REGULATION OF T CELL FATES BY THE TRANSCRIPTION FACTOR NFAT5 IN DIFFERENT MICROENVIRONMENTS

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Etxekoari,

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THESIS ABSTRACT

CD4$^+$ T cells can adopt specific functional programs in response to changes in microenvironment variables, such as cytokine availability, cell-to-cell contacts and stress conditions. It has been described that the elevation of the extracellular concentrations of sodium can stimulate T cells to enhance the expression of diverse cytokines and receptors, some of which are regulated by the transcription factor NFAT5. NFAT5 is a general regulator of osmotic stress responses in mammals, although it also has hypertonicity-independent roles, such as in the regulation of thymocyte development and the activation of macrophages by Toll-like receptors. Here we have used mice that lack NFAT5 specifically in T cells to analyze the involvement of NFAT5 in the acquisition of polarized functions in T cells when they are activated in osmostress conditions and in the absence of hypertonicity. We describe that exposure of CD4$^+$ T cells to hypertonic stress during stimulation with signals that mimic their T cell receptor enhances the expression of various genes associated with a T helper 17 (Th17) polarization profile, and analyze the role of NFAT5 and the Th17 master transcription factor ROR-γt in the hypertonicity-induced Th17 bias. We have also explored the potential relevance of NFAT5 as a regulator of pathogenic T cell responses *in vivo* in an experimental mouse model of inflammatory disease. Altogether, our results indicate that NFAT5 can modulate T cell polarization in different microenvironments, and that this capability may play a relevant role in inflammatory responses *in vivo*.

RESUM DE LA TESI

Les cèl·lules T CD4$^+$ poden adoptar programes funcionals diferents com a resposta a canvis en l'entorn com ara la disponibilitat de citocines, contactes entre cèl·lules i condicions d'estrès. Per exemple, s'ha descrit que un increment en la concentració extracel·lular de sodi promou en les cèl·lules T l'augment de l'expressió de gens que codifiquen per citocines i receptors, alguns dels quals són regulats pel factor de transcripció NFAT5. Aquesta proteïna és un regulador general de la resposta a l'estrès osmòtic en mamífers, encara que també té un paper en la regulació del desenvolupament dels timòcits i l'activació de receptors de tipus Toll en macròfags. En aquest estudi hem fet servir ratolins que no expressen NFAT5 en les cèl·lules T per tal d'analitzar quin és el paper d'aquest factor de transcripció en la polarització de les cèl·lules T activades en condicions isotòniques o d'estrès osmòtic. Hem descrit que l'exposició de les cèl·lules T CD4$^+$ a estrès hipertònic durant la seva activació mitjançant l'estimulació del receptor de cèl·lules T, augmenta l'expressió de gens associats al perfil de polarització Th17. Hem analitzat el paper de l'NFAT5 i del factor de transcripció que regula la diferenciació de les cèl·lules Th17, el ROR-γt, en la polarització a cèl·lules Th17 induïda per hipertonicitat. També hem explorat el paper de l'NFAT5 en la resposta *in vivo* de les cèl·lules T en un model experimental de malaltia inflamatòria murina. En resum, els nostres resultats indiquen que l'NFAT5 pot modular la polarització de cèl·lules T en diferents microambients, i que aquesta capacitat pot jugar un paper important en respostes inflamatòries *in vivo*.

PREFACE

Immune responses occur in a great variety of niches that are in a constant change. Leukocytes have to be able to integrate different inputs such as the cytokines that are present, nutrient and oxygen availability or extracellular tonicity level variations. Those conditions change considerably at inflamed tissues, infected wounds or tumors compared to blood or the lymphoid organs. $CD4^+$ T cells can adopt different functional properties depending on the environmental factors. They are divided into seven different T cell subtypes. There is increasing information about how environmental factors alter the outcome of $CD4^+$ T cells, and which are the mechanisms involved in the plasticity of these cells. Our work provides novel insights on how T cells differentiate in the presence of extracellular high salt concentrations and in specific T cell differentiation conditions and the way NFAT5 regulates T cell responses *in vivo*.

ABREVIATIONS

2-DG	2-deoxyglucose
AAD	aromatic I-amino acid decarboxylase
AED	auxiliary export domain
AhR	aryl hydrocarbon receptor
AKT	protein kinase B
APC	antigen-presenting cell
AQP	aquaporin
AR	aldose reductase
ATA2	sodium-coupled neutral amino acid transporter 2
BAFF	B cell-activating factor
BATF	basic leucine zipper transcription factor ATF-like
Bcl2	B-cell lymphoma
BGT	betaine/γ-aminobutyric acid transporter
CCL	C-C chemokine ligand
CCR	C-C chemokine receptor
CD	cluster of differentiation
CD40L	CD40 ligand
CNS	conserved smad and NFAT element
COX	cyclooxygenase
CTLA	cytolytic T lymphocyte-associated antigen
CXCL	CXC chemokine ligand
CXCR	CXC chemokine receptor
DBD	DNA-binding domain
DC	dendritic cell
DN	double negative
Dnmt	DNA methyltransferase
DSS	dextrane sulfate sodium
EAE	experimental autoimmune encephalomyelitis
ELISA	enzyme-linked immunosorbent assay
ERK	extracellular signal-regulated kinase
Foxp3	forkhead box P3
GATA3	GATA binding protein 3
GALT	gut-associated lymphoid tissue
GC	germinal center

Gfi	growth factor independent
GM-CSF	granulocyte-macrophage colony-stimulating factor
GPC	glycerophosphocholine
HICC	hypertonicity-induced cation channel
HIF	hypoxia-inducible factor
HIV	human immunodeficiency virus
HSP	heat shock protein
IBD	inflammatory bowel disease
ICOS	inducible T-cell co-stimulator
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IL-23R	interleukin-23 receptor
iNOS	inducible nitric oxide synthase
IRF	interferon regulatory factor
iTreg cell	induced T regulatory cell
JAK	Janus kinase
KCC	K^+-Cl^- cotransporter
KO	knockout
LAT	linker for activation of T cells
LT	lymphotoxin
MAPK	mitogen-activated protein kinase
MCP	monocyte chemoattractant protein
MHC	major histocompatibility complex
MIP	macrophage-inflammatory protein
mLN	mesenteric lymph node
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NES	nuclear export signal
NFAT	nuclear factor of activated T cells
NF-κB	nuclear factor κB
NHE	Na^+/H^+ exchanger
NK cell	natural killer cell
NKCC	Na^+-K-2Cl^- cotransporter
NLS	nuclear localization signal
NO	nitric oxide
OREBP	osmotic response element-binding protein

OS	osmotic stress
OSPHOS	oxidative phosphorylation
PI3K	phosphatidylinositol 3-kinase
PSGL	P-selectin glycoprotein ligand
RHR	Rel homology region
ROR	RAR-related orphan receptor
RT-qPCR	real-time quantitative polymerase chain reaction
RUNX	runt-related transcription factor
RVD	regulatory volume decrease
RVI	regulatory volume increase
SAP	SLAM-associated protein
SGK1	serum and glucocorticoid-regulated kinase 1
SLAM	signaling lymphocytic activation molecule
SLO	secondary lymphoid organ
SMIT	sodium/myo-inositol cotransporter
STAT	signal transducer and activator of transcription
TAD	transactivation domain
TauT	sodium and chloride-dependent taurine transporter
T-bet	T-box transcription factor TBX21
TCA	tricarboxylic acid cycle
TCR	T cell receptor for antigen
TF	transcription factor
TGF	transforming growth factor
Th cell	T helper cell
Thy	thymus cell antigen
TLR	Toll-like receptor
TNF	tumor necrosis factor
TonEBP	tonicity-responsive enhancer-binding protein
Treg cell	regulatory T cell
TSLP	thymic stromal lymphopoietin
UT-A	urea transporter-A
VEGFC	vascular endothelial growth factor-C
WT	wild-type

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INTRODUCTION

1. Adaptive immune responses

Immune responses are generated when invading agents enter the host, or certain cellular stress signals are present. The first line of defense is the innate immunity, which fights against the infection immediately. It activates the adaptive immune response, which is antigen-specific, and is driven by T and B lymphocytes. Each lymphocyte has a unique antigen receptor and the whole lymphocyte population can recognize specifically almost any invading agent. Adaptive immunity is slower than innate immunity, but highly specific and strong, and is also responsible for the immunological memory; a subset of T and B memory cells "remember" the antigens they have encountered before and provide enhanced protection against reinfection. This complex network requires a tight coordination of the different cell types and signals involved in both innate and adaptive immune response for efficient removal of the pathogen.

Each of the different cell types involved in innate and adaptive immunity in turn comprise various subsets with specific phenotypic and functional properties. This introduction focuses on T lymphocytes. T lymphocytes express the T cell receptor (TCR) that recognizes antigens presented by MHC molecules expressed by antigen-presenting cells and numerous other cells in the body. T cells are divided into cytotoxic T cells (or $CD8^+$ T cells) which kill infected or abnormal cells directly, and helper T cells ($CD4^+$ T cells) that regulate numerous immune cell types by secreting specific cytokines.

2. T cell differentiation

2.1. CD4$^+$ T cells

Morphologically indistinguishable from other lymphocytes, these cells express the CD4 molecule at the cell surface. They play a very important role in the coordination of immune responses: they are necessary for B cells to make antibodies, they stimulate CD8$^+$ T cell functions, they regulate innate lymphoid cell function, they enhance anti-pathogen responses, and are able to suppress or downregulate autoimmune reactions. Therefore, they orchestrate both innate and adaptive immune responses (Zhu & Paul 2008).

CD4$^+$ T cells differentiate from hematopoietic stem cells in the bone marrow and they acquire the mature phenotype in the thymus, where they undergo positive and negative selection and exit as CD4$^+$ naïve T cells to the periphery (Koch & Radtke 2011). When a naïve T cell encounters an antigen-presenting cell (APC) and recognizes the specific antigen that is presented by its major histocompatibility complex class II (MHCII), it will activate, grow, divide (clonal expansion) and differentiate into an effector (Th) or induced regulatory (iTreg, hereafter referred as Treg) CD4$^+$ T cell (**Figure I**). Depending on the strength of the interaction of the TCR with the MHCII, the cytokine milieu and other signals present in the environment, T cells will differentiate towards a different subset: Th1, Th2, Th17, Treg, Tfh, Th22 or Th9. These subsets are characterized by the cytokines they produce, the master transcription factor regulator they express and the function they develop (Zhu et al. 2010).

Depending on the stimulus they encounter, CD4$^+$ T cells can differentiate towards one or another subset. For instance, a bacterial infection will be controlled by Th1 cells, and a helminth infestation by Th2 cells. The deregulation of each subset would also give rise to a different type of disease. Th1 and Th17 subsets are related to autoimmune diseases while Th2 cell overreaction drives allergies.

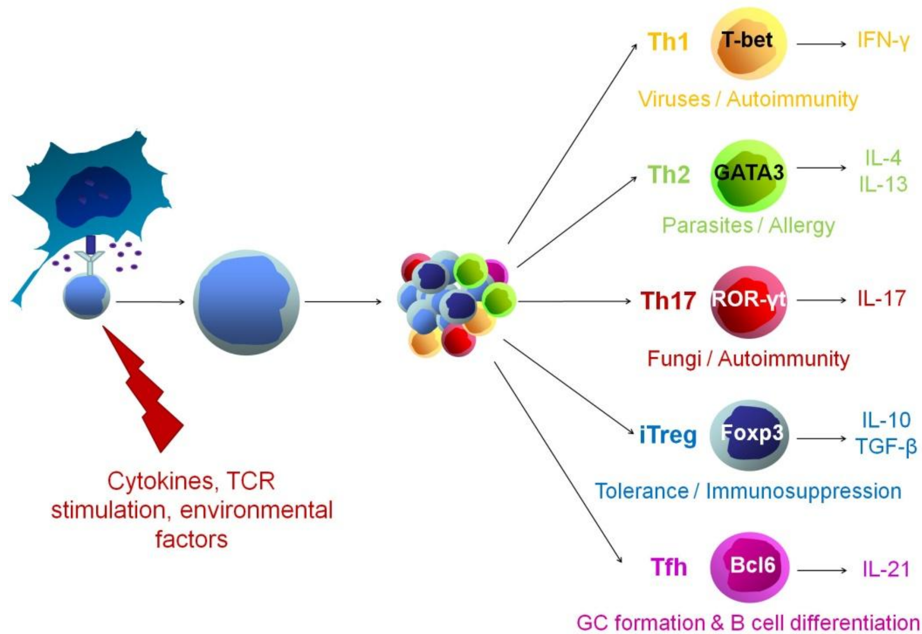


Figure I: T cell activation and differentiation. An antigen-presenting cell stimulates a naïve T cell, and depending on the environmental factors, the CD4$^+$ T cell grows, divides and differentiates into different subtypes, expressing specific transcription factors and cytokines for carrying out different immune-related functions. Their deregulation leads to different types of immunological problems.

2.2. Naïve T cells

These cells are defined by the expression on their surface of L-selectin (CD62L) molecule and the lack of the activation markers CD44 and CD25. Naïve T cells are considered quiescent cells because they do not undergo cell division, they have low metabolic rate (they produce energy slowly and have reduced ability to generate macromolecules), their chromatin is highly condensed and they have few organelles (Frauwirth & Thompson 2004; Rawlings et al. 2011). These cells must be actively stimulated in order to maintain homeostasis (Takada & Jameson 2009).

Naïve T cells are tightly regulated because it is fundamental to preserve their cell number, functional competence and clonal diversity for a proper immune

response. TCR engagement by self-peptide-MHC complexes and interleukin 7 receptor (IL-7R) stimulation by interleukin 7 (IL-7) promote naïve T cell homeostasis. Mature T cells continually exit the thymus, but cell numbers in the periphery must remain constant. Homeostasis requires a careful balance between cell division and lymphocyte survival. The mechanisms for maintaining the naïve T cell pool in the periphery constant, ensuring the diversity of the T cell pool, are not clear yet. Several hypotheses of T cell competition have been suggested. Some studies agree that T cells that have the same reactivity compete for the self-peptide-MHC complexes (intraclonal competition) (Troy & Shen 2003; Hataye et al. 2006), while others argue that the competition occurs between T cells of different reactivities (interclonal competition) (Hao et al. 2006). The latter study suggests that there is a positive selection towards the naïve cells that can bind to different self-peptide-MHC complexes (this would imply an advantage for more promiscuous T cells). A third model states that there is a competition for the self-peptide-MHC complex between the cells that have the same clonality, and that there is also competition for limited survival factors (Agenès et al. 2008). One of the most important survival cytokines for naïve T cell homeostasis is IL-7 (Hassan & Reen 1998; Vivien et al. 2001), which is involved in the activation of pro-survival factors in T cells such as B cell lymphoma-2 (Bcl2) (Akashi et al. 1997) as well as cell migration (Kerdiles et al. 2009).

Naïve T cells circulate between the lymph, the blood and secondary lymphoid organs (SLO). Self-peptide-MHC complexes are presented to naïve cells in the T cell zone of SLO, where they also receive the necessary survival factors. The C-C chemokine receptor 7 (CCR7) and the C-C chemokine ligand 19 (CCL19) and CCL21 are necessary for the recruitment of these cells in the T cell zones (Dai & Lakkis 2001; Link et al. 2007; Okada & Cyster 2007).

Besides being a key niche for homeostasis control, SLO are the place where the activation of naïve T cells occurs. During the immune response, APCs

capture and process the antigen and travel to the SLO where the antigens are presented by the MHCII to naïve T cells. When a T cell encounters its cognate antigen presented by the MHC, it gets activated (Banchereau & Steinman 1998).

2.3. T cell activation

For the proper activation of T cells, two stimuli are required: TCR complex recognition of antigen-MHCII complex from the APC and co-stimulatory molecule recognition. The TCR is formed by α/β subunits that recognize specifically the peptide-MHC complex, and by the signal transducing CD3 complex (formed by ε, γ, δ and ζ subunits). For signal transduction a co-receptor in the TCR complex is needed. The co-receptor of $CD4^+$ T cells is CD4 which also binds to the MHC-II of the APC (Acuto & Cantrell 2000).

An example of a co-stimulatory molecule is the CD28, which interacts with CD80 or CD82 receptors of the APC. Many co-stimulatory and co-inhibitory molecules that control the outcome of TCR signaling can be found on the surface of T cells. Their expression and function are context dependent (Chen & Flies 2013). Signaling only through the TCR results in anergy, a nonresponsive state in which T cells are intrinsically inactivated and therefore are refractory to be reestimulated (stimulated again) (Schwartz 2003).

For an optimal activating signaling both stimuli are needed. TCR/CD28 activates the linker of activated T cells signalosome (LAT signalosome) which propagates the signal through various signaling pathways: the Ca^{2+} and calcineurin branch (which involves NFATc proteins), the mitogen activated protein kinase (MAPK), and the nuclear factor κB (NF-κB). These pathways are critical for gene expression and T cell growth, proliferation and differentiation. Signals from the TCR also promote actin reorganization and activation of integrins for cell adhesion (Brownlie & Zamoyska 2013). TCR signaling strength is important for determining T cell fate, as will be discussed later.

The TCR also activates the phosphatidyl inositol-3-kinase (PI3K)/protein kinase B (AKT)/mammalian target of rapamycin (mTOR) signaling pathway. This pathway responds to cytokines, such as IL-2 and is required for T cell activation, being involved in cell cycle regulation and the metabolic switch from catabolism to anabolism of naïve T cells (Pearce 2010; Chappert & Schwartz 2010). Signaling by mTOR is also regulated by environmental cues such as nutrient and oxygen availability, which allows T cells to monitor not only signals from surface receptors but also nutritional variables in their microenvironment (Chappert & Schwartz 2010).

2.4. T cell subsets

Depending on the strength and length of TCR-antigen-MHCII presentation, the cytokine milieu and other environmental factors such as nutrient availability, naïve T cells may differentiate into different T helper lineages, including Th1, Th2, Th17, Tfh and iTreg. Each subset is determined by the set of transcription factor it expresses, a cytokine production pattern and a function. The $CD4^+$ T cell differentiation process is understood as a two-phase process; the first one is the TCR-driven induction phase in which key transcription factors are activated, and the second phase is cytokine driven, important to establish the T cell fate.

Originally, $CD4^+$ T cells were divided into two different fates designated by Mossman and Coffman (Mosmann et al. 1986): Th1 and Th2 cells. They were distinguished by the cytokines they produced. This dichotomy was then changed due to *in vivo* evidences of other types of effector T cells and thus, Th17 cells broke the Th1/Th2 paradigm. Nowadays the view about T helper cell differentiation has evolved. It is not seen as a non-return differentiation, it is a dynamic process. Depending on the microenvironment, cellular conditions or chromatin modifications T cells can change their gene expression profile (Zhou et al. 2009; Nakayamada et al. 2012; Reiner & Adams 2014).

2.4.1. Th1

Th1 cells mediate immune responses against intracellular pathogens and play a role in the progression of diverse autoimmune diseases. IL-12 and interferon γ (IFN-γ) induce polarization towards Th1 function, through the activation of the signal transducer and activator of transcription 4 (STAT4) and STAT1 respectively. The master regulator that governs Th1 responses is T box transcription factor Tbx21 (T-bet, encoded by the *Tbx21* gene). The signature cytokines are IFN-γ, tumor necrosis factor-α (TNF-α) and lymphotoxin-α (LT-α). IL-2 is also produced by these cells, but it is not a Th1-exclusive cytokine. IFN-γ is the cytokine that facilitates the development of Th1 cells, generating a positive feedback loop (Zhu & Paul 2008) (**Figure II**).

APCs produce IL-12, but the first wave of T-bet induction is not IL-12 dependent, as TCR activation inhibits the expression of IL-12Rβ2 subunit. T-bet expression is firstly induced by TCR and IFN-γ produced by natural killer cells (NK cells) and T cells, via IFN-γ receptor signaling and STAT1 (Afkarian et al. 2002; Schulz et al. 2009). T-bet binds to the *Ifng* locus and induces the expression of IFN-γ (Zhu et al. 2012), generating a positive feedback loop through STAT-1 (**Figure II**).

When the TCR stimulation stops, IL-2 (that is produced by T cells due to TCR ligation) induces IL-12Rβ expression through IL-2R-STAT5, and T cells can uptake the extracellular IL-12 (Liao et al. 2011; Schulz et al. 2009). IL-12 induces the second wave of T-bet expression via STAT4, which is needed for Th1 fate stabilization (Thieu et al. 2008). T-bet together with STAT4 induces IFN-γ production (Zhu & Paul 2010) (**Figure II**). STAT4 is also necessary for upregulating IL-12Rβ2 and IL-18R. IL-18 can synergize with IL-12 in inducing IFN-γ (Robinson et al. 1997; Yang et al. 2001). T-bet directly activates the expression of around half of Th1-characteristic genes: cytokines as IFN-γ, LT-α and TNF-α, chemokines as CCL3 and CCL4, and receptors as IL-12Rβ2, CXC chemokine receptor 3 (CXCR3) and CCR5 that are necessary

for the function and migration of Th1 cells and the recruitment of other immune cells to the inflammation sites (Lord et al. 2005; Thieu et al. 2008; Jenner et al. 2009; Zhu et al. 2012). Although not exclusive, CXCR3 and CCR5 receptors are preferentially expressed on Th1 cells (Bromley et al. 2008; Loetscher et al. 1998).

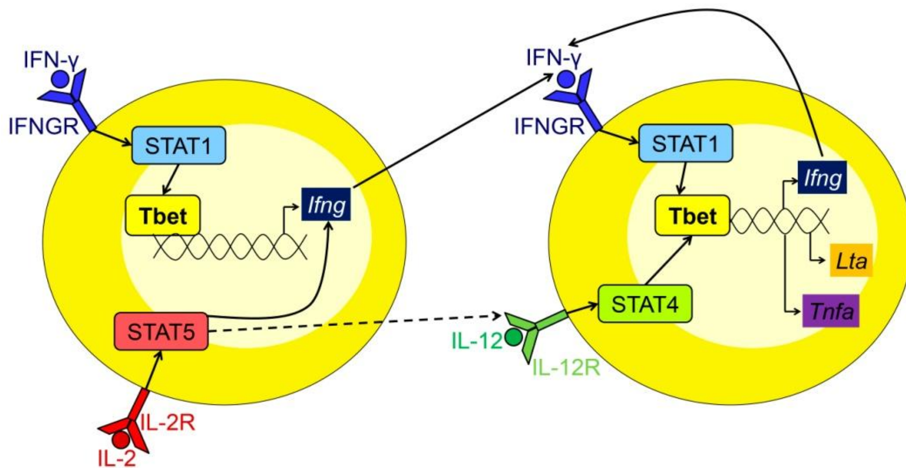


Figure II: Th1 cell differentiation. Schematic representation of the cytokine and transcription factor networks involved in Th1 cell fate initiation and stabilization.

Suppressor of cytokine signaling 1 (SOCS1) is a key negative regulator of the IFN-γ and STAT1 signaling pathway (Alexander et al. 1999). The removal of this protein in $CD4^+$ T cells augments the generation of Th1 (Eyles et al. 2002). SOCS3 inhibits STAT4 signaling by binding to the IL-12Rβ2 chain, the STAT4 docking site (Yamamoto et al. 2003).

2.4.2. Th2

Th2 cells mediate host defense against extracellular parasites and are also responsible for the development of allergic diseases such as asthma (Zhu et al. 2010). Th2 fate is promoted by IL-4, which is the positive feedback cytokine. These cells produce IL-4, IL-5, IL-9, IL-13 and IL-25 (also known as IL-17E). Th2 cells regulate immunoglobulin (Ig) class switching to IgE in B cells and promote alternative macrophage activation which is induced by IL-4

(Finkelman et al. 1988; Kopf et al. 1993; Mosser & Edwards 2008). IL-5 recruits eosinophils, IL-9 attracts mast cells and acts on epithelial cells for producing mucin during allergic reactions (Longphre et al. 1999). Th2 cells can act on several other cells, such as smooth muscle cells or macrophages, through IL-13 and IL-4 (Paul & Zhu 2010). In T cells, IL-4 induces the master regulator of Th2 fate, GATA binding protein 3 (GATA3) through the activation of STAT6 (Kurata et al. 1999; Zhu et al. 2001). IL-10, IL-2 and IL-21 can be also produced by Th2, although they are not exclusive of this T cell fate.

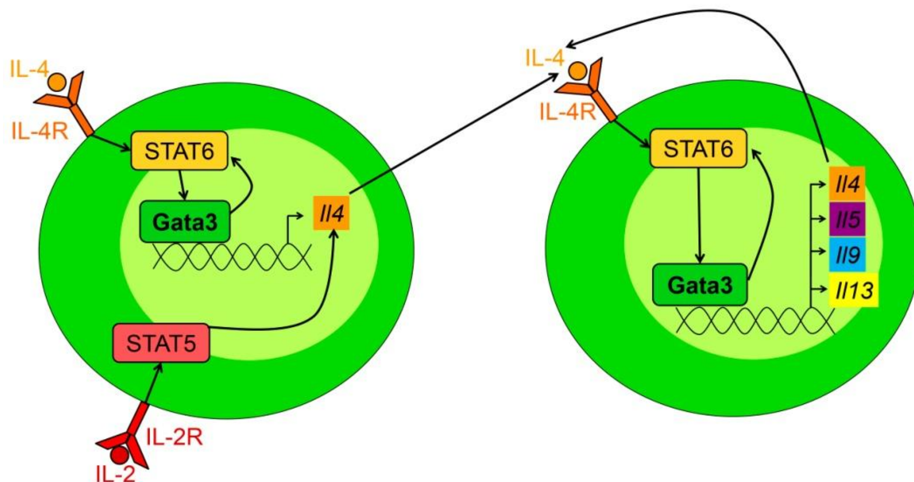


Figure III: Th2 cell differentiation. Schematic representation of the cytokine and transcription factor networks involved in Th2 cell fate initiation and stabilization.

TCR activation promotes IL-4 and GATA3 expression, and GATA3 induces many Th2 genes: IL-4, IL-5, IL-13 and IL-24 among others (Horiuchi et al. 2011). IL-4 activates both Janus kinase-1 (JAK1) and JAK3 and the transcription factor STAT6 which directly controls the expression of GATA3. This generates a positive feedback loop to maintain Th2 gene expression active (Knosp & Johnston 2012). IL-2 is a key cytokine for Th2 polarization; its neutralization generates the inhibition of IL-4 production (Cote-Sierra et al. 2004) and the overexpression of STAT5 bypasses IL-4 requirements (Zhu et

al. 2003) (**Figure III**). In Th2 responses, c-Maf is upregulated and induces IL-4 production (Kim et al. 1999). Other transcription factors required for these responses are interferon regulatory factor-4 (IRF-4) and growth factor independent-1 (Gfi-1) (Lohoff et al. 2002; Zhu et al. 2002).

IL-4 has extensively been considered the key cytokine for Th2 differentiation, a concept supported by *in vitro* models and some *in vivo* models of Th2-associated diseases. However, as more *in vivo* Th2 models have been developed, it has been shown that the STAT6-IL-4 pathway is dispensable for Th2 cell generation (Jankovic et al. 2000), and other Th2-polarizing cytokines can be found *in vivo*, such as the thymic stromal lymphopoietin (TSLP), IL-25 and IL-33.

TSLP is produced by epithelial cells, mast cells and basophils. It induces Th2-like responses through STAT5 activation (Isaksen et al. 1999). TSLP deficient mice do not develop allergic responses to inhaled antigens, have strong Th1 responses (Al-Shami et al. 2005), and Th2 cells from these mice produce less of Th2-associated cytokines and present an impaired allergic skin inflammation (He et al. 2008). IL-33 together with TSLP is able to induce TCR-independent IL-13 production (Miller 2011).

Administration of IL-25 to naïve T cells induces IL-4, IL-5 and IL-13 production and Th2 functions (Paul & Zhu 2010). Mice that lack IL-25 fail to expel helminths, correlating with low Th2 and enhanced Th1 responses. Blocking IFN-γ and IL-12 restores the deregulation of IL-25-deficient mice (Owyang et al. 2006), which indicates that IL-25 has a role in regulating the balance of Th1-Th2 responses. Th2 cell-associated receptors are CCR4 that promotes the recruitment of cells to the skin and lungs, and CCR8, for trafficking to skin (Griffith et al. 2014).

The Th2 fate is also regulated by SOCS proteins; SOCS1 and SOCS5 inhibit IL-4 signaling (Seki et al. 2002; Losman et al. 1999). SOCS2 also negatively regulates Th2 fate, inhibiting IL-2 signaling (Knosp et al. 2011). SOCS3

favors Th2 fate preventing Th1 and Th17 cell development (Knosp & Johnston 2012).

2.4.3. Th17

Th17 cells mediate immune responses against extracellular pathogens, bacteria and fungi, particularly at mucosal surfaces such as the intestine or airways. They participate in autoimmune diseases, as multiple sclerosis, psoriasis, inflammatory bowel disease and rheumatoid arthritis. Th17 cell produce IL-17A, IL-17F, IL-21 and IL-22, and are induce by the synergistic action of several cytokines, including IL-1β, IL-6, IL-21, IL-23 and transforming growth factor-β (TGF-β). The RAR-related orphan receptor-γt (ROR-γt) is the master regulator of this subset, which is induced by STAT3. Th17 cytokines induce recruitment of neutrophils (Wu et al. 2007), enhancement of bactericidal properties of macrophages (Higgins et al. 2006) and antimicrobial responses in mucosal surfaces (Aujla et al. 2008).

This subset of helper T cells was discovered because an IL-23-dependent pathogenic T cell subtype which produced mainly IL-17 and was different from Th1 and Th2 cells was found *in vivo* (Cua et al. 2003; Langrish et al. 2005; Harrington et al. 2005; Park et al. 2005). The following year, three independent groups discovered that this Th17 cells could be generated *in vitro* with IL-6 and TGF-β stimulation (Veldhoen et al. 2006; Mangan et al. 2006; Bettelli et al. 2006). IL-6 activates STAT3, which induces the transcription of several Th17 genes such as *Rorc*, *Il17a* and *Il23r* (Durant et al. 2010) as well as suppresses Foxp3 expression, which is activated by TGF-β (Bettelli et al. 2006) (**Figure IV**). Foxp3 is the master regulator of Treg cells, so STAT3 blocks this fate. Mice that lack IL-6 have reduced but not absent numbers of Th17 cells (Korn et al. 2007), suggesting that *in vivo* there are different combinations of cytokines that can promote Th17 cells, although IL-6 is very important.

Differentiation of Th17 cells was initially thought to be TGF-β-dependent. Later on, there appeared some studies that claimed TGF-β to be dispensable for Th17 generation and others that stated that TGF-β was essential for T cell differentiation in humans (Acosta-Rodriguez et al. 2007; Volpe et al. 2008; Yang et al. 2008). The discrepancies could be due to different ways of obtaining and culturing $CD4^+$ T cells. There are several hypotheses about the exact role of TGF-β in T cells; it could be a Th17 fate promoter or an inhibitor of Th1 and Th2 polarizations. Using mice that lack STAT6 and T-bet, only IL-6 was required for full Th17 phenotype (Das et al. 2009), which suggests that TGF-β could act as a negative regulator of Th1 and Th2 fates in Th17 differentiation (Gaffen et al. 2014).

The initiation phase of Th17 differentiation is driven by IL-6 and TGF-β, and the cytokine responsible for the positive feedback loop for this fate is IL-21. It is produced by T cells in response to IL-6 and also by NK cells, binds to its receptor (formed by IL-21R and the γc chain) and signals through STAT3 (Nurieva et al. 2007; Korn et al. 2007). IL-21 amplifies an autocrine loop to induce more IL-21 and IL-23R. Both IL-21 and IL-23 with TGF-β can induce IL-17 expression independently of IL-6 (Zhou et al. 2007) (**Figure IV**). IL-1β also promotes Th17 generation. It binds to IL-1R, which is induced by IL-6 in Th17 cells, and activates IRF-4 to promote ROR-γt expression (Chung et al. 2009).

When T cells are cultured under TGF-β and IL-6 alone they are not as pathogenic as the Th17 proinflammatory cells that are found *in vivo*. For developing this pathogenicity IL-23 is required. In fact, Th17 cells can be divided into two different groups regarding function and cytokine profile: a proinflammatory Th17 subtype, close to Th1 cells and producing IL-17, IL-22, GM-CSF and IFN-γ (Langrish et al. 2005; Zheng et al. 2007), and a more anti-inflammatory group, related to Treg cells, expressing IL-10 and IL-17 and involved in mucosal defense and barrier tissue integrity (McGeachy et al. 2007; Esplugues et al. 2011). IL-10-producing Th17 cells express higher

levels of the Th17 transcription factors aryl hydrocarbon receptor (AhR) and c-Maf (Lee et al. 2012) than pathogenic Th17 cells.

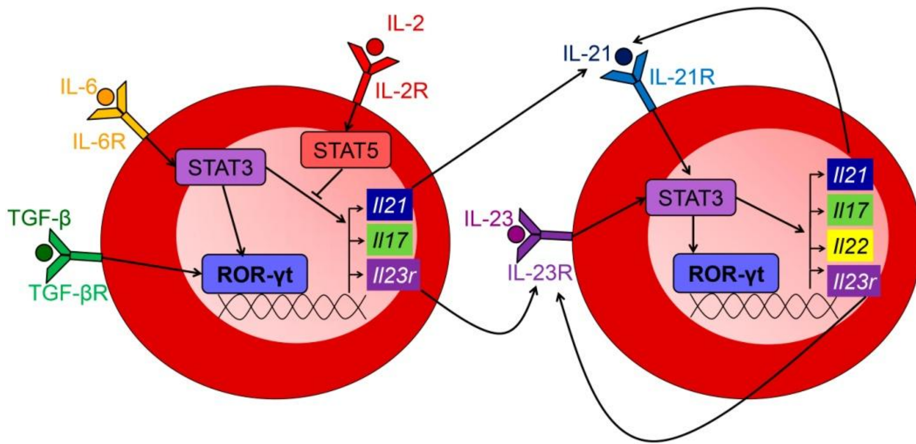


Figure IV: Th17 cell differentiation. Schematic representation of the main cytokine and transcription factor networks involved in Th17 cell fate initiation and stabilization.

A report showed that pathogenic Th17 cells could be obtained in the presence of IL-6, IL-23 and IL-1β in a TGF-β-independent manner (Ghoreschi et al. 2010). IL-23 promotes the maintenance of ROR-γt and IL-17 expression, as well as inducing GM-CSF, IFN-γ and IL-22 (Codarri et al. 2011; El-Behi et al. 2011; Ghoreschi et al. 2010; Hirota et al. 2011), so it generates a positive feedback loop by upregulating the IL-23R-STAT3 axis (Ghoreschi et al. 2010). Moreover, IL-23 downregulates some genes that repress the Th17 fate as IL-2 and IL-27 (Diveu et al. 2009; Laurence et al. 2007). The activation of STAT5 by IL-2 inhibits the development of Th17 cells. It binds to the promoter of *Il17* and antagonizes STAT3 transcriptional activity (Yang et al. 2011). The molecular signature of IL-23-induced pathogenic Th17 cells is different from that of Th17 cells induced by IL-6 plus TGF-β (Lee et al. 2012).

Although the master regulator of Th17 fate is ROR-γ, it cooperates with other transcription factors: STAT3, IRF-4, AhR, c-Maf, runt-related transcription

factor-1 (RUNX1) and basic leucine zipper transcription factor ATF-like (BATF). STAT3 activation through IL-6, IL-21 or IL-23 directly induces several Th17-genes: *Il17a*, *Il17f*, *Il23r*, *Rorc*, *Batf* and *Irf4* (Durant et al. 2010). BATF also induces the expression of *Il17*, *Il21* and *Il22* (Schraml et al. 2009). Recent studies have shown that BATF and IRF-4 govern the accessibility to chromatin of Th17, thus they would be initiating factors (Li et al. 2012; Ciofani et al. 2012). In fact, a chromatin immunoprecipitation and sequencing (ChIP-Seq) analysis showed a big overlap between BATF and IRF-4 promoter occupancy not only in polarized Th17 cells, but also TCR-stimulated Th0 cells (Ciofani et al. 2012).

SOCS3 protein is a STAT3 inhibitor, it inhibits IL-6 and IL-23 signaling in $CD4^+$ T cells (Chen et al. 2006). SOCS1 and SOCS2 deficiency have similar effects in IL-17 expression. SOCS2 deficient T cells have impaired IL-17 secretion and elevated SOCS3 levels (Knosp et al. 2011). SOCS1 promotes Th17 differentiation through TGF-β signaling and regulating SOCS3 levels (Tanaka et al. 2008).

2.4.4. Treg

There are two major types of regulatory T cells that are distinguished regarding their origin: nTreg (natural Tregs) and iTregs (induced Tregs). The first ones differentiate in the thymus and iTreg cells differentiate from naïve T cells upon antigen presentation in the periphery. The iTreg subset (hereafter referred as Treg) is discussed next in the context of T cell subset polarization after antigen presentation.

Treg cells mediate the suppression of the immune response, being the negative regulators of immune inflammation. These cells are important for the control of autoimmunity, inflammatory disorders, allergy, cancer, acute and chronic infection and metabolic inflammation (Josefowicz et al. 2012). TGF-β induces Treg cell polarization upon TCR activation, together with IL-2. The master regulator of this fate is forkhead box P3 (Foxp3) and they produce IL-

10, IL-35 and TGF-β (Zhu et al. 2010) (**Figure V**). Treg cells have several mechanisms for suppressing immune responses; the suppression by inhibitory cytokines, which consists on secreting IL-10, IL-35 or TGF-β, suppression by cytolysis, inducing granzymes and perforins to mediate the cytolysis of different cells, suppression by metabolic disruption (Vignali et al. 2008), and suppression by targeting dendritic cells (DC) through modulating the maturation or function of dendritic cells via the interaction of CD223 of the Treg cell with the MHC-II of the DC, or the induction of the immunosuppressive molecule indoleamine 2,3-dioxygenase (IDO) as a result of the interaction between CD80/CD86 of the DCs and the Treg upregulated receptor cytolytic T lymphocyte-associated antigen-4 (CTLA-4) (Onodera et al. 2009). CTLA-4 also directly downregulates the expression of CD80 and CD86 receptors on DCs (Wing et al. 2008) for suppressing the antigen presentation capacity of APCs.

Upon TCR stimulation Treg cells upregulate the CD25 (IL-2Rα) subunit of the high affinity receptor for IL-2. As they express high levels of CD25, they consume high quantities of IL-2, and so, they reduce available IL-2 for other T cells (Boyman & Sprent 2012). The suppression by metabolic disruption consists on this effect, leaving Th1 and Th2 cells without their growth factor. In fact, regulatory T cells are highly dependent on IL-2 produced by other cells (mainly effector $CD4^+$ T cells) for their induction, survival and Foxp3 expression (Rubtsov et al. 2010; Chen et al. 2011), because they are not able to produce significant amounts of IL-2 by themselves (Thornton & Shevach 1998). IL-2 signaling is also important because it activates STAT5 which induces Foxp3 directly, and it also inhibits Th17 cell fate, because, STAT5 competes with STAT3 for the same locus on *Il17a* gene, thus IL-2 inhibits IL-17A expression (Yang et al. 2011) (**Figure V**).

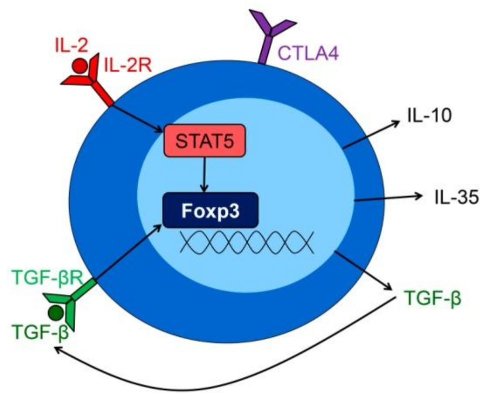


Figure V: Schematic representation of a Treg cell. Treg cells are induced by IL-2 and TGF-β which activate Foxp3. These cells express the receptor CTLA-4 and produce IL-10, IL-35 and TGF-β, among other cytokines.

TGF-β signaling induces the differentiation towards Treg by different mechanisms. It promotes the Smad-mediated expression of Foxp3 through the binding of Smad to *Foxp3*-conserved Smad and NFAT element-1 (CNS1) (Zheng et al. 2010) and it opposes the inactivation of Foxp3 driven by the DNA methyltransferase-1 (Dnmt1) (Josefowicz et al. 2009).

The lack of Foxp3 in human and mice results in a severe lymphoproliferative disorder. The expression levels of Foxp3 are critical for its suppressive function, experimental reduction of Foxp3 amounts resulting in a decreased suppressive activity (Wan & Flavell 2007). Furthermore, this expression has to be maintained because the deletion of Foxp3 in mature Treg cells induces a loss of Treg cell characteristics (suppressor function and cell surface markers) and effector T cell properties, as production of IFN-γ, IL-4 and IL-17, appearance (Williams & Rudensky 2007). Altogether, these experiments show that Foxp3 is essential for Treg cell differentiation and function.

Strong TCR signaling and suboptimal costimulation (increased CTLA-4 signaling and thus a decreased CD28 signaling) are required for proper Treg phenotype acquisition (Benson et al. 2007; Josefowicz et al. 2012).

Intriguingly, Treg cells have different TCR specificities depending on the tissue or SLO where are found. For example, Treg cells that are close to the gut-associated lymphoid tissue (GALT) recognize antigens derived from commensal microbiota (Lathrop et al. 2011). This means that the antigen specificity Treg cells have is critical for their function in the niche (Josefowicz et al. 2012).

SOCS1 has a positive effect on Treg cell generation, as it is required for proper Treg suppressor activity (Lu et al. 2010). SOCS2 is highly induced in Treg cells and it is required for the stable expression of Foxp3 in vitro and in vivo (Knosp et al. 2013).

To sum up, sustained expression of Foxp3 in Treg cells depends on a strong TCR signaling with low costimulation and the effect of different cytokines as TGF-β and IL-2. These conditions are found in particular niches as mucosal surfaces.

2.4.5. Tfh

A more recently discovered subset of T cells is the follicular helper T cell subset. These cells are required for germinal center formation, affinity maturation and the development of antibodies and memory B cells. Tfh differentiation is a multisignal process with different mechanisms involved. Upon TCR activation by a DC in the lymph node, if the T cell expresses CXCR5 receptor during the first divisions, these early Tfh cells will migrate to the border of the B cell follicle and continue with Tfh cell differentiation. This early phase is regulated by IL-6, IL-21 and the inducible T cell co-stimulator (ICOS). IL-6 stimulation through IL-6R transiently induces Bcl6 expression (Nurieva et al. 2009), which is required for CXCR5 expression (Choi et al. 2011). ICOS is required for Tfh cell differentiation and migration (Xu et al. 2013) (**Figure VI**).

The second level of differentiation occurs when the T cell interacts with antigen-specific B cells in the follicle. For reaching the zones of the spleen where T and B cells can interact, T cells upregulate CXCR5 and downregulate CCR7 and P-selectin glycoprotein ligand-1 (PSGL-1) whose ligands are in the T cell zone. B cells are completely required for Tfh cell differentiation (Crotty 2014). These B cells present antigen to Tfh cells and serve as a source of ICOS ligand (ICOSL) for T cell migration (Choi et al. 2011; Haynes et al. 2007; Nurieva et al. 2008) (**Figure VI**).

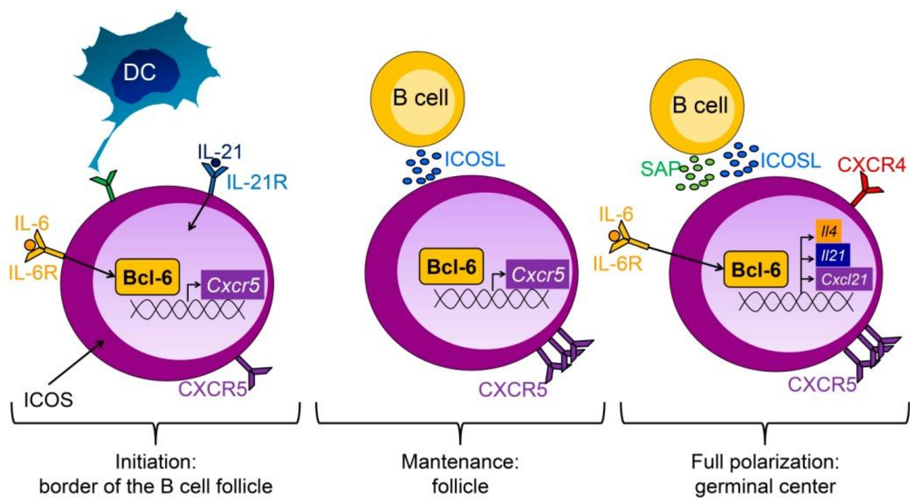


Figure VI. Tfh cell differentiation. Schematic representation of the initiation, maintenance and polarization of Tfh cells regarding the main cytokine and transcription factor networks involved and the stimuli required for full differentiation.

The full differentiation of Tfh cells occurs in the germinal center (GC). Adhesion molecules are very important in GC Tfh cells for regulating the interactions with B cells and their localization in the GC. There are many molecules involved in this process that are completely required for proper Tfh function, such as lymphocyte activation molecule (SLAM) family receptors expressed on Tfh or B cells from the GC. SLAM-associated protein (SAP) is necessary for signal transduction between T and B cells, and thus for B and Tfh cell differentiation in the GC. At this site Tfh cells secrete CXCL13, IL-

21 and IL-4 and express CXCR5 and CXCR4, receptors required for staying in the GC, close to B cells. Fully differentiated Tfh cells can exit the GC and enter a different GC, temporally reside in the adjacent B cell follicle of exit a GC (Crotty 2014) (**Figure VI**).

Bcl6 is the master regulator of this subset, and c-Maf and STAT3 are also required for Tfh differentiation. Bcl6 is sufficient to block Th1, Th2, Treg and Th17 fates, through the downregulation of the receptors necessary for these subsets, and also inhibiting some of the master regulators. The amounts of Bcl6 are lower in the peripheral Tfh cells, than GC Tfh cells (Nurieva et al. 2009; Crotty 2014).

2.4.6. Th22

Th22 cells mediate host defense at barrier sites such as skin and respiratory and intestinal tracts. Th22 cells are induced by cytokines signaling through STAT3; IL6, IL-21 and IL-23, which are sufficient to induce IL-22 expression. However, IL-23 alone cannot induce IL-22 in naïve T cells because they do not express the IL-23R. TCR stimulation in the presence of IL-6 activates STAT3 and induces the expression of IL-23R. STAT3 alone can induce IL-22, but not ROR-γt. TGF-β potently inhibits IL-22 expression through c-Maf (Rutz et al. 2013). IL-22 expression can be rescued in the presence of AhR ligands such as retinoic acid. The transcription factor AhR is a Th22 fate regulator (Rutz et al. 2013).

2.4.7. Th9

Although their function has not been fully elucidated, they play a role in allergic and autoimmune inflammation and in anti-tumor immunity. IL-4 and TGF-β induce the Th9 cell fate, through the activation of STAT6, IRF-4, GATA3 and PU.1 transcription factors, and produce IL-9. These cells have also been shown to express IL-10 and IL-21 (Schmitt et al. 2014).

2.5. Cross-regulation between subsets

T cell differentiation involves not only the acquisition of a given polarized function, but also the active inhibition of main cytokines and transcription factors of the rest of the subsets. IFN-γ and IL-4 are mutually suppressive, as shown in early studies of T cell differentiation (Th1 fate needs to block IL-4 production and Th2 fate also needs IFN-γ to be absent (Mosmann et al. 1986). IFN-γ blocks IL-23R expression, and thus Th17 stabilization. IL-4 also inhibits Th17 fate (Harrington et al. 2005). In Th1-inducing conditions, T-bet has been shown to bind to the *Gata3* locus and promote repressive chromatin modifications and thus inhibition of GATA3 expression (Zhu et al. 2012). Furthermore, T-bet interacts physically with GATA3 and redistributes GATA3 away from Th2 genes (Hwang, Szabo, et al. 2005). T-bet, together with RUNX3, also binds to the *Il4* silencer preventing IL-4 expression (Djuretic et al. 2007). T-bet can limit IL-2 production through RelA, and as IL-2 is needed for upregulating IL-4Rα, it inhibits also Th2 signaling (Hwang, Hong, et al. 2005). T-bet also inhibits the development of the Th17 lineage, blocking RUNX1-mediated activation of ROR-γ (Lazarevic et al. 2011).

GATA3 suppresses Th1 development by downregulating the expression of STAT4, which is required for IL-12 signaling (Usui et al. 2003). It also induces repressive histone modifications in *Ifng* and *Tbx21* genes (Wei et al. 2011). GATA3 inhibits RUNX3 and as a consequence, it blocks IFN-γ production (Yagi et al. 2010). IL-4 suppresses Th1, Treg and Th17 responses through the upregulation of Gfi1 which inhibits the expression among others of IFN-γ or IL-17 (Zhu et al. 2006; Zhu et al. 2009).

TGF-β suppresses Th1 and Th2 responses, in part by repressing STAT4, T-bet and GATA3 (Li et al. 2006). Th17 and Treg cells share the requirement of TGF-β for their differentiation. High concentrations of this cytokine inhibit Th17-related gene expression of IL-23R, IL-22 or IL-17. STAT3 and STAT5 transcription factors, required for Th17 and Treg fate respectively, compete

for binding to the promoter of IL-17 (Yang et al. 2011), so that IL-2-activated STAT5 inhibits IL-17 production (Liao et al. 2011).

Foxp3 represses production of the proinflammatory cytokines IL-2, TNF-α, IFN-γ and IL-4 (Williams & Rudensky 2007). It can also interact directly with ROR-γt and inhibit its function (Zhou et al. 2008). Moreover, Foxp3 can inhibit ROR-γt expression and RUNX1 mediated IL-17 expression (Zhang et al. 2008). TGF-β-induced Foxp3 expression is inhibited in part, but not completely, by IL-6, IL-21 and IL-23, as shown by the existence of IL-17^{+} Foxp3^{+} cells (Nurieva et al. 2007).

Bcl6 is known to be a negative inhibitor of Th1, Th2, Th17 and the Treg fate. It reduces the expression of IFN-γ, IL-4, IL-17 or T-bet (Nurieva et al. 2009; Mondal et al. 2010).

Other gene products that are not master regulators or specific cytokines can be found to inhibit different fates. For example TNF receptor superfamily member 6 is expressed in Th17 cells and inhibits the expression of IFN-γ, Mina (a chromatin regulator from the Jumonji C family) promotes Th17 fate, inhibiting Foxp3 program (Yosef et al. 2013). Gfi has shown to be required in the context of Th2 differentiation program, for inhibiting Th17 and Treg fates (Zhu et al. 2009).

2.6. TCR stimulation strength and T cell fate

T cell differentiation is not only determined by the concentration of different cytokines in the environment during and after antigen presentation. There are other important factors, such as the strength of the T cell stimulation, which is determined by the dose of antigen, costimulatory interactions and the avidity and affinity of the TCR to the peptide-MHCII complex (O'Garra et al. 2011) (**Figure VII**).

High antigen dose favors the polarization towards Th1 subset while weak signaling favors Th2 differentiation (Constant et al. 1995). High antigen doses

with sustained antigen signaling lead to the induction of Th1 cells that produce IFN-γ independently of IL-12. Low antigen doses result in a weak and transient extracellular signal-regulated kinase (ERK) activation and cells rapidly produce GATA3 and IL-4 (Yamane et al. 2005). Mutations on genes encoding TCR signaling molecules that reduce the TCR activation strength are related to Th2-related diseases (Aguado et al. 2002) which goes in line with the observation that weak TCR signaling favors Th2 cell fate.

TCR costimulation is also important for the T cell fate. The interaction between the CD28 molecule of the T cell with the B7 protein that is expressed in the surface of the APCs is required for Th1 differentiation (what provides a stronger TCR signaling), but not for the Th2 fate (Tao et al. 1997). Moreover, CD40-CD40L interaction has been shown to be important for the cell fate in a similar way. A strong TCR stimulation induces an upregulation of CD40L, thus promoting Th1 cell development, whereas a weak stimulation fails to induce CD40L and favors Th2 cell polarization (Ruedl et al. 2000). A high TCR stimulation strength and the CD40-CD40L interaction have also been shown to be important for Th17 cell development (Iezzi et al. 2009).

Foxp3 is more efficiently induced upon stimulation by a low dose of a high affinity antigen (Gottschalk et al. 2010). CTLA-4 and weak CD28 signaling are necessary for Treg induction (Zheng et al. 2006; Josefowicz et al. 2012). Thus, a high affinity TCR signaling with a suboptimal costimulation (strong CTLA-4 and weak CD28 signaling) favors Treg cell generation.

A recent study has shown a close correspondence between the strength of the TCR signaling and Th1 or Th2 cell fates. It shows that antigen concentration is more relevant than adjuvant or pathogen-induced cytokines for the polarization. Adjuvants influence T cell fate through costimulation on the TCR signaling and the strength of the TCR stimulation, and costimulatory proteins control the expression of cytokine receptors, which are needed for cytokine uptake and thus, polarization (van Panhuys et al. 2014).

2.7. Epigenetic control on T cell fate

T cell polarization is not determined by a single transcription factor (TF). A highly coordinated network of TFs binds to the regulatory elements of genes that encode cytokines and TFs. In addition to expressing subset-specific cytokines, lineage-inappropriate cytokine genes must be silenced. Epigenetic programs help to coordinate all the lineage requirements, and facilitate a heritable and stable program of gene expression, in which a potential to change is also ensured.

In brief, there are different ways of affecting gene expression without altering the DNA sequence: histone tail modifications (including acetylation or methylation), DNA methylation, nucleosome compaction or chromosome conformation. Histone acetylation as well as methylation of the lysine 4 of the histone 3 (H3K4me), and the trimethylation of the H3K36 are generally involved with active transcription of chromatin. Trimethylation of H3K9 and H3K27 are related to silenced chromatin. DNA methylation occurs in the cytosine residues of CpG islands and also other non-CG contexts and it is associated with promoters and actively transcribed regions. Most genomic DNA is tightly compacted in nucleosomes, and it is related to non transcribed genes. The most transcribed regions have reduced nucleosome occupancy over the promoters. All these elements act in cis (the regulatory regions are in the same chromosome as the gene regulated), but there is an epigenetic mechanism that can be acting in cis or trans: the chromosome conformation. Chromatin looping permits increasing the number of regulatory elements, because regions that are not close in the same chromosome can regulate loci that are physically (in a tridimensional structure) close (Kanno et al. 2012).

Experiments with the DNA methylation inhibitor 5' azacytidine revealed that epigenetic modifications played an important role in T cell cytokine expression, as it caused a constitutive production of IL-2 and IFN-γ (Ballas 1984; Young et al. 1994). Deletion of Dnmt1 enhanced IL-4 transcription in

CD4 T cells (Makar et al. 2003). There is increasing evidence of the important roles that epigenetic modifications have in T cell differentiation. Histone acetylation and methylation, DNA methylation or nucleosome compaction have been reported to be necessary for proper T cell function (Deaton et al. 2011; Valouev et al. 2011; Bettini et al. 2012; Boucheron et al. 2014).

Naïve CD4$^+$ T cells express low levels of cytokines and transcription factors, the chromatin of these cells is condensed, and loci for T cell polarized transcription factors and genes is inactive or in a poised state (with activation and repressive marks together). These marks include high levels of DNA methylation, poised or repressive histone modifications, and more compact chromatin. When cells are activated, there is an increase of permissive histone modifications and loss of repressive histone modifications and DNA demethylation (Lee & Rao 2004; Zhang & Boothby 2006; Baguet & Bix 2004; Tripathi & Lahesmaa 2014; Rawlings et al. 2011).

High throughput technologies have facilitated the analysis of several epigenetic marks simultaneously. An article showing genome wide maps of H3K4me3 and H3K27me3 in naïve CD4 T$^+$ cells and the different T cell subsets (Th1, Th2, Th17 and Treg) revealed that, in accordance with gene expression profiles for each lineage, the subset-specific cytokines have active marks while the rest of the cytokines are silenced. However, the master regulators of the different fates have bivalent poised marks (accessible and repressive marks together), in the conditions which are not expressed (Wei et al. 2009), for example, *Tbx21* gene locus has both H3K4me3 and H3K27me3 modifications in cells differentiated towards Th2. The master regulators, although not being expressed in the different fates, are not silenced. This gives evidence of T cell plasticity.

Under differentiation conditions that are not specific for their fate, there are some subset-specific cytokine genes that present a poised state in their chromatin. In a Th17 differentiation context, *Ifng* locus has bivalent

modifications. Adding IL-12 to the Th17-differentiating cytokines induces additional modifications for high expression of IFN-γ (Mukasa et al. 2010).

Global chromatin state analysis of Th1 and Th2 showed that around 30% of the enhancers of Th1 and Th2 subset specific genes were active at 72 hours and at 7 days of differentiation, and 99% of Th2 and 76% of Th1 enhancers that were active at day 3 continued active at day 7. This suggests that epigenetic status is established before lineage commitment. The analysis of the sequences of the enhancers of Th1 and Th2 fate revealed that Th1-specific enhancers were enriched with binding sites for Th1 TFs as STAT4 or STAT1. The specific enhancers at Th2 fate were enriched in binding sites for STAT6, GATA3 or Gfi-1 (Hawkins et al. 2013; Tripathi & Lahesmaa 2014).

STAT4 and STAT6 are known to be key TF for Th1 and Th2 fate respectively. An analysis of active and repressive histone modifications (H3K4me3, H3K36me3 and H3K27me3) in STAT4 and STAT6 wild-type and deficient T cells revealed that these TFs modified the histone marks on the 25% of the genes that were bound. While STAT4 promoted active epigenetic marks, STAT6 antagonized repressive marks (Wei et al. 2010; Kanno et al. 2012).

iTreg and nTreg have different epigenetic modifications of the *Foxp3*, *Rorc* and *Il17a* loci. In both cases, *Il17a* has repressive marks (H3K27me3). However, the *Rorc* locus in iTregs has active histone marks (H3K4me3) while in nTregs it has repressive marks (Wei et al. 2009). Regarding the methylation of DNA, the *Foxp3* locus is methylated in naïve and iTreg cells while it is demethylated in nTregs (Baron et al. 2007). Furthermore, it has been established that Foxp3 itself drives epigenetic modifications cooperating with histone acetyl transferases (Bettini et al. 2012).

There is increasing evidence showing that epigenetic mechanisms are a second layer of T cell fate regulation. There are excellent review articles in which many studies are reported (Wilson et al. 2009; Kanno et al. 2012;

Tripathi & Lahesmaa 2014). However, there is still much to investigate about the role of different TFs establishing relevant epigenetic marks, and how epigenetic marks influence the profile of cytokine expression of each subset under different environmental conditions.

2.8. Plasticity of T cell subsets

When the Th1/Th2 dichotomy was established, T cell fate was thought to be an end-stage commitment process, because those subsets produced cytokines and chemokine receptors selectively, and had differentiated functions that were stable through many passages (Murphy et al. 1996). However, in depth analyses of Th17 cells have shown that initially equally differentiated Th17 cells can change to a pathogenic or a regulatory subset, with different functions and cytokine profile expression. Moreover, it has been described that during a experimental autoimmune encephalomyelitis (EAE), Th17 cells that expressed IL-17A in early stages of the disease change their cytokine expression and produce IFN-γ (Hirota et al. 2011). Furthermore, conversion of Treg cells into Th17 in autoimmune arthritis (Komatsu et al. 2014), conversion of Th17 cells into Th1 (Kurschus et al. 2010), and stably committed Th2 cells that can express also IFN-γ in addition to IL-4 upon viral infection have been reported (Hegazy et al. 2010).

Those data show that T helper cells can change their phenotype (**Figure VII**). There is increasing evidence that differentiated T cell populations can alter their transcriptional program and coexpress different subset-specific TFs (Zhang et al. 2008; Koch et al. 2009; Wang et al. 2011). Indeed, most cytokines can be produced by different lineages; IL-10 is produced by Th1, Th2 and Tregs and IL-21 is expressed by Tfh, Th17 and Th1 cells. Moreover, depending on the conditions of T cell activation, Tfh cells may produce Th1, Th2 or Th17 subset-specific cytokines (Crotty 2011).

Epigenetic information further supports the idea of the flexibility of T cell subsets. In the different committed subsets, there are bivalent histone marks in

the master regulators of the rest of the fates, which indicates that these genes are "ready" to be transcribed upon a small change on the chromatin (Wei et al. 2009).

Thus, T cell polarization does not seem to be a fully determined state. It might be reasonable to think about a polarization that can be changed upon different environmental circumstances. As T cells migrate and must deal with changing conditions, irreversibility might be disadvantageous (O'Shea & Paul 2010). There are several reviews discussing the flexibility versus commitment of T helper cell subsets (Murphy & Stockinger 2010; O'Shea & Paul 2010; Reiner & Adams 2014). A general consensus is that more information about transcriptional regulation and analyses at the single-cell level are required to fully understand T cell differentiation processes.

2.9. T cell differentiation under changing environmental conditions

When an immune response occurs, T cells move from the lymphoid organs, where environmental conditions are optimal for growth, to sites of inflammation where they might find limiting concentrations of growth factors, oxygen or nutrients.

T cell differentiation also is influenced by different environmental cues that can be found throughout the organism. Not all the subsets have the same requirements for differentiating, and depending on the extracellular milieu they will polarize towards one or another subset (**Figure VII**). Nutrient availability, hypoxia (Shi et al. 2011; Dang et al. 2011), UV radiation (Wang et al. 2008; Wang et al. 2010) or hypertonic stress (Kleinewietfeld et al. 2013; Wu et al. 2013), are some of the factors that influence T cell outcomes.

T cell metabolism changes upon activation. Naïve T cells, as other non-proliferating cells, have low rates of biosynthesis and nutrient uptake. They have a catabolic metabolism by which glucose is fully oxidized through the tricarboxylic acid (TCA) cycle and oxidative phosphorylation (OXPHOS).

Upon activation, as T cells grow and divide they require energy and biomass accumulation for the synthesis of DNA, proteins and membranes for organelle biogenesis. TCR signaling induces a metabolic switch to aerobic glycolysis, in which most of the pyruvate produced is converted to lactate instead of being oxidized in the mitochondria (Warburg effect). Conversion of pyruvate to lactate yields NAD^+ that enhances the speed of glycolysis and the rate of glucose uptake to sustain it. In addition, glucose is not simply metabolized for ATP generation, but yields valuable building blocks for the biosynthesis of lipids, nucleic acids, and amino acid required for cell growth (Pearce et al. 2013; Maciver et al. 2013; Vander Heiden, M. G., Cantley, L.C., Thompson 2009). Nonetheless, although activated T cells are highly glycolytic, they still use mitochondrial respiration to produce ATP by oxidizing substrates such as glutamine. Metabolic inhibition or inadequate nutrient supply can impair T cell activation and proliferation (Maciver et al. 2013).

T cell subsets depend on specific metabolic signature for their function and maintenance. Effector $CD4^+$ T cell subsets are highly glycolitic and use less mitochondrial metabolism (OXPHOS) than Treg cells, which display a mixed metabolism involving glycolysis, OXPHOS and lipid oxidation (Michalek et al. 2011). The addition of the glycolysis inhibitor 2-deoxyglucose (2-DG) or the withdrawal of glucose inhibits T effectors *in vitro*, while inhibition of mitochondrial lipid oxidation inhibits Treg differentiation (Shi et al. 2011; Michalek et al. 2011).

TCR and CD28 stimulation activate the PI3K/AKT/mTOR pathway involved in the regulation of cell survival, growth and proliferation. mTOR inhibition by rapamycin or deletion of mTOR in T cells, that would to a certain extent be similar to nutrient deprivation, inhibits cell expansion and polarization of Th1, Th2 and Th17 cells promoting Treg generation (Delgoffe et al. 2009). mTOR is part of two different complexes, mTORC1 and mTORC2. Specific deletion of the component of the mTORC1 complex Rheb prevents Th1 and Th17 polarization while specific deletion of the component of mTORC2 RICTOR,

has been shown to inhibit Th2 generation, which goes in line with the observation that the metabolic signature of the different subsets is different (Delgoffe et al. 2011). However, a recent study has shown that inhibition of Raptor (a mTORC1 component) not only prevents Th1 and Th17 fates, but also Th2 differentiation (Yang et al. 2013).

Th17 cell differentiation is favored by transcription factors that promote glycolytic pathways: hypoxia inducible factor-1α (HIF-1α) is highly expressed in Th17 cells and it is required for the development of Th17. HIF-1α promotes Th17 cell differentiation by inducing glycolytic genes and enhancing ROR-γt and IL-17 expression. HIF-1α-deficiency favors Treg development (Shi et al. 2011; Dang et al. 2011). HIF-1α can be regulated by the activation of PI3K/AKT/mTOR pathway. Other important role of this protein is the regulation of the stress response upon oxygen deprivation. As there are many hypoxic areas where immune responses occur, as the gut or inflamed tissues, HIF-1α may have an important role regulating T cell differentiation at these sites (Maciver et al. 2013).

When an antigen-presenting cell interacts with a regulatory T cell, Treg cells induce pathways required for consuming essential amino acids. This results in a depletion of these nutrients. Limitation of essential amino acids attenuates mTOR signaling, which promotes an increase in Treg cells (Cobbold et al. 2009) and a decrease in the numbers of effector T cells. Treg cells use several mechanisms for reducing T helper cell responses, as the inhibition of dendritic cell-induced extracellular cysteine (Cys) accumulation (DCs produce and export glutathione and it is degraded to Cys outside the cell). Extracellular Cys, supports glutathione synthesis in T cells and generates a reducing microenvironment which promotes T cell proliferation (Yan & Banerjee 2010; Yan et al. 2010). With this strategy, effector T cell responses are diminished.

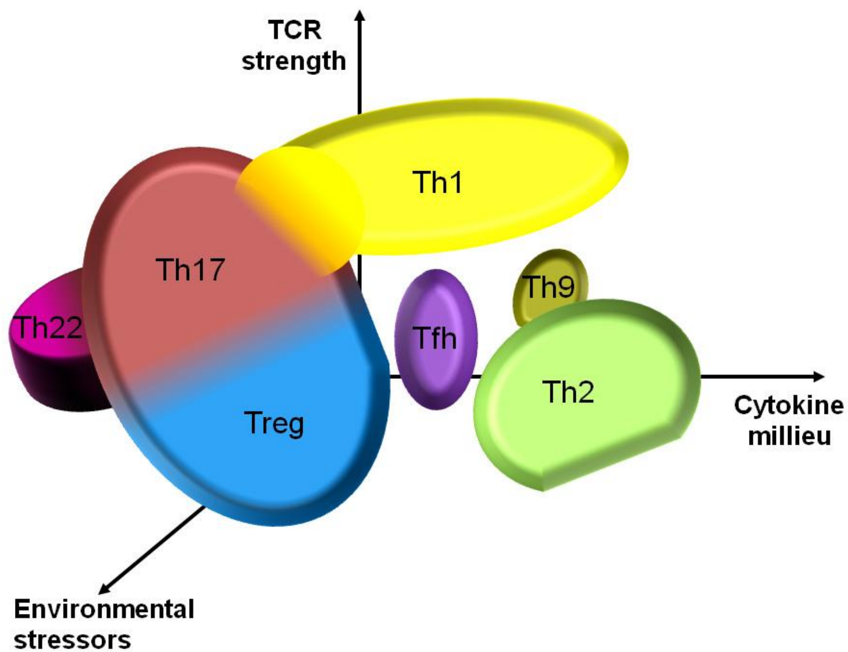


Figure VII. New paradigm of T cell polarization. Qualitative representation of T cell “subsets”. T cell differentiation is not only driven by the availability of cytokines. TCR signaling strength and different environmental factors also dictate the gene expression profile of a $CD4^+$ T cell. Moreover, genes that were thought to be subset-specific can be expressed by other subsets, supporting the idea that T cell specification is not an end-stage process, it is a flexible program.

3. Osmotic stress

Constant cellular volume maintenance is required for normal cell function and survival. Perturbations in the extracellular or intracellular solute concentration (osmotic stress) need to be carefully regulated, as they can result in cell volume alterations. Cell volume is important for defining the intracellular solute concentration and shape of the cell. Maintaining equilibrated ionic strength in the cell is necessary for cell growth, migration, survival, protein stability and the regulation of the intracellular metabolism (Wehner et al. 2003).

3.1. Osmotic stress response

Almost all mammalian cell membranes are highly permeable to water. Water flow across the cytoplasmic membrane occurs by diffusion, although some cells have transmembrane proteins called aquaporins for rapid water conduction in and outside the cell (Strange 2004).

Water diffuses through the membrane by osmosis, which refers to the water movement through a semi-permeable membrane (in this case the cell membrane) for equalizing the total solute concentrations on both sides of the membrane. The osmotic pressure is the hydrostatic force needed for preventing osmotic water flow across the membrane, and it depends on the total number of particles that are dissolved in the solution. Osmolarity and osmolality designate the number of particles present in a liter of solution and kilogram of solvent respectively. As a liter of plasma weights approximately one kilogram, when referring to living organisms these terms are used interchangeably. The magnitudes used for expressing the osmolarity and osmolality are mOsm/L and mOsm/kg respectively (Lang et al. 1998; Strange 2004).

The extracellular and intracellular fluids are usually in thermodynamic equilibrium, they both have the same osmolality. Osmotic stress means that

there is an increase or decrease of solute concentration in one of the sides. An increase of osmolality in the extracellular fluid (hypertonicity) or a decrease (hypotonicity) results in a cell response for adapting to the new scenario. Cells behave as osmometers as a result of their semi-permeable membrane. When the osmolality of the extracellular milieu is higher than the intracellular osmolality, cells lose water to maintain an osmotic equilibrium and thus, cells shrink. If the intracellular osmolality is higher than the extracellular, there is a swelling of the cell as a result of the gain of water. Cells can sense and respond to changes even lower than 3% of their volume, as rapid changes in cellular volume are deleterious for the cells. For overcoming these fast reactions, organisms have developed two mechanisms that start within minutes of exposure to stress: regulatory volume increase (RVI) for shrunken cells and regulatory volume decrease (RVD) for swollen cells (**Figure VIII**).

Cell osmolarity can only be regulated by the increase or decrease of the concentrations of osmotically active solutes, which can be inorganic ions such as Na^+, K^+ and Cl^-, and in latter phases of osmoregulation, small organic molecules termed compatible osmolytes (Strange 2004).

RVI consists on the gain of ions for water influx to cell. The ion uptake is paralleled by inhibition of ion release. The objective is to increase the intracellular concentration of Na^+, K^+ and Cl^- for creating an influx of water and increasing cell volume to normal values. This is achieved through the activation of Na^+/H^+ exchangers (NHEs) and Cl^-/HCO_3^- exchangers, the Na^+-K^+-$2Cl^-$ cotransporters (NKCCs) and nonselective hypertonicity-induced cation channels (HICCs) (Strange 2004; Hoffmann et al. 2009; Alfieri & Petronini 2007) (**Figure VIII**).

The RVD process is characterized by the active release of ions, for water efflux from the cell and thereby decreasing cell volume. Swelling-activated Cl^- and K^+ channels together with the K^+-Cl^- cotransporters (KCCs) reduce the intracellular content of these ions and promote water loss driven by osmotic

pressure (Strange 2004; Alfieri & Petronini 2007; Hoffmann et al. 2009) (**Figure VIII**).

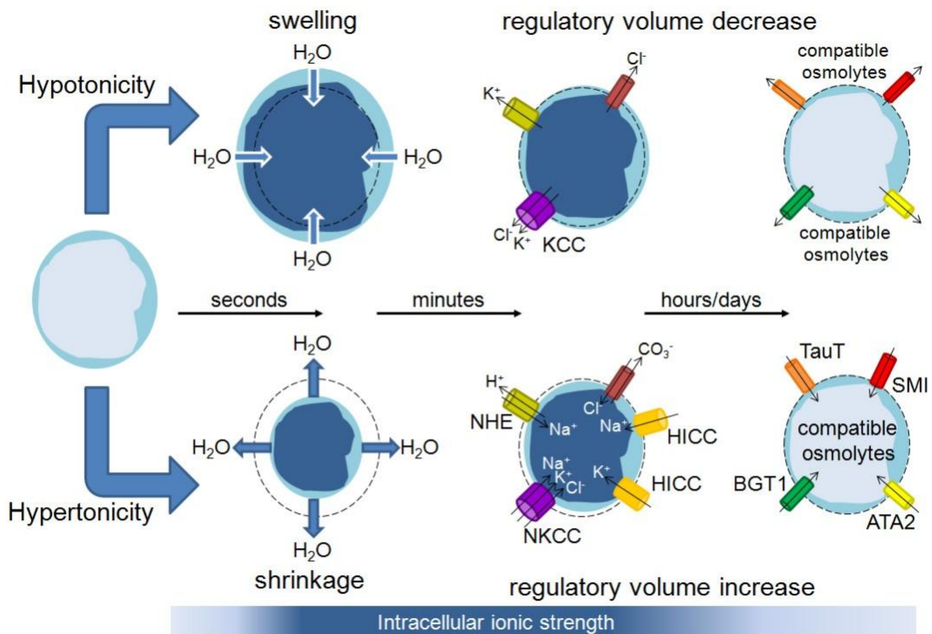


Figure VIII. Regulatory volume decrease and regulatory volume increase representation. Upon hypotonicity cells gain water and swell, and for recovering their initial volume undergo the regulatory volume decrease. Within minutes cells release ions and for avoiding harmful intracellular ionic strength, they adapt to hypotonicity releasing compatible osmolites. Upon hypertonicity, cells lose water and shrink. For recovering the initial volume they undergo regulatory volume increase. Within minutes cells uptake ions and for avoiding excessive intracellular ionic strength, they synthesize several channels for increasing the intracellular concentration of organic osmolytes and thus adapt to hypertonicity.

In hypertonic environments, electrolyte accumulation upon cell shrinkage must be limited because high ionic strength causes structural and molecular damage to macromolecules altering many cell functions. To avoid these perturbing effects, cells produce compatible osmolytes (nonionic molecules). These organic molecules increase solute concentration inside the cell without disturbing its functions. They include methylamines such as betaine and

glycerophosphocholine (GPC), polyalcohols such as sorbitol and *myo*-inositol and amino acids and their derivatives such as taurine and glutamic acid. The specificity of accumulated osmolytes varies with the cell type. This process is slower than the uptake of ions. Activation of compatible osmolyte accumulation pathways require transcription and translation of several genes involved in the synthesis and transport of osmolytes. Betaine, myo-inositol, neutral aminoacids and taurine accumulation are mediated by Na^+ coupled transport through different symports: Betaine/γ-aminobutyric acid transporter (BGT), sodium/myo-inositol cotransporter (SMIT), sodium- and chloride-dependent taurine transporter (TauT), and neutral amino acid transporter. Furthermore, glucose conversion to sorbitol is catalyzed by the aldose reductase (AR) enzyme. Additional processes involved in RVI are the increase of the amount of neutral amino acids in the cell, through the increase of proteolysis and decrease of protein synthesis (Hoffmann et al. 2009; Burg et al. 2007; Strange 2004; Wehner et al. 2003; Lang et al. 1998) (**Figure VIII**).

Hypertonicity stress can cause many perturbing effects, such as cell cycle arrest, DNA damage, oxidative stress, mitochondrial depolarization, inhibition of transcription and translation and apoptosis (Michea et al. 2002; Alfieri et al. 2002; Bortner & Cidlowski 1996; Michea et al. 2000; Dmitrieva et al. 2003; Dmitrieva et al. 2004; Robbins et al. 1970; Pederson & Robbins 1970). The severity of these effects depends on the intensity and duration of the stress. There is not a specific value for which the osmotic stress is considered lethal, because each cell type can respond differently to a determined NaCl concentration. Many of the effects are common to other stressors such as UV radiation, hypoxia or oxidative stress: apoptosis, protein, RNA and genomic instability and growth inhibition. However, cells that adapt to osmotic stress show impairments that are not observed in cells adapted to other stressors such as the double strand breaks or oxidative stress-induced DNA lesions (Dmitrieva et al. 2004; Zhang et al. 2004).

3.2. When do cells encounter hypertonic stress?

Homeostasis:

Under normal physiological conditions, plasma osmolality is maintained constant around 285-295 mOsm/kg H_2O. Most cells are exposed to isotonic plasma and the usual volume changes are due to intracellular hypertonicity: as a result of the accumulation of metabolites due to the transepithelial transport or autocrine production of molecules.

The function of the kidney is to control sodium and water levels in plasma, thus the osmolality, modulating their reabsortion and excretion. Renal medullary cells are exposed to very high concentrations of NaCl and urea mainly during antidiuresis, when the osmolality can reach levels higher than 1000 mOsm/Kg (Burg et al. 2007). Blood cells in kidney medulla capillaries are also exposed to these levels hypertonicity. Although the osmolality of the renal medulla is high, the elevated urea levels present in this microenvironment compensate the elevated concentrations of sodium cations. Thus the harmful effects of the hypertonicity are compensated in this specific site.

Nucleus pulposus cells from the intervertebral discs are also exposed to hypertonicity, because in the cartilage there are high levels of free cations (Na^+). These sites can reach 450 mOsm/kg (Urban et al. 1993). Other sites where osmolality is moderately higher than plasma are liver, thymus and spleen, around 330 mOsm/kg (Go et al. 2004). However these levels of extracellular solute concentration are not as high to induce an osmotic stress response (Morancho et al. 2008; Berga-Bolaños et al. 2013).

Hypernatremia and other hyperosmotic stress situations:

Hypernatremia is a pathologic situation that occurs when the levels of sodium in plasma are increased over 145 mmol/L. The osmolality in plasma is closely controlled by water homeostasis through the thirst, the secretion of

vasopressin (antidiuretic hormone) and renal reabsortion or secretion of free water (Shoker 1994). When some of these mechanisms are impaired, hypernatremia can develop. The majority of cases of hypernatremia are due to net water loss that can occur in the absence (pure water loss) or presence of sodium (hypotonic fluid loss). Pure water loss can be caused in cases of diabetes insipidus or dehydration. The hypotonic fluid loss can be due to renal, gastrointestinal or cutaneous causes such as osmotic diuresis, intrinsic renal disease, vomiting, diarrhea, excessive sweating or tissue burns. The fewer cases are because of an hypertonic sodium gain that occurs during clinical interventions (as a result of using parenteral hypertonic saline) or when there is an accidental sodium loading (Adrogué & Madias 2000). The reported cases of hypernatremia reach sodium levels in plasma around 155 mmol/L with osmolalities from 320 to 360 mOsm/kg in adults (Shoker 1994).

There are some groups of patients for whom the risk for developing hypernatremia is higher due to the inability for accessing to water or impairment of thirst perception: intubated patients, elderly persons, patients with altered mental status and infants. Thirst impairments are usual in elderly persons and infants can develop hypernatremia due to diarrhea (Bruck et al. 1968; Phillips et al. 1991; Adrogué & Madias 2000). In fact, plasma osmolality of 430 mOsm/kg has been reported in a case of an infant with diarrhea (Papadimitriou et al. 1997). Besides systemic hypernatremia, local hyperosmotic stress can occur in specific pathologies. Inflammatory bowel diseases (IBD) include different chronic inflammatory diseases affecting gastrointestinal tract. The most commons are ulcerative colitis (UC) and Crohn's disease (CD). It has been shown that the fecal fluid in the colon of these patients has higher osmolarity than controls and that the high osmolarity levels in Crohn's disease patients correlates with disease severity (Vertzoni et al. 2010; Schilli et al. 1982).

The effects of hypertonicity have been analyzed mainly in kidney-derived cells, due to the high tonicity present in the medulla. However it has been

shown that other cell types that are not usually in a hypertonic environment maintain similar responses to osmotic stress, such as: chondrocytes (Tsai et al. 2006), macrophages (Denkert et al. 1998; Jantsch et al. 2015), lymphocytes, endothelial cells (Petronini et al. 2000), mesothelial cells (Matsuoka et al. 1999), astrocytes (Olsen et al. 2005) and neurons (Loyher et al. 2004). That the response to osmotic stress is such a widely conserved mechanism suggests that there might exist biological contexts in which these responses are required more frequently than only in specific disorders. Although the physiological relevance of osmotic stress responses outside of the renal medulla has remained elusive, recent works have shown that dietary intake of large quantities of salt and bacterial infections in skin wounds can cause local osmotic stress in tissues (Machnik et al. 2009; Jantsch et al. 2015). Experiments have shown that under a prolonged high salt diet, macrophages in the skin, a site of natural accumulation of Na^+, activate osmotic stress responses that influence vascular function and blood pressure (Machnik et al. 2009). A high salt diet can also exacerbate Th17 inflammatory responses in the brain in mouse experimental models, although whether this effect is connected with the activation of skin macrophages has not been elucidated (Kleinewietfeld et al. 2013; Wu et al. 2013). Finally, in normal feeding conditions, infected wounds in the skin of both humans and mice have been shown to be hypertonic sites where Na^+ concentration can reach sufficiently elevated levels as to elicit osmostress responses in local immune cells (Jantsch et al. 2015).

4. NFAT5

The nuclear factor of activated T cells (NFAT5) also known as tonicity-responsive enhancer-binding protein (TonEBP) is a transcription factor that was first isolated by Lopez-Rodriguez and colleagues and Miyakawa and colleagues. Those groups independently cloned the protein based in different criteria; Lopez-Rodriguez identified a protein that was related to the calcineurin-regulated NFAT transcription factors, while Miyakawa isolated a hypertonicity-induced protein using the yeast one hybrid technology (Miyakawa et al. 1999; Lopez-Rodríguez et al. 1999), which resulted to be the same protein. Ko and colleagues also cloned it a year later and named it osmotic response element binding protein (OREBP) (Ko et al. 2000).

With close to 1500 amino acids, NFAT5 is the largest protein of the Rel family of transcription factors. The other members of the Rel family are the NF-κB proteins and the calcium and calcineurin-dependent NFAT proteins (NFATc) that comprises NFAT1, NFAT2, NFAT3 and NFAT4. This protein family is defined by the conserved DNA binding domain (DBD) known as the Rel homology region (RHR), a domain of about 200 amino acids. Outside this region, there is no recognizable similarity between NFAT5, NFATc and NF-κB (Miyakawa et al. 1999; Lopez-Rodríguez et al. 1999) (**Figure IX**).

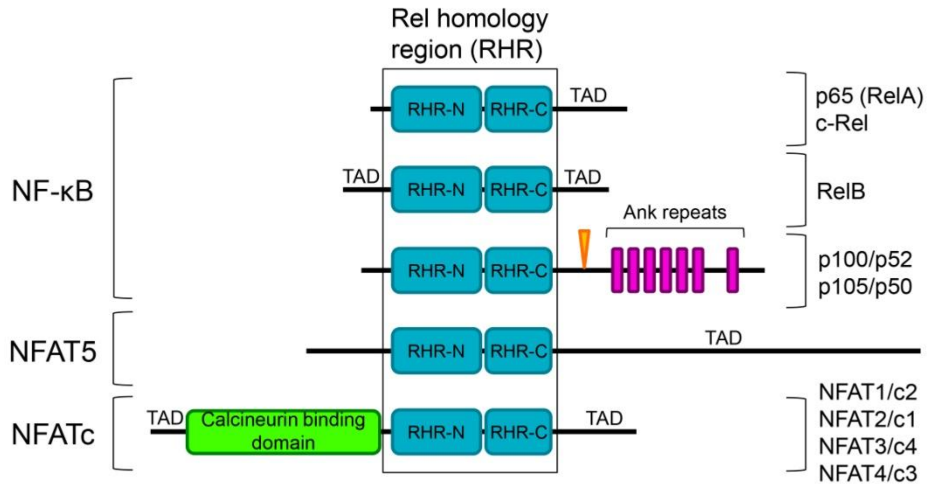


Figure IX. Schematic diagram of the members of the Rel family of transcription factors. Rel proteins share the structurally conserved Rel homology region (RHR, in blue), that comprises the DNA binding loop in the amino-terminal region (RHR-N) and the dimerization domain in the carboxy-terminal region (RHR-C). NF-κB family comprises five members: RelA, c-Rel, RelB, p100/p52 and p105/p50. RelA and c-Rel have a short amino-terminal domain and a transactivation domain (TAD) in the carboxy-terminal region. RelB protein has two TAD; one in the amino-terminal and the other in the carboxy-terminal region. p100/p52 and p105/p50 have ankirin repeats (shown in purple) and contain a proteolytic cleavage site (in orange). NFAT5 has the TAD in the carboxy-terminal region. The NFATc protein family that comprises four different proteins that contain a calcineurin binding domain in the amino-terminal region (in green) and two TADs, located in the amino- and carboxy-terminal regions.

NFAT5 has a long carboxy-terminal domain of 900 amino acids long that includes two activation domains and two modulatory domains that are involved in its transactivation, and two glutamine repeats (absent in other members of the Rel family) (Lee et al. 2003). The amino-terminal region has two motifs that control its nuclear translocation: a nuclear localization signal (NLS) and an auxiliary export domain (AED) that is necessary for its export under normal tonicity conditions. In the case of the NFAT5c isoform, there is another motif: a Crm1-interacting, leucine-rich nuclear export signal (NES) that allows the full translocation of NFAT5 when hypertonicity inactivates the

NLS and AED (Tong et al. 2006). The NFAT5c isoform has also another activation domain in the amino-terminal. This region differs from the NFATc proteins, because it lacks the calcineurin-binding regulatory domain that includes the NLS, and thus, nuclear import is not regulated by calcineurin (Aramburu et al. 2006; Lee et al. 2003) (**Figure X**).

The *Nfat5* gene encodes for three different isoforms in mammals, named NFAT5a, b and c. They differ in the amino-terminal region. NFAT5a is the predominant and shortest isoform, and NFAT5c which is the longest, has an extra nuclear export signal (NES) and a transactivation domain as mentioned above (Aramburu et al. 2006) (**Figure X**).

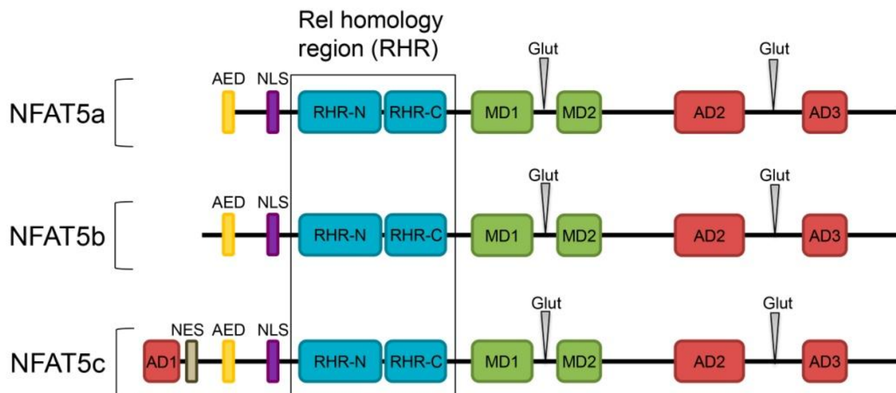


Figure X. Schematic diagram of the NFAT5 isoforms. The three isoforms of NFAT5 (NFAT5a, NFAT5b and NFAT5c) contain the nuclear localization signal (NLS, in purple) and an auxiliary export domain (AED, in orange) in the amino-terminal region, the Rel homology region (RHR, in blue) and the carboxy-terminal region comprises two activation domains (AD, in red), two modulatory domains (MD, in green) and two glutamine repeats (Glut, in grey). The three isoforms differ in their amino-terminal region. NFAT5b has a longer amino-terminal region than NFAT5a, and NFAT5c, has the longest amino-terminal region which includes an AD and a nuclear export signal (NES).

The DNA binding domain of NFAT5 is a hybrid between NFATc and NF-κB proteins. NFAT5 binds to DNA elements that are similar to those recognized

by NFATc proteins but are different from the palindromic elements bound by NF-κB. The consensus sequence for NFAT5 is TGGAAANNYNY (where N represents any nucleotide and Y represents a pirimidine) and NFATc proteins bind to the sequence GGAA. Thus, although the sequence requirement for NFAT5 is stricter, there are some NFATc sites that can be bound by NFAT5. Although NFAT5 has a lower affinity for the DNA than the calcineurin-dependent NFATc, once it binds DNA it is more stably bound than them (Stroud et al. 2002). It is widely described that NFATc proteins form stable cooperative complexes with AP1 (formed by Jun and Fos) and bind to composite NFATc:AP1 binding sites for regulating immune-related genes. NFAT5 lacks Fos/Jun contact residues that are present in NFATc proteins, suggesting that it does not bind cooperatively with Fos and Jun to DNA (Lopez-Rodríguez et al. 1999; Miyakawa et al. 1999; Ko et al. 2000; Hogan et al. 2003) (**Figure XI**).

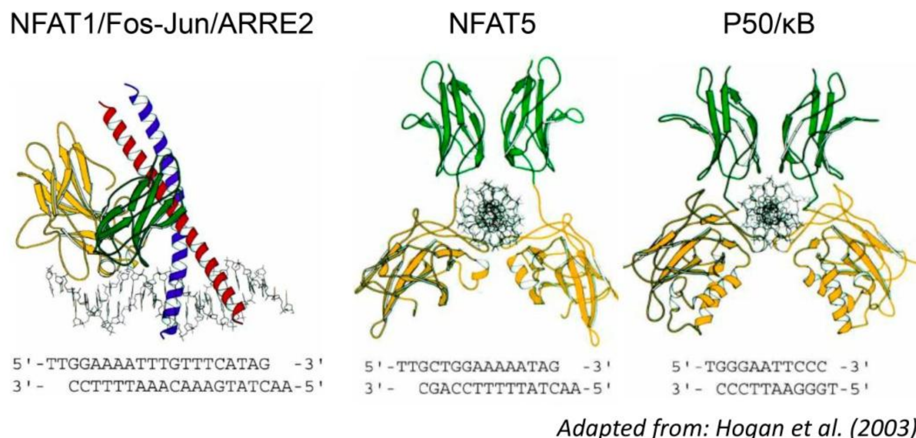


Figure XI. Crystal structures of NFATc, NFAT5 and NF-κB dimeric DNA/protein structures. NFAT1/Fos-Jun complex bound to ARRE2 site of IL-2 gene, NFAT5 homodimer bound to a tonicity response element and p50 homodimer bound to a κB site are shown. The amino-terminal region of the RHR is shown in yellow and the carboxy-terminal region of the RHR domain is shown in green. Below each structure the DNA sequences are indicated.

Similar to NF-κB, NFAT5 is an obligate dimer and the dimerization domain is located in the carboxy-terminal region of the DBD. It adopts an NF-κB-like structure upon binding DNA ("butterfly"-like structure). However, only a contact of one monomer to the DNA binding sequence is required for transcriptional regulation of its target genes. The other half of the dimer can bind to a non-consensus site. Curiously, the NFAT5 dimer forms a complete circle around the DNA. NFAT5 has not been described to dimerize with other Rel proteins (Stroud et al. 2002; Aramburu et al. 2006; Lopez-Rodríguez et al. 1999) (**Figure XI**).

The structural similarities of the members of the Rel family of transcription factors reflect evolutionary relationships. This family is found from arthropods to mammals, and is absent in unicellular eukaryotes and nematodes. Insects have two types of Rel proteins: Rel, which is similar to NF-κB, and dNFAT that has over 50% of amino acid identity to NFAT5 in the DNA binding domain. NFAT5 and dNFAT also have in common the glutamine repeats and the lack of calcineurin-binding sites (Huang & Rubin 2000; Aramburu et al. 2006). dNFAT has been described to be involved in the osmotic stress response in *Drosophila melanogaster* (Keyser et al. 2007).

NFAT5 mRNA expression has been detected in many tissues in the adult mice such as kidney, brain, heart, liver, pancreas, small intestine, colon, lung, skeletal muscle, spleen, thymus and peripheral blood lymphocytes (Lopez-Rodríguez et al. 1999; Miyakawa et al. 1999; Trama et al. 2000), and also at different stages of embryonic development (Maouyo et al. 2002). NFAT5 protein appears to be more abundant in certain tissues and cell types, such as in thymus, brain, testis, lung and lymphocytes (Trama et al. 2000; Trama et al. 2002). Although initially described as an osmoregulatory protein, its expression at sites where a hypertonic challenge is not probable suggests that it could be involved in other functions independent of osmostress.

4.1. Osmotic stress-dependent functions of NFAT5

Cells require NFAT5 to activate the gene expression program in response to hypertonicity. Several approaches as RNA interference, dominant negative NFAT5 constructs and NFAT5-deficient mice have proven it (Miyakawa et al. 1999; López-Rodríguez et al. 2001; Trama et al. 2002; López-Rodríguez et al. 2004; Go et al. 2004). Under hypertonic stress NFAT5 is activated by a combination of mechanisms that enhance its NFAT5 protein synthesis, its nuclear translocation, and its transcriptional activity (Tong et al. 2006; Gallazzini et al. 2011; Ferraris et al. 2002; Lee et al. 2003; Chen et al. 2007; Irarrazabal et al. 2006; Zhou et al. 2011; Cai et al. 2005). NFAT5 then induces the transcription of many osmoprotective genes that are required to compensate the increase in the intracellular ionic strength. These genes include transporters, enzymes and stress-related proteins that promote the increase of compatible osmolyte concentration and protein stability. Among the transporters that are induced by NFAT5 can be found: betaine/γ-aminobutyric acid transporter (BGT1), sodium/myo-inositol cotransporter (SMIT) (Woo et al. 2002; López-Rodríguez et al. 2004), sodium- and chloride-dependent taurine transporter (TauT) (Ito et al. 2004; Tsai et al. 2006) and sodium-coupled neutral amino acid transporter 2 (ATA2) (Trama et al. 2002) that are required for the uptake of betaine, myo-inositol, neutral amino acids and taurine respectively, urea transporter A (UT-A) (Lam et al. 2004; Nakayama et al. 2000) for urea transport and aquaporins AQP1, AQP2, AQP3 and AQP4 (Lanaspa et al. 2010; Kasono et al. 2005; Yi et al. 2013; López-Rodríguez et al. 2004; Lam et al. 2004) that are water channels. NFAT5 also increases the abundance of aldose reductase (AR) (López-Rodríguez et al. 2004; Na et al. 2003; Woo et al. 2002), aromatic I-amino acid decarboxylase (AAD) (Hsin et al. 2011), and β1,3-Glucuronosyltransferase-I (Hiyama et al. 2009) that are enzymes required for the synthesis of sorbitol, dopamine and glycosaminoclycans respectively. In addition, NFAT5 upregulates the induction of the chaperone heat shock protein 70 (HSP70)

required for protein folding (Woo et al. 2002; Na et al. 2003; Tsai et al. 2006). NFAT5 is also involved in enhancing the expression of the serum and glucocorticoid-regulated kinase 1 (Sgk1), a kinase required for the regulation of dehydration natriuresis (Chen et al. 2009).

Furthermore, hypertonicity induces cytokines, growth factors and other molecules related with the immune response such as the cell adhesion molecule CD24 (Berga-Bolaños et al. 2010), TNF-α (López-Rodríguez et al. 2001; Esensten et al. 2005) and LT-β (López-Rodríguez et al. 2001) in T cells, monocyte chemoattractant protein-1 (MCP-1, also known as CCL-2) (Kojima et al. 2010; Küper et al. 2012) in a rat kidney cell line and mesothelial cells, cyclooxygenase-2 (COX2) (Favale et al. 2009; Woo et al. 2002) in renal epitelial cells or vascular endothelial growth factor-C (VEGFC) (Machnik et al. 2009) and inducible nitric oxide synthase (iNOS) in macrophages. NFAT5 also regulates salt dependent chemotaxis in macrophages (Müller et al. 2013) (**Table 1**).

Table 1. NFAT5 target genes upon hypertonicity

Type	Name	Function	Cell type	Reference
Transporters	Betaine/γ-aminobutyric acid transporter (BGT1)	Betaine uptake	MDCK Kidney cells	(Woo et al. 2002; López-Rodríguez et al. 2004)
	Sodium/myo-inositol cotransporter (SMIT)	Myo-inositol uptake	HeLa Kidney cells MDCK	(Na et al. 2003; López-Rodríguez et al. 2004; Woo et al. 2002)
	Sodium-coupled neutral amino acid transporter 2 (ATA2)	Neutral amino acid uptake	T cells	(Trama et al. 2002)
	Sodium- and chloride-dependent taurine transporter (TauT)	Taurine uptake	Nucleus pulposus cells HepG2 cells	(Tsai et al. 2006; Ito et al. 2004)

	Vasopressin regulated urea transporter (UT-A)	Transport of urea	Renal epithelial cells MDCK cells	(Lam et al. 2004; Nakayama et al. 2000)
	Aquaporin 1 (AQP-1)	Water channel	mIMCD3 renal epithelial cells	(Lanaspa et al. 2010; Lam et al. 2004)
	Aquaporin 2 (AQP-2)	Water channel	MDCK cells Renal epithelial cells mpkCCD cells	(Kasono et al. 2005; Lam et al. 2004; López-Rodríguez et al. 2004; Hasler et al. 2006)
	Aquaporin 3 (AQP-3)	Water channel	Kidney	(López-Rodríguez et al. 2004)
	Aquaporin 4 (AQP-4)	Water channel	Astrocytes	(Yi et al. 2013)
Enzymes	Aldose reductase (AR)	Synthesis of sorbitol	MEF HeLa Kidney MDCK	(López-Rodríguez et al. 2004; Na et al. 2003; Woo et al. 2002)
	Aromatic I-amino acid decarboxylase (AAD)	Synthesis of dopamine	HK-2 cells	(Hsin et al. 2011)
	B1,3-Glucuronosyltransferase I	Synthesis of glycosaminoglycans	Nucleus pulposus cells	(Hiyama et al. 2009)
Chaperone	Heat shock protein 70 (HSP70)	Protein stability	HeLa mIMCD Nucleus pulposus cells	(Na et al. 2003; Woo et al. 2002; Tsai et al. 2006)
Kinases	Serum and glucocorticoid regulated kinase 1 (SGK1)	Kinase	IMCD cells	(Chen et al. 2009)

Immune function related	CD24	T cell proliferation	T cells	(Berga-Bolaños et al. 2010)
	Lymphotoxin β (LT-β)	Cytokine	Jurkat T cells	(López-Rodríguez et al. 2001)
	Tumor necrosis factor α (TNF-α)	Cytokine	Jurkat T cells L929 fibroblasts	(López-Rodríguez et al. 2001; Esensten et al. 2005)
	Monocyte chemoattractant protein-1 (MCP-1)	Macrophage and T cell recruitment	NRK52E cells Mesothelial cells	(Kojima et al. 2010; Küper et al. 2012)
	Inducible nitric oxide synthase (iNOS)	Synthesis of NO	Macrophages	(Jantsch et al. 2015)
	B cell-activating factor (BAFF)	Cytokine	Splenocytes	(Kino et al. 2010)

4.2. Osmotic stress-unrelated functions of NFAT5

NFAT5 is also involved in different functions that are not related to osmotic stress. It is required for embryonic development, HIV replication, skeletal muscle myogenesis, integrin-induced cellular migration and innate immune responses induced by TLRs in macrophages.

NFAT5 expression is detected in almost all the organs of the mouse embryo, and mice that lack NFAT5 show reduced embryonic viability between days 13.5 and 17.5, which indicates a role for NFAT5 in embryonic development that is most likely independent from osmoregulation (Maouyo et al. 2002; López-Rodríguez et al. 2004; Go et al. 2004). In this regard, it has been described that this mortality is in part due to improper heart development and impaired cardiac function not associated with osmostress responses (Mak et al. 2011).

NFAT5 is also implicated in HIV regulation in monocytes. It functionally interacts with a specific binding site that is conserved in human and simian immunodeficiency viruses, which suggests a regulatory role in HIV-1 replication for NFAT5 (Ranjbar et al. 2006).

Skeletal muscle regeneration depends on the migration and differentiation of myoblasts and the formation of myofibers. NFAT5 is relevant in muscle regeneration because the inhibition of NFAT5 transcription generates migratory and differentiation defects in myoblasts. It has been described that NFAT5 acts through Cyr61 for promoting muscle regeneration (O'Connor et al. 2007).

NFAT5 has been linked to migration also in other contexts. Integrins are critical regulators of the cell migration. Specifically, α6β4 integrin has been linked to epithelial motility, cell survival and carcinoma invasion, features observed in metastatic tumors. Clustering of α6β4 integrin induces NFAT5 transcription in breast carcinoma cell lines, which results in enhanced cell migration (Jauliac et al. 2002).

It has been shown that NFAT5 is a regulator of mammalian anti-pathogen responses. Specifically, TLR stimulation in macrophages promotes the production of IL-6, iNOS and TNF-α in an NFAT5-dependent manner. NFAT5 binds to the loci of *Il6, Nos2* and *Tnfa*, inducing their transcription in a dose-dependent manner. Moreover, *in vivo* NFAT5 is required for effective immunity against the parasite *Leishmania major* (Buxade et al. 2012; Jantsch et al. 2015).

In addition, NFAT5 elevated expression has been detected in fibroblast-like synoviocytes of rheumatoid arthritis patients, suggesting a role for NFAT5 in this disease (Masuda et al. 2002).

4.3. NFAT5 in T cells.

The immunological significance of osmostress-driven responses has remained elusive, and apart from certain diseases and mouse mutant models of systemic hypernatremia, not much was known about physiopathologic situations where this response could be relevant for immune function (Ka et al. 2003; Dogan et al. 2005; Papadimitriou et al. 1997; Berga-Bolaños et al. 2010; Ma et al. 1998; Yun et al. 2000; Machnik et al. 2009; Kleinewietfeld et al. 2013; Jantsch et al. 2015). Recent works, though, have shed light on the immunological relevance of hyperosmotic stress by showing that hypernatremia occurring naturally in infected wounds, or as result of prolonged dietary salt intake, can promote inflammatory functions in macrophages and exacerbate Th17 responses in models of autoimmune diseases. In parallel, the transcription factor NFAT5, known to be a central regulator of adaptive responses to osmostress in mammalian cells, has been also found to modulate diverse functions in immune cells, such as macrophages and T cells, when they are exposed to hyperosmotic conditions (Jantsch et al. 2015; Machnik et al. 2009; Kleinewietfeld et al. 2013; Wu et al. 2013; Berga-Bolaños et al. 2010).

Tonicity levels of 380 mOsm/kg are enough to activate NFAT5 in T cells (Morancho et al. 2008). NFAT5-deficient T cells cultured under physopathologic levels of hypertonic stress show an increase in the proportion of effector memory cells, decreased proliferative capacity, increased cell death and cell cycle arrest (Berga-Bolaños et al. 2010; Drews-Elger et al. 2009). Both in vivo and in vitro, NFAT5 promotes the expression of the homeostatic proliferation regulator CD24 in response to hypertonic stress, a function that allows T cells to survive under osmostress (Berga-Bolaños et al. 2010).

Different reports agree that NFAT5 is required for T cell-related immune functions, some of which are particularly relevant in the context of hypertonic stress. Mice with a whole-body deficiency in NFAT5 exhibit lymphopenia

and reduced cellularity of thymus and spleen (Go et al. 2004; López-Rodríguez et al. 2004). NFAT5-null mice also exhibit altered CD8 naïve/memory cell homeostasis, inability to reject tumors and constitutive hypernatremia (Berga-Bolaños et al. 2010). By contrast, mice with a T cell-specific conditional deletion of *Nfat5* have normal cellularity in thymus and spleen and exhibit only a mild bias in CD8 T lymphocytes towards memory cells. Also, NFAT5-deficient T cells can reject allogeneic tumors in vivo as efficiently as wild-type T cells, indicating that some of the T cells defects observed in the NFAT5-null mice are not due directly to the lack of NFAT5 in mature T cells (Berga-Bolaños et al. 2010). The observation that NFAT5-null mice suffered a constitutive hypernatremia suggested that some of the T cell defects observed in them could result from inappropriate adaptation to osmostress. This was confirmed in adoptive transfer experiments in which NFAT5-deficient T cells cultured in the absence of stress were inoculated in hypernatremic NFAT5-null mice and found to proliferate less than when transferred into normal mice, and less than wild-type T cells transferred into hypernatremic mice. Moreover, wild-type T cells transferred into hypernatremic mice upregulated CD24, whereas NFAT5-deficient cells showed a poorer induction of this receptor (Berga-Bolaños et al. 2010).

The group of Stephan Ho described that heterozygous mice had also impaired antigen-specific antibody responses. In wild-type mice, they described a mildly hypertonic (around 330 mOsm/kg) osmolality of spleen and thymus and argued that a possible explanation for the heterozygous mice to have lower levels of cells could be due to an impaired response to osmotic stress (Go et al. 2004). However, other recent report suggests that the tonicity of the thymus microenvironment was unlikely to be responsible of the reduced cell numbers of NFAT5 heterozygous mice. In this regard, NFAT5 was found to regulate early stages of thymocyte differentiation by acting as a prosurvival factor downstream the pre-TCR in an osmostress-independent manner (Berga-Bolaños et al. 2013). NFAT5-deficient conditional mice that lose NFAT5 at

the double negative (DN) stage (specifically at DN2) of thymic differentiation, show reduced number of T cells in the periphery with an arrest in the DN3 stage of the thymic differentiation (Berga-Bolaños et al. 2013). An earlier work had described that mice expressing a thymocyte and mature T cell-restricted dominant negative protein of NFAT5 showed impaired thymocyte maturation (Trama et al. 2002). In view of recent findings, this effect seems more likely explained by the role of NFAT5 in early steps of thymocyte differentiation than by an impaired capacity to respond to osmostress (Berga-Bolaños et al. 2013).

These observations have shown that in addition to its role in osmostress-sensitive immunomodulatory processes, NFAT5 can contribute to T cell differentiation in osmostress-independent contexts. Although very little is known about osmostress-independent functions of NFAT5 in mature T cells, some early reports showed that T cell activation induced NFAT5 nuclear translocation and its expression under isotonic conditions (López-Rodríguez et al. 2001; Trama et al. 2000; Trama et al. 2002). NFAT5 upregulation was observed in naïve T cells as well as in activated Th1 or Th2 cells. Since the increase in NFAT5 levels upon T cell stimulation did not coincide with any obvious osmostress response, it was possible that it might reflect other functions.

OBJECTIVES

OBJECTIVES

$CD4^+$ T cells have the ability to polarize into different subsets depending on the cytokine milieu or environmental stressors they encounter. The response to hypertonic stress caused by elevations of the extracellular concentration of NaCl is regulated by the transcription factor NFAT5 in mammalian cells. In T cells exposed to hypertonic stress, NFAT5 not only regulates adaptation responses but also induces the expression of different receptors and cytokines. On the other hand, NFAT5 has been shown to regulate T cell development in thymus in an osmostress-independent context. These observations led us to ask whether NFAT5 could regulate polarization responses in $CD4^+$ T lymphocytes exposed to osmotic stress, and also in response to hypertonicity-independent stimulation. To test this hypothesis, we outlined the following specific objectives:

1. To explore the role of NFAT5 in the expression of polarization genes in $CD4^+$ T lymphocytes stimulated under osmotic stress conditions or through combinations of different cytokines.

2. To dissect the mechanisms by which NFAT5 modulates $CD4^+$ T cell polarization in response to hypertonic stress.

3. To characterize the role of NFAT5 in the acquisition of polarized characteristics by T cells *in vivo*.

MATERIALS AND METHODS

MATERIALS AND METHODS

- Mouse model. *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice (Berga-Bolaños et al. 2010; Drews-Elger et al. 2009) were bred and housed in specific pathogen-free conditions at the animal facility of Parc de Recerca Biomèdica de Barcelona. For the DSS-induced colitis experiments, mice were kept at the animal facility of Universidad de Cantabria. Animal handling and experiments were in accordance with approved protocols by the ethic al committee of Parc de Recerca Biomèdica de Barcelona Animal Care and Use Committee or the Universidad de Cantabria Institutional Laboratory Animal Care and Use Committee and carried out in accordance with the Declaration of Helsinki and the European Communities Council Directive (86/609/EEC).

- CD4$^+$ T cell isolation and culture. Mouse CD4$^+$ T cells were obtained from spleens and lymph nodes of *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice. Spleen and lymph nodes were disgregated and CD4$^+$ T cells were isolated with the magnetic positive CD4 separation system CD4$^+$ Dynabeads®/Detachabead (Invitrogen, catalog 11445D and 12406D respectively) according to the manufacturer's instructions. Cells were activated with hamster anti-mouse CD3 (1 µg/10^6 cells) plus hamster anti mouse CD28 antibodies (1 µg/10^6 cells) (BD Biosciences, catalogue 553058 and 553295) in plates coated with goat anti-hamster IgG (9.5 µg/cm^2) and cultured in DMEM culture medium (Life Technologies) supplemented with 10% FBS (Life Technologies, catalogue 10270-106), non-essential amino acids (Life Technologies), 2 mM L-glutamine (Life Technologies), 50 µM β-mercaptoethanol (Life Technologies), 10 mM Hepes (Lonza), 1 mM sodium pyruvate (Life Technologies) and penicillin and streptomycin (Life Technologies). For cultures lasting over 48 hours, 5 ng/ml recombinant human IL-2 (Proleukin) was added at 48 hours. For Th17 conditions, 10 ng/ml IL-6 (ImmunoTools, catalogue 12340063) and 2.5 ng/ml TGF-β (PeproTech, catalogue 100-21)

were added. For Th1 conditions 5 ng/ml IL-12 (PeproTech, catalogue 210-12) was added. Digoxin (Sigma) was used at 10 µM.

- **Splenocyte culture.** Splenocytes were obtained from the spleens of *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice upon disgregation and density gradient centrifugation with Lymphoprep™ (StemCell technologies, catalogue 07801). Cells were activated with hamster anti-mouse CD3 (1 µg/10^6 cells) plus hamster anti mouse CD28 antibodies (1 µg/10^6 cells) (BD Biosciences, catalogue 553058 and 553295) in plates coated with goat anti-hamster IgG (9.5 µg/cm^2) and cultured in DMEM culture medium (previously described) for the times indicated. After the cell culture, $CD4^+$ T cells were isolated with the magnetic positive CD4 separation system Dynabeads® (Invitrogen, catalog 11445D). Rapamycin (Calbiochem) was used at 50 nM and FK506 (Calbiochem) at 100 nM.

- Hypertonic stress. The osmolality of the culture medium was measured in a VAPRO 5520 vapor pressure osmometer (Wescor). As the osmolality of the T cell medium with supplements was 330 mOsm/kg, 10% sterile water was added for adjusting it to 300 mOsm/kg. This medium was made hypertonic by adding sterile NaCl from 4M stock. Over the baseline of 300 mOsm/kg (isotonic conditions), addition of 40mM NaCl raised the osmolality to 380 mOsm/kg, the addition of 60 mM NaCl raised the osmolality to 420 mOsm/kg, and the addition of 100 mM NaCl raised the osmolality to 500 mOsm/kg.

- mRNA analysis. Total RNA was isolated using the High Pure RNA isolation kit (Roche, catalog 11 828 665 001), quantified in a NanoDrop (ND-1000) spectrophotometer and 100 ng to 1 µg of total RNA was retro-transcribed to cDNA using Transcriptor First Strand cDNA Synthesis Kit (Roche, catalog 04 897 030 001). Real-time quantitative PCR was performed with LightCycler 480 SYBR Green I mix (Roche, catalog 04 887 352 001) and the LightCycler 480 Real-Time PCR System according manufacturer's

instructions. Samples were normalized to *L32* (L32 ribosomal protein gene) mRNA levels using the LightCycler Software, version 1.5 (Roche). Primer sequences for the PCR reactions are described in **Table 1**.

Table 2. primers for mRNA analysis.

Genes	Sequence
*Akr1b3 (*AR)	Forward: 5'-TGA GCT GTG CCA AAC ACA AG-3'
	Reverse: 5'-GGA AGA AAC ACC TTG GCT AC-3'
Ccr6	Forward: 5'-CCT CAC ATT CTT AGG ACT GGA GC-3'
	Reverse: 5'-GGC AAT CAG AGC TCT CGG A-3'
Cd4	Forward: 5'-GTA CCA GCC TGT TGC AAG G-3'
	Reverse: 5'-AGA AGT CGC TGT CCT GAA CC-3'
Cd8a	Forward: 5'-TCT TCC AGA ACT CCA GCT CC-3'
	Reverse: 5'-CCT TGC TGA ACT TGT TCA GG-3'
Ctla4	Forward: 5'-TTC TCT GAA GCC ATA CAG GTG-3'
	Reverse: 5'-TCT TCT CTG TGA ATG TCG TGG-3'
Foxp3	Forward: 5'-CAA GGG CTC AGA ACT TCT AG-3'
	Reverse: 5'-AGC TGA TGC ATG AAG TGT GG-3'
Gata3	Forward: 5'-TGG GCT GTA CTA CAA GCT TC-3'
	Reverse: 5'- GCG TCA TGC ACC TTT TTG C-3'
Ifng	Forward: 5'-CTC AAG TGG CAT AGA TGT GG-3'
	Reverse: 5'-CAG GTG TGA TTC AAT GAC GC-3'
Il10	Forward: 5'-TGA ATT CCC TGG GTG AGA AG-3'
	Reverse: 5'-TAG ACA CCT TGG TCT TGG AG-3'
Il12rb2	Forward: 5'-ACA TTA CTG CCA TCA CAG AG AA-3'
	Reverse: 5'-TCA CGA ATG CTT TCC AGT TG-3'
Il17a	Forward: 5'-TCA GAC TAC CTC AAC CGT TC-3'
	Reverse: 5'-AAT TCA TGT GGT GGT CCA GC-3'
Il2	Forward: 5'-AGC TGT TGA TGG ACC TAC AG-3'
	Reverse: 5'-AAA TCC AGA ACA TGC CGC AG-3'
Il22	Forward: 5'-TTG AGG TGT CCA ACT TCC AGC A-3'
	Reverse: 5'-AGC CGG ACA TCT GTG TTG TT-3'

Il23r	Forward: 5'-TCA AGA GAC ACT GAT TTG TGG G-3'
	Reverse: 5'-TGT CTC CAA ACT CTT CAC ATG C-3'
Il4	Forward: 5'-CTC ACA GCA ACG AAG AAC AC-3'
	Reverse: 5'-GCT TAT CGA TGA ATC CAG GC-3'
Il6	Forward: 5'-GAA GTT CCT CTC TGC AAG AGA C-3'
	Reverse: 5'-GCC TCC GAC TTG TGA AGT GG-3'
Itgam (Cd11b)	Forward: 5'-AGA GAA TGT CCT CAG CAG GAG-3'
	Reverse: 5'-TCC GAG TAC TGC ATC AAA GAG-3'
Klrb1c (NK1.1)	Forward: 5'-ACA CAG GTT GGC TCT GAA GC-3'
	Reverse: 5'-TCC TCC CAA GTG TTG GAA AC-3'
Nfat5	Forward: 5'-CAG CCA AAA GGG AAC TGG AG-3'
	Reverse: 5'-GAA AGC CTT GCT GTG TTC TG-3'
Rorc	Forward: 5'-CCG CTG AGA GGG CTT CAC-3'
	Reverse: 5'-TGC AGG AGT AGG CCA CAT TAC A-3'
Sgk1	Forward: 5'-CCA AAC CCT CCG ACT TTC AC-3'
	Reverse: 5'-CCTTGTGCCTAGCCAGAAGAA-3'
Tbx21 (Tbet)	Forward: 5'-TAC CAG AAC GCA GAG ATC AC-3'
	Reverse: 5'-TGG GAA CAG GAT ACT GGT TG-3'
Tcra	Forward: 5'- CAA TGT GCC GAA AAC CAT GG-3'
	Reverse: 5'- TTC TCA GTC AAC GTG GCA TC-3'
Thy1	Forward: 5'-CCA TCC AGC ATG AGT TCA GC-3'
	Reverse: 5'-CAC ACT TGA CCA GCT TGT CTC-3'
Tnfa	Forward: 5'-TCG TAG CAA ACC ACC AAG TG-3'
	Reverse: 5'-GGA GTA GAC AAG GTA CAA CC-3'

- Chromatin immunoprecipitation (ChIP). $CD4^+$ T cells grown for 5 days were cleaned of dead cell and debris by centrifugation on Lymphoprep™ (StemCell technologies, catalogue 07801) washed, activated with hamster anti-mouse CD3 (1 μg/10^6 cells) plus hamster anti mouse CD28 antibodies (1 μg/10^6 cells) in plates coated with goat anti-hamster IgG (9.5 μg/cm^2), replated and cultured for 12 hours in the presence of 300 or 500 mOsm/kg.

Cells (16 x 10^6) were fixed with 1% formaldehyde for 10 min at room temperature. Formaldehyde was quenched with glycine (final concentration of 125 mM) for 5 min. After washing cells once with cold PBS and once with PBS with 1 mM PMSF, cells were lysed in 175 µl of lysis buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 mM NaF, 10 mM sodium orthovanadate and 10 mM β-glycerophosphate) for 30 min on ice and stored at -80ºC for at least 1 hour. Lysates were sonicated with a bath sonicator (Bioruptor; Diagenode) for 6 cycles of 30 s on and 30 s off on high power to obtain DNA fragments between 500 and 1000 bp, and centrifuged at 16000 x g to remove insoluble debris. Supernatants were collected and 6% of each sample was separated to use as a measure of chromatin imput for normalization. The rest of the sample was diluted 10 times in ChIP dilution buffer (1% TX-100, 20 mM Tris-HCl pH 8, 2 mM EDTA, 150 mM NaCl, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 mM NaF, 10 mM sodium orthovanadate and 10 mM β-glycerophosphate). Samples were precleared for one hour with protein A-sapharose beads (Amersham, catalog 17-0780-01) that were previously preadsorbed with fish sperm DNA (Roche, catalog 11 467 001) and bovine serum albumin (New England Biolabs, catalog B9001S) overnight at 4ºC. After removing the preclearing beads, 20 µl preimmune serum, or a mixture of two rabbit polyclonal NFAT5-specific antibodies that recognize its N-terminal and DNA binding domain regions (Lopez-Rodríguez et al. 1999) (10 µl of each) were added to the lysates and incubated overnight at 4ºC. Preadsorbed protein A-sepharose beads were then added, incubated for 1 hour at 4ºC, and then washed three times with washing buffer (0.1% SDS, 0.1% TX-100, 20 mM Tris-HCl pH 8, 2 mM EDTA and 150 mM NaCl) and once with final washing buffer (0.1% SDS, 1% TX-100, 20 mM Tris-HCl pH 8, 2 mM EDTA and 500 mM NaCl). For DNA elution, beads were incubated with elution buffer (1% SDS and 100 mM $NaHCO_3$) for 15 min at room temperature in a shaker. To reverse the cross-linking, samples were incubated overnight at 65ºC with 6

ng/µl RNase (Roche, catalog 11 119 915 001) and DNA was purified using the phenol/chloroform protocol. DNA was subjected to real time quantitative PCR. DNA from each sample was normalized to its respective chromatin input. Primer sequences for the PCR reactions were: *Rorc* promoter—forward: 5'- TGT ACC ACA CTG GTT ATG GC-3', reverse: 5'- CTT CAC CAA GTG ACA TCA GG-3'; *Akr1b3* (Aldose reductase) promoter—forward: 5'-CAC CAG AAT TTC CAC ATG CC-3', reverse: 5'-AGG GAC AAC TGC ATC TGC AA-3'.

- Protein sample preparation and Western blot analysis. $CD4^+$ T cells grown for 5 days were cleaned of dead cell and debris by centrifugation on Lymphoprep™ (StemCell technologies, catalogue 07801), activated with hamster anti-mouse CD3 (1 µg/10^6 cells) plus hamster anti mouse CD28 antibodies (1 µg/10^6 cells) in plates coated with goat anti-hamster IgG (9.5 µg/cm^2) and cultured in the conditions and time indicated in the figures. Cells were washed twice with cold PBS at the same osmolality as the culture medium and lysed (8x10^6 cells in 50 µl) for 30 min at 4ºC in Nonidet P-40 lysis buffer (50 mM HEPES pH 7.4, 80 mM NaCl, 5 mM $MgCl_2$, 10 mM EDTA, 5 mM NaPPi, 1% Nonidet P-40, 2mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 1 µg/ml pepstatin A, 10 mM sodium orthovanadate and 20 mM β-glycerophosphate). Lysates were cleared by centrifugation at 16000g for 8 min at 4ºC and supernatants were collected. Protein concentration was measured using the BCA Protein Assay (Pierce, catalogue 23227) to ensure loading equal amounts of protein per sample in the gels. Lysates were then boiled in reducing 1X Laemmli buffer and equal amounts of protein from each sample were separated in SDS-polyacrylamide gels and transferred to nitrocellulose Protran membranes (Whatman, catalogue 10401396) in 25mM Tris, 192mM glycine and 20% methanol. Membranes were blocked in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 150 mM NaCl) containing 5% BSA (bovine serum albumin) for 1 hour at room temperature. Membranes were probed overnight at 4ºC with specific antibodies: rabbit polyclonal anti-

phospho-STAT3 (Tyr705) (Cell Signaling, Catalogue 9131) and for loading control, goat anti-pyruvate kinase (Abcam, Catalogue ab6191). Proteins were detected with HPR-labeled secondary antibodies and enhanced chemiluminescence (Supersignal West Pico Chemiluminescent Substrate) (Pierce, 34080).

- ELISA assay. Cell-free supernatants from cultures of isolated $CD4^+$ T cells (1×10^6 cells/ml) were harvested and IFN-γ or IL-17A were measured by enzyme-linked immunosorbent assay (ELISA) (eBiosciences, catalog 88-7314 and 88-7371 respectively).

- Anti-CD3 injection. *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice were injected intraperitoneally with 0.8 µg anti-CD3/g of mouse weight at 0 hours and again at 48 hours. Mice were analyzed 52 hours after the first injection. $CD4^+$ T cells were isolated from mesenteric lymph nodes and spleen with magnetic isolation with Dynabeads® (Invitrogen, catalog 11445D). The antibody used was clone 145-2C11 (BD Biosciences, catalogue 553058).

- DSS-induced colitis. Colitis was induced by 3% (w/v) dextran sodium sulfate (DSS; molecular weight 36-50 kDa, ICN Biochemicals) added to the drinking water for 4 or 5 days and colonic inflammation was assessed 2, 4 or 6 days after DSS treatment. Mice were weighted every day during and after DSS treatment and were sacrificed at days 4 and 6 for the first experiment and days 2 and 4 for the second experiment for further analysis. Colon length was measured for assessing inflammation and proximal and distal regions were processed for histology and mRNA extraction. Cells from mesenteric lymph nodes were also obtained for mRNA analysis. For the histological analyses, sections (4 µm) of paraffin embedded tissues were stained with hematoxylin and eosin. Images were captured and quantified with Axio Scan Z1 equipped with the ZEN 2'12 (blue edition) software (Zeiss Iberica).

- Reagents. Formaldehyde, sodim chloride, Trizma base, glycine, EDTA, sodim orthovanadate, β-glycerophospate, phenylmethanesulfonyl fluoride (PMSF), leupeptin, pepstatin A, aprotinin, sodium dodecyl sulphate (SDS), sodium pyrophosphate (NaPPi), Nonidiet P-40, methanol, bovine serum albumin (BSA) and Triton X-100 (TX-100) were purchased from Sigma-Aldrich. Sodium fluoride (NaF) was from Merck. Hepes was from Lonza.

- Statistical analysis. Statistical analyses were done with GraphPad Prism 5. Significance of the differences between sets of experimental data was determined with unpaired or paired Student's *t* tests, or non-parametric Mann Whitney tests.

RESULTS

RESULTS

1. NFAT5 drives the upregulation of some Th17-specific genes in $CD4^+$ T cells activated in hypertonic stress conditions.

The transcription factor NFAT5 is fundamental for the response of mammalian cells to hypertonic stress (Ko et al. 2000; López-Rodríguez et al. 2001; Miyakawa et al. 1999). It has been described that high levels of osmotic stress upregulate cytokines such as IL-1, IL-8, TNF-α, LT-β and BAFF (Kino et al. 2010; López-Rodríguez et al. 2001; Shapiro and Dinarello 1997), of which at least TNF-α, LT-β and BAFF are induced by NFAT5 (Kino et al. 2010; López-Rodríguez et al. 2001). Recently, two independent groups have described that hypertonicity (around 400 mOsm/kg) can increase Th17 responses (Kleinewietfeld et al. 2013; Wu et al. 2013). Kleinewietfeld and colleagues described that human $CD4^+$ T cells activated in Th17 polarizing conditions in hypertonic medium had a more pathogenic phenotype (increased levels of Th17-specific genes and also IL-2, TNF-α and CFS2). They also showed that NFAT5 and the serum and glucocorticoid-regulated kinase 1 (SGK1) enhanced the expression of IL-17A in this context (Kleinewietfeld et al. 2013). On the other hand, Wu and colleagues demonstrated that murine $CD4^+$ T cells cultured under hypertonic conditions upregulated the expression of Th17 cell-specific genes. In this case, SGK1 was required to enhance IL-17A induction only when hypertonic stress was combined with IL-23 stimulation, but not upon hypertonic stress in combination with IL-6 and TGF-β (Wu et al. 2013). Although in both cases the authors showed a relationship between the increase of some Th17 genes when T cells were polarized to Th17 under high salt conditions, it was not known what role was played specifically by osmotic stress alone and NFAT5 in the absence of polarizing cytokines.

To address this question, we used T-cell specific NFAT5 conditional knockout mice. Specifically, we took advantage of a conditional mouse that deletes NFAT5 when the *Cd4* gene is expressed in the double positive stage of thymic differentiation, *Nfat5* $^{fl/fl\ CD4\text{-}cre}$ mice (Drews-Elger et al. 2009). This strategy permits to avoid effects derived from the thymic differentiation of T cells that lose NFAT5 before this stage (Berga-Bolaños et al. 2013).

We first activated splenocytes with anti-CD3 plus anti-CD28 antibodies (CD3+CD28) and cultured them for 24 and 48 hours in non-polarizing (Th0) conditions in isotonic or hypertonic medium. Splenocytes were subjected to two different salt concentrations: 380 mOsm/kg or 420 mOsm/kg without any exogenously added polarizing cytokine. These hypertonicity levels are in the range found in pathological conditions of hypernatremia in humans (Bruck, Abal, and Aceto 1968; Papadimitriou et al. 1997; Phillips et al. 1991; Shoker 1994), and in animal models that have defects in osmoregulation or hypernatremic disorders (Drews-Elger et al. 2009). At 24 and 48 hours, $CD4^+$ T cells were isolated and mRNA levels of main cytokines and transcription factors of differentiated T cell subsets analyzed. We measured the expression of IFN-γ for Th1, IL-4 for Th2, Foxp3 for Treg, and ROR-γt and IL-17 for Th17. IL-2 was also analyzed as it is important for T cell growth (**Figure 1**). Both osmotic stress conditions caused a downregulation of IFN-γ and IL-4 mRNA, but enhanced the NFAT5-dependent expression of IL-2 and ROR-γt. At 420 mOsm/kg, the Th17 subset-specific cytokine Th17 was also upregulated, although its induction was NFAT5-independent (**Figure 2**).

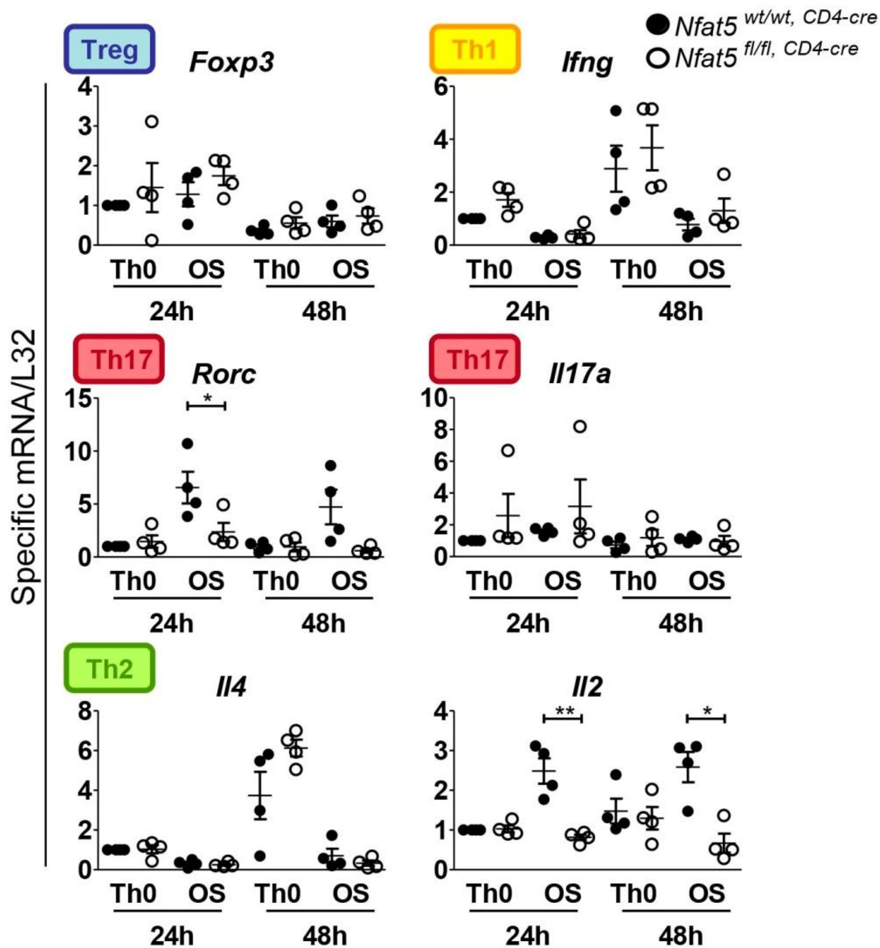


Figure 1. T cell subset-specific gene expression levels in CD4^{+} T cells isolated from splenocyte cultures under isoosmotic or hypertonic conditions at 380 mOsm/kg. Splenocytes from *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice were activated with antibodies to CD3 and CD28 in neutral conditions with isotonic osmolality (300 mOsm/kg) or in hypertonic medium (OS) and CD4^{+} T cells were isolated at 24 and 48 hours. Relative mRNA abundance for each gene was determined by RT-qPCR and values were normalized to their respective L32 mRNA levels. Circles represent single experiments, line represents the mean and the error bars represent the SEM. (n=4) Significance was determined by a paired *t* test (*=p<0.05; **=p<0.01).

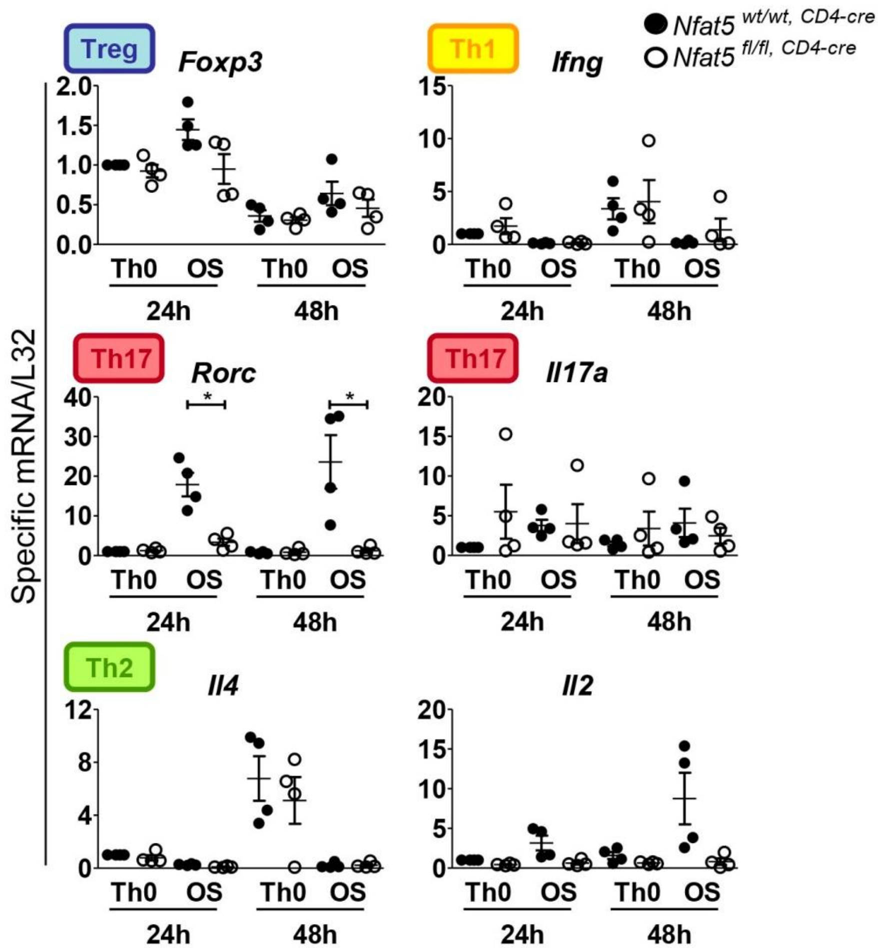
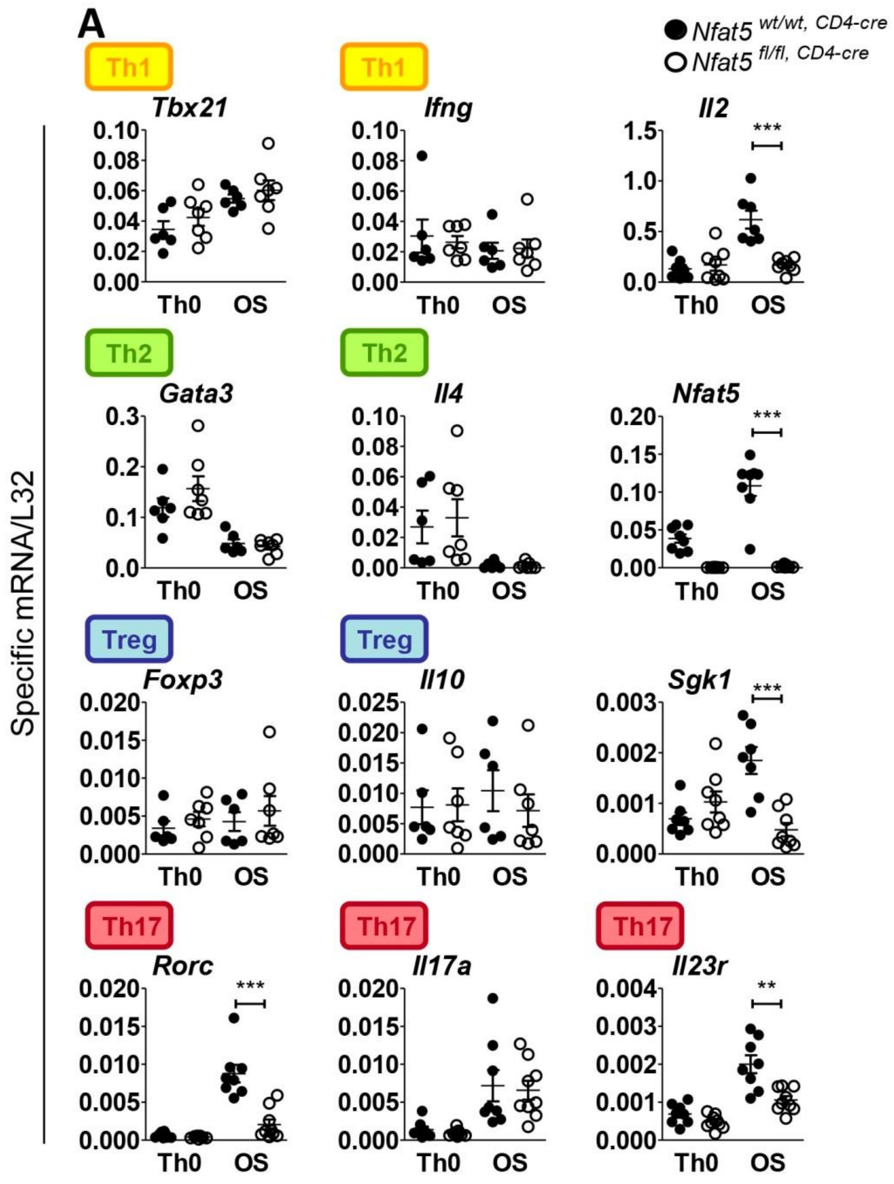


Figure 2. T cell subset-specific gene expression levels in CD4^{+} T cells isolated from splenocyte cultures under isoosmotic or hypertonic conditions at 420 mOsm/kg. Relative mRNA abundance for each gene was determined by RT-qPCR and values were normalized to their respective L32 mRNA levels. Splenocytes from *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice were activated with antibodies to CD3 and CD28 in neutral conditions with isotonic osmolality (300 mOsm/kg) or hypertonic medium (OS, 420 mOsm/Kg) and CD4^{+} T cells were isolated at 24 and 48 hours (n=4). Circles represent single experiments, line represents the mean and the error bars represent the SEM. Significance was determined by a paired *t* test (*=p<0.05).

To elucidate whether the effects of osmotic stress were intrinsic to T cells, we isolated $CD4^+$ T cells from *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice, activated them with antibodies to CD3 and CD28 during 48 to 72 hours in isotonic (300 mOsm/kg) or hypertonic medium (420 mOsm/kg), and analyzed mRNA levels of characteristic transcription factors and cytokines representing main $CD4^+$ T cells functional phenotypes. As shown in **Figure 3A**, osmotic stress induced the expression of NFAT5, IL-2, ROR-γt, IL-17 and IL-23R but downregulated GATA3 and IL-4 (Th2 markers). Again, induction of IL-2, IL-23R and ROR-γt by hypertonic stress in isolated $CD4^+$ T cells was NFAT5-dependent, whereas IL-17A did not require NFAT5. We also found that a more prolonged exposure to osmotic stress, 72 hours, enhanced the expression of IL-22 mRNA in an NFAT5-dependent manner (**Figure 3B**). We next analyzed the protein levels of IL-17. In agreement with the mRNA results, osmotic stress increased IL-17 protein levels after 48 hours of culture (**Figure 3C**). However, we found that the upregulation of IL-17 protein levels was dependent on NFAT5 (**Figure 3C**), while NFAT5-deficient lymphocytes had not shown an impairment in IL-17A mRNA induction. It is possible that the reduced ability to produce IL-17 protein by NFAT5-deficient T cells could be due to a lower capacity to maintain biosynthetic activity under prolonged osmostress (Drews-Elger et al. 2009; Ortells et al. 2012). In view of earlier works (Chen et al. 2009; Kleinewietfeld et al. 2013), we also analyzed the dependence of SGK1 expression on NFAT5 in T cells. Basal expression of SGK-1 in T cells stimulated through CD3 and CD28 did not require NFAT5 but its upregulation upon osmostress was NFAT5-dependent (**Figure 3B**). This result and the observation that NFAT5-deficient cells were also capable of expressing low levels of ROR-γt upon hypertonic stress (**Figure 3**) suggested that perhaps ROR-γt could suffice to induce IL-17A mRNA in NFAT5-deficient T cells. To address this question we used a chemical inhibition strategy (**Figures 4** and **5**).


A
Nfat5 wt/wt, CD4-cre
Nfat5 fl/fl, CD4-cre
Th1
Tbx21
Th1
Ifng
Il2
Th2
Gata3
Th2
Il4
Nfat5
Treg
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Il23r
Specific mRNA/L32
Th0 OS

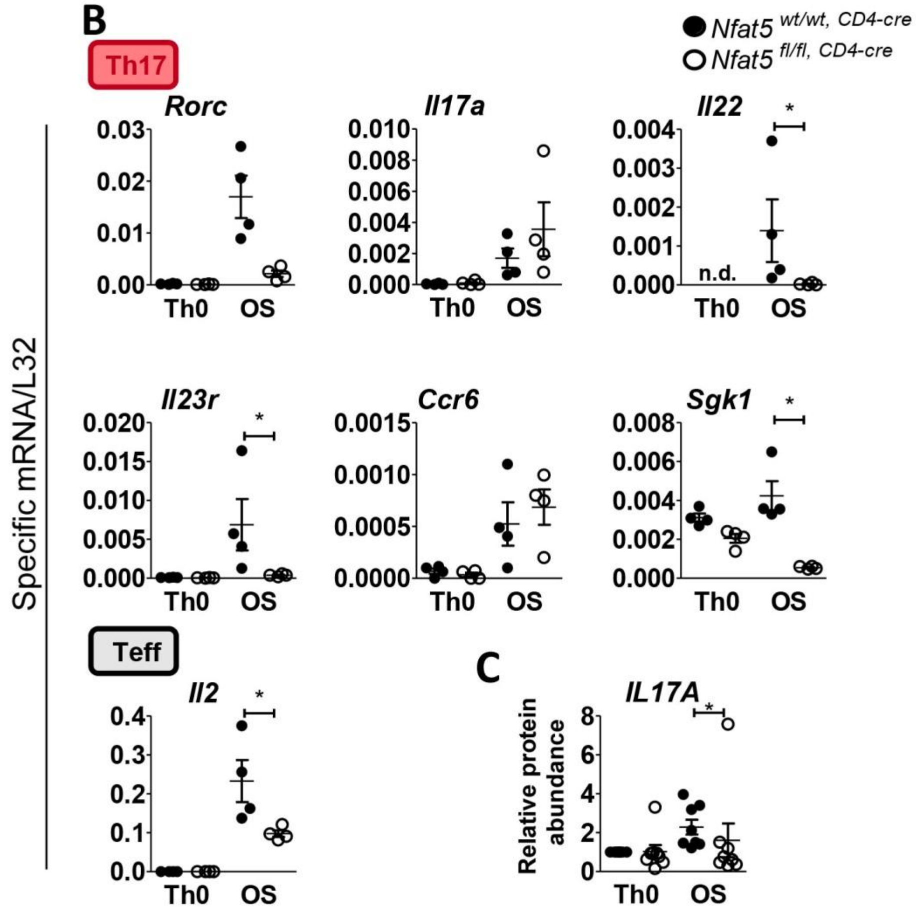


Figure 3. T cell subset-specific transcription factor and cytokine expression levels in CD4^{+} T cells cultured under isoosmotic or hypertonic conditions. CD4^{+} T cells were isolated from the spleens and lymph nodes of *Nfat5* $^{\text{wt/wt, CD4-cre}}$ and *Nfat5* $^{\text{fl/fl, CD4-cre}}$ mice and activated with antibodies to CD3 and CD28 in neutral conditions with isotonic osmolality (300 mOsm/kg) or hypertonic medium (OS, 420 mOsm/kg) and harvested at 48 hours **A)** and **C)** or 72 hours **B)**. **A), B)** Relative mRNA abundance for each gene was determined by RT-qPCR and values were normalized to their respective L32 mRNA levels. (n=6-8 in **A)** and n=4 in **B)**) **C)** IL-17A was measured in the supernatants after 48 hours. Data from 8-9 experiments is shown. Circles represent single experiments, line represents the mean and the error bars represent the SEM. Significance was determined by a nonparametric unpaired *t* test (*=p<0.05; **=p<0.01; ***=p<0.01).

We cultured CD4$^+$ T cells for 48 or 72 hours (**Figures 4** and **5** respectively) in the presence of hypertonicity and digoxin, an inhibitor that targets specifically the transcriptional activity of ROR-γt (Huh et al. 2011). As shown in **Figure 4** and **Figure 5**, in the presence of osmotic stress (OS), digoxin inhibited the expression of ROR-γt target genes: IL-17A, IL-23R, CCR6 and IL-22 without affecting the expression of the osmotic stress-responsive genes NFAT5, SGK-1, IL-2 or ROR-γt itself. These results showed that ROR-γt was needed to induce the Th17 markers IL-17A, IL-23 and IL-22 but not NFAT5, SGK-1 and IL-2.

Figure 4 (next page)**. Effect of inhibiting ROR-γt transcriptional activity on the response of CD4$^+$ T cells to osmotic stress for 48 hours.** CD4$^+$ T cells were isolated from the spleens and lymph nodes of *Nfat5* $^{\text{wt/wt, CD4-cre}}$ and *Nfat5* $^{\text{fl/fl, CD4-cre}}$ mice and activated with antibodies to CD3 and CD28 in the absence (300 mOsm/kg) or presence of hypertonic stress (OS, 420 mOsm/kg) for 48 hours. Digoxin was added at 100 nM as indicated. mRNA expression of the different markers was analyzed by RT-qPCR. Relative mRNA abundance values were normalized to their respective L32 mRNA levels. Data from 4 experiments are shown. Circles represent independent experiments, the line represents the mean and the error bars represent SEM. Significance was determined by a nonparametric unpaired *t* test (*=p<0.05). n.d.= not detected.

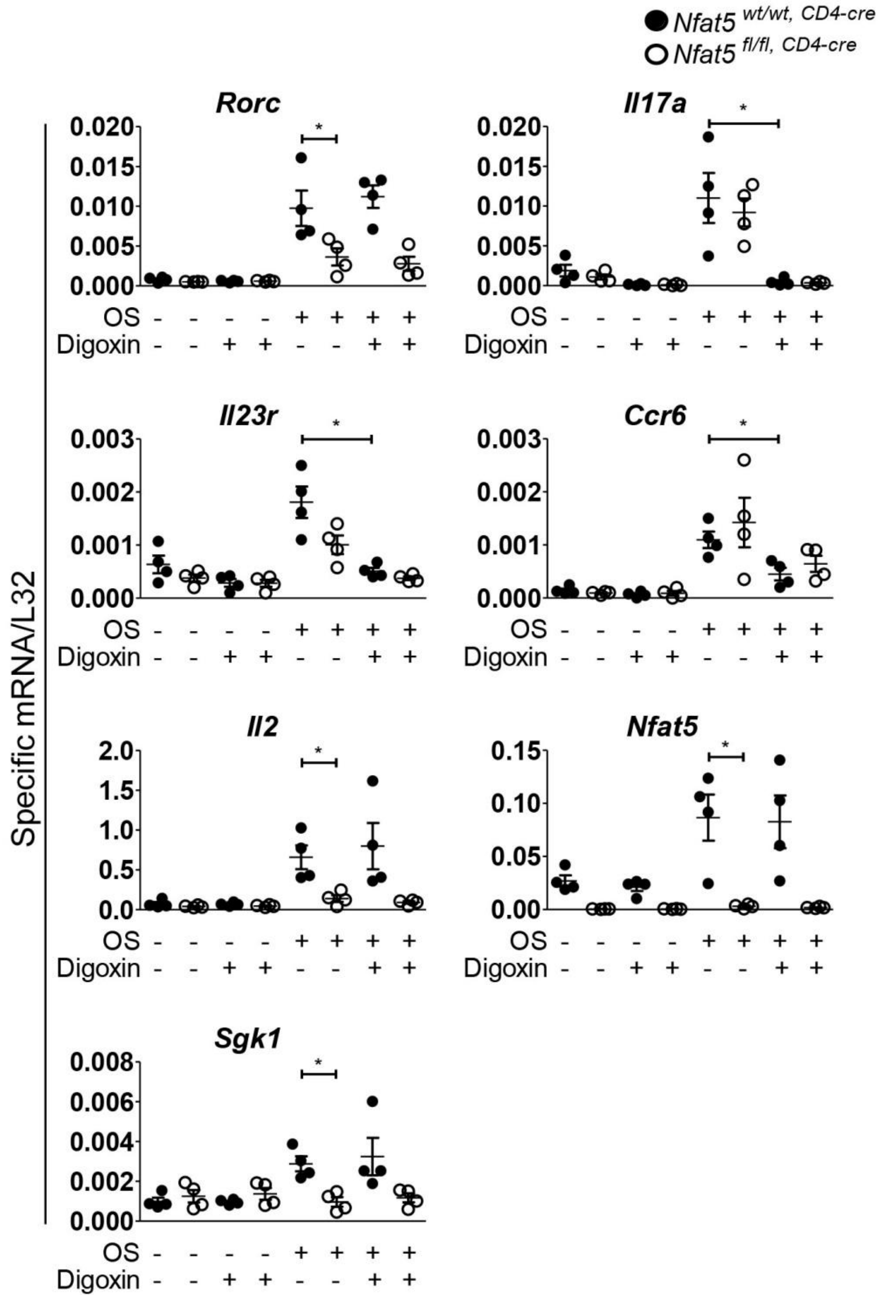


Figure 4. Effect of inhibiting ROR-γt transcriptional activity on the response of CD4⁺ T cells to osmotic stress for 48 hours

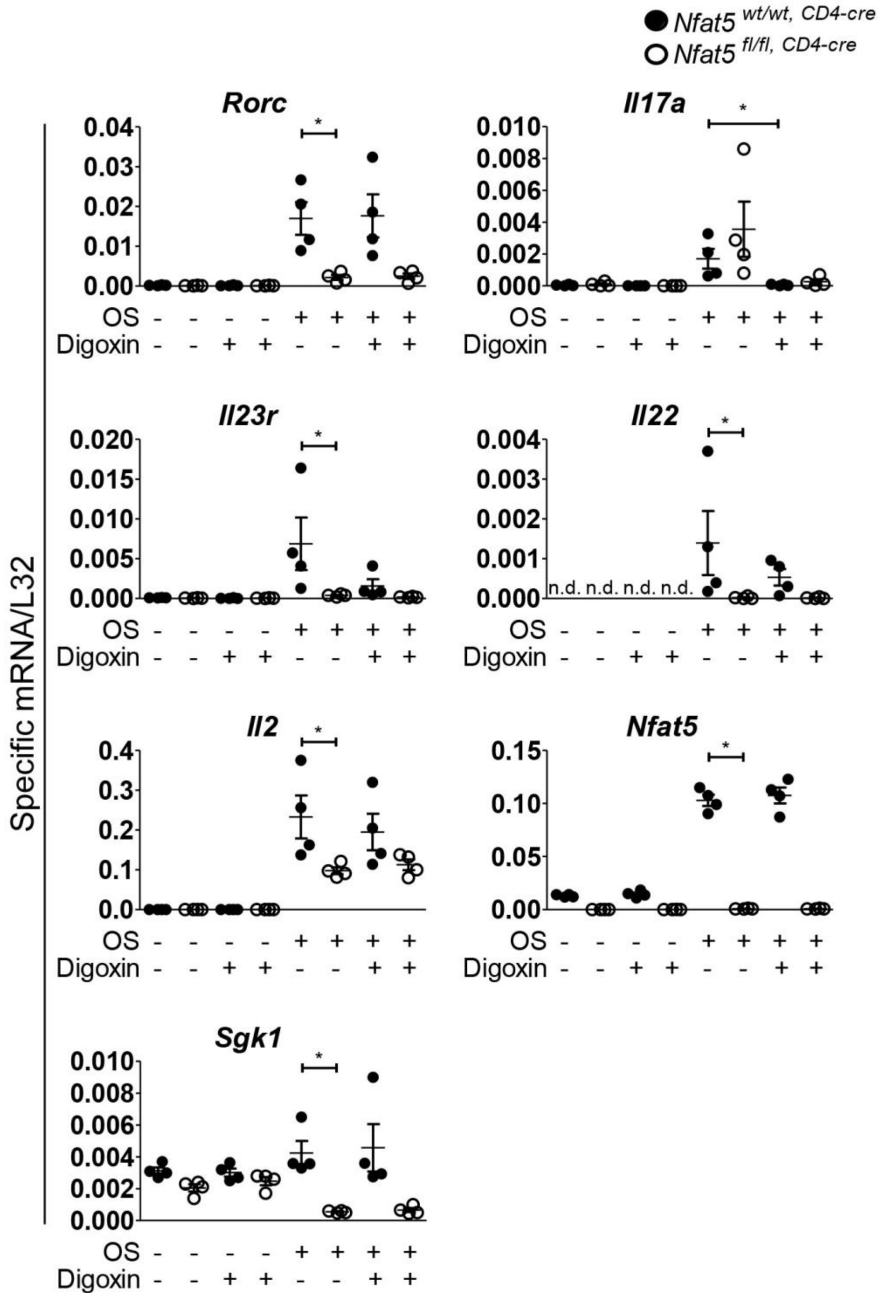


Figure 5. Effect of inhibiting ROR-γt transcriptional activity on the response of CD4⁺ T cells to osmotic stress for 72 hours. CD4⁺ T cells were isolated from the spleens and lymph nodes of *Nfat5* wt/wt, CD4-cre and *Nfat5* fl/fl, CD4-cre mice and activated with antibodies to CD3 and CD28 in the absence (300 mOsm/kg) or presence of

hypertonic stress (OS, 420 mOsm/kg) at 72 hours. Digoxin was added at 100 nM as indicated. mRNA expression of the different markers was analyzed by RT-qPCR. Relative mRNA abundance values were normalized to their respective L32 mRNA levels. Data from 4 experiments are shown. Circles represent independent experiments, the line represents the mean and the error bars represent SEM. Significance was determined by a nonparametric unpaired *t* test (*=p<0.05). n.d.= not detected.

Our results were consistent with the interpretation that NFAT5 might induce the expression of various Th17-associated genes through its ability to enhance ROR-γt expression, which has been defined as a master regulator of the Th17 phenotype (Ivanov et al. 2006). We found that the *Rorc* promoter contained various elements that fit the consensus binding site for NFAT5 (TGGAAAC/A/T) (Lopez-Rodríguez et al. 1999; Stroud et al. 2002) and were conserved in different species (**Figure 6A** in orange). We then tested whether NFAT5 could bind to the *Rorc* promoter by using chromatin immunoprecipitation assays. We observed that NFAT5 could bind to the proximal region of *Rorc* promoter in hypertonic conditions (**Figure 6**) comparably to the promoter of *Akr1b3*, the gene that encodes for aldose reductase, which is a gene induced by NFAT5 under osmotic stress conditions and it has been shown that NFAT5 binds to this site (Ko et al. 2000; Ortells et al. 2012).

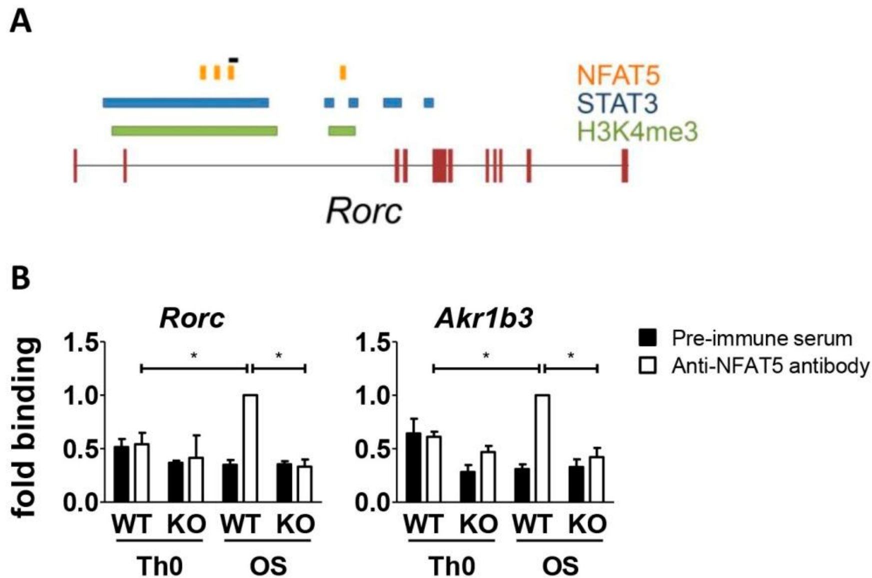


Figure 6. NFAT5 binding to the *Rorc* promoter. A) Schematic representation of the binding sites for STAT3 (in blue), sites where H3K4 is trimethylated (in green) and consensus sites for NFAT5 binding (in orange) in the *Rorc* promoter region. The exons are represented by red boxes in the gene. A black line marks the region where *Rorc* primers were designed. **B)** $CD4^+$ T cells isolated from the spleens and lymph nodes of *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$ (WT) and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ (KO) mice were expanded for 3 days in the absence of osmotic stress and reestimulated with antibodies to CD3 and CD28 in isotonic (300 mOsm/kg) or hypertonic (500 mOsm/kg) conditions during 12 hours. Formaldehyde cross-linked chromatin was immunoprecipitated with preimmune rabbit serum or a mixture of two rabbit polyclonal antibodies specific for NFAT5. A genomic region from de promoter of the aldose reductase gene (*Akr1b3*) to which NFAT5 binds was used as a positive control. Graphics represent the enrichment in chromatin immunoprecipitated by the NFAT5-specific antibodies in each sample. Data from 3 to 5 experiments for the *Rorc* promoter and from 2 to 4 experiments for the *Akr1b3* promoter are shown. The bars represent the mean and the error bars represent SEM. Statistical significance was determined by an unpaired *t* test ($p<0.05$).

Our results so far suggested that osmotic stress could induce a Th17-like phenotype in activated T cells through NFAT5 and RORγ-t. Another transcription factor that has been shown to be important for Th17 polarization in response to IL-6 is STAT3, which binds to several Th17 genes, including *Rorc*, *Il17a* and *Il23r* to promote their expression (Durant et al. 2010; Ghoreschi et al. 2010). STAT3 is also necessary for the expression of IL-22 (Yeste et al. 2014). High salt concentrations (around 300 mM, equivalent to 600 mOsm/kg) have been described to phosphorylate STAT3 at short time points (Gatsios et al. 1998), so we wondered if less extreme hypertonicity levels could contribute to the phosphorylation of this factor and therefore to the induction of the Th17-like phenotype. T cells proliferating under isotonic conditions were reestimulated with antibodies to CD3 and CD28 in normal conditions (Th0), the presence of osmotic stress (420 mOsm/kg) or Th17-polarizing cytokines IL-6 and TGF-β for short time points. As shown in **Figure 7**, the phosphorylation of STAT3 at Tyr 705 was rapidly induced in Th17 conditions, reaching a peak at 4 hours after stimulation. STAT3 was also activated, albeit with a slower kinetics, by CD3 and CD28 costimulation without additional cytokines, and this activation was partially impaired when cells were simultaneously exposed to hypertonic stress. This result suggested that the stimulatory effect of hypertonic stress on the induction of several Th17-associated genes was not mediated by enhanced activation of STAT3.

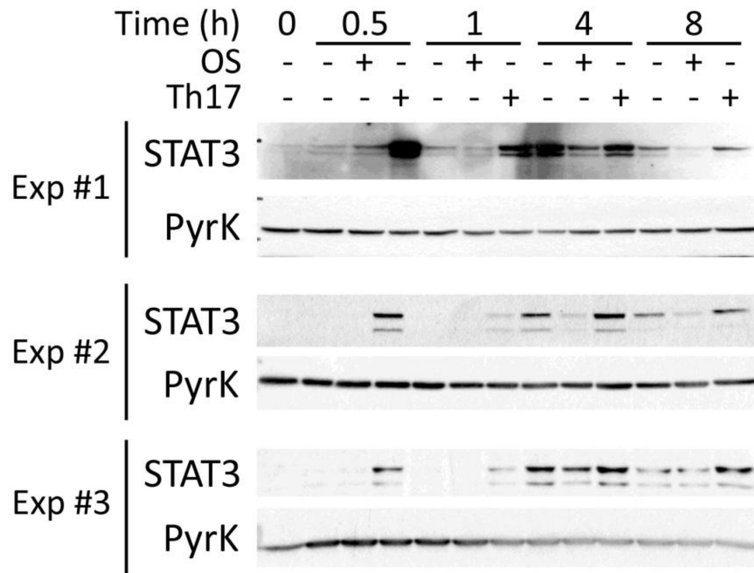


Figure 7. **STAT3-Tyr705 phosphorylation in activated T cells.** Phosphorylation of Tyr-705 of STAT3 was analyzed by Western blot in lysates of $CD4^+$ T cells expanded for 6 days in isotonic conditions and reestimulated with antibodies to CD3 and CD28 were cultured under indicated conditions: OS (osmotic stress, 420 mOsm/kg) and Th17 (TGF-β and IL-6). Pyruvate kinase (PyrK) is shown as a protein loading control. The figure shows three independent experiments.

We also analyzed two additional pathways activated downstream the T cell receptor that had been previously shown to be relevant in osmostress responses and Th17 differentiation: the mTOR pathway (Delgoffe et al. 2009, 2011; Lee et al. 2010; Ortells et al. 2012) and the calcineurin pathway (Hermann-Kleiter and Baier 2010; Macian 2005). As shown in **Figure 8,** the mTOR inhibitor rapamycin strongly reduced the expression of IL-17A, IL-2 and IL-23R mRNA induction by hypertonic stress but caused a weaker inhibition of ROR-γt. The calcineurin inhibitor FK506 strongly inhibited IL-2 and impaired ROR-γt and IL-17A mRNA induction.

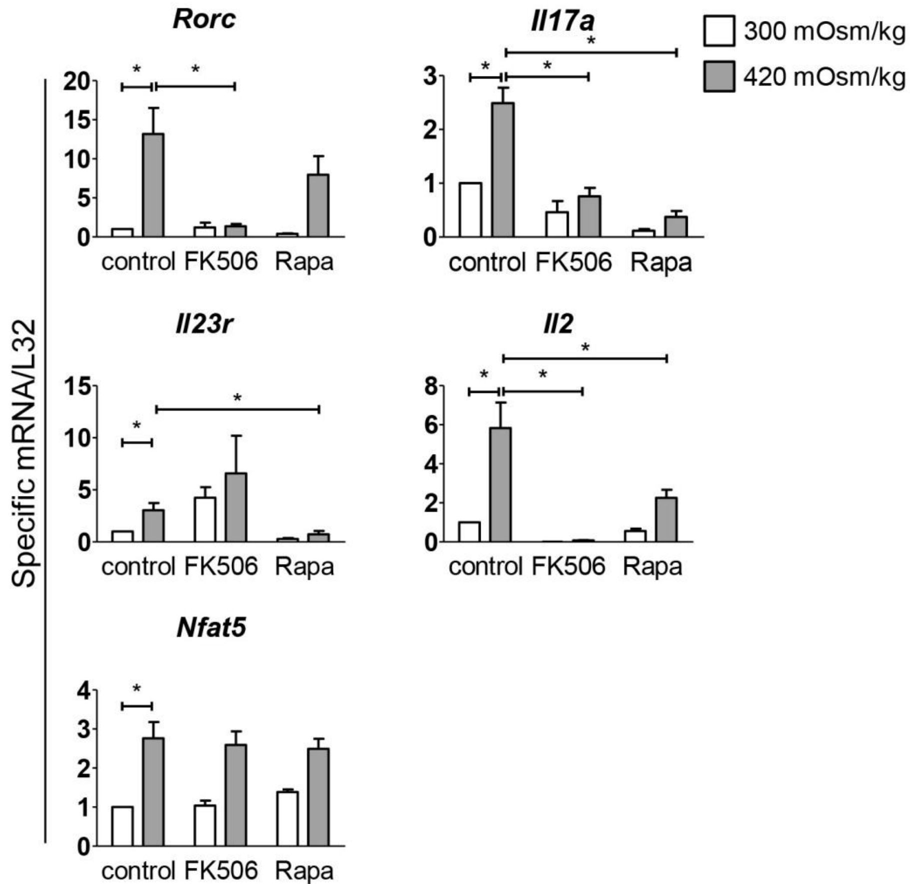


Figure 8. Involvement of calcineurin and mTOR in the induction of Th17 cell-associated genes by osmotic stress. Splenocytes were stimulated for 24 hours with antibodies to CD3 and CD28 in isotonic or hypertonic medium, without or with the calcineurin inhibitor FK506 at 100 nM or the mTOR inhibitor rapamycin (Rapa) at 50 nM. $CD4^+$ T cells were isolated to determine the mRNA abundance by RT-qPCR. Values were normalized to their respective L32 mRNA levels. The values are shown as relative induction respective to the levels of the control in isotonic conditions. Data from 4 experiments is shown. The bars represent the mean and the error bars represent SEM. Significance was determined by nonparametric *t* test (*=p<0.05).

The finding that NFAT5 could regulate several Th17-associated genes in T cells exposed to hypertonic stress led us to analyze whether it also contributed to Th17 cell differentiation in isotonic conditions. We stimulated wild-type

and NFAT5-deficient CD4+ T cells with antibodies to CD3 and CD28 in the presence of the Th17-differentiating cytokines IL-6 and TGF-β for 48 hours (**Figure 9A and 9C**) and 96 hours (**Figure 9B**). As shown in **Figure 9**, cells that lacked NFAT5 had an impaired ability to induce IL-17A mRNA and protein at both time points. Moreover, at longer time points NFAT5 also contributed to IL-23R expression. However, NFAT5 did not regulate ROR-γt expression in these stimulatory conditions.

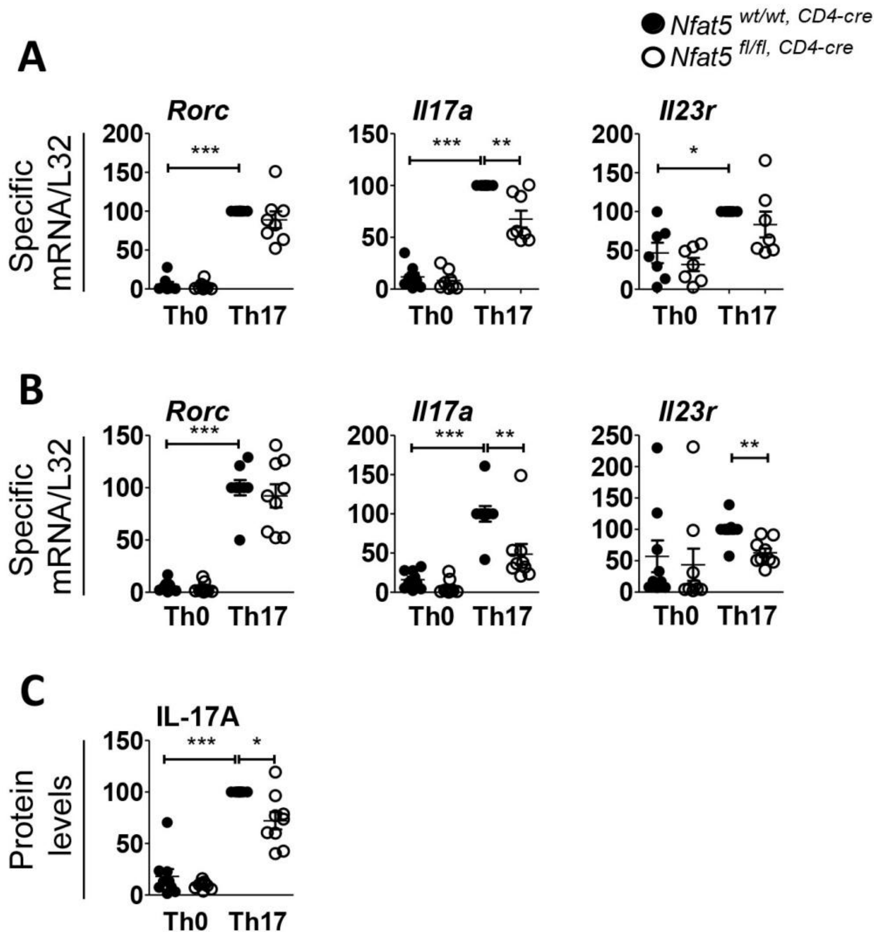


Figure 9. Th17 cell polarization in NFAT5-deficient CD4+ T cells in the absence of osmotic stress. CD4+ T cells were isolated from the spleens and lymph nodes of *Nfat5* wt/wt, CD4-cre and *Nfat5* fl/fl, CD4-cre mice, and stimulated with antibodies to CD3 and CD28, plus IL-6 and TGF-β. **A)** and **B)** mRNA expression of the indicated markers

was analyzed by RT-qPCR. Relative mRNA abundance values were normalized to their respective L32 mRNA levels. **A)** $CD4^+$ T cells were cultured for 48 hours. Circles represent independent experiments (n=8), the line represents the mean and the error bars represent SEM. Significance was determined by a paired *t* test (*=p<0.05; **=p<0.01; ***=p<0.01). **B)** $CD4^+$ T cells were cultured for 96 hours. Circles represent independent experiments (n=9), the line represents the mean and the error bars represent SEM. Significance was determined by an unpaired *t* test (**=p<0.01; ***=p<0.01). **C)** IL-17A protein measurement of the supernatant after 48 hour culture by ELISA. Circles represent independent experiments (n=9), the line represents the mean and the error bars represent SEM. Significance was determined by a paired *t* test (*=p<0.05; ***=p<0.01).

Altogether, these results indicated that NFAT5 was involved in the induction of a Th17-like phenotype by osmotic stress, and it could also regulate Th17 polarization induced by TGF-β and IL-6 in the absence of osmotic stress. Under osmotic stress, NFAT5 was recruited to the *Rorc* promoter and increased its expression, and enhanced IL-23R and IL-22 mRNA expression. Osmotic stress also increased IL-17 production, although transcription of *Il17a* was not dependent on NFAT5. Under Th17 differentiating conditions and independently of osmotic stress, NFAT5 could contribute to the induction of IL-17 and IL-23R, although it did not participate in inducing ROR-γt.

2. Analysis of the response of *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice in the experimental model of dextran sulphate sodium-induced colitis.

We sought to determine if NFAT5 deficiency in T cells might alter the outcome of Th17-sensitive inflammatory responses *in vivo*. We used the model of dextran sulphate sodium (DSS)-induced colitis. DSS disrupts the intestinal epithelial barrier and causes an inflammatory response driven by Th17 and Th1 cells, among other leukocytes (Neurath 2014; Shale, Schiering, and Powrie 2013; Strober, Fuss, and Blumberg 2002). We first did a trial to set up the DSS-induced colitis experiment. DSS (MW = 32-50 KDa) was given with drinking water to the mice for four days, and they were sacrificed at days 4 and 6 after the treatment. Mice were monitored for body weight along the duration of the experiment. In this trial we found a moderate severity of the disease judged by loss of body weight, although there was a clear effect of DSS on colon shortening (**Figure 10**). Surprisingly, colons of *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice were shorter than those of wild-type mice (**Figure 10B**). Analysis of mRNA levels of the mesenteric lymph nodes (mLN) of wild-type and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice revealed higher levels of IFN-γ, IL-17 and IL-22 mRNA in mLN of mice whose T cells lacked NFAT5 at day 4 but not at day 6 after DSS treatment (**Figure 10C**). We did not observe differences between both mouse genotypes in ROR-γt mRNA and other markers associated with polarized T cell subsets.

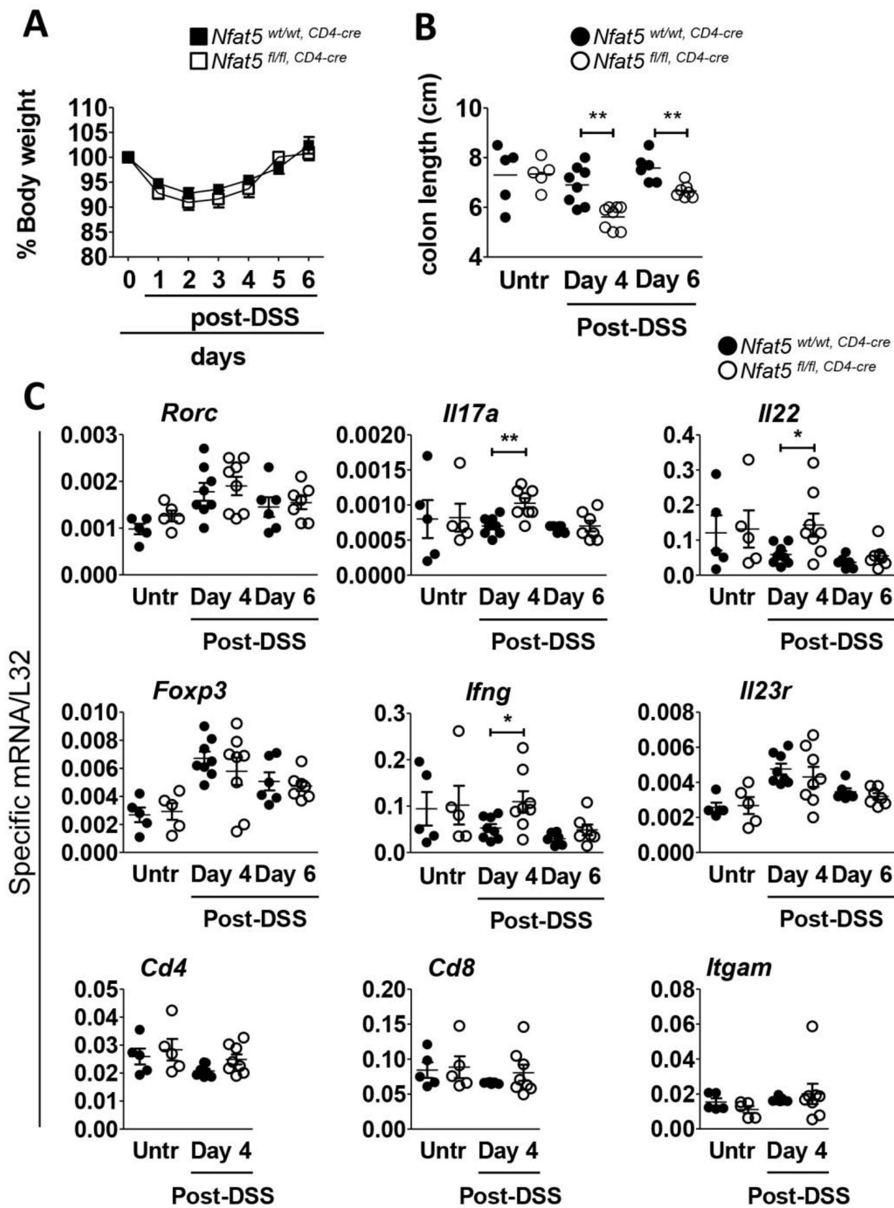


Figure 10. DSS-induced colitis in wild-type and *Nfat5* fl/fl, CD4-cre mice. *Nfat5* wt/wt, CD4-cre and *Nfat5* fl/fl, CD4-cre mice were administered 3% DSS by drinking water for 4 days. **A)** Body weight changes after DSS treatment. **B)** Colon length was measured after euthanizing mice at the days indicated. **C)** mRNA analysis of mesenteric lymph nodes of *Nfat5* wt/wt, CD4-cre and *Nfat5* fl/fl, CD4-cre mice upon 4-day DSS treatment. Relative mRNA abundance for each gene was determined by RT-qPCR and values

were normalized to their respective L32 mRNA levels. Circles represent single mice and the bars represent the mean±SEM of each group. Statistical significance was determined by a nonparametric unpaired t test (*=p<0.05; **=p<0.01) (n=5 for controls, n=8 for day 4 and n=6 for *Nfat5* $^{wt/wt, CD4-cre}$ day 6 and n=7 for *Nfat5* $^{fl/fl, CD4-cre}$ day 6). Post-DSS means days after finishing the 4-day DSS treatment; day 0 means the moment before the treatment was started.

We repeated the experiment using a longer DSS treatment (3% DSS, 5 day-treatment) and a larger number of mice. This time, the loss of body weight was more pronounced, and we found it to be more severe in *Nfat5* $^{fl/fl, CD4-cre}$ mice than in wild-type littermates (**Figure 11A**). By day 3-4 post treatment wild-type mice started recovering weight, while *Nfat5* $^{fl/fl, CD4-cre}$ mice did not. Colon shortening was again more pronounced in *Nfat5* $^{fl/fl, CD4-cre}$ mice at both time points after DSS treatment (**Figure 11B**). Histopathology analysis of colon sections revealed the presence of similar lesions (both in extension and morphology) in wild-type and *Nfat5* $^{fl/fl, CD4-cre}$ mice at day 2 post-DSS (**Figure 11C**). These lesions were characterized by severe inflammation, with a diffused distribution involving mucosa and submucosa, epithelial erosion and crypt disappearance, loss of goblet cells and areas of mucosal ulceration. However, although mice began to recover on day 4 post-DSS, the extension of colon lesions was significantly higher in *Nfat5* $^{fl/fl, CD4-cre}$ mice than in wild-type controls (**Figure 11C**). Altogether, these results indicated that a deficiency of NFAT5 in T cells could exacerbate the disease in the DSS-induced colitis model.

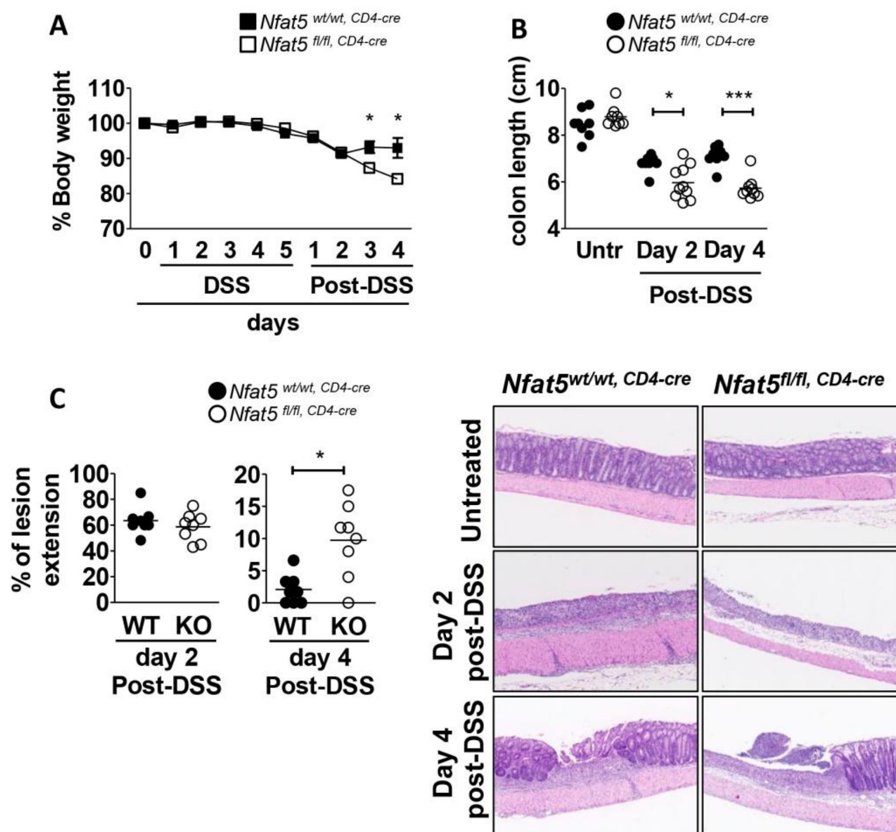


Figure 11. Susceptibility of *Nfat5* $^{fl/fl, CD4\text{-}cre}$ mice to acute DSS-induced colitis. *Nfat5* $^{wt/wt, CD4\text{-}cre}$ and *Nfat5* $^{fl/fl, CD4\text{-}cre}$ mice were administered 3% DSS in drinking water for 5 days. **A)** Body weight changes after DSS treatment of each group at different time points are shown (mean±SEM) as percentage of body weight at day 0 before starting the DSS treatment (100%). **B)** Colon length was measured after euthanizing mice at the days indicated. **C)** DSS-induced lesion extension was assessed by histological analysis. Circles represent single mice and the bars represent the mean of each group. Statistical significance was determined by a nonparametric unpaired *t* test (*=p<0.05; ***=p<0.001) (n=8 for all the groups except *Nfat5* $^{fl/fl, CD4\text{-}cre}$ day 4, n=9). Post-DSS means days after finishing the 5-day DSS treatment; day 0 means the moment before the treatment was started.

We then analyzed mRNA levels in colon of various genes whose expression could inform about the activity of different T cell subsets: Th17 and Th1 as disease-promoting, and Treg as disease-attenuating cells (Alex et al. 2009; Bird et al. 1998; Feng et al. 2011; Globig et al. 2014; Izcue, Coombes, and Powrie 2009; Kumawat et al. 2013; Perše and Cerar 2012; Rovedatti et al. 2009). We also analyzed the expression of aldose reductase mRNA, as a readout of local activation of an osmostress response. We analyzed two different fragments of the colon: the proximal colon (close to the cecum) (**Figure 12**) and the distal colon (close to the rectum) (**Figure 13**).

There was an increase in the mRNA levels of different cytokines and transcription factors upon DSS treatment in both colon regions (**Figure 12 and 13**). In the case of the proximal colon (**Figure 12**) ROR-γt, IL-22 and Foxp3 were mainly induced at day 2 while IFN-γ increased along the time. There was no difference between wild type and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice. Expression of the osmosensitive gene aldose reductase was not elevated in DSS-induced mice compared to the untreated mice. On the other hand, we found a greater expression of mRNAs of IFN-γ, IL-17A and IL-22 in distal colon sections of *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice than in wild-type ones (**Figure 13**). The levels of these cytokines have been shown to be increased in the colon of animal models of colitis and patients with inflammatory bowel disease (Alex et al. 2009; Perše and Cerar 2012; Strober et al. 2002). That IL-22 and IL-17A mRNA were upregulated in the colons of mice whose T cells lacked NFAT5 was intriguing, as we had previously found that in T cells cultured in a hypertonic environment NFAT5 promoted, not inhibited, the expression of IL-22 and other Th17 genes.

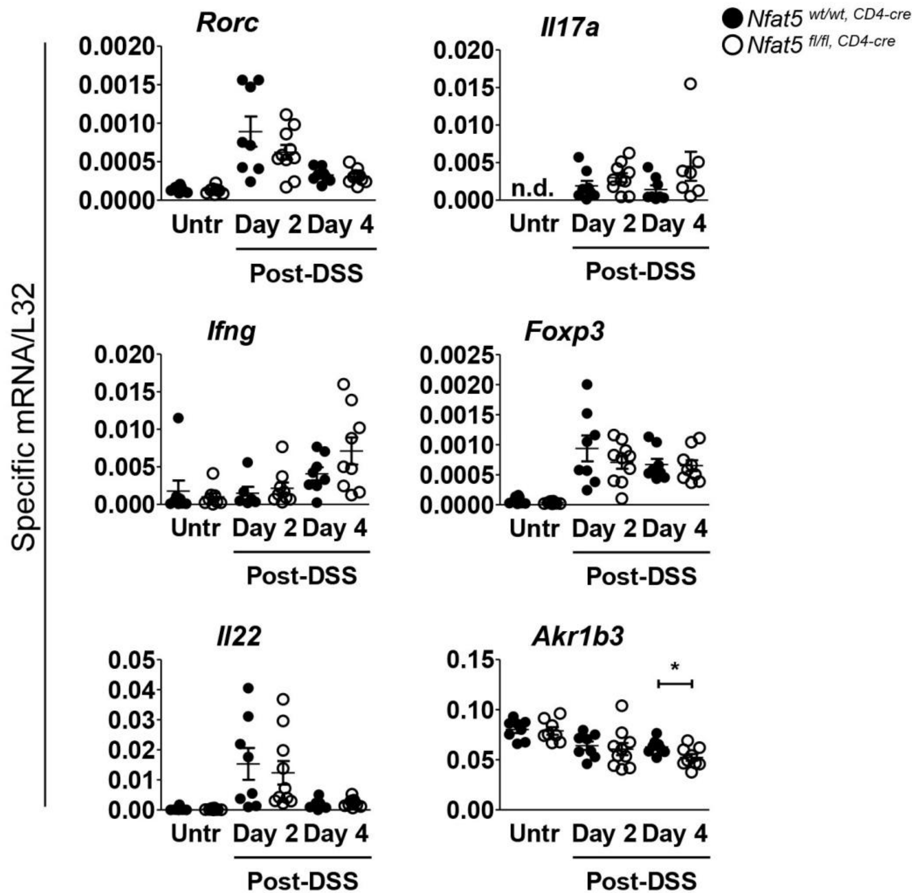


Figure 12. Cytokine mRNA levels in distal colon upon DSS treatment of wild-type and *Nfat5* fl/fl, CD4-cre mice. mRNA analysis of whole cell types in the distal colon of *Nfat5* wt/wt, CD4-cre and *Nfat5* fl/fl, CD4-cre mice upon 5-day DSS treatment. Relative mRNA abundance for each gene was determined by RT-qPCR and values were normalized to their respective L32 mRNA levels. Circles represent single mice and the bars represent the mean±SEM of each group (n=8 in all groups except *Nfat5* fl/fl, CD4-cre day 4, n=9). Statistical significance was determined by a nonparametric unpaired *t* test (*=p<0.05). Post-DSS means days after finishing the 5-day DSS treatment.

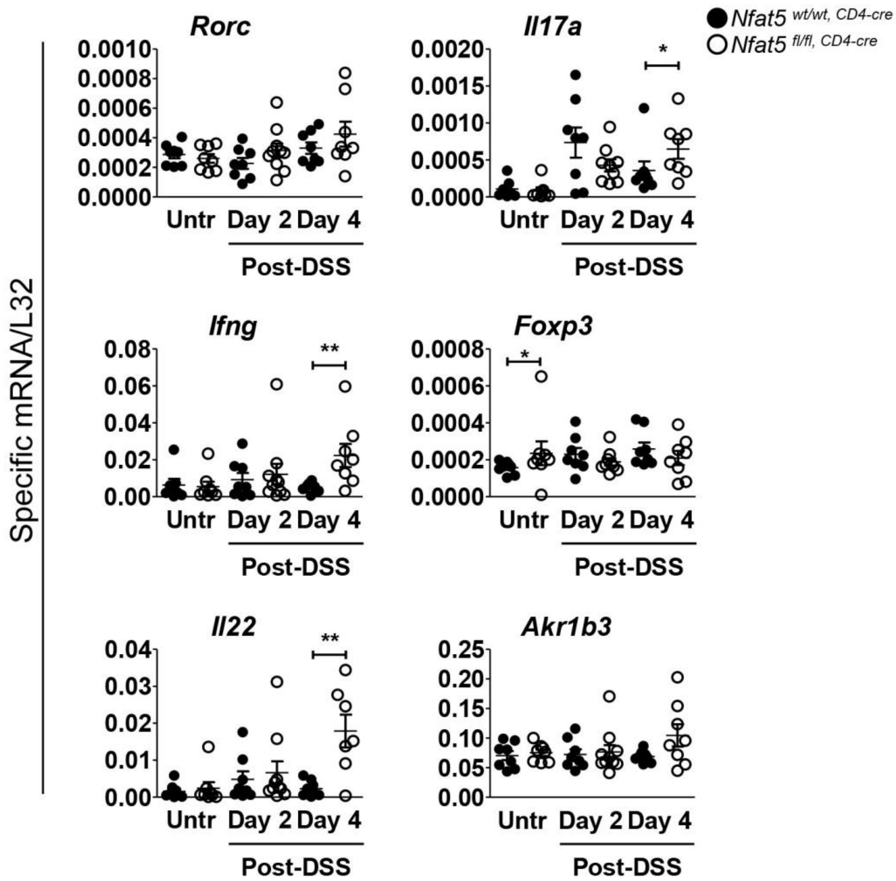
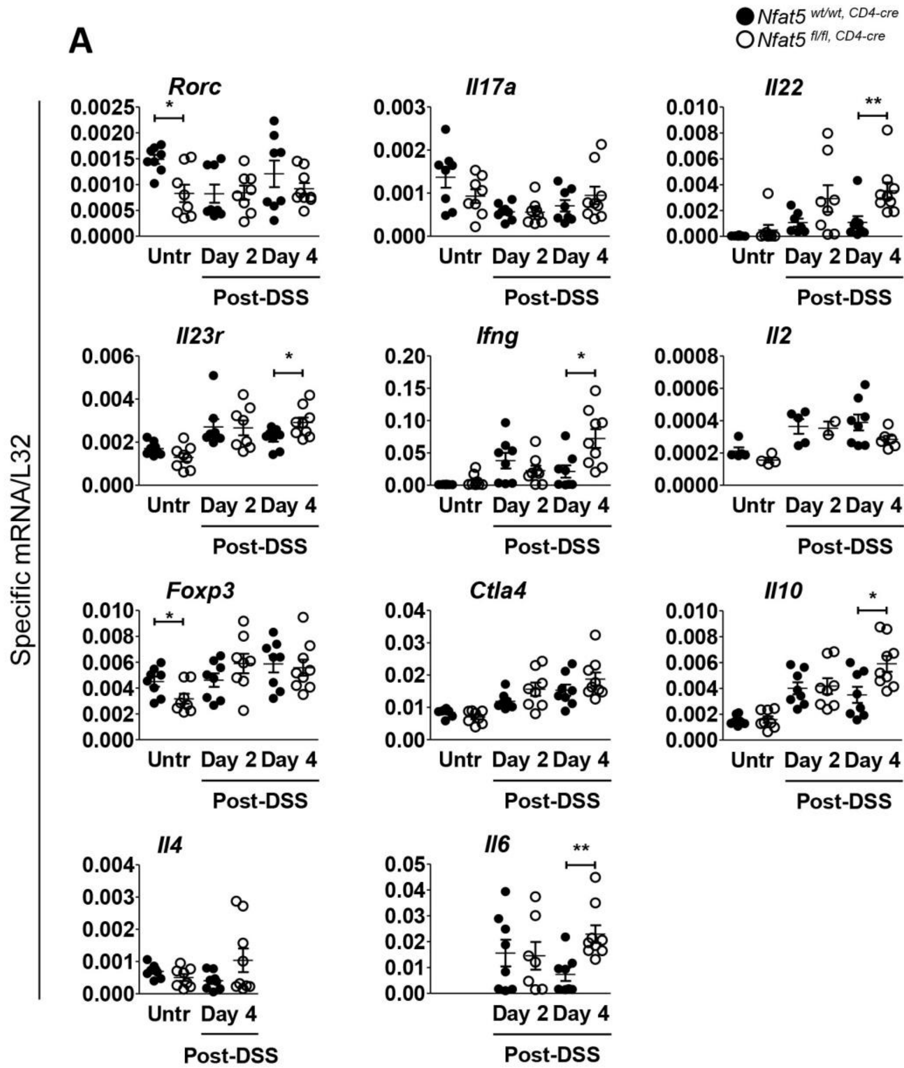


Figure 13. Cytokine mRNA levels in proximal colon upon DSS treatment of wild-type and *Nfat5* fl/fl, CD4-cre mice. mRNA analysis of whole cell types in the proximal colon of *Nfat5* wt/wt, CD4-cre and *Nfat5* fl/fl, CD4-cre mice upon 5-day DSS treatment. Relative mRNA abundance for each gene was determined by RT-qPCR and values were normalized to their respective L32 mRNA levels. Circles represent single mice and the bars represent the mean±SEM of each group (n=8 in all groups except *Nfat5* fl/fl, CD4-cre day 4, n=9). Statistical significance was determined by a nonparametric unpaired *t* test (*=p<0.05; **=p<0.01). Post-DSS means days after finishing the 5-day DSS treatment.

We also found that the mLN of DSS-treated mice had increased mRNA levels for IL-22, IL-6 and IFN-γ, and other genes important for immune responses, such as IL-23R and CTLA4 (**Figure 14A**) (Alex et al. 2009; Brand et al. 2006; Eken et al. 2014; Fais et al. 1991; Feng et al. 2011; Fuss et al. 1996; Globig et al. 2014; Ito et al. 2006; Maul et al. 2005; Maynard et al. 2007; Monteleone et al. 2011; Mudter et al. 2008; Sakuraba et al. 2009; Sugimoto and Ogawa 2008; Waldner and Neurath 2014; Zenewicz et al. 2008; Zindl et al. 2013). These differences were more pronounced at day 4 post treatment than at day 2. There were some genes whose expression was significantly different between wild-type and *Nfat5* $^{fl/fl, CD4-cre}$ mice. The Th17-related genes *Il23r* and *Il22*; and the Th1 gene *Ifng* showed enhanced expression in *Nfat5* $^{fl/fl, CD4-cre}$ mice at day 4 after DSS treatment (**Figure 14A**).

As the sample analyzed was the whole mesenteric lymph node (mLN), the differences observed between wild-type and *Nfat5* $^{fl/fl, CD4-cre}$ mice could be due to the a different proportion of the cell types involved in colitis. Since the sample we had was mRNA from the whole tissue, we analyzed the expression of specific receptors for different cell types as an approximate surrogate of their relative distribution: Thy 1, CD8, CD4 and TCR-α for T cells, NK1.1 (*Klbr1c* gene) for NK cells, and CD11b (*Itgam* gene) for monocytes (**Figure 14B**). We found no substantial differences between both mouse genotypes, other than a mild increase of CD4 and NK1.1 at day 2 in mLN of *Nfat5* $^{fl/fl, CD4-cre}$ mice, although without a parallel increase in TCR-α or Thy1.1, and enhanced TCR-α expression in mLN of *Nfat5* $^{fl/fl, CD4-cre}$ mice at day 4.


A
Nfat5 wt/wt, CD4-cre
Nfat5 fl/fl, CD4-cre
Specific mRNA/L32
Rorc
Il17a
Il22
Il23r
Ifng
Il2
Foxp3
Ctla4
Il10
Il4
Il6
Untr
Day 2
Day 4
Post-DSS
*
**

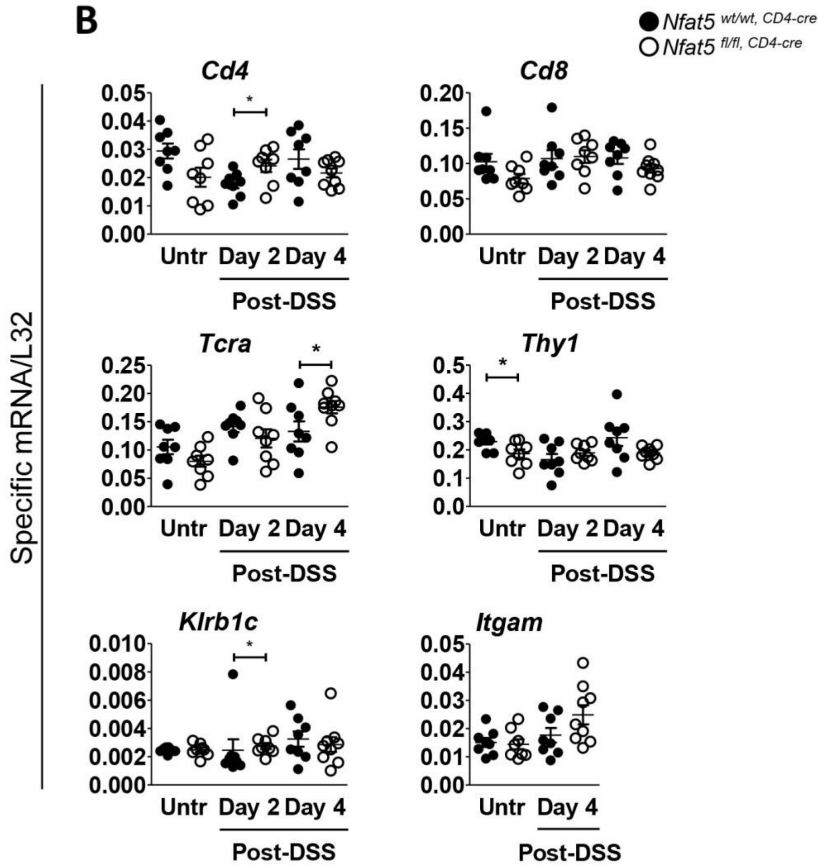


Figure 14. mRNA levels of T cell subset-specific genes and other leukocyte markers upon DSS treatment in wild-type and *Nfat5* $^{fl/fl, CD4\text{-}cre}$ mice. mRNA analysis of whole mesenteric lymph nodes of *Nfat5* $^{wt/wt, CD4\text{-}cre}$ and *Nfat5* $^{fl/fl, CD4\text{-}cre}$ mice upon 5-day DSS treatment. Relative mRNA abundance for each gene was determined by RT-qPCR and values were normalized to their respective L32 mRNA levels. **A)** T cell subset specific mRNA analysis: ROR-γt, IL-17A, IL-23R and IL-22 for Th17; Foxp3, CTLA4 and IL-10 for Treg; IFN-γ and IL-2 for Th1, and IL-4 for Th2. **B)** Analysis of leukocyte markers: *Thy1, Tcra, Cd8* and *Cd4* for T cells*, Klrb1c* (NK1.1) for NK cells and *Itgam* (CD11b) for monocytes and macrophages. Circles represent single mice and the bars represent the mean±SEM of each group (n=8 in all groups except *Nfat5* $^{fl/fl, CD4\text{-}cre}$ day 4, n=9). Statistical significance was determined by a nonparametric unpaired *t* test (*=p<0.05). Post-DSS means days after finishing the 5-day DSS treatment.

In summary, our analysis of the colitis experiments revealed that the lack of NFAT5 in T cells had a disease-exacerbating effect, evidenced by a more pronounced weight loss and colon shortening in *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice. Analysis of different markers in colon tissue and draining mesenteric lymph nodes showed that *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice also exhibited elevated levels of IFN-γ and IL-22 in comparison with wild-type mice. Since IFN-γ has been shown to contribute to the pathogenesis of colitis (Ito et al. 2006; Yamashita et al. 2013), it is possible that the delayed recovery of body weight and colon length observed in *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice after DSS removal could be associated with the greater induction of IFN-γ. On the other hand, we do not have an obvious explanation for the possible role of the elevated IL-22 also observed in *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice. Although IL-22 has been shown to be protective in mouse models of experimental colitis (Brand et al. 2006; Monteleone et al. 2011; Zindl et al. 2013), it is induced in response by cytokines such as IL-1β and IL-23 often found in inflammatory milieus (Dudakov, Hanash, and van den Brink 2015; Liang et al. 2006; Ma et al. 2008; Zheng et al. 2007). Among various interpretations (which will be elaborated in the discussion section), it is possible that the greater expression of IL-22 in mLN and colon in *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice could either reflect a tissue-healing response elicited as result of local damage, or a persistence of inflammatory conditions.

3. Lack of NFAT5 in T cells enhances their expression of IFN-γ both *in vivo* and *in vitro*.

NFAT5 promoted a Th17-like response in T cells under hypertonicity conditions. Nonetheless, the DSS-induced colitis experiments revealed unexpected effects of NFAT5 in the immune response: the lack of NFAT5 only in T cells induced a more exacerbated disease and a proinflammatory response in mLNs. It has been previously described that increased concentrations of IFN-γ promote a more severe pathology of colitis (Ito et al. 2006; Yamashita et al. 2013). The RNA analyzed in the experiment was from the whole mLN tissue, so we could not determine whether this increase on IFN-γ levels occurred specifically in T cells.

To test this, we used the injection of anti-CD3 antibody as an *in vivo* model of generalized T cell activation (Esplugues et al. 2011; Hirschi et al. 1989; Scott et al. 1990). Mice were injected intraperitoneally with anti-CD3 antibody at time 0 and again at 48 hours, and T cells were isolated from mLN 4 hours after the second injection (**Figure 15**). The expression of Th1 and Th17 gene products such as T-bet, IFN-γ, IL-12Rβ2, ROR-γt, IL-17A, IL-22 and IL-23R increased upon anti-CD3 treatment. NFAT5 mRNA levels were also higher upon reactivation. Regarding the differences between the two mouse genotypes, there was an enhanced expression of IFN-γ and IL-17A mRNA in *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice.

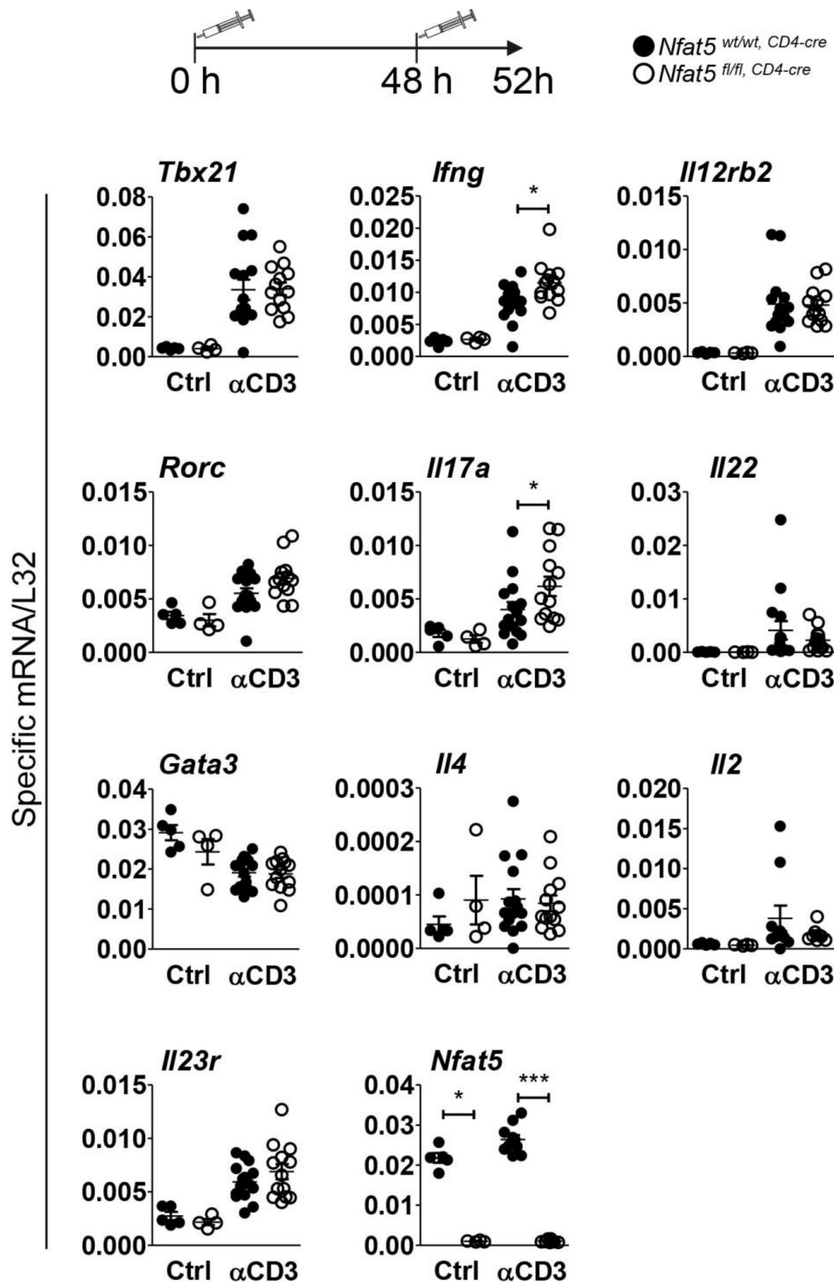


Figure 15. Expression of Th1- and Th17-associated genes in wild-type and *Nfat5* $^{fl/fl,\ CD4\text{-}cre}$ mice upon in vivo treatment with an antibody to CD3. Schematic representation of the experimental methodology. Mice were injected intraperitoneally with anti-CD3, a recall injection was given after 48 hours, and mice were sacrificed 4 hours later. mRNA analysis of $CD4^+$ T cells isolated from mLN of *Nfat5* $^{wt/wt,\ CD4\text{-}cre}$

and *Nfat5* $^{fl/fl, CD4-cre}$ mice. Relative abundance for each gene was determined by RT-qPCR. Values were normalized to their respective L32 mRNA levels. Each point represents one mouse and the bars represent the mean±SEM of each group. Two experiments were done; the first with 5 wild-type and 4 *Nfat5* $^{fl/fl, CD4-cre}$ untreated mice plus 5 wild-type and 5 *Nfat5* $^{fl/fl, CD4-cre}$ treated mice and the second with 10 wild-type and 8 T *Nfat5* $^{fl/fl, CD4-cre}$ treated mice. Significance was determined by a nonparametric *t* test. (*=$p<0.05$; ***=$p<0.001$).

Upon direct activation *in vivo*, $CD4^+$ T cells from *Nfat5* $^{fl/fl, CD4-cre}$ mice showed greater induction of IFN-γ mRNA than T cells from wild-type mice, whereas T cells from both mouse genotypes showed comparable induction of IL-22. This result supported the notion that upregulation of IFN-γ levels in colon and mesenteric lymph nodes in *Nfat5* $^{fl/fl, CD4-cre}$ mice in the DSS-induced colitis experiments might be due to direct enhanced IFN-γ expression by T cells lacking NFAT5.

We next determined whether the lack of NFAT5 in lymphocytes promoted the induction of IFN-γ in T cells *in vitro*. In the absence of polarizing cytokines, NFAT5-deficient T cells expressed similar levels of IFN-γ compared to wild-type T cells (**Figure 3** and **Figure 16**). However, when cultures were supplemented with the Th1-polarizing cytokine IL-12, both induced similar levels of IFN-γ mRNA at 48 hours, but at 96 hours NFAT5-deficient T cells maintained IFN-γ expression while wild-type ones downregulated it (**Figure 16A**). In 5 out of 6 experiments, the NFAT5-deficient T cells also produced more IFN-γ than the wild-types (**Figure 16B**). Another Th1-associated cytokine, TNF-α, was increased in Th1 conditions but there were no differences between wild-type and NFAT5-deficient T cells (**Figure 16A**). These results indicated that NFAT5 may limit the duration of IFN-γ production by $CD4^+$ T cells stimulated in Th1 conditions.

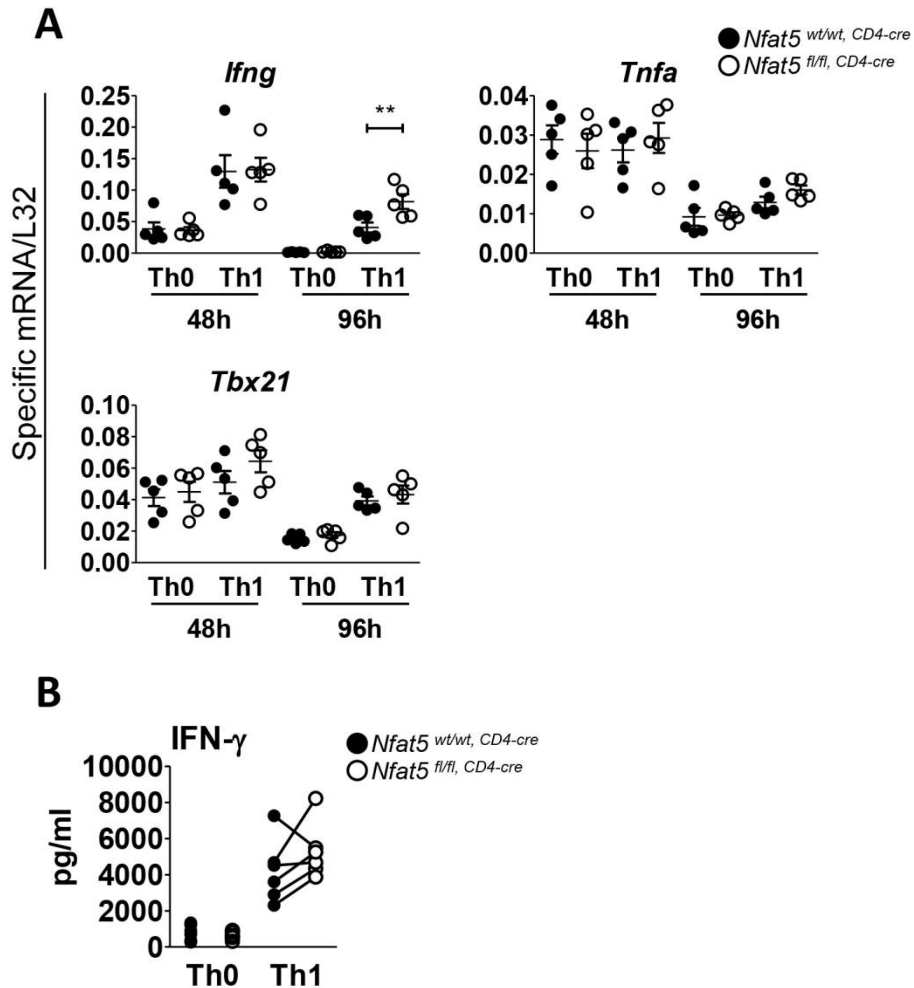


Figure 16. Induction of Th1 genes in wild-type and NFAT5-deficient CD4+ T cells in response to IL-12. *Nfat5* $^{wt/wt, CD4-cre}$ and *Nfat5* $^{fl/fl, CD4-cre}$ CD4+ T cells were activated with antibodies to CD3 and CD28 and polarized with IL-12. **A)** RNA analysis by RT-qPCR at 48 and 96 hours. The values were normalized to L32 mRNA levels. Circles represent independent experiments, the bars represent the mean±SEM (n=5). Significance was determined by an unpaired t test. (*=p<0.05). **B)** IFN-γ protein measurement of the supernatant after 96 hour culture by ELISA. Circles represent independent experiments (n=6). Each pair of wild-type and NFAT5-deficient T cell cultures is shown connected by lines.

DISCUSSION

DISCUSSION

NFAT5 has been shown to regulate diverse functions in T cells in response to hyperosmotic stress conditions, including the balance of naïve/memory T cells, their homeostatic survival, and cytokine expression (Berga-Bolaños, Drews-Elger, Aramburu, & López-Rodríguez, 2010; Kleinewietfeld et al., 2013; López-Rodríguez et al., 2001). On the other hand, NFAT5 has been recently shown to regulate thymocyte development in an osmotic stress-independent manner, an observation that led us to ask whether NFAT5 could regulate other functions in mature T cells (Berga-Bolaños, Alberdi, Buxadé, Aramburu, & López-Rodríguez, 2013).

Here we have analyzed how the lack of NFAT5 affected the expression of gene products associated with the acquisition of polarized functions in T cells when they were activated under hypernatremia as well as in the absence of osmotic stress. Our results revealed that NFAT5 enhanced the expression of the Th17 subtype transcription regulator ROR-γt in response to hypernatremia, and that both transcription factors could regulate different Th17 genes in T cells activated under osmotic stress without exogenous polarizing cytokines. In addition, using an experimental model of intestinal inflammation we have found that a deficiency in NFAT5 in T cells can have pathological consequences *in vivo*, and further results supported an association between this effect and an enhanced capacity of NFAT5-deficient T cells to produce IFN-γ in the absence of osmotic stress. Our results indicate that NFAT5 could play different roles in T cell polarization responses depending on the cytokine milieu and stress conditions in their microenvironment.

Our data show that hypertonic stress can promote the acquisition of Th17 cell features in T lymphocytes activated through their TCR in the absence of exogenous cytokines. It had been previously described that exposure to osmotic stress of T cells simultaneously stimulated with Th17-polarizing

cytokines enhanced the expression of genes related to the Th17 polarization program, but it was not known whether osmotic stress alone could directly promote Th17 polarization (Wu et al., 2013). Our results reveal a more general scenario in which ROR-γt, directly regulated by NFAT5, could function as an osmosensitive factor, cooperating with NFAT5 to further induce the expression of IL-22 and IL-23R in conditions of hypertonic stress. Notably, we also found that ROR-γt, without additional cooperation from NFAT5, could enhance IL-17A mRNA expression in activated T cells exposed to hypertonic stress. In this regard, it was intriguing to observe that while the upregulation of mRNA expression in response to osmostress was NFAT5-independent, NFAT5 was required for the production of IL-17A protein (**Figure 3**). A possible explanation for this dichotomy might be that even if NFAT5 may not be directly involved in IL-17A transcription, it may indirectly influence the biosynthetic capacity of cells under osmotic stress, since our group had previously shown that lymphocytes lacking NFAT5 suffered cell cycle arrest and a greater loss of viability than their wild-type counterparts under salt stress (Drews-Elger, Ortells, Rao, López-Rodriguez, & Aramburu, 2009). We also found that IL-2, a cytokine required for T cell growth, was upregulated upon hypertonicity in an NFAT5-dependent manner, which goes in the line with the general notion that NFAT5 enables proliferative competency of T cells upon osmotic stress, and also promotes their homeostatic survival by inducing CD24 (Berga-Bolaños et al., 2010; Drews-Elger et al., 2009). Altogether, these results emphasize the relevance of NFAT5 for the survival of T cells in hypertonic environments.

Th17 cells are most abundant at mucosal surfaces such as intestine, and can also be found in the skin (Cheroutre & Madakamutil, 2004; Clark, 2010; Mucida & Salek-Ardakani, 2009). Cells in these niches can be exposed to variations in environmental conditions, including changes in local tonicity, which can lead to the activation of NFAT5 (Jantsch et al., 2015; Machnik et al., 2009; Schilli et al., 1982; Vertzoni et al., 2010). Therefore, a high local

concentration of sodium at barrier sites could function as a proinflammatory stimulus to protect the organism from external microorganisms, and it is interesting to observe that osmostress enhances the expression of diverse gene products that have been associated with inflammatory responses: TNF-α, LT-β, iNOS, IL-17A, IL-8, IL-1, BAFF, IL-2 (Jantsch et al., 2015; Kino et al., 2010; Kleinewietfeld et al., 2013; López-Rodríguez et al., 2001; Shapiro & Dinarello, 1997).

We have also found that NFAT5 can regulate the polarization of $CD4^+$ T cells in the absence of hypertonic stress. In T cells stimulated with the Th17-promoting cytokines TGF-β and IL-6, NFAT5 regulated a different set of genes than in response to osmostress, contributing to the expression of IL-23R and IL-17A, but not to ROR-γt. We have not address how NFAT5 may promote IL-23R expression in both conditions, osmostress or cytokine stimulation, but it was differently required for the induction of IL-17A and ROR-γt depending on the stimulatory context. One possibility is that hypertonic stress and cytokine stimulation activate different combinations of transcription factors which would result in different gene expression patterns. This is illustrated by the greater induction of NFAT5 by osmostress than through cytokine stimulation (**Figure 4** and data not shown), and by the inhibitory effect of osmostress on the activation of STAT3 (**Figure 7**). STAT3, which is strongly activated by IL-6 and is essential for the expression of ROR-γt and IL-17A in canonical Th17 cells (Yang et al., 2007), could suffice to induce ROR-γt regardless of NFAT5 in cytokine-stimulated Th17 cells, whereas NFAT5 would play a more significant role under osmostic stress when STAT3 is less active. Regarding IL-17A, its low-level induction by osmostic stress could be sustained by ROR-γt, but its optimal induction by IL-6 might involve the combination of ROR-γt, STAT3 and NFAT5. Further experiments will be needed to address whether NFAT5 can regulate directly the *Il17a* and *Il23r* genes in IL-6-stimulated T cells or contributes to their expression through indirect mechanisms.

We have addressed the potential role of NFAT5 in T cells in an experimental model of intestinal inflammation induced by DSS, in which Th17 and Th1 cells have a disease-promoting effect. We observed that *Nfat5* $^{fl/fl, CD4-cre}$ mice, which lack NFAT5 in their T cell compartment, suffered a more pronounced disease, characterized by a greater shortening of the colons and later recovery of body weight than wild-type mice. Although it has been reported that the osmolality of the feces in DSS-induced colitis mice is higher than in untreated controls (Laroui et al., 2012), an osmotic imbalance does not seem to explain our results. First, we did not find that the colons and mLN of DSS-treated mice had elevated expression of the mRNA of NFAT5 and aldose reductase, both markers being general hallmarks of osmostress responses. Although we cannot rule out an osmostress component during the first days of DSS treatment, in which we did not analyze stress-associated markers, the cytokine expression patterns in colon and mLN of DSS-treated wild-type and *Nfat5* $^{fl/fl, CD4-cre}$ mice did not coincide with the cytokine profile elicited by osmotic stress in T cells, either wild-type or NFAT5-deficient. In view of these observations, and as it will be discussed below, we favor the interpretation that the exacerbated pathology associated with the deficiency of NFAT5 in T cells was rather T cell-intrinsic, and not dependent on hypertonic stress.

Analysis of mRNA expression for diverse T cell-differentiation markers in mLN and colon in the DSS-induced mice showed that lack of NFAT5 in T cells led to an increase in IL-22 and IFN-γ mRNA in both tissues. These cytokines are significantly increased in patients with inflammatory bowel disease (IBD) and in mouse models of colitis (Brand et al., 2006; Fais et al., 1991; Fuss et al., 1996; Ito et al., 2006; Perše & Cerar, 2012; Sugimoto & Ogawa, 2008; Zenewicz et al., 2008). Our findings that mice with more pronounced pathology have increased IFN-γ mRNA expression levels are in accordance with the literature, as it has been described that IFN-γ has a proinflammatory role in colon inflammation, and neutralization of this cytokine improves the pathological symptoms in mice (Ito et al., 2006;

Powrie et al., 1994; Yamashita et al., 2013). In this regard, a treatment for colitis based on the blockade of IFN-γ was developed for CD patients, although it was not therapeutically as effective as the antibody in mice (Reinisch et al., 2006). On the other hand, IL-22 has been generally linked to protective functions in colon inflammation (Kamanaka et al., 2011; Pickert et al., 2009; Zenewicz et al., 2008). It has been described to contribute to the improvement of the mucus layer and restitution of goblet cells in the colon (Sugimoto & Ogawa, 2008) and to induce antimicrobial peptide secretion in a DSS-induced model of colitis (Zindl et al., 2013). However, other reports have shown that IL-22 can have disease-promoting or attenuating roles, depending on the colitis model used (Eken, Singh, Treuting, & Oukka, 2014; Kamanaka et al., 2011). The model that these last reports propose for the pathology driven by IL-22 is an enhanced mucus production and epithelial hyperplasia. Nonetheless, in both articles IFN-γ expression was modulated in parallel to IL-22, suggesting a role for IFN-γ in the mechanism of the IL-22 driven pathogenesis. In this regard, our experiments support the interpretation that the *in vivo* exacerbated pathology of mice that lack NFAT5 in T cells could be mediated, at least in part, by their T cells either producing more of the proinflammatory cytokines IFN-γ and IL-22, or causing other cells to enhance their expression. Also, as the pathogenesis of the DSS-induced colitis model is complex, we cannot rule out that other mediators besides IFN-γ and IL-22 could be involved in the exacerbation of the inflammation in *Nfat5* $^{fl/fl, CD4\text{-}cre}$ mice. Further experiments blocking IFN-γ or IL-22 should be done to provide stronger evidences of the involvement of these cytokines in this model. Finally, it is interesting to note that recently published data show that patients with active ulcerative colitis (UC) and Crohn's disease (CD) express less NFAT5 mRNA in colon tissue compared to healthy individuals (Boland et al., 2015). This finding, together with our results described here, suggests that in both cases NFAT5 seems to be protective against intestinal inflammation.

Besides the DSS colitis model, we also found direct evidence that NFAT5-deficient T cells can produce more IFN-γ than wild-type ones during i*n vivo* T cell activation with an antibody to CD3 and in *in vitro* Th1 polarization assays. These results suggest that the increased expression of IFN-γ mRNA in colon and mLN in DSS-treated *Nfat5*$^{fl/fl,\ CD4\text{-}cre}$ mice could be T cell intrinsic. Altogether, our results reveal an unknown role for NFAT5 as a regulator of IFN-γ expression in an osmostress-unrelated way. However, the mechanism underlying the enhanced IFN-γ expression in NFAT5-deficient T cells remains unknown. The Th1 cell polarization assays showed that IFN-γ expression was downregulated at late time points in NFAT5-sufficient T cells, but lasted longer in NFAT5-deficient ones. On the other hand, NFAT5 did not affect the early induction of IFN-γ during Th1 polarization. Thus, NFAT5 might play a role in limiting the excessive duration of Th1 responses, an effect that would attenuate pathogenic inflammatory responses.

The lack of NFAT5 in T cells had different effects depending on the scenario, for which different explanations can be discussed. In T cells, NFAT5 is found in both the cytoplasm and nucleus constitutively (Lopez-Rodríguez, Aramburu, Rakeman, & Rao, 1999; López-Rodríguez et al., 2001), which could facilitate its access to target genes. That the nuclear residency of NFAT5 may allow it to bind to certain target genes in the absence of apparent stimulation has been shown in macrophages, in which NFAT5 is constitutively bound to the *Tnf* promoter, although this does not cause NFAT5-dependent TNF-α expression unless appropriate stimulation is provided (Buxade et al., 2012). By contrast, NFAT5 is only recruited to another target gene, *Nos2*, upon Toll-like receptor (TLR) stimulation and TLR-dependent activation of histone modifiers (Buxade et al., 2012). NFAT5 itself can regulate epigenetic marks, and for instance lack of NFAT5 caused an increase in the accumulation of trimethylated H3K27 in the promoter of *Cd24* in T cells (Berga-Bolaños et al., 2010). These observations suggest that a constitutive level of nuclear residency could facilitate the access of NFAT5

to target genes, but its ability to influence their expression would be subjected to a further layer of control by environmental and stimulatory conditions regulating a permissive chromatin configuration. A combination of whether NFAT5 is pre-bound to potential target genes with the co-occurrence of appropriate signalling could explain how it may regulate different genes in different contexts. Other possible mechanisms would be that NFAT5 acted as an indirect regulator of Th17 and Th1 genes, for instance through modulating the expression of, or cooperating with, other context-activated transcription factors. Further experiments need to be done to elucidate the precise mechanisms of regulation of the different NFAT5 target genes in each stimulatory condition.

Our data show that NFAT5 signaling can bias T cell differentiation towards different subsets depending on environmental cues. Under hypertonic stress or Th17-polarization conditions, NFAT5 promoted Th17-specific gene expression, while in Th1-differentiating conditions or *in vivo* models of inflammation it downregulated the expression of some proinflammatory genes. Thus, NFAT5 could have a dual effect in T cell regulation. There are other transcription factors that can regulate T cell fate differently. For instance c-Maf promotes Th17 and Th2 cell fates, being required for the expression of IL-4, ROR-γt and IL-21, but inhibiting IL-22 production (Kim, Ic, Grusby, & Lh, 1999; Rutz et al., 2011; Tanaka et al., 2014); and Gfi-1, which inhibits Th17 and Treg differentiation, promotes Th2 cell polarization (Zhu et al., 2009; Zhu, Jankovic, Grinberg, Guo, & Paul, 2006). In summary, we have shown that NFAT5 could help T cells to integrate different stimulatory and stress conditions in their microenvironment, and could play a relevant role in attenuating pathogenic responses driven by activated T cells.

CONCLUSIONS

CONCLUSIONS

1. Hypertonic stress caused by extracellular NaCl induces the mRNA expression of IL-2 and the Th17 markers IL-17A, IL-22, IL-23R and ROR-γt in activated $CD4^+$ T cells.

2. NFAT5 is required for the upregulation of ROR-γt, IL-23R, IL-22 and IL-2 in activated $CD4^+$ T cells under hypertonic stress conditions.

3. ROR-γt is necessary for the induction of IL-17A, IL-23R and IL-22 but not NFAT5 and IL-2 upon hypertonic stress.

4. NFAT5 binds to the promoter region of *Rorc* in activated $CD4^+$ T cells under hypertonic stress.

5. Calcineurin and the mTOR pathway regulate to varying degrees the increase in the mRNA expression of IL-2, ROR-γt, IL-17A and IL-23R induced by hypertonic stress in activated $CD4^+$ T cells.

6. In the absence of hypertonic stress, NFAT5 contributes to the expression of IL-17A and IL-23R in activated $CD4^+$ T cells costimulated with TGF-β and IL-6.

7. $Nfat5^{fl/fl,\ CD4\text{-}Cre}$ mice suffer an exacerbated colitis in comparison with wild-type ones in an experimental model of colitis induced by dextran sulfate sodium (DSS).

8. $Nfat5^{fl/fl,\ CD4\text{-}Cre}$ mice express higher levels of IFN-γ and IL-22 mRNA than wild-type mice in mesenteric lymph nodes and colon upon DSS-induced colitis.

9. NFAT5-deficient $CD4^+$ T cells stimulated *in vivo* with an antibody to CD3 express higher levels of IFN-γ mRNA than wild-type T cells.

10. NFAT5-deficient $CD4^+$ T cells polarized towards Th1 maintain a more prolonged expression of IFN-γ mRNA and protein than wild-type T cells.

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