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The role of tumor necrosis factor receptor family (TNFRF) members in regulatory T cell biology

Martina Lubrano Di Ricco

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Ecole doctorale 394 « Physiologie, Physiopathologie et Thérapeutique »

Centre d'Immunologie et des Maladies Infectieuses de Paris

Equipe « Biologie des lymphocytes T régulateurs et Thérapie »

Thèse de doctorat d'Immunologie

The role of Tumor Necrosis Factor Receptor Family (TNFRF) members in regulatory T cell biology

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To my parents, Luca and Ilaria,

To my sibling, Lolli and Zack

And to my love, Dodo.

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ABBREVIATIONS

AIRE	AutoImmune Regulator
AP-1	Activator Protein 1
APCs	Antigen Presenting cells
CD	Cluster of differentiation
CNS	Conserved Non-coding sequence
CTLA-4	Cytotoxic T-Lymphocytes-associated Antigen 4
DCs	Dendritic cells
EAE	Experimental Autoimmune Encephalomyelitis
EMSA	Electrophoretic Mobility Shift Assay
Foxp3	Forkhead box P3
GFP	Green Fluorescent Protein
GITR	Glucocorticoid-Induced TNF Receptor
HAT	Histone Acetyl Transferase
ICOS	Inducible T-cell COStimulator
IDO	Indoleamine 2 3-dioxygenase
Ig	Immunoglobuline
IKK	IkB kinases
IL	InterLeukine
IFN	Interferon
IPEX	Immune dysregulation Polyendocrinopathy Enteropathy X-linked
KO	knock-Out
LAG-3	Lymphocyte Activation Gene-3
LN	Lymph Nodes
MFI	Mean Fluorescence Intensity
MHC	Major Histocompatibility Complex
NFAT	Nuclear Factor of Activated T cells
NF-kB	Nuclear Factor-kB
NK	Natural killer cells
NKT	Natural killer T cells
NOD	Non-Obese Diabetic mice

Nrp1	Neuropiline 1
PBS	Phosphate Buffered Saline
PD-1	Programmed Death 1
PKB	Protein Kinase B (or Akt)
STAT	Signal Transducer and Activator of Transcription
TCR	T cell receptor
Tconv	Conventional T cells
Teffs/Tconv	Effector/Conventional T cells
Tfh	Follicular T helper cells
TGF- β	Transforming Growth Factor β
Th	T helper cells
TNF	Tumor Necrosis Factor
TNFR	TNF Receptor
iTreg	In vitro induced regulatory T cells
pTreg	Peripheral regulatory T cells
tTreg	Thymic regulatory T cells
WT	Wild Type

PREFACE

Very often I try to give a definition of “autoimmunity”, but I still find it hard. I imagine autoimmunity as a mosaic, as suggested by Shoenfeld and Isenberg in 1989. A mosaic is a “picture or pattern produced by arranging together small pieces of stone, glass, etc.”. If you move any of each piece you might have a different picture. Autoimmunity could be compared to a mosaic to explain two major features that render autoimmune diseases so complicated. First, although different mechanisms are involved in driving autoimmunity, there are shared factors among several autoimmune diseases. Second, this can explain why there is a high heterogeneity among people classified with the “same autoimmune disease”.

The interest in autoimmune diseases has grown recently because of the rising incidence of many of them which is still unexplained. Several hypotheses have been proposed: the too hygienic lifestyle which does not challenge enough the immune system, or the western diet with a low fiber content.

But the truth is that the immune system will remain a problem as long as variation and natural selection remain the laws of nature. Indeed, more than a century ago Darwin and Wallace taught us that life depends upon adaptation of an organism to its ever-changing environment. And the immune system needs to distinguish component of the environment that promote survival from those that threaten it.

The tools used by the human immune system for recognition have been refined, specialized and expanded and many have been reshaped from invertebrate predecessors.

The way of doing research and interpreting the immune system have been changed during the years. For example, historically “self” was used to describe an entity fixed during early development and responsible for imposing and maintaining natural immune tolerance in the primary lymphoid tissues. However, several new findings rendered the self and non-self distinction more difficult: should we consider the microbiota self? And products derived from apoptotic cells?

Among various mechanisms for establishing and sustaining immunological self-tolerance and immune homeostasis, T-cell-mediated suppression of immune responses toward self

and non-self antigens has always attracted enormous interest.

The idea of the existence of suppressor cells, now regulatory T cells (Treg), came in the early 70s. Gershon and Kondo found that T cells not only augmented but also dampened immune responses and that this down-regulation was mediated by T cells different from the helper cells. However, in the following years the interest for these cells collapsed due to technical issues such as absence of markers to distinguish suppressor cells from the others, ambiguity in the molecular basis of suppression and difficulty in preparing antigen-specific suppressor T-cell clones. But also immunologists failed to proof a direct connection between immunological diseases and anomaly of suppressor cells.

Therefore, for some years it was thought that the suppressor activity was due to T cells that start to produce immunosuppressive cytokines such as IL-10.

Then, in the 90s, many researchers decided to focus more on studying self-tolerance and how it can be inhibited and this approach led to the finding that the immune system naturally harbors T cells and thymocytes with suppressive activity, later called Treg.

The immune system is composed of several tissues and cells and it is important that each component works perfectly to avoid unbalanced responses.

The Treg are key component of this system because they are involved in keeping the tolerance against self-antigens and therefore their dysfunction can lead to the development of autoimmunity. These cells also need to switch off the responses of the immune system once the threat is eliminated to avoid pathologic inflammation. On the opposite, Treg are a barrier for cancer immunotherapy because they block the specific response against tumor cells. Therefore, it is highly important to enrich our awareness of these cells to be able to manipulate them to control different immunopathologies and to develop promising therapies.

In addition, in the last years the majority of the research on autoimmune diseases has been based on the reductionist principle of isolating a single variable within complex background. This idea is now challenged and now scientists have started to study the immune system as a complex network in which changes in one parameter can alter the whole system.

With this new way of studying biology in general, the wet lab work is not sufficient anymore if we want to enlarge our knowledge and be able to connect a biological finding

with a clear explanation.

Indeed, one of the major revolutions is the use of Next Generation Sequencing which dramatically changed the way of doing research. It permits us to take a shot of what is going on in even a single cell in a particular condition. It can generate high amount of data which then brings to the problem of how to interpret them.

Being passionate of immunology and eager to learn and use new bioinformatics tools to arrive where the lab research is not enough, I decided to start this thesis project. Nowadays, there are new techniques, such as the –omics, that could help to fasten the research driving to new findings. With this work I hope I have contributed, at least in a small part, to the missing information regarding the immune system in general and the regulatory T cells in particular.

INTRODUCTION

1. The immune tolerance

Tolerance refers to the prevention of an immune response against a particular antigen. Normally, the immune system is tolerant against self-antigens to avoid to attack body's own cells and tissues, therefore there is a sort of balance between self-antigen-driven tolerance and pathogen-driven immunity. A shift toward the extremes such as lack of response (immunodeficiency) or an excessive response (autoimmunity or allergy) results in pathophysiological conditions [1].

The thymus is the site where the central tolerance of T cells takes place, which is one of the most important mechanisms that lead to the elimination of autoreactive T cells. However, some autoreactive cells might escape the central checkpoints and circulate in the tissues. This is the reason why there is also a peripheral tolerance that protects us.

1.1 Central tolerance

Thymus is the site of central tolerance, which occurs throughout life even if this organ undergoes significant atrophy during aging. T cell precursors are produced in the bone marrow, and then they migrate to the thymus to complete their development becoming CD4⁺ or CD8⁺ T cells and acquiring the expression of the T cell receptor (TCR) which permits the selection of T cells. During postnatal life, in humans, around 10-100 hematopoietic precursors enter the thymus per day. Then there are several rounds of division resulting in the generation of about 5×10^7 T cells daily.

Central tolerance consists in positive selection of lymphocytes that express a functional TCR and then negative selection based on the affinity of TCR-MHC (Major Histocompatibility Complex) binding and together they cause the death of around 90%-95% of thymocytes [1]. This process ensures that only T cells with a functional TCR and that does not recognize self-antigen with high or intermediate avidity, are released in the circulation.

The TCR comes in two different versions, $\alpha\beta$ or $\gamma\delta$, each chain of the dimer having one variable domain and one constant. T cells are able to produce hypervariable TCRs thanks to the recombination of variable (V) and joining (J) gene segments from the pools of

α and γ TCR genes and V, diversity (D) and J gene segments from the pool of β and δ TCR genes. There are genes that encode the enzymes RAG-1 and RAG-2 which mediate these processes. Splicing inaccuracies and insertion of nucleotides around the V(D)J junctions further increase diversity. This permits to have cells that can recognize a broad spectrum of foreign antigens but some of them also recognize self-molecules.

Initially, bone-marrow derived progenitors enter the thymus as double negative (DN) thymocytes, defined by the choice of CD4 or CD8 expression (Figure 1).

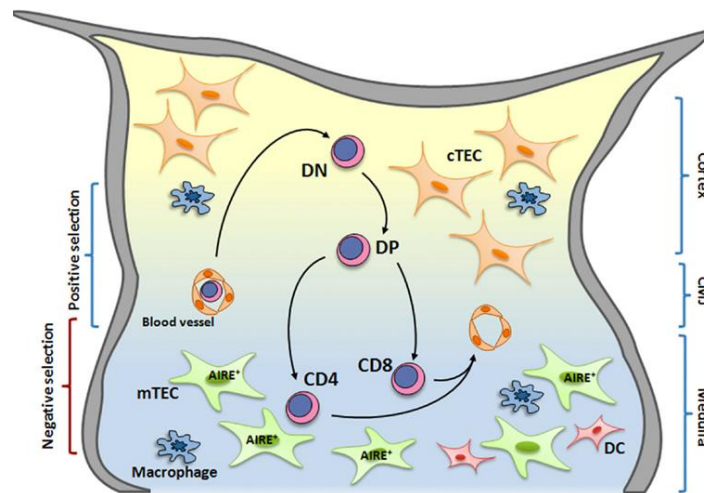


Figure 1 – Schematic view of the different steps occurring during central tolerance in the thymus

From: Passos, G.A., D.A. Mendes-da-Cruz, and E.H. Oliveira. *Front Immunol*, 2015. 6: p. 352

Thymocyte development and interactions with the microenvironment. Thymocytes migrate and interact with microenvironmental components; these interactions are responsible for selective processes. Medullary thymic epithelial cells (mTECs) express AIRE, to present all peripheral antigens to thymocytes.

In humans, they stay in the cortex around 2 weeks undergoing approximately 20 divisions.

Then, the TCR β -chain rearrangement permits the survival of only DN thymocytes with a functionally rearranged chain (positive selection). At this step, they become double positive (DP) thymocytes, defined by the co-expression of CD4 and CD8.

Following, there is the lineage commitment because they become single positive (SP) thymocytes expressing either CD4 or CD8.

Now, thymocytes translocate to the medulla, in a CCR7-dependent way [2], where they reside for 4-5 days before receiving the “exit permit” [3].

In the medulla there is the rearrangement of the other chain of the TCR, the α , and the

subsequent negative selection during which thymocytes are selected based on the affinity of the binding TCR-MHC. Indeed, both rearrangements are random which gives the possibility to recognize a wide array of antigens, including self-molecules. That is why the thymocytes are subjected to the positive selection, where only cells with a TCR that is able to bind to MHC molecules survive, and negative selection where cells that bind MHC with a strong affinity are eliminated. In this way if the affinity is too low, the TCR might not be functional and cells die within a few days; however, if it is too high, they might be potential autoreactive cells and they are eliminated by clonal deletion. Thymocytes that bind the MHC with low to intermediate affinity complete this development and enter the periphery as mature, naïve T cells.

One issue regarding the central tolerance was how T cells can be tolerized in the thymus for antigens which are not expressed there. Later, it was shown that thymic medullary epithelial cells (mTECs) express a wide array of antigens representing essentially all organs, including genes expressed in a spatially-restricted fashion and developmentally and temporally regulated genes [1]. It was indeed discovered a gene called AutoImmune REgulator (AIRE), expressed in mTECs, that functions as a transcription factor to drive the expression of peripheral tissues antigens in those cells [4]. The negative selection is operated by both mTECs but also dendritic cells (DCs) since mTECs are poor antigen presenting cells for ubiquitous antigens and very few mTECs express a given antigen. DC may be resident in the thymus (CD11c^{hi} and pDC) or coming from the periphery (CD11b⁺), and they can cooperate with mTECs acquiring antigens from them [5], or alternatively, they can transport peripheral antigens, in a CCR9-dependent way, into the thymus, contributing to immune tolerance [6].

Some of T cells, that have a high affinity TCR, that survive negative selection differentiate into CD4⁺Foxp3⁺ Treg that prevent autoimmunity and plays an important role in modulating inflammatory responses [7].

1.2 Peripheral tolerance

Although central tolerance is a very efficient mechanism, it cannot eliminate all self-reactive lymphocytes. For this reason, there is the peripheral tolerance, which is important when lymphocytes encounter self-antigens for the first time, such as food

antigens, developmental antigens and antigens displayed during chronic infections [8]. In fact, there is a clear association between autoimmunity and defects in genes involved in peripheral tolerance confirming its importance. It is possible to divide it into two categories: one that act directly on T cells (T-cell intrinsic) and the other which is mediated by different types of suppressive cells, and among these, Treg are the most important (T-cell extrinsic).

For the T-cell intrinsic part, it is possible to identify 4 mechanisms [9]:

- Ignorance: when T cells cannot detect the antigen and this can happen either because it is unaccessible, like in immune-privileged sites (such as eyes and brain), or because the amount of antigen is very low and not enough to mount a response.
- Anergy: which means that T cells are functionally inactivated because of the lack of all the necessary signals. This happens because T cells to be activated need an antigen-specific signal (signal 1) but also a costimulatory one (signal 2), mediated mainly by CD28 [10] and when the latter is missing it can impair T cell activation. But the lack of costimulatory signals is not the only mechanism, indeed the expression of some inhibitory molecules, such as Cytotoxic T-Lymphocytes-associated Antigen 4 (CTLA4) and Programmed Death-1 (PD-1), can induce anergy in T cells. CTLA4 can compete with CD28 for the binding of CD80 and CD86 expressed on Antigen Presenting Cells (APCs) [11], [12] while PD-1 binds its ligand and inhibits T cell proliferation and function [13].
- Phenotype skewing: the cytokine environment present during the encounter with the antigen highly influences T cell phenotype. Indeed, there are several subsets of CD4⁺ T cells, with different phenotype and functions, that can be induced with a different cytokine milieu. A common example is the shift versus T-helper 2 cells which downregulates autoimmunity in some mouse models [14].
- Apoptosis: this is perhaps the most important mechanism and involves the receptor Fas expressed on T cell and its ligand (FasL) present on APCs [15].

As mentioned above, Treg are the most important mediators of T-cell extrinsic suppression. There are some Foxp3⁺CD4⁺ T cells, namely T_R1 cells, which are generated

when naïve CD4⁺T cells are cultivated with IL-10 and suppress T cells by producing IL-10 [16], and T_h3 cells which suppress by producing Transforming Growth Factor-β (TGF-β) [17]. But the ones that seem the most important in peripheral tolerance are the Foxp3⁺CD4⁺ T cells which can be divided in tTreg or nTreg (thymic or natural Treg) or pTreg (peripheric Treg) based on their generation. The nTreg are generated from SP CD4⁺ T cells which recognize the MHC with high affinity and require also CD28/CD80/CD86 signal for their induction while the pTreg derive from naïve T cells. They keep tolerance by several ways, mainly acting by inhibiting other cells[9].

2. Foxp3⁺CD4⁺Regulatory T cells

Foxp3⁺CD4⁺ T cells are the best characterized population of regulatory cells and, as already mentioned, there are several types of these cells based on their development. They are indispensable for the maintenance of self-tolerance and immune homeostasis. And indeed their defects cause autoimmune diseases, immunopathologies and allergies [18].

Their discovery was one of the major findings in immunology and helped to acquire more knowledge regarding the immune system and autoimmunity; it also opened the door to cell therapy based on Treg expansion *ex vivo* and their transfer into patients to treat autoimmune diseases and inhibit graft-versus-host disease after bone marrow transplantation [19].

2.1 Discovery

The first evidences for the existence of Foxp3⁺CD4⁺ T cells came in 1969 when a neonatal thymectomy at day 3 after birth resulted in autoimmune damage of various organs; and this condition was reverted with the transplantation of thymus 7 days later [20]. One year later, it was shown that cells derived from the thymus can suppress immune responses [21].

Then, in the following years, it was shown that adult thymectomy causes an autoimmune disease called thyroiditis [22] which can be reverted with the injection of CD4⁺ T cells from syngeneic mice [23]. These results showed that not only there are pathogenic autoreactive T cells but also T cells that suppress autoimmunity. The key problem was to identify a marker to enrich these regulatory cells.

2.2 Phenotypic markers

The growing interest for the possibility to identify markers to define Treg pushed researchers to conduct several experiments. One of the most important was to deplete a CD4⁺ T cell suspension of a population of CD5^{high} cells and to transfer the rest to T-cell-deficient athymic nude mice; the result was the development of autoimmunity in several organs [24]. Reconstitution of depleted CD4⁺ T cell subpopulation inhibited autoimmunity. Indeed, the first identified marker of Treg was CD5 or Lyt-1.

More efforts to search for something more specific led to the identification of CD25 which is the α chain of the IL-2 receptor. Transfer of T cells depleted of CD25⁺ T cell causes autoimmunity in athymic nude mice, while a cotransfer of CD25⁺CD4⁺ T cell inhibits the development of autoimmunity [25].

Indeed IL-2 is a key cytokine for Treg development, survival and function and mice lacking IL-2 develop T cell-mediated fatal lymphoproliferative/inflammatory disease with autoimmune features [26].

Mice deficient in CD25 or CD122 (the β chain of the IL-2 receptor) succumb to a similar disease.

In humans, CD25 deficiency is indistinguishable from IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome), an autoimmune disease characterized by defective Treg [27]. Evidences suggest that the syndrome is due to deficiency or dysfunction of Foxp3⁺Treg for 3 reasons: the number of these cells is reduced in mice lacking either CD25 or IL-2 [28], T cell-specific deficiency of Signal Transducer and Activator of Transcription (STAT) 5a and b (which mediate signaling from IL-2 receptor) abrogates the development of Foxp3⁺Treg causing autoimmunity [29] and lastly, high dose of neutralizing anti-IL-2 antibody to normal neonatal mice reduces the number of Foxp3⁺CD25⁺CD4⁺ T cells for a limited period [30].

It is possible to conclude that IL-2 has a pivotal role in immune homeostasis since it is produced by activated non-regulatory T cells (Treg are unable to produce it), and it contributes to differentiation, maintenance, expansion and activation of Treg which in turn limits the expansion of non-regulatory T cells (*Figure 2*). Disruption of this feedback loop promotes the development of autoimmunity.

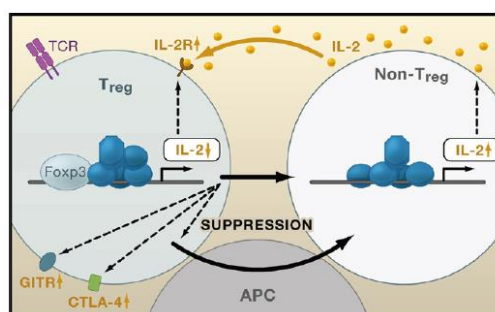


Figure 2 – Key role of IL-2 in immune homeostasis

From: Sakaguchi, S., et al. Cell, 2008. 133(5): p. 775-87

IL-2 signaling in Treg, non-Treg and APCs. Foxp3 represses IL-2 transcription in Treg, making them highly dependent on exogenous IL-2 (produced by non-Treg). Foxp3 regulates, as well, the expression of Treg-associated molecules as CD25, GITR and CTLA4.

But it was only in the early 2000s that was discovered the main marker of Treg: Forkhead box P3 (Foxp3). It is a member of head/winged-helix family of transcription factors and is a master regulator gene of Treg development and function. It was identified as a defective gene in Scurfy mice, which exhibit an X-linked recessive disease that causes death one month after birth. This mice show hyperactivation of CD4⁺ T cell and overproduction of proinflammatory cytokines [31]. Furthermore, mutations of the human *FOXP3* gene causes the human counterpart of Scurfy which is called IPEX [32]. Given the similarities of the diseases caused by Foxp3 mutations among the species, it was hypothesized that it must have a key role in Treg development and function [33], [34], [35].

To confirm this, it was shown that ectopic transduction of *Foxp3* gene in CD25⁻CD4⁺ T cell can convert them to CD25⁺CD4⁺Treg-like cells that were suppressive and could control autoimmunity [33]. Moreover, Foxp3-deficient mice and Scurfy mice have few CD25⁺CD4⁺Treg, and inoculation of CD25⁺CD4⁺ T cell prevents systemic inflammation in Scurfy mice [35].

Foxp3 interacts with several transcription factors: Nuclear Factor of Activated T cells (NFAT), Runt-related transcription factor 1 (AML1/Runx1), the histone acetyl transferase (HATs)/histone deacetyl transferase(HDAC), Nuclear Factor-κB (NF-κB) and many others (Figure 3A).

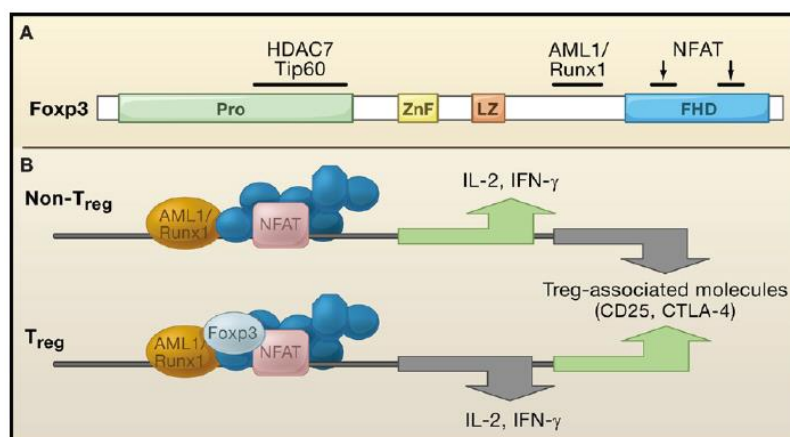


Figure 3 – Control of Treg by Foxp3

From: Sakaguchi, S., et al. Cell, 2008. 133(5): p. 775-87

In A, the structure of Foxp3 locus. Black bars are binding sites for other transcription factors or chromatin-remodelling enzymes. In B, NFAT and AML1/Runx1 regulate genes encoding molecules in Treg and non-Treg, depending on the presence of Foxp3.

Upon activation, NFAT forms a complex with Activator Protein 1 (AP-1) and NF- κ B and promotes the expression of *IL-2*, *Il4*, *Ctla4*, and other genes in conventional T cells contributing to their differentiation in effector T cells [36]. In these cells, AML1/Runx1 binds to the *IL-2* promoter facilitating the assembly of transcriptional activation complexes driving IL-2 production. In contrast, in Treg, AML1/Runx1 binds physically to Foxp3 and disruption of this binding impairs Foxp3-dependent suppression of IL-2 production.

There are other markers of Treg related to their function that are differentially expressed compared to Tconv, in both humans and mice. I will list the mains [37], which will be further explained in the “function” chapter of Treg:

- CTLA-4: which downregulates T cell activation by competing with CD28 for B7 binding
- CD103: which is expressed mainly by mouse Treg and identify IL-10 producing Treg and is a marker of skin and mucosal retention of Treg
- CCR6: which is a marker of Th17 cells
- CD127: Treg have low expression of this marker compared with conventional T cells
- ICOS (Inducible T-cell COStimulator): which is expressed by the majority of mouse Treg and by half of human Treg, namely those that produce IL-10 and TGF- β , while ICOS⁻ cells produce only TGF- β .
- LAG-3 (Lymphocyte Activation Gene-3), a ligand of MHC II, which is expressed on activated cells
- CD39: which characterize functional Treg and contribute to their suppression consuming extracellular ATP
- TNFR2: which binds TNF and is expressed at very low level on conventional T cells

Here there are two tables resuming, in a more extensive way, the main markers of mouse and human Treg (*Table 1*).

Markers of mouse functional Tregs.

Marker	Function/comments
CD25 ⁺	CD4 ⁺ CD25 ⁺ T cells in mouse are generally FoxP3 ⁺ Tregs which are heterogenous and contain both highly and minimally suppressive subsets. However, CD25 can be expressed on activated Teffs and memory T cells, and CD25 ⁻ T cells can be potent suppressive FoxP3 ⁺ Tregs
FoxP3 ⁺	Master regulator and to date the best intracellular marker of Tregs. FoxP3 ⁺ Tregs are phenotypically and functionally heterogenous, including both highly suppressive and minimally suppressive Tregs
CTLA4 ⁺	Constitutively and preferentially expressed intracellularly by Tregs. Expression levels may indicate the suppressive potency of Tregs
CD103 ⁺	Memory/effector Tregs with potential to produce IL-10. Marker of skin- and mucosal-homing Tregs
CCR6 ⁺	Identifies effector-memory Tregs with potential to produce IL-10
CD127 ^{-/low}	Mouse Tregs express relative lower levels of CD127 (IL-7R α) than Teffs. However, ICOS ⁺ and CD103 ⁺ Tregs express high level of CD127; its expression can be up-regulated on Tregs upon in vitro and in vivo activation
ICOS ⁺	Expressed on most of Tregs in normal mice. High expression identifies IL-10 producing Tregs or Ag-specific Tregs which share features of Tr1/Th1/Th17 cells
LAG-3 ⁺	Expressed on TCR-activated Tregs and contributes to Treg function. Also expressed on activated Teffs
CD39 ⁺	Constitutively expressed on majority of mouse Tregs. Moderately up-regulated upon in vitro TCR stimulation, but markedly enhance suppressive activity of Tregs on APT-mediated maturation of DCs.
TNFR2 ⁺	Predominantly expressed by 30–40% of highly suppressive peripheral Tregs and ~80% thymic Tregs. Transduce activating effect of TNF on Tregs and shed by Tregs as suppressive molecule

Markers of human functional Tregs.

Marker	Function/comments
CD25 ^{high}	Top 1–2% of brightest CD25-expressing, FoxP3 ^{high} and CD45RO ⁺ CD4 cells are highly suppressive Tregs
FoxP3 ⁺	FoxP3 expression is necessary but not sufficient to identify human Tregs. Teffs can express FoxP3 upon activation, especially in the presence of TGF β . Functional suppressive FoxP3 ⁺ Tregs can be identified by using other markers, e.g.: CD45RO ⁺ FoxP3 ^{high}
CTLA-4 ⁺	Constitutively and preferentially expressed intracellularly by Tregs. Expression levels correlate with the suppressive potency of Tregs
CD103 ⁺	Expressed very low levels on human Tregs, which may be up-regulated in inflammatory responses or by TGF β
HLA-DR ⁺	Identify mature, terminally differentiated, high FoxP3-expressing Tregs responsible for contact dependent inhibition.
CCR6 ⁺	Identifies memory Tregs and IL-17-producing suppressive Tregs
CD127 ^{-/low}	Human Tregs are characterized by low CD127 expression. CD4 ⁺ , CD25 ⁺ and CD127 ⁻ are generally used to define human Tregs. This combination may be further improved by additional marker, such as CD62L ⁺ . Teffs also down-regulate their CD127 expression upon activation
CD45RO ⁺ (or CD45RA ⁻)	CD45RO ⁺ CD25 ^{high} FoxP3 ⁺ identify effector Tregs with highly potent suppressive capacity; CD45RA ⁺ CD25 ⁺ FoxP3 ^{low} identify resting Tregs with moderate suppressive function; CD45RO ⁺ CD25 ⁺ FoxP3 ^{low} identify non-suppressive FoxP3 ⁺ T cells which can produce proinflammatory cytokines
ICOS ⁺	ICOS identifies memory-like Tregs. ICOS ⁺ Tregs produce IL-10 and express membrane bound TGF β
LAG-3 ⁺	Constitutively expressed by ~3% of PB Tregs and expanded by activation in tumor patients. Identifies an activated and terminally differentiated Tregs with superior suppressive potential
GARP ⁺	Upon activation, GARP is selectively expressed on highly potent suppressive Tregs, but not expressed on activation-induced FoxP3 ⁺ non-suppressive cells
LAP ⁺ /CD121a ⁺ / CD121b ⁺	After TCR stimulation, expression of LAP, CD121a and CD121b can identify functional suppressive FoxP3 ⁺ Tregs
CD39 ⁺	Expressed on effector/memory-like FoxP3 ⁺ functional Tregs
CD49d ⁺	Absence of CD49d excludes CD25 ⁺ Teffs from Tregs. CD4 ⁺ CD25 ^{high} CD49d ⁻ CD127 ⁻ identifies highly pure FoxP3 ⁺ functional Tregs
TNFR2 ⁺	Constitutively and preferentially expressed by ~70% of PB FoxP3 ⁺ Tregs and all of human thymic Tregs. Highly expressed by memory/effector Tregs, while naïve Tregs also express higher TNFR2 than naïve Teffs. Combination of CD25 ⁺ and TNFR2 ⁺ can identify more FoxP3 ⁺ Tregs in human PB than CD25 ^{high} . Combination with additional marker, such as CD127 ^{-/low} , may yield the better identification of Tregs. Also expressed by tolerogenic DC-induced Ag-specific CD4 Tregs and anti-CD3 induced CD8 Tregs. Up-regulated on activated Tregs by inflammation and promotes survival of Tregs in inflammatory environment

Table 1 – Markers of mouse and human Treg

From: Chen, X. and J.J. Oppenheim. *Int Immunopharmacol*, 2011. **11**(10): p. 1489-96.

2.3 Features of Treg stability and activation

Recently, researchers have put a lot of efforts to couple NGS techniques to Treg to unravel their core signature meaning the major genes characterizing their phenotype.

To this regard, Benoist et al. [38] compared the transcriptome of Treg and conventional T cells (Tconv) from mice and humans at the steady state using single cell RNA-sequencing. They saw two separated clusters for Treg and Tconv, as expected. However, surprisingly, they saw some so-called “furtive Treg” with a transcriptome clustering with the Tconv (26% in mice and 55% in humans). *Foxp3* was detected in those cells at similar levels to

those of other Treg and canonical Treg-signature genes were also overexpressed in these cells compared to surrounding Tconv. However, some of these key genes were present at lower levels compared to other Treg, suggesting that they could be “weaker” Treg with a poorly suppressive phenotype.

In addition, they also saw Tconv and Treg clustering together in three separated minor clusters. One of these contains cells expressing genes associated with residence in B cell follicles (T follicular helper and regulatory cells) and the others contain cells that upregulate a set of genes associated with early response to TCR engagement. These observations mean that even if Treg are generally distinguishable from Tconv, the two populations were imbricated at different levels with an important degree of overlap and that TCR signaling may drive similar programs in both Treg and Tconv. To go deeper with the analysis, they systematically compared Treg with closest Tconv and identified a small set of genes (*IL-2ra*, *IL-2rb*, *Ikzf2*, *Ctla4*, *Capg*, *Tnfrsf4*, *Tnfrsf18*, *Izumo1r*, *Chchd10*, *Gpr83* and *Foxp3*) overexpressed by Treg in all clusters. Moreover, they also pointed out that in the big cluster of Treg it is possible to identify six clusters, half of which contain resting and the other half activated Treg according to *Sell* (CD62L) and *Ccr7* expression. Among the activated ones, there were Treg expressing genes of early cell activation thus evoked TCR-mediated activation, while others characterized by a diverse set of genes.

These data describe well Treg identity which is not so separated from Tconv but also their heterogeneity suggesting that in inflammatory conditions or in different tissues we might find Treg expressing different sets of genes.

There are several papers supporting this heterogeneity investigating the transcriptomes of intratumoral Treg.

One of this [39] compares the transcriptome of Th1, Th17 and Treg infiltrating colorectal or non-small-cell lung cancers to the same subsets from normal tissues. It was noticed that tumor infiltrating Treg cluster together in both tumors suggesting common features in these cells dictated probably by the tumor microenvironment. They up-regulated, also at protein level, several immune checkpoints (GITR, OX40, TIGIT, LAG-3, TIM-3) and their ligands (OX40L, Galectin-9, CD70). In general, they showed an enrichment in genes related to lymphocyte activation and with increased suppressive

activity (OX40, CTLA4 and GITR). It was also noticed that tumor infiltrating Treg express high amounts of 4-1BB, a costimulatory molecule that seems to mark antigen-activated Treg.

Moreover, Treg display the most pronounced differences in gene expression among CD4+ T cell subsets infiltrating normal and tumor tissues. This suggests that tumor microenvironment influences specific gene expression in Treg and this correlates with the evidence that Treg from different tissues are instructed by environmental factors to display different gene-expression profiles.

Another work [40] compared Treg from untreated human breast carcinomas, normal mammary gland and peripheral blood and showed that tumor infiltrating Treg are very similar to tissue-resident Treg but very different from Treg from peripheral blood. Thus, residence in a non-lymphoid tissue, regardless of whether it is healthy or with oncogenic transformation, serve as a major determinant of gene-expression characteristics. Once again tumor Treg were enriched in genes for cytokines signaling, defense response, inflammatory response and lymphocyte activation compared to cells from peripheral blood. Of these genes, a subset was more highly expressed by Treg in tumor compared to tissue-resident cells, including *EBI3*, *OX40* and *IL1R2*.

Lastly, it was investigated the impact of Helios in Treg identity. This transcription factor might be dispensable for Treg activity at the steady state but seems to be essential during inflammation to maintain a stable phenotype and potentiate suppressive function. Indeed, Helios deficiency in Treg leads to enhanced antitumor immunity that reflects the induction of an unstable phenotype and conversion of Treg into Tconv within the tumor microenvironment [41].

It was shown that Helios-deficient intratumoral Treg displayed increased levels of some receptors associated with final Treg differentiation as *Icos*, *Tnfrsf18*, *Tnfrsf9*, *Klrg1* and *Ilr1*. But they also up-regulated factors involved in Th lineage determination and cytokine production. In general Helios-deficient Treg resemble the so-called "tissue Treg". Moreover, the expression levels of GITR and OX40 have been positively correlated with the strength of TCR signaling during Treg development and in mature Treg. Increased expression of those receptors promotes Treg maturation. Since GITR is highly up-

regulated in Helios-deficient Treg this might implicate that the TCR repertoire in those cells is skewed toward increased self-reactivity [41].

Interestingly, recently it was demonstrated an important role of the TNFSF in the generation of effector Treg (eTreg) from central Treg (cTreg) [42]. Indeed, Treg generated in the thymus recirculate through secondary lymphoid organs as cTreg. In this state they already have regulatory functions, however they do not express the full repertoire of molecules associated with their suppressive capacity. This means they undergo further differentiation in the periphery to acquire a fully suppress phenotype, like downregulation of CD62L, upregulation of Blimp-1, ICOS, TIGIT, CD73, PD-1 and IL-10 production. Maturation and functional specialization of Treg are associated with their propensity to localize to non-lymphoid tissues, and indeed the majority of Treg in those tissues are eTreg. IRF4 is required for this differentiation and its deletion in Treg impairs their ability to suppress inflammation [43]. IRF4 can be activated by TCR signaling which is required for Treg functional maturation in the periphery. However, Treg survive and suppress without TCR, suggesting that there should be additional signals promoting Treg survival and function [44]. In fact, other signals can be driven through TNFR family members, which are highly expressed on Treg. It was demonstrated that they activate RelA which has a critical role in development and maintenance of eTreg. Deficiency of RelA compromised the fitness of eTreg in lymphoid and non-lymphoid tissues [42] (See also Annex 1).

All these data underlie the main genes involved in Treg biology but also highlight the heterogeneity of these cells based on their development, residency, activation status and impact of the surrounding environment. One of the major driver of Treg diversity is surely the site of their generation, which will be discussed in the next paragraph.

2.4 Treg development

As already mentioned, it is possible to distinguish 2 major types of Foxp3⁺CD4⁺ T cells based on the site of development: thymic Treg (tTreg) generated in the thymus and peripheral Treg (pTreg) for peripherally generated cells. Then, there is a third class, called induced Treg (iTreg) for *in vitro* generated cells. They are generated starting from naïve

CD4⁺ T cells (defined by Foxp3⁻CD44^{low}CD62L^{hi}) isolated and cultured with anti-CD3/anti-CD28 in the presence of IL-2 and TGF-β.

2.4.1 Development of tTreg

Foxp3⁺ thymocytes are detectable from a late DP stage to SP stage, constituting around 5% of CD4⁺ thymocytes and less than 1% of CD8⁺ thymocytes (Figure 4A).

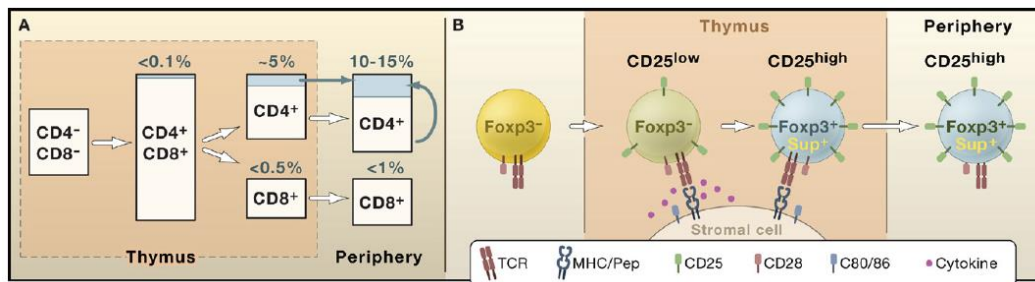


Figure 4 – Thymic development of nTreg

From: Sakaguchi, S., et al. Cell, 2008. **133**(5): p. 775-87

In A, the percentage of Foxp3⁺ cells in thymocytes subpopulation. In B, Development of Foxp3⁺ Treg in the thymus involves interaction with stromal cells. Foxp3⁻ thymocytes turn on a Treg differentiation program when they receive signals produced by the interaction between their TCRs and MHC/self-peptide complexes on stromal cells. The signal involves also accessory molecules and stromal cell-derived humoral factors.

Evidences suggest that tTreg may have a TCR with higher affinity for thymic MHC/self-peptide ligands than those of other T cells and that those self-reactive cells are selected as tTreg precursors. This was confirmed in double-transgenic mice expressing a transgene-encoded peptide in thymic stromal cells at high level and in this case T cells expressing the TCR specific for the peptide differentiate into Treg [7],[45].

In contrast, Treg fail to develop when these mice express either low-affinity transgenic TCR or a high concentration of the peptide, presumably due to insufficient positive selection or strong negative selection, respectively. The self-reactivity of Treg could be a consequence of positive selection of highly self-reactive T cells or a resistance to negative selection [46].

In addition to the affinity of the TCR/MHC interaction, the intensity of the binding between accessory molecules contributes to tTreg generation. Deficiency of CD28, CD40, CD11a/CD18, or CD80 and CD86 results in a reduction of tTreg [47].

At a cellular level, both mTEC and DCs in the thymus contribute to tTreg generation. Interestingly, Aire-deficient mice develop an autoimmune disease similar to the one

produced by Treg depletion. A study suggests that Aire⁺ mTECs expressing tissue antigens induce the development of Treg specific to recognize tissue specific antigens [48].

An interesting feature is that it seems that Foxp3 is not required for this initial cell fate determination in the thymus [49],[50]. In mice where the Foxp3 encoding region was substitute with a reporter GFP protein, GFP-positive thymocytes, which do not express Foxp3, have a Treg phenotype although they are not suppressive *in vitro*. This suggests that the interaction between thymocytes and stromal cells turns on a program of transcriptional regulation (with or) upstream Foxp3; and once it is on, Foxp3 stabilizes and sustains Treg phenotype and confers suppression activity (*Figure 4B*).

Recent genome-wide analysis showed different patterns of DNA methylation or histone modification between Tconv and Treg.

For example, Treg-specific DNA hypomethylation (associated to open chromatin and therefore accessibility to transcription factors) occurs in a limited number of loci, half of which are located in genes important for Treg phenotype and functions [51].

Furthermore, some changes in DNA methylation are highly stable, such as the demethylation of the conserved noncoding sequence 2 (CNS2) in the intron 1 of *Foxp3* which is bound by Foxp-3, Gata-3 and STAT5 in Treg and enhances the expression of Foxp3 [52],[53],[54]. In contrast, DNA methylation of *Iira* varies with the activation status of Treg.

Treg-specific epigenetic pattern appears in developing Treg, already in the SP stage, and progresses from the thymus to the periphery. Treg-specific DNA hypomethylation and Foxp3 expression have distinct roles in tTreg development. For example, Foxp3 represses some molecules important for Treg functions (IL-2 and IFN- γ), whereas Treg-specific epigenetic changes do not [51].

Therefore it seems that there is a cooperation between Foxp3 and epigenetic modifications, supported by the fact that Treg-specific DNA hypomethylated regions in the genome are different from Foxp3-binding sites [55].

Moreover, chromatin accessibility of Foxp3-binding sites is similar between Treg and Tconv [56], suggesting that Foxp3-dependent regulation is independent of epigenetic changes.

The two have distinct roles in tTreg development but they are both required for a mature

and stable phenotype. TCR stimulations is important for both events, but while Foxp3 expression seems to depend on the intensity of TCR stimulation, the epigenetic changes depend more on the duration of TCR stimulation [51]. Therefore, only thymocytes that have acquired the Treg-specific epigenetic changes become poised to express Foxp3, and if they do express Foxp3 they will become mature Treg. While thymocytes that have acquired the Treg-specific epigenetic pattern, but do not express Foxp3, can potentially become Treg, Foxp3-expressing thymocytes without the correct epigenome lose Foxp3 and fail to differentiate into Treg [57] (Figure 5).

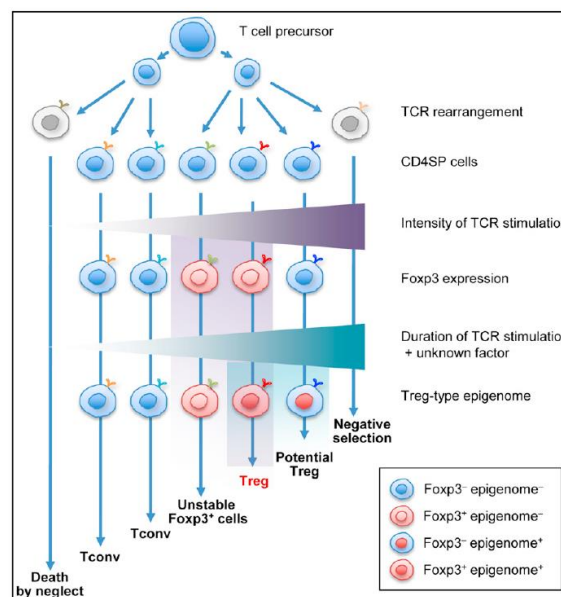


Figure 5 – A model for nTreg development in the thymus

From: Ohkura, N., Y. Kitagawa, and S. Sakaguchi. 2013. **38**(3): p. 414-23.

TCR rearrangement generates diverse TCRs that recognize with different intensities and durations self-ligands (shown as gradients). Foxp3 expression is driven by high intensity TCR stimulation, while DNA hypomethylation is induced by high duration of TCR stimulation. When the signal is high and persistent, stable Treg develop. Foxp3+ cells without the right epigenome are unstable Treg, while T cells with the right Treg-type epigenome without Foxp3 expression are potential Treg. Too strong or too weak intensity of TCR stimulation leads to apoptosis (negative selection) and death by neglect (positive selection).

It is possible to conclude that thymic Treg development is a two-step process. An initial signal driven by TCR and CD28 leads to the expression of IL-2R and chromatin remodeling. A second, TCR-independent step, occurs when Treg progenitors respond to IL-2 stimulation, driving the expression of Foxp3.

However, there are additional signals that play a role in thymic Treg development and since a hallmark of Treg progenitors is the high expression of TNFRSF members, their role was investigated. GITR expression precedes the induction of Foxp3, therefore it might support

the conversion into mature Treg. In addition to GITR, it was found the expression of OX40 and TNFR2 on Treg progenitors. Moreover, mTECs express the corresponding ligands GITRL, OX40L and TNF. It was also shown that thymic progenitors that receive the strongest TCR signaling during development have the highest GITR, OX40, TNFR2 expression. The expression of these receptors seems to be driven by TAK1, downstream of the TCR and CD28 signaling.

How the TNFRSF members promote the development of Treg is not clear. One possibility could be that they enhance sensitivity to IL-2, allowing Treg to respond to lower concentration of IL-2. An alternative mechanism could be the induction of antiapoptotic proteins. Of course it might also be a cooperation of different mechanisms [58].

2.4.2 Development of pTreg

Some of the early evidences of peripheral conversion of naïve conventional CD4⁺ cells into Treg originated from adoptive transfer experiments in which polyclonal CD25⁺CD4⁺ naïve T cells were injected into lymphopenic mice or mice containing a monoclonal T cell repertoire devoid of tTreg. In this condition, part of donor population became CD25⁺CTLA-4⁺GITR⁺ and acquired Foxp3 expression and suppressive activity [59],[60].

The conditions for the development of pTreg vary a lot, for instance they appear in mesenteric LN during induction of oral tolerance [61], they continuously differentiate in the lamina propria of the gut in response to microbiota and food antigens [62], but they are also generated in chronically inflamed tissues [63] and transplanted tissues [64]. The requirements necessary for pTreg development are the following: TCR stimulation, IL-2 and TGF- β . TGF- β induces Foxp3 transcription cooperating for the binding of NFAT and STAT5 on the Foxp3 gene [65]. *In vivo*, neutralization of TGF- β impairs [66] oral tolerance and inhibits the differentiation of antigen-specific pTreg. In contrast, the role of IL-2 in the development of pTreg is not very clear. IL-2 is required in cultures of naïve CD4⁺ T cells, stimulated with anti-CD3 and TGF- β , for the TGF- β -mediated Foxp3 transcription and suppressive activity of iTreg [67]. IL-2 signaling activates STAT5 which binds to the Foxp3 gene and may cooperate for Foxp3 induction [29].

As for tTreg development, there also other signals that impact on pTreg generation. Some researchers have showed that TNFRSF members might actually inhibit the differentiation

of pTreg. Zhang et al., starting from observations that TNF inhibits iTreg differentiation *in vitro*, decided to investigate its impact *in vivo*. They saw, in a model of EAE, that neutralization of TNF ameliorates the disease increasing the levels of Treg but not their function. And they showed that the increased percentage of Treg is due to the increased number of iTreg while nTreg were not affected. Moreover, depletion of iTreg abolished the effect of the anti-TNF treatment. The proposed mechanism involves the binding of TNF to the TNFR2 which inhibits *Foxp3* transcription through inhibition of TGF- β -induced Smad3 phosphorylation, decreasing its binding to the *Foxp3* promoter during iTreg generation. The inhibition of Smad3 phosphorylation is mediated by Akt, directly activated by the TNF signaling [68].

Given the negative role of TNFR2 in iTreg and pTreg generation, other receptors of the family have been investigated. It was recently showed that OX40 inhibits iTreg differentiation *in vitro* and this mechanism was studied. Gene array analysis of activated CD4⁺ T cells with and without OX40 costimulation revealed the upregulation of *Batf3* and *Batf* following OX40 triggering. BATF3 and BATF are members of the AP-1 family transcription factors and in the *Foxp3* locus there are five putative AP-1 binding sites and indeed one way of inhibition of iTreg generation through OX40 involves repression of *Foxp3* expression by BATF3 and BATF. However, this is not the only way for OX40 to inhibit iTreg because in the absence of BATF3 and BATF it activates AKT-mTOR pathway that suppresses *Foxp3* expression[69].

(See fig.1 in “Additional Results” for further details of the role of TNFRs in iTregs).

2.5 Treg stability and plasticity

The molecular and cellular bases of Treg stability remain a key issue of Treg research. To use Treg in clinical for immunosuppression, it is important to know exactly how they develop in terms of *Foxp3* expression and epigenetic changes. What does Treg stability mean, first of all? It could be defined by the maintenance of all the following characteristics: *Foxp3* expression, suppressive activity and lack of effector activity. Stable *Foxp3* expression is due mainly to specific epigenetic modifications. As already mentioned, the CNS2 at the 5'UTR of *Foxp3* must be hypomethylated in Treg allowing transcription [70]; and this pattern is maintained mainly by IL-2.

Many studies, in the beginning, have suggested that Treg development leads to a terminally differentiated population [71], suggesting Treg are a highly stable lineage. However, others have suggested that Treg can lose *Foxp3* expression and develop a pro-inflammatory, memory-like phenotype in certain disease states [72].

Indeed tTreg have been shown to convert to different helper T cells such as Th1, Th17 or Tfh cells [73]. Under lymphopenic conditions, many Treg were found to lose *Foxp3* and start to produce IL-2 and IFN- γ [74].

The topic is still controversial in part because of different strains of mouse used, then because of different methods used to sort Treg and lastly, it is also possible that T cells transiently upregulate *Foxp3* during development leading to a “false labeling” of cells [75].

2.5.1 Treg stability

Most reports have suggested that these unstable *Foxp3*⁺ cells constitute a minor fraction under normal conditions. Thus, there must be mechanisms that prevent the pathogenic conversion of Treg.

Once *Foxp3* is up-regulated, its expression must be kept to assure Treg function and stability. One important molecule is STAT5 (which is downstream of the IL-2 signaling), which binds the *Foxp3* promoter, stabilizing the locus [29].

But also *Foxo1* and *Foxo3a* are involved because they prevent Treg from taking effector functions [76]; indeed in the absence of *Foxo1*, Treg development is diminished and they are unable to suppress *in vitro* leading to autoimmunity *in vivo* [77]. Stable Treg have reduced Akt expression, leading to enhanced *Foxo1* and *Foxo3* in the nucleus [78] (Figure 6).

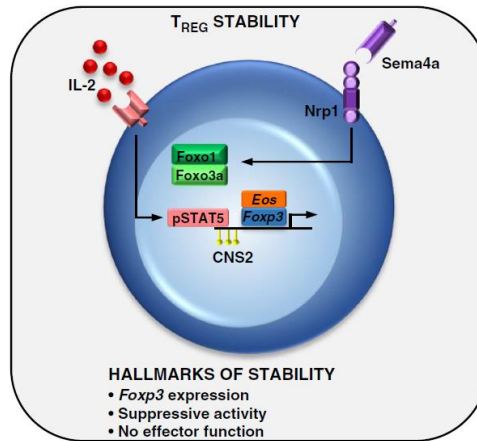


Figure 6 – Maintenance of Treg stability *in vivo*

From: Overacre, A.E. and D.A. Vignali. *Curr Opin Immunol*, 2016. **39**: p. 39-43.

IL-2 signaling leads to phosphorylation of STAT5 that contributes to Foxp3 expression. Moreover, Nrp1 increases the suppressive activity of Treg. All these are hallmark of stability of Treg.

Eos, a zinc-finger transcription factor, was identified as a key factor for Treg stability [79]. Overexpression of *Eos* avoids Treg reprogramming even in destabilizing environments.

Another important pathway is mediated by Neuropilin-1 (Nrp1):Semaphorin-4a (Sem4) axis. Most of Treg express high levels of Nrp1 and it increases function and stability of Treg [80]. Indeed, Nrp1-deficient Treg lose stability and suppressive capacity in tumor microenvironment and blocking antibodies to Nrp1 or Sem4 were found to limit tumor growth. This pathway seems to activate Phosphatase and Tensin Homolog (PTEN), which in turn limits Protein Kinase B (PKB/Akt) activity, leading to increase nuclear translocation of *Foxo1* and *Foxo2*, stabilizing *Foxp3* expression.

As already said, the hypomethylation of CNS2 is also critical, not for Foxp3 induction but maintenance. Indeed *in vitro* generated Treg with TGF- β and IL-2 does not present this pattern and are indeed more unstable; while pTreg generated *in vivo* present the Treg specific demethylated region (TSDR) [70].

In addition to the transcriptional and epigenetic mechanisms controlling Treg stability, there are also MicroRNAs (miRNA). In model of Dicer-deficiency, Treg are unstable and ineffective suppressor, producing effector cytokines and losing Treg-associated proteins [81]. MiR-155 is important for maintaining Treg fitness downregulating the STAT signaling inhibitor SOCS1, augmenting responsiveness to IL-2 [82].

Foxp3⁺ Treg have always been considered a stable lineage committed to suppression but the formal proof was missing. Therefore, Komatsu et al. [74], using Foxp3-reporter mice, transferred Foxp3⁺ T cells in T cell-deficient mice and saw that after 4 weeks, around 50% of the donor cells lost Foxp3. These cells lost the whole Treg signature (CD25, GITR and CTLA-4), produced abundant effector cytokines and failed to suppress *in vitro*. Interestingly, they saw more ex-Foxp3 cells in the Peyer's patches than in other lymph nodes and spleen; and many showed a follicular helper T (Tfh) phenotype.

Because the cytokine environment is critical for Th differentiation, Foxp3⁺ T cells were activated with anti-CD3/CD28 mAb plus different cytokines to see how they influence ex-Foxp3 generation. IL-6 resulted in a Th17-like phenotype, IL-4 in a Th2-like phenotype and neutralization of TGF- β led to ex-Foxp3 generation. This conversion of Treg was then seen also in non-lymphopenic mice [83].

2.5.2 Class control

In certain circumstances, Foxp3⁺ T cells acquire Th cell-like functions without losing Foxp3 expression, showing a "hybrid" phenotype. For example in humans [84] and mice [85] have been identified Foxp3⁺ROR γ t⁺IL-17⁺ cells; and some Foxp3⁺ T cells acquire T-bet and Interferon- γ (IFN- γ) expression when stimulated under Th1 polarizing conditions *in vitro* [86].

It was shown that Treg can express T-bet which drives the expression of CXCR3, two key markers of Th1 cells. In this state Treg can migrate to the sites of inflammation and control Th1 cells [87]. It was also confirmed, in the Experimental Autoimmune Encephalomyelitis (EAE) model, that the expression of CXCR3 permits the Treg to migrate to the CNS and suppress Tconv cells [88].

Similarly, they can express Irf4, a key transcription factor expressed by Th2 cells. Its expression is important to produce IL-10, granzyme B and CCR8 to permit them to migrate to the site of inflammation mediated by Th2 cells and control them [89].

Treg can also express STAT3, involved in Th17 differentiation. STAT3-deletion in Treg causes less expression of IL-10, IL-35 and CCR6 which are required to migrate to inflammation sites created by Th17 cells. Moreover, these Treg are unable to control the development of colitis which is mainly due to Th17 cells [90].

2.6 Role of tTreg and pTreg

The role of Treg at the steady state was shown with the depletion of Foxp3⁺ T cells, which led to the development of autoimmunity [91], [92]. In addition, the depletion of Treg in new born mice causes the development of an autoimmune disease similar to the scurfy mice. Therefore, in normal condition, Treg regulate the activation of the immune system, controlling potentially auto-reactive Tconv. For this reason, any kind of dysfunction of Treg may lead to pathogenic conditions in which we have over- or under- activation of the immune system causing several different diseases.

Indeed, although Treg have a pivotal role in preventing autoimmunity, such as type I diabetes, and limiting chronic inflammatory diseases, such as inflammatory bowel disease, they also block beneficial responses in cancer and infections.

2.6.1 Autoimmunity

Treg are important to control the activation of the immune system and their quantitative and qualitative defects have been observed in several autoimmune diseases.

In type I diabetes for example, it was reported the failure of suppression of autoreactive T cells. In Non Obese Diabetic (NOD) mice, which is a spontaneous model of type I diabetes, it was reported a reduced function of Treg and a reduced susceptibility of conventional T cells to Treg-mediated suppression [93], [94].

Their importance was seen also in multiple sclerosis patients who have a functional decline of Treg activity. The frequency and number in the periphery are normal but they are no functional *in vitro* [95], [96]. This reduced activity is correlated with reduced Foxp3 protein levels. And consistent with this, recovery from symptoms of multiple sclerosis is associated with the presence of Treg in the central nervous system [97].

2.6.2 Allergies and asthma

The importance of Treg was shown with the depletion of Treg in allergen-sensitized mice, causing increased levels of Th2 cytokines, IgE and airway hypersensitivity reaction [98].

In humans, Treg from allergic donors have been found to be functionally defective [99]. Both tTreg and antigen-induced T_R1 cells have been implicated in controlling the allergen-induced Th2 response in mice and humans. And also an increase in their number was associated with the disappear of the allergy to cow milk [100].

2.6.3 Infections

The consequences of Treg suppression during infections are controversial. Studies suggest that Treg, while limiting local tissue damage, prevent sterilizing immunity against pathogens and so causing persistence of infections. In turn, this persistent infection results in protective immunity against subsequent challenge with the pathogen [101].

2.6.4 Pregnancy

In this case they are important to keep tolerance against paternal antigens, avoiding the rejection of the fetus. It was observed indeed an increased number of Treg during pregnancy [102] and their elimination in pregnant mice causes the rejection of the embryos carrying paternal antigens [103].

2.6.5 Tumors

Tumors are seen as self and thus Treg try to prevent anti-tumor specific T cell function and they are therefore a barrier for effective immunotherapy. The presence of Treg inside the tumor was already demonstrated some years ago and this was correlated with poor prognosis. And in mice it was seen that in the absence of Treg, a proper immune response against the tumor can be mounted [104]. Indeed, several anti-cancer therapies use antibodies to target Treg (anti-CCR4, anti-GITR and anti-CTLA4).

2.6.6 *tTreg* and *pTreg*

Firstly, there was a consensus for the role of tTreg in preventing autoimmunity, but now data support also the importance of pTreg in mucosal tolerance and in responses against foreign antigens, neo-antigens in tumors and transplants. However, it is difficult to study the contribution of each population because of the lack of suitable surface markers or reporter mice that enable the distinction of the two populations.

Haribhai and colleagues examined the roles of tTreg and pTreg by adoptive transfer immunotherapy of newborn Foxp3-deficient mice. They firstly tried a monotherapy with tTreg alone, but both failed to maintain tolerance. On the contrary, the injection with a combination of tTreg and Tconv, capable to differentiate in pTreg, rescued the phenotype of the mice. Then they compared the gene expression profile of pTreg and tTreg, but they were similar to each other and to the published tTreg genetic signatures. However, other factors, such as differences in antigenic specificity, might underlie the requirement for

pTreg; for this reason they analyzed the TCR repertoires of tTreg and pTreg isolated from the mice, which was very different between the two populations [105]. In addition, it was shown that the generation of pTreg in the intestine depends on the presence of gut microbiota suggesting that they are likely biased to recognize preferentially antigens expressed by the gut microbiota.

2.7 Impact of inflammatory cytokines on Treg

A lot of efforts have been put to characterize Treg, meaning their signature, phenotype and functions. However, it was seen that it might be difficult to define a unique subset of Treg. They are different in all the aforementioned aspects based on their localization, activation state and exposure to environmental factors. Of course inflammation is a crucial factor that impact on Treg differentiation and homeostasis. Some cytokines have been further analyzed in relation to their impact on Treg biology.

IL-7 is a cytokine produced by lymph node stromal cells important for naïve T cell survival and activation during adaptive immune responses. The binding to its receptor causes STAT5 phosphorylation and expression of antiapoptotic proteins important for T cell development and proliferation. The role of this cytokine on Treg is less clear. It was shown that injections of IL-7 complexes optimize their reactivity to IL-2. Mice deficient for IL-7R have reduced Treg and they exhibit impaired proliferation. In a model of skin allograft tolerance it was seen that IL-7 augments CD25 expression, increasing Treg sensibility to IL-2, depriving Tconv from IL-2 and therefore inhibiting strong allogenic T cell response. Indeed, IL-7 stabilizes Treg suppressive phenotype increasing the expression of CD25, GITR and ICOS. Normally, cTreg express highly CD25 relying deeply on IL-2, while eTreg express less CD25 but higher amount of IL-7R. Therefore, in the presence of inflammation and therefore of high amounts of IL-7, they rapidly upregulate the CD25 consuming more IL-2. This shift in IL-2 consumption could then impact on the cTreg/eTreg balance during inflammation[106].

IL-33 is part of the IL-1 family and its receptor exists in two forms: membrane-bound and soluble. It is usually considered an epithelial cytokine that promotes type 2 immune responses, however recent studies showed its role in basal tissue regulation, organ injury and repair and immunity to microbes.

Certain Treg express IL-33R at high level and they are highly suppressive and express activation markers such as IL-10, ICOS, GITR and KLRG1. IL-33 increases the proliferation of Treg that express its receptor and stabilizes Foxp3 expression [107]. In a model of collagen-induced arthritis it was shown that injections of IL-33 in the initial or developing phase, dramatically inhibit the disease. This is mediated by a type 2 immune response which includes expansion of eosinophils and Th2 cells. In addition, it was seen that IL-33 treatment promotes Treg expansion and increases the expression of IL-33R and CD39, which is related to their suppressive activity [108].

The increase of Treg by IL-33 seems to be mediated by the group 2 of innate lymphoid cells, which then in turn co-stimulate Treg mainly through ICOS-ICOSL [109]. In addition, it was shown that IL-33 stimulates IL-2 production by CD11c⁺ DC, that in turn drives Treg expansion [110].

Moreover, transcriptional profiling of Treg showed that IL-33 signaling is essential for Treg development and maintenance in visceral adipose tissue. BATF and IRF4 are involved in Treg differentiation in that tissue through direct regulation of IL-33R and PPAR- γ which regulates cellular metabolism in adipose tissue and is required for Treg development [111].

Type I IFN are a family of cytokines with both pro- and anti-inflammatory effects, because they can inhibit T cell egress from lymph nodes promoting DC/T cell interaction but they can also induce anti-inflammatory cytokines, including IL-10 and IL-27. They have an important role in controlling Treg homeostasis in the intestine. In a model of colitis it was demonstrated that IFN signaling is important to control T cell accumulation and Treg function and stability [112]. The signal mediated by IFNs may directly or indirectly affect STAT5 phosphorylation since KO mice for IFNR have Treg expressing lower CD25 and phosphorylated STAT5 [113].

IL-27 is another important cytokine that impact on Treg homeostasis. It induces IL-10 production by Tconv and is important to attenuate Th17-mediated inflammation. It interferes with TGF- β during iTreg generation blocking the conversion of T cells into Treg. It also promotes Tbet and CXCR3 expression in Treg. In a model of colitis, it was shown a protective role of this cytokine inhibiting T cell expansion and differentiation into Th1/Th17 colitogenic cells. But also improving Treg expansion *in vitro* and *in vivo*. IL-27

induces Lag3 expression in Treg which is important to control early T-cell activation and intestinal inflammation [114].

IL-27 is also essential to control EAE, indeed Treg-specific IL-27ra^{-/-} mice develop more severe EAE. However, it is not the number of Treg to be affected by IL-27 signaling suggesting an impact on the function of Treg. Furthermore, systemic administration of recombinant IL-27 in Treg-specific IL-27ra^{-/-} mice fails to ameliorate EAE even in the presence of IL-27-responsive Tconv [115].

Also some inflammatory cytokines, such as IL1 or IL6, are able to impact on the stability of Treg [116]. The role of the members of the TNFSF on Treg will be discussed later.

2.8 Mechanisms of suppression

Defining how Treg suppress is of crucial importance not only to provide insight into the peripheral tolerance but also to identify new therapeutic targets. It can be identified 4 main “mode of action”: suppression by inhibitory cytokines, cytolysis, metabolic disruption and modulation of DC maturation and function (Figure 7).

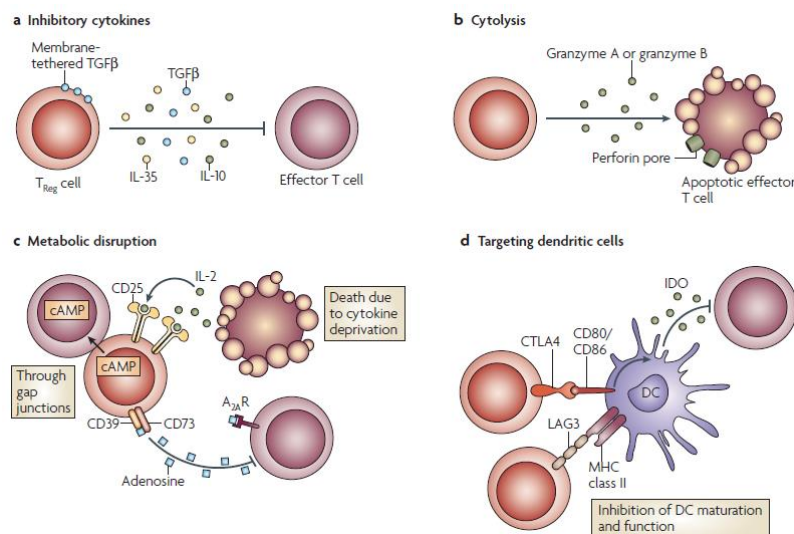


Figure 7 – Basic mechanisms used by Treg cells

From: Vignali, D.A., L.W. Collison, and C.J. Nat Rev Immunol, 2008. 8(7): p. 523-32.

Description of Treg four basic modes of action. A) Production of inhibitory cytokines. B) Cytolysis of effector cells. C) Metabolic disruption through CD25-mediated IL-2 deprivation, cAMP-mediated inhibition and CD39/CD73-mediated immunosuppression. D) Inhibition of DC maturation and function through LAG3, CTLA4 and IDO.

2.8.1 Suppression by inhibitory cytokines

The two main cytokines involved are IL-10 and TGF- β which are not only part of suppression mediated by Treg but also stimulate the development of pTreg, which renders them attractive therapeutic targets.

In allergy and asthma models, evidences suggest both tTreg and pTreg control these diseases which depend, in part, on IL-10 [117]. After antigen challenge, adoptively transferred Treg stimulate Tconv to produce high amount of IL-10 and therefore to control the disease. However, after transfer of IL-10-deficient Treg, the disease was still controlled by IL-10 which means that the production of this cytokine by Treg is not necessary for the suppression observed [118]. In contrast, IL-10 production by Treg seems essential for the prevention of colitis in mouse model of IBD [119]. Moreover, the tumor microenvironment promotes the generation of Treg that mediate IL-10-dependent, cell-contact independent suppression [120]. In addition, papers suggest a role for Treg-derived IL-10 in the induction of fetomaternal tolerance and B-cell enhanced recovery from EAE [121].

The importance of TGF- β for tTreg has been also a controversial topic. There are studies suggesting that TGF- β produced by Treg may directly participate in the suppression of Tconv. For instance, Tconv resistant to TGF- β -mediated suppression cannot be controlled by Treg [122]. In addition, TGF- β produced by Treg has been found important in the control of immune responses against *M. tuberculosis* [123], suppression of allergic responses [124] and prevention of colitis in an IBD model [125].

A new inhibitory cytokine was then added in mediated Treg suppression, IL-35. Indeed IL-35 is sufficient for Treg activity as ectopic expression of IL-35 confers regulatory activity on naïve T cells and recombinant IL-35 suppresses Tconv proliferation *in vitro* [126].

2.8.2 Suppression by cytotoxicity

Cytotoxicity has long been considered a characteristic of natural killer and CD8⁺ cells. However, it was shown that activated tTreg express granzyme A and that Treg-mediated killing was mediated by granzyme A and perforin through adhesion of CD137 [127]. Moreover, granzyme B-deficient mouse Treg have reduced suppressive activity *in vitro*, and this granzyme B-dependent suppression appears to be perforin-independent [128].

Galectin-1 also, which can induce T cell apoptosis, has been shown to be up-regulated by mouse and human Treg and galectin 1-deficient Treg have reduced regulatory activity in vitro [129].

2.8.3 Suppression by metabolic disruption

Other mechanisms used by Treg to suppress Tconv involve the deprivation of important molecules for Tconv survival and function, as IL-2 and ATP, and the production of a compound that blocks Tconv activity as cAMP.

One feature of Treg is that they are not able to produce IL-2 but they rely on it for their survival, proliferation and function. Indeed, as already said, Treg express at high level CD25, which is part of the IL-2 receptor. For this reason they depend on exogenous IL-2 for their homeostasis, which is produced mainly by Tconv [60]. Since Treg express high levels of IL-2R they are able to consume it more than Tconv, depriving them of IL-2 [130]. In this way they block the proliferation of Tconv and cause their apoptosis.

Another mechanism involves ATP consumption. Indeed, ATP is normally released from damaged or activated cells and it enhances immune responses. Treg can produce enzymes that reduce local concentration of extracellular ATP. CD39, nucleoside triphosphate diphosphohydrolase-1, is an enzyme that degrades ATP to AMP. CD73 is an ecto-5'-nucleotidase, which degrades AMP to adenosine. Both CD39 and CD73 are preferentially expressed by mouse and human Treg [131],[132]. Therefore Treg cause the production of adenosine which then binds to its receptor, adenosine receptor 2A, inhibiting Tconv functions and enhancing the generation of pTreg promoting TGF- β secretion [133].

Finally, it was shown that Treg express at low level the enzyme that degrades the cAMP, c-AMP cleaving enzyme phosphodiesterase 3B, causing its accumulation. Then they can transfer the cAMP, a potent inhibitor of proliferation, inside Tconv through membrane gap junctions [134].

2.8.4 Suppression by targeting dendritic cells

Treg can also modulate the maturation and/or function of DCs, which are required for the activation of effector T cells. Studies using intravital microscopy have revealed direct

interactions between Treg and DC *in vivo*. These interactions reduce Tconv activation by DCs, which is mediated mainly by CTLA-4. This molecule is constitutively expressed on Treg and competes with CD28 for the binding of CD80/CD86 in the immunological synapse. Indeed the use of anti-CTLA-4 blocking antibodies or CTLA-4-deficient Treg, reduced Treg mediated suppression showing the importance of CTLA-4 [135]. It was also shown that Treg could induce DC to produce indoleamine 2,3-dioxygenase (IDO), a potent molecule which induces the production of pro-apoptotic metabolites from the catabolism of tryptophan, resulting in the suppression of Tconv through a mechanism involving CTLA-4 and CD80/CD86 [136].

In addition, Treg may downregulate the expression of the co-stimulatory molecules CD80 and CD86 by DC *in vitro*, or even capture CD80/CD86 from APC by a process of trans-endocytosis [137], reducing their ability to activate Tconv [138].

Another molecule, LAG3, can block DC maturation. It is a homologue that binds MHC class II with high affinity and is required for Treg suppression. Binding of LAG3 to MHC II on immature DCs induces an ITAM-mediated inhibitory signaling, which involves ERK and SHP1, suppressing DCs maturation and their immunostimulatory functions [139].

It has also been shown that neuropilin-1, expressed on Treg, promotes prolonged interactions between Treg and DCs, giving an advantage over naïve T cells in modulating DCs functions [140].

These are the main observed mechanisms, but this list is not exhaustive and others will be discovered. And also, there is still the question if Treg require all of them to suppress. The first possibility is that a single mechanism is used by all Treg. But this is unlikely since none of the mechanisms result in absence of regulatory activity when blocked or deleted. The second possibility is that multiple, non-redundant mechanisms are required for maximal Treg function, with each molecule contributing to the whole suppression. It is likely that Treg use different mechanisms of suppression depending on the tissue and level and type of inflammation. The third possibility is that multiple, redundant mechanisms are required for maximal Treg function. At present, the second is the most plausible. In addition, there is the question if there is a “hierarchical” order of them, meaning Treg tend to use one or two that are the most important. Or if it is the context

and background in which Treg reside that influence the type of mechanism to choose [141].

3. Role of Tumor Necrosis Factor Receptor Super Family (TNFRSF) in T cells

Tumor necrosis factor (TNF) is a major participant in the initiation and orchestration of events in inflammation and immunity. However, it is only one of the members of the TNF super family (TNFSF), which consists of proteins that organise lymphoid tissue development, co-stimulate lymphocyte activation and can either increase lymphocyte survival or induce cell death.

Moreover, TNF was found to be involved in autoimmune diseases such as rheumatoid arthritis (RA) and psoriasis [90], indeed anti-TNF antibodies are beneficial for most of these patients. Conversely, a worsening of the disease was seen with those drugs in case of multiple sclerosis suggesting a more complex role of TNF in the immune system [142]. Therefore, it would be important to improve the knowledge of the role of these receptors on T cells in order to improve treatments for autoimmunity, cancer and inflammation.

3.1 The discovery of the TNFSF

In 1890, Coley treated a woman with a pain in her right hand, but despite the operation she died a few months later of an aggressive round cell sarcoma. Coley, shocked of the event, became interested in the case of a man with a sarcoma that disappeared after a post-operative bacterial infection. For this reason, he started to infect cancer patients with bacterial isolates and then created the “Coley’s mixed toxin” with extracts from *Streptococcus pyogenes* and *Serratia marcescens*.

Later, scientists interested in his results tried to reproduce in animal model his experiments. In 1931, Gratia and Linz showed that bacterial extracts caused tumor necrosis in a guinea pig model of sarcoma. In 1944 Shear *et al.* isolated LPS from bacterial extracts and showed that it was responsible for tumor regression. O’Malley *et al.* took serum from endotoxin-treated animal and transferred it into animal models of cancer and showed that it caused tumor necrosis [143]. A major finding was in 1975 when Carswell *et al.* reported that it was a factor made by the host cells in response to endotoxin and not a bacterial endotoxin that destroys tumors [144]. Because of its activity, it was called “tumor necrosis factor”.

The first indication that there might be a family of related proteins came when TNF and lymphotoxin were found to bind the same cell receptor [145]. The availability of the protein sequence led to gene cloning of the 2 proteins and in the following years all the other members of the family were discovered and cloned.

3.2 Structure

The TNFSF is composed of 19 ligands and 29 receptors and plays diversified roles in the body. The majority of them exhibit pro-inflammatory activity, mainly through NF- κ B activation; but others show proliferative function on hematopoietic cells, apoptotic or survival activity or induction of differentiation and morphogenetic changes.

The existence of more receptors than ligands implies that some of the ligands can interact with more than one receptor. For instance, TRAIL binds to DR4, DR5, DcR1 and DcR2. Furthermore, the receptors are characterized by an extracellular cysteine-rich domain to which the ligands bind.

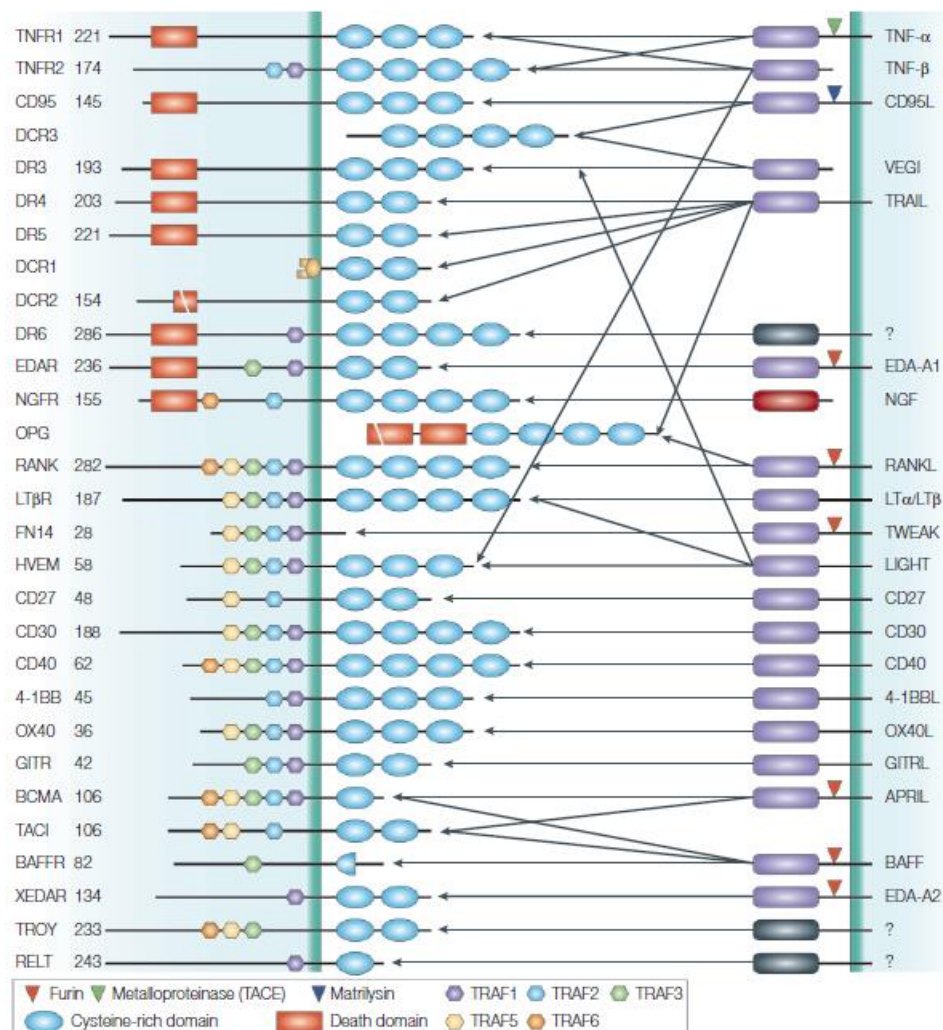


Figure 8 – The TNFSF

From: Aggarwal, B.B. Nat Rev Immunol, 2003 3(9): p. 745-56

The figure shows the members of the TNFSF. On the right the ligands can bind to different receptors, on the left, through the cysteine-rich domain. Not all the receptors have an intracellular domain and some have an intracellular death domain (DD).

Most of the receptors can be divided into 2 categories: those that possess an intracellular death domain (DD) (as CD95, DR6, TNFR1, TRAILR1, etc.) and those that do not (as TNFR2, GITR, OX40, 4-1BB, CD30, CD27, CD40, HVEM, RANK, etc.).

TNF can bind to 2 different receptors, TNFR1, which has a DD, is expressed on most cell types while TNFR2, which lacks the DD, is expressed mainly on cells of the immune system, endothelial and some nerve cells.

There also is a third category composed of receptors lacking cytosolic domain or that possess a non-functional cytosolic domain, called decoy receptors (as TRAILR3, TRAILR4, DcR3 and OPG)

The TNFRSF receptors function as preformed trimeric oligomer complexes either before ligand binding or oligomerized after ligand binding. They do not have intrinsic enzymatic activity and bind to adaptors ubiquitin ligases and kinases to induce a signal [143].

Almost all ligands of the TNFSF are expressed by cells of the immune system. Some ligands, such as FasL, CD27L, CD30L, CD40L, OX40L and 4-1BBL, are primarily expressed as transmembrane proteins on the cell surface and interact with cells expressing the corresponding receptor. Others, like TNF- α , are expressed as both transmembrane and soluble forms, however, the TNFR2 binds primarily the membrane-bound form, whereas TNFR1 bind to both soluble and membrane-bound TNF. Finally some, as TNF- β , also known as LT α 3, are expressed only as soluble ligands because they lack the transmembrane domain [143].

3.3 Signaling

The majority of the published data, have been collected in cell lines. Only some data are available regarding the signaling in primary Tconv and very few in Treg.

Signaling through TNF has been a paradigm for most of other members of the family and it activates all the major pathways involved in immune cells: as NF- κ B, apoptosis

pathways and the three MAPK pathways: extracellular signal-regulated kinase (ERK), p38 mitogen-activated protein kinase (p38MAPK) and c-Jun N-terminal kinase (JNK).

Almost all the members signal by binding one or more TNFR-associated factors (TRAFs)[146]. TRAF1, TRAF2 and TRAF5 interact with the same intracellular domain of TNFRSF, which may differ between different TNFRSF members they bind, while TRAF6 uses a different motif. Furthermore, TRAF3 may not bind directly to TNFRSF members but indirectly via others TRAFs.

TRAFs can already exist as trimers in the cytosol before binding the tails of TNFRSF members, suggesting that oligomerization of the receptors will additionally result in oligomerized multiple TRAF molecules [147]. Interestingly, a TRAF2/1 trimer binds one cIAP1 molecule, which is required for downstream signaling. Thus, two TRAF2/1 trimers are required for cIAP1 transactivation.

Collectively, TRAFs function as integrative platforms regulating diverse signaling pathways triggered by TNFRSF members that exert pleiotropic effects on T cells.

In general, TRAFs lead to NF- κ B activation and survival promotion in TNFRs with and without DD.

However, it was shown that TRAF2 activation, downstream GITR triggering, inhibits the activation of the alternative NF- κ B pathway and therefore it might differentially regulate signaling pathways. Upon GITR triggering, there is the activation of TRAF2, which inhibits NF- κ B activation, and TRAF4 which augments NF- κ B. Therefore, the effects of these TRAFs counteract each other. TRAF5 KO T cells showed that this molecule is critical to activate NF- κ B, ERK and p38 upon GITR triggering [148].

Moreover, TRAF1KO in T cells, lead to enhanced NF- κ B activation, suggesting that the TRAFs interact and regulate each other and this influence the consequent NF- κ B activation [149].

TNFR1, can signal in 2 different ways, leading to survival or apoptosis. It recruits TNF receptor-associated death domain (TRADD) adapter proteins, which then in turn recruit TRAF2. The following events lead to NF- κ B and JNK/SAPK activation. Specifically, there is a complex of proteins composed of TRAF2 or TRAF5, c-IAP1/2, and ubiquitin-conjugated

protein Ubc13 bound to TRADD and RIP. C-IAPs and TRAFs contribute to the ubiquitination of RIP1. This leads to activation of the catalytic I κ B kinase (IKK) complex, composed of three proteins, IKK α , IKK β and IKK γ , driving phosphorylation of inhibitor of kappaB-alpha (I κ B α), the NF- κ B inhibitory protein. Poly-ubiquitinated RIP1 can alternatively activate IKK complex via interaction of the TAK1 complex, consisting of the TAK1 kinase and its associated proteins TAK1-binding protein 1/2/3 (TAB1,2,3). Translocation of NF- κ B subunits leads to transcription of anti-apoptotic factors as cIAPs and TRAFs, thus promoting survival.

However, TRADD and RIP can also associate with Fas-associated death domain (FADD) protein and caspase 8 forming a cytoplasmatic complex, resulting in apoptosis.

As mentioned before, NF- κ B is one of the most important pathways involved in signal transduction in most, if not all, TNFRSF receptors. In cell lines, TNFR2 activates both the canonical and non-canonical NF- κ B pathways (Figure 9). TRAF1/2, downstream of TNFR2, activates PI3K/Akt which then activates the canonical NF- κ B pathway, leading to the translocation of dimers of p50/RelA and p50/cRel.

Moreover, normally TRAF2 and c-IAP1/2 interact with TRAF3 and NF- κ B-inducing kinase (NIK) resulting in c-IAP1/2-mediated ubiquitination of NIK and subsequent proteosomal degradation of it. NIK activates IKK α , which phosphorylates NF- κ B precursor, and usually it is constitutively degraded, preventing NF- κ B translocation. However, it was shown, in both cell lines and primary immune cells, that TNFR2 prevents NIK degradation, leading to the activation of the non-canonical NF- κ B pathway with nuclear translocation of dimers of p52/RelB [149].

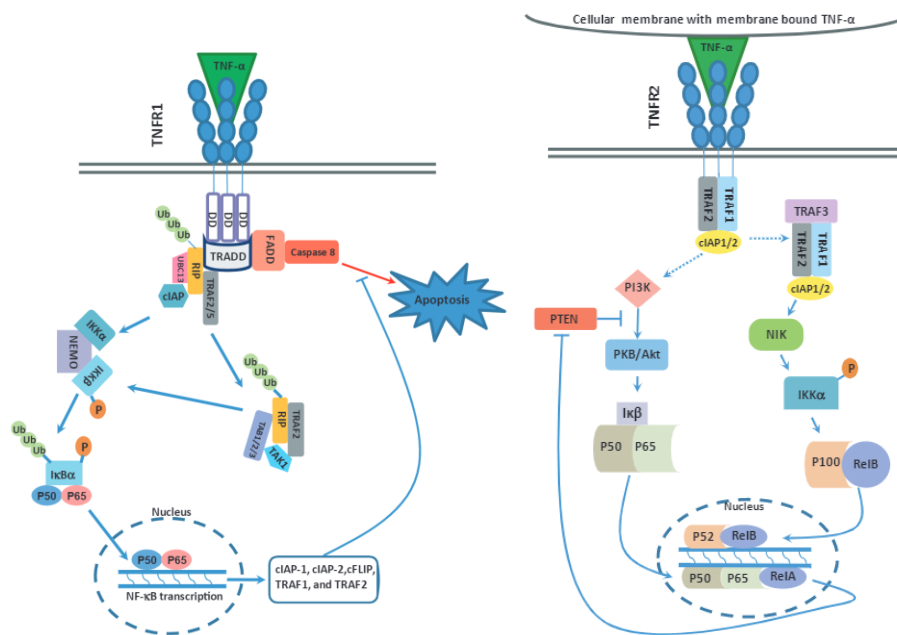


Figure 9 – TNFR2-mediated NF-kB activation

From: Naude, P.J., et al., *Tumor necrosis factor receptor cross-talk*. FEBS J, 2011

On the left it is shown the pathway triggered by TNFR1, which is activated by soluble TNF. It can activate both NF-kB driving survival signals or apoptosis through caspases. On the right, the signaling driven by membrane-bound TNF which binds to TNFR2 and, through canonical and non-canonical NF-kB, drives survival.

Even if there is a belief that TNFR1 induces apoptosis and TNFR2 survival, the reality is more complex because of cross-talks between the two receptors. TRAF2 is critical in TNFR1-mediated survival and this pathway can be inhibited by TNFR2-mediated TRAF2 degradation by the proteasome. Then, TRAF2 degradation prevents cIAP1/2 to be recruited to TNFR1 which may promote cell death, as cIAP1/2 interfere with caspase 8 activation and apoptosis. Also TRAF1 prevents this process by reducing TNFR2-induced TRAF2 depletion and therefore increasing TNFR1-mediated NF-kB and JNK activation [149].

Translocation into lipid-raft may be an important feature of some of these receptors, as shown for OX40 [150] and 4-1BB in T cells [151]. Lipid-rafts may play a critical role in triggering PI3K-Akt by facilitating the localization of oligomerized TNFR members and TRAFs with PI3K, PIP2 and PDK1.

3.4 General features on the roles of TNFRSF members

3.4.1 TNFRSF members and Tconv

Signals through TNFRs have been shown to promote clonal expansion and accumulation of high numbers of antigen-specific effector T cells in mouse models. Often this is also

accompanied by an effect on the frequency of memory T cells that develop. Years ago it was proposed that TNFRs act in a temporal manner on T cells, one after another, allowing the response to be sustained in the short-term and long-term [152]. For example, CD40L can be induced rapidly on T cells after antigen recognition and ligate CD40 on APCs. CD40 signals in turn can induce molecules like OX40L and CD70 that would then ligate their receptors on T cells.

Certain molecules like CD27, DR3, TNFR2, HVEM and GITR are constitutively expressed on some CD4 and CD8 T cells. Among these, some are further up-regulated (TNFR2 and GITR) and others down-regulated (DR3 and HVEM) upon activation. Other molecules, such as OX40, 4-1BB and CD30, are expressed only after antigen encounter [143].

We are lacking in our knowledge of the expression profile of most of the TNFR molecules *in vivo*. Data from animals are useful, but data from human samples during the course of immune diseases may be essential. Most TNFSF molecules are not found constitutively and they have been hard to analyze because they are often weakly and transiently expressed either due to rapid cycling and endocytosis, cleavage from cell membrane or simply tight regulation.

Apart from T cell expansion and accumulation, TNFSF members might be important also for the development of subpopulations of effector and memory T cells. Some literature suggests 4-1BB and CD27 are more important for the accumulation of CD8 T cells compared to CD4 T cells and that OX40 is more important for CD4 T cells [152].

Another important step is the development of memory T cells cells, in which we can distinguish effector memory T cells, which recirculate through extra-lymphoid tissues and exert rapid functions, and central memory T cells, which home to secondary lymphoid tissues.

It is still not clear if TNFRs contribute to lineage or subset decisions during T cell responses and if all of them are involved or not. For instance, there is a study in which mice lacking OX40 showed a selective impairment in the generation of CD4 effector memory T cells but no effect on central memory cells [153]. Moreover, the first patient with an OX40 deficiency showed reduced CD4 effector memory T cells and poor CD4 T cell recall

reactivity to several antigens [154]. Therefore, there can be indeed a selective or differential activity in the TNFR family in contributing T cell priming

TNFRs are important not only during the earlier phases of T cell responses but they may influence also T cell responses at later time after the peak of the effector responses. Studies of 4-1BB and OX40 showed they can exert survival activity in CD4 and CD8 T cells independently of antigen recognition and TCR signaling [155].

3.4.2 TNFRSF members and Treg

It is important to notice that some TNFRSF receptors are part of the core signature that identify Treg. Transcriptomic analysis comparing Treg and Tconv of lymphoid tissues showed that TNFR2, OX40 and GITR are part of the Treg signature and they are also regulated at the epigenetic level, since their expression correlated with epigenetic regulation in Treg. By Chip-Seq to detect H3K27Ac up-regulated in eTreg (compared to Tconv) only, 10 are conserved in human and mice. Among the 10, there is Foxp3, CD25, Helios, GARP, 4-1BB, TNFR2 and CCR8. [156]

In addition, TNFRSF members seem to have a role also in differentiation of Treg, which was already discussed in the previous chapter. While they are important for tTreg development, they block iTreg and pTreg differentiation.

3.4.3 TNFRSF members and disease

It is therefore clear that TNFSF members influence biology of immune and non-immune cells, and, for this reason, they are involved in several pathologies.

TNF was discovered because of its ability to cause tumor necrosis. However, as mentioned before, TNF through TNFR1 can lead to 2 different outcomes: survival or apoptosis. Indeed, it was also reported that TNF has carcinogenic activity due to its ability to activate the pro-inflammatory transcription factor NF- κ B, which upregulates the expression of genes linked to tumor survival, proliferation, invasion and metastasis [157]. In contrast, other members of the family exhibit anticancer potential. For example, TRAIL seems to be able to induce apoptosis in tumorigenic or transformed cells but not in normal cells or tissues [158].

Besides their role in tumor cells, TNFRs are also expressed by T cells involved in antitumor immunity. For instance, OX40 and 4-1BB are currently tested in clinical trials for cancer immunotherapy because of their boost of Tconv.

TNF- α and its receptors are also expressed by microglial cells in the brain. And there, it seems to induce pro-inflammatory activity through NF- κ B activation [159, 160]. In contrast, as previously said, there are evidences that TNF- α might boost Treg survival and proliferation in the context of EAE protecting mice from the disease.

TNF- α and other molecules of the family play also a role in initiation and progression of cardiovascular diseases. Indeed, normal heart does not express TNF- α , but a failing heart produces massive amounts of TNF- α . The earliest indication of this connection emerged in 1990 when levels of circulating TNF- α were found elevated in sera of patients with chronic heart failure [161].

TNF- α has been shown to play a role in various pulmonary diseases, including asthma, chronic bronchitis, chronic obstructive pulmonary disease and others [162]. TNF- α is expressed in asthmatic airways and has been shown to play a role in amplifying asthmatic inflammation through the activation of NF- κ B, AP-1 and other transcription factors [163]. Interestingly, signaling through DR3 has been linked to expansion of Treg, which play a protective role against allergic lung inflammation in a mouse model of asthma [164].

Lastly, the major area where TNFSF members seem to play a major role is autoimmunity. Many autoimmune diseases, such as uveitis, multiple sclerosis, SLE, arthritis, psoriasis, are connected with dysregulation of various members of this family. And indeed many drugs acting on them are currently being tested [165].

I will now give a brief overview of some TNFRs reporting what is known in the literature about them and about their role on Treg. During my PhD thesis we have focused our study on the analysis of 5 TNFRSF members (TNFR2, GITR, 4-1BB, DR3 and OX40) because of their specific interest in Treg biology. So I will pay specific attention to these receptors in my review.

3.5 TNFR

3.5.1 Expression and main features

Upon stimulation by pathogens and inflammatory signals, TNF is produced primarily by immune and somatic cells. Initially transmembrane TNF is synthesized, and it can be subsequently released as soluble TNF upon cleavage. Both forms of TNF are biologically active in their trimeric form [166].

The effects of TNF are mediated by two structurally related, but functionally different receptors, TNFR1 and TNFR2. They can be released as soluble forms by proteolysis and have the capacity to neutralize the action of TNF.

As already said, TNFR1 owns a DD and is present on many cell types driving pro-inflammatory, cytotoxic and apoptotic effects classically attributed to TNF [167]. TNFR2 lacks a DD and is expressed mainly by lymphocytes and promotes their survival and proliferation [168].

Interestingly, the majority of thymic Treg in normal mice express TNFR2. Compared to thymic Treg, TNFR2 expression by Treg in peripheral lymphoid organs and in the circulation is reduced; nevertheless, TNFR2 is still preferentially expressed by Treg [169]. It was observed that the expression of TNFR2 identifies the maximally suppressive subset of mouse Treg. Indeed, TNFR2 is expressed on 30-40% of peripheral activated/memory Treg which are the most suppressive. In contrast, TNFR2⁻ Treg have a naïve phenotype and minimal suppressive activity. In addition, the majority of tumor infiltrating Treg are highly suppressive TNFR2⁺ cells [169] and depletion of this subset results in tumor eradication. Moreover Treg deficient in TNFR2 are not able to control inflammatory responses in vivo [170].

In humans also it was seen that CD4⁺CD25⁺TNFR2⁺ T cells express high levels of CTLA-4, CD45RO, CCR4 and low levels of CD45RA and CD127, a phenotype characteristic of effector Treg. Upon TCR stimulation, these cells inhibited proliferation and cytokine production of co-cultured Teff cells. In contrast, CD4⁺CD25⁺TNFR2⁻ T cells did not show inhibitory activity [171].

Even if TNFR2 is mainly expressed by Treg, it is present also on <10% of resting Tconv and it can be quickly up-regulated by TCR stimulation. And it was shown that TNFR2 is

associated with greater suppression when expressed by Treg but it is also associated with greater resistance when expressed by Tconv [172].

3.5.2 Role in AD

TNF has well-documented pro-inflammatory effects, that is likely due to its interaction with TNFR1, but it has also anti-inflammatory and immunosuppressive functions, that is likely due to its interaction with TNFR2. For instance, NOD mice over-expressing TNF in their pancreatic islets failed to develop autoimmune diabetes [173] and repeated injections of TNF suppressed both type I diabetes in NOD mice and lupus nephritis in susceptible mouse strains [174]. However, TNF KO mice develop prolonged and exacerbated EAE, although with delayed onset, after EAE induction [175]. Therefore, in models of autoimmunity, TNF can either promote or inhibit inflammatory responses, depending on its interaction with TNFR1 or TNFR2, respectively.

Moreover, in my team it was demonstrated that in a model of autoimmune diabetes, conventional T cells increase Treg expansion and activity, and that it is TNF-dependent [176]. Other data in the lab supported a role of TNF on Treg during EAE (see annex 2). It was firstly showed a worsening of EAE with the injection of an anti-TNF antibody; and the same effect was then observed with an anti-TNFR2 antibody, confirming that TNF binds mainly to that receptor on Treg. To better understand the underlying mechanism of this action, *Foxp3^{Cre}Tnfr2^{fl/fl}* mice have been generated. These mice lack the TNFR2 only in Treg and no differences have been reported at the steady state compared with *Foxp3^{CRE}* control mice. However, they develop a more severe EAE that was associated to decreased number and activation of Treg in the central nervous system. It is therefore likely that in this condition, in this tissue, TNFR2 has a pivotal role to maintain immune homeostasis supporting Treg number and function. It is possible that in other inflammatory conditions and in other tissues, other receptors of the family might have central roles.

3.5.3 Role in cancer

Antagonistic antibodies to TNFR2 are currently being testing to target Treg within tumors. As already mentioned, TNFR2 is highly expressed in tumor-infiltrating Treg, and a promising strategy is to target Treg with a specific antibody, to enforce the action of the immune system against tumor cells. It was seen with colon cancer cell lines, that mice

lacking TNFR2 faced complete tumor regression after 2 weeks of inoculation, associated with lower proportion of Foxp3⁺ Treg [177].

3.5.4 Role in Tconv

Interaction of TNF with TNFR2, but not TNFR1, is directly co-stimulatory to TCR-mediated T cell activation. TNFR2 deficiency resulted in the reduced proliferative ability of CD4 cells as well as CD8 cells, and decreased production of IFN- γ , TNF and IL-2 in response to TCR stimulation [178]. TNFR2 is also important for the survival of T cells driving the production of anti-apoptotic molecules [179].

In human Tconv, it was shown a synergy between TNF and IL-2 to increase CD25 expression at the protein and RNA levels but that only TNF activates NF- κ B [180]. Indeed, it was seen by biochemical analysis, using TNFR1 and TNFR2 KO and agonists and antagonists, that TNFR2 triggering on T cells leads to canonical and non-canonical NF- κ B activation [181].

3.5.5 Role in Treg

Chen *et al.* showed that *in vitro* TNF boosts Treg proliferation and suppressive activity, with increased expression of CD25, Foxp3 and p-STAT5 [182] and that it amplifies its effect inducing the expression of other TNFRSF members (OX40, 4-1BB and GITR) [183].

In vivo experiments of co-transfer of Treg/Tconv showed that Treg from TNFR2KO are not able to control colitis and that after 8 weeks in colon lamina propria there are less KO Treg compared to WT. Moreover, co-transfer of WT and TNFRKO Treg led to less KO Treg after 10 weeks with a less stable phenotype, proven by reduced Foxp3 expression [184].

It was also reported that TNF promotes the survival and function of Treg in an inflammatory environment. Indeed, Treg are resistant to oxidative stress-induced cell death compared with Tconv, thanks to the secretion of thioredoxin-1, which is increased by TNF [185].

Only a few data are available regarding the signaling pathways triggered downstream TNFR2 in Treg. In one paper, human Treg were stimulated with TNF for 2 and 24 hours, without TCR stimulation, followed by a transcriptomic analysis. The numbers and types of genes up-regulated were basically the same between the two time points. Especially, it is

possible to see genes of the NF- κ B family (NF- κ B2, RelB and NFKBIA) and activation markers including several members of the TNFSF (TNF, LTA, Fas, 4-1BB, OX40). It is possible to see the increase of TRAF1, 2 and 3 confirming the data generated previously in cell lines. By FACS it was also assessed the phosphorylation of RelA in Treg stimulated with TNF [186].

Biochemical studies confirmed that triggering of TNFR2 may activate canonical and non-canonical NF- κ B. However, these studies were not convincing because purified Treg were contaminated with other cells [187].

3.6 GITR

3.6.1 Expression and main features

Murine GITR is a 228-residue type I transmembrane protein identified in 1997 with a constitutive high-level expression on Treg [188]. GITR is constitutively expressed on all Treg and its expression is up-regulated by Foxp3. Several studies suggest that GITR is a marker of actively suppressing Treg because of its association with the expression of activation markers as CTLA-4 but also with the production of cytokines as IL-10 and TGF- β .

It is also expressed at low to moderate levels on conventional naïve and memory T cells but then it is rapidly up-regulated upon activation [189]. In conventional T cells NF- κ B regulates its expression. The ligand is expressed mainly by APCs and after activation it increases but after 24h it is reduced [190].

3.6.2 Role in AD

Models of inflammation, autoimmunity and tissue transplantation suggest that the blocking of GITR signaling is beneficial, mitigating immune responses.

Several papers report an important role of GITR in inflammation and GITR deficient mice exhibit reduced leukocyte infiltration, inflammatory cytokines and disease severity compared to WT mice in the context of lung inflammation [191], [192].

Moreover, EAE exacerbation was seen after GITR agonist (DTA-1) injection at day 0 and 9, even in mice where Treg were depleted, suggesting that it acts more by boosting Tconv than eliminating Treg [193].

In NOD mice, GITR agonist (2F8) injection in 2-4 weeks old mice accelerated type 1 diabetes (T1D) development, while GITR antagonist (YGL 386.2) injected in 10-14 weeks old NOD mice suppressed T1D [194].

Lastly, a SNP in GITR gene is associated with autoimmune thyroid disease [195]. Similarly, levels of plasma GITRL positively correlate with IgG levels and clinical parameters in patients with primary Sjogren's syndrome [196], systemic lupus erythematosus [197] and rheumatoid arthritis [198].

3.6.3 Role in cancer

Conversely to what has been shown in inflammation and autoimmunity, GITR stimulation might be beneficial in cancer. Its antitumor activity is due to expansion of CD4⁺ and CD8⁺ T cells and inhibition or depletion of intratumoral Treg. Indeed, several GITR-targeting agents are currently being tested in preclinical cancer studies and the most widely used is the agonistic antibody DTA-1. DTA-1 injection after 5 days of tumor inoculation (CT26), induced tumor regression. The proposed mechanism is that GITR signaling in CD4⁺ cells activates TRAF6 and NF- κ B which drive Th9 differentiation and IL9 production that favor cytotoxic lymphocytes by activating DCs [199].

Moreover, it was demonstrated that DTA-1 antitumor activity is also due to deletion of Treg in tumors (not dLN). Specifically, DTA-1 injected at day 6-8 of tumor inoculation (CT26) induced tumor regression by depleting Treg rather than activating CD8⁺ cells. Within 3 days, DTA-1 injection led to severe, moderate and minor deletion of Treg, CD4⁺ and CD8⁺ T cells, respectively, which correlates with levels of GITR expression [200].

However, it was not clear if the Treg loss was due to Foxp3 loss or real Treg depletion. In MC38 tumor model, DTA-1 induced complete tumor rejection and this was associated to real tumor Treg deletion, not instability. With these changes in Treg, CD8⁺ T cells acquired a more functional phenotype with downregulation of exhaustion markers as PD1 and LAG3 [201].

3.6.4 Role in Tconv

In the periphery, engagement of GITR on T cells enhances their activation with upregulation of CD25, IL-2 and IFN- γ expression and increased proliferation. It was demonstrated that GITR co-stimulation increases early survival and then proliferation and IL-2 production in Tconv. The mechanism, investigated in cell lines, depends on TRAF4

and 5 but is negatively regulated by TRAF2, indeed, as mentioned before, it seems inhibitory after GITR triggering. GITR activates NF- κ B and the 3 MAPK pathways (p38, ERK, JNK) [202].

Moreover, naïve CD4⁺ T cells stimulated by anti-CD3/CD28 and DTA-1, have increased activation of canonical and alternative NF- κ B pathways shown by Western blot [203].

3.6.5 Role in Treg

Regarding Treg, it was shown in many papers that *in vitro* it increases dramatically Treg proliferation in the presence of APCs, anti-CD3 and IL-2. *In vivo*, it may play a role at the steady state because GITR KO mice have decreased proportion of Treg in LN and spleen. Moreover, injection of agonistic antibodies led to preferential Treg expansion without affecting their function [204].

It was proposed that GITR activation has two distinct effects on Treg and Tconv: transient inhibition of Treg regulatory activity and decreased sensitivity of effector T cells to Treg suppression.

Indeed, it seems that increased proliferation of effector cells in response to GITR triggering renders them more resistant to Treg suppression. Regarding the influence of Treg function, the situation is still unclear: some papers showed partial inhibition of Treg function with GITR triggering [199], while others observed no modification in their suppressive activity [205]. The fully activity of Treg in GITRL-transgenic mice suggests the inhibition of Treg suppression is transient and may be due to overstimulation of GITR in a non-physiological condition. Indeed, GITR-dependent decrease in Foxp3 protein is not due to changes in the levels of mRNA, confirming it is a transient effect [206].

Regarding Treg differentiation, GITR has opposite roles in thymus and periphery. In the thymus, GITR is expressed during T cell development and plays a crucial role in thymic Treg differentiation and expansion; triple KO for TNFR2, GITR and OX40 caused reduced thymic Treg. Oppositely, agonists for 4-1BB, GITR and TNFR2 inhibits iTreg differentiation probably through activation of PI3K-Akt which suppress Foxp3 by downregulating Foxo1 and Foxo3 [207].

3.7 4-1BB

3.7.1 Expression and main features

4-1BB is expressed on several hematopoietic cells and can be transiently up-regulated on CD8 T cells, CD4 helper T cells, B cells, Treg, NK, NKT, DCs and others [208] and it is already expressed on around 20% of freshly purified Treg.

4-1BB signaling evokes robust effector responses being useful in infectious diseases. However, at the same time, it has also the potential to alleviate autoimmune diseases. This double action may be explained by its capacity of promoting Th1 type responses while inhibiting Th2- and Th17-relating pathologies [209].

3.7.2 Role in AD

It was demonstrated that the administration of an agonistic anti-4-1BB antibody reduces the incidence and severity of EAE decreasing the infiltration of T cells in the brain. However, anti-4-1BB treatment after adoptive transfer of encephalitogenic T cells fails to prevent EAE, suggesting that the treatment acts in the induction phase, inhibiting autoreactive T cell responses rather than preventing T cell trafficking into the CNS [210]. Its amelioration of EAE seems to be due to the inhibition of Th17 cell generation, altering the environment necessary for Th17 differentiation from CD4⁺ T cells reducing IL-6 levels [209].

Moreover, in patients with multiple sclerosis it was observed a decreased 4-1BB expression on Treg and increased plasma s4-1BB protein levels. These plasma levels inversely correlate with 4-1BB surface expression on Treg. It is likely that the decreased 4-1BB expression on Treg may be involved in the impaired immunoactivity of these Treg [211].

Anti-4-1BB treatment also protected NOD mice from autoimmune diabetes but did not prevent insulinitis nor eliminate pathogenic cells. This protection is not mediated by CD8⁺ regulatory T cells, which are known to be induced by this receptor, neither by the increased IL-10 production by NKT cells. Diabetes protection seemed to be due to increase Treg population which binds specifically the anti-4-1BB antibody [212].

Furthermore, injection of a 4-1BB agonistic antibody suppressed arthritis, which seemed to be due to increased IFN- γ production by CD8⁺ T cells and consequently IDO induction in DCs, since its effect was abolished when IFN- γ and IDO were neutralized [213].

Collectively, agonists of 4-1BB suppress many types of autoimmune diseases when injected at disease onset or later, by three mechanisms:

- 4-1BB drives IFN- γ production by CD8⁺ cells, which drives IDO on DCs that creates an immunosuppressive environment;
- 4-1BB decreases levels of IL6 reducing Th17 generation and increasing pTreg differentiation;
- 4-1BB boosts Treg in NOD mice.

3.7.3 Role in cancer

On the other side, 4-1BB is highly expressed on tumor-infiltrating lymphocytes (TILs) and therefore different antibodies are currently being tested in clinical trials for cancer treatment. Unfortunately, first evidences show liver inflammation with 4-1BB agonist antibodies [214].

The preferential expression of 4-1BB in TILs may be due to hypoxia and HIF1a activation, since TIL of HIF1aKO mice were 4-1BB negative [215].

4-1BB agonists, alone or in combination with anti-PD1 and/or anti-CTLA4, are efficient to decrease tumor growth in TC1, MC38 or B16 models. In treated mice there was increased proportion of CD8⁺ cells expressing IFN- γ and Eomes within tumors and increased proportion of central and effector memory cells and tumor-specific cells among whole CD8⁺ T cells in the spleen [216].

It was shown that 4-1BBL is also expressed on T cells and is down-regulated by autocrine 4-1BB; it has the ability to reverse signaling on T cells, inhibiting IL-2, expansion and differentiation. For this reason, anti-4-1BB treatment might enforce anti-tumor immunity also by blocking reverse signaling [217].

Moreover, 4-1BB has a beneficial effect also by increasing cell metabolism of CD8⁺ cells, adapting them to survive in the tumor environment. 4-1BB agonists increase glycolysis

and fatty acid oxidation supporting the demand for energy and biomass. It was observed that agonists increase mitochondria mass and their membrane potential in mouse and human CD8⁺ T cells [218].

3.7.4 Role in Tconv

In T cells, 4-1BB increases proliferation through IL-2 production, inhibits activation-induced cell death (AICD) [219], promotes survival through Bcl-xL and Bfl-1 and is critical for immunological memory formation through upregulation of anti-apoptotic genes [220]. 4-1BB enhances function of T cells as it drives IFN- γ and TNF production and induces DC maturation upregulating B7 co-stimulatory ligands, increasing DC survival and boosting the production of inflammatory cytokines [221].

Upon receptor ligation, TRAF-1 and TRAF-2 are recruited, generating a docking site for multiple kinases. It was observed that 4-1BB increased TCR signaling by recruiting in lipid rafts a signalosome composed of several phosphorylated proteins and this led to activation of NF- κ B, JNK, ERK, β -catenin and AKT [222]. Most of these proteins are shared between other TNFRs but none of the members can replicate the phenotypic changes associated to 4-1BB stimulation. Therefore, it is likely that additional pathways are triggered by 4-1BB.

3.7.5 Role in Treg

There are not many data on 4-1BB and Treg but its triggering promotes *in vitro* Treg expansion in combination with TCR and IL-2 stimulation and the expanded cells seem to remain suppressive and maintain Foxp3 expression [223-225].

In vivo, injection of a 4-1BB agonist induced Treg expansion in spleen and liver, associated with strong production of IL-2 by Tconv. This Treg expansion was abolished using blocking anti-IL-2 [226].

Regarding the function, Treg boosted by 4-1BB seem to keep their suppressive function, however, Tconv seem to be more refractory to Treg suppression, partly because of increased IL-2.

3.8 OX40

3.8.1 Expression and main features

OX40 is a type I transmembrane glycoprotein that can be induced on activated CD4 and CD8 T cells, but it is not found on resting conventional naïve T cells. OX40 is expressed constitutively on murine Treg especially on cells present at tumor sites. Its upregulation can peak from 24h up to 4-5 days after stimulation with a much more transient expression on CD8 than CD4 T cells. Its expression is increased with simultaneous CD28 triggering and favorable cytokine milieu. Analogously, OX40L is only induced after stimulation on APCs [227].

3.8.2 Role in AD

OX40 signaling plays a double role in the pathogenesis of EAE. Early injections of agonistic antibody decreased EAE and this was associated to increased Treg expansion in dLN. Conversely, injections during the disease onset worsened EAE probably because of boosting pathogenic T cells, expressing up-regulated OX40 during the onset of EAE [228]. In addition, OX40L-deficient mice poorly develop EAE [229]. Moreover, blocking OX40 using an anti-OX40 slightly decreased active EAE and efficiently passive EAE. The effect did not seem to be due to reduced priming but to reduced migration in the CNS [230].

Furthermore, injections of anti-OX40 agonistic antibody (OX86) in NOD mice slightly reduced diabetes. This was associated with a small Treg expansion and proliferation in the spleen and pancreatic LN [231].

At the same time, it was demonstrated that OX40 signaling is important for Treg accumulation in the colon but not in peripheral lymphoid organs. And that under inflammatory conditions it plays an essential role for Treg-mediated suppression of colitis [232].

Moreover, the OX40L-OX40 seems to play a role also in rheumatoid arthritis. In general it could possibly be involved in the pathogenesis of diseases where activated CD4 and CD8 T cells orchestrate the immune response [233].

3.8.3 Role in cancer

The capacity of OX40 to regulate both CD4 and CD8 T cells makes it also a promising candidate for tumor therapy. It was shown that tumor infiltrating Treg express OX40 at high level and that its triggering suppress their function [234]. Moreover, anti-OX40 antibody depletes Treg at tumor sites by antibody dependent cell cytotoxicity or phagocytosis. Indeed, FcRs present on myeloid and NK cells recognize the anti-OX40 antibody bound to the OX40 expressed at the cell surface and kill Treg [235].

3.8.4 Role in Tconv

The most recognized function of OX40 is to enhance proliferation and survival of CD4 and CD8 T cells. Using models of OX40 deficiency, it was shown that it does not control initial activation or proliferation (day 3) but provides signals to maintain late proliferation (after day 6) and prolong cell survival through effector phase, associated with increased Bcl2 and BclXL [236].

It was reported a critical role of survivin in the defect of late survival of OX40-deficient T cells; survivin expression alone was indeed sufficient to restore proliferation and to antagonize apoptosis of OX40-deficient T cells [237].

Stimulation of naïve CD4 T cells with anti-CD3 and OX40L transduced APCs showed that OX40 inhibits iTreg generation and induced Th9. It triggered transient canonical NF- κ B activation (nuclear translocation of p50 and RelA at 12-24h) and strong and sustained non-canonical NF- κ B activation (translocation of p52 and RelB at 12-72h). Gain and loss experiments showed that TRAF6 and non-canonical NF- κ B were critical for Th9 differentiation [238].

TRAF 2, 3 and 5 seem to be also important because they interact with the NF- κ B inhibitor, I κ B α , triggering both the canonical and non-canonical pathways. NF- κ B activation increases the expression of anti-apoptotic proteins of the Bcl-2 family, causing suppression of apoptosis and enhanced cell survival [239]. This pathway can be triggered even without concomitant TCR stimulation and in this way OX40 acts as a separate unit. In this case one triggered pathway is PI3K/PKB which is involved in cycle progression through survivin and aurora B. These proteins promote the activity of cyclin dependent kinases allowing for G1 to S phase progression in the cell cycle and maintenance of

mitosis in T cells [240].

Lastly, in conjunction with TCR signaling, OX40 can increase calcium influx and enhance NFAT activation leading to the production of cytokines as IL-2, IL-4, IL-5 and others [241].

3.8.5 Role in Treg

In OX40 KO mice, it was seen normal proportion of Treg in lymphoid tissues but a strong reduction in lamina propria (2-3 fold) and moderate reduction in liver and lung. Also, these Treg did not control colitis because of early defect in survival (but normal proliferation) [232]. In addition, it was observed reduced expansion of OX40 KO Treg when in a competition with WT Treg in lymphopenic mice [242]. Collectively, OX40 seems to be important for Treg homeostasis only in specific situations, such as competition with WT Treg, in lymphopenic mice, or in non-lymphoid tissues.

It was observed a synergy between IL-2 and OX40. Indeed, the Treg boost induced by low-dose IL-2 was no more observed in OX40KO mice. Treg boost can also be increased by combining anti-OX40 and IL-2 complexes. The synergistic action was associated to increased p-STAT5 and p-Akt after 1h *in vitro*. Moreover, the IL-2 prevented the down-modulation of Foxp3 and CD25 which is observed with anti-OX40 alone [243]. Indeed, OX40 promotes miR155 and inhibits SOCS1, rendering Treg more sensible to IL-2 [242].

No effect was seen of OX40 triggering (preincubated 2 hours and then washed), compared to GITR, on the proliferation of purified Treg *in vitro* stimulated with APC and anti-CD3 for 3 days. However, this might also be due to low expression of OX40 on fresh cells [244]. Conversely, *in vivo* injection of agonistic anti-OX40 caused strong Treg expansion in the spleen, however, they had decreased Foxp3 and CD25 expression associated with decreased suppressive function [243].

Recently, data showed that the role of OX40 in Treg development should be assessed in relation with other receptors of the family. Mahumud et al. used conditional KOs of GITR, TNFR2 and OX40 to show that individually these genes had a limited impact on Treg generation but the combination of all three KOs caused a significant inhibition of Treg development.

In contrast, in iTreg generation OX40 plays a central role since OX40 KO cells showed increased conversion. OX40 might affect iTreg development by altering the cytokine

environment but also downregulating Foxp3 expression. One way of interfering with Foxp3 is altering the binding of SMAD3 and STAT3 on the Foxp3 promoter [245].

3.9 DR3

3.9.1 Expression and main features

DR3 is expressed mainly on memory T cells. It is up-regulated after TCR stimulation on naïve T cells, with a peak after 3 days in CD4 T cells.

The signaling through DR3 is controlled by the availability of its ligand, TL1A, which is very low at baseline in most cell types but is highly inducible by proinflammatory stimuli [246]. Autocrine feedback via DR3 may control TL1A levels because DR3-deficient T cells have reduced induction of TL1A following TCR stimulation [247].

3.9.2 Role in AD

TL1A might be considered a broad costimulator of different T cells and its upregulation is a characteristic of a variety of human inflammatory diseases.

Patients with Crohn's disease and colonic involvement have elevated serum TL1A levels [248]. A parallel observation was that TL1A expression correlates with the presence and severity of inflammation in mucosal tissues isolated from patients with ulcerative colitis and Crohn's disease. Therefore it was proposed that it could play a role in the generation and/or severity of inflammatory bowel disease [249]. In rheumatoid arthritis, increased soluble TL1A is found in both synovial fluid and serum, particularly in patients with severe or terminal arthritis [250]. Increased TL1A has been found also in autoimmune diseases such as psoriasis [251].

EAE was almost absent in DR3KO mice with reduced proportion of CD4⁺ T cells in the spinal cord and of IFN- γ production [247]. Moreover, in DR3KO mice, colitis was more severe compared to WT mice and this was associated with reduced proportion of Treg in mLN and lamina propria.

3.9.3 Role in Tconv

The effect of DR3 costimulation differs from cell to cell but in general it promotes IL-2 signaling. During T cell activation, TL1A increases IL-2 production, CD25 (IL-2RA) and IL-2RB expression. And the downstream effect is to promote T cell proliferation, particularly under suboptimal conditions [252, 253].

Studies show that it promotes Th1-associated cytokines such as IFN- γ , especially under conditions of suboptimal TCR activation [254]. But TL1A effects are not confined to Th1 cells. Indeed, even if in human cells TL1A does not enhance Th2 cytokines [255], it increases IL4 and other Th2 cytokines in murine T cells [256]. DR3 is highly up-regulated in Th17 cells and it promotes their proliferation in the presence of IL-2-signaling inhibition [257].

Regarding the signaling triggered by DR3, there are at least two major pathways that can lead either to NF- κ B activation or to caspase activation and apoptosis. The distinction between the two cascades is dictated by the recruitment of TRADDs or FADDs proteins in the cytosolic tail of DR3. Despite its name of death receptor, DR3 stimulates only proliferation, cell activation and effector function in primary T cells. To date, the only setting in which it drives apoptosis is in transformed or immortalized cell lines. Indeed, in TRADDKO T cells, it was shown that TRADD is important to recruit TRAF2 and RIP1, which is ubiquitinated, stimulating MAPK signaling and activation of NF- κ B [258].

In vivo experiments showed that TL1A enhances Th9 differentiation through IL-2 and STAT5, which promotes Th9 through IL4 and STAT6 [259].

3.9.4 Role in Treg

TL1A has also been reported to influence the generation and function of Treg. Triggering of DR3 increases Treg proliferation both *in vitro* [260] and *in vivo* [164].

Injection of a DR3 agonist (4C12) induced a high preferential expansion of Treg with a CD62L⁺CD44⁺ central memory phenotype. After 4 days, DR3-boosted Treg had increased expression of Ki67, TIGIT, PD1, KLRG1, ICOS, LAP and decreased CD25 [261].

However, despite this expansion, it was reported that TL1A dampens the suppressive capacity of Treg in *in vitro* assays, in part because of its ability to costimulate also effector T cells but potentially also because of a direct effect on Treg [260].

In contrast to nTreg, during iTreg generation from naïve T cells, TL1A inhibits the production of Foxp3⁺ T cells [259]. The inhibition of iTreg generation raises the question whether TL1A might divert Treg toward another fate. Indeed, Richard et al. showed that the addition of TL1A to iTreg cultures promoted the generation of T cells secreting IL9 and lacking Foxp3.

3.10 Other TNFRSF members

As mentioned before, the TNFRSF is composed of many receptors. However, not all of them have been extensively studied and especially not much is known regarding their role in T cells in general and Treg in particular.

Here, I selected some receptors that seem to be the most important in Treg biology but that were not included in my thesis project because of lack of specific ligands or time or effect.

3.10.1 CD30

CD30 is expressed on activated T and B cells as well as Treg. The expression of CD30L on APCs induces either cell death and cell-cycle arrest or survival and proliferation. It utilizes TRAF2 and TRAF3 to activate pathways including non-canonical NF- κ B, p38MAPK, ERK, AP-1 and AKT.

CD30 regulates primarily CD4⁺ T cells responses but can affect adaptive immunity more broadly.

Clinically, measurements of soluble CD30 are used for monitoring immune activation and high levels are associated with chronic infections and poor prognosis.

It has also been associated with Th2 responses in autoimmunity and it is also a cancer-associated surface antigen expressed in some lymphomas, leukemia and solid tumors [262].

3.10.2 HVEM

The biology of this receptor is very complicated because it has four different ligands: LIGHT, LT α , BTLA and CD160. It can promote or block cell activation depending on the situation.

BTLA and HVEM combined in *trans* during cell-to-cell interactions drives cell activation; in contrast, HVEM and BTLA, when co-expressed in lymphocytes, can combine in *cis* to limit cellular activation, through reverse inhibitory signaling of BTLA.

HVEM is expressed across all the hematopoietic lineages while BTLA varies across different cell types with B cells expressing the highest.

LIGHT and CD160 act to counterregulate HVEM-BTLA inhibitory pathway but on different cell types. The membrane form of LIGHT disrupts HVEM-BTLA complexes in an

uncompetitive mode promoting HVEM activation. In contrast the soluble form of LIGHT promotes HVEM-BTLA interactions enhancing the inhibitory activity of the BTLA checkpoint.

CD160 engages HVEM in the same site as BTLA. It acts as a co-stimulatory signal (in combination with cytokines) for NK cells in the context of inflammation, however other studies have implicated CD160 in controlling T cell exhaustion.

LIGHT promotes systemic autoimmune pathology, as demonstrated by transgenic animals with enforced T cell expression. LIGHT in tumors drives NK and T cell activation that can eradicate tumor expansion with sustained memory. The enhancing of immune responses by LIGHT is mediated by the binding of both HVEM and LT β R.

HVEM activation of BTLA serves as a counterpoint to LIGHT-activated inflammation. The higher expression of HVEM in Treg than in Tconv was also proposed to act as a mechanism by which these cells could mediate suppression [262].

3.10.3 CD40

CD40 is a stimulatory receptor expressed on dendritic cells, macrophages and B cells. It drives activation, maturation, survival and inflammatory cytokine production. It is crucial to induce IgG autoantibodies and immunoglobulin class switching. After the binding to its ligand, CD40L, on T cells, it increases antigen presentation and activation of T cells by upregulating MHC molecules and inducing the expression of stimulatory ligands, including those belonging to the TNFSF.

CD40 has been linked to rheumatic disease pathogenesis. And soluble CD40L in serum, or CD40L in inflamed tissue, epithelial or T cells, is up-regulated in patients with RA, psoriatic arthritis, SLE and systemic sclerosis correlating with disease severity or levels of autoantibodies [263].

Animal studies showed that neutralization of CD40L has a suppressive effect on pathogenic T cell development and antibody response, which renders it an attractive therapeutic target. For example, disruption or blocking of CD40L-CD40 binding inhibits clinical manifestation and ameliorates EAE in mice and monkeys [264].

3.10.4 CD27

CD27 is constitutively expressed on most T cells and the interaction with its ligand, CD70, control T cell accumulation and reactivity. In addition, CD70 is inducible on dendritic and

B cells and can induce reverse signaling in these APCs inducing their activation. In mice with collagen-induced arthritis, blocking CD27-CD70 interactions reduces bone and cartilage erosion and inflammatory infiltrates in the joints. In the synovial fluids of RA patients, soluble CD27 and CD27⁺ T cells are elevated [263].

Moreover, it plays a role in other pathological conditions. Soluble CD27 correlate also with disease activity in SLE patients and the proportion of plasma cells expressing CD27 correlates with SLE disease indices. Treatment of animals with an anti-CD70 antibody suppress EAE. CD70-transgenic mice showed increased number of IFN- γ -producing CD4⁺ cells suggesting a role in controlling Th1 responses [264].

Elevated concentrations of soluble CD27 were found in different tumor conditions predicting poor outcome. Increasing amount of iTreg were seen in the presence of CD70-expressing malignant cells. Blockade of CD70-CD27 pathway abrogated the induction of Foxp3 expression in intratumoral T cells. Likewise, chronic lymphocytic leukemia cells with high CD70 expression were shown capable of inducing Treg in a CD27-dependet manner. But also in solid tumors, the interaction augmented the frequency of Treg, reduced tumor-specific T cell responses and promoted tumor growth [265].

3.10.5 DR5

This receptor drives cell death when binds to its ligand, TRAIL. DR5 is expressed in neurons and oligodendrocytes, but TRAIL is absent in a healthy CNS. Signaling from TRAIL/DR5 is implicated in the pathogenesis of MS and EAE inducing death of neuronal cells. Blocking brain-specific TRAIL leads to a reduction of EAE [264].

TRAIL might be a promising novel anticancer molecule because it specifically kills tumor cells by apoptosis. Local TRAIL treatment decreased intratumoral Treg but did not affect Treg in draining lymph nodes and spleen. Indeed, TRAIL induced apoptosis of Treg but not of Tconv and, in addition, activated Treg in the tumor expressed high level of DR5 while naïve Treg did not [266].

AIM OF WORK

It is therefore increasing the interest in the receptors of the TNF family, focusing on their role on Treg given their high expression and upregulation on these cells. However, there are not many data on this topic and most of them have been achieved with KOs models, injection of agonists or antagonists or transgenic animals which render difficult to point out their role on Treg. Indeed, truly they are highly expressed on Treg but they are express also on other cell types and the observed effects on Treg might be indirectly achieved.

Our hypothesis is that triggering TNFRs on Tregs can impact differently in their biology in term of survival, proliferation, stability and function. To assess this, Foxp3^{GFP} mice have been used to easily and quickly isolate highly purified Treg.

The project could be divided into 3 main parts, *in vitro* and *in vivo* characterization and signaling pathway analysis.

For the *in vitro* part, short-term cultures of Treg plus agonists for TNFRs family members have been done.

The phenotype of Treg in term of survival, proliferation and surface markers expression was analysed by flow cytometry.

The cytokines production was also measured to see if the TNFRs triggering causes a change of phenotype in cultured Treg.

The function of Treg stimulated with agonists for TNFRs members was measured with a suppression assay combining Treg, Tconv and APCs.

For the *in vivo* part, Treg, stimulated *in vitro* with agonists for TNFRs members, have been injected in wild type mice to investigate their survival, proliferation, stability and migration at the steady state.

Moreover, boosted Treg were also injected in mouse model of EAE to investigate all these parameters in an inflamed environment.

To investigate their ability to suppress autoimmunity they have been injected, after *in vitro* stimulation, with Tconv in immune-deficient mice to compare their ability to control colitis.

Another important part of the thesis is the study of pathways triggered by TNFRs members. The idea is to link a biological finding with a mechanistic explanation. For this reason, it was performed the RNA-sequencing analysis of Treg stimulated *in vitro* with agonists for TNFRs members for 18h and 36h.

The aim of the sequencing is to identify the impact of TNFR triggering in key genes and/or pathways important for Treg biology. We expected to see some genes specific of some receptors but also common pathways that could be even more activated with synergic activation of different receptors of the family.

Because of previous data in the literature highlighting the importance of NF- κ B downstream several receptors, we decided to study it by biochemical analysis. For this reason, *in vitro* stimulated Treg were used to investigate NF- κ B activation, with the EMSA and supershift technique, following receptor triggering.

One of the goals of this project would be to generate “super Treg” triggering TNFRs member with higher survival, proliferation, stability and function. These Treg could be used in a cell-based therapy, starting from Treg from patients with autoimmune diseases. The treatment with specific agonists for TNFRs would permit to expand them and potentiate their suppression activity *ex vivo* and them to re-inject them into patients.

PAPER

Tumor-necrosis factor receptor superfamily co-stimulation increased regulatory T cells activation via the canonical NF- κ B pathway

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Abstract

Drugs targeting tumor-necrosis factor (TNF) superfamily (TNF-SF) or TNF receptor superfamily (TNFR-SF) are widely used in medicine and are under intensive investigations in clinical trials and pre-clinical studies. Their mechanisms of action, which are diverse and poorly known, could be partly mediated by their effect on Foxp3 regulatory T cells (Treg) that preferentially expressed some of them, such as TNFR2, 4-1BB, GITR or OX40. Here, we showed that Treg co-stimulation with agonists of TNFR2, 4-1BB, GITR or DR3, but not of OX40, increased their proliferation and survival. There were additive effects when combined some of these co-stimulations. This TNFR-SF co-stimulation improved Treg expansion in vivo and increased their capacity to control an inflammatory disease. Triggering these receptors induced a similar signature at the transcription level, showing that they share signal transduction. Using a DNA binding assay and loss of function approach, we showed a critical role of the canonical NF- κ B pathway in the TNFR-SF Treg co-stimulation. Thus, TNFR-SF may play a major role in Treg biology and part of the beneficial effects of drugs targeting TNF-SF or TNFR-SF molecules may be Treg-mediated.

Introduction

TNF superfamily (TNF-SF) is composed of 19 structurally related cytokines that play a major role in the immune system. There is an increasing interest in targeting several members of the TNF-SF or TNF receptor superfamily (TNFR-SF) in medicine. Several TNFR-SF members (4-1BB, GITR, CD27, OX40, CD40, CD30, BAFFR or TACI) are emerging targets in cancer immunotherapy beyond anti-PD1 and anti-CTLA4 monoclonal antibodies (mAb), which are currently tested in clinical trials ([1], [2], [3]). Drugs used are often mAb with agonist activity. TNF-SF and TNFR-SF members are also important targets for autoimmune or inflammatory bowel diseases. Some of them are clinically approved, such as anti-TNF drugs, and many others targeting various TNF-SF and TNFR-SF members are under investigation in clinical trials. Most of these drugs are being used as blocking reagents to dampen inflammation induced by these cytokines.

Mouse preclinical experiments have started to elucidate the mechanism of action of some TNFR-SF agonists in cancer. 4-1BB agonists seem to act mainly by stimulating tumor infiltrating CD8 T cells ([4], [5]). GITR or OX40 agonists act by depleting or inhibiting tumor infiltrating regulatory T cells (Treg) and by stimulating conventional T cells (Tconv) ([6], [7], [8]). Quite intriguingly, agonists of 4-1BB or OX40 that are able to increase anti-tumor immunity are also able to suppress autoimmune diseases in mice, even sometimes when using the same mAb clone ([6], [7], [9], [4]). These paradoxical findings are not yet elucidated but some mechanisms have been proposed. 4-1BB agonists would increase IFN- γ production by Tconv, leading to protective IDO production in an autoimmune context and anti-tumor rejection in cancer ([10], [11]). OX40 agonists would promote protective Treg expansion in an autoimmune context and depletion of Treg infiltrating tumors mediated by myeloid cells expressing activating Fc γ R present in high density ([7], [12]). Thus, whereas the therapeutic potential of drugs targeting TNF-SF or TNFR-SF is major, their mechanism of action is still poorly defined.

Transcriptomic analyses clearly showed that several members of the TNFR-SF are preferentially expressed on human and mouse Treg. For instance, Tnfrsf1b (TNFR2), Tnfrsf9 (4-1BB), Tnfrsf18 (GITR) and Tnfrsf4 (OX40) belong to the very restrictive core Treg signature expressed by all Treg subsets, composed of the top 10 genes that are the most highly expressed by Treg compared to Tconv, which is correlated with low DNA-methylation suggesting regulation at the epigenetic level ([13], [14]). Also, Tnfrsf9 (4-1BB), Tnfrsf18 (GITR) and Tnfrsf4 (OX40) were among the top genes that were the most highly expressed by tumor infiltrating Treg compared to healthy tissue Treg and tumor infiltrating Tconv in mice and human ([15], [16]). This is confirmed at the protein level

since for instance GITR expression is often considered as a prototypic Treg marker and early upregulation of 4-1BB is used to identify human activated Treg, excluding activated Tconv ([17]).

The intrinsic functions of TNFR-SF on Treg biology are poorly known. Experiments using full knockout mice were difficult to interpret because the impact on Treg could be due to a direct or indirect effect by non-Treg and conditional knockout in Treg are lacking. Injections of agonists of TNFR2, 4-1BB, GITR, DR3 or OX40 led to Treg expansion in mice, some of them associated with therapeutic effects in autoimmunity ([18], [19], [20], [21], [12]). Again, it was not clear whether their effect was direct or indirect. In vitro experiments showed that agonists of TNFR2, 4-1BB, GITR or DR3 increased Treg expansion ([22], [23], [24], [25]). Finally, the effect of several TNFR-SF agonists on Treg suppressive function led to some apparent contradictory findings. It was suggested that agonists of TNFR2, GITR or OX40 inhibited Treg suppressive function. However, these agonists rendered Tconv refractory to Treg-mediated suppression. When studies used Treg pre-incubation with the agonists or a mix of WT Treg and KO Tconv, it was usually found that most TNFR-SF agonists did not inhibit Treg suppressive function in vitro ([26], [27], [28], [29], [6]).

Most of what we know on signal transduction induced by TNFR-SF triggering came from cell lines, showing activation of the NF- κ B, PI3K and MAPK pathways ([30], [31]). Some studies have been performed with fresh Tconv and very few (less than 5 for all TNFR-SF) on fresh Treg, suggesting also activation of the same pathways ([32], [33], [34], [35]). Also, only two studies have analyzed the effect of TNFR-SF agonists in vitro on the Treg transcriptome, one for TNFR and one for OX40 ([36], [37]). And there is not a single study that has compared the effect of different TNFR-SF agonists on Treg biology and function. So we know nothing about what is common and what is different on the role of different TNFR-SF members on Treg. Here, we analyzed the in vitro effect of different TNFR-SF members on purified Treg in different directions. We analyzed their capacity to increase Treg proliferation, survival, suppressive function and assessed their mechanism of action by studying the NF- κ B pathway and performing transcriptomic analyses.

Results

Increased Treg proliferation and survival by agonists of TNFR2, 4-1BB, GITR and DR3.

Among the TNFR-SF members that have been studied in Treg biology, they are expressed at low levels on resting Treg, except for GITR. For most of them, their expression appears to be rapidly up-regulated upon TCR/CD28 activation. This is the case for TNFR2, 4-1BB, GITR or OX40 with high expression at the protein levels as soon as 24h after initial activation (Suppl Fig 1). To address the direct effect of agonists of different TNFR-SF members on Treg biology, we chose to stimulate highly purified Treg by anti-CD3 and CD28 mAbs with different concentrations of agonists of various TNFR-SF members. Since we expect to see an effect on Treg proliferation and survival, we quantified the numbers of living Treg at day 3. Compared to the control culture, the addition of agonists of TNFR2, 4-1BB, GITR or DR3 increased the numbers of living Treg by 3 to 4 fold at their optimal concentration. In contrast, addition of agonists of OX40, DR5, HVEM, CD27 or RANK had no or minimal effects and the agonist of Fas killed all Treg (Suppl Fig 2). At the RNA level, *Tnfrsf1b* (TNFR2), *Tnfrsf9* (4-1BB), *Tnfrsf18* (GITR), and *Tnfrsf4* (OX40) were highly expressed, *Tnfrsf25* (DR3), *Tnfrsf10b* (DR5), *Cd27*, *Fas* and *Tnfrsf14* (HVEM) were expressed at low level and *Tnfrsf11a* (RANK) was undetectable 18h after initial Treg activation (Suppl Fig 3).

For the rest of the study, we thus analyzed the effect of agonists of TNFR2, 4-1BB, GITR or DR3, as well as the agonist of OX40 because of its well-characterized capacity to boost Treg *in vivo* and to suppress autoimmune diseases and promote tumor rejection (see above). Compared to the control culture, TNFR2 and 4-1BB agonists were the most effective to increase Treg survival and proliferation with GITR and DR3 agonists having a weaker effect and OX40 agonist having no effect (Fig1A, B). We then addressed whether additive effect would be observed by combining two agonists. Associating agonists of TNFR2 and 4-1BB or 4-1BB and DR3 or TNFR2 and DR3 or GITR and DR3 had clearly additive effect on survival and proliferation whereas combining agonists of TNFR2 and GITR or 4-1BB and GITR had no or minimal additive effects (Fig 1C, D and Suppl Fig 4A). When Treg were co-stimulated by OX40 agonist, agonists of the four other TNFR-SF members increased survival and proliferation (Suppl Fig 4B). To assess whether these additive effects were due to increased expression of TNFR-SF members, we quantified their expression at the RNA level. None of the agonists increased expression of their own or the other TNFR-SF members (Suppl Fig 5). In conclusion, agonists of TNFR2, 4-1BB, GITR and DR3 had a direct effect on Treg to increase their survival and proliferation with some additive effects when they were combined.

Shared transcriptomic signature induced by different TNFR-SF agonists on Treg.

We performed a transcriptomic analysis to obtain a more global view on the effect of these agonists on Treg biology. To synchronize Treg activation, cells were stimulated with coated anti-CD3/CD28 mAb in the presence or not of agonists of TNFR2, 4-1BB, DR3 and OX40 for 18 and 36 hours before RNA-Seq analysis. Using this antigen presenting cell-free assay, we limited the risk to have TNFR signals triggered by other sources than the added agonists, with the notable exception of TNFR2 since Treg could produce TNF. GITR agonist was omitted in this analysis because of its relative weak capacity to co-stimulate Treg, whereas OX40 agonist was included as a control, because of the absence of an effect on Treg proliferation and survival. Unsupervised PCA analysis showed that Treg co-stimulated by all four agonists had a specific transcriptomic signature that were clearly separated from the control Treg. In addition, the signatures induced by the TNFR2 and 4-1BB agonists were the farthest from the control (Fig 2A). To document the level of similarities of the effect of the different TNFR-SF agonists on Treg, we analyzed fold change expressions compared to control. There were strong positive correlations of the effects of TNFR2 and 4-1BB agonists on Treg ($r=0.82$ with $p < 2 \times 10^{-16}$ using a Pearson correlation) (Fig 2B and Fig Sup 6). Similar strong similarities between the effect of TNFR2 and the effect of DR3 or OX40 were observed at the whole transcriptome level (Fig Sup 6). Similarities and differences were further documented by looking at differentially expressed (DE) genes compared to control Treg. Among all the DE genes ($FDR < 0.05$), about half were shared between at least two agonists (123 out of 253 at 18h and 101 out of 209 at 36h) (Fig Sup 7). The results were even more striking when looking at the top DE genes. 4-1BB and TNFR2 agonists induced the strongest difference compared to the control, with DR3 and OX40 agonists somewhere in between the effect of TNFR2 or 4-1BB agonists and control at both 18 and 36h (Fig 2C and Fig Sup 2). Many of the top DE genes induced by the TNFR2 agonist were also induced by the 4-1BB agonist. Quite surprisingly, the OX40 agonist that did not increase Treg proliferation and survival seemed to generate an effect with an impact comparable to the one of DR3 agonist (Fig 2A, C). Also, several of the top DE genes induced by the TNFR-SF agonists belong to the Treg transcriptomic signature to be either up-regulated (Dennd5a, Cd83, Scin, Irf5, Stx11, Bhlhe40 and Mxd1) or down-regulated (Gpr83, Nt5e or Entpd1) (Fig 2C).

Because of the strong similarities in the transcriptomic signatures induced by the four TNFR-SF agonists, we had to exclude that all four triggered the same TNFR, directly or indirectly. None of the agonists increased the expression of the other TNFR-SF (Fig Sup 5). Because Treg can express

TNF at high level but not the other natural ligands, we focused our attention to TNF and TNFR2. Definitive evidence came from the analysis of Treg proliferation and survival of Tnf and Tnfrsf1b (coding for TNFR2) knockout Treg. Similar increased Treg proliferation and survival induced by agonists of TNFR2, 4-1BB, GITR and DR3 were observed with wild type and Tnf knockout Treg, showing that their effect was not TNF-mediated. When using Tnfrsf1b knockout Treg, the increased Treg proliferation and survival was abolished only with the TNFR2 agonist, showing that agonists of 4-1BB, GITR or DR3 did not co-stimulate Treg via TNFR2 (Fig 2D). In conclusion, triggering all four TNFR-SF members induced a share signature on Treg with the strongest effect observed with agonists of TNFR2 and 4-1BB.

Impact of TNFR-SF agonists on Treg function.

The classical Treg suppressive assay, studying the capacity of Treg to inhibit proliferation of conventional T cells, is an accurate and quantitative way to measure Treg function, although only a small fraction of the multitude in vivo suppressive mechanisms are tested. Using this in vitro assay, we found that Treg that were previously co-stimulated by agonists of TNFR2, 4-1BB, GITR or DR3 had similar suppressive activity as control Treg (Fig 3A). When looking at molecules that have been described to have effector function in Treg, we found that some were down-regulated and others were up-regulated at the RNA level by agonists of TNFR2 or 4-1BB (Fig 3B). We also analyzed cytokine produced by Treg after 3 days of culture. Control Treg mainly produced IL-10, low levels of IFN- γ , IL-17 and IL-23 p19 and very low level of TNF, IL-4, IL-5 and IL-13. Agonists of TNFR2, 4-1BB, GITR or DR3 increased Th2 cytokines (IL-4, IL-5 and IL-13) but had no reproducible effect on the other cytokines (Fig 3C and Fig Sup 9). Thus, these TNFR-SF agonists seem to favor the differentiation of type 2 Treg. To further document this finding, we analyzed cytokines, chemokine receptors and transcription factors that characterize the different types of Th cells. Evidence of Th2 type differentiation of Treg co-stimulated by TNFR-SF agonists was observed at the RNA level with increased expression of Il5, Ccr4, Ccr6, and Jun and Fosl2 (both involved in Th2 differentiation) and decreased expression of Tnf, Il10 or Cxcr3 (Fig 3D). Differential expressions were also observed at the protein level for CXCR3, CCR6 and GATA3 (Fig 3E). Finally, we assessed the effect of agonists of TNFR-SF members on Treg function in vivo in a colitis model. We first used Treg that were co-stimulated during a 4-day culture with no positive results, which was likely due to the poor survival of pre-activated Treg after their transfer (not shown). That is why we used freshly purified Treg that were incubated for only 2h with the agonists. We used only agonists of TNFR2 and GITR because these receptors were significantly expressed already in

freshly purified Treg (Sup Fig 1). Remarkably, compared to control Treg, Treg that were pre-incubated with agonists of TNFR2 or GITR increased significantly their capacity to control the disease (Fig 3F). In conclusion, triggering TNFR induced differentiation of Type 2 Treg and increased their capacity to suppress colitis, at least with TNFR2 and GITR agonists.

TNFR-SF triggering increased in vivo Treg expansion.

To assess the effect of TNFR2, 4-1BB, GITR and DR3 agonists on Treg homeostasis, cells were first co-stimulated in vitro and then co-transferred with the same number of control Treg to compare the two Treg subsets for their capacity to migrate, survive and proliferate in different tissues within the same mouse. Migration was first analyzed at the early time point (12 hours after cell transfer). Co-stimulated Treg were present at about the same ratio as control Treg in various tissues except in the blood in which they were almost absent (Sup Fig 10A,B). This could not be explained by lower RNA expression of S1pr1 or higher RNA expression of Ccr7 or Cd69, molecules involved in the lymph node to blood recirculation (Sup Fig 10C). Interestingly, CD62L expression was up-regulated in co-stimulated Treg, suggesting that their entry in lymph nodes may be increased, impoverishing blood Treg (Sup Fig10D).

Migration, survival and proliferation were then analyzed 7 days after co-transfer of control and co-stimulated Treg. Overall, co-stimulated Treg were present in higher numbers than control Treg in lymphoid tissues, lung or liver, with variations depending on the tissue and TNFR-SF agonists (Fig 4A). The 4-1BB agonist was the one that induced the strongest effect, followed by GITR, TNFR2 and DR3 agonists. Also, the lung was the tissue where the higher number of co-stimulated Treg (compared to control Treg) was the most apparent. We compared the expression of key markers to tackle the mechanism of the higher number of co-stimulated Treg. Compared to controls, the latter cells expressed higher levels of the Ki67 proliferative marker in all tissues (Fig 4B). This difference was not yet observed at 12h after transfer (Sup Fig 10D), which indicated that co-stimulated Treg had either increased proliferation or increased survival of proliferating cells after transfer. The increased expression of Bcl2l1 (Bcl-XL) and decreased expression of Bcl2l11 (Bim) in co-stimulated Treg, two molecules critical in Treg survival, may indicate an effect on cell survival (Supp Fig 11).

Compared to control Treg, co-stimulated Treg expressed higher levels of CD62L and CCR6 and lower levels of CXCR3 at 7d after transfer (Fig 4B), a phenomenon that was already observed at 12h after transfer (Sup Fig 10D). Before cell transfer, these differences were marginally observed

(Sup Fig 12) Thus, co-stimulated Treg rapidly changed their biology, acquiring a modified phenotype that should impact on Treg recirculation and tissue retention depending on the context. To address this question in an inflamed environment, we induced experimental autoimmune encephalomyelitis (EAE) that generates an inflammation in the draining lymph nodes and the spleen, followed by T cell migration in the central nervous system that is partly CCR6 dependent. Mice were transferred with control or co-stimulated Treg at the time of immunization (Day 0) or at day 7. At day 30, there were increased numbers of co-stimulated Treg compared to controls in the spleen, draining lymph nodes and inflamed central nervous system (Fig 4C). In conclusion, Treg that were co-stimulated with TNFR-SF agonists modified their phenotype in vivo after transfer and acquired rapidly increased capacity to expand in non-inflamed and inflamed lymphoid and non-lymphoid tissues.

Role of NF- κ B in Treg boost induced by agonists of TNFR-SF members.

Most reports on signal transduced by TNFR-SF agonists highlighted the role of canonical and non-canonical NF- κ B pathways. These studies were obtained mostly with cell lines. The most reliable biochemical analysis to study this pathway consists in measuring the capacity of nuclear extract proteins to bind to a consensus NF- κ B sequence (EMSA assay), combined with supershift to assess the activation status of the different NF- κ B subunits. This technique was performed on Treg pre-stimulated with coated anti-CD3 and anti-CD28 mAb for 24 hours to synchronize their activation and induced TNFR-SF up-regulation, followed by co-stimulation with agonists of TNFR2, 4-1BB, GITR, DR3 and OX40. Compared to the first four agonists, OX40 agonist was the least efficient to activate NF- κ B by far. The four others induced strong NF- κ B activation 30 minutes after initial co-stimulation with sustained activation for at least 4 hours (Fig 5A). When looking at the identity of the different NF- κ B members by supershift, RelA and cRel were present in cells co-stimulated by TNFR2, 4-1BB and DR3 agonists whereas cRel was predominant in cells co-stimulated by GITR agonist.

Since binding of RelA to its DNA target by agonists of TNFR-SF members in Treg was consistently observed, we analyzed whether the effect of these agonists on Tregs would be lost in the absence of RelA. Compared to controls, RelA-deficient Treg had reduced survival and proliferation, reaching the levels of the culture in the absence of agonists only for survival with agonists of DR3 and GITR (Fig 5B). This suggested that the stimulatory effect of agonists of TNFR2 and 4-1BB was partially dependent on RelA and the effect of agonists of GITR and DR3 was fully dependent on

RelA, at least for Treg survival. To further assess the role of RelA and cRel, we compared genes that were up-regulated by the TNFR2 agonists with genes that were down-regulated in Treg that were knockout for RelA, cRel or both. Interestingly, most of the genes up-regulated by TNFR2 were down-regulated in one or the other knockout Treg (142 out of 245) (Fig 5C). When looking at the NF- κ B pathway, many genes that were differentially expressed by TNFR2 triggering project at different levels in the pathway (membrane-bound proteins, adaptors, kinases or transcription factors) (Fig Sup 13). The same was observed with 4-1BB triggering (not shown). Altogether, these experiments showed that agonists of TNFR2, 4-1BB, GITR and DR3 activated Treg with a critical role of the canonical NF- κ B pathway.

Discussion

There are multiple drugs targeting TNF-SF are TNFR-SF members. Some are widely used to treat autoimmune diseases (ie of anti-TNF) and many others are in clinical trials in cancers, autoimmune and inflammatory diseases. Although their mechanisms of action are likely diverse, there is strong evidence that part of them are Treg-mediated for different reasons: (i) Treg specifically expressed at high levels several TNFR-SF members (ie TNFR2, 4-1BB, GITR, OX40...), (ii) different agonists of TNFR-SF members increased Treg expansion in vitro, and (iii) injecting agonists of several TNFR-SF induced Treg expansion, although it could be via an indirect mechanism. Besides these findings, we know surprisingly very little on the direct biologic effect of these molecules on Treg.

Our study showed for the first time the following new findings: (i) Agonists of 4-1BB, GITR and DR3 increased Treg survival, (ii) there was an additive effect of combining agonists of DR3 with TNFR2, 4-1BB or GITR or combining agonists of 4-1BB with TNFR2 or GITR for Treg proliferation and survival, (iii) at the whole transcriptome level, agonists of TNFR2, 4-1BB, DR3 and OX40 induced a surprisingly comparable signature, although the global effect was stronger with agonists of TNFR2 and 4-1BB, (iv) agonists of 4-1BB, GITR and DR3 did not act simply by increasing TNF/TNFR2 signaling in Treg, (v) agonists of TNFR2, 4-1BB, GITR and DR3 induced a type 2 Treg profile, (vi) agonists of TNFR2 and GITR increased the capacity of Treg to control colitis, (vii) agonists of TNFR2, 4-1BB, GITR and DR3 had a direct effect to increase Treg proliferation and expansion in vivo in lymphoid and non-lymphoid organs, (viii) agonists of TNFR2, 4-1BB, GITR and DR3 activated the canonical NF- κ B pathway by increasing bindings of RelA and cRel to its DNA target, (iv) part of the increased Treg proliferation and survival induced by agonists of TNFR2, 4-1BB, GITR and DR3 was lost when using RelA-deficient cells. Overall, we showed here that triggering several members of the TNFR-SF in Treg modified their biology, increasing their capacity to proliferate and survived due to increased canonical NF- κ B activity.

One of the major surprises of our findings was the strong similarities of the effects of agonists of TNFR2, 4-1BB, GITR and OX40 when looking at the whole transcriptome level or on some gene sets using heatmap representations. Since we excluded the possibility that this was due to triggering the same receptor (TNFR2), this indicates that their downstream signaling pathway are somehow very closed to each others. There is clearly something special about OX40 triggering that did not co-stimulate Treg proliferation and survival, which was likely due to the absence of canonical NF- κ B activation.

It was shown previously that triggering TNFR2, 4-1BB, GITR and DR3 increased Treg proliferation in vitro ([22], [23], [24], [25]). We confirmed here these findings. It was also showed that injection of agonists of these receptors also increased Treg expansion in vivo. However, this effect could be indirect since these agonists increased activation and IL-2 produced by Tconv that may boost Treg proliferation ([38]). This seems to be the case for OX40 since injection of an agonist of this receptor induced strong Treg proliferation ([12]) whereas we did not see any effect in vitro. Mechanistically, it was proposed that OX40 triggering increased Treg sensitivity to IL-2, due to decreased SOCS1 and increased miR155 expression ([39]). Thus, the absence of an effect of OX40 agonist in vitro could be due to high IL-2 level in the culture. Importantly, since we pre-incubated Treg with the agonist before their adoptive transfer (instead of injecting the agonists to the mice), we show here for the first time that agonists of TNFR2, 4-1BB, GITR and DR3 increased Treg proliferation also in vivo by a direct effect.

We showed here that agonists of TNFR2, 4-1BB, GITR, and DR3 increased Treg survival. This was shown previously for conventional T cells but not for Treg ([31]). In conventional T cells, it was observed that increased survival was associated with increased expression of pro-survival molecules, such as surviving, Bcl2, Bcl-XL, cFlipshort or Bfl-1 and decreased expression of pro-apoptotic molecules, such as Bim and Bad ([31], [40], [41]). The mechanism of Treg survival induced by TNFR-SF may be only partly similar to Tconv since, from one side, we also observed increased expression of Bcl2l1 (coding for Bcl-XL) and decreased expression of Bcl2l11 (coding for Bim) but, on the other side, we had reduced expression of Bcl2 and no significant difference for the other described molecules. Other molecules may play a role since we found modifications of the expression of caspases (Casp2 and 7), Ripk1 and Ripk3 or TRAF molecules (Traf1, 3, 6 and 7), all involved in T cell survival or apoptosis. Interestingly, Treg co-stimulated by TNFR-SF agonists expressed lower level of Il6ra and Il6st (coding for Gp130), both involved in IL-6 signaling transduction, lower level of Il1a and higher level of Sema4a and Nrp1. Since it was shown that IL-6 and IL-1 rendered Treg unstable whereas Sema4a and Nrp1 would increase their stability ([42], [43]), co-stimulated Treg could be more stable in inflammatory contexts.

In general, it has been shown that TNFR-SF agonists did not modified Treg function in vitro, as we confirm here. However, these in vitro assays only reflect the “tip of the iceberg” of the suppressive potential of Treg that are able to use very divers mechanisms of suppression in vivo. Our findings that Treg, pre-incubated for only 2 hours with agonists of TNFR2 or GITR before transfer, were more efficient to suppress colitis suggest that such co-stimulation would improve their suppressive function in vivo. Alternatively, their increased efficacy could be well due to their increased proliferation and expansion, as we showed in another setting 7 days after transfer.

Whatever the involved mechanism, our findings are important in regards to cell therapy using Treg. This is a therapeutic approach that is intensively assessed in pre-clinical experiments and some clinical assays to limit graft rejection, GvHD or autoimmune and inflammatory diseases ([44]). The major challenge is to obtain high amounts of pure, stable and functional Treg obtained after cell culture. Adding agonists of TNFR-SF to the culture might help to reach this goal given their capacity to increase Treg proliferation, expansion and maybe in vivo suppressive function. The tendency toward a type 2 profile of Treg co-stimulated by the TNFR-SF agonists was not expected. It might be due to the increased expression of Gata3, Stat5, Stat6 and AP-1 factors (Jun and Fos12), all involved in Th2 differentiation of Tconv ([45]), although decreased expression of Maf and Irf4 may limit type 2 Treg differentiation. Our findings may suggest that Treg co-stimulated by TNFR-SF agonists would be more adapted to control Th2 type rather than Th1 type diseases because of increased expression of CCR4 and decreased expression of CXCR3. Also, looking at the suppressive molecules down-regulated by TNFR-SF agonists (for example Furin, Lrrc32, Tigit, Nt5e, Fgl2, Entpd1, Icos, Il10 or Il2ra) or up-regulated by TNFR-SF agonists (for example Il2rg, Ebi3 or Areg) may indicate that these co-stimulated Treg would be more appropriate to control certain types of diseases rather than others.

The signal transduction induced by TNFR-SF agonists in vitro has been hardly studied in Treg. Transcriptomic analyses have been reported in only two papers. In one, they studied the effect of TNF on human Treg but there was no TCR/CD28 stimulation ([36]). In the other, they studied the effect of an agonist of OX40 in combination with TCR activation on purified Treg in vitro ([37]), which is the condition that resembles the most to our experimental setting. However, they detected only 21 DE genes, suggesting that co-stimulation was maybe not optimal since we could detect over 100 DE genes induced by OX40 triggering (FRD<0.5, FC>2). Also, we observed that OX40 is the one, among the 5 studied TNFR-SF, that maybe the least interesting because its triggering had no effect on Treg proliferation and survival. Thus, using our transcriptomic analysis, comparing the effect of agonist of TNFR2, 4-1BB, DR3 and OX40 on Treg, opens a new field of investigation. As for the transcriptome, few biochemical data have been generated to explore the signal transduced by TNFR-SF on Treg. Looking at quantity of I κ B α , and NF- κ B members (NF κ B1, NF κ B2, RelA, cRel and RelB) by Western blot, it was suggested that triggering TNFR2, GITR and DR3 activate canonical and non-canonical NF- κ B pathways ([32], [33], [34], [35], [46]). However, these assays are less informative than the EMSA and supershift assays that we did since amounts of a transcription factor does not reflect necessarily that they actually bind to their DNA target. Our study challenges previous findings and indicates that TNFR-SF agonists activate the canonical NF- κ B pathway with DNA binding of cRel and RelA. We do not find any evidence of the activation

of the non-canonical pathway, which may be even inhibitory for Treg since GITR-costimulation induced an increased proliferation when cells were deficient for NF- κ B-inducing kinase, an upstream activator of this pathway ([35]). This was confirmed using RelA-deficient Treg in which part of the biological activity induced by TNFR-SF co-stimulation was lost. This was also supported by comparing the signatures induced by TNFR-SF co-stimulation and by the deficiency of RelA or cRel. The other signaling pathways that have been proposed for Treg were activation of the three MAPK pathways (p38, ERK and JNK), all analyzed by Western blot ([33], [34]). Gene ontology analysis of our transcriptomes did not support MAPK activation of Treg co-stimulated by TNFR-SF agonists. Indeed, whereas NF- κ B, TNFR2, CD40, 4-1BB, RANK, Th2, apoptosis signaling pathways showed-up with very low p values, the MAPK signaling pathways were never observed.

Materials and Methods

Mice. Foxp3-CRE-IRES-YFP (Foxp3Cre) ([47]) and Foxp3-IRES-GFP ([48]) (Foxp3GFP) knock-in mice were kindly given by Prs. Alexander Rudensky and Bernard Malissen, respectively. Foxp3-DTR (Foxp3^{tm3}(DTR/GFP)^{Ayr/J}), Tnf^{-/-} (Tnftm1Gk1/J), Tnfrsf1b^{-/-} (Tnfrsf1btm1Mwm/J) were obtained from the Jackson laboratory. Cd3^{-/-} (CD3^{etm1}Mal) and RAG2^{-/-} mice were obtained from the cryopreservation distribution typing and animal archiving department (Orléans, France). Relaflox and Relflox mice were previously described ([49], [50]). All mice were on a C57Bl/6 background. Mice were housed under specific pathogen-free conditions. All experimental protocols were approved by the local ethics committee and are in compliance with European Union guidelines.

Cell preparation from tissues. For lymphoid tissues, cells were isolated by mechanical dilacerations. For non-lymphoid tissues, anesthetized mice were perfused intracardially with cold PBS. Small pieces of livers and lungs were digested in type IV collagenase (0.3 mg/ml) and DNase I (100 µg/ml) for 30 min at 37°C, followed by Percoll gradient (30–70%) separation. For CNS analysis, spinal cord and brain were harvested from mice. Tissues were digested in type IV collagenase (1 mg/ml) and DNase I (100 µg/ml) for 30 min at 37°C, followed by Percoll gradient (80-40%).

Antibodies and flow cytometry analysis. The following mAbs from BD Biosciences were used: anti-CD45 (30-F11), anti-CD4 (RM4-5), anti-CD62L (MEL-14), anti-CD90.1 (OX-7), anti-CD90.2 (30-H12), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD25 (PC61 or 7D4), anti-ICOS (7E.17G9), anti-GITR (DTA-1), anti-CD103 (M290), anti-CTLA-4 (UC10-4F10-11), anti-GATA3 (L50-823), anti-CD49d (R1-2), anti-CD11a (2D7), anti-CXCR3 (CXCR3-173), anti-CCR6 (140706). Anti-GFP antibody was purchased from Life Technologies. Anti-CD3 (145-2C11), anti-Foxp3 (FJK-16s), anti-CD44 (IM7), anti-Ki-67 (SOLA15), anti-CD73 (TY/11.8) were purchased from eBioscience, and Foxp3 staining was performed using the eBioscience kit and protocol. Cell survival was assessed with fixable viability dyes (e780 and e506). Cells were acquired on a BD LSRII and a BD Fortessa X20 cytometers and analyzed using FlowJo software.

Treg and Tconv cell purification. Tregs were purified from spleen and lymph nodes after enrichment of CD25⁺ cells using biotinylated anti-CD25 mAb (7D4) and anti-biotin microbeads (Miltenyi Biotec), followed by CD4 staining (RM4.5) and cell sorting of CD4⁺ Foxp3/YFP⁺ cells or CD4⁺ Foxp3/GFP⁺ using the BD FACSAria II. Tconv cells were purified after enrichment of CD25-

cells using biotinylated anti-CD25 mAb (7D4) or of CD8-CD19-CD11b- cells using biotinylated anti-CD8 (53-6.7), CD19 (1D3) and CD11b (M1/70) mAbs and anti-biotin microbeads (Miltenyi Biotec), followed by CD4 staining (RM4.5) and cell sorting of CD4⁺ Foxp3/YFP⁻ cells or CD4⁺ Foxp3/GFP⁻ using the BD FACSAria II.

Cell cultures. Purified Tregs were cultivated with RPMI 1640 w/glutamax-FBS 10% with IL-2 (10 ng/ml, Peproteck). They were stimulated either with an anti-CD3 antibody (1 or 2 µg/ml, 2C11, BioXcell) and anti-CD28 (2 µg/ml) in a flat plate (15 x 10⁴ cell/well) or with irradiated splenocytes isolated from CD3^{-/-} mice (7.5 x 10⁴ cell/well) and an anti-CD3 antibody (0.05 µg/ml) in a round plate (2.5 x 10⁴ cell/well). The following compounds were used soluble to stimulate Tregs: anti-41BB (10 µg/ml, 3H3, BioXcell), anti-GITR (3 µg/ml, DTA-1, BioXcell), OX40L (100 ng/ml, AdipoGen), TNFR2L (STAR2) (12 ng/ml, [18]), TL1A (12 ng/ml, ?), LIGHT (AdipoGen), CD70 (?), RANKL (Adipogen), FASL (Adipogen), TRAIL (Adipogen), anti-DR5 (MD5-1, BioXCell). Proliferation was assessed with labeling of Tregs with CellTrace Violet (following the protocol of Life Technologies) and the following formula was used to calculate the “Relative Division”= [(MFI CT CTRL Tregs – MFI CT TNFR Tregs)/ MFI CT CTRL Tregs] x 100.

For the suppression assay, Tconv (2.5 x 10⁴ cell/well), labeled with CellTrace Violet (following the protocol from Life technologies), were incubated with different concentration of Tregs and splenocytes from CD3^{-/-} mice (7.5 x 10⁴ cell/well) with an anti-CD3 antibody (0.05 µg/ml) in a round plate with RPMI 1640 w/glutamax-FBS 10%.

Cytokine quantification. Cytokine production was assessed using supernatants from 3 days cultures of Tregs stimulated with coated antibodies and agonists as explained in “cell culture”. It was used the Luminex kit from R&D systems according to manufacturer’s procedure.

Colitis. Tconv cells (CD4+GFP⁻, 1 x 10⁵ cells) and Tregs (CD4+YFP⁺, 2 x 10⁴ cells) were injected intravenously into sex-matched RAG2^{-/-} mice. Tregs were pre-incubated 2h at 37°C in cRPMI with IL-2 and anti-GITR antibody (12 µg/ml) and STAR2 (48 ng/ml). The clinical evaluation was performed three times a week by measuring body weight.

T-cell adoptive transfer. Tregs from Foxp3-IRES-GFP and Foxp3-DTR mice were purified and cultivated from 3 days as explained before. Cells were rested for 24h in cRPMI and IL-2 and then co-injected intravenously (5 x 10⁵ of each group) in C57BL/6 mice (CD90.2/CD45.2). Mice were sacrificed after 12 hours and 7 days post injection. Spleen, pLN, mLN, lung and liver were harvested.

RNA-sequencing. Tregs were cultivated in a flat plate (see conditions above) for 18 and 36 hours. Cells were harvested and RNA extracted using the NucleoSpin RNA XS kit from Macherey-Nagel following their protocol. Illumina paired-end sequencing was performed. Quality of raw data has been evaluated with FastQC. Poor quality sequences have been trimmed or removed with Trimmomatic software to retain only good quality paired reads. Star v2.5.3a [51] has been used to align reads on reference genome mm10 using standard options. Quantification of gene and isoform abundances has been done with rsem 1.2.28 [52], prior to normalisation on library size with DESeq2 bioconductor package. Finally, differential analysis has been conducted with edgeR bioconductor package. Multiple hypothesis adjusted p-values were calculated with the Benjamini-Hochberg procedure to control FDR.

Electrophoretic Mobility Shift Assays (EMSA) combined with supershift assays. Tregs from Foxp3-IRES-GFP were stimulated for 24h with anti-CD3/CD28 and rested for 1.5 or 4 hours. Cells were stimulated with and without TNFSF members and IL-2 for 30 minutes, 1h and 4h. Nuclear extracts were prepared and analyzed for DNA binding activity using the HIV-LTR tandem κ B oligonucleotide as κ B probe (42). For supershift assays, nuclear extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe.

EAE induction and evaluation. For EAE, Tregs were cultured for 3 days with coated antibodies and agonists (see "cell culture"). Cells were rested for 24h in complete RPMI and IL-2, washed in PBS and injected intravenously in mice (106/mouse). Two different protocols were tested: EAE induced after 1 days of Treg injection or Treg injection after 7 days of EAE induction. EAE was induced in the following way: mice were injected subcutaneously in the flanks with 100 μ g of MOG35-55 peptide (Polypeptide) emulsified in 100 μ l of CFA (Sigma-Aldrich) supplemented with 50 μ g of heat-killed Mycobacterium tuberculosis H37Ra (BD Biosciences). Animals were additionally injected intravenously with 200 ng of Bordetella pertussis toxin (Enzo) at the time of, and two days following immunization.

The clinical evaluation was performed on a daily bases by a 6-point scale ranging: 0, no clinical sign; 1, limp tail; 2, limp tail, impaired righting reflex, and paresis of one limb; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, moribund/death. A score of 5 was permanently attributed to dead animals.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Software. For the statistical analysis, multiple t-test was used for data following a normal distribution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means \pm SEM were used throughout the figures.

Acknowledgments

References

1. Assal, A., et al., Emerging targets in cancer immunotherapy: beyond CTLA-4 and PD-1. *Immunotherapy*, 2015. 7(11): p. 1169-86.
2. Croft, M., C.A. Benedict, and C.F. Ware, Clinical targeting of the TNF and TNFR superfamilies. *Nat Rev Drug Discov*, 2013. 12(2): p. 147-68.
3. Burugu, S., A.R. Dancsok, and T.O. Nielsen, Emerging targets in cancer immunotherapy. *Semin Cancer Biol*, 2018. 52(Pt 2): p. 39-52.
4. Kocak, E., et al., Combination therapy with anti-CTLA-4 and anti-4-1BB antibodies enhances cancer immunity and reduces autoimmunity. *Cancer Res*, 2006. 66(14): p. 7276-84.
5. Menk, A.V., et al., 4-1BB costimulation induces T cell mitochondrial function and biogenesis enabling cancer immunotherapeutic responses. *J Exp Med*, 2018. 215(4): p. 1091-1100.
6. Piconese, S., B. Valzasina, and M.P. Colombo, OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection. *J Exp Med*, 2008. 205(4): p. 825-39.
7. Bulliard, Y., et al., OX40 engagement depletes intratumoral Tregs via activating FcγRs, leading to antitumor efficacy. *Immunol Cell Biol*, 2014. 92(6): p. 475-80.
8. Bulliard, Y., et al., Activating Fcγ receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies. *J Exp Med*, 2013. 210(9): p. 1685-93.
9. Sun, Y., et al., Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis. *J Immunol*, 2002. 168(3): p. 1457-65.
10. Choi, B.K., et al., 4-1BB-mediated amelioration of experimental autoimmune uveoretinitis is caused by indoleamine 2,3-dioxygenase-dependent mechanisms. *Cytokine*, 2006. 34(5-6): p. 233-42.
11. Chen, S., et al., Combination of 4-1BB agonist and PD-1 antagonist promotes antitumor effector/memory CD8 T cells in a poorly immunogenic tumor model. *Cancer Immunol Res*, 2015. 3(2): p. 149-60.
12. Ruby, C.E., et al., Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right. *J Immunol*, 2009. 183(8): p. 4853-7.
13. Zemmour, D., et al., Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nat Immunol*, 2018. 19(3): p. 291-301.
14. Arvey, A., et al., Genetic and epigenetic variation in the lineage specification of regulatory T cells. *Elife*, 2015. 4: p. e07571.

15. Magnuson, A.M., et al., Identification and validation of a tumor-infiltrating Treg transcriptional signature conserved across species and tumor types. *Proc Natl Acad Sci U S A*, 2018. 115(45): p. E10672-E10681.
16. Plitas, G., et al., Regulatory T Cells Exhibit Distinct Features in Human Breast Cancer. *Immunity*, 2016. 45(5): p. 1122-1134.
17. Bacher, P., et al., Regulatory T Cell Specificity Directs Tolerance versus Allergy against Aeroantigens in Humans. *Cell*, 2016. 167(4): p. 1067-1078 e16.
18. Chopra, M., et al., Exogenous TNFR2 activation protects from acute GvHD via host T reg cell expansion. *J Exp Med*, 2016. 213(9): p. 1881-900.
19. Irie, J., et al., Modulating protective and pathogenic CD4+ subsets via CD137 in type 1 diabetes. *Diabetes*, 2007. 56(1): p. 186-96.
20. Ephrem, A., et al., Modulation of Treg cells/T effector function by GITR signaling is context-dependent. *Eur J Immunol*, 2013. 43(9): p. 2421-9.
21. Schreiber, T.H., et al., Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation. *J Clin Invest*, 2010. 120(10): p. 3629-40.
22. Chen, X., et al., Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J Immunol*, 2007. 179(1): p. 154-61.
23. Zheng, G., B. Wang, and A. Chen, The 4-1BB costimulation augments the proliferation of CD4+CD25+ regulatory T cells. *J Immunol*, 2004. 173(4): p. 2428-34.
24. Valzasina, B., et al., Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR. *Blood*, 2005. 105(7): p. 2845-51.
25. Taraban, V.Y., et al., Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia. *Mucosal Immunol*, 2011. 4(2): p. 186-96.
26. Chen, X., et al., Expression of costimulatory TNFR2 induces resistance of CD4+FoxP3- conventional T cells to suppression by CD4+FoxP3+ regulatory T cells. *J Immunol*, 2010. 185(1): p. 174-82.
27. Zaragoza, B., et al., Suppressive activity of human regulatory T cells is maintained in the presence of TNF. *Nat Med*, 2016. 22(1): p. 16-7.
28. Stephens, G.L., et al., Engagement of glucocorticoid-induced TNFR family-related receptor on effector T cells by its ligand mediates resistance to suppression by CD4+CD25+ T cells. *J Immunol*, 2004. 173(8): p. 5008-20.
29. Takeda, I., et al., Distinct roles for the OX40-OX40 ligand interaction in regulatory and nonregulatory T cells. *J Immunol*, 2004. 172(6): p. 3580-9.

30. Croft, M. and R.M. Siegel, Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases. *Nat Rev Rheumatol*, 2017. 13(4): p. 217-233.
31. So, T. and M. Croft, Regulation of PI-3-Kinase and Akt Signaling in T Lymphocytes and Other Cells by TNFR Family Molecules. *Front Immunol*, 2013. 4: p. 139.
32. Wang, J., et al., TNFR2 ligation in human T regulatory cells enhances IL2-induced cell proliferation through the non-canonical NF-kappaB pathway. *Sci Rep*, 2018. 8(1): p. 12079.
33. Bittner, S. and M. Ehrenschwender, Multifaceted death receptor 3 signaling-promoting survival and triggering death. *FEBS Lett*, 2017. 591(17): p. 2543-2555.
34. Joetham, A., et al., Loss of T regulatory cell suppression following signaling through glucocorticoid-induced tumor necrosis receptor (GITR) is dependent on c-Jun N-terminal kinase activation. *J Biol Chem*, 2012. 287(21): p. 17100-8.
35. Lu, L.F., et al., NF kappa B-inducing kinase deficiency results in the development of a subset of regulatory T cells, which shows a hyperproliferative activity upon glucocorticoid-induced TNF receptor family-related gene stimulation. *J Immunol*, 2005. 175(3): p. 1651-7.
36. Nagar, M., et al., TNF activates a NF-kappaB-regulated cellular program in human CD45RA- regulatory T cells that modulates their suppressive function. *J Immunol*, 2010. 184(7): p. 3570-81.
37. Burocchi, A., et al., Intratumor OX40 stimulation inhibits IRF1 expression and IL-10 production by Treg cells while enhancing CD40L expression by effector memory T cells. *Eur J Immunol*, 2011. 41(12): p. 3615-26.
38. Grinberg-Bleyer, Y., et al., Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Tregs. *J Clin Invest*, 2010. 120(12): p. 4558-68.
39. Piconese, S., et al., A non-redundant role for OX40 in the competitive fitness of Treg in response to IL-2. *Eur J Immunol*, 2010. 40(10): p. 2902-13.
40. Starck, L., et al., Costimulation by CD137/4-1BB inhibits T cell apoptosis and induces Bcl-xL and c-FLIP(short) via phosphatidylinositol 3-kinase and AKT/protein kinase B. *Eur J Immunol*, 2005. 35(4): p. 1257-66.
41. Song, J., et al., Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion. *Immunity*, 2005. 22(5): p. 621-31.
42. Zhou, X., et al., Plasticity of CD4(+) FoxP3(+) T cells. *Curr Opin Immunol*, 2009. 21(3): p. 281-5.
43. Delgoffe, G.M., et al., Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis. *Nature*, 2013. 501(7466): p. 252-6.
44. Brusko, T.M., A.L. Putnam, and J.A. Bluestone, Human regulatory T cells: role in autoimmune disease and therapeutic opportunities. *Immunol Rev*, 2008. 223: p. 371-90.

45. Li, P., et al., Complex interactions of transcription factors in mediating cytokine biology in T cells. *Immunol Rev*, 2014. 261(1): p. 141-56.
46. Vasanthakumar, A., et al., The TNF Receptor Superfamily-NF-kappaB Axis Is Critical to Maintain Effector Regulatory T Cells in Lymphoid and Non-lymphoid Tissues. *Cell Rep*, 2017. 20(12): p. 2906-2920.
47. Rubtsov, Y.P., et al., Regulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity*, 2008. 28(4): p. 546-58.
48. Wang, Y., et al., Th2 lymphoproliferative disorder of LatY136F mutant mice unfolds independently of TCR-MHC engagement and is insensitive to the action of Foxp3+ regulatory T cells. *J Immunol*, 2008. 180(3): p. 1565-75.
49. Grinberg-Bleyer, Y., et al., NF-kappaB c-Rel Is Crucial for the Regulatory T Cell Immune Checkpoint in Cancer. *Cell*, 2017. 170(6): p. 1096-1108 e13.
50. Powolny-Budnicka, I., et al., RelA and RelB transcription factors in distinct thymocyte populations control lymphotoxin-dependent interleukin-17 production in gammadelta T cells. *Immunity*, 2011. 34(3): p. 364-74.
51. Dobin, A., et al., STAR: ultrafast universal RNA-seq aligner. *Bioinformatics*, 2013. 29(1): p. 15-21.
52. Li, B. and C.N. Dewey, RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics*, 2011. 12: p. 323.

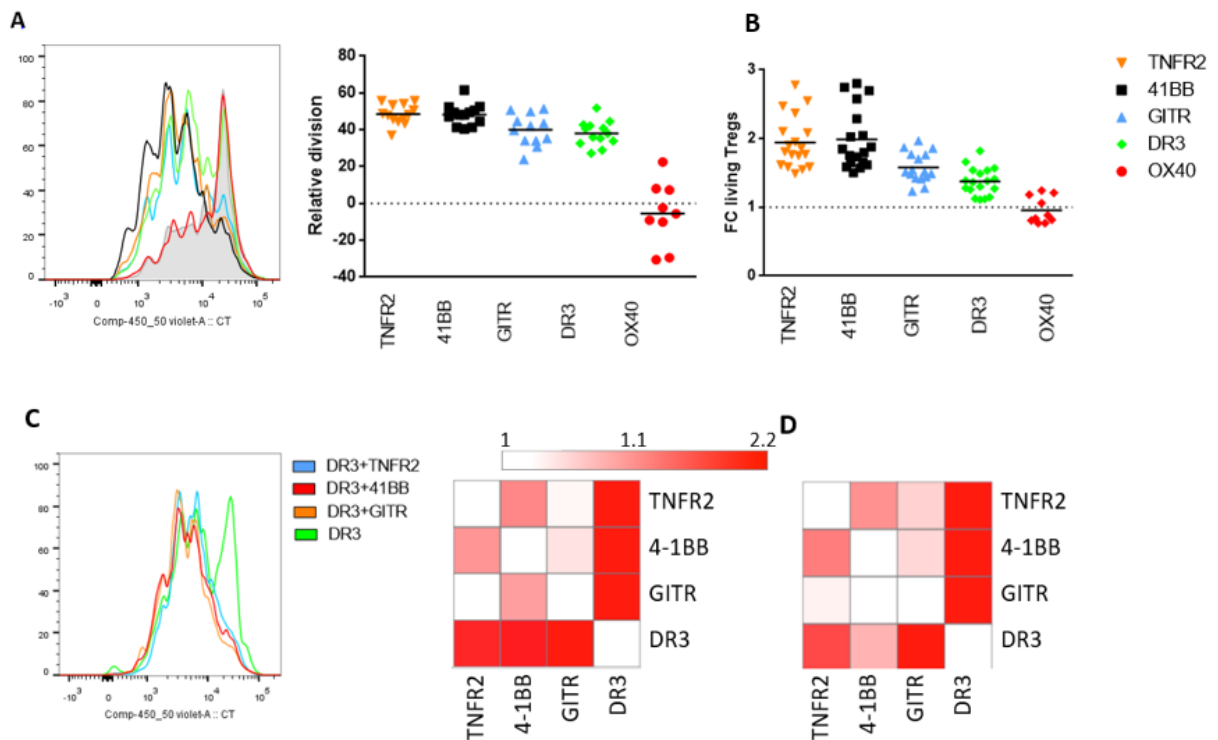


Figure 1 – Increased Treg proliferation and survival by agonists of TNFR2, 4-1BB, GITR and DR3. Tregs were cultivated with APCs/anti-CD3 and agonists for TNFRs for 3 days and proliferation and survival were quantified. (A, B) Cell trace profile and quantification of increased proliferation (A) and increased percentage of living cells (B), compared to CTRL tregs, when agonists are administrated individually. The left panel shows a representative experiment and right panels a pool of at least 9 experiments per condition. Each dot is an independent experiment. (C, D) TNFR agonists were combined one by one to measure increased proliferation (C) and survival (D), compared to the agonists alone (X axis of the heatmap). The left panel shows a representative cell trace profile with the DR3 agonist and the right panels a pool of 3 independent experiments.

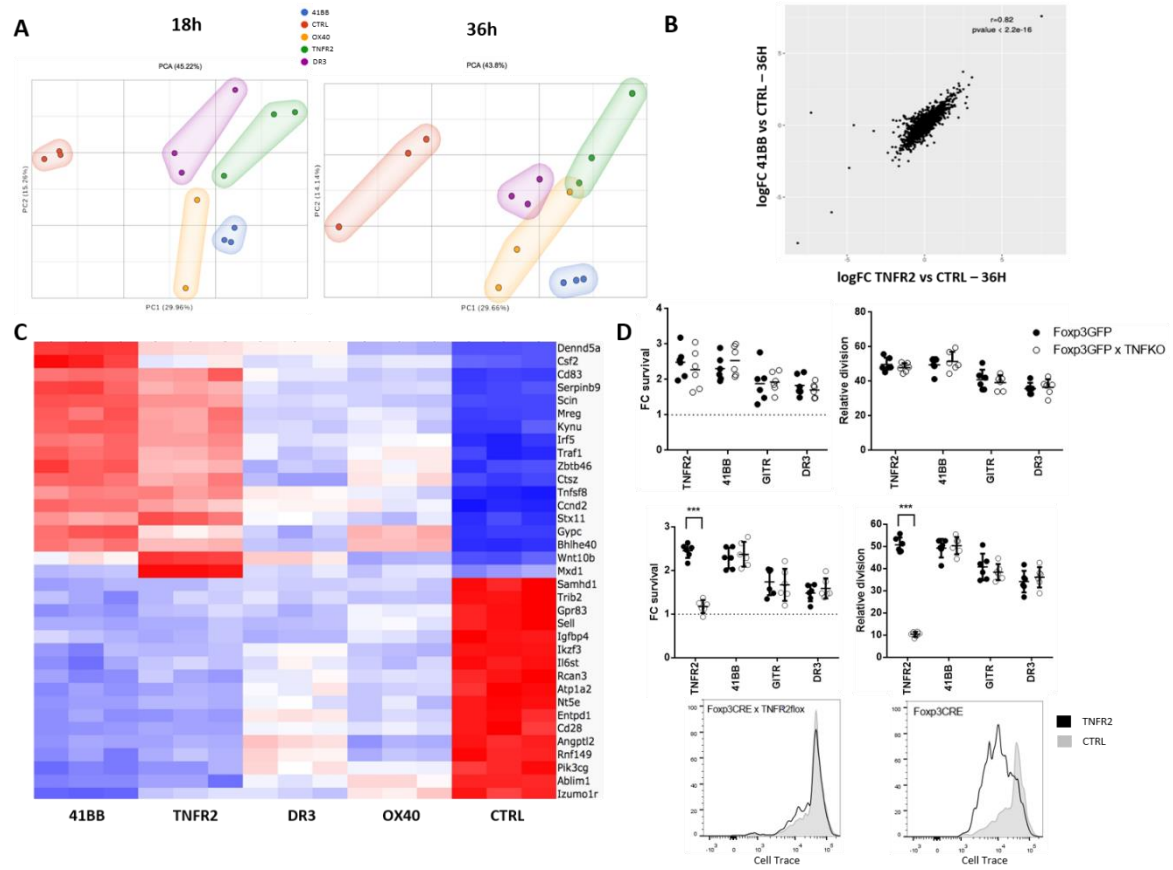


Figure 2 – Different TNFR-SF agonists induced a similar transcriptomic signature. RNA-sequencing was performed on Tregs stimulated with anti-CD3/CD28 mAb and agonists for TNFR-SF for 18 and 36 hours. (A) PCA analysis of the biological triplicates at both time points. (B) Correlation of DE genes between TNFR2vsCTRL (x axis) and 41BBvsCTRL (y axis) at 36h. (C) Top DE genes at 36 hours comparing the different TNFR-SF co-stimulation. (D) The effect of TNFR2, 41BB, GTR and DR3 agonists on Treg was assessed when cells did not express TNF or TNFR2. Pool of 3 experiments is shown.

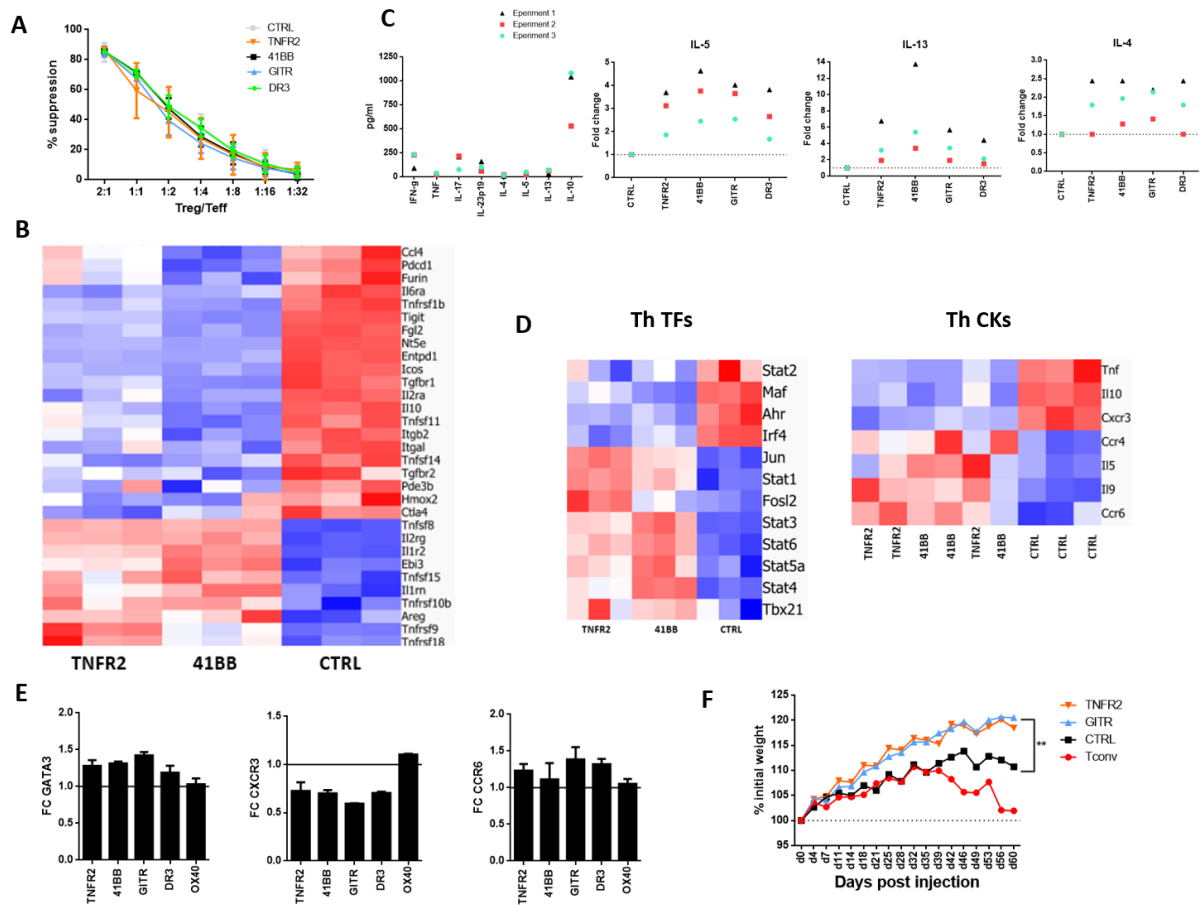


Figure 3 – TNFR-SF co-stimulation of Treg modified their suppressive molecules and increased their capacity to control an inflammatory disease. (A) Tregs, pre-activated with anti-CD3/CD28 mAb and TNFR-SF agonists, were tested for their *in vitro* suppressive function. This is representative of 3 independent experiments. (B) DE genes among a gene sets of Treg effector molecules on cells activated as in Figure 2. (C) Cytokines in the supernatants were measured after 3 days of culture of Treg activated with anti-CD3/CD28 mAb and TNFR-SF agonists. The left panel shows values in the control culture and the right panels show the fold-change compared to the control culture. Each dots represent 3 independent experiment. (D) DE genes among gene sets of Th transcription factors and Th cytokine and chemokine receptors on cells activated as in Figure 2. (E) Fold change compared to control Treg of GATA3, CXCR3 and CCR6 expression, assessed by flow cytometry, after a 3 days culture with TNFR agonists and coated anti-CD3/CD28. (F) Treg pre-incubated with agonists for GITR or TNFR2 where injected in RAG KO mice to assess their ability to control colitis compared to CTRL Tregs. Statistical significance was investigated with the ANOVA analysis. Pool of 2 independent experiments with 6 mice per group.

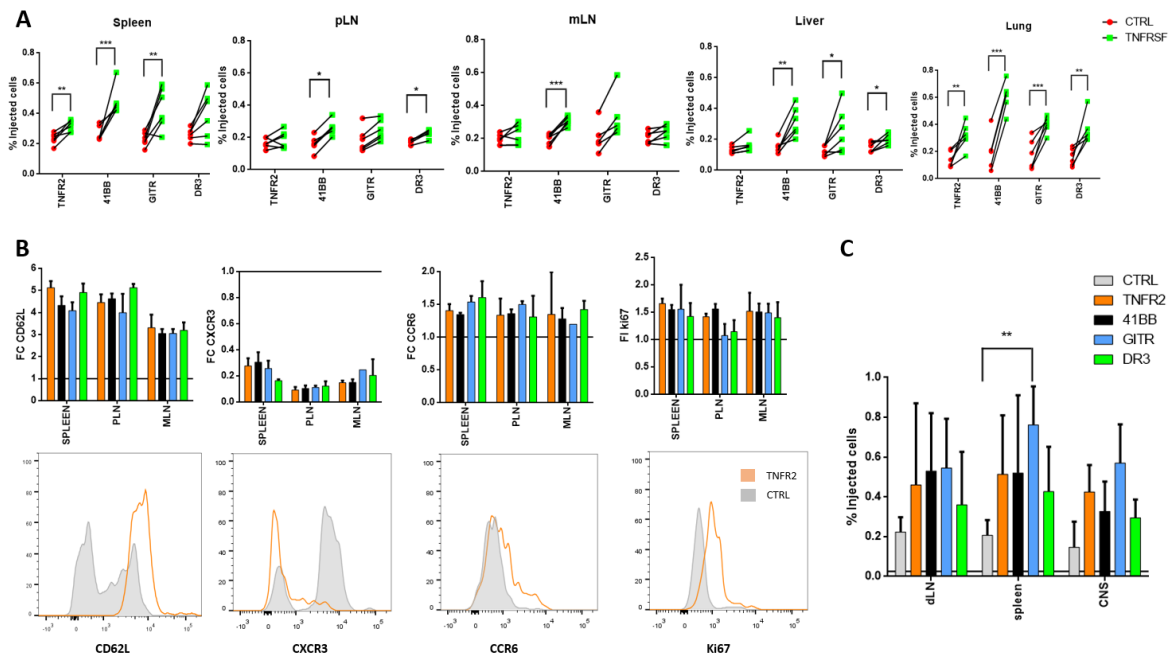


Figure 4 – TNFR-SF co-stimulation of Treg increased their in vivo expansion and modified their tissue re-circulation. Tregs, pre-activated with anti-CD3/CD28 mAb and TNFR-SF agonists, were tested for their in vivo homeostasis. (A, B) Control Treg and Treg co-stimulated with a TNFR-SF agonist were co-injected in equal numbers and analyzed 7 days later. Data show 2 independent experiments with 6 mice per group. (A) Proportion of co-injected Treg in various organs. Each dot is a mouse and lines connect cells from the same mouse. (B) CD62L, CXCR3, CCR6 and Ki67 expression among injected cells. Upper panels show FC of co-stimulated Treg compared to control Treg for each individual mouse. Lower panels show representative overlays for CTRL and TNFR2 co-stimulated Treg in the spleen. (C) TNFR-SF co-stimulated Treg were injected in mice at day 0 or day 7 of EAE induction. Percentage of injected Treg at day 30 post injection. Pool of 2 independent experiments with 6 mice per group. Statistical significance was assessed by ANOVA analysis.

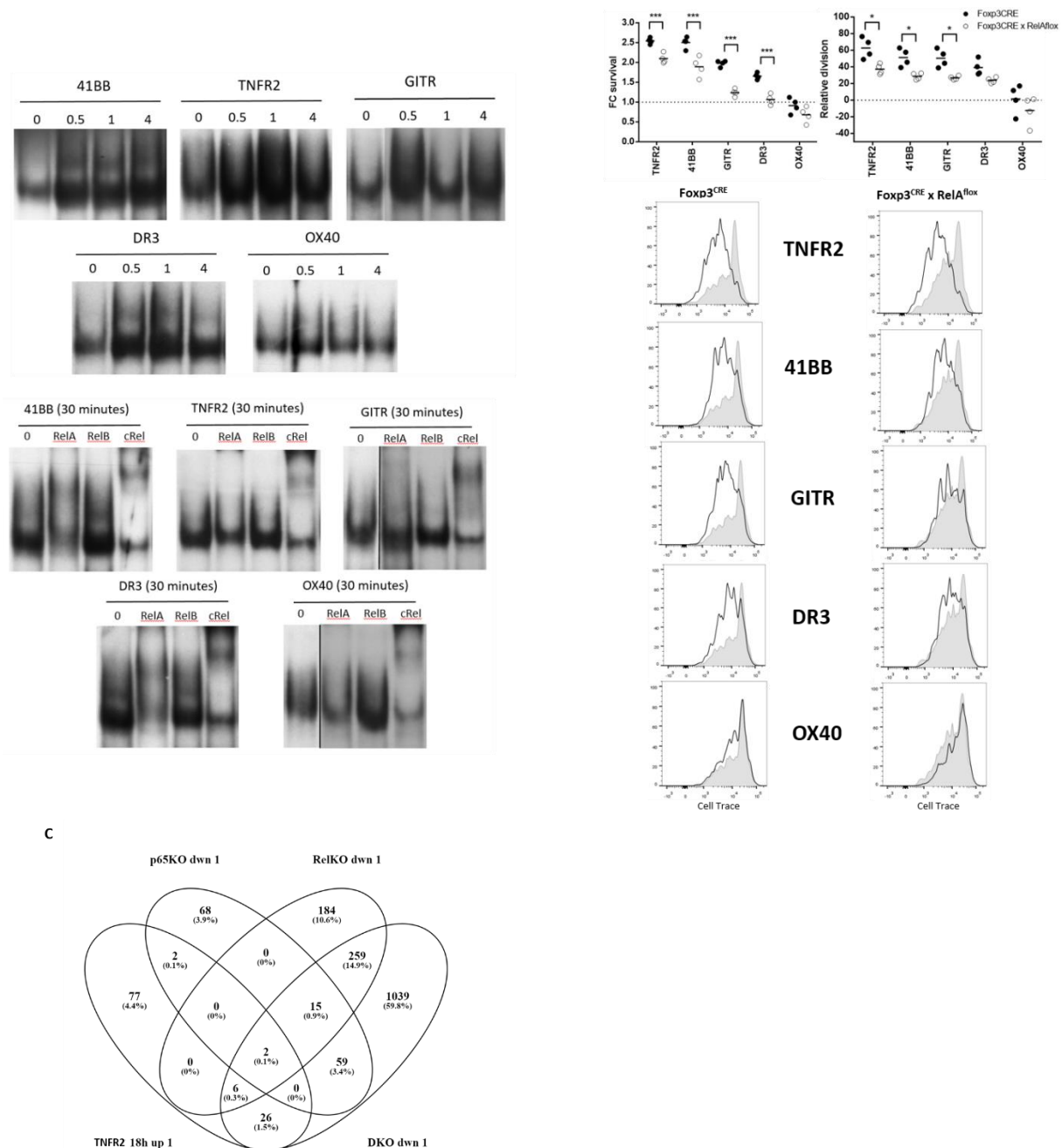
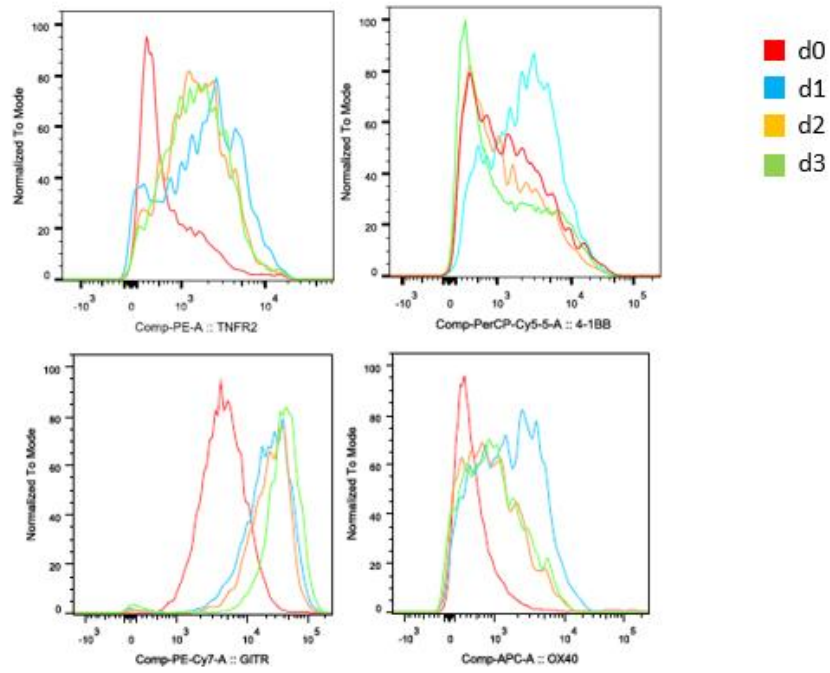
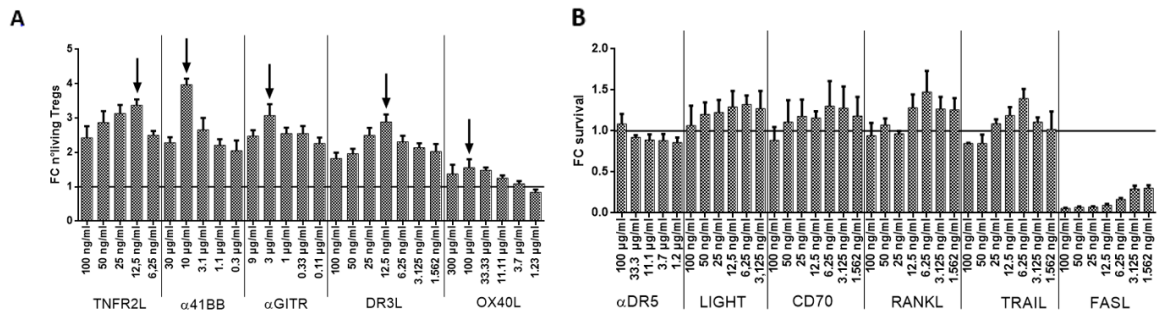


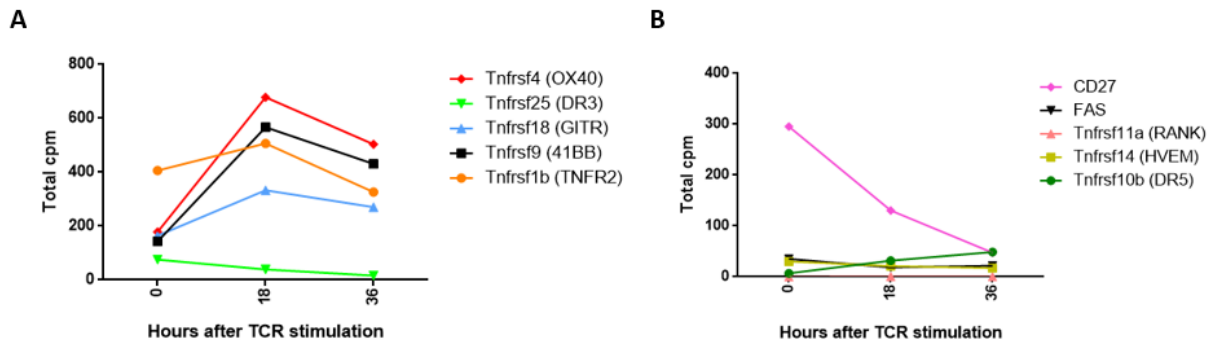
Figure 5 – TNFR-SF co-stimulation of Treg activated the canonical NF-κB pathway (A) Treg, pre-activated with anti-CD3/CD28 mAb, were re-stimulated with and TNFR-SF agonists alone. EMSA (upper panels) and supershift (lower panels) analyses at 30 minutes, 1h and 4h to investigate NF-κB activation. A representative of 3 independent experiments is shown. (B) Treg from control *Foxp3^{CRE}* or *Foxp3^{CRE} x RelA^{lox}* mice or were stimulated with anti-CD3/CD28 mAb and TNFR-SF agonists. (Upper panels) Fold change of survival and proliferation compared to control Treg stimulated without TNFR-SF co-stimulation. Pool of 2 independent experiments. Each dot is a mouse. (Lower panels) Representative proliferation overlays of Treg stimulated with or without TNFR-SF agonist. (C) Venn diagram showing genes up-regulated by TNFR2 triggering (STAR) and down-regulated by the KO of RelA (p65) or cRel (Rel) on Treg activated with anti-CD3/CD28 mAb for 18h (STAR) or 4h (RelA and cRel KO).



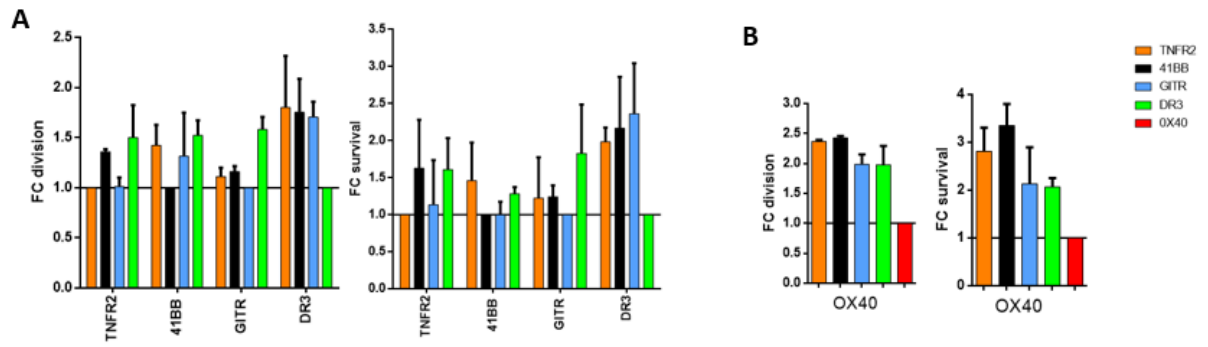
Supplemental figure 1 – Kinetic expression of TNFR2, 41BB, GITR and OX40. Expression of TNFR2, 41BB, GITR and OX40 was assessed by flow cytometry on freshly purified Tregs and after 24, 48 and 72 hours of culture with agonists and beads coated with anti-CD3/CD28.



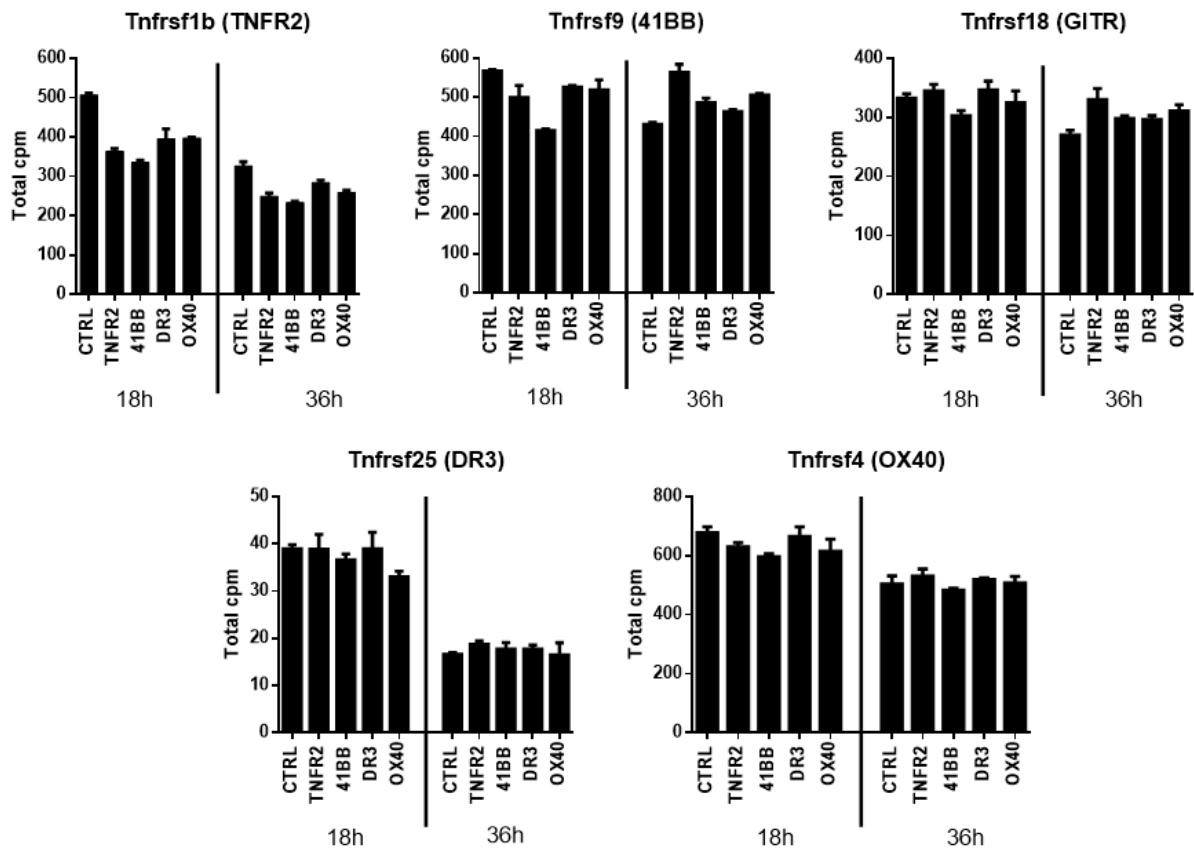
Supplemental figure 2 – Titration of agonists for TNFR-SF. Tregs were cultivated for 3 days with beads coated with anti-CD3/CD28 and different doses of agonists for TNFRs. (A) The four receptors that boosted Treg survival and proliferation (not shown) and OX40. The dose with the highest survival was selected for the rest of the experiments. (B) TNFR-SF agonists that did not boost Treg survival and proliferation.



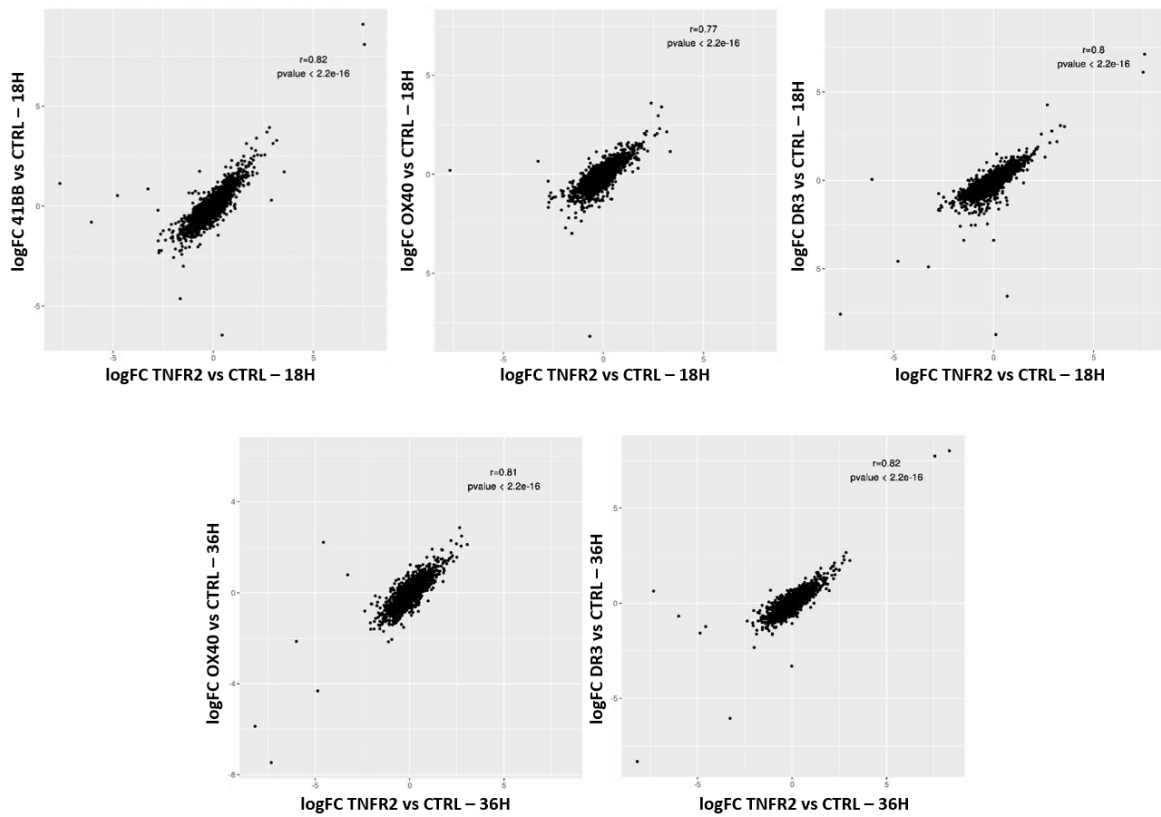
Supplemental figure 3 – RNA quantification of TNFRs expression on fresh and cultivated Tregs. Total count per millions (cpm) of reads, obtained by RNA-sequencing, were used to quantify the expression of different TNFR-SF members on freshly purified Treg and Treg cultivated for 18 and 36 hours with coated anti-CD3/CD28.



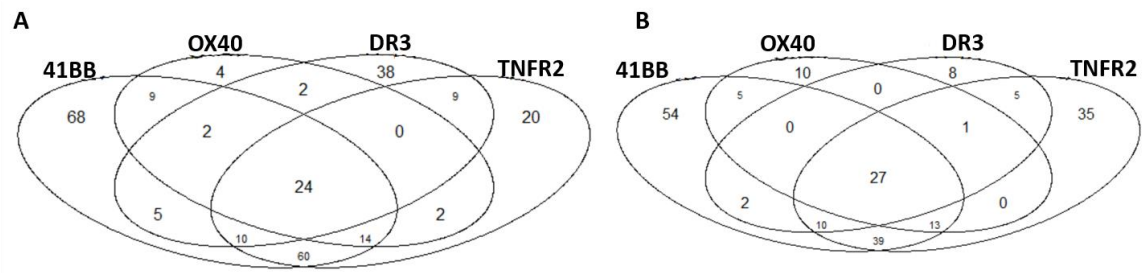
Supplemental figure 4 – Quantification of increased proliferation and survival with combination of agonists for TNFRs. (A) Quantification of the heatmaps (Figure 1C, D) showing increased proliferation and survival when agonists were combined. (B) Increased proliferation and survival when agonists were combined with OX40.



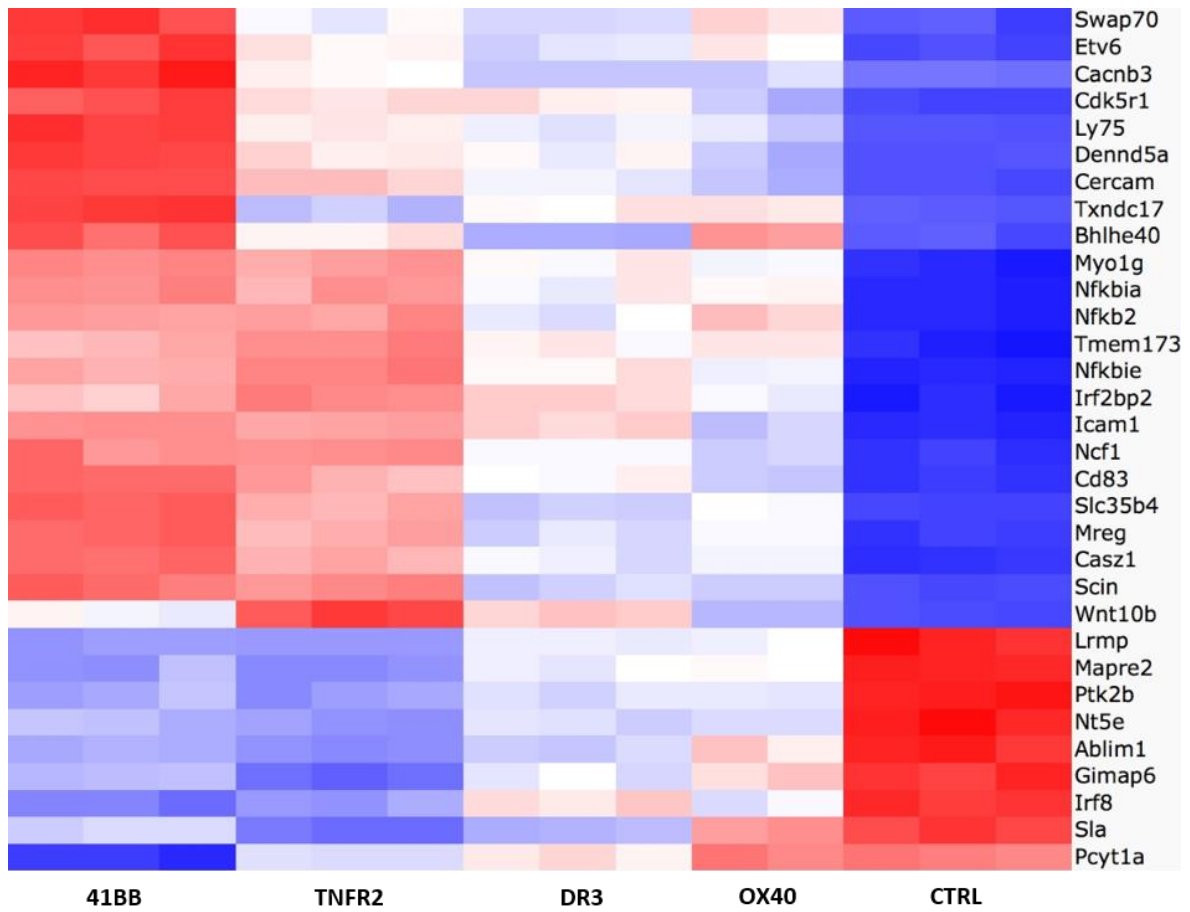
Supplemental figure 5 – TNFR-SF agonists did not modify TNFR-SF expression on Treg. Total count per millions (cpm) of reads, obtained by RNA-sequencing, were used to quantify the expression of TNFR-SF on Treg cultivated for 18 and 36 hours with coated anti-CD3/CD28 and agonists (indicated on the x axis).



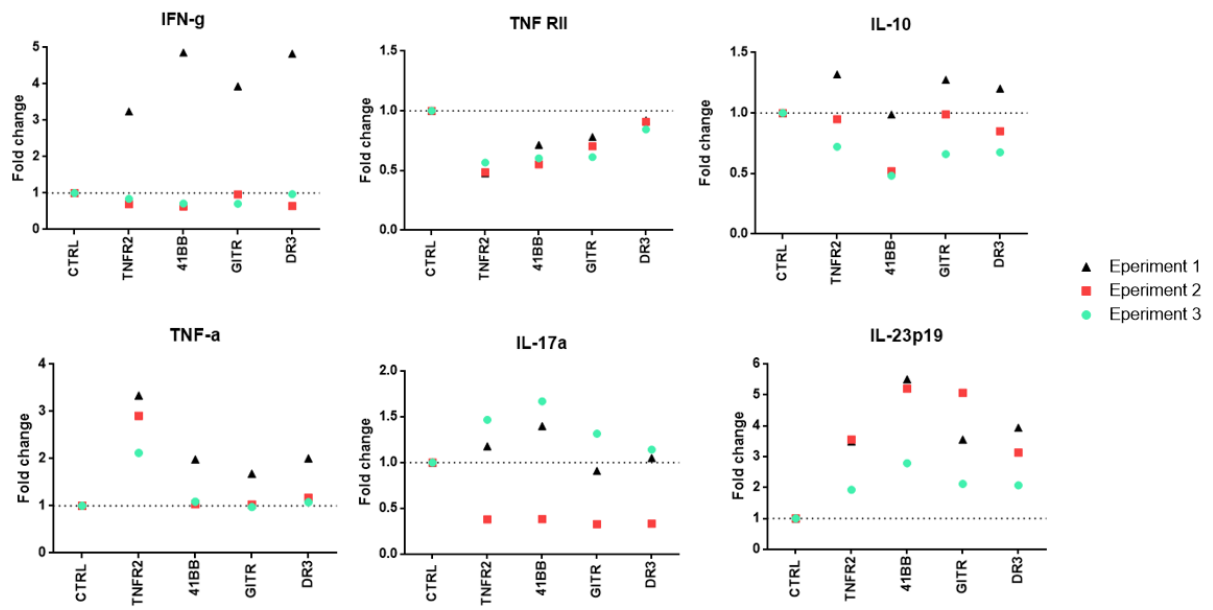
Supplemental figure 6. Similarities in the signature induced by the different TNFR-SF agonists on Treg. Treg were cultured for and processed as in Figure 2. Comparisons of the fold change expressions of DE genes compared to control of Treg co-stimulated by TNFR2, 4-1BB, DR3 or OX40.



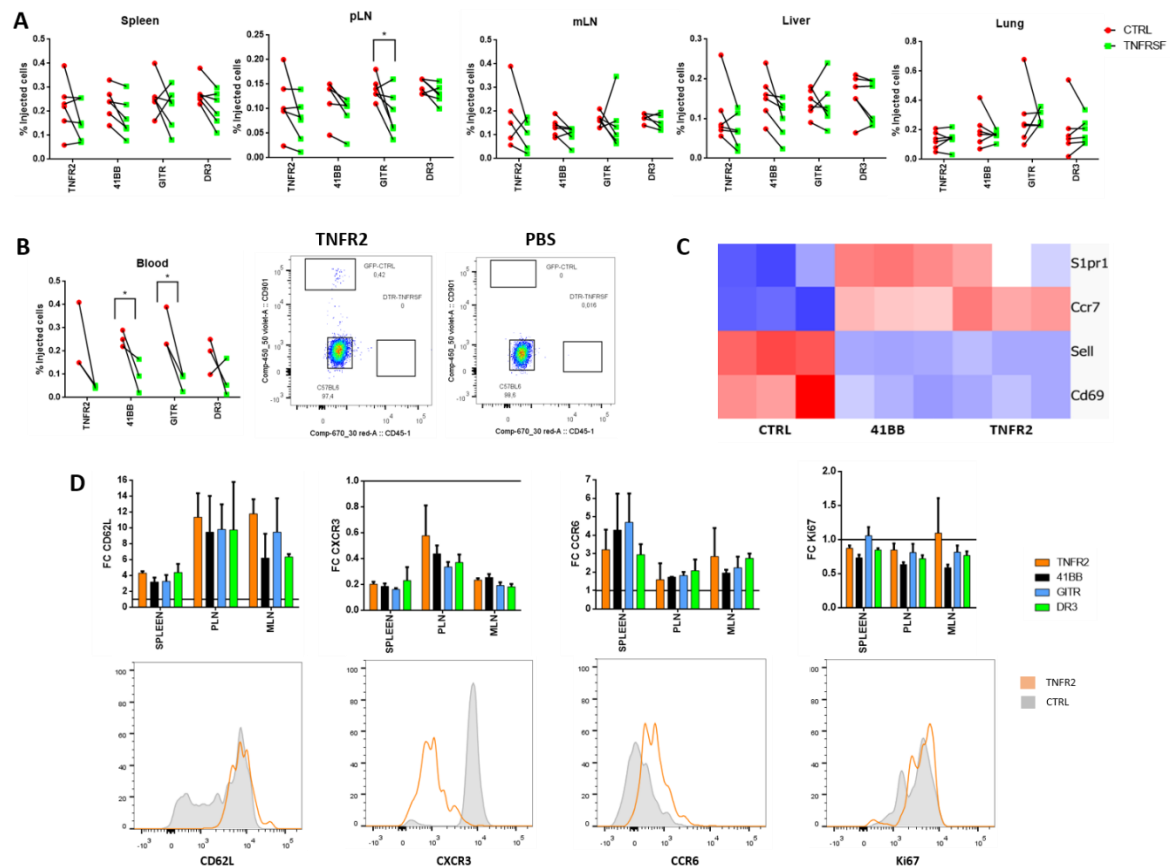
Supplemental figure 7 – Venn diagram of differentially expressed genes between the different TNFRs and CTRL, shared by other TNFRs. Treg were cultured for 18 (left) and 36 hours (right) and processed as in Figure 2.



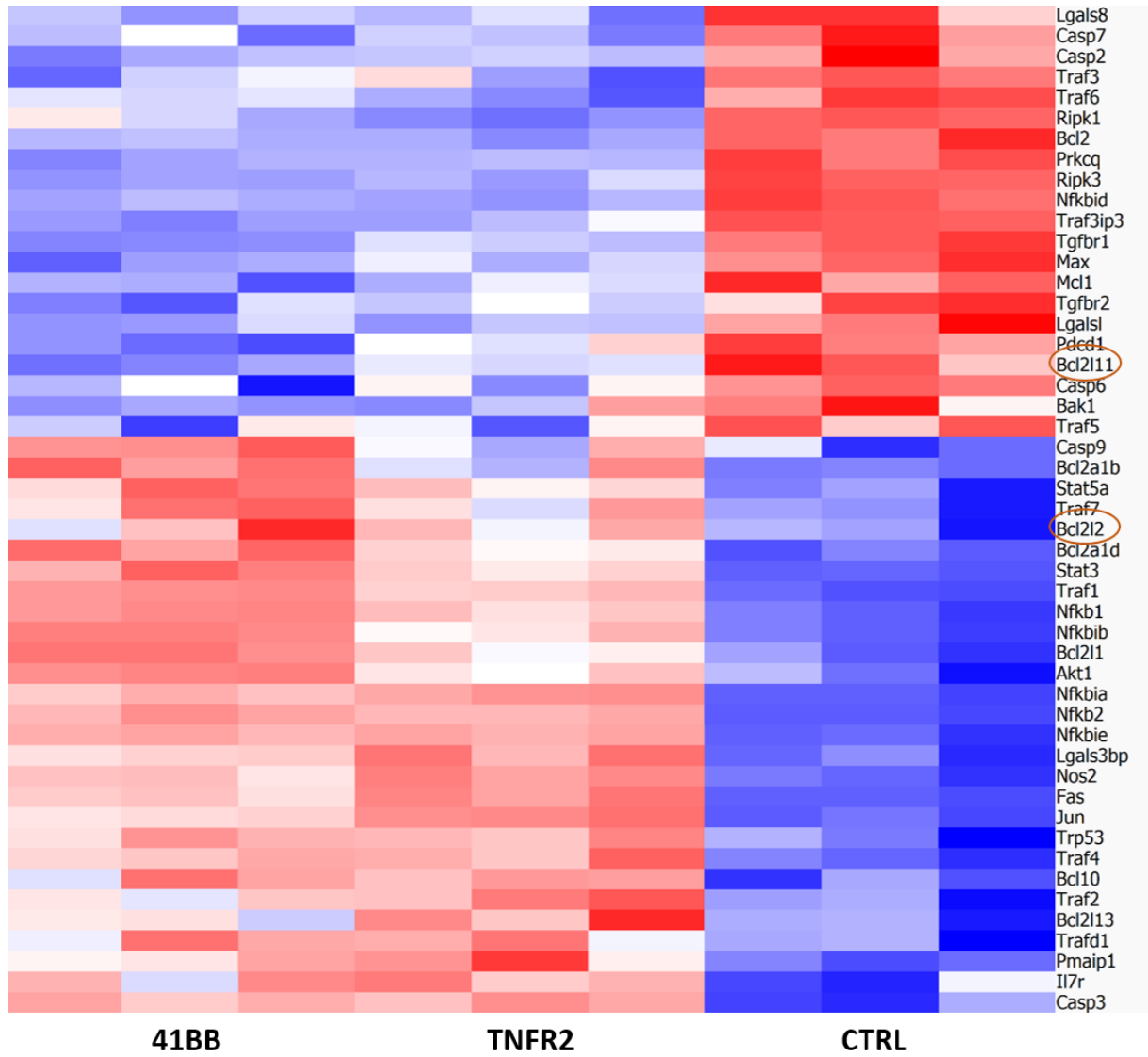
Supplemental figure 8 – Top DE genes at 18 hours comparing the different TNFR-SF co-stimulation. Treg were cultured and processed as in Figure 2.



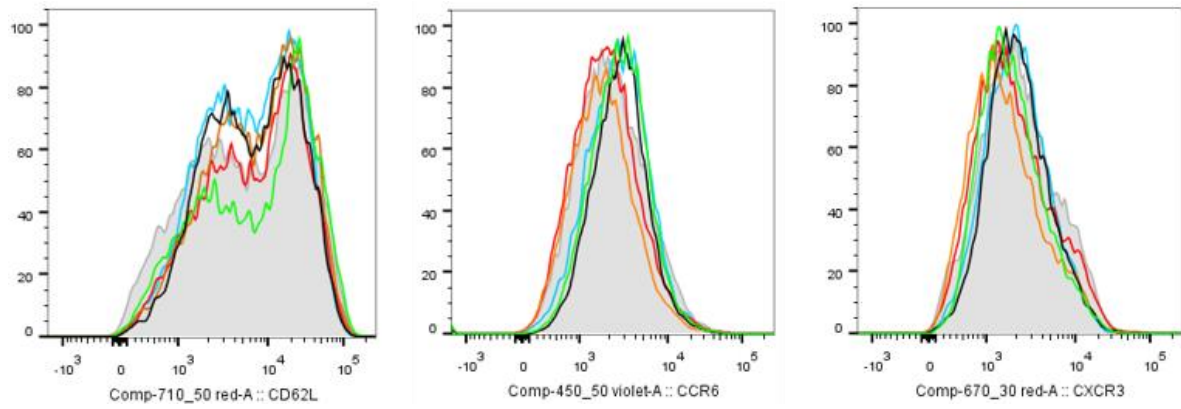
Supplemental figure 9 – Cytokine production by boosted Tregs. Levels of cytokine production by Tregs boosted for 3 days with agonists for TNFR2, 41BB, GITR and DR3 and coated anti-CD3/CD28.



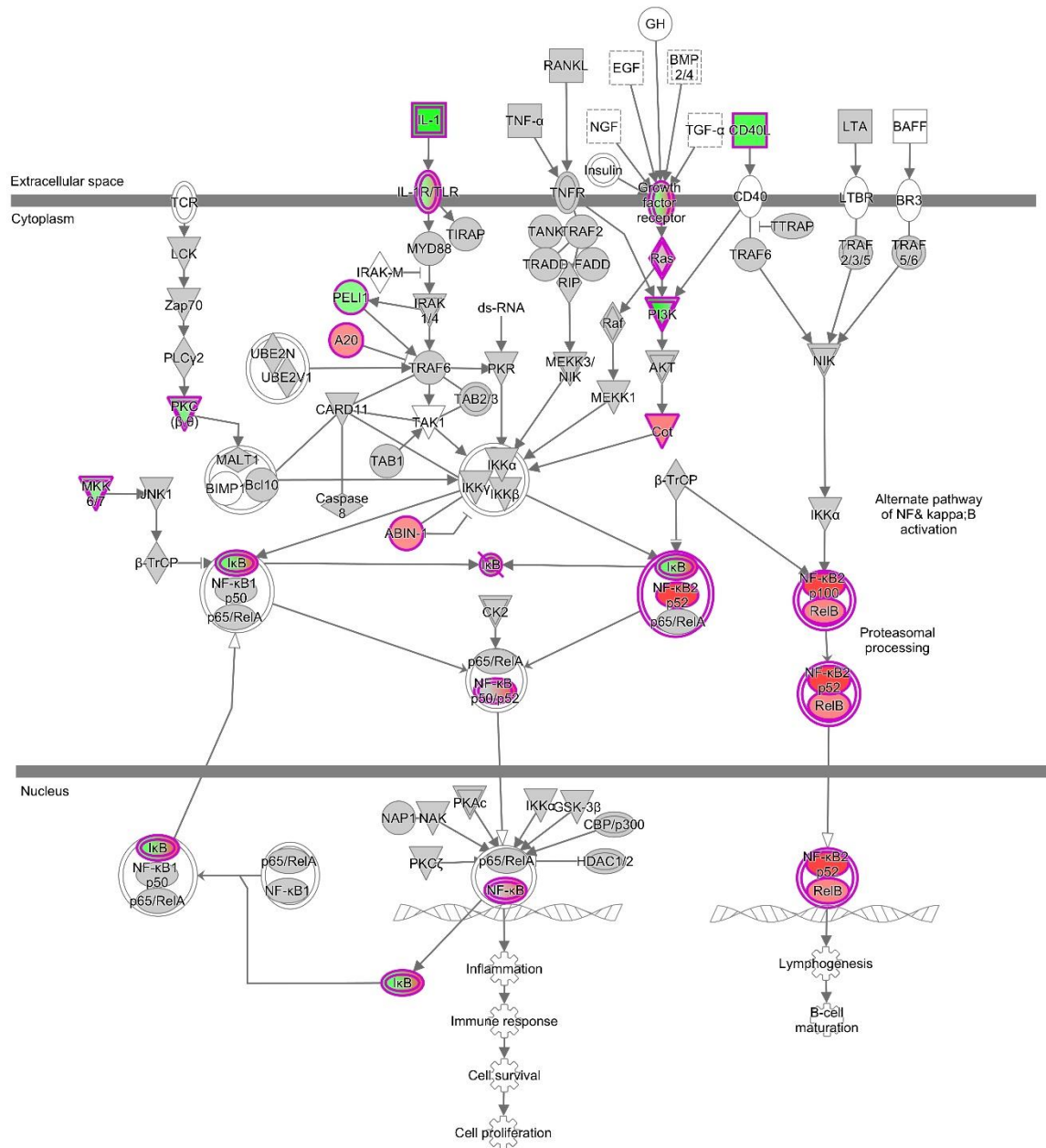
Supplemental figure 10 – Early cell homeostasis of Treg co-stimulated by TNFR-SF agonists. Tregs, pre-activated with anti-CD3/CD28 mAb and TNFR-SF agonists, were tested for their in vivo homeostasis at 12 hours. (A, B) Control Treg and Treg co-stimulated with a TNFR-SF agonist were co-injected in equal numbers. Data show 2 independent experiments with 6 mice per group. Proportion of co-injected Treg in various organs. Each dot is a mouse and lines connect cells from the same mouse. (C) RNA levels among a set of genes involved in blood to LN re-circulation in Treg activated for 36h and processed as in Figure 2. (D) CD62L, CXCR3, CCR6 and Ki67 expression among injected cells. Upper panels show FC of co-stimulated Treg compared to control Treg for each individual mouse. Lower panels show representative overlays for CTRL and TNFR2 co-stimulated Treg in the spleen.



Supplemental 11– RNA levels among a set of genes involved in T cell survival and apoptosis in Treg activated for 36h and processed as in Figure 2.

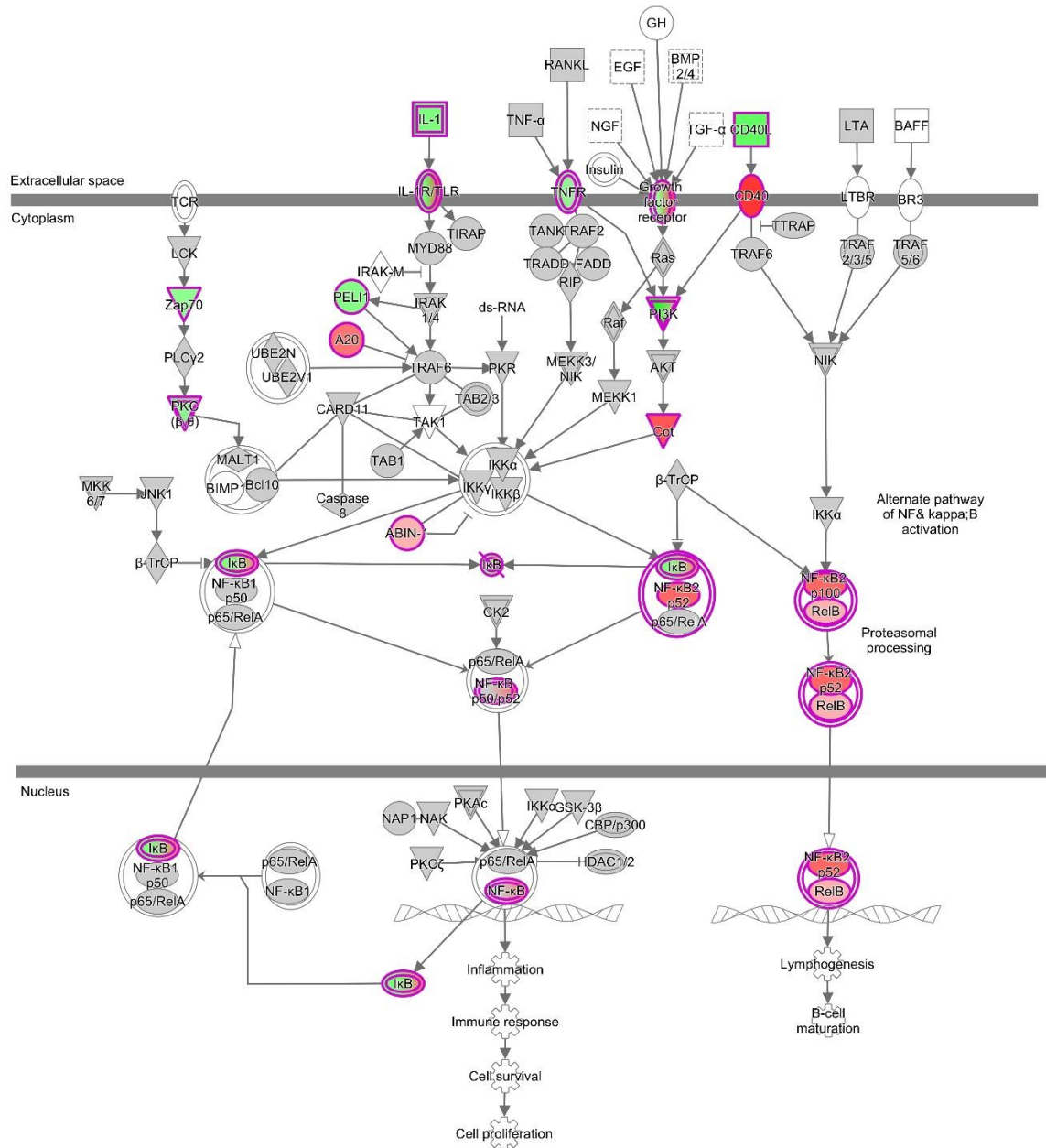


Supplemental 12 – Tregs were stimulated for 3 days with coated anti-CD3/CD28 and agonists, followed by a one day of rest before adoptive transfer. Markers before injection were assessed by flow cytometry. Legend is as in Figure 1A.



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Supplemental 13A. NF-κB pathway analysed using Ingenuity. Treg were stimulated for 18h and processed as in figure 2. Genes up-regulated by the TNFR2 agonists are in red and genes down-regulated by the TNFR2 agonists are in green.



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Supplemental 13B. NF-κB pathway analysed using Ingenuity. Treg were stimulated for 18h and processed as in figure 2. Genes up-regulated by the 41BB agonists are in red and genes down-regulated by the 41BB agonists are in green.

ADDITIONAL RESULTS

As discussed in the introduction, many studies reported an inhibitory role of TNFRs during iTreg and pTreg development. We therefore wanted to compare the impact of the agonists on already generated iTreg compared to nTreg (Fig.1).

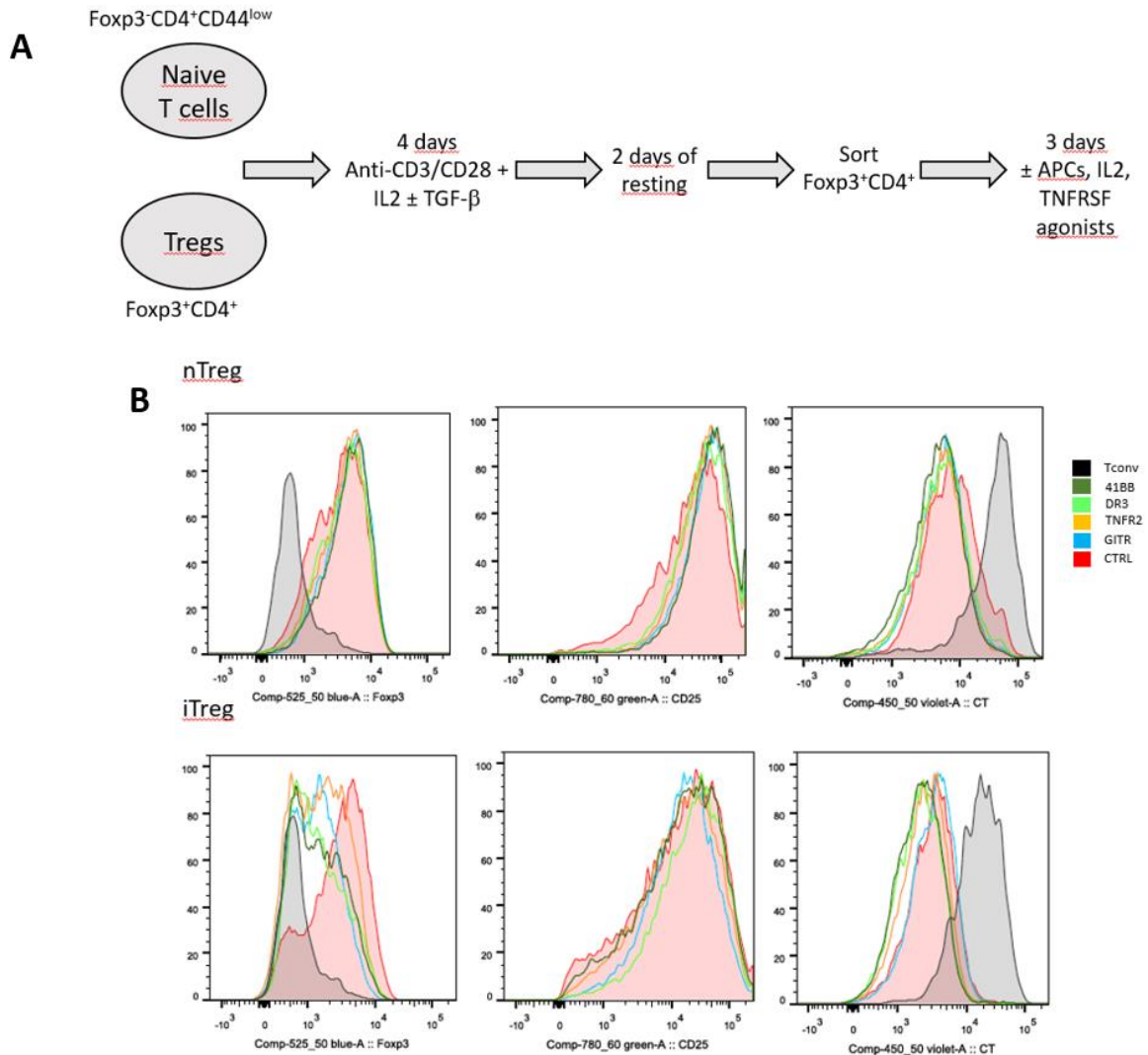


Figure 1 – The impact of TNFRSF members on iTreg. In figure A is presented the protocol used to assess the impact of triggering TNFRs in iTreg compared to nTreg. Naïve T cells ($CD4^+GFP^-CD44^{low}$) and Treg ($CD4^+GFP^+$) were sorted from $Foxp3^{GFP}$ mice. They were cultivated for 4 days with IL-2 and TGF- β to induce iTreg and then $CD4^+GFP^+$ were sorted from both populations. TNFRs were triggered as usual for 3 days with APCs and anti-CD3. In figure B, the overlays of Foxp3, CD25 and cell trace in nTreg and iTreg when boosted with and without the agonists, in comparison to Tconv (black).

DISCUSSION

1. The importance of Treg and TNFRs

The general aim of my thesis project was to investigate the role of TNFRSF members in Treg biology, with the goal of boosting their survival, proliferation, function and stability to develop new treatments for autoimmune diseases.

Indeed, a correct function of the immune system is fundamental to avoid autoimmune disorders, due to an excess of Tconv and/or deficiency of Treg, and cancer, in which the presence of Treg inhibits anti-tumoral immunity. Decreased number and function of Treg are indeed observed in many autoimmune conditions, and many treatments aim to boost their expansion and/or suppressive activity.

Moreover, a better knowledge of Treg biology could improve their use in therapy opening possibilities to new treatments.

It has been a while that the TNF family is under the loop to characterize the different members. However, the family is big and the members are expressed on different cell types, therefore it is difficult to study each of them separately. Despite their pleiotropic expression, many of them are highly present on fresh Treg (TNFR2, GITR, 4-1BB, ...) and others are up-regulated after TCR stimulation on these cells. Moreover, it was shown that agonists for TNFRs increased Treg expansion *in vitro*. These observations raised the possibility that these members might play an important role in Treg biology and opened the way to several studies.

It is worth to notice that the majority of the studies, in which it was seen a boost of Treg expansion *in vivo*, have been conducted with injections of agonists or antagonists of the receptors of the TNFSF, and since their expression is not restricted to Treg, it is impossible to unravel if their action was direct or indirect. Furthermore, people did see several effects when these receptors are triggered but there are only a few studies investigating signaling pathways downstream their activation in Treg, and the majority of signaling data are acquired in cell lines.

Currently, some receptors are being studied in clinical trial for cancer treatment. Indeed, many boost proliferation and survival of CD4 and CD8 T cells stimulating anti-tumoral

response. Also, some, seem to target Treg within tumors. Therefore, injection of agonistic antibodies seems a promising therapy for some cancers.

Moreover, anti-TNF drugs are already used for some autoimmune diseases as psoriasis. However, in the case of multiple sclerosis it was observed a worsening of the disease with this treatment. This might be due to the importance of TNF signal in Treg in the context of multiple sclerosis, as explained before.

This could mean that in different tissues and conditions, different receptors play an important role and that the presence of many members of the family is not redundant and each one has a function depending on the environment.

Therefore, it might be helpful to further investigate the role of these receptors in the immune system to envisage treatments for autoimmune diseases on one side and cancer on the other.

2. Role of TNFRs members in Treg biology

The picture is indeed very confusing because of the difficulty to study these molecules. A few human data are available because of the transient expression of the receptors. This is the first study aiming to compare the role of different receptors in Treg biology simultaneously. The goal was to start from *in vitro* evidences of action and to confirm them *in vivo*. Moreover, we focused on signaling pathways triggered by the receptors to give a mechanistic explanation to biological effects.

Because of the expression of TNFRs on different cell types, we conducted all the experiments on purified Treg, to be sure to investigate only direct effects.

TNFRs are many and not for all are available agonistic antibodies or ligands. We therefore selected some of them based on the boosting of Treg survival and proliferation *in vitro* and decided to focus on TNFR2, 4-1BB, GITR, DR3 and OX40. The latter does not enhance Treg proliferation and survival in our conditions, however, some papers showed boosting of Treg with *in vivo* injection of OX40 [228], but this effect might be mediated by other cells since there are no evidences of boosting in purified Treg. In addition, TNFR2 and 4-1BB have a stronger effect compared to GITR and DR3.

Other members of the family have been tested, but they did not increase proliferation

and survival *in vitro* and for this reason they were excluded from the study. When we checked the expression of all these receptors by RNA-sequencing, we saw that the TNFRs that did not boost Treg were expressed at low level or down-regulated by TCR stimulation and this could explain the lack of effect.

Almost all the experiments have been done in concomitant TCR stimulation because not all the receptors are already highly expressed in fresh cells, as shown in the kinetics study.

Dose-responses have been conducted to identify for each receptor the best window of agonist concentration, which may depend on receptor expression and ligand affinity.

Since it is known that Treg produce TNF, which binds to TNFR2 and enhances proliferation, we wanted to confirm that the effects of the agonists were not mediated by an overproduction of TNF when other receptors of the family are triggered. To validate our system, we verified to have the same boosting of Treg in TNFKO mice, proving the effect is not mediated by TNF produced by Treg, but that each receptor has its own activity.

Moreover, even if Treg express mainly TNFR2, we wanted to be sure to trigger only this receptor and not TNFR1, therefore we used a modified version of TNF which binds only to TNFR2. We confirmed its specificity because of the complete loss of Treg boosting in TNFR2-deficient Treg. Additionally, the maintenance of the effects by other TNFRs is another confirmation that their boost is not mediated by TNFR2.

It was reported a cross-talk and positive feedback among the members of the family. For instance, TNF does drive the expression of other receptors. To investigate if the receptors have synergistic activities, we combined the agonists one by one. The combination of DR3 with the other members (TNFR2, 4-1BB and GITR), showed an increase survival and proliferation of Treg compared to DR3 alone but also with other agonists alone. This means there are synergistic activities, possibly due to different pathways triggered by the receptors which can combine when two agonists are used together.

Moreover, the addition of OX40 to the other members did not decrease neither increase their boosting, meaning it does not have any inhibitory neither additive activity.

The RNA-sequencing analysis of boosted Treg *in vitro*, was performed to unravel key pathways triggered by TNFRs.

Looking at the most DE genes is possible to notice that the transcriptome of 41BB and TNFR2 are similar and farther to CTRL transcriptome compared to DR3. This correlates well with the huge boost of proliferation and survival observed *in vitro* with triggering of 41BB and TNFR2 while less increase is observed with DR3.

Surprisingly, OX40 itself has a different transcriptome compared to CTRL Treg, even if no biological effects were observed *in vitro*.

The increases survival in Tconv, associated to TNFR triggered, reported to increase the expression of pro-survival molecules, as Bcl2, Bcl-XL, cFlip_{short} or Bfl-1 and decreased expression of pro-apoptotic molecules, such as Bim and Bad [263], [237, 267].

In our context, the increased survival might be explained by increased expression of pro-survival molecules (*Bcl2l1*) and decreased expression of pro-apoptotic molecules (*Bcl2l11*). However, a more complex signaling seems to be involved, as we found modified expression of caspases (*Casp2* and *7*), *Ripk1* and *Ripk3* or TRAFs molecules (*Traf1*, *3*, *6* and *7*).

In addition, TNFRs triggering lowered the expression of *Il6ra* and *Il6st*, both involved in IL6 signaling, and *Il1a* and higher levels of *Sema4a* and *Nrp1*. Since it was shown that IL6 and IL1 decreased Treg stability while *Sema4a* and *Nrp1* increased Treg stability [72], [80], we can conclude that TNFR triggering increases Treg stability.

Opposite data are published regarding the impact of the TNFRs on Treg function, some showed their suppressive action is reduced while others saw it as unchanged. To investigate this aspect, cytokine production after 3 days of culture was measured. Boosted Treg seem to produce more Th2-type cytokines as we saw increased levels of IL4, IL5 and IL13. This Th2 tendency was not expected and it might be due to increased expression of *Gata3*, *Stat5*, *Stat6* and AP-1 factors (*Jun* and *Fosl2*), involved in Th2 differentiation of Tconv [268], even though lower expression of *Maf* and *Irf4* may limit type 2 Treg differentiation.

To further investigate the suppressive potential of Treg, we conducted an *in vitro* suppression assay, pre-incubating Treg with the agonists. Indeed, in the literature there are some data regarding the role of TNFRs triggering, but in many cases, agonists are added during the assay and they can therefore act also on Tconv. Our suppression assay showed no differences in function between control Treg and Treg boosted with the agonists. However, this is a very reductive system as Treg can use many different mechanisms of suppression *in vivo*.

We therefore decided to test their function *in vivo* with a colitis model. Tconv alone, Tconv with control Treg and Tconv with Treg pre-incubated with GITR or STAR, were injected in immune-deficient mice. We saw that GITR and TNFR2 triggering increased the capacity of Treg to control colitis.

We also investigated the effect of TNFR triggering *in vivo*. To assess this, Treg were boosted *in vitro* with the agonists, to be sure to directly act on Treg. Then, control Treg and TNFSF Treg, were co-injected in mice that were sacrificed after 12 hours and 7 days post-injection. After 12 hours, only control cells were detected in the blood, suggesting that TNFR triggering might change their migration properties. In particular, the increased expression of CD62L, observed by flow cytometry might be responsible for their migration into the LNs and absence in the blood.

It was noticed that boosted Treg have an advantage *in vivo*, as we can see higher number of injected cells compare to control cells after 7 days. Especially in the lung we see higher presence of cells. Flow-citometry analysis permitted us to investigate the phenotype of the boosted cells, and clearly the triggering of the receptors changed the phenotype of Treg, especially related to the expression of migration markers. Boosted Treg showed decreased CXCR3 expression, which is involved in the trafficking of Th1 cells to peripheral sites of Th1-type inflammation. Moreover, they had increased CD62L, a marker of central memory cells, that favors their migration to lymph nodes, and a small increase in CCR6 which was reported to be important for Treg recruitment in inflamed sites [269].

Moreover, because of the important role of TNFR2 in the EAE context, we decided to inject pre-activated Treg in mice to which EAE was induced. At the end of the disease, the

harvest of draining lymph nodes, spleen and CNS permitted to see that TNFR triggering conferred an advantage, as we saw more cells in all organs.

Collectively, not only at the steady state, but also in the context of inflammation, TNFR triggering boost Treg survival and proliferation *in vivo*, influencing their migration properties. In addition, we saw increased ability to control colitis. These characteristics are all important in regards to cell therapy using Treg. This is a therapeutic approach tested in several pre-clinical assays to limit graft rejection, GvHD or autoimmune and inflammatory diseases [270]. However, the limit factor has always been the capacity to obtain high amounts of pure, stable and functional Treg. Adding TNFR agonists might finally help to reach this goal.

Moreover, because of cytokine production of boosted Treg, their preferential migration to the lung *in vivo*, and increased expression of *Ccr4* and decreased *Cxcr3*, these cells might be more able to control Th2-mediated diseases. Further work would be to test the effect of TNFRSF triggering on Treg in the context of allergy or asthma.

As mentioned before, we sought to investigate signaling downstream the receptors since transcriptomic analysis have been reported in only 3 papers. In one, human Treg were stimulated with TNF but there was not concomitant TCR stimulation [186]. In another one, they injected an agonist for DR3 and analyzed the effect on ex-vivo Treg, but as discussed earlier, it is impossible to dissociate direct and indirect effects [271]. In the last one, the most similar to my study, purified Treg were stimulated *in vitro* with TCR triggering and agonist for OX40 [272]. However, only a few genes have been described as differentially regulated which correlates with my analysis showing that OX40 is the receptor that influence less Treg biology.

From literature analysis, it was frequently reported, the activation of NF- κ B for several members of the family [187], [273], [274], [275]. However, the majority of the data are related to Western blot analysis and amounts of transcription factors not always correlate with their binding to DNA.

To assess this, we decided to analyse by EMSA and supershift the activation of NF- κ B. Quantitatively, the receptors showed no differences in NF- κ B, except for OX40 that did show decreased NF- κ B activation. However, the different TNFRSF members showed

different kinetics of NF- κ B activation. Because of the previous TCR stimulation and consequent upregulation of the receptors, the different kinetics should not be explained by different receptor expression. Moreover, all the agonists activate the canonical pathway with DNA binding of cRel and RelA. We did not find activation of the non-canonical pathway.

Because of these interesting findings, we compared the effects of the agonists in purified Treg deficient for RelA. In all cases, we observed a partial reduction of boost of proliferation and survival. The effect is not massive and this could be due to the possibility that in those Treg there is a hyper activation of c-Rel to compensate RelA deficiency and therefore the receptors might in part overcome that absence.

The genes down-regulated in RelA KO, c-Rel KO and double KO Treg were compared to the up-regulated genes by TNFR2 triggering. Interestingly, the majority of up-regulated genes by TNFR2 were down-regulated in the KOs, suggesting the importance of the canonical NF- κ B pathway downstream this receptor.

Another confirmation of the importance of NF- κ B, derives from pathway analysis in Gene Ontology. When triggering TNFR2 or 4-1BB, it comes up NF- κ B as differentially expressed compared to CTRL Treg at different levels.

With NF- κ B activation was also reported, in the literature, the activation of MAPK and IP3K, that were not investigated here with biochemical analysis. However, the Gene Ontology analysis of pathways differentially expressed showed no differences between Treg boosted with the agonists and CTRL Treg. Actually, as differentially expressed, came up, with NF- κ B, also TNFR2, CD40, 4-1BB, RANK, Th2 and apoptosis signaling.

3. Role of TNFRs in iTreg

While TNFRs seem to be important during nTreg development in the thymus, it has been reported several times an inhibitory action of TNF in iTreg and pTreg generation.

It was shown, in an iTreg differentiation assay, that when TCR stimulation is strong there is inhibition of iTreg generation, due to increased production of IFN- γ and TNF, since iTreg development is restored when these cytokines are blocked [276].

Furthermore, using an anti-TNF in co-transfer of Tconv and Treg in SCID mice, it was seen an inhibitory effect of TNF in pTreg generation. This inhibitory effect was lost using Tconv from TNFR2 KO mice [68].

Moreover, in my lab it was observed that TNF inhibits Treg differentiation both *in vitro* and *in vivo* using TCR transgenic mice.

However, we lack information regarding the role of TNFRs in already developed iTreg or pTreg.

Some human studies suggest that anti-TNF treatments favor pTreg (rather than tTreg). In the context of colitis with TNFR2 KO mice, it was shown that nTreg, but not iTreg, require TNFR2 to control the disease [277].

These evidences raise the question if TNFRs might have a different effect on nTreg and iTreg.

To assess this, after their induction, iTreg were boosted with the agonists as usual. We found a drastic decrease of Foxp3 expression in boosted compared to control Treg, with a slight decrease in CD25 expression.

Further work is fundamental to understand if the reduction of Foxp3 expression is observed also in recently differentiated pTreg *in vivo* to see how TNFRs impact on Treg stability.

Also it would be important to address if the reduction of Foxp3 occurs at the RNA or protein levels.

In conclusion, here we demonstrate a role for different TNFR family members in boosting Treg survival, proliferation and function. These effects are mediated by the canonical pathway of NF- κ B, but also other genes and pathways are involved, given the different transcriptome of boosted Tregs. Further analysis is needed to unravel the role of other members and the key genes involved in the boosting of Tregs, as we expect to have common pathways shared across the members but also individual features of single receptors. These studies can lead to the development of new cell therapies involving Treg to treat autoimmune diseases, as this approach could be very promising but it is still poorly studied and tricky.

BIBLIOGRAPHY

1. Kyewski, B. and L. Klein, *A central role for central tolerance*. *Annu Rev Immunol*, 2006. **24**: p. 571-606.
2. Kurobe, H., et al., *CCR7-dependent cortex-to-medulla migration of positively selected thymocytes is essential for establishing central tolerance*. *Immunity*, 2006. **24**(2): p. 165-77.
3. Klein, L., et al., *Antigen presentation in the thymus for positive selection and central tolerance induction*. *Nat Rev Immunol*, 2009. **9**(12): p. 833-44.
4. Anderson, M.S., et al., *Projection of an immunological self shadow within the thymus by the aire protein*. *Science*, 2002. **298**(5597): p. 1395-401.
5. Gallegos, A.M. and M.J. Bevan, *Central tolerance to tissue-specific antigens mediated by direct and indirect antigen presentation*. *J Exp Med*, 2004. **200**(8): p. 1039-49.
6. Hadeiba, H., et al., *Plasmacytoid dendritic cells transport peripheral antigens to the thymus to promote central tolerance*. *Immunity*, 2012. **36**(3): p. 438-50.
7. Jordan, M.S., et al., *Thymic selection of CD4+CD25+ regulatory T cells induced by an agonist self-peptide*. *Nat Immunol*, 2001. **2**(4): p. 301-6.
8. Xing, Y. and K.A. Hogquist, *T-cell tolerance: central and peripheral*. *Cold Spring Harb Perspect Biol*, 2012. **4**(6).
9. Walker, L.S. and A.K. Abbas, *The enemy within: keeping self-reactive T cells at bay in the periphery*. *Nat Rev Immunol*, 2002. **2**(1): p. 11-9.
10. Harding, F.A., et al., *CD28-mediated signalling co-stimulates murine T cells and prevents induction of anergy in T-cell clones*. *Nature*, 1992. **356**(6370): p. 607-9.
11. Krummel, M.F. and J.P. Allison, *CD28 and CTLA-4 have opposing effects on the response of T cells to stimulation*. *J Exp Med*, 1995. **182**(2): p. 459-65.
12. Walunas, T.L. and J.A. Bluestone, *CTLA-4 regulates tolerance induction and T cell differentiation in vivo*. *J Immunol*, 1998. **160**(8): p. 3855-60.
13. Freeman, G.J., et al., *Engagement of the PD-1 immunoinhibitory receptor by a novel B7 family member leads to negative regulation of lymphocyte activation*. *J Exp Med*, 2000. **192**(7): p. 1027-34.
14. Young, D.A., et al., *IL-4, IL-10, IL-13, and TGF-beta from an altered peptide ligand-specific Th2 cell clone down-regulate adoptive transfer of experimental autoimmune encephalomyelitis*. *J Immunol*, 2000. **164**(7): p. 3563-72.
15. Watanabe-Fukunaga, R., et al., *Lymphoproliferation disorder in mice explained by defects in Fas antigen that mediates apoptosis*. *Nature*, 1992. **356**(6367): p. 314-7.
16. Groux, H., et al., *A CD4+ T-cell subset inhibits antigen-specific T-cell responses and prevents colitis*. *Nature*, 1997. **389**(6652): p. 737-42.
17. Chen, Y., et al., *Regulatory T cell clones induced by oral tolerance: suppression of autoimmune encephalomyelitis*. *Science*, 1994. **265**(5176): p. 1237-40.
18. Sakaguchi, S., et al., *Regulatory T cells and immune tolerance*. *Cell*, 2008. **133**(5): p. 775-87.
19. Miyara, M., K. Wing, and S. Sakaguchi, *Therapeutic approaches to allergy and autoimmunity based on FoxP3+ regulatory T-cell activation and expansion*. *J Allergy Clin Immunol*, 2009. **123**(4): p. 749-55; quiz 756-7.
20. Nishizuka, Y. and T. Sakakura, *Thymus and reproduction: sex-linked dysgenesis of the gonad after neonatal thymectomy in mice*. *Science*, 1969. **166**(3906): p. 753-5.
21. Gershon, R.K. and K. Kondo, *Cell interactions in the induction of tolerance: the role of thymic lymphocytes*. *Immunology*, 1970. **18**(5): p. 723-37.
22. Penhale, W.J., et al., *Spontaneous thyroiditis in thymectomized and irradiated Wistar rats*. *Clin Exp Immunol*, 1973. **15**(2): p. 225-36.
23. Sakaguchi, S., T. Takahashi, and Y. Nishizuka, *Study on cellular events in post-thymectomy autoimmune oophoritis in mice. II. Requirement of Lyt-1 cells in normal female mice for the prevention of oophoritis*. *J Exp Med*, 1982. **156**(6): p. 1577-86.

24. Sakaguchi, S., et al., *Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease.* J Exp Med, 1985. **161**(1): p. 72-87.
25. Sakaguchi, S., et al., *Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases.* J Immunol, 1995. **155**(3): p. 1151-64.
26. Malek, T.R. and A.L. Bayer, *Tolerance, not immunity, crucially depends on IL-2.* Nat Rev Immunol, 2004. **4**(9): p. 665-74.
27. Caudy, A.A., et al., *CD25 deficiency causes an immune dysregulation, polyendocrinopathy, enteropathy, X-linked-like syndrome, and defective IL-10 expression from CD4 lymphocytes.* J Allergy Clin Immunol, 2007. **119**(2): p. 482-7.
28. Antony, P.A., et al., *Interleukin-2-dependent mechanisms of tolerance and immunity in vivo.* J Immunol, 2006. **176**(9): p. 5255-66.
29. Burchill, M.A., et al., *IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells.* J Immunol, 2007. **178**(1): p. 280-90.
30. Setoguchi, R., et al., *Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization.* J Exp Med, 2005. **201**(5): p. 723-35.
31. Brunkow, M.E., et al., *Disruption of a new forkhead/winged-helix protein, scurf, results in the fatal lymphoproliferative disorder of the scurfy mouse.* Nat Genet, 2001. **27**(1): p. 68-73.
32. Bennett, C.L. and H.D. Ochs, *IPEX is a unique X-linked syndrome characterized by immune dysfunction, polyendocrinopathy, enteropathy, and a variety of autoimmune phenomena.* Curr Opin Pediatr, 2001. **13**(6): p. 533-8.
33. Hori, S., T. Nomura, and S. Sakaguchi, *Control of regulatory T cell development by the transcription factor Foxp3.* Science, 2003. **299**(5609): p. 1057-61.
34. Khattri, R., et al., *An essential role for Scurfin in CD4+CD25+ T regulatory cells.* Nat Immunol, 2003. **4**(4): p. 337-42.
35. Fontenot, J.D., M.A. Gavin, and A.Y. Rudensky, *Foxp3 programs the development and function of CD4+CD25+ regulatory T cells.* Nat Immunol, 2003. **4**(4): p. 330-6.
36. Rao, A., C. Luo, and P.G. Hogan, *Transcription factors of the NFAT family: regulation and function.* Annu Rev Immunol, 1997. **15**: p. 707-47.
37. Chen, X. and J.J. Oppenheim, *Resolving the identity myth: key markers of functional CD4+FoxP3+ regulatory T cells.* Int Immunopharmacol, 2011. **11**(10): p. 1489-96.
38. Zemmour, D., et al., *Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR.* Nat Immunol, 2018. **19**(3): p. 291-301.
39. De Simone, M., et al., *Transcriptional Landscape of Human Tissue Lymphocytes Unveils Uniqueness of Tumor-Infiltrating T Regulatory Cells.* Immunity, 2016. **45**(5): p. 1135-1147.
40. Plitas, G., et al., *Regulatory T Cells Exhibit Distinct Features in Human Breast Cancer.* Immunity, 2016. **45**(5): p. 1122-1134.
41. Yates, K., et al., *Comparative transcriptome analysis reveals distinct genetic modules associated with Helios expression in intratumoral regulatory T cells.* Proc Natl Acad Sci U S A, 2018. **115**(9): p. 2162-2167.
42. Vasanthakumar, A., et al., *The TNF Receptor Superfamily-NF-kappaB Axis Is Critical to Maintain Effector Regulatory T Cells in Lymphoid and Non-lymphoid Tissues.* Cell Rep, 2017. **20**(12): p. 2906-2920.
43. Cretney, E., et al., *The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells.* Nat Immunol, 2011. **12**(4): p. 304-11.
44. Levine, A.G., et al., *Continuous requirement for the TCR in regulatory T cell function.* Nat Immunol, 2014. **15**(11): p. 1070-8.

45. Kawahata, K., et al., *Generation of CD4(+)CD25(+) regulatory T cells from autoreactive T cells simultaneously with their negative selection in the thymus and from nonautoreactive T cells by endogenous TCR expression.* J Immunol, 2002. **168**(9): p. 4399-405.
46. van Santen, H.M., C. Benoist, and D. Mathis, *Number of T reg cells that differentiate does not increase upon encounter of agonist ligand on thymic epithelial cells.* J Exp Med, 2004. **200**(10): p. 1221-30.
47. Sakaguchi, S., *Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self.* Nat Immunol, 2005. **6**(4): p. 345-52.
48. Aschenbrenner, K., et al., *Selection of Foxp3+ regulatory T cells specific for self antigen expressed and presented by Aire+ medullary thymic epithelial cells.* Nat Immunol, 2007. **8**(4): p. 351-8.
49. Lin, W., et al., *Regulatory T cell development in the absence of functional Foxp3.* Nat Immunol, 2007. **8**(4): p. 359-68.
50. Gavin, M.A., et al., *Foxp3-dependent programme of regulatory T-cell differentiation.* Nature, 2007. **445**(7129): p. 771-5.
51. Ohkura, N., et al., *T cell receptor stimulation-induced epigenetic changes and Foxp3 expression are independent and complementary events required for Treg cell development.* Immunity, 2012. **37**(5): p. 785-99.
52. Rudra, D., et al., *Transcription factor Foxp3 and its protein partners form a complex regulatory network.* Nat Immunol, 2012. **13**(10): p. 1010-9.
53. Yao, Z., et al., *Nonredundant roles for Stat5a/b in directly regulating Foxp3.* Blood, 2007. **109**(10): p. 4368-75.
54. Zheng, Y., et al., *Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate.* Nature, 2010. **463**(7282): p. 808-12.
55. Zheng, Y., et al., *Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells.* Nature, 2007. **445**(7130): p. 936-40.
56. Samstein, R.M., et al., *Foxp3 exploits a pre-existent enhancer landscape for regulatory T cell lineage specification.* Cell, 2012. **151**(1): p. 153-66.
57. Ohkura, N., Y. Kitagawa, and S. Sakaguchi, *Development and maintenance of regulatory T cells.* Immunity, 2013. **38**(3): p. 414-23.
58. Mahmud, S.A., et al., *Costimulation via the tumor-necrosis factor receptor superfamily couples TCR signal strength to the thymic differentiation of regulatory T cells.* Nat Immunol, 2014. **15**(5): p. 473-81.
59. Curotto de Lafaille, M.A., et al., *CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion.* J Immunol, 2004. **173**(12): p. 7259-68.
60. Furtado, G.C., et al., *Interleukin 2 signaling is required for CD4(+) regulatory T cell function.* J Exp Med, 2002. **196**(6): p. 851-7.
61. Mucida, D., et al., *Oral tolerance in the absence of naturally occurring Treg.* J Clin Invest, 2005. **115**(7): p. 1923-33.
62. Sun, C.M., et al., *Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid.* J Exp Med, 2007. **204**(8): p. 1775-85.
63. Curotto de Lafaille, M.A., et al., *Adaptive Foxp3+ regulatory T cell-dependent and -independent control of allergic inflammation.* Immunity, 2008. **29**(1): p. 114-26.
64. Cobbold, S.P., et al., *Induction of foxP3+ regulatory T cells in the periphery of T cell receptor transgenic mice tolerized to transplants.* J Immunol, 2004. **172**(10): p. 6003-10.
65. Fantini, M.C., et al., *Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7.* J Immunol, 2004. **172**(9): p. 5149-53.
66. Miller, A., et al., *Suppressor T cells generated by oral tolerization to myelin basic protein suppress both in vitro and in vivo immune responses by the release of transforming growth factor beta after antigen-specific triggering.* Proc Natl Acad Sci U S A, 1992. **89**(1): p. 421-5.

67. Zheng, S.G., et al., *IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells.* J Immunol, 2007. **178**(4): p. 2018-27.
68. Zhang, Q., et al., *TNF-alpha impairs differentiation and function of TGF-beta-induced Treg cells in autoimmune diseases through Akt and Smad3 signaling pathway.* J Mol Cell Biol, 2013. **5**(2): p. 85-98.
69. Zhang, X., et al., *OX40 Costimulation Inhibits Foxp3 Expression and Treg Induction via BATF3-Dependent and Independent Mechanisms.* Cell Rep, 2018. **24**(3): p. 607-618.
70. Floess, S., et al., *Epigenetic control of the foxp3 locus in regulatory T cells.* PLoS Biol, 2007. **5**(2): p. e38.
71. Feuerer, M., et al., *Foxp3+ regulatory T cells: differentiation, specification, subphenotypes.* Nat Immunol, 2009. **10**(7): p. 689-95.
72. Zhou, X., et al., *Plasticity of CD4(+) FoxP3(+) T cells.* Curr Opin Immunol, 2009. **21**(3): p. 281-5.
73. Tsuji, M., et al., *Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches.* Science, 2009. **323**(5920): p. 1488-92.
74. Komatsu, N., et al., *Heterogeneity of natural Foxp3+ T cells: a committed regulatory T-cell lineage and an uncommitted minor population retaining plasticity.* Proc Natl Acad Sci U S A, 2009. **106**(6): p. 1903-8.
75. Overacre, A.E. and D.A. Vignali, *T(reg) stability: to be or not to be.* Curr Opin Immunol, 2016. **39**: p. 39-43.
76. Ouyang, W., et al., *Foxo proteins cooperatively control the differentiation of Foxp3+ regulatory T cells.* Nat Immunol, 2010. **11**(7): p. 618-27.
77. Ouyang, W., et al., *Novel Foxo1-dependent transcriptional programs control T(reg) cell function.* Nature, 2012. **491**(7425): p. 554-9.
78. Harada, Y., et al., *Transcription factors Foxo3a and Foxo1 couple the E3 ligase Cbl-b to the induction of Foxp3 expression in induced regulatory T cells.* J Exp Med, 2010. **207**(7): p. 1381-91.
79. Pan, F., et al., *Eos mediates Foxp3-dependent gene silencing in CD4+ regulatory T cells.* Science, 2009. **325**(5944): p. 1142-6.
80. Delgoffe, G.M., et al., *Stability and function of regulatory T cells is maintained by a neuropilin-1-semaphorin-4a axis.* Nature, 2013. **501**(7466): p. 252-6.
81. Zhou, X., et al., *Selective miRNA disruption in T reg cells leads to uncontrolled autoimmunity.* J Exp Med, 2008. **205**(9): p. 1983-91.
82. Lu, L.F., et al., *Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein.* Immunity, 2009. **30**(1): p. 80-91.
83. Miyao, T., et al., *Plasticity of Foxp3(+) T cells reflects promiscuous Foxp3 expression in conventional T cells but not reprogramming of regulatory T cells.* Immunity, 2012. **36**(2): p. 262-75.
84. Beriou, G., et al., *IL-17-producing human peripheral regulatory T cells retain suppressive function.* Blood, 2009. **113**(18): p. 4240-9.
85. Zhou, L., et al., *TGF-beta-induced Foxp3 inhibits T(H)17 cell differentiation by antagonizing RORgammat function.* Nature, 2008. **453**(7192): p. 236-40.
86. Dominguez-Villar, M., C.M. Baecher-Allan, and D.A. Hafler, *Identification of T helper type 1-like, Foxp3+ regulatory T cells in human autoimmune disease.* Nat Med, 2011. **17**(6): p. 673-5.
87. Koch, M.A., et al., *The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation.* Nat Immunol, 2009. **10**(6): p. 595-602.
88. Muller, M., et al., *CXCR3 signaling reduces the severity of experimental autoimmune encephalomyelitis by controlling the parenchymal distribution of effector and regulatory T cells in the central nervous system.* J Immunol, 2007. **179**(5): p. 2774-86.
89. Zheng, Y., et al., *Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control T(H)2 responses.* Nature, 2009. **458**(7236): p. 351-6.

90. Chaudhry, A., et al., *CD4+ regulatory T cells control TH17 responses in a Stat3-dependent manner*. *Science*, 2009. **326**(5955): p. 986-91.
91. Fontenot, J.D., et al., *Regulatory T cell lineage specification by the forkhead transcription factor foxp3*. *Immunity*, 2005. **22**(3): p. 329-41.
92. Kim, J.M., J.P. Rasmussen, and A.Y. Rudensky, *Regulatory T cells prevent catastrophic autoimmunity throughout the lifespan of mice*. *Nat Immunol*, 2007. **8**(2): p. 191-7.
93. Lawson, J.M., et al., *Increased resistance to CD4+CD25hi regulatory T cell-mediated suppression in patients with type 1 diabetes*. *Clin Exp Immunol*, 2008. **154**(3): p. 353-9.
94. You, S., et al., *Autoimmune diabetes onset results from qualitative rather than quantitative age-dependent changes in pathogenic T-cells*. *Diabetes*, 2005. **54**(5): p. 1415-22.
95. Viglietta, V., et al., *Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis*. *J Exp Med*, 2004. **199**(7): p. 971-9.
96. Haas, J., et al., *Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis*. *Eur J Immunol*, 2005. **35**(11): p. 3343-52.
97. McGeachy, M.J., L.A. Stephens, and S.M. Anderton, *Natural recovery and protection from autoimmune encephalomyelitis: contribution of CD4+CD25+ regulatory cells within the central nervous system*. *J Immunol*, 2005. **175**(5): p. 3025-32.
98. Lewkowich, I.P., et al., *CD4+CD25+ T cells protect against experimentally induced asthma and alter pulmonary dendritic cell phenotype and function*. *J Exp Med*, 2005. **202**(11): p. 1549-61.
99. Ling, E.M., et al., *Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease*. *Lancet*, 2004. **363**(9409): p. 608-15.
100. Karlsson, M.R., J. Rugtveit, and P. Brandtzaeg, *Allergen-responsive CD4+CD25+ regulatory T cells in children who have outgrown cow's milk allergy*. *J Exp Med*, 2004. **199**(12): p. 1679-88.
101. Belkaid, Y., et al., *CD4+CD25+ regulatory T cells control Leishmania major persistence and immunity*. *Nature*, 2002. **420**(6915): p. 502-7.
102. Somerset, D.A., et al., *Normal human pregnancy is associated with an elevation in the immune suppressive CD25+ CD4+ regulatory T-cell subset*. *Immunology*, 2004. **112**(1): p. 38-43.
103. Aluvihare, V.R., M. Kallikourdis, and A.G. Betz, *Regulatory T cells mediate maternal tolerance to the fetus*. *Nat Immunol*, 2004. **5**(3): p. 266-71.
104. Curiel, T.J., et al., *Specific recruitment of regulatory T cells in ovarian carcinoma fosters immune privilege and predicts reduced survival*. *Nat Med*, 2004. **10**(9): p. 942-9.
105. Haribhai, D., et al., *A requisite role for induced regulatory T cells in tolerance based on expanding antigen receptor diversity*. *Immunity*, 2011. **35**(1): p. 109-22.
106. Schmalzer, M., et al., *IL-7R signaling in regulatory T cells maintains peripheral and allograft tolerance in mice*. *Proc Natl Acad Sci U S A*, 2015. **112**(43): p. 13330-5.
107. Molofsky, A.B., A.K. Savage, and R.M. Locksley, *Interleukin-33 in Tissue Homeostasis, Injury, and Inflammation*. *Immunity*, 2015. **42**(6): p. 1005-19.
108. Biton, J., et al., *In Vivo Expansion of Activated Foxp3+ Regulatory T Cells and Establishment of a Type 2 Immune Response upon IL-33 Treatment Protect against Experimental Arthritis*. *J Immunol*, 2016. **197**(5): p. 1708-19.
109. Molofsky, A.B., et al., *Interleukin-33 and Interferon-gamma Counter-Regulate Group 2 Innate Lymphoid Cell Activation during Immune Perturbation*. *Immunity*, 2015. **43**(1): p. 161-74.
110. Matta, B.M., et al., *IL-33 is an unconventional Alarmin that stimulates IL-2 secretion by dendritic cells to selectively expand IL-33R/ST2+ regulatory T cells*. *J Immunol*, 2014. **193**(8): p. 4010-20.

111. Vasanthakumar, A., et al., *The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells.* Nat Immunol, 2015. **16**(3): p. 276-85.
112. Kole, A., et al., *Type I IFNs regulate effector and regulatory T cell accumulation and anti-inflammatory cytokine production during T cell-mediated colitis.* J Immunol, 2013. **191**(5): p. 2771-9.
113. Metidji, A., et al., *IFN-alpha/beta receptor signaling promotes regulatory T cell development and function under stress conditions.* J Immunol, 2015. **194**(9): p. 4265-76.
114. Do, J.S., et al., *An IL-27/Lag3 axis enhances Foxp3+ regulatory T cell-suppressive function and therapeutic efficacy.* Mucosal Immunol, 2016. **9**(1): p. 137-45.
115. Do, J., et al., *Treg-specific IL-27Ralpha deletion uncovers a key role for IL-27 in Treg function to control autoimmunity.* Proc Natl Acad Sci U S A, 2017. **114**(38): p. 10190-10195.
116. Barbi, J., D. Pardoll, and F. Pan, *Treg functional stability and its responsiveness to the microenvironment.* Immunol Rev, 2014. **259**(1): p. 115-39.
117. Hawrylowicz, C.M. and A. O'Garra, *Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma.* Nat Rev Immunol, 2005. **5**(4): p. 271-83.
118. Kearley, J., et al., *Resolution of airway inflammation and hyperreactivity after in vivo transfer of CD4+CD25+ regulatory T cells is interleukin 10 dependent.* J Exp Med, 2005. **202**(11): p. 1539-47.
119. Asseman, C., et al., *An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation.* J Exp Med, 1999. **190**(7): p. 995-1004.
120. Bergmann, C., et al., *Expansion and characteristics of human T regulatory type 1 cells in co-cultures simulating tumor microenvironment.* Cancer Immunol Immunother, 2007. **56**(9): p. 1429-42.
121. Mann, M.K., et al., *B cell regulation of CD4+CD25+ T regulatory cells and IL-10 via B7 is essential for recovery from experimental autoimmune encephalomyelitis.* J Immunol, 2007. **178**(6): p. 3447-56.
122. Fahlen, L., et al., *T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells.* J Exp Med, 2005. **201**(5): p. 737-46.
123. Kursar, M., et al., *Cutting Edge: Regulatory T cells prevent efficient clearance of Mycobacterium tuberculosis.* J Immunol, 2007. **178**(5): p. 2661-5.
124. Joetham, A., et al., *Naturally occurring lung CD4(+)CD25(+) T cell regulation of airway allergic responses depends on IL-10 induction of TGF-beta.* J Immunol, 2007. **178**(3): p. 1433-42.
125. Li, M.O., Y.Y. Wan, and R.A. Flavell, *T cell-produced transforming growth factor-beta1 controls T cell tolerance and regulates Th1- and Th17-cell differentiation.* Immunity, 2007. **26**(5): p. 579-91.
126. Collison, L.W., et al., *The inhibitory cytokine IL-35 contributes to regulatory T-cell function.* Nature, 2007. **450**(7169): p. 566-9.
127. Grossman, W.J., et al., *Differential expression of granzymes A and B in human cytotoxic lymphocyte subsets and T regulatory cells.* Blood, 2004. **104**(9): p. 2840-8.
128. Gondek, D.C., et al., *Cutting edge: contact-mediated suppression by CD4+CD25+ regulatory cells involves a granzyme B-dependent, perforin-independent mechanism.* J Immunol, 2005. **174**(4): p. 1783-6.
129. Garin, M.I., et al., *Galectin-1: a key effector of regulation mediated by CD4+CD25+ T cells.* Blood, 2007. **109**(5): p. 2058-65.
130. O'Gorman, W.E., et al., *The initial phase of an immune response functions to activate regulatory T cells.* J Immunol, 2009. **183**(1): p. 332-9.
131. Kobie, J.J., et al., *T regulatory and primed uncommitted CD4 T cells express CD73, which suppresses effector CD4 T cells by converting 5'-adenosine monophosphate to adenosine.* J Immunol, 2006. **177**(10): p. 6780-6.

132. Deaglio, S., et al., *Adenosine generation catalyzed by CD39 and CD73 expressed on regulatory T cells mediates immune suppression*. J Exp Med, 2007. **204**(6): p. 1257-65.
133. Zarek, P.E., et al., *A2A receptor signaling promotes peripheral tolerance by inducing T-cell anergy and the generation of adaptive regulatory T cells*. Blood, 2008. **111**(1): p. 251-9.
134. Bopp, T., et al., *Cyclic adenosine monophosphate is a key component of regulatory T cell-mediated suppression*. J Exp Med, 2007. **204**(6): p. 1303-10.
135. Serra, P., et al., *CD40 ligation releases immature dendritic cells from the control of regulatory CD4+CD25+ T cells*. Immunity, 2003. **19**(6): p. 877-89.
136. Fallarino, F., et al., *Modulation of tryptophan catabolism by regulatory T cells*. Nat Immunol, 2003. **4**(12): p. 1206-12.
137. Qureshi, O.S., et al., *Trans-endocytosis of CD80 and CD86: a molecular basis for the cell-extrinsic function of CTLA-4*. Science, 2011. **332**(6029): p. 600-3.
138. Cederbom, L., H. Hall, and F. Ivars, *CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells*. Eur J Immunol, 2000. **30**(6): p. 1538-43.
139. Huang, C.T., et al., *Role of LAG-3 in regulatory T cells*. Immunity, 2004. **21**(4): p. 503-13.
140. Sarris, M., et al., *Neuropilin-1 expression on regulatory T cells enhances their interactions with dendritic cells during antigen recognition*. Immunity, 2008. **28**(3): p. 402-13.
141. Vignali, D.A., L.W. Collison, and C.J. Workman, *How regulatory T cells work*. Nat Rev Immunol, 2008. **8**(7): p. 523-32.
142. Kollias, G., et al., *The role of TNF/TNFR in organ-specific and systemic autoimmunity: implications for the design of optimized 'anti-TNF' therapies*. Curr Dir Autoimmun, 2002. **5**: p. 30-50.
143. Aggarwal, B.B., *Signalling pathways of the TNF superfamily: a double-edged sword*. Nat Rev Immunol, 2003. **3**(9): p. 745-56.
144. Carswell, E.A., et al., *An endotoxin-induced serum factor that causes necrosis of tumors*. Proc Natl Acad Sci U S A, 1975. **72**(9): p. 3666-70.
145. Aggarwal, B.B., T.E. Eessalu, and P.E. Hass, *Characterization of receptors for human tumour necrosis factor and their regulation by gamma-interferon*. Nature, 1985. **318**(6047): p. 665-7.
146. Arch, R.H., R.W. Gedrich, and C.B. Thompson, *Tumor necrosis factor receptor-associated factors (TRAFs)--a family of adapter proteins that regulates life and death*. Genes Dev, 1998. **12**(18): p. 2821-30.
147. Park, Y.C., et al., *Structural basis for self-association and receptor recognition of human TRAF2*. Nature, 1999. **398**(6727): p. 533-8.
148. Esparza, E.M., et al., *Tumor necrosis factor receptor (TNFR)-associated factor 5 is a critical intermediate of costimulatory signaling pathways triggered by glucocorticoid-induced TNFR in T cells*. J Biol Chem, 2006. **281**(13): p. 8559-64.
149. Naude, P.J., et al., *Tumor necrosis factor receptor cross-talk*. FEBS J, 2011. **278**(6): p. 888-98.
150. So, T., H. Choi, and M. Croft, *OX40 complexes with phosphoinositide 3-kinase and protein kinase B (PKB) to augment TCR-dependent PKB signaling*. J Immunol, 2011. **186**(6): p. 3547-55.
151. Nam, K.O., et al., *Cross-linking of 4-1BB activates TCR-signaling pathways in CD8+ T lymphocytes*. J Immunol, 2005. **174**(4): p. 1898-905.
152. Croft, M., *Co-stimulatory members of the TNFR family: keys to effective T-cell immunity?* Nat Rev Immunol, 2003. **3**(8): p. 609-20.
153. Soroosh, P., et al., *Differential requirements for OX40 signals on generation of effector and central memory CD4+ T cells*. J Immunol, 2007. **179**(8): p. 5014-23.
154. Byun, M., et al., *Inherited human OX40 deficiency underlying classic Kaposi sarcoma of childhood*. J Exp Med, 2013. **210**(9): p. 1743-59.

155. So, T., et al., *Antigen-independent signalosome of CARMA1, PKCtheta, and TNF receptor-associated factor 2 (TRAF2) determines NF-kappaB signaling in T cells*. Proc Natl Acad Sci U S A, 2011. **108**(7): p. 2903-8.
156. Arvey, A., et al., *Genetic and epigenetic variation in the lineage specification of regulatory T cells*. Elife, 2015. **4**: p. e07571.
157. Balkwill, F., *Tumour necrosis factor and cancer*. Nat Rev Cancer, 2009. **9**(5): p. 361-71.
158. Wiley, S.R., et al., *Identification and characterization of a new member of the TNF family that induces apoptosis*. Immunity, 1995. **3**(6): p. 673-82.
159. Murashima, Y.L., J. Suzuki, and M. Yoshii, *Role of cytokines during epileptogenesis and in the transition from the interictal to the ictal state in the epileptic mutant EL mouse*. Gene Regul Syst Bio, 2008. **2**: p. 267-74.
160. Swardfager, W., et al., *A meta-analysis of cytokines in Alzheimer's disease*. Biol Psychiatry, 2010. **68**(10): p. 930-41.
161. Levine, B., et al., *Elevated circulating levels of tumor necrosis factor in severe chronic heart failure*. N Engl J Med, 1990. **323**(4): p. 236-41.
162. Matera, M.G., L. Calzetta, and M. Cazzola, *TNF-alpha inhibitors in asthma and COPD: we must not throw the baby out with the bath water*. Pulm Pharmacol Ther, 2010. **23**(2): p. 121-8.
163. Kips, J.C., et al., *The potential role of tumour necrosis factor alpha in asthma*. Clin Exp Allergy, 1993. **23**(4): p. 247-50.
164. Schreiber, T.H., et al., *Therapeutic Treg expansion in mice by TNFRSF25 prevents allergic lung inflammation*. J Clin Invest, 2010. **120**(10): p. 3629-40.
165. Vinay, D.S. and B.S. Kwon, *The tumour necrosis factor/TNF receptor superfamily: therapeutic targets in autoimmune diseases*. Clin Exp Immunol, 2011. **164**(2): p. 145-57.
166. Black, R.A., et al., *A metalloproteinase disintegrin that releases tumour-necrosis factor-alpha from cells*. Nature, 1997. **385**(6618): p. 729-33.
167. Chen, G. and D.V. Goeddel, *TNF-R1 signaling: a beautiful pathway*. Science, 2002. **296**(5573): p. 1634-5.
168. Grell, M., et al., *TNF receptor type 2 mediates thymocyte proliferation independently of TNF receptor type 1*. Eur J Immunol, 1998. **28**(1): p. 257-63.
169. Chen, X., et al., *Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse CD4+CD25+FoxP3+ T regulatory cells: applicability to tumor-infiltrating T regulatory cells*. J Immunol, 2008. **180**(10): p. 6467-71.
170. van Mierlo, G.J., et al., *Cutting edge: TNFR-shedding by CD4+CD25+ regulatory T cells inhibits the induction of inflammatory mediators*. J Immunol, 2008. **180**(5): p. 2747-51.
171. Chen, X., et al., *Co-expression of TNFR2 and CD25 identifies more of the functional CD4+FOXP3+ regulatory T cells in human peripheral blood*. Eur J Immunol, 2010. **40**(4): p. 1099-106.
172. Chen, X., et al., *Expression of costimulatory TNFR2 induces resistance of CD4+FoxP3-conventional T cells to suppression by CD4+FoxP3+ regulatory T cells*. J Immunol, 2010. **185**(1): p. 174-82.
173. Grewal, I.S., et al., *Local expression of transgene encoded TNF alpha in islets prevents autoimmune diabetes in nonobese diabetic (NOD) mice by preventing the development of auto-reactive islet-specific T cells*. J Exp Med, 1996. **184**(5): p. 1963-74.
174. Jacob, C.O., et al., *Prevention of diabetes in nonobese diabetic mice by tumor necrosis factor (TNF): similarities between TNF-alpha and interleukin 1*. Proc Natl Acad Sci U S A, 1990. **87**(3): p. 968-72.
175. Kassiotis, G. and G. Kollias, *Uncoupling the proinflammatory from the immunosuppressive properties of tumor necrosis factor (TNF) at the p55 TNF receptor level: implications for pathogenesis and therapy of autoimmune demyelination*. J Exp Med, 2001. **193**(4): p. 427-34.

176. Grinberg-Bleyer, Y., et al., *Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Treg*. J Clin Invest, 2010. **120**(12): p. 4558-68.
177. Williams, G.S., et al., *Phenotypic screening reveals TNFR2 as a promising target for cancer immunotherapy*. Oncotarget, 2016. **7**(42): p. 68278-68291.
178. Kim, E.Y. and H.S. Teh, *TNF type 2 receptor (p75) lowers the threshold of T cell activation*. J Immunol, 2001. **167**(12): p. 6812-20.
179. Chen, X. and J.J. Oppenheim, *Contrasting effects of TNF and anti-TNF on the activation of effector T cells and regulatory T cells in autoimmunity*. FEBS Lett, 2011. **585**(23): p. 3611-8.
180. Pimentel-Muinos, F.X., M.A. Munoz-Fernandez, and M. Fresno, *Control of T lymphocyte activation and IL-2 receptor expression by endogenously secreted lymphokines*. J Immunol, 1994. **152**(12): p. 5714-22.
181. Miller, P.G., M.B. Bonn, and S.C. McKarns, *Transmembrane TNF-TNFR2 Impairs Th17 Differentiation by Promoting Il2 Expression*. J Immunol, 2015. **195**(6): p. 2633-47.
182. Chen, X., et al., *Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells*. J Immunol, 2007. **179**(1): p. 154-61.
183. Hamano, R., et al., *TNF optimally activates regulatory T cells by inducing TNF receptor superfamily members TNFR2, 4-1BB and OX40*. Eur J Immunol, 2011. **41**(7): p. 2010-20.
184. Chen, X., et al., *TNFR2 is critical for the stabilization of the CD4+Foxp3+ regulatory T cell phenotype in the inflammatory environment*. J Immunol, 2013. **190**(3): p. 1076-84.
185. Mougiakakos, D., et al., *Increased thioredoxin-1 production in human naturally occurring regulatory T cells confers enhanced tolerance to oxidative stress*. Blood, 2011. **117**(3): p. 857-61.
186. Nagar, M., et al., *TNF activates a NF-kappaB-regulated cellular program in human CD45RA- regulatory T cells that modulates their suppressive function*. J Immunol, 2010. **184**(7): p. 3570-81.
187. Wang, J., et al., *TNFR2 ligation in human T regulatory cells enhances IL2-induced cell proliferation through the non-canonical NF-kappaB pathway*. Sci Rep, 2018. **8**(1): p. 12079.
188. Nocentini, G., et al., *A new member of the tumor necrosis factor/nerve growth factor receptor family inhibits T cell receptor-induced apoptosis*. Proc Natl Acad Sci U S A, 1997. **94**(12): p. 6216-21.
189. Shimizu, J., et al., *Stimulation of CD25(+)CD4(+) regulatory T cells through GITR breaks immunological self-tolerance*. Nat Immunol, 2002. **3**(2): p. 135-42.
190. Zhan, Y., et al., *Glucocorticoid-induced TNF receptor expression by T cells is reciprocally regulated by NF-kappaB and NFAT*. J Immunol, 2008. **181**(8): p. 5405-13.
191. Cuzzocrea, S., et al., *Proinflammatory role of glucocorticoid-induced TNF receptor-related gene in acute lung inflammation*. J Immunol, 2006. **177**(1): p. 631-41.
192. Krausz, L.T., et al., *GITR-GITRL system, a novel player in shock and inflammation*. ScientificWorldJournal, 2007. **7**: p. 533-66.
193. Kohm, A.P., J.S. Williams, and S.D. Miller, *Cutting edge: ligation of the glucocorticoid-induced TNF receptor enhances autoreactive CD4+ T cell activation and experimental autoimmune encephalomyelitis*. J Immunol, 2004. **172**(8): p. 4686-90.
194. You, S., et al., *Key role of the GITR/GITRLigand pathway in the development of murine autoimmune diabetes: a potential therapeutic target*. PLoS One, 2009. **4**(11): p. e7848.
195. Tomizawa, R., et al., *Association of functional GITR gene polymorphisms related to expression of glucocorticoid-induced tumour necrosis factor-receptor (GITR) molecules with prognosis of autoimmune thyroid disease*. Clin Exp Immunol, 2011. **165**(2): p. 141-7.
196. Gan, X., et al., *Correlation of increased blood levels of GITR and GITRL with disease severity in patients with primary Sjogren's syndrome*. Clin Dev Immunol, 2013. **2013**: p. 340751.

197. Gu, L., et al., *Correlation of circulating glucocorticoid-induced TNFR-related protein ligand levels with disease activity in patients with systemic lupus erythematosus*. Clin Dev Immunol, 2012. **2012**: p. 265868.
198. Wang, S., et al., *Glucocorticoid-induced tumor necrosis factor receptor family-related protein exacerbates collagen-induced arthritis by enhancing the expansion of Th17 cells*. Am J Pathol, 2012. **180**(3): p. 1059-67.
199. Kim, Y.H., et al., *Authentic GITR Signaling Fails To Induce Tumor Regression unless Foxp3+ Regulatory T Cells Are Depleted*. J Immunol, 2015. **195**(10): p. 4721-9.
200. Bulliard, Y., et al., *Activating Fc gamma receptors contribute to the antitumor activities of immunoregulatory receptor-targeting antibodies*. J Exp Med, 2013. **210**(9): p. 1685-93.
201. Mahne, A.E., et al., *Dual Roles for Regulatory T-cell Depletion and Costimulatory Signaling in Agonistic GITR Targeting for Tumor Immunotherapy*. Cancer Res, 2017. **77**(5): p. 1108-1118.
202. Esparza, E.M. and R.H. Arch, *Glucocorticoid-induced TNF receptor, a costimulatory receptor on naive and activated T cells, uses TNF receptor-associated factor 2 in a novel fashion as an inhibitor of NF-kappa B activation*. J Immunol, 2005. **174**(12): p. 7875-82.
203. Kim, I.K., et al., *Glucocorticoid-induced tumor necrosis factor receptor-related protein co-stimulation facilitates tumor regression by inducing IL-9-producing helper T cells*. Nat Med, 2015. **21**(9): p. 1010-7.
204. Nishioka, T., et al., *In vivo expansion of CD4+Foxp3+ regulatory T cells mediated by GITR molecules*. Immunol Lett, 2008. **121**(2): p. 97-104.
205. Mahesh, S.P., et al., *Expression of GITR ligand abrogates immunosuppressive function of ocular tissue and differentially modulates inflammatory cytokines and chemokines*. Eur J Immunol, 2006. **36**(8): p. 2128-38.
206. van Olfen, R.W., et al., *GITR triggering induces expansion of both effector and regulatory CD4+ T cells in vivo*. J Immunol, 2009. **182**(12): p. 7490-500.
207. Merckenschlager, M. and H. von Boehmer, *PI3 kinase signalling blocks Foxp3 expression by sequestering Foxo factors*. J Exp Med, 2010. **207**(7): p. 1347-50.
208. Vinay, D.S. and B.S. Kwon, *4-1BB signaling beyond T cells*. Cell Mol Immunol, 2011. **8**(4): p. 281-4.
209. Kim, Y.H., et al., *4-1BB triggering ameliorates experimental autoimmune encephalomyelitis by modulating the balance between Th17 and regulatory T cells*. J Immunol, 2011. **187**(3): p. 1120-8.
210. Sun, Y., et al., *Administration of agonistic anti-4-1BB monoclonal antibody leads to the amelioration of experimental autoimmune encephalomyelitis*. J Immunol, 2002. **168**(3): p. 1457-65.
211. Liu, G.Z., et al., *Decreased 4-1BB expression on CD4+CD25 high regulatory T cells in peripheral blood of patients with multiple sclerosis*. Clin Exp Immunol, 2008. **154**(1): p. 22-9.
212. Irie, J., et al., *Modulating protective and pathogenic CD4+ subsets via CD137 in type 1 diabetes*. Diabetes, 2007. **56**(1): p. 186-96.
213. Seo, S.K., et al., *4-1BB-mediated immunotherapy of rheumatoid arthritis*. Nat Med, 2004. **10**(10): p. 1088-94.
214. Dubrot, J., et al., *Treatment with anti-CD137 mAbs causes intense accumulations of liver T cells without selective antitumor immunotherapeutic effects in this organ*. Cancer Immunol Immunother, 2010. **59**(8): p. 1223-33.
215. Palazon, A., et al., *The HIF-1alpha hypoxia response in tumor-infiltrating T lymphocytes induces functional CD137 (4-1BB) for immunotherapy*. Cancer Discov, 2012. **2**(7): p. 608-23.
216. Berrien-Elliott, M.M., et al., *Checkpoint blockade immunotherapy relies on T-bet but not Eomes to induce effector function in tumor-infiltrating CD8+ T cells*. Cancer Immunol Res, 2015. **3**(2): p. 116-24.

217. Kang, S.W., et al., *Anti-CD137 Suppresses Tumor Growth by Blocking Reverse Signaling by CD137 Ligand*. *Cancer Res*, 2017. **77**(21): p. 5989-6000.
218. Teixeira, A., et al., *Mitochondrial Morphological and Functional Reprogramming Following CD137 (4-1BB) Costimulation*. *Cancer Immunol Res*, 2018. **6**(7): p. 798-811.
219. Hurtado, J.C., Y.J. Kim, and B.S. Kwon, *Signals through 4-1BB are costimulatory to previously activated splenic T cells and inhibit activation-induced cell death*. *J Immunol*, 1997. **158**(6): p. 2600-9.
220. Lee, H.W., et al., *4-1BB promotes the survival of CD8+ T lymphocytes by increasing expression of Bcl-xL and Bfl-1*. *J Immunol*, 2002. **169**(9): p. 4882-8.
221. Kuang, Y., et al., *Effects of 4-1BB signaling on the biological function of murine dendritic cells*. *Oncol Lett*, 2012. **3**(2): p. 477-481.
222. Sabbagh, L., et al., *ERK-dependent Bim modulation downstream of the 4-1BB-TRAF1 signaling axis is a critical mediator of CD8 T cell survival in vivo*. *J Immunol*, 2008. **180**(12): p. 8093-101.
223. Zheng, G., B. Wang, and A. Chen, *The 4-1BB costimulation augments the proliferation of CD4+CD25+ regulatory T cells*. *J Immunol*, 2004. **173**(4): p. 2428-34.
224. Elpek, K.G., et al., *Ex vivo expansion of CD4+CD25+FoxP3+ T regulatory cells based on synergy between IL-2 and 4-1BB signaling*. *J Immunol*, 2007. **179**(11): p. 7295-304.
225. Zhang, P., et al., *Agonistic anti-4-1BB antibody promotes the expansion of natural regulatory T cells while maintaining Foxp3 expression*. *Scand J Immunol*, 2007. **66**(4): p. 435-40.
226. Kim, J., et al., *Host CD25+CD4+Foxp3+ regulatory T cells primed by anti-CD137 mAbs inhibit graft-versus-host disease*. *Biol Blood Marrow Transplant*, 2012. **18**(1): p. 44-54.
227. Croft, M., *Control of immunity by the TNFR-related molecule OX40 (CD134)*. *Annu Rev Immunol*, 2010. **28**: p. 57-78.
228. Ruby, C.E., et al., *Cutting Edge: OX40 agonists can drive regulatory T cell expansion if the cytokine milieu is right*. *J Immunol*, 2009. **183**(8): p. 4853-7.
229. Weinberg, A.D., et al., *Selective depletion of myelin-reactive T cells with the anti-OX-40 antibody ameliorates autoimmune encephalomyelitis*. *Nat Med*, 1996. **2**(2): p. 183-9.
230. Nohara, C., et al., *Amelioration of experimental autoimmune encephalomyelitis with anti-OX40 ligand monoclonal antibody: a critical role for OX40 ligand in migration, but not development, of pathogenic T cells*. *J Immunol*, 2001. **166**(3): p. 2108-15.
231. Bresson, D., et al., *Antigen-specific prevention of type 1 diabetes in NOD mice is ameliorated by OX40 agonist treatment*. *J Autoimmun*, 2011. **37**(4): p. 342-51.
232. Griseri, T., et al., *OX40 is required for regulatory T cell-mediated control of colitis*. *J Exp Med*, 2010. **207**(4): p. 699-709.
233. Yoshioka, T., et al., *Contribution of OX40/OX40 ligand interaction to the pathogenesis of rheumatoid arthritis*. *Eur J Immunol*, 2000. **30**(10): p. 2815-23.
234. Piconese, S., B. Valzasina, and M.P. Colombo, *OX40 triggering blocks suppression by regulatory T cells and facilitates tumor rejection*. *J Exp Med*, 2008. **205**(4): p. 825-39.
235. Aspeslagh, S., et al., *Rationale for anti-OX40 cancer immunotherapy*. *Eur J Cancer*, 2016. **52**: p. 50-66.
236. Croft, M., et al., *The significance of OX40 and OX40L to T-cell biology and immune disease*. *Immunol Rev*, 2009. **229**(1): p. 173-91.
237. Song, J., et al., *Sustained survivin expression from OX40 costimulatory signals drives T cell clonal expansion*. *Immunity*, 2005. **22**(5): p. 621-31.
238. Xiao, X., et al., *OX40 signaling favors the induction of T(H)9 cells and airway inflammation*. *Nat Immunol*, 2012. **13**(10): p. 981-90.
239. Rogers, P.R., et al., *OX40 promotes Bcl-xL and Bcl-2 expression and is essential for long-term survival of CD4 T cells*. *Immunity*, 2001. **15**(3): p. 445-55.
240. Song, J., et al., *The kinases aurora B and mTOR regulate the G1-S cell cycle progression of T lymphocytes*. *Nat Immunol*, 2007. **8**(1): p. 64-73.
241. So, T., et al., *Signals from OX40 regulate nuclear factor of activated T cells c1 and T cell helper 2 lineage commitment*. *Proc Natl Acad Sci U S A*, 2006. **103**(10): p. 3740-5.

242. Piconese, S., et al., *A non-redundant role for OX40 in the competitive fitness of Treg in response to IL-2*. Eur J Immunol, 2010. **40**(10): p. 2902-13.
243. Xiao, X., et al., *New insights on OX40 in the control of T cell immunity and immune tolerance in vivo*. J Immunol, 2012. **188**(2): p. 892-901.
244. Valzasina, B., et al., *Triggering of OX40 (CD134) on CD4(+)CD25+ T cells blocks their inhibitory activity: a novel regulatory role for OX40 and its comparison with GITR*. Blood, 2005. **105**(7): p. 2845-51.
245. So, T. and M. Croft, *Cutting edge: OX40 inhibits TGF-beta- and antigen-driven conversion of naive CD4 T cells into CD25+Foxp3+ T cells*. J Immunol, 2007. **179**(3): p. 1427-30.
246. Richard, A.C., et al., *The TNF-family cytokine TL1A: from lymphocyte costimulator to disease co-conspirator*. J Leukoc Biol, 2015. **98**(3): p. 333-45.
247. Meylan, F., et al., *The TNF-family receptor DR3 is essential for diverse T cell-mediated inflammatory diseases*. Immunity, 2008. **29**(1): p. 79-89.
248. Bamias, G., et al., *Differential expression of the TL1A/DcR3 system of TNF/TNFR-like proteins in large vs. small intestinal Crohn's disease*. Dig Liver Dis, 2012. **44**(1): p. 30-6.
249. Bamias, G., et al., *Role of TL1A and its receptor DR3 in two models of chronic murine ileitis*. Proc Natl Acad Sci U S A, 2006. **103**(22): p. 8441-6.
250. Sun, X., et al., *Elevated serum and synovial fluid TNF-like ligand 1A (TL1A) is associated with autoantibody production in patients with rheumatoid arthritis*. Scand J Rheumatol, 2013. **42**(2): p. 97-101.
251. Li, L., et al., *TNF-like ligand 1A is associated with the pathogenesis of psoriasis vulgaris and contributes to IL-17 production in PBMCs*. Arch Dermatol Res, 2014. **306**(10): p. 927-32.
252. Jin, S., et al., *TL1A/TNFSF15 directly induces proinflammatory cytokines, including TNFalpha, from CD3+CD161+ T cells to exacerbate gut inflammation*. Mucosal Immunol, 2013. **6**(5): p. 886-99.
253. Reichwald, K., T.Z. Jorgensen, and S. Skov, *TL1A increases expression of CD25, LFA-1, CD134 and CD154, and induces IL-22 and GM-CSF production from effector CD4 T-cells*. PLoS One, 2014. **9**(8): p. e105627.
254. Migone, T.S., et al., *TL1A is a TNF-like ligand for DR3 and TR6/DcR3 and functions as a T cell costimulator*. Immunity, 2002. **16**(3): p. 479-92.
255. Papadakis, K.A., et al., *TL1A synergizes with IL-12 and IL-18 to enhance IFN-gamma production in human T cells and NK cells*. J Immunol, 2004. **172**(11): p. 7002-7.
256. Fang, L., et al., *Essential role of TNF receptor superfamily 25 (TNFRSF25) in the development of allergic lung inflammation*. J Exp Med, 2008. **205**(5): p. 1037-48.
257. Pappu, B.P., et al., *TL1A-DR3 interaction regulates Th17 cell function and Th17-mediated autoimmune disease*. J Exp Med, 2008. **205**(5): p. 1049-62.
258. Pobezinskaya, Y.L., et al., *The adaptor protein TRADD is essential for TNF-like ligand 1A/death receptor 3 signaling*. J Immunol, 2011. **186**(9): p. 5212-6.
259. Richard, A.C., et al., *The TNF-family ligand TL1A and its receptor DR3 promote T cell-mediated allergic immunopathology by enhancing differentiation and pathogenicity of IL-9-producing T cells*. J Immunol, 2015. **194**(8): p. 3567-82.
260. Taraban, V.Y., et al., *Sustained TL1A expression modulates effector and regulatory T-cell responses and drives intestinal goblet cell hyperplasia*. Mucosal Immunol, 2011. **4**(2): p. 186-96.
261. Kim, B.S., et al., *Treatment with agonistic DR3 antibody results in expansion of donor Treg and reduced graft-versus-host disease*. Blood, 2015. **126**(4): p. 546-57.
262. Ward-Kavanagh, L.K., et al., *The TNF Receptor Superfamily in Co-stimulating and Co-inhibitory Responses*. Immunity, 2016. **44**(5): p. 1005-19.
263. Croft, M. and R.M. Siegel, *Beyond TNF: TNF superfamily cytokines as targets for the treatment of rheumatic diseases*. Nat Rev Rheumatol, 2017. **13**(4): p. 217-233.
264. Sonar, S. and G. Lal, *Role of Tumor Necrosis Factor Superfamily in Neuroinflammation and Autoimmunity*. Front Immunol, 2015. **6**: p. 364.

265. Jacobs, J., et al., *CD70: An emerging target in cancer immunotherapy*. *Pharmacol Ther*, 2015. **155**: p. 1-10.
266. Diao, Z., et al., *TRAIL suppresses tumor growth in mice by inducing tumor-infiltrating CD4(+)CD25 (+) Treg apoptosis*. *Cancer Immunol Immunother*, 2013. **62**(4): p. 653-63.
267. Starck, L., et al., *Costimulation by CD137/4-1BB inhibits T cell apoptosis and induces Bcl-xL and c-FLIP(short) via phosphatidylinositol 3-kinase and AKT/protein kinase B*. *Eur J Immunol*, 2005. **35**(4): p. 1257-66.
268. Li, P., et al., *Complex interactions of transcription factors in mediating cytokine biology in T cells*. *Immunol Rev*, 2014. **261**(1): p. 141-56.
269. Yamazaki, T., et al., *CCR6 regulates the migration of inflammatory and regulatory T cells*. *J Immunol*, 2008. **181**(12): p. 8391-401.
270. Brusko, T.M., A.L. Putnam, and J.A. Bluestone, *Human regulatory T cells: role in autoimmune disease and therapeutic opportunities*. *Immunol Rev*, 2008. **223**: p. 371-90.
271. Nishikii, H., et al., *DR3 signaling modulates the function of Foxp3+ regulatory T cells and the severity of acute graft-versus-host disease*. *Blood*, 2016. **128**(24): p. 2846-2858.
272. Burocchi, A., et al., *Intratumor OX40 stimulation inhibits IRF1 expression and IL-10 production by Treg cells while enhancing CD40L expression by effector memory T cells*. *Eur J Immunol*, 2011. **41**(12): p. 3615-26.
273. Joetham, A., et al., *Loss of T regulatory cell suppression following signaling through glucocorticoid-induced tumor necrosis receptor (GITR) is dependent on c-Jun N-terminal kinase activation*. *J Biol Chem*, 2012. **287**(21): p. 17100-8.
274. Bittner, S. and M. Ehrenschwender, *Multifaceted death receptor 3 signaling-promoting survival and triggering death*. *FEBS Lett*, 2017. **591**(17): p. 2543-2555.
275. Lu, L.F., et al., *NF kappa B-inducing kinase deficiency results in the development of a subset of regulatory T cells, which shows a hyperproliferative activity upon glucocorticoid-induced TNF receptor family-related gene stimulation*. *J Immunol*, 2005. **175**(3): p. 1651-7.
276. Molinero, L.L., et al., *High TCR stimuli prevent induced regulatory T cell differentiation in a NF-kappaB-dependent manner*. *J Immunol*, 2011. **186**(8): p. 4609-17.
277. Housley, W.J., et al., *Natural but not inducible regulatory T cells require TNF-alpha signaling for in vivo function*. *J Immunol*, 2011. **186**(12): p. 6779-87.

ANNEX 1

The NF- κ B RelA transcription factor is critical for regulatory T cell activation and stability

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ABSTRACT

Regulatory T cells (Treg) play a major role in immune homeostasis and in the prevention of autoimmune diseases. It has been shown that c-Rel is critical in Treg thymic differentiation but little is known on the role of NF- κ B on mature Treg biology. We generated mice with a specific knockout of RelA, a key member of NF- κ B, in Treg. These mice developed a severe autoimmune syndrome with multi-organ immune infiltration and high activation of lymphoid and myeloid cells. Phenotypic and transcriptomic analyses showed that RelA-deficient Treg have a clear reduction of the signature of activated cells, suggesting a non-redundant intrinsic role of RelA in acquisition of the effector Treg state. Unexpectedly, RelA-deficient Treg had also reduced stability and cells that had lost Foxp3 produced inflammatory cytokines. Thus, RelA appears to be critical in Treg biology by both allowing their capacity to become effector Treg and by maintaining their integrity.

KEYWORDS

Regulatory T cells, NF- κ B, autoimmunity

INTRODUCTION

CD4⁺ CD25⁺ Foxp3⁺ regulatory T cells (Treg) play a critical role in immune homeostasis and in the prevention of autoimmune diseases by regulating immune responses. In humans and mice, it is well established that forkhead box protein 3 (Foxp3) deficiency conducts to the development of an autoimmune syndrome leading to early death (1). Although Foxp3 plays a critical role in differentiation, suppressive function and stability of Treg, other transcription factors (TFs), some of which interacting with Foxp3 in multi-molecular complexes, are also involved in different aspects of their biology. Some, such as c-Rel, are involved in Treg differentiation (2, 3). Others, such as NFAT, RunX1, BACH2 or Eos are critical to maintain their suppressive activity (4–7). Another group of TFs, such as Blimp1, Myb, STAT3, Tbet, IRF4, Bcl6 or PPAR γ are involved in further differentiation of activated Treg and in their capacity to suppress different types of immune responses (8–14). Other TFs, such as STAT5, TET, GATA3, p300/CBP or Ezh2 are involved in maintaining Treg integrity and stability by controlling Foxp3 transcription and epigenetics (15–19). Although it has been reported that NF- κ B is able to bind to the regulatory sequence of Foxp3 and to interact with a complex containing Foxp3 (2, 3, 20), the role of this TF in Treg biology is poorly defined.

The NF- κ B TFs consist of homo or heterodimeric molecules of NF- κ B1 (p105/50), RelA (p65) and c-Rel subunits for the canonic pathway and of NF- κ B2 (p100/52) and RelB subunits for the non-canonic pathway. It has been reported that c-Rel is essential for thymic Treg development by binding to the promoter sequence and the conserved non-coding sequence (CNS) 3 of Foxp3 (2, 3, 21). The role of NF- κ B in mature Treg biology has been addressed by knocking-out upstream activators of the pathway. IKK α and IKK β are kinases that activate NF- κ B. Mice with a conditional knockout (KO) in Treg of either Ubc13, an E2 ubiquitin ligase activating IKK β , or of IKK β itself develop a spontaneous autoimmune syndrome, associated with conversion of Treg into effector-like T cells without Foxp3 loss or reduced Treg survival respectively (22, 23). Mice with a conditional KO of IKK α in whole CD4⁺ T cells have a decreased proportion of Treg in lymphoid organs, which seem to have a defective suppression and proliferation capacities *in vivo* (24). The specific role of RelA in Treg, which is considered as the main factor of NF- κ B members in conventional T cells (25), has been recently studied.. By interacting with RelA and other TFs, such as Helios and p300, Foxp3 forms a multimolecular complex localized in active nuclear areas to act primary as a transcriptional activator (Kwon...Benoist Nat Immunol 18:1238). Mice with a conditional KO of RelA in Treg developed a severe and early spontaneous autoimmune syndrome

that was associated with a defect of effector Treg (26-28). Here, we confirmed these latter findings and added further information on the nature of the disease with extensive description of lymphoid and myeloid cell activation in lymphoid and non-lymphoid tissues. Importantly, we showed that RelA-deficient Treg were unstable, lost Foxp3 expression and produced inflammatory cytokines, revealing that RelA was also critical to maintain Treg integrity, an observation that was not previously reported.

RESULTS

Mice with RelA deficient Treg develop systemic inflammation

To assess the role of RelA in Treg biology, we generated Foxp3Cre Relalox mice that have a specific deletion of RelA in Treg by crossing mice expressing CRE in Treg with mice expressing a RelA floxed allele. In these mice, Treg expressed a non-functional truncated form of RelA (Fig. 1A), as expected using this floxed allele (26). From 5-10 weeks of age, Foxp3Cre Relalox mice developed a spontaneous disease characterized by localized alopecia and skin lesions (epidermal hyperplasia, hyperparakeratosis, cystic hair), and reduced weight growth compared to Foxp3Cre control mice (Fig. 1B, C). This pathology had high penetrance and was severe since all females and most males died by 40 weeks of age (Fig. 1D, E). At 10-12 weeks of age, Foxp3Cre Relalox mice exhibited adenomegaly and macroscopic signs of mild colon inflammation (Fig. 1F, G). Histological analyses showed moderate immune cell infiltration in the lung, stomach and colon and massive infiltration in the skin (Fig. 1H). The liver and small intestine were not or minimally infiltrated. Thus, mice with RelA-deficient Treg developed a severe and systemic inflammatory syndrome.

Foxp3Cre Relalox mice develop a spontaneous autoimmune syndrome

We then analyzed the lymphocyte compartment of 10-12 week-old Foxp3Cre Relalox mice. Numbers of CD45⁺ leukocytes were significantly increased in the skin draining lymph nodes (sdLN), the intern LN (iLN) and the inflamed non-lymphoid tissues (lung, colon and skin) but not in the spleen, mesenteric LN (mLN) or the non-inflamed non-lymphoid tissues (liver, small intestine) (Fig. 2A). This leukocyte expansion was due to increased numbers of CD8⁺ and CD4⁺ T cells, B cells (Fig. 2B and not shown) and myeloid cells (see below). Moreover, the proportions of CD44^{high}CD62L^{low} and Ki67⁺ activated/memory CD8⁺ and CD4⁺ T cells were significantly increased in the sdLN and lung of Foxp3Cre Relalox mice compared to Foxp3Cre control mice (Fig. 2C, D). The same tendency was observed in the colon and skin, although it was not significant, probably because basal levels of activated cells were already high in Foxp3Cre control mice. Interestingly, an increased proportion of activated/memory T cells was observed in the spleen, iLN and mLN as well as in the non-inflamed liver and small intestine, demonstrating a global systemic T cell activation in Foxp3Cre Relalox mice (Fig. 2C, D and data not shown). Systemic inflammation was confirmed by quantifying cytokines in the serum since levels of IFN γ , IL-4, IL-10, IL-17, IL-6 and TNF α were highly increased (Fig. 2E). Also, serum levels of IgM, IgG1, IgG2b, IgA

and IgE (Fig. 2F) and of anti-DNA autoantibodies (Fig. 2G) were increased in 12-14 week-old sick Foxp3Cre Relalox mice compared to Foxp3Cre control mice. The systemic inflammation was further documented by analyzing myeloid cells, characterized as shown in figure 3A, since their numbers were strongly increased in the spleen and sdLN as well as in the non-lymphoid tissues (lung, colon and skin) in Foxp3Cre Relalox mice compared to controls (Fig. 3B). This increase of myeloid cells was due to an increase of neutrophils in all these tissues and of eosinophils and monocytes in all tissues except the lung (Fig. 3C). Only part of this inflammatory phenotype was observed in 4-6 week-old Foxp3Cre Relalox mice. Increased numbers of whole CD45+ leukocytes were observed in sdLN and iLN but not yet in the lung, colon and skin (Sup. Fig. 1A). A trend for higher proportion of activated/memory T cells, defined by expression of CD44, CD62L and Ki67, was observed in all analyzed lymphoid and non-lymphoid tissues of young mice (Sup. Fig. 1B). Finally, inflammatory cytokines, natural antibodies and anti-DNA antibodies were not or minimally increased in 4-6 week-old Foxp3Cre Relalox compared to control mice (Fig. 2E-G). In conclusion, Foxp3Cre Relalox mice developed a severe systemic autoimmune syndrome, already uncovered at 4-6 weeks of age, followed, 1-3 months later, by massive activation of lymphoid and myeloid cells, immune infiltration of several tissues and high rise of serum inflammatory cytokines, immunoglobulins and auto-antibodies.

Homeostasis and function of Treg in Foxp3Cre Relalox mice

We then analyzed Treg homeostasis in 12 week-old Foxp3Cre Relalox mice. Strikingly, Treg proportion was significantly increased in lymphoid organs, except in mLN, whereas it was decreased in the colon and skin and unchanged in the liver, lung and small intestine compared to Foxp3Cre control mice (Fig. 4A). The proportion of activated/memory CD44^{hi}CD62L^{low} Treg was decreased in all LN, the liver and the skin, unchanged in the spleen, colon and small intestine and increased in the lung (Fig. 4B and Sup. Fig. 2A). Finally, Treg of Foxp3Cre Relalox mice had a more activated phenotype, based on the expression of the ICOS, GITR and CD103 Treg activation markers, in most lymphoid and non-lymphoid organs (Fig. 4C and Sup. Fig. 2B). Foxp3 and CD25 expressions were unchanged (data not shown). In 4-5 week-old Foxp3Cre Relalox mice, Treg proportion and markers of activation (CD44, ICOS and Ki67) were not significantly increased in lymphoid tissues (Sup. Fig. 2C, D). Interestingly, Treg proportion was already decreased in the intestine and skin of these young mice (Sup. Fig. 2C).

The severe disease of Foxp3Cre Relalox mice in the absence of major Treg quantitative defect suggests that Treg may be dysfunctional. In vitro assays showed that RelA-deficient Treg, purified

from 5-6 week-old mice, were able to suppress proliferation of conventional T cells almost as efficiently as control Treg (Fig. 4D). To further analyze their function, we assessed their capacity to suppress colitis induced by effector T cells transferred in lymphopenic mice, measured by weight loss and histology. Surprisingly, not only RelA-deficient Treg were unable to control colitis but the disease was even more severe compared to mice transferred with effector T cells alone (Fig. 4E, F). This exacerbated colitis was not associated with increased number of cells from Tconv origin or to their lower propensity to differentiate in peripheral Treg (data not shown). Instead, the severe colitis was rather due to the fact that most RelA-deficient Treg lost Foxp3 expression in the colon and mLN, potentially differentiating in pathogenic effector T cells (Fig. 4G). In conclusion, Foxp3Cre Relalox mice had higher numbers of Treg in lymphoid tissues (probably due to systemic inflammation) but lower numbers of Treg in the colon and skin, which could be due to Treg instability and Foxp3 loss since RelA-deficient Treg lost Foxp3 expression in a lymphopenic environment.

Reduced expression of activation markers in RelA-deficient Treg at steady state

Foxp3Cre Relalox mice developed systemic inflammation, which in return impact on Treg biology. To assess the intrinsic role of RelA in Treg at steady state, we generated Foxp3Cre/wt Relalox heterozygote females, in which theoretically half of Treg expressed RelA and the other half were RelA-deficient because the Foxp3 locus is on the X chromosome. We first observed that these mice did not have any sign of disease and inflammation, which was most likely due to the presence of functional RelA-sufficient Treg. Indeed, numbers of leukocytes, CD4, CD8, and Treg and proportions of activated Tconv cells were similar in Foxp3Cre/wt Relalox females and Foxp3Cre/wt controls (Sup. Fig. 3). In the Foxp3Cre/wt control females, CRE-expressing Treg were present in lower proportion and with slightly lower expression of activation markers compared to Treg not expressing CRE, suggesting that the CRE transgene moderately impact on Treg biology in this competitive condition (Fig. 5A, B). That is why it was critical to compare Foxp3Cre/wt Relalox mice with Foxp3Cre/wt controls. Compared to these controls, the knockout of RelA slightly but not significantly reduced the proportion of Treg in spleen and LNs (Fig. 5A). The absence of RelA expression had a more severe impact on Treg activation since the proportions of CD44^{high}CD62L^{low}, Ki67⁺ and CD103⁺ Treg were strongly reduced in all tissues and the ones of ICOS, CTLA-4 and GITR were reduced in some tissues (Fig. 5B and Sup. Fig. 4A). Also, the proportion of Nrp1⁺, Helios⁺ thymic derived Treg and FoxP3 expression were slightly but not

significantly reduced in the absence of RelA (Sup. Fig. 4B). In conclusion, in the absence of inflammation, RelA expression appears critical to promote proper Treg activation.

Lack of stability of RelA-deficient Treg.

We tested whether RelA had any role in maintenance of Treg stability and integrity by analyzing Foxp3 expression after transfer of RelA-deficient Treg. Cells were purified from Foxp3Cre/wt Relalox mice (Foxp3Cre/wt for controls) and not from Foxp3Cre Relalox mice since systemic inflammation in these latter mice would modify Treg biology in addition to the impact of the RelA defect. We co-transferred RelA-deficient and -sufficient Treg with Tconv cells, to sustain viability and expansion of injected Treg, in CD3 KO mice (Fig. 6A). Sixteen days after transfer, the proportions of RelA-deficient cells were much reduced compared to the ones of RelA-sufficient cells (Fig. 6B), particularly in the colon, a location subjected to high inflammation in this setting. Importantly, a large fraction of RelA-deficient Treg became ex-Treg in all lymphoid and non-lymphoid tissues (Fig. 6C). In addition, the remaining low level of Foxp3 was even further reduced in RelA-deficient ex-Treg compared to control ex-Treg (Fig. 6D). Importantly, RelA-deficient ex-Treg expressed higher amounts of the pro-inflammatory cytokines IFN γ and TNF α in the spleen and mLN (Fig. 6E).

We then investigated the level of CpG methylation in the conserved noncoding sequence 2 (CNS2) of Foxp3 since its hypomethylation is associated with Treg stability (30, 32). However, we did not observe any difference between RelA-deficient and WT Treg, whether they were sorted from Foxp3Cre and Foxp3Cre Relalox mice or from Foxp3Cre/wt and Foxp3Cre/wt Relalox mice (data not shown).

To further explore the mechanism of Treg instability, we performed EMSA combined with supershift to assess the activation status of the different NF- κ B subunits in Treg of Foxp3Cre and Foxp3Cre Relalox mice (Fig. 6F). In control Treg, there was mainly an activation of RelA, rather than RelB or cRel. As expected, we did not observe this phenomenon in RelA-deficient Treg, confirming that the truncated RelA protein was not functional. However, there was a massive activation of c-Rel in Treg of Foxp3Cre Relalox mice, showing that c-Rel, rather than RelA, was the dominant NF- κ B member bound to its DNA target in RelA-deficient Treg. As discussed below this increased c-Rel activation may be involved in Treg instability. In conclusion, the lack of RelA activation affected Treg stability leading to Foxp3 loss and increased differentiation of ex-Treg, which produced inflammatory cytokines.

RelA deficient Treg have a major activation defect

To characterize more extensively the effects of the RelA deficiency on Treg, we purified CRE-expressing Treg from *Foxp3Cre/wt* (WT) and *Foxp3Cre/wt Relalox* (RelA KO) mice and profiled their transcriptomes by low-input RNAseq as described (27). Overall, transcriptome differences were modest (Fig. 7A), with only 180 differentially expressed genes at an arbitrary fold change cutoff of 2.0 (and false discovery rate <0.05). The most biased transcript was *Klrg1*, as previously reported (28), but several other transcripts expressed in Treg showed a significant bias (e.g. *Ccr4*, *Ccr6* or *Cd86*). Gene ontology analysis did not reveal any evocative common pathway, so we projected various Treg-specific signatures onto the comparison of WT vs. RelA KO Treg profiles (Fig. 7B). RelA deficit significantly but modestly reduced the canonical signature of genes differentially expressed in Treg vs Tconv cells (29) (Fig. 7B, left). But, consistent with the phenotype described above showing reduced proportion of activation markers in RelA-deficient Treg, a stronger bias was observed for signatures typical of activated Treg (from comparison of CD44^{hi} vs CD62L^{hi} Treg, or from *Blimp1*- WT vs KO Treg (11)) (Fig. 7B, right). This effect was not unique to Treg activation, as GSEA analysis showed a strong bias of generic signatures of activation of CD4⁺ or CD8⁺ Tconv cells (30) (Fig. 7C). For further resolution, we cross-matched the RelA WT/KO difference to a curated series of 289 signatures that distinguish different sub-phenotypes of Treg (31) (Fig. 7D). Compared with WT counterparts, RelA KO Treg were impoverished in several signatures of activated Treg (lower region of Fig. 7D), consistent with Fig. 7B. Interestingly, however, RelA-deficient Treg were enriched in several signatures resulting from the expression of TF that have inhibitory roles in Treg, and most markedly for *Bach2* (upper region of Fig. 7D). Indeed, the changes found here in response to RelA deficiency were largely anti-correlated with changes provoked by the absence of *Bach2* in a previous report (7) (Fig. 7E, $r = -0.13$ with $p < 10^{-15}$ using a Pearson correlation). Overall, these results suggest an inability of RelA-deficient Treg to adopt fully active configurations.

DISCUSSION

Here, we show that RelA plays a major role in Treg biology since its specific deletion leads to the development of a spontaneous, severe and systemic autoimmune syndrome. The disease recapitulates some of the symptoms observed in Treg-deficient scurfy mice, although with a slower kinetics (1). Our data suggest that this disease is initially due to a major activation defect of RelA-deficient Treg. Indeed, in the *Foxp3Cre/wt Relalox* non-inflamed mice, we observed reduced numbers of effector Treg and suppressive molecules among the RelA-deficient Treg. We thus speculate that in the *Foxp3Cre Relalox* mice, and more specifically in tissues that are in contact with external environment and microbiota like the intestine and skin, effector T cells and myeloid cells become highly activated because of insufficient control by effector Treg. Then, inflammatory factors may alter drastically stability of RelA-deficient Treg most of them becoming pathogenic ex-Treg, as we observed in the colitis model and cell co-transfer in lymphopenic mice experiments, precipitating local inflammation. The combination of reduced Treg proportion in the intestine and the skin, reduced Treg activation and the generation of pathogenic ex-Treg may be the driving forces of the autoimmune syndrome of *Foxp3Cre Relalox* mice.

Recent reports describe similar conditional KO mice developing a related autoimmune syndrome (28, 32, 33). They observed that *Foxp3Cre Relalox* mice developed inflammation of the skin, stomach, lung and colon, massive activation of effector T cells and myeloid cells in lymphoid organs and high levels of inflammatory cytokines, immunoglobulins and anti-DNA in the serum. We confirmed these data and got deeper into the analysis of the disease since we showed that the effector T cells and myeloid cells were also drastically activated in multiple non-lymphoid organs. These data suggest a major defect of RelA-deficient Treg. In addition, the injection of WT Treg before 7 days of age was sufficient to stop the development of the pathology (data not shown). Surprisingly, we and others observed an increase of Treg proportion in lymphoid organs and *in vitro* assays did not reveal Treg suppressive defect. However, our extensive analysis enabled to point out a decrease of Treg proportion in the inflamed non-lymphoid tissues, such as the colon and skin. As discussed above, this initial event may ignite the whole immune system, leading to widespread activation of the lymphoid and myeloid compartments and release of inflammatory cytokines that will boost global Treg activation and expansion, which remains insufficient to control the pathology.

Investigating initial events that led to disease could not be properly analyzed in Foxp3Cre Relalox mice since inflammation has major impact on Treg migration, survival, activation, suppressive function or stability (34, 35), confounding the interpretation of what was due to inflammation or to the intrinsic RelA deficit. Using LckCre Relalox mice, Messina et al. suggested that a major alteration of RelA-deficient Treg was their defect to differentiate in effector Treg (28). However, in this work, this defect was only partial, observed in LN and not in the spleen, and mostly analyzed in a quite irrelevant model since RelA was knockout in whole T cells. Vasanthakumar et al. showed a more global activation defect of RelA-deficient Treg using Foxp3Cre/wt Relalox mice or mixed bone marrow chimeric mice (28). We confirmed and completed these results since we observed a downregulation of CD44, ICOS, TIGIT, CD103, Ki67, GITR and CTLA4 not only in the lymphoid organs but also in the liver and lung of Foxp3Cre/wt Relalox mice. Moreover, our transcriptomic analysis highlighted the major activation defect of RelA-deficient Treg, since a strong bias was observed for signatures typical of activated Treg. This poor Treg activation may be Bach2 dependent. Indeed, the signature of RelA-deficient Treg was largely anti-correlated with the signature of Bach2-deficient Treg, suggesting a possible over-activity of Bach2 in RelA-deficient Treg. High activity of Bach2 may limit their capacity to be properly activated and to become effector Treg since this TF represses the effector program in Treg (7).

What was more consistent and unexpected was the increased un-stability of RelA-deficient Treg. This was first suggested in the colitis model. However, one cannot conclude from this finding that RelA plays an intrinsic role in Treg stability. Indeed, compared to lymphopenic mice co-transferred with Teff and WT Treg, the ones co-transferred with Teff and RelA-deficient Treg developed very severe colitis. Increased Treg un-stability could be well due to increased inflammation, and not RelA deficiency, since it is well established that different inflammatory factors precipitate Foxp3 loss (34). Direct evidence came from studies when we compared RelA-sufficient and -deficient Treg in the same environment after cell co-transfer in lymphopenic mice. We clearly showed that most RelA-deficient Treg became ex-Treg, contrary to control Treg. Although with reduced intensity, increased un-stability of RelA-deficient Treg was also observed in the absence of inflammation, as measured after transfer in lymphoreplete mice (data not shown). Moreover, we revealed that these newly RelA-deficient ex-Treg expressed inflammatory cytokines, suggesting that they could become pathogenic because of their intrinsic autoreactivity. This phenomenon may explain the increased severity observed in the colitis experiment and support our hypothesis that this ex-Treg contribute to the pathology of Foxp3Cre Relalox mice. What are the molecular mechanisms explaining un-stability of RelA-deficient Treg? Foxp3 stability is controlled by histone

and protein acetylation and by DNA methylation in the CNS 2 of Foxp3. RelA activity may impact on these epigenetic modulations by different ways. RelA interacts with CBP and p300 histone/protein acetyltransferases, which seems to be critical for the recruitment of CBP and p300 to their target promoter sites, as shown in fibroblasts (36). Because CBP and p300 promote Foxp3 transcription, Foxp3 stability at the level of CNS2 and prevent Foxp3 degradation (17, 37), RelA-deficient Treg may have major un-stability. It has also been recently reported that RelA binds to genes involved in histone modification (33). Surprisingly, we did not observe any difference in the level of CNS2 CpG methylation between WT and RelA-deficient Treg. This result may be explained by a possible rapid loss of Foxp3 in unstable RelA-deficient Treg, precluding identification of precursor of ex-Treg. Also, Foxp3 and RelA seem to cooperate to promote Foxp3 and CD25 expression by binding to their regulatory sequences (38, 39), which may favor Treg stability given the known role of IL-2 receptor signaling pathway in maintenance of Treg identity (16). Furthermore, Oh et al. recently reported that Foxp3 expression was down-regulated on Treg of Foxp3CreInd cRellox mice and even more in the Foxp3CreInd cRellox RelAlox mice, suggesting that RelA favors Foxp3 expression (33). Interestingly, we observed a dramatic increased binding of c-Rel to its target DNA sequence in Treg of Foxp3Cre Relalox mice. This phenomenon may hide the genuine role of RelA in Treg and may further increase its conversion in pathogenic Th17 cells since c-Rel has been reported to be involved in Th1 and Th17 differentiation (44, 45). Overall, our study further confirms the non-redundant role of RelA in Treg biology, highlighting its critical role in Treg stability.

EXPERIMENTAL PROCEDURES

Mice. Foxp3-CRE-IRES-YFP (Foxp3Cre) (40), RelAflox (26) and Foxp3-IRES-GFP (41) knock-in (Foxp3GFP) mice were kindly given by Prs. Alexander Rudensky, Falk Weih and Bernard Malissen, respectively. CD3etm1Mal (CD3^{-/-}), CD45.1, CD90.1 and RAG2^{-/-} mice were obtained from the cryopreservation distribution typing and animal archiving department (Orléans, France). All mice were on a C57Bl/6 background. Mice were housed under specific pathogen-free conditions. All experimental protocols were approved by the local ethics committee and are in compliance with European Union guidelines.

Western blot. Cells were lysed for 20 min on ice in extraction buffer (0.4 M NaCl, 25 mM Hepes pH 7.7, 1.5 mM MgCl₂, 0.2 mM EDTA, 1% NP40, 20 mM glycerol phosphate, 0.2 mM Na₃VO₄, 10 mM PNPP, 2mM DTT, 0.1 M PMSF). Whole cell extract was harvested after centrifuging the lysate for 10 min at 9500 X g. 20 µg of whole cell extract were separated on 7.5% SDS–polyacrylamide gels and transferred to nitrocellulose membranes (GE Healthcare). Immunoblotting was performed with anti-RelA (C20) polyclonal antibodies (Santa Cruz Biotechnology) and anti-β-actin antibody (Sigma Aldrich) and visualized using the ECL Western blotting detection kit (Pierce).

Histology. Organs were collected and fixed in PBS containing 4% formaldehyde for 48 hours and then transferred in 70% ethanol. Five-micrometer paraffin-embedded sections were cut and stained with hematoxylin and eosin and then blindly analyzed.

Cell preparation from tissues. For lymphoid tissues, cells were isolated by mechanical dilacerations. For non-lymphoid tissues, anesthetized mice were perfused intracardially with cold PBS. Small pieces of livers and lungs were digested in type IV collagenase (0.3 mg/ml) and DNase I (100 µg/ml) for 30 min at 37°C, followed by Percoll gradient (30–70%) separation. Small pieces of intestines, removed of their Peyer patches and epithelium, were digested in type IV collagenase (1 mg/ml) and DNase I (10 µg/ml) for 30 min at 37°C, followed by Percoll gradient (40–80%) separation. Small pieces of skin were digested in liberase DL (0.4mg/ml), collagenase D (0.05 mg/ml) and DNase I (10µg/ml) for 1h at 37°C, followed by Percoll gradient (40–80%) separation.

Antibodies and flow cytometry analysis. The following mAbs from BD Biosciences were used: anti-CD45 (30-F11), anti-CD8 (53-6.7), anti-CD4 (RM4-5), anti-CD62L (MEL-14), anti-CD90.1 (OX-7), anti-CD45.1 (A20), anti-CD45.2 (104), anti-CD25 (PC61 or 7D4), anti-ICOS (7E.17G9), anti-GITR (DTA-1), anti-CD103 (M290), anti-Helios (22F6), anti-CTLA-4 (UC10-4F10-11), anti-CD11b (M1/70), anti-CD11c (HL3), anti-CD19 (1D3), anti-IA/E (M5/114.15.2), anti-Ly6C (AL-21), anti-Ly6G (1A8). Anti-GFP antibody was purchased from Life Technologies. Anti-CD3 (145-2C11), anti-Foxp3 (FJK-16s), anti-CD44 (IM7), anti-Ki-67 (SOLA15), anti-Nrp1 (3DS304M), anti-NKp46 (29A1.4) and anti-F4/80 (BM8) were purchased from eBioscience, and Foxp3 staining was performed using the eBioscience kit and protocol. Cells were acquired on a BD LSRII and a BD Fortessa X20 cytometers and analyzed using FlowJo software.

Cytokine quantification. Serum cytokines were quantified using the mouse Th1/Th2/Th17 Cytokine CBA Kit (BD Biosciences) according to manufacturer's procedure. Datas were analyzed using FCAP array software.

Immunoglobulin and autoantibody quantification by ELISA. 96-well flat plates were coated with either salmon sperm DNA (Sigma) or with goat anti-mouse IgM, IgA, IgE, IgG1, IgG2b (Southern Biotech). After washes, they were saturated with BSA and first incubated with mice sera, then with biotinylated goat anti-mouse IgG (Southern Biotech) or goat anti-mouse IgM, IgA, IgE, IgG1, IgG2b (Southern Biotech). A streptavidin-horseradish conjugate (Sigma) was added followed by the addition of TMB (eBioscience). The reaction was stopped with HCl (1N) and revealed with an ELISA plate reader DTX880 Multimode Detector (Beckman Coulter).

Treg and Tconv cell purification. Treg were purified after enrichment of CD25⁺ cells using biotinylated anti-CD25 mAb (7D4) and anti-biotin microbeads (Miltenyi Biotec), followed by CD4 staining (RM4.5) and cell sorting of CD4⁺ Foxp3⁺/YFP⁺ cells or CD4⁺ Foxp3⁺/GFP⁺ using the BD FACS Aria II. Tconv cells were purified after enrichment of CD25⁻ cells using biotinylated anti-CD25 mAb (7D4) or of CD8⁻CD19⁻CD11b⁻ cells using biotinylated anti-CD8 (53-6.7), CD19 (1D3) and CD11b (M1/70) mAbs and anti-biotin microbeads (Miltenyi Biotec), followed by CD4 staining (RM4.5) and cell sorting of CD4⁺ Foxp3⁻/YFP⁻ cells or CD4⁺ Foxp3⁻/GFP⁻ using the BD FACS Aria II.

Cell cultures. Purified Treg (CD4+YFP+, 25 x 10³ cells/well) were cultured with or without whole splenocyte from CD3KO mice (7.5 x 10⁴ cells/well), anti-CD3 mAb (0.05µg/ml, BioXcell), TNF (50ng/ml, Protein Service Facility, VIB, Belgium) and IL-2 (10ng/ml, Peproteck) in a 96-well round plate in RPMI 1640 10% FCS. For suppression assays, after labeling with CellTrace Violet Proliferation Kit (Life technologies), Tconv cells (CD4+YFP-, 2.5 x 10⁴ cells/well) were co-cultures with various Treg (CD4+YFP+) numbers and stimulated by splenocytes from CD3 KO mice (7.5 x 10⁴ cells/well) and soluble anti-CD3 (0.05µg/ml 2C11, BioXCell) in RPMI 1640-10% FCS.

Colitis. Tconv cells (CD4+GFP-, 1 x 10⁵ cells) and Treg (CD4+YFP+, 2 x 10⁴ cells) were injected intravenously into sex-matched RAG2^{-/-} mice. The clinical evaluation was performed three times a week by measuring body weight.

T-cell adoptive transfer. CD3 KO mice were co-transferred with Treg (CD4+YFP+, 1 x 10⁵ each) purified from age and sex-matched CD45.1/2 Foxp3Cre/+ and CD45.2/2 Foxp3Cre/+ Relalox mice and Tconv cells (CD4+GFP-, 8 x 10⁵) purified from CD90.1 Foxp3GFP mice.

Pyrosequencing-based bisulfite PCR analysis. 250ng of genomic DNA was treated with EZ DNA methylation-Gold kit according manufacturer's protocol (Zymo Research Corporation). We PCR-amplified the bisulfite treated DNA using unbiased nested primers: 5'-TTTTGGGTTTTTTGGTATTTAAGA-3' and 5'-Biotin-TTAACCAAATTTTTCTACCATTAAC-3'. Biotin-labeled single stranded amplicons were isolated according manufacturer's protocol using the Qiagen PyroMark Q24 WorkStation (Qiagen). Pyrosequencing was next performed on a PyroMark Q24 Instrument (Qiagen) with 0.5µM of each sequencing primers: 5'- ATTCTATCTTTCTTAAATACC-3' and 5'-AAAATAACTAATCTATCCT-3'. The percent methylation for each of the CpGs within the target sequence was calculated using the PyroQ CpG Software (Qiagen).

Electrophoretic Mobility Shift Assays (EMSA) combined with supershit assays. Nuclear extracts were prepared and analyzed for DNA binding activity using the HIV-LTR tandem κB oligonucleotide as κB probe (42). For supershift assays, nuclear extracts were incubated with specific antibodies for 30 min on ice before incubation with the labeled probe.

Gene-Expression Profiling and Analysis. Treg (1,000) were double-sorted into TRIzol (Invitrogen). Subsequent sample processing was followed by Ultra-low input RNAseq protocol as described (27). Normalized data were analyzed with Multiplot Studio, GSEA and Gene-e modules in Genepattern. For signature enrichment analysis, each signature was curated from published datasets and computed by comparison between two conditions (e.g. WT vs KO). Data were downloaded from GEO containing replicates were used. To reduce noise, genes with a coefficient of variation between biological replicates > 0.6 in either comparison groups were selected. Up- and down-regulated transcripts were defined as having a fold change in gene expression > 1.5 or $< 2/3$ and a t.test p-value < 0.05 .

Statistical analysis. Statistical analyses were performed using GraphPad Prism Software. Statistical significance was determined using a log-rank (Mantel- Cox) test for the mouse survival data. For all the other statistical analysis, the two-tailed unpaired nonparametric Mann–Whitney U test was used for data not following a normal distribution and the t-test was used for data following a normal distribution. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$. Means \pm SEM were used throughout the figures.

AUTHOR CONTRIBUTIONS

B.L.S. and E.R. designed the research. E.R. performed almost all the experiments and analyzed the data. M.L.R, R.V., J.D., S.G. A.R. and D.F. helped E.R. on some experiments. H.K. and C.B. performed and analyzed the RNA-seq data. D.C. and V.B. performed and analyzed the western blot and EMSA. E.R. and B.L.S. wrote the manuscript using comments from all authors.

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COMPETING FINANCIAL INTERESTS.

The authors declare no competing financial interests.

REFERENCES

1. Sakaguchi, S., M. Ono, R. Setoguchi, H. Yagi, S. Hori, Z. Fehervari, J. Shimizu, T. Takahashi, and T. Nomura. 2006. Foxp3+CD25+CD4+ natural regulatory T cells in dominant self-tolerance and autoimmune disease. *Immunol. Rev.* 212: 8–27.
2. Isomura, I., S. Palmer, R. J. Grumont, K. Bunting, G. Hoyne, N. Wilkinson, A. Banerjee, A. Proietto, R. Gugasyan, L. Wu, A. McNally, R. J. Steptoe, R. Thomas, M. F. Shannon, and S. Gerondakis. 2009. c-Rel is required for the development of thymic Foxp3+ CD4 regulatory T cells. *J. Exp. Med.* 206: 3001–3014.
3. Long, M., S.-G. Park, I. Strickland, M. S. Hayden, and S. Ghosh. 2009. Nuclear Factor- κ B Modulates Regulatory T Cell Development by Directly Regulating Expression of Foxp3 Transcription Factor. *Immunity* 31: 921–931.
4. Wu, Y., M. Borde, V. Heissmeyer, M. Feuerer, A. D. Lapan, J. C. Stroud, D. L. Bates, L. Guo, A. Han, S. F. Ziegler, D. Mathis, C. Benoist, L. Chen, and A. Rao. 2006. FOXP3 Controls Regulatory T Cell Function through Cooperation with NFAT. *Cell* 126: 375–387.
5. Ono, M., H. Yaguchi, N. Ohkura, I. Kitabayashi, Y. Nagamura, T. Nomura, Y. Miyachi, T. Tsukada, and S. Sakaguchi. 2007. Foxp3 controls regulatory T-cell function by interacting with AML1/Runx1. *Nature* 446: 685–689.
6. Pan, F., H. Yu, E. V. Dang, J. Barbi, X. Pan, J. F. Grosso, D. Jinasena, S. M. Sharma, E. M. McCadden, D. Getnet, C. G. Drake, J. O. Liu, M. C. Ostrowski, and D. M. Pardoll. 2009. Eos Mediates Foxp3-Dependent Gene Silencing in CD4+ Regulatory T Cells. *Science* 325: 1142–1146.
7. Roychoudhuri, R., K. Hirahara, K. Mousavi, D. Clever, C. A. Klebanoff, M. Bonelli, G. Sciumè, H. Zare, G. Vahedi, B. Dema, Z. Yu, H. Liu, H. Takahashi, M. Rao, P. Muranski, J. G. Crompton, G. Punkosdy, D. Bedognetti, E. Wang, V. Hoffmann, J. Rivera, F. M. Marincola, A. Nakamura, V. Sartorelli, Y. Kanno, L. Gattinoni, A. Muto, K. Igarashi, J. J. O’Shea, and N. P. Restifo. 2013. BACH2 represses effector programs to stabilize Treg-mediated immune homeostasis. *Nature* 498: 506–510.
8. Chaudhry, A., D. Rudra, P. Treuting, R. M. Samstein, Y. Liang, A. Kas, and A. Y. Rudensky. 2009. CD4+ Regulatory T Cells Control TH17 Responses in a Stat3-Dependent Manner. *Science* 326: 986–991.
9. Koch, M. A., G. Tucker-Heard, N. R. Perdue, J. R. Killebrew, K. B. Urdahl, and D. J. Campbell. 2009. The transcription factor T-bet controls regulatory T cell homeostasis and function during type 1 inflammation. *Nat. Immunol.* 10: 595–602.
10. Zheng, Y., A. Chaudhry, A. Kas, P. deRoos, J. M. Kim, T.-T. Chu, L. Corcoran, P. Treuting, U. Klein, and A. Y. Rudensky. 2009. Regulatory T-cell suppressor program co-opts transcription factor IRF4 to control TH2 responses. *Nature* 458: 351–356.

11. Cretney, E., A. Xin, W. Shi, M. Minnich, F. Masson, M. Miasari, G. T. Belz, G. K. Smyth, M. Busslinger, S. L. Nutt, and A. Kallies. 2011. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat. Immunol.* 12: 304–311.
12. Linterman, M. A., W. Pierson, S. K. Lee, A. Kallies, S. Kawamoto, T. F. Rayner, M. Srivastava, D. P. Divekar, L. Beaton, J. J. Hogan, S. Fagarasan, A. Liston, K. G. C. Smith, and C. G. Vinuesa. 2011. Foxp3+ follicular regulatory T cells control the germinal center response. *Nat. Med.* 17: 975–982.
13. Cipolletta, D., M. Feuerer, A. Li, N. Kamei, J. Lee, S. E. Shoelson, C. Benoist, and D. Mathis. 2012. PPAR- γ is a major driver of the accumulation and phenotype of adipose tissue Treg cells. *Nature* 486: 549–553.
14. Dias, S., A. D'Amico, E. Cretney, Y. Liao, J. Tellier, C. Bruggeman, F. F. Almeida, J. Leahy, G. T. Belz, G. K. Smyth, W. Shi, and S. L. Nutt. 2017. Effector Regulatory T Cell Differentiation and Immune Homeostasis Depend on the Transcription Factor Myb. *Immunity* 46: 78–91.
15. Wohlfert, E. A., J. R. Grainger, N. Bouladoux, J. E. Konkel, G. Oldenhove, C. H. Ribeiro, J. A. Hall, R. Yagi, S. Naik, R. Bhairavabhotla, W. E. Paul, R. Bosselut, G. Wei, K. Zhao, M. Oukka, J. Zhu, and Y. Belkaid. 2011. GATA3 controls Foxp3+ regulatory T cell fate during inflammation in mice. *J. Clin. Invest.* 121: 4503–4515.
16. Feng, Y., A. Arvey, T. Chinen, J. van der Veecken, G. Gasteiger, and A. Y. Rudensky. 2014. Control of the Inheritance of Regulatory T Cell Identity by a cis Element in the Foxp3 Locus. *Cell* 158: 749–763.
17. Liu, Y., L. Wang, R. Han, U. H. Beier, T. Akimova, T. Bhatti, H. Xiao, P. A. Cole, P. K. Brindle, and W. W. Hancock. 2014. Two Histone/Protein Acetyltransferases, CBP and p300, Are Indispensable for Foxp3+ T-Regulatory Cell Development and Function. *Mol. Cell. Biol.* 34: 3993–4007.
18. DuPage, M., G. Chopra, J. Quiros, W. L. Rosenthal, M. M. Morar, D. Holohan, R. Zhang, L. Turka, A. Marson, and J. A. Bluestone. 2015. The Chromatin-Modifying Enzyme Ezh2 Is Critical for the Maintenance of Regulatory T Cell Identity after Activation. *Immunity* 42: 227–238.
19. Yang, R., C. Qu, Y. Zhou, J. E. Konkel, S. Shi, Y. Liu, C. Chen, S. Liu, D. Liu, Y. Chen, E. Zandi, W. Chen, Y. Zhou, and S. Shi. 2015. Hydrogen Sulfide Promotes Tet1- and Tet2-Mediated Foxp3 Demethylation to Drive Regulatory T Cell Differentiation and Maintain Immune Homeostasis. *Immunity* 43: 251–263.
20. Bettelli, E., M. Dastrange, and M. Oukka. 2005. Foxp3 interacts with nuclear factor of activated T cells and NF- κ B to repress cytokine gene expression and effector functions of T helper cells. *Proc. Natl. Acad. Sci. U. S. A.* 102: 5138–5143.
21. Ruan, Q., V. Kameswaran, Y. Tone, L. Li, H.-C. Liou, M. I. Greene, M. Tone, and Y. H. Chen. 2009. Development of Foxp3+ Regulatory T Cells Is Driven by the c-Rel Enhanceosome. *Immunity* 31: 932–940.

22. Chang, J.-H., Y. Xiao, H. Hu, J. Jin, J. Yu, X. Zhou, X. Wu, H. M. Johnson, S. Akira, M. Pasparakis, X. Cheng, and S.-C. Sun. 2012. Ubc13 maintains the suppressive function of regulatory T cells and prevents their conversion into effector-like T cells. *Nat. Immunol.* 13: 481–490.
23. Heuser, C., J. Gotot, E. C. Piotrowski, M.-S. Philipp, C. J. F. Courrèges, M. S. Otte, L. Guo, J. L. Schmid-Burgk, V. Hornung, A. Heine, P. A. Knolle, N. Garbi, E. Serfling, C. Evaristo, F. Thaiss, and C. Kurts. 2017. Prolonged IKK β Inhibition Improves Ongoing CTL Antitumor Responses by Incapacitating Regulatory T Cells. *Cell Rep.* 21: 578–586.
24. Chen, X., J. Willette-Brown, X. Wu, Y. Hu, O. M. Z. Howard, Y. Hu, and J. J. Oppenheim. 2015. IKK α is required for the homeostasis of regulatory T cells and for the expansion of both regulatory and effector CD4 T cells. *FASEB J.* 29: 443–454.
25. Oh, H., and S. Ghosh. 2013. NF- κ B: roles and regulation in different CD4+ T-cell subsets. *Immunol. Rev.* 252: 41–51.
26. Algül, H., M. Treiber, M. Lesina, H. Nakhai, D. Saur, F. Geisler, A. Pfeifer, S. Paxian, and R. M. Schmid. 2007. Pancreas-specific RelA/p65 truncation increases susceptibility of acini to inflammation-associated cell death following cerulein pancreatitis. *J. Clin. Invest.* 117: 1490–1501.
27. Zemmour, D., A. Pratama, S. M. Loughhead, D. Mathis, and C. Benoist. 2017. Flicr, a long noncoding RNA, modulates Foxp3 expression and autoimmunity. *Proc. Natl. Acad. Sci.* 114: E3472–E3480.
28. Messina, N., T. Fulford, L. O’Reilly, W. X. Loh, J. M. Motyer, D. Ellis, C. McLean, H. Naeem, A. Lin, R. Gugasyan, R. M. Slattery, R. J. Grumont, and S. Gerondakis. 2016. The NF- κ B transcription factor RelA is required for the tolerogenic function of Foxp3+ regulatory T cells. *J. Autoimmun.* 70: 52–62.
29. Hill, J. A., M. Feuerer, K. Tash, S. Haxhinasto, J. Perez, R. Melamed, D. Mathis, and C. Benoist. 2007. Foxp3 Transcription-Factor-Dependent and -Independent Regulation of the Regulatory T Cell Transcriptional Signature. *Immunity* 27: 786–800.
30. Kaech, S. M., S. Hemby, E. Kersh, and R. Ahmed. 2002. Molecular and Functional Profiling of Memory CD8 T Cell Differentiation. *Cell* 111: 837–851.
31. Zemmour, D., R. Zilionis, E. Kiner, A. M. Klein, D. Mathis, and C. Benoist. 2018. Single-cell gene expression reveals a landscape of regulatory T cell phenotypes shaped by the TCR. *Nat. Immunol.* 19: 291–301.
32. Oh, H., Y. Grinberg-Bleyer, W. Liao, D. Maloney, P. Wang, Z. Wu, J. Wang, D. M. Bhatt, N. Heise, R. M. Schmid, M. S. Hayden, U. Klein, R. Rabadan, and S. Ghosh. An NF- κ B Transcription-Factor-Dependent Lineage-Specific Transcriptional Program Promotes Regulatory T Cell Identity and Function. *Immunity* .
33. Vasanthakumar, A., Y. Liao, P. Teh, M. F. Pascutti, A. E. Oja, A. L. Garnham, R. Gloury, J. C. Tempany, T. Sidwell, E. Cuadrado, P. Tuijnenburg, T. W. Kuijpers, N. Lalaoui, L. A. Mielke, V. L. Bryant, P. D. Hodgkin, J. Silke, G. K. Smyth, M. A. Nolte, W. Shi, and A. Kallies. 2017. The TNF

Receptor Superfamily-NF- κ B Axis Is Critical to Maintain Effector Regulatory T Cells in Lymphoid and Non-lymphoid Tissues. *Cell Rep.* 20: 2906–2920.

34. Zhou, X., S. L. Bailey-Bucktrout, L. T. Jeker, C. Penaranda, M. Martínez-Llordella, M. Ashby, M. Nakayama, W. Rosenthal, and J. A. Bluestone. 2009. Instability of the transcription factor Foxp3 leads to the generation of pathogenic memory T cells in vivo. *Nat. Immunol.* 10: 1000–1007.

35. van der Veeken, J., A. J. Gonzalez, H. Cho, A. Arvey, S. Hemmers, C. S. Leslie, and A. Y. Rudensky. 2016. Memory of Inflammation in Regulatory T Cells. *Cell* 166: 977–990.

36. Mukherjee, S. P., M. Behar, H. A. Birnbaum, A. Hoffmann, P. E. Wright, and G. Ghosh. 2013. Analysis of the RelA:CBP/p300 Interaction Reveals Its Involvement in NF- κ B-Driven Transcription. *PLOS Biol.* 11: e1001647.

37. van Loosdregt, J., and P. J. Coffey. 2014. Post-translational modification networks regulating FOXP3 function. *Trends Immunol.* 35: 368–378.

38. Soligo, M., C. Camperio, S. Caristi, C. Scottà, P. D. Porto, A. Costanzo, P.-Y. Mantel, C. B. Schmidt-Weber, and E. Piccolella. 2011. CD28 costimulation regulates FOXP3 in a RelA/NF- κ B-dependent mechanism. *Eur. J. Immunol.* 41: 503–513.

39. Camperio, C., S. Caristi, G. Fanelli, M. Soligo, P. D. Porto, and E. Piccolella. 2012. Forkhead Transcription Factor FOXP3 Upregulates CD25 Expression through Cooperation with RelA/NF- κ B. *PLOS ONE* 7: e48303.

40. Rubtsov, Y. P., J. P. Rasmussen, E. Y. Chi, J. Fontenot, L. Castelli, X. Ye, P. Treuting, L. Siewe, A. Roers, W. R. Henderson Jr., W. Muller, and A. Y. Rudensky. 2008. Regulatory T Cell-Derived Interleukin-10 Limits Inflammation at Environmental Interfaces. *Immunity* 28: 546–558.

41. Wang, Y., A. Kissenpfennig, M. Mingueneau, S. Richelme, P. Perrin, S. Chevrier, C. Genton, B. Lucas, J. P. DiSanto, H. Acha-Orbea, B. Malissen, and M. Malissen. 2008. Th2 Lymphoproliferative Disorder of LatY136F Mutant Mice Unfolds Independently of TCR-MHC Engagement and Is Insensitive to the Action of Foxp3+ Regulatory T Cells. *J. Immunol.* 180: 1565–1575.

42. Jacque, E., K. Billot, H. Authier, D. Bordereaux, and V. Baud. 2013. RelB inhibits cell proliferation and tumor growth through p53 transcriptional activation. *Oncogene* 32: 2661–2669.

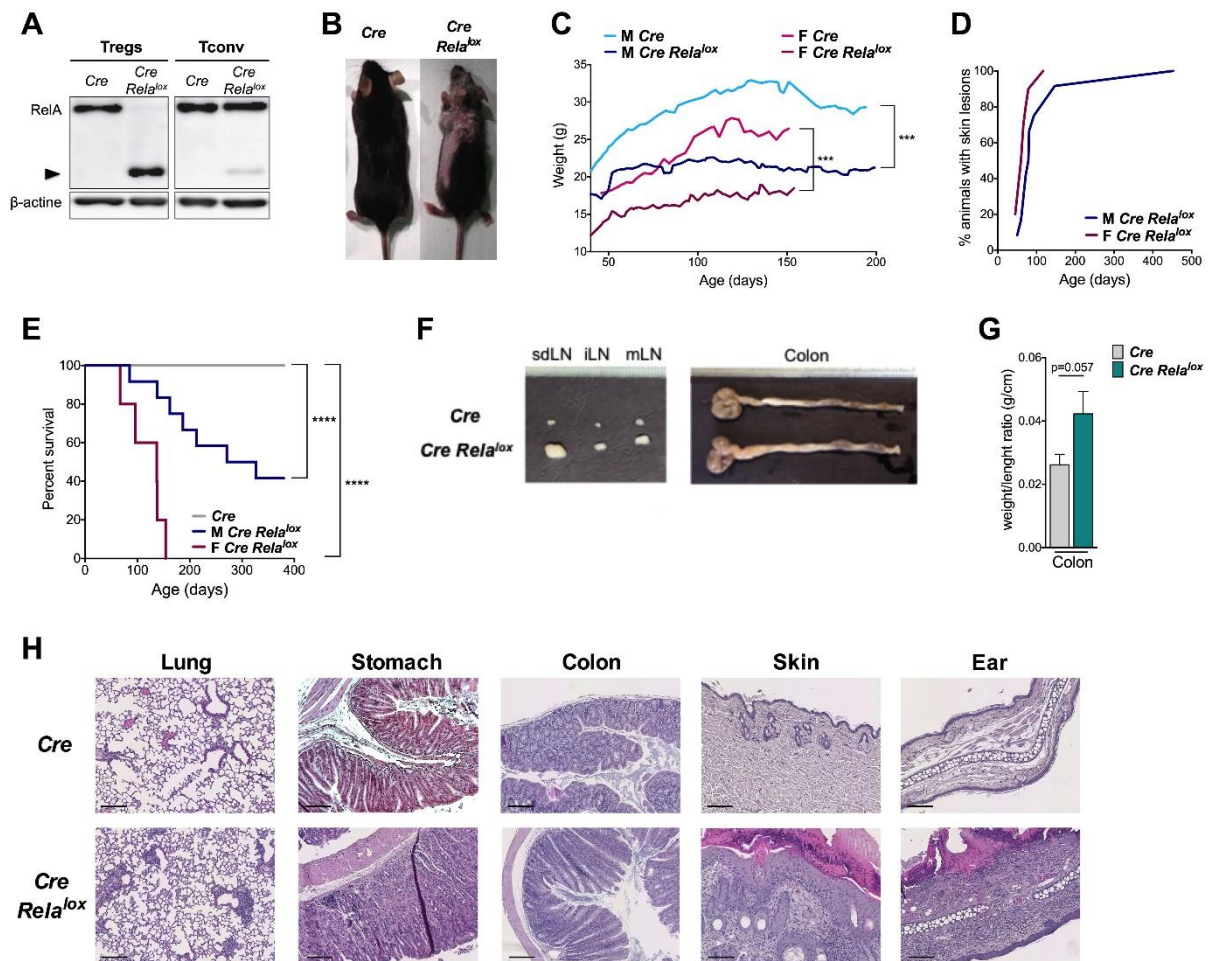


Figure 1. Mice with RelA deficient Treg develop systemic inflammation. (A) Western blot analysis of RelA expression in Treg and CD4⁺ conventional T cells (Tconv) isolated from *Foxp3*^{Cre} (Cre) and *Foxp3*^{Cre} *Rela*^{lox} (Cre *Rela*^{lox}) mice. The blot is representative of 2 independent experiments. (B) Representative pictures of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. (C) Body weight monitoring of *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} males and females. (D) Percentages of *Foxp3*^{Cre} *Rela*^{lox} males and females with skin lesions. (E) Survival monitoring of *Foxp3*^{Cre} *Rela*^{lox} males and females. (F) Representative pictures from 20 mice of the LN and colon of 12 week-old *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox} mice. (G) Weight/length ratio of colon of 12 week-old *Foxp3*^{Cre} (n=3) and *Foxp3*^{Cre} *Rela*^{lox} (n=4) mice. Data are representative of 2 independent experiments (H) Representative histology from four 12 week-old mice of the lung, stomach, colon, skin and ear of *Foxp3*^{Cre} and *Foxp3*^{Cre} *Rela*^{lox}. Scale bars represent 200μm (lung, *Foxp3*^{Cre} stomach, colon), 150μm (*Foxp3*^{Cre} *Rela*^{lox} stomach) and 100μm (skin, ear).

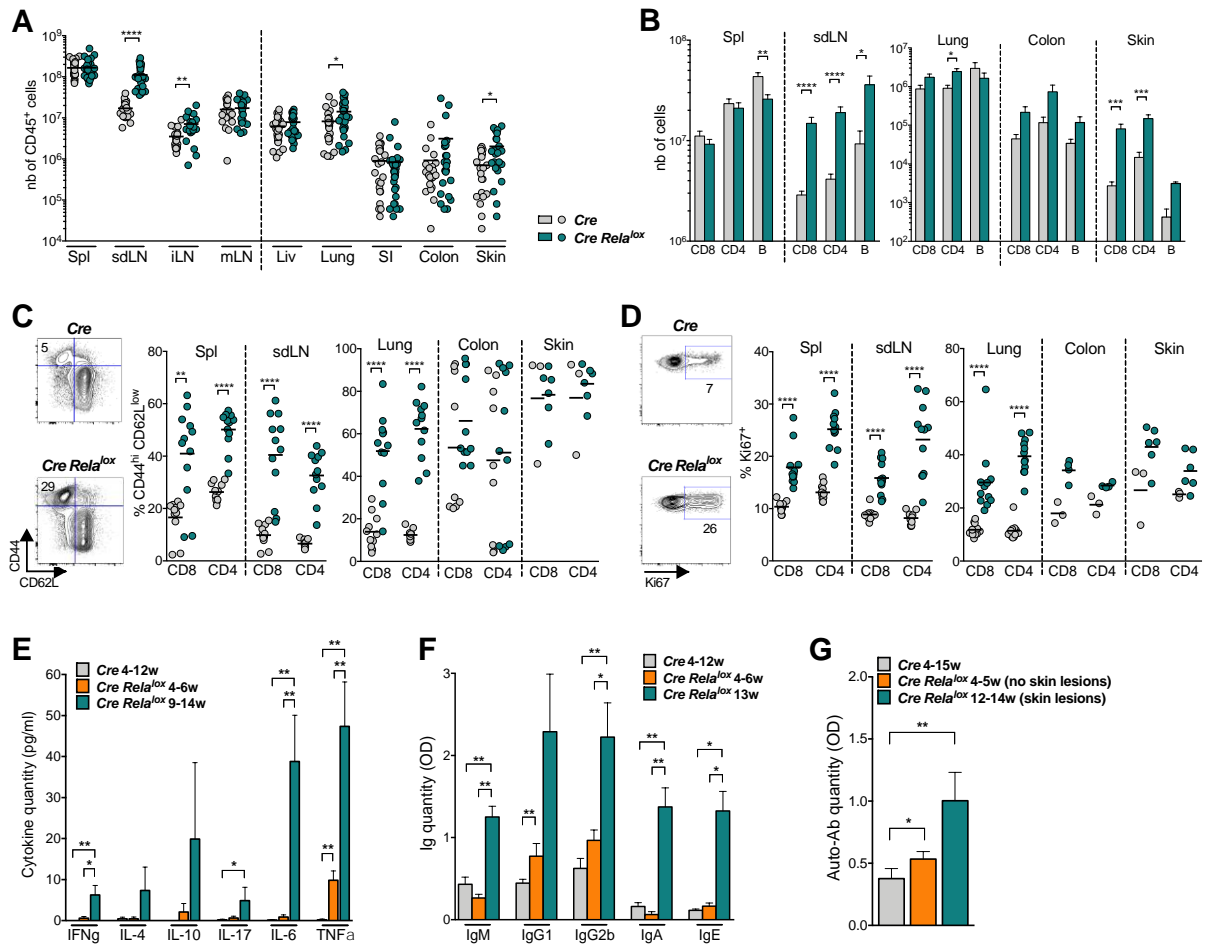


Figure 2. High activation of T and B lymphocytes in *Foxp3^{Cre} Rela^{lox}* mice. (A, B) Number of CD45⁺ (A), CD8⁺, CD4⁺ and B cells (B) in the indicated organs (spl=spleen, liv=liver, SI= small intestine) of 12 week-old *Foxp3^{Cre}* (*Cre*) and *Foxp3^{Cre} Rela^{lox}* (*Cre Rela^{lox}*) mice. Each dot represents a mouse, collected from 8 independent experiments. (C, D) Representative dot plots and proportion of CD44^{hi} CD62L^{low} (C) and Ki67⁺ (D) among CD8⁺ and CD4⁺ Tconv in the indicated organs of 12 week-old *Foxp3^{Cre}* and *Foxp3^{Cre} Rela^{lox}* mice. Each dot represents a mouse, collected from 5 (spleen, sdLN and lung) and 2 (colon and skin) independent experiments. (E) Cytokine quantification in the serum of 4-12 week-old *Foxp3^{Cre}*, and 4-6 week-old and 9-14 week-old *Foxp3^{Cre} Rela^{lox}* mice (n=5). (F) Immunoglobulin quantification in the serum of 4-12 week-old *Foxp3^{Cre}* mice, and 4-6 week-old and 13 week-old *Foxp3^{Cre} Rela^{lox}* mice (n=5). (G) Anti-DNA antibody quantification in the serum of 4-15 week-old *Foxp3^{Cre}* mice (n=15), and 4-12 week-old (n=6) and 12-14 week-old (n=9) *Foxp3^{Cre} Rela^{lox}* mice. Data were pooled from 2 independent experiments.

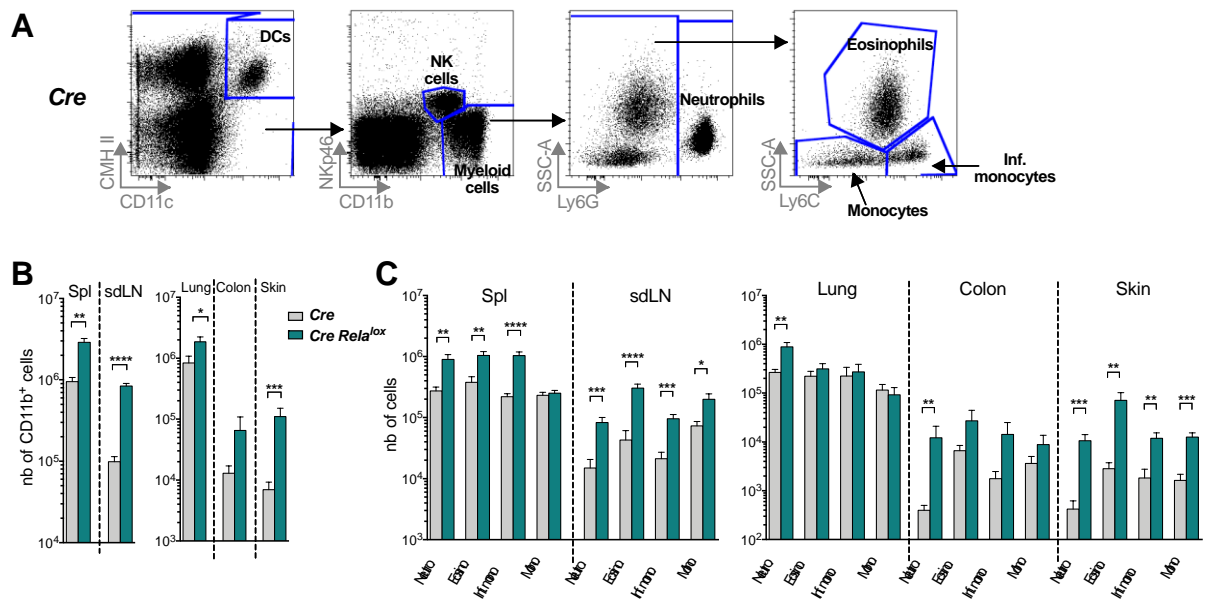


Figure 3. Increased numbers of myeloid cells in *Foxp3^{Cre} Relα^{lox}* mice. (A) Gating strategy of myeloid and NK cells. (B, C) Number of CD11b⁺ (B) and of neutrophils, eosinophils, inflammatory monocytes and monocytes (C) in the indicated organs (spl=spleen) of 12 week-old *Foxp3^{Cre}* (*Cre*) and *Foxp3^{Cre} Relα^{lox}* (*Cre Relα^{lox}*) mice. Data were from 3 independent experiments (n=8 to 10).

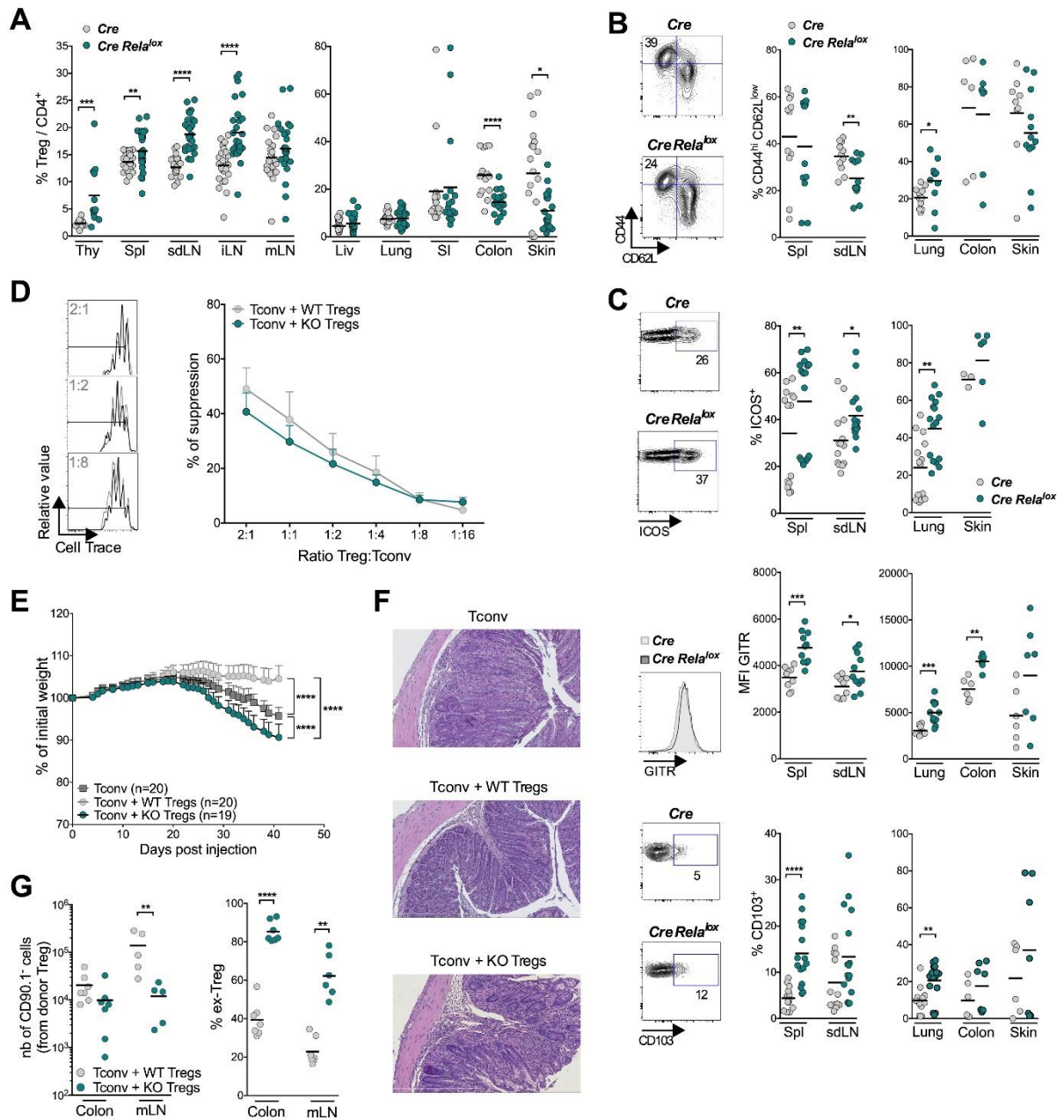


Figure 4. Homeostasis and function of Treg in *Foxp3^{Cre} Rela^{lox}* mice. (A) Proportion of Treg among the CD4⁺ T cells in the indicated organs (thy= thymus, spl=spleen, liv=liver, SI= small intestine) of 12 week-old *Foxp3^{Cre}* (*Cre*) and *Foxp3^{Cre} Rela^{lox}* (*Cre Rela^{lox}*) mice. (B, C) Representative dot plots or histograms and proportions of CD44^{hi} CD62L^{low} (B) and ICOS⁺, CD103⁺ and MFI GTR (C) among the Treg of 12 week-old *Foxp3^{Cre}* and *Foxp3^{Cre} Rela^{lox}* mice. Each dot represents a mouse, collected from 4 to 8 independent experiments. (D) *In vitro* suppressive activity of Treg cells from *Foxp3^{Cre}* (WT Treg) and *Foxp3^{Cre} Rela^{lox}* (KO Treg) 5-6 week-old mice. Representative data at 2:1, 1:2 and 1:8 (left) and different (right) Treg:Tconv ratios of 4 independent experiments. (E-G) *In vivo* suppressive activity of Treg cells from *Foxp3^{Cre}* (WT Treg, 6 week-old mice) and *Foxp3^{Cre} Rela^{lox}* (KO Treg, 6 week-old mice) mice, determined in a colitis model stopped at 6 weeks for analyses. (E) Percentage of initial body weight pooled from 4 independent experiments. (F) Representative histology of the colon from 4 mice. (G) Numbers of CD4⁺CD90.1⁻ cells derived from Treg and proportions of ex-Treg defined by their CD4⁺CD90.1⁻Foxp3⁻ phenotype in the mLN and colon. Each dot represents a mouse, collected from 2 independent experiments.

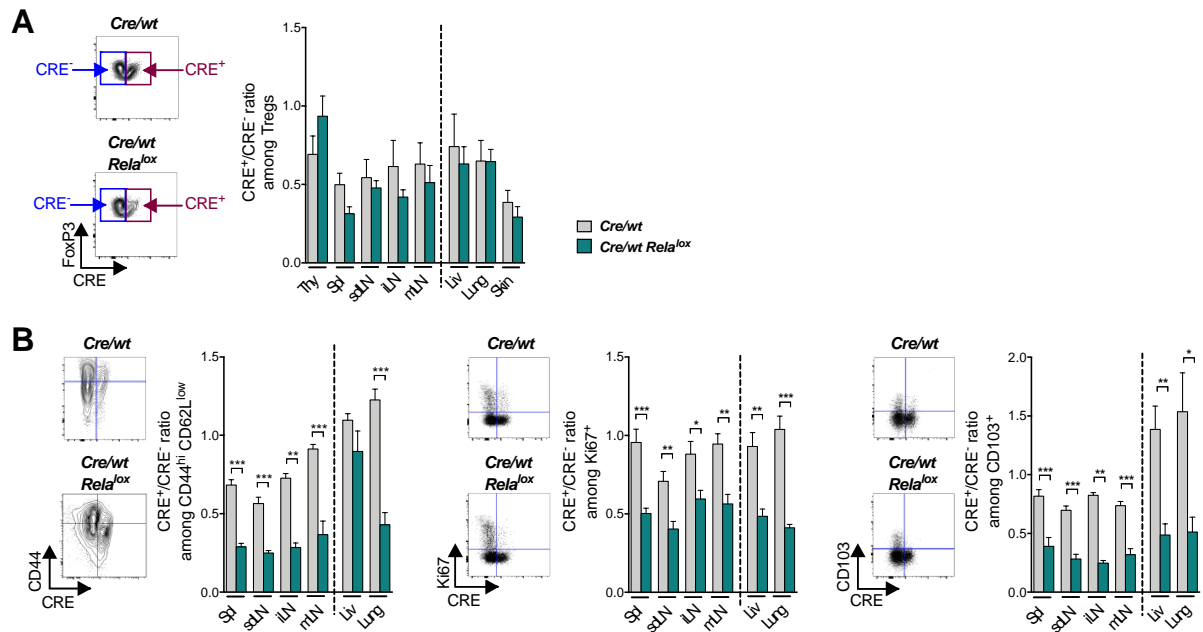


Figure 5. Reduced expression of activation markers in RelA deficient Treg at steady state. Analyses in the indicated organs (thy= thymus, spl=spleen, liv=liver) of 8 week-old $Foxp3^{Cre/wt}$ (Cre/wt) and $Foxp3^{Cre/wt} RelA^{lox}$ ($Cre/wt RelA^{lox}$) mice. Representative dot plots and ratios of the percentage of Treg expressing (CRE^+) or not (CRE^-) CRE among Treg (A) and among $CD44^{hi} CD62L^{low}$, $Ki67^+$ and $CD103^+$ Treg (B). Data are from 3 independent experiments (n=6 to 13).

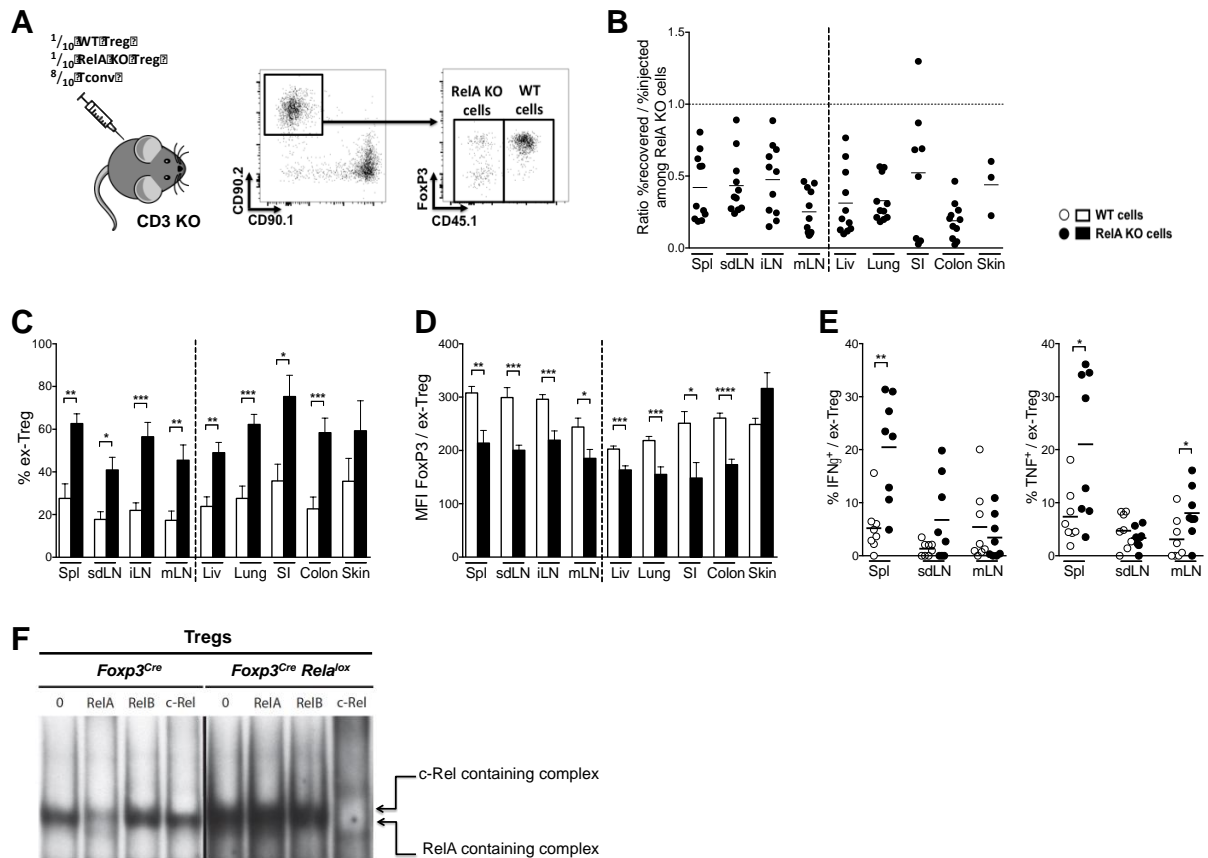


Figure 6. RelA deficient Treg have a defect of stability and turn pathogenic. (A-E) Adoptive transfer of a 1:1:8 ratio of a mix of CRE-expressing Treg from *Foxp3^{Cre/wt}* (CD45.1/2 CD90.2 WT Treg), *Foxp3^{Cre/wt} RelA^{lox}* (CD45.2 CD90.2 RelA KO Treg) mice and CD4⁺ conventional T cells (CD90.1 Tconv) into CD3 KO mice and analysis of donor cells 16 days later. Data are from 3 independent experiments. (A) Experimental scheme and representative gating strategy. (B) Ratio of %recovered RelA KO cells to %injected RelA KO cells in the indicated organs (spl=spleen, liv=liver, SI= small intestine). (C) Proportion of ex-Treg from injected WT cells and RelA KO cells in different tissues. (D, E) Foxp3 MFI (D) and proportions of IFN γ ⁺ and TNF⁺ cells (E) among WT ex-Treg and RelA KO ex-Treg. Each dot represents a mouse, collected from 2 or 3 independent experiments. (F) EMSA combined with supershift assay analysis of NF- κ B subunits activation in Treg isolated from *Foxp3^{Cre}* and *Foxp3^{Cre} RelA^{lox}* mice. The results are representative of 2 independent experiments.

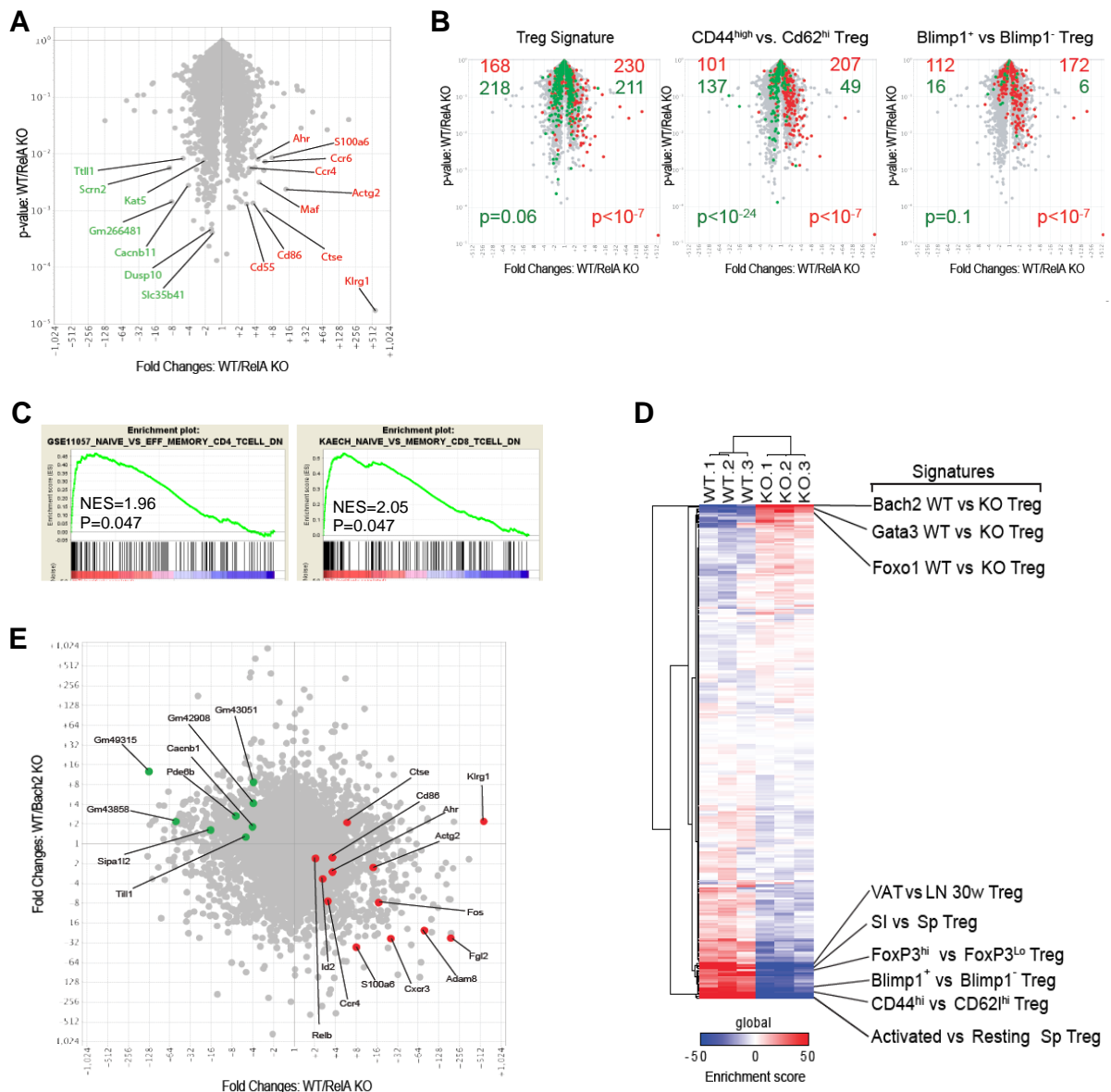
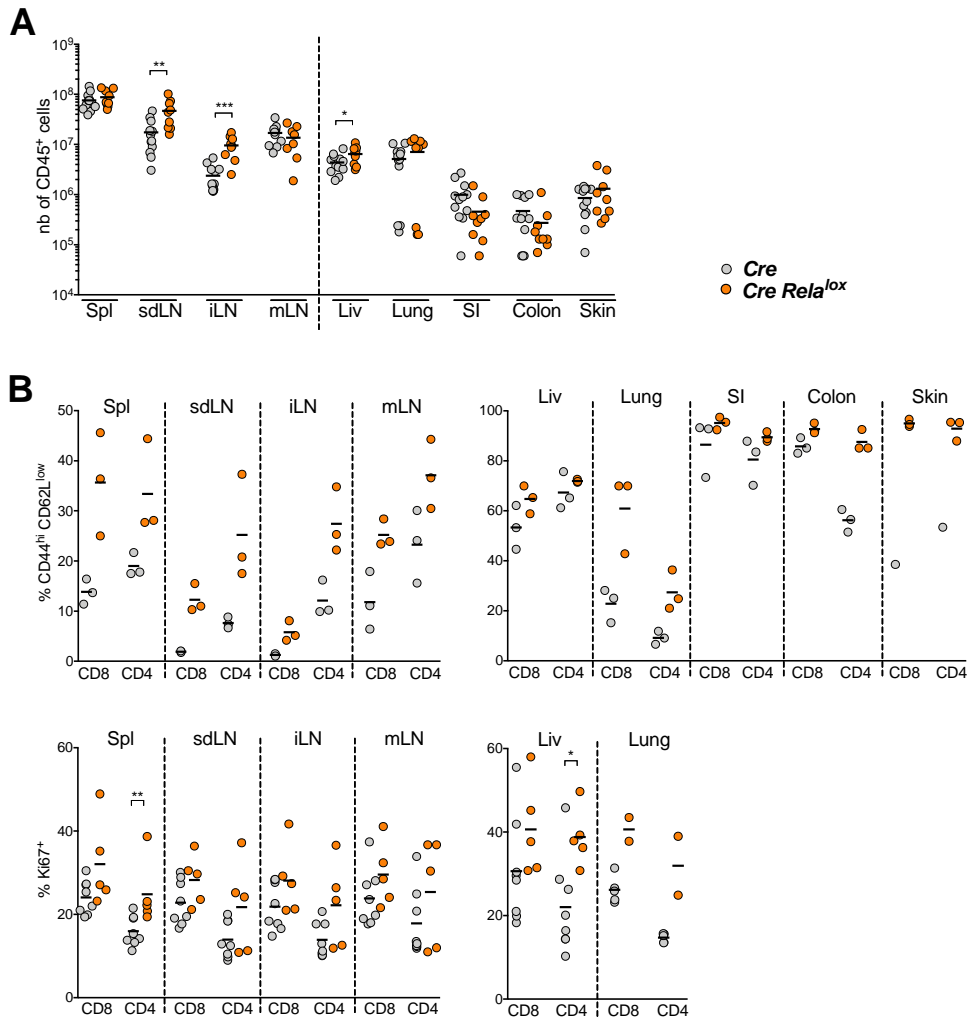
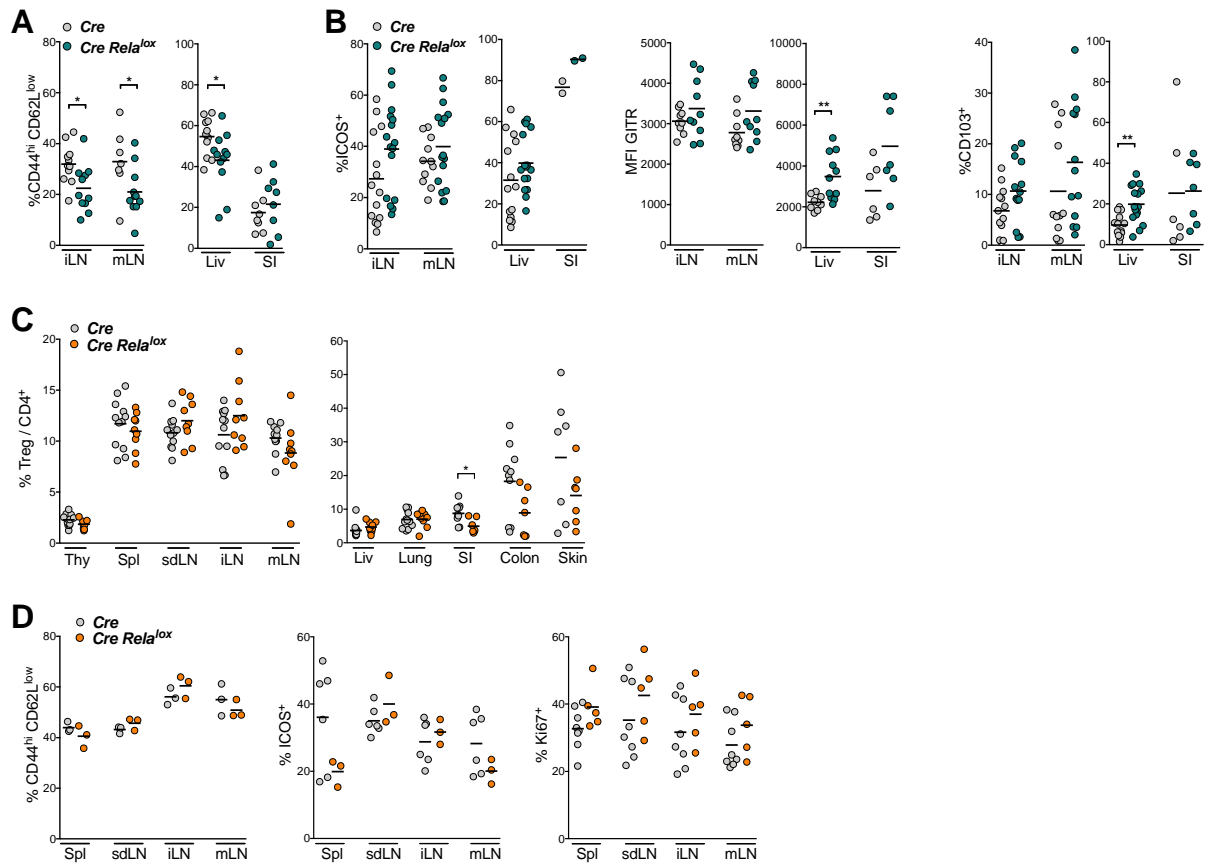


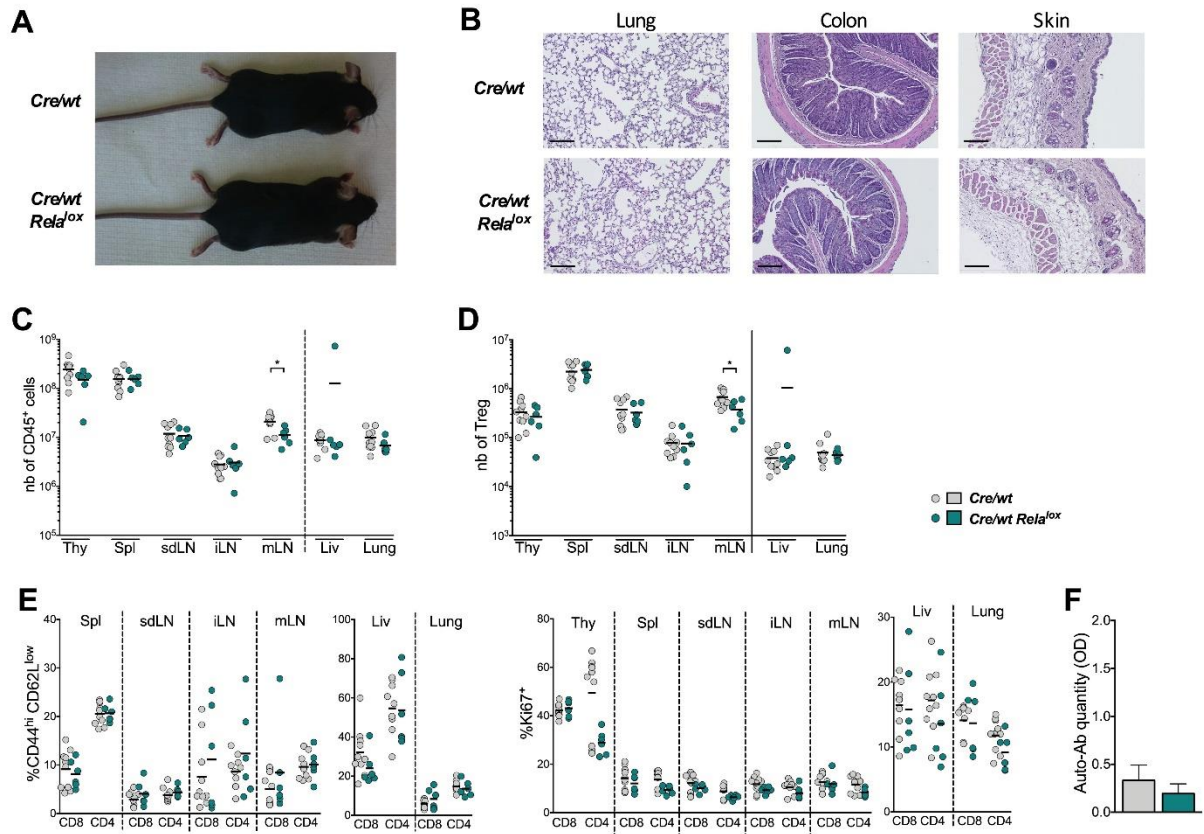
Figure 7. RelA deficient Treg have integrity and activation defects. (A) Volcano plot of WT vs. RelA KO Treg. Red and green indicate transcripts up- and down-regulated, respectively, by WT Treg cells. (B) WT vs. RelA KO Treg (as in A) overlaid with various Treg signatures. Red and green indicate genes up- and down-regulated, respectively, in each signature (chi-squared test for p-value). (C) GSEA enrichment plots. (D) Heatmap for the enrichment score of each gene signature. (E) Fold change-fold change plot of WT vs RelA KO Treg (x-axis) and WT iTreg vs WT Bach2 KO iTreg (y-axis, from published data (Roychoudhuri *et al.*, 2013)). Red and green transcripts from (A).



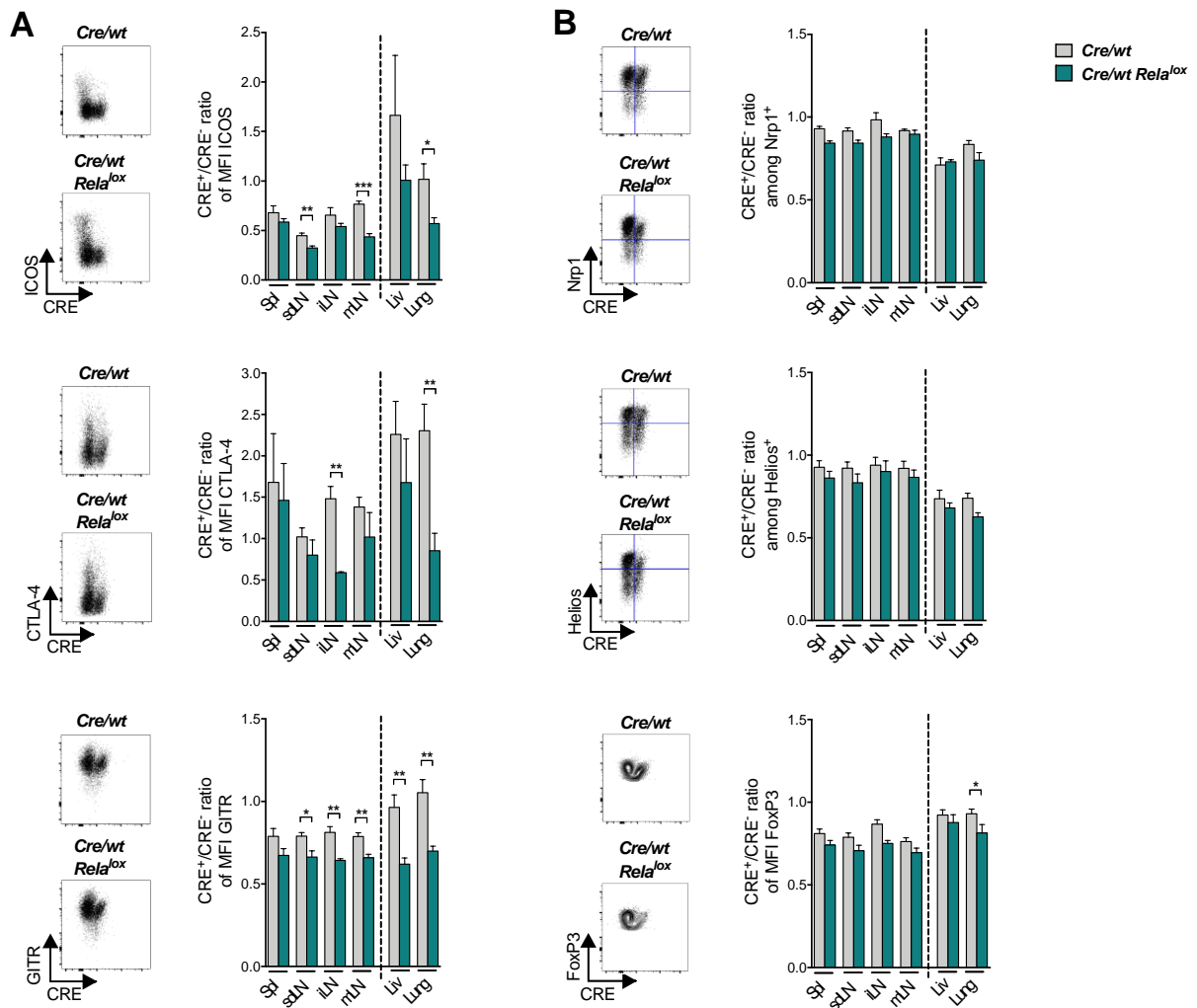
Supplementary Figure 1. Homeostasis and phenotype of T cells in 5 week-old *Foxp3^{Cre} Relα^{lox}* mice. Number of CD45⁺ (A) and proportion of CD44^{hi}CD62L^{low}, ICOS⁺ and Ki67⁺ among CD8⁺ and CD4⁺ conventional T cells (B) in different tissues (spl=spleen, liv=liver, SI= small intestine) of *Foxp3^{Cre}* (*Cre*) and *Foxp3^{Cre} Relα^{lox}* (*Cre Relα^{lox}*). Each dot represents a mouse, collected from 2 or 3 independent experiments in 5 week-old mice.



Supplementary Figure 2. Homeostasis and function of Treg in *Foxp3^{Cre} Rela^{lox}* mice. (A, B) Proportion of CD44^{hi}CD62L^{low} (A) and of ICOS⁺ and CD103⁺ and MFI GITR (B) among Treg in the indicated organs (thy=thymus, spl=spleen, liv=liver, SI= small intestine) of 12 week- *Foxp3^{Cre}* (*Cre*) and *Foxp3^{Cre} Rela^{lox}* (*Cre Rela^{lox}*). (C, D) Proportion of Treg among CD4⁺ cells (C) and of CD44^{hi}CD62L^{low}, ICOS⁺ and CD103⁺ among Treg (D) in 5 week-old *Foxp3^{Cre}* and *Foxp3^{Cre} Rela^{lox}* mice. Each dot represents a mouse, collected from 4-5 (A, B) or 2-3 (C, D) independent experiments.



Supplementary Figure 3. *Foxp3*^{Cre/wt} *RelA*^{lox} mice have no sign of disease. (A) Representative pictures of 8 week-old mice. (B) Representative histology of lung, colon and skin of 8 week-old. Scale bars represent 100 μ m. Number of CD45⁺ (C), of Treg among CD4⁺ T cells (D) and proportion of CD44^{hi}CD62L^{low} and Ki67⁺ among CD8⁺ and CD4⁺ conventional T cells (E) in different tissues (thy=thymus, spl=spleen, liv=liver, SI= small intestine) of 8 week-old *Foxp3*^{Cre/wt} (*Cre/wt*) and *Foxp3*^{Cre/wt} *RelA*^{lox} (*Cre/wt RelA*^{lox}) mice. Each dot represents a mouse, collected from 3 independent experiments. (F) Anti-DNA auto-antibodies quantification in the serum of 8 week-old *Foxp3*^{Cre/wt} and *Foxp3*^{Cre/wt} *RelA*^{lox} mice (n=5 and 10, respectively).



Supplementary Figure 4. *Rela* deficient Treg have reduced expression of some activation markers at steady state. Analyses in the indicated organs (spl=spleen, liv=liver) of 8 week-old *Foxp3*^{Cre/wt} (*Cre/wt*) and *Foxp3*^{Cre/wt} *Rela*^{lox} (*Cre/wt Rela*^{lox}) mice. Representative dot plots and ratios of (A) the MFI of ICOS, CTLA-4, GITR and (B) the MFI of Foxp3 and the percentage of Nrp1⁺ and Helios⁺ among the CRE⁺ Treg by the same MFI or percentage among the CRE⁻ Treg.

ANNEX 2

REGULATORY T CELLS CONTROL CENTRAL NERVOUS SYSTEM AUTOIMMUNITY THROUGH THE TNF/TNFR2 PATHWAY

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ABSTRACT

Anti-TNF therapy has improved medical care of many autoimmune diseases. However, these treatments may paradoxically initiate signs of multiple sclerosis (MS) and are formally contraindicated in MS patients because of risk of disease exacerbation. Here we bring a mechanistic explanation to this long-term unresolved clinical conundrum. Anti-TNF drugs exacerbated experimental autoimmune encephalomyelitis (EAE), a murine model of MS, replicating the observation in patients. Strikingly, mice with conditional deletion of TNF receptor type 2 (TNFR2) in Treg cells were highly sensitive to EAE, and displayed altered Treg numbers and phenotype in the inflamed central nervous system (CNS). Cell transfer experiments suggested a CNS-restricted effect of TNF- α on Treg cells. Thus, TNF- α directly stimulates the expansion and activation of Treg cells in the inflamed central nervous system during EAE to control the disease, which may explain some of the adverse effects of anti-TNF therapy.

INTRODUCTION

The inflammatory properties of TNF- α have been extensively studied and are well recognized. This cytokine plays a central role in inflammation and has detrimental effects on several autoimmune diseases¹. Anti-TNF therapies have significantly improved the treatment of rheumatoid arthritis, Crohn disease, psoriasis and other inflammatory diseases. However, several unexplained observations limit the utilization of these drugs. Some patients develop paradoxical aggravation of their autoimmunity or a new form of demyelinating disease at a prevalence of 0.075%^{2,3}. Also, these therapies are formally contra-indicated in multiple sclerosis (MS) patients because of disease exacerbation^{4,5}. The mechanism of this long-term conundrum, well known to clinicians, remains essentially unexplained.

Aggravation of autoimmune diseases upon anti-TNF therapy reveals an immuno-regulatory facet of TNF- α . This is further supported by mouse data, as TNF-deficient mice exhibit increased Th1 immune responses⁶ and TNF- α administration inhibits lupus, type 1 diabetes and T-cell immune responses⁷. Several mechanisms have been proposed to explain the immunosuppressive properties of TNF- α . The cytokine may promote tolerogenic innate cells, such as myeloid-derived stem cells or semi-mature dendritic cells⁸⁻¹⁰ or down-modulate inflammatory cytokine production by myeloid cells¹¹. TNF- α may also kill or inhibit the activation of conventional T-cells (T_{conv} cells)^{7,12}. Alternatively, the immuno-regulatory property of TNF- α may be due to its effects on Treg cells that play a central role in the control of autoimmune diseases¹³. Indeed, our group and others reported that TNF- α increased the proliferation and suppressive function of Treg cells by signaling through its type 2 receptor (TNFR2)¹⁴⁻¹⁶. Here, we tested whether MS aggravation by anti-TNF therapy could be explained by an effect of the cytokine on Treg cells, using the experimental autoimmune encephalomyelitis (EAE) mouse disease model. We first validated the model by showing that anti-TNF treatment indeed induced EAE exacerbation. Then, we showed that TNF- α directly stimulated the accumulation and activation of Treg cells in the inflamed central nervous system (CNS) to control the disease.

RESULTS

Anti-TNF treatment induces EAE exacerbation and limits Treg cell expansion in the CNS

We first assessed whether MS exacerbation following anti-TNF therapy could be recapitulated in the EAE mouse model. Mice were treated with an anti-TNF mAb from day 10, when they developed the first neurological signs, up to day 20. Compared to isotype-control treated mice, they developed an aggravated form of EAE after day 20 leading to death in 35% of the mice (Fig. 1a). Similar findings were obtained when the treatment was extended up to day 30, or when using a clinically-approved soluble TNF receptor (Fig. 1b and Supplementary Fig. 1). We then analysed Treg cells in the inflamed CNS. In control mice, the proportion of Treg cells was 15% of CD4⁺ T-cells at the beginning of CNS infiltration (day 7), and rapidly reached 40-50% after the peak of disease (day 20) (Supplementary Fig. 2). This high Treg cell proportion was likely due to their earlier and higher proliferation compared to Tconv cells (Supplementary Fig. 2). In anti-TNF treated mice, Treg cell accumulation and proliferation were decreased at late time points in the CNS, but unchanged in LN draining the site of immunization (dLN), thereby suggesting a CNS-restricted role of TNF- α (Fig. 1c-e). Because TNF- α stimulates Treg cell proliferation through its interaction with TNFR2 rather than TNFR114, we analysed expression of this receptor. TNFR2 was higher expressed on Treg than Tconv cells at steady state and in the dLN and the inflamed CNS after EAE induction (Fig. 1f). As with anti-TNF, blocking TNFR2 induced EAE worsening (Fig. 1g). Together, blocking TNF/TNFR2 after disease onset induced EAE exacerbation, reminiscent of the human situation, which was associated with reduced Treg cell accumulation in the inflamed CNS.

TNFR2 expression by hematopoietic cells is required to limit EAE severity and promotes Treg cell intrinsic expansion

To further study the role of the TNF/TNFR2 pathway in EAE, we performed experiments with mice deficient in Tnfr2 (Tnfr2^{-/-}). As previously observed¹⁷⁻¹⁹, Tnfr2^{-/-} mice that have a significant defect of Treg cells in the spleen (Supplementary Fig. 3), developed more severe EAE than WT controls (Fig. 2a,b). This was associated with reduced Treg cell accumulation in the inflamed CNS at day 10-15 (Fig. 2c). We next generated bone marrow chimeric mice to assess the role of TNFR2 expression by hematopoietic and non-hematopoietic cells. In WT mice reconstituted with TNFR2-deficient hematopoietic cells, EAE was more severe and Treg cell expansion in the CNS was reduced when compared to mice reconstituted with WT hematopoietic cells (Fig. 2d-f). In

contrast, EAE severity was similar in *Tnfr2*^{-/-} and WT recipients reconstituted with WT hematopoietic cells (Fig. 2g,h). To evaluate the intrinsic role of TNFR2 expression by Treg cells, we reconstituted WT animals with a mix of bone marrow cells from WT and *Tnfr2*^{-/-} mice (Fig. 2i). After EAE induction, the expansion of TNFR2-deficient Treg cells was highly reduced in the CNS compared to TNFR2-sufficient Treg cells (Fig. 2j,k). In conclusion, aggravation of EAE in *Tnfr2*^{-/-} mice was due to lack of TNFR2 expression by hematopoietic cells, which was associated with a cell intrinsic deficit of TNFR2-deficient Treg cell expansion in the CNS.

TNFR2 is dispensable in Treg cell biology at steady state

To further analyse the intrinsic role of TNFR2 in Treg cell biology, we generated mice with a conditional ablation of TNFR2 in Treg cells by crossing mice expressing CRE-recombinase in Treg cells (*Foxp3*CRE) with mice expressing a floxed *Tnfr2* allele (*Tnfr2*^{fl/fl}). Specific ablation of TNFR2 in Treg cells was confirmed by flow cytometry (Fig. 3a,b). These mice had no sign of spontaneous autoimmunity, their weight growth and litter size were normal (Supplementary Fig. 4) and transmission of mutated alleles followed Mendel's law. In the steady state, Treg cell numbers and *Foxp3* expression in lymphoid tissues were similar in *Foxp3*Cre*Tnfr2*^{fl/fl} mice compared to controls (Fig. 3c,d). Their in vitro suppressive capacity was slightly reduced. Moreover, in an in vivo suppression assay, TNFR2-deficient Treg cells were partially but not fully functional to prevent colitis (Fig. 3e,f). Finally, we utilized *Foxp3*Cre/wt heterozygote female mice, in which only roughly half of Treg cells carry a functional CRE recombinase because of its localization on the X chromosome, to assess the role of TNFR2 in mature Treg cells in a competitive environment. In *Foxp3*Cre/wt*Tnfr2*^{wt} control mice, CRE-expressing cells represented 50% of Treg cells as expected, whereas in *Foxp3*Cre/wt*Tnfr2*^{fl/fl} mice they represented only 30-35% of Treg cells (Fig. 3g). Interestingly, this Treg cell unbalance was observed in 8 week-old but not 10 day-old mice. In conclusion, TNFR2 is mainly dispensable for Treg cell homeostasis and impacts partially on their suppressive function at steady state.

Intrinsic expression of TNFR2 by Treg cells is required to maintain their proper activation in the CNS and control EAE

We then explored the intrinsic role of the TNF/TNFR2 signalling in Treg cells during CNS inflammation. Strikingly, compared to their WT littermates, *Foxp3*Cre*Tnfr2*^{fl/fl} mice developed a very severe EAE leading to death in half of the mice by day 15 (Fig. 4a). Remarkably, Treg cell

proportions were significantly reduced in *Foxp3CreTnfr2fl/fl* mice compared to controls in the CNS at day 10 (Fig. 4b). Also, CNS Treg cells of *Foxp3CreTnfr2fl/fl* mice expressed lower levels of CTLA4, ICOS and Blimp-1 (Fig. 4b), which are markers of activation and effector Treg cells²⁰⁻²². These quantitative and phenotypic Treg cell alterations, observed in the CNS, were not present in the spleen or dLN (Fig. 4b). The expression of active effector caspase 3 and Ki67⁺ cells among Treg cells was similar in the 2 groups of mice (Supplementary Fig. 5). Intriguingly, the reduced proportions of Treg cells were not yet observed at day 7, when the first immune cells started infiltrating the CNS, and were abolished at day 15, when *Foxp3CreTnfr2fl/fl* mice were very sick (Supplementary Fig. 6). Thus, *Foxp3CreTnfr2fl/fl* mice that developed a severe EAE had a specific alteration of the numbers and activated phenotype of Treg cells in the CNS at day 10.

We then assessed the impact of this Treg cell deficit on pathogenic Th1 and Th17 cells²³ and on GM-CSF, the major pathogenic cytokine in the EAE²⁴. We performed the analysis in the CNS at day 15, when *Foxp3CreTnfr2fl/fl* mice exhibited severe disease compared to controls. We could not find reproducible differences in the numbers of Tconv cells (Supplementary Fig. 7) or of their production of IFN γ , IL-17 or GM-CSF at single or polyfunctional levels (Fig. 4c,d). Also, the expression levels of the master transcription factors of Th1 and Th17, Tbet and ROR γ t, were similar in the 2 groups of mice (Fig. 4e). Finally, we analyzed the proportions of the various myeloid cell populations (defined in Supplementary Fig. 8) in the CNS at day 15. They were similar in *Foxp3CreTnfr2fl/fl* and control mice (Supplementary Fig. 9). In conclusion, TNFR2 expression by Treg cells is critical in the control of EAE and plays a non-redundant role on their expansion and proper activation in the inflamed CNS at day 10.

Expression of TNFR2 by Treg cells is critical to control the autoimmune process in the CNS rather than in lymphoid tissues.

In the course of active EAE, the disease is initiated in dLN. Then, from day 5-6, pathogenic T-cells start migrating to the CNS, leading to a progressive increase of inflammation and accumulation of infiltrating immune cells (Supplementary Fig. 7), perpetuating locally the disease process²⁵. We performed experiments to assess whether TNFR2-expressing Treg cells control EAE by limiting the priming of pathogenic T-cells in dLN or by regulating the disease in the inflamed CNS. To quantify early T-cell priming, we transferred T-cell receptor-transgenic Tconv cells specific for the myelin oligodendrocyte glycoprotein (MOG)³⁵⁻⁵⁵ peptide in *Foxp3CreTnfr2fl/fl* and control mice, immunized the mice as for EAE induction, and measured proliferation of donor cells in dLN 3 days later. The level of T-cell proliferation was similar in the 2 groups of mice (Fig. 5a). To assess more

directly the pathogenicity of MOG-specific T-cells primed in dLN, we measured their capacity to induce EAE after adoptive transfer in naive mice. Remarkably, cells primed in *Foxp3CreTnfr2fl/fl* and control mice induced similar passive EAE (Fig. 5b). Then, to measure the control of EAE by TNFR2-expressing Treg cells within the inflamed CNS, we transferred WT pathogenic T-cells into *Foxp3CreTnfr2fl/fl* and control naive mice since injected cells rapidly migrated into the CNS to damage the neural tissue during passive EAE. Strikingly, EAE was much more severe in *Foxp3CreTnfr2fl/fl* than control recipients (Fig. 5c). We obtained similar findings when we transferred pathogenic T-cells in WT recipients that were treated with an anti-TNF blocking mAb (Fig. 5d). In conclusion, TNFR2 expression by Treg cells appears to be critical to control EAE within the inflamed CNS rather than limiting the priming of pathogenic T-cells in dLN.

DISCUSSION

A series of pre-clinical studies in mice conducted in the 90s concluded that blocking TNF was beneficial in EAE (review in 26). These findings led to initiate 2 clinical trials in MS patients that had to be rapidly stopped because of disease aggravation^{4,5}. Here, we revisited this question in mice with better-defined reagents and found that blocking TNF- α induced EAE exacerbation, as in MS patients. The discrepancy between our results and previous studies has 2 explanations: spectrum of action the timing of injection. In early experiments, the drugs blocked both TNF- α and lymphotoxin- α , which is pathogenic in EAE²⁷. Here, we used a mAb that blocked only TNF- α . In some early experiments, anti-TNF drugs were administered at disease induction. When we performed similar timing, EAE was not exacerbated, and even slightly delayed (Supplementary Fig. 10). At disease initiation, TNF- α is likely pathogenic by activating antigen-presenting cells via TNFR1, whereas TNF- α would regulate the disease by activating Treg cells via TNFR2 later in the inflamed CNS. In this line, blocking TNFR1 at disease induction attenuated EAE²⁸, whereas we showed that blocking TNFR2 at day 10 induced disease exacerbation. Thus, aggravation of MS by anti-TNF drugs was recapitulated in the EAE mouse model as long as relevant reagents and timing are used.

It has been proposed that aggravated EAE in *Tnfr2*^{-/-} mice, which was previously described¹⁷⁻¹⁹, was due to the absence of TNFR2 on oligodendrocytes since TNF α promotes proliferation of their progenitors via TNFR2²⁹, or to a direct killing of autoreactive pathogenic Tconv cells by TNF α via TNFR2¹². Our results clearly exclude these 2 hypotheses.

Different results concur to strongly suggest that the control of EAE by TNFR2-expressing Treg cells is taking place in the inflamed CNS rather than in the dLN. (i) Treg cell proportions and phenotypes (CTLA4, ICOS, Blimp-1) were altered in the CNS but not in the dLN of *Foxp3CreTnfr2fl/fl* mice compared to controls. (ii) Anti-TNF treatment induced EAE aggravation when administered after day 10 but not at disease induction. Also, clinical scores were similar in *Foxp3CreTnfr2fl/fl* and control mice before day 13-14. These latter two findings suggest that the early autoimmune process that was initiated in dLN was not altered by the absence of TNFR2 in Treg cells, which was confirmed by showing that the priming of MOG-specific T-cells in dLN was similar in *Foxp3CreTnfr2fl/fl* and control mice as well as their capacity to induce passive EAE. (iii) Finally, after transfer of pathogenic T-cells in passive EAE, the disease was much more severe in *Foxp3CreTnfr2fl/fl* than in control recipients. These findings suggest that TNFR2-expressing Treg cells controlled the disease in the CNS since donor cells rapidly migrate to this tissue after

transfer. Our work brings the first evidence that the control of EAE by Treg cells is at least partially regulated in the inflamed CNS.

Our work reveals a non-redundant and critical role of TNF- α acting on Treg cells in the control of EAE. Quite remarkably, the quantitative and phenotypic alteration of Treg cells in the CNS of Foxp3CreTnfr2fl/fl mice was observed at day 10 after disease induction but not at day 7 or day 15, pinpointing that TNFR2 expression by Treg cells is critical for disease control at this precise time-period in the CNS. This may be due to a boost of TNF- α or a high reactivity of Treg cells to the cytokine at this specific time. Alternatively, the defect of Treg cells in the CNS of Foxp3CreTnfr2fl/fl mice at day 10 may trigger the release of other inflammatory factors, able to boost Treg cell expansion and activation, which led to catch up and compensate their defect at day 15. But this late Treg cell boost was inefficient to control the disease maybe because of irreversible tissue damage.

Several mechanisms may be responsible for the reduced numbers of Treg cells in Foxp3CreTnfr2fl/fl mice at day 10 in the CNS. (i) Decreased proliferation. Our group and the one of Oppenheim showed that TNF- α triggered Treg cell proliferation and expansion via TNFR2^{14,16}. This seems unlikely since proportion of Ki67 among Treg cells was not reduced. However, one cannot exclude a transient decrease of Treg cell proliferation, not detectable with the Ki67 marker that is expressed not only during the S phase but also during the G1 and G2 phases of the cellular cycle. (ii) Increased cell death. We did not find evidence of increased cell death using an active caspase 3 marker. However, it is possible that dying cells would be very rapidly phagocytosed by myeloid cells that represented over 50% of the leukocytes, precluding their proper detection. (iii) Decreased stability. It was shown that TNFR2 was critical for maintaining stability and Foxp3 expression of Treg cells in the inflammatory environment of colitis³⁰. We did not see any decrease in the intensity of Foxp3 expression in Treg cells in the CNS at day 10 (Supplementary Fig. 11). However, it cannot be formally excluded that some TNFR2-deficient Treg cells would rapidly and entirely lose Foxp3 expression. (iv) Finally, reduced Treg cell numbers might be due to a defect of migration into the CNS, which was unlikely since their numbers were comparable to controls at day 7, or alternatively to an increased exclusion from the CNS at day 10.

In addition to be present in reduced numbers, Treg cells of Foxp3CreTnfr2fl/fl mice at day 10 in the CNS had an altered phenotype with lower expression of CTLA4, ICOS and Blimp-1. This may impact on their suppressive activity since CTLA4 is one of the major mechanisms of Treg-mediated suppression and ICOS and Blimp-1 expression are critical to promote IL-10 production²⁰⁻²². It has been shown that these 3 molecules are up-regulated in Treg cells by T-cell activation and

inflammatory cytokines, such as IL-2 or IL-6^{21,22}, but the effect of TNF α has not been described. In conclusion, the role of TNFR2 expressed by Treg cells in the control of EAE is likely due to its capacity to promote their expansion and/or to increase their suppressive activity in the inflamed CNS.

We were not able to determine which pathogenic cells or molecules were increased in Foxp3CreTnfr2fl/fl mice to explain their exacerbated EAE. Since GM-CSF is now considered as the major pathogenic cytokine in the disease²⁴, it will be important in the future to refine our analyses of myeloid cells to assess their capacity to produce molecules that are highly pathogenic for oligodendrocytes and neurones, such as reactive-oxygen species or IL-1.

It is increasingly clear that Treg cells are functionally heterogeneous. Various Treg cell populations acquire different biological properties under the influence of environment cues. At steady state, homeostasis of central Treg cells present in lymphoid tissues depends on IL-23. We show here that TNF- α has no role at steady state, except in the non-physiological context of competition between TNFR2-sufficient and deficient cells after weaning. Recent findings emphasized the critical role of IL-33 in the accumulation of Treg cells residing in the intestine and the adipose tissues³²⁻³⁴. Factors responsible for the high Treg cell accumulation in the inflamed CNS have not been described. Here, we reveal a non-redundant function of TNF- α in this phenomenon. Thus, depending on the tissue and type of inflammation, Treg cells may rely on different environment cues (IL-33, TNF- α) for their homeostasis and function. This may partly explain why anti-TNF therapy would be beneficial in some autoimmune diseases and detrimental in others. We found that TNFR2-deficient Treg cells were partially able to control colitis. It would be interesting to test their role in arthritis or psoriasis as well because of the utilisation of anti-TNF therapy in these diseases. Our data indicate that the TNF/TNFR2 pathway is critical to reach an optimal Treg cell function and accumulation in the CNS during EAE, which is essential to control the disease. These results bring new insights in Treg cell biology, provide an explanation for the failure of anti-TNF therapy in MS patients and may provide new tools to treat autoimmune diseases.

Methods

Mice. C57BL/6J (WT) mice were purchased from Janvier Labs (France). *Tnf^{f/-}* and *Tnfrsf1b^{-/-}* (*Tnfr2^{-/-}*) mice and 2D2 T cell receptor transgenic mice, specific for myelin oligodendrocyte glycoprotein, were purchased from the Jackson Laboratory. B6-Cd3e^{tm1Mal} (*Cd3^{-/-}*) and *RAG2^{-/-}* mice were provided by Cryopreservation, Distribution, Typing and animal Archiving department (Orléans, France). Foxp3-IRES-GFP knock-in (*Foxp3^{GFP}*) mice were kindly provided by Pr. Bernard Malissen. Foxp3-CRE-IRES-YFP (*Foxp3^{Cre}*) mice were a gift from Pr. Alexander Rudensky. *Tnfrsf1b*-floxed (*Tnfr2^{f/f}*) mice were obtained from the EMMA consortium. 2D2 and Foxp3^{GFP} mice were crossed with CD90.1 congenic animals. All mice were on a C57Bl/6 background. Mice were housed under specific pathogen-free conditions and were studied at 7–14 weeks of age. All experimental protocols were approved by the local ethics committee and are in compliance with European Union guidelines.

Reagents. Anti-TNF- α (XT3-11) and Anti-TNFR2 (TR75-54.7) were purchased from BioXCell and were injected at a dose of 500ug by intraperitoneal route every two days for 8 days. Etanercept was provided by Wyeth and was administered at a dose of 1mg by intraperitoneal route every two days for 8 days.

EAE induction. For active EAE, mice were injected subcutaneously in the flanks with 100 μ g of MOG₃₅₋₅₅ peptide (Polypeptide) emulsified in 100 μ l of CFA (Sigma-Aldrich) supplemented with 50 μ g of heat-killed *Mycobacterium tuberculosis* H37Ra (BD Biosciences). Animals were additionally injected intravenously with 200 ng of *Bordetella pertussis* toxin (Enzo) at the time of, and two days following immunization. For the passive EAE model, we first induced active EAE in donor mice as described above to generate pathogenic cells. 10 days post-immunization, cells from spleen and dLN were cultured in RPMI (Gibbco) at 5 x 10⁶ cells/ml with 20 μ g/ml of MOG₃₅₋₅₅ peptide, 10 μ g/ml of anti-IFN γ (XMG1.2, BioXCell) and 5 ng/ml of IL-23 (R&D Systems). After 3 days, cells were harvested and dead cells eliminated with a Ficoll gradient, then 2 x 10⁶ cells were injected intravenously to recipient mice, leading to passive EAE. The clinical evaluation was performed on a daily bases by a 6-point scale ranging: 0, no clinical sign; 1, limp tail; 2, limp tail,

impaired righting reflex, and paresis of one limb; 3, hindlimb paralysis; 4, hindlimb and forelimb paralysis; 5, moribund.

Preparation of cell suspensions. For isolation of CNS-infiltrating leukocytes, mice were anesthetized with a Ketamine (Merial) / Xylazine (Bayer) solution and perfused with 20 ml of cold PBS. Spinal cords were removed by intrathecal hydrostatic pressure. Brain and spinal cords were cut into small pieces and digested in DMEM (Gibbco) supplemented with 1 mg/ml collagenase type IV (Sigma) and 1 mg/ml DNase I (Sigma) for 30 min at 37°C followed by mechanical desegregation. Single cell suspensions were washed once, re-suspended in Percoll 40% and laid on a Percoll 80% solution. Mononuclear cells were prepared from the interface of the 40:80% Percoll gradient after centrifugation for 20 min at 2,000 rpm at room temperature and washed two times in a PBS-3% FCS buffer. Cells were isolated from spleen and LN by mechanical dilacerations.

Antibodies and flow cytometry analysis. The following mAbs from BD Biosciences were used: anti-CD45 (30-F11), anti-CD4 (RM4-5), anti-Foxp3 (MF23), anti-Ki-67 (SOLA15), anti-active caspase 3 (C92-605), anti-CD90.1 (OX-7), anti-CD90.2 (30-H12), anti-ICOS (17G9), anti-CTLA-4 (UC10-4B9), anti-Blimp1 (5E7), anti-TNFR2 (TR75-89), anti-V β 11 (RR3-15), anti-IFN- γ (XMG1.2), anti-IL-17 (TC11-18H10), anti-CD11b (M1/70), anti-CD11c (HL3), anti-IA/E (M5/11.15.2), anti-Ly6C (AL-21), anti-Ly6G(1AB), anti-NKp46 (29A1.4), anti-F4/80 (BM8). Anti-CD25 (PC61), anti-T-bet (eBio4B10), anti-ROR γ t (q31-378), anti-GM-CSF (MP1-22E9) and anti-Foxp3 (FJK16s) were purchased from eBioscience, and Foxp3 staining was performed using the eBioscience kit and protocol. For intracellular cytokine staining, cells were re-stimulated with 1 μ g/ml PMA (Sigma) and 0.5 μ g/ml Ionomycin (Sigma) for 3 hours in the presence of 1 μ l/ml GolgiPlug (BD Biosciences). After cell surface staining, intracellular staining was performed using the Intracellular Fixation & Permeabilization Buffer Set from eBioscience. Cells were acquired on a BD LSRII cytometer and analyzed using FlowJo software.

Bone marrow chimeras. Bone marrow cells were isolated from tibia and femur of donor mice. Recipient mice were lethally irradiated (10.5 Gray) and transplanted intravenously with 10 x 10⁶ bone marrow cells.

Treg suppression assays. Cells were isolated from the spleen and LN of *Foxp3^{YFP-Cre}Tnfr2^{fl/fl}* and *Foxp3^{YFP-Cre}* mice. Cell suspensions were obtained as described above and stained with an anti-CD4 antibody. CD4⁺YFP⁻ cells (Tconv) and CD4⁺YFP⁺ cells (Treg) were then sorted using a BD FACSAria II.

For the in vitro suppressive assay, purified Tconv were labeled with CellTrace Violet Proliferation Kit (Life technologies) and plated in 96-well plate at 2.5×10^4 cells/well with splenocytes from *Cd3^{-/-}* mice at 7.5×10^4 cells/well. Culture medium was supplemented with anti-CD3 (2C11) from BioXCell at 0.05 µg/ml. Purified Treg were then added at different ratios from 1:1 to 1:8. At day 3, CellTrace dilution was assessed by flow cytometry. For the colitis model, purified Tconv and Treg cells (1×10^5 cells) were injected intravenously into sex-matched *RAG2^{-/-}* mice. The clinical evaluation was performed twice a week by measuring body weight. 8 weeks after transfer, mice were sacrificed and colons were collected and fixed in PBS containing 4% formaldehyde. Five-micrometer paraffin-embedded sections were cut and stained with haematoxylin and eosin and then blindly analyzed.

T-cell priming evaluation. LN and spleen cells from 2D2 CD90.1 mice were stained with the following biotin-labeled antibodies: anti-CD19 (6D5), anti-CD11b (M1/70), anti-CD11c (N418), anti-CD8 (53-6.7) and anti-CD25 (7D4) and then were coated with anti-biotin microbeads (Miltenyi Biotec). After magnetic sorting, cells of the CD4⁺ enriched negative fraction were labeled with CellTrace Violet Proliferation Kit and were intravenously injected in naive mice (10^6 cells/mouse). The following day, mice were immunized with MOG₃₅₋₅₅ peptide as for EAE induction. CellTrace dilution of CD4⁺ CD90.1⁺ Vβ11⁺ (T cell receptor transgene) cells was assessed by flow cytometry from brachial and inguinal LNs at day 3.

Statistical analysis. Statistical analyses were performed using GraphPad Prism Software. Statistical significance was determined using the two-tailed nonparametric Mann–Whitney U test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Means \pm s.e.m. were used throughout the figures.

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COMPETING FINANCIAL INTERESTS.

The authors declare no competing financial interests.

References

1. Ware, C.F. Network communications: lymphotoxins, LIGHT, and TNF. *Annu Rev Immunol* 23, 787-819 (2005).
2. Bosch, X., Saiz, A., Ramos-Casals, M. & Group, B.S. Monoclonal antibody therapy-associated neurological disorders. *Nat Rev Neurol* 7, 165-172 (2011).
3. Kodama, S., Davis, M. & Faustman, D.L. The therapeutic potential of tumor necrosis factor for autoimmune disease: a mechanistically based hypothesis. *Cell Mol Life Sci* 62, 1850-1862 (2005).
4. Study-group. TNF neutralization in MS: results of a randomized, placebo-controlled multicenter study. The Lenercept Multiple Sclerosis Study Group and The University of British Columbia MS/MRI Analysis Group. *Neurology* 53, 457-465 (1999).
5. van Oosten, B.W., et al. Increased MRI activity and immune activation in two multiple sclerosis patients treated with the monoclonal anti-tumor necrosis factor antibody cA2. *Neurology* 47, 1531-1534 (1996).
6. Zganiacz, A., et al. TNF-alpha is a critical negative regulator of type 1 immune activation during intracellular bacterial infection. *J Clin Invest* 113, 401-413 (2004).
7. Cope, A.P., et al. Chronic tumor necrosis factor alters T cell responses by attenuating T cell receptor signaling. *J Exp Med* 185, 1573-1584 (1997).
8. Zhao, X., et al. TNF signaling drives myeloid-derived suppressor cell accumulation. *J Clin Invest* 122, 4094-4104 (2012).
9. Sade-Feldman, M., et al. Tumor Necrosis Factor-alpha Blocks Differentiation and Enhances Suppressive Activity of Immature Myeloid Cells during Chronic Inflammation. *Immunity* 38, 541-554 (2013).
10. Menges, M., et al. Repetitive injections of dendritic cells matured with tumor necrosis factor alpha induce antigen-specific protection of mice from autoimmunity. *J Exp Med* 195, 15-21 (2002).

11. Notley, C.A., et al. Blockade of tumor necrosis factor in collagen-induced arthritis reveals a novel immunoregulatory pathway for Th1 and Th17 cells. *J Exp Med* 205, 2491-2497 (2008).
12. Ban, L., et al. Selective death of autoreactive T cells in human diabetes by TNF or TNF receptor 2 agonism. *Proc Natl Acad Sci U S A* 105, 13644-13649 (2008).
13. Josefowicz, S.Z., Lu, L.F. & Rudensky, A.Y. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30, 531-564 (2012).
14. Chen, X., Baumel, M., Mannel, D.N., Howard, O.M. & Oppenheim, J.J. Interaction of TNF with TNF receptor type 2 promotes expansion and function of mouse CD4+CD25+ T regulatory cells. *J Immunol* 179, 154-161 (2007).
15. Chen, X., et al. Cutting edge: expression of TNFR2 defines a maximally suppressive subset of mouse CD4+CD25+FoxP3+ T regulatory cells: applicability to tumor-infiltrating T regulatory cells. *J Immunol* 180, 6467-6471 (2008).
16. Grinberg-Bleyer, Y., et al. Pathogenic T cells have a paradoxical protective effect in murine autoimmune diabetes by boosting Treg. *J Clin Invest* 120, 4558-4568 (2010).
17. Probert, L., et al. TNFR1 signalling is critical for the development of demyelination and the limitation of T-cell responses during immune-mediated CNS disease. *Brain* 123 (Pt 10), 2005-2019 (2000).
18. Suvannavejh, G.C., et al. Divergent roles for p55 and p75 tumor necrosis factor receptors in the pathogenesis of MOG(35-55)-induced experimental autoimmune encephalomyelitis. *Cell Immunol* 205, 24-33 (2000).
19. Tsakiri, N., Papadopoulos, D., Denis, M.C., Mitsikostas, D.D. & Kollias, G. TNFR2 on non-haematopoietic cells is required for Foxp3+ Treg-cell function and disease suppression in EAE. *Eur J Immunol* 42, 403-412 (2012).
20. Wing, K., et al. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322, 271-275 (2008).
21. Cretney, E., et al. The transcription factors Blimp-1 and IRF4 jointly control the differentiation and function of effector regulatory T cells. *Nat Immunol* 12, 304-311 (2011).
22. Neumann, C., et al. Role of Blimp-1 in programming Th effector cells into IL-10 producers. *J Exp Med* 211, 1807-1819 (2014).

23. Stromnes, I.M., Cerretti, L.M., Liggitt, D., Harris, R.A. & Goverman, J.M. Differential regulation of central nervous system autoimmunity by T(H)1 and T(H)17 cells. *Nat Med* 14, 337-342 (2008).
24. Codarri, L., Greter, M. & Becher, B. Communication between pathogenic T cells and myeloid cells in neuroinflammatory disease. *Trends Immunol* 34, 114-119 (2013).
25. Bailey, S.L., Schreiner, B., McMahon, E.J. & Miller, S.D. CNS myeloid DCs presenting endogenous myelin peptides 'preferentially' polarize CD4⁺ T(H)-17 cells in relapsing EAE. *Nat Immunol* 8, 172-180 (2007).
26. Dendrou, C.A., Bell, J.I. & Fugger, L. A clinical conundrum: the detrimental effect of TNF antagonists in multiple sclerosis. *Pharmacogenomics* 14, 1397-1404 (2013).
27. Suen, W.E., Bergman, C.M., Hjelmstrom, P. & Ruddle, N.H. A critical role for lymphotoxin in experimental allergic encephalomyelitis. *J Exp Med* 186, 1233-1240 (1997).
28. Williams, S.K., et al. Antibody-mediated inhibition of TNFR1 attenuates disease in a mouse model of multiple sclerosis. *PLoS One* 9, e90117 (2014).
29. Arnett, H.A., et al. TNF alpha promotes proliferation of oligodendrocyte progenitors and remyelination. *Nat Neurosci* 4, 1116-1122 (2001).
30. Chen, X., et al. TNFR2 is critical for the stabilization of the CD4⁺Foxp3⁺ regulatory T. cell phenotype in the inflammatory environment. *J Immunol* 190, 1076-1084 (2013).
31. Smigielski, K.S., et al. CCR7 provides localized access to IL-2 and defines homeostatically distinct regulatory T cell subsets. *J Exp Med* 211, 121-136 (2014).
32. Schiering, C., et al. The alarmin IL-33 promotes regulatory T-cell function in the intestine. *Nature* 513, 564-568 (2014).
33. Kolodin, D., et al. Antigen- and cytokine-driven accumulation of regulatory T cells in visceral adipose tissue of lean mice. *Cell Metab* 21, 543-557 (2015).
34. Vasanthakumar, A., et al. The transcriptional regulators IRF4, BATF and IL-33 orchestrate development and maintenance of adipose tissue-resident regulatory T cells. *Nat Immunol* 16, 276-285 (2015).

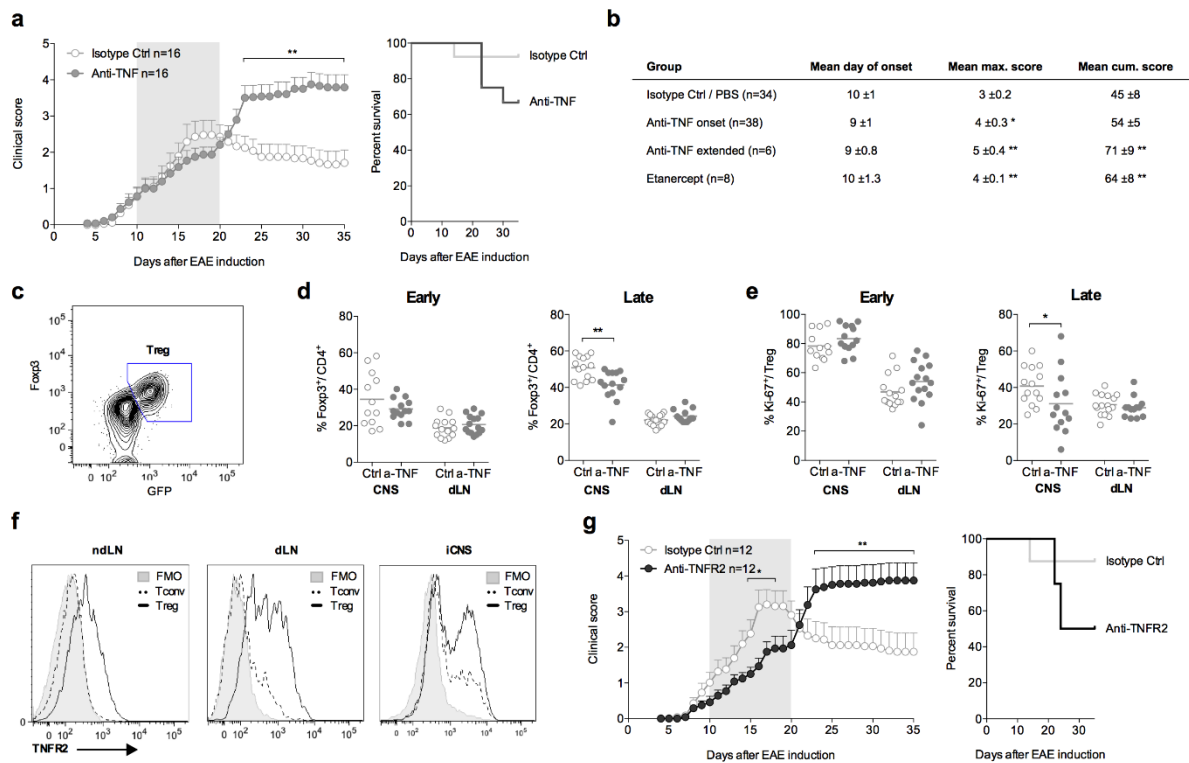


Figure 1. Anti-TNF treatment induces EAE exacerbation and limits Treg cell expansion in the CNS. (a) Clinical score of EAE and percent survival in mice immunized to induce EAE at day 0 and treated with anti-TNF or isotype control mAbs at day 10-18. Mean ± SEM from 3 independent experiments. (b) Mean day of EAE onset ± SEM, mean of maximum EAE score ± SEM and mean of cumulative EAE score ± SEM in mice immunized to induce EAE at day 0 and then treated with isotype control mAb or PBS at day 10-18 or with anti-TNF mAb at disease onset for 8 days (onset) or 18 days (extended) or with etanercept at disease onset for 8 days. Proportions of Treg cells among CD4⁺ cells (d), determined as in (c), and of Ki67⁺ among Treg cells (e) were measured at day 15-19 (early) or day 29-45 (late) in the CNS and dLN of *Foxp3*^{GFP} mice immunized to induce EAE and treated at day 10-18 with anti-TNF (a-TNF) mAb or isotype control mAbs or PBS (Ctrl). Each dot represents a mouse, collected from 5 independent experiments. (f) Representative TNFR2 expression in Tconv (CD4⁺Foxp3⁻) and Treg cells in non-draining LN (ndLN), dLN and CNS at day 15 after EAE induction. Control staining is the fluorescence minus one (FMO). (g) Clinical score of EAE and percent survival in mice immunized to induce EAE at day 0 and treated with anti-TNFR2 or isotype control mAbs at day 10-18. Mean ± SEM from 2 independent experiments. **p*>0.05, ***p*>0.01, two-way unpaired Mann-Whitney test.

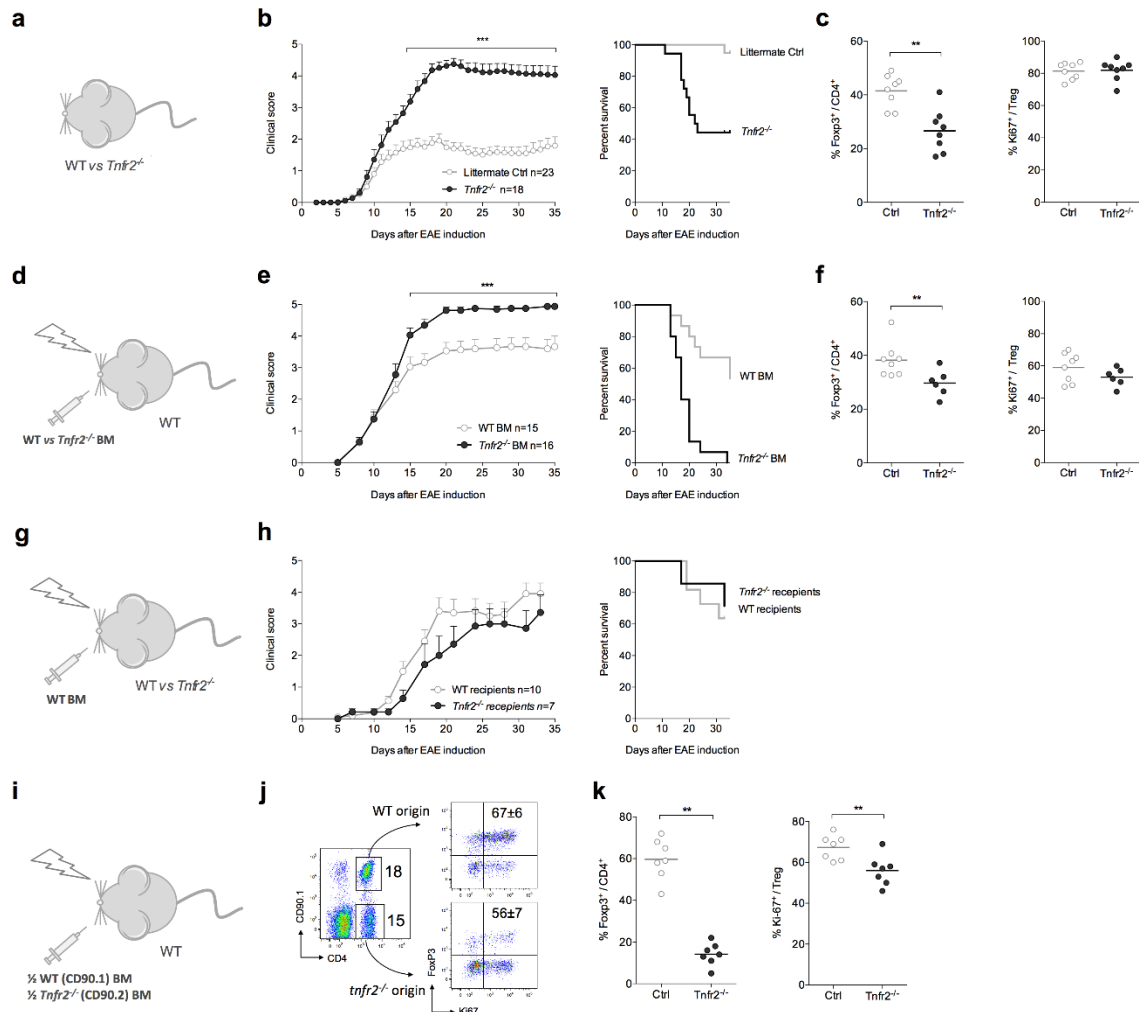


Figure 2. Mice with TNFR2-deficient hematopoietic cells develop severe EAE and cell intrinsic role of TNFR2 in Treg cell homeostasis in the CNS. (a-c) Mean clinical score of EAE ± SEM and percent survival (b) and proportions of Treg cells and Ki67⁺ among Treg cells in the CNS at day 10-15 (c) in *Tnfr2*^{-/-} and WT littermate control mice (a) immunized to induce EAE. (d-f) Mean clinical score of EAE ± SEM and percent survival (e) and proportions of Treg cells and Ki67⁺ among Treg cells in the CNS at day 16-19 (f) in WT recipients reconstituted with *Tnfr2*^{-/-} or WT bone marrow cells (d) and immunized to induce EAE. (g,h) Mean clinical score of EAE ± SEM and percent survival (h) in *Tnfr2*^{-/-} or WT recipients reconstituted with *Tnfr2*^{-/-} or WT bone marrow cells (g) and immunized to induce EAE. (i-k) Proportions of Treg cells within cells of the WT and *Tnfr2*^{-/-} origin and of Ki67⁺ cells among Treg cells within cells of the WT and *Tnfr2*^{-/-} origin was determined in the CNS at day 20 (k), as shown in (j), in WT recipients reconstituted with 1:1 ratio of a mix of *Tnfr2*^{-/-} CD90.2⁺ and WT CD90.1⁺ bone marrow cells (i). Data were collected from 4 (b, c) or 2 (e, f, h, j, k) independent experiments. □**p>0.01, ***p>0.001, two-way unpaired Mann-Whitney test. □□

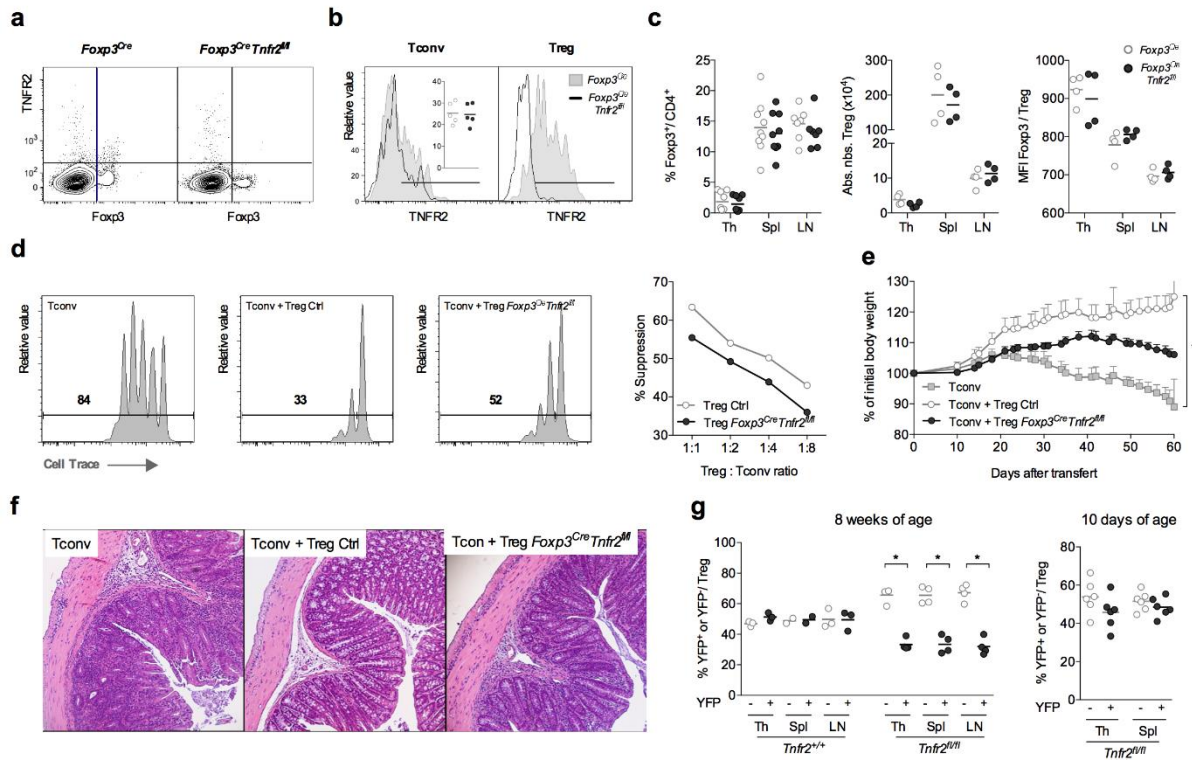


Figure 3. TNFR2 is dispensable in Treg cell biology at steady state. (a) TNFR2 expression in resting CD4⁺ cells and (b) in *in vitro* activated Treg and Tconv cells of *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* control mice. (c) Proportion of Fcγ3⁺ cells among CD4⁺ cells, absolute numbers of Treg cells and MFI of Fcγ3 expression among Treg cells of *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* control mice in the thymus (Th), spleen (Spl) and LN. Each dot represents a mouse, collected from 5 independent experiments. (d) *In vitro* suppressive activity of Treg cells from *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* control mice. Representative data at a 1:2 (left) and different (right) Treg:Tconv ratios of 3 independent experiments. (e,f) *In vivo* suppressive activity of Treg cells from *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* control mice, determined in a colitis model. Percentage of initial body weight pooled from 2 independent experiments (n=6) (e) and representative histology of the colon (f). (g) Percentage of CRE/YFP⁺ (+,) and CRE/YFP⁻ (-,) cells among Treg cells of 8-week and 10-day old *Foxp3^{CRE/+}Tnfr2^{fl/fl}* and *Foxp3^{CRE/+}Tnfr2^{+/+}* females in the thymus (Th), spleen (Spl) and LN. Each dot represents a mouse, collected from 2 independent experiments.

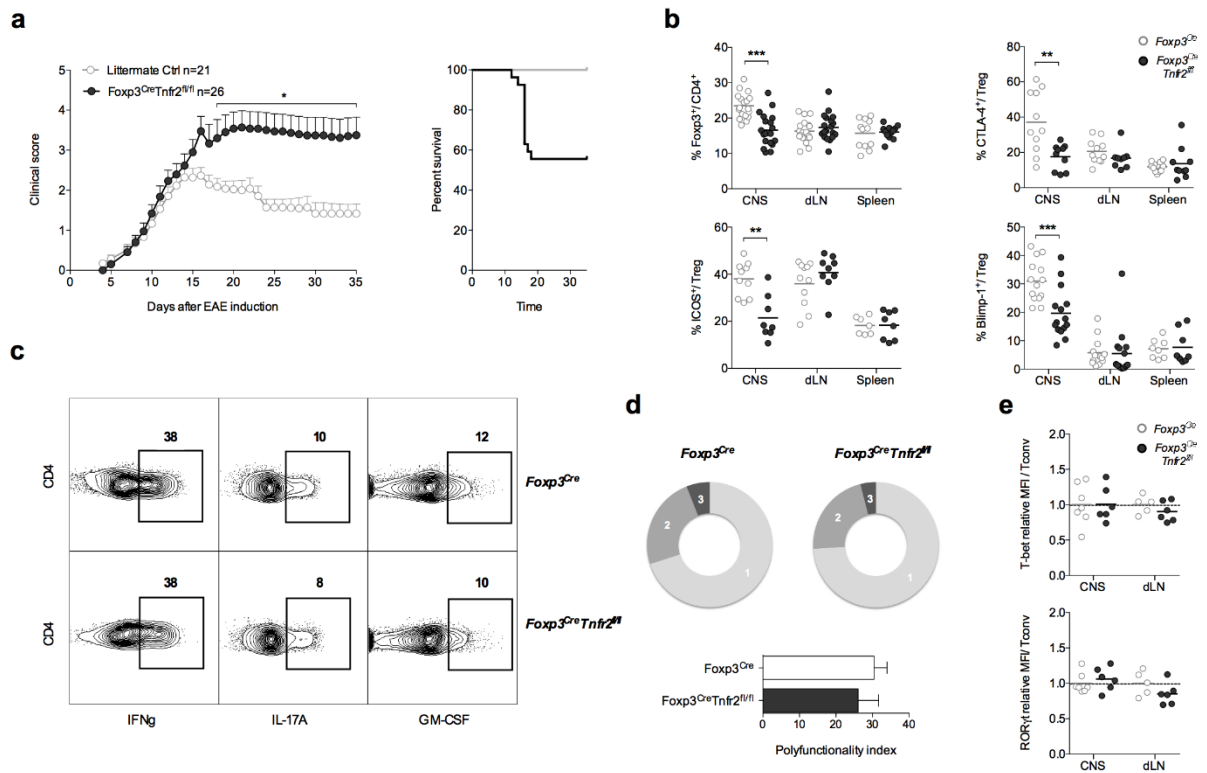


Figure 4. Control of EAE by Treg cells depends on their expression of TNFR2, which impacts on their numbers and effector phenotype in the inflamed CNS. (a) Mean clinical score of EAE \pm SEM and percent survival of *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* mice immunized to induce EAE. **(b)** Proportions of Treg cells among CD4⁺ T cells and of CTLA4, ICOS and Blimp-1 in the CNS, dLN and spleen at day 10 of *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* mice immunized to induce EAE. Each dot represents a mouse, collected from 4 independent experiments. **(c,d)** Representative expression of IFN γ , IL-17A and GM-CSF **(c)**, proportion of cells co-expressing 1, 2 or 3 of these cytokines and polyfunctionality index (% cells expressing 3 cytokines) **(d)** among CD4⁺*Foxp3⁺* cells at day 15 in the CNS of *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* mice immunized to induce EAE. Data are from 2 independent experiments (n=6). **(e)** Relative mean fluorescence intensity (MFI) of ROR γ t and Tbet among CD4⁺*Foxp3⁺* cells at day 15 in the CNS of *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* mice immunized to induce EAE. The values correspond to each MFI divided by the mean MFI of *Foxp3^{CRE}* mice. Each dot represents a mouse, collected from 2 independent experiments. * $p > 0.05$, ** $p > 0.01$, *** $p > 0.001$, two-way unpaired Mann-Whitney test.

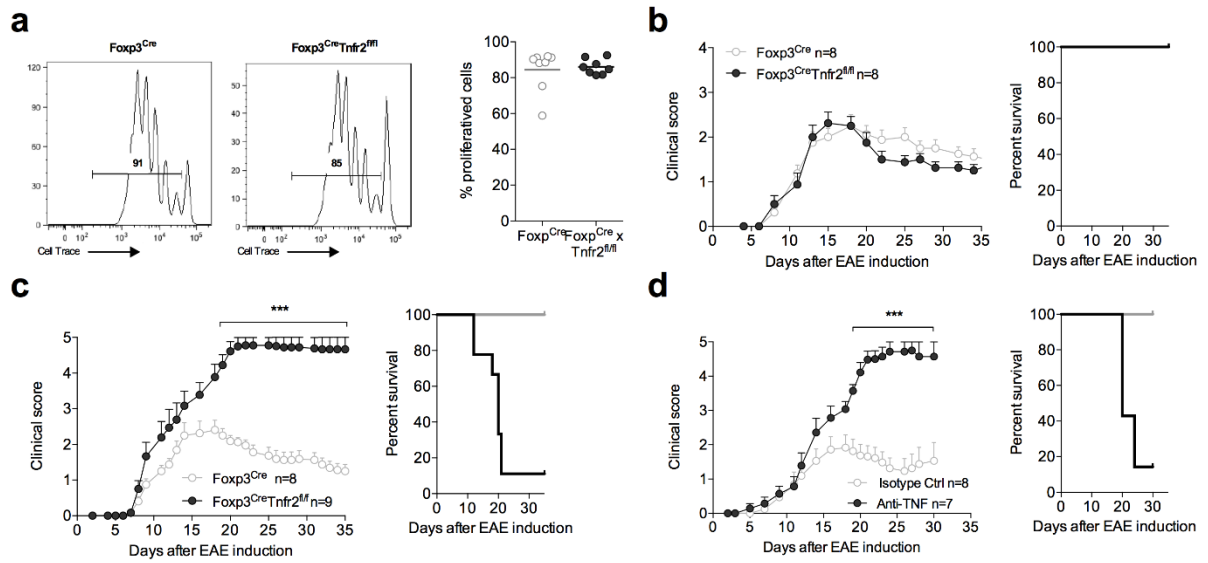
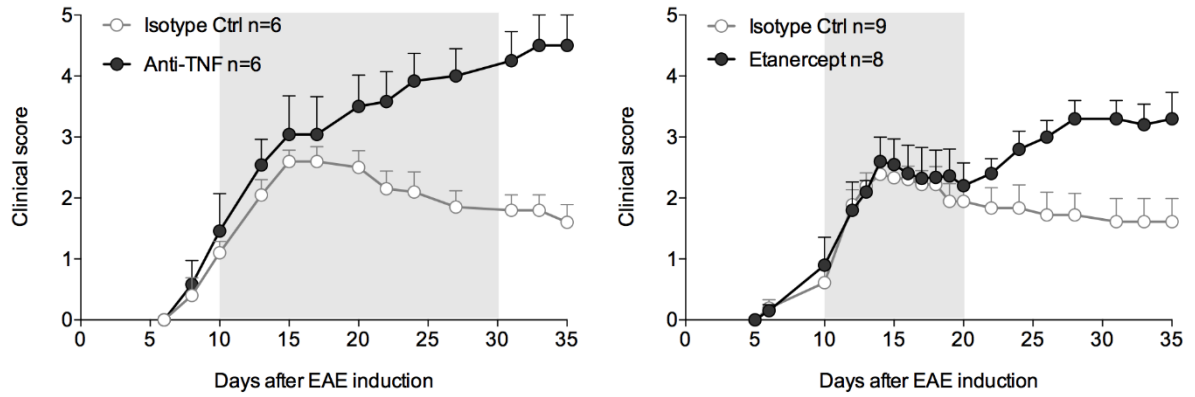
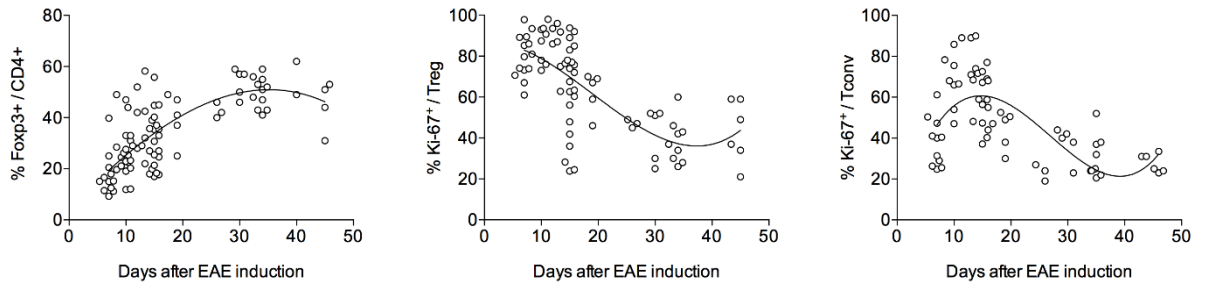


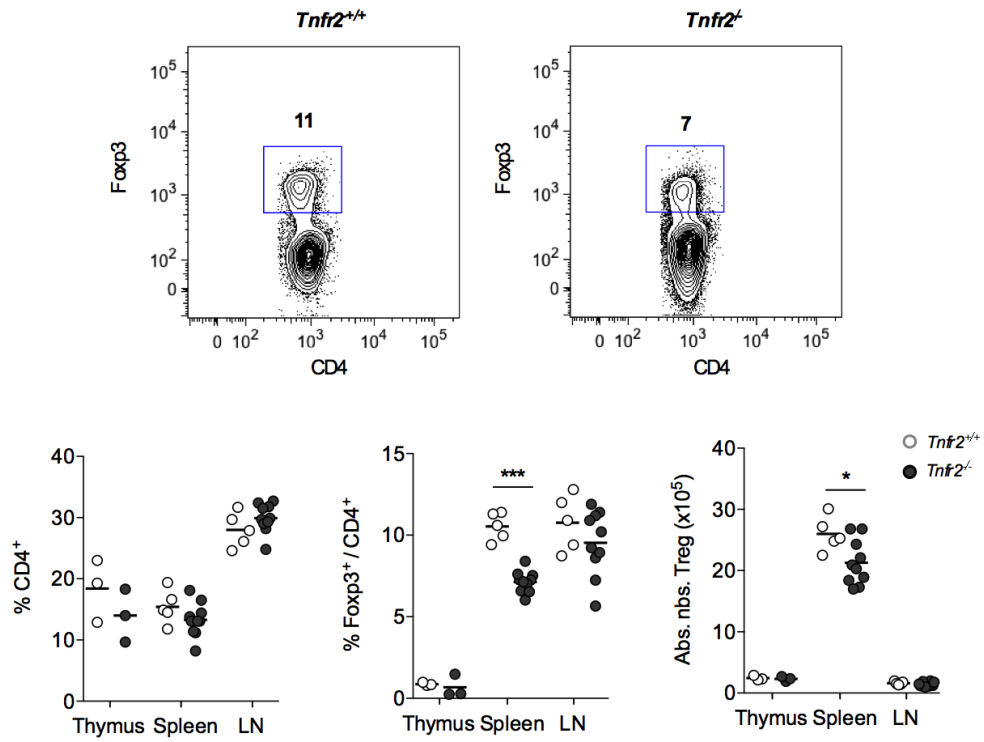
Figure 5. Expression of TNFR2 by Treg cells is critical to control EAE in the CNS. (a) Representative division profile (left) and cumulative percentages of divided cells (right) of T cell receptor transgenic myelin oligodendrocyte glycoprotein reactive Tconv cells 3 days after their transfer in *Foxp3^{CRE}Tnfr2^{fl/fl}* and *Foxp3^{CRE}* mice immunized to induce EAE. (b) Mean clinical score of passive EAE ± SEM and percent survival in WT recipients transferred with pathogenic cells obtained from dLN of *Foxp3^{CRE}Tnfr2^{fl/fl}* or *Foxp3^{CRE}* mice immunized 10 earlier to induce EAE. (c) Mean clinical score of passive EAE ± SEM and percent survival in *Foxp3^{CRE}Tnfr2^{fl/fl}* or *Foxp3^{CRE}* recipients transferred with pathogenic cells obtained from dLN of WT mice immunized 10 earlier to induce EAE. (d) Mean clinical score of passive EAE ± SEM and percent survival in WT recipients transferred with pathogenic cells obtained from dLN of WT mice immunized 10 earlier to induce EAE. Recipients were treated with an anti-TNF mAb or PBS from day 0 to 10 after cell transfer. Data are from 4 (a) and 2 (b,c,d) independent experiments.



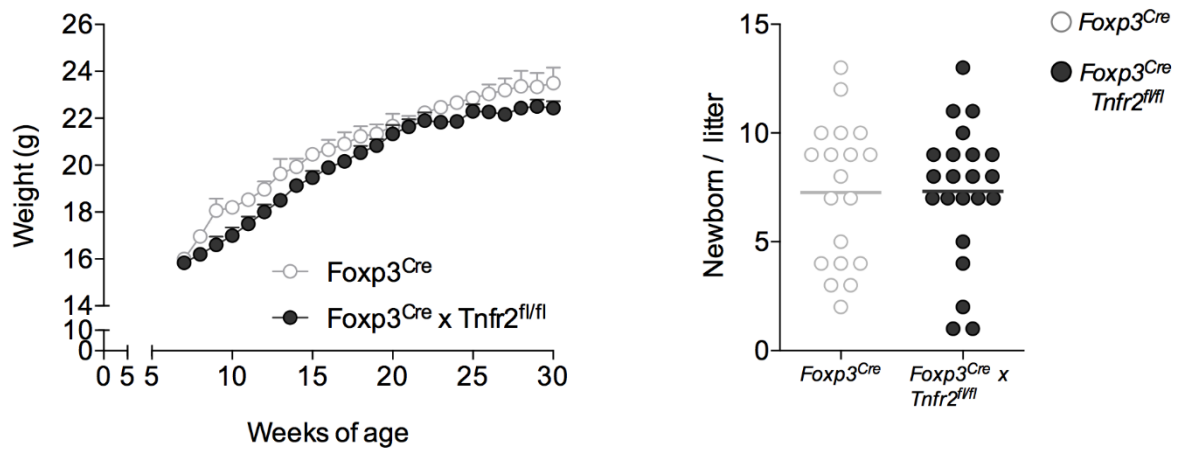
Supplementary Figure 1. Anti-TNF treatment induces EAE exacerbation. Active EAE was induced at day 0. Clinical score of EAE in WT mice treated with anti-TNF mAb or with isotype control mAb or PBS (Ctrl) at day 10-28 (left) or with Etanercept at disease onset for 8 days (right). Mean from 2 (left) and 3 (right) independent experiments.



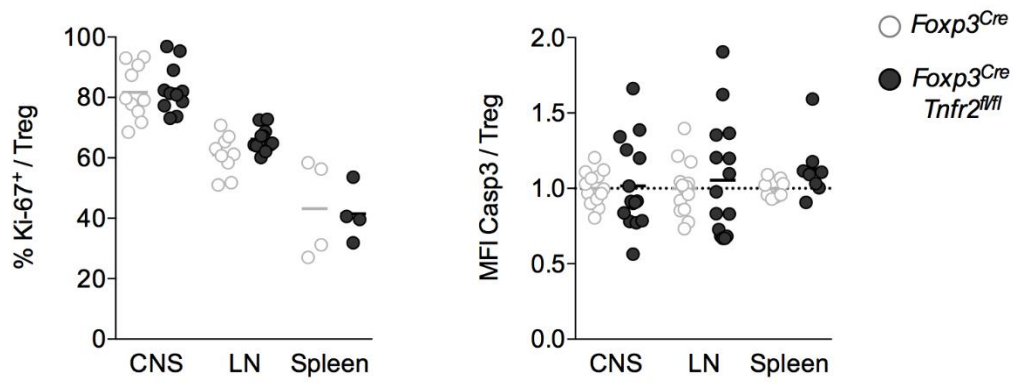
Supplementary Figure 2. Treg cell expansion in the CNS during EAE. Proportion and proliferation of Treg and Tconv cells in the CNS different times after induction of EAE. Each dot represents a mouse. Curves show third order polynomial non-linear regression.



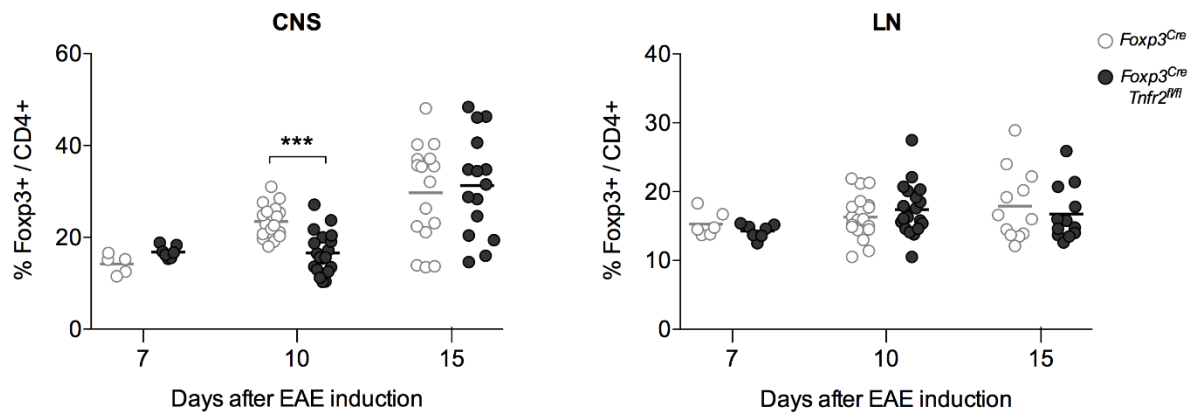
Supplementary Figure 3. Partial defect of Treg cells in the spleen of *Tnfr2*^{-/-} mice. Proportions and absolute numbers of CD4⁺ and Treg cells in *Tnfr2*^{-/-} and control mice in the spleen (upper and lower panels), thymus and LN (lower panels).



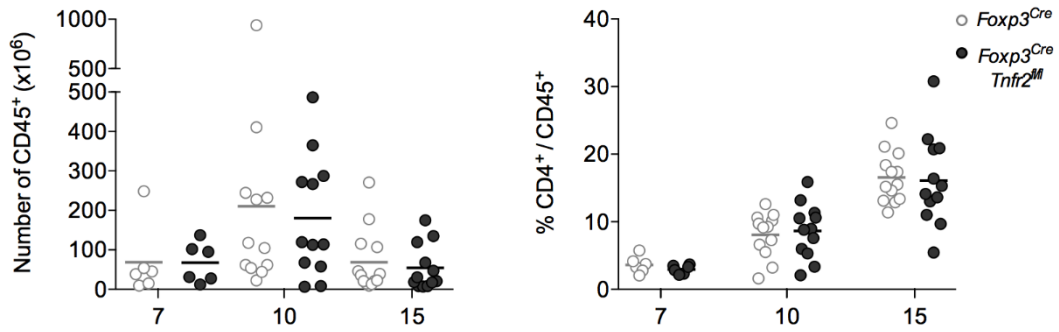
Supplementary Figure 4. Normal weight growth and litter size in *Foxp3^{Cre}Tnfr2^{fl/fl}* mice.



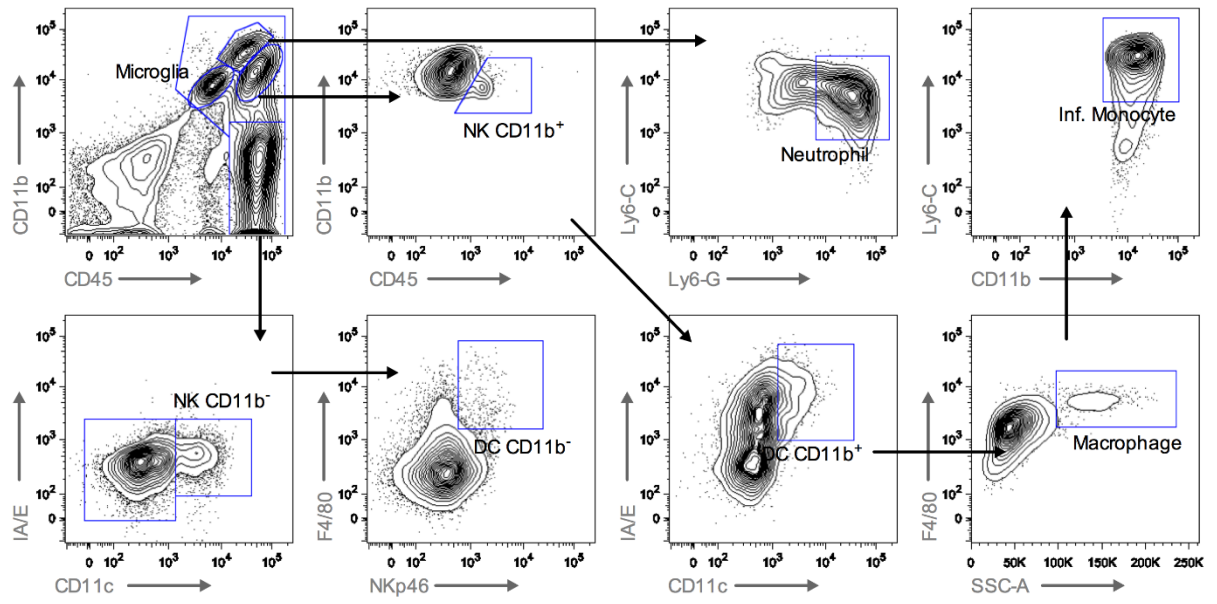
Supplementary Figure 5. Normal expression of Ki-67 and active caspase 3 in CNS Treg cells of *Foxp3^{Cre}Tnfr2^{fl/fl}* mice in EAE. Proportion of Ki-67+ cells (left) and relative mean fluorescence intensity (MFI) of active caspase 3 (right) among Treg cells in the CNS at day 10 after EAE induction. For caspase 3 expression, the values correspond to each MFI divided by the mean MFI of *Foxp3^{CRE}* control mice. Each dot represents a mouse, collected from 2 independent experiments.



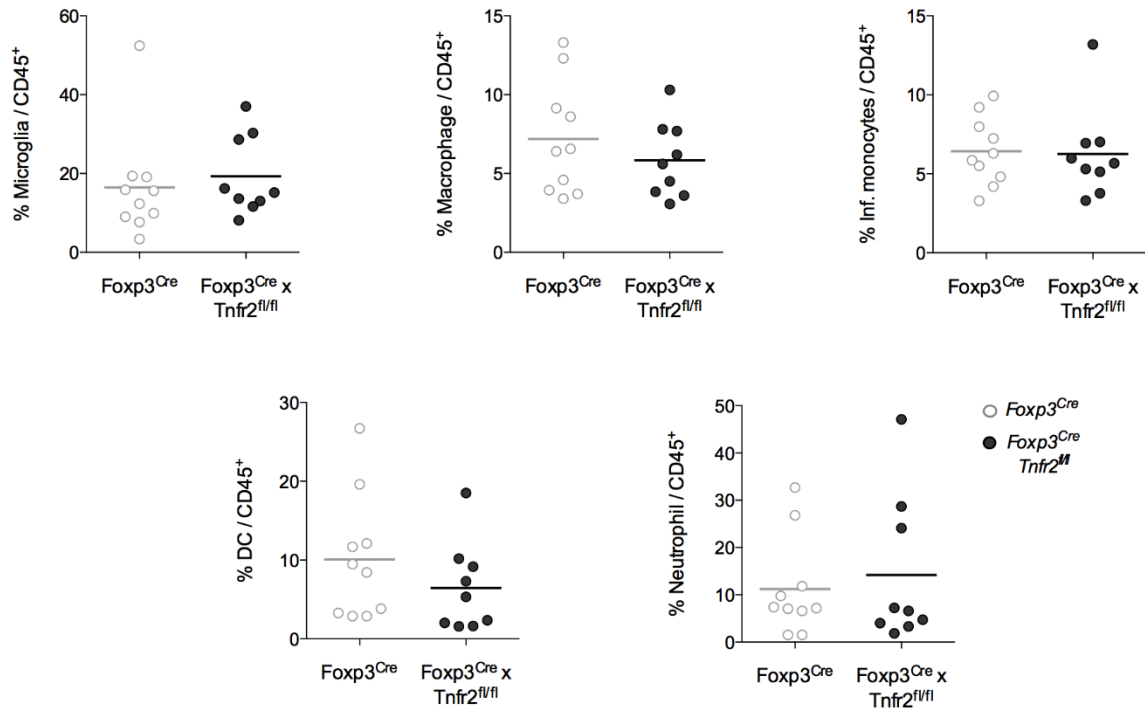
Supplementary Figure 6. Reduced proportions of Treg cells in *Fosp3^{CRE}Tnfr2^{fl/fl}* mice during EAE is restricted to the CNS at day 10. Proportion of Treg cells in the CNS and dLN at day 7, 10 and 15 after EAE induction. Each dot represents a mouse, collected from 2 (day 7), 4 (day 10) and 5 (day 15) independent experiments. At day 10, data are the same as shown in figure 4b.



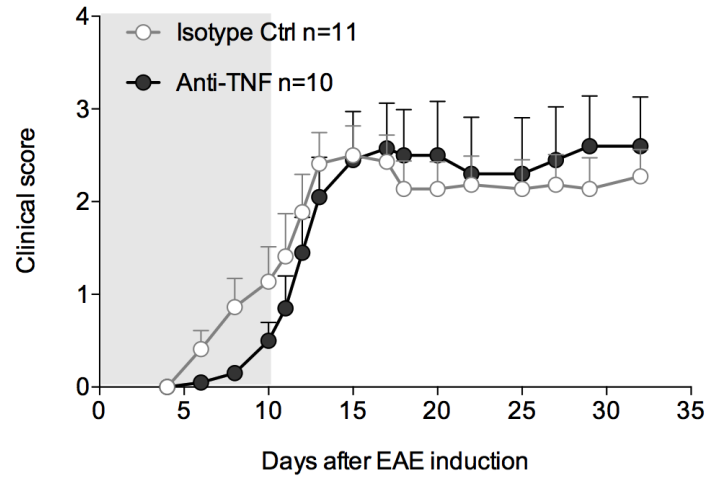
Supplementary Figure 7. Normal accumulation of leukocytes and CD4⁺ cells in the CNS of *Foxp3^{Cre}Tnfr2^{fl/fl}* mice during EAE. Absolute number of leukocytes (left) and proportion of CD4⁺ cells (right) in the CNS at day 7, 10 and 15 after EAE induction. Each dot represents a mouse, collected from 2 (day 7), 4 (day 10) and 5 (day 15) independent experiments.



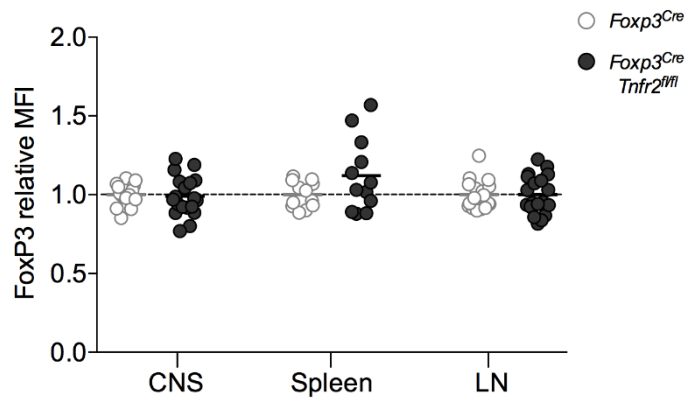
Supplementary Figure 8. Gating strategy to identify myeloid cell subsets in the CNS during EAE.



Supplementary Figure 9. Normal proportion of myeloid cells subsets in the CNS of *Foxp3^{Cre}Tnfr2^{fl/fl}* mice during EAE. Proportion of the indicated myeloid cells subsets in the CNS at day 15 after EAE induction. Each dot represents a mouse, collected from 3 independent experiments.



Supplementary Figure 10. Early anti-TNF treatment did not induce EAE exacerbation. Active EAE was induced at day 0. Clinical score of EAE in WT mice treated with anti-TNF or with isotype mAbs at day 0-8. Mean from 2 independent experiments.



Supplementary Figure 11. Normal Foxp3 expression in Treg cells of *Foxp3^{Cre}Tnfr2^{fl/fl}* mice during EAE. Relative mean fluorescence intensity (MFI) of Foxp3 among Treg cells in the CNS, spleen and dLN at day 10 after EAE induction. The values correspond to each MFI divided by the mean MFI of *Foxp3^{CRE}* control mice. Each dot represents a mouse, collected from 4 independent experiments.