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Gustavo Javier Martinez

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**MOLECULAR MECHANISMS UNDERLYING THE TRANSCRIPTIONAL
REGULATION OF T HELPER 17 AND REGULATORY T CELLS**

by

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**MOLECULAR MECHANISMS UNDERLYING THE TRANSCRIPTIONAL
REGULATION OF T HELPER 17 AND REGULATORY T CELLS**

A

DISSERTATION

**Presented to the Faculty of
The University of Texas
Health Science Center at Houston
and
The University of Texas
M. D. Anderson Cancer Center
Graduate School of Biomedical Sciences
in Partial Fulfillment**

of the Requirements

for the Degree of

DOCTOR OF PHILOSOPHY

by

Gustavo J. Martinez, M.S.

Houston, Texas

May 2011

DEDICATION

To

my twin brother, Juan, for his endless support, for keeping me motivated when I needed it the most, for encouraging me to be the best I can possibly be and for being my best friend;

my parents, Juan and Alicia, for instilling the concept that hard work and education can guide you somewhere in life;

my parents, my twin brother, my sisters (Patricia, Veronica and Valeria) and my nephews (Floencia, Vanesa, Matias, Romina, Tobias and Martin), for inspiring me to achieve my goals, even though it meant being physically away from them.

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**MOLECULAR MECHANISMS UNDERLYING THE TRANSCRIPTIONAL
REGULATION OF T HELPER 17 AND REGULATORY T CELLS**

Publication No. _____

Gustavo J. Martinez, Ph.D.

Supervisory Professor: Chen Dong, Ph.D.

CD4⁺ T helper (Th) lymphocytes are vital for integrating immune responses by orchestrating the function of other immune cell types. Naïve Th cells can differentiate into different effector subsets that are characterized by their cytokine profile and immune regulatory functions. These subsets include Th1, Th2, Th17, natural and inducible regulatory T cells (nTreg and iTreg respectively), among others. We focused our investigation on two Th lineages, Th17 and regulatory T cells, with opposing functions in the immune system. These subsets have been suggested to be reciprocally regulated since they both require TGF- β for their development. We investigated the role of the Treg-associated master transcription factor Foxp3, and found that Foxp3 inhibits Th17 cell generation by preventing the transcriptional activity of the two main Th17-specific transcription factors, nuclear orphan receptors ROR α and ROR γ t. At the molecular level, we identified two different functional domains in Foxp3 required for such inhibition: the LQALL sequence in exon 2 and the TIP60/HDAC7 binding domain. These domains could be crucial to either prevent the association of the nuclear receptors to coactivators or to recruit histone deacetylases to ROR α - or ROR γ t-target genes. Since TGF- β is a common cytokine required for the commitment towards both Th lineages, we

determined the role of the TGF- β -dependent signaling pathway in the generation of each subset. By using mice with deficiencies in signaling molecules downstream of TGF- β , we found that while Smad2, Smad3 and Smad4 are required for the generation of iTreg cells, only Smad2 is indispensable for the induction of IL-17-producing cells, suggesting that TGF- β induces these T helper lineages through differential signaling pathways. Thus, our findings describe novel transcriptional regulatory mechanisms that control the generation of two T helper lineages with opposing functions. These findings could provide novel therapeutic targets to treat diseases where the balance of these T cells is dysregulated, such as in autoimmunity, chronic infectious diseases and cancer.

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CHAPTER I - INTRODUCTION

The immune system has evolved to not only recognize and fight against invading pathogens, but also to generate immunological memory. For this particular response, the adaptive branch of the immune system plays a crucial role. Within the adaptive immune response, CD4⁺ T helper (Th) cells are vital for integrating immune responses by orchestrating the function of other immune cell types from either the adaptive or innate immune system, and thus have a major role in cancer, autoimmune and infectious diseases.

Commitment towards the T cell lineage occurs in the thymus. Once a hematopoietic precursor exits the bone marrow and enters the thymus, it loses the potential to become non-T hematopoietic cell types (lineage exclusion) and adopts T cell-properties (reviewed in (Rothenberg et al., 2010)). After a cell commits to the T cell fate, it undergoes positive and negative selection, so that T cells expressing a T cell receptor (TCR) with intermediate affinity towards major histocompatibility complexes (MHC) are selected (reviewed in (Starr et al., 2003)). Having succeeded at these stages, naïve CD4⁺ T helper cells exit the thymus and migrate to the periphery. These naïve Th cells circulate through the body and home to lymph nodes until they become activated. Upon activation, naïve CD4⁺ Th cells are able to differentiate into effector cells that are characterized by their cytokine profile and immune regulatory functions. This differentiation process depends on several factors including the strength of the signals induced through the TCR, costimulatory molecules and the cytokines present in the microenvironment during T cell activation. These cytokines are mainly produced by cells from the innate immune system, especially from antigen-presenting cells (APCs) upon recognition of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Hsieh et al., 1993; Seder et al., 1993). Thus, the type of effector CD4⁺ Th cell induced will ultimately depend on the activation status of the innate immune cells, the type of PRRs activated, and the balance between the different cytokines present in the local microenvironment. In the present chapter we will discuss the different T helper lineages identified and focus on the cytokine and transcription factors that control the commitment towards Th cell types,

in particular Th17 and regulatory T cells. Then, throughout this dissertation, we will determine the requirement of several transcription factors that regulate the differentiation to two Th lineages: Th17 and regulatory T cells.

1.1. T helper cell differentiation: Th1-Th2 paradigm

More than twenty years ago, it became evident that naïve Th cells, once activated, could adopt two destinies: support B cell activation and humoral immunity or macrophage activation and thus cell-mediated immunity (CMI). This led Mosmann and Coffman to propose the Th1-Th2 paradigm (Mosmann et al., 1986; Mosmann and Coffman, 1989) where Th1 cells induced delayed type hypersensitivity (DTH) and CMI responses and Th2 cells promoted humoral/allergic immune responses. The identification of these two T cell lineages demonstrated that CD4⁺ Th cells are heterogeneous and capable of adopting differential fates depending on the microenvironment during T cell activation, indicating a previously unidentified versatility of the adaptive immune system.

By analysis of the cytokine profile produced by these cells, it was established that Th1 cells produce mainly interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α). IFN- γ induces macrophage activation and is thus crucial for the host immune defense against intracellular pathogens, and also mediates immunoglobulin G2a (IgG2a) class switching in B cells. By contrast, Th2 cells produce interleukin (IL)-4, IL-5 and IL-13, which mediate immunoglobulin class switching in B cells (class switch to IgG1 and IgE), induce recruitment of eosinophils and lead to mucosal activation. Furthermore, Th2 cells are important in the host immune responses against extracellular bacteria as well as parasites.

Commitment towards Th1 or Th2 cell fate depends on many factors including signals induced by costimulatory molecules, type of antigen presenting cells priming T cells, and most importantly, cytokines present in the local microenvironment during the T cell activation. It has been demonstrated that Th1 cell generation depends on IL-12, produced by macrophages or dendritic cells upon recognition

of pathogen associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) (Hsieh et al., 1993; Seder et al., 1993). Th2 development, on the other hand, depends on IL-4 (Hsieh et al., 1992; Seder et al., 1992) which is produced by Th2 cells, eosinophils, mast cells, NKT cells and basophils (reviewed in (Paul, 2010)). Indeed, it has been recently demonstrated that Th2 priming *in vivo* requires antigen presentation by basophils which also produce IL-4 and thymic stromal lymphopoietin (TSLP) (Perrigoue et al., 2009; Sokol et al., 2009; Yoshimoto et al., 2009). Then, IL-4 produced by Th2 cells can act in a positive feedback loop to further induce Th2 cell development.

Once the key molecules driving differentiation of Th1 and Th2 cells were identified, molecular approaches were designed to investigate the transcription factors involved in the commitment towards each cell lineage. Since Th1/Th2 differentiation requires cytokines belonging to the class I cytokine superfamily (reviewed in (Leonard and O'Shea, 1998)), the role of Signal Transducers and Activators of Transcription (STATs) was determined. It has been demonstrated that development of Th1 cells requires T cell receptor (TCR) and STAT1 signal transductions. IFN- γ , produced by NK cells in response to IL-12 secreted by innate immune cells such as macrophages or dendritic cells, induce STAT1 activation. This, in turn, leads to the expression of the Th1 master transcription factor T-bet (Szabo et al., 2000). T-bet is highly expressed in Th1 cells and induces IFN- γ production as well as upregulation of IL-12 receptor β 2 chain (Szabo et al., 2000). Upon upregulation of the latter, IL-12 induces activation of STAT4, which further enhances IFN- γ expression and thus acts in an autocrine loop. Interestingly, STAT4 or T-bet deficient mice are unable to induce Th1 cells (Kaplan et al., 1996; Szabo et al., 2002). T-bet not only stimulates the expression of IFN- γ , but is also capable of redirecting developing Th2 cells into Th1 cells, demonstrating that T-bet is required to initiate commitment towards Th1 cells and is also capable of repressing Th2 cell induction (Szabo et al., 2000).

Th2 cell generation requires IL-4, which signals through STAT6 to induce expression of the master transcription factor GATA-3 (Zheng and Flavell, 1997). GATA-3, similar to T-bet, not only induces epigenetic modifications in *il4*, *il5* and *il13* loci that lead to their expression, but also represses

Th1 cell generation by interfering with STAT4 and IL-12R β 2 expression (Ouyang et al., 2000; Ouyang et al., 1998; Pai et al., 2004; Ranganath et al., 1998).

1.2. Gaps in Th1/Th2 paradigm: Identification of Th17 cells as an independent T cell lineage

The Th1/Th2 paradigm has prevailed in the immunology field for over two decades. However, several reports indicated that CD4⁺ T helper cells can produce other cytokines, such as IL-17 and IL-17F, that were not co-expressed by IFN- γ -(Th1) or IL-4-(Th2) producing cells (Aggarwal et al., 2003; Infante-Duarte et al., 2000). Furthermore, even though Th1 cells have been associated with autoimmune diseases, mice lacking IFN- γ R, IFN- γ or STAT1 (all required for Th1 cell development) were still susceptible to the autoimmune disease models collagen-induced arthritis (CIA) or experimental autoimmune encephalomyelitis (EAE) (Bettelli et al., 2004; Ferber et al., 1996; Kageyama et al., 1998; Willenborg et al., 1996). On the other hand, *Icos*^{-/-} or *Il23*^{-/-} mice were resistant to CIA development, which correlated with reduced IL-17 expression (Dong, 2003; Murphy et al., 2003). Furthermore, it was shown that IL-23 alone could expand Th cells expressing IL-17 and IL-17F, which played a pathogenic role in the induction of EAE (Langrish et al., 2005). These observations led to the identification of Th17 cells as an independent lineage from Th2 or Th1 cells (Fig. 1), since IFN- γ , IL-12 or IL-4, which are required for Th1 or Th2 cell development, respectively, were dispensable for the generation of IL-17-producing T cells both *in vitro* and *in vivo* (Harrington et al., 2005; Park et al., 2005). Other Th subsets have also been identified in recent years, including Th9 cells, regulatory T cells (Treg cells), and follicular helper T cells (Tfh), among others (Fig. 1). Although effector T cells were originally considered to be terminally differentiated, a growing body of evidence has challenged this view and suggested that the phenotype of effector T cells is not completely fixed but is more flexible or plastic (discussed in Chapter IV).

Th17 cells have been characterized to produce mainly IL-17A, IL-17F, IL-22 and IL-21 cytokines as well as the chemokine C-C motif ligand 20 (CCL20). IL-17A and IL-17F can bind to two

receptors: IL-17RA and IL-17RC. In mice, IL-17RA was found to be expressed in lungs, kidneys, liver, spleen, epithelial cells, fibroblasts (Yao et al., 1995), whereas in humans it has been observed in epithelial cells, B and T lymphocytes and eosinophils, among others (Cheung et al., 2008; Moseley et al., 2003). IL-17RC, on the other hand, was found on human muscle, cartilage, liver, heart and eosinophils (Cheung et al., 2008; Moseley et al., 2003). IL-21 binds to IL-21 receptor (IL-21R), which is expressed on many immune cell types such as B, T and NK cells, dendritic cells, macrophages and epithelial cells (reviewed in (Leonard and Spolski, 2005)). IL-22R is expressed in non-immune cells, predominantly epithelial cells (reviewed in (Trivella et al., 2010; Wolk et al., 2010)). Upon binding to their receptors, Th17 cytokines can act on different target cell types to induce the secretion of granulopoietic factors (stem cell factor (SCF) and granulocyte colony-stimulating factor (G-CSF)), CC and CXC chemokines, matrix metalloproteases, anti-microbial peptides and pro-inflammatory cytokines (reviewed in (Dong, 2008)). This combination of factors leads to an increase in the recruitment of immune cell types, including neutrophils, and the induction of a local inflammatory and/or antimicrobial immune response (Fig. 2). Therefore, Th17 cells are crucial regulators of host immune responses, but also show an important role in inducing inflammatory responses.

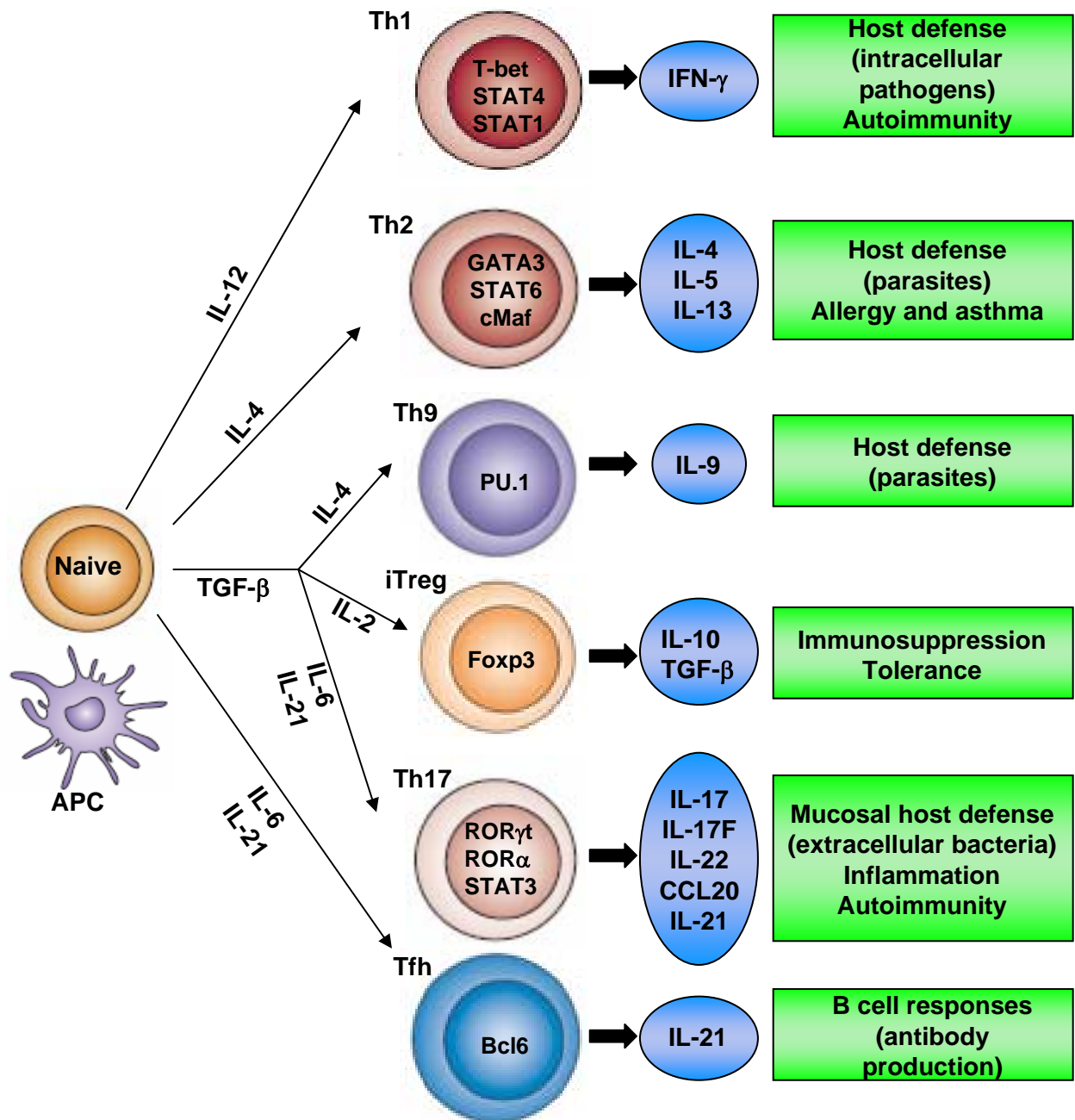


Figure 1. Schematic representation of T helper subsets. Naïve CD4⁺ Th cells, upon activation can differentiate into different effector Th subsets. Each effector cell expresses specific transcription factors (shown inside the cells). These effector subsets are characterized by the secretion of different soluble mediators (shown in blue circles) and therefore display different effector functions (shown in green rectangles). Th1 cells produce IFN- γ and regulate antigen presentation and immunity against intracellular pathogens. Th2 cells produce IL-4, IL-5 and IL-13, and mediate humoral responses and immunity against parasites and are important mediators of allergic diseases. Th17 cells express IL-17, IL-17F, IL-21 and IL-22 (and IL-26 in humans), regulate mucosal host immune responses and participate in inflammation and autoimmunity processes. Th9 cells produce IL-9 and primarily mediate host immune responses against parasites. Tfh cells produce IL-21, and provide help to B cells, participating in germinal center reactions. iTregs express Foxp3 transcription factor and mediate immune suppression by secretion of TGF- β , IL-10. Adapted from Dong, 2008; Yoshimura et al., 2010.

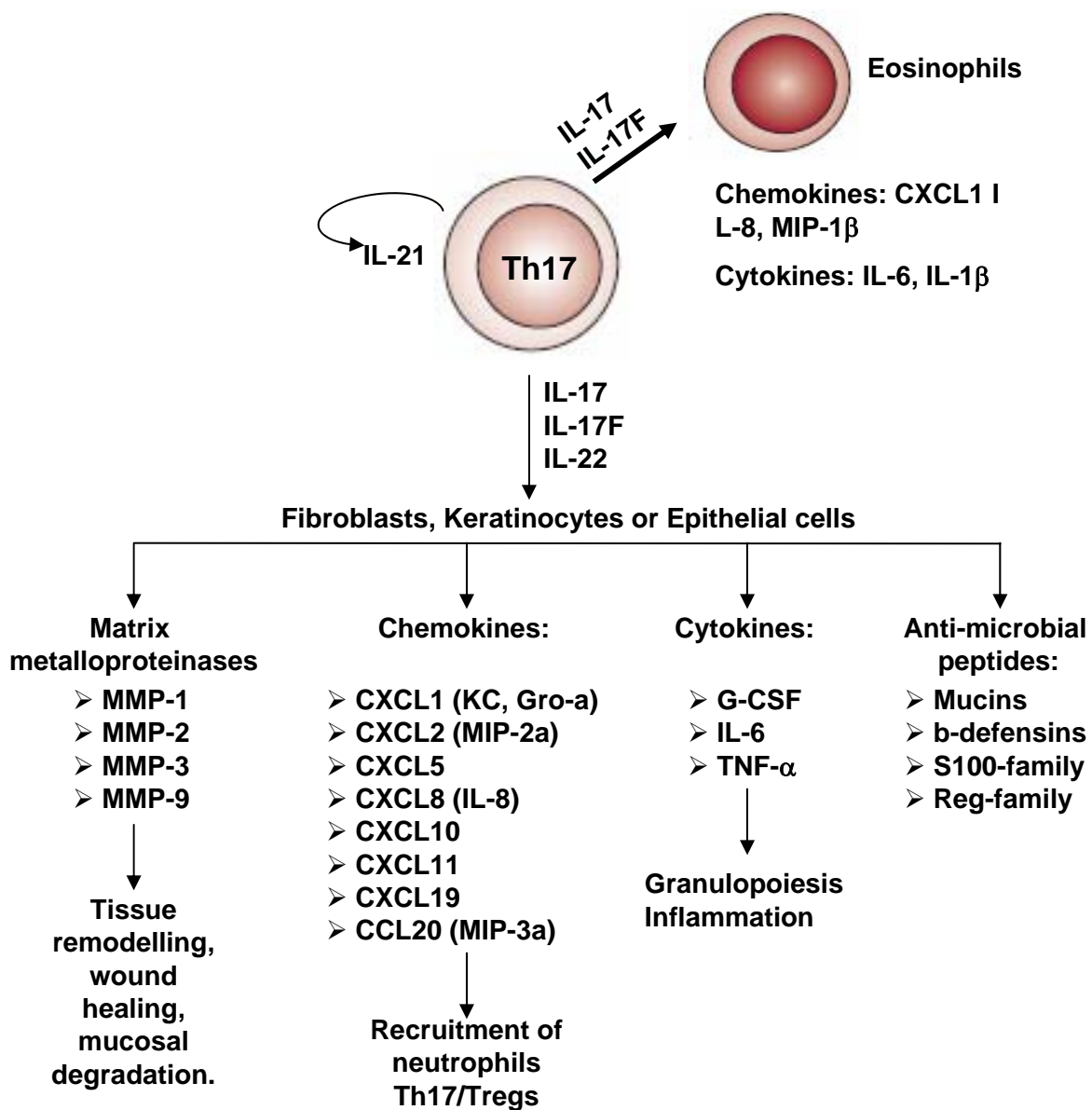


Figure 2. Role of Th17 cytokines. On immune cells, Th17-produced IL-21 further induces Th17 cell differentiation creating a positive feedback loop in an autocrine/paracrine manner. Moreover, both IL-17 and IL-17F induce production of cytokines and chemokines by eosinophils, leading to allergic inflammation. In non-immune cells, Th17 cytokines collectively induce the secretion of cytokines, chemokines, anti-microbial compounds and matrix metalloproteases from epithelial cells, fibroblasts and keratinocytes, leading to an increase in inflammation by recruitment of neutrophils and macrophages and tissue remodeling. Reprinted from “Regulation and function of proinflammatory Th17 cells” Gustavo J. Martinez, Roza Nurieva, Xuexian O. Yang and Chen Dong. Annals of the New York Academy of Sciences 2008. 1143:188-211 with permission from John Wiley and Sons.

I.3. Cytokine requirement for the generation of Th17 cells

Similar to the commitment of Th1 or Th2 cells, Th17 cells also require a specific combination of cytokines for their induction. In this section we will describe the positive as well as negative regulators of Th17 cell induction.

I.3.a. Positive regulators in the generation of Th17 cells

Upon determination of Th17 cells as an independent Th cell lineage, several groups determined that transforming growth factor- β (TGF- β) and IL-6 are responsible for initiation of Th17 cell responses (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a). Stockinger's group demonstrated that while TGF- β and IL-6 support the acquisition of IL-17 expression from naïve T cells, IL-1 β and TNF- α further amplify the expression of this cytokine (Veldhoen et al., 2006a). The requirement of TGF- β in the induction of a pro-inflammatory T cell lineage was unexpected in view of the role of TGF- β as an immunosuppressive cytokine that can directly inhibit T cell activation (Gorelik and Flavell, 2002) or induce the generation of Foxp3⁺ regulatory T cells (Bettelli et al., 2006; Chen et al., 2003). Furthermore, Bettelli et al. demonstrated that IL-6 can inhibit TGF- β -dependent induction of Foxp3⁺ regulatory T cells and favor the generation of IL-17-producing T cells, suggesting that not only is there functional antagonism between these two T cell lineages, but also that they are reciprocally regulated (see below, and discussed in Chapter II) (Bettelli et al., 2006). Even though TGF- β has been demonstrated to be crucial in the induction of Th17 cells, Zhou and colleagues recently demonstrated that increasing concentrations of TGF- β *in vitro* decreases IL-23R expression and induces regulatory T cells, thus shifting the balance from Th17 to regulatory T cells (Zhou et al., 2008a). Combined, these reports demonstrate that the balance between pro-inflammatory cytokines and TGF- β will determine the final outcome towards the induction of T cell lineages with opposing functions.

The requirement for TGF- β and IL-6 in driving Th17 cell generation was also confirmed *in vivo*. Kuchroo and colleagues showed that TGF- β transgenic mice immunized with myelin oligodendrocyte glycoprotein (MOG) in Complete Freund's Adjuvant (CFA) responded with a higher Th17 response and presented exacerbated EAE (Bettelli et al., 2006). Further supporting the role of TGF- β in Th17 cell differentiation, Mangan et al. showed that mice deficient in this cytokine were unable to produce IL-17 *in vivo* (Mangan et al., 2006). Deletion of TGF- β 1, specifically in T cells, also leads to resistance to EAE and inability to mount Th17 responses *in vivo*, suggesting that secretion of TGF- β by T cells is essential for the proper regulation of effector T cell generation (Li et al., 2007c). As for the requirement of IL-6 *in vivo*, it has been demonstrated that IL-6-deficient mice are unable to mount Th17 responses and are therefore resistant to EAE disease development, and also show a higher frequency of Foxp3⁺ regulatory T cells (Korn et al., 2007a; Yang et al., 2007).

Recently, it has also been established both *in vitro* and *in vivo* that IL-21, which is produced by Th17 cells, can act in an autocrine manner to induce further Th17 cell development similar to IL-4 and IFN- γ amplification of Th2 and Th1 responses respectively (Korn et al., 2007a; Nurieva et al., 2007; Zhou et al., 2007). IL-21 is induced by IL-6 in a STAT3-dependent manner, supported by the observation that T cells from IL-6-deficient mice fail to produce IL-21 or IL-17 *in vivo* (Nurieva et al., 2007). Furthermore, IL-21 in the presence of TGF- β induces expression of IL-23R and the transcription factor ROR γ t (see below), leading to the generation of IL-17-producing T cells (Nurieva et al., 2007; Zhou et al., 2007). Indeed, mice deficient in either IL-21 or IL-21 receptor fail to generate Th17 cells *in vivo* (Korn et al., 2007a; Nurieva et al., 2007). However, IL-21 can substitute for IL-6 to generate Th17 cells in an IL-6-independent manner (Korn et al., 2007a).

IL-1 has also been shown to be a crucial cytokine in the generation of Th17 cells. IL-1, which has two isoforms (IL-1 α and IL-1 β), can bind similarly to two receptors, IL-1RI and IL-1RII. IL-1RI is involved in cell activation by binding to IL-1R accessory protein (IL-1RAcP), whereas IL-1RII acts only as a decoy receptor. Initially, it had been shown that mice lacking IL-1RI were resistant to EAE induction (Schiffenbauer et al., 2000). Indeed, Sutton and colleagues demonstrated that generation of

antigen-specific Th17 cells is abrogated in IL-1RI deficient mice, whereas Th1 or Th2 responses were not affected (Sutton et al., 2006). Furthermore, Veldhoen et al. demonstrated that IL-1 β can promote expansion/development of Th17 cells stimulated in the presence of TGF- β and IL-6 (Veldhoen et al., 2006a). Recently, Chung and colleagues have also demonstrated that the combination of IL-1, IL-6 and IL-23 can initiate Th17 genetic programming, although this polarizing condition still required TGF- β since neutralization of endogenous TGF- β inhibited Th17 cell induction (Chung et al., 2009). Importantly, IL-1 was shown to regulate the induction of the transcription factors ROR γ t and IRF4 during generation of Th17 cells (Chung et al., 2009). Furthermore, IL-1 can also suppress the inhibitory effects IL-2 has on IL-17 production, by inducing the expression of IL-23R, IL-1R and ROR γ t (Kryczek et al., 2007a).

As previously described, IL-23 is another important cytokine for Th17 cell generation/maintenance. IL-23, a heterodimer composed of IL-12 p19 and p40 chains (Oppmann et al., 2000), binds a heterodimeric receptor complex containing IL-12R β 1 and IL-23R (Parham et al., 2002). *In vivo*, it has been demonstrated that IL-23 is required for sustained inflammation since mice deficient in IL-23 are resistant to EAE (Murphy et al., 2003), inflammatory bowel disease (IBD) (Yen et al., 2006) and CIA (Murphy et al., 2003), all of which correlate with decreased IL-17 production by T cells. IL-23 has been shown to expand Th cells expressing IL-17 and IL-17F (Langrish et al., 2005). However, the exact direct role of IL-23 on Th17 cell differentiation has been unclear since IL-23R is not expressed on naïve T cells. Later work has shown that IL-6 and IL-21, cytokines that initiate Th17 cell generation (Nurieva et al., 2007; Yang et al., 2007; Zhou et al., 2007), are able to induce IL-23R (Bettelli et al., 2006; Mangan et al., 2006; Veldhoen et al., 2006a), indicating that IL-23 plays an important role in the maintenance of these cells. Furthermore, IL-23 synergizes with IL-6 in Th17 cell induction (Yang et al., 2007) and cooperates with IL-1 to enhance IL-17 expression, independent of TCR stimulation (Chung et al., 2009; Sutton et al., 2006). Recently, Cua and colleagues have demonstrated that *il23r*^{-/-} mice have no defect in the early generation of antigen-specific Th17 cells *in vivo* after immunization protocols. However, they demonstrated that IL-23 is required for Th17 cell expansion and migration to peripheral

tissues, whereas it is not necessary for Th1 cells (McGeachy et al., 2009). Thus, these results demonstrate a role of IL-23 not in the initiation but in the maintenance and expansion of Th17 cell responses.

1.3.b. Negative regulators of Th17 cell development

Two independent groups demonstrated that neutralization of Th1 and Th2 cytokines, IFN- γ and IL-4 respectively, markedly increased the production of IL-17 by CD4⁺ Th cells (Harrington et al., 2005; Park et al., 2005). Indeed, IFN- γ -deficient mice showed enhanced frequency of IL-17-producing cells as well as IL-17 protein expression (Harrington et al., 2005; Park et al., 2005), which correlates with previous reports showing that IFN- γ R- and IFN- γ -deficient mice displayed higher susceptibility to EAE induction (Hunter, 2005; Iwakura and Ishigame, 2006; McKenzie et al., 2006). However, in many autoimmune and inflammatory conditions, cells co-expressing IL-17 and IFN- γ have been observed (Abromson-Leeman et al., 2009). The exact role of these cells in the pathogenesis of these diseases has not been determined yet, nor is it known whether they are generated from Th1 or Th17 cells.

The Th1-promoting cytokine IL-27 has also been shown to negatively regulate Th17 cell generation (Batten et al., 2006; Stumhofer et al., 2006). IL-27, which belongs to the IL-12 cytokine family, is formed by two subunits: IL-12 p35-related protein p28 and IL-12 p40-related protein EBI-3 (EBV-induced gene 3). IL-27 binds to a receptor composed of gp130, shared by the IL-6 receptor complex, and a unique subunit named WSX1 (Hunter, 2005). IL-27 has been recently shown to suppress autoimmune disease models. Mice deficient in WSX1 developed more severe EAE, which correlated with enhanced frequency of IL-17-producing cells infiltrating the central nervous system (Batten et al., 2006; Stumhofer et al., 2006). In addition, these mice chronically infected with *Toxoplasma gondii* had no significant differences in IFN- γ -producing cells, whereas higher frequency of IL-17-producing cells were detected in the brain compared to control animals (Stumhofer et al., 2006). Moreover, exogenous addition of IL-27 reduced the EAE disease severity (Fitzgerald et al., 2007). *In*

vitro, it has been shown that IL-27 suppressed induction of Th17 cells both directly through a STAT1 dependent mechanism and indirectly by inducing IL-10 expression, which was associated with a reduction in IL-17-producing cells (Batten et al., 2006; Stumhofer et al., 2006). Also, IL-27 is able to induce expression of SOCS3 (Suppressor of Cytokine Signaling 3), which inhibits Stat3, a transcription factor required for Th17 cell generation (see below) (Owaki et al., 2006; Villarino et al., 2006).

Recently, the T cell growth factor IL-2 has also been shown to suppress development of Th17 cells (Laurence et al., 2007). Laurence et al. showed that suppression of IL-17-producing cells and induction of Foxp3 by IL-2 is mediated through activation of STAT5, which binds directly to the *il17a* promoter competing with Smad3 binding (Laurence et al., 2007). Furthermore, addition of IL-2 resulted in reduction of ROR γ t levels and enhanced TGF- β -induced expression of Foxp3, suggesting that IL-2/STAT5 pathway might shift the balance between Th17 and regulatory T cells.

1.4. Transcriptional regulation of Th17 cells

As mentioned for Th1 and Th2 cell generation, the Th lineage commitment is determined by signaling through cytokine receptors and subsequent induction or activation of specific transcription factors, which ultimately initiate and maintain the expression of Th lineage-specific genes. Similar regulatory mechanisms also occur during Th17 cell commitment. In the present section, we will discuss the role of several transcription factors that have been shown to play either positive or negative regulatory functions for Th17 cell generation (summarized in Figure 3). However, it is noteworthy mentioning that many of these transcription factors have also been shown to regulate the generation of other Th cell subsets or T cell development.

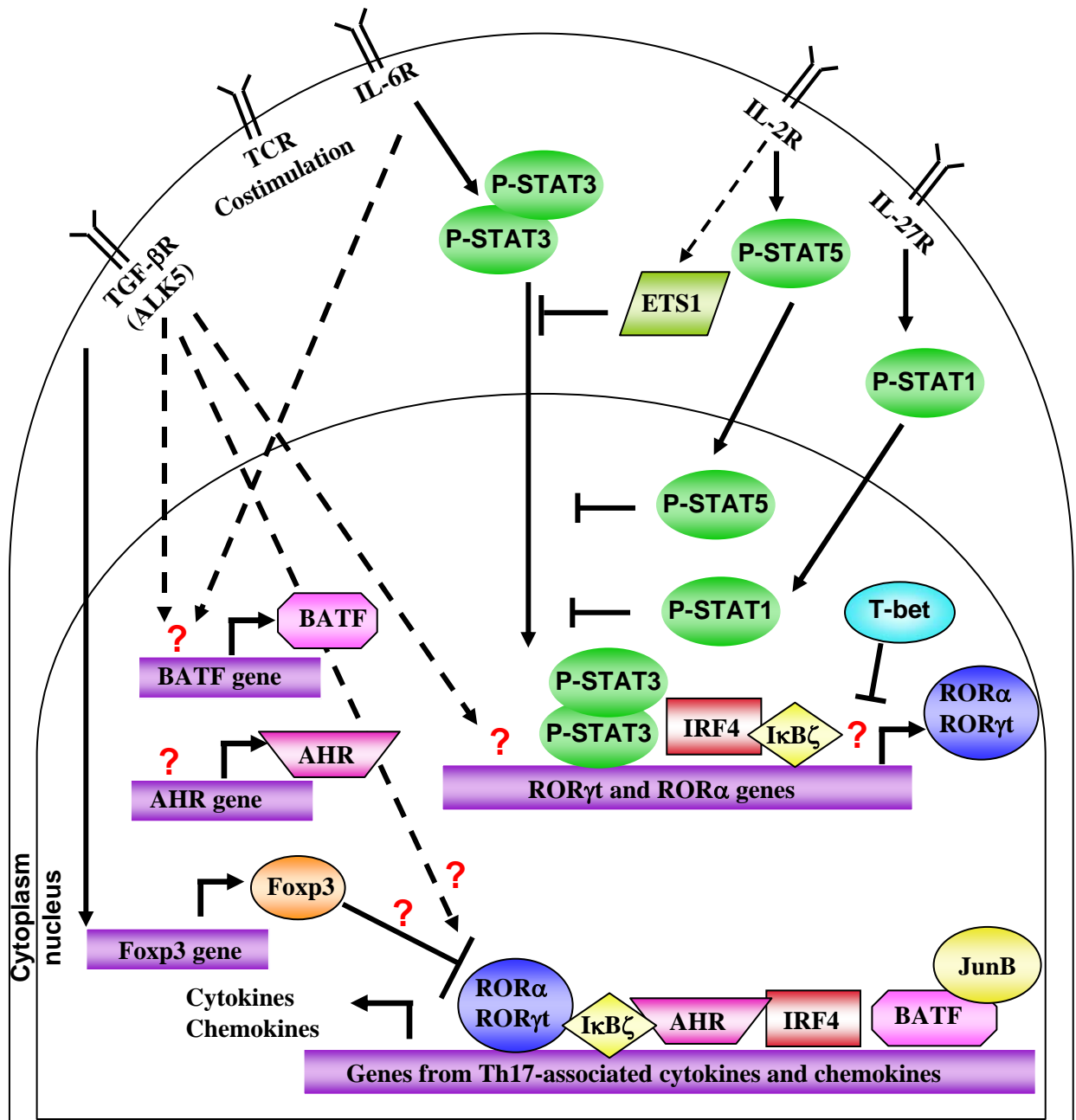


Figure 3. Transcriptional regulation of Th17 cell differentiation. Naïve CD4⁺ T cells stimulated under the presence of IL-6 and/or IL-21 induce activation of the signal transducer and activator of transcription 3 (STAT3). Activation of STAT3 induces the expression of retinoic-acid-receptor-related orphan receptor- α (ROR α) and ROR γ t, which establish the expression of Th17-specific gene program. The role of STAT3 in directly inducing IRF4 remains unclear. And whether IRF4 also directly regulates RORs expression is currently unknown. STAT1, downstream of IFN- γ and IL-27 signalling, or STAT5, downstream of IL-2 signalling, as well as ETS1, negatively regulate Th17 cell generation. Moreover, the transcription factor forkhead box P3 (Foxp3), induced by transforming growth factor- β (TGF- β) signalling, antagonizes the Th17 developmental program by directly binding to ROR α or ROR γ t. Several transcription factors regulate the expression of Th17-associated cytokines in synergism with ROR α /ROR γ t including BATF, which binds to JunB, I κ B ζ and AHR, which mainly control IL-22 production. How or whether TGF- β signalling regulates the expression of Th17-associated transcription factors (I κ B ζ , BATF, AHR, ROR α and ROR γ t) or Th17-specific cytokines and chemokines needs further clarification.

I.4.a. STATs

Most of the cytokines required for differentiation of Th lineages bind to Type I/II cytokine receptors. Unlike receptors with intrinsic enzymatic activity, these receptors associate with Janus kinases (Jaks), which become activated upon binding of cytokines to their cognate receptors. After becoming activated, Jaks phosphorylate tyrosine residues in the cytokine receptor cytoplasmic domain providing a docking site for SH2 (Src homology 2)-containing proteins such as STATs. Once they interact with the receptor, STATs also become phosphorylated, then dimerize and translocate to the nucleus to bind DNA target sequences (reviewed in (O'Shea and Murray, 2008))

It has been recently shown that STAT1, activated by IL-27 and type I/II IFNs, and IL-2-induced STAT5 activation negatively regulate Th17 cell differentiation (Amadi-Obi et al., 2007; Batten et al., 2006; Harrington et al., 2005; Laurence et al., 2007; Stumhofer et al., 2006), whereas no role has been shown for STAT4 or STAT6 (Harrington et al., 2005; Park, 2005). However, it has been recently suggested that STAT4 is required for the synergistic effect of IL-18 and IL-23 in IL-17 production (Mathur et al., 2007).

The requirement of STAT3 in Th17 cell differentiation was originally suggested by studies performed using mice deficient in SOCS3, where increased IL-17 production was observed. This phenotype was associated with enhanced STAT3 activity and binding to *il17a* and *il17f* promoters in response to IL-23 (Chen et al., 2006b). Furthermore, over-expression of a constitutively active STAT3 was shown to increase Th17 cell generation whereas deficiency in STAT3 results in impaired Th17 cell differentiation *in vitro* (Laurence et al., 2007; Yang et al., 2007) and *in vivo* (Zhou et al., 2007). Consistent with these findings, patients with STAT3 mutations (in Hyper IgE syndrome) fail to induce Th17 responses (Milner et al., 2008). Moreover, STAT3 is also required for IL-21 expression induced by IL-6, and similarly for IL-21-induced Th17 differentiation (Nurieva et al., 2007). Even though STAT3 can bind to *il-17a* gene promoter (Chen et al., 2006b), STAT3 seems to regulate more than IL-17 expression in Th17 cells. It is plausible that STAT3, similar to what occurs with STAT1 and STAT6

in Th1 and Th2 differentiation respectively, can regulate the expression of the two Th17 master transcription factors retinoic acid receptor (RAR)-related orphan receptor gamma-t (ROR γ t) (Ivanov et al., 2006; Laurence et al., 2007; Yang et al., 2007) and ROR α (Yang et al., 2008c).

I.4.b. ROR γ t and ROR α .

ROR γ t was the first master transcription factor identified for Th17 cells (Ivanov et al., 2006). Both ROR γ and T-cell expressed ROR γ (ROR γ t) are encoded by the same gene *Rorc* but are expressed using alternative promoters. Together with ROR α and ROR β , ROR γ and ROR γ t belong to the RAR (retinoic acid receptor) orphan nuclear hormone receptor family (reviewed in (Jetten, 2009) and summarized in Figure 4).

Over-expression of ROR γ t in Th cells leads to the generation of Th17 cells when development of Th1 and Th2 cells is inhibited. ROR γ t, which is regulated by transcription factor STAT3 (Laurence et al., 2007; Yang et al., 2007), is required for induction of Th17-specific cytokines IL-17 and IL-17F (Ivanov et al., 2006). Conversely, mice deficient in ROR γ t have reduced induction of Th17 cells *in vivo* and therefore displayed reduced susceptibility to EAE disease (Ivanov et al., 2006). Moreover, reduced Th17 cells have also been observed in intestinal lamina propria from ROR γ t-deficient mice compared to wild type controls.

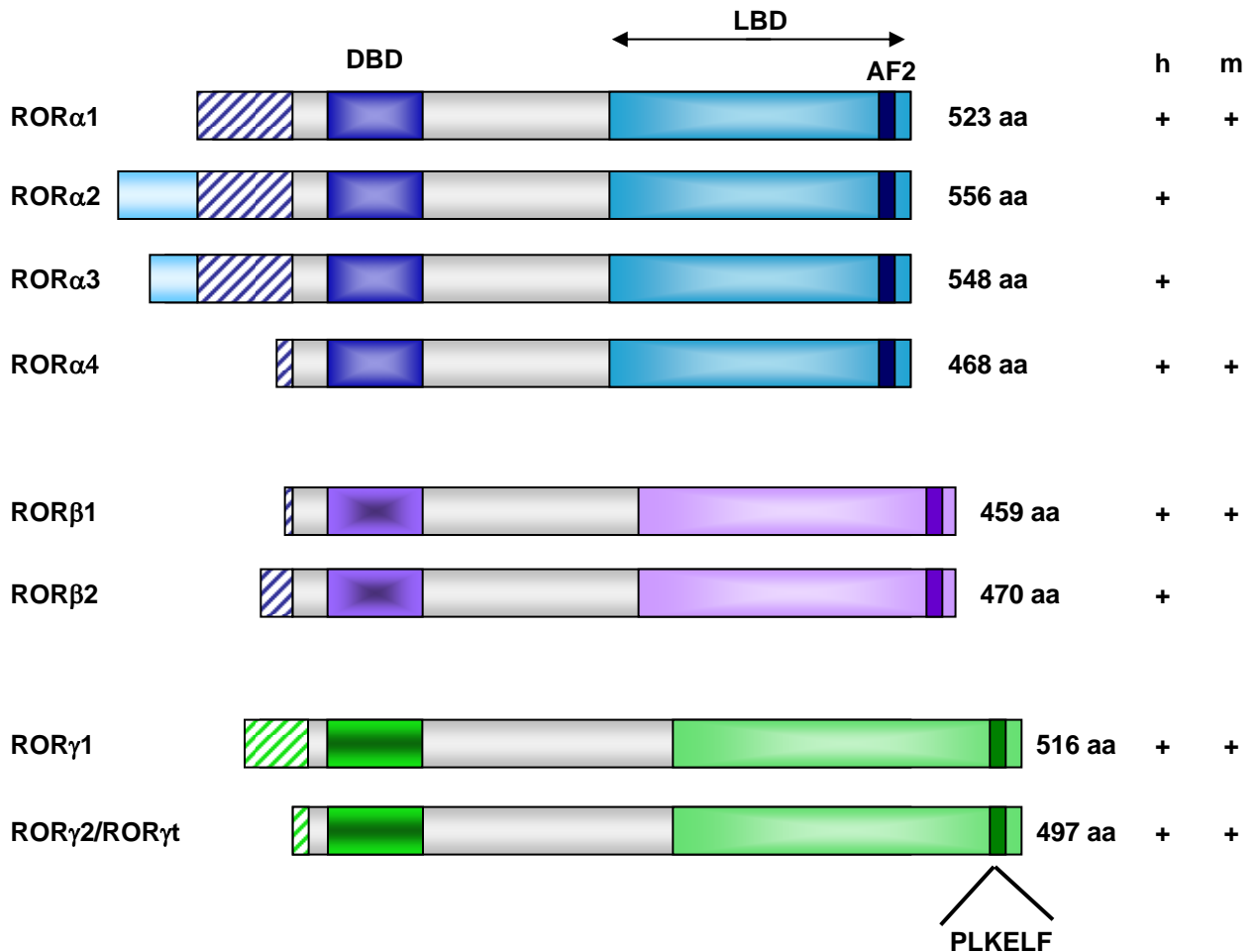


Figure 4. Schematic illustration of ROR family members. ROR family members include ROR α , ROR β and ROR γ . Several isoforms of each member have been described, due to either alternative promoter usage or alternative splicing (sequences shown with patterns in the N-terminal region of each ROR family member). Also indicated are the ligand binding domain (LBD), the DNA binding domain (DBD) and the activation function 2 (AF2) domain, with the amino acid sequence PLKELF. In the right, the expression of these members in human or mouse is indicated by a +. Adapted from Jetten, 2009.

Besides ROR γ t, Th17 cells show high expression of the orphan nuclear receptor ROR α . Over-expression of ROR α also promotes Th cells to become Th17 cells (Yang et al., 2008c). Deficiency in ROR α leads to reduced IL-17 expression both *in vivo* and *in vitro*. Whereas either ROR γ t- or ROR α -deficient mice exhibit reduced EAE disease severity and Th17 cell generation, deficiency in both ROR α and ROR γ t leads to complete resistance to EAE and impaired induction of Th17 cells *in vitro* and *in vivo* (Yang et al., 2008c). Indeed, over-expression of both ROR α and ROR γ t resulted in a synergistic induction of Th17 cells, even under unfavorable settings in which cells were stimulated under Th1-, Th2- or iTreg-skewing conditions (Yang et al., 2008c). Thus, these results confirm the requirement of these two transcription factors in the induction of Th17 cells. How the two transcription factors coordinately regulate Th17 cell responses though remains unclear.

I.4.c. BATF

The AP-1 transcription factor BATF (basic leucine zipper transcription factor, ATF-like) is also an important factor for the generation of Th17 cells (Schraml et al., 2009). BATF was found to associate with JunB and directly bind to *Il21*, *Il22* and *Il17a* promoters in addition to two intergenic regions between *Il17a-Il17f* genes (Schraml et al., 2009). By using BATF-deficient T cells, Schraml et al. showed that BATF is dispensable for Th1 or Th2 cell induction *in vitro* despite BATF expression in these two T cell lineages. However, T cells lacking BATF were unable to become Th17 cells *in vivo* or upon stimulation with TGF- β and IL-6 *in vitro* (Schraml et al., 2009). Consistent with these observations, BATF-deficient mice were resistant to EAE disease. At the molecular level, Th cells lacking BATF were able to induce ROR α and ROR γ t expression at early time points during Th17 cell generation (Schraml et al., 2009). However, these cells were unable to maintain the expression of these transcription factors, suggesting that BATF participates directly or indirectly in the maintenance of RORs expression in Th17 cells. Furthermore, Schraml et al also established by over-expression studies that BATF and ROR γ t synergize in the induction of Th17 cells, and that these two transcription factors

bind to overlapped conserved sequence in the *il17a* gene (Schraml et al., 2009). However, over-expression of ROR γ t in cells lacking BATF failed to induce Th17 cells, suggesting that the synergism between these two transcription factors is indispensable for proper generation of Th17 cells. BATF has been previously considered as an AP-1 repressor (Dorsey et al., 1995; Echlin et al., 2000). Thus, even though Schraml et al. demonstrated that binding of BATF with JunB directly regulates Th17-associated genes, it still remains unclear whether AP-1 might have an inhibitory role in the induction of Th17 genes.

I.4.d. IRF4

Interferon Regulatory Factor 4 (IRF4) has been previously shown to regulate development of Th2 cells by activation of GATA-3 (Hu et al., 2002; Lohoff et al., 2002; Rengarajan et al., 2002). Recently, Brustle et al. also demonstrated that IRF4 is crucial in directing Th17 cell differentiation (Brustle et al., 2007). Th cells deficient in IRF4, or treated with IRF4 siRNA, failed to commit towards the Th17 cell lineage. Indeed, IRF4-deficient Th cells showed impaired ROR γ t expression while induction of Foxp3 was enhanced (Brustle et al., 2007). Also, *Irf4*^{-/-} mice were resistant to EAE induction. However, transfer of wild type Th cells into IRF4 KO mice rescued Th17 cell generation and rendered these mice susceptible to EAE disease, demonstrating a T cell-intrinsic role of IRF4 in this process (Brustle et al., 2007).

I.4.e. AHR

Another transcription factor shown to regulate Th17 cell generation is the Aryl Hydrocarbon Receptor (AHR), a type I nuclear receptor which interacts with Hsp90 upon binding to its ligand (Schmidt and Bradfield, 1996). Even though AHR is expressed both in regulatory T cells as well as

Th17 cells, in comparison its expression is significantly enhanced in Th17 cells (Quintana et al., 2008; Veldhoen et al., 2008a). Veldhoen et al demonstrated that AHR-deficient Th cells have defective IL-22 production, although other Th17 cytokines were not dramatically affected (Veldhoen et al., 2008a). IL-22 has been shown to protect against hepatic damage in an acute inflammation model (Zenewicz et al., 2007). Consistently, mice lacking AHR develop hepatic defects (Schmidt et al., 1996).

Whereas Th17 and Treg levels were not affected in AHR-deficient mice, an increase in either Tregs or Th17 cells was observed upon ligation of this receptor with different ligands. Binding of AHR to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) led to an increase in Foxp3 levels, due to a direct binding of AHR transcription factor to *Foxp3* promoter (Quintana et al., 2008). Consistently, administration of one dose of TCDD *in vivo* before MOG immunization significantly inhibited EAE induction and reduced disease severity (Quintana et al., 2008). On the other hand, ligation of AHR with 6-formylindolo[3,2-b]carbazole (FICZ) *in vitro* during generation of Th17 cells significantly increased expression of Th17 cytokines IL-17, IL-17F and most dramatically IL-22 (Quintana et al., 2008; Veldhoen et al., 2008a). Moreover, mice treated with FICZ at the time of MOG immunization showed enhanced Th17 cell generation and more severe EAE disease development (Quintana et al., 2008; Veldhoen et al., 2008a). Thus, AHR is an important transcription factor that can regulate Th17/Treg balance depending on environmental factors/ligands that bind to it.

1.4.f. T-bet and GATA3

As previously mentioned, terminal differentiation of Th subsets is distinguished by the selective expression of lineage-specific transcription factors. Th1 cells express the master transcription factor T-bet, whereas Th2 cells express GATA3 (Dong and Flavell, 2000). Mice lacking T-bet showed enhanced IL-17-producing Th cells upon immunization with MOG peptide (Park et al., 2005). Moreover, T-bet-deficient mice developed enhanced experimental autoimmune myocarditis compared to wild type counterparts, which correlated with increased IL-17-producing T cells infiltrating the heart (Rangachari

et al., 2006). Furthermore, using an allergic airway inflammation model, Fujiwara et al. recently demonstrated that T-bet not only inhibits eosinophil recruitment by Th2 cells but also Th17-mediated neutrophilic inflammation (Fujiwara et al., 2007). The same group later demonstrated that whereas T-bet deficient mice had enhanced Th17 cell generation, mice lacking STAT4 or both STAT4 and T-bet had diminished antigen-specific IL-17-producing cells (Furuta et al., 2008). It has also been demonstrated using *Trypanozoma cruzi* infection model that T-bet is able to regulate in a T cell-intrinsic fashion the generation of antigen-specific Th17 cells (Guo et al., 2009). However, recently it has been shown that Th17 cells infiltrating the central nervous system during EAE are able to express T-bet and that indeed T-bet expression is crucial for encephalitogenicity of Th1 and Th17 cells (Yang et al., 2009). Consistent with this report, Dorf and colleagues have recently shown that CNS infiltrating T cells that express both T-bet and ROR γ t produce IFN- γ but vary in their IL-17 expression (Abromson-Leeman et al., 2009). However, Lovett-Racke and colleagues recently reported that suppression of T-bet reduced EAE disease severity by decreasing Th1 autorreactive T cells as well as restraining generation of Th17 cells through the regulation of IL-23R (Gocke et al., 2007). More recently, Glimcher and colleagues have demonstrated that T-bet inhibits the expression of the master transcription factor ROR γ t by preventing Runx1-induced transcription of *Rorc* gene. However, even though T-bet inhibits the expression of ROR γ t, and thus the development of Th17 cells, over-expression of both T-bet and ROR γ t still led to inhibition of IL-17-producing cells, clearly indicating that there is still an unidentified mechanism by which T-bet inhibits commitment to Th17 cells (Lazarevic et al., 2011). Thus, the precise role of T-bet, particularly in cells expressing both IFN- γ and IL-17 found in autoimmune diseases still remains controversial.

The role of GATA3 in Th17 cells has also been addressed. By utilizing arthritis model in transgenic mice overexpressing GATA3 on T cells (CD2-promoter driven), van Hamburg et al. recently showed that GATA3 inhibits the generation of IL-17-producing T cells systemically and in the local inflammation site (van Hamburg et al., 2009). Moreover, GATA3 over-expression led to reduced expression of the Th17 master transcription factor ROR γ t (van Hamburg et al., 2009). However, since

enhanced Th2 cytokines were also observed, it still remains unclear whether the GATA3 effect on Th17 cells is direct.

I.5. Th cells with suppressive function: natural and inducible regulatory T cells

The immune system in higher organisms has evolved to be able to recognize and fight against pathogens. However, side effects such as immune responses against self-tissues (autoimmunity), commensal microorganisms or even environmental antigens often develop. Thus, mechanisms to prevent such unwanted responses are needed. Several cell types have been shown to control inflammatory responses due to their suppressive nature, including tolerogenic dendritic cells, NKT cells, myeloid suppressor cells as well as certain subtypes of B and T cells. Based on their origin, CD4⁺ T cells with suppressive function can be divided into two groups: i) cells with a thymic origin which have never been exposed to their cognate antigen prior to their generation, constituted only by thymus-derived natural regulatory T cells (nTregs), or ii) cells generated after exposure to their cognate antigen, constituted by inducible regulatory T cells (iTregs), IL-10-producing Th cells (Tr1 cells) and TGF- β -producing Th cells (Th3 cells). One hallmark of both natural and inducible regulatory T cells is the expression of the master transcription factor Foxp3 (Fig. 5).

The identification of thymus-derived natural regulatory T cells became evident in experiments using neonatal thymectomized mice, which develop severe inflammatory responses. However, transfer of T cells from euthymic mice (expressing the IL-2R α chain, CD25) prevented disease development (Sakaguchi et al., 1995; Suri-Payer et al., 1998). Furthermore, Powrie and Mason showed that adoptive transfer of naïve T cells (CD4⁺CD45RB^{high}) into lymphopenic mice caused development of inflammatory bowel disease, while co-transfer of CD4⁺CD45RB^{low} cells prevented disease induction (Powrie and Mason, 1990). These results clearly demonstrate the existence of a CD4⁺ T cell population with suppressive phenotype.

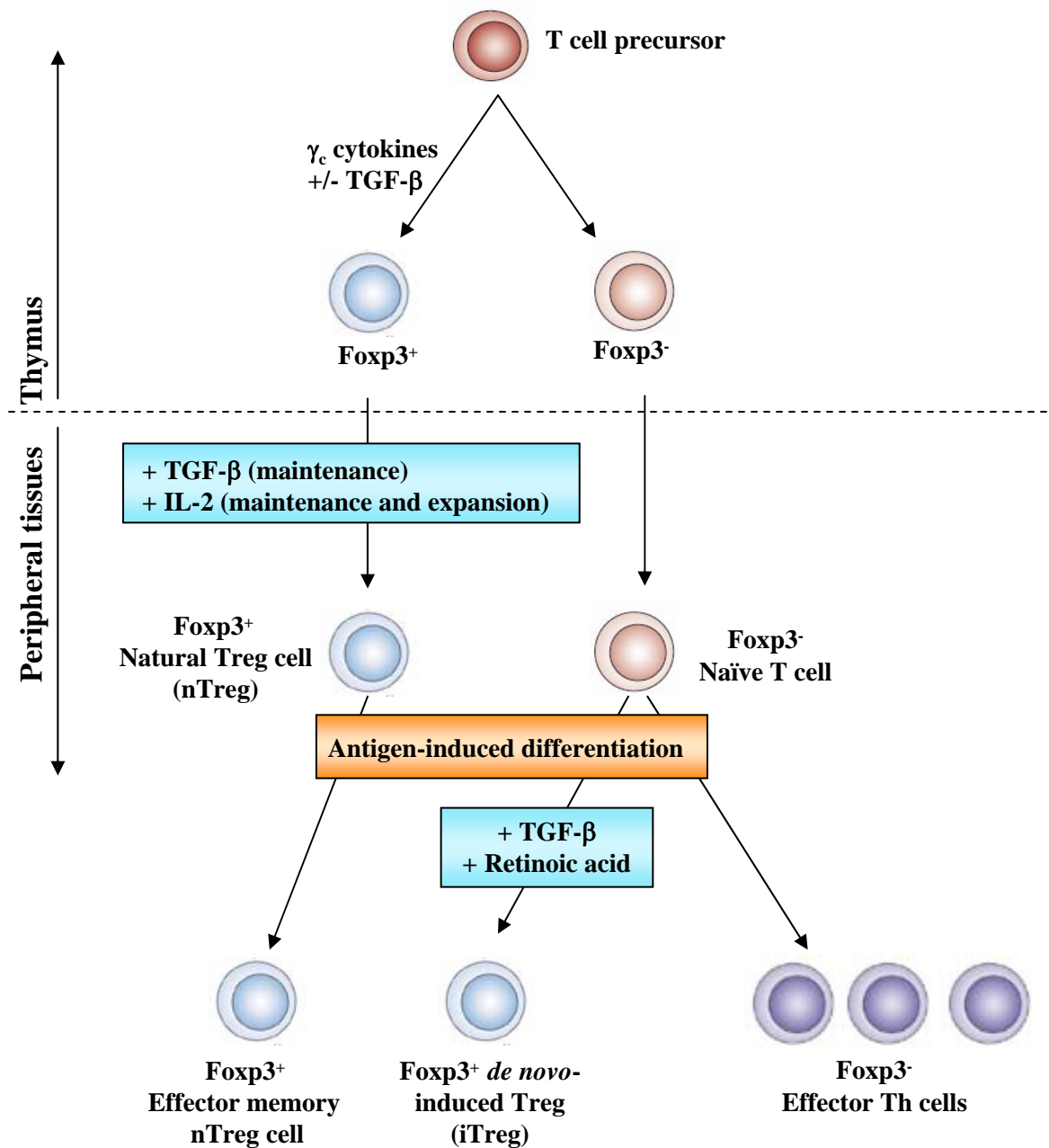


Figure 5. Development of natural and inducible regulatory T cells. The expression of the master transcription factor forkhead box P3 (Foxp3) defines the regulatory Th subset. Foxp3 expression can be acquired in the thymus, and these cells are termed natural Treg cells (nTreg). The exact mechanism by which Foxp3 is induced in thymic CD4 single positive T cells is not entirely understood, but cytokines that utilize the common γ -chain subunit receptor, such as IL-2, are required for nTreg development. The role of TGF- β in this process is still contradictory. However, both TGF- β and IL-2 are required in peripheral tissues for nTreg cell maintenance. Foxp3 expression can also be induced in the periphery from naïve Th cells, in a TGF- β -dependent manner upon antigen-induced activation of naïve T cells. Also, retinoic acid, a vitamin A metabolite, can induce Foxp3 expression in peripheral tissue, primarily the gut. Adapted from Huehn et al., 2009.

In an attempt to further characterize these regulatory T cells, Foxp3 was then identified as the transcription factor required for their generation and suppressive function (Fontenot et al., 2003; Hori et al., 2003; Khattri et al., 2003). Indeed, mice deficient in Foxp3 (scurfy mice) or patients with IPEX (Immune-dysregulation Polyendocrinopathy Enteropathy X-linked) syndrome, who show several mutations in *Foxp3* gene, display a similar autoimmune phenotype as the one observed in neonatal thymectomized mice (Brunkow et al., 2001; Chatila et al., 2000; Wildin et al., 2001). Both mice and human diseases are characterized by lymphoproliferation, and infiltration of lymphocytes to several tissues. The role of Foxp3 as the master transcription factor involved in regulatory T cells function has been widely confirmed by independent laboratories throughout the world.

Besides the thymic origin of Foxp3-expressing natural regulatory T cells, cumulative evidence has shown that Foxp3 can be acquired in the periphery in a TGF- β -dependent manner from naïve CD4⁺ Th cells (reviewed in (Curotto de Lafaille and Lafaille, 2009), Fig. 1 and Fig. 5). TGF- β plays an important role in the development, maintenance, and induction of regulatory T cells (Chen et al., 2003; Curotto de Lafaille et al., 2008; Fantini et al., 2004). TGF- β -induced activation of Smad3 leads to its recruitment to a cis-regulatory region in *Foxp3* gene, and the induction of Foxp3 expression in synergism with NFAT (Nuclear Factor of Activated T cells) (Tone et al., 2008). Furthermore, this conserved non-coding sequence (CNS) region has been shown to be crucial for the development of iTreg cells, whereas it is completely dispensable for induction of nTreg cells in mice (Zheng et al., 2010).

Foxp3 expression is crucial and required for regulatory T cell function. Indeed, forced over-expression of Foxp3 in CD4⁺ Th cells leads to enhanced expression of Treg-associated markers such as CTLA-4 (Cytotoxic T-Lymphocyte Antigen 4), GITR (Glucocorticoid-Induced TNFR-Related protein), and acquisition of a suppressive phenotype (Fontenot et al., 2003; Hori et al., 2003). Furthermore, as a master regulator of this lineage commitment, Foxp3 controls its own expression by binding to the regulatory element CNS2 in *Foxp3* gene in a Cb β -Runx1 dependent manner (Zheng et al., 2010).

The relative abundance of effector and suppressor cells will then determine the outcome of an immune response. Excessive control of inflammatory responses by regulatory T cells may promote enhanced pathogen survival and/or growth whereas defective suppressive function might lead to “collateral” injury to the host tissues due to an increased inflammatory response. Furthermore, this sustained inflammatory response might provoke the induction of autoimmunity. Indeed, defective regulatory T cell activity or numbers has been documented in several autoimmune disorders (reviewed in (Jager and Kuchroo, 2010; Littman and Rudensky, 2010; Vila et al., 2009)). In the following section we will focus our attention to the role of inflammatory and regulatory cells in inflammation, autoimmunity and cancer.

I.6. Role of Th17 and regulatory T cells in inflammation, autoimmunity and cancer

Over the past few years, Th17 and regulatory T cells, with opposing functions in the immune system, have been a major focus of research in several types of diseases, ranging from infectious diseases to cancer, inflammation and autoimmunity (reviewed in (Jager and Kuchroo, 2010; Littman and Rudensky, 2010; Martinez et al., 2008)). In this section, we will center our attention to the latest findings and controversies on the role of Th17 and regulatory T cells in cancer and autoimmune diseases.

I.6.a. Role of Th17 and Treg cells in cancer

Regulatory T cells inhibit T cell proliferation (Wing et al., 2006) and play important roles in hindering anti-tumor immune responses in mouse models (Danese and Rutella, 2007). Indeed, depletion of CD4⁺CD25⁺ nTreg cells allows the rejection of a colon tumor (Golgher et al., 2002). Furthermore, high frequency of regulatory T cells have been detected in peripheral blood, draining lymph nodes and in tumor sites from patients with cancer (Danese and Rutella, 2007).

Several tumors directly secrete IL-10 and TGF- β , two cytokines that can either induce or maintain the generation of regulatory T cells. TGF- β is an important cytokine driving the generation of both regulatory and Th17 cells, by inducing the expression of Foxp3 and ROR γ t master transcription factors respectively (Ivanov et al., 2006; Marie et al., 2005). Therefore, TGF- β secretion by tumor cells or the tumor microenvironment might lead to the development of regulatory T cells or Th17 cells, depending on the presence and relative abundance of pro-inflammatory cytokines. In fact, the tumor microenvironment has been found to directly induce the *de novo* generation of Treg cells (Danese and Rutella, 2007), and similar kinetics in recruitment of both Th17 and Treg cells have been observed in several tumor microenvironments (Kryczek et al., 2007b).

The role of Th17 cells in tumor progression/destruction is controversial. IL-17, produced among others by Th17 cells, can stimulate the production of chemokines or cytokines associated with an inflammatory process ((Kawaguchi et al., 2004) and reviewed in (Martinez et al., 2008)), and certain cancers take advantage of inflammatory mediators for their own benefit to induce angiogenesis and tissue destruction/remodeling (Coussens and Werb, 2002; Numasaki et al., 2005). Also, IL-23, which is required to promote Th17 cell expansion and survival, has been shown to be an important factor in tumor cell growth and development (Langowski et al., 2006). On the contrary, recruitment of CD8⁺ T lymphocytes with cytotoxic activity against the tumor can also be enhanced by IL-17 (Benchetrit et al., 2002). Moreover, transfer of melanoma-specific Th17 cells into mice confers resistance to tumor growth, indicating Th17 cells have an important role in the induction of anti-tumor immune responses (Martin-Orozco et al., 2009b; Muranski et al., 2008). Thus, it still remains controversial whether inflammatory responses induced by Th17 cells have pro- or anti-tumor capacities.

1.6.b. Role of Th17 and Treg cells in autoimmune/inflammatory diseases

Th17 cells have been shown to have major pathogenic roles in several autoimmune and inflammatory diseases. While in this section we will focus only on multiple sclerosis, Th17 cells play important roles in the induction of other diseases including psoriasis (Lowes et al., 2008), rheumatoid arthritis (Agarwal et al., 2008; Hirota et al., 2007a; Hirota et al., 2007b; Joosten et al., 2008; Kohno et al., 2008; Nistala et al., 2008), Sjögren's syndrome (Nguyen et al., 2008), systemic lupus erythematosus (Herber et al., 2007; Sawalha et al., 2008; Wong et al., 2008) and systemic sclerosis (Deleuran and Abraham, 2007; Murata et al., 2008), among others (reviewed in (Martinez et al., 2008)). Also, recently it was demonstrated that in BDX2 mice, which spontaneously develop autoimmune disease, Th17 cells induce autoreactive germinal centers (Hsu et al., 2008). Furthermore, Th17-associated cytokines IL-17A, IL-17F and IL-23 can induce the expression of chemokines such as GRO-alpha/CXCL1, MIP-1beta/CCL4 and IL-8/CXCL8 by human eosinophils, possibly implicating these cytokines in allergic inflammation (Cheung et al., 2008) (Fig. 2).

Multiple sclerosis is a human disease characterized by an ascending paralysis due to an inflammatory demyelinating response. T cells, primarily Th17 cells, play a crucial role in the disease onset and progression. In rodents, ascending paralysis can be induced upon immunization with peptides derived from myelin protein, or by adoptive transfer of myelin-specific T cells. This model in rodents is termed experimental autoimmune encephalomyelitis (EAE), and is the mouse homologue to human multiple sclerosis. Before the description of Th17 cells as an independent Th lineage, Th1 cells were believed to play an important role in the pathogenesis of this disease. However, mice deficient in STAT1, IL-12 p35 or IFN- γ showed enhanced susceptibility to EAE, contradicting the current paradigm at the time (Bettelli et al., 2004; Ferber et al., 1996; Segal et al., 1998). It is now well established that Th17 cells, rather than Th1 cells, are the primary reactive cells inducing this disease (Cua et al., 2003; Langrish et al., 2005; Park et al., 2005; Yang et al., 2008a).

The role of Th17-associated cytokines has been evaluated in the induction and/or maintenance of EAE disease. Even though Th17 cells express IL-22, IL-17 and IL-17F in the central nervous system (CNS), IL-17 but not IL-22 or IL-17F is directly required for disease induction (Kreymborg et al., 2007; Yang et al., 2008a). Another cytokine expressed by cells infiltrating the CNS is IL-21, which is primarily produced by Th17 cells. Mice lacking IL-21 or its receptor have delay in EAE disease onset and have an overall decrease in disease severity (Korn et al., 2007a; Nuriyeva et al., 2007).

Even though IL-22 seems to be dispensable for induction of EAE, IL-17R and IL-22R are highly expressed on CNS vessels within highly infiltrated lesions in brain samples from multiple sclerosis patients (Kebir et al., 2007). IL-17 and IL-22 can increase the permeability of endothelial cells. Consistent with this observation, human Th17 cells were able to migrate more avidly across human brain-derived microvascular endothelial cells when compared to Th1 cells, indicating that by producing IL-17 and IL-22, Th17 cells can access the CNS parenchyma (Kebir et al., 2007). Also, CD4⁺CD45RO⁺ cells expressing both IL-22 and IL-17 have been found in highly infiltrated multiple sclerosis lesions compared to non-inflamed brain samples (Kebir et al., 2007; Tzartos et al., 2008).

Due to the controversies on whether Th1 or Th17 cells are required in the induction or maintenance of disease, Goverman's group evaluated the role of these cells in EAE and found that inflammation in brain or spinal cord depends on the Th1:Th17 ratio of antigen-specific cells (Stromnes et al., 2008). Low and high ratios of Th17:Th1 cells infiltrate the spinal cord and brain meninges at early stages during disease development. However, at later stages infiltration of cells into brain parenchyma occurs only at high Th17:Th1 ratios, while infiltration into spinal cord parenchyma occurs at high Th1:Th17 ratios (Stromnes et al., 2008).

Regulatory T cells restrict over-reactive responses by inhibiting T cell proliferation, and thus have crucial roles in preventing the induction or maintenance of autoimmune diseases (reviewed in (Littman and Rudensky, 2010)). In EAE settings, Kuchroo and colleagues have demonstrated that while antigen-specific regulatory T cells are generated in peripheral tissues and accumulate in the CNS, they do not prevent EAE disease onset (Korn et al., 2007b). However, these regulatory T cells infiltrating the CNS are capable of suppressing *in vitro* naïve T cell proliferation, but are unable to suppress

encephalitogenic effector T cells (Korn et al., 2007b). In human multiple sclerosis, however, no Foxp3⁺ Treg cells were observed in either active or inactive areas of brain tissues (Tzartos et al., 2008), indicating a possible difference between human and mouse systems. On the other hand, in an arthritis model, enhanced Th17 cell levels were detected as disease progressed from the mild form of persistent oligoarthritis to the more severe form of disease (extended oligoarthritis), whereas an inverse correlation was found with regulatory T cells (Nistala et al., 2008). Thus, even though Treg cells have been clearly shown to mediate immune suppression, their exact role in autoimmune diseases in human *versus* mouse systems needs further investigation.

1.7. Objective, hypothesis and specific aims of the present work

The main goal of the present dissertation is to understand the events involved in the induction of inflammatory versus suppressive responses by investigating the developmental pathways and factors required for the commitment towards Th17 or regulatory T cells, two Th cell lineages with opposing functions in the immune system. In particular, the objective of this dissertation is to further understand the reciprocal regulation of Th17 and regulatory T cells so that we can identify key target molecules that drive or repress differentiation of each cell lineage at the transcriptional level. The identification of these molecules will aid the development of tools for possible therapies to enhance the commitment to one cell lineage while inhibiting the commitment towards the other. This approach could be used for targeting diseases where the balance of these T cells is dysregulated, such as in autoimmunity, chronic infectious diseases and cancer.

We thus hypothesized that there is cross-regulation between Th17 and Treg genetic programs and that TGF- β induces Th17 or Treg cells through differential signaling pathways. For this purpose, in Chapter II, we have evaluated the role of the Treg-specific transcription factor, Foxp3, in development of Th17 cells. Then, in Chapter III, we determined the requirement of the Smad-dependent TGF- β signaling pathway in the generation of Th17 and Treg cells.

CHAPTER II – RECIPROCAL GENERATION OF TH17 AND REGULATORY T CELLS¹

BACKGROUND

In the presence of TGF- β , naïve CD4⁺ T helper (Th) cells can differentiate into either suppressor or effector cells (Fig. 1). TGF- β plays an important role in the development of both iTreg and Th17 lineages by inducing the expression of the master transcription factors Foxp3 and ROR γ t, respectively (Ivanov et al., 2006; Marie et al., 2005). The requirement of TGF- β in the induction of iTreg and Th17 cells suggested that these two Th cell lineages, with opposing functions in the immune system, may be reciprocally regulated (Fig. 6 and (Bettelli et al., 2006)). The commitment towards either cell lineage therefore depends on additional cytokines present in the microenvironment during T cell priming. Whereas IL-2 regulates the generation of inducible regulatory T (iTreg) cells, IL-6 and/or IL-21 induces the generation of Th17 cells (Bettelli et al., 2006; Korn et al., 2007a; Mangan et al., 2006; Nurieva et al., 2007; Setoguchi et al., 2005; Veldhoen et al., 2006a).

In a TGF- β -rich environment like the gut, presence of a pathogen would induce the activation of antigen-presenting cells such as dendritic cells, through the recognition of PAMPs by PRRs. Consequently, these activated antigen presenting cells would secrete pro-inflammatory cytokines such as IL-6 and IL-23 (Fig. 6). Thus TGF- β and IL-6 would induce the generation of IL-17-producing T cells, and IL-23 would further expand these cells. Moreover, Th17 cells produce IL-21, which would act in an autocrine manner to further amplify the induction of Th17 cells in the local microenvironment. Th17 cells also secrete IL-17 and IL-17F, which induce the production of G-CSF and IL-8 by epithelial cells and fibroblast, leading to the recruitment of neutrophils and therefore enhancing the inflammatory

¹ The results presented in this Chapter have been published in Yang et al., 2008b.

process (Fig. 6). Also, Th17-associated cytokines can induce the secretion of anti-microbial peptides from epithelial cells to help fight the ongoing infection (Fig. 6).

On the contrary, if the naïve T cell priming occurs in the absence of “danger signals” (homeostatic conditions), then the mucosal dendritic cells presenting antigens would produce retinoic acid. This vitamin A derivative inhibits TGF- β -dependent Th17 cell generation through inhibition of ROR γ t expression while promoting Foxp3⁺ Treg cell differentiation (Elias et al., 2008; Kang et al., 2007; Mucida et al., 2007; Schambach et al., 2007; Sun et al., 2007). Retinoic acid was found to enhance Smad3 protein and phosphorylation levels, resulting in increased TGF- β signaling, which would then lead to increased Foxp3 expression and reduced expression of Th17-associated genes IL-6R, IL-23R and IRF4 in a Stat3- and Stat5-independent manner (Elias et al., 2008; Xiao et al., 2008). Thus, retinoic acid, together with TGF- β would favor the commitment towards a regulatory T cell, which produces IL-10, IL-35 and TGF- β , playing important roles in immune-suppression (Fig. 6).

Foxp3 is a member of the forkhead/winged-helix family of transcription factors (reviewed in (Zhou et al., 2008b)). It has been demonstrated that Foxp3 can interact with other transcription factors, including AP-1 (Activator Protein 1) (Lee et al., 2008), NF- κ B (Nuclear Factor of kappa light polypeptide gene enhancer in B cells) (Bettelli et al., 2005), NFAT (Bettelli et al., 2005; Wu et al., 2006), Forkhead box Protein P1 (Foxp1) (Li et al., 2007b) and Runx1/AML1 (Runt-related transcription factor 1/acute myeloid leukemia 1) (Ono et al., 2007). Furthermore, Foxp3 can also bind to proteins with enzymatic activity such as histone deacetylases HDAC1, HDAC9 and HDAC7 as well as HIV-1 Tat interacting protein 60 KDa (TIP60) (Li et al., 2007a). Interestingly though, it has been suggested that HDAC7 and TIP60, with opposing enzymatic activity, might compete for Foxp3 binding, implying a dynamic process (Zhou et al., 2008b). Consistent with this idea, it has been shown that over-expression of Foxp3 in T cells can lead to induction of transcription of some genes while inhibition of another group of genes. The enhanced transcription was directly associated with acetylation of histone H3 whereas downregulation of transcription correlated with reduced acetylation of histon H3 (Chen et

al., 2006a). Accordingly, Foxp3 can be found in two distinct molecular complexes upon over-expression which might explain these opposite functions (Liang et al., 2006).

Foxp3 has been shown to require Runx1 for its transcriptional activity (Ono et al., 2007). Interestingly, ROR γ t, the Th17-specific transcription factor, also relies on Runx1 to induce transcription of *il17a* gene, suggesting that both Foxp3 and ROR γ t might compete for Runx1 binding, which in turn would define the outcome of a cell becoming a regulatory T cell or a Th17 cell (Zhang et al., 2008). Furthermore, while TGF- β stimulation leads to enhanced binding of Foxp3 to chromatin, stimulation of T cells with TGF- β together with IL-6 results in reduced binding of Foxp3 to DNA (Samanta et al., 2008), suggesting that the reciprocal regulation of these Th lineages might occur at the transcriptional level.

Even though it is clear that Th17 and regulatory T cells are reciprocally regulated during their development, the exact molecular regulation of Th17 and iTreg cell genetic programs remains unclear. We thus analyzed how Foxp3, the Treg-specific master transcription factor, can regulate the developmental process of the opposing Th17 lineage. Our results have identified a molecular antagonism of Th17 and regulatory T cell genetic programs.

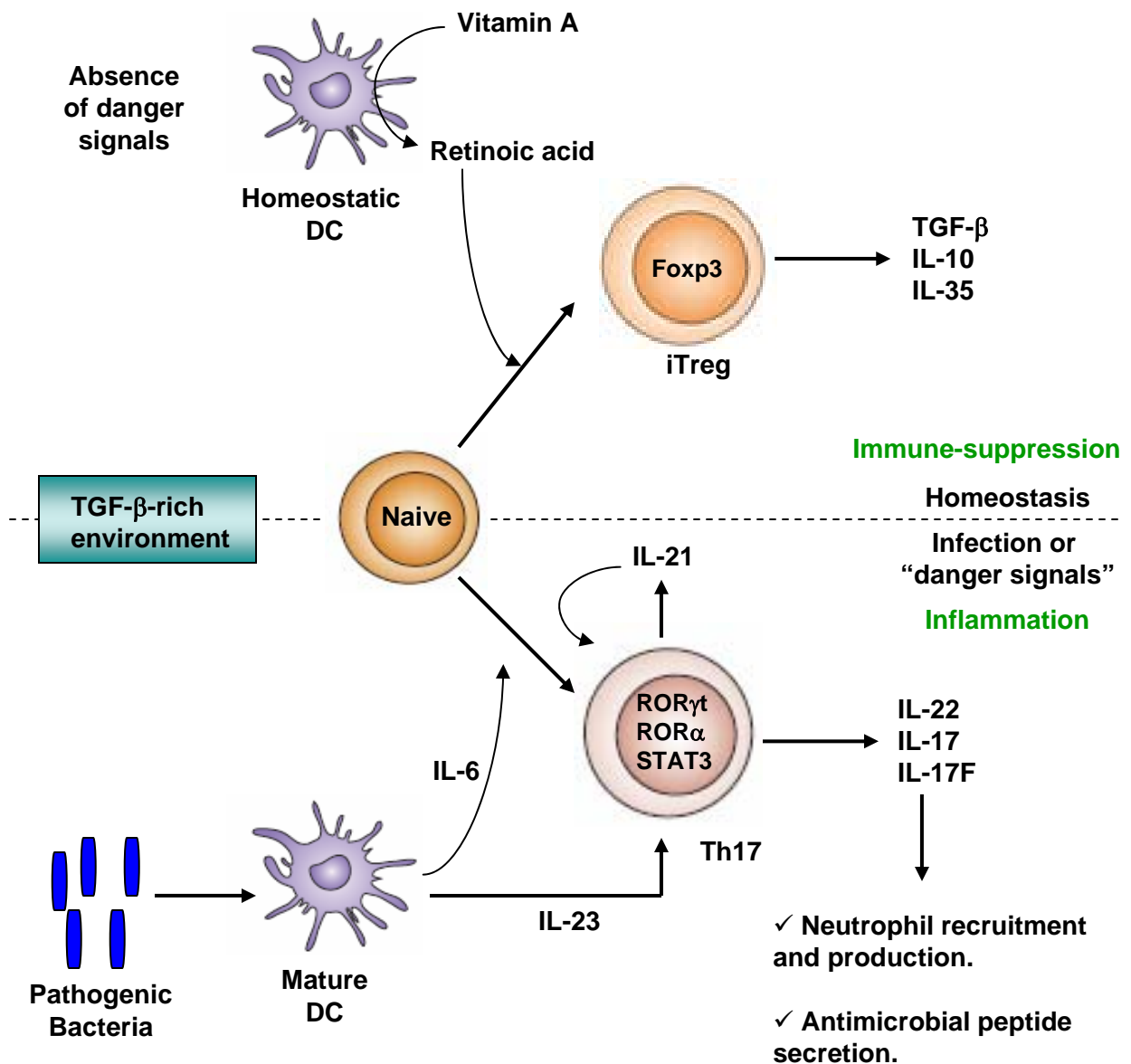


Figure 6. Reciprocal generation of Th17 and regulatory T cells. In a TGF-β-rich environment, naïve CD4⁺ Th cells can differentiate into either Th17 or regulatory T cells, depending on whether T cell priming occurs in the presence of “danger signals” or under homeostatic conditions. In the absence of “danger signals” (top part), homeostatic dendritic cells present in the gut can convert vitamin A from the diet into retinoic acid, which would favor Foxp3 induction and therefore generation of inducible regulatory T cells (iTreg). This scenario leads to an environment where immune suppression prevails. In contrast, in the presence of danger signals, activated (mature) dendritic cells would produce pro-inflammatory cytokines such as IL-6, which together with TGF-β, induce Th17 cell generation (bottom part). Furthermore, these mature dendritic cells produce IL-23 which further expands this IL-17-producing T cells. Th17 cells, through the production of IL-22, IL-17 and IL-17F induce neutrophil recruitment and production as well as the secretion of antimicrobial peptides from epithelial cells. This scenario then leads to an inflammatory condition. Adapted from Weaver and Hatton, 2009.

RESULTS

II.1. Foxp3 inhibits differentiation of Th17 cells independently of its DNA binding domain

Previous work has shown that Th17 and regulatory T cells are reciprocally regulated by TGF- β (Bettelli et al., 2006). Thus, we determined the kinetics for the induction of Th17 and iTreg cells by utilizing an IL-17F-RFP and Foxp3-GFP double reporter mouse model. Naïve T cells from these mice were stimulated under Th17 or iTreg polarizing conditions *in vitro* and RFP and GFP expression was determined by flow cytometry (Fig. 7). We found that during the first two days of *in vitro* differentiation, Foxp3 expression was induced independently of the polarizing condition utilized. On the other hand, a significant proportion of IL-17F-RFP⁺ cells were observed the second day of culture upon Th17-polarizing stimuli. However, on days three and four, Foxp3 expression was down-regulated under Th17-skewing conditions, corresponding with an enhancement of IL-17F-RFP⁺ cells (Fig. 7). Similar results were also obtained when expression of IL-17 and Foxp3 was determined by intracellular staining (data not shown).

Given that Th17-polarizing conditions induced the expression of Foxp3, which was then down-regulated as the differentiation proceeded, we next evaluated the role of sustained Foxp3 expression on Th17 cell differentiation. For this purpose, Foxp3 was over-expressed in activated T cells by retroviral transduction with bicistronic virus containing IRES-GFP, which allows transduced cells to be identified based on GFP expression. Naïve OT-II TCR transgenic T cells were cultured with irradiated T-cell depleted splenocytes as antigen presenting cells (APCs) under Th17-skewing conditions and after one day, cells were infected with viral supernatants. Four days later, cells were analyzed for Foxp3 and IL-17 expression by intracellular staining in GFP⁻ (non-infected) or GFP⁺ (infected) cells. Over-expression of Foxp3 greatly reduced the generation of IL-17-producing T cells compared to cells infected with a control virus (Fig. 8A).

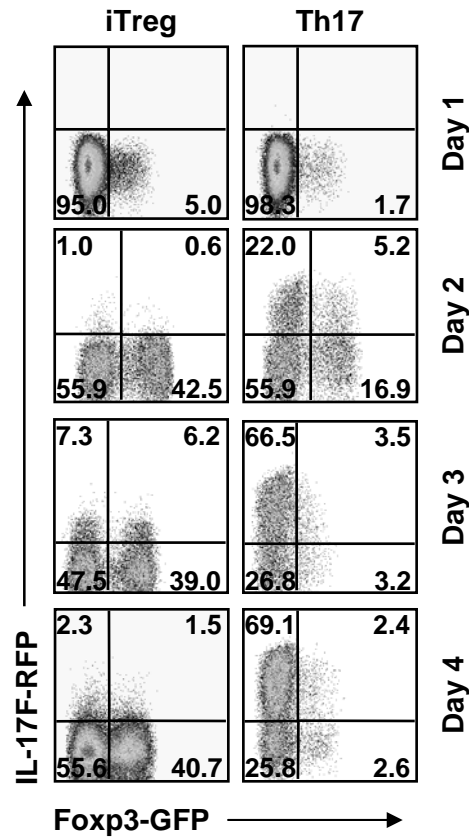


Figure 7. Transient induction of Foxp3 expression in developing Th17 cells. FACS-sorted naïve T cells from IL-17F-RFP x Foxp3-GFP mice were activated under Th17 (TGF- β , IL-6, anti-IFN- γ , anti-IL-4) or iTreg (TGF- β , IL-2, anti-IFN- γ , anti-IL-4) conditions. GFP and RFP expression was assessed daily by flow cytometry. A representative experiment out of three is shown. Similar results were also obtained by performing intracellular cytokine staining. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

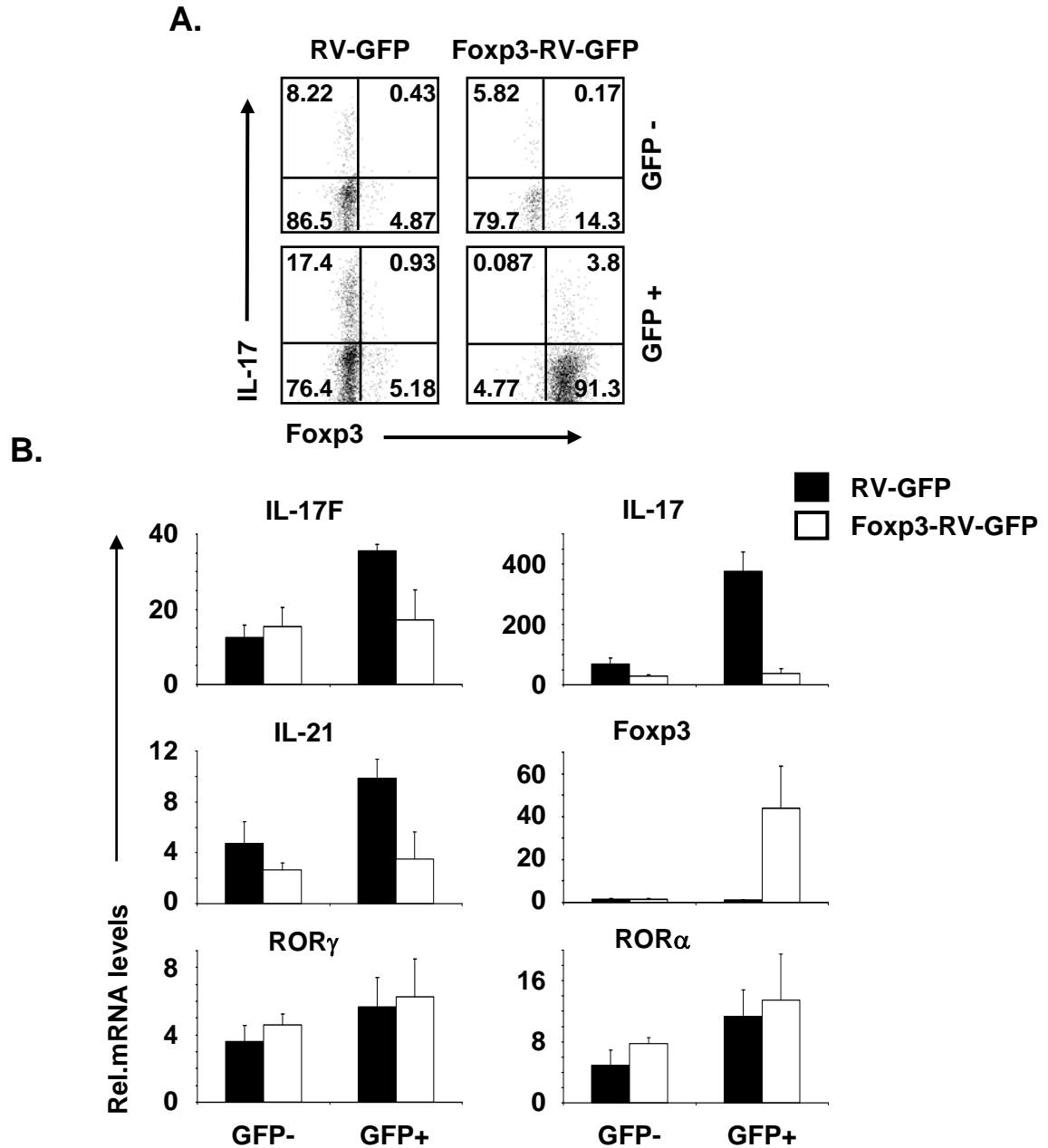


Figure 8. Foxp3 over-expression inhibits Th17 cell generation. FACS-sorted naïve T cells from OT-II TCR transgenic mice were activated under Th17 conditions (TGF- β , IL-6, anti-IFN- γ , anti-IL-4) and infected with an IRES-GFP-containing bicistronic retrovirus expressing Foxp3 or a vector control virus. GFP⁻ and GFP⁺ cells were FACS-sorted, and IL-17- and Foxp3-expressing cells were measured by intracellular staining on each population. The experiments were repeated at least three times with similar results. (B) GFP⁻ or GFP⁺ cells from (A) were restimulated for 4 hours with anti-CD3. mRNA expression of indicated genes was analyzed by real-time RT-PCR. The data shown were normalized to expression of a reference gene β -actin. The lowest expression levels for each gene were referred as 1. Data shows mean \pm S.D. A representative experiment out of four is shown. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

To identify the mechanism by which Foxp3 inhibits the generation of IL-17-producing T cells, cells were purified by FACS-sorting based on GFP expression and their gene expression profile was determined by real time RT-PCR. Compared to cells infected with a control virus, cells over-expressing Foxp3 exhibited lower IL-17, IL-17F and IL-21 mRNA levels, while the expression of the two major Th17 transcription factors ROR α and ROR γ was not affected (Fig. 8B). These results suggested that Foxp3 might affect Th17 cell generation by directly hindering with the function of these Th17-associated transcription factors.

To test the hypothesis that Foxp3 interferes with ROR α or ROR γ transcriptional activity, we used a luciferase reporter assay. EL-4 thymoma cells were transfected with a luciferase reporter vector where Firefly Luciferase was cloned under the control of an *Il17* promoter and *conserved non-coding sequence 2 (CNS2)* region (Yang et al., 2008c) in the presence or absence of vectors coding for ROR α , ROR γ and/or Foxp3. Expression of either ROR α or ROR γ alone led to substantial luciferase activity while co-expression of Foxp3 inhibited this induction (Fig. 9A and B). Furthermore, Foxp3 also inhibited ROR γ activation of a RORE (ROR response element) luciferase reporter in a dose-dependent manner (Fig. 9C). Although no putative Foxp3-binding sites were identified in *Il17* promoter, *CNS2* region or RORE luciferase vector, we tested whether Foxp3 DNA binding or homo-dimerization domains were required for the suppression of ROR γ or ROR α function. By using site-directed mutagenesis, two Foxp3 mutants were cloned into the retrovirus vectors: i) Foxp3 Δ FKH/NLS, a mutant containing the SV40 nuclear localization sequence but lacking the forkhead domain (Lee et al., 2008), or ii) Foxp3 Δ E250, a mutant that contains a single amino acid deletion in the leucine-zipper domain which impairs its ability to homo-dimerize and thus reduces its ability to bind DNA (Chae et al., 2006). Co-expression of Foxp3 mutants were still found to inhibit both ROR α and ROR γ -induced luciferase activity (Fig. 9A and B). Also, Foxp3 Δ E250 and Foxp3 Δ FKH/NLS were able to inhibit Th17 cell generation in primary T cells (Fig. 9D). Together, these data indicated that sustained Foxp3 expression decreased Th17 cell development through inhibition of ROR α and ROR γ transcriptional activity in a process that is independent of DNA binding.

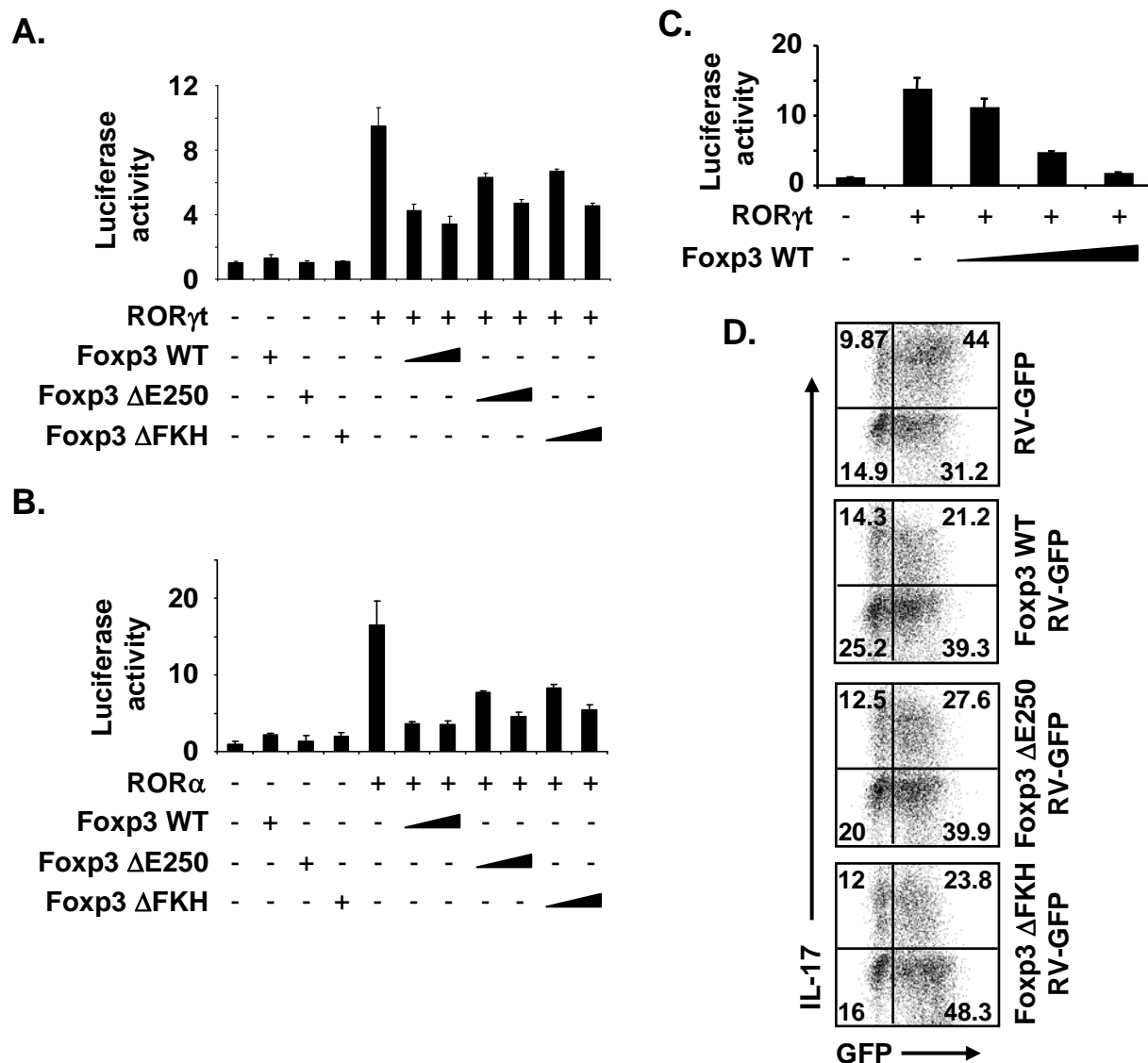


Figure 9. Foxp3 inhibits ROR α and ROR γ t transcriptional activity independent of DNA binding or homo-dimerization. (A-B) EL-4 cells were transfected with a vector containing the firefly luciferase gene under the control of the *Il17* promoter-CNS2 region, a vector expressing Renilla luciferase, and IRES-GFP-containing bicistronic vectors expressing ROR γ t (A) or ROR α (B), Foxp3 wild-type (WT) or various Foxp3 mutants, or vector alone. Cells were stimulated with PMA and ionomycin, and luciferase activity was determined and normalized to Renilla luciferase. Values were also normalized to vector alone. The data represent at least four independent experiments with consistent results. (C) HEK 293T cells were co-transfected with RORE-Luciferase reporter vector in the presence or absence of ROR γ t and Foxp3 WT with increasing concentrations. Then, luciferase activity was determined and normalized to β -galactosidase activity. Values were also normalized to vector alone. The data represent at least three independent experiments with consistent results. (A-C) data shows mean \pm S.D. (D) FACS-sorted naïve OT-II TCR transgenic T cells were stimulated under Th17 polarizing conditions (TGF- β , IL-6, anti-IFN- γ , anti-IL-4) and infected with an IRES-GFP-containing bicistronic retrovirus expressing Foxp3 (WT or mutants) or a vector control virus. IL-17-expressing cells were measured by intracellular staining. The experiments were repeated at least two times with similar results. Reprinted from "Molecular antagonism and plasticity of regulatory and inflammatory T cell programs." Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

II.2. Foxp3 LxxLL and TIP60/HDAC7 domains are required for inhibition of RORs transcriptional activity

Nuclear receptors such as ROR α and ROR γ t bind to LxxLL motifs (where L means leucine residues and x means any other amino acid) in nuclear corepressor (NCOR) or nuclear coactivator (NCOA) family members through their AF2 transactivation domain (reviewed in (Xu et al., 2009)). Since Foxp3 contains the sequence LQALL in its second exon (LxxLL structure), we hypothesized that Foxp3 directly interacts with ROR α or ROR γ t, competing with the ability of coactivators to bind to these nuclear receptors. To examine this hypothesis, we first determined the ability of Foxp3 to bind ROR α by co-immunoprecipitation. Flag-tagged ROR α was over-expressed in HEK 293T cells in the presence or absence of full length Foxp3 or several Foxp3 mutants, and immunoprecipitation was performed using anti-Flag. We found that Foxp3 interacted with ROR α upon over-expression (Fig. 10A). However, a Foxp3 variant with a mutation in the LxxLL sequence (LL-AA mutant where the last two consecutive leucine residues were mutated to alanine residues) showed a severe reduction in its association with ROR α (Fig. 10A). Furthermore, a Foxp3 mutant that contains only the first two exons of Foxp3 (which includes the LxxLL motif) but lacks the dimerization and DNA binding domains was capable of inhibiting ROR γ t transcriptional activity (Fig. 10B).

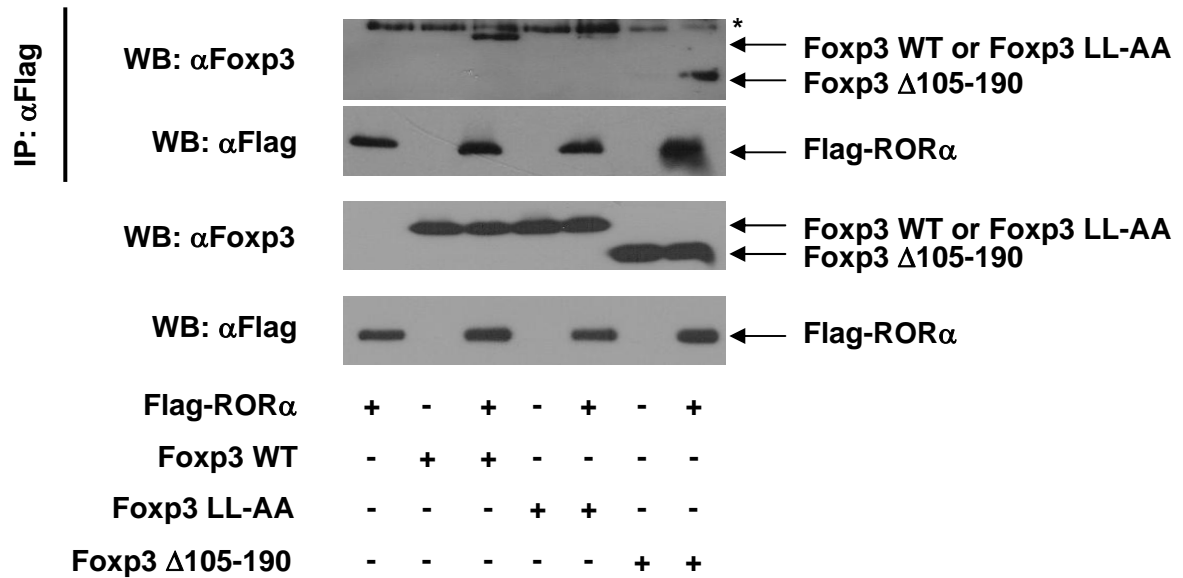
Given that Foxp3 can be found in a molecular complex containing ROR α , and since Foxp3 contains an LxxLL sequence, we next determined whether Foxp3 could prevent the functional association between coactivators and RORs. We utilized a mammalian two-hybrid system where luciferase reporter activity is induced upon binding of ROR γ to a peptide (termed EBIP96) containing a high-affinity LxxLL motif (Kurebayashi et al., 2004) (Fig. 11A). In this system, ROR γ was fused to the VP16 transactivation domain (VP16 TD-ROR γ) while the EBIP96 peptide was fused to the Gal4 DNA binding domain (Gal4 DBD- EBIP96) (Fig. 11A). Surprisingly, increasing concentrations of Foxp3 inhibited luciferase activity induction in a dose-dependent manner (Fig. 11B), suggesting that Foxp3 might interfere with the association between ROR γ and nuclear coactivators. We next evaluated the

function of the LxxLL domain in Foxp3 by using the LL-AA mutant and found decreased inhibition of luciferase activity compared to WT Foxp3 (Fig. 11B), indicating that LxxLL domain is at least partially, but not completely required for the displacement of association of ROR γ to nuclear coactivators. To further evaluate the functional role of Foxp3 LxxLL sequence, we determined whether mutation in this domain renders Foxp3 incapable of inhibiting ROR γ t transcriptional activity. To this end, we utilized the IL-17 promoter-CNS2 luciferase reporter assay, and observed as in our previous experiments down-regulation of luciferase activity when WT Foxp3 was coexpressed with ROR γ t or ROR α (Fig. 12A and B). Even though the Foxp3 LL-AA mutant had severe reduction in the binding ability to ROR α (Fig. 10A), it was still able to inhibit the luciferase activity induced by either ROR α or ROR γ t, although to a lesser extent to that compared to WT Foxp3 (Fig. 12A and B). Together, these results suggest that the Foxp3 LxxLL domain is required for optimum binding to RORs. However, the lack of this functional domain does not completely abolish the inhibition of RORs activity by Foxp3, indicating that additional domains in Foxp3 might still be required for interfering with RORs transcriptional activity.

Previous reports have shown that Foxp3 can interact with the acetyltransferase TIP60 and the histone deacetylase 7 (HDAC7) through amino acids 105-190 (Li et al., 2007a). Thus, we next investigated whether this domain is functionally required for Foxp3 to dampen the transcriptional activity of RORs by utilizing a Foxp3 Δ 105-190 mutant (Li et al., 2007a). Even though this mutant was capable of binding to ROR α upon over-expression in HEK 293T cells (Fig. 10A), it only partially repressed RORs transcriptional activity compared to WT Foxp3 (Fig. 12A and B). Interestingly, simultaneous disruption of LxxLL and TIP60/HDAC7 binding domains completely abolished Foxp3-mediated inhibition of ROR γ t or ROR α transcriptional activity (Fig. 12A and B). To further evaluate the role of these two domains in Th17 cell generation, Foxp3 WT or mutants were over-expressed by retroviral transduction. Naïve OT-II cells were stimulated under Th17-polarizing conditions and infected with Foxp3-expressing constructs (WT or mutants), after which the amount of IL-17-producing cells was determined by intracellular staining. Over-expression of WT Foxp3, LL-AA or Δ 105-190 Foxp3 mutants greatly reduced the frequency of IL-17-producing cells in GFP⁺ infected cells compared

to mock-infected cells (Fig. 12C). However, consistent with the luciferase reporter assay, the Foxp3 mutant lacking both domains was incapable of blocking Th17 cell generation (Fig. 12C). Thus, these results demonstrate that Foxp3 suppresses the generation of IL-17-producing cells by inhibiting ROR α and ROR γ t transcriptional activity through the LxxLL and TIP60/HDAC7 binding domains.

A.



B.

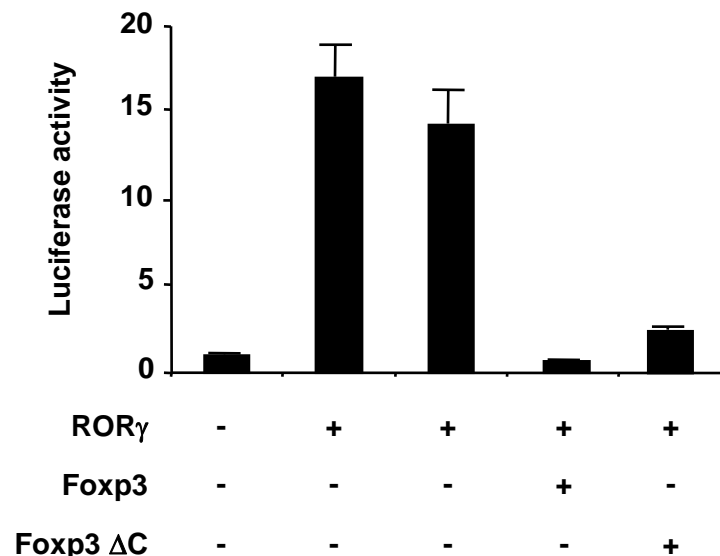
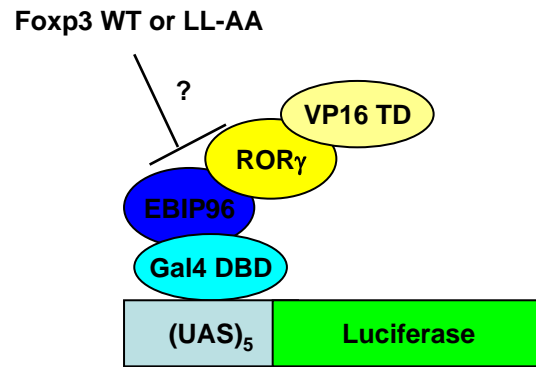


Figure 10. Fxp3 LxxLL sequence is required for binding to RORα and inhibition of RORγt transcriptional activity. (A) HEK 293T cells were transiently transfected with pCMV-Flag-tagged RORα in the presence or absence of bicistronic vectors expressing wild-type (WT) Fxp3, or Fxp3 mutants as indicated. After 48 h, lysates were prepared and immunoprecipitated with an anti-FLAG mAb (IP), followed by immunoblotting with mouse anti-Fxp3, or rabbit anti-FLAG (top two panels). Bottom two panels indicate western blot of whole cell lysates. *, non-specific. (B) HEK 293T cells were co-transfected with RORE-Luciferase reporter vector in the presence or absence of RORγ and Fxp3 WT or Fxp3 ΔC (containing only the first two exons). Then, luciferase activity was determined and normalized to β-galactosidase activity. Values were also normalized to vector alone. Data shows mean ± S.D. The data represent at least three independent experiments with consistent results. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

A.



B.

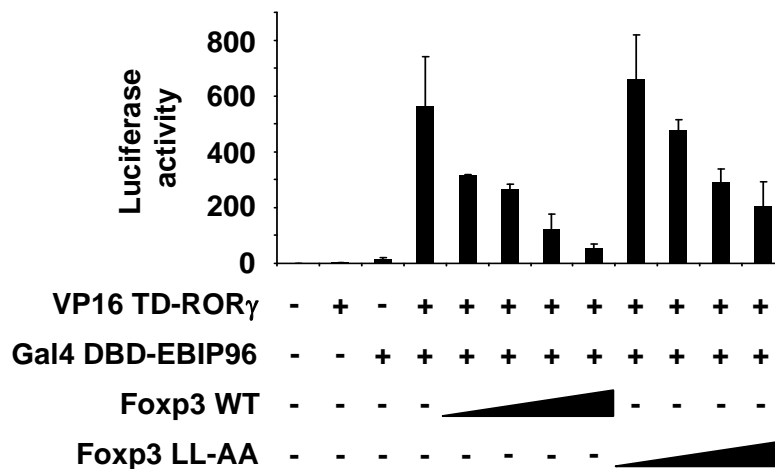


Figure 11. Fo xp3 prevents ROR γ functional association with a peptide containing LxxLL sequence partially through its LQALL sequence. (A) Schematic representation of the mammalian two hybrid system utilized. (B) CHO cells were transfected with (UAS)₅-Luciferase construct, and the indicated vectors, and luciferase assay was determined to assess the binding of ROR γ to a peptide containing a high-affinity LxxLL sequence (termed EBIP96). The luciferase activity was normalized to β -galactosidase activity. Data shows mean \pm S.D. The data represents at least two independent experiments with similar results. The experiment was performed by Dr. Kang at NIEHS-NIH. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

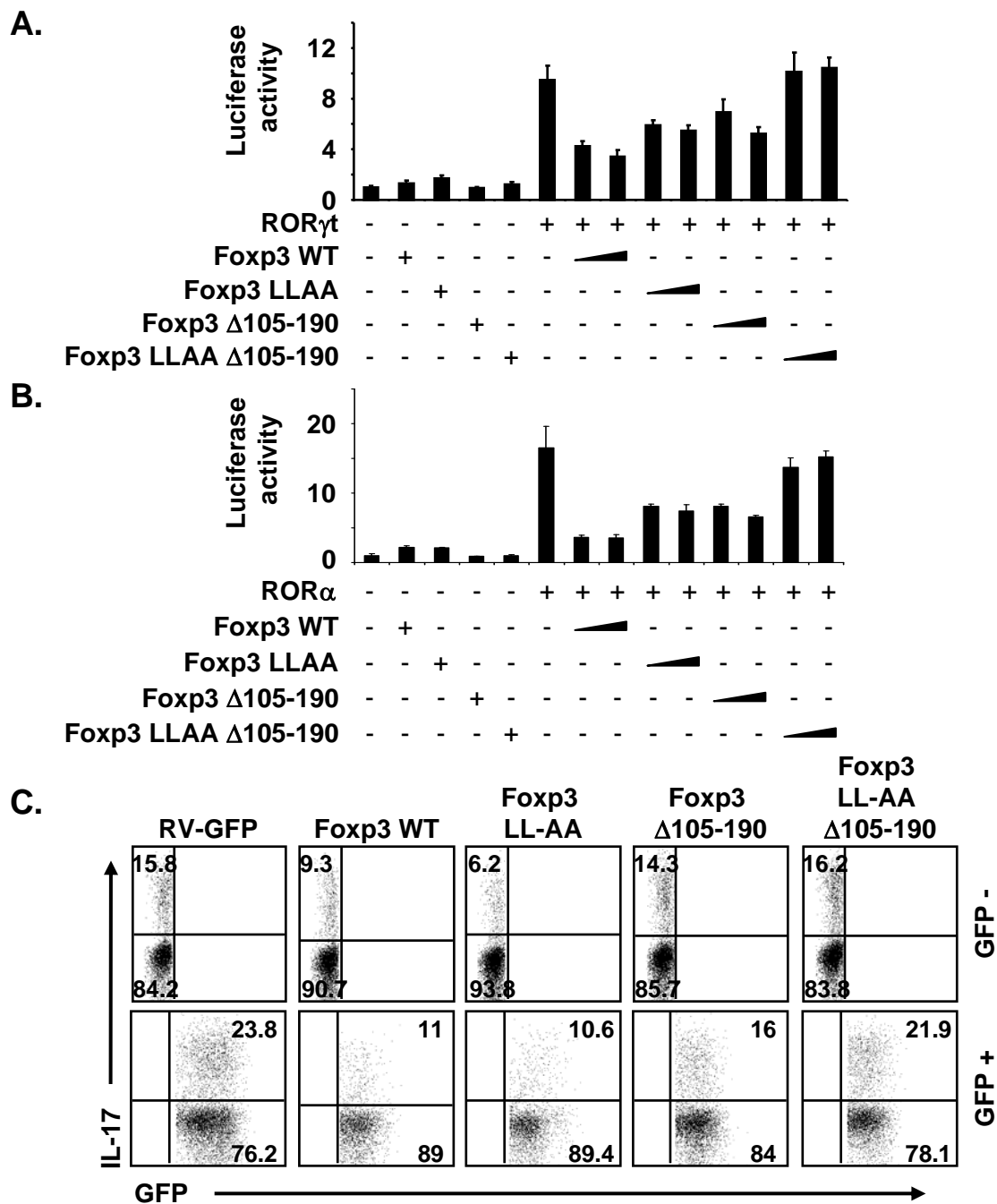


Figure 12. Foxp3 requires both LxxLL and TIP60/HDAC7 binding domains to inhibit ROR α and ROR γ t transcriptional activity. (A-B) EL-4 cells were transfected with a vector containing the firefly luciferase gene under the control of the *Il17* promoter-CNS2 region, a vector expressing Renilla luciferase, and IRES-GFP-containing bicistronic vectors expressing ROR γ t (A) or ROR α (B) and Foxp3 wild-type (WT) or various Foxp3 mutants, or vector alone. Then, cells were stimulated with PMA and ionomycin for the last 5h of culture, and luciferase activity was determined and normalized to Renilla luciferase. Values were also normalized to vector alone. Data shows mean \pm S.D. The data represent at least four independent experiments with consistent results. (C) FACS-sorted naïve OT-II T cells were activated under Th17 conditions and infected with an IRES-GFP-containing bicistronic retrovirus expressing Foxp3 WT or Foxp3 mutants or a vector control virus. IL-17-expressing cells were measured by intracellular staining on the GFP⁻ and GFP⁺ population. The experiments were repeated at least three times with similar results. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

II.3. Lack of Foxp3 does not result in enhanced development of IL-17-producing T cells

To further understand the role of Foxp3 in Th17 cell generation, we utilized mice with deficiency in Foxp3. These mice (Scurfy mice) possess a frameshift mutation in the Foxp3 gene that results in a product that lacks the forkhead domain (DNA binding domain) and thus develop a severe autoimmune syndrome (Brunkow et al., 2001). To minimize the autoimmune phenotype, we crossed these mice with OT-II TCR transgenic mice. These TCR transgenic Scurfy mice still developed an autoimmune phenotype although delayed compared to non-TCR transgenic Scurfy mice (data not shown). In our experiments, 5-6 week old mice were utilized, and naïve CD4⁺ T cells were isolated by FACS-sorting. These cells were then cultured with irradiated T cell-depleted splenocytes as antigen presenting cells and OT-II peptide in the presence of various cytokines. Similar to their WT counterparts, T cells lacking functional Foxp3 were not able to produce IL-17 when stimulated with TGF- β alone (Fig. 13), indicating that IL-6 signaling, in combination with TGF- β , is necessary for the proper induction of Th17 cells even in the absence of Foxp3. When Scurfy T cells were activated in the presence of TGF- β and IL-6, reduced IL-17-producing cells were detected compared to WT controls (Fig. 13A). Moreover, we observed lower expression of Th17-associated cytokines IL-17 and IL-17F, while no differences in IL-21 production were detected (Fig. 13B and C). In contrast, IFN- γ expression was enhanced in cells lacking functional Foxp3 compared to WT controls stimulated under this condition (Fig. 13B and C), which may account for the reduced expression of Th17 cytokines. The fact that no differences in IL-21 production were detected could be accounted based on the notion that IL-21 can also be produced by Th1 cells (Ma et al., 2009). Given that enhanced Th1 and Th2 cytokines were detected (Fig. 13 and data not shown), we then neutralized endogenous IFN- γ and IL-4 levels by using blocking antibodies and found a similar IL-17 production in Scurfy and WT OT-II Th17 cells (Fig. 13). Real time RT-PCR analysis of activated Foxp3-deficient T cells also showed decreased expression of Th17 cytokines as well as the Th17 transcription factor ROR γ when cells were stimulated in the presence of TGF- β and IL-6. However, the addition of blocking antibodies against IFN- γ and IL-4

restored the expression of Th17-associated genes (Fig. 13B). These results suggest that deficiency in the master transcription factor Foxp3 leads to enhanced Th1/Th2 differentiation, but does not alter Th17 cell generation upon optimal stimulation conditions.

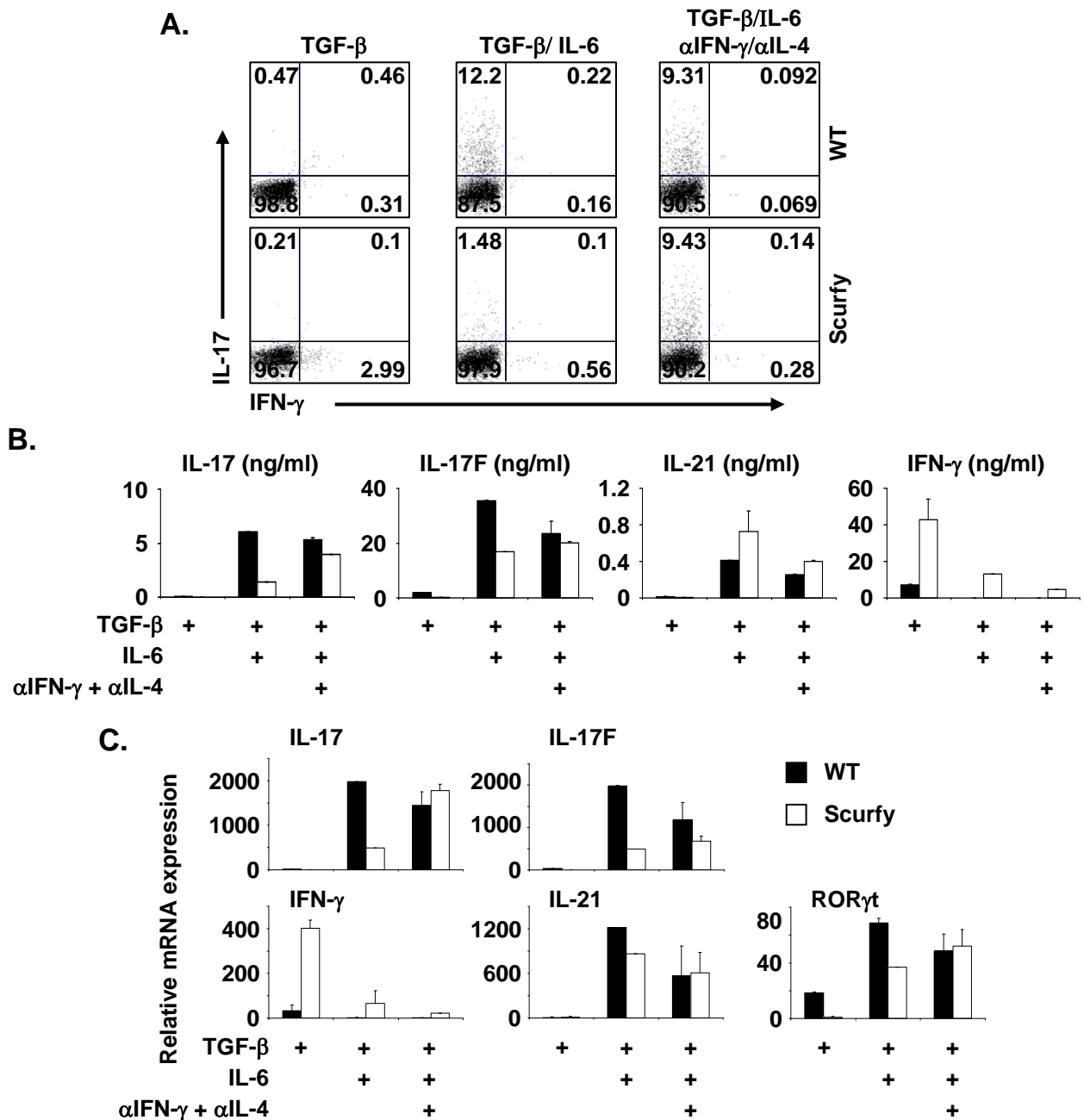


Figure 13. Lack of Foxp3 does not result in enhanced Th17 cell commitment when induction of other Th lineages is inhibited. FACS-sorted naïve T cells from 5-6 weeks old OT-II TCR transgenic Foxp3 WT or Foxp3-deficient (Scurfy) mice were activated with plate-bound anti-CD3 and anti-CD28 under indicated conditions for 4 days. (A) Cells were then restimulated with PMA and ionomycin in the presence of Golgi inhibitor for 4h and IL-17- and IFN- γ -expressing cells were assessed by intracellular staining. (B) Cells were then restimulated with anti-CD3 for 16h, and cytokine concentration in culture supernatants was measured by ELISA. (C) Cells were restimulated with anti-CD3 for 4h and cDNA was prepared. Gene expression profile was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The lower expression level for each gene was referred as 1. (B-C) Data shows mean \pm S.D. A representative experiment out of two is shown. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

DISCUSSION

TGF- β plays an important role in the development of Th17 cells while contributing also to the generation and maintenance of regulatory T cells by inducing the master transcription factors ROR γ t and Foxp3, respectively (Ivanov et al., 2006; Marie et al., 2005). Therefore, Kuchroo and colleagues have suggested a reciprocal regulation of these two cell lineages (Bettelli et al., 2006). In the present chapter we have evaluated the molecular regulation of regulatory T and Th17 cell genetic programs. Using double reporter mice containing IL-17F-RFP and Foxp3-GFP, we found that during *in vitro* generation of Th17 cells, a transient population expresses both RFP and GFP markers. These data suggest that the Treg and Th17 genetic programs can simultaneously arise in cells that are not yet fully differentiated.

We evaluated the role of Foxp3 induction during Th17 cell generation by over-expression studies and found that ectopic expression of Foxp3 led to a reduction in the generation of IL-17-producing cells. When determining the gene expression profile from these cells over-expressing Foxp3, we observed reduced induction of Th17-associated cytokines but not the Th17-specific transcription factors ROR α and ROR γ t. These results indicated that Foxp3 might block the induction of the Th17-transcriptional program by directly interfering with RORs. In support of this idea, Foxp3 was capable of suppressing the transcriptional activity of RORs, as assessed by luciferase reporter assays containing ROR binding sites (RORE or *Il17p-CNS2* luciferase reporters). Interestingly, such inhibition was independent of Foxp3 DNA binding or homo-dimerization domains. Using mutagenesis analysis, we concluded that Foxp3 can interfere with the transcriptional activity of RORs via two independent, and most likely non-exclusive, mechanisms: first, Foxp3 would bind to RORs through its LQALL sequence coded in the second exon, and thus prevent the association of RORs with coactivators such as the Steroid Receptor Coactivator molecules; second, through the recruitment of TIP60/HDAC7 complex, Foxp3 may impede the direct binding of nuclear co-activators via components in this complex or may induce deacetylation of specific molecules including histones, preventing the transcription of ROR-

target genes (Fig. 14). Interestingly, deficiency of either the LQALL or TIP60/HDAC7 domains partially impaired Foxp3-mediated block of development of IL-17-producing cells. However, lack of both domains completely abrogated the inhibitory function of Foxp3 in the context of Th17 cell development. Thus, our results indicate that the presence of both domains is required to impede the generation of IL-17-producing T cells. Further investigation is needed to thoroughly comprehend how these two mechanisms cooperate in the repressive activity of Foxp3, and whether there might be redundancy amongst them. Also, whether Foxp3 utilizes the same machinery to target other Th-specific genes still remains unclear.

Recently, the laboratories of Drs. Yoshimura, Littman and Ziegler have also independently demonstrated that Foxp3 can directly interact with ROR γ t and/or ROR α , hindering with their transcriptional activity (Du et al., 2008; Ichiyama et al., 2008; Zhou et al., 2008a). Indeed, using ROR γ t-GFP reporter mice, Littman's group showed that cells co-expressing Foxp3 and ROR γ t in the lamina propria produced lower levels of IL-17 compared to cells expressing ROR γ t alone (Zhou et al., 2008a), consistent with our *in vitro* data demonstrating that ectopic expression of Foxp3 leads to reduced generation of IL-17-producing T cells. Moreover, Foxp3 exon 2 (amino acids 71-105), which contains the LxxLL sequence, was shown to associate with the Th17-specific transcription factors ROR α and ROR γ t (Du et al., 2008; Zhou et al., 2008a). However, unlike our observations, these authors showed that complete deletion of exon 2 resulted in impaired suppressive activity by Foxp3 (Du et al., 2008; Ichiyama et al., 2008; Zhou et al., 2008a). Interestingly, Foxp3 has two different isoforms in humans, one isoform containing the full protein while the other isoform has a deletion of exon 2 by alternative splicing. The differential role of these two isoforms in human regulatory T cell functions still remains unclear but suggests that a fraction of these regulatory T cells (expressing a Foxp3 Δ exon 2 isoform) might not fully antagonize the Th17 program efficiently. Further research needs to be performed in mouse studies to try to elucidate the role of regulatory T cells expressing only this short isoform. Also, the exact role of the Tip60/HDAC7-binding domain in Foxp3 still remains unclear. Whether Foxp3 is able to recruit complexes containing HDAC7 to ROR α /ROR γ t-target loci needs

confirmation using genomic-wide studies. Thus, these combined results suggest a potential role of Foxp3 in the suppression of Th17 development *in vivo* by restraining both ROR α and ROR γ t activity.

We further determined the requirement of Foxp3 in Th17 differentiation by using mice deficient in Foxp3 (Scurfy mice). Cells lacking functional Foxp3 did not spontaneously differentiate into Th17 cells when stimulated with TGF- β alone, suggesting that TGF- β signaling itself is not sufficient to force the commitment towards Th17 cell differentiation in the absence of a commitment towards the regulatory T cell program. IL-6 and IL-21, through STAT3 activation (Nurieva et al., 2007; Yang et al., 2007), are thus also required for Th17 commitment indicating that the role of these factors extends beyond inducing Foxp3 down-regulation (Bettelli et al., 2006; Yang et al., 2008b), supporting the idea of a synergism between IL-6 and TGF- β signaling for the induction of Th17 cells. Interestingly, stimulation of Scurfy naïve CD4⁺ T cells with TGF- β resulted in enhanced Th1 and Th2 cytokines, suggesting that a functional Foxp3 is required to inhibit Th1 and Th2 cell induction. Future research needs to be performed to clarify how Foxp3 deficiency results in enhanced Th1 and Th2, but not Th17, cell development. It is possible that Foxp3 could inhibit the generation of these cell lineages by directly antagonizing T-bet or GATA-3 expression, respectively. Also, Foxp3 could directly inhibit the induction of Th1/Th2 cytokines by directly binding to DNA in these cytokine gene loci.

When Scurfy naïve CD4⁺ T cells were stimulated only with TGF- β and IL-6, we found reduced expression of the Th17-associated cytokines IL-17 and IL-17F, as well as the transcription factor ROR γ t, compared to wild-type controls. This reduced expression of Th17-specific genes correlated with enhanced Th1 and Th2 cytokine secretion. Since blockade of Th1 and Th2 pathways can enhance the commitment towards Th17 cells (Harrington et al., 2005; Park et al., 2005), we used neutralizing antibodies against IFN- γ and IL-4. Surprisingly, we found that the expression of Th17-specific genes was restored in functional Foxp3-deficient cells. Several different explanations could account for the observed results:

i) Our data showing inhibition of ROR α /ROR γ t transcriptional activity by Foxp3 is based on over-expression studies, which could be artificial since over-expression studies exclude the endogenous

regulatory mechanisms by which these transcription factors are subjected. However, given that Littman and colleagues have shown that cells co-expressing Foxp3 and ROR γ t in lamina propria show reduced expression of IL-17 compared to cells expressing ROR γ t alone, we believe this is not the most appropriate explanation.

ii) Although Foxp3 is transiently induced in developing Th17 cells *in vitro* and coexpressed with ROR γ t *in vivo* (Zhou et al., 2008a), Foxp3 expression is inhibited by IL-6 in a STAT3-dependent manner (Bettelli et al., 2006; Yang et al., 2008b). Littman and colleagues have shown that enhanced TGF- β stimulation would favor the induction of Foxp3⁺ regulatory T cells while inhibit the commitment towards Th17 cells (Zhou et al., 2008a). In our *in vitro* stimulation conditions, IL-6 signaling could be strong enough to induce complete Foxp3 downregulation, and thus lack of a functional Foxp3 does not lead to enhanced Th17 cell development. Therefore, experiments using titration of both TGF- β and IL-6 concentrations could help address this controversy.

iii) Naïve CD4⁺ T cells isolated from Scurfy mice express a Foxp3 mutant that has a deletion of the forkhead domain, but still conserve an intact exon 2 (containing LxxLL sequence) and Tip60/HDAC7 domains. These two domains could then prevent the enhanced induction of IL-17-producing cells by interfering with ROR α /ROR γ t transcriptional activity as previously described.

In conclusion, our results contributed to our understanding of the reciprocal regulation of Th17/Treg cell development through the identification of an inhibitory role of Treg-specific transcription factor Foxp3 in the generation of Th17 cells. Thus, we have demonstrated a molecular antagonism between regulatory T cell and Th17 cell genetic programs.

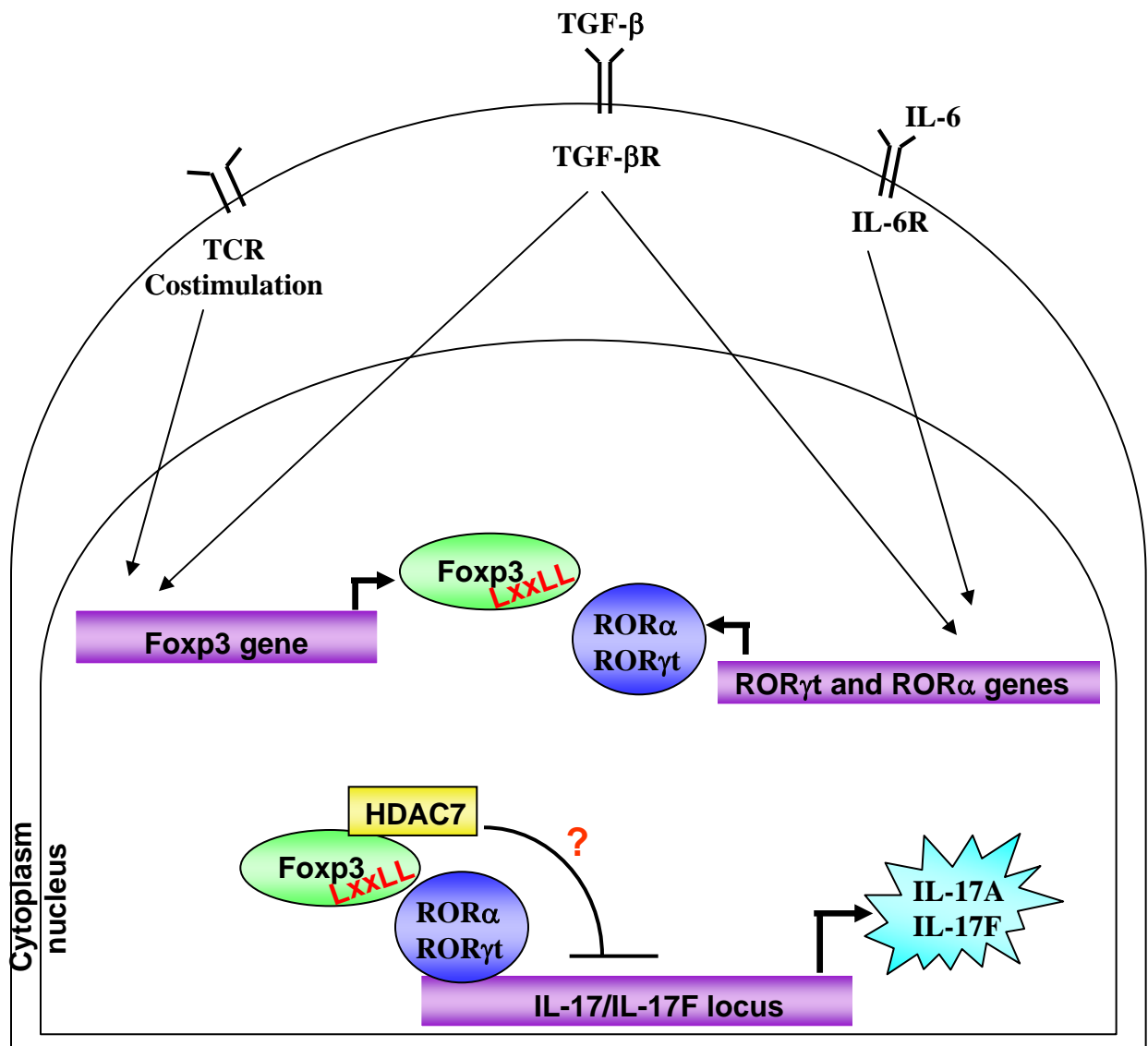


Figure 14. Model of inhibitory function of Foxp3 in developing Th17 cells. TGF-β stimulation of naïve T cells leads to the expression of both Foxp3 and RORγt transcription factors. Under high TGF-β concentrations, Foxp3 expression is maintained. In turn, Foxp3 would bind to RORα/RORγt through its LxxLL sequence, preventing the binding of coactivators to these nuclear receptors. Also, Foxp3 might recruit histone deacetylase 7 (HDAC7), which might also prevent the transcription of ROR-target genes through repressive chromatin modifications. The direct recruitment of Foxp3 to ROR-target genes, as well as the role of HDAC7 in Foxp3 repression still needs further elucidation.

CHAPTER III – ROLE OF TGF- β SIGNALING PATHWAY IN TH17/TREG DIFFERENTIATION²

BACKGROUND

The transforming growth factor beta (TGF- β) superfamily is a group of secreted proteins that regulate cellular processes such as proliferation, differentiation, apoptosis and migration, among others. This family of proteins is composed of over 50 members including TGF- β s, Activins, Nodals and Bone Morphogenic Proteins (BMPs). Within the TGF- β family, there are three different isoforms: TGF- β 1, TGF- β 2 and TGF- β 3 (reviewed in (Feng and Derynck, 2005; Schmierer and Hill, 2007)). TGF- β 1 (referred to as TGF- β throughout this work) but not TGF- β 2 or TGF- β 3, is mainly expressed in the immune system (Chang et al., 2002; Govinden and Bhoola, 2003). TGF- β has either positive or negative regulatory properties in several immune cell types, and is thus a crucial cytokine with many immunological functions (reviewed in (Wan and Flavell, 2007)).

TGF- β superfamily cytokines signal through tetrameric serine/threonine kinase transmembrane receptors. There are two functional classes of TGF- β superfamily receptors, TGF β RI and TGF β RII, with seven type I receptors, termed activin receptor-like kinases (ALK) 1-7, and five type II receptors: TGF β RII, AMHR-II, BMPR-II, ActR-II and ActR-IIB (reviewed in (Feng and Derynck, 2005; Schmierer and Hill, 2007)). Even though there are considerably fewer receptors compared to ligands, signaling mechanisms are more flexible than expected. This versatility is the result of the combinatorial interactions between the two classes of receptors, a variety of different signaling pathways that can be activated, and the interaction of these signaling pathways (such as Smad molecules) with different types of transcription factors or coactivators (see below).

² The results presented in this Chapter have been published in Yang et al., 2008b; Martinez et al., 2009; Martinez et al., 2010.

Binding of a TGF- β homodimer to the pre-formed TGF β RII dimer leads to the association of these molecules with the pre-formed homodimer of TGF β RI. The TGF β RII is thought to be constitutively active and, upon formation of a stable receptor complex, TGF β RII kinase activity induces the phosphorylation of the glycine-serine rich region (GS sequence) in TGF β RI, which is highly conserved among all the TGF β RI members (reviewed in (Feng and Derynck, 2005; Schmierer and Hill, 2007)). Upon phosphorylation of this regulatory domain, TGF β RI becomes activated allowing for the recruitment and subsequent phosphorylation of receptor-associated Smads (mothers against decapentaplegic homologue; R-Smads: Smad1, Smad2, Smad3, Smad5 and Smad8) at two Serine residues in the distal C-terminus SXS motif. Once phosphorylated, R-Smads interact with the Common-Smad (C-Smad: Smad4), exposing the nuclear localization signal which allows the complex to translocate to the nucleus (reviewed in (Feng and Derynck, 2005; Schmierer and Hill, 2007)) (Fig. 15).

Smads are not thought to recruit the basal transcription factor machinery to target genes. Rather, they are believed to recruit chromatin remodeling complexes, such as Swi/Snf and Brg1, and histone acetyltransferases such as p300 and CBP (Ross et al., 2006). Furthermore, the binding of Smads to Smad Binding Elements (SBE) in DNA is relatively weak. However, association of Smads with other transcription factors, which contain DNA binding sequences adjacent to SBE sequences, leads to enhanced binding of the Smad-transcription factor complex to their respective cognate DNA sequences. For instance, upon interaction of Smad3 with Sp1 and c-Jun, their DNA binding capacity is synergistically increased (Feng et al., 2000; Qing et al., 2000).

It is commonly accepted that TGF- β , through binding to TGF β RI ALK5, induces the phosphorylation of Smad2 and Smad3 (reviewed in (Feng and Derynck, 2005)). However, recent reports have suggested that TGF- β can induce phosphorylation of Smad1 and Smad5 through ALK1 and ALK5 in a cell-type specific manner (Bharathy et al., 2008). Also noteworthy is the fact that TGF- β can signal through Smad-independent pathways (Fig. 15). Studies using Smad4-deficient or a TGF β RI mutant that lacks the Smad-binding domain L45 loop, have shown that TGF- β stimulation leads to the

activation of MAPK in different cell types (Goumans et al., 2003; Goumans et al., 2002; Itoh et al., 2003; Lebrin et al., 2004; Pannu et al., 2007; Wrighton et al., 2009; Yu et al., 2002). The role of TGF- β -induced phosphorylation of Smad2/3 and/or Smad1/5, as well as the Smad-independent TGF- β signaling pathway, in the commitment towards Th17/iTreg cells remains unclear.

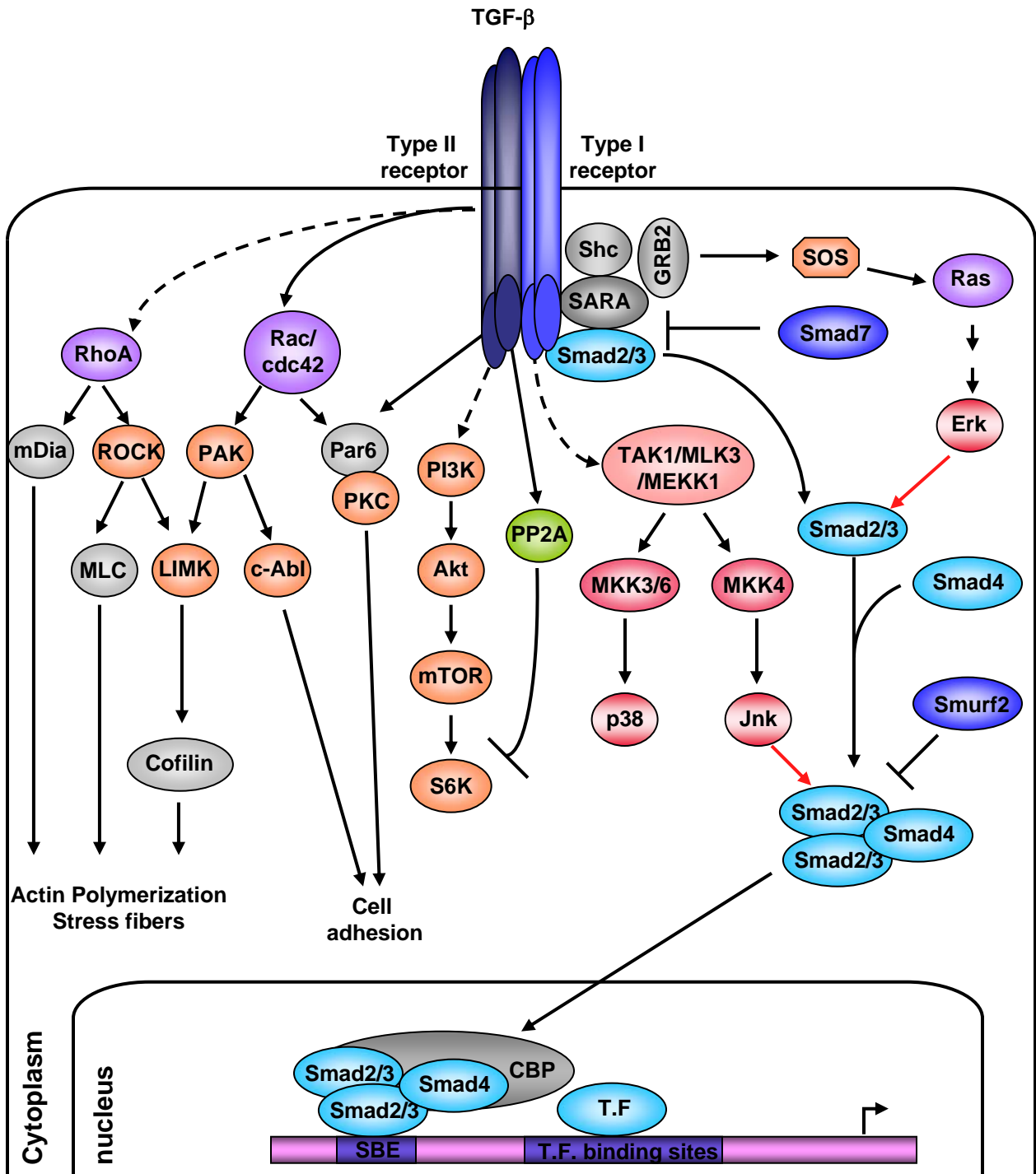


Figure 15. Schematic representation of TGF- β signaling pathway. Upon binding to its receptor, TGF- β induces activation of Smad-dependent or Smad-independent signaling pathways. The Smad-dependent pathway is constituted by activation (phosphorylation) of Smad2 and Smad3, which then bind to Smad4 and translocate to the nucleus and bind to Smad Binding Elements (SBE) in DNA. The Smad-independent pathway includes several pathways, mostly mitogen-activated protein kinases (MAPKs) and Akt-mTOR (mammalian target of Rapamycin) pathways. Interestingly, there is cross-talk between the Smad-dependent and Smad-independent pathways, as indicated with red arrows where both Jnk and Erk MAPK can phosphorylate Smad molecules. Upon binding to target sequences, Smad molecules recruit histone modifying complexes such as the histone acetyltransferase CBP and synergize with other adjacent transcription factors (T.F.) in the transcription of target genes. Adapted from cell signaling technology (<http://www.cellsignal.com/pathways/tgf-beta-smad.jsp>)

RESULTS

III.1. TGF- β signaling through TGF β RI is required for the differentiation of Th17 and inducible regulatory T cells

Th17 and regulatory T cells both require TGF- β stimulus for their generation (Bettelli et al., 2006; Veldhoen et al., 2006a). Development of iTreg cells depends on TGF- β and IL-2 signaling, whereas Th17 cell differentiation is regulated by TGF- β in combination with IL-6 and/or IL-21. Our group and others have demonstrated that IL-6-mediated STAT3 activation is essential for the generation of Th17 cells (Chen et al., 2006b; Laurence et al., 2007; Yang et al., 2007; Zhou et al., 2007), whereas STAT5 activation by IL-2 is indispensable for the generation of regulatory T cells (Burchill et al., 2007; Yao et al., 2007). However, the signaling pathway induced by TGF- β in the differentiation of either cell lineage is still unclear. Additionally, whether Th17 or iTregs require TGF- β -mediated activation of Smads or MAPKs during their development has not been completely established. Finally, whether the TGF- β Smad-dependent or -independent signaling pathway crosstalk with STAT3/STAT5 and/or regulate the lineage-specific transcription factors needs to be determined.

To address these aspects of TGF- β biology, we investigated the molecular mechanisms induced by TGF- β that lead to the commitment towards either cell lineage. We first isolated naïve CD4⁺ T cells (CD4⁺CD25⁻CD62L^{hi}CD44^{lo}) from C57BL/6 mice and stimulated these cells with TGF- β and/or IL-6 in the presence of a TGF β RI kinase inhibitor, SB431542 (Inman et al., 2002). To confirm the inhibition of TGF β RI kinase activity by this compound, we evaluated the phosphorylation levels of Smad2 and Smad3 by Western blot and indeed found reduction in their activation upon SB431542 treatment (Fig. 16).

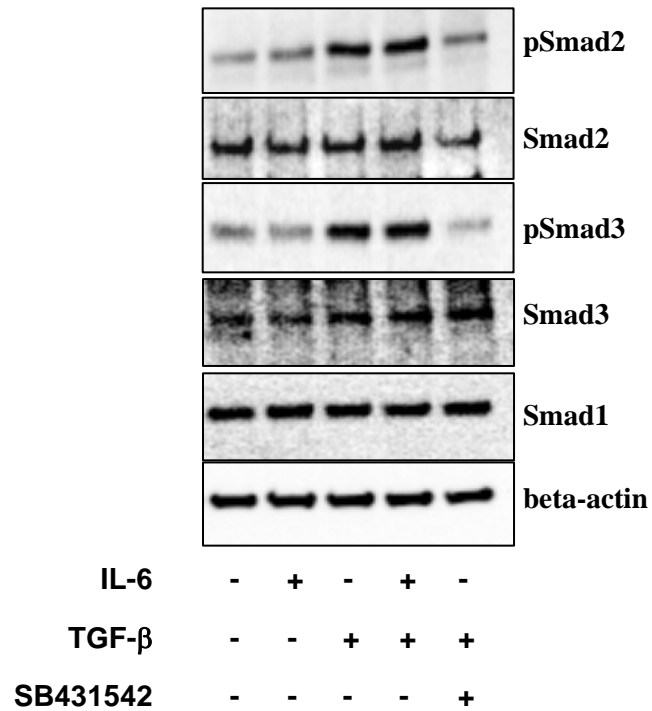


Figure 16. TGFβRI signaling induces activation of Smad2 and Smad3. FACS-sorted naïve T cells from B6 mice were activated with plate-bound anti-CD3 and anti-CD28 in the presence of indicated cytokines or TGFβRI kinase inhibitor (SB431542, 5μM) for 1 hour. Then, cell extracts were prepared and expression and phosphorylation of Smad molecules was determined by Western blot.

To evaluate the kinetics of TGF- β signaling during Th17 differentiation, naïve CD4⁺ T cells were stimulated with Th17-skewing conditions for four days and the TGF β RI kinase inhibitor was added in the culture at different time points. When TGF- β signaling pathway was inhibited on day zero or day one, the generation of IL-17-producing cells was completely abolished (Fig. 17A and B). Furthermore, a 50% reduction in Th17 cells was observed when the TGF β RI kinase inhibitor was added on day two, whereas no differences were observed on day three (Fig. 17A and B). The decrease in IL-17-producing cells in the presence of the TGF β RI inhibitor was confirmed by analysis of gene expression in these cells. Reduction of IL-17, IL-17F, ROR α , ROR γ t and IL-23R mRNA was detected following addition of SB431542 on day zero and day one (Fig. 17B). Similar kinetics requirements for TGF- β signaling were obtained for the induction of Foxp3 expression and thus the generation of inducible regulatory T cells (Fig. 18). Thus, our data suggests that TGF- β signaling is indispensable during the first two days of *in vitro* generated Th17 and iTreg cells.

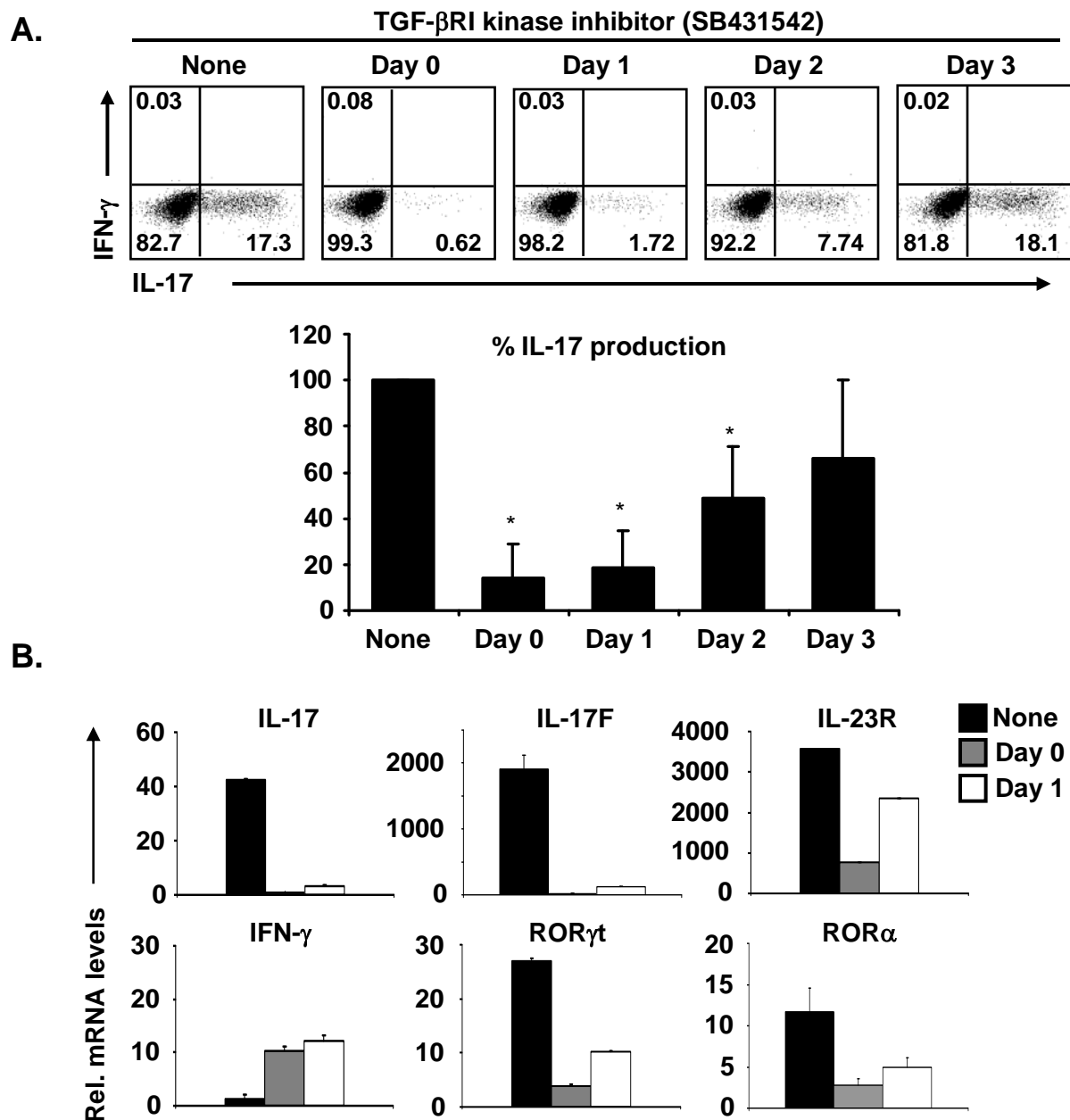


Figure 17. TGF β RI signaling is required for the generation of Th17 cells. FACS-sorted naïve T cells from B6 mice were activated with plate-bound anti-CD3 and anti-CD28 under Th17 (TGF- β , IL-6, anti-IFN- γ , anti-IL-4) conditions, and a TGF β RI kinase inhibitor (SB431542, 5 μ M) was added at different time points as indicated. (A) 4d after differentiation, cells were stimulated with PMA and ionomycin in the presence of Golgi Stop for 4h, and intracellular cytokine staining performed. A representative dot plot graph is shown in the top panel, and the numbers in quadrants represent the percentages. In the bottom panel, the inhibition of IL-17-producing cells for six independent experiments are indicated. *, $p < 0.05$, Wilcoxon signed rank test. (B) 4d after differentiation, cells were stimulated with plate bound anti-CD3 (1 μ g/ml) for 4h, and then mRNA expression of indicated genes was analyzed by real-time RT-PCR. The data shown was normalized to expression of a reference gene β -actin. The lowest expression levels for each gene were referred as 1. (A-B) Data shows mean \pm S.D. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

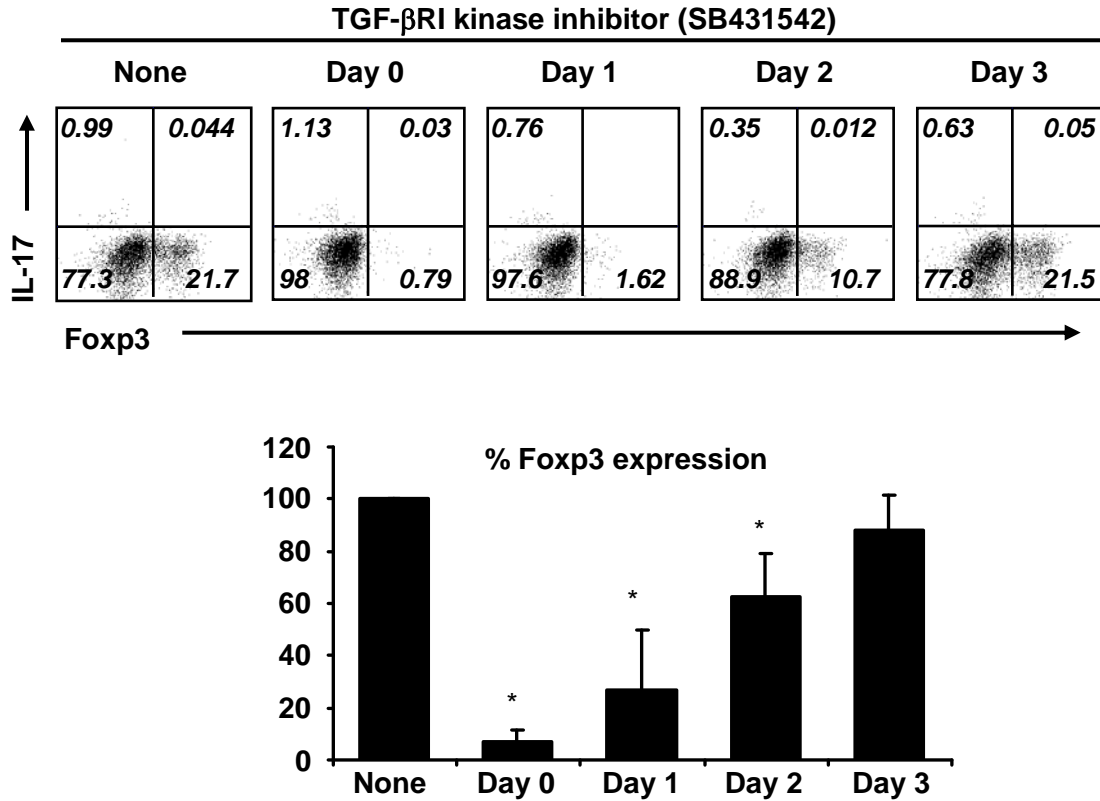


Figure 18. TGF β RI signaling is required for the generation of inducible Treg cells. FACS-sorted naïve T cells from B6 mice were activated with plate-bound anti-CD3 and anti-CD28 under iTreg conditions (TGF- β , IL-2, anti-IFN- γ , anti-IL-4), and a TGF β RI kinase inhibitor (SB431542, 5 μ M) was added at different time points as indicated. Cells were assessed for Foxp3 expression after 4 days of stimulation using intracellular staining. A representative dot plot graph is shown in the top panel, and the numbers in quadrants represent the percentages. In the bottom panel, the inhibition of Foxp3⁺ cells for six independent experiments are indicated. Data shows mean \pm S.D. *, $p < 0.05$, Wilcoxon signed rank test. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

III.2. Role of the Smad-signaling pathway in the generation of regulatory T cells

III.2.a. Smad4 partially regulates the generation of iTreg cells

Given that TGF- β signaling is indispensable for iTreg generation, we next investigated the downstream signaling molecules that could participate in such commitment. Since Smad4 is the common Smad factor (Feng and Derynck, 2005), the role of this molecule in Treg biology was initially determined. Mice with T cell-specific Smad4 deficiency were generated by crossing mice with floxed Smad4 alleles (Smad4^{f/f}) (Chu et al., 2004) with mice bearing the Cre recombinase under the control of *Cd4* promoter (CD4-Cre mice) (Lee et al., 2001). Appropriate deletion of the *Smad4* gene was confirmed by PCR strategy in CD4⁺ T cells by analyzing thymus of Smad4^{f/f} CD4Cre⁺ (Smad4 tKO) and WT control mice (Fig. 19A). Smad4 tKO mice exhibited normal levels of CD4⁺ and CD8⁺ T cells, as well as natural regulatory T cells (nTregs; CD4⁺CD25⁺Foxp3⁺) in all the lymphoid organs analyzed (spleen, peripheral and mesenteric lymph nodes, and thymus) (Fig. 19 and 20A respectively). Furthermore, Smad4-deficient nTregs exhibited the same suppressive activity *in vitro* as their WT counterparts (Fig. 20B). Together these results indicate that Smad4 is dispensable for the generation or function of nTreg cells.

Then, we determined whether the generation of iTreg cells *in vitro* required Smad4 expression. Naïve T cells from either Smad4^{f/f} CD4Cre⁻ (WT) or Smad4^{f/f} CD4Cre⁺ (tKO) mice were stimulated with iTreg-polarizing conditions (TGF- β , IL-2 and neutralizing antibodies against IFN- γ and IL-4) for four days. We found that cells lacking Smad4 exhibited a 50% reduction in Foxp3 expression compared to their WT counterparts (Fig. 20C). Thus, these results indicate that the Smad-dependent TGF- β signaling pathway is at least partially required for the generation of inducible regulatory T cells. However, given that Smad2/3 can bind factors other than Smad4, and that Smad4 deficiency does not fully impair Smad2/3 transcriptional activity in other cell types (Descargues et al., 2008; Doisne et al., 2009; He et al., 2006), we evaluated the role of the other Smad molecules in Treg biology.

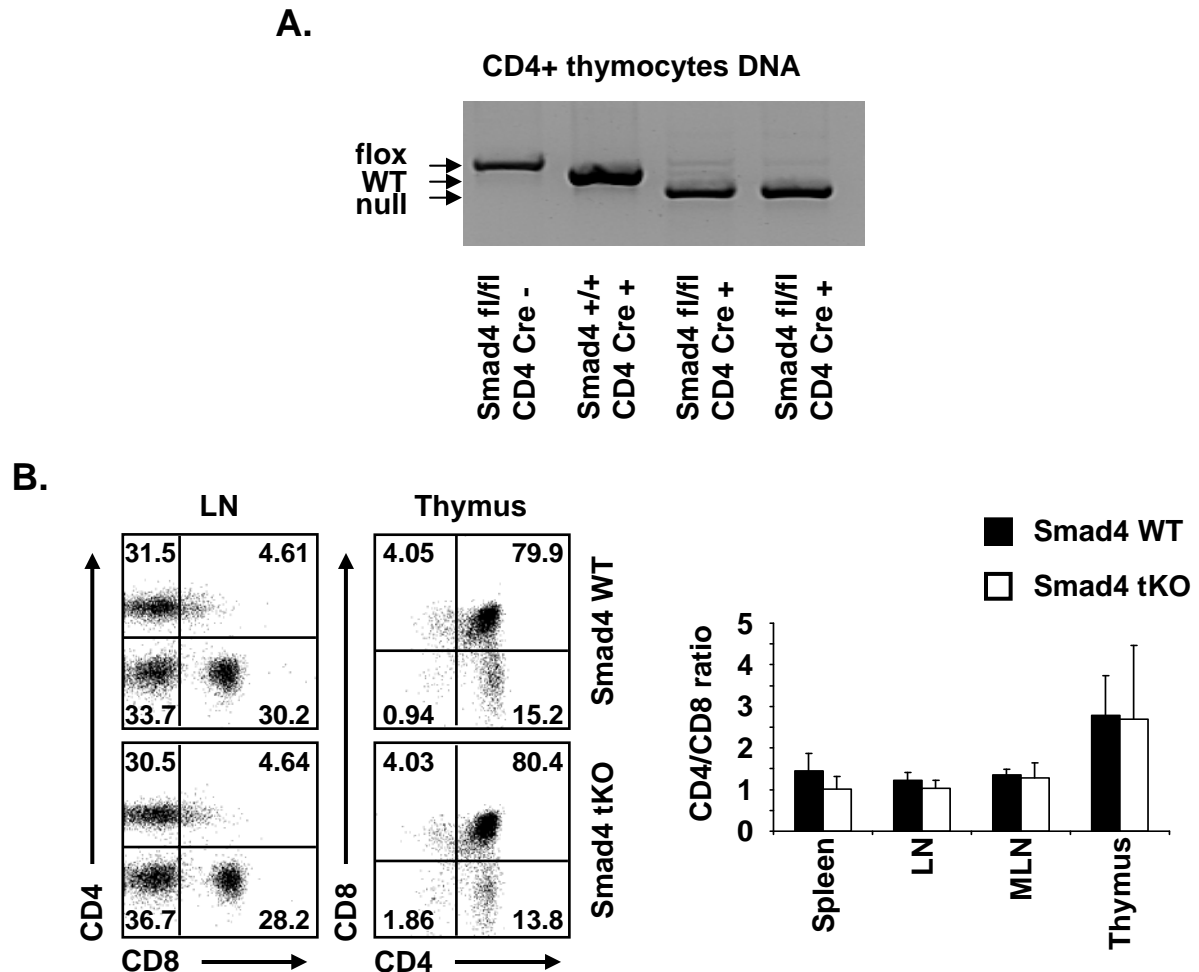


Figure 19. Normal T cell development in mice with a specific deletion of Smad4 in T cells. (A) Genomic DNA from MACS sorted CD4⁺ thymocytes was analyzed for Smad4 gene deletion by PCR. (B) CD4⁺ and CD8⁺ T cells levels were determined in peripheral lymph nodes, spleen, mesenteric lymph nodes and thymus. A representative dot plot example is shown for peripheral lymph nodes and thymus (left panel), and the combined analysis for seven mice each group is also indicated (right panel). Right panel shows mean \pm S.D. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

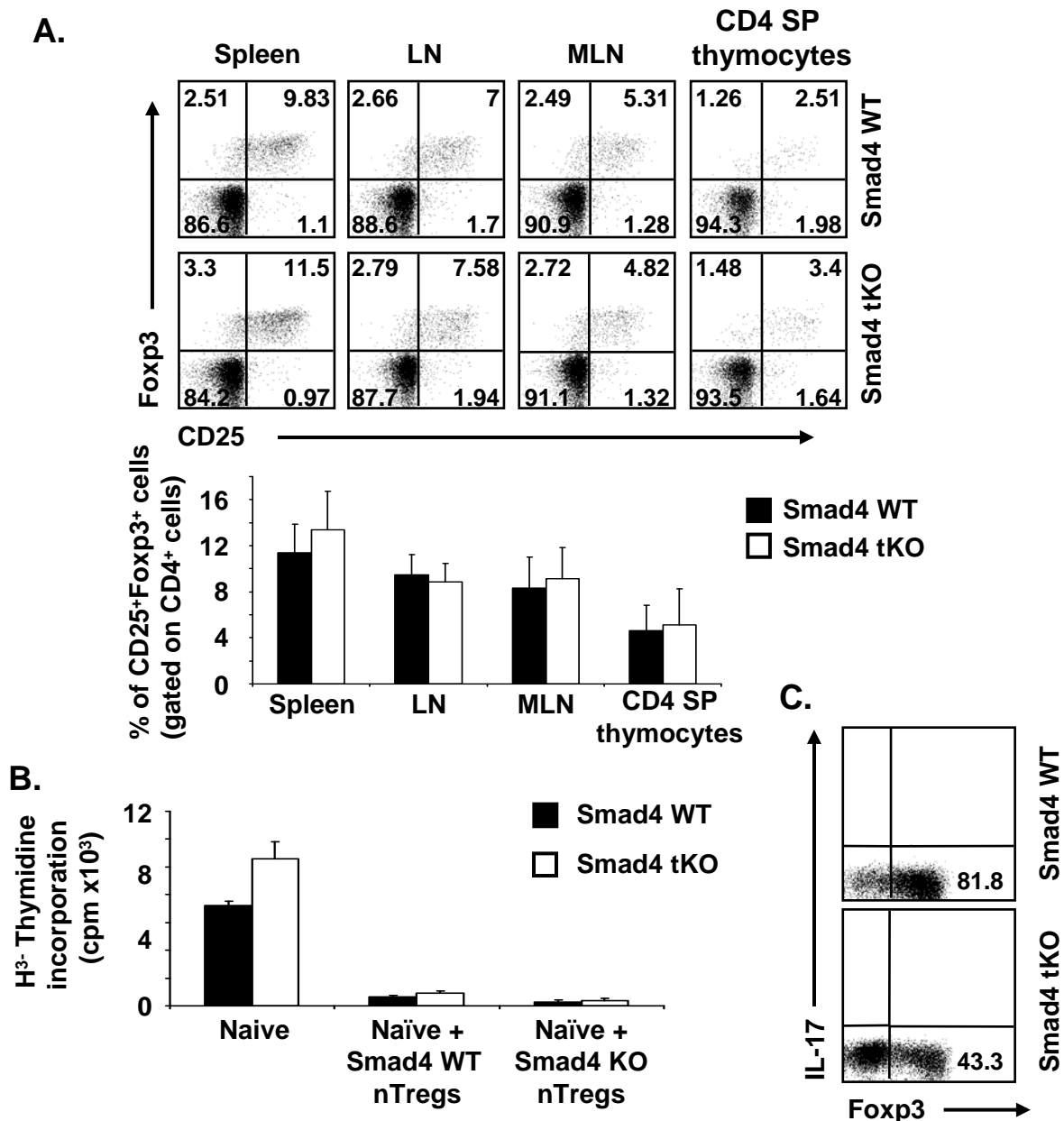


Figure 20. Differential role of Smad4 in the generation of natural *versus* inducible regulatory T cells. (A) Presence of CD25⁺Foxp3⁺ cells in a CD4⁺ T cell gate was analyzed in different tissues. A representative dot plot example is shown for each group in each tissue, and the combined results for seven mice each group is indicated (mean \pm S.D.). (B) CD4⁺CD25⁻CD62L^{hi}CD44^{lo} naïve T cells from Smad4^{fl/fl}CD4Cre⁻ (WT) or Smad4^{fl/fl}CD4Cre⁺ (tKO) mice were cultured with or without WT or KO CD4⁺CD25^{hi} natural regulatory T cells in triplicate wells with irradiated APCs and stimulated with 2 μ g/ml of anti-CD3. Proliferation was assayed 72 h after treatment by adding [³H]-thymidine to the culture for the last 8 h. Data shows mean \pm S.D. A representative example of three independent experiments is shown. (C) CD4⁺CD25⁻CD62L^{hi}CD44^{lo} naïve T cells from Smad4 WT or Smad4 tKO mice were activated with anti-CD3/CD28 in the presence of TGF- β , IL-2, anti-IFN- γ and anti-IL-4. After 4d, Foxp3 expression was analyzed by intracellular staining. Numbers in quadrants represent the percentages. The experiments were repeated three times with consistent results. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

III.2.b. Smad3 partially induces the generation of Foxp3-expressing T cells

To further delineate the role of the TGF- β signaling pathway in Treg cell generation, Smad3 KO mice were utilized (Datto et al., 1999). Similar to the Smad4 tKO mice, these mice presented normal levels of CD4⁺ and CD8⁺ T cells *in vivo* as well as nTreg cells in spleen, peripheral and mesenteric lymph nodes (Fig. 21A and B). However, a significantly lower frequency of nTreg cells was observed in Smad3-deficient thymi, indicating either reduced Treg generation or homeostasis in this organ (Fig. 21A and B). Nevertheless, the function of peripheral nTreg cells was unaffected as evaluated by *in vitro* suppressive activity (Fig. 21C). Altogether, these results suggest that the maintenance or suppressive activity of nTreg cells in the periphery does not depend on Smad3.

We next evaluated whether the generation of inducible regulatory T cells *in vitro* require Smad3. Naïve T cells from Smad3 KO or WT littermates were activated with plate bound anti-CD3 and anti-CD28 together with TGF- β and IL-2 for four days, and the expression of Foxp3 was determined by intracellular staining. Deficiency of Smad3 led to a 50-60% reduction in Foxp3-expressing cells compared to WT counterparts (Fig. 22A and B), similar to what was observed for Smad4-deficient T cells. Furthermore, the expression of iTreg-associated genes, *Gpr83* (G- protein coupled receptor 83) and *Ecm1* (Extracellular matrix protein 1), was also reduced in Smad3-deficient T cells (Fig. 22B).

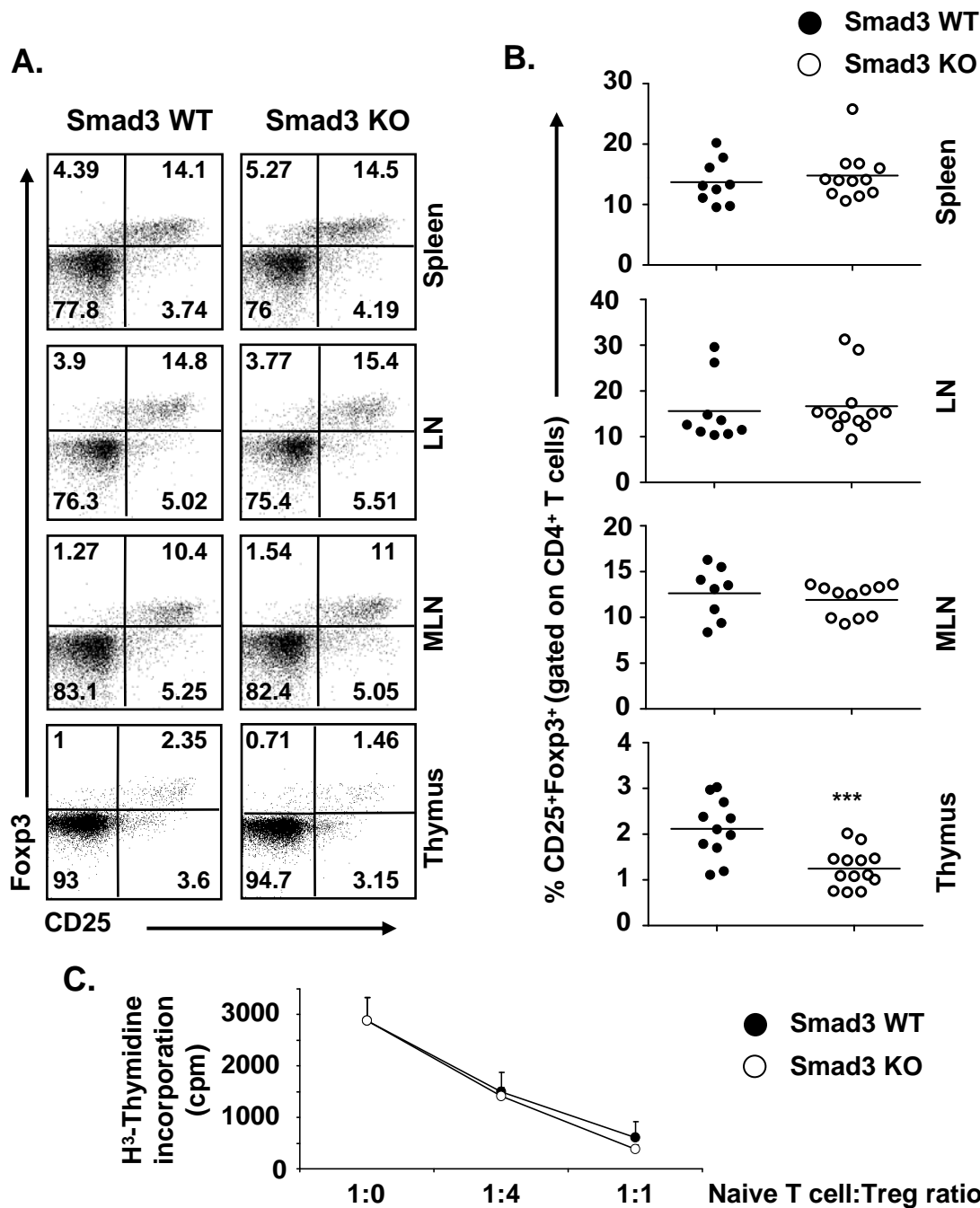


Figure 21. Smad3 is not required for nTreg generation or suppressive activity, although there is slight decrease in nTreg numbers in thymus. (A) Presence of CD25⁺Foxp3⁺ cells in a CD4⁺ T cell gate was analyzed in different tissues from Smad3 WT or KO mice. A representative dot plot example is shown for each group in each tissue (A), and the combined results for seven-ten mice each group is indicated (B). Data shows mean \pm S.D. p values were calculated using Student t test. ***, p < 0.001. (C) CD4⁺CD25⁺CD62L^{hi}CD44^{lo} naïve T cells from Smad3 WT mice were cultured with or without WT or KO CD4⁺CD25⁺ natural regulatory T cells in triplicate wells with irradiated APCs and stimulated with 1 μ g/ml of anti-CD3. Proliferation was assayed 72 h after treatment by adding [³H]-thymidine to the culture for the last 8 h. Data shows mean \pm S.D. A representative example of three independent experiments is shown. This research was originally published in “Smad3 Differentially Regulates the Induction of Regulatory and Inflammatory T Cell Differentiation.” Gustavo J. Martinez, Zhengmao Zhang, Yeonseok Chung, Joseph M. Reynolds, Xia Lin, Anton M. Jetten, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2009 284: 35283-35286. © the American Society for Biochemistry and Molecular Biology.

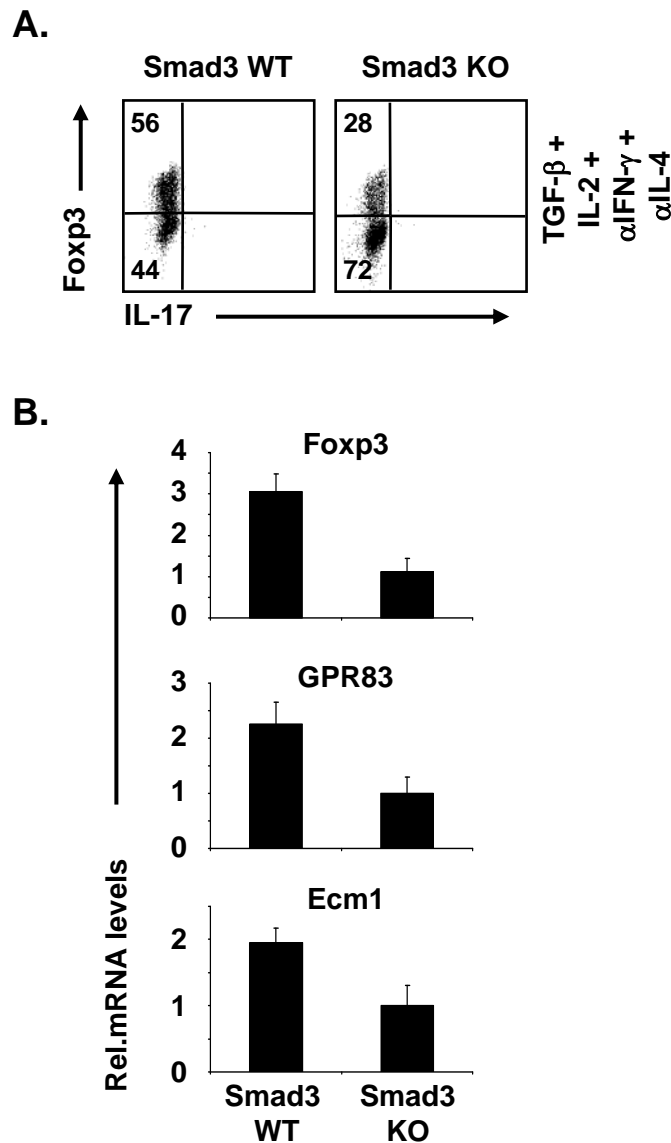


Figure 22. Smad3 is partially required for the generation of inducible regulatory T cells *in vitro*. (A) FACS-sorted naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from Smad3 WT or KO mice were activated under the indicated inducible regulatory T cell polarizing conditions with plate-bound anti-CD3 and anti-CD28 for 4 days and Foxp3 expression was analyzed by intracellular staining. A representative experiment from four independent experiments is shown. (B) Cells were differentiated as in (A), then restimulated with anti-CD3 for 4 h and cDNA was prepared. Gene expression profile was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The lower expression level for each gene was referred as 1. Data shows mean \pm S.D. This research was originally published in “Smad3 Differentially Regulates the Induction of Regulatory and Inflammatory T Cell Differentiation.” Gustavo J. Martinez, Zhengmao Zhang, Yeonseok Chung, Joseph M. Reynolds, Xia Lin, Anton M. Jetten, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2009 284: 35283-35286. © the American Society for Biochemistry and Molecular Biology.

III.2.c. Smad2 deficiency results in reduced generation of Foxp3⁺ iTreg cells

Given that Smad3 and Smad4 have been shown to partially regulate TGF- β -induced Foxp3 expression, we next determined the role of Smad2 in the generation of inducible regulatory T cells. Mice lacking Smad2 in T cells (Smad2 tKO) were generated by breeding Smad2 floxed mice (Vincent et al., 2003) with CD4-Cre transgenic mice (Lee et al., 2001) (Fig 23). Smad2 tKO mice exhibited normal development of CD4⁺ and CD8⁺ T cells in thymus compared to wild type littermate controls (Fig. 24). Moreover, Smad2 tKO mice showed similar frequencies and absolute cell numbers of CD8⁺ and CD4⁺ T cells in peripheral tissues compared to WT controls, with only a modest reduction in the frequency of CD8⁺ T cells in spleens (Fig 25 and data not shown). Furthermore, when we analyzed the phenotype of CD4⁺ T cells in the periphery, we observed an increase of effector memory (CD44^{hi}CD62L^{low}) T cells in Smad2 tKO mice compared to wild type controls (Fig. 26).

We next determined whether the generation of natural regulatory T cells would be affected in the absence of Smad2. Unlike the phenotype observed for Smad4 tKO or Smad3 KO mice, Smad2 tKO mice showed increased frequency of nTreg cells in all the lymphoid tissues studied, including the thymus (Fig. 27A). Because of the enhanced levels of memory/activated CD4⁺ T cells in spleen, we then hypothesized that nTregs deficient in Smad2 may have reduced suppressive activity. To evaluate this, we analyzed peripheral nTreg cell function by using an *in vitro* suppressive assay, in which naïve CD4⁺ T cells were stimulated in the presence of wild type or Smad2-deficient nTreg cells. Interestingly, Smad2-deficient nTreg cells displayed similar suppressive capacity compared to their wild type counterparts (Fig. 27B). Thus, these results, combined with our data from Smad3- and Smad4-deficient nTreg cells, suggest that nTreg suppressive activity might be Smad-independent. However, whether compensatory mechanisms exist for Smad function still remains to be determined.

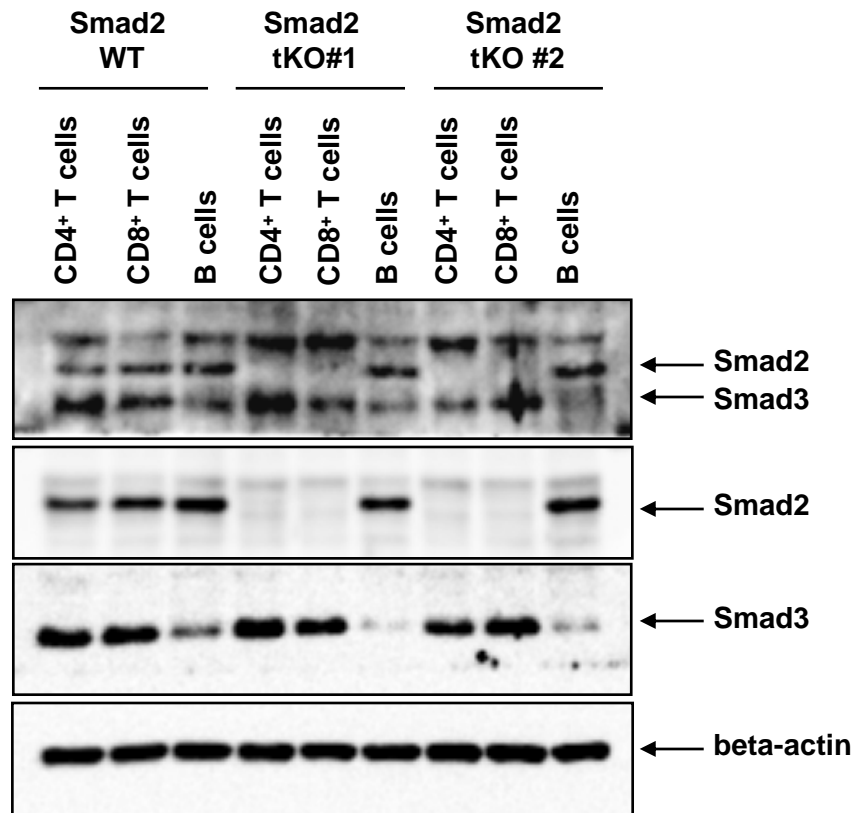


Figure 23. Mice with a Smad2-specific deletion in T cells show deficiency of Smad2 but not Smad3 in T cells. Splenocytes and peripheral lymph nodes were harvested from Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice. Cells were sorted based on expression of the following markers: B220⁺ (B cells), CD4⁺ (CD4 T cells) or CD8⁺ (CD8 T cells). Then, cell extracts were prepared and expression of Smad2, Smad3 and β -actin was analyzed by western blot. This research was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

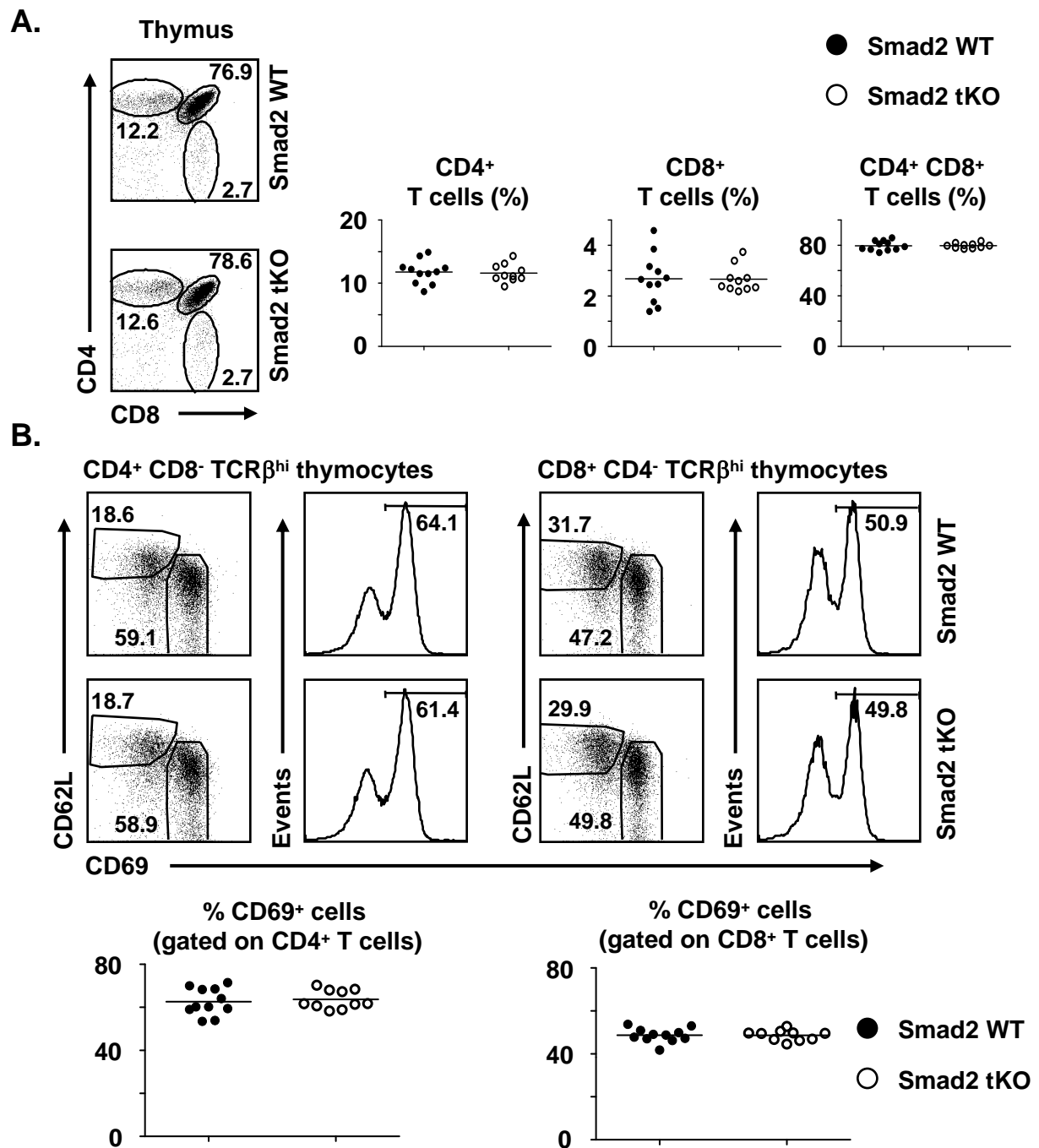


Figure 24. Normal development and maturation of CD4⁺ and CD8⁺ T cells in mice lacking Smad2 in T cells. Thymocytes from 6-8 weeks old Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice were harvested. (A) Frequency of CD4⁺ and CD8⁺ T cells was determined by flow cytometry. (B) Expression of CD62L and CD69 was determined in CD4⁺CD8⁻TCRβ^{hi} and CD8⁺CD4⁻TCRβ^{hi} T cells by flow cytometry. (A-B) A representative dot plot is shown for each group, and the combined results for 10-15 mice each group are indicated. p values were calculated using Student t test. This research was originally published in "Smad2 positively regulates the generation of Th17 cells" Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

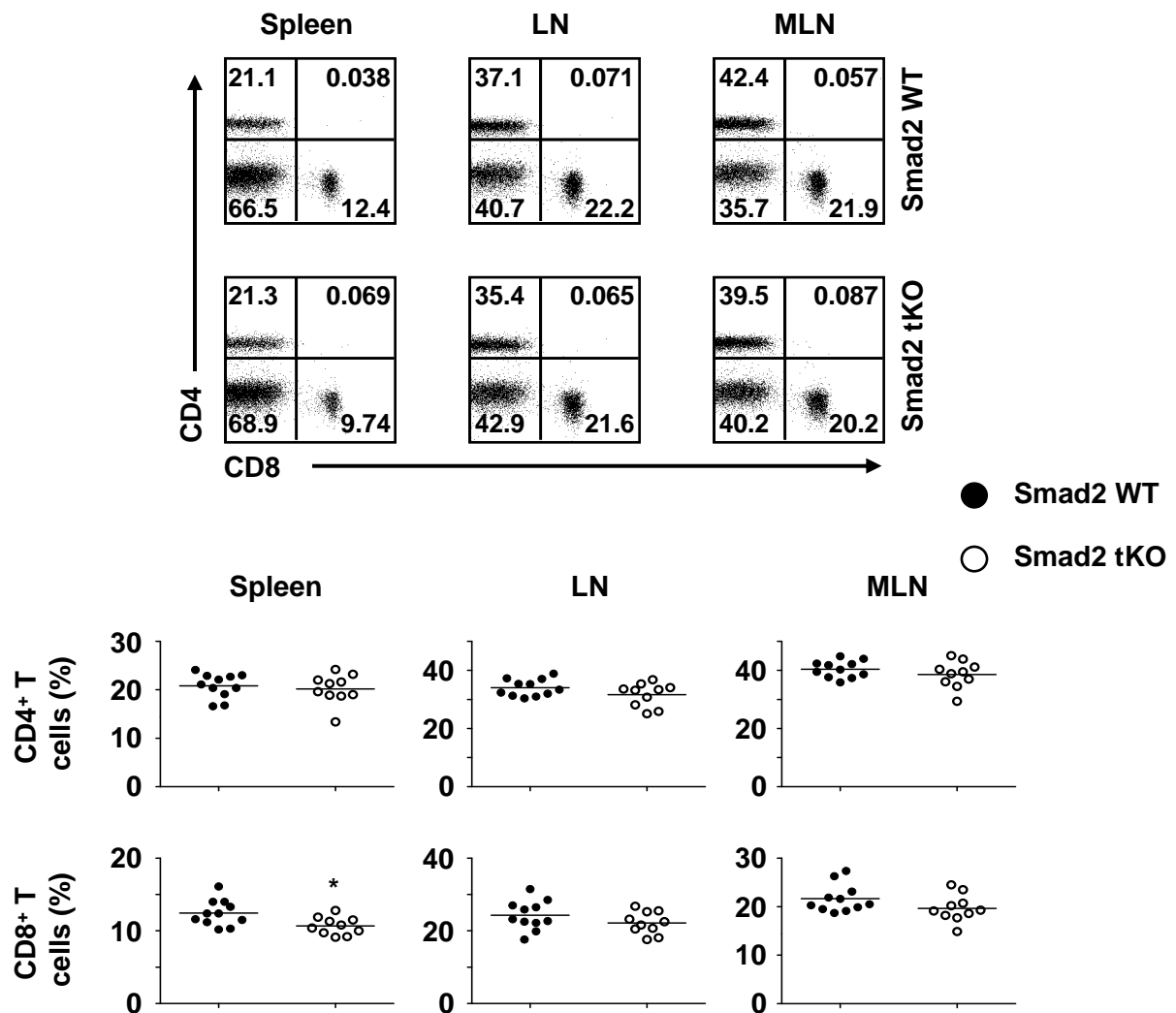


Figure 25. Normal levels of CD4⁺ and CD8⁺ T cells in mice lacking Smad2 in T cells in peripheral tissues. Spleen, peripheral and mesenteric lymph nodes from 6-8 weeks old Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice harvested. Expression of CD4 and CD8 markers was determined by flow cytometry. A representative dot plot is shown for each group (top panels), and the combined results for 10-15 mice each group are indicated (bottom panels). p values were calculated using Student t test. *, p < 0.05. This research was originally published in "Smad2 positively regulates the generation of Th17 cells" Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

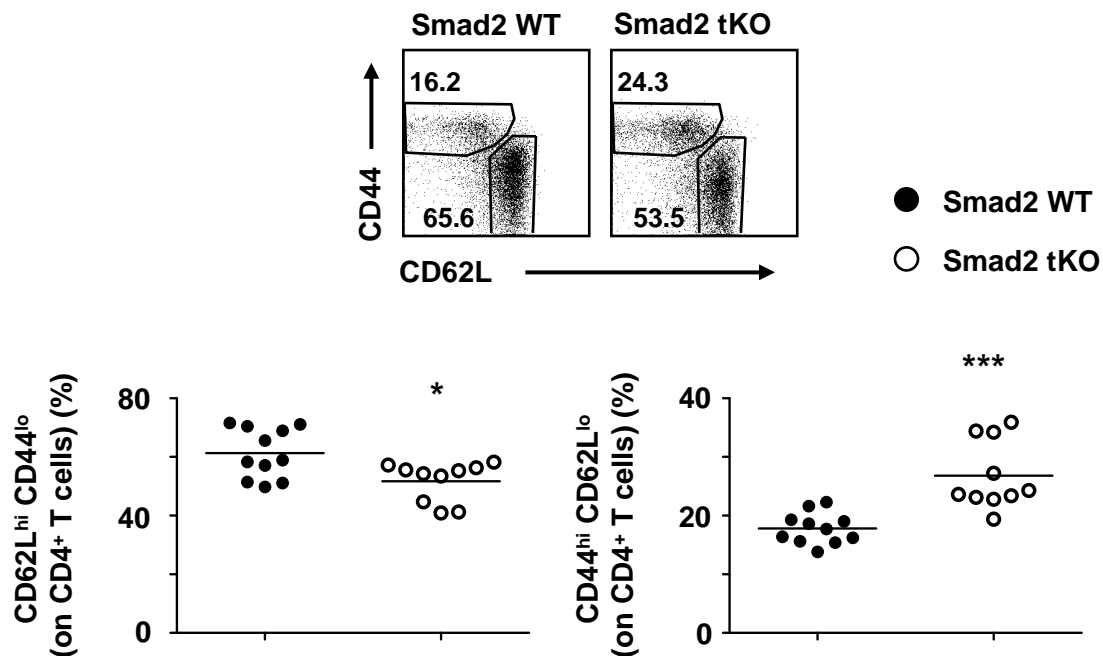


Figure 26. Decreased naïve and enhanced memory CD4⁺ T cells in spleens from mice lacking Smad2 in T cells. Spleens from 6-8 weeks old Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice were harvested. Expression of CD62L and CD44 was determined in CD4⁺CD8⁻ T cells by flow cytometry. A representative dot plot is shown for each group (top panels), and the combined results for 10-15 mice each group are indicated (bottom panels). p values were calculated using Student t test. * p < 0.05; *** p < 0.001. This research was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

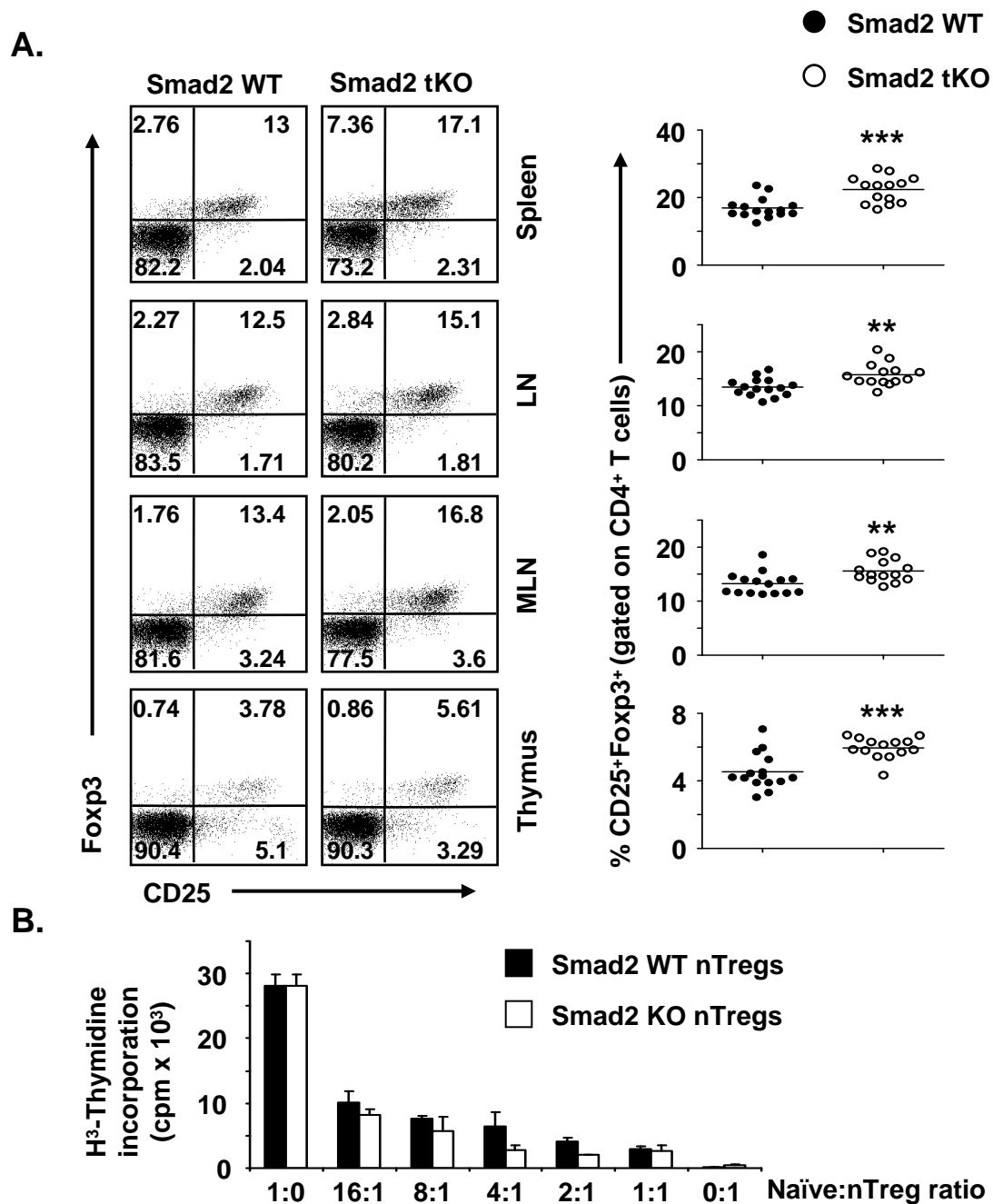


Figure 27. Smad2 is not required for development or function of natural regulatory T cells. (A) CD25⁺Foxp3⁺ cells in a CD4⁺ T cell gate were analyzed in different tissues from Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice. A representative dot plot is shown for each group in each tissue (left panels), and the combined results for 10-15 mice each group are indicated (right panel). *p* values were calculated using Student *t* test. * *p* < 0.05; ** *p* < 0.01; *** *p* < 0.001. (B) CD4⁺CD25⁻CD62L^{hi}CD44^{lo} naïve T cells from Smad2 WT mice were cultured in the presence or absence of different ratios of Smad2 KO or WT CD4⁺CD25^{hi} natural regulatory T cells in triplicate wells with irradiated APCs and stimulated with 1 µg/ml of anti-CD3. Proliferation was assayed 72 h after treatment by adding [³H]-thymidine to the culture for the last 8 h. Data shows mean ± S.D. A representative of three independent experiments is shown. This research was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xinhua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

Recently, Tone et al. reported that Smad3 directly induces *Foxp3* expression by synergizing with NFAT (Tone et al., 2008). Furthermore, the authors showed that Smad3, but not Smad2, was able to induce luciferase activity in a reporter construct containing the *Foxp3* enhancer region (Tone et al., 2008). However, the Smad2 isoform expressed in T cells contains exon 3 (data not shown), which shows reduced or complete lack of DNA-binding capacity (Dennler et al., 1998; Zawel et al., 1998). Therefore, Smad2 by itself may not be able to induce transactivation of a reporter gene, so it is still plausible that Smad2 regulates *Foxp3* expression by associating with Smad3 and Smad4 or other factors. We then evaluated the role of Smad2 in the induction of *Foxp3*. To this end, FACS-sorted naïve CD4⁺ T cells were stimulated with plate-bound anti-CD3 and anti-CD28 under iTreg-skewing conditions. We observed decreased generation of *Foxp3*-expressing cells in Smad2-deficient T cells compared to WT counterparts (Fig. 28A). Such reduction in *Foxp3* expression was observed at the protein level as well as the mRNA level (Fig. 28B). These results suggest that Smad2, Smad3 and Smad4, by either forming a molecular complex with each other, or by binding to other transcription factors, regulate TGF- β -induced *Foxp3* gene transcription. However, while single deficiency in a Smad molecule results in partial defect in iTreg cell generation, they are not essential for nTreg development/homeostasis. However, we cannot rule out possible compensatory mechanisms in these single Smad-deficient mice.

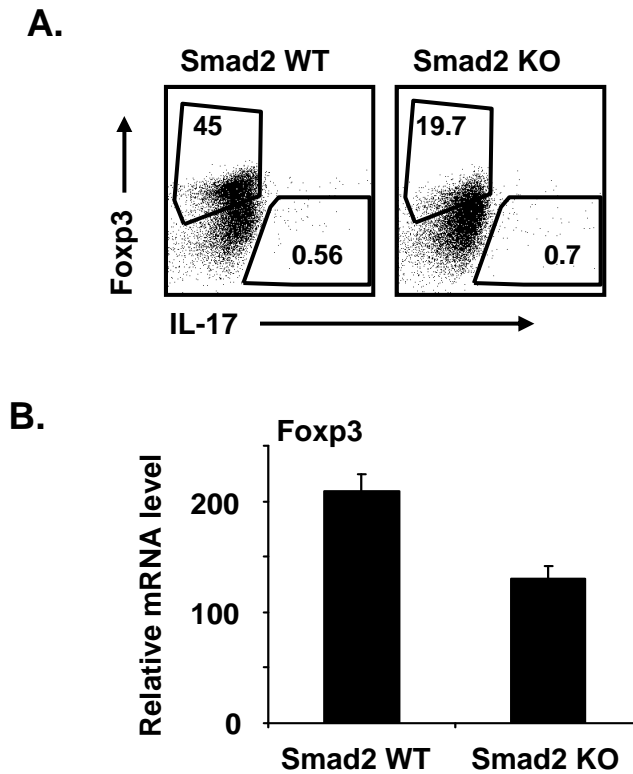


Figure 28. TGF- β -induced Foxp3 expression is regulated by Smad2. FACS-sorted naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice were stimulated with plate bound anti-CD3 and anti-CD28 under inducible regulatory T cell (iTreg) conditions (TGF- β , IL-2, anti-IFN- γ and anti-IL-4) for 4 days. (A) Foxp3 expression was analyzed by intracellular staining. (B) After differentiation, cells were restimulated with anti-CD3 for 4 h and cDNA was prepared. Foxp3 gene expression was analyzed by real-time RT-PCR, and normalized to a reference gene *Actb*. The lower expression level for Foxp3 gene in naïve T cells was referred as 1. Data shows mean \pm S.D. A representative of three independent experiments is shown. This research was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

III.3. Role of Smad-signaling pathway in the generation of Th17 cells

III.3.a. Dispensable role of Smad4 in Th17 cell differentiation

Given that we found impaired generation of Th17 cells in the presence of a TGF β RI kinase inhibitor, demonstrating Th17 reliance on TGF- β signaling pathway, we then investigated the downstream signaling molecules important for such commitment. We first determined the role of Smad4 during Th17 differentiation by isolating naïve T cells from either Smad4^{fl/fl} CD4Cre⁻ (WT) or Smad4^{fl/fl} CD4Cre⁺ (tKO) mice. Cells were stimulated with Th17-polarizing conditions (TGF- β , IL-6, and neutralizing antibodies against IFN- γ and IL-4) for four days. Interestingly, similar amounts of IL-17-producing cells were found upon Th17 differentiation in Smad4-deficient and -sufficient T cells (Fig. 29A). To further demonstrate that Smad4 does not play a role in the generation of IL-17-producing cells *in vivo*, we utilized an immunization protocol. Mice were immunized with Keyhole Limpet Hemocyanin (KLH) protein emulsified in Complete Freund's Adjuvant (CFA). This immunization protocol elicits the induction of antigen-specific Th17 responses *in vivo* (Chung et al., 2009; Nurieva et al., 2007). Seven days after immunization, splenocytes were harvested and restimulated *ex vivo* with increasing concentrations of KLH protein for three days and cytokine expression was determined by ELISA. Consistent with our *in vitro* results, no differences were observed in the production of Th17-specific cytokines IL-17, IL-17F and IL-22 between WT and Smad4-deficient cells (Fig. 29B). Overall, these results suggest that Smad4 differentially regulates the generation of Th17 and iTreg cells.

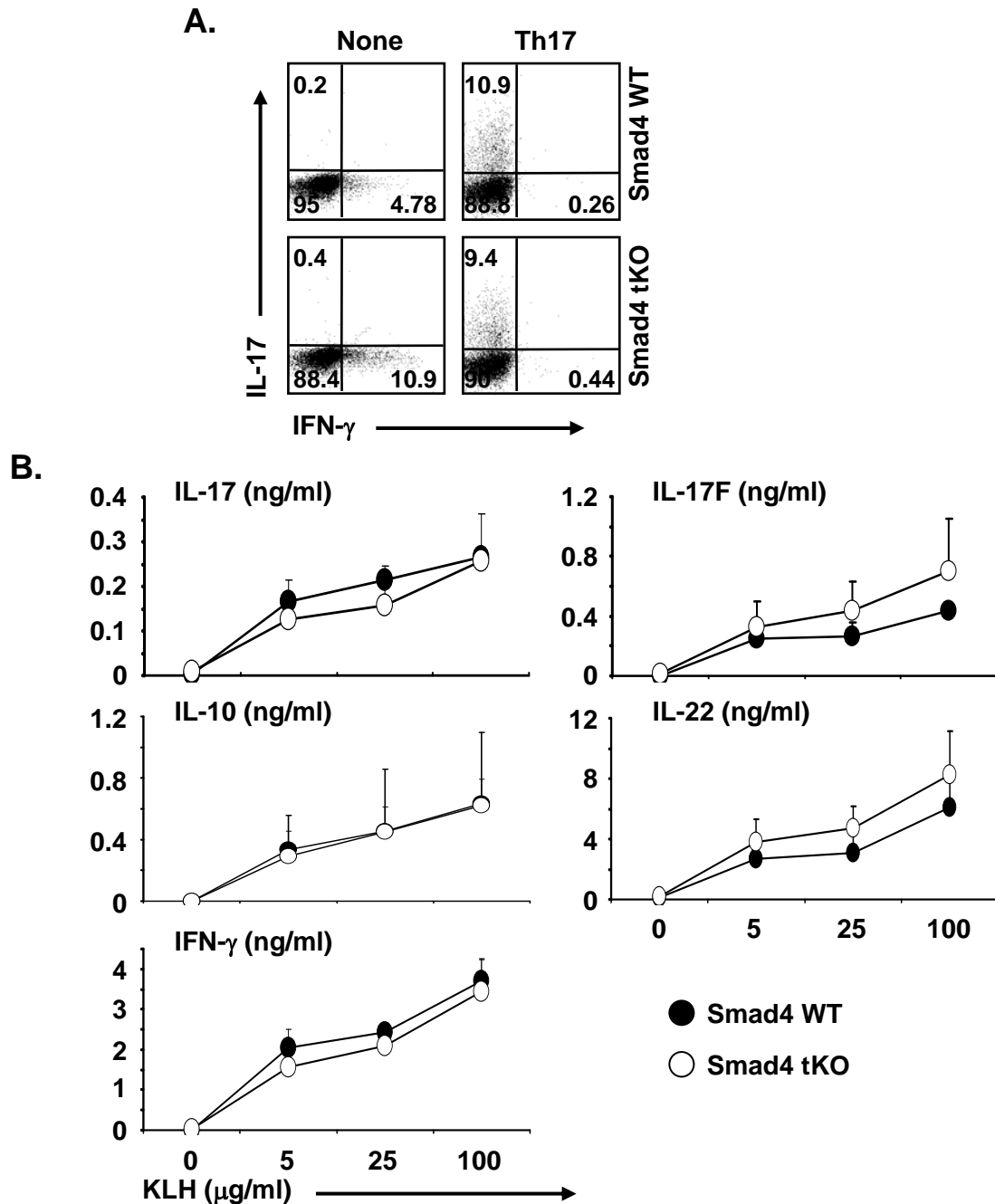


Figure 29. Smad4 is dispensable for Th17 cell generation *in vitro* and *in vivo*. (A) CD4⁺CD25⁻CD62L^{hi}CD44^{lo} naïve Smad4-sufficient or -deficient T cells were activated under Th17 conditions (TGF- β , IL-6, anti-IFN- γ , anti-IL-4). After 4d, cells were restimulated with PMA and ionomycin in the presence of Golgi Stop for 4h and IL-17/IFN- γ expression was analyzed by intracellular staining. Numbers in quadrants represent the percentages. The experiments were repeated three times with consistent results. (B) Smad4^{fl/fl} CD4Cre⁺ (tKO) or Smad4^{fl/fl} CD4Cre⁻ (WT) mice were immunized with KLH in CFA. Seven days later, splenocytes were re-stimulated with increasing concentration of KLH *ex vivo* for 3d and cytokine production was measured by ELISA in the culture supernatant. Data shows mean \pm S.D. (n=4). The results shown are a representative example of two independent experiments. Reprinted from “Molecular antagonism and plasticity of regulatory and inflammatory T cell programs.” Xuexian O. Yang, Roza Nurieva, Gustavo J. Martinez, Hong Soon Kang, Yeonseok Chung, Bhanu P. Pappu, Bhavin Shah, Seon Hee Chang, Kimberly S. Schluns, Stephanie S. Watowich, Xin-Hua Feng, Anton M. Jetten and Chen Dong. *Immunity*. 2008. 29(1):44-56 with permission from Elsevier.

III.3.b. Smad3 deficiency leads to enhanced Th17 cell differentiation

Since Smad4 was found to be dispensable for the generation of Th17 cells *in vivo* and *in vitro*, we next evaluated whether Smad3 could regulate the differentiation of Th17 cells. Thus, naïve T cells from Smad3 KO or WT littermates were stimulated with Th17-polarizing conditions as previously mentioned. Interestingly, an increase in frequency of Th17 cells was observed when Smad3-deficient naïve T cells were stimulated with TGF- β , IL-6 and neutralizing antibodies against IFN- γ and IL-4 compared to WT counterparts (Fig. 30A). Gene expression analysis by real time RT-PCR showed not only increased IL-17A but also IL-17F, CCR6 and CCL20 mRNA in T cells lacking Smad3 (Fig. 30B). However, no differences were observed for IL-22 or IL-21 mRNA, indicating that their expression is not regulated by TGF- β . We also measured cytokine secretion levels by ELISA from *in vitro*-generated WT or Smad3-deficient Th17 cells after anti-CD3 restimulation for 16h. We found increased IL-17 and IL-17F cytokine production, however no differences were observed for IL-21 in Smad3-deficient Th17 cells (Fig. 30C). We next analyzed the expression of transcription factors by real time RT-PCR and detected a slight increase in ROR α , but lower AHR and IRF4 mRNA expression in Smad3-deficient Th17 cells compared to WT counterparts. However, no differences were noticed for ROR γ t expression (Fig. 30B).

Our lab has recently shown that the combination of IL-1, IL-6 and IL-23 also initiates the Th17 genetic programming. This polarizing condition still requires low TGF- β concentration since neutralization of endogenous TGF- β levels inhibits Th17 cell induction (Chung et al., 2009). Thus, we next determined if Smad3 deficiency would still affect Th17 cell generation under low TGF- β concentrations. For this purpose, Smad3-deficient or –sufficient naïve T cells were activated with anti-CD3 and anti-CD28 in the presence of IL-1, IL-6 and IL-23. Even though stimulation with exogenous TGF- β led to enhanced numbers of IL-17-producing cells, activation of naïve T cells with IL-1, IL-6 and IL-23 resulted in similar levels of Th17 cells in Smad3 KO T cells compared to WT controls (Fig. 30A and B). These results suggested that in the presence of high TGF- β concentrations, Smad3 is

capable of inhibiting Th17 cell generation. To further assess this hypothesis, Smad3 KO or WT naïve T cells were cultured with IL-6 and neutralizing antibodies against IFN- γ and IL-4 with increasing concentrations of TGF- β . At low concentrations of TGF- β , low but similar levels of IL-17-producing cells were observed in Smad3-deficient or -sufficient T cells. However, with increasing concentrations of TGF- β , higher frequency of IL-17-producing cells was observed in cells lacking Smad3 (Fig. 31). Interestingly, at TGF- β concentrations that were not sufficient to induce Foxp3 expression, enhanced Th17 cells were still detected in Smad3-deficient cells (Fig. 31) indicating that the inhibitory role of Smad3 on Th17 cells might be independent of the induction of Foxp3.

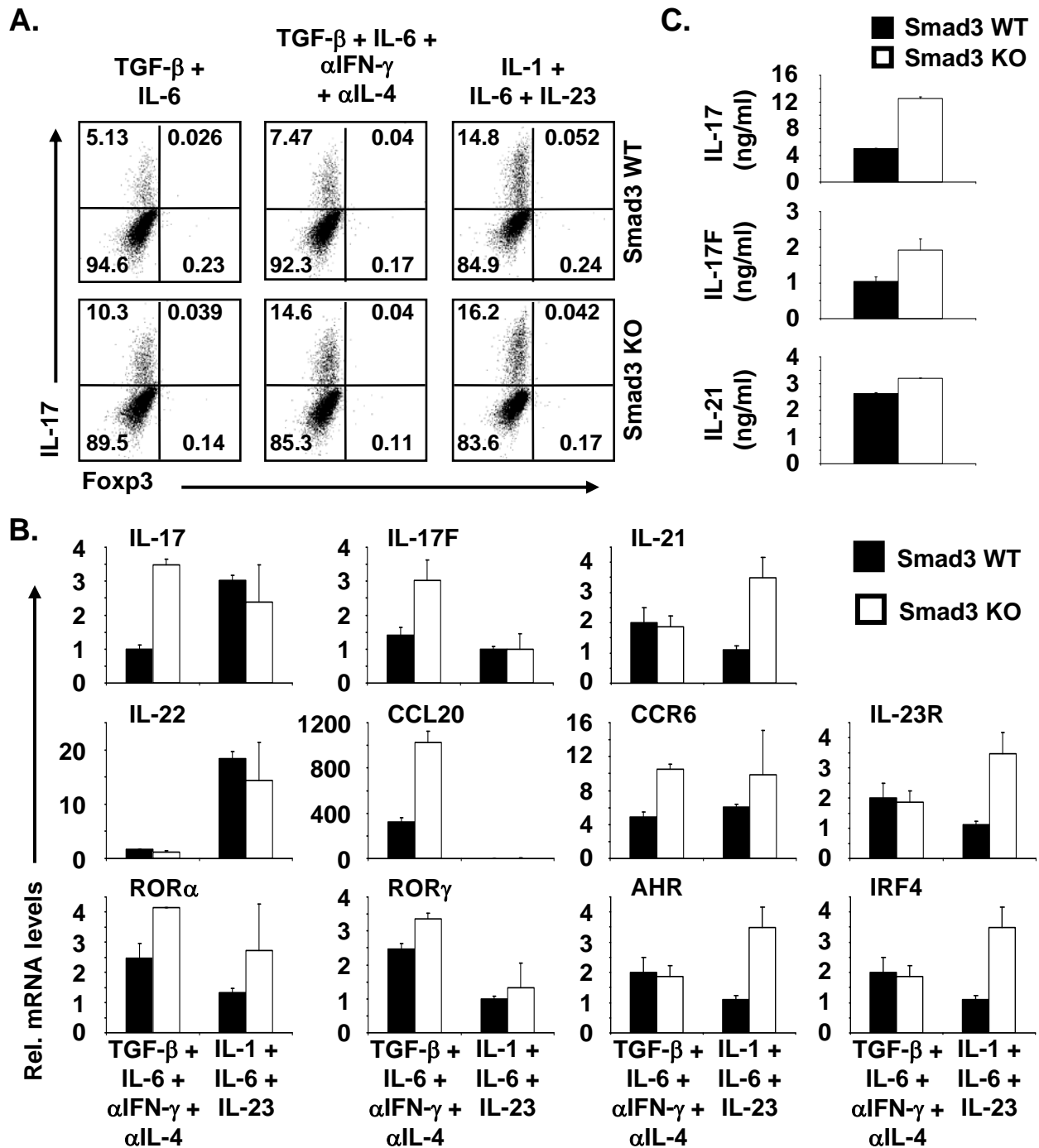


Figure 30. Smad3 deficiency leads to enhanced Th17 cells upon stimulation with exogenous TGF- β . FACS-sorted naïve T cells from Smad3 WT or KO mice were activated with plate-bound anti-CD3 and anti-CD28 under indicated Th17 conditions for 4 days. (A) Cells were then restimulated with PMA and ionomycin in the presence of Golgi inhibitor for 4h and IL-17- and Foxp3-expressing cells were assessed by intracellular staining. (B) Cells were restimulated with anti-CD3 for 4h and cDNA was prepared. Gene expression profile was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The lower expression level for each gene was referred as 1. (C) Cells were then restimulated with anti-CD3 for 16h, and cytokine concentration in culture supernatants was measured by ELISA. A representative experiment out of four is shown. (B-C) Data shows mean \pm S.D. This research was originally published in “Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation” Gustavo J. Martinez, Zhengmao Zhang, Yeonseok Chung, Joseph M. Reynolds, Xia Lin, Anton M. Jetten, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2009 284: 35283-35286. © the American Society for Biochemistry and Molecular Biology.

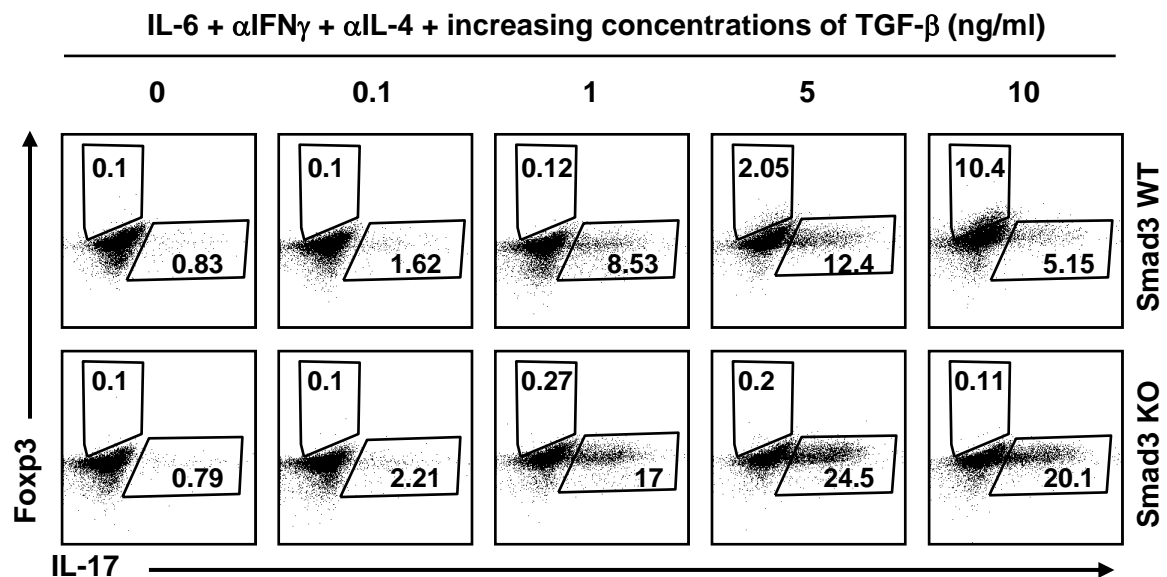


Figure 31. Smad3 inhibits Th17 differentiation in a TGF- β -dose dependent manner. FACS-sorted naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from Smad3 WT or KO mice were activated with plate-bound anti-CD3 and anti-CD28 under indicated Th17-skewing conditions for 4 days. Cells were then restimulated with PMA and ionomycin in the presence of Golgi inhibitor for 5 h and IL-17- and Foxp3-expressing cells were assessed by intracellular staining. A representative experiment out of three is shown. This research was originally published in “Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation.” Gustavo J. Martinez, Zhengmao Zhang, Yeonseok Chung, Joseph M. Reynolds, Xia Lin, Anton M. Jetten, Xin-Hua Feng, and Chen Dong. *J. Biol. Chem.* 2009 284: 35283-35286. © the American Society for Biochemistry and Molecular Biology.

III.3.c. Smad3 associates with ROR γ t to inhibit its transcriptional activity

Given that Smad3 KO T cells showed an enhanced capability to generate Th17 cells even in conditions that were not sufficient to induce Foxp3 expression, and that Smad3-deficient Th17 cells had similar mRNA levels of the Th17 master transcription factor ROR γ t, we next determined the regulation of ROR γ t function by Smad3. HEK 293T cells were transfected with the RORE luciferase reporter system previously mentioned (Yang et al., 2008b) in the presence or absence of vectors expressing ROR γ t and/or a constitutively active form of Smad3. Transfection of ROR γ t alone induced high luciferase activity, whereas expression of ROR γ t with increasing concentrations of constitutively active Smad3 led to a significant reduction in luciferase activity (Fig. 32A).

Since Smad3 is capable of partially inhibiting ROR γ t transcriptional activity, we then investigated whether Smad3 could directly or indirectly associate with ROR γ t. Smad3 was found to be in a complex bound to ROR γ t when the two molecules were co-expressed in HEK 293T cells (Fig. 32B). Interestingly, this binding seemed to be enhanced upon co-expression of a constitutively active form of rat TGF- β receptor I (TGF β RI T202D) (Feng et al., 2000) (Fig. 32B), indicating that phosphorylated-Smad3 might show a higher affinity either directly for ROR γ t or for other molecules that also bind to ROR γ t.

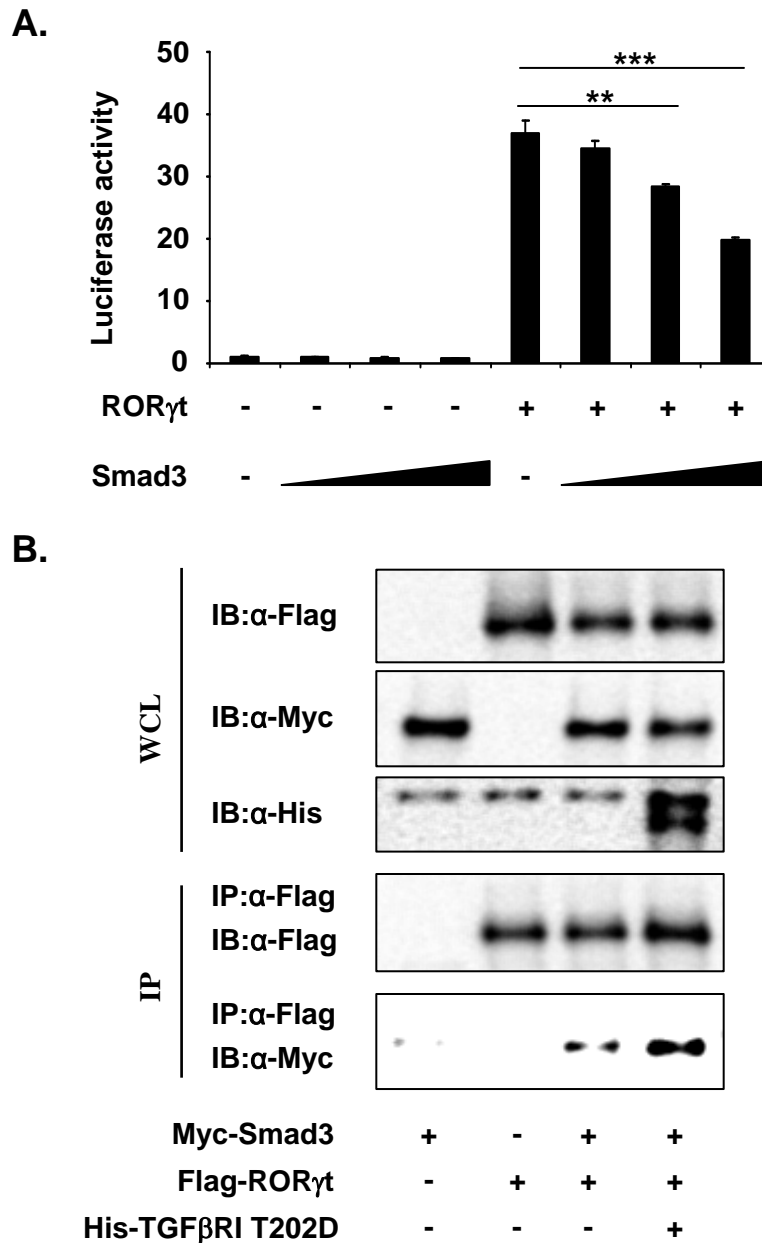


Figure 32. Smad3 binds to ROR γ t and inhibits its transcriptional activity. (A) HEK 293T cells were transfected with RORE-Luciferase and the indicated vectors. Firefly Luciferase activity was normalized to Renilla luciferase activity. Data shows mean \pm S.D. The data represents at least two independent experiments with triplicate measurements with similar results. p values were calculated using Student t test. **, $p < 0.005$; ***, $p < 0.0005$. (B) HEK 293T cells were transiently transfected with Myc-tagged Smad3, Flag-tagged ROR γ t and/or His-tagged TGF β RI T202D. After 48h, lysates were prepared and immunoprecipitated with an anti-FLAG mAb (IP), followed by immunoblotting with anti-FLAG or anti-Myc (bottom two panels). Top three panels indicate western blot of whole cell lysates. This experiment was performed by Dr. Zhang at Baylor College of Medicine. This research was originally published in “Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation.” Gustavo J. Martinez, Zhengmao Zhang, Yeonseok Chung, Joseph M. Reynolds, Xia Lin, Anton M. Jetten, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2009 284: 35283-35286. © the American Society for Biochemistry and Molecular Biology.

III.3.d. Lack of Smad3 leads to enhanced Th17 cell generation in vivo

To further demonstrate that Smad3 deficiency leads to enhanced Th17 cell generation, we next analyzed the role of Smad3 *in vivo*. Since Smad3-deficient mice develop colorectal adenocarcinomas starting at four month of age (Zhu et al., 1998), we utilized bone marrow chimeras for our experiments. Sublethally irradiated RAG1 (recombination activating gene 1) KO mice were adoptively transferred with WT or Smad3-deficient bone marrow cells. Eight weeks after reconstitution, mice were immunized with MOG peptide emulsified in CFA. Seven days after immunization, splenocytes were restimulated *ex vivo* with increasing concentrations of MOG peptide for three days and then cytokine levels were determined in the culture supernatant. Increased Th17 cytokines (IL-17, IL-17F and IL-22) but not Th1 cytokine IFN- γ , were observed in splenocytes containing Smad3-deficient T cells compared to WT controls (Fig. 33 and data not shown). Thus, these results confirm our *in vitro* data and suggest that Smad3 deficiency leads to enhanced Th17 cell generation.

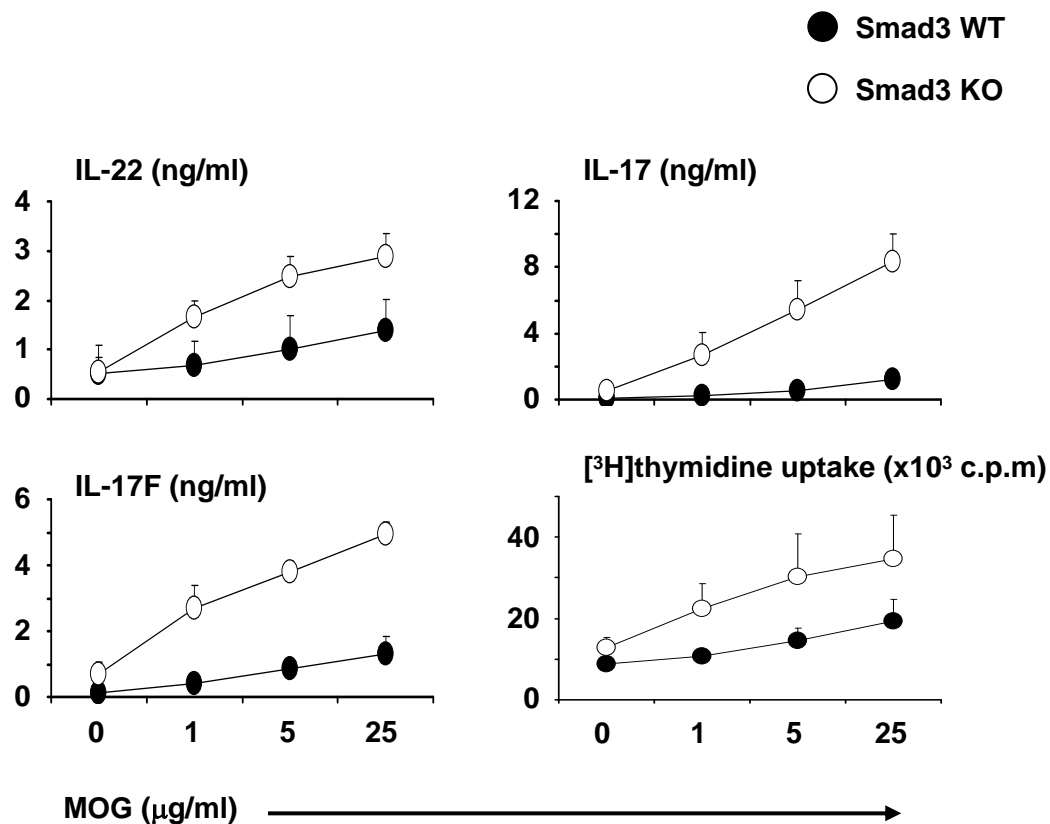
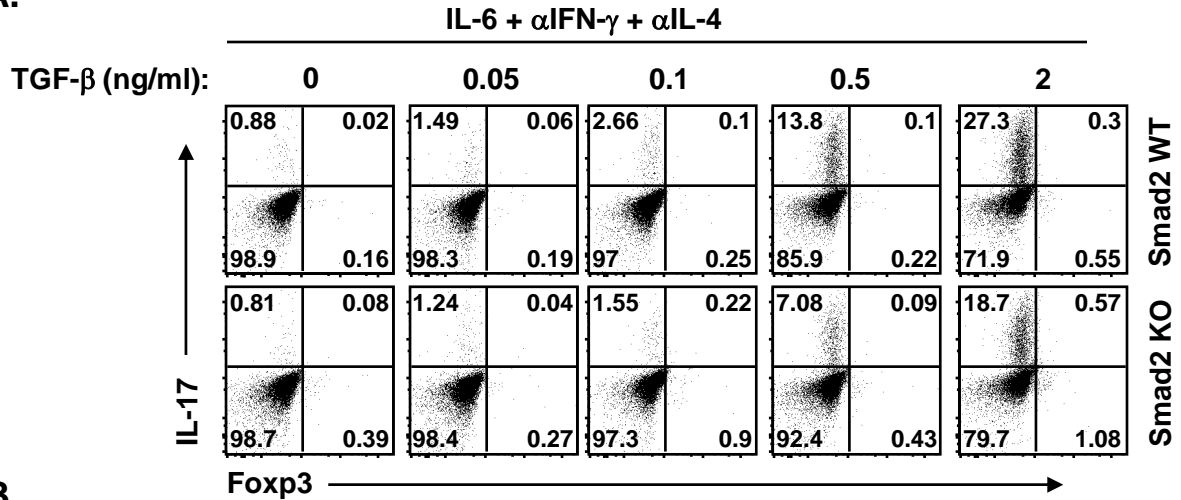


Figure 33. Smad3 deficiency leads to enhanced Th17 cell differentiation *in vivo*. Bone marrow cells from Smad3 WT or KO mice were intravenously transferred to sub-lethally irradiated RAG1 KO mice. After 8 weeks, the recipient mice were immunized subcutaneously with 150 μg of MOG₃₅₋₅₅ peptide emulsified in CFA. Seven days later, splenocytes were isolated and restimulated with MOG peptide *ex vivo* for 3 days. Cytokine production was measured from cell-free supernatants by ELISA. Data shows mean ± S.D. A representative experiment with five mice in each group out of two is shown. This research was originally published in “Smad3 differentially regulates the induction of regulatory and inflammatory T cell differentiation.” Gustavo J. Martinez, Zhengmao Zhang, Yeonseok Chung, Joseph M. Reynolds, Xia Lin, Anton M. Jetten, Xin-Hua Feng, and Chen Dong. *J. Biol. Chem.* 2009 284: 35283-35286. © the American Society for Biochemistry and Molecular Biology.

III.3.e. Smad2 deficiency results in a reduction of Th17 cells *in vitro*

Since TGF- β has been shown to be an essential cytokine in driving Th17 cell responses independently of direct involvement of Smad3 or Smad4, we next determined the requirement of Smad2 in Th17 cell generation. Differentiation into IL-17-producing T cells *in vitro* was partially impaired in cells lacking Smad2 compared to their WT counterparts (Fig. 34A and 35A). When analyzing the expression profile of lineage-specific genes by real-time RT-PCR, we found a reduction in IL-17, IL-17F and CCL20 mRNA expression in Smad2-deficient T cells (Fig. 34B and 35B). However, expression of other Th17-related cytokines, such as IL-22 or IL-21, was not impaired in Smad2-deficient T cells (Fig. 34B and 35B), suggesting that the regulation of these cytokines is independent of Smad proteins. Moreover, we did not detect any significant differences in the expression of the Th17-associated transcription factors ROR α , ROR γ , IRF4, AHR, I κ B ζ and BATF (Fig. 34B, 35B and data not shown). Smad2-deficient T cells also showed similar expression of the Th1, Th2 and iTreg transcription factors T-bet, GATA3 and Foxp3, respectively, compared to WT cells (Fig. 34B and data not shown), indicating that the Th17 deficiency is not a reflection of enhanced commitment to alternative Th lineages. Furthermore, reduced IL-17-producing cells were also observed using a combination of IL-1, IL-6 and IL-23 cytokines in the absence of Smad2 (Fig. 35). Similarly, decreased IL-17, IL-17F and CCL20, but not IL-21, IL-22 or Th17-specific transcription factors ROR α and ROR γ t were observed in Smad2-deficient Th17 cells when stimulated with IL-1, IL-6 and IL-23 (Fig. 35B and data not shown). Thus, our data suggest that Smad2 may function as an important factor in inducing Th17-specific cytokines IL-17 and IL-17F as well as Th17-associated chemokine CCL20.

A.



B.

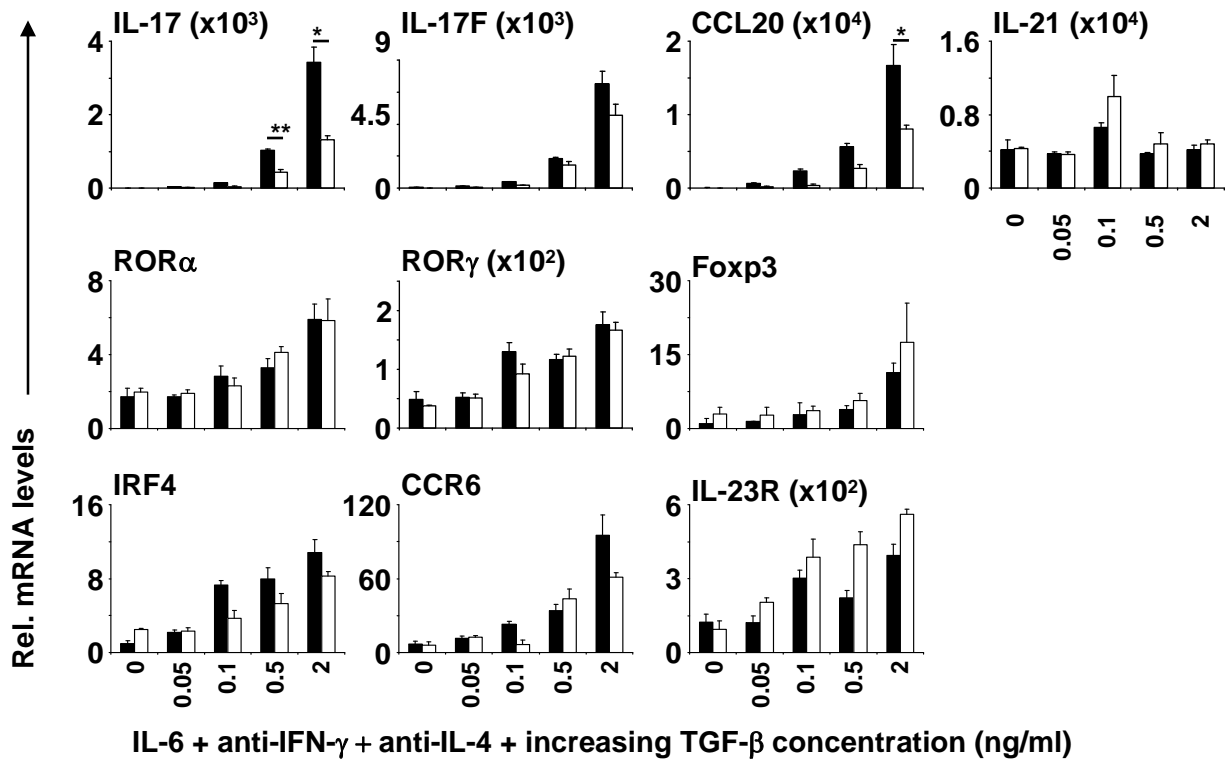


Figure 34. T cells lacking Smad2 show decreased ability to differentiate into Th17 cells *in vitro*. FACS-sorted naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice were stimulated with plate bound anti-CD3 and anti-CD28 under indicated Th17-skewing conditions for 4 days. (A) Cells were then re-stimulated with PMA and ionomycin in the presence of Golgi inhibitor for 4h and IL-17- and Foxp3-expressing cells were assessed by intracellular staining. (B) After differentiation, cells were re-stimulated with anti-CD3 for 4h and cDNA was prepared. Gene expression profile was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The lower expression level for each gene was referred as 1. A representative example of four independent experiments is shown. p values were calculated using Student t test. *, p < 0.05; **, p < 0.01.

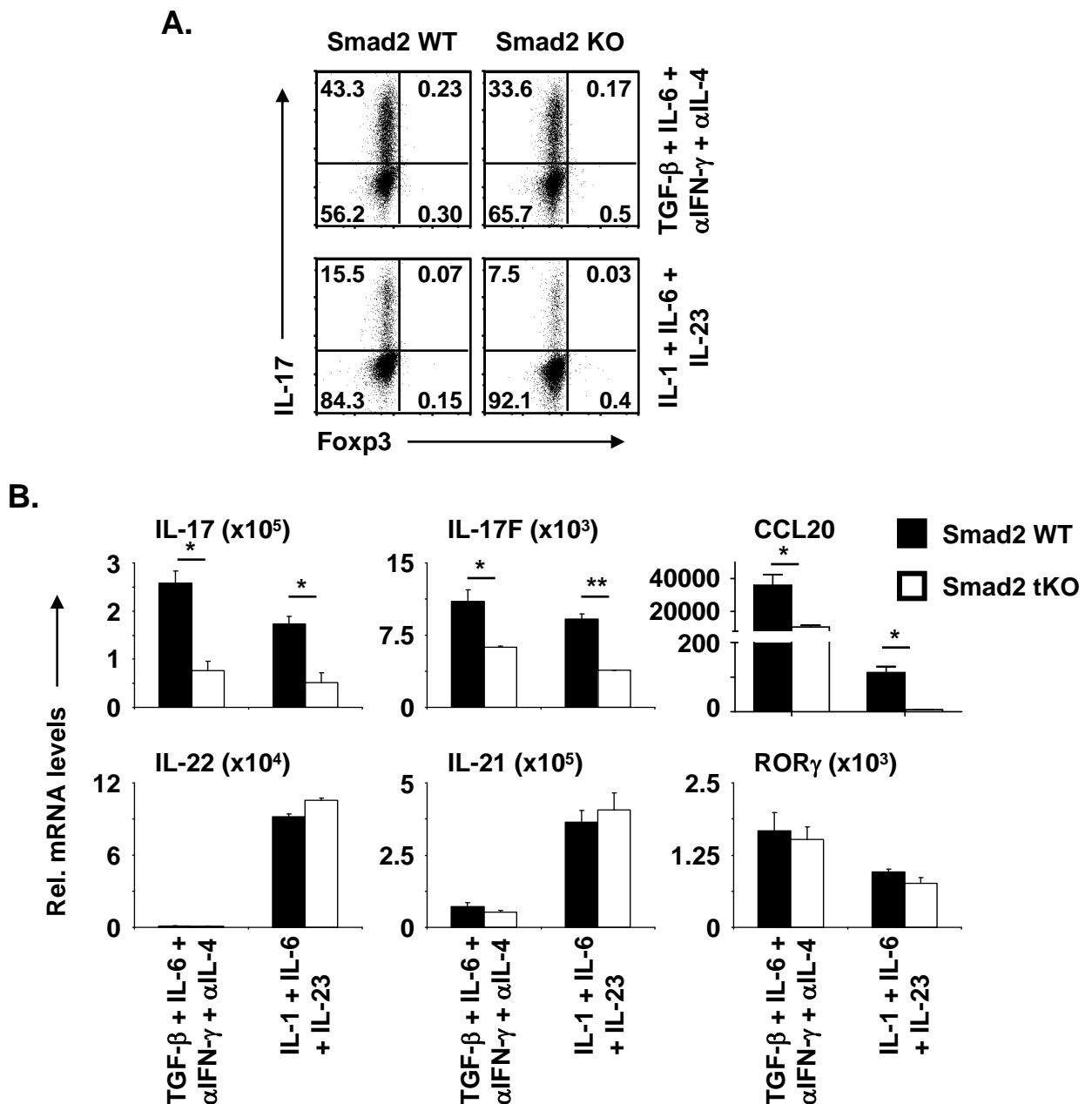


Figure 35. Reduced Th17 cell induction in cells lacking Smad2 when stimulated in the presence of IL-1, IL-6 and IL-23. FACS-sorted naïve CD4⁺CD25⁻CD62L^{hi}CD44^{lo} T cells from Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice were stimulated with plate bound anti-CD3 and anti-CD28 in the presence of TGF-β, IL-6 and neutralizing antibodies against IFN-γ and IL-4, or IL-1, IL-6 and IL-23 for 4 days. (A) Cells were then re-stimulated with PMA and ionomycin in the presence of Golgi inhibitor for 4h and IL-17- and Foxp3-expressing cells were assessed by intracellular staining. (B) After differentiation, cells were re-stimulated with anti-CD3 for 4h and cDNA was prepared. Gene expression profile was analyzed by real-time RT-PCR. Data were normalized to a reference gene *Actb*. The lower expression level for each gene was referred as 1 (expression level in naïve T cells). Data shows mean ± S.D. A representative example of three independent experiments is shown. p values were calculated using Student t test. *, p < 0.05; **, p < 0.005. This research (partially) was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

III.3.f. Smad2 is necessary for the generation of Th17 cells in vivo

Since Smad2-deficient naïve T cells exhibited reduced capacity to differentiate into Th17 cells *in vitro*, we next analyzed if the same holds true for *in vivo* models. Smad2 tKO or WT control mice were first immunized with KLH emulsified in CFA. Seven days later, cytokine production by cells residing in the draining lymph nodes and spleen was determined. A significant reduction in IL-17-producing cells (frequency and absolute cell numbers) was observed in Smad2 tKO mice compared to WT littermates when cells from draining lymph nodes were restimulated with KLH *ex vivo* (Fig. 36A). Furthermore, significantly lower IL-17 and IL-17F cytokines were observed in culture supernatants when cells from draining lymph nodes and spleen were restimulated *ex vivo* with increasing concentrations of KLH (Fig. 36B and C). However, IL-22 and IFN- γ expression was similar between WT and KO cells, suggesting that Smad2 regulates IL-17 and IL-17F expression but not other Th-specific cytokines.

Th17 cells play important roles in autoimmune diseases and inflammatory responses (reviewed in (Martinez et al., 2008)). In particular, Th17 cells have been shown to be the main pathogenic population in experimental autoimmune encephalomyelitis (EAE), an inflammatory disease that affects the central nervous system (CNS) (Langrish et al., 2005; Park et al., 2005). Thus, to further comprehend the role of Smad2 during *in vivo* autoimmune settings, we used the EAE model. Smad2 WT or tKO mice were immunized with MOG peptide emulsified in CFA on days zero and seven, and then injected with pertussis toxin on days one and eight. Disease scores were monitored daily following the second immunization as detailed in material and methods. We found that both groups of mice showed signs of disease at a similar time point and disease severity further progressed until day nine after the second immunization (Fig. 37A). While Smad2 tKO mice showed signs of recovery from disease on day ten onward after the second immunization, WT littermates showed increased or sustained disease severity. Mice were euthanized fourteen days following the second immunization, and cell infiltration in the CNS was determined. We observed a significant reduction in the frequency of CD4⁺

T cells in Smad2 tKO mice compared to WT littermates (Fig. 37B). Furthermore, we found decreased total CD4⁺ T cells in the CNS, although this comparison was not statistically significant (Fig. 37B). We then analyzed the cytokines produced by the CNS-infiltrating CD4⁺ T cells by intracellular staining and observed significantly lower IL-17⁺ and IL-17⁺IFN- γ ⁺ cells (frequency and absolute cell number) (Fig. 37C). However, the amount of IFN- γ -producing CD4⁺ T cells was similar between WT and Smad2 tKO mice (Fig. 37C), further confirming that Smad2 specifically regulates Th17, but not Th1, generation *in vivo*. Moreover, when splenocytes from Smad2 tKO mice were restimulated with increasing concentrations of MOG peptide *ex vivo*, we found reduced Th17 cytokine secretion, albeit similar proliferation potential, compared to WT cells (Fig. 37D). Taken together, our results suggest that Smad2 expression is necessary for normal Th17-mediated immune responses *in vitro* and *in vivo*.

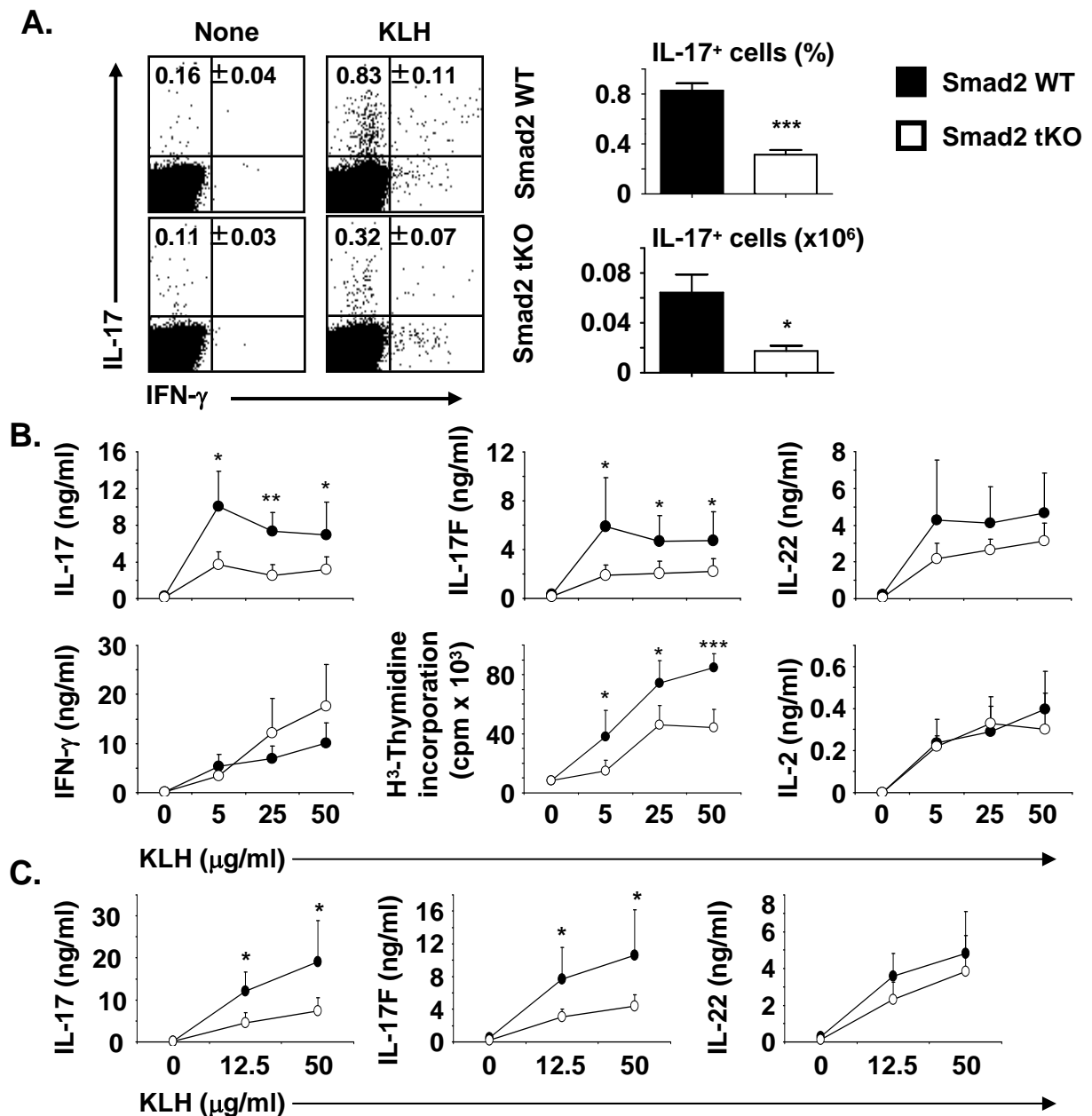


Figure 36. Mice with Smad2 deficiency in T cells have impaired Th17 cell responses *in vivo*. Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) mice were immunized subcutaneously with keyhole limpet haemocyanin (KLH) emulsified in CFA. Seven days after immunization, spleen and draining lymph nodes were harvested. (A) Cells from draining lymph nodes were restimulated with 100 μg/ml KLH protein, or media alone for 16h. Golgi Stop was added during the last 6h of culture, and cytokine expression in CD4⁺ T cells was determined by intracellular staining. A representative dot plot is shown (right), and the combined results (frequency or total cell number) for four mice in each group are indicated (left). Left panel shows mean ± S.D. p values were calculated using Student t test. * *p* < 0.05, *** *p* < 0.001. (B-C) Cells from spleens (B) or draining lymph nodes (C) were isolated and restimulated with increasing concentrations of KLH *ex vivo* for 3 days. Cytokine production was measured from cell-free supernatants by ELISA. Data shows mean ± S.D. p values were calculated using Student t test. * *p* < 0.05, ** *p* < 0.01, *** *p* < 0.001. A representative experiment out of two is shown. This research was originally published in "Smad2 positively regulates the generation of Th17 cells" Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

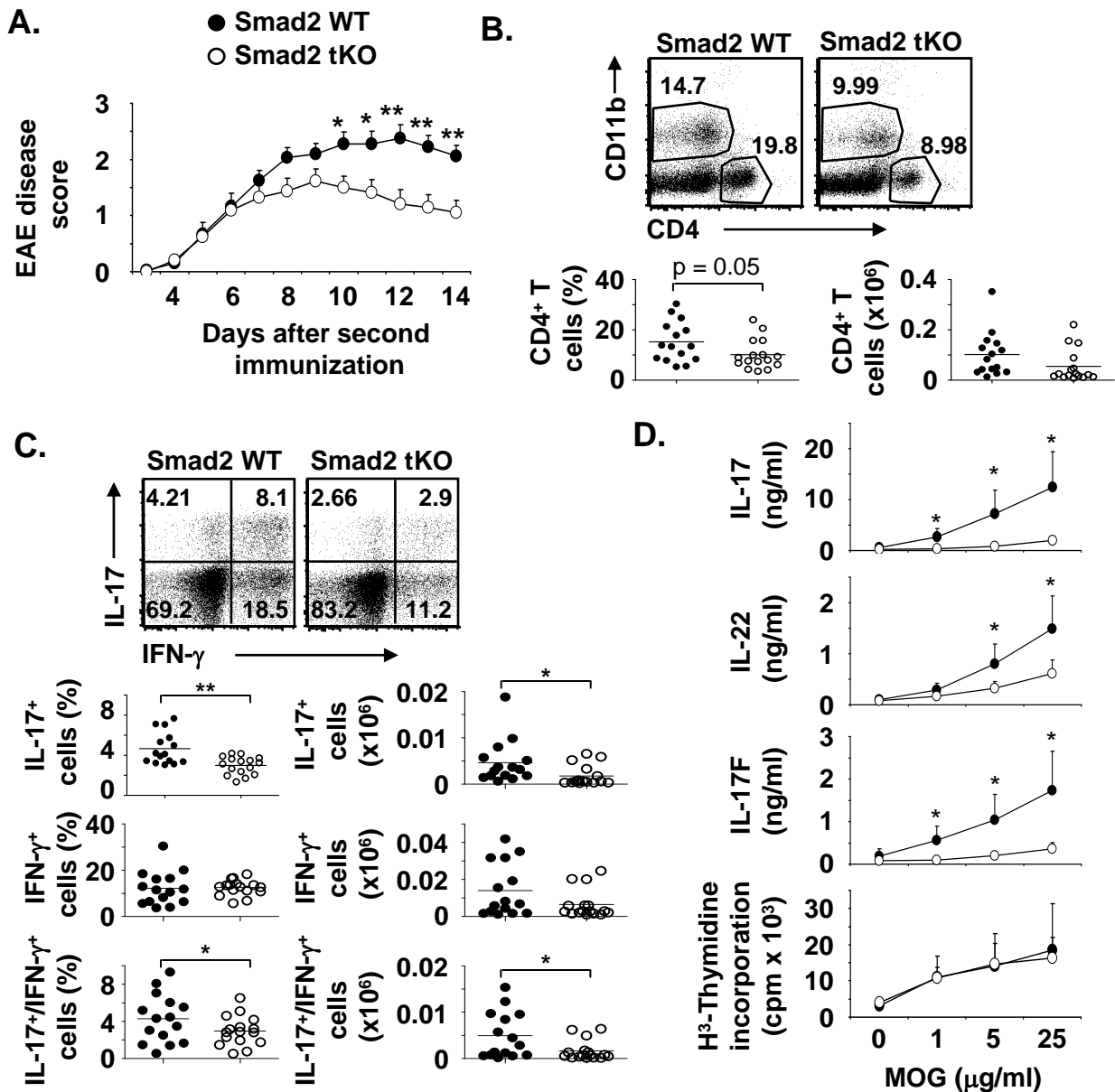


Figure 37. Smad2 deficiency in T cells ameliorates EAE disease development. EAE was induced in Smad2^{fl/fl}CD4Cre⁻ (Smad2 WT) or Smad2^{fl/fl}CD4Cre⁺ (Smad2 tKO) female mice. (A) Disease score (mean \pm SEM) measured after the second MOG immunization (see Materials and methods) combining three independent experiments (Smad2 WT, n=10; Smad2 tKO, n=13). p values were calculated using student t test comparing the disease score within each day. *, $p < 0.05$; **, $p < 0.005$. (B-C) Mononuclear cells infiltrating the central nervous system from the EAE mice were isolated on day 14 after the 2nd immunization and restimulated with PMA, ionomycin and Golgi Stop for 6h. CD4 and CD11b expression was determined (B) and IL-17- or IFN- γ -expressing cells were measured by intracellular staining on a CD4⁺ T cell gate (C). (B-C) A representative dot plot is shown, and the combined results (frequency or total cell number) for each group are indicated. p values were calculated using Student t test. *, $p < 0.05$, **, $p < 0.005$, ***, $p < 0.001$. (D) Splenocytes from the above mice were stimulated with MOG peptide *ex vivo* and cytokine expression levels were measured by ELISA or proliferation by H³-thymidine incorporation. Data shows mean \pm S.D. Data shown is a representative example of three independent experiments with consistent results. p values were calculated using Student t test. *, $p < 0.05$. This research was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

III.3.g. Smad2 binding to ROR γ t results in a synergistic induction of Th17 cells

Given that Th17 cells lacking Smad2 display similar levels of ROR α and ROR γ t transcription factors compared to their WT controls but show reduced cytokine responses, we hypothesized that Smad2 is not necessary for the induction of Th17-associated transcription factors but rather required for their function. Since we observed binding of Smad3 to ROR γ t (Fig. 32A), we evaluated whether Smad2 could also associate with this transcription factor. Indeed, by using a co-immunoprecipitation approach, we found that Smad2 as well as Smad3, but not Smad4, is found in a molecular complex associated with ROR γ t upon co-expression of these molecules in HEK 293T cells (Fig. 38A). Furthermore, we observed that this association was enhanced upon co-expression of a constitutively active form of rat TGF- β type I receptor (TGF β RI T202D) (Feng et al., 2000) (Fig. 38A). Thus, these data indicate that Smad2 and/or Smad3 can bind to complexes containing ROR γ t, and further activation of these molecules by TGF β R signaling can increase such binding.

Smad2 as well as Smad3 can be present in a molecular complex with ROR γ t. However, Smad2 and Smad3 have opposing functions in the generation of Th17 cells. Thus, we investigated the binding affinity of each Smad molecule to ROR γ t by performing a competitive co-immunoprecipitation experiment. We over-expressed constant amounts of Smad2 or Smad3 molecules in the presence of increasing concentrations of the other Smad member, in the presence of ROR γ t. We observed that over-expression of increasing concentrations of Smad2 did not affect Smad3 binding to ROR γ t (Fig. 38B). On the contrary, upon increasing concentrations of Smad3, Smad2 binding to ROR γ t was diminished (Fig. 38B). Thus, these results showed that Smad3 competed with Smad2 for ROR γ t binding, blocking Smad2 binding to ROR γ t. These results then suggest that Smad3 may actually play an indirect inhibitory role in Th17 cell generation by restricting ROR γ t function through the prevention of Smad2 binding.

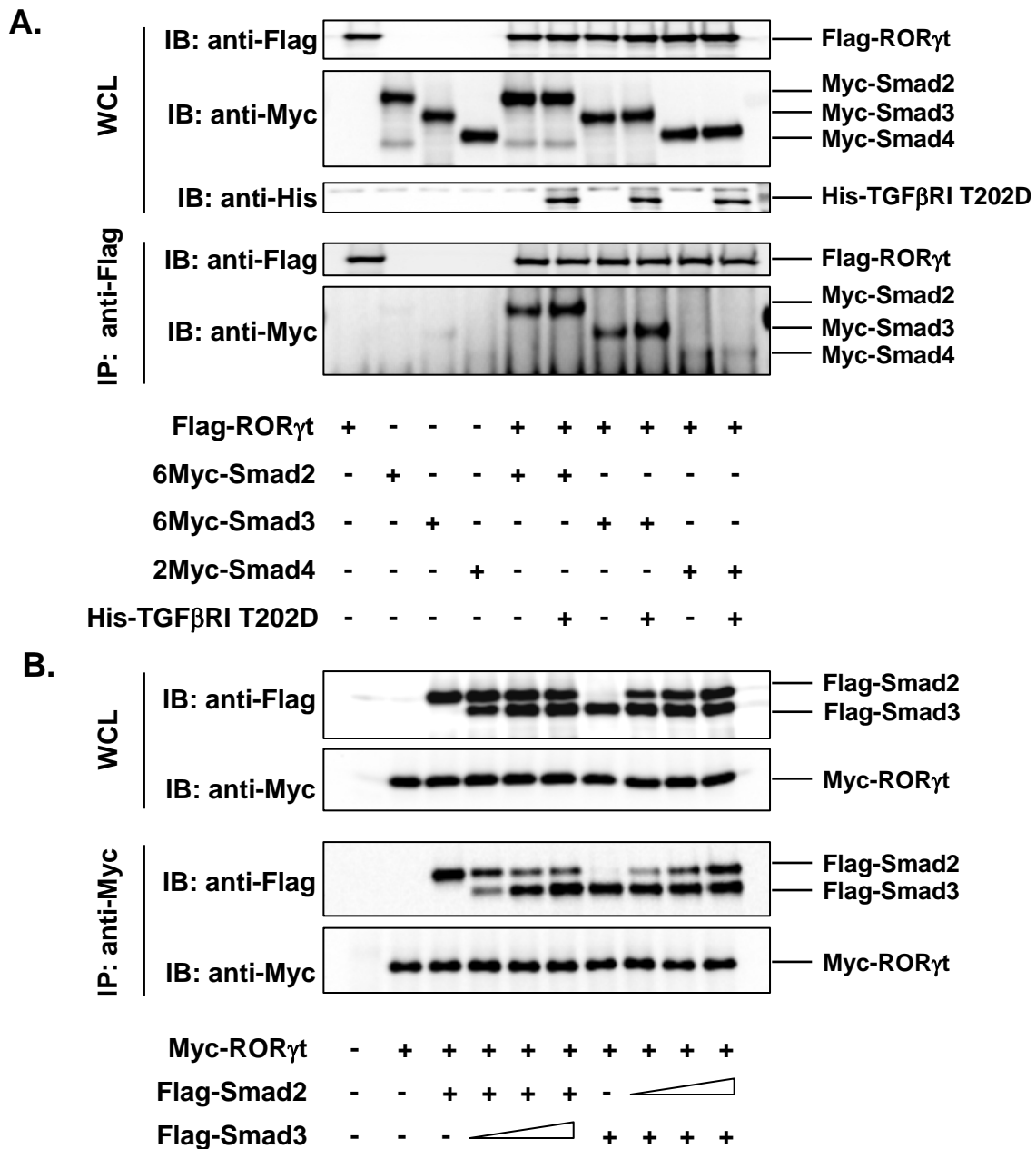


Figure 38. Smad3 inhibits Smad2 binding to ROR γ t. (A) HEK 293T cells were transiently transfected with 6xMyc-tagged Smad3, 6xMyc-tagged Smad2, 2xMyc-tagged Smad4, Flag-tagged ROR γ t and/or His-tagged TGF β RI T202D. After 48h, lysates were prepared and immunoprecipitated with an anti-FLAG mAb (IP), followed by immunoblotting with anti-FLAG or anti-Myc (bottom two panels). Top three panels indicate western blot of whole cell lysates. (B) HEK 293T cells were transiently transfected with Flag-tagged Smad3, Flag-tagged Smad2 and/or cMyc-tagged ROR γ t. Lanes 4-6, constant Smad2 and increasing concentrations of Smad3 plasmid (0.25X, 0.5X and 1X) were transfected. Lanes 8-10, constant Smad3 and increasing concentrations of Smad2 plasmid (0.25X, 0.5X and 1X) were transfected. After 48h, lysates were prepared and immunoprecipitated with an anti-Myc mAb (IP), followed by immunoblotting with anti-FLAG or anti-Myc (bottom two panels). Top two panels indicate western blot of whole cell lysates. The experiments were performed twice with similar results by Dr. Zhang at Baylor College of Medicine. This research was originally published in "Smad2 positively regulates the generation of Th17 cells" Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

Given that Smad2 can be found in a molecular complex together with ROR γ t, and that it has reduced DNA binding capacity, we next hypothesized that Smad2 could act as a coactivator for ROR γ t and therefore synergize with this transcription factor in driving the generation of Th17 cells. To examine this question, we over-expressed ROR γ t and/or constitutively active Smad2 (Smad2-2SD) in T cells by retroviral transduction. The retroviruses also contained IRES-GFP or IRES-hCD2, so that cells that have been infected by both viruses can be recognized based on GFP and hCD2 co-expression. While over-expression of control vectors alone or Smad2 by itself did not induce Th17 cells (Fig. 39A and B), ROR γ t over-expression led to the induction of IL-17-producing cells and increased expression of genes related to a Th17 phenotype (Fig. 39A and B). Interestingly, over-expression of both Smad2-2SD and ROR γ t greatly enhanced the generation of IL-17-producing cells in comparison to ROR γ t over-expression alone (Fig. 39A). To further comprehend the molecular mechanism by which Smad2 could synergize with ROR γ t in the induction of IL-17-producing cells, we next determined the gene expression profile of these cells. We found increased ROR γ t-dependent mRNA expression of IL-17, IL-17F and CCL20 upon co-expression with Smad2-2SD (Fig. 39B). Moreover, endogenous ROR γ expression was augmented upon ROR γ t over-expression, and such induction was even higher when Smad2-2SD and ROR γ t were co-expressed (Fig. 39B and data not shown). However, we did not observe any differences in other Th17-specific transcription factors such as ROR α , BATF and I κ B ζ (Fig. 39B and data not shown). Thus, our data suggest that Smad2 could function as a coactivator for ROR γ t which, in turn, would lead to enhanced Th17 cell generation.

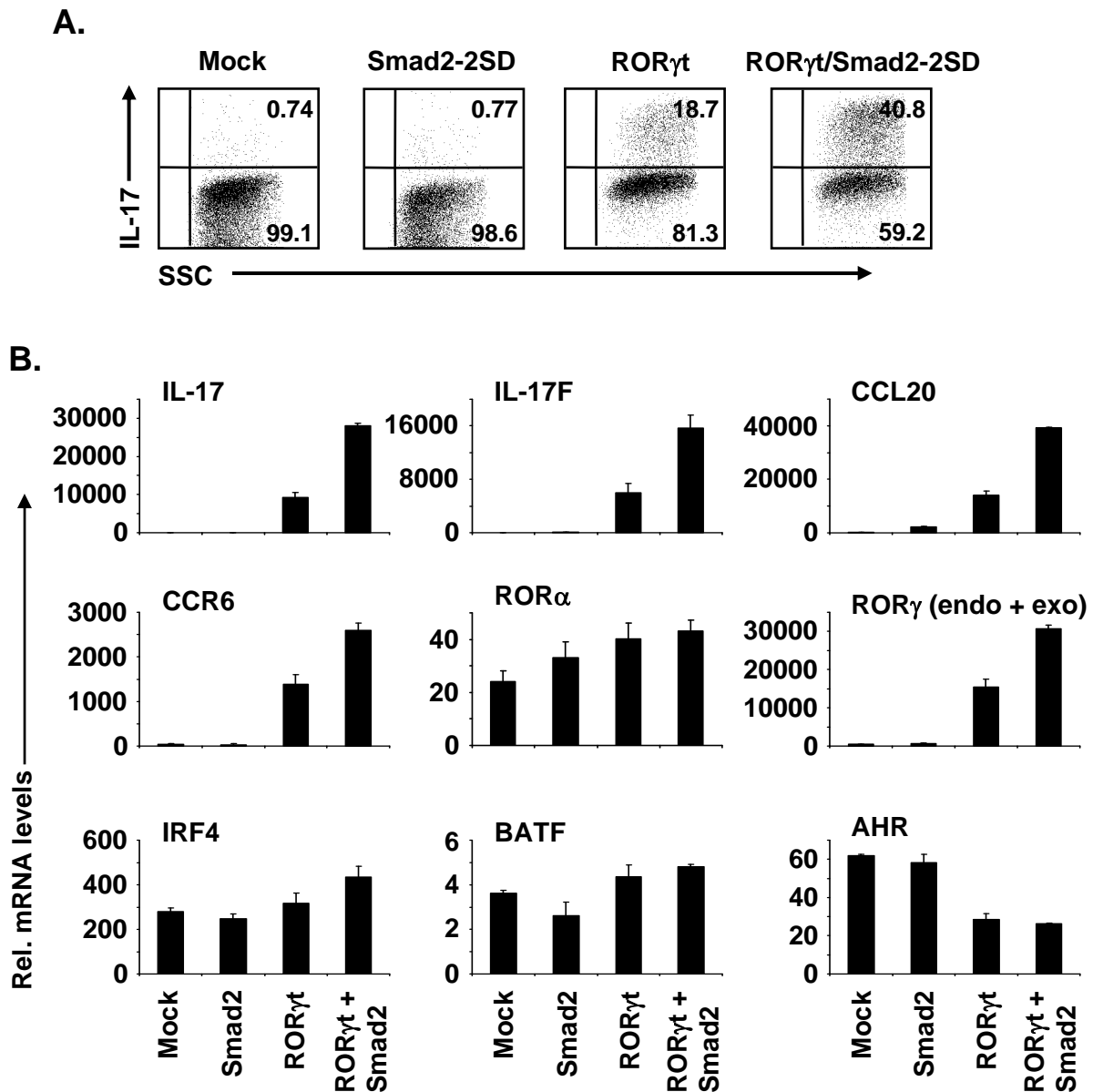


Figure 39. Smad2 synergizes with ROR γ t in the generation of Th17 cells. FACS-sorted naïve OT-II CD4⁺ T cells were activated with OVA peptide-pulsed splenic APCs under neutral (anti-IL-4, anti-IFN- γ and anti-TGF- β) conditions and co-infected with two bicistronic retroviruses (IRES-GFP or IRES-hCD2) expressing ROR γ t-GFP, Smad2-2SD-hCD2 and/or GFP or hCD2 vector controls. After 4d, GFP⁺ hCD2⁺ infected cells were FACS-sorted. (A) GFP⁺ hCD2⁺ cells were stimulated with PMA, ionomycin and Golgi Stop for 4h, and IL-17-producing cells were determined by intracellular staining. (B) GFP⁺ hCD2⁺ cells were re-stimulated for 4h with anti-CD3, and mRNA expression of the indicated genes was analyzed by real time RT-PCR. The data shown were normalized to expression of a reference gene *Actb*. The lowest expression of each gene was referred to as 1, and corresponds to naïve T cells. Data shows mean \pm S.D. The data represents at least three independent experiments with consistent results. This research was originally published in “Smad2 positively regulates the generation of Th17 cells” Gustavo J. Martinez, Zhengmao Zhang, Joseph M. Reynolds, Shinya Tanaka, Yeonseok Chung, Ting Liu, Elizabeth Robertson, Xia Lin, Xin-Hua Feng, and Chen Dong. J. Biol. Chem. 2010 285: 29039-29043. © the American Society for Biochemistry and Molecular Biology.

DISCUSSION

In the present chapter we have investigated the role of TGF- β -induced Smad-dependent signaling pathway in the generation of two T cell lineages with opposing functions in the immune system: Th17 and regulatory T cells. The role of each Smad molecule in the generation of Th17 or regulatory T cells is indicated in Table 1. In this section, we will first discuss the role of this signaling pathway in the regulation of Foxp3 expression and the subsequent generation of regulatory T cells, and then we will discuss the Smad-dependent signaling pathway in the commitment towards Th17 cells.

Role in generation of:	nTreg cells	iTreg cells	Th17 cells
Smad4	Dispensable (no defect in nTreg levels in any tissue analyzed)	Partially required	Dispensable
Smad2	Inhibitory (enhanced nTreg cell levels in thymus and peripheral tissues)	Partially required	Partially required
Smad3	Required in thymus but not in peripheral tissues, suggesting role in generation but not in homeostasis	Partially required	Inhibitory

Table 1. Role of Smad molecules in the generation of regulatory T and Th17 cells. The requirement of each Smad member studies is indicated based on the phenotype observed in cells or animals deficient in each molecule. The effect observed could be direct or indirect.

III.i. Role of the TGF- β signaling pathway in the generation and function of natural regulatory T cells

We first evaluated the role of Smad molecules in the generation of nTreg cells. We found that whereas Smad4 deficiency in T cells does not affect generation or maintenance of nTreg cells, mice with Smad2 deficiency in T cells exhibit enhanced nTreg cells in the thymus and peripheral tissues including spleen, lymph nodes and mesenteric lymph nodes (summarized in Table 1). Takimoto et al. and Malhotra et al. have also recently found enhanced CD4⁺Foxp3⁺ nTreg cells in thymus, spleen and mesenteric lymph nodes of mice lacking Smad2 in T cells as well, confirming our findings (Malhotra and Kang, 2010; Takimoto et al., 2010). These results suggest a mechanism of enhanced generation of regulatory T cells and/or homeostatic proliferation in peripheral tissues in the absence of Smad2. Indeed, TGF β RI conditional KO mice also showed enhanced nTreg levels in the thymus one week after birth due to an increased responsiveness to IL-2 (Liu et al., 2008). Furthermore, Smad2 has been shown to be phosphorylated and translocated into the nuclei of CD4⁺CD8⁻ SP thymocytes, which also co-expressed the TGF β RI (Rosendahl et al., 2003). Thus, these results indicate that Smad2 activation by TGF β RI in thymocytes may allow for enhanced IL-2 responsiveness. Further characterization of mice deficient in Smad2 at different ages and biochemical analysis of thymic regulatory T cells isolated from those mice could help identify the exact role Smad2 plays in Treg cell development. Moreover, to fully comprehend the role of Smad2 in Treg homeostasis, adoptive transfer experiments could be utilized. For this purpose, regulatory T cells from either thymus or peripheral tissues could be isolated and transferred into wild type congenic mice to identify whether these cells can expand, leading to enhanced numbers, compared to adoptive transfer of wild type Treg cells.

We also determined the role of Smad3 in nTreg biology and found that Smad3-deficient mice exhibited reduced CD4⁺CD25⁺Foxp3⁺ T cells in the thymus compared to WT mice, but no significant differences were detected in peripheral tissues. In contrast to our results, Wang and colleagues have found enhanced CD4⁺CD25⁺ T cells in peripheral tissues such as spleen, lymph nodes and thymus in

mice lacking Smad3 (Wang et al., 2006). However, in this report, the authors did not evaluate Foxp3 expression in these animals. Similarly, Takimoto et al. observed enhanced nTreg cells in peripheral tissues including mesenteric lymph nodes and spleens in Smad3 KO mice, while no differences were detected in the thymus (Takimoto et al., 2010). The discrepancies between our data and the other reports could be due to the conditions in which these mice were housed, since all these mice are on C57BL/6 background. It would be important to confirm this statement by exchanging the strains between the laboratories and confirming each phenotype.

More recently, Waisman and colleagues have shown that mice lacking the inhibitory Smad, Smad7, in T cells do not show enhanced generation of natural regulatory T cells (Kleiter et al., 2010). Interestingly, though, mice lacking both Smad2 and Smad3 showed significant reduction in CD4⁺Foxp3⁺ nTreg cells in peripheral tissues but not in the thymus and display an autoimmune phenotype, suggesting that Smad2 and Smad3 have somewhat overlapping roles in the maintenance of natural regulatory T cells *in vivo* (Takimoto et al., 2010). Overall, these results identify the Smad-dependent signaling pathway as an important regulator of nTreg cell homeostasis *in vivo*. Similar results were shown in mice with a T cell-specific deletion of TGF- β RII, which show enhanced nTreg cell generation in the thymus, but reduced peripheral regulatory T cells, demonstrating an important role of TGF- β signaling in nTreg cell homeostasis.

We also investigated the function of natural regulatory T cells in the absence of Smad molecules and found that Smad2, Smad3 or Smad4 are not required for nTreg suppressive function *in vitro*. Other groups have also demonstrated that Smad2-deficient nTreg cells have similar suppressive activity compared to wild type counterparts *in vitro* and *in vivo*, supporting our findings (Malhotra and Kang, 2010; Takimoto et al., 2010). In contrast to our results, Maggio-Price et al. have recently shown that Smad3-deficient nTreg cells lack suppressive function *in vivo*. Recent work has demonstrated that Smad3-deficient mice develop colorectal adenocarcinomas spontaneously (Zhu et al., 1998). However, whether this disease is caused by an inflammatory process induced by T cells still remains unclear. Maggio-Price et al. evaluated this question by using Smad3^{-/-} RAG^{-/-} mice and showed an increase in the incidence of adenocarcinoma in these mice compared to either single KO mice, suggesting that cancer

development as a result of Smad3 deficiency is indeed independent of B and T lymphocytes (Maggio-Price et al., 2009). These mice also exhibited enhanced pro-inflammatory cytokine production following bacterial-induced disease, suggesting a prominent role of Smad3 in innate immune cells. Interestingly, adoptive transfer of WT nTregs prevented disease, while transfer of Smad3-deficient nTregs into Smad3^{-/-}Rag1^{-/-} mice did not restore bacterial-induced cancer in these mice, suggesting that nTregs lacking Smad3 are not functional *in vivo* in this particular model (Maggio-Price et al., 2009). However, the authors have not analyzed the role of Smad3-deficient nTreg cells *in vitro*. Thus, the discrepancy between our results and the findings from Maggio-Price et al. suggest that further research should be performed to fully elucidate the role of Smad3 in Treg cell function in different disease settings. A conditional KO mouse where Smad3 is specifically deleted in regulatory T cells (by utilizing Foxp3-Cre transgenic mice) would also be beneficial to address these questions.

III.ii. Induction of *de novo* Foxp3 expression by naïve T cells

TGF- β plays an important role in the development, maintenance, and induction of regulatory T cells (Chen et al., 2003; Curotto de Lafaille et al., 2008; Fantini et al., 2004). Upregulation of Foxp3 expression in naïve T cells, induced by TGF- β , correlates with the acquisition of a suppressive phenotype in these cells (Hori et al., 2003). We first investigated the role of the TGF- β signaling pathway in the induction of regulatory T cells by using a TGF β RI kinase inhibitor. We found that *in vitro* induction of Foxp3 required TGF- β signaling during the first two days of culture.

When analyzing the role of Smads during iTreg induction, we observed that naïve CD4⁺ T cells lacking Smad2, Smad3 or Smad4 have a partial impairment in the *de novo* expression of Foxp3 transcription factor upon stimulation with TGF- β (Fig. 40, and summarized in Table 1). Similar to our results, several groups have also demonstrated a partial requirement for either Smad2 or Smad3 in iTreg cell generation (Jana et al., 2009; Malhotra and Kang, 2010; Takimoto et al., 2010). These results

suggest that either there may be redundant functions between Smad molecules, or that the TGF- β Smad-independent signaling pathway is also required for activating *Foxp3* gene transcription.

In support of the notion of a redundancy between Smad molecules, Takimoto et al. have recently reported that both Smad2 and Smad3 are partially required for induction of Foxp3⁺ iTreg cells (Takimoto et al., 2010). However, by using Smad2/Smad3 double-deficient T cells, the authors have demonstrated that TGF- β induction of Foxp3 is completely abrogated, suggesting a synergistic and overlapping role of these two molecules in Foxp3 induction (Takimoto et al., 2010). Consistent with these reports and our results, T cells deficient in Smad7 (an inhibitory Smad molecule) showed enhanced commitment towards the generation of iTreg cells, whereas cells overexpressing Smad7 have impaired induction of Foxp3 expression (Kleider et al., 2010).

Smad3 has been shown to synergize with NFAT in the induction of Foxp3 expression upon TGF- β and TCR stimulation by binding to an enhancer element in *Foxp3* gene (Tone et al., 2008; Zheng et al., 2010). Rudensky and colleagues demonstrated the importance of this enhancer element, termed conserved non-coding sequence 1 (CNS1), as deletion of this cis-acting sequence prevented the generation of iTreg cells, but was dispensable for the induction of thymus-derived regulatory T cells (Zheng et al., 2010). However, these results are inconsistent with the fact that Smad2/Smad3 double deficient mice show defective nTreg peripheral maintenance (Takimoto et al., 2010). Thus, the exact mechanism by which Smads regulate Foxp3 expression *in vivo* remains to be investigated. One possible explanation would be that other unidentified cis-regulatory elements containing Smad-binding sites are required for Smads-dependent Foxp3 maintenance. It is also plausible that Smad molecules bind to other transcription factors, and regulate Foxp3 expression independently of their DNA binding. Finally, we cannot rule out an indirect regulation of Foxp3 expression by Smads, in which Smad complexes would directly regulate the expression of other transcription factors which would then bind to regulatory elements in *Foxp3* gene.

In support of the idea of a TGF- β -induced Smad-independent signaling pathway controlling Foxp3 expression, Huber et al. have suggested that p38 MAPK signaling is crucial for induction of

Foxp3 expression (Huber et al., 2008), whereas Lee and colleagues have proposed that TGF- β -mediated inactivation of Erk is a requirement for Foxp3 induction (Luo et al., 2008). However, recently Lu et al. have suggested that Erk activation is needed for generation of iTreg cells, while p38 and JNK play no role (Lu et al., 2010). Furthermore, this group also proposed that neither Smad2 nor Smad3 play a significant role in the induction of Foxp3 expression by naïve T cells (Lu et al., 2010). However, in this report the authors did not show evidence of full deletion of either Smad2 or Smad3 in T cells, possibly indicating that the lack of deficiency in Foxp3 expression might be due to residual levels of Smad proteins (Lu et al., 2010).

Overall, our results together with recent publications suggest that the Smad-dependent signaling pathway is indispensable for the induction of *de novo* transcription of *Foxp3* gene and maintenance of Foxp3 expression in nTreg cells (Fig. 40). Smad3 has been shown to directly bind to Foxp3 CNS1 region (Tone et al., 2008). Even though Smad2 and Smad4 are also required for *de novo* induction of Foxp3 expression, their direct association to this cis-acting regulatory region needs further investigation (Fig. 40). Furthermore, it is still plausible that the Smad-independent signaling pathways also collaborate with the Smad-dependent pathway for optimal Foxp3 expression. Indeed, several reports have suggested a crosstalk between MAPKs and Smads signaling pathways (Burch et al., 2010; Carta et al., 2009; Jiang et al., 2010; Li et al., 2009; Li et al., 2010). Further work using mice deficient in both signaling pathways could help address the remaining controversies in the field. However, since the MAPK signaling pathway is also required by costimulatory and TCR signals, understanding their exact contribution in Treg cell generation or maintenance will be a major challenge. One possible way of studying the MAPK-dependent *versus* the Smad-dependent contribution induced by TGF- β would be to utilize cells with a TGF β R that has a mutation either in the domain that is required for MAPK activation or in the L45 loop required for Smads activation (Yu et al., 2002). By utilizing knock-in mice with such mutations, one could separately study the contribution of each signaling pathway induced by TGF- β in the induction or maintenance of Foxp3 expression.

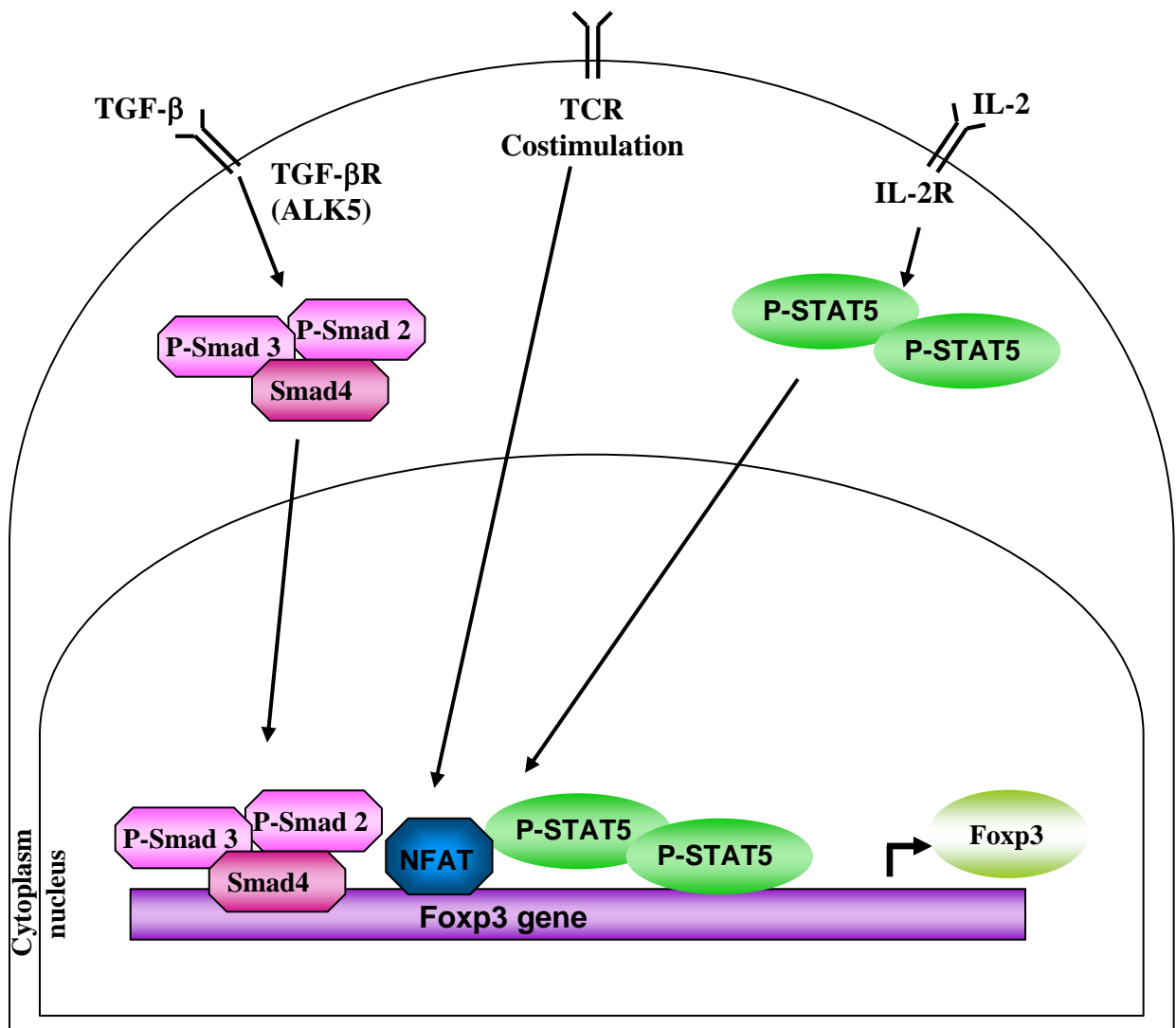


Figure 40. Summary of the role of TGF- β signaling in induction of Foxp3 expression. TGF- β induces activation of Smad2 and Smad3 (phosphorylation), which then bind to Smad4 and translocate to the nucleus. Smad3 directly binds to Foxp3 CNS1 region and synergizes with NFAT in induction of Foxp3 (Tone et al. 2008). We found that Smad2, Smad3 and Smad4 are partially required for TGF- β -induced Foxp3 expression. Also, deficiency in Smad2 and Smad3 completely abrogates Foxp3 expression (Takimoto et al. 2010), confirming our results on the role of the Smad-dependent signaling pathway in the generation of iTreg cells.

III.iii. Role of the Smad-dependent signaling pathway in Th17 cell generation

TGF- β has also been shown to be required for the induction of Th17 cells (Veldhoen et al., 2006a). In fact, mice with TGF β RI-deficient T cells are unable to generate Th17 cells *in vivo* and thus fail to develop EAE, albeit the fact that Th1 cells infiltrating the CNS were still detected (Veldhoen et al., 2006b). We have also confirmed the role of TGF β RI requirement *in vitro* by demonstrating that inhibition of TGF β RI kinase activity also impairs the induction of Th17 cells. Thus, these results suggest that active TGF- β signaling is indeed required for induction of these inflammatory T cells.

It has been suggested that TGF- β primarily inhibits Th1 and Th2 differentiation rather than directly inducing the commitment towards Th17 cells (Das et al., 2009; Santarlasci et al., 2009; Yang et al., 2008d). While we agree that the TGF- β -induced Smad-dependent pathway is not required for Th17 cells commitment (Smad-deficient cells do not have defect in the expression of Th17-associated transcription factors), we still believe that TGF- β signaling is required for full commitment towards Th17 cells generation since inhibition of TGF β RI kinase activity also results in complete inhibition of ROR γ t expression. Das and colleagues have shown that cells lacking STAT6 and T-bet, which are unable to differentiate into Th1 or Th2 cells, only require IL-6 to become IL-17-producing cells. Furthermore, when using the EAE model, they observed that STAT6^{-/-}T-bet^{-/-} mice were highly susceptible to EAE disease development (Das et al., 2009). Moreover, they showed that blocking endogenous TGF- β *in vitro* still led to the generation of Th17 cells (Das et al., 2009). Yang et al. have also demonstrated that cells lacking T-bet and STAT6 have a lower TGF- β threshold required for Th17 cell generation, and that IL-6 alone can promote the differentiation into IL-17-producing T cells. However, IL-6-induced Th17 cells do not express IL-10 and are more pathogenic than TGF- β /IL-6-generated Th17 cells in a colitis model. Furthermore, whereas Das and colleagues showed no significant differences in the generation of Th17 cells between IL-6 and IL-6/TGF- β stimulation conditions, Ding and colleagues observed enhanced Th17 commitment upon addition of TGF- β (Das et al., 2009; Yang et al., 2008d). Thus, these reports suggest that even though TGF- β can play an

important role in inhibiting Th1 and Th2 generation, it is still required for optimal Th17 cell induction *in vitro* and *in vivo* (Veldhoen et al., 2006b).

We examined the TGF- β -induced Smad-dependent signaling pathway in the induction of Th17 cells and found that although Smad4 was dispensable, Smad2 was required for their generation (summarized in Table 1). Recently Takimoto et al. and Malhotra et al. have also shown that Smad2-deficient T cells have impaired Th17 cell development *in vitro*. Furthermore, using two *in vivo* disease models where Th17 cells have been shown to play crucial roles, they found reduced EAE disease development and enhanced *Citrobacter rodentium*-induced weight loss, respectively (Malhotra and Kang, 2010; Takimoto et al., 2010). At the molecular level, there are some discrepancies as to what role Smad2 plays. Takimoto et al. suggest that the reduction in Th17 generation is due to enhanced IL-2 and IFN- γ production in the absence of Smad2 (Takimoto et al., 2010). On the other hand, Malhotra et al. found that Smad2-deficient Th17 cells have reduced IL-6R and thus reduced STAT3 activation (Malhotra and Kang, 2010). We have failed to detect any enhanced T-bet, GATA-3, IFN- γ , IL-4, or reduced IL-6R expression (data not shown) in Th17 cells lacking Smad2, which suggests to us that Smad2 plays a direct role in the induction of several Th17-specific genes rather than acting to prevent Th1 or Th2 cell generation. Indeed, a constitutively active form of Smad2 cooperated in the ROR γ t-induced Th17 cell generation.

On the other hand, we found that cells lacking Smad3 had enhanced induction of Th17 cells, suggesting that Smad molecules have differential requirements for Th17 cell generation, contrary to their role in Foxp3 induction. Previous work has demonstrated that Smad3-deficient mice have enhanced pro-inflammatory cytokines compared to WT mice in a model of contact hypersensitivity induced by oxazolone (Anthoni et al., 2008). Anthoni et al. also found that Smad3-deficient mice showed higher IL-17 mRNA levels compared to WT mice upon oxazolone treatment. However, given that these mice also had higher TGF- β , IL-6, IL-1 and TNF- α levels in inflamed skin, the authors could not rule out the possibility that enhanced IL-17 production was due to an increase in Th17-promoting factors. By contrast, we have shown that Smad3 deficiency in T cells intrinsically led to enhanced Th17

cell differentiation. Consistent with this idea, other groups have reported that retinoic acid, important in inducing Foxp3 expression and inhibiting Th17 generation, enhances Smad3 protein expression as well as its phosphorylation levels. This enhanced Smad3 activation in turn leads to increased Foxp3 expression and reduced IL-6R, IL-23R and IRF4 expression, in a Stat3- and Stat5-independent manner (Elias et al., 2008; Xiao et al., 2008), leading to inhibition of Th17 cell development.

We have observed that both Smad2 and Smad3 can be found in a molecular complex associated with ROR γ t. We then determined the relative affinity of each molecule to ROR γ t and found that Smad3 inhibits Smad2 binding to ROR γ t, suggesting that it might play an indirect inhibitory role in Th17 cell generation (Fig. 41). Even though it is widely accepted that TGF β R induces similar phosphorylation levels of Smad2 and Smad3, Heikkinen et al. have recently demonstrated that the Protein Phosphatase 2A (PP2A) differentially regulates Smad2 and Smad3 phosphorylation by directly dephosphorylating Smad3 under hypoxic conditions (Heikkinen et al., 2010). Furthermore, Tu et al. have also shown that Smad2 and Smad3 molecules can be distinctively acetylated which results in an enhanced nuclear retention of Smad2 over Smad3 (Tu and Luo, 2007). Thus, these studies suggest that even though TGF β R similarly phosphorylates Smad2 and Smad3, their activation status may rely independently on other molecules/pathways as well. It is plausible that developing Th17 cells utilize some mechanism to guarantee an enhanced Smad2/Smad3 activation ratio that would then allow for full commitment to this T helper lineage. However, it still remains to be determined whether Th17 cells have differential activation of Smad2 over Smad3. For this purpose, future work evaluating the nuclear translocation, protein stability and/or post-translational modifications of Smad molecules in Th17 cells throughout their development needs to be performed.

In contrast to our results, Lu et al. have recently suggested that neither Smad2 nor Smad3 play a direct role in the induction of Th17 cells (Lu et al., 2010). Furthermore, the authors showed normal EAE disease development in the absence of Smad3 (Lu et al., 2010). The authors also investigated the MAPK signaling pathway required for generation of Th17 cells, and found that p38 and JNK inhibition led to reduced differentiation into IL-17-producing cells (Lu et al., 2010). Indeed, by using a p38

inhibitor *in vivo* they found reduced EAE disease severity, suggesting that p38 might be important in the induction of pathogenic T cells (Lu et al., 2010). However, given that TCR signaling also induces MAPK activation, and the crosstalk between MAPK and Smad pathways, the direct role of TGF- β induced MAPK activation during Th17 differentiation still remains controversial.

We have observed that Smad-deficient T cells exhibited no defect in the expression of Th17-associated transcription factors (Fig. 41). Consistent with our results, Yoshimura and colleagues have recently demonstrated that Smad3, Smad2 or double-deficient T cells showed no significant differences in the expression of ROR α and ROR γ t compared to WT controls, suggesting a Smad-independent induction of these transcription factors (Takimoto et al., 2010) (Fig. 41). Thus, these results indicate that the TGF- β -induced Smad-dependent signaling pathway is not required for the commitment towards Th17 cell generation, but rather is necessary for the induction of cytokines or chemokines produced by these cells. However, by using TGF β RI-specific kinase inhibitor, we found that the generation of IL-17-producing cells and the induction of the ROR γ t transcription factor was impaired, suggesting that even though the Smad-dependent signaling pathway might be dispensable for Th17 cell commitment, TGF- β signaling is still required. Thus, how TGF- β directly regulates the induction of such transcription factors, and whether other unidentified signaling pathways are required for the *de novo* expression of Th17-associated transcription factors need further investigation. For example, agonistic ligation of PPAR γ (Peroxisome Proliferator-Activated Receptor gamma) leads to a selective inhibition of Th17 cell generation by interfering with ROR γ t expression through the impaired removal of the nuclear corepressor SMRT (silencing mediator of retinoid and thyroid receptors) from ROR γ t promoter. However, in the absence of PPAR γ agonists and upon TGF- β and IL-6 stimulation, the nuclear corepressor SMRT complex is displaced from ROR γ t promoter allowing for the opening of the chromatin structure and transcription of this gene (Klotz et al., 2009). Additionally, the MAP3K NIK was shown to be required for induction of Th17 cells and ROR γ t expression (Jin et al., 2009). NIK is responsible for the synergistic activation of STAT3 by TCR and IL-6R signaling (Jin et al., 2009), and several investigations have shown that STAT3 is required for induction of ROR α and ROR γ t (Chen et

al., 2006b; Yang et al., 2007). Therefore, it still remains plausible that TGF- β and IL-6 signaling pathways cooperate to induce Th17-associated transcription factors. In fact, STAT3 has been shown to bind to the TGF- β promoter, inducing TGF- β transcription (Kinjyo et al., 2006). Furthermore, TGF- β also cooperates with the STAT3 signaling pathway by inhibiting IL-6- and IL-21-induced SOCS3 expression (Qin et al., 2009). Thus, TGF- β might prolong STAT3 activation which in turn would favor Th17 cell development. Whether this documented TGF- β inhibition of SOCS3 expression is Smad-dependent still remains unclear, but recent evidence suggests that Smad2 might be important for sustaining STAT3 activation (Malhotra and Kang, 2010).

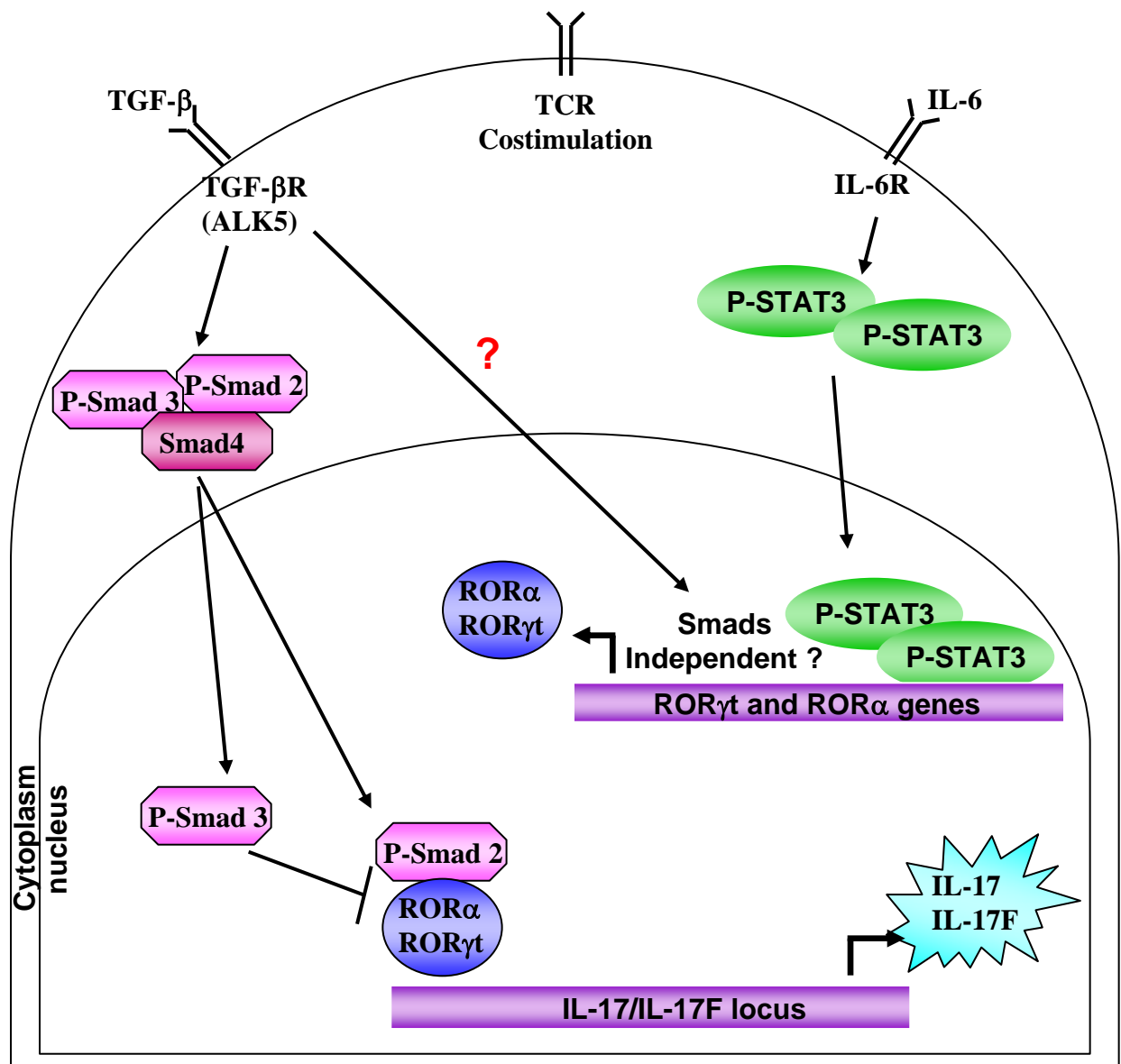


Figure 41. Summary of the role of TGF- β signaling in the generation of Th17 cells. TGF- β induces activation of Smad2 and Smad3 (phosphorylation), which then bind to Smad4 and translocate to the nucleus. Smad2, Smad3 or Smad4 are not directly required to induce expression of the Th17-associated transcription factors ROR α and ROR γ t, while IL-6-induced STAT3 activation is. How TGF- β regulates ROR α or ROR γ t expression needs further understanding. Smad2 directly binds to ROR γ t, the master transcription factor for Th17 cells, cooperating in ROR γ t-induced IL-17 and IL-17F expression. On the contrary, Smad3 inhibits Th17 cell generation by interfering with Smad2 binding to ROR γ t, among other possible mechanisms.

In summary, we have found that Smad molecules differentially regulate the generation of iTreg and Th17 cells. Our results are important in understanding the reciprocal regulation of these two cell lineages with opposing functions in the immune system, and may allow for the development of novel immunotherapies to target these cell types *in vivo*.

CHAPTER IV – GENERAL DISCUSSION AND FUTURE DIRECTIONS

Naïve CD4⁺ T helper cells can differentiate into different effector subsets as previously described in Chapter I. Herein, we have focused on the transcriptional regulation of Th17 and regulatory T cells, two T helper lineages with opposing functions in the immune system. We analyzed the reciprocal generation of these cells by investigating the role of the Treg-associated master transcription factor Foxp3 in Th17 cell development. We found that Foxp3 inhibits Th17 cell generation by suppressing the transcriptional activity of the two main Th17-associated transcription factors, nuclear receptors ROR α and ROR γ t. At the molecular level, we identified two different functional domains in Foxp3 required for such inhibition: the LxxLL sequence in exon 2 and the TIP60/HDAC7 binding domain. These domains could be crucial for preventing the association of the coactivators to nuclear receptors ROR α or ROR γ t (through LxxLL sequence) and/or for the recruitment of histone deacetylases to ROR-target genes (through the HDAC7 binding domain) (Chapter II). Additionally, we investigated the role of TGF- β , a common cytokine required for the generation of the Th17 and regulatory T cell lineages. We identified a differential signaling pathway induced by TGF- β in the generation of Th17 and regulatory T cells. We observed that while Smad2, Smad3 and Smad4 are required for TGF- β -induced Foxp3 expression and the subsequent development of inducible regulatory T cells, only Smad2 is indispensable for the induction of IL-17-producing T cells. Interestingly, we determined that Smad2 is capable of enhancing ROR γ t-induced Th17 cell generation, while Smad3 inhibits Th17 cell generation by preventing the binding of Smad2 to ROR γ t (Chapter III). In this chapter, we will relate our results to recent reports on the transcriptional regulation of these two Th lineages, as well as the heterogeneity and plasticity of T helper subsets.

IV.1. The roles of TGF- β and its signaling pathway in inducing Th responses

TGF- β can control the generation of inducible regulatory T cells (Chen et al., 2003). In the present work, we demonstrated that the Smad-dependent signaling pathway is crucial for the induction of Foxp3 expression in naïve T cells. In support of our findings, Takimoto et al. have also demonstrated that deficiency in both Smad2 and Smad3 leads to a complete inhibition of Foxp3 expression (Takimoto et al., 2010). TGF- β -induced Smad-independent signaling pathways may also be involved in the generation of inducible regulatory T cells, primarily through activation of MAPK. However, the role of these kinases in the TGF- β -induced *Foxp3* transcription remains controversial (Huber et al., 2008; Lu et al., 2010; Luo et al., 2008). Interestingly, while it is clear that Smads directly control Foxp3 expression (Chapter III and (Takimoto et al., 2010; Tone et al., 2008)), it has been recently shown that other Fox-family proteins, specifically Foxo1 and Foxo3, can directly regulate Foxp3 expression as deficiency of both molecules also abrogates the induction of Foxp3 (Kerdiles et al., 2010; Ouyang et al., 2010). Cells deficient in both Foxo proteins showed no defect in phosphorylation of Smads (Kerdiles et al., 2010), suggesting that activation of Smads is required but not sufficient for proper induction of Foxp3 expression. Foxo1 and Foxo3 have been shown to directly bind the MH1 domains of Smad3 and Smad4, but not Smad2 or Smad1, and regulate TGF- β -dependent induction of p21Cip1 (Seoane et al., 2004). Thus, based on this previous report, a similar synergism between Smad and Foxo proteins might be required for optimal transcription of *Foxp3* gene. To address this issue, sequential chromatin immunoprecipitation experiments assessing the binding of Smads and Foxo proteins in the same *Foxp3* promoter/enhancer regions should be performed. Moreover, overexpression of Foxo proteins in Smad-deficient cells should determine whether Foxo proteins require the presence of Smad molecules for directly inducing Foxp3 expression.

Several conserved non-coding sequences (CNS) containing different epigenetic marks in a cell-type specific manner have been recently identified in *Foxp3* gene (Zheng et al., 2010). The exact combination of different transcription factors and chromatin remodeling complexes that bind to these

CNS regions still remains unclear. For instance, Smad3 has been shown to synergize with NFAT in *Foxp3* transcription by directly binding to one of such CNS regions (Tone et al., 2008). Whether Smad3 and NFAT require an “opened” chromatin structure to bind to their target sequences is currently unknown. Furthermore, the sequential events in the binding of Smad3, NFAT and other transcription factors to *Foxp3* locus are still poorly understood. Thus, analysis of epigenetic changes in several transcription factor-deficient Treg cells (using either Treg-specific conditional or germline knock-out mice) will help in our understanding of which transcription factors might act as pioneer transcription factors by recruiting chromatin remodeling complexes, and which transcription factors require an open chromatin structure prior to their binding to *Foxp3* gene.

TGF- β is also important for the generation of Th17 cells (Bettelli et al., 2006; Veldhoen et al., 2006a). However, the contribution of the TGF- β signaling pathway in the differentiation of Th17 cells is still not completely understood. We and others have shown that Smad2 is vital for the proper induction of IL-17-producing cells *in vitro* and *in vivo* (Chapter III and (Malhotra and Kang, 2010; Takimoto et al., 2010)). Interestingly, neither of the Smad molecules analyzed were required for the expression of Th17-associated transcription factors ROR α , ROR γ t, BATF, I κ B ζ , among others (Chapter III and (Malhotra and Kang, 2010; Takimoto et al., 2010)). While we found that Smad2 synergizes with ROR γ t in the generation of IL-17-producing T cells, Malhotra et al. suggest that Smad2 controls IL-6R expression through STAT3 activation (Malhotra and Kang, 2010). By contrast, Yoshimura and colleagues argue that the role of Smad2 is simply to downregulate inhibitory cytokines such as IL-2 and IFN- γ (Takimoto et al., 2010). Furthermore, even though TGF- β signaling pathway is crucial for the induction of ROR γ t expression, as assessed by inhibition of TGF β RI kinase activity (Chapter III and (Ivanov et al., 2006)), the precise mechanism by which this occurs remains unknown. It is plausible that the Smad-independent signaling pathway, in synergism with IL-6 signaling, contributes to the up-regulation of ROR γ t and other Th17-associated transcription factors. However, most of the molecules activated by TGF- β from the Smad-independent pathway (Figure 14) can also be activated by costimulatory molecules, cytokines and TCR signaling. Thus, understanding the direct contribution of

the Smad-independent pathway in a TGF- β -dependent manner will be arduous since these cells also require TCR and costimulatory signals for their commitment. One possible way of addressing this point, as previously mentioned, would be the utilization of cells with different mutations in TGF β R so that only the Smad-dependent or the MAPK pathways can be induced upon TGF- β stimulation. For instance, it has been previously demonstrated that mutation in L45 loop in TGF β RI prevents Smads phosphorylation without affecting the activation of MAPKs (Yu et al., 2002). Thus, in order to address the contribution of each signaling pathway in the generation of Th17 cells, we can either utilize mice with a knock-in mutation in L45 loop, or with mutations in the domain required for activation of MAPK.

In addition to Th17 and Treg cells, TGF- β has been more recently linked to the generation of other Th lineages such as Tr1 and Th9 cells. Tr1 cells, characterized by the production of IL-10, can be generated in the presence of TGF- β and IL-27 (Awasthi et al., 2007). These cells, which depend on cMaf and AHR expression for their generation, are believed to play important roles in tolerance (Apetoh et al., 2010). By contrast, Th9 or IL-9-producing T helper cells require TGF- β and IL-4 (Dardalhon et al., 2008; Veldhoen et al., 2008b). More recently it has also been proposed that TGF- β together with IL-1-family members can also induce IL-9 expression in an IL-4-independent fashion (Uyttenhove et al., 2010) or that TGF- β in combination with IL-25 can drive IL-9 expression in T cells expressing the IL-25 receptor, IL-17RB (Angkasekwinai et al., 2010). Th9 cells depend on PU.1 and IRF4 transcription factors for their generation (Chang et al., 2010; Staudt et al., 2010), two transcription factors that have been also shown to regulate Th2 cell differentiation (Chang et al., 2005; Lohoff et al., 2002; Rengarajan et al., 2002). IL-10, originally identified as a Th2-specific cytokine, has later been shown to be produced by other Th subsets including Th1, Th17, Th9 and regulatory T cells (reviewed in (Saraiva and O'Garra, 2010)). Similarly, IL-9 is produced by Th2, Th17 and Treg cells (Beriou et al., 2010; Eller et al., 2011; Nowak et al., 2009) and can further regulate both Th17 and Treg cells (Elyaman et al., 2009). Because of the production of these cytokines by other Th subsets, the definition of Tr1 and Th9 cells as independent Th lineages is debatable. Regardless of whether these cells are indeed an independent lineage, it is clear that TGF- β is important for the instruction of these cytokine-producing

cells, or as suggested by several groups, for the inhibition of the generation of alternative T cell lineages (primarily Th1 and Th2 cell differentiation). Even though the molecules downstream of TGF- β required for the generation of these cytokine-producing T cells have not been identified, we believe that the TGF- β -induced Smad-dependent signaling pathway could potentially regulate the induction of these cytokine-producing cells. Thus, this lays the foundations for new exciting investigation on the role of the TGF- β signaling pathway, in combination with other cytokine-induced signaling pathways, in the regulation of several cytokine-producing Th cells.

When we analyzed the role of the Smad-dependent signaling pathway in the generation of the reciprocally regulated Th17/Treg cells, we observed a differential requirement of Smad molecules in the commitment towards these opposing cell lineages. While Smad4 showed a partial requirement in the generation of inducible regulatory T cells, it was dispensable for the induction of IL-17-producing T cells *in vitro* and *in vivo*. On the other hand, Smad2 was required for both Th17 and iTreg cell development while Smad3 was only necessary for iTreg generation. Recent reports have shown that Smad2 and Smad3 can form complexes with other molecules besides the common Smad, Smad4. For instance, TIF1 γ (Transcriptional Intermediary Factor 1 gamma, also termed Trim33) as well as IKK α (IkappaB kinase alpha) can replace Smad4 and associate in molecular complexes with Smad2 and Smad3 (Descargues et al., 2008; He et al., 2006). Furthermore, it was suggested that TIF1 γ and Smad4 may have differential roles during TGF- β -dependent invariant NKT cell development (Doisne et al., 2009). Thus, since Th17 cell generation requires Smad2 but not Smad4, it is plausible that TIF1 γ and/or IKK α , both of which are expressed in T cells, might regulate their development. Indeed, recently it has been shown that IKK α has an important role in the generation of IL-17-producing CD4⁺ T cells in an NF- κ B-independent manner (Li et al., 2011). Similar to what we have observed in Smads-deficient T cells, IKK α deficiency does not impact the induction of the Th17-associated transcription factors ROR α or ROR γ t, but rather dramatically affects the expression of IL-17 but not IL-17F (Li et al., 2011). While the authors did not evaluate the putative association of IKK α with Smad molecules, in particular Smad2, it is plausible that it regulates IL-17 production by complexing with this molecule. Future research

evaluating the molecular complexes found bound to *il17* and *il17F* locus will help understand the transcriptional regulation of these cytokines. Also, whether Smad2 or Smad3 differentially bind to other signaling molecules particularly in Th17 cells remains poorly understood. Thus, evaluating the different complexes containing Smad2 and/or Smad3 during Th17 cell development might provide insight into the exact contribution of each molecule in the generation of this Th lineage. Genome-wide chromatin immunoprecipitation experiments can also be crucial for better understanding the genes that are differentially regulated by these two signaling molecules.

Smad3 is directly required for induction of Foxp3 expression, while it inhibits the generation of IL-17-producing cells. Since enhanced levels of regulatory T cells have been shown to occur in some models of cancer, while Th17 cells showed a protective role in melanoma mouse models, from a therapeutic point of view, Smad3 could be an interesting target in some cancers. Thus, one would expect that Smad3 inhibition would result in a reduction of regulatory T cells and a reciprocal increase in the generation of IL-17-producing Th cells. One caveat for the targeting of Smad3 is that TGF- β is required in several biological processes, and thus the development of a therapy where Smad3 is only targeted in T cells would be desirable. Specific Smad3-inhibitors have already been described (Jinnin et al., 2006; Liu et al., 2006), so the major challenge would be to develop a strategy to specifically deliver these compounds to T cells without affecting other cell types in the body.

IV.2. Plasticity and heterogeneity of T helper cells: How can we adequately define Th subsets?

As widely discussed in Chapter I, Th subsets produce different cytokines, which dictate their immune regulatory functions. However, at the single cell level, a particular Th subset rarely produces all of its associated cytokines at the same time. For instance, only a limited amount of Th1 cells can produce IFN- γ , IL-2, TNF- α and LT α at the same time (Darrah et al., 2007). Additionally, Th1 cells can produce IL-10 under certain conditions, but again not every Th1 cell produces IL-10. The same is true for Th2 cells where IL-4, IL-5 and IL-13 are differentially regulated and not always produced in the same cell, even though their genes are present in the same locus in mouse chromosome 11 (Tanaka et al., 2011). Th17 cells are not the exception either since the genes for the cytokines IL-17 and IL-17F, which are also adjacent to one another in a tail-to-tail configuration, are differentially regulated (data not shown and (Gomez-Rodriguez et al., 2009)) and IL-17- or IL-17F-single producing cells are readily detected (Chung et al., 2009; Gomez-Rodriguez et al., 2009; Yang et al., 2008a). Also, expression of the Th17-associated cytokine IL-22 seems to be independent of IL-17 or IL-17F and indeed it has been suggested that IL-22-producing Th cells or Th22 cells might represent an independent Th lineage in the human system (Eyerich et al., 2009; Fujita et al., 2009). Thus, these reports indicate that the Th cell lineages can be more heterogenous than previously thought, and a more careful and accurate description of the cells under study should be considered.

The expression of Th-associated transcription factors at the population level as a distinguishing cell-intrinsic characteristic is also unclear. For instance, PU.1 expression is augmented in Th2 cells with low expression of IL-4 (Chang et al., 2005), but PU.1 is also indispensable for the generation of IL-9-producing T cells (Th9 cells) (Chang et al., 2010; Staudt et al., 2010). Moreover, in Th17 cells, the expression of ROR α and ROR γ t leads to the induction of Th17-associated cytokines. However, not all ROR γ t⁺ or ROR α ⁺ cells express IL-17 (Ivanov et al., 2006; Yang et al., 2008c). Furthermore, while Th17 cells express ROR γ t and AHR, the relationship between these cells and IL-17 or IL-22 production

remains unclear. Also, IRF4, which is required for the development of Th2 and Th9 cells, can also regulate the generation of Th17 cells. These reports indicate that the same transcription factors are required for the development of different Th cell subsets, which suggests that these cells might share the same developmental commitment process. Furthermore, how the combination of expression of transcription factors at a single-cell level leads to the acquisition of a particular phenotype is also not completely understood.

Recent work has demonstrated that Th cell subsets can be more plastic than previously thought. For instance, IL-17-producing T cells, when transferred into lymphopenic hosts, can convert into IFN- γ -producing T cells (Bending et al., 2009; Lee et al., 2009; Martin-Orozco et al., 2009a; Nurieva et al., 2009). Moreover, the conversion of Th17 cells into Th1 cells upon IL-12 treatment has also been reported in studies *in vitro* (Lee et al., 2009). However, whether the reverse conversion of Th1 into Th17 cells can also occur, *in vitro* or *in vivo*, remains elusive. Interestingly, Th cells expressing both IL-17 and IFN- γ have been documented in several autoimmune and inflammatory manifestations. Whether these cells indeed represent the *in vivo* conversion of Th17 into Th1 cells, or vice-versa or whether they are generated directly into double-producer cells needs further demonstration. Transfer experiments in several disease settings using cells obtained from IL-17 and IFN- γ double reporter mice, or using fate-mapping systems such as IL-17-Cre or IFN- γ -Cre mice crossed with Rosa26-YFP locus could help solve this uncertainty. Indeed, recently Stockinger and colleagues have shown that cells expressing IFN- γ in the central nervous system of mice with experimental autoimmune encephalomyelitis disease had expressed IL-17 before converting into IFN- γ -producing cells (Hirota et al., 2011).

Regulatory T cells have also been shown to convert to other Th lineages. We and others have shown that stimulation of regulatory T cells with IL-6 leads to the conversion of these cells into IL-17-producing T cells, although some remaining cells coexpressed both IL-17 and Foxp3 (Lochner et al., 2008; Voo et al., 2009; Xu et al., 2007; Yang et al., 2008b). Foxp3⁺ Treg cells can also convert into IFN- γ -producing T cells when cultured under Th1-skewing conditions or in a *Toxoplasma* infection

model (Oldenhove et al., 2009; Wei et al., 2009). Furthermore, recently Foxp3⁺ T cells have been shown to become follicular helper T cells in Peyer's patches *in vivo* (Tsuji et al., 2009).

Th2 cells have not been the exception to the conversion to other Th lineages. For instance, human Th2 memory/effector cells have been shown to also express IL-17, which leads to an enhanced allergic asthma phenotype (Wang et al., 2010). Furthermore, memory Th2 cells can become regulatory T cells expressing Foxp3, and these converted cells are crucial in inhibiting airway hyperreactivity caused by Th2 memory cells (Kim et al., 2010). Finally, while Th1 and Th2 cells have been always considered fixed and stable populations, recently Löhning and colleagues challenged this view and demonstrated that *in vivo* LCMV infection can induce a Th2 cell to stably coexpress GATA3 and T-bet with combined characteristics of Th1 and Th2 cells (Hegazy et al., 2010).

Although effector T cells were originally considered to be terminally differentiated, a growing body of evidence discussed in the present section has challenged this view and suggested that the phenotype of effector T cells is not completely fixed but is more flexible or plastic. Th cells can have 'mixed' phenotypes (that is, have characteristics usually associated with more than one Th cell subset) and can interconvert from one subset phenotype to another. Th cell plasticity can be important for adaptation of immune responses in different microenvironments and might be particularly relevant for host defence against pathogens that colonize different tissues. Thus, the immune system has developed different strategies to adapt to the demands of the body. While we understand from a biology perspective that this adaptation of the immune system is extremely important to generate an appropriate host defense response in a tissue-specific manner, we (immunologists) should be cautious with the way we denominate Th subsets according to the expression of "master" transcription factors or specific cytokines. For instance, we have demonstrated that in Smad- as well as in Steroid Receptor Coactivator (SRC)-deficient cells, expression of the Th17 master transcription factors ROR α and ROR γ t was not significantly affected, albeit an altered pattern of cytokine production was observed (Chapter III and data not shown). Thus, according to a definition in terms of the expression of "master" transcription factors, we would have concluded that both Smad and SRC molecules play no role in Th17 cell development. Moreover, Th17 cells were termed on the basis of IL-17 production. However, in SRC1-

and IKK α -deficient Th17 cells, only IL-17A production is defective, while the expression of other Th17-associated cytokines remains unchanged ((data not shown and (Li et al., 2011)). In this scenario, would it be appropriate to say there is a defect in Th17 cell generation or commitment? A more accurate statement would be to just describe the role of SRC1 or Smad2 in IL-17 production rather than discussing their role in Th17 cell development/commitment.

Thus, our work, together with recent reports on the plasticity and heterogeneity of Th subsets, urge for a more stringent interpretation of results, and therefore a more rigorous definition of these subsets. It is understandable that as scientists we need a framework to study our fields, so that we can advance our discoveries and refer to the same universal definitions. However, a re-consideration in our nomenclature of CD4⁺ T helper subsets seems inevitable based on the recent discoveries. Probably, we should refer to our populations on the basis of cytokine production or on their role or characteristics in immune responses. To this effect, Dong and colleagues, in their identification of the currently-termed Th17 cells, initially named these cells Thi or inflammatory T helper cells (Park et al., 2005). This name better reflected the role of these cells in immune responses. With the development of new flow cytometry antibodies, the expansion of markers that can be used to analyze CD4⁺ T helper populations at the single cell level and the generation of cytokine or transcription factor reporter mice, we would be able to better characterize these populations. This phenotypification will help develop a better and more accurate nomenclature system.

IV.3. Reciprocal generation of Th17 and regulatory T cells

In 2006, it was suggested that Th17 and regulatory T cells are reciprocally regulated since both lineages depend on TGF- β for their generation (Bettelli et al., 2006; Veldhoen et al., 2006a). The requirement of TGF- β in Th17 cell generation *in vivo* was demonstrated in mice deficient in TGF- β 1 that were unable to produce IL-17 cytokine in T cells (Mangan et al., 2006). Recently, Zhou and colleagues showed that TGF- β levels are crucial for determining the balance between Th17 and regulatory T cells. The authors demonstrated that increasing concentrations of TGF- β shifts the balance towards the induction of regulatory T cells (Zhou et al., 2008a). Furthermore, we have demonstrated that Foxp3, the master transcription factor for Treg cells that requires TGF- β for its expression, is transiently induced in developing Th17 cells *in vitro*. Also, we found that forced expression of Foxp3, which mimics environments with high TGF- β levels, leads to the suppression of Th17 cell generation through the repression of ROR α and ROR γ t transcriptional activity. Littman and colleagues have also identified a cell population in the gut that co-expresses ROR γ t and Foxp3 and has lower IL-17 production compared to ROR γ t single positive cells (Zhou et al., 2008a).

The identification of regulatory T cells that express ROR γ t in the gut suggests that either these cells could be progenitors of Th17/Treg cells or that Foxp3⁺ regulatory T cells consist of a heterogeneous population (Zhou et al., 2008a). Our *in vitro* data showing co-expression of Foxp3 and IL-17F in developing Th17 cells (Chapter II) would argue in favor of the former. However, recent reports have identified other distinct subpopulations within regulatory T cells that coexpress Foxp3 and other Th-associated markers, supporting the idea that regulatory T cells consist of a heterogeneous population, thus challenging the idea of a fixed and completely stable population. Foxp3⁺ regulatory T cells can express T-bet, IRF4 or Stat3 and control Th1, Th2 or Th17-dependent responses respectively (Chaudhry et al., 2009; Koch et al., 2009; Zheng et al., 2009). Cells co-expressing Foxp3 and T-bet show high expression of the Th1-associated chemokine receptor CXCR3. Acquisition of CXCR3 expression in a T-bet-dependent manner by regulatory T cells allows them to migrate to inflammatory regions and

enables them to control Th1-mediated inflammatory and autoimmune responses (Koch et al., 2009). Similarly, cells coexpressing IRF4 and Foxp3 were required to control Th2 responses since mice with a specific deletion of *Irf4* gene in regulatory T cells showed enhanced plasma cell levels and antibody production (Zheng et al., 2009). Furthermore, unpublished data from our laboratory shows that Foxp3⁺ T cells can express the follicular helper T cell (Tfh)-specific transcription factor Bcl6 and acquire CXCR5 expression. The expression of Bcl6 and CXCR5 by regulatory T cells allows them to migrate to the B cell area in germinal center reactions, and seems to be crucial for controlling germinal center responses (Chung Y et al. unpublished data). Also, recently Foxp3⁺ T cells have been shown to convert into Tfh cells in Peyer's patches (Tsuji et al., 2009). Thus, these combined studies would suggest that the population of regulatory T cells present in the gut that co-express RORγt would behave in a similar way as cells expressing other Th-associated transcription factors. Indeed, it has been recently shown that these cells coexpressing Foxp3 and RORγt can show an inhibitory function in the development of autoimmune diabetes (Tartar et al., 2010). These publications indicate that regulatory T cells might acquire the expression of Th-associated transcription factors so that they can migrate to sites where an ongoing immune response is occurring, and thus control such response. It still remains unclear whether other subsets of regulatory T cells expressing other Th-associated transcription factors exist. These recent findings in the regulatory T cell field opens up an exciting area of research to further understand the exact role regulatory T cells play in health and diseases. Furthermore, the molecular mechanisms behind the acquisition of these transcription factors in regulatory T cells still remain to be determined.

Overall, the findings presented in this work provide novel mechanisms that regulate the generation of two T helper lineages with opposing functions in the immune system, and thus could result in possible therapeutic targets to treat diseases where the balance of these T cells is dysregulated, such as in autoimmunity, chronic infectious diseases and cancer.

CHAPTER V - EXPERIMENTAL PROCEDURE

Mice. C57BL/6, B6.SJL (CD45.1), RAG1-deficient, Scurfy (Foxp3 deficient), OT-II and 2D2 TCR transgenic mice were purchased from Jackson Laboratories. Foxp3-GFP mice were kindly provided by Dr. Ruslan Medzhitov with permission of Dr. Alexander Rudensky (Fontenot et al., 2005), and crossed with IL-17F-RFP knockin mice, generated by Dr. Xuexian Yang (Yang et al., 2008a; Yang et al., 2008b). Smad3 KO mice were kindly provided by Dr. Xin-Hua Feng with permission from Dr. Xiao-Fan Wang (Datto et al., 1999), Smad4^{n/n} and Smad2^{n/n} mice by Dr. Xin-Hua Feng and Dr. Martin Matzuk with permission of Dr. Elizabeth Robertson (Chu et al., 2004; Vincent et al., 2003). Floxed mice were bred with CD4-Cre mice provided by Dr. Christopher Wilson (Lee et al., 2001). Mice were housed in the SPF animal facility at M. D. Anderson Cancer Center and the animal experiments were performed at the age of 6-10 weeks using protocols approved by Institutional Animal Care and Use Committee.

T cell differentiation. Naïve CD4⁺ T cells (CD4⁺CD25⁻CD62L^{hi}CD44^{lo}) were sorted by FACS (Fluorescent Activated Cell Sorting) and stimulated with plate-bound anti-CD3 (0.5 µg/ml, clone 2C11) and anti-CD28 (0.5 µg/ml, clone 37.51) in the presence or absence of the following cytokines, antibodies or compounds: IL-2 (50 units/ml), TGF-β (1-20 ng/ml, R&D system), IL-6 (1-30 ng/ml, R&D system), IL-23 (40 ng/ml, R&D system), IL-1β and IL-1α (10 ng/ml, Peprotech), TNF-α (10 ng/ml, R&D system), anti-IL-4 (10 µg/ml, clone 11B11), anti-IFN-γ (10 µg/ml, clone XMG 1.2), anti-TGF-β (20 µg/ml, clone 1D11) and TGFβRI kinase inhibitor SB431542 (5µM, Sigma Aldrich). Four days after stimulation, cells were washed and restimulated with 50 ng/ml of PMA and 500 ng/ml of ionomycin in the presence of Golgi Stop (BD Pharmingen) for 5 hours. Then, cells were permeabilized with Cytofix/Cytoperm Plus Kit (BD Pharmingen) or Foxp3-staining kit (eBioscience) according to the manufacturer's protocols and Foxp3-, IL-17-, IL-17F- and IFN-γ-producing cells were analyzed using intracellular staining. Samples were analyzed using a FACSCalibur or LSRII (BD Biosciences) and

analyzed with FlowJo (Tristar). Also, after differentiation cells were restimulated with anti-CD3 (1 $\mu\text{g/ml}$, clone 2C11) for 16h after which cytokines were measured in the cell-free culture supernatants by enzyme-linked immunosorbent assay (ELISA), or with anti-CD3 for 4h and cDNA was prepared as described below.

In vitro regulatory T cells suppression assay. FACS-sorted naïve $\text{CD4}^+\text{CD25}^-\text{CD62L}^{\text{hi}}\text{CD44}^{\text{lo}}$ T cells were stimulated in the presence or absence of FACS-sorted $\text{CD4}^+\text{CD25}^{\text{hi}}$ natural regulatory T cells (nTregs) at different ratios with 3000 Rads irradiated T-cell depleted splenocytes as antigen presenting cells and soluble anti-CD3 (0.5 $\mu\text{g/ml}$, clone 2C11) for three days. Proliferation was determined by addition of [^3H]-thymidine to the culture for the last 8h.

Cloning into retrovirus. Bicistronic retroviral vectors pGFP-RV (provided by Dr. Murphy at Washington University (Ouyang et al., 1998)) or pMIG-hCD2 (provided by Dr. Bevan at Washington University (Deftos et al., 1998)) containing *IRES*-regulated *GFP* and human *CD2*, respectively were utilized. The coding sequences for the following proteins were cloned in those vectors: $\text{ROR}\alpha$ and $\text{ROR}\gamma\text{t}$ (Yang et al., 2008c), constitutively active Smad2 (Smad2 2SD) (Dai et al., 2009), Smad3 2SD and $\text{TGF}\beta\text{RI T202D}$ (Feng et al., 2000). Also, full length Foxp3 was amplified with BglII and XhoI sites and cloned into pGFP-RV. Site directed mutagenesis using specific primers carrying deletions or mutations were utilized to generate Foxp3 mutants (Foxp3 $\Delta\text{FKH/NLS}$, Foxp3 ΔE250 , Foxp3LL-AA, Foxp3 $\Delta\text{105-190}$ or Foxp3 LL-AA $\Delta\text{105-190}$), and cloned into pGFP-RV vector.

Transduction of T cells by retrovirus. For the generation of retroviral supernatants, HEK 293T cells were transfected with the retrovirus constructs together with packaging vector pCL-Eco using calcium phosphate precipitation. Supernatant containing retroviral particles were collected 48h after transfection. For transduction, $\text{CD4}^+\text{CD25}^-\text{CD62L}^{\text{hi}}\text{CD44}^{\text{lo}}$ T cells from either C57BL/6 or OT-II TCR transgenic mice were stimulated with anti-CD3 and anti-CD28 or irradiated T-cell depleted splenocytes

and OT-II peptide (10 µg/ml), respectively in the presence of various cytokine/antibodies as previously indicated for 1d. Then, supernatants containing retroviral particles and polybrene (8 µg/ml) were added to activated-T cells and centrifuged at 1800 rpm for 50 min. Cells were then washed and incubated with various cytokine/antibodies. Four days after infection, cells were sorted based on GFP and/or hCD2 expression by FACS, and stimulated with PMA and ionomycin in the presence of Golgi Stop for 4h to measure cytokine or transcription factor expression by intracellular staining. Also, FACS-sorted populations were restimulated with anti-CD3 for 4h, after which cDNA was prepared and gene expression determined by real time RT-PCR (see below).

Quantitative real-time PCR. Total RNA was extracted from activated or naïve T cells using TriZol reagent (Invitrogen). Then, cDNA was synthesized using Superscript reverse transcriptase and oligo(dT) primers (Invitrogen) and gene expression was examined with a Bio-Rad iCycler Optical System using iQ™ SYBR green real-time PCR kit (Bio-Rad Laboratories, Inc.). The data were normalized to *β-actin* reference. The list of primers utilized is shown in Table 2.

Table 2: List of real time PCR primers

a) Transcription Factors			
<i>Gene</i>	<i>Primers sequence forward</i>	<i>Primers sequence reverse</i>	<i>Reference</i>
ROR γ	CACGGCCCTGGTTCTCAT	CAGATGTTCCACTCTCCTCTTCTCT	(Kang et al., 2006)
ROR α	TCCAAATCCCACCTGGAAAC	GGAAGGTCTGCCACGTTATCTG	(Akashi and Takumi, 2005)
T-bet	CAACAACCCCTTTGCCAAAG	TCCCCAAGCAGTTGACAGT	(Grogan et al., 2001)
GATA-3	AGAACCGGCCCTTATGAA	AGTTCGCGCAGGATGTCC	(Grogan et al., 2001)
Foxp3	TACTTCAAGTTCCACAACATGCGACC	CGCACAAAGCACTTGTGCAGACTCA G	(Chen et al., 2008)
IRF4	TCCTCTGGATGGCTCCAGATGG	CACCAAAGCACAGAGTCACCTG	(Chung et al., 2009)
AHR	AGCATCATGAGGAACCTTGG	GGATTTCGTCCGTTATGTCTG	(Reynolds et al., 2010)
Twist1	CGCACGCAGTCGCTGAACG	GACGCGGACATGGACCAGG	(Niesner et al., 2008)
Twist2	GCATCCTGGCCAACGTGC	TCCATGCGCCACACGGAG	(Niesner et al., 2008)
BATF	AGTGAGGACCTGGAGAAAC	GCTCAGCACTGATGTGAAGTA	(Horisawa et al., 2008)
b) Cytokines and chemokines			
<i>Gene</i>	<i>Primers sequence forward</i>	<i>Primers sequence reverse</i>	<i>Reference</i>
IL-17A	CTCCAGAAGGCCCTCAGACTAC	AGCTTTCCTCCGCATTGACACAG	(Ivanov et al., 2006)
IL-17F	CCCATGGGATTACAACATCACTC	CACTGGGCCTCAGCGATC	(Harrington et al., 2005)
IL-21	TCATCATTGACCTCGTGGCCC	ATCGTACTTCTCCACTTGCAATCCC	(Nurieva et al., 2007)
IL-22	CATGCAGGAGGTGGTACCTT	CAGACGCAAGCATTTCTCAG	(Chung et al., 2006)
CCL20	ATGGCCTGCGGTGGCAAGCGTCTG	TAGGCTGAGGAGGTTACAGCCCT	(Park et al., 2005)
IFN- γ	GATGCATTGATGAGTATTGCCAAGT	GTGGACCACTCGGATGAGCTC	(Nurieva et al., 2003)
IL-4	AGATCACGGCATTGTAACG	TTTGGCACATCCATCTCCG	(Grogan et al., 2001)
IL-5	CGCTCACCGAGCTCTGTTG	CCAATGCATAGCTGGTGATTTTT	(Grogan et al., 2001)
IL-13	GCTTATTGAGGAGCTGAGCAACA	GGCCAGGTCCACACTCCATA	(Grogan et al., 2001)
IL-10	ATAACTGCACCCACTTCCCAGTC	CCCAAGTAACCCTTAAAGTCCTGC	(Lang et al., 2002)
CCL2	CTCAGCCAGATGCAGTTAACGCCC	GGTGCTGAAGACCTTAGGGCAGAT	(Reynolds et al., 2010)
Gro- α	CGCTTCTCTGTGCAGCGCTGCTGCT	AAGCCTCGCGACCATTCTTGAGTG	(Park et al., 2005)
c) Cytokine and chemokine receptors			
<i>Gene</i>	<i>Primers sequence forward</i>	<i>Primers sequence reverse</i>	<i>Reference</i>
CCR6	CCTCACATTCTTAGGACTGGAGC	GGCAATCAGAGCTCTCGGA	(Yamazaki et al., 2008)
IL-23R	GCCAAGAGAACCATTCCCGA	TCAGTGCTACAATCTTCAGAGGACA	(Harrington et al., 2005)
CCR2	CCTTGGGAATGAGTAACTGTGTGAT	ATGGAGAGATACCTTCGGAATTCT	(Ishibashi et al., 2004)
CXCR5	ACTCCTTACCACAGTGCACCT	GGAAACGGGAGGTGAACCA	(Wu and Hwang, 2002)

d) Treg-associated markers			
<i>Gene</i>	<i>Primers sequence forward</i>	<i>Primers sequence reverse</i>	<i>Reference</i>
GPR83	GAAGATGCTGGTGCTTGTGGTAGTC	AAGTGGTGATTAGGTAGTGGAGCCC	(Sugimoto et al., 2006)
Glutaredoxin	GGACATCACAGCCACTAACAACACC	ATCTGCTTCAGCCGAGTCATCAG	(Sugimoto et al., 2006)
Ecm1	ACTACCTGCTCCGACCCTGC	CCTGTTCTGGATATGGAAGCTCG	(Sugimoto et al., 2006)
Helios	ACTCCTCAGAAGTTTGTGGGGG	GCTGGGCTTTGTTTCCTCTTG	(Sugimoto et al., 2006)
SOCS2	TCTGGGGACTGCCTTTACCAAC	CCTCTGGGTTCTCTTTCACATAGC	(Sugimoto et al., 2006)
GITR	GACGGTCACTGCAGACTTTG	GCCATGACCAGGAAGATGAC	(Wang et al., 2005)
f) Housekeeping gene			
<i>Gene</i>	<i>Primer sequence forward</i>	<i>Primer sequence reverse</i>	<i>Reference</i>
β-Actin	TCCTTCGTTGCCGGTCCAC	ACCAGCGCAGCGATATCGTC	(Nurieva et al., 2003)

Bone marrow reconstitution. Female RAG1 KO mice were reconstituted with T cell-depleted bone marrow cells from different KO mice or their respective WT controls. Eight weeks later, appropriate reconstitution was determined by measuring CD4⁺ and CD8⁺ T cells and B cell populations in blood. Then, these mice were analyzed for immune cell populations in different tissues or used for *in vivo* animal experimental models (immunizations or EAE, see below)

Myelin Oligodendrocyte Glycoprotein (MOG) or Keyhole Limpet Hemocyanin (KLH) immunization. Mice at 5-8 weeks of age were immunized subcutaneously at the dorsal flanks with 150 µg MOG peptide (amino acids 35–55; MEVGWYRSPFSROVHLYRNGK) emulsified in 500 µg Complete Freund's Adjuvant (CFA) or 200 µg KLH protein emulsified in 50 µg CFA. Seven days later, cells from spleens and/or draining lymph nodes were isolated and restimulated with 25 µg/ml MOG₃₅₋₅₅ peptide or 50 µg/ml KLH protein or media alone for 24 hr. Golgi Stop and/or PMA and ionomycin was added the last 6h of culture, and cytokine expression in CD4⁺ T cells was determined by intracellular cytokine staining as previously described. Also, cells were restimulated with increasing concentrations of KLH protein or MOG₃₅₋₅₅ peptide for three days, and cytokine production was determined in cell-free supernatants by ELISA (Pharmingen). Also, after KLH protein or MOG₃₅₋₅₅ peptide restimulation for three days, proliferation was determined by addition of [³H]-thymidine to the culture for the last 8h.

Experimental Autoimmune Encephalomyelitis (EAE). Female mice at 6-8 weeks of age were immunized subcutaneously at the dorsal flanks with 150 µg MOG peptide (amino acids 35–55; MEVGWYRSPFSROVHLYRNGK) emulsified in 500 µg Complete Freund's Adjuvant (CFA) on day 0 and day 7. Pertussis toxin (500 ng/mouse) was given intraperitoneally at day 1 and day 8. Clinical signs of EAE were assigned scores on a scale of 1–5 as follows: 0, none; 1, limp tail or waddling gait with tail tonic; 2, wobbly gait; 3, hind limb paralysis; 4, hind limb and forelimb paralysis; 5, death. To analyze central nervous system infiltrates, mice were perfused with heparin 0.6%, brain and spinal cord were collected after perfusion, and mononuclear cells were prepared by percoll gradient.

Co-Immunoprecipitation. HEK 293T cells were transfected with expression vectors encoding Flag-ROR α , Flag-ROR γ t, Myc-ROR γ t (Yang et al., 2008c), 6xMyc-Smad3, 6xMyc-Smad2, 2xMyc-Smad4, Flag-Smad2, Flag-Smad3, His-TGF- β RI T202D (Feng et al., 2000) and wild-type or mutant Foxp3. Forty eight hours after transfection, cells were washed with cold-PBS and lysed with lysis buffer (50 mM Tris-HCl [pH 8.0], 1% Nonidet P-40, 120 mM NaCl, 4 mM EDTA, 1 mM Na₃VO₄, 50 mM NaF, 1 µg/ml leupeptin, 2 µg/ml aprotinin, 1 µg/ml pepstatin A). Lysates were generated by centrifugation and pre-cleared with protein A/G-sepharose (Sigma-Aldrich) for 2 h. Then, immunoprecipitation was performed by incubating the pre-cleared lysate with 2 µg of anti-FLAG-M2 or anti-Myc antibodies (Sigma-Aldrich) for 2h after which protein A/G-Sepharose was added. Immunoprecipitates were finally obtained by centrifugation. Similar protein concentration from whole cell lysates or immunoprecipitates were analyzed by Western blot using anti-FLAG-M2, anti-myc, anti-His (Sigma-Aldrich) or anti-human/mouse Foxp3 (eBioscience) antibodies.

Mammalian Two-hybrid reporter assay. Transfection of Chinese Hamster Ovary (CHO) cells was performed using Fugene 6 transfection reagent (Roche, Indianapolis, IN). The following vectors were cotransfected: 0.1 µg of (UAS)5-Luciferase reporter vector, which contains 5 copies of the GAL4

upstream-activating sequence (UAS), and 0.05 μ g of VP16-ROR γ , 0.1 μ g pM-EBIP96, encoding an LXXLL motif derived from SRC-1 (Kurebayashi et al., 2004), and Foxp3 expression vectors cloned into the pGFP-RV vectors as previously described. Cells were incubated for forty hours and luciferase activity was then determined with a luciferase kit (Promega). Transfection efficiency was normalized by β -galactosidase activity.

IL-17 transcription reporter assay. ROR γ t (Yang et al., 2008c), ROR α (Yang et al., 2008c), wild-type or mutant Foxp3 vectors were transfected into EL-4 cells with a luciferase reporter vector containing IL-17 minimal promoter region together with CNS2 element (Akimzhanov et al., 2007; Yang et al., 2008c). Luciferase activity was determined using the dual-luciferase reporter system (Promega) to assay Firefly and Renilla luciferase activity in each sample.

RORE reporter assay. HEK 293T cells were co-transfected with 1-10 μ g of the (RORE)₃-Luciferase reporter (provided by Dr. A. Jetten) in the presence or absence of 1-10 μ g of ROR γ t-RV and increasing concentrations of Foxp3-RV or Smad3 2SD-RV. Cells were incubated for 16 h with complete medium, and then for 24 h with 0.5% FBS containing medium to reduce the endogenous TGF- β concentration present in FBS. Furthermore, transfection of Chinese Hamster Ovary (CHO) cells was performed using Fugene 6 transfection reagent (Roche, Indianapolis, IN). CHO cells were co-transfected with (RORE)₃-Luciferase reporter and 0.05 μ g pZeoSV-ROR γ (provided by Dr. A. Jetten) in the presence or absence of Foxp3 wild-type or mutant expression vectors for forty hours. Luciferase activity was determined using the dual-luciferase reporter system (Promega) to assay Firefly and Renilla luciferase activity in each sample. Renilla luciferase or β -galactosidase activities were used to normalize transfection efficiency.

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