferation of polarized Th1 and increased production of Th1 cytokines. This is partially similar to our *in vitro* results, suggesting that THEMIS may repress IFN γ production in Th1 cells as described in **Model 1**, by recruiting SHP-1 into TCR signalling complexes or by enhancing its PTP activity. However, our results show that, THEMIS deficiency does not affect the proliferation and survival of Th1 cells, suggesting that a different mechanism might be engaged. SHP-1 deficiency also leads to exacerbated Th2 responses

(Johnson *et al.*, 2013), which is not the case of THEMIS deficiency. In addition, naïve CD4⁺ T cells with either natural mutation on *ptpn6*, the gene encoding for SHP-1 or deficient for SHP-1 exhibit normal signalling responses following TCR stimulation, contrasting with our data showing that THEMIS deficiency leads to increased ERK1/2 activity upon TCR stimulation. Furthermore, our results show that THEMIS deficiency leads to increased production of IFN γ in Th1 particularly upon strong TCR stimulations (high doses of anti-CD3 or high affinity peptides). This is different from what has been described in studies supporting model 1, which detected an increase in TCR signalling events when using weak TCR stimulation (Fu *et al.*, 2013). Altogether, these considerations suggest that THEMIS does not inhibit Th1 responses *in vitro* by promoting or by repressing SHP-1.

Studies based on SHP-1 deficient mice support model 2 more than model 1

More generally, model 1 which is mostly based on *in vitro* studies has been challenged by several *in vivo* observations (Choi, Cornall, *et al.*, 2017). A prediction from Model 1 is that SHP-1 deficiency should lead to similar effect on T cell development than THEMIS deficiency. This is not the case since positive selection is mildly exacerbated in mice expressing inactive form of SHP-1 (Johnson *et al.*, 2013; Kamata *et al.*, 2003; Mercadante & Lorenz, 2017). The absence of similarity was explained by authors of Model 1, by the potential redundancy between SHP-1 and SHP-2 in thymocytes (Fu *et al.*, 2013; Mehta *et al.*, 2018). However, this explanation was challenged recently by the phenotype of mice with double deficiencies in THEMIS and SHP-1. According to model 1, SHP-1 deficiency in a THEMIS deficient background should either aggravate the effect of THEMIS on positive selection or have no additional consequences as the ones observed in the context of THEMIS deficiency alone. However studies from Paul Love's team showed that SHP-1 deficiency rescued the impaired positive selection of thymocytes due to THEMIS deficiency, arguing against model 1 and in favour of model 2 (Choi, Warzecha, *et al.*, 2017). Analysis of TCR signal strength using the TCR signalling sensor transgenic mouse model, Nur-77 promoter-driven GFP, reveals that TCR signalling is reduced in the absence of THEMIS *in vivo* rather than increased as predicted by model 1 (Zvezdova *et al.*, 2016). Altogether those suggest are in favour model 2 rather than of model 1.

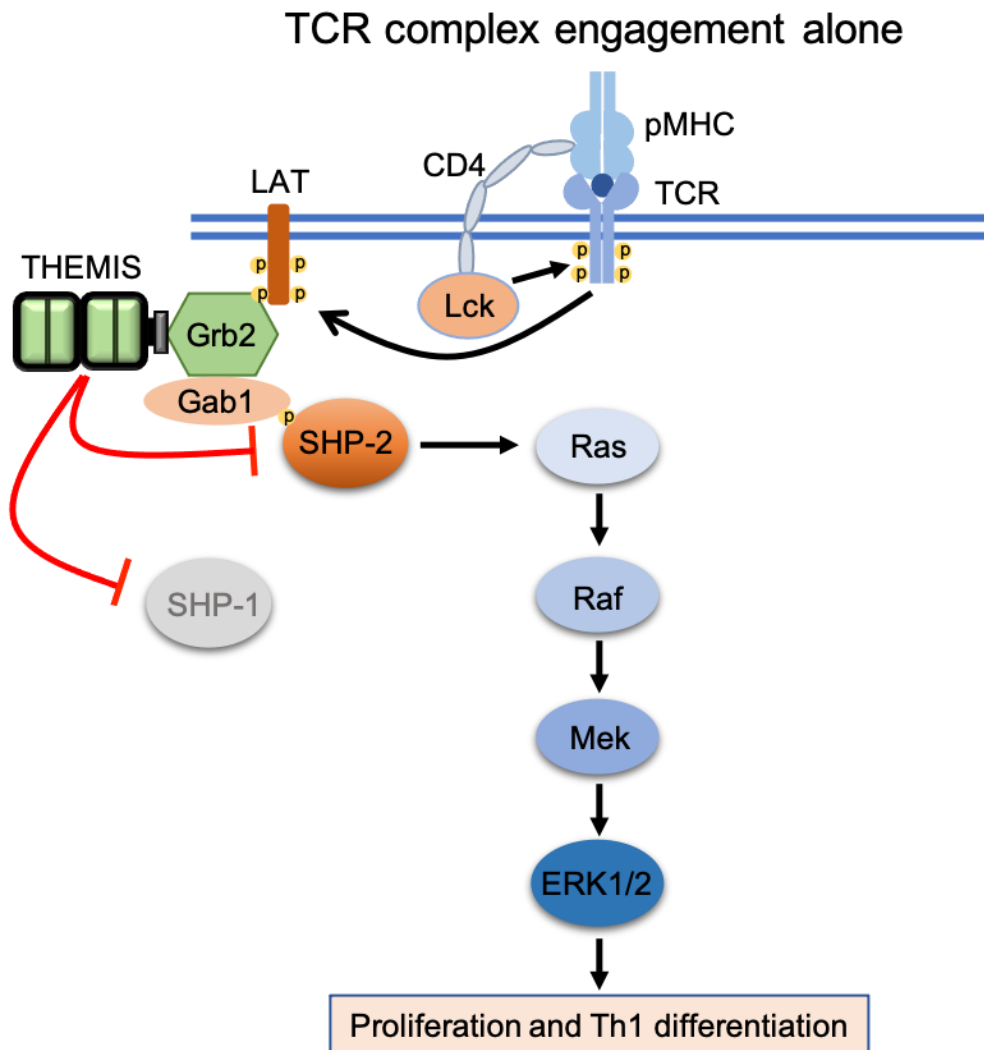


Figure 15 . Hypothetical model of THEMIS function in CD4⁺ T cells following TCR stimulation *in vitro*

TCR stimulation-induced phosphatase SHP-2 is able to positively regulate Th1 cell responses, while SHP-1 is not TCR signaling dependent. THEMIS may inhibit ERK-mediated Th1 responses by blocking the PTP activity of SHP-2 upon TCR stimulation alone. (Chang *et al.*, 2012; Johnson *et al.*, 2013; Nguyen *et al.*, 2006)

THEMIS would repress Th1 responses in vitro by blocking SHP-2 activity

The inactivation of SHP-2 activity in Jurkat cells and SHP-2 deficiency in thymocytes lead to reduced ERK activation following TCR engagement *in vitro* (Fearnson & Alexander, 1998; Nguyen *et al.*, 2006). SHP-2 participates in a multimeric signaling complex that regulates TCR coupling to the Ras/MAPK pathway in Jurkat T cells. It suggests that THEMIS may inhibits ERK1/2-mediated IFN γ production in Th1 cells by repressing the PTP activity of SHP-2 (**Figure 15**).

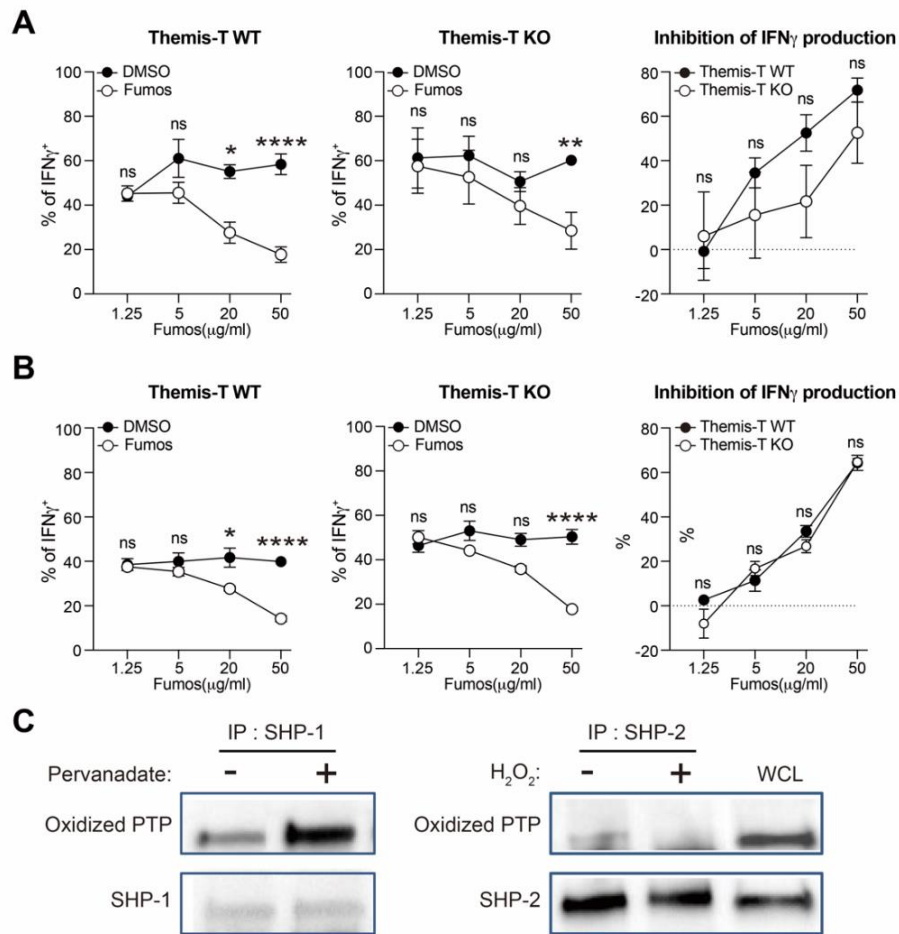


Figure 16. SHP-2 phosphatase activity and IFN γ production in polarized Th1 cells *in vitro*

(A, B) Effect of THEMIS deficiency on the inhibition of IFN γ production in Th1 cells following treatment with the SHP-2 inhibitor Fumos. Naïve CD62L^{high}CD25- CD4⁺ T cells from *themis*-T^{WT} and *themis*-T^{KO} mice were stimulated with anti-CD3 antibodies in Th1 polarizing conditions in the presence of the indicated doses of SHP-2 inhibitor (Fumos) or DMSO. (A) and (B) show the results of the first and second experiments, respectively. Left and middle curve graphs represent the production of IFN γ by CD4⁺ T cells analysed by flow cytometry after intracellular staining. Right curve graph represents the inhibition of IFN γ produced by CD4⁺ T cells following treatment with the indicated doses of Fumos. Data are mean \pm S.E.M, including n = 3 mice per group in each experiment. Unpaired t test. *P < 0.05; **P < 0.01; ***P < 0.001; ****P < 0.0001. (C) Oxidation of phosphatase SHP-1 and SHP-2 in peripheral CD4⁺ T cells. Total CD4⁺ T cells from C57BL/6 mice were stimulated with pervanadate or H₂O₂. SHP-1 (Left) and SHP-2 (right) were immunoprecipitated from cellular extracts of the stimulated cells and analysed by western blot with antibodies against oxidized PTP, SHP-1 and SHP-2. WCL: cellular lysates of total CD4 T cells from C57BL/6 mice.

In support of this interpretation, we found that SHP-2 phosphorylation is reduced in THEMIS deficient CD4⁺ T cells. The reduced phosphorylation of SHP-2 might be the consequences of an hyperactivated state of this phosphatase which could presumably dephosphorylate itself through auto- or trans-phosphatase activity. We tried to test this hypothesis further by treating naïve CD4⁺ T cells with Fumosorine (Fumos), a specific SHP-2 inhibitor, under Th1-polarizing conditions, in two distinct experiments (**Figure 16 – A, B**). Result from one experiment suggests that higher doses of inhibitor were required to inhibit the production of IFN γ in THEMIS deficient Th1 cells to the same extent as compared to that in control cells (**Figure 16 – A**). However, this was not confirmed in the second experiment (**Figure 16 – B**). We are planning to perform additional experiments to resolve this question.

The stimulation of immune cell surface receptors has been shown to generate intracellular reactive oxygen species (ROS), which may modulate signal transduction by inactivating the PTP domain of phosphatases (Pao *et al.*, 2007). In thymocytes, THEMIS inhibits SHP-1 activity by promoting or stabilizing the oxidation of SHP-1 catalytic cysteine in the presence of ROS (Choi, Warzecha, *et al.*, 2017). In Jurkat T cells, SHP-2 has been shown to regulate T cell adhesion in a redox-dependent manner by downregulating the phosphorylation of ADAP and its recruitment to SLP-76 (Choi, Warzecha, *et al.*, 2017). These findings suggest that THEMIS may inhibit the PTP activity of SHP-2 by regulating its susceptibility to ROS oxidation. Therefore, we analysed the effect of THEMIS deficiency on the oxidation level of SHP-2 in CD4⁺ T cells treated with pervanadate using specific antibodies which recognize the oxidated form of the catalytic cysteine contained in the catalytic site of several tyrosine phosphatases. Unfortunately, we were not able to detect an oxidation of SHP-2, by contrast to SHP-1, after pervanadate treatment. It remains possible that this antibody does not efficiently recognize oxidized-SHP-2 (**Figure 16 – C**) by contrast to other oxidized forms of tyrosine phosphatases.

B. What is the mechanism by which THEMIS regulates Th1 responses *in vivo*?

Although our *in vitro* data indicate that THEMIS inhibits the secretion of Th1 cytokines, results obtained *in vivo* suggest that THEMIS promotes the differentiation of naïve CD4⁺ T cells into Th1 cells. One potential explanation to this apparent discrepancy could be that THEMIS regulates additional signals that are triggered specifically *in vivo* and which may not be recapitulated *in vitro* in a reductionist model. CD4⁺ T cell responses induced *in vitro* are triggered either exclusively by anti-CD3 stimulation or by

irradiated splenic cells which may not express the same set of co-stimulatory and co-inhibitory molecules expressed by APC in the draining lymph nodes following immunization. The phosphatases SHP-1 and SHP-2 are recruited to several co-inhibitory and cytokines receptors which may be engaged by their ligands *in vivo* but not *in vitro* (Rhee & Veillette, 2012). Several of those receptors were shown to be important in the regulation of Th1 responses, suggesting that THEMIS may enhance type 1 responses *in vivo* by operating downstream of those receptors rather than downstream of the TCR.

A major tumour escape mechanism relies on the stimulation of inhibitory receptors expressed on T cells, so called immune checkpoints (IC), leading to the inhibition of signalling events triggered by the TCR and by addition co-stimulatory receptors (Rudd, 2019), and ultimately to the development of a state of exhaustion in which T cell required higher TCR stimulation to respond to antigen challenge. In the context of cancer treatment, blocking the activation of immune checkpoints with antagonist antibodies results partly in an increase in the production of IFN γ by Th1 and other T cells, which is associated with a better anti-tumour response (Chen *et al.*, 2015; Curran *et al.*, 2010; Laheurte *et al.*, 2019; Wei *et al.*, 2017).

Blocking the stimulation of CTLA4 and PD-1 enhances the co-stimulatory effects of ICOS on Th1 anti-tumor response (**Figure 17**). CD4⁺ICOS^{hi}IFN- γ ⁺ cells show strong anti-tumor activity in the context bladder cancer and melanoma in humans, and are shown to produce high level of Th1-cytokines IFN γ and TNF α (Fu *et al.*, 2011; Liakou *et al.*, 2008). Two studies showed that this population could be effectively expanded by blocking CTLA-4 alone or in combination with PD-1, resulting in decreased tumour expansion (Fan *et al.*, 2014; Wei *et al.*, 2019). A study of James P. Allison's team based on t-SNE analysis found that blocking CTLA-4 led to an expansion of tumor infiltrating Th1-like cells which highly express T-bet (Wei *et al.*, 2017). According to previous studies, CTLA-blockade enhances T-bet expression induced by ICOS-mediated PI3K/Akt signaling (Chen *et al.*, 2014). THEMIS may thus suppress the CTLA-4/SHP-2-mediated inhibition of PI3K pathway, thereby enhancing ICOS-mediated signaling associated with T-bet expression in CD4⁺ T cells.

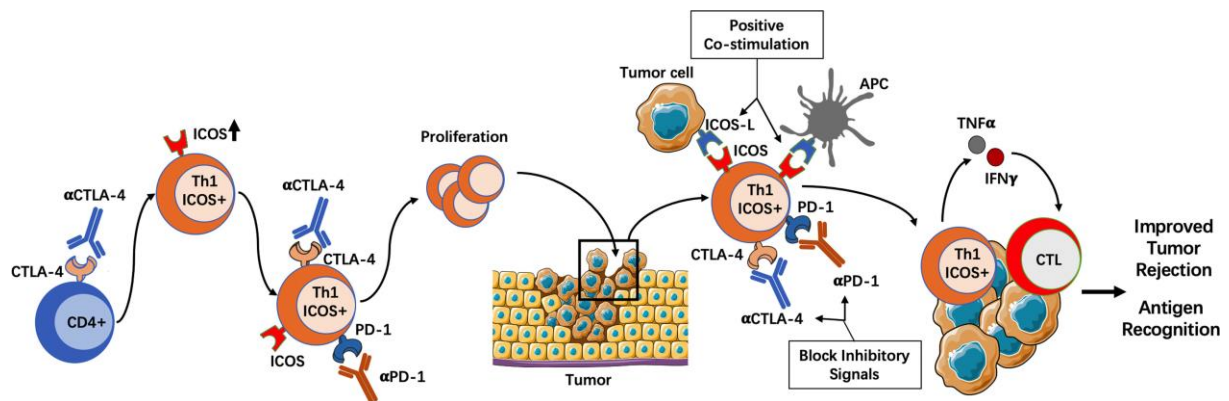


Figure 17. Enhanced Th1 cells production following immune checkpoints blockade

The engagement of the co-stimulatory receptor ICOS by its ligand ICOS-L induces the production of ICOS⁺Th1 cells, a population that suppress tumor expansion with high production of IFNγ and TNFα. Blocking the activation of the co-inhibitory receptors CTLA-4 and PD-1 with antibodies enhance the differentiation of this population. (Lee *et al.*, 2021)

The immune checkpoint PD-1 has also been shown to exert selective effect on Th1 responses in the context of cancer. Robert L. Ferris's team found that the expression of SHP-2 and PD-1 are significantly higher in tumor-infiltrating lymphocytes (TILs) than in peripheral blood lymphocytes [10]. Their results showed that stimulation of TILs by PD-L1 suppresses the STAT1/T-bet signalling axis in a SHP-2 dependent fashion, resulting in decreased production of Th1 cytokines by those cells. These reduced Th1 responses were restored by anti-PD-1 blocking antibodies.

Another interesting finding of this study is that the stimulation of PD-1 represses the production of Th1 cytokines but not Th2 cytokines by those TILs upon TCR stimulation, indicating a preferential inhibitory effect of PD-1 on the Th1 subset. These suggest that THEMIS may enhance T-bet-mediated Th1 responses by suppressing the PD1/SHP-2/pSTAT1.

Additional inhibitory receptors that recruit SHP-1 and SHP-2 were shown to be important in the regulation of Th1 responses *in vivo*. The co-inhibitory receptor BTLA which operates mainly by recruiting SHP-1 (Celis-Gutierrez *et al.*, 2019), has been described as preferentially expressed on Th1 cells (Loyet *et al.*, 2005). Mice deficient for BTLA exhibit exacerbated EAE development due to enhanced auto-immune CD4⁺ T cell activity (Watanabe *et al.*, 2003). The Leukocyte-associated Ig-like receptor 1 (LAIR1), another inhibitory co-receptor which functions by recruiting both SHP-1 and SHP-2, is associated with reduced Th1 responses [22].

Based on those evidences, we speculate that THEMIS may enhance Th1 responses *in vivo* by blocking the inhibitory activity of one or several of those receptors. THEMIS would operate by blocking the PTP activity of SHP-1 and SHP-2 which are required for the signaling function of those receptors. Imaging studies suggest that THEMIS is recruited to the Human T-cell leukaemia virus type 1 (HTLV-1) bZIP factor (HBZ) and modulates in this context the interaction of PD-1 with SHP-2, resulting in enhanced T cell proliferation (Kinosada *et al.*, 2017). Molecularly speaking, we believe that THEMIS operates similarly *in vitro* and *in vivo* by blocking the PTP activity of SHP-1 and SHP-2.

However, the respective importance of those phosphatases on T cell responses might be different in the context of *in vitro* stimulation, when signals emanate almost exclusively from the TCR, and *in vivo* stimulation, when signals integrate multiple additional inputs triggered by additional receptors. SHP-1 exerts relatively modest effect on T cell responses *in vitro* following T cell stimulation with anti-CD3 antibodies (Johnson *et al.*, 2013), by contrast to SHP-2 which exerts strong positive regulatory roles on T cell activation (Nguyen *et al.*, 2006). The effect of SHP-2 on TCR signaling is almost the opposite according to whether it operates downstream of the TCR (stimulatory function) or downstream of immune checkpoints (inhibitory functions). Thus, it's possible that THEMIS represses TCR signals that emanate directly from the TCR, but that repressing effects is overcome *in vivo* by positive effect due to THEMIS-mediated inhibition of co inhibitory receptors that negatively regulate Th1 responses.

In addition, strong TCR signals are correlated with increased development of Th1 responses (van Panhuys *et al.*, 2014; Yamane & Paul, 2013), while the engagement of SHP-1 and SHP-2 by several inhibitory receptors dampens TCR signal strength (Egen & Allison, 2002; Hannier *et al.*, 1998; Xu, 2019; Yokosuka *et al.*, 2012). The effect of THEMIS deficiency on TCR signal strength is reflected *in vivo* by the reduced expression of CD5 at the surface of antigen-specific CD4⁺ T cells, whereas the expression of CD5 was not affected *in vitro* following TCR engagement (data not shown), suggesting that THEMIS may enhance TCR signals and consequently Th1 responses by repressing inhibitory receptors which may recruit SHP-1 and SHP-2.

An alternative possibility to explain the effect of THEMIS on CD4⁺ T cell responses *in vivo* may rely on the inhibitory effect of SHP-1 on cytokine receptor-mediated signals driving Th1 responses. TGF- β 1 is an anti-inflammatory cytokine which can be produced by Treg cells and is able to inhibit Th1 development by suppressing T-bet expression in CD4⁺ T cells (Gorelik *et al.*, 2002; Lin *et al.*, 2005). By using T cells with motheaten-derived mutation of SHP-1, the team of James D. Gorham found that the inhibition of TGF- β 1-mediated inhibition of T-bet depends on SHP-1. TGF- β 1 rapidly induced the expression of SHP-1 in Th1 cells in the presence of IFN γ , which then dephosphorylate the kinases Jak1 and the transcription factor STAT1, leading to reduced expression of Th1 transcription factors T-bet and IRF-1 (I. K. Park *et al.*, 2005).

It has been reported that SHP-2 also impairs IFN γ induced JAK1/STAT1 signaling by binding with unphosphorylated form of STAT1, inhibiting the recruitment of STAT1 to IFN γ R (Wu *et al.*, 2012). Moreover, PD-1 ligation can enhance this inhibition of IFN γ -induced pathway by SHP-2 (Li *et al.*, 2015). Therefore, one possibility would be that THEMIS enhances T-bet-mediated Th1 development by inhibiting the recruitment of SHP-1 and SHP-2 to IFN γ -induced JAK1/STAT1/T-bet signaling.

Taken together, based on model 2, suggesting that THEMIS regulates T activation by blocking the phosphatase activity of SHP-1 and SHP-2, we speculate that THEMIS may enhance T-bet-mediated Th1 responses *in vivo* by regulating TCR signal strength and other signalling pathways independent of the TCR (**Figure 18**).

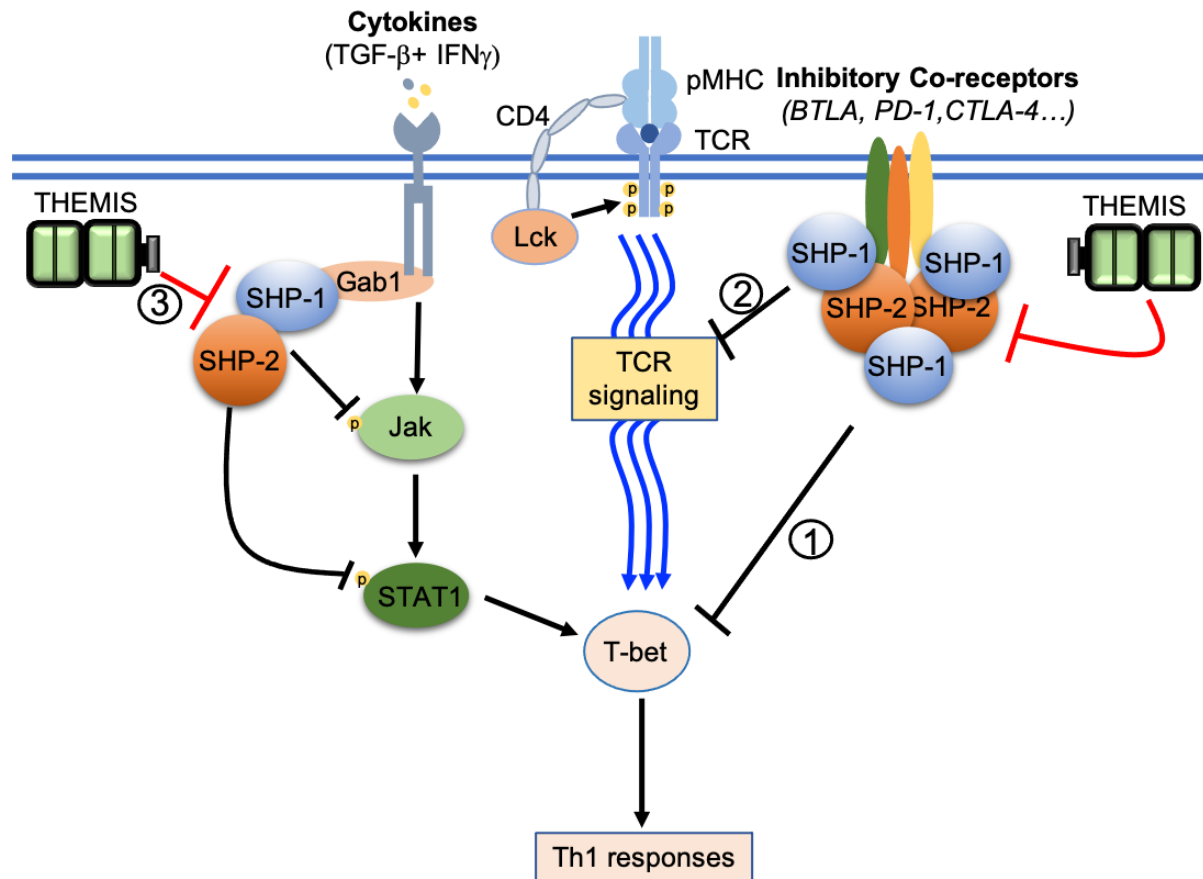


Figure 18. Hypothetical model of THEMIS function in CD4⁺ T cells upon antigen stimulation *in vivo*

In vivo, the phosphatases SHP-1 and SHP-2 can be recruited to several signaling pathways. By blocking the phosphatase activity of SHP-1 and SHP-2, THEMIS may represses the inhibitory effects of immune checkpoints (1) on T-bet expression and (2) on TCR signal strength, and (3) enhances the signals mediated by cytokine/ cytokine receptor, leading to enhanced T-bet mediated Th1 responses *in vivo*. (Chen *et al.*, 2014; I.-K. Park *et al.*, 2005; Wu *et al.*, 2012; Yu *et al.*, 2005)

2. Role of THEMIS in the pathophysiology of EAE

Our study demonstrated that THEMIS enhances the susceptibility to the development of EAE. The absence of THEMIS is correlated with the decreased expression of T-bet in MOG₃₅₋₅₅ specific CD4⁺ T cells and the reduced production of IFN γ following re-stimulation with encephalitogenic peptides *ex vivo*. This is associated with the decreased infiltration of effector CD4⁺ T cells in the CNS. The expression of T-bet is also decreased in CD4⁺ T cells following immunization with a foreign antigen 1W1K, and is associated with a reduction in the production of IFN γ . Given the central role of T-bet in the development of EAE, this suggests that THEMIS promotes EAE by positively regulating T-bet expression.

A. Effect of THEMIS on T-bet and IFN γ in the pathogenicity of CD4⁺ T cell in EAE

The absence of T-bet in CD4⁺ T cells leads to a selective defect in the differentiation of naïve T cells into Th1 cells *in vitro* and *in vivo* (Szabo *et al.*, 2002). Moreover, T-bet is expressed in CD8⁺ T cells, B cells and NK cells in addition to CD4⁺ T cells but its effect on IFN γ production in these cells is not as pronounced as in CD4⁺ T cells (Lugo-Villarino, 2003; Sullivan *et al.*, 2003; Szabo *et al.*, 2002), suggesting a pivotal role of T-bet in Th1 cells. Regarding the effect of T-bet in EAE development, initial studies showed that the silencing or the invalidation of T-bet in mice confers a strong resistance to the development of MOG₃₅₋₅₅ peptide-induced EAE (Bettelli *et al.*, 2004; Lovett-Racke *et al.*, 2004). T-bet^{-/-} splenocytes had remarkably reduced secretion of IFN γ but increased production of IL-10 in response to either MOG₃₅₋₅₅ or PLP₁₈₀₋₁₉₉ *ex vivo* (Bettelli *et al.*, 2004; Nath, 2006). Even though this was challenged by a later study, in which the absence of T-bet affected more mildly the development of EAE (O'Connor *et al.*, 2013). However, in this study, compared with the wild type control, the peripheral and CNS-infiltrated T-bet^{-/-} CD4⁺ T cells produced very low amounts of IFN γ but significantly higher amounts of IL-17, which is related to the inhibition of Th17 responses by T-bet (Lazarevic *et al.*, 2011; O'Connor *et al.*, 2013).

In addition, the same study also showed that adoptive transfer of C57BL/6L mice with MOG₃₅₋₅₅ pre-activated T-bet^{-/-} Th1 cells failed to induce EAE, while the control mice developed severe disease, indicating that T-bet is required for the encephalitogenic potential of Th1 cells (O'Connor *et al.*, 2013).

The reason for the difference in the impact of T-bet on the development of EAE between different reports is still unclear. It may be related to the different backgrounds of mice. One of the earlier studies used antisense RNAs to silence *TBX21* gene *in vivo*, while this technology might have bystander effects (Lovett-Racke *et al.*, 2004). T-bet KO mice used in the other two studies, were generated in 129/Sv ES cells and backcrossed to C57BL/6 for only 5 generations (Bettelli *et al.*, 2004; Szabo *et al.*, 2002). By comparison, the T-bet^{-/-} mice used in the later studies, showing that T-bet has mild effect on EAE induction, have been further backcrossed to C57BL/6, reflecting a different genetic background (Grifka-Walk *et al.*, 2013; O'Connor *et al.*, 2013).

The mechanism by which T-bet controls EAE is not clear but it may be dependent at least partly on its ability to positively regulate the production of IFN γ in CD4⁺ T cells. The effect of IFN γ in the context of CNS autoimmunity is complex since it produces both stimulatory and inhibitory outcomes on the disease. In initial studies, IFN γ was identified as an aggravating factor in EAE and MS, as the increased production of IFN γ was associated with increased demyelination in the CNS (O'Connor *et al.*, 2008; Olsson, 1992; Panitch *et al.*, 1987; Pettinelli & McFarlin, 1981). However, this theory was challenged by later studies, in which mice lacking IFN γ or IFN γ R or mice treated with IFN γ neutralizing mAbs developed severer EAE, suggesting a beneficial role of IFN γ in this autoimmune disease (Sosa *et al.*, 2015). Gradually, further explorations have shown that IFN γ may have distinct effects on EAE according to the stage of EAE at which it operates. It is proposed that IFN γ enhances the severity of the disease during the initiation phase, while it has beneficial role on the pathology during the chronic phase and in the context of therapy (Furlan *et al.*, 2001; Naves *et al.*, 2013). Accordingly, the production of IFN γ in CD4⁺ T cells was decreased at the initiation stage of EAE (6 days after the immunization) in the absence of THEMIS, suggesting that the reduced incidence and disease severity of EAE could be partly due to the reduced production of this inflammatory cytokines.

The role of IFN γ produced by Th1 cells in the CNS is also complex. On one hand, IFN γ aggravate the disease by enhancing Th1 responses and the infiltration of immune cells into the spinal cord (Sanvito *et al.*, 2010; Stromnes *et al.*, 2008).

On the other hand, IFN γ attenuates the disease by inhibiting Th9 and Th17-mediated pathogenic responses and positively regulating the number and function of Treg cells (Murugaiyan *et al.*, 2012; Wang *et al.*, 2006; Wildbaum *et al.*, 2010).

The absence of THEMIS led to reduced number of antigen-specific CD4⁺ T cells in the CNS, both in the 2D2 transfer model and in active EAE with CD4-CreERT2-*themis*^{fox/fox} mice, suggesting that the reduced production of IFN γ in THEMIS deficient CD4⁺ T cells in dLN may lead to a reduced infiltration of the CNS by immune cells. The percentages of 2D2+CD4⁺ T cells infiltrating the CNS were not affected by THEMIS expression, suggesting also that THEMIS may promote the recruitment of endogenous CD4⁺ T cells or other leukocyte subsets through bystander effects using similar effector mechanism. Regarding the cytokines production in CNS, our study showed that the absence of THEMIS did not affect the percentages of cells producing IFN γ , GM-CSF and IL-17 in antigen-specific CD4⁺ T cells (data not shown), but their cell numbers exhibit decreased due to the general reduction in the number of antigen-specific *themis*-deficient CD4⁺ T cells. One possible reason is that THEMIS would operate early by controlling the polarization of naïve CD4⁺ T cells into Th1 cells and by modulating the ability of those cells to migrate into the CNS. The percentage of IFN γ -producing CD4⁺ T cells remains possibly unchanged because only cells that successfully develop into Th1 without THEMIS manage to migrate to CNS.

B. Effect of THEMIS on GM-CSF in the pathogenicity of CD4⁺ T cell in EAE

Although the effect was not as pronounced as that on IFN γ production, we also show that the expression of THEMIS is positively associated with the production of GM-CSF following MOG₃₅₋₅₅ restimulation of 2D2+CD4⁺T cells. It has been reported that GM-CSF promotes neuroinflammation in EAE by inducing the activation and proliferation of microglia and by enhancing the production of additional proinflammatory cytokines that favor demyelination (Parajuli *et al.*, 2012; Ponomarev *et al.*, 2007). Previous studies showed that Th1 cells primed with MOG₃₅₋₅₅, which produce IFN γ and GM-CSF, transfer a severe EAE in recipient mice accompanied by enrichment of proinflammatory cytokines and infiltrated monocytes, in a GM-CSF-dependent fashion (El-Behi *et al.*, 2011; Grifka-Walk *et al.*, 2015).

In addition, GM-CSF is produced by Th17 and ThGM cells (a newly identified pro-neuroinflammatory population) (Y. K. Lee *et al.*, 2009; Rasouli *et al.*, 2020). Although we did not detect any differences in Th17 cells development based on our analysis on ROR γ t expression and IL-17 secretion, we could not exclude that THEMIS might also be important for the development of ThGM cells in the context of EAE. Interestingly the encephalitogenic potential of ThGM cells is dependent on T-bet, suggesting that THEMIS might be important to enhance the pathogenic potential of those cells.

One study suggests that NFAT positively regulates the expression of GM-CSF by binding to the enhancer upstream of the transcription start site of the gene encoding for GM-CSF and enhancing the formation of DNase I hypersensitive site within this enhancer to induce transactivation (Bert *et al.*, 2007). Recent investigation on THEMIS by the team of Nicholas Gascoigne show that CD4⁺ T cells bearing a germline mutation in *themis* gene exhibited a reduction of NFAT translocation upon TCR stimulation (Prasad *et al.*, 2020), suggesting that THEMIS may enhance the production of GM-CSF by peripheral CD4⁺ T cells *in vivo* by upregulating NFAT translocation.

C. Effect of THEMIS on the infiltration of the CNS by CD4⁺ T cells

Analysis of T cell infiltration in mice after the appearance of symptoms suggests that the expression of THEMIS is positively associated with the infiltration of CD4⁺ T cells in the CNS. The reduced expression of T-bet in THEMIS-deficient 2D2⁺ T cells transferred in recipient mice was associated with a decreased expression of the chemokine receptor CXCR3. It has been shown that T-bet is required for Th1 cell migration to inflammatory sites by controlling the binding of CD4⁺ T cells to E-selectin and P-selectin on inflamed endothelium and by enhancing the expression of CXCR3 on the T cells (Lord *et al.*, 2005; Underhill *et al.*, 2005) .

It has long been recognized that the infiltrated T cells in MS lesions exhibit high expression of the chemokine receptor CXCR3 (Balashov *et al.*, 1999; Simpson *et al.*, 2000; Sørensen *et al.*, 1999). In EAE mouse models, the CNS infiltrates are characterized by large percentages of CXCR3⁺IFN γ ⁺-producing T cells, which are correlated with the upregulation of CXCL10 (a ligand of CXCR3) on adjacent astrocytes (O'Connor *et al.*, 2012; Sporici & Issekutz, 2010). Also, an early study showed that

CXCR3 blockade resulted in decreased migration of peripheral T cells into the CNS (Sporici & Issekutz, 2010), suggesting a positive association between CXCR3 expression on T cells and their ability to migrate into the CNS. Although CXCR3 is highly expressed on Th1 cells (Loetscher *et al.*, 1998; Sallusto *et al.*, 1998), active EAE in CXCR3^{-/-} mice and adoptive transfer of EAE with polarized-CXCR3^{-/-} Th1 cells showed that CXCR3 expression does not affect the disease severity and the number of CNS-infiltrated T cells (Lalor & Segal, 2013). One possible reason of this observation may be the development of compensatory effect mediated by other chemokine receptors. Accordingly, a previous publication showed that, compared with targeting CXCR3 alone, blocking CXCR3 and CXCR4 at the same time has a therapeutic effect on adoptively transferred EAE (Kohler *et al.*, 2008).

D. Does THEMIS regulate CD4⁺ T cell responses *in vivo* through T-bet-independent mechanism?

Results of both active EAE and transferring EAE in our study indicate that the expression of THEMIS is positively associated with the infiltration of CD4⁺ T cells to CNS. Based on the discussion above, we speculate that THEMIS positively regulates CD4⁺ T cell infiltration in the CNS through a T-bet dependent process. However, in the transfer model of EAE, we also observed that THEMIS deficiency results in significantly lower percentages and numbers of 2D2⁺ CD4⁺ T cells in the dLNs and the SPL of recipient mice at the stage of T cell priming after EAE induction, suggesting that the effect of THEMIS on EAE could result from insufficient expansion or survival of encephalitogenic T cells after transfer into recipient mice.

The analysis of CD4⁺ T cells following staining with MHC tetramers coupled to MOG₃₅₋₅₅ peptide suggest that THEMIS does not affect the proportions of CD4⁺ T cells that recognize encephalitogenic peptide following immunization (data not shown), ruling out the possibility that the reduced severity of EAE in THEMIS deficient mice could be exclusively the consequence of a lack of antigen recognition. Moreover, by tracing the CTV-stained naïve CD4⁺ T cells in response to MOG₃₅₋₅₅ stimulation *in vitro* and *in vivo* and analyzing the expression of the proliferative marker Ki-67⁺CD4⁺ T cells, we could not detect an effect of THEMIS on the proliferation of CD4⁺ T cells, suggesting that the reduced numbers of THEMIS-deficient CD4⁺ T cells does not result from a defective priming of CD4⁺ T cells.

We could not exclude however that the reduced EAE, observed following transfer of THEMIS deficient 2D2+ T cells in recipient mice, could be the consequence of the reduced ability of those cells to survive efficiently in their host or to migrate into LN prior immunization.

Recent studies suggest that THEMIS is important for the homeostatic maintenance of CD8+ T cells by enhancing signaling events resulting from the integration of TCR and cytokine receptor stimulation (Brzostek *et al.*, 2020). Although authors in this study could not detect significant effect of THEMIS on CD4+ T cells maintenance using their mouse model (Lck-Cre *themis*^{flox/flox}) and although numbers of CD4+ T cells were also normal in the absence of THEMIS in our model (CD2-Cre *themis*^{flox/flox}), we cannot exclude that THEMIS-deficient CD4+ may present a disadvantage for homeostatic maintenance over wild-type cells in a competitive environment where endogenous CD4+ T cells reside. An additional study from Nicholas Gascoigne's team showed that naïve CD4+ T cells from germline deficient *themis*^{-/-} mice result in poor expansion of effector cells following injection into RAG^{-/-} mice suggesting that THEMIS might be important to promote the survival or the proliferation of those cells in this experimental setting (Prasad *et al.*, 2020). This study suggests that THEMIS enhances the expansion of effector cells by promoting metabolic reprogramming through a NFAT dependent process (Prasad *et al.*, 2020). However, those results are to be interpreted with caution, since they were obtained using a germline THEMIS-deficient line which exhibit a strong block in T cell development causing lymphopenia in peripheral organs, which could result in aberrant changes due to functional compensation in the thymus and in indirect effects on T cells caused by the lymphopenic environment. Likewise, the defective metabolism caused an overall defect in CD4+ T cell responses, leading to impaired expansion and function of both Th1 and Th17 cells in RAG^{-/-} mice (Prasad *et al.*, 2020). However, our study shows that THEMIS deficiency in peripheral CD4 T cells does not affect their proliferation or differentiation into Th17 cells *in vitro* and *in vivo*, suggesting that THEMIS is not essential for the metabolic events associated to those T cell responses.

In addition, according to the reduced numbers of THEMIS-deficient antigen-specific CD4⁺ T cells and the decreased expression of CD5 on those cells in our study, a previous study on cell survival in CD5^{-/-} mice may need to be mentioned. This study demonstrated that CD5 enhances EAE severity by positively regulating the survival of activated T cells in response to MOG₃₅₋₅₅ stimulation (Axtell *et al.*, 2004). Due to the rapid activation and proliferation of T cells (increased at day 4 but sharply decreased at day 10), CD5^{-/-} mice exhibited enhanced activation-induced cell death (AICD) and low numbers of effector T cells in the spleen and dLN, leading to markedly delayed onset and reduced severity of EAE. This suggests that THEMIS may enhance the survival of activated antigen-specific CD4⁺ T cells by upregulating CD5 and preventing the elimination of effector T cells *in vivo*. While, in fact, our analysis did not show a rapid activation and proliferation of antigen-specific CD4⁺ T cells in the absence of THEMIS *in vivo*, indicating that THEMIS does not regulate the stability of activated CD4⁺ T cells mediated by CD5.

One can wonder therefore whether the reduced numbers of THEMIS-deficient CD4⁺ T cells observed in the draining lymph nodes could result from an impaired ability of those cells to circulate from the blood to lymphoid organs. The chemokine receptor CCR7 has been identified as a critical receptor of T cell entry into peripheral lymph organs. Some previous adoptive transfer experiments showed that CCR7^{-/-} T cells had reduced migration from peripheral tissues to dLN compared to the control (Bromley *et al.*, 2005; Debes *et al.*, 2005). In addition, the interaction of CD44 with the macrophage mannose receptor (MRC) is also important for the migration of T cells into dLN (Salmi *et al.*, 2013). Our study shows that THEMIS deficiency has no obvious effect on the expression of CCR7 (data not shown) and CD44 on the transferred CD4⁺ T cells. In addition, we analysed the cell number of injected CD4⁺ T cells in the dLN before proliferation (day 2 after the EAE induction), and found no statistical difference between the number of *themis*-T^{WT} and *themis*-T^{KO} antigen-specific CD4⁺ T cells (data not shown). These results suggest that THEMIS may be not required for the entry of T cells to secondary lymphoid organs.

3. Outlook for THEMIS function in other peripheral T-cell subsets in regard of our results

In peripheral CD8⁺ T cells: recent studies from Nicholas Gascoigne's team suggest that THEMIS promotes the maintenance and function of CD8⁺ T cells by enhancing signaling events resulting from the co-stimulation of the TCR and cytokine receptors involved in CD8⁺ T cell homeostasis such as the IL-7R (Brzostek *et al.*, 2020). Interestingly, the absence of THEMIS had no detectable effect on CD8⁺ T cell responses when signals only emanate from TCR stimulation, recapitulating results obtained in CD4⁺ T cells in unpolarized conditions and supporting the hypothesis that THEMIS might be important to modulate TCR signals through other receptors than the TCR itself. The reduced maintenance of CD8⁺ T cells was rescued by SHP-1 deficiency, suggesting that decreased CD8⁺ T cell-maintenance signals might result from exacerbated SHP-1 activity. Thus, those results suggest that THEMIS operates by blocking, rather than enhancing, SHP-1 phosphatase activity. Besides, a very recent publication from this team showed that *themis* germline deficiency reduces the diversity of TCR α repertoire and in counterpart results in an increase in the proportions of CD8⁺ T cells expressing V α 3.2⁺ TCR, which represent a virtual memory subpopulation that exhibit higher activation and stronger response to self-peptide and cytokines, than V α 3⁻ CD8⁺ T cells (Prasad *et al.*, 2021). Of note, authors did not detect alteration of the TCR repertoire in CD4⁺ T cells, consistent with the idea that the reduced EAE in THEMIS-deficient mice does not result from a major change of TCRs diversity in this cell compartment.

In Treg cells: Compared to conventional CD4⁺ and CD8⁺ T cells, the expression of THEMIS in Treg cells is much lower (Duguet *et al.*, 2017). An initial study, performed on the rat Brown-Norway (BN) line by the team of Abdelhadi Saoudi in our institute, identified a spontaneous invalidating mutation on *themis* gene which led to impaired inhibitory function of Treg cells and to the spontaneous development of IBD in the mice (Chabod *et al.*, 2012; Duguet *et al.*, 2017). Later studies by the same group showed that the impact of THEMIS deficiency on Treg suppressive functions depends on a 117-kb interval coding for a R63W polymorphism which leads to reduced expression of the rac-GTP exchange factor VAV1 (Pedros *et al.*, 2015). Interestingly, the analysis of THEMIS interactome identified VAV1 as one of THEMIS preferential binding partner in thymocytes.

THEMIS deficiency also results in the decreased phosphorylation of VAV1 following TCR stimulation and reduced production of Rac1-GTP in thymocytes (Zvezdova *et al.*, 2016). Of note, VAV1 is dephosphorylated by SHP-1 in T cells (Stebbins *et al.*, 2003) and NK cells (Mesecke *et al.*, 2011), suggesting that THEMIS may enhance VAV1 activity by repressing the inhibitory effect of SHP-1 on VAV1. Thus, the decreased suppressive function of Treg cells was only observed when THEMIS deficiency was combined with a genetic alteration resulting in lower VAV1 expression. Recent studies from the same group show that combined mutations lead to a profound defect in the activation of RelA, a NF- κ B transcription factors which cooperate with FOXP3 to promote Treg responses (Benamar *et al.*, Science signaling, 2021, in press). The activity of RelA and the suppressive function of Treg cells could be restored by treatment with SHP-1 inhibitors, suggesting once again that THEMIS enhances Treg responses by blocking SHP-1 activity.

the further analysis showed that, by binding to the VAV1 that have full adaptor functions, THEMIS enhances the suppressive function of Treg cells both *in vitro* and *in vivo*, whereas THEMIS deficiency alone has no impairment of on the suppressive function of Treg cells. VAV1 is a positive regulator of TCR signaling, in thymocytes, THEMIS enhances the activity of the VAV1(Zvezdova *et al.*, 2016). As THEMIS inhibits SHP-1 PTP activity in thymocytes, SHP-1 was identified as an endogenous inhibitor of the suppressive function of Treg cells and VAV1 is a predominant target of SHP-1, it's speculated that THEMIS may enhances VAV1 activity by blocking SHP-1(Duguet *et al.*, 2017; Iype *et al.*, 2010; Stebbins *et al.*, 2003). While further research is needed for the verification of this hypothesis.

4. THEMIS and human diseases

Variants inside or in proximity of THEMIS have been associated to several autoimmune disorders and inflammatory diseases. So far, studies identify *themis* gene as a potential susceptibility locus in IBD and atopic dermatitis (Kim *et al.*, 2015; Momozawa *et al.*, 2018). Higher expression of THEMIS was identified in the intestinal mucosa of patients suffering from Celiac disease (Bondar *et al.*, 2014). The gene encoding for THEMIS was also characterized as a potential susceptibility locus in patients suffering from multiple sclerosis (Beecham *et al.*, 2013; Sawcer *et al.*, 2011).

More recently the group of Joanne Jones identified 16 novel isoforms of *themis* mRNA in human T cells, and show that the expression of the exon 1-containing mRNA isoforms is associated with a genotype that is protective against Multiple Sclerosis (Davies *et al.*, 2016). Contrasting with results obtained in mouse models, these data suggest that the expression of full-length *themis* might be beneficial in the context of this auto-immune disease. However, authors could not confirm a significant increase of exon 1-containing THEMIS expression at the protein level and were unable to identify specific changes of CD4⁺ T cell responses associated to the expression of this isoform, indicating that further investigations will be required to characterize THEMIS as a protective factor in this disease.

In addition, THEMIS with a full function is required for pathogenesis of Cerebral Malaria and protection against Pulmonary Tuberculosis, a typical intracellular infection mainly inhibited by Th1 proinflammatory cytokines (Berghout *et al.*, 2013; Torre *et al.*, 2015). ENU-induced *themis*^{I23N} mutation could protect more than 80% of mice from the experimental cerebral malaria (ECM) induced by *Plasmodium berghei* ANKA, corresponding to a significantly reduced cerebral cellular infiltration and pro-inflammatory cytokines production (Torre *et al.*, 2015). This presents a similar effect of THEMIS deficiency on Th1 responses during EAE development in our study, further enhancing the connection between THEMIS expression and the development of autoimmune diseases. However, those results are to be taken with caution since the ENU-induced *themis*^{I23N} mutation caused a general decrease in peripheral T cells, presumably resulting from a block in T cell development similar to what is seen in germline knockout mice, which might be responsible of the observed effect.

Several autoimmune diseases have been associated with a Th1 dominant state. T-bet, as the key transcription factor of Th1 cells, is considered as a strong biomarker of Th1-mediated autoimmune disease in humans and mouse models, including Type I diabetes (Sasaki *et al.*, 2004), collagen II-induced arthritis (Xue *et al.*, 2014), Th1 cell-transfer mediated Colitis (Neurath *et al.*, 2002) and EAE (O'Connor *et al.*, 2013). As well, T-bet is also considered as a predictor for the treatment of the related diseases. Some studies showed that, compared with patients in remission or healthy subjects, T-bet is one of the transcription factors upregulated in PBMCs of MS patients at the relapsing stage (Frisullo *et al.*, 2006; Iorio *et al.*, 2009).

According to the positive effect of THEMIS on T-bet expression and on encephalitogenic CD4⁺ T cell responses during EAE development, one can suspect that THEMIS expression might constitute an interesting predicting factor of disease progression and responses to various treatments of CNS autoimmune diseases.

5. Conclusion and perspectives

To conclude (**Figure 19**), by using a mouse model with conditional deletion for *themis* gene in peripheral T cells, our study identified the function of THEMIS in peripheral CD4⁺ T cell responses. We found that THEMIS deficiency leads to opposite effects on CD4⁺ T cell responses upon TCR triggering *in vitro* and antigen recognition *in vivo*. Based on our results and previous evidences suggesting that THEMIS blocks SHP-1 and SHP-2 PTP activity, we propose that THEMIS inhibits ERK-dependent Th1 responses *in vitro* by blocking SHP-2, which operates as a positive regulator of TCR signals when the TCR is stimulated alone. We speculate that this effect is overcome *in vivo* by additional effects of THEMIS on receptors which engage SHP-1 and SHP-2 to negatively regulate TCR signals leading to T-bet expression.

Those results provide new insights to explain the previous discrepancy on THEMIS function in TCR signaling. More generally, they suggest that the function of signaling proteins may depend on the signaling context in which a particular protein is engaged and ultimately on the integration of multiple signals which are received in a given immunological context. It remains unclear whether the dual effect of THEMIS on CD4⁺ T cell responses is biologically relevant. We cannot exclude that THEMIS may, on the one hand repress the responsiveness of polarized Th1 cells to TCR stimulation to avoid excessive Th1 responses that may lead to detrimental effects and, on the other hand enhance the magnitude of Th1 cells polarization by blocking immune checkpoints to enable efficient responses to foreign antigens. An important future axe of research on THEMIS will be to investigate the role of THEMIS in the signaling function of co-receptors, such as PD-1 and BTLA, which were characterized for their ability to recruit SHP-1 and SHP-2.

Conclusion

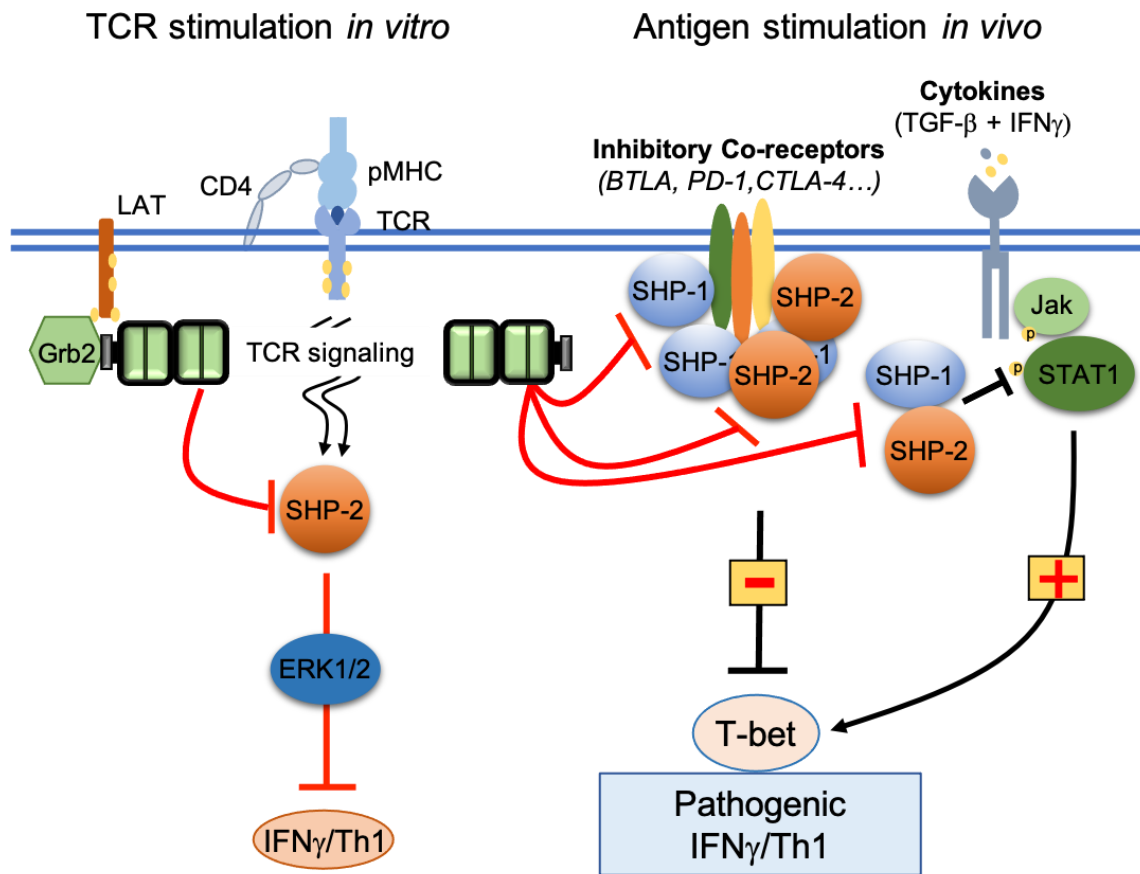


Figure 19. Effect of THEMIS on CD4⁺ T cell responses *in vitro* and *in vivo*

In context of the TCR stimulation alone (*in vitro*): THEMIS inhibits Th1 responsiveness by suppressing SHP-2/ERK1/2 pathway

Antigen stimulation *in vivo*: THEMIS enhances T-bet mediated Th1 responses by blocking the recruitment of SHP-1 and SHP-2 to co-inhibitory receptors-induced inhibitory signaling and by blocking these two phosphatases-mediated inhibition of cytokine/STAT pathway.

Our *in vivo* analysis highlights a specific effect of THEMIS on the production of T-bet and IFN γ by CD4⁺ T cells. We speculated from this observation that THEMIS modulates Th1 responses. However, both Th1-like Th17 cells and type I Tfh cells co-express T-bet and IFN γ (Cunill *et al.*, 2017; Kamali *et al.*, 2019; Ma & Phan, 2017; Unger, 2018), suggesting that THEMIS may exert a specific effect on T-bet and IFN γ but not a broad effect on the development of Th1 cells. Therefore, a more extensive RNA-Seq characterization of 2D2 T cells *in vivo* would be interesting to investigate further the effect of THEMIS on the signature of those effector cells in the context of EAE.

Given the important role of Th1 cells in the context of cancer and given the key role of immune checkpoints blocking therapies, an interesting perspective would be to explore the role of THEMIS in a cancer model which relies on Th1 cells, such as bladder cancer and melanoma and to address whether THEMIS expression affects the susceptibility to immune checkpoint blockade therapy.

Portfolio

Scientific publications during this thesis

THEMIS enhances the magnitude of normal and neuroinflammatory Th1 responses by promoting TCR-independent signals

Cui Yang, Gaëtan Blaize*, Rémi Marrocco*, Nelly Rouquié*, Cyrielle Bories, Mylène Gador, Suzanne Mélique, Emeline Joulia, Mehdi Benamar, Anne S. Dejean, Hélène Daniels-Treffandier, Paul E. Love, Nicolas Fazilleau, Abdelhadi Saoudi, Renaud Lesourne.

Under review

CD5 signalosome coordinates antagonist TCR signals to control the generation of Treg cells induced by foreign antigens

Blaize G., Daniels-Treffandier H., Aloulou M., Rouquié N., **Yang C.**, Marcellin M., Gador M., Benamar M., Ducatez M., Song K., Burlet-Schiltz O., Saoudi A., E. Love P, Fazilleau N., Gonzalez de Peredo A., & Lesourne R.

PNAS, 2020 June 9

(Annexes)

Awards and honors

2021 – *EFIS-EJI Abstract Grant* at the 15th World Immune Regulation Meeting

Workshop presentation

2020 – 2021 *Fondation ARC pour la recherche sur le cancer* for the 4th year of PhD student

2017 – 2020 *China scholarship council* for PhD student

Experimental skills acquired

- **Flow cytometry analysis** (LSR-II and LSR-FORTESSA): Extracellular and intracellular staining for the analysis of T cell phenotype, proliferation, and differentiation.
- **Animal experiment**: Induction of EAE in mice, transfer of mice with T cells, and isolation of leukocytes from the central nervous system of mice.
- **Cell culture** of mouse primary lymphocytes (LT) and stimulation of LT with antibodies and with peptides *in vitro*.
- Protein biochemistry: Analysis of phosphorylated proteins, immunoprecipitation and Western blot.

Courses and Training attended

- Fluorescence microscopy - Level 1
- Transmission electron microscopy for in situ localizations. Obtaining and interpreting images
- French speaking and writing - Level A2
- Speak English Professionally: In Person, Online & On the Phone

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Annexes

CD5 signalosome coordinates antagonist TCR signals to control the generation of Treg cells induced by foreign antigens

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CD5 is characterized as an inhibitory coreceptor with an important regulatory role during T cell development. The molecular mechanism by which CD5 operates has been puzzling and its function in mature T cells suggests promoting rather than repressing effects on immune responses. Here, we combined quantitative mass spectrometry and genetic studies to analyze the components and the activity of the CD5 signaling machinery in primary T cells. We found that T cell receptor (TCR) engagement induces the selective phosphorylation of CD5 tyrosine 429, which serves as a docking site for proteins with adaptor functions (c-Cbl, CIN85, CRKL), connecting CD5 to positive (PI3K) and negative (UBASH3A, SHIP1) regulators of TCR signaling. c-CBL acts as a coordinator in this complex enabling CD5 to synchronize positive and negative feedbacks on TCR signaling through the other components. Disruption of CD5 signalosome in mutant mice reveals that it modulates TCR signal outputs to selectively repress the transactivation of *Foxp3* and limit the inopportune induction of peripherally induced regulatory T cells during immune responses against foreign antigen. Our findings bring insights into the paradigm of coreceptor signaling, suggesting that, in addition to providing dualistic enhancing or dampening inputs, coreceptors can engage concomitant stimulatory and inhibitory signaling events, which act together to promote specific functional outcomes.

T cells | signaling | coreceptors

T cells have the ability to develop a wide variety of cellular responses following the stimulation of a single receptor, namely the T cell antigen receptor (TCR). The recognition by TCRs of self or foreign peptides bound to the major histocompatibility complex (pMHC) triggers multiple signaling pathways, which lead to the activation of specific effector proteins involved in the transmission of distinct signaling responses. The relative intensity and the persistency by which signals are transmitted in each pathway play a critical role in specifying and driving specific T cell responses. Because different pathways may have either synergistic or antagonist effects on these responses, their coordination in time and space (signaling patterns) is also critical to shape T cell effector profiles and determine specific outcomes.

Signals transmitted by the TCR can be regulated by coreceptors that are engaged differentially based on their relative expression on the T cell surface and on the availability of their cognate ligands in the extracellular environment. Initial work on coreceptor signaling led to the classification of these proteins into two main functional categories, depending on their overall effect on T cell activity: Stimulatory coreceptors—such as CD28, ICOS, or OX40—which promote naïve T cell activation and amplify effector T cell responses, and inhibitory coreceptors—such as CTLA-4, PD-1, or BTLA—which prevent the potential activation of T cells by self-antigens and contribute to terminate or tune down effector T cell

responses following antigen clearance. More recent investigations indicate that many coreceptors act more selectively on specific signaling pathways and contribute to shape the effector profile of T cells according to the immunological context (1–3). Although the mechanisms by which coreceptors positively or negatively regulate T cell activity have been well documented (1, 4–7), the molecular processes by which they convey signals to selectively modulate T cell responses remain poorly understood.

CD5 is a type 1 transmembrane cell surface glycoprotein that is essentially expressed in T cells. Initial characterization of *Cd5*^{−/−} mice indicated an inhibitory function for this receptor on TCR signaling (8). Later work showed that CD5 surface levels on thymocytes are correlated to the strength of TCR signaling that is dictated during positive selection by the affinity of the TCR for self-pMHC (9). The increased expression of CD5 in thymocytes that express TCRs with greater self-reactivity dampens TCR signals, possibly enabling some thymocytes that would otherwise

Significance

T cell coreceptors are often described as molecular switches that broadly regulate T cell activation by enhancing or repressing T cell receptor (TCR) signaling according to the immunological context. However, many coreceptors act more selectively by instructing or restricting specific T cell responses. The molecular mechanisms by which these subtle regulations occur remain incompletely defined. In this study, we show that CD5 coreceptors engage a multimeric signaling complex, which synchronize positive and negative feedback on TCR signaling to limit the induction of inopportune regulatory T cells during immune response. Our findings suggest that rather than exclusively acting as stimulators or inhibitors of TCR signaling, coreceptors may coordinate antagonist TCR signals that act together to promote specific T cell responses.

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be negatively selected to avoid activation-induced cell death and instead complete their maturation and be exported to peripheral lymphoid organs (10). Through this mechanism CD5 would enable the selection of T cells with higher self-reactivity, which are presumed to be more effective responders to foreign antigens (11).

Whereas the role of CD5 during thymic selection has been well characterized, its function in peripheral T cells is less clear. The expression level of CD5 remains correlated with the affinity of TCR for self-pMHC in peripheral T cells (11), suggesting that CD5 could be important for the maintenance of self-tolerance by dampening homeostatic TCR signals that could otherwise cause activation and autoimmunity. However, *Cd5*^{-/-} mice do not exhibit signs of spontaneous autoimmune or inflammatory pathology and, in contrast, show a reduced susceptibility to active experimental autoimmune encephalomyelitis (12) and inflammatory bowel disease (13). This suggests that in the absence of CD5 compensatory mechanisms might prevent the expansion and the full activation of T cells expressing TCRs with relatively high affinity to self-pMHC. Notably, previous studies showed that the numbers and suppressive function of regulatory T (Treg) cells are increased in CD5-deficient mice (13, 14). However, more recent findings indicate that CD5 plays an instructive role in the generation of peripherally induced Treg cells (iTreg) in response to tolerizing antigens (15), suggesting that CD5 could have different influences on this T cell subset according to the immunological context.

Although several ligands have been reported for CD5 (16–18), it was shown that its extracellular domain is not required for negative regulation of TCR signaling in thymocytes (19), indicating that CD5 is engaged in a feedback loop that tunes down TCR signals following TCRs engagement. Accordingly, CD5 is constitutively associated with the TCR subunits at the cell surface (20) and contains several phospho-tyrosine binding (PTB) sites that are phosphorylated by SRC kinases following TCR engagement (21). Although many CD5-interacting partners have been reported, the relative importance of these interactions remains unclear because most of these binding partners were identified in independent studies or within distinct cellular models through approaches that do not always enable global comparisons of protein–protein interactions. Interestingly, whereas some of these proteins are well-characterized inhibitors of TCR signaling (22, 23), others are known to be positive effectors (24–27), suggesting that CD5-mediated feedback on TCR signaling might be more complex than what was initially presumed.

In this study, we combined quantitative mass spectrometry (MS) and mouse genetics to analyze the composition, the mode of assembly, and the molecular function of the CD5 transduction machinery in primary T cells. We found that CD5 coordinates the recruitment of a signaling complex composed of proteins with adapter functions (c-CBL, CIN85, and CRKL) that connect CD5 to positive (PI3K) and negative (UBASH3A and SHIP1) regulators of TCR signaling. The recruitment of this complex is entirely dependent on the Y429 of CD5, which is predominantly phosphorylated following TCR engagement and serves as a docking site for c-CBL. Disruption of Y429 phosphorylation site in primary T cells shows that this signaling complex promotes, on the one hand, AKT-mediated inhibition of FOXO1 and represses, on the other hand, ERK kinase activity to selectively dampen the transactivation of *Foxp3* gene expression. Analysis of antigen-specific Treg cells in CD5-Y429F mutant mice show that CD5 signaling selectively represses the generation of these cells presumably to promote the development of optimal immune responses.

Results

MS Analysis of the CD5 Interactome. To investigate the molecular mechanism by which CD5 regulates TCR signaling, we performed an MS-based analysis of CD5-containing complexes in thymocytes. CD5 was immunoprecipitated from WT or *Cd5*^{-/-}

thymocytes that were treated with pervanadate for 1 min to induce widespread activation of protein tyrosine kinases. CD5 protein complexes were eluted, and the components of the different purified complexes were characterized by nanoflow liquid chromatography combined with tandem MS. To discriminate CD5-binding molecules from the background of contaminant proteins, a thorough quantitative comparison based on MS intensity values was performed for each identified protein between samples immunoprecipitated from WT versus *Cd5*^{-/-} thymocytes. Candidate proteins were selected based on their significant enrichment in WT samples (fold-change > 2 and Student *t* test *P* < 0.001, *n* = 8 replicate experiments) (see *Materials and Methods* for details). On this basis, we identified 11 proteins as potential interacting partners of CD5 in thymocytes (Fig. 1A, *SI Appendix*, Fig. S1A, and *Dataset S1*). Among these, six proteins were previously identified as regulators of TCR-mediated signaling (c-CBL, UBASH3A/STS-2, CIN85/SH3KBP1, SHIP1, CRKL, and PI3K), two proteins are known components of the AP2 complex that is involved in clathrin-mediated internalization of CD5 (AP2a1, AAK1) (28), and three proteins have diverse reported functions not directly associated with TCR signaling (IGH, TRIM21, CYB5). The interactions of CD5 with c-CBL, UBASH3A, PI3K, CRKL, and AP2 were reported previously in independent studies (22, 24, 25, 28, 29) but not its interaction with SHIP1 and CIN85. Among these proteins, three are cytosolic adaptors (CRKL, CIN85, and AP2) and five are effector molecules with enzymatic function (c-CBL, UBASH3A, SHIP1, AAK1, and PI3K). The association of these proteins with CD5 was still detected after 10 min of stimulation and no additional interactors were identified at this later time of stimulation (*SI Appendix*, Fig. S1B and *Dataset S2*).

To estimate the relative abundance of these signaling partners in the immunoprecipitated samples, we used the intensity-based absolute quantification (iBAQ) metric, which corresponds to the sum of all of the peptide intensities divided by the number of theoretically observable tryptic peptides of a protein. Analysis of these interactions (with normalized iBAQ intensities) shows that UBASH3A (iBAQ = 604×10^4) and c-CBL (iBAQ = 560×10^4) are more abundantly recruited to CD5 than CIN85 (iBAQ = 147×10^4), CRKL (iBAQ = 143×10^4), SHIP1 (iBAQ = 55×10^4), and PI3K (iBAQ = 18×10^4), suggesting an essential role for c-Cbl and UBASH3A in CD5-mediated regulation of T cell activation (*SI Appendix*, Fig. S1A). Analysis by Western blot showed that CD5 interacts with c-CBL, UBASH3A, SHIP1, CIN85, and PI3K both in thymocytes and in peripheral CD4⁺ T cells, suggesting that CD5 engages similar signaling processes upon T cell development and primary T cell responses (Fig. 1B). Further analysis showed that c-CBL, UBASH3A, SHIP1, CRKL, CIN85, and PI3K interacted poorly with CD5 in resting cells but were recruited to CD5 upon TCR+CD4 cross-linking (Fig. 1C and *Dataset S3*), suggesting that CD5 contributes to TCR signaling through the activity of these proteins. Note that RAS-GAP (22), SHP-1 (23), CBL-b (30), and CK2 (27), which were previously reported to interact with CD5 in thymocytes or in T cell lines, were either undetected (Ras-GAP), detected at the same level in immunoprecipitated samples from WT and *Cd5*^{-/-} controls (SHP-1), or inconsistently detected across biological replicates without a statistically significant enrichment ratio in immunoprecipitated samples versus controls (CBL-b and CK2). Thus, these proteins were not selected in the list of major CD5-interacting proteins (*Dataset S1*).

Tyrosine 429 of CD5 Is Essential for Assembly of the CD5 Signalingome.

We next investigated the mechanism by which CD5 recruits these proteins following TCR stimulation. Previous studies performed on cell lines identified three potential tyrosine-phosphorylation sites on the intracytoplasmic domain of human CD5: Tyrosine 429 (pY429), tyrosine 441 (pY441), and tyrosine 463 (pY463)

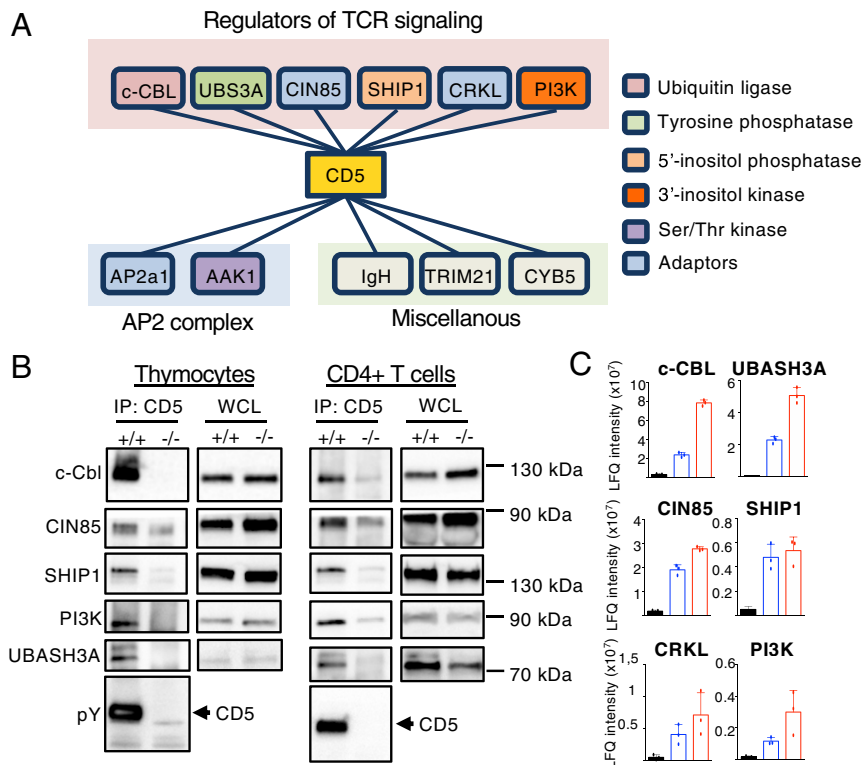


Fig. 1. MS analysis of CD5 interactome. (A) Schematic of those proteins that preferentially interact with CD5 in thymocytes stimulated with pervanadate for 1 min. Layouts indicate the classification of these proteins according to their function (pink layout: TCR signaling; blue layout: AP2 complex; green layout: unknown function in T cell signaling). Data represent eight independent experiments with $n = 8$ mice per group. (B) Thymocytes and peripheral CD4⁺ T cells from $Cd5^{+/+}$ ($+/+$) or $Cd5^{-/-}$ ($-/-$) mice were stimulated with pervanadate for 5 min. Samples were then subjected to immunoprecipitation (IP) with antibodies specific for CD5 and then analyzed by Western blotting with antibodies specific for the indicated proteins. WCL, whole cell lysate. (C) Relative abundance of principal CD5 interacting partners after co-IP in thymocytes either nonstimulated (black bars) or stimulated with anti-CD3 and anti-CD4 antibodies (blue bars) or pervanadate (red bars). Protein abundances were estimated using the LFQ metric calculated by MaxQuant based on MS peptide signals intensities. Data are means \pm SD of three MS measurement and are representative of three independent experiments containing $n = 1$ mouse per group.

(31, 32). To address which of these tyrosines is preferentially phosphorylated following TCR engagement in primary thymocytes, we analyzed the relative MS intensity of CD5 phosphorylated peptides encompassing these three modification sites compared to their respective unmodified forms. It must be noted that this percentage does not strictly measure the phosphorylation stoichiometry of each site (as phosphorylated and unphosphorylated forms may have different ionization efficiencies in the mass spectrometer). We nevertheless used this metric to qualitatively illustrate the phosphorylation occupancy before and after stimulation. Whereas the phosphorylation of these residues was barely detectable in unstimulated thymocytes, we found that CD5 was nearly exclusively phosphorylated on Y429 after TCR cross-linking with CD4 upon different times of stimulation (Fig. 2A and [Datasets S3](#) and [S4](#)). All three tyrosine residues of CD5 were phosphorylated following pervanadate treatment, suggesting that Y441 and Y463 might be phosphorylated independently of TCR engagement upon additional costimulatory signals (Fig. 2A).

To determine the role of Y429 in the context of CD5 signaling, we expressed a WT ($CD5^{tg^{WT}}$) or a mutated form of CD5, containing a tyrosine to phenylalanine substitution at position 429 ($CD5^{tg^{Y429F}}$), as transgenes under the control of the T cell-specific hCD2 promoter/enhancer and crossed both transgenes into the $Cd5^{-/-}$ background ($Cd5^{-/-}; CD5^{tg^{WT}}$ or $Cd5^{-/-}; CD5^{tg^{Y429F}}$, hereafter designated $CD5^{tg^{WT}}$ or $CD5^{tg^{Y429F}}$, respectively). We verified that the WT and Y429F CD5 transgenes were similarly expressed in thymocyte subsets and in peripheral CD4⁺ and CD8⁺ T cells ([SI Appendix, Fig. S2A](#)). The surface levels of CD5 remained unchanged following stimulation with

anti-CD3 antibodies and were comparable in $CD5^{tg^{WT}}$ and $CD5^{tg^{Y429F}}$ thymocytes ([SI Appendix, Fig. S2B](#)). Moreover, the proportions and numbers of thymocytes and peripheral T cells in each subset were similar in $CD5^{tg^{WT}}$, $CD5^{tg^{Y429F}}$, and $Cd5^{-/-}$ mice ([SI Appendix, Fig. S2C](#) and [D](#)). Percentages of FOXP3⁺ T cells in the thymus and the spleen ([SI Appendix, Fig. S2E](#)) and surface levels of activation/memory markers, such as CD44, CD69, and PD-1 ([SI Appendix, Fig. S2F](#) and [G](#)) were also comparable between these three lines. To investigate the impact of this mutation on the formation of CD5 signaling complexes, we compared the interactome of the WT and the mutated form of CD5 in thymocytes stimulated with pervanadate. We verified that c-CBL, UBASH3A, PI3K, CRKL, SHIP1, and CIN85 interacts with WT CD5 in $CD5^{tg^{WT}}$ thymocytes similarly to what we observed in the C57BL/6 background (Fig. 2B and [Dataset S5](#)). IGH, TRIM21, CYB5 were not detected in this interactome, suggesting that they represent low affinity or nonspecific interactions. Notably, the mutation of tyrosine Y429 disrupted the association of c-CBL, UBASH3A, PI3K, CRKL, SHIP1, and CIN85 with CD5 following pervanadate treatment (Fig. 2B and [Dataset S5](#)) and TCR cross-linking (Fig. 2C), despite comparable phosphorylation of tyrosine Y463 of $CD5^{tg^{WT}}$ and $CD5^{tg^{Y429F}}$ (Fig. 2B and [Dataset S5](#)). Quantitative analysis of the LFQ intensity metrics showed that the amount of each protein partner detected in the $CD5^{tg^{Y429F}}$ interactome was drastically decreased compared to $CD5^{tg^{WT}}$, and nearly comparable to those detected in $Cd5^{-/-}$ thymocytes ([SI Appendix, Fig. S1C](#)), suggesting that the mutation of Y429 almost entirely disrupted the assembly of the CD5 signalosome. No additional proteins were

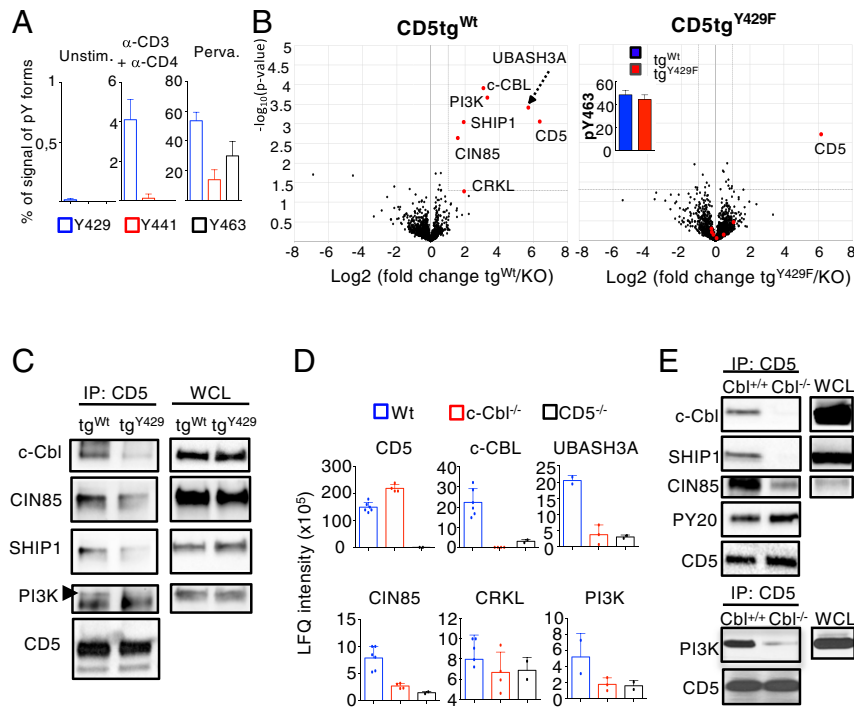


Fig. 2. c-CBL is recruited to CD5 Y429 and is connecting CD5 with its signaling partners. (A) MS analysis of CD5 phosphorylation sites. Thymocytes from WT mice were left unstimulated (Unstim.) or were stimulated with anti-CD3 + anti-CD4 antibodies or pervanadate (Perva.) for 1 min. Samples were then subjected to IP with antibodies specific for CD5. MS intensity of peptide ions containing the three main phosphotyrosine residues of the protein were used to calculate for each site the percentage of MS signal deriving from modified phosphotyrosine forms. MS intensity values of phosphorylated ions were averaged for MS replicate measurements of each sample. Data are the means \pm SD and represent three independent experiments containing $n = 1$ mouse per group. (B) Volcano plots [$-\log_{10}(\text{Student } t \text{ test } P \text{ value})$ versus $\log_2(\text{fold change})$] showing abundances difference of proteins identified by MS analysis of samples obtained by IP of CD5 from cellular extracts of thymocytes stimulated with pervanadate. (Left) Comparison CD5tg^{Wt} vs. CD5^{-/-} thymocytes ($n = 6$ independent samples); (Right) comparison CD5tg^{Wt} versus CD5tg^{Y429F} thymocytes ($n = 5$ independent samples). Protein labeled in red show significant enrichment in CD5tg^{Wt} samples compared to control CD5^{-/-} samples (fold-change > 2 and $P < 0.05$). The same proteins plotted in red show no significant enrichment in CD5tg^{Y429F} samples versus controls. Bar graph (Inset) in the CD5tg^{Y429F} volcano plot represent the MS intensity (log₁₀) of CD5 phosphorylated peptides containing the Y463 of CD5. (C) Thymocytes from CD5tg^{Wt} or CD5tg^{Y429F} mice were stimulated with anti-CD3 + anti-CD4 for 1 min. Samples were then subjected to IP with antibodies specific for CD5 and then analyzed by Western blotting with antibodies specific for the indicated proteins. (D and E) CD5 was immunoprecipitated from cellular extracts of total thymocytes from the indicated genotypes stimulated with pervanadate. Samples were analyzed by MS (D) or Western blot (E). Abundance of specific interacting partners was estimated based on MS signal intensity. Data are means \pm SD from at least two MS measurements and are representative of two experiments containing $n = 1$ mouse per group. Western blots are representative of two independent experiments. WCL, whole cell lysate.

detected in the interactome of CD5 when tyrosine 429 was mutated (Fig. 2B).

c-CBL Harbors a Connective Function that Links CD5 to Other Regulators of TCR Signaling. We next investigated the mechanism by which CD5 interacting proteins are recruited by tyrosine phosphorylation of a single residue (Y429) on the cytoplasmic tail of CD5. Y429 is included in a D(N/D)XpY motif predicted to be a potential binding site for the PTB domain of c-CBL (33). In addition, UBASH3A, SHIP1, CRKL, PI3K, and CIN85 were also previously characterized as direct or indirect c-CBL-interacting proteins, suggesting that c-CBL might be important to recruit these molecules to CD5 (34–36). To investigate this possibility, we immunoprecipitated CD5 in WT, *c-Cbl*^{-/-}, and *Cd5*^{-/-} thymocytes and evaluated if UBASH3A, SHIP1, CRKL, PI3K, and CIN85 were coimmunoprecipitated with CD5 by MS (Fig. 2D and Dataset S3) and Western blot (Fig. 2E). The amount (Fig. 2D and E) and the phosphorylation level (Fig. 2E) of immunoprecipitated CD5 were slightly increased in *c-Cbl*^{-/-} thymocytes compared to that in WT cells. Nevertheless, we found that the amounts of UBASH3A, SHIP1, CRKL, PI3K, and CIN85 that coimmunoprecipitated with CD5 were strongly reduced in *c-Cbl*^{-/-} thymocytes compared to those in WT thymocytes and were similar to those observed in *Cd5*^{-/-} thymocytes, suggesting that c-CBL is required to recruit

these molecules to CD5. In addition, we found that CD5 was not required for the phosphorylation of c-CBL (SI Appendix, Fig. S3A) or for its interaction with PI3K, SHIP1, and CIN85 (SI Appendix, Fig. S3B), suggesting that CD5 is important for the recruitment of these molecules at the membrane but not for their assembly with c-CBL.

CD5 Integrates Both Costimulatory and Coinhibitory Signaling. To elucidate the mechanism by which the CD5 regulates TCR signals, we next focused our study on c-CBL, which has well characterized inhibitory functions on TCR signaling (37). It has been proposed that c-CBL, an E3 ubiquitin-protein ligase, negatively regulates TCR signals by targeting TCRs and other components of the TCR signaling machinery to lysosomal or proteasomal degradation through ubiquitination. Consequently, TCR surface expression is increased in *c-Cbl*^{-/-} CD4⁺CD8⁺ thymocytes (referred to as double-positive [DP] thymocytes) compared to WT (*c-Cbl*^{+/+}) DP thymocytes (SI Appendix, Fig. S3C). Contrasting with this observation, we found that CD5 deficiency did not affect TCR surface expression in DP thymocytes, suggesting that CD5 is not required for c-CBL-mediated TCR turnover. Supporting this conclusion, the degradation of the TCR ζ -chain, which is induced following TCR cross-linking, was reduced in *c-Cbl*^{-/-} thymocytes but not in *Cd5*^{-/-} thymocytes (SI Appendix,

Fig. S3 D and E). Expression of LCK, which was previously identified as a target of c-CBL in T cells (38), was slightly enhanced in *c-Cbl*^{-/-} thymocytes but was also unaffected in *Cd5*^{-/-} thymocytes (SI Appendix, Fig. S3F). Finally, we found that PI3K, SHIP1, and CIN85 are expressed similarly in WT, *c-Cbl*^{-/-}, and *Cd5*^{-/-} thymocytes, suggesting that c-CBL does not drive the

degradation of the other CD5-interacting proteins (SI Appendix, Fig. S3F).

We next hypothesized that CD5 could use c-CBL, CIN85, and CRKL adapter functions to recruit positive (PI3K) and negative (UBASH3A, SHIP1) regulators of TCR signaling that would act more selectively to regulate specific signaling pathways. Recent

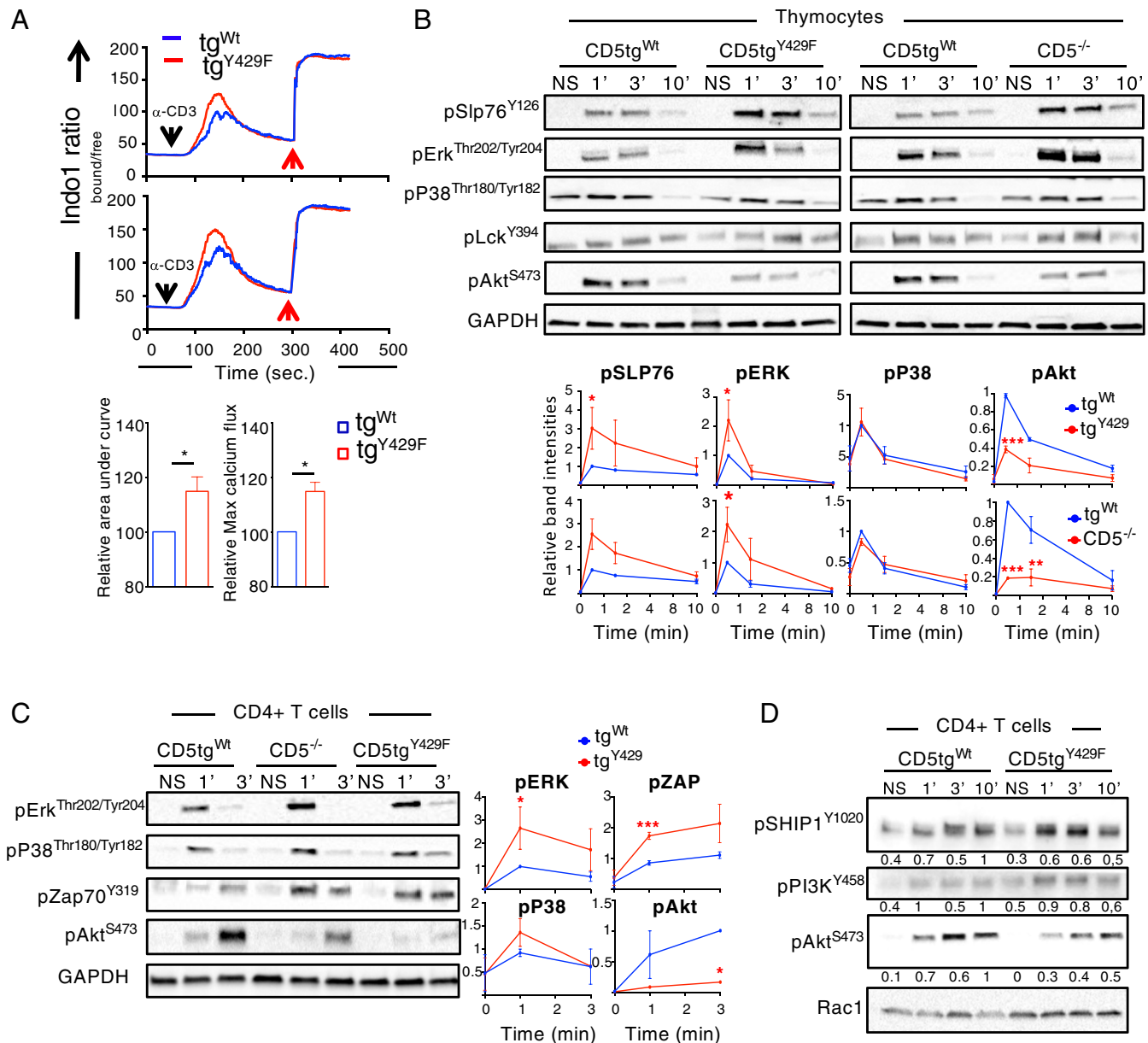


Fig. 3. CD5 coordinates TCR signals in thymocytes and peripheral T cells. (A) Analysis of calcium flux in DP thymocytes from CD5^{tg}^{Wt} and CD5^{tg}^{Y429F} mice stimulated with anti-CD3 antibodies at 5 or 10 μ g/mL (Upper and Lower graph, respectively) in the presence of anti-CD4 antibodies. Red arrows indicate maximum responses following stimulation with ionomycine. Bar graphs represent the area under the curve and the maximum peak intensity of calcium flux normalized to the values obtain in DP thymocytes from CD5^{tg}^{Wt} mice. Data are means \pm SD and are representative of two independent experiments with $n = 1$ to 2 mice per group. (B) Thymocytes from CD5^{tg}^{Wt}, CD5^{tg}^{Y429F}, and CD5^{-/-} mice were stimulated with anti-CD3 + anti-CD4 antibodies for the indicated times. Total cytoplasmic extracts of the cells were then analyzed by Western blotting with antibodies against phosphorylated forms of SLP76, ERK, P38, LCK, and AKT. Curves show the relative abundances of the indicated phosphorylated proteins as determined by calculating the ratios of the intensities of the bands corresponding to the phosphorylated proteins to those corresponding to GAPDH, the loading control. The y axes represent means \pm SD of the relative values calculated after normalization to the highest value in the CD5^{tg}^{Wt} cells. Data are representative of three to five independent experiments, each including one mouse of the indicated genotype. (C and D) Peripheral CD4⁺ T cells from CD5^{tg}^{Wt}, CD5^{tg}^{Y429F} and CD5^{-/-} mice were stimulated with anti-CD3 antibodies for the indicated times. Total cytoplasmic extracts of the cells were then analyzed by Western blotting with antibodies against phosphorylated forms of SLP76, ERK, P38, ZAP-70, PI3K, SHIP1, and AKT. Curves or values next to the blots show the relative abundances of the indicated phosphorylated proteins calculated as in B. Data are representative of two to four independent experiments each including one mouse of the indicated genotype. Unpaired two-tailed t test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

studies show that CIN85 negatively regulates the phosphorylation of ZAP-70, SLP-76, and ERK by recruiting UBASH3A into TCR microclusters (39). CIN85 also interacts with SHIP1, which also exerts an inhibitory effect on ZAP-70 activity through the proteins Dok-1 and Dok-2 (40) that presumably act through CSK to inhibit SRC kinases activity (41, 42). This suggested that SHIP1 and UBASH3A could act together to repress ZAP-70 activity. In addition, SHIP1 negatively regulates calcium responses (43) and the phosphorylation of Tec kinases (44, 45), which play an essential role in PLC γ 1 and ERK kinases activation. PI3K may interact directly with c-Cbl (46) or indirectly through CRKL (47). The corecruitment of SHIP1 and PI3K by CD5 was surprising since these two proteins were reported to have antagonistic effect on AKT activity (40, 48). To analyze whether these effector proteins are involved in CD5 signaling function, we stimulated thymocytes or peripheral CD4⁺ T cells from CD5tg^{wt}, CD5tg^{Y429F}, and *Cd5*^{-/-} mice with anti-CD3 and anti-CD4 antibodies and compared the intracellular increase of calcium and the phosphorylation of several of their downstream signaling targets. The calcium response was increased in CD5tg^{Y429F} DP thymocytes compared to that in CD5tg^{wt} DP thymocytes (Fig. 3A). Accordingly, Western blot analysis showed that the phosphorylation of ERK and SLP-76 was increased in CD5tg^{Y429F} thymocytes and peripheral CD4⁺ T cells similarly to what was observed in *Cd5*^{-/-} cells (Fig. 3B and C). Although we could not detect significant ZAP-70 phosphorylation in CD5tg^{wt} thymocytes with the stimulation conditions used in these experiments, we found that TCR stimulation induced phosphorylation of ZAP-70 was increased in both CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells compared to that in CD5tg^{wt} CD4⁺ T cells (Fig. 3C). In contrast, the phosphorylation of AKT was strikingly reduced in thymocytes and peripheral CD4⁺ T cells from CD5tg^{Y429F} and *Cd5*^{-/-} mice compared to that in similar T cell subsets from CD5tg^{wt} mice (Fig. 3B and C). In comparison, the phosphorylation of LCK and P38 were similar in thymocytes and CD4⁺ T cells from the three different lines of mice (Fig. 3B and C). Finally, we found that the phosphorylation of CD5-interacting proteins, such as SHIP1 and PI3K, were not significantly impaired in CD5tg^{Y429F} CD4⁺ T cells despite a consistent decreased of Akt phosphorylation in those cells, suggesting that CD5 is important to relocate those proteins close to their molecular targets rather than directly controlling their activity (Fig. 3D). Altogether, these results suggest that CD5 acts through c-CBL binding proteins to exert both enhancing and inhibitory effects on specific TCR-generated signals.

CD5-Mediated Signaling Negatively Regulates CD4⁺ T Cell Activation and Restrains the Generation of Treg Cells Induced by Foreign Antigens. We next analyzed the functional consequences of these CD5-mediated signaling modulations on thymocyte and naïve CD4⁺ T cell activation. We found that expression of Nur77, a quantitative sensor of TCR signal strength (49), was enhanced in CD5tg^{Y429F} and *Cd5*^{-/-} DP and CD4-SP thymocytes as compared to that in CD5tg^{wt} thymocytes following TCR cross-linking (Fig. 4A). TCR stimulation-induced surface expression of CD25 on CD4⁺ T cells and the proportions of CD4⁺ T cells expressing CD69 were also similarly augmented in CD5tg^{Y429F} and *Cd5*^{-/-} T cells as compared to those in CD5tg^{wt} thymocytes (Fig. 4B and C). The up-regulation of CD25 surface expression was also increased in CD5tg^{Y429F} CD4⁺ T cells when cells were costimulated with anti-CD28 antibodies, indicating that CD28 signaling does not compensate for CD5 signaling deficiency (Fig. 4B). Finally, we found that CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells proliferated more than CD5tg^{wt} CD4⁺ T cells in response to anti-CD3 stimulation alone (Fig. 4D) or with anti-CD4 antibodies (Fig. 4E), suggesting that CD5 negatively regulates peripheral CD4⁺ T cell activation independently of CD4 costimulation.

The analysis of TCR signaling suggested that, rather than repressing broadly TCR signals, CD5 acts more selectively by

promoting AKT and repressing ERK kinases and calcium response activities through a dedicated set of effector molecules. We thus suspected that CD5 signaling could exert a more selective effect on T cell responses in addition to its role in controlling the threshold for T cell activation. Among the many reported effects of AKT in CD4⁺ T cells, it is well known that strong AKT-mediated signals reduce their ability to differentiate into FOXP3⁺ Treg cells in presence of TGF- β (50). One proposed mechanism to explain this effect is that AKT stimulates the cytoplasmic retention of FOXO1 through the phosphorylation of two residues (Thr24 and Ser256), inhibiting the translocation of FOXO1 to the nucleus and the subsequent transactivation of *Foxp3* gene (51). The activation of ERK kinases was shown, in similar experimental settings, to have positive effect on *Foxp3* transactivation in vitro (52), suggesting that CD5 could coordinate these signaling pathways to selectively repress the induction of Treg cells.

To address this possibility, we first compared FOXP3 expression in CD5tg^{wt}, CD5tg^{Y429F}, and *Cd5*^{-/-} CD4⁺ T cells stimulated with different doses of anti-CD3 antibodies in the presence of TGF- β . We found that the percentages of CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells expressing FOXP3 were enhanced, compared to that in CD5tg^{wt} CD4⁺ T cells (Fig. 5A), upon weak but not high TCR stimulations, suggesting that CD5 may contribute to enhance the threshold at which the development of iTreg is engaged. By comparison, we found that CD5tg^{wt}, CD5tg^{Y429F}, and *Cd5*^{-/-} CD4⁺ T cells differentiated similarly into ROR γ t⁺IL-17⁺ cells following TCR stimulation under Th17 polarizing conditions (Fig. 5B). Numbers of CD5tg^{Y429F} and *Cd5*^{-/-} FOXP3⁺ cells were enhanced independently of the concentration of anti-CD3 antibodies (Fig. 5A), suggesting that CD5 operates both by controlling the ability of CD4⁺ T cells to differentiate into iTreg and by repressing the proliferation/survival of conventional T cells prior or during their engagement into the Treg lineage. Accordingly, the percentages of cells expressing FOXP3 were higher in nondivided (CTV^{hi}) CD5tg^{Y429F} CD4⁺ T cells compared to the same cell populations from CD5tg^{wt} CD4⁺ T cells (SI Appendix, Fig. S4). Confirming the positive effect of CD5 on AKT activity, we found that the phosphorylation of FOXO1 on Thr24 and Ser256 was impaired in CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells (Fig. 5C). The phosphorylation of ERK, which is also correlated with efficient generation of induced Treg cells (52), was enhanced in CD5tg^{Y429F} and *Cd5*^{-/-} CD4⁺ T cells compared to that in CD5tg^{wt} CD4⁺ T cells, showing further that CD5 delivers both costimulatory and coinhibitory signals to modulate a specific T cell response, namely the induction of *Foxp3* and the generation of iTreg cells. Finally, the numbers of FOXP3⁺ cells were similar in CD5tg^{wt} and CD5tg^{Y429F} CD4⁺ T cells when cells were incubated with high concentrations of FOXO1 inhibitors in vitro assays, suggesting that the increased activity of ERK observed in CD5tg^{Y429F} CD4⁺ T cells was not sufficient alone to enhance the generation of Treg cells (Fig. 5D). In comparison, the numbers of CD5tg^{Y429F} CD4⁺FOXP3⁺ T cells, reflecting T cell expansion, remained increased compared to those in CD5tg^{wt} CD4⁺FOXP3⁺ T cells when cells were treated with similar doses of FOXO1 inhibitors (Fig. 5D). Altogether, these results suggested that CD5 signaling shape TCR signals to selectively repress FOXP3 expression.

The surface level of CD5 correlates with TCR signal intensity, which is dictated by the affinity of the TCR for self-ligands (9). To determine whether quantitative variations of CD5 surface expression within normal physiological ranges influences the generation of induced FOXP3⁺ cells, we next sorted naïve CD62L^{hi} CD25⁻ CD4⁺ T cells expressing either high or low surface levels of CD5 and compared their ability to differentiate into FOXP3⁺ cells in Treg-cell polarizing conditions. We found that CD5^{lo}CD4⁺ T cells differentiate more efficiently into FOXP3⁺ cells than CD5^{hi}CD4⁺ T cells under identical TCR stimulation conditions, suggesting that physiologically high levels of CD5 on naïve CD4⁺

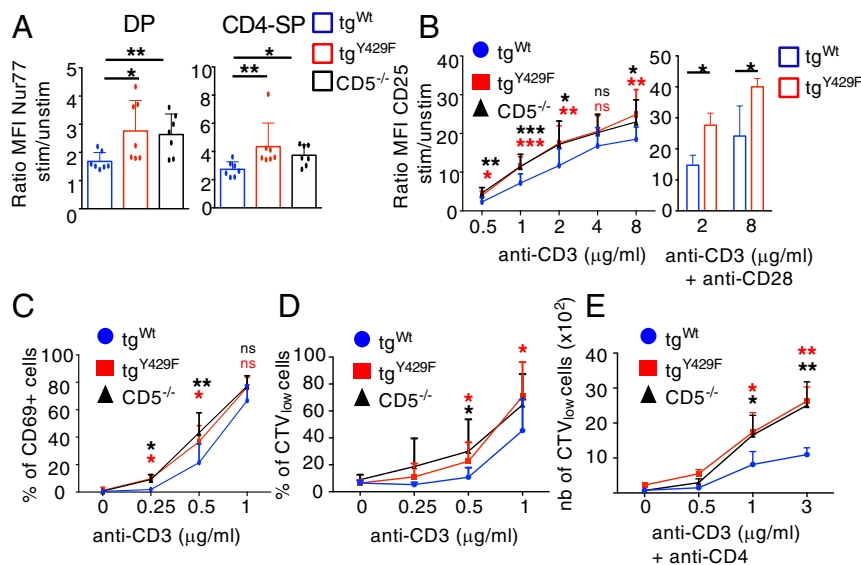


Fig. 4. The CD5 signalosome negatively regulates the activation and the proliferation of CD4⁺ T cells. (A) Thymocytes from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice were stimulated or not with anti-CD3 and anti-CD28 antibodies for 4 h. Nur77 protein abundance was then analyzed by flow cytometry in DP and CD4-SP cells after intracytoplasmic staining with anti-Nur77 antibodies. Bar graphs represents the ratio of mean fluorescent intensities (MFI) of Nur77 in stimulated cells to unstimulated cells. Data are means \pm SD and are representative of two independent experiments, with $n = 3$ mice per group. (B) Peripheral CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice were stimulated with the indicated doses of anti-CD3 antibodies alone (curves) or with anti-CD28 antibodies (bar graphs) for 24 h. Curves and bar graphs represents the ratio of MFI of CD25 in stimulated cells to unstimulated cells. Data are mean \pm SD and represent, for anti-CD3 alone, four independent experiments with $n = 12$ mice per group or, for anti-CD3 + anti-CD28, two experiments with $n = 4$ mice per group. (C) Peripheral CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice were stimulated with the indicated doses of anti-CD3 antibodies for 24 h. Curve graph represents the percentages of CD69⁺ cells. Data are mean \pm SD and represent three independent experiments with $n = 4$ to 8 mice per group. (D and E) Peripheral CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice previously stained with cell trace violet (CTV) were stimulated with anti-CD3 antibodies alone (D) or with anti-CD4 antibodies (E) for 72 h. Curve graphs represent the percentages (D) or the numbers (E) of CTV^{low} CD4⁺ T cells following stimulation with variable doses of anti-CD3 antibodies for 72 h. Data are mean \pm SD and represent three independent experiments with $n = 7$ to 11 mice per group. Unpaired two-tailed Mann–Whitney t test. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

T cells might reduce the ability of these cells to differentiate into Treg cells upon antigenic recognition (Fig. 6A). Contrasting with those results, previous studies suggested that CD5 promotes, rather than represses, the differentiation of peripherally induced Treg cells when those cells are generated in a tolerogenic context such as the gut mucosa (15). Accordingly, we found that the percentages of FOXP3⁺CD4⁺ T cells and of FOXP3⁺Neuropilin^{low}CD4⁺ T cells, which are essentially composed of iTreg cells (53), were decreased in the Peyer patches of CD5^{-/-} and CD5tg^{Y429F} mice as compared to those in CD5tg^{Wt} mice (SI Appendix, Fig. S5). We thus hypothesized that CD5 signalosome might be influenced by the environment in which T cells are localized and that CD5 may operate differently in a nontolerogenic environment when CD4⁺ T cells encounter foreign antigens or pathogens. To examine this possibility, we first immunized CD5tg^{Wt} and CD5tg^{Y429F} mice with a peptide variant (EAWGALANKAVDKA, called 1W1K peptide hereafter) of the I-E α -chain immunodominant peptide 52–68 in the presence of incomplete Freund's adjuvant (IFA), which was shown to favor the polarization of iTreg cells (54). To follow antigen-induced FOXP3⁺ T cells, we stained cells from the draining lymph nodes with 1W1K-pMHCII tetramer and analyzed FOXP3 expression in tetramer⁺CD4⁺ T cells. We found that the percentages of CD44⁺tetramer⁺CD4⁺ T cells were similar in CD5tg^{Wt} and CD5tg^{Y429F} mice, indicating that CD5 signaling does not significantly impact the clonal expansion of antigen-specific CD4⁺ T cells in this experimental setting (Fig. 6B). We observed that the percentages and numbers of tetramer⁺FOXP3⁺CD4⁺ T cells were increased in CD5tg^{Y429F} mice compared to those in CD5tg^{Wt} mice (Fig. 6C). In comparison, the percentages and numbers of tetramer⁺T-BET⁺CD4⁺ T cells were similar in CD5tg^{Wt} and CD5tg^{Y429F} mice (Fig. 6D). Proportions and numbers of tetramer⁺FOXP3⁺Neuropilin^{low}CD4⁺ T cells were

also higher in CD5tg^{Y429F} mice compared to those in CD5tg^{Wt} mice, suggesting that CD5 signaling restrains the generation of iTreg cells following immunization with foreign antigens.

To confirm this observation in a more pathophysiological model, we next analyzed whether CD5 signaling could repress the generation of Treg cells following mice infection with the respiratory virus influenza A, which was shown to drive important antigen-specific Treg cell responses (55). Mice were infected intranasally and pathogen-specific Treg were analyzed with NP311–325-IA^b tetramer in the draining lymph nodes 5 d following infection. We found that the percentages of virus-specific CD4⁺ T cells were comparable in CD5tg^{Wt} and CD5tg^{Y429F} mice, indicating that CD5 signaling does not influence the overall expansion of antigen-specific CD4⁺ T cell following infection (Fig. 6E). The proportions and numbers of virus-specific FOXP3⁺CD4⁺ T cells and of FOXP3⁺Neuropilin^{low}CD4⁺ T cells were increased in CD5tg^{Y429F} mice as compared to those in CD5tg^{Wt}, showing that CD5 represses the development of those cells during pathogenic infection (Fig. 6F).

Discussion

In this study, we identified a multimeric signaling complex that acts through CD5 to limit the induction of Treg cells. This complex is composed of proteins with adaptor functions (c-CBL, CIN85, CRKL) that connect CD5 to distinct effector proteins (UBASH3A, SHIP1, PI3K), which acts cooperatively by repressing ERK and promoting AKT activity to inhibit the transactivation of *Foxp3*. This signalosome is recruited to CD5 Y429 following TCR engagement, suggesting that it is part of a feedback loop that is differentially engaged according to the strength of TCR signals that gradually regulates CD5 expression at the cell surface. It is generally admitted that stimulation of CD4⁺ T cells with a high dose of strong

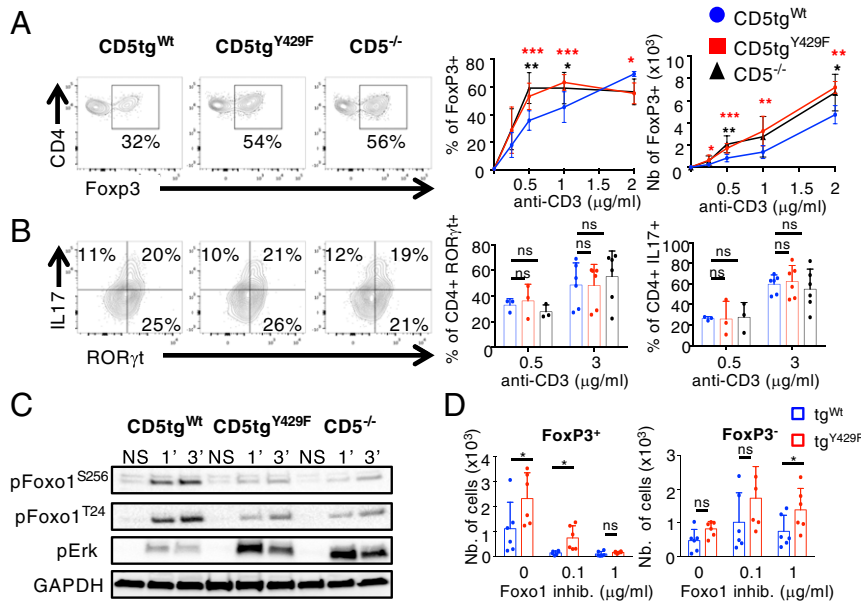


Fig. 5. CD5 signaling restrains the generation of induced Treg cells by promoting AKT-mediated inhibition of FOXO1 and repressing ERK activity. (A) Naïve CD62L^{hi}CD25^{lo} CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice were stimulated with anti-CD3 antibodies in presence of TGF-β for 3 d. Contour plots represent CD4 vs. FOXP3 staining profiles of CD4⁺ T cells from the indicated genotypes. Curve graphs represent the percentages and numbers of FOXP3⁺ CD4⁺ T cells from mice of the indicated genotypes stimulated with the indicated doses of anti-CD3 antibodies in presence of TGF-β. Data are means ± SD and represent two independent experiments with *n* = 6 to 9 mice per group. (B) Naïve CD62L^{hi}CD25^{lo} CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice were stimulated with anti-CD3 antibodies in presence of TGF-β and IL-6 for 5 d. Contour plots represent CD4 versus RORγt staining profiles of CD4⁺ T cells from the indicated genotypes. Bar graphs represent the percentages of RORγt⁺ and IL-17⁺ CD4⁺ T cells from mice of the indicated genotypes stimulated with the indicated doses of anti-CD3 antibodies in presence of TGF-β and IL-6. Data are means ± SD and represent two independent experiments, with *n* = 3 (0.5 μg/mL anti-CD3) and *n* = 6 (3 μg/mL anti-CD3) mice per group. (C) Peripheral CD4⁺ T cells from CD5tg^{Wt}, CD5tg^{Y429F}, and CD5^{-/-} mice were stimulated or not (NS) with anti-CD3 antibodies for the indicated times. Total cytoplasmic extracts of the cells were then analyzed by Western blotting with antibodies against phosphorylated forms of FOXO1 and ERK. Western blots are representative of two independent experiments. (D) Naïve CD62L^{hi}CD25^{lo} CD4⁺ T cells from CD5tg^{Wt} and CD5tg^{Y429F} mice were stimulated with anti-CD3 antibodies in presence of TGF-β with or without variable doses of FOXO1 inhibitors for 3 d. The expression of FOXO1 was then analyzed by flow cytometry after intracytoplasmic staining of the cells with anti-FOXO1 antibodies. Bar graphs represent the numbers of FOXO1⁺ (Left) and FOXO1⁻ CD4⁺ T cells from mice of the indicated genotypes stimulated with anti-CD3 antibodies in presence of TGF-β with or without the indicated doses of FOXO1 inhibitors. Data are means ± SD and represent two experiments with *n* = 6 mice per group. Unpaired two-tailed Mann-Whitney *t* test, except for D, where paired *t* test with Welch's correction was performed. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

agonist exerts a repressive effect on the generation of iTreg cells, presumably because it promotes strong AKT-mediated signals that prevent the transactivation of *Foxp3* (50). A molecular model to explain this effect was that strong, but not weak, TCR signals repress the expression of the PtdIns(3,4,5)P3 phosphatase and tensin homolog (PTEN), which is an inhibitor of the AKT signaling pathway (56). Our study also suggests that CD4⁺ T cells could finely tune AKT signaling by titrating the surface expression of CD5 to reduce the generation of iTreg cells upon TCR stimulation with a relatively high dose of high-affinity ligands. It was shown that CD5^{hi}CD4⁺ T cells respond better than CD5^{lo}CD4⁺ T cells to diverse foreign antigens (11). This was explained by the enrichment, in the CD5^{hi} population, of CD4⁺ T cells with greater self-reactivity, which exhibit enhanced TCR signaling potential (57). An additional explanation inferred from our study is that CD5 could act by reducing the emergence of inopportune antigen-specific Treg cells that might occur during the recognition of foreign antigens and which may lead to the development of ineffective immune responses. This interpretation could also bring an explanation to the reduced development of active autoimmunity observed in CD5 deficient mice models (12).

The modes of interaction of c-CBL with UBASH3A, SHIP, CIN85, CRKL, and PI3K have been extensively studied particularly in the context of EGF receptor (EGFR) signaling for which a similar molecular machinery as the one described in this study for CD5 has been described (36, 58, 59). PI3K can bind directly to c-CBL through a PTB-site located at the C-terminal end of c-Cbl (Y731) (60), or indirectly, through CRKL, which

binds to two PTB sites located in the same region (Y700 and Y774) (61). CIN85 binds directly with SHIP1 and was shown to compete with UBASH3A for binding to c-CBL, suggesting that two distinct CD5 signaling subsets might coexist in T cells (62). Previous studies have shown that CIN85/c-CBL complexes drive EGFR internalization and degradation in lysosomal compartments, whereas UBASH3A/c-CBL complexes prevent these processes and sustain EGFR-mediated signals (36, 58). Thus, these distinct complexes could contribute to regulate the turnover of CD5 in addition to their regulatory function on TCR signaling. Although c-CBL was shown to mediate CD5 ubiquitylation, resulting in its degradation in lysosomes following TCR/CD5 cocross-linking (63), we found that the substitution of tyrosine 429 by phenylalanine does not significantly modify CD5 surface expression either before or after TCR engagement, suggesting that the direct engagement of CD5 by external ligands might be required for its degradation.

Several proteins previously identified as CD5-interacting proteins, such as Ras-Gap (22), SHP-1 (23), Vav1 (25), and ZAP70 (64), were not detected as CD5 interactors in our MS analysis. Although initial studies suggested that SHP-1 interacts with CD5 in thymocytes (65) and Jurkat cells (23), several studies since then have failed to reproduce this interaction (22, 66). Moreover, both the phosphatase activity associated to CD5 immunoprecipitates and the reduction in positive selection conferred by CD5 overexpression were shown to be unaffected by SHP-1 deficiency, suggesting that CD5 operates independently of this phosphatase (67). CK2 (26) and Cbl-b (30), also previously described as

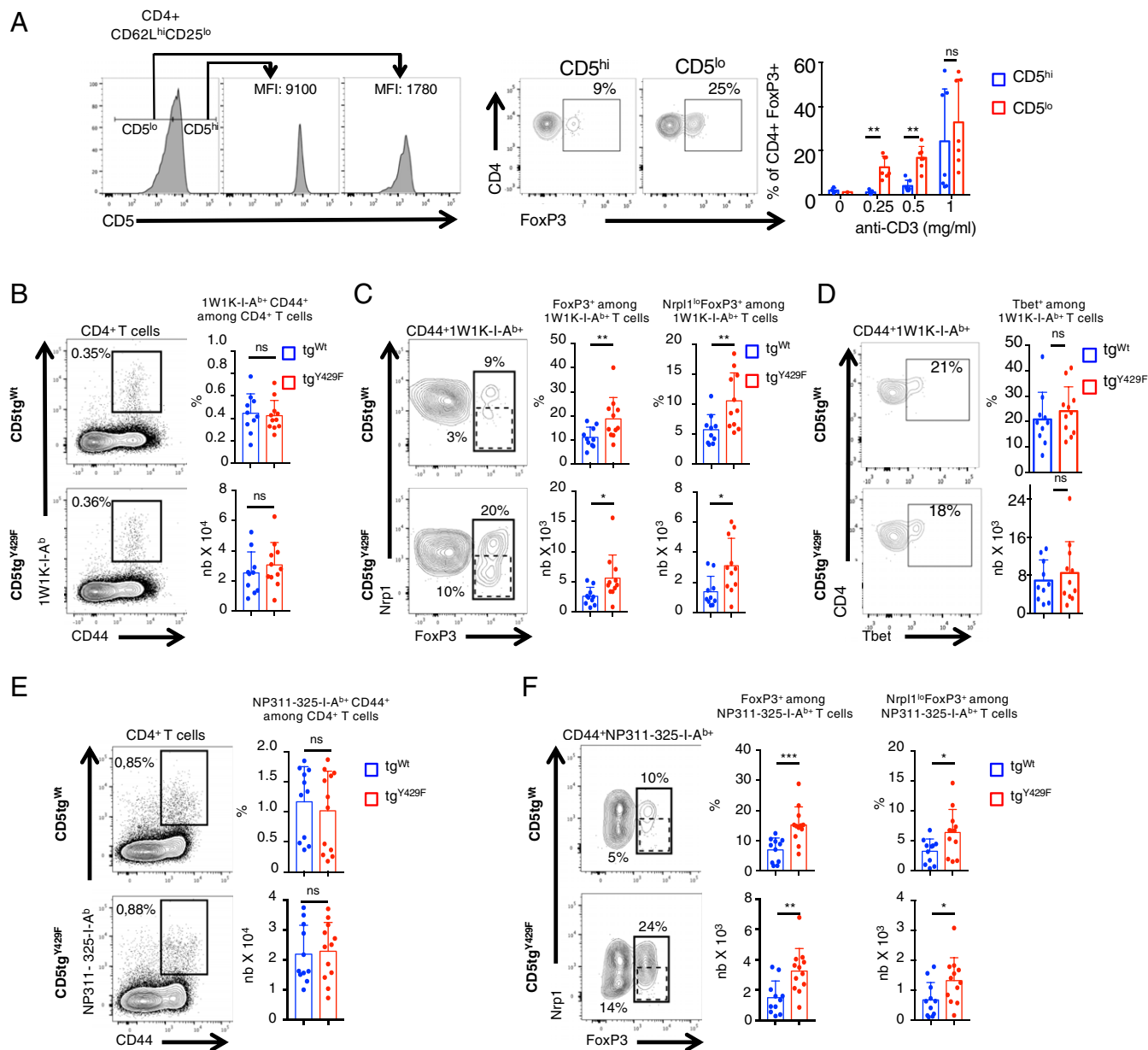


Fig. 6. CD5-mediated signaling restrains the generation of peripherally induced Treg cells following immunization with foreign antigens and viral infection. (A) CD5^{lo} and CD5^{hi} naive CD62L^{hi}CD25^{lo} CD4⁺ T cells from C57BL/6 mice were sorted prior to stimulation with anti-CD3 antibodies in presence of TGF- β for 3 d. Histograms show the sorting strategy. Contour plots represent CD4 versus FOXP3 staining profiles of CD5^{lo} and CD5^{hi} CD4⁺ T cells. Bar graphs represent the percentages of FOXP3⁺ CD4⁺ T cells following stimulation with the indicated doses of anti-CD3 antibodies in presence of TGF- β . Data are means \pm SD and represent two independent experiments with $n = 7$ mice per group. (B–D) CD5tg^{Wt} and CD5tg^{Y429F} mice were immunized with 1W1K peptide in presence of IFA. Draining lymph nodes were collected after 7 d and analyzed by flow cytometry after staining of the cells with 1W1K-conjugated MHC tetramers. (E and F) CD5tg^{Wt} and CD5tg^{Y429F} mice were intranasally infected with PR8 virus. Draining lymph nodes were collected after 5 d and analyzed by flow cytometry after staining of the cells with NP311-325-conjugated MHC tetramers. (B and E) Contour plots represent CD44 versus tetramer staining profile on gated CD4⁺ T cells. Bar graphs represent the percentages and absolute numbers of tetramer⁺CD44⁺ T cells among CD4⁺ T cells. (C and F) Contour plots represent FoxP3 vs. Neuropilin1 (Nrp1) staining profile on gated tetramer⁺CD44⁺ CD4⁺ T cells. Bar graphs represent the percentages and absolute numbers of either FoxP3⁺ (solid gate) or FoxP3⁺ neuropilin^{low} (dashed gate) among tetramer⁺CD44⁺ CD4⁺ T cells. Data are representative of two independent experiments and are means \pm SD of at least 10 mice for each genotype. Nonparametric t test * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

CD5-binding proteins, were recruited to CD5 but not as robustly as other interactors, suggesting that CD5 could engage these proteins but in other molecular or cellular contexts than the one used in this study. It was shown that CD5 enhances Th17 responses when cross-linked to the TCR but not when the TCR is engaged alone (68, 69), suggesting that CD5 could recruit distinct signaling effectors whether or not it is engaged by extracellular ligands. Accordingly, the deletion of a CK2

binding site on CD5 impairs the polarization of CD4⁺ T cells into Th17 cells following stimulation with antigen-presenting cells but not following anti-CD3 antibodies (69).

We show that CD5 exerts a combined effect on TCR signaling, reducing ZAP-70 and ERK activity, presumably through the joint action of UBASH3A and SHIP1, which are known regulators of these signaling molecules (40, 44, 45, 70, 71), and enhancing AKT activity, likely through PI3K, which facilitates the

retention of FOXO1 in the cytoplasm and prevents the consequent transactivation of *Foxp3* (51). This signaling complex does not detectably modulate the expression of ROR γ t or T-BET in activated CD4⁺ T cells in vitro and in vivo, respectively, indicative of a selective effect of CD5 on the generation of iTreg cells. Remarkably, studies using SHIP1- and PI3K-deficient murine models identified repressive functions for both proteins on the generation of induced Treg cells (72, 73). Whether UBASH3A is also directly involved in the control of this population has not yet been studied. However, a recent investigation performed in human T cells has shown that it represses NF- κ B signaling (74), a key pathway for the generation of induced Treg cells (75). Thus, CD5 could act as a scaffold that selectively engages specific signaling effectors involved in the generation of peripheral Treg cells to selectively control the expansion of this population during immune responses.

Previous studies have shown that CD5 deficiency on the BALB/c but not on the C57BL/6 genetic background leads to increased numbers of thymic Treg cells, suggesting that CD5 may also repress the generation of these cells according to the molecular context in which CD5 operates in the thymus (13, 14). CD5 is highly expressed at the surface of thymic Treg cells, indicating that the ability of CD5 to negatively regulate FoxP3 expression in the thymus could also be overcome by additional signals (such as high TCR affinity for self-ligand) that impose FoxP3 expression despite the opposite force exerted by CD5. We thus speculate that CD5-mediated inhibition may be sufficient to block Foxp3 expression and Treg lineage commitment in thymocytes that express TCRs that bind with intermediate affinity to self-ligands, preventing their commitment to the Treg lineage. In addition, coreceptors, such as GITR, OX40, and TNFR2, are highly expressed by thymic Treg progenitors and were also shown to trigger costimulatory signals that induce thymic Treg differentiation (76). In contrast, these coreceptors are poorly expressed by naive peripheral CD4 T cells, suggesting that the negative effect of CD5 on FoxP3 expression may become predominant in this subset.

A recent study reported an effect of CD5 opposite to that shown in our study on the generation of peripherally induced Treg cells in a mouse model in which tolerance to experimental autoimmune encephalomyelitis is induced by direct delivery of encephalitogenic peptides to antigen-presenting dendritic cells in the absence of adjuvant (15). Those authors found that CD5 prevents the inhibition of Treg cell induction potentially mediated by effector cell cytokines, such as IL-6, IL-4, and IFN- γ . Although they do not provide a clear signaling mechanism by which CD5 operates here, they show that CD5 promotes the generation of Treg cells by inhibiting IL-6-mediated AKT signaling leading to mammalian target of rapamycin activation. One potential explanation for the apparent discrepancy between those results and our current findings is that CD5 might exert opposite effects on PI3K/AKT signaling in tolerized and non-tolerized T cells. A recent proteomic study showed that CD5 interacts with both c-CBL and CBL-b in peripheral CD4⁺ T cells (30), suggesting that distinct pools of CD5, connected either to a

c-CBL or CBL-b signalosome, could be preferentially assembled under specific stimulation conditions. Whereas c-CBL has known positive effects on PI3K/AKT signaling (46), CBL-b blocks this pathway by inducing PI3K ubiquitylation (77) and by blocking Nedd4-mediated ubiquitylation of PTEN (78). Because CBL-b expression is up-regulated in CD4⁺ T cells after tolerizing signals (79) and degraded in CD4⁺ T cells that have received appropriate costimulatory signals (80), we speculate that CD5 signaling pools may vary in size or composition, tuning up or down the PI3K/AKT signaling axis according to the immunological context in which cells are stimulated.

Altogether, the findings of our study suggest that CD5 engages a polymorphic signaling machinery that can transduce both stimulatory and inhibitory signals to selectively control specific T cell responses depending on the immunological context. Our results also provide new insights into the paradigm of coreceptor signaling, suggesting that, in addition to providing classic enhancing or dampening inputs, coreceptors coordinate TCR signals that may have antagonist effects to promote specific functional outcomes, such as the generation of iTreg cells.

Materials and Methods

Full details of materials and methods, including mice, antibodies, cell stimulation, immunoprecipitations, MS analysis, calcium flux, Western blot, immunization and influenza virus infection, statistical analysis are provided in *SI Appendix*.

Mice. All the experiments were conducted with sex- and age-matched mice between 6- and 12-wk-old housed under specific pathogen-free conditions at the INSERM Zootechnie US-006 animal facility, which is accredited by the French Ministry of Agriculture to perform experiments on live mice (accreditation number A-31 55508). All experimental protocols were approved by the local ethics committee and are in compliance with the French and European Union regulations on care and protection of laboratory animals (EC Directive 2010/63).

Data Availability Statement. The data generated or analyzed during this study are included in the article and its *SI Appendix* or dataset files. The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifier PXD017343. Raw data from Figs. 1 A and C and 2 A, B, and D and *SI Appendix*, Fig. S1 A–C can be found in *Datasets S1–S3* and *S5*.

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Normal and pathological functions of the T cell signaling protein, THEMIS, in CD4⁺ T cells

CD4⁺ T cells or T helper (Th) cells play a pivotal role in triggering and shaping adaptive immune responses. Naïve CD4⁺ T cells may differentiate into multiple effector subsets with distinct lineages and specific immune functions which help to orchestrate host defense against various classes of pathogens. The mechanism that controls cell fate decision depends on the integration of multiple signals triggered by the T cell antigen receptor (TCR), which recognize Self or foreign peptide associated with the molecules of the Major Histocompatibility Complex (MHC), and by an array of cytokines receptors which are stimulated according to the cytokines present in the extracellular environment. In addition to those cell fate decision signals, additional signals are important to control the magnitude of T cell effector responses and set the balance between effective and pathologic immune responses.

THEMIS is a T cell lineage protein that plays an essential role during the positive selection of T cells in the thymus. THEMIS has been characterized as a TCR signaling protein, but whether it operates as an inhibitor or enhancer of TCR signals has been controversial. Its function in peripheral T cells, especially in peripheral CD4⁺ T cells, remains poorly characterized. The objective of this study was to investigate the role of THEMIS in normal and pathological CD4⁺ T cell responses.

By using a mouse model which is selectively deficient for THEMIS after the stage of positive selection (lateCD2-Cre *themis*^{flax/flax} mice; named *themis*-T^{KO}), we observed that THEMIS deficiency has opposite consequences on CD4⁺ T cell responses upon TCR stimulation *in vitro* and following immunization with foreign- and self-antigens *in vivo*. *In vitro*, THEMIS deficiency leads to a selective increase of interferon-gamma production in Th1 polarized CD4⁺ T cells. The absence of THEMIS did not affect pro-Th1 signals mediated by IL-12 and IFN γ receptors but results in a specific increase of the phosphorylation of ERK1/2 MAP kinases following TCR stimulation which is responsible for the increased production of Th1 cytokines observed in THEMIS deficient CD4⁺ T cells. *In vivo*, immunization of *themis*-T^{KO} with a foreign antigen leads to decreased production of IFN γ by antigen-specific CD4⁺ T cells associated with a lower expression level of the transcription factor T-bet. Also, susceptibility to the development of Experimental Autoimmune Encephalomyelitis (EAE), the murine model of Multiple Sclerosis, is decreased in *themis*-T^{KO} mice. This is associated with an attenuated encephalitogenic potential of *themis*-T^{KO} CD4⁺ T cells, which express lower levels of the pro-Th1 transcription factor T-bet and of the chemokine receptor CXCR3 and produce reduced amounts of interferon-gamma. Altogether, these results suggest that THEMIS exerts inhibitory effects on TCR-mediated signaling and CD4⁺ T cell responses *in vitro*, which are overcome *in vivo* by TCR-independent signals that promote normal and neuro-inflammatory Th1 responses.

In conclusion, this thesis work presents insights that may explain the previous discrepancy associated with the function of THEMIS on TCR signaling and identifies a deleterious effect of THEMIS on the regulation of Central Nervous System autoimmunity.

Keywords: THEMIS, T cell antigen receptor, CD4⁺ T cells, TCR signaling, Experimental autoimmune encephalomyelitis

Fonctions normales et pathologiques de la protéine de signalisation des lymphocytes T, THEMIS, dans les lymphocytes T CD4+

Les lymphocytes T (LT) CD4⁺ (aussi appelés LT helper ou LTh) jouent un rôle central dans le déclenchement et le contrôle des réponses immunitaires adaptatives. Les LT CD4⁺ dits naïfs peuvent se différencier en différentes sous-populations de LT effecteurs ayant des fonctions immunitaires spécifiques, qui contribuent à orchestrer la défense de l'hôte contre diverses classes d'agents pathogènes. Ce processus de différenciation dépend de l'intégration de plusieurs signaux déclenchés par le récepteur d'antigène des LT (TCR), qui reconnaît des peptides du Soi ou étrangers associés aux molécules du complexe majeur d'histocompatibilité (CMH), et par un ensemble de récepteurs aux cytokines plus ou moins stimulés selon les cytokines présentes dans le milieu extracellulaire. En plus de signaux contrôlant la différenciation des LT effecteurs, des signaux supplémentaires sont importants pour contrôler l'amplitude des réponses effectrices des LT et établir un équilibre optimal entre une bonne efficacité des réponses immunitaires et la prévention de réponses potentiellement pathologiques.

THEMIS est une protéine de la lignée des LT qui joue un rôle essentiel lors de leur sélection positive dans le thymus. THEMIS a été identifiée comme une protéine de signalisation du TCR mais sa fonction dans ce contexte est encore controversée. Son rôle dans les LT périphériques, en particulier dans les LT CD4⁺, reste mal caractérisé. L'objectif de cette étude a été d'étudier le rôle de THEMIS dans les réponses normales et pathologiques des lymphocytes T CD4⁺.

En utilisant un modèle murin sélectivement déficient pour THEMIS après l'étape de sélection positive dans le thymus (souris lateCD2-Cre *themis*^{flx/flx}; nommées *themis*-T^{KO}), nous avons observé que le déficit en THEMIS a des conséquences opposées sur les réponses des LT CD4⁺ lors de la stimulation du TCR *in vitro* et après immunisation avec des antigènes étrangers et/ou du Soi *in vivo*. *In vitro*, le déficit en THEMIS entraîne une augmentation sélective de la production d'IFN γ dans les LTh1 due à une augmentation spécifique de la phosphorylation des MAP kinases ERK1/2 suite à la stimulation du TCR. En revanche, l'absence de THEMIS n'a pas d'effet sur les signaux pro-Th1 déclenchés par les récepteurs à l'IL-12 et à l'IFN γ . *In vivo*, la déficience en THEMIS entraîne une diminution de la production d'IFN γ par les LT CD4⁺ spécifiques d'antigènes exogènes associée à des niveaux d'expression plus faible du facteur de transcription pro-Th1 T-bet. En outre, la susceptibilité au développement de l'encéphalomyélite auto-immune expérimentale (EAE), le modèle murin classique de sclérose en plaques, est diminuée chez les souris *themis*-T^{KO}. Les LT CD4⁺ déficients en THEMIS sont caractérisés par un potentiel encéphalitogénique atténué, de faibles niveaux d'expression du facteur de transcription T-bet et du récepteur CXCR3 ainsi qu'une moindre capacité à produire de l'IFN γ . Dans l'ensemble, ces résultats suggèrent que THEMIS exerce des effets inhibiteurs sur la signalisation du TCR et les réponses des lymphocytes T CD4⁺ *in vitro*; effets contrebalancés *in vivo* par des signaux indépendants du TCR qui amplifient les réponses Th1 normales et neuro-inflammatoires.

En conclusion, ce travail de thèse apporte un nouvel éclairage sur les fonctions de THEMIS sur la signalisation du TCR et identifie des effets potentiellement pathologiques de THEMIS dans les maladies auto-immunes affectant le système nerveux central.

Mots-clés : THEMIS, Récepteur d'antigène des lymphocytes T, Lymphocytes T CD4⁺, Signalisation du TCR, Encéphalomyélite Auto-immune Expérimentale.