ROLE OF THE SERINE-THREONINE KINASE TPL2 IN REGULATORY T CELL DEVELOPMENT AND FUNCTION

by

XIN LI

(Under the Direction of Wendy T. Watford)

ABSTRACT

The serine-threonine kinase, tumor progression locus 2 (Tpl2, also known as Map3k8/Cot), is a potent inflammatory mediator that drives the production of tumor necrosis factor (TNFα), Interleukin-1β (IL-1β), and Interferon γ (IFNγ). We previously demonstrated that Tpl2 regulates T cell receptor (TCR) signaling and modulates T helper cell differentiation. However, very little is known about how Tpl2 modulates the development of regulatory T cells (Tregs). Tregs are a specialized subset of T cells that express forkhead box P3 (FoxP3) and possess immunosuppressive properties to limit excess inflammation. Because of Tpl2's documented role in promoting inflammation, we hypothesized that Tpl2 antagonizes Treg development and immunosuppressive function. Herein, we demonstrate that Tpl2 constrains the development of inducible Tregs (iTregs). *Tpl2*$^{-/-}$ naïve CD4^{+} T cells preferentially develop into FoxP3^{+} iTregs *in vitro* as well as *in vivo* in a murine model of OVA-induced systemic tolerance. Treg biasing of *Tpl2*$^{-/-}$ T cells depended upon the TCR signal strength and corresponded with reduced activation

of the mammalian target of rapamycin (mTOR) pathway. Importantly, *Tpl2*$^{-/-}$ Tregs have basally increased expression of FoxP3 and immunosuppressive molecules, IL-10, IL-35 subunit Ebi3 and cytotoxic T lymphocyte-associated protein 4 (CTLA-4). Furthermore, they were more immunosuppressive *in vivo* in a T cell transfer model of colitis, as evidenced by reducing effector T cell accumulation, systemic production of inflammatory cytokines, and colonic inflammation. These results demonstrate that Tpl2 promotes inflammation in part by constraining FoxP3 expression and Treg immunosuppressive functions. Overall these findings suggest that Tpl2 inhibition could be used to preferentially drive Treg induction and thereby limit inflammation in a variety of autoimmune diseases.

INDEX WORDS: T cell, Cell differentiation, Serine/threonine protein kinase, mTOR complex, S6 kinase, Immunosuppression, Tolerance, Autoimmune Diseases.

ROLE OF THE SERINE-THREONINE KINASE TPL2 IN REGULATORY T CELLS DEVELOPMENT AND FUNCTIONS

by

XIN LI

B.V.M ., South China Agricultural University, Guangzhou, China, 2011

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2016

ROLE OF THE SERINE-THREONINE KINASE TPL2 IN REGULATION OF REGULATORY T CELLS DEVELOPMENT AND FUNCTION

by

XIN LI

Major Professor:	Wendy T. Watford
Committee:	Donald Harn
	Kimberly D. Klonowski
	David J. Hurley
	Balazs Rada

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
August 2016

Dedicated

To

My parents

With love and gratitude

ACKNOWLEDGEMENTS

I came to the University of Georgia to pursue a Ph.D. degree in virology with a dream of becoming a doctor without borders to prevent the outbreak of Zoonotic diseases in developing areas. Interestingly, I completely changed my mind to pursue my Ph.D. in immunology after meeting Dr. Wendy Watford who showed me how fascinating immunology is.

First and foremost, my sincerest thanks to Wendy for accepting me in her lab and also for providing immeasurable guidance and training during the last five years. She acts like a "Science Mom" for us, providing her constant support and encouragement which has been invaluable to my growth as an immunologist. I am also truly grateful to my committee members, Drs. Kimberly Klonowski, Balazs Rada, David Hurley and Donald Harn for their time, support, encouragement and advice throughout my Ph.D. program, without which this work would not have been possible.

Furthermore, I wish to express my thanks to the members of the Watford lab, Rebecca Kirkland, Angela Peeks, Nicole Acuff, Teneema Kuriakose, Sean Rowley, Monica LaGatta, Jessica Elmore, Kara Wyatt and Chris Slade for their help in conducting experiments and daily lab activities.

Dozens of people have aided my research at the University of Georgia. I would like to express my gratitude to Julie Nelson, Tamas Nagy, Barbara Reaves, Jamie Barber, Lisa Shollenberger McEwen, Hillary Shane, Hong Qiu as well as the staff in the Central Animal Facility.

I took part in many other activities, with countless people teaching and helping me at every step. Thanks for everyone from the UGA Chapter of the Blue Key Honor Society, especially for E. Farah Smali, Kausar Nadim Samli, Janet Lance, Nathan Howell, Ben Jacobs, Kasey Darley, Mugdha Joshi, Ashley Maccray, and Chelsea Fitzhugh. Thanks to everyone from the Emerging Leaders Program at the University of Georgia. Your friendship was irreplaceable. I would also like to thank Shelley Fincher for being my mentor at the UGA Innovation Gateway summer program. Her support and inspiration has been invaluable.

Last but not least, I would like to express my gratitude to my best friends who have supported me through my good and bad times during these five years of my Ph.D. studies: Yuge Wang, Hanbo Chen, Runqing Diao, Yuxiao Wang, Weihao Zhang, Shuyuan Zhu, Tian Wang and Charles Chi.

TABLE OF CONTENTS

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The immune system is tasked with the recognition and elimination of foreign pathogens. It is comprised of non-specific innate immune cells and antigen-specific adaptive lymphocytes. T lymphocytes derived from the thymus, along with B lymphocytes derived from the bone marrow express somatically rearranged antigen receptors that mediate specific recognition of antigens and are responsible for immunologic memory to antigens upon a second encounter. Host immune responses to pathogens require some degree of inflammation, however, dysregulated or inappropriate inflammation can lead to the development of autoimmune diseases. For example, lymphocytes recognizing host-derived or "self" antigens mount intense pathologic immune responses that drive systemic inflammation and autoimmunity (1). Therefore, the immune system has devised multiple mechanisms to ensure that the host repertoire of lymphocytes is educated to tolerate self-antigens. First, during T cell development in the thymus, immature high-affinity, self-reactive thymocytes are eliminated via clonal deletion (or negative selection) by a mechanism known as central tolerance (2). However, self-reactive T cells cannot be fully eliminated by negative selection, which is an imperfect process. Therefore, a second mechanism, known as peripheral tolerance is in place to control self-reactive T cells in peripheral tissues (3). Importantly, peripheral tolerance is indispensable for preventing inflammation triggered by environmental antigens or self-antigens that are not abundant in the thymus (4).

Immune suppression mediated by regulatory T (Treg) cell is an essential component of peripheral tolerance (5, 6). Therefore, a better understanding of the mechanisms that regulate Treg development and immunosuppressive functions is needed to facilitate the development of novel treatments for autoimmune diseases.

T helper cell development

$CD4^+$ T helper (Th) cells are a subset of T cells that are essential for modulating adaptive immune responses. The T cell receptor (TCR) and CD4 co-receptor enable the recognition of antigen presented in the context of major histocompatibility complex (MHC) II molecules by antigen presenting cells (APC), leading to $CD4^+$ T cell activation (7, 8). In addition to antigens, T cells require a second signal provided by co-stimulatory molecules and cytokines. The CD28 co-stimulator is expressed on the surface of naïve T cells and interacts with its ligands CD80 (B7-1) and CD86 (B7-2) expressed on APC (8). T helper cell lineage specification depends on TCR activation along with local cytokine production (Figure 1.1). $CD4^+$ helper T cells can differentiate into distinct lineages of effector T cells, including T helper 1 (Th1) cell, T helper 2 (Th2) cell, T helper 17 (Th17) cell, and regulatory T cells (Treg) (7-10) (Figure 1.1). These T helper subsets are characterized by the expression of unique transcription factors, cytokine expression profiles and effector functions (Reviewed in (7)). Upon TCR signals activation, interleukin (IL)-12 can drive naïve T cells into the Th1 cell lineage, whereas IL-4 converts naïve T cells into the Th2 lineage (Reviewed in (7)). Th1 and Th2 cells express lineage-specifying transcriptional factors, T-bet (encoded by *Tbx21*) and GATA Binding Protein 3 (GATA3) respectively (11). In addition, Th1 and Th2 cells protect against intracellular bacterial infection and extracellular parasitic infections, respectively, through producing the signature cytokine interferon (IFN)-γ (Th1) or IL-4, IL-5 and IL-13 (Th2). Additional lineages have been

described, including Th17 cells and Tregs, which have their own master transcriptional regulators. The orphan retinoid receptor (Rorγt) is critical for Th17 differetiation, whereas Forkhead Box P3 (FoxP3) promotes Treg differetiation (12-15). In the presence of TCR signals, transforming growth factor beta 1 (TGF-β) and IL-6 can drive naïve T cells into Th17 cell lineage, a pro-inflammatory subset that provides protection against extracellular bacterial and fungal infections, whereas IL-2 and TGF-β promote the generation of Treg (Reviewed in (7)), a specialized subset of T cell that have potent immunosuppressive activity that maintains peripheral tolerance to self-antigens (16).

Tumor Progression Locus 2

Tumor progression locus 2 (Tpl2, also known as Map3k8) was identified as an oncogene in a human thyroid carcinoma cell line in 1991 and was therefore designated Cancer Osaka Thyroid (Cot) (17). Subsequently, provirus integration within the last intron of the *Tpl2* gene led to Moloney murine leukemia virus (MoMuLV)-induced T cell lymphomas in rodents (18, 19). Tpl2 is translated into 58- and 52-kDa protein isoforms from alternate translation initiation sites (20). In addition to its serine/threonine kinase domain, Tpl2 also contains an amino-terminal region with unknown function and a carboxy-terminal tail with regulatory function over Tpl2's kinase activity (21). Tpl2 is involved in signal transduction downstream of multiple receptors, including Toll-like receptors (TLRs), cytokine receptors, antigen receptors (B cell receptor and T cell receptor) and G protein-coupled receptors, to regulate the production of a variety of immune mediators. Tp2 is through to function primarily by activating the mitogen-activated protein (MAP) kinase pathway downstream of nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (22, 23). In the steady state, Tpl2 is constitutively associated with NF-κB p105 and A20-binding inhibitor of NF-κB (ABIN-2) in an inactivate form (24) (Figure 1.2). The

death domain (DD) of NF-κB p105 directly interacts with the kinase domain of Tpl2, inhibiting its kinase activity (25). Stimulus-induced proteolysis of NF-κB p105 releases Tpl2 to phosphorylate its substrate mitogen-activated protein kinase kinase (MEK), which subsequently induces extracellular signal–regulated kinases 1/2 (ERK1/2) phosphorylation and signaling (22, 23). Tumor necrosis factor (TNF)- or Toll-like receptor 4 (TLR4)-mediated activation of the IκB kinase (IKK) complex induces Tpl2 serine 400 phosphorylation and dissociation from NF-κB p105 in macrophages (26, 27). Once Tpl2 is free from NF-κB p105 inhibition, it functions as a MAP kinase kinase kinase (MAP3K) and phosphorylates MEK, leading to ERK activation, before undergoing rapid proteosomal degradation (23). In addition to ERK1/2 signaling, overexpressed Tpl2 can also induce the activation of c-Jun N-terminal kinase (JNK), p38γ and ERK5 MAPKs (19). Despite the multitude of MAP3Ks, Tpl2 serves a critical, non-redundant role in TLR-dependent ERK activation leading to expression of inflammatory mediators, including TNF, IL-1β and cyclooxygenase-2 (COX-2) (23, 28, 29). Because of its essential role in contributing to the expression of proinflammatory mediators, Tpl2 is considered to be a potential target for immunotherapy of autoimmune diseases.

Tpl2 has been shown to play an important role in innate and adaptive immune regulation of host defenses and inflammation. The generation of Tpl2-deficient mice by Philip Tsichlis' laboratory has made it possible to determine numerous immunological functions of Tpl2 *in vivo*. First, Tpl2-deficient mice are resistant to endotoxin-induced septic shock due to a defect in TNF secretion (28). Watford *et al.* demonstrated that *Tpl2*$^{-/-}$ mice were more susceptible to *Listeria monocytogenes* infection, which correlated with a defect of the induction of IL-1β (30). In innate immune cells, such macrophages and dendritic cells, Tpl2 is required for Toll like receptor (TLR)-induced activation of MEK and ERK MAP kinases in macrophages and bone marrow-

derived dendritic cells (BMDCs) but is dispensable for NF-κB, p38 and JNK activation (30, 31). Interestingly, *Tp2*$^{-/-}$ macrophages displayed abrogated TNF alpha production in response to lipopolysaccharides (LPS) stimulation. However, *Tp2*$^{-/-}$ BMDC activated with either LPS (TLR4 ligand), polyinosinic:polycytidylic acid (Poly (I:C)) (TLR3 ligand) or CpG (TLR9 ligand) displayed only a partial reduction in TNF-α production (30) demonstrating cell type specific functions of Tpl2. In addition, Tpl2 has been showed to maintain the expression of C-C chemokine receptor type 2 (CCR2) and CCR5 and the migration of macrophages (32). Tpl2 can also regulate the immunoregulatory cytokines, such as IL-10. Tpl2 is required for maximal IL-10 production following stimulation of macrophages and myeloid dendritic cells with LPS or CpG (30, 33).

Tpl2 also plays important roles in the adaptive immune response. Eliopoulos *et al*. showed that Tpl2 transduced CD40 signals in B cells via ERK signaling (34). Importantly, Tpl2-deficient T cells also exhibit a defect in TCR-induced ERK activation (35-37). In particular, Tsatsanis *et al*. showed Tpl2 deficient murine $CD8^+$ T cells were defective in ERK activation and exhibited enhanced proliferation after TCR stimulation (35-37). In addition, they also showed that *Tpl2*$^{-/-}$ $CD8^+$ T cells were also defective in the induction of cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) (35). Watford *et al*. demonstrated that Th cell differentiation into Th1 and Th2 lineages is also altered by Tpl2 ablation (36, 37). The differentiation of Tpl2-deficient Th1 cells was reduced, and this was due to impaired expression of the Th1 transcription factors, signal transducer and activator of transcription 4 (STAT4) and T-bet, resulting in impaired immune responses to the intracellular parasite *Toxosplasma gondii* (36). In contrast, Th2 responses and immunopathology were enhanced in *Tpl2*$^{-/-}$ mice in an ovalbumin (OVA)-induced model of allergic asthma (37). They found that Tpl2 negatively regulates Th2 cells development via

promoting IFN-γ production (37). Watford *et al.* also found that Tpl2 was required for maximal differentiation of IL-17^{+} Th17 cells *in vitro* through negative regulation of the Treg transcription factor, FoxP3 (38). Because a number of autoimmune diseases and cancers are believed to be T cell-mediated (39), understanding how Tpl2 contributes to the TCR signaling of T helper cell lineages and development, is warranted.

Regulatory T cells

Regulatory T cells (Tregs) are a subset of T helper cells with immunosuppressive properties that maintain immunological self-tolerance by controlling self-reactive T cells. In 1985, Sakaguchi *et al.* showed that CD4^{+} splenic cells depleted of CD5^{high}CD4^{+} and transferred to T cell-deficient athymic nude mice induced spontaneous systemic autoimmune diseases. However, simultaneous co-transfer of total CD4^{+} splenocytes prevented disease development (40). Powrie *et al.* and Morriset *et al.* subsequently independently demonstrated that transfer of CD45RB^{high} CD4^{+} T cells into congenic severe combined immunodeficient (SCID) mice also induced inflammatory bowel disease (41, 42). These findings proved that a fraction of T cells possess dominant immune-suppressive functions required for immune homeostasis. Sakaguchi *et al*. further identified CD25 (the IL-2 receptor α-chain) as a surface marker to distinguish this population of suppressor T cells (43). Indeed, they demonstrated that adoptive transfer of CD4^{+}CD25^{-} T cells induced lethal, multi-organ autoimmune disease, whereas co-transfer of CD4^{+}CD25^{+} T cells fully protected the mice. Furthermore, they demonstrated that depletion of CD4^{+}CD25^{+} T cells from wild-type mice was sufficient to break peripheral self-tolerance and induce autoimmune diseases. In 2003, both Sakaguchi *et al.* and Rudensky *et al.* independently revealed that the transcription factor forkhead box P3 (FoxP3) controls the development and functions of CD25^{+}CD4^{+} Tregs (14, 15). FoxP3, encoded by the X chromosome, specifies the Treg lineage

and exhibits a critical, non-redundant role in maintaining peripheral self-tolerance in mice and humans (44). Humans with mutations in *Foxp3*, exhibit impaired development and dysfunction of Tregs in a condition known as immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX). This devastating, life-threatening condition is accompanied by severe multi-organ autoimmune diseases (44). Acute depletion of FoxP3 in murine model similarly results in Treg deficiency and the development of fatal systemic autoimmunity, whereas overexpression of FoxP3 confers its immunosuppressive functions (16). Therefore, FoxP3 is considered to be the "master regulator" transcription factor of the Treg lineage (15, 16, 45). $CD4^+$ $Foxp3^+$ Tregs can develop as either natural regulatory cells (nTreg) in the thymus or inducible regulatory T cells (iTreg) derived from naïve conventional T cells (Tcon) in the periphery (Figure 1.3) (4, 46).

Generation of natural regulatory T cells and inducible regulatory T cells

The development of distinct Treg subsets (natural nTregs versus peipheral iTregs) occurs in different anatomical locations and through different mechanisms. TCR signals are clearly indispensable for nTreg development in thymus. The development of nTregs is highly influenced by TCR signal strength, being strongly induced by high avidity TCR interactions with self-peptide presented in the context of MHC class II molecules in the thymic environment (47). In addition to TCR signals, co-stimulatory signals are also critical for thymic nTreg generation. CD28 is a prominent T cell co-stimulator that is crucial for naïve T cell activation and expansion in the periphery through interactions with B7 (B7-1 and B7-2) co-stimulatory molecules expressed by antigen presenting cells. Mice deficient in CD28 or its ligands, B7-1 and B7-2, develop more severe autoimmune diabetes with dramatically reduced numbers of Tregs in the thymus and periphery (48). Furthermore, a recent study revealed that the numbers of $FoxP3^+$

nTregs were reduced in CD28 conditional knockout mice, but that the Foxp3$^+$ Treg percentages in spleens and lymph nodes were similar to those of the control mice (49). All of these findings confirm that the CD28/B7 co-stimulatory pathway is required for the generation of nTregs in the thymus.

Several studies have demonstrated the essential role IL-2 and TGF-β signaling in Treg development and maintenance (47, 50, 51). Mice deficient in IL-2, IL-2Rα or IL-2Rβ lead to the development of lymphoproliferative diseases associated with the accumulation of activated CD4$^+$ and CD8$^+$ T cells (52-54). In particular, IL-2Rβ deficient mice exhibit drastically reduced numbers of Tregs (53-55). Similarly, mice lacking signal transducer and activator of transcription 5 (STAT5), a latent cytosolic transcription factor activated by IL-2 signaling, are also severely impaired in Treg development, however, blockade of IL-2 results in a dramatic reduction of the numbers of FoxP3$^+$ Tregs the spleen, despite similar numbers of nTregs in the thymus (56, 57). However, mice deficient in IL-2 or IL2R-α maintain similar numbers of Treg cells (53-55). Therefore, the role of IL-2 in thymic FoxP3$^+$ nTreg cell generation is still incompletely understood (45, 51, 58, 59). However, IL-2 and TGF-β clearly synergistically contribute to the induction of iTregs from naïve CD4$^+$ T cells in the presence of TCR stimulation *in vivo* and *in vitro* (47, 50, 51). *In vitro* and *in vivo*, either neutralization or genetic ablation of IL-2 significantly reduced FoxP3 transcription and suppressive activity of iTregs (60-62). Disruption of IL-2 signaling by deletion of STAT5 also lead to significant reduction of FoxP3 expression of iTreg *in vivo* (62). TGF-β has also been implicated in the generation and immunosuppressive functions of Tregs (46, 47, 50). However, iTreg and nTreg differ in their developmental requirements for TGF-β as well as IL-2. For iTreg development, TGF-β activates mothers against DPP homolog 2/3 (Smad2/Smad3) signaling to induce FoxP3 transcription (63).

In contrast to the critical role of TGF-β in iTreg development, the role of TGF-β in the development of nTregs is less well established. In TGF-β receptor II-deficient mice, the number of nTregs is unaffected (64). However, a recent study showed that conditional deletion of TGF-β receptor I resulted in a dramatic reduction of thymic nTreg generation in mice between postnatal days 3 and 5 (65). Collectively, these studies clearly demonstrate that IL-2 and TGF-β are essential for FoxP3$^+$ iTreg generation (47, 50, 51, 60-62). It is generally believed that iTregs are the result of co-evolution of the adaptive immune system with commensal bacteria and food antigens in the gut (4). There has been considerable interest in understanding the mechanisms regulating iTreg differentiation in gut-associated lymphoid tissues. In addition to the required cytokines signals, many studies have confirmed that the local microenvironment significantly influences the development and differentiation of iTregs in gut-associated lymphoid tissues (66-70). The major vitamin A metabolite all-trans retinoic acid (RA) has been shown to regulate the conversion of TGF-β-mediated FoxP3$^+$ iTreg cells (66, 71-74). For example, Belkaid and colleagues found that CD103$^+$ dendritic cells from the small intestines and mesenteric lymph nodes produce retinoic acid (RA) that can synergistically enhance the conversion of naïve T cells into FoxP3$^+$ Tregs cells in the presence of TGF-β (66). Kuchroo *et al.,* further demonstrated that RA can enhance TGF-β signaling by promoting the expression and phosphorylation of Smad3, subsequently increasing FoxP3 expression (73). Microbial metabolites, such as short chain fatty acids from clostridial species, including acetic acid, propionic acid, and butyric acid, have also been shown to contribute to the differentiation and expansion of colon FoxP3$^+$ Tregs (68, 70, 75).

The T cell transfer model of colitis

Immune suppression mediated by Tregs is a dominant mechanism to control systemic immune homeostasis (76). This is perhaps most apparent in the gastrointestinal tract where abundant Treg accumulation is necessary to maintain peripheral tolerance to ingested antigens (via oral tolerance) and the vast numbers of resident commensal flora. Enrichment of $FoxP3^+$ Tregs has been observed in mesenteric lymph nodes (MLN) as well as intestinal lamina propria following adoptive transfer $CD25^+CD4^+$ T cell into recombination-activating gene 1- deficient (*Rag1*$^{-/-}$) recipients (77, 78). In addition, a recent study indicated that $CD4^+CD25^+$ Tregs within the lamina propria are sufficient to control intestinal inflammation, as these cells maintain peripheral tolerance even in splenectomized lymphotoxin-α-deficient mice that lack secondary lymphoid organs (79). Because of their paramount importance in maintaining intestinal homeostasis, a number of inflammatory bowel disease (IBD) models have been developed to investigate Treg functionality. In a well characterized murine model of inflammatory bowel disease developed by Powrie and colleagues, $CD4^+CD45RB^{hi}CD25^{lo}$ naïve T cells are transferred into T cell-deficient hosts and, in the absence of Treg-mediated peripheral tolerance, induce inflammation in the colon (41). The induction of local and systemic inflammation is a complex process with mixed Th1 and Th17 pathology (76, 80, 81). Following adoptive transfer, the naïve T cells migrate to the secondary lymphoid organs where they undergo lymphopenia-induced expansion. In the absence of Treg-mediated peripheral tolerance, the colitogenic T cells migrate to the colon where they are triggered by self-antigens primarily derived from commensal bacteria (82). After further division of reactive T cells, inflammatory T effectors accumulate in the colonic lamina propria and isolated lymphoid follicles (ILFs). This T cell-driven inflammation also recruits effectors of the innate immune system to the laminia propria, isolated lymphoid follicles (ILFs) and

secondary lymph nodes. The cross-talk between central and local lymphoid structures continues to amplify the inflammation. T cell-driven colitis exhibits local and systemic clinical manifestations, including wasting disease, splenomegaly and increased pro-inflammatory cytokines such as TNF, IFNγ and IL-6 (76). Importantly, colitis can be prevented by co-transfer of $CD4^+CD25^+$ Treg cells (83, 84) that mediate immune suppression in this model primarily through the production of the anti-inflammatory molecules, such as CTLA-4 and IL-10. CTLA-4 deficient Tregs could prevent CD4+ T cells proliferation and cytokine production *in vitro*, however, they were unable to restrict the colitis development due to lymphopenia-induced expansion and cytokines production of $CD4^+$ T cells in the T cell adaptive transfer model of colitis (85). The role of IL-10 remains controversial in the T cell transfer model of colitis. Several studies have suggested that IL-10 is required for the prevention of colitis (77, 85-87). Powrie *et al.* revealed anti-IL-10 receptor monoclonal antibody (mAb) abrogates the ability $CD4^+CD45RB^{low}$ cells to restrict the development of colitis (86). In addition, IL-10 deficient $CD4^+CD45RB^{low}CD25^+$ Tregs failed to control the expansion of $CD4^+CD45RB^{high}$ T cells and colitis development upon transfer compared to their WT counterparts in T cells transfer model of colitis (87). However, some studies have demonstrated IL-10 deficient Tregs are still fully suppressive and able to prevent colitis as well as WT Tregs, suggesting that Treg-derived IL-10 is dispensable for the prevention of colitis development (82, 88). One explanation for these studies is that host IL-10 is important for maintaining the stability of $FoxP3^+$ Tregs, which in turn limits the inflammation and colitis development in the T cells adaptive transfer model of colitis (88). This model is therefore extremely useful for studying the suppressive functions of Treg cells during intestinal inflammation (83, 84).

Mechanisms of immunosuppression mediated by Tregs

Treg-mediated suppression can be classified into four basic 'modes of action': (1) suppression by anti-inflammatory cytokines, (2) suppression by cytolysis, (3) suppression by metabolic disruption, and (4) suppression by modulation of dendritic cell (DC) maturation or function (89) (Figure 1.4). The immunosuppressive cytokines, IL-10, IL-35 and TGF-β, have all been implicated in Treg suppressive functions (90, 91). IL-10 is an anti-inflammatory cytokine expressed by a variety of cell-types (92), including Tregs. IL-10 derived from Tregs is critically important in inhibiting Th1 and Th2 cells by down-regulating the expression of co-stimulatory molecules on dendritic cells and macrophages (93-95). In addition, IL-10 is required to maintain FoxP3 expression and suppressive function of Tregs (88). However, specific deletion of Treg-derived IL-10 did not lead to the development of spontaneous systemic autoimmunity, indicating that Treg-derived IL-10 is not absolutely required for immune tolerance (96). Therefore, other major cellular sources of IL-10, including macrophages and dendritic cells, are also important for IL-10-mediated immune homeostasis (93-95). In addition, these data also suggest that other Treg-derived factors may compensate for IL-10 deficiency.

TGF-β is a highly pleiotropic cytokine that is required for both the maintenance of FoxP3 expression and suppressor function of Tregs (97). TGF-β suppresses effector T cell activation, differentiation and proliferation by inhibiting the expression of lineage-specifying transcription factors, such as T-bet and Gata3 (76). Furthermore, TGF-β also modulates the maturation and functions of other immune cells, such as dendritic cells and macrophages (98). In particular, TGF-β promotes the induction of indoleamine 2,3-dioxygenase (IDO) in dendritic cells. IDO inhibits effector T cells immune responses against infectious mircoorgansims (99). Similarly, TGF-β dampens macrophage functions by inhibiting inducible nitric-oxide synthase (iNOS)

(100). TGF-β blockade abrogates the protection from colitis afforded by co-transfer of nTregs, indicating that TGF-β has an essential role in Treg-mediated immune suppression (76, 101).

IL-35, a heterodimeric protein comprised of IL-12a p35 subunit (IL-12a) and Epstein-Barr virus induced gene 3 (Ebi3), was recently discovered as a new inhibitory cytokine preferentially expressed by FoxP3$^+$ Tregs (91). In addition, the result of co-immunoprecipitation of Ebi3 with IL-12a p35 confirmed that IL-35 was secreted from activated Tregs, but not from activated effector T cells (91). Co-transfer of Tregs deficient in either *Ebi3* or *Il12a* failed to suppress colitis development in a T cell transfer model of inflammatory bowel disease, indicating that IL-35 was required for Treg-mediated peripheral tolerance. Vignali *et al.* also demonstrated that ectopic expression of IL-35 by conventional CD4$^+$ T cells can inhibit T cell proliferation and cytokine production by acquiring a regulatory function. Another studies further revealed that IL-35 could also inhibit the Th17 differentiation from CD4$^+$ T cells (102). IL-35 is a very important suppressive mediator to maintain mucosal immune homeostasis, since the recombinant IL-35 significantly reduced the development of experimental of colitis by inhibiting Th1 and Th17 cells accumulation (103). Thus, IL-35 is a novel anti-inflammatory cytokine that can be expressed both nTregs and iTregs and is required for maintenance of maximal immunosuppressive function of Tregs (91, 104).

In contrast to the active secretion of anti-inflammatory cytokines, IL-2 consumption by Tregs is also a documented mechanism of immune suppression (89). IL-2 is comprised of three receptor chains: IL-2 receptor α, β and γ. The IL-2 receptor expressed on resting naïve T cells consists of IL-2Rβ and IL-2Rγ and displays intermediate affinity for IL-2, whereas Tregs and activated T cells express all three chains, including IL-2Rα (CD25), and display high affinity for IL-2 (105). Tregs express characteristically high levels of IL-2Rα (CD25) and therefore are at a competitive

advantage to consume local IL-2 (89). Because IL-2 is necessary for T cell survival and clonal expansion (106), Treg-imposed IL-2 deprivation of effector T cells leads to their apoptosis (107, 108).

In addition to soluble mediators of immune suppression, Tregs express immunosuppressive molecules that are essential for cell contact-dependent suppression by Tregs though regulating co-stimulation or cytolysis, such as CTLA-4, programmed cell death protein 1 (PD-1), granzymes and perforin. CTLA-4 has a higher affinity for B7 molecules (CD80 and CD86) on antigen presenting cells compared to CD28, CTLA-4 competes with CD28 for binding their shared ligands CD80 and CD86, resulting in the downregulation of CD80 and CD86 expression and antigen presenting cells activation (review in (109). In this case, CTLA-4 can transmits inhibitory signals that interfere with the maturation of antigen presenting cells and impairs their ability to stimulate effector T cells (76, 89, 110). Mice deficient in the CTLA-4 exhibit massive lymphoproliferation and tissue destruction, leading to lethality around 3 weeks of age (111, 112). Treatments with anti-CTLA-4 monoclonal antibodies accelerates and exacerbates mouse models of autoimmunity, such as experimental autoimmune encephalomyelitis (EAE), colitis, type 1 diabetes (T1D) (113), and could even induces autoimmune manifestations in normal mice, including gastritis, oophoritis, and mild sialoadenitis (114, 115). In conclusion, CTLA-4 constitutively expression on Tregs are crucial for their suppressive functions (114).

PD-1 is a member of the B7/CD28 family of co-stimulatory receptors. It regulates T cell activation through binding to its ligands, programmed death ligand 1(PD-L1) and programmed death ligand 2 (PD-L2) (116). PD-1 is expressed by many immune cells types, such as T cells, B cells, natural killer T cells, activated monocytes, and dendritic cells (DCs), and inhibits the proliferation of effector T cells by preventing maturation of antigen presenting cells and

negatively regulates T cells survival through promoting apoptoisis of T cells (116, 117). Recent studies demonstrated that PD-1 and PD-L1 signaling enhances the development of $FoxP3^+$ iTregs development and maintain their suppressive functions by restricting Akt-mammalian target of rapamycin (mTOR) signaling (118, 119). PD-1 blockade also resulted in down-regulation of intracellular FoxP3 expression by Tregs and reversed Treg-mediated suppression of effector T cells(120, 121). Therefore, PD-1 has also been importantly implicated in maintaining the immunosuppressive function of Tregs.

Granzyme B and perforin, are well-known for their high levels of expression in natural killer (NK) cells and cytotoxic $CD8^+$ T lymphocytes (CTLs), where they execute cytotoxic activity over target cells displaying non-self or "altered self" antigens (122, 123). Recent studies have demonstrated that Tregs also exhibit cytolytic activity, which they utilize to suppress effector B cells and T cells via induction of their apoptosis (123-125). Reduced cell contact-mediated suppressive activity was observed in granzyme B-deficient murine Tregs and perforin-deficient Tregs *in vitro* (124, 125). Particularly, activated murine Treg cells suppressed B cell proliferation through induction of cell death in a granzyme and perforin-dependent manner (125). Therefore, granzymes and perforin are considered key components of Treg-mediated suppression.

Regulation of FoxP3 expression.

TCR activation in cooperation with co-stimulatory molecules and cytokines (TGF-β and IL-2) activates the transcription of *Foxp3* (47, 50, 51, 126). TCR-derived signals are required for *Foxp3* expression in both nTregs and iTregs. However, intermediate TCR ligation and co-stimulation are necessary for the generation of nTregs in the thymus, while the development of iTregs in the periphery is favored by low affinity antigen or weak TCR signals and inefficient co-stimulation (67, 127, 128). The *Foxp3* locus (Figure 2) is comprised of three highly conserved

non-coding regulatory regions including the *Foxp3* promoter, TGF-β sensor (conserved non-coding sequence 1; CNS1), and Treg cell-specific demethylation regions (TSDR/CNS2), the pioneer element (CNS3) (129).

TCR ligation triggers the activation of a number of intracellular signaling pathways, including, NF-κB, nuclear factor of activated T cells (NFAT), phosphoinositide 3-kinase (PI3K) and MAPK pathways (Figure 1.4) (126). TCR activation promotes cyclic-AMP response element binding protein (CREB)/activating transcription factor (ATF) to an intronic enhancer element in the *Foxp3* gene, which results in a decreased methylation of the intronic CpG island and is inversely correlated with FoxP3 expression (130). In nTregs, protein kinase C-θ (PKC-θ), NFAT and activator protein 1 (AP-1) all promote *Foxp3* expression in response to TCR and/or co-stimulation signals (131). In this pathway, PKC-θ acts as an upstream regulator of *Foxp3* expression by activating the Ca^{2+}-calcineirin-NFAT pathway (132, 133). Understandably, stronger TCR signals therefore promote increased *Foxp3* expression through these pathways. In contrast, studies examining the differentiation of iTregs *in vitro* have demonstrated that both PKCθ and the MEK-ERK pathway inhibit of *Foxp3* expression, as inhibition of these pathways enhance the expression of *Foxp3* and Treg-associated genes (134-136). Another signaling pathway activated in response to TCR and/or co-stimulation signals, the PI3K/Akt/mammalian target of rapamycin (mTOR) pathway, has been shown to inhibit FoxP3 expression and Treg function through regulating Treg metabolism (134, 137-140). The mTOR pathway consists of two complexes, mammalian target of rapamycin complex 1 (mTORC1) and mammalian target of rapamycin complex 1 (mTORC2), with scaffold proteins regulatory-associated protein of mTOR (Raptor) and rapamycin-insensitive companion of mTOR (Rictor) as their defining components, respectively (Review in (141)). The immunosuppressive drug rapamycin preferentially inhibits

mTORC1, but also interferes with mTORC2 activity and promotes iTreg conversion from naïve T cells (137). T cell deficient of both of mTORC1 and mTORC2 failed to differentiate into Th1, Th2 and Th17 cells (138). However, T cells deficient of mTOR of T cells preferentially differentiate into iTregs, even in absence of iTreg polarizing cytokines, such as IL-2 and TGF-β (138). Together, these studies indicate that the strength of TCR and co-stimulatory signals are key determinants of *Foxp3* expression, and that *Foxp3* expression is differentially regulated by TCR signals during T cell development.

In addition to TCR and co-stimulatory signals, IL-2 and TGF-β are also essential for Foxp3 expression during the induction of iTregs in the periphery (59, 97, 142) (Figure 3). Binding of IL-2 to its receptor activates Janus kinase 1 (JAK1), JAK3 and the latent cytosolic transcription factor, signal transducer and activator of transcription 5 (STAT5). Once activated, STAT5 translocates into the nucleus to bind to the *Foxp3* promoter and Treg cell-specific demethylation regions (TSDR) for the induction of Foxp3 expression (53). In contrast, TGF-β activates activating cell surface receptors, which in turn phosphorylate the downstream Smad2/3 complex (Reviewed in(63)). Phosphorylated Smad2 and Smad3, form a new complex with Smad4, which is translocated into the nucleus. This ultimately results in the binding of the Smad3 transcription factor to the TGF-β sensor region (Figure 2) of the *Foxp3* locus to induce FoxP3 expression (47, 143).

Regulatory T cells in autoimmune diseases

A number of studies have demonstrated that dysregulation of immune homeostasis or a break in immune tolerance can lead to autoimmune conditions, such as type 1 diabetes (T1D), rheumatoid arthritis (RA), inflammatory bowel diseases (IBD), and other disorders (144, 145). A variery of therapies have been developed for treatments of autoimmune diseases, including traditional

corticosteroids, disease modifying drugs, biologic therapies and new immunomodulation therapies. The traditional therapies usually are the first-line therapy for the autoimmune diseases, but they are associated with high toxicity and incomplete efficacy. The development of new biological therapies, mainly dominated by proinflammatory cytokines monoclonal antibodies (mAbs), such as infliximab, adalimumab, and certolizumab, open up a new era for autoimmune diseases treatments. These new therapies provide better efficacy and safety for many patients, particularly for those in whom traditional therapies fail to control the diseases (reviewed in (146)). Recently, small molecule oral inhibitors have been discovered as a new class of inhibitors that modulate pro-inflammatory immune responses on a cellular level. Notably, tofacitinib, an oral inhibitor of Janus kinase (JAK), has been approved by Food and Drug Administration (FDA) for patients with moderate to severe rheumatoid arthritis who do not respond to a disease-modifying anti-rheumatic drug (DMARD) (147, 148). Tofacitib interferes with Janus kinase (JAK)-STAT pathway to inhibit pro-inflammatory cytokines production and transduction (148, 149). More selective JAK inhibitors such as filgotinib and baricitinib, are being investigated for treatment of Crohn's disease and rheumatoid arthritis in clinical trials (150, 151). These biologic or immunomodulatory therapies generally exhibit more specificity and lower toxicity compared to corticosteroids and other disease modifying drugs (146, 152-154). These new therapies usually alleviate autoimmunity by a variery of mechanisms, such as pro-inflammatory cytokine blockade, depeletion of T cells or B cells or cellular signaling pathway modulation in immune cells (146). However, none of them can restore the immune balance after withdrawal of drug treatment. T cell-mediated immune homeostasis was identified as a critical regulatory mechanism for autoimmune disease developmement (155). Tregs are an essential component in regulating immune tolerance to prevent autoimmunity (5, 6). Many studies have described

abnormalities in the functions of Tregs in patients with autoimmune conditions (156). Therefore, Treg-based therapeutic approaches are being investigated as potential treatments for autoimmune conditions (157). Adoptive transfer of Tregs into mice and patients with inflammatory disorders can restore immune tolerance in pro-inflammtory environments (78, 158, 159). *Ex vivo* expansion and adoptive transfer of Tregs into diabetes-susceptible NOD mice can restore immune tolerance and reverse diabetes (158). A recent report also demonstrated that *ex vivo*-expansion and transfer of autologous polyclonal Tregs is safe and well tolerated in adult patients with recent-onset T1D (157). Therefore, targeting or utilizing Tregs is now being evaluated in human clinical trials for therapeutic potential in autoimmune diseases (160, 161), but clinicians face significant hurdles in maintaining Tregs of highly purify or functional stability for autoimmune diseases treatment. Targeting Treg signaling pathways has been shown to aid in the expansion and/or functionality of Tregs *ex vivo* for treating autoimmune diseases (162). Inhibition of the PI3K-Akt-mTOR pathway can enhance Treg cell development and function *in vitro* and *in vivo* (162). Histone deacetylases (HDACs) and histone acetyltransferases (HATs) are suggested as potential targets to epiegentically modulate of Treg cells function (163, 164). Therefore, understanding how Treg cell signaling alters Treg development and function is necessary to advance Treg based immunotherapy in autoimmune diseases treatment.

CONCLUSION

Autoimmune diseases are approaching epidemic levels, estimated to affect 5-8% of the U.S. population. These encompass a broad range of conditions, including multiple sclerosis, rheumatoid arthritis, inflammatory bowel disease, type-1 diabetes, psoriasis and chronic graft-versus-host disease associated with tissue transplants. In many cases, pathogenesis is attributed to self-reactive T cells that recognize tissue-specific auto-antigens and trigger destruction of

tissues through the production of inflammatory cytokines (39). Disease management varies widely, but consists primarily of neutralizing effector cytokines or reducing T cell receptor (TCR) and co-stimulatory signaling. Although some treatments have shown promise, low efficacy, off-target effects and increased susceptibility to infections are routinely observed and demand the search for additional, more precisely targeted, therapeutic candidates. Of particular interest are therapeutic strategies that favor the development of immunosuppressive T regulatory cells (Tregs) which favor self-tolerance at the expense of self-reactivity and inflammation. Because TCR and cytokine signal integration influence effector lineages commitment, intervention at this level represents a potential means to deviate pathologic immune responses. Watford *et al.* recently demonstrated that *Tpl2*$^{-/-}$ T cells failed to up-regulate T helper 1 (Th1)-specific transcription factors responsible for IFNγ induction, including STAT4 and T-bet, in response to TCR signaling and *Tpl2*$^{-/-}$ mice were more susceptible to infection with the intracellular parasite *Toxoplasma gondii* (36). These findings suggest that Tpl2 normally promotes the development of T helper 1 cells that secrete IFN γ and are implicated in autoimmunity. While it represents a theoretical target for control of autoimmune disease, significant gaps in our understanding of Tpl2 function remain. Although we found Tpl2 might be required for ERK activation in activated CD4^{+} T cells in response to TCR stimulation (36, 37), it is not clear precisely how Tpl2 impinges upon TCR and cytokine signaling pathways and to what extent Tpl2 contributes to the differentiation and function of T helper cells lineages. In addition, a recent study indicated that Cot/Tpl2 mediates P-Ser473 Akt phosphorylation, and subsequently leads to activation of the Akt-ribosomal protein S6 kinase beta-1 (p70 S6k)-mTOR pathway in macrophages (165). However, we do not know whether Tpl2 is required for Akt-p70 S6k-mTOR pathway in T cells in response to TCR and/or co-stimulatory signals or how Tpl2 influences the

development and function of immunosuppressive T regulatory cells. These are important problems, because a more complete understanding of how TCR signaling pathways are regulated could reveal novel therapies for T cell-dependent autoimmune diseases. Clearly, a better understanding of the mechanisms by Tpl2 that regulates Treg development and immunosuppressive functions is needed to facilitate new Treg-based immunotherapies. Therefore, the objective of this doctoral thesis is to determine how Tpl2 regulates the development and function of immunosuppressive T regulatory (Treg) cells. Our core hypothesis is that Tpl2 normally promotes inflammation by inhibiting regulatory T cell development and their anti-inflammatory functions. In Chapter 2, we investigated how Tpl2 regulates Treg differentiation *in vitro* and development *in vivo. I*n Chapter 3, we evaluated whether Tpl2 regulates Treg immunosuppressive functions. We believe this study provides key insights into the mechanism(s) by which Tpl2 genetic ablation (or ultimately clinical inhibition) enhances Treg cell development and anti-inflammatory activities. This study provides novel information that may lead to a method to deviate pathologic inflammatory immune responses in human autoimmune diseases by promoting the differentiation of immunosuppressive Tregs through the use of Tpl2 inhibitors.

FIGURES

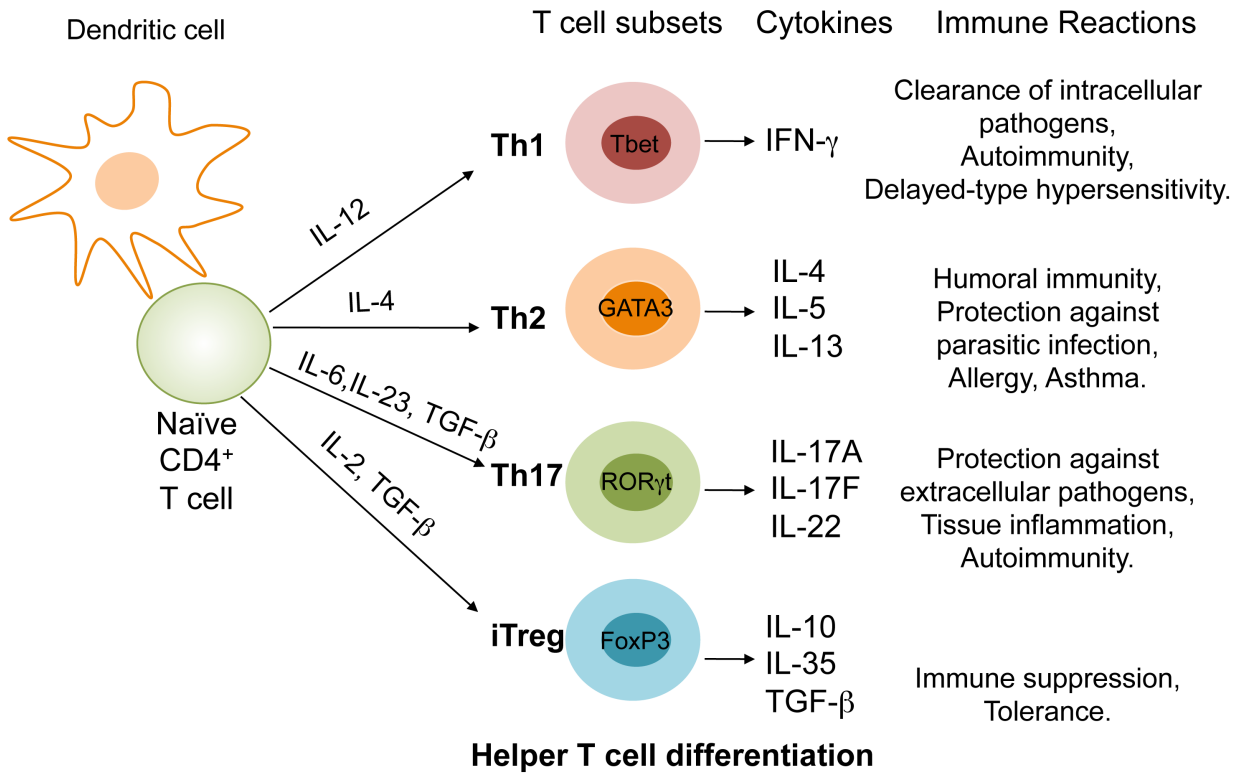

Figure 1.1: Helper T cell differentiation.

TCR signals drive naïve T cells differentiate into distinct T helper cells (Th) lineages under the direction of different cytokines signals in the local environment. These Th cells lineages can be characterized by their cytokines production profiles and by transcription factor expression. Th1 cells that are specified by the transcription factor, T-bet, produce IFN-γ to provide protection against intracellular pathogens. Th2 cells express GATA3 and secrete IL-4, IL-5 and IL-13, which are important for humoral immunity and clearance of helminth infections. Th17 cells express ROR-γt and produce IL-17 and IL-22 to clear infections by extracellular pathogens. Regulatory T (Treg) cells express FoxP3 and produce anti-inflammatory cytokines, IL-10, IL-35 and TGF-β, which inhibit pro-inflammatory immune responses and maintain immune tolerance.

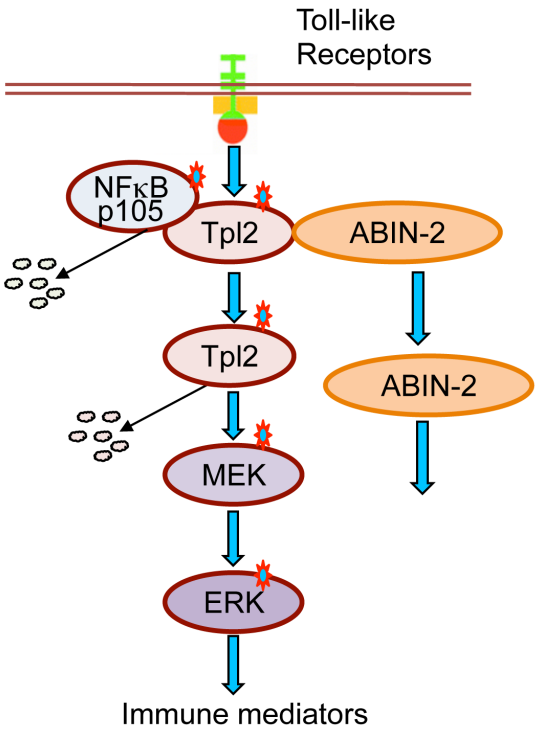

Figure 1.2: Tpl2 activation by Toll-like receptors (TLRs).

Tpl2 is activated by a variety of receptors, including Toll like receptors, G-protein coupled receptors, cytokine and antigen receptors. In un-stimulated cells, Tpl2 is associated with NF-κB p105 and A20-binding inhibitor of NF-kB (ABIN-2) in an inactivate form. NF-κB p105 and Tpl2 mutually inhibit each other. Upon activation, p105 is degraded, releasing Tpl2 from inhibition to activate its downstream substrate, MEK, which then activates ERK. This leads to the production of pro-inflammatory mediators.

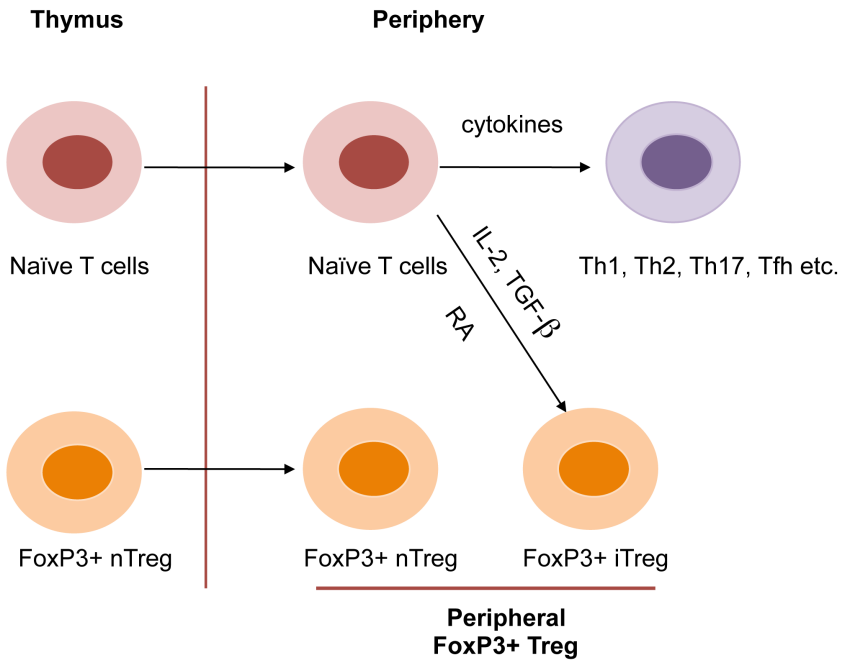

Figure 1.3: Model for generation of thymic derived natural regulatory T cells (nTregs) and inducible regulatory T cells (iTregs).

Natural Treg cells (nTregs) or thymic Treg cells (tTregs) differentiate in the thymus and migrate to peripheral tissue to maintain immune tolerance. Inducible Treg cells (iTregs) can be induced from naïve T cells under influence of the cytokines or retinoic acid (RA) and TCR signals in secondary lymphoid organs and tissues. Tregs residing in the periphery comprise both nTregs and iTregs.

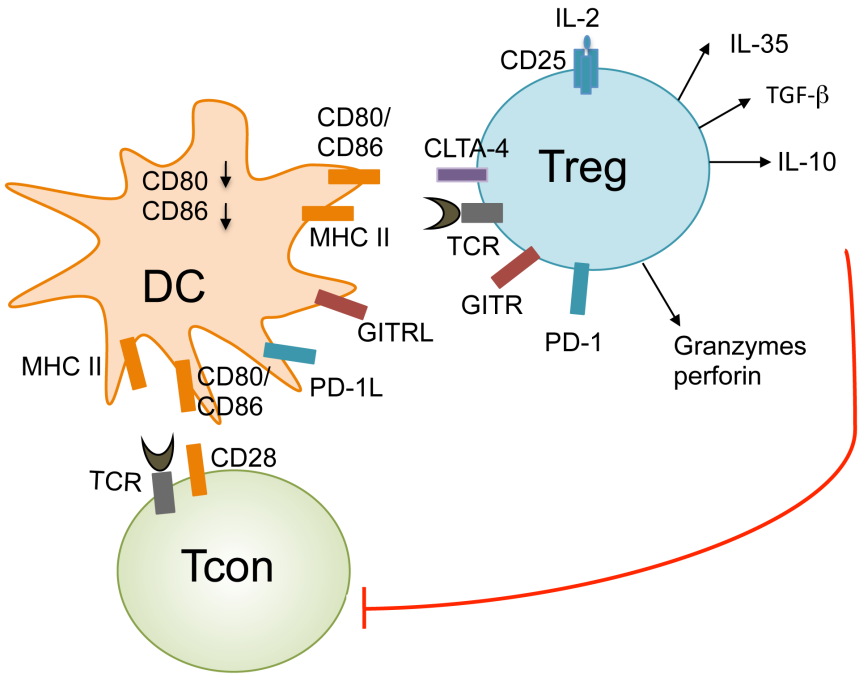

Figure 1.4: Mechanisms of Treg-mediated suppression of conventional T (Tcon) cells.

Several mechanisms have been implicated in Treg-mediated suppression of conventional T (Tcon) cells. Tregs can produce immunosuppressive cytokines, IL-10, IL-35 and TGF-β to inhibit Tcon activation and proliferation. Tregs also suppress Tcon cells survival and expansion by competitive IL-2 consumption. Granzyme and perforin also can be produced by Tregs to induce Tcon cell death. Furthermore, inhibitory co-stimulation molecules, such as CTLA-4 and PD-1 that are expressed on the Treg surface can downregulate the activation and function of antigen presenting cell (APC), thereby inhibiting Tcon activation.

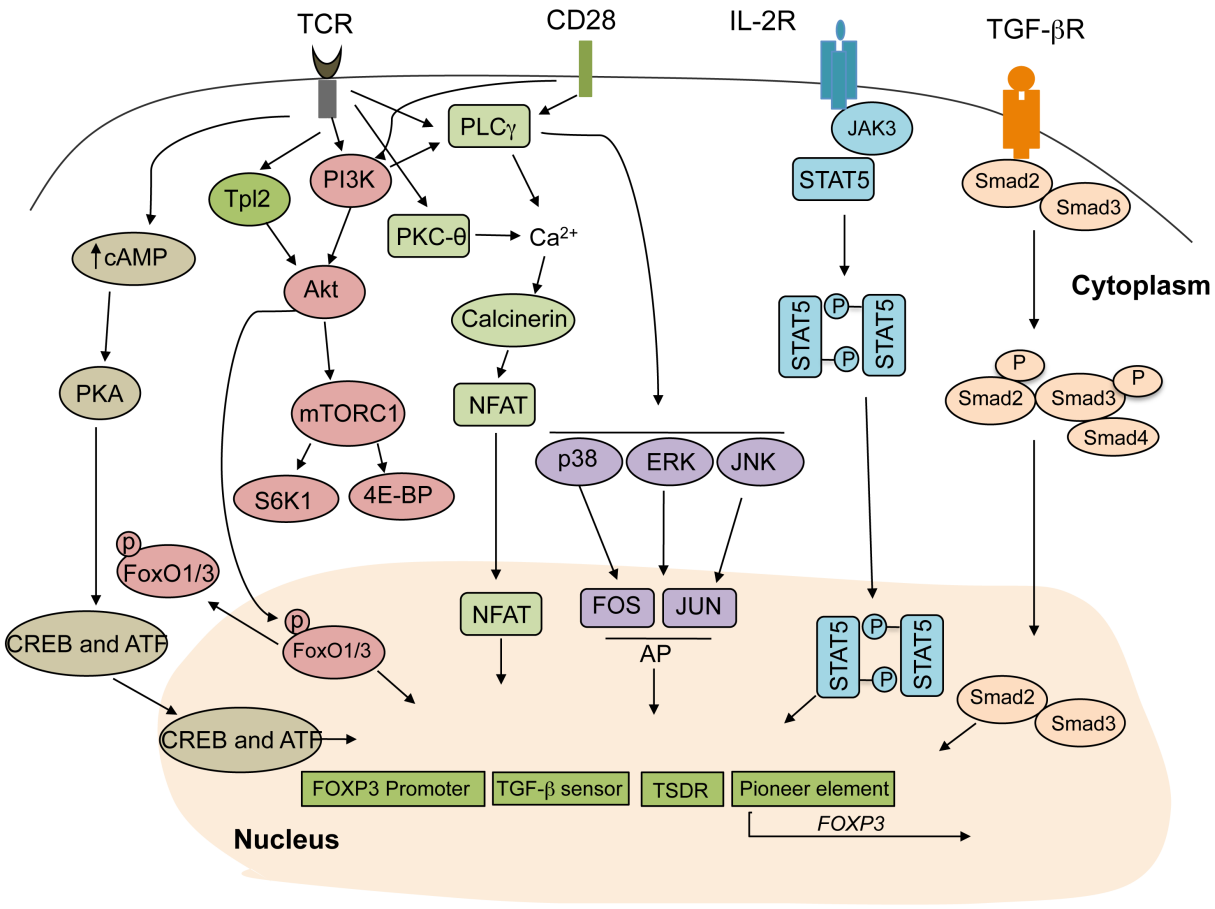

Figure 1.5: Multiple signaling pathways regulate the induction of FoxP3 expression.

Treg commitment can be determined by TCR, CD28 and cytokine receptor signals. IL-2 and TGF-β trigger activation of transcriptional factors: STAT5 and SMADs, respectively, that drive FoxP3 expression in the nucleus. TCR and co-stimulation signals can lead to the activation of multiple signaling pathways that result in the activation of transcription factors and influence the regulation of FoxP3 expression, including cAMP-responsive-element-binding protein (CREB), activating transcription factor (ATF), nuclear factor of activated T cells (NFAT), Forkhead box protein O1/3 (FoxO1/3) and activator protein 1 (AP1). However, each pathway contributes to the differential regulation (either beneficial or inhibitory) of FoxP3 expression between the different types of Tregs. For example, co-stimulation signal is required for the development of thymic

FoxP3$^+$ Tregs, whereas the activation of the phosphoinositide 3-kinase (PI3K)–AKT–mammalian target of rapamycin (mTOR) pathway inhibits FoxP3 expression during iTreg conversion.

CHAPTER 2

TUMOR PROGRESSION LOCUS 2 NEGATIVELY REGULATES INDUCIBLE T REGULATORY CELL DIFFERENTIATION

Li X., Acuff N. V., Peeks A. R., Kirkland R., Wyatt K. D., Nagy T, Watford W. T. Accepted by the *Journal of Biological Chemistry*. Reprinted here with permission of publisher, 2016.

ABSTRACT

The serine-threonine kinase, tumor progression locus 2 (Tpl2, also known as Map3k8/Cot), is a potent inflammatory mediator that drives the production of TNFα, IL-1β, and IFNγ. We previously demonstrated that Tpl2 regulates TCR signaling and modulates T helper cell differentiation by promoting IFNγ production and inhibiting IL-4 production by $CD4^+$ T cells. However, very little is known about how Tpl2 modulates the development of regulatory T cells (Tregs). Tregs are a specialized subset of T cells that express FoxP3 and possess immunosuppressive properties to limit excess inflammation to self- or non-self antigens. Because of Tpl2's documented role in promoting inflammation, we hypothesized that Tpl2 antagonizes the differentiation of immunosuppressive Tregs. Herein, we demonstrate that Tpl2 constrains the development of inducible Tregs (iTregs). *Tpl2*$^{-/-}$ naïve $CD4^+$ T cells cultured *in vitro* preferentially developed into $FoxP3^+$ iTregs *in vitro* as well as *in vivo* in a murine model of OVA-induced systemic tolerance. Treg biasing in *Tpl2*$^{-/-}$ T cells depended upon the TCR signal strength and corresponded with reduced activation of the Akt/mammalian target of rapamycin (mTOR)/S6 ribosomal protein (S6) pathway in the absence of Tpl2. These results demonstrate that Tpl2 has an important role in limiting FoxP3 expression and Treg differentiation. Overall these findings suggest that Tpl2 inhibition could be used to preferentially drive Treg induction or expansion and thereby antagonize inflammation in variety of autoimmune diseases.

INTRODUCTION

Pathogenesis of a number of autoimmune diseases is attributed to self-reactive T cells that recognize self-antigens and trigger organ-specific damage (166). Regulatory T cells are a specialized lineage of T cells with immunosuppressive properties. The forkhead box transcription factor FoxP3 is important in specifying this lineage (15, 167), and humans with mutations in the *Foxp3* gene develop severe multi-organ autoimmune disease including autoimmune enteropathy, dermatitis, thyroiditis, and typle I diabetes (44). This syndrome is highly homologous to scurfy mice that also harbor mutations within the *Foxp3* gene (168). Tregs arise naturally in the thymus (nTregs or tTregs) or can be induced from naïve conventional T cells in the periphery (inducible regulatory T cells; iTregs) (4, 90, 169, 170). Both types of $FoxP3^+$ Tregs exhibit critical immunoregulatory functions to maintain immune tolerance (4, 169). Treatment with immunosuppressive iTregs is now being evaluated for therapeutic potential in autoimmune diseases like type 1 diabetes (TID) and graft-versus host disease (GvHD) (157, 171, 172), but clinicians face significant obstacles in obtaining enough highly purified and stably immunosuppressive Tregs for treatment protocols. Therefore, a better understanding of the mechanisms that regulate Treg development and immunosuppressive functions is clearly warranted.

One molecule that has recently gained interest as a potential therapeutic target is the serine-threonine kinase, tumor progression locus 2 (Tpl2), also known as Map3k8/Cot. Tpl2 is essential for the processing, secretion and even signal transduction of TNFα (28), an inflammatory cytokine implicated in diverse autoimmune diseases including rheumatoid arthritis, inflammatory bowel diseases, psoriasis and lupus (173). Tpl2 shows low homology to other kinases, is not inhibited by the nonspecific kinase inhibitor staurosporine and is the only known human kinase

to have a proline instead of a glycine in its ATP binding region, all of which make it an attractive drug target for selective inhibition (174). In macrophages, Tpl2 is maintained in an inactive form through a stoichiometric interaction with NFκB1/p105 (175). TNF- or TLR4-mediated activation of the IκB kinase complex leads to phosphorylation of Tpl2 and its release from p105 inhibition. Phosphorylated Tpl2 is released to activate the MEK-ERK signaling pathway (23). Despite the multitude of MAP kinases, Tpl2 serves a critical, non-redundant role in TLR-dependent ERK activation leading to expression of inflammatory mediators, including TNF, IL-1β and COX-2 (28-30).

Importantly, *Tpl2*$^{-/-}$ T cells also exhibit altered T helper cell differentiation into Th1 and Th2 lineages (36, 37). We demonstrated that *Tpl2*$^{-/-}$ Th1 cells were impaired in expression of the Th1 transcription factors, STAT4 and T-bet, and secretion of IFN-γ (36). In contrast, Th2 responses and immunopathology were enhanced in *Tpl2*$^{-/-}$ mice in an OVA-induced model of allergic asthma (37). Because a number of autoimmune diseases and cancers are believed to be T cell-mediated (39), understanding how Tpl2 contributes to other T helper cell lineages, especially the development and function of Treg cells, is necessary.

Several TCR-induced signaling pathways are known to regulate iTreg development, which is favored by weak TCR signals and limited co-stimulation in peripheral T cells (67, 127, 128). In particular, inhibition of either the MEK-ERK pathway or the PI3K/Akt/mammalian target of rapamycin (mTOR)/S6 ribosomal protein (S6) pathway promotes the expression of FoxP3 and Treg-associated genes (134, 135, 137, 138). Because Tpl2 promotes the activation of ERK and the PI3K/Akt/mTOR pathway in a variety of innate cell types and in response to multiple stimuli (35-37, 165), Tpl2 deficiency might therefore be predicted to enhance FoxP3 expression. However, a previous study demonstrated that Tpl2 ablation increased inflammation-induced

intestinal tumorigenesis in APC^{min} mice, and this correlated with reduced IL-10 expression and impaired Treg generation (176). In contrast, another study demonstrated that Tpl2 was a negative regulator of Tregs, since Tpl2 destabilized the Treg lineage through inhibition of FoxP3 DNA binding activity in a MEK-ERK-dependent manner (177).

To address this apparent discrepancy in the regulation of Treg differentiation by Tpl2, we investigated the mechanisms by which Tpl2 regulates iTreg cell development *in vitro* and *in vivo* via TCR and/or cytokine-induced signals. We observed that differentiation to the iTreg lineage preferentially occurred in *Tpl2*$^{-/-}$ T cells in a T cell autonomous manner. Furthermore, we observed increased proportions of $FoxP3^+$ iTregs induced from naïve *Tpl2*$^{-/-}$ $CD4^+$ T cells *in vivo* in a murine model of OVA-induced systemic tolerance, indicating that Tpl2 plays an important role in restricting FoxP3 expression. This inhibition of FoxP3 expression by Tpl2 depended upon the strength of signal sensed by the TCR and correlated with decreased activation of the Akt-mTOR-S6 pathway in Tpl2-deficient $CD4^+$ T cells. Collectively, our data suggest that Tpl2 inhibition may provide a means to deviate pathologic immune responses, not only by impairing *de novo* induction of pathogenic Th1 cells (36), but also by promoting the differentiation and development of immunosuppressive iTregs.

MATERIALS AND METHODS

Mice

C57BL/6 and *Rag1*$^{-/-}$ mice were obtained from the Jackson Laboratory. *Tpl2*$^{-/-}$ mice backcrossed more than ten generations onto the C57BL/6 genetic background were kindly provided by Thomas Jefferson University and Dr. Philip Tsichlis (Tufts University). All wild-type (WT) and *Tpl2*$^{-/-}$ mice were bred as littermate controls. *Tpl2*$^{-/-}$ mice were also intercrossed with OT-II TCR transgenic mice obtained from the NIH repository. C57BL/6-Ly5.1 (CD45.1) mice were

purchased from Charles River Laboratories. Animals were used at six-to-sixteen weeks of age as indicated and were age- and sex-matched for individual experiments. Animals were maintained in sterile microisolator cages on the same housing rack of the Central Animal Facility of the College of Veterinary Medicine. All experiments involving mice were performed according to the University of Georgia guidelines for laboratory animals and were approved by the UGA Institutional Animal Care and Use Committee.

Cell isolation and purification

Spleens, lymph nodes or thymi were disaggregated by pressing through a 70 μm filter. For spleens, red blood cells were lysed with ACK lysing buffer (Invitrogen). Naïve $CD4^+$ T cells and Tregs were isolated from spleens and lymph nodes of mice as follows. First, $CD4^+$ T cells were enriched using magnetic separation with a $CD4^+$ T cell isolation kit (Mitenyi Biotec). Untouched naïve T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^-$) and Tregs ($CD4^+CD25^+$) were further purified by FACS using antibodies recognizing CD4, CD44, CD62L and CD25 (eBiosciences). Lamina propria lymphocytes (LPLs) were purified from colons of mice as described previously (66). Bone marrow-derived dendritic cells (BMDCs) were generated as described previously (30). BMDCs were harvested after 7 days and purified by magnetic positive selection with CD11c microbeads (Mitenyi Biotec).

Cell culture

For T cell/dendritic cell co-cultures, 100,000 naïve WT OT-II^+ or *Tpl2*$^{-/-}$ OT-II^+ T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^-$) were co-cultured with either 10,000 WT or *Tpl2*$^{-/-}$ BMDCs in a volume of 200 μL medium under neutral (medium alone) or iTreg-inducing conditions (5 ng/mL rhTGF-β + 40 IU/mL rhIL-2, Peprotech) and in the presence of increasing concentrations of $OVA_{323-339}$ peptide (Peptides International). Alternatively, 100,000 WT naïve OT-II or *Tpl2*$^{-/-}$

OT-II T cells were co-cultured with 10,000 WT BMDCs in a volume of 200 μL complete medium alone (Th0) or in the presence of iTreg-inducing conditions (0, 1.25, 2.5, 5, or 10 ng/mL rhTGF-β + 40 IU/mL rhIL-2) along with 3 μM OVA ($OVA_{323-339}$, Peptides International). On day three of culture, FoxP3 expression was determined by intracellular staining using the FoxP3 Fix/Perm Kit (eBiosciences) followed by flow cytometry or by real-time PCR for *Foxp3* mRNA expression as described below. For stimulation of T cells with anti-CD3 and anti-CD28, 1 x 10^6 WT or *Tpl2*$^{-/-}$ naïve $CD4^+$ T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^-$) were plated in complete RPMI medium on wells pre-coated with the indicated concentrations of anti-CD3 and anti-CD28 as indicated concentations. Cells were cultured under iTreg-inducing conditions (40 IU/mL rhIL-2 + 2.5-10 ng/mL rhTGF-β) for 3 days and then harvested for RT-PCR, Flow cytometry or Western blot analysis.

Cell stimulation for phospho-flow staining

Splenocytes were isolated from WT and *Tpl2*$^{-/-}$ mice. After a 2-hour resting period in complete medium, cells were stained on ice with 5 μg/mL biotinylated anti-CD3 and 2.5 μg/mL biotinylated anti-CD28 (eBioscience) followed by cross-linking with 50 μg/mL streptavidin (Thermo Scientific). Cells then were stimulated for 2, 5, or 30 min in complete medium at 37°C. ERK phosphorylation within the gated naïve $CD4^+$ T cells population ($CD4^+TCR\beta^+CD25^-CD44^{lo}$) was detected by intracellular staining for anti-ERK1/2 (pT202/pY204) (BD Biosciences) and flow cytometry. For measurement of phosphorylation of ribosomal protein S6, splenocytes were stimulated with 2.5 μg/mL each of soluble anti-CD3 and anti-CD28 for 10, 20, or 30 min, and phosphorylation of S6 (Ser235/236; Cell Signaling Technology) was determined by intracellular staining within the gated naïve $CD4^+$ T cell population ($CD4^+TCR\beta^+CD25^-CD44^{lo}$).

Flow cytometry

For analysis of cell surface markers, cells were stained in PBS containing in either 5% fetal bovine serum (FBS) or 0.1% (wt/vol) bovine serum albumin (BSA) with antibodies directed against: CD4 (eBioscience, RM4-5), CD25 (eBioscience, PC61.5), CD62L (eBioscience, MEL-14), CD44 (eBioscience, IM7), TCRβ (eBioscience, H57-597), ICOS (eBioscience, C398.4A), GITR (eBioscience, DTA-1), CD45.1 (eBioscience, A20) and CD45.2 (eBioscience, 104). Annexin V-FITC (eBioscience) was stained following the manufacturer's instructions (eBioscience). Intracellular FoxP3 (eBioscience, FJK-16s) and CTLA-4 (eBioscience, UC10-4B9) were stained using the FoxP3 Fix/Perm Kit (eBioscience) following surface staining.

Measurement of mRNA expression and cytokine secretion

mRNA expression was determined as described previously using RT-PCR. SensiFAST Probe Hi-ROX kit (Bioline, Taunton, MA) and specific TaqMan probes (Applied Biosystems, Grand Island, NY). Samples were run on a StepOnePlus qPCR machine (Applied Biosystems). Results given are relative to actin control and wild type cells ($\Delta\Delta C_T$). In some cases, wild type cells were assigned a C_T value of 40 when no amplification occurred within 40 cycles. The following probe/primer sets were purchased from Applied Biosystems: *Map3k8 (Mm00432637)*, *Foxp3 (Mm00475165_m1)*, *Tgfbr1 (Mm00436964) Actinb (4352341E-1112017) and 18s (4310893E-0802039)*.

Carboxyfluorescein succinimidyl ester (CFSE) labeling

1×10^7 WT OT-II$^+$ or *Tpl2*$^{-/-}$ OT-II$^+$ naïve T cells were incubated for 8 minutes at room temperature in 1 mL PBS with 2.5 μM CFSE (Cayman Chemical). The incubation was terminated by addition of an equal volume of FBS for 1 min. Cells were washed twice with complete medium and counted. 100,000 CFSE-labeled naïve WT OT-II$^+$ or *Tpl2*$^{-/-}$ OT-II$^+$ naïve

T cells were co-cultured with 10,000 WT BMDC and 1 μM OVA ($OVA_{323-339}$, Peptides International) in a volume of 200 μL media under iTreg-inducing conditions (5 ng/mL rhTGF-β + 40 IU/mL rhIL-2, Peprotech) for 3 days. Cells were harvested and stained intracellularly for FoxP3 following surface staining.

Cell apoptosis assay

100,000 naïve WT OT-II+ or *Tpl2*$^{-/-}$ OT-II+ naïve T cells were co-cultured with 10,000 WT BMDC and 1 μM OVA ($OVA_{323-339}$, Peptides International) in a volume of 200 μL medium under iTreg-inducing conditions (5 ng/mL rhTGF-β + 40 IU/mL rhIL-2, Peprotech) for 3 days. Cells were harvested and surface stained for Annexin V following the instructions of the FITC Annexin V apoptosis detection Kit I (BD Biosciences) and stained intracellularly for FoxP3 (eBioscience, FJK-16s) using the FoxP3 Fix/Perm Kit (eBioscience).

Calcium Flux Assay

Splenocytes were harvested from WT or Tpl2-deficient mice as described above. Cells were washed once in Hank's Balanced Salt Solution (HBSS) supplemented with 0.2 mM EDTA and resuspended in HBSS containing 0.5% BSA. Cells were incubated with Fluo-4 dye (Molecular Probes) at a final concentration of 0.5 μM for 30 min at 37°C. Cells were washed twice with HBSS plus 0.5% BSA and incubated 1 hr more at 37°C. Ten million cells/mL were stained with 10 μg/mL biotinylated anti-CD3 antibody (eBioscience) for 15 minute on ice in HBSS + 0.5% BSA, washed in HBSS + 0.5% BSA containing 2 mM $CaCl_2$ and resuspended in 1 mL of the same buffer. For measurement of calcium flux, samples were heated at 37°C for 3 min, and a baseline reading was taken for 1 min on an LSRII flow cytometer (BD Biosciences). Then, 50 μL of a 100 ng/mL stock of streptavidin (Thermo Scientific) was added, and the CD3-triggered calcium flux was measured over the next 3 min. Calcium ionophore (5 μl of a 5 μg/mL stock)

was added, and the maximal calcium flux was measured for an additional 2 min.

Western Blotting

WT or *Tpl2*$^{-/-}$ CD4^{+} T cells were stimulated with the indicated ligands over a timecourse at 37°C. Cells were lysed in protein lysis buffer containing 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, 200 M Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2.5 μM nitrophenyl p-guanidinobenzoate. Proteins were separated on Bis-Tris 4-12% gradient gels (Life Technologies) and probed with antibodies to detect Tpl2 (Santa Cruz Biotechnology), phospho-Smad2 (Ser465/467), phospho-STAT5 (Tyr694) (Cell Signaling Technology), Smad2 (Cell Signaling Technology), STAT5 (Santa Cruz Biotechnology), and β-Actin (Santa Cruz Biotechnology) followed by HRP-labeled secondary antibodies. Western blots were visualized by ECL (Lumigen, Inc.).

In vivo differentiation of iTregs using a systemic tolerance model

Systemic tolerance was induced as previously described (136, 178). Briefly, 4 million sorted naïve WT OT-II^{+} or *Tpl2*$^{-/-}$ OT-II^{+} T cells (CD4^{+}CD44^{lo}CD62L^{hi}CD25^{-}) were adoptively transferred into C57BL/6-Ly5.1 (CD45.1^{+}) congenic recipient mice. After 24 hours, each recipient was injected with 5 μg $OVA_{323-339}$ peptide or an equal volume of PBS alone. At day 8 post-injection, spleens and mesenteric lymph nodes (MLNs) were harvested and stained for the quantitation of FoxP3^{+}CD4^{+} iTreg conversion.

Statistical analysis.

Unless otherwise indicated, *p* values were calculated using two-tailed Student's *t*-test, and error bars represent means ± SEM.

RESULTS

Tpl2 is dispensable for nTreg development under homeostatic conditions.

In order to determine whether Tpl2 regulates Treg development or functions, we first measured the relative expression of Tpl2 in Tregs isolated from spleens and lymph nodes of C57BL/6 (WT) mice. Compared to sorted $CD4^+CD25^-$ naïve T cells, $CD4^+CD25^+$ Tregs expressed approximately six-fold more Tpl2 mRNA and protein (Figure 2.1A-B). Because Tregs have previously received a TCR signal to differentiate, we next investigated whether the increased Tpl2 expression in Tregs is a consequence of prior TCR stimulation. Tpl2 gene expression was measured in activated T cells and iTregs. Indeed, Tpl2 was significantly induced by approximately 37-fold in naïve T cells that had been activated for three days with anti-CD3 and anti-CD28 (Th0; Figure 2.1A-B). In contrast, Tpl2 expression in activated T cells cultured under iTreg-inducing conditions showed significantly lower Tpl2 expression similar to that observed in freshly isolated Tregs. These data suggest that (1) TCR signals induce Tpl2 expression and (2) TGF-β likely negatively regulates Tpl2 expression in iTregs because Tpl2 may be counter-productive for Treg functions.

We next evaluated whether Tpl2 regulates Treg development *in vivo* under homeostatic conditions. Thymi, spleens, mesenteric lymph nodes (MLNs) and lamina propria lymphocytes (LPLs) were isolated from sex-matched littermate C57BL/6 or *Tpl2*$^{-/-}$ mice derived from heterozygous matings and analyzed for Tregs. No differences were observed for the total cell numbers, proportions or absolute numbers of $FoxP3^+$ Tregs in the thymus, spleen, MLNs or LPLs between WT and *Tpl2*$^{-/-}$ mice (Figure 2.2A-E). Therefore, Tpl2 ablation does not alter the proportion or absolute number of Tregs in the central or peripheral lymphoid organs under homeostatic conditions. Helios (*Ikzf2*) is a transcription factor of the Ikaros family that is

expressed in the majority of $CD4^+FoxP3^+$ nTregs, but not in iTregs (179); therefore, Helios has been utilized as a marker to distinguish natural Tregs and peripherally derived iTregs in naïve mice. It is important to note that the majority of Tregs under homeostatic conditions are natural Tregs derived from the thymus (179). Similar proportions of $Helios^+$ $FoxP3^+$ Tregs were observed in the thymus and peripheral lymphoid organs in WT and *Tpl2*$^{-/-}$ mice (Figure 2.3A-C), suggesting that Tpl2 ablation does not alter nTreg development.

Tpl2 inhibits FoxP3 expression and iTreg differentiation *in vitro* via a T cell autonomous mechanism.

Treg differentiation is orchestrated by both T cell intrinsic factors such as TCR signaling pathways and T cell extrinsic factors, such as co-stimulatory or cytokine signals provided by accessory cells (4, 51, 170, 180). TCR signals, in combination with the cytokines IL-2 and TGF-β are important for iTreg differentiation (51, 63). To delineate the T cell intrinsic role of Tpl2 in iTreg development and differentiation, we investigated whether *Tpl2*$^{-/-}$ naïve $CD4^+$ T cells differentiate normally into iTregs *in vitro* by performing co-culture experiments. OT-II^+ TCR-transgenic naïve $CD4^+$ T cells derived from WT OT-II^+ or *Tpl2*$^{-/-}$ OT-II^+ mice were stimulated with an MHC class II-restricted OVA peptide ($OVA_{323-339}$) in the presence of WT BMDCs or *Tpl2*$^{-/-}$ BMDCs under neutral (Th0) or iTreg (TGF-β+IL-2) conditions. In co-culture with WT BMDCs, *Tpl2*$^{-/-}$ T cells preferentially developed into FoxP3-expressing iTregs compared to WT T cells via a T cell-intrinsic mechanism under iTreg conditions (Figure 2.4A; Figure 2.4C, left panel; Figure 2D, bottom). A similar trend was observed in the presence of *Tpl2*$^{-/-}$ BMDCs (Figure 2.4C, right panel), demonstrating that the dominant role for Tpl2 in iTreg development occurred within the T cell compartment. Importantly, this bias in FoxP3 expression was also observed in *Tpl2*$^{-/-}$ T cells cultured under Th0 conditions, although WT and *Tpl2*$^{-/-}$ T cells had

similarly low levels of FoxP3 expression (Figure 2.4B; Figure 2D, top). These data confirm a T cell intrinsic requirement for Tpl2 in suppressing FoxP3 expression. CTLA-4, inducible T-cell costimulator (ICOS), glucocorticoid-induced TNFR-related protein (GITR) and CD25 are all considered to be cell surface markers of Tregs (169, 181-183). Notably, CTLA-4 supports FoxP3 expression and iTreg differentiation in response to TGF-β and IL-2 (184, 185). Consistent with increased FoxP3 expression, our data revealed that *Tpl2*$^{-/-}$ iTregs also exhibit higher CTLA-4 induction than WT iTregs (Figure 2.5). However, the expression of other Treg markers, such as CD25, ICOS, and GITR (181, 183), was not different between WT and *Tpl2*$^{-/-}$ cultured iTregs (Figure 2.5). In addition, purified Tpl2-deficient T cells also displayed an increased propensity to differentiate into FoxP3^{+} iTregs even in response to stimulation with low dose anti-CD3 and anti-CD28 in the presence of IL-2 and TGF-β (Figure 2.6, left panel). These data confirm that increased FoxP3 expression in *Tpl2*$^{-/-}$ T cells is a consequence of iTreg-inducing signals, i.e. TCR plus cytokines. FoxP3 expression was favored in *Tpl2*$^{-/-}$ T cells with decreasing TCR signal strength (i.e. decreasing OVA peptide or anti-CD3 and anti-CD28 stimulations), whereas strong TCR signals compensated for Tpl2-deficiency in iTreg cultures (Figure 2.4D, and Figure 2.6 left panel). These data confirm a T cell intrinsic requirement for Tpl2 in suppressing FoxP3 expression in response to TCR signals.

Tpl2 inhibits differentiation of FoxP3^{+} iTregs independent of cell proliferation and survival.

A previous study demonstrated that *Tpl2*$^{-/-}$ CD8 T cells exhibit enhanced proliferation compared to WT CD8^{+} T cells upon TCR stimulation (35). In order to determine whether enhanced FoxP3 expression may be attributed to increased proliferation and outgrowth of FoxP3^{+} iTregs in *Tpl2*$^{-/-}$ cultures, we examined the proportion of FoxP3^{+} iTregs within WT and *Tpl2*$^{-/-}$ T cells at

individual cell divisions. WT and *Tpl2*$^{-/-}$ OT-II^{+} CD4 T cells were labeled with CFSE and cultured under iTreg conditions. Over the culture period, more *Tpl2*$^{-/-}$ T cells underwent 3-4 cell divisions (25%) compared to WT T cells (13%), confirming a modest increase in proliferation within *Tpl2*$^{-/-}$ CD4 T cells (Figure 2.7A). However, increased proliferation was unable to account for the increased frequency of *Tpl2*$^{-/-}$ iTregs, because within each cell division, there was a consistent increase in the proportion of *Tpl2*$^{-/-}$ FoxP3^{+} iTregs (Figure 2.7B-C). This demonstrates increased conversion of *Tpl2*$^{-/-}$ T cells to the iTreg lineage independent of proliferation. Furthermore, we also investigated whether increased iTreg generation may be attributed to increased survival in *Tpl2*$^{-/-}$ iTregs. WT and *Tpl2*$^{-/-}$ OT-II^{+} CD4 T cells cultured for three days under iTreg conditions were stained with Annexin V and anti-FoxP3 antibody to examine both cell survival and apoptosis of iTreg cells. There was no difference in the proportion of viable cells observed between WT and *Tpl2*$^{-/-}$ iTreg cultures within the gated FoxP3^{+} population or within all gated CD4^{+} T cells (Figure 2.8). Overall, these results demonstrate that enhanced FoxP3 expression in *Tpl2*$^{-/-}$ T cells is due to increased conversion to the iTreg lineage rather than increased outgrowth or survival of already committed iTregs.

Tpl2 inhibits FoxP3 expression by promoting activation of the mTOR signaling pathway in response to TCR signals.

In order to determine which pathways are altered in *Tpl2*$^{-/-}$ CD4^{+} T cells, we performed biochemical analyses to measure the contribution of Tpl2 to both TCR- and cytokine-induced pathways that regulate iTreg differentiation. Because of the early identification of Tpl2 as a regulator of nuclear factor of activated T cells (NFAT) signaling in T cells (186), we first examined the intracellular calcium signaling that drives NFAT activation during TCR signaling. Our data showed similar calcium release between WT and *Tpl2*$^{-/-}$ T cells (Figure 2.9) confirming

that Tpl2 does not contributed to very early TCR signaling events (Figure 2.9). This finding is consistent with a previous report showing similar IL-2 secretion, which is regulated by NFAT (28), by WT and *Tpl2*$^{-/-}$ CD4^{+} T cells. ERK inhibition is known to promote FoxP3 expression (134), and a recent study revealed that Tpl2 activates the MEK-ERK pathway to impair FoxP3 stability (177). Therefore, we first examined whether Tpl2 modulates ERK phosphorylation in naïve CD4^{+} T cells upon TCR stimulation with anti-CD3 and anti-CD28. However, acute ERK activation proceeded normally in Tpl2-deficient naïve CD4^{+} T cells (Figure 2.10). Because the AKT-mTOR-S6 pathway is another TCR signaling axis that negatively regulates iTreg differentiation (134, 137, 138), we similarly examined the phosphorylation of ribosomal protein S6, a translational regulator and robust indicator of mTOR activation (187). Significantly impaired phosphorylation of S6 was observed in Tpl2-deficient naïve CD4^{+} T cells upon TCR activation for 20 or 30 min with anti-CD3 and anti-CD28 compared to WT T cells (Figure 2.11A). A trend towards reduced S6 activation was also noted at the earlier 10 min time point. These data suggest that reduced mTOR signaling contributes to enhanced iTreg differentiation in the absence of Tpl2. Because S6 is the substrate of ribosomal protein S6 kinase beta-1 (S6K1) that is phosphorylated by mammalian target of rapamycin complex 1 (mTORC1), reduced S6 activation in *Tpl2*$^{-/-}$ T cells suggests that Tpl2 promotes mTORC1 activation. We further tested whether treatment with rapamycin, an inhibitor of mTORC1, leads to similar enhancement of iTregs in WT and *Tpl2*$^{-/-}$ T cells. Indeed, rapamycin treatment increased the proportion of FoxP3^{+} WT iTregs to a level comparable to *Tpl2*$^{-/-}$ iTregs treated with the vehicle control (Figure 2.11B). Furthermore, rapamycin treatment did not significantly enhance the proportion of FoxP3^{+} cells in *Tpl2*$^{-/-}$ iTregs. These data further support a role for Tpl2 in enhancing FoxP3 expression via inhibition of the mTOR pathway.

We also examined whether alterations in cytokine signaling contribute to the iTreg bias in *Tpl2*$^{-/-}$ T cells. Naïve CD4^{+} T cells from WT or *Tpl2*$^{-/-}$ mice were first activated with anti-CD3 and anti-CD28 to induce expression of the high affinity IL-2Rα receptor, CD25, and then re-stimulated with IL-2 over a brief time course to analyze IL-2-induced STAT5 activation by Western blotting. Activation of STAT5 occurred with normal kinetics and magnitude in *Tpl2*$^{-/-}$ T cells (Figure 2.12). Next, we examined TGF-β responsiveness of *Tpl2*$^{-/-}$ T cells. *Tpl2*$^{-/-}$ T cells displayed increased functional sensitivity to TGF-β compared to WT T cells, since the proportion of Foxp3^{+} iTregs was significantly increased in *Tpl2*$^{-/-}$ T cells cultured with increasing concentrations of TGF-β (Figure 2.13A). A similar trend was observed in purified T cell cultures activated with low dose (2.5 μg/mL) anti-CD3 and anti-CD28 (Figure 2.6B). Increased functional sensitivity to TGF-β was not due to increased TGF-β receptor expression, because the signaling chain, *Tgfbr1,* was expressed at similar levels in naïve T cells isolated from WT and *Tpl2*$^{-/-}$ mice (Figure 2.13B). To test the effect of Tpl2 ablation on Smad2 activation, Th0 cells were stimulated over a time course under iTreg-inducing conditions (10 ng/mL TGF-β in combination with 100 IU/mL IL-2 and 10 μg/mL anti-CD3), and Smad2 activation was assessed. Modestly increased levels of phosho-Smad2 activation were observed very early after stimulation in *Tpl2*$^{-/-}$ CD4^{+} T cells based on three individual experiments (Figure 2.13C), although this modest effect is unlikely to contribute to the observed phenotypic changes. These data may suggest Tpl2 somehow integrates TGF-β signals with TCR signal to suppress FoxP3 expression during iTreg conversion. Collectively, biochemical studies clearly demonstrate that Tpl2 promotes TCR signaling through mTOR to suppress FoxP3 expression during iTreg conversion.

Tpl2 inhibits iTreg development *in vivo* in a murine model of systemic tolerance.

We next evaluated whether Tpl2 inhibits iTreg development *in vivo* in a murine model of OVA-induced systemic tolerance. In this model, iTregs are induced in response to OVA antigen administered systemically in the absence of adjuvant (136, 178). Donor naïve $CD4^+$ T cells were purified from WT OT-II^+ or *Tpl2*$^{-/-}$ OT-II^+ transgenic mice expressing the CD45.2 congenic marker and adoptively transferred into C57BL/6-Ly5.1 recipient mice expressing CD45.1. Twenty four hours later, $OVA_{323-339}$ peptide was injected intravenously into $CD45.1^+$ recipients in the absence of adjuvant. After 8 days, a subpopulation of donor OT-II^+ transgenic T cells could be seen to differentiate from naïve $CD4^+$ T cells into $FoxP3^+$ iTregs ((178) and Figure 2.14A-B). FoxP3 was nearly undetectable in either WT or *Tpl2*$^{-/-}$ transferred $CD45.2^+$ OT-II^+ T cells without systemic administration of OVA antigen (Figure 2.14B). However, upon OVA administration, *Tpl2*$^{-/-}$ OT-II^+ donor T cells differentiated significantly more efficiently into iTregs in the spleen and MLN than did WT OT-II^+ donor cells (Figure 2.14B-C). These results are consistent with the observed Treg bias in *Tpl2*$^{-/-}$ $CD4^+$ T cells *in vitro* and confirm the role for Tpl2 and TCR signaling in regulating FoxP3 expression and iTreg induction *in vivo*.

DISCUSSION

Our data demonstrate that Tpl2 is dispensable for nTreg development but promotes iTreg conversion via a T cell intrinsic role. First, naïve *Tpl2*$^{-/-}$ OT-II^+ T cells preferentially differentiate into FoxP3-expressing iTregs when co-cultured with either WT or *Tpl2*$^{-/-}$ dendritic cells and OVA peptide antigen, demonstrating that the dominant functional role for Tpl2 reside within the T cell compartment. *In vivo*, we also observed a significant increase in the proportions of $FoxP3^+$ iTregs in the spleen and MLNs in an OVA antigen-induced murine model of systemic tolerance. Enhanced iTreg conversion of *Tpl2*$^{-/-}$ $CD4^+$ T cells correlated with impaired activation of the Akt-mTOR-S6 axis upon TCR activation. Futhermore, rapamycin corrected the iTreg bias by

inducing similarly increased iTreg frequencies in both WT and *Tpl2*$^{-/-}$ T cells, comfirming that Tpl2 inhibits FoxP3^{+} iTreg cell differentiation through promoting activation of the Akt-mTOR-S6 pathway.

The host complement of Tregs is comprised of both nTregs derived from the thymus and iTregs that differentiate from naïve T cells in the periphery (4, 90, 169, 170). Development of these distinct Treg subsets occurs in different anatomical locations and through different mechanisms (4, 90, 169, 170). Our data demonstrate that nTregs develop normally in *Tpl2*$^{-/-}$ mice. Because the majority of peripheral Tregs in most organs of naïve mice are thymus-derived nTregs (179, 188), normal development of nTreg in *Tpl2*$^{-/-}$ mice likely also explains the similar pool of peripheral Tregs observed in naïve Tpl2-deficient and WT mice. In order to specifically interrogate iTreg conversion by naïve antigen-specific WT and *Tpl2*$^{-/-}$ CD4^{+} T cells, a murine model of systemic tolerance was utilized. Analysis of iTreg cell conversion from naïve CD4^{+} T cells *in vivo* in response to antigen stimulation in the absence of adjuvants clearly revealed that induction of iTreg differentiation was enhanced by Tpl2 ablation via a T cell intrinsic mechanism.

Development of nTregs in the thymus requires strong TCR signals (reviewed in (189)), whereas weak TCR signals and limited co-stimulation favor iTreg development in the periphery (72, 127, 128). Several TCR signaling molecules and pathways have been implicated in the integration of TCR signals to restrict iTreg differentiation, including PKC-θ, mTOR, Akt and MEK-ERK (134, 135, 138). For instance, PKC-θ is critically important for thymic Treg development and function by activating the calcineurin/nuclear factor of activated T-cells (NFAT) pathway, but inhibition of PKC-θ promotes iTreg differentiation via the Akt-FoxO1/3a pathway (132, 136). In addition, activation of MEK-ERK pathway or the Akt-mTOR-S6 pathway inhibits FoxP3 expression

during iTreg conversion (134, 135, 137, 138). Although prior studies have shown a defect in TCR-induced ERK activation in previously activated Tpl2-deficient T cells (35-37), no defect in ERK phosphorylation was observed in the current study in freshly isolated naïve Tpl2-deficient $CD4^+$ T cells upon anti-CD3 and anti-CD28 stimulation. This finding is consistent with another recently published report (190). Consistent with a previous report that demonstrated Tpl2 is required for the activation of the Akt-p70S6k pathway in LPS-stimulated macrophages (165), our data reveal a novel role for Tpl2 in activating the mTOR signaling pathway in TCR-stimulated T cells. Reduced S6 activation in *Tpl2*$^{-/-}$ T cells suggests that Tpl2 promotes mTORC1 and S6K activation. As expected, treatment with the mTORC1 inhibitor, rapamycin, eliminated the iTreg bias by inducing similarly increased iTreg frequencies in both WT and *Tpl2*$^{-/-}$ T cells. Therefore, enhanced iTreg differentiation by *Tpl2*$^{-/-}$ T cells, as observed *in vitro* and *in vivo,* likely results initially from reduced activation of Akt-mTOR-S6 pathway by *Tpl2*$^{-/-}$ T cells in response to TCR stimulation. Consistent with altered TCR signaling, FoxP3 expression was favored in *Tpl2*$^{-/-}$ T cells with decreasing TCR signal strengths (i.e. decreasing OVA peptide), whereas strong TCR signals partially compensated for Tpl2-deficiency in iTreg cultures. Although our data suggest that Tpl2 ablation leads to enhanced proliferation of CD4 T cells, which was similarly observed in CD8 T cells (35), enhanced FoxP3 expression in *Tpl2*$^{-/-}$ T cells is due to increased conversion to the iTreg lineage rather than increased outgrowth (or survival) of already committed iTregs. We also demonstrated that Tpl2 inhibits CTLA-4 expression in iTregs. The fact that AKT-mTOR-S6 pathway activation inhibits FoxP3 expression and CTLA-4 induction during *de novo* Treg conversion (191), is consistent with our observation of reduced mTOR activation in *Tpl2*$^{-/-}$ $CD4^+$ T cells.

TGF-β is also clearly important in the induction and maintenance of FoxP3 expression by

working in concert with TCR-derived signals to activate the *Foxp3* enhancer (63, 192). Accordingly, TGF-β-induced phosphorylation of Smad2 was modestly increased in *Tpl2*$^{-/-}$ Tregs, which correlated with an increase in TGF-β-dependent FoxP3 expression. The increased functional sensitivity to TGF-β by *Tpl2*$^{-/-}$ T cells is consistent with our previous finding that high concentrations of TGF-β impaired Th17 differentiation of *Tpl2*$^{-/-}$ naïve CD4^{+} T cells by inappropriately inducing FoxP3 expression under classical Th17 differentiation conditions of IL-6 + TGFβ (38). Given the increased functional sensitivity of *Tpl2*$^{-/-}$ T cells to TGF-β and the antigen dose-dependent requirement for Tpl2 during Treg development, Tpl2 may functions to integrate both TCR and TGF-β signals to suppress FoxP3 expression during iTreg conversion. Further studies are needed to understand the integration between these two signaling pathways.

One study reported that Tpl2 ablation resulted in modestly reduced Treg proportions *in vivo* in an *Apc*^{min} model of intestinal tumorigenesis, and this reduction correlated with decreased IL-10 secretion in the intestinal mucosa (176). The same study also demonstrated that Tpl2 was required for iTreg generation in response to TGF-β *in vitro* (176). In contrast, a more recent study demonstrated that Tpl2 inhibits the DNA binding activity of FoxP3 through a MEK-ERK-dependent pathway (177). Consistent with the findings of the latter study, our data further demonstrate that Tpl2 inhibits FoxP3^{+} iTreg differentiation *in vitro* and *in vivo*. The reasons for the discordant findings between the former and latter studies, including our own, are currently unclear but may relate to the relative contribution of Tpl2 to nTregs versus iTregs in the model systems used. In the former study, the majority of measurements were made within the intestines of mice on the APC$^{min/+}$ genetic background of intestinal inflammation, which is likely to alter Treg proportions. Therefore, additional studies are needed to clarify the tissue- and cell type-

specific functions for Tpl2 in the generation and maintenance of specific Treg populations *in vivo* under specific inflammatory conditions.

In conclusion, our data demonstrate a T cell-intrinsic role for Tpl2 in promoting TCR activation through enhancing Akt-mTOR-S6 signaling activation, which corresponds with impaired FoxP3 expression and iTreg differentiation in the presence of Tpl2. These findings provide important information about the therapeutic potential of Tpl2 inhibitors; Tpl2 inhibitors might be a means to enhance the expansion of more stable iTregs *in vitro* for use in Treg-based immunotherapies to treat autoimmune diseases.

FIGURES

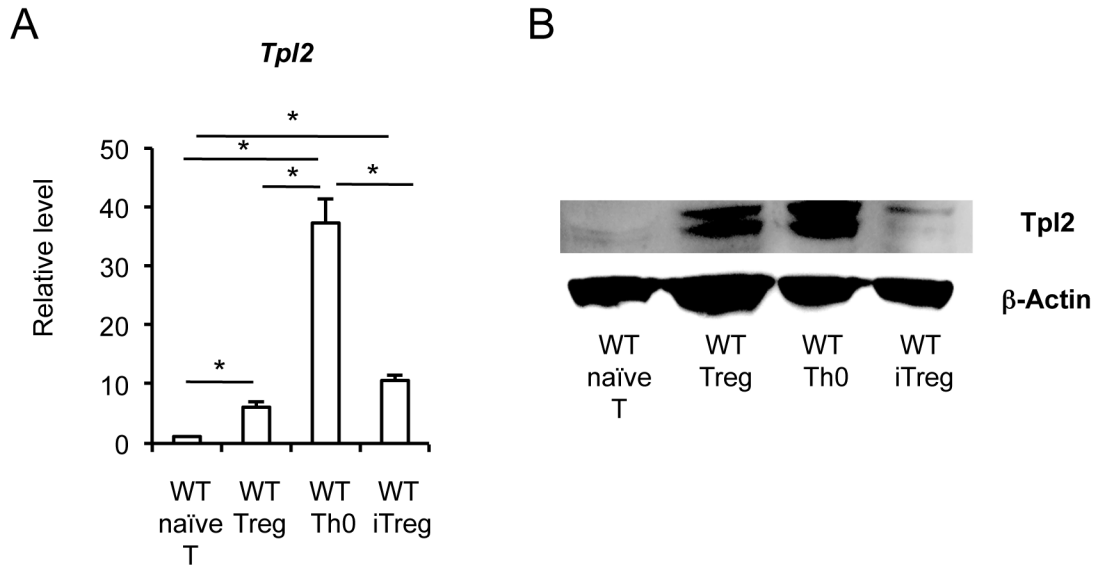

Figure 2.1:Tpl2 expression is inhibited in iTreg cells compared to activated Th0 cells.

(**A**) Sorted naïve $CD4^+$ T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^-$) and Tregs ($CD4^+CD25^+$) were isolated from WT mice. WT naïve T cells were cultured with 5 μg/mL immobilized anti-CD3 and CD28 in neutral (Th0) or iTreg (40 IU/mL rhIL-2 + 10 ng/mL rhTGF-β) conditions for 3 days. *Tpl2* mRNA expression was measured by real-time RT-PCR for freshly isolated WT naïve T cells, freshly isolated WT Tregs, day 3 cultured WT Th0 and day 3 cultured WT iTreg cells. Data are pooled from 3 or more independent experiments. *, $p<0.01$, two-tailed Student's *t*-test. (**B**) WT naïve $CD4^+$ T cells, freshly isolated Tregs, day 3 cultured WT Th0 and day 3 cultured WT iTregs were immunoblotted for Tpl2 and β-Actin. Data are representative of 2 independent experiments.

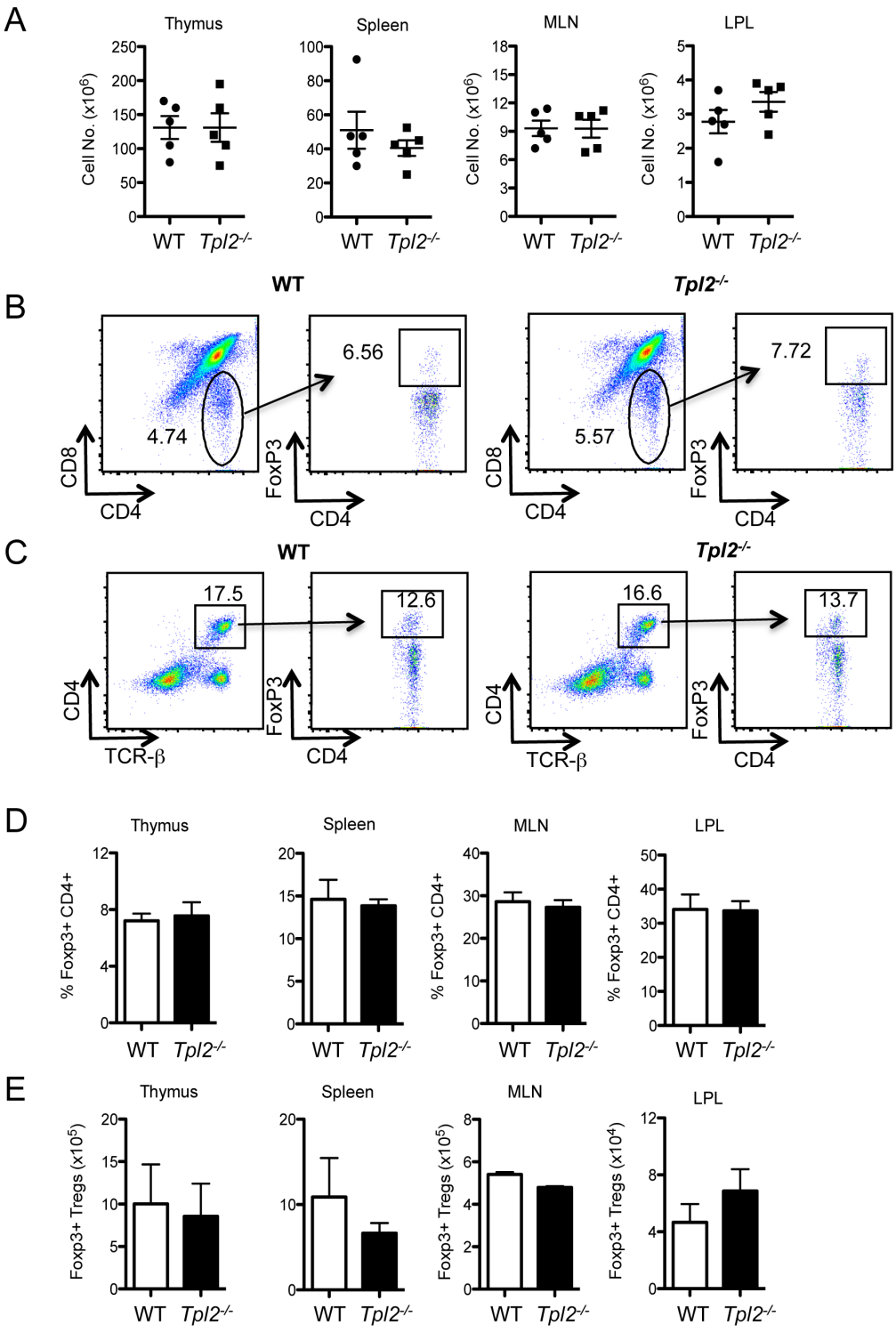

Figure 2.2: Tpl2 is dispensable for FoxP3$^+$ Treg development under homeostatic conditions.

Thymi, spleens, MLNs and LPLs were harvested from littermate control WT or *Tpl2*$^{-/-}$ mice at 16 weeks of age. **(A)** Organ total cell numbers. **(B-E)** Cells from thymi, spleens, MLNs and LPLs were stained for CD4^{+} FoxP3^{+} Tregs. The representative data and gating strategy for FoxP3^{+} Treg frequencies within CD90.2^{+}CD4^{+}CD8^{-} T cell population in thymi (**B**) and spleens (**C**) are shown. (**D**) Frequencies of FoxP3^{+} Tregs, (**E**) Absolute numbers of FoxP3^{+} Tregs. N=5 mice. Data are representative of 2 independent experiments. Two-tailed Student's *t*-test.

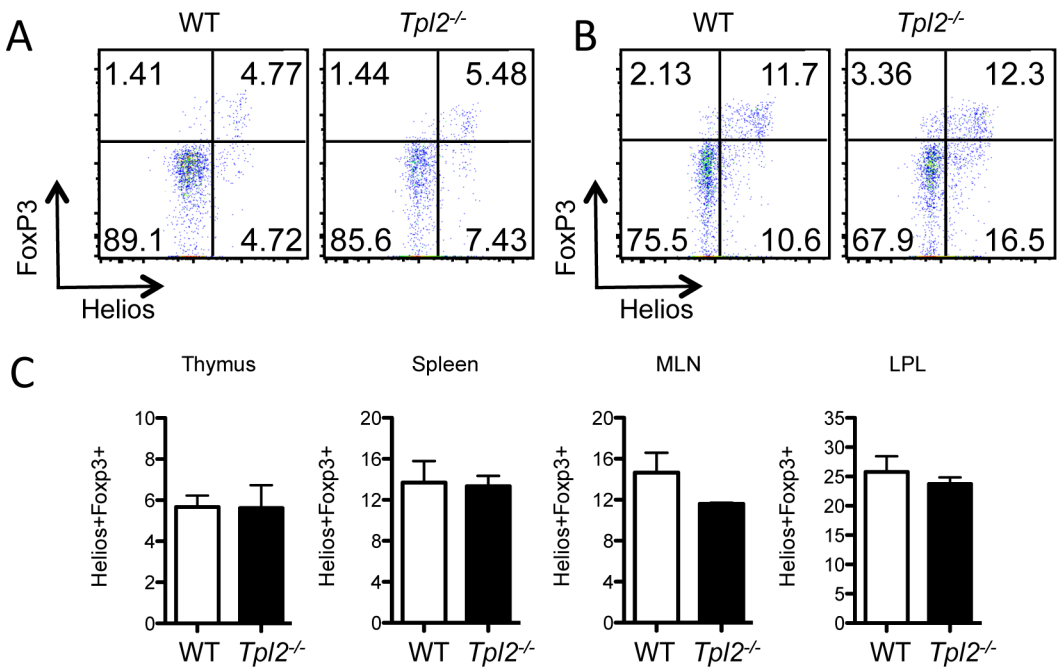

Figure 2.3: Tpl2 is dispensable for nTreg development under homeostatic conditions.

Thymi, spleens, MLNs and LPLs were harvested from littermate control WT or *Tpl2*$^{-/-}$ mice at 16 weeks of age. **(A-C)** Cells from thymi, spleens, MLNs and LPLs were stained for CD4^{+} FoxP3^{+} Helios^{+} Tregs. (**A-B**) The representative data and gating strategy for Helios^{+}FoxP3^{+} nTreg frequencies within CD90.2^{+}CD4^{+}CD8^{-} T cell population in thymi (**A**) and spleens (**B**) are shown. (**C**) Frequencies of Helios^{+}FoxP3^{+} nTregs. N=5 mice. Data are representative of 2 independent experiments. Two-tailed Student's *t*-test.

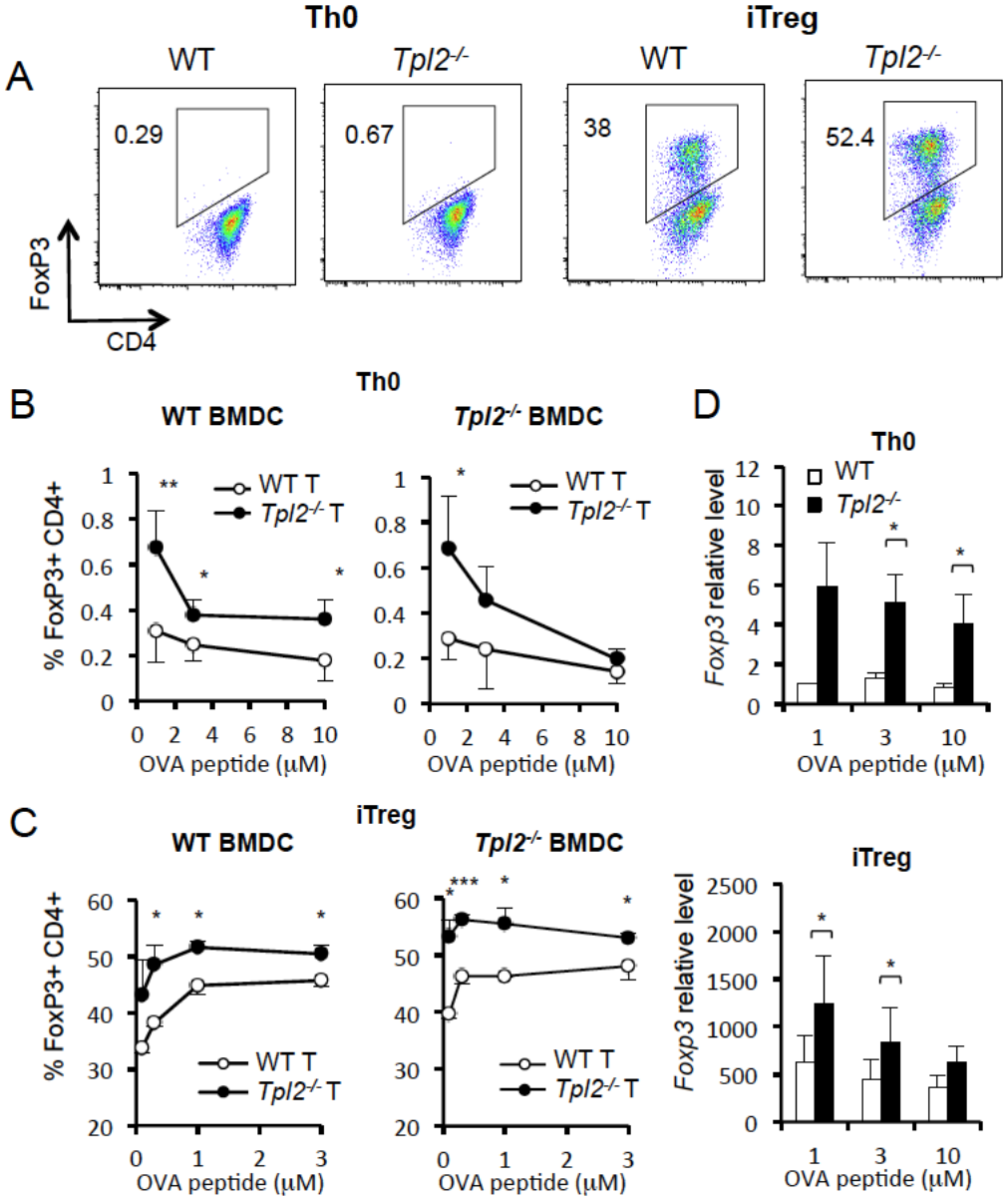

FIGURE 2.4: Tpl2 inhibits Foxp3 expression in vitro via a T cell autonomous mechanism.

(**A**) Representative strategy for gating TCRβ$^+$CD4$^+$ Foxp3$^+$ iTreg cells *in vitro* under Th0 and iTreg culture conditions with dose of 0.3 μM OVA peptide. CD4$^+$TCRβ$^+$ cells were first gated and then analyzed for the proportion of Foxp3$^+$ cells. (**B**) Either 100,000 WT OT-II or *Tpl2*$^{-/-}$ OT-II naïve CD4$^+$ T cells were co-cultured with 10,000 WT or *Tpl2*$^{-/-}$ BMDCs with increasing

doses of OVA (0.1, 0.3, 1, 3, or 10 μM) in a volume of 200 μL for three days under neutral Th0 conditions or iTreg conditions (40 IU/mL rhIL-2 + 10 ng/mL rhTGF-β). Cells cultured with WT BMDCs (left) and KO BMDCs (right) were harvested and stained intracellularly for FoxP3, and the percentage of $Foxp3^+$ gated $CD4^+$ T cells was measured by flow cytometry. N≥3 replicates. Data are presented as means ± SD. *, $p<0.05$; **, $p<0.01$, ***, $p<0.001$, two-tailed Student's *t*-test. Data are representative of three or more experiments with similar results. (**C**) FoxP3 mRNA expression was measured from cell pellets in C. Th0, N=3 individual experiments; iTreg, N=4 individual experiments. *, $p<0.05$; one-tailed paired Student's *t*-test.

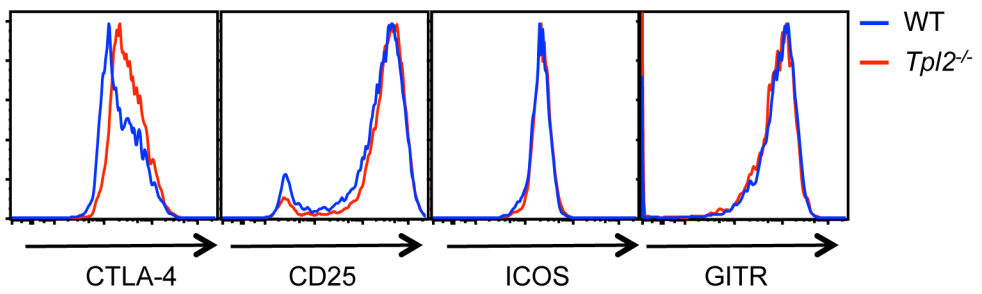

Figure 2.5: Tpl2 inhibits CTLA-4 expression of iTreg.

Either 100,000 WT OT-II^{+} or *Tpl2*$^{-/-}$ OT-II^{+} naïve CD4^{+} T cells were co-cultured with 10,000 WT BMDCs with 1μM OVA in a volume of 200 μL for three days under iTreg conditions (40 IU/mL rhIL-2 + 10 ng/mL rhTGF-β). Cells were harvested and stained for CTLA-4, CD25, ICOS and GITR. Data are representative of 3 independent experiments.

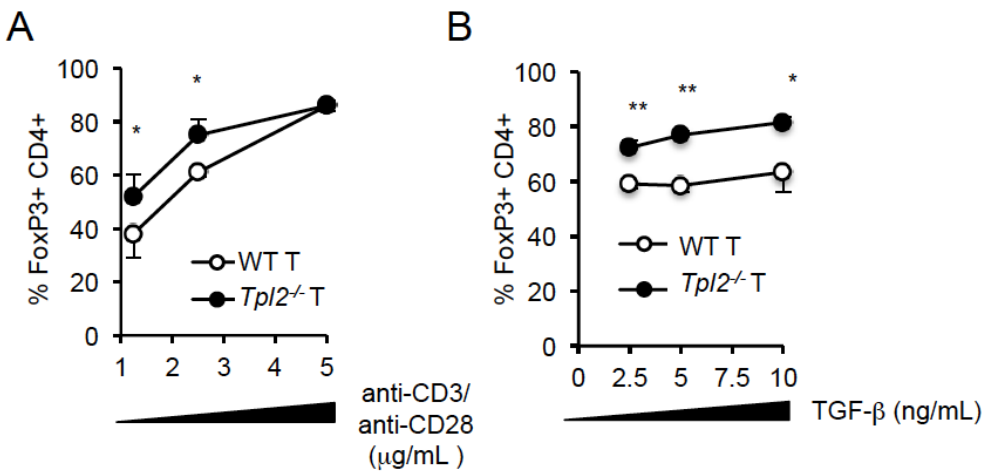

Figure 2.6: FoxP3 expression is favored in *Tpl2*$^{-/-}$ T cells with decreasing TCR signals.

(**A**) Naïve CD4^{+} T cells (CD4^{+}CD62L^{hi}CD44^{lo}CD25^{-}) were harvested from WT or Tpl2-deficient mice and cultured with increasing doses of immobilized anti-CD3 and anti-CD28 (1.25 μg/mL, 2.5 μg/mL, 5 μg/mL, or 10 μg/mL each) under iTreg-inducing conditions (40 IU/mL rhIL-2 + 10 ng/mL rhTGF-β) for 3 days Cells were harvested and stained intracellularly for FoxP3. The percentage of CD4^{+} FoxP3^{+} T cells in the gated TCR-β^{+}CD4^{+} population was measured by flow cytometry. (**B**) Naïve CD4^{+} T cells (CD4^{+}CD62L^{hi}CD44^{lo}CD25^{-}) were harvested from WT or Tpl2-deficient mice and cultured with 2.5 μg/mL anti-CD3 and anti-CD28, 40 IU/ml rhIL-2 and increasing concentrations of rhTGF-β (2.5 ng/mL, 5.0 ng/mL or 10 ng/mL) and analyzed similarly (**A**). N=5 individual experiments. Error bars represent means ± SEM. *, $p<0.05$; **, $p<0.005$; two-tailed paired Student's *t*-test.

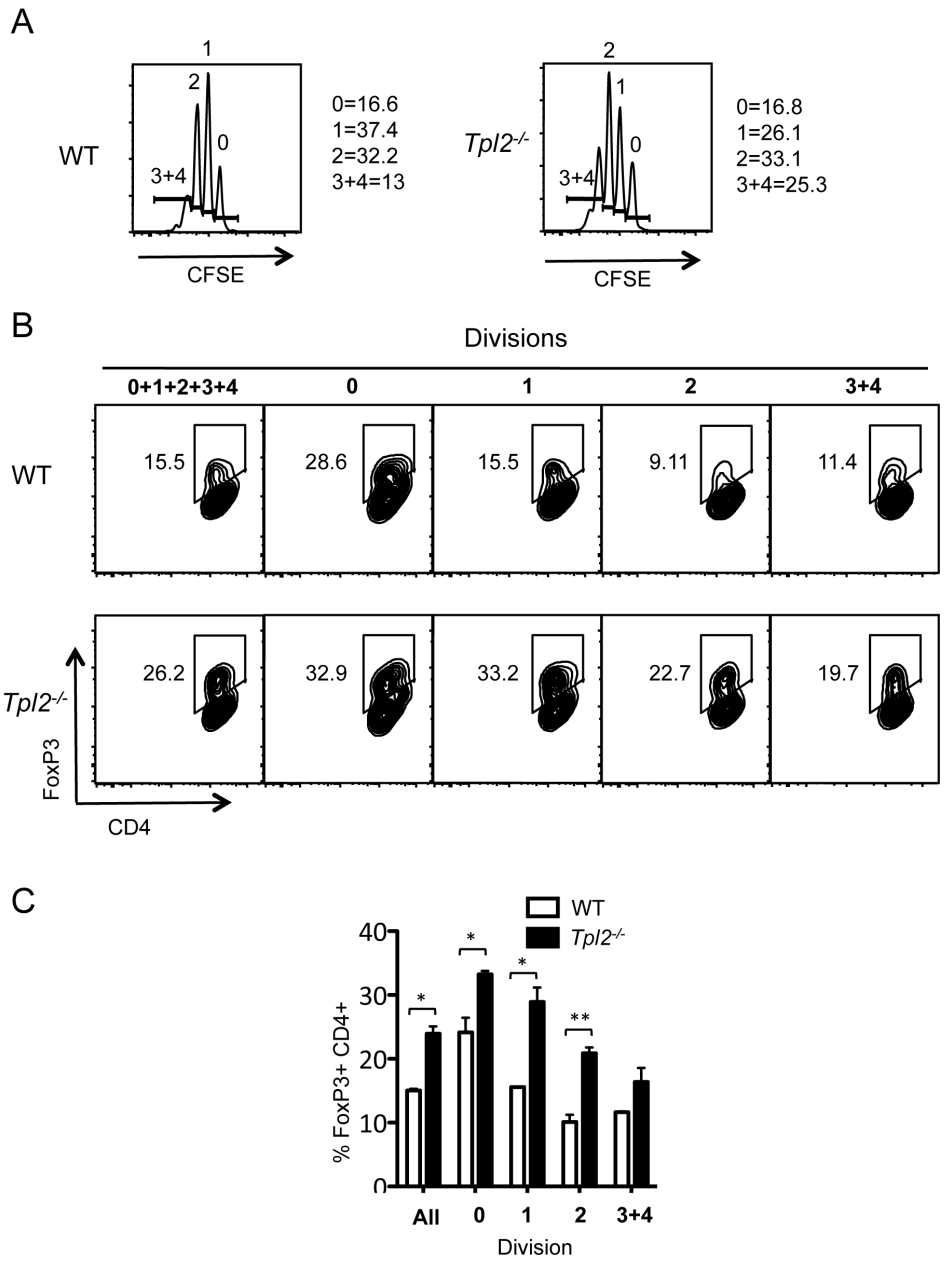

FIGURE 2.7: Tpl2 inhibits differentiation of FoxP3$^+$ iTregs independent of cell proliferation.

Either 100,000 sorted naïve WT OT-II$^+$ or *Tpl2*$^{-/-}$ OT-II$^+$ T naïve cells (CD4$^+$CD44^{lo}CD62L^{hi}CD25$^-$) were labeled with 2.5 μM CFSE and co-cultured with 1 μM OVA peptide and 10,000 WT BMDCs for 3 days under iTreg conditions (40 IU/mL rhIL-2 + 5 ng/mL rhTGF-β). WT T cells and *Tpl2*$^{-/-}$ T cells were harvested and stained for CD4, TCR-β and FoxP3.

(**A**) The proliferation of gated $CD4^+$ TCR-β^+ T cells was measured by CFSE. (**B**) Representative data showing the proportion of $FoxP3^+$ iTreg within each gated cell division of $CD4^+$TCR-β^+ T cells in (**A**). (**C**) Pooled data showing the percentage of $FoxP3^+$ iTregs within each gated cell division. N=3 replicates. Data are presented as means ± SD. *, $p<0.05$; **, $p<0.01$, two-tailed Student's *t*-test. Data are representative of three experiments with similar results.

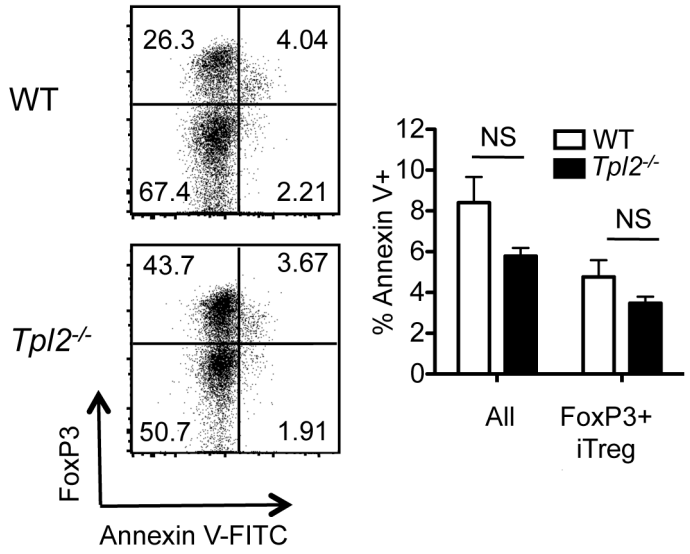

Figure 2.8: Tpl2 inhibits differentiation of FoxP3$^+$ iTregs independent of cell survival.

Either 100,000 sorted naïve WT OT-II$^+$ or *Tpl2*$^{-/-}$ OT-II$^+$ naïve T cells were co-cultured with 10,000 WT BMDCs and 1 μM OVA peptide for 3 days under iTreg conditions (40 IU/mL rhIL-2 + 5 ng/mL rhTGF-β). Cells were harvested and stained for Annexiv V-FITC and FoxP3 within the gated CD4$^+$TCR-β$^+$ T cells. Left: Representative data and gating strategy. Right: Pooled data showing the frequencies of Annexin V$^+$ T cells and Annexin V$^+$ iTregs. N=3 replicates. Data are presented as means ± SD. No significant differences were identified using a two-tailed paired Student's *t*-test. Data are representative of 2 experiments with similar results.

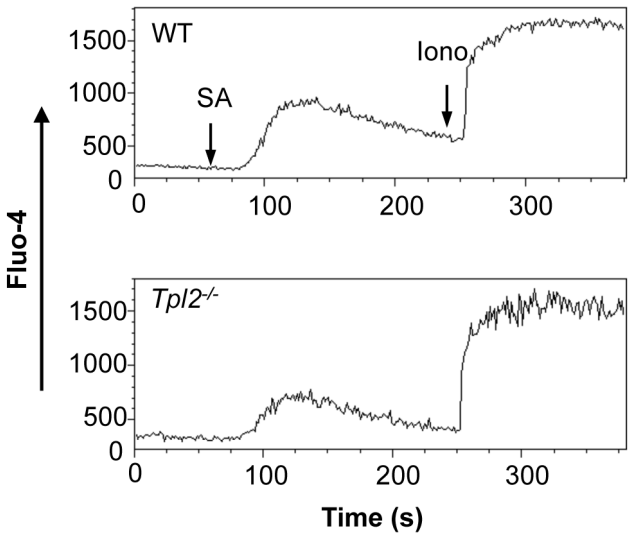

Figure 2.9: Tpl2 is dispensable for calcium release in CD4$^+$ T cells upon TCR activation.

Splenocytes were loaded with Fluo-4 calcium-sensitive dye, and cells were stimulated with anti-CD3 as described in the *Materials and Methods*. For measurement of calcium flux, samples were heated at 37°C for 3 min, and a baseline reading was taken for 1 min on an LSRII flow cytometer. Then, 50 mL streptavidin at a concentration of 100 ng/mL was added, and the CD3-triggered calcium flux was measured over the next 3 min. Calcium ionophore (5 ml of a 5 mg/mL stock) was added, and the maximal calcium flux was measured for an additional 2 min.

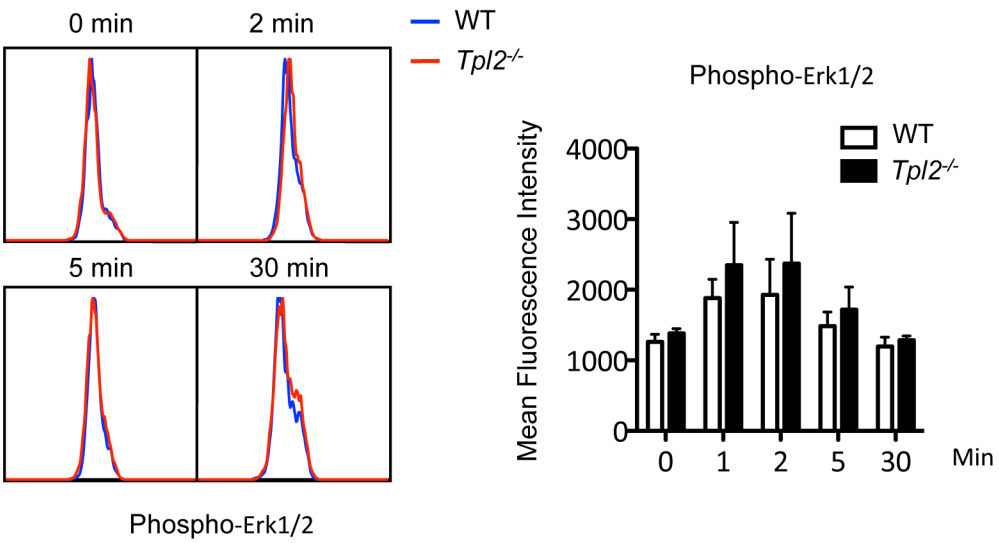

Figure 2.10: Tpl2 is not required for ERK activation in CD4$^+$ naïve T cell upon TCR stimulation.

Splenocytes were isolated from WT and *Tpl2*$^{-/-}$ mice and rested for 2 h in complete medium at 37°C and 5% CO_2. Cells were stimulated with anti-CD3 and anti-CD28 for 2, 5 or 30 min, and ERK phosphorylation within the gated naïve CD4$^+$ T cell population was detected by intracellular staining and flow cytometry (Left). The mean fluorescence intensity (MFI) of phospho-ERK1/2 within the gated naïve CD4$^+$ T cell population (Right). Data are pooled from 3 independent experiments.

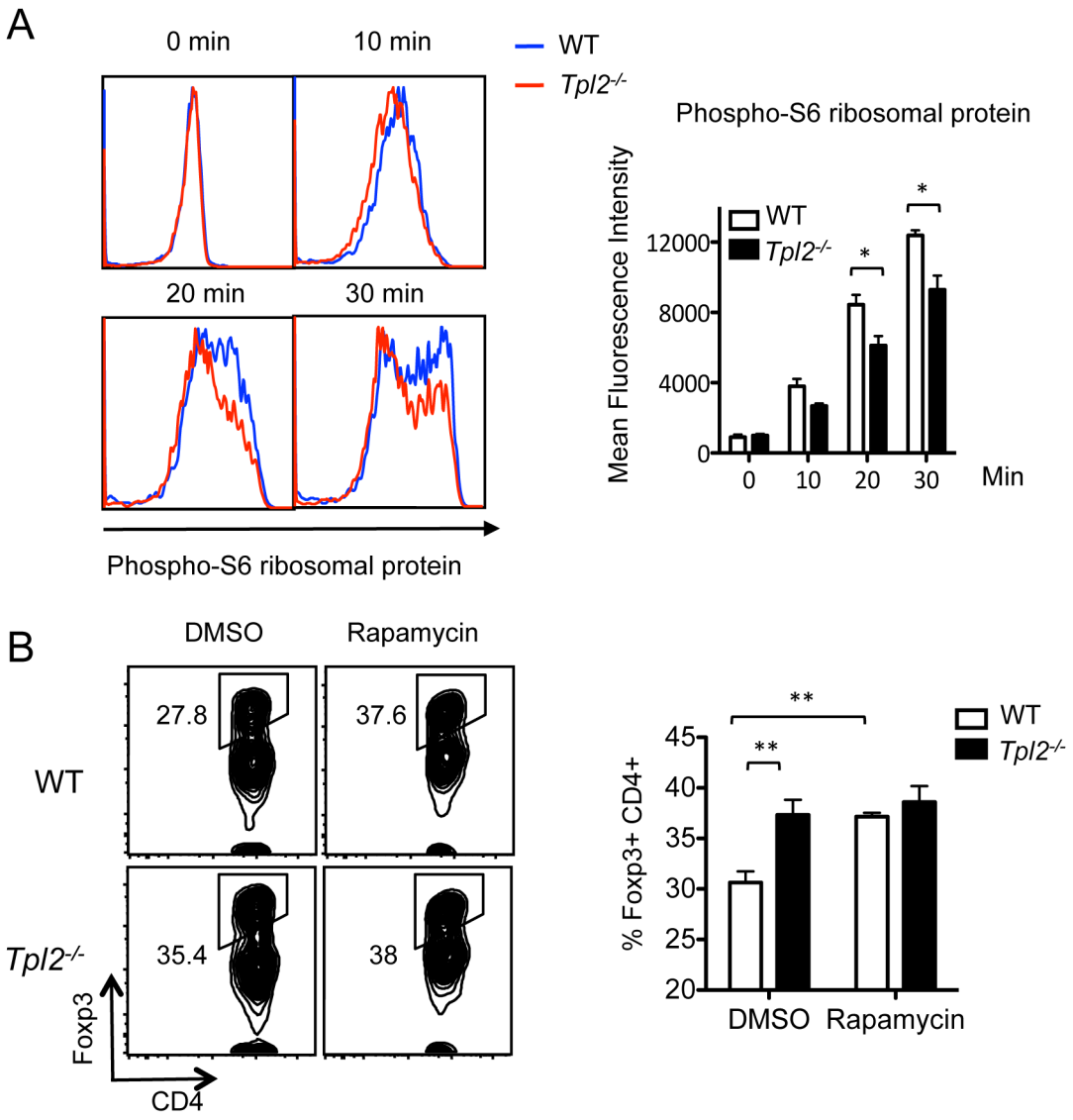

Figure 2.11: Tpl2 promotes the activation of the mTOR pathway in response to TCR signals.

(**A**) Splenocytes were isolated from WT and *Tpl2*$^{-/-}$ mice and rested for 2 h in complete medium at 37°C and 5% CO2. Cells were stimulated with anti-CD3 and anti-CD28 for 10, 20 or 30 min, and phosphorylation of S6 was determined by intracellular staining within the gated naïve CD4^{+} T cell population (Left). The mean fluorescence intensity (MFI) of phospho-S6 within the gated naïve CD4^{+} T cell population is shown (Right). Data are pooled from 3 independent experiments.

*, $p<0.05$, two-tailed paired Student's *t*-test. (**C**) Either 100,000 sorted naïve WT OT-II$^+$ or *Tpl2*$^{-/-}$ OT-II$^+$ T cells (CD4$^+$CD44^{lo}CD62L^{hi}CD25$^-$) were co-cultured with 10,000 WT BMDC and 1 μM OVA peptide for 3 days in Treg conditions (40 IU/mL rhIL-2 + 5 ng/mL rhTGF-β). WT and *Tpl2*$^{-/-}$ cultures were also treated with either 25 nM Rapamycin or DMSO vehicle control. Left: Representative data showing the proportions of FoxP3$^+$ iTregs within the gated CD4$^+$ TCR-β$^+$ T cells. Right: Pooled data from 4 or more replicates are presented as means ± SD, **, $p<0.01$, two-tailed paired Student's *t*-test. Data are representative of 3 experiments with similar results.

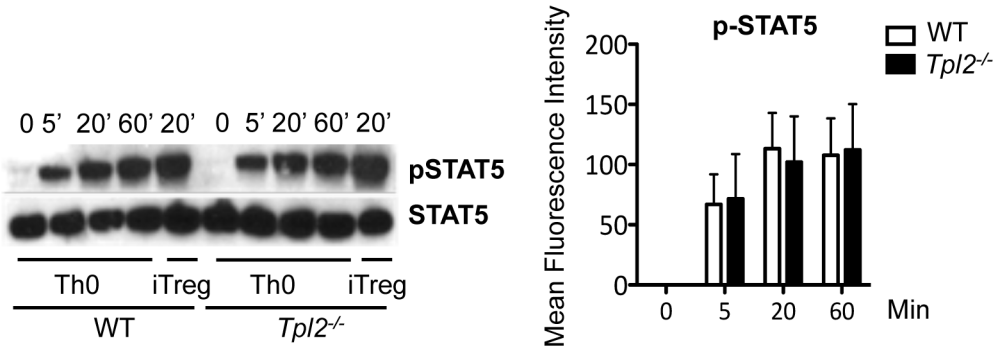

Figure 2.12: Tpl2 is dispensable for IL-2 induced STAT5 activation in CD4$^+$ cells.

Sorted WT and *Tpl2*$^{-/-}$ naïve CD4$^+$ T cells were cultured in neutral (Th0) or iTreg conditions for 3 days, followed by expansion in 100 IU/mL IL-2 for 4 more days. Cells were re-stimulated with 100 IU/mL IL-2 for 5, 20, or 60 min and immunoblotted for phospho-STAT5 and total STAT5. Left: data are representative of three experiments with similar results. Right: quantitative densitometry analysis of the phospho-STAT5 activation normalized relative to STAT5 in WT and *Tpl2*$^{-/-}$ Th0 cells pooled from three experiments. No significant differences were observed between groups; Two-tailed paired Student's *t*-test.

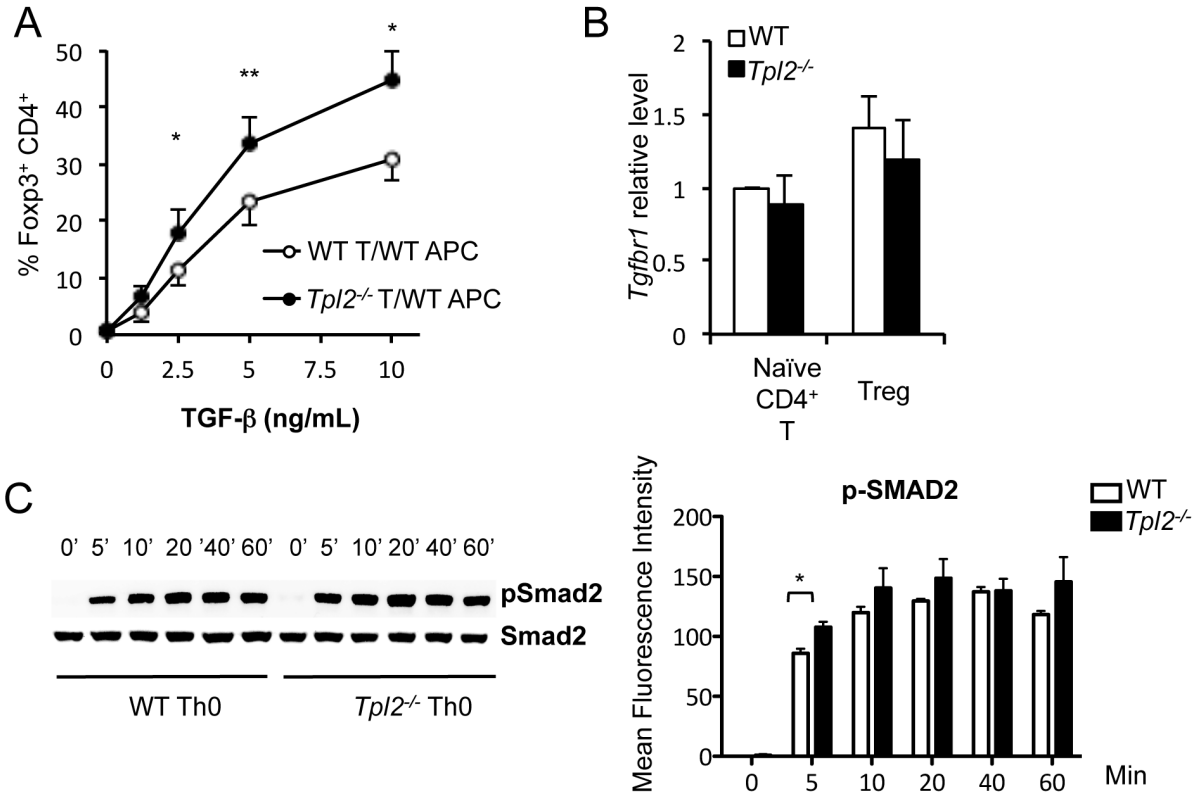

Figure 2.13: Tpl2$^{-/-}$ T cells enhances functional responsiveness to TGF-β in CD4^{+} T cells.

(**A**) Naïve CD4^{+} T cells sorted from WT OT-II or *Tpl2*$^{-/-}$ OT-II mice expressing a transgenic TCR for OVA were co-cultured with 10,000 WT BMDCs (WT APC) for three days in the presence of 3 μM OVA, 40 IU/ml rhIL-2 and increasing concentrations of rhTGF-β (0, 1.25, 2.5, 5, 10 ng/mL rhTGF-β). The proportions of FoxP3^{+} iTreg were quantitated by intracellular staining. N=4 experiments. *, $p<0.05$; **, $p<0.01$, two-tailed paired Student's *t*-test. (**B**) TGFβR1 mRNA expression was measured by RT-PCR in sorted WT and *Tpl2*$^{-/-}$ naïve CD4^{+} T cells and Tregs. N=3 experiments. No significant differences were observed between groups; two-tailed Student's *t*-test. (**C**) WT and *Tpl2*$^{-/-}$ naïve CD4 T cells were cultured in neutral (Th0) conditions for 3 days, followed by expansion in 100 IU/mL IL-2 for 2 more days. Th0 cells were

harvested and rested for 4 h. After 4 h resting, cells were stimulated with 10 ng/mL TGF-β in combination with 100 IU/mL IL-2 and 10 μg/mL anti-CD3 for 5, 10, 20, 40 or 60 min and immunoblotted for phospho-Smad2 and Smad2. Left: data are representative of three experiments with similar results. Right: quantitative densitometry analysis of the phospho-SMAD2 activation normalized relative to SMAD2 in WT and *Tpl2*$^{-/-}$ Th0 cells pooled from three experiments. *, $p<0.05$, two-tailed paired Student's *t*-test.

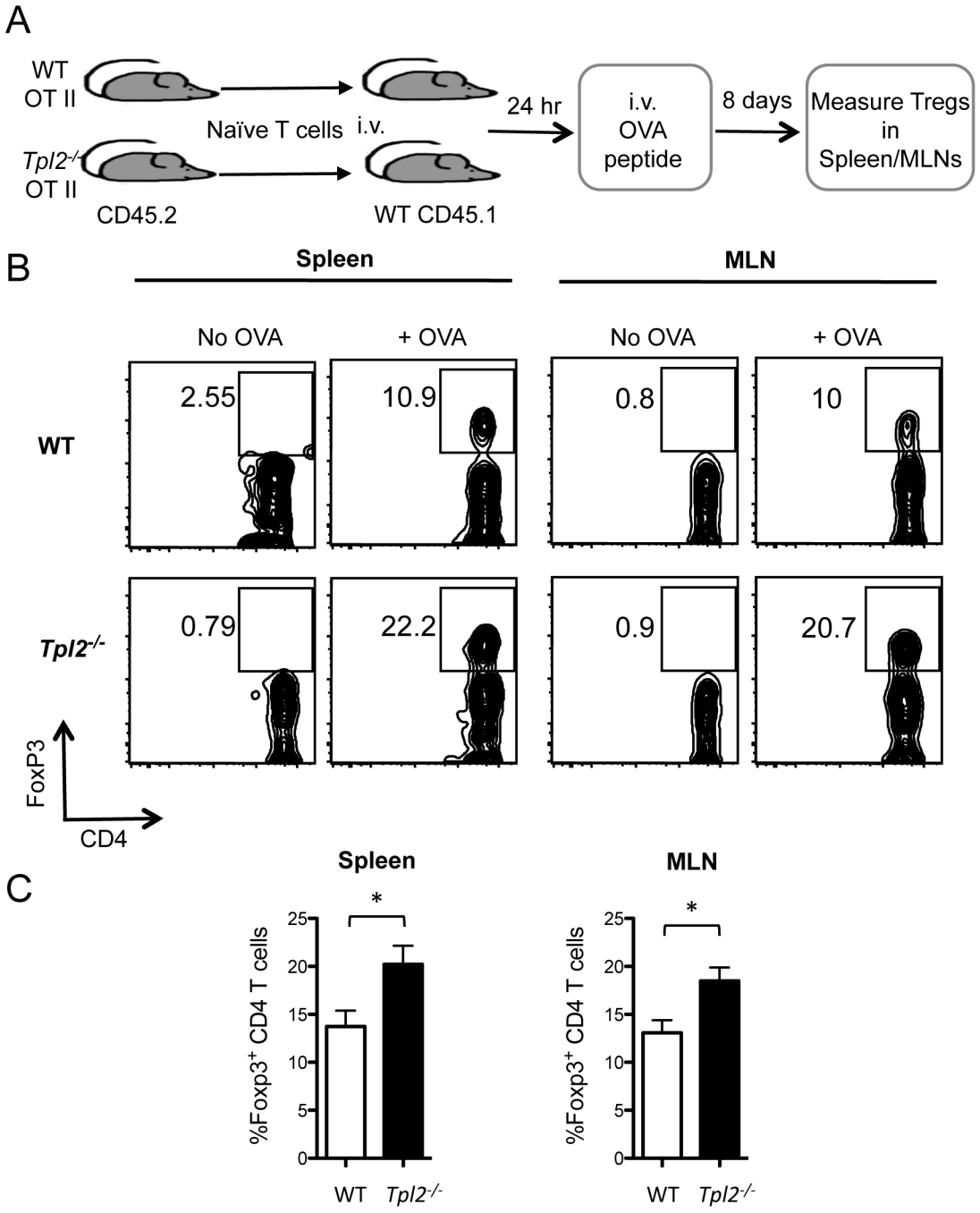

Figure 2.14: Tpl2 inhibits iTreg cell development *in vivo* in a murine model of systemic tolerance.

(**A**) 4,000,000 sorted naïve WT OT-II^{+} or *Tpl2*$^{-/-}$ OT-II^{+} T cells (CD4^{+}CD44^{lo}CD62L^{hi}CD25^{-}) were adoptively transferred into C57BL/6-Ly5.1 (CD45.1^{+}) recipient mice. 5 μg $OVA_{323-339}$

peptide or an equal volume of PBS alone was injected into CD45.1^{+} recipients 24 hours post-T cell transfer. At day 8 post-OVA peptide injection, spleens and mesenteric lymph nodes (MLNs) were harvested. (**B**) Representative data for WT or *Tpl2*$^{-/-}$ FoxP3^{+} iTreg cells within the gated CD4^{+}TCR-β^{+}CD45.2^{+} donor cell population in spleen and MLN. (**C**) Pooled data from 6 mice. Data are presented as the percentage of converted FoxP3^{+} iTreg cells within the donor population. Data are representative of 2 independent experiments. *, $p<0.05$, 2-tailed Student's *t*-test.

CHAPTER 3

THE SERINE/THREONINE KINASE, TPL2 (MAP3K8), INHIBITS REGULATORY T CELL IMMUNOSUPPRESSIVE FUNCTIONS

Li X., Acuff N. V., Peeks A. R., Kirkland R., Wyatt K. D., Nagy T, Watford W. T. Accepted by the *Journal of Biological Chemistry*. Reprinted here with permission of publisher, 2016.

ABSTRACT

Regulatory T cells (Tregs) are a specialized subset of T cells that function to maintain peripheral tolerance by limiting immune responses to self-antigens. We previously demonstrated that the serine-threonine kinase, tumor progression locus 2 (Tpl2, also designated Cot/Map3k8), regulates TCR signaling and inflammatory cytokine secretion in $CD4^+$ T cells. Herein, we reveal that *Tpl2*$^{-/-}$ Tregs have increased expression of the transcription factor FoxP3 and immunosuppressive molecules, IL-10, Epstein-Barr virus induced gene 3 (Ebi3), and cytotoxic T lymphocyte-associated protein 4 (CTLA-4). Furthermore, they were more immunosuppressive *in vivo* in a T cell transfer model of colitis, as evidenced by their enhanced ability to limit effector T cell accumulation, systemic production of inflammatory mediators, such as TNFα, IL-6, IFNγ, and monocyte chemoattractant protein-1 (MCP-1), and colonic inflammation. These results demonstrate that Tpl2 has an important role in limiting FoxP3 expression and immunosuppressive functions in Tregs. Therefore, Tpl2 inhibition could potentially deviate pathological immune responses in a variety of autoimmune diseases towards a protective, tolerogenic response through restricting Treg immunosuppressive functions.

INTRODUCTION

Inflammatory bowel diseases (IBD) are estimated to affect 1-1.3 million people in the United States (193). Although the causes of IBD still remain obscure, recent studies have demonstrated that aberrant, excessive effector T cell responses to host microbiota antigens play a central role in driving the inflammatory process (193). Regulatory T cells are a specialized subset of T cells with immunosuppressive properties that are critical in maintaining peripheral tolerance. Consequently, humans and mice with mutations in *Foxp3,* the gene that specifies the Treg lineage, develop a fatal multi-organ lymphoproliferative condition (15, 44, 167). Regulatory T cell-based immunotherapy has shown potential in the treatment of IBD due to its regulatory capacity in prevention of T cell mediated inflammation (78). Seminal studies by Powrie *et al.* (78, 84) demonstrated that treatment with $CD4^+CD25^+$ Tregs can effectively prevent the development of colitis in a murine model based upon naive T cell transfer into Rag-deficient hosts. However, clinicians face significant obstacles in obtaining Tregs of high purity, adequate numbers and functional stability for treatment protocols. Therefore, a better understanding of the mechanisms that regulate Treg immunosuppressive functions, expansion and stability is needed to facilitate Treg-based immunotherapies.

Recent attention has focused on the serine-threonine kinase, tumor progression locus 2 (Tpl2, also known as Cot/Map3k8) as a potential target for immunotherapy, because of its essential role in regulating the secretion of inflammatory cytokines, such as TNFα, IFNγ, and IL-1β (28, 30, 36), which have been implicated in diverse autoimmune diseases including inflammatory bowel diseases, rheumatoid arthritis, psoriasis and lupus (194). Tpl2 is held inactive through a stoichiometric interaction with NF-κB1-p105 (175). Inflammatory stimuli that activate the NF-κB pathway induce p105 degradation and Tpl2 phosphorylation (23). Tpl2 is transiently free to

phosphorylate MEK, leading to ERK activation, before undergoing rapid proteosomal degradation (23). A recent study demonstrated that Tpl2 may serve a critical role in destabilizing FoxP3 expression via activation of the Tpl2-MEK-ERK pathway in response to external inflammatory stimuli (177). However, information about how Tpl2 regulates Treg immunosuppressive functions is currently lacking. In this study, we observed that *Tpl2*$^{-/-}$ Tregs are more protective in a T cell transfer model of colitis, and this correlated with both reduced accumulation of effector T cells and systemic production of the inflammatory meditors TNFα, IL-6, IFNγ and MCP-1. Enhanced immunosuppressive activity also correlated with increased expression of the transcription factor FoxP3 and the immunosuppressive molecules IL-10, IL-35 subunit Ebi3, and CTLA-4 in *Tpl2*$^{-/-}$ Tregs on a per cell basis *in vitro*. Collectively, our data suggest that Tpl2 inhibition may provide a means to deviate pathologic immune responses during chronic inflammatory conditions by enhancing the immunosuppressive functions of Tregs.

MATERIALS AND METHODS

Mice

C57BL/6 and *Rag1*$^{-/-}$ mice were obtained from the Jackson Laboratory. *Tpl2*$^{-/-}$ mice backcrossed more than ten generations onto the C57BL/6 genetic background were kindly provided by Thomas Jefferson University and Dr. Philip Tsichlis (Tufts University). C57BL/6-Ly5.1 mice were purchased from Charles River. Animals were used at six-to-sixteen weeks of age as indicated, and were age- and sex-matched for individual experiments. Animals were maintained in sterile microisolator cages on the same housing rack of the Central Animal Facility of the College of Veterinary Medicine. All experiments involving mice were performed according to the University of Georgia guidelines for laboratory animals and were approved by the UGA Institutional Animal Care and Use Committee.

Cell isolation and purification

Naïve $CD4^+$ T cells and Tregs were isolated from spleens and lymph nodes of mice as follows. First, $CD4^+$ T cells were enriched using magnetic separation with a $CD4^+$ T cell isolation kit (Mitenyi Biotec). Naïve T cells ($CD4^+CD44^{lo}CD62L^{hi}CD25^-$) and Tregs ($CD4^+CD25^+$) were further purified by FACS using antibodies recognizing CD4, CD44, CD62L and CD25. Lamina propria lymphocytes (LPLs) were purified from colons of mice as described previously (66).

Cell culture

Naïve T cells and Tregs were stimulated with 40 or 100 IU/mL IL-2, respectively, in combination with 10 μg/mL immobilized anti-CD3 and 2 μg/mL soluble anti-CD28 (eBioscience) for two days. Cells were harvested and subjected to either RT-PCR or Western blot analysis.

Flow cytometry

For analysis of surface markers, cells were stained in PBS containing in either 5% fetal bovine serum (FBS) or 0.1% (w/vol) BSA with antibodies: CD4 (eBioscience, RM4-5), CD25 (eBioscience, PC61.5), CD62L (eBioscience, MEL-14), CD44 (eBioscience, IM7), CD45RB (eBioscience, C363.16A), TCRβ (eBioscience, H57-597), CD45.1(eBioscience, A20), CD45.2 (eBioscience, 104). Intracellular FoxP3 (eBiosicence, FJK-16s) was stained with the Foxp3 Fix/Perm Kit (eBiosciences) following surface staining.

Measurement of mRNA expression and cytokine secretion

Gene expression was determined by RT-PCR. SensiFAST Probe Hi-ROX kit (Bioline, Taunton, MA) and specific TaqMan probes (Applied Biosystems, Grand Island, NY). Samples were run on a StepOnePlus qPCR machine (Applied Biosystems). Results given are relative to actin control and wild type cells ($\Delta\Delta C_T$). In some cases, wild type cells were assigned a C_T value of 40 when no amplification occurred within 40 cycles. The following probe/primer sets were purchased from Applied Biosystems: *Map3k8 (Mm00432637)*, *Foxp3 (Mm00475165_m1)*, *Il10 (Mm01288386_m1)*, *Il12a (Mm00434165_m1)*, *Ebi3 (Mm00469294_m1), Ctla4 (Mm00486849). Actinb (4352341E-1112017) and 18s (4310893E-0802039)*. IL-10 concentrations in cell culture supernatants were measured by ELISA (IL-10 Ready-Set-Go ELISA, eBioscience).

Western Blotting

WT or *Tpl2*$^{-/-}$ CD4^{+} CD25^{-} Tregs were stimulated with the indicated ligands over a timecourse at 37°C. Cells were lysed in protein lysis buffer containing 0.5% Triton X-100, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 2 mM EDTA, 200 M Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 2.5 μM nitrophenyl p-guanidinobenzoate. Proteins were separated on Bis-Tris 4-12% gradient gels (Life Technologies) and probed with antibodies. Antibodies were used to detect Ebi3 (Santa Cruz Biotechnology) and β-Actin (Santa Cruz Biotechnology) in combination with

HRP-labeled secondary antibodies (Cell Signaling Techonology).

T cell transfer model of colitis

For *in vivo* suppression assays, 6- to 7- week old *Rag1*$^{-/-}$ mice were injected with 300,000 FACS-sorted naïve WT CD4^{+}CD45RB^{hi}CD25^{-} T cells alone, or in combination with 100,000 WT or *Tpl2*$^{-/-}$ CD4^{+}CD25^{+} Treg cells, intravenously in a total volume of 200 μL PBS. Mice were weighed prior to injection and weekly thereafter. Blood was drawn at three-week intervals post T cell transfer. Where indicated, the following modified protocol was used, in which a suboptimal number of Tregs was administered to specifically assess defects in Treg immunesuppresive functions. 500,000 FACS-sorted naïve WT CD4^{+}CD45RB^{hi}CD25^{-} T cells alone, or in combination with 12,500 WT or *Tpl2*$^{-/-}$ CD4^{+}CD25^{+} Treg cells, were injected intravenously into *Rag1*$^{-/-}$ mice in a total volume of 200 μL PBS according to a published report (195). Mice were weighed prior to injection and twice weekly thereafter. Blood was collected from the tail vein at the indicated intervals post transfer as well as by terminal cardiac puncture at the time of euthanasia. **S**erum cytokines were quantified using a murine Th1/Th2/Th17 or inflammation cytokine bead array (BD Biosciences).

Histopathology

Colonic sections from mice were collected and fixed in 10% neutral buffered formalin for 24 h at room temperature. Complete cross sections of formalin-fixed intestinal sections were placed in cassettes, embedded in paraffin, sectioned at 4 μm thickness, mounted on glass slides, and stained with haematoxylin and eosin (H&E). Histological sections were evaluated by a veterinary pathologist (TN) and scored according according to the following criteria: (A) Distribution of inflammation: 0=None, 1=Focal, 2=Multifocal, 3=Diffuse; (B) Degree of inflammation: 0=None, 1=Mild, 2=Moderate, 3=Severe; (C) Extent of erosion and/or ulceration:

0=None, 1=Superficial (lamina propria only), 2: Moderate (extends to the submucosa), 3: Severe (transmural). Scores were pooled to give a total inflammation score.

Statistical analysis.

P values were calculated using two-tailed Student's *t*-test, one or two-tailed paired Student's *t*-test, or 1-tailed Mann-Whitney test as indicated in the figure legends. Error bars represent means ± SEM unless otherwise indicated.

Results

***Tpl2*$^{-/-}$ Tregs offer greater protection in a T cell transfer model of colitis.**

In Chapter 2, we demonstrate that Tpl2 inhibits FoxP3 expression and the generation of inducible Tregs (iTregs). In addition, significantly increased FoxP3 expression was observed in freshly isolated *Tpl2*$^{-/-}$ Tregs (Figure 3.1A). Because FoxP3 promotes and maintains the immunosuppressive properties of Tregs (196), these findings led us to examine Tpl2's role in the regulation of Treg immunosuppressive functions. To investigate whether *Tpl2*$^{-/-}$ Tregs are more functionally suppressive, we employed an *in vivo* suppression assay. This model, based on CD4^{+} T cell transfer into *Rag1*$^{-/-}$ mice, is believed to faithfully demonstrate immune suppressive functions of Tregs *in vivo* (41, 83, 84). Colitis induced by naïve effector T cells can be prevented by co-transfer of CD4^{+}CD45RB^{low}CD25^{+} Tregs (83, 84, 195). As expected, *Rag1*$^{-/-}$ mice that received naïve T cells alone developed colitis characterized by weight loss and increased serum inflammatory cytokines, including TNF, IL-6 and IFNγ, due to a break in peripheral tolerance (Figure 3.2A-B). On the contrary, co-administration of either WT or *Tpl2*$^{-/-}$ Tregs completely blocked the development of colitis (Figure 3.2A-B). We further evaluated the immunopathology of these mice colons. We found that *Tpl2*$^{-/-}$ Tregs were as effective as WT Tregs at reducing ulcerations and the extent and distribution of inflammation along the colonic epithelium (Figure

3.2 C-D). In fact, there was modestly increased immunosuppression by *Tpl2*$^{-/-}$ Tregs *in vivo* based on body weights (Figure 3.2A). Together, these data demonstrate that Tpl2 normally functions within Tregs to limit their differentiation, proliferation and functions. Increased suppressive activity of *Tpl2*$^{-/-}$ Tregs may have been obscured by the fact that WT Tregs were already fully suppressive under the experimental conditions used (300,000 WT effector cells + 100,000 Tregs). Therefore, a modified protocol was employed in which colitis was induced by transfer of 500,000 WT naïve effector cells, and colitis was 'rescued' by co-transfer of 12,500 WT or *Tpl2*$^{-/-}$ Tregs (195) (Figure 3.3A). As expected, *Rag1*$^{-/-}$ mice that received naïve T cells alone developed colitis characterized by weight loss as early as 2-3 weeks post transfer (Figure 3.3B). On the contrary, co-administration of 12,500 WT or *Tpl2*$^{-/-}$ Tregs (Figure 3.3B) with 500,000 WT naive T cells either partially or fully blocked weight loss (Figure 3.3B). WT Tregs were only partially immunosuppressive and protected young mice from weight loss but failed to support weight gain. *Tpl2*$^{-/-}$ Tregs, however, were fully suppressive and supported significant weight gain in age-matched recipients (Figure 3.3B). Furthermore, *Tpl2*$^{-/-}$ Tregs, but not WT Tregs, were fully able to inhibit the increase in spleen and MLN total cell numbers (Figure 3.3C) and colonic inflammation (Figure 3.4A-B). These findings also correlated with systemic pro-inflammatory cytokines. *Tpl2*$^{-/-}$ Tregs effectively prevented the induction of serum pro-inflammatory cytokines TNF, IL-6, IFNγ and monocyte chemoattractant protein-1 (MCP-1) MCP-1 (Figure 3.5), which contribute to colitis development (197-200), whereas WT Tregs were less effective at reducing their induction. Consistent with this, *Tpl2*$^{-/-}$ Tregs significantly reduced the proportion of inflammatory effector T cells (T_{eff}) within spleen, MLN and lamina propria lymphocytes (LPLs) (Figure 3.6A), as well as the absolute number of inflammatory T_{eff} cells in spleen and MLN (Figure 3.6B). Although the proportions of *Tpl2*$^{-/-}$ Tregs within the lymphocyte

gate were elevated in spleen, the absolute number of Tregs remained similar or trended towards a decrease in *Tpl2*$^{-/-}$ Tregs compared to WT Tregs in the spleen and MLN (Figure 3.7A-B). Collectively, these findings demonstrate that *Tpl2*$^{-/-}$ Tregs offer superior protection in a T cell transfer model of colitis and suggest that this is achieved by increased suppressive functions of *Tpl2*$^{-/-}$ Tregs on a per cell basis.

***Tpl2*$^{-/-}$ Tregs secrete increased levels immunosuppressive cytokines.**

Next, we investigated the nature of the increased immunosuppressive activities of *Tpl2*$^{-/-}$ Tregs. Tregs inhibit effector cell activation and proliferation through cell contact-independent and contact-dependent mechanisms as described in Chapter 1 and reviewed in (90). Particularly, both IL-10 and CTLA-4 have been implicated in maintaining Treg suppressive functions in the T cell transfer model of colitis (85, 88, 201). Although Tpl2 is required for IL-10 production by antigen presenting cells in response to Toll-like receptor stimulation (33), whether Tpl2 functions similarly in Tregs is unknown. In this regard, stimulus- and cell type-specific functions of Tpl2 have been reported previously (202). To evaluate IL-10 production by *Tpl2*$^{-/-}$ Tregs, we first performed real-time PCR analysis on either freshly isolated or TCR-stimulated T cells. IL-10 expression was greater in freshly isolated Tregs compared to naïve T cells. Furthermore, IL-10 expression was basally increased in freshly isolated *Tpl2*$^{-/-}$ Tregs relative to WT Tregs (Figure 3.8A). Increased expression and secretion of IL-10 by *Tpl2*$^{-/-}$ Tregs upon activation was also confirmed by RT-PCR and ELISA (Figure 3.8A-B). IL-35, a novel heterodimeric cytokine comprising interleukin-12p35 (*Il12a*) and Epstein-Barr virus induced gene 3 (*Ebi3*), is also highly expressed in FoxP3^{+} Treg cells and is required for maximal Treg suppressive activity *in vivo* and *in vitro* (91, 203). Neither IL-12p35 nor Ebi3 basal expression was significantly altered in freshly isolated *Tpl2*$^{-/-}$ Tregs compared to WT Tregs (Figure 3.9A). However, after TCR

stimulation with anti-CD3 and anti-CD28, Ebi3 mRNA and protein expression were significantly increased in *Tpl2*$^{-/-}$ Tregs compared to WT Tregs (Figure 3.9B-C). There was also a trend towards increased IL-12p35 expression in the *Tpl2*$^{-/-}$ Tregs, although this was not statistically significant (Figure 3.9B). Consistent with increased CTLA-4 induction in iTreg cells (Figure 2.5), activated *Tpl2*$^{-/-}$ Tregs expressed higher levels of *Ctla4* compared to WT Tregs (Figure 3.10). Collectively, these results suggest that Tpl2 normally inhibits Treg suppressive functions, at least in part, through restricting the expression of the immunosuppressive cytokine IL-10, IL-35 subunit Ebi3, and the cell surface inhibitory molecule, CTLA-4.

DISCUSSION

Our data demonstrate that *Tpl2*$^{-/-}$ Tregs provide better protection against T cell-mediated colitis compared to WT Tregs in a murine model. *Tpl2*$^{-/-}$ Tregs effectively restrict systemic pro-inflammatory cytokine production and colonic inflammation. The significantly reduced inflammation correlated with reduced inflammatory T_{eff} cell accumulation in mice receiving WT naïve T_{eff} cells co-transferred with *Tpl2*$^{-/-}$ Tregs compared to mice receiving only WT naïve effector T cells or those co-transferred with WT Tregs. However, enhanced immunosuppressive activity of *Tpl2*$^{-/-}$ Tregs was not due to increases in total Treg cell numbers. Instead, the enhanced immunosuppression correlated with increased expression of immunosuppressive molecules, IL-10, IL-35 (Ebi3 subunit) and CTLA-4 *in vitro*. Collectively, our findings demonstrate that Tpl2 restricts Treg immunosuppressive functions in a T cell transfer model of colitis, suggesting that Tpl2 inhibitors may be useful for treating chronic inflammatory conditions, such as colitis.

Lymphopenia-induced rapid proliferation in the T cell transfer colitis model is thought to be driven by TCR recognition of self- and non-self antigen within the lymphopenic host (204). Co-

transfer of Tregs can rescue T cell-mediated colitis mainly through the immunoregulatory molecules, IL-10 and CTLA-4 (85, 201). IL-10 is an anti-inflammatory cytokine with critical roles in inhibiting inflammatory Th1 responses by acting on macrophages and dendritic cells to reduce antigen presentation (93). In addition, IL-10 maintains FoxP3 expression and contributes to the immunosuppressive functions of Tregs (88). Considering the published requirement for Tpl2 in IL-10 secretion by macrophages and dendritic cells (33), we were surprised by the increased production of IL-10 by *Tpl2*$^{-/-}$ Tregs. These findings further highlight the cell-type specific immunomodulatory functions of Tpl2 previously observed by Das *et al.* (202). IL-35, a heterodimeric cytokine consisting of IL-12p35 and Ebi3, is a recently identified inhibitory cytokine expressed by Foxp3^{+} Tregs (91). The Ebi3 subunit is itself a downstream target of FoxP3 and is critically required to maintain immunosuppressive activity of Tregs (91). IL-35 is a very important suppressive mediator to maintain mucosal immune homeostasis, since the recombinant IL-35 significantly reduced the development of experimental of colitis by inhibiting Th1 and Th17 cells accumulation (103). Consistent with the increased IL-10 expression, we also observed increased expression of Ebi3 and a trend towards increased expression of IL-12p35 in *Tpl2*$^{-/-}$ Tregs. CTLA-4 is a co-inhibitory molecule highly expressed by FoxP3^{+} Tregs to maintain their immunosuppressive functions (114, 182). We demonstrated that Tpl2 inhibits CTLA-4 expression in both iTregs (Chapter 2) and activated Tregs. The fact that AKT-mTOR-S6 pathway activation inhibits FoxP3 expression and CTLA-4 induction during *de novo* Treg conversion (191), is consistent with our observation of reduced mTOR activation in *Tpl2*$^{-/-}$ CD4^{+} T cells. In addition, Tpl2 was shown to promote the destabilization of FoxP3 in a MEK-ERK-dependent manner (177). Importantly, our data revealed that Tpl2 ablation positively regulates FoxP3 expression in inducible Treg (iTreg) development. Therefore, in addition to initial

induction of FoxP3 expression, Tpl2 likely exerts a tolerogenic effect on limiting effector T cells accumulation and inflammation in a lymphopenic environment.

In conclusion, our data support a T cell-intrinsic role for Tpl2 in inhibiting Treg immunosuppressive function by constraining the expression of FoxP3 and immunosuppressive molecules IL-10, Ebi3 and CTLA-4. These findings provide important information about how the therapeutic inhibition of Tpl2 might be used to deviate pathogenic immune response in variety of autoimmune diseases, including those dominated by Th1 or Th17 profiles.

ACKNOWLEDGEMENTS

We thank Monica LaGatta and Rebecca Kirkland for excellent technical support and maintenance of the animal colonies. We thank UGA's Center for Tropical and Emerging Global Diseases (CTEGD) flow cytometry core facility and Julie Nelson for FACS and the College of Veterinary Medicine's Central Animal Facility for animal care. Research reported in this publication was supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI099058 to W.T.W. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This study is also supported by the *American Association of Immunologists Careers in Immunology Fellowship* to X.L. and W.T.W.

Conflicts of Interest Disclosures

The authors have no financial conflicts of interest.

Author Contributions

X.L., W.T.W. designed the research; X.L., N.V.A., A.R.P., R.K., K.D.H., and W.T.W. performed experiments; X.L., N.V.A., T.N. and W.T.W. analyzed the data; X.L. and W.T.W. wrote the manuscript.

FIGURES

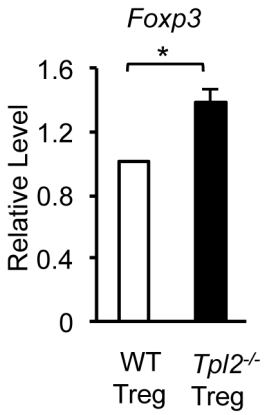

Figure 3.1: *Tpl2*$^{-/-}$ Tregs expresses higher levels of FoxP3 mRNA compared to that of WT Tregs.

FoxP3 mRNA expression was measured by RT-PCR in freshly sorted WT and *Tpl2*$^{-/-}$ Tregs. N=5 individual experiments. *, $p<0.05$, two-tailed paired Student's *t*-test.

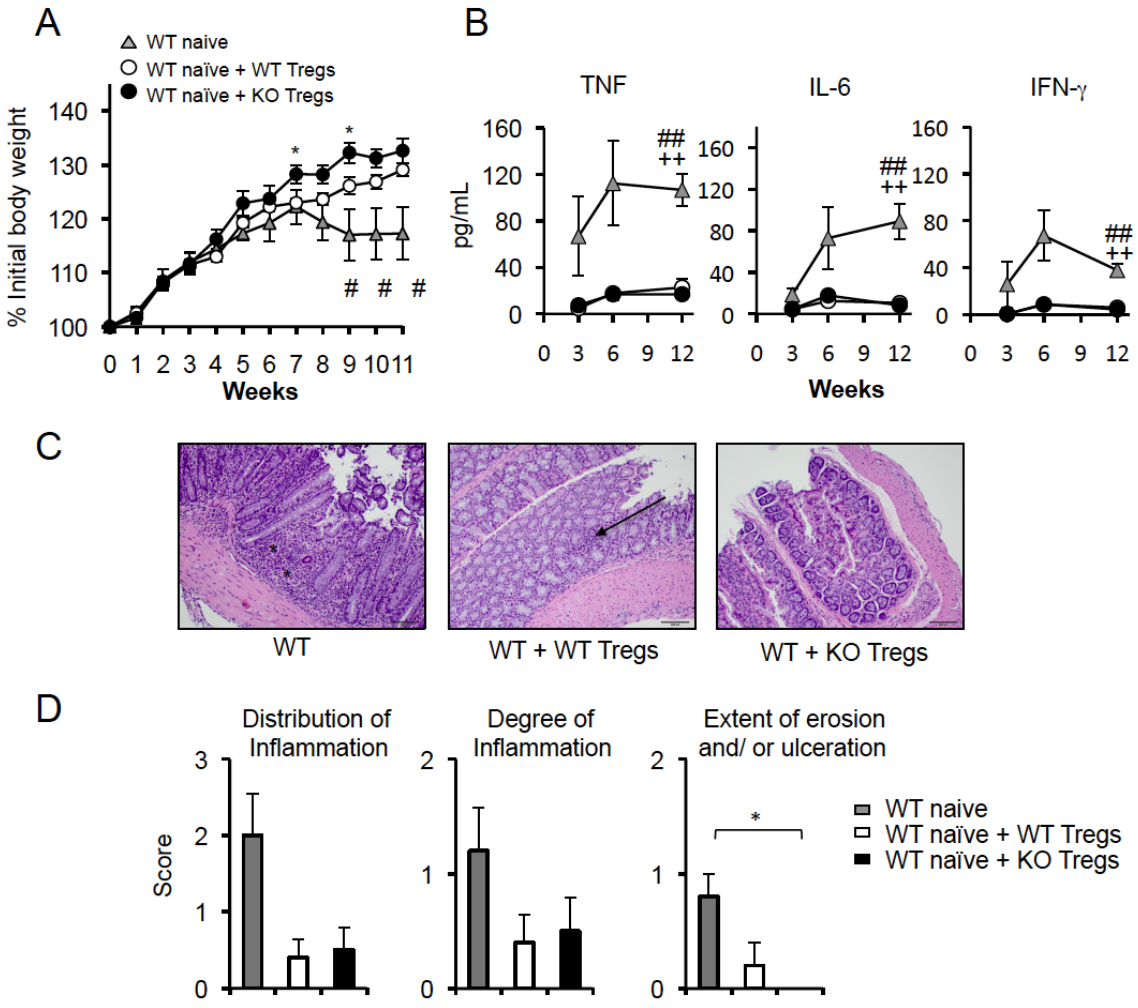

Figure 3.2: *Tpl2*$^{-/-}$ Tregs are functionally suppressive *in vivo*.

(**A)** Colitis was induced by T cell transfer into *Rag1*$^{-/-}$ mice. *Rag1*$^{-/-}$ recipients received 300,000 WT sorted CD4^{+}CD45RB^{hi}CD25^{-} naïve T cells alone or in combination with 100,000 purified WT or *Tpl2*$^{-/-}$ CD4^{+}CD25^{+} (KO) Tregs. Mice were weighed weekly. N=5 mice per experiment and are representative of two independent experiments. **#,** $p<0.05$; two-tailed Student's *t*-test, WT naïve versus WT naïve + KO Treg. ***,** $p<0.05$; two-tailed Student's *t*-test, WT Treg versus KO Treg. **(B)** Serum TNF, IL-6 and IFN-γ were measured using a Th1/Th2/Th17 cytokine bead array at 3, 6 and 12 weeks. N=5 mice per group. **++,** $p<0.01$; two-tailed Student's *t*-test, WT naïve T versus WT Treg. **##,** $p<0.01$; two-tailed Student's *t*-test, WT naïve T versus KO Treg.

(C) Colon sections obtained from mice in (A) were used for H&E staining and clinical scoring. Representative histological images (H&E, 100X magnification). Photomicrograph of a colitic intestine from a mouse receiving WT naïve T cells alone shows that infiltrating inflammatory cells separate the intestinal glands, and intestinal glands are absent focally (asterisks). In a mouse receiving WT Treg co-transfer, focal areas were evident where the intestinal glands were replaced by a small focus of inflammatory cells (arrow). In a mouse receiving KO Treg co-transfer, normal colonic mucosa was depicted. (**D**) Clinical scoring of colon sections. Development of colitis was monitored by distribution of inflammation (left), degree of inflammation (middle) and extent of erosion/ulceration (right). N≥4 mice for a group. ***,** $p<0.05$; 1-tailed Mann-Whitney test.

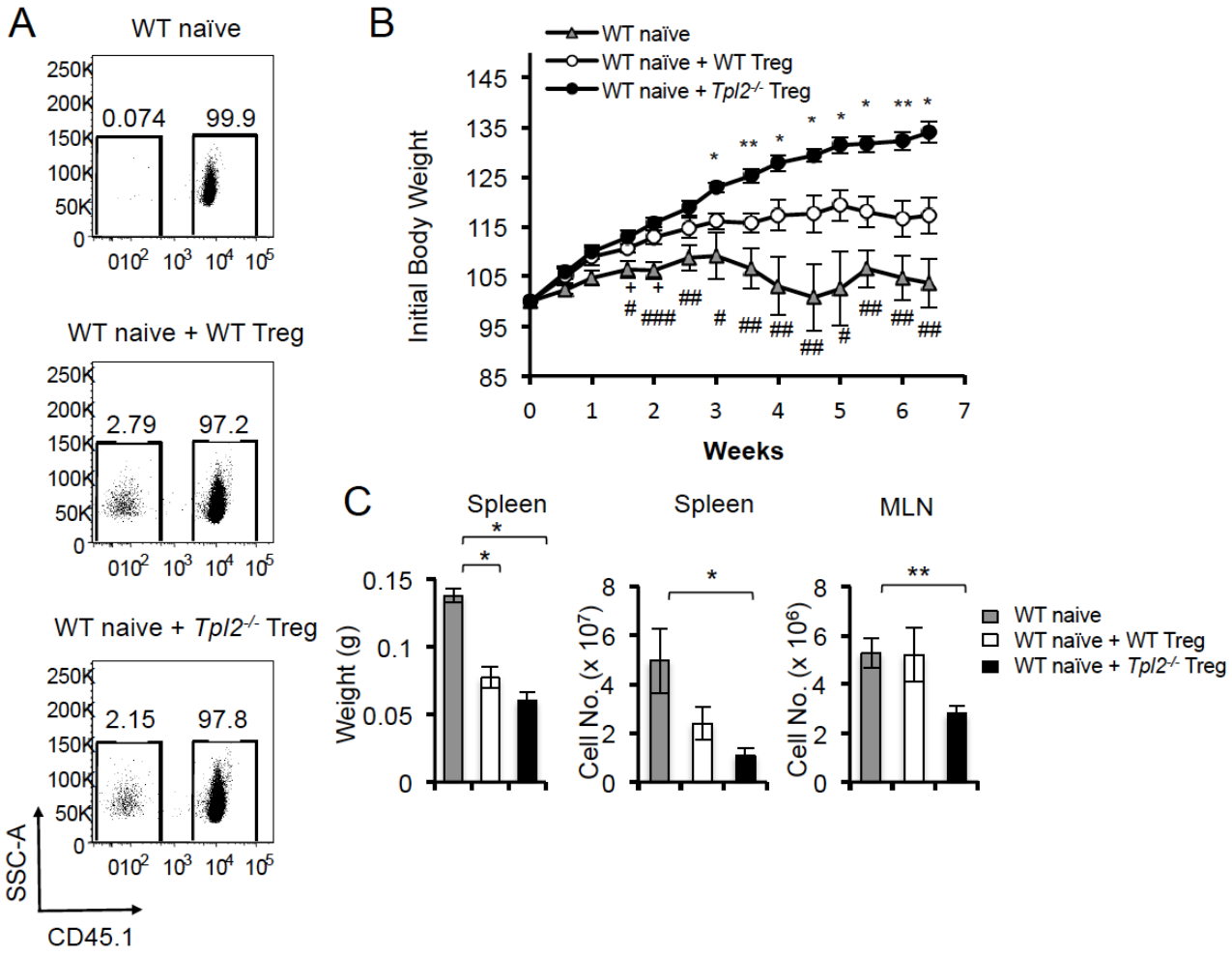

Figure 3.3: Co-transferred *Tpl2*$^{-/-}$ Tregs offer greater protection than WT Tregs in a T cell transfer model of colitis.

(**A**) Representative data showing the starting ratios of cells used in the T cell transfer model of colitis performed as described below. 500,000 WT sorted CD4^{+}CD45RB^{hi}CD25^{-} naïve T cells (CD45.1^{+}) alone or in combination with 12,500 purified WT or *Tpl2*$^{-/-}$ CD4^{+}CD25^{+} Tregs (CD45.2^{+}) were injected into *Rag1*$^{-/-}$ recipients. An unused portion of the injected cell mixture was analyzed for CD45.1 expression by flow cytometry to confirm the ratio of injected effectors:Tregs. (**B**) 6 to 7 week old *Rag1*$^{-/-}$ recipients received 500,000 WT sorted CD4^{+}CD45RB^{hi}CD25^{-} naïve T cells alone or in combination with 12,500 purified WT or *Tpl2*$^{-/-}$

$CD4^+CD25^+$ Tregs. Mice were weighed twice weekly. N=7, 4, and 8 for WT naïve, WT naïve + WT Treg, WT naïve + *Tpl2*$^{-/-}$ Treg groups, respectively. Data are pooled from 2 independent experiments. **+,** $p<0.05$; two-tailed Student's *t*-test, WT naïve versus WT Treg. **#,** $p<0.05$; **##,** $p<0.01$; **###,** $p<0.001$, two-tailed Student's *t*-test, WT naïve versus *Tpl2*$^{-/-}$ Treg, ***,** $p<0.05$; ******, $p<0.01$, two-tailed Student's *t*-test, WT Treg versus *Tpl2*$^{-/-}$ Treg. (**C**) Spleens were harvested and weighed (left), and total cell numbers of spleen (Middle) and MLN (Right) were quantitated. ***,** $p<0.05$; ******, $p<0.01$ two-tailed Student's *t*-test.

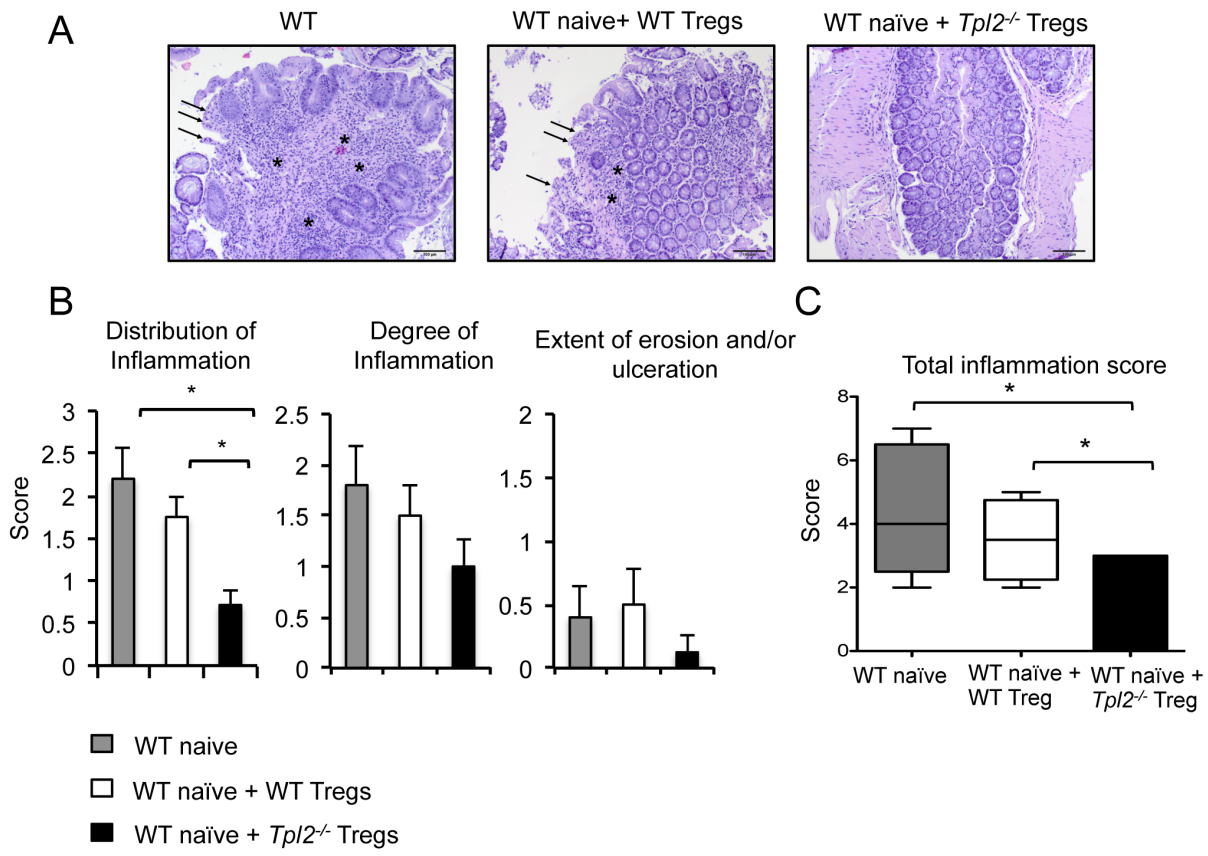

Figure 3.4: *Tpl2*$^{-/-}$ Tregs are more cacapble of limiting colon inflammation.

(**A**) Colon sections were used for H&E staining and clinical scoring. Representative histological images (H&E, 100X magnification). Photomicrographs of inflamed colons from mice receiving WT naïve T cells alone or in combination with co-transferred WT Tregs. Images show focal erosions (solid arrows), and the intestinal glands in the underlying lamina propria were replaced by inflammatory cells (asterisks). In mice receiving WT naïve T cells and co-transferred *Tpl2*$^{-/-}$ Tregs, normal colonic mucosa was observed. (**B-C**) Clinical scoring of colon sections. Development of colitis was monitored by distribution of inflammation (**B**, left), degree of inflammation (**B**, middle) and extent of erosion/ulceration (**B**, right). Total clinical scores (**C**) were the sum of: distribution of inflammation, degree of inflammation and extent of erosion/ulceration of colon sections. ***,** $p<0.05$, 1-tailed Mann-Whitney test.

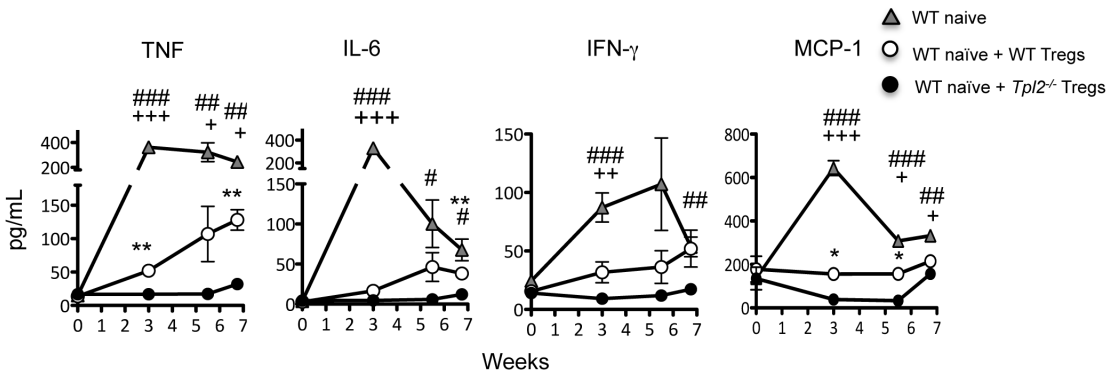

Figure 3.5: *Tpl2*$^{-/-}$ Tregs are superior at limiting systemic pro-inflammatory cytokine production.

Serum TNF, IL-6, IFN-γ and MCP-1 were measured by a cytokine bead array (BD Parmingen) at 3, 5.5 or 6.75 weeks. N=7, 4, and 8, respectively. **+,** $p<0.05$; ++, $p<0.01$; +++, $p<0.001$, two-tailed Student's *t*-test, WT naïve versus WT Treg. **#,** $p<0.05$; ##; $p<0.01$ **###,** $p<0.001$, two-tailed Student's *t*-test, WT naïve versus *Tpl2*$^{-/-}$ Treg, **, $p<0.01$, two-tailed Student's *t*-test, WT Treg versus *Tpl2*$^{-/-}$ Treg. Data are pooled from 2 independent experiments. Samples were collected from mice in Figure 3.5B.

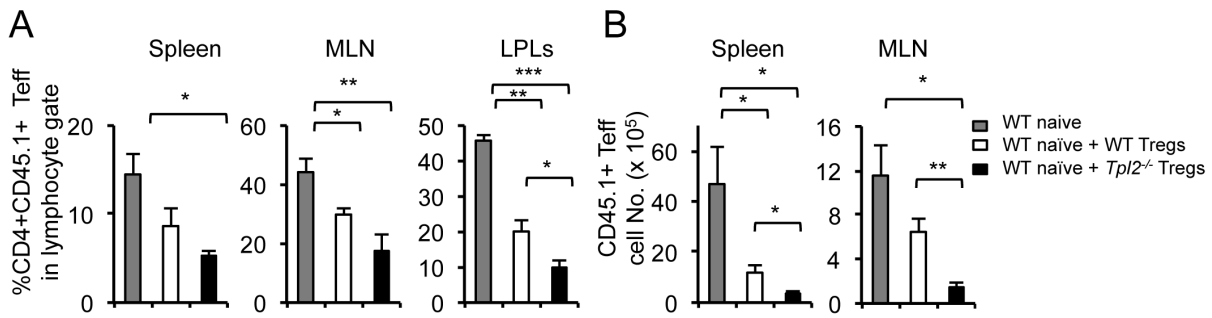

Figure 3.6: *Tpl2*$^{-/-}$ Tregs are superior at limiting effector T cell accumulation.

(**A-B**) Spleens, MLNs, and colon LPLs were harvested, and the proportions of $CD4^+CD45.1^+$ inflammatory effector T cells within the lymphocyte population are shown in (**A**). Absolute numbers of $CD4^+CD45.1^+$ T_{eff} cells are shown in (**B**). ***,** $p<0.05$; ****,** $p<0.01$; *****,** $p<0.001$, A; two tailed Student's *t*-test, B; one-tailed Student's *t*-test; Data are pooled from 2 independent experiments.

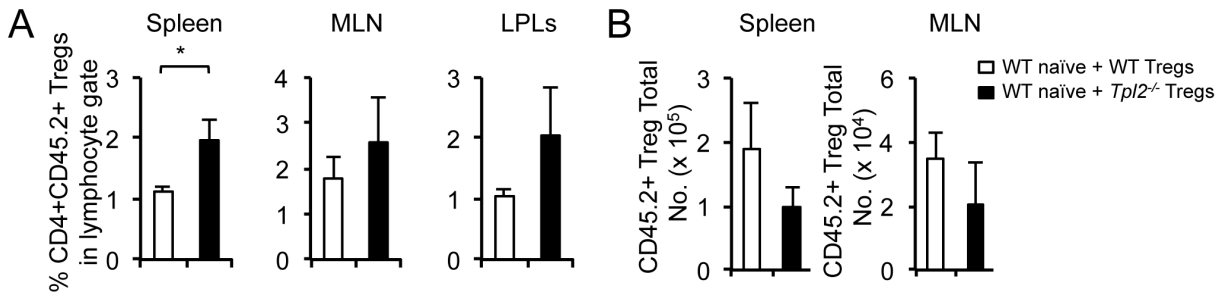

Figure 3.7: Normal accumulation of *Tpl2*$^{-/-}$ Tregs in the colitis model.

(**A-B**) Spleens, MLNs, and colon LPLs were harvested. The proportions of $CD4^+CD45.2^+$ Tregs within recipients of WT naïve T cells co-transferred with either WT Tregs or *Tpl2*$^{-/-}$ Tregs are shown in (**A**). Absolute $CD4^+CD45.2^+$ Treg numbers are shown in (**B**). ***,** $p<0.05$, A; two tailed Student's *t*-test, B; one-tailed Student's *t*-test; Data are pooled from 2 independent experiments.

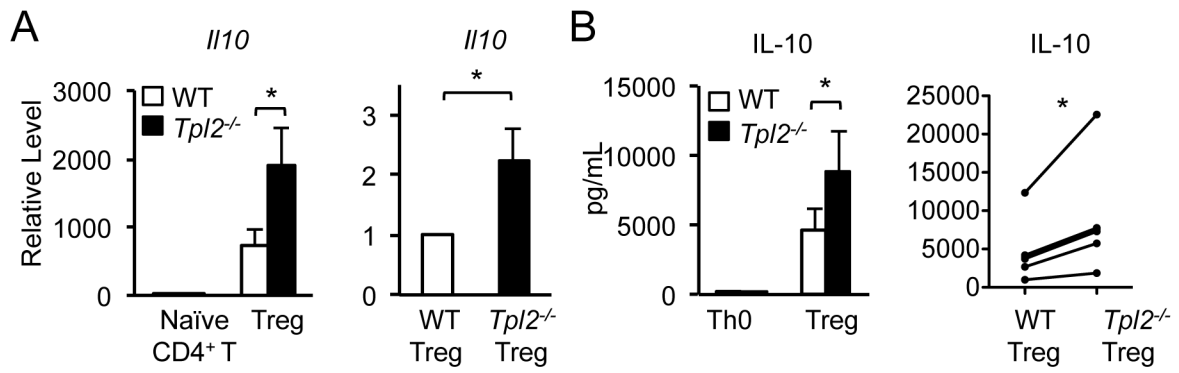

Figure 3.8: Tpl2 inhibits the expression of the Treg immunosuppressive cytoine, IL-10, by Tregs.

(**A**) Left: IL-10 mRNA expression was measured by RT-PCR in sorted WT and *Tpl2*$^{-/-}$ naïve CD4^{+} T cells and Tregs. N=4 individual experiments. *, $p<0.05$, two-tailed paired Student's *t*-test. Right: activated WT or *Tpl2*$^{-/-}$ CD25^{+} Tregs were re-stimulated with 40 IU/mL IL-2, 10 μg/mL immobilized anti-CD3 and 2 μg/mL soluble anti-CD28 for 2 days. IL-10 expression was measured by RT-PCR. N=5 individual experiments. *, $p<0.05$, one-tailed Student's *t*-test. (**B**) WT and T*pl2*$^{-/-}$ naïve T cells and Tregs were activated as indicated in (**A**), and supernatants were collected at 48 h. IL-10 secretion was measured by ELISA. Left panel, N≥5 individual experiments. Right panel, N=6 individual experiments; paired measurements within an experiment are connected by a line. *, $p<0.05$, two-tailed paired Student's *t*-test.

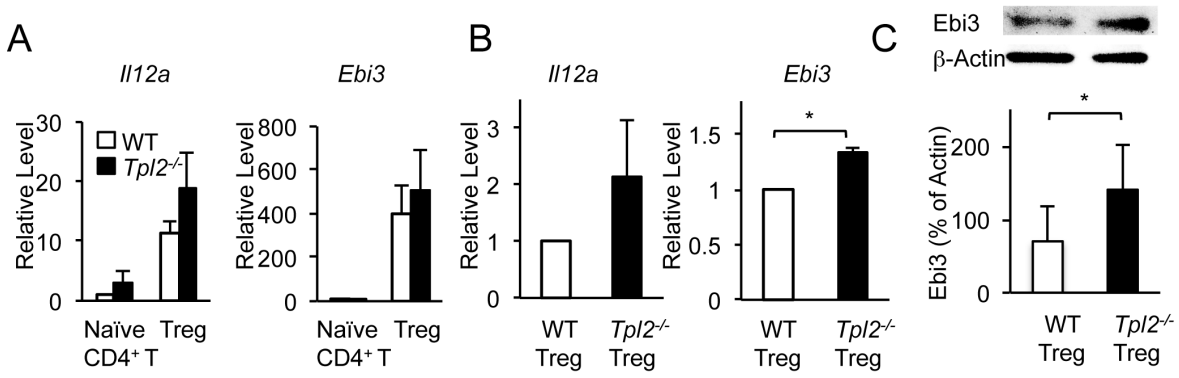

Figure 3.9: Tpl2 inhibits the expression of the immusuppressive cytokine IL-35 subunit Ebi3, by Tregs.

(**A**) *IL-12p35 (Il12a,* left) and *Ebi3* (right) mRNA expression was measured in naïve $CD4^+$ T cells and Tregs. N=3 individual experiments. No significant differences were identified using a two-tailed paired Student's *t*-test. (**B**) Activated WT or *Tpl2*$^{-/-}$ Tregs were re-stimulated with 40 IU/mL IL-2 and 10 mg/mL immobilized anti-CD3 and 2 mg/mL soluble anti-CD28 for two days. mRNA expression of *Il12a* (left) and *Ebi3* (right) was measured by RT-PCR in the cell pellets. N=4 individual experiments. *, $p<0.05$, two-tailed Student's *t*-test. (**C**) WT or *Tpl2*$^{-/-}$ Tregs were stimulated with 100 IU/mL IL-2 and 10 mg/mL immobilized anti-CD3 and 2 mg/mL soluble anti-CD28 for two days. Top: Western blot for Ebi3 and β-Actin; Bottom: Ebi3 densitometry relative to β-Actin loading control. N=3 individual experiments. *, $p<0.05$, two-tailed paired Student's *t*-test.

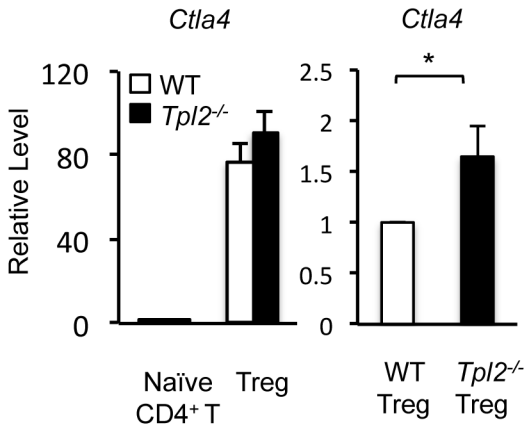

Figure 3.10: *Tpl2*$^{-/-}$ Tregs express higher levels of *Ctla4* mRNA than WT Tregs.

Left: *Ctla4* mRNA was measured by RT-PCR in freshly sorted WT and *Tpl2*$^{-/-}$ naïve CD4^{+} T cells and Tregs. Right: *Ctla4* mRNA was measured in activated WT or *Tpl2*$^{-/-}$ CD25^{+} Tregs as indicated in (**A**). *, $p<0.05$, one-tailed Student's *t*-test.

CHAPTER 4

CONCLUSION

Immune cells function to program and coordinate pro-inflamatory and anti-inflammatory immune responses through complex cytokine signaling networks. Inflammtion is a 'necessary evil' for eradication of infectious microorganisms. The immune system responds rapidly to invading pathogens with a strong pro-inflammatory immune response designed to eradicate the pathogen but it may also inadvertently damage host cells and tissues. Therefore, understanding the molecular mechanisms regulating this immunological balance is urged for advancing new treatments for both infectious and autoimmune diseases. A particular focus has been to elucidate the molecular mechanisms by which the serine-threonine kinase, tumor progress locus 2 (Tp2), regulates both innate and adaptive immune responses. Tpl2 is required for the expression of the prominent pro-inflammatory mediator, TNFα, by innate immune cells (28). Therefore, Tpl2 small molecule inhibitors are being developed as potential therapeutic treatments for chronic autoimmune conditions in which TNFα plays a pathogenic role, such as rheumatoid arthritis and Crohn's diseases (173, 205). Watford *et al.* further identified Tpl2 as factor expressed in T cells that is required for optimal host immunity against intracellular parasites by promotine the production of the pro-inflammatory cytokine, IFNγ (36). Tpl2 is expressed at low levels in naïve T cells but is inducible with activation via T cell receptors and cytokine receptors (36-38, 206). However, prior to this study it was not clear precisely how Tpl2 impinges upon TCR and cytokine signaling pathways and to what extent Tpl2 contributes to the differentiation of Treg

lineage. The overall goal of this study was to determine how Tpl2 regulates T helper cell differentiation and effector functions, especially for the specialized lineage of regulatory T cells. Our study revealed important information about basic immunological functions of Tpl2 and whether Tpl2 inhibition might provide a therapeutic benefit to patients suffering from autoimmune diseases. Our findings are summarized as follows.

1. Tpl2 is upregulated upon TCR and co-stimulatory activation; however, TGF-β restricts Tpl2 expression in iTreg cells. These data suggest that Tpl2 may negatively regulate FoxP3^{+} iTreg development and differentiation.
2. Naïve *Tpl2*$^{-/-}$ T cells preferentially differentiate into FoxP3^{+} iTregs when cultured with either purified anti-CD3 + anti-CD28 or when co-cultured with WT dendritic cells and OVA peptide antigen. Increased iTreg differentiation of *Tpl2*$^{-/-}$ naïve CD4 T cells was not attributed to defects in *Tpl2*$^{-/-}$ dendritic cells. These data clearly demonstrate that Tpl2 inhibits the generation of FoxP3^{+} iTregs *in vitro* during iTreg conversion from naïve CD4 T cells in a cell-intrinsic manner.
3. A significant increase in the proportions of FoxP3^{+} iTregs in the spleens and MLNs was observed *in vivo* in an OVA antigen-induced murine model of systemic tolerance. These data underscore the physiological role of Tpl2 in suppressing iTreg conversion *in vivo*.
4. Although enhanced proliferation was observed within *Tpl2*$^{-/-}$ CD4^{+} T cells compared to WT CD4^{+} T cells, elevated frequencies of FoxP3^{+} iTreg of *Tpl2*$^{-/-}$ T cells was due to increased conversion rather than increased outgrowth (i.e. increased survival and/or increased proliferation) of *Tpl2*$^{-/-}$ FoxP3^{+} iTregs.

5. Tpl2 inhibits FoxP3 expression by enhancing activation of the Akt-mTOR-S6 pathway rather than by activating the ERK MAP kinase pathway, cytokine signaling pathways or early TCR signals (Figure 4.1).
6. *Tpl2*$^{-/-}$ Tregs are more immunosuppressive *in vivo* in a T cell transfer model of colitis than WT Tregs. *Tpl2*$^{-/-}$ Tregs effectively restrict the production of systemic pro-inflammatory meditors and colonic inflammation. Reduced inflammation correlated with reduced accumulation of inflammatory effector T cells in mice receiving *Tpl2*$^{-/-}$ Tregs compared to WT Tregs.
7. Tpl2 inhibits the immunosuppressive capacity of Tregs by inhibiting the expression of FoxP3 and the immunosuppressive molecules, IL-10, Ebi3 and CTLA-4.

Overall, our findings provide key insights into the mechanism by which Tpl2 ablation (or potential therapeutic inhibition) enhances Treg cell development and anti-inflammatory activities. This study also provides a novel underlying mechanism by which Tpl2 controls T helper cell differentiation and function through the mTOR signaling pathway.

A more complete model of T helper cell differentiation

Our findings in the current study have provided a more complete picture of the complex regulatory pathways by which Tpl2 regulates T helper cell differentiation and functions (Figure 4.2). Watford *et al*. first demonstrated that T helper cell differentiation into Th1 and Th2 lineages could be regulated by Tpl2 ablation (36, 37). Tpl2 promotes Th1 cell differentiation, and Tpl2 ablation reduced the expression of STAT4 and T-bet transcription factors in response to TCR stimulation, which led to impaired T cell immune responses to the intracellular parasite *Toxosplasma gondii* infection (36). In contrast, Tpl2 ablation enhanced the production of IgE and the development of bronchoalveolar eosinophilic inflammation in OVA-immunized mice. In this

model, *Tpl2*$^{-/-}$ mice exhibited enhanced Th2 responses, as evidenced by elevated levels of IL-4 and IL-5, suggesting that Tpl2 inhibits Th2 development (37). Enhanced Th2 cell development in *Tpl2*$^{-/-}$ mice correlated with functional impairments in the production of the Th1 signature cytokine, IFN-γ (37). We also found that Tpl2 impaired the production of IL-17A and IL-17F by CD4^{+} T cells under Th17 prolarzing conditions (TGF-β + IL-6) (38). Reduced expression of IL-17A correlated with increased FoxP3 expression by *Tpl2*$^{-/-}$ CD4^{+} T cells but was independent of *Rorc*, *Rora*, *Irf4* and *IL-23r* expression or STAT3 activation (38). Futhermore, either removing TGF-β or neutralizaing IL-2 during Th17 culture inhibited FoxP3 expression and restored IL-17A production in *Tpl2*$^{-/-}$ Th17 cells to WT levels (38). These data suggest that the IL-17A defect in *Tpl2*$^{-/-}$ T cells was due to aberrantly high FoxP3 expression. In the current study, we have extended our observations of how Tpl2 regulates T helper cell differentiation by demonstrating that Tpl2 inhibits FoxP3 expression under iTreg polarizing conditions in a T cell-intrinsic manner (Figure 4.2). Mechanistically, Tpl2 promotes the Akt-mTOR-S6 signaling pathway to inhibit FoxP3 expression during Treg conversion from naïve T cells (206). These data are consistent with the previsous finding that Tpl2 is required for the activation of the Akt-mTOR-S6 pathway in macrophages upon TLRs stimulation (165). Currently, Tpl2 inhibitors are being developed to treat inflammatory diseases (205, 207). Therefore, our data provide novel insights into the molecular mechanism(s) by which Tpl2 inhibition may globally alter T helper cell development.

In conclusion, this study, taken together with our previous studies, has revealed that Tpl2 systematically regulates T helper cell development by promoting inflammatory lineages at the expense of anti-inflammatory Tregs. These findings suggest that Tpl2 inhibitors may be useful to

treat human autoimmune diseases by deviating pathologic pro-inflammatory T cell responses, such as Th1 and Th17 reponses, by favoring the development of immunosuppressive Tregs.

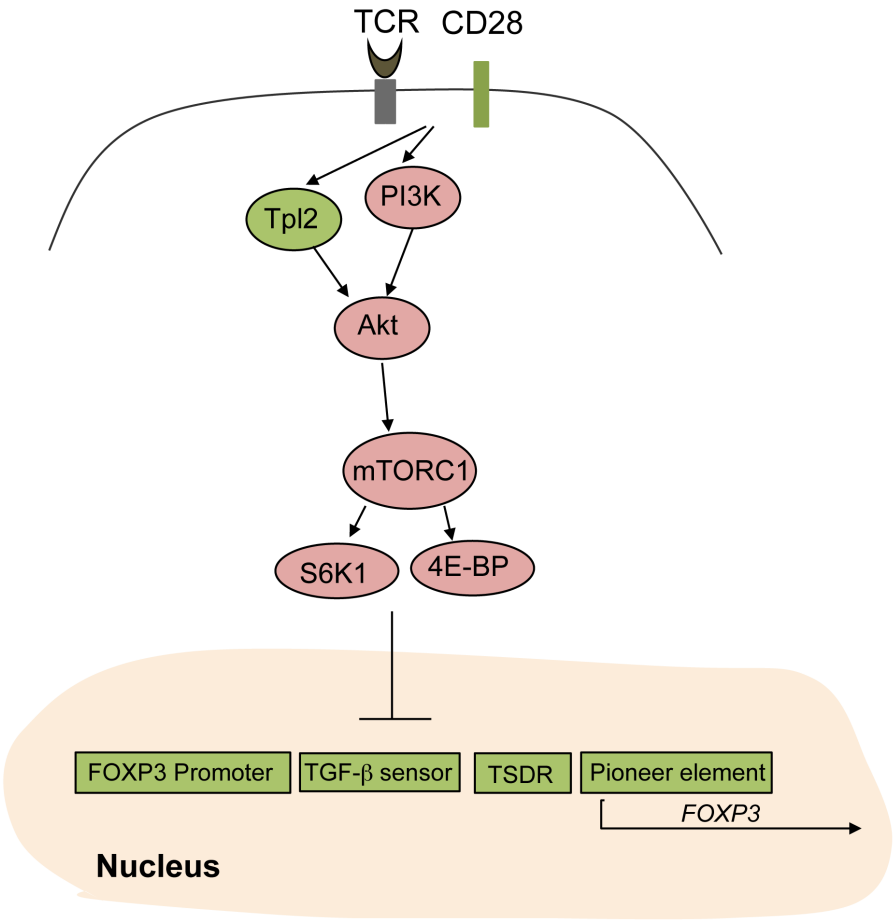

Figure 4.1: Tpl2 inhibits FoxP3 expression via enhancing mTOR pathway in response to TCR and co-stimulatory signals.

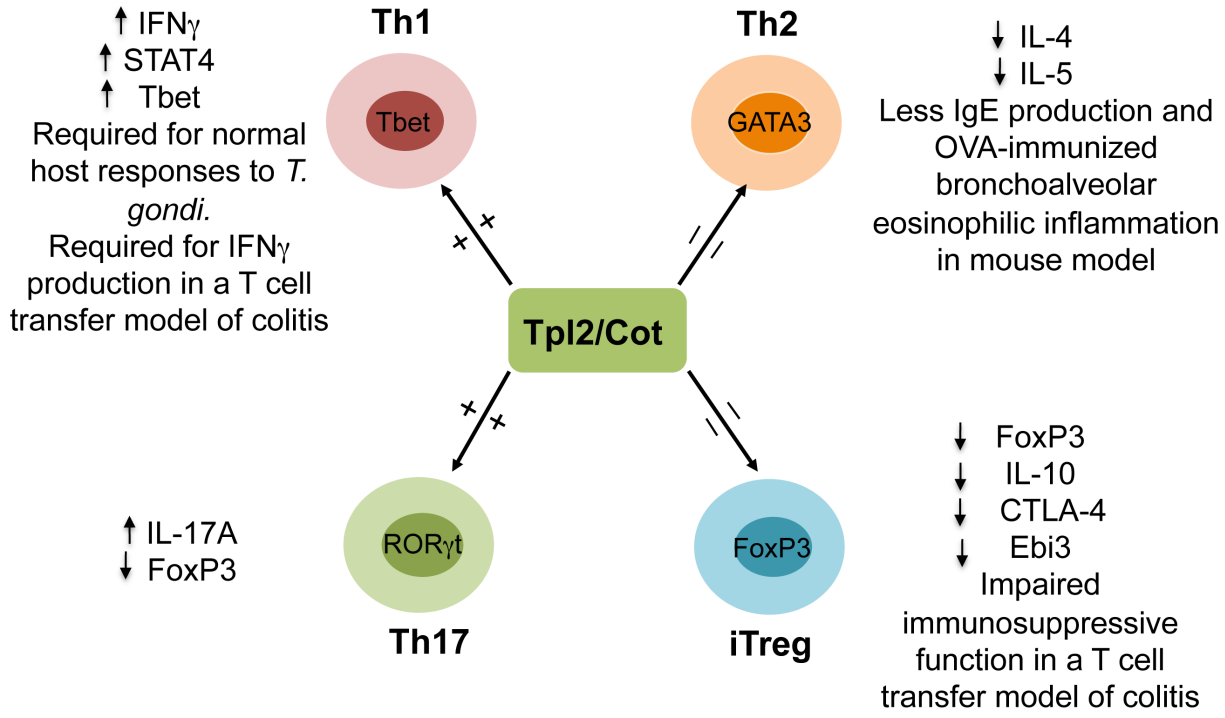

Figure 4.2: The role of Tpl2 in T helper cells development and function.

REFERENCES

1. Josefowicz SZ, Lu LF, Rudensky AY. 2012. Regulatory T cells: mechanisms of differentiation and function. *Annu Rev Immunol* 30: 531-64
2. Starr TK, Jameson SC, Hogquist KA. 2003. Positive and negative selection of T cells. *Annu Rev Immunol* 21: 139-76
3. Mueller DL. 2010. Mechanisms maintaining peripheral tolerance. *Nat Immunol* 11: 21-7
4. Bilate AM, Lafaille JJ. 2012. Induced CD4+Foxp3+ regulatory T cells in immune tolerance. *Annu Rev Immunol* 30: 733-58
5. Curotto de Lafaille MA, Lafaille JJ. 2009. Natural and adaptive foxp3+ regulatory T cells: more of the same or a division of labor? *Immunity* 30: 626-35
6. Sakaguchi S. 2003. Control of immune responses by naturally arising CD4+ regulatory T cells that express toll-like receptors. *J Exp Med* 197: 397-401
7. Zhu J, Paul WE. 2008. CD4 T cells: fates, functions, and faults. *Blood* 112: 1557-69
8. Murphy KM, Reiner SL. 2002. The lineage decisions of helper T cells. *Nat Rev Immunol* 2: 933-44
9. Weaver CT, Hatton RD, Mangan PR, Harrington LE. 2007. IL-17 family cytokines and the expanding diversity of effector T cell lineages. *Annu Rev Immunol* 25: 821-52
10. Ansel KM, Djuretic I, Tanasa B, Rao A. 2006. Regulation of Th2 differentiation and Il4 locus accessibility. *Annu Rev Immunol* 24: 607-56

11. Baron U, Floess S, Wieczorek G, Baumann K, Grutzkau A, Dong J, Thiel A, Boeld TJ, Hoffmann P, Edinger M, Turbachova I, Hamann A, Olek S, Huehn J. 2007. DNA demethylation in the human FOXP3 locus discriminates regulatory T cells from activated FOXP3(+) conventional T cells. *European Journal of Immunology* 37: 2378-89

12. Stockinger B, Veldhoen M, Martin B. 2007. Th17 T cells: linking innate and adaptive immunity. *Semin Immunol* 19: 353-61

13. Weiss JM, Bilate AM, Gobert M, Ding Y, Curotto de Lafaille MA, Parkhurst CN, Xiong H, Dolpady J, Frey AB, Ruocco MG, Yang Y, Floess S, Huehn J, Oh S, Li MO, Niec RE, Rudensky AY, Dustin ML, Littman DR, Lafaille JJ. 2012. Neuropilin 1 is expressed on thymus-derived natural regulatory T cells, but not mucosa-generated induced Foxp3+ T reg cells. *J Exp Med* 209: 1723-42, S1

14. Fontenot JD, Gavin MA, Rudensky AY. 2003. Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. *Nat Immunol* 4: 330-6

15. Hori S, Nomura T, Sakaguchi S. 2003. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 299: 1057-61

16. O'shea JJ, Paul WE. 2010. Mechanisms Underlying Lineage Commitment and Plasticity of Helper CD4(+) T Cells. *Science* 327: 1098-102

17. Miyoshi J, Higashi T, Mukai H, Ohuchi T, Kakunaga T. 1991. Structure and transforming potential of the human cot oncogene encoding a putative protein kinase. *Mol Cell Biol* 11: 4088-96

18. Makris A, Patriotis C, Bear SE, Tsichlis PN. 1993. Genomic organization and expression of Tpl-2 in normal cells and Moloney murine leukemia virus-induced rat T-cell lymphomas: activation by provirus insertion. *J Virol* 67: 4283-9

19. Patriotis C, Makris A, Bear SE, Tsichlis PN. 1993. Tumor progression locus 2 (Tpl-2) encodes a protein kinase involved in the progression of rodent T-cell lymphomas and in T-cell activation. *Proc Natl Acad Sci U S A* 90: 2251-5

20. Aoki M, Hamada F, Sugimoto T, Sumida S, Akiyama T, Toyoshima K. 1993. The human cot proto-oncogene encodes two protein serine/threonine kinases with different transforming activities by alternative initiation of translation. *J Biol Chem* 268: 22723-32

21. Ceci JD, Patriotis CP, Tsatsanis C, Makris AM, Kovatch R, Swing DA, Jenkins NA, Tsichlis PN, Copeland NG. 1997. Tpl-2 is an oncogenic kinase that is activated by carboxy-terminal truncation. *Genes & Development* 11: 688-700

22. Gantke T, Sriskantharajah S, Sadowski M, Ley SC. 2012. IkappaB kinase regulation of the TPL-2/ERK MAPK pathway. *Immunol Rev* 246: 168-82

23. Gantke T, Sriskantharajah S, Ley SC. 2011. Regulation and function of TPL-2, an IkappaB kinase-regulated MAP kinase kinase kinase. *Cell Res* 21: 131-45

24. Lang V, Symons A, Watton SJ, Janzen J, Soneji Y, Beinke S, Howell S, Ley SC. 2004. ABIN-2 forms a ternary complex with TPL-2 and NF-kappa B1 p105 and is essential for TPL-2 protein stability. *Mol Cell Biol* 24: 5235-48

25. Beinke S, Deka J, Lang V, Belich MP, Walker PA, Howell S, Smerdon SJ, Gamblin SJ, Ley SC. 2003. NF-kappaB1 p105 negatively regulates TPL-2 MEK kinase activity. *Mol Cell Biol* 23: 4739-52

26. Roget K, Ben-Addi A, Mambole-Dema A, Gantke T, Yang HT, Janzen J, Morrice N, Abbott D, Ley SC. 2012. IkappaB kinase 2 regulates TPL-2 activation of extracellular signal-regulated kinases 1 and 2 by direct phosphorylation of TPL-2 serine 400. *Mol Cell Biol* 32: 4684-90

27. Robinson MJ, Beinke S, Kouroumalis A, Tsichlis PN, Ley SC. 2007. Phosphorylation of TPL-2 on serine 400 is essential for lipopolysaccharide activation of extracellular signal-regulated kinase in macrophages. *Mol Cell Biol* 27: 7355-64

28. Dumitru CD, Ceci JD, Tsatsanis C, Kontoyiannis D, Stamatakis K, Lin JH, Patriotis C, Jenkins NA, Copeland NG, Kollias G, Tsichlis PN. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. *Cell* 103: 1071-83

29. Eliopoulos AG, Dumitru CD, Wang CC, Cho JH, Tsichlis PN. 2002. Induction of COX-2 by LPS in macrophages is regulated by Tpl2-dependent CREB activation signals. *Embo Journal* 21: 4831-40

30. Mielke LA, Elkins KL, Wei L, Starr R, Tsichlis PN, O'Shea JJ, Watford WT. 2009. Tumor Progression Locus 2 (Map3k8) Is Critical for Host Defense against Listeria monocytogenes and IL-1 beta Production. *Journal of Immunology* 183: 7984-93

31. Rousseau S, Papoutsopoulou M, Symons A, Cook D, Lucocq JM, Prescott AR, O'Garra A, Ley SC, Cohen P. 2008. TPL2-mediated activation of ERK1 and ERK2 regulates the processing of pre-TNF alpha in LPS-stimulated macrophages. *J Cell Sci* 121: 149-54

32. Rowley SM, Kuriakose T, Dockery LM, Tran-Ngyuen T, Gingerich AD, Wei L, Watford WT. 2014. Tumor progression locus 2 (Tpl2) kinase promotes chemokine receptor expression and macrophage migration during acute inflammation. *J Biol Chem* 289: 15788-97

33. Kaiser F, Cook D, Papoutsopoulou S, Rajsbaum R, Wu X, Yang HT, Grant S, Ricciardi-Castagnoli P, Tsichlis PN, Ley SC, O'Garra A. 2009. TPL-2 negatively regulates interferon-beta production in macrophages and myeloid dendritic cells. *J Exp Med* 206: 1863-71

34. Eliopoulos AG, Wang CC, Dumitru CD, Tsichlis PN. 2003. Tpl2 transduces CD40 and TNF signals that activate ERK and regulates IgE induction by CD40. *Embo Journal* 22: 3855-64

35. Tsatsanis C, Vaporidi K, Zacharioudaki V, Androulidaki A, Sykulev Y, Margioris AN, Tsichlis PN. 2008. Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells. *Proc Natl Acad Sci U S A* 105: 2987-92

36. Watford WT, Hissong BD, Durant LR, Yamane H, Muul LM, Kanno Y, Tato CM, Ramos HL, Berger AE, Mielke L, Pesu M, Solomon B, Frucht DM, Paul WE, Sher A, Jankovic D, Tsichlis PN, O'Shea JJ. 2008. Tpl2 kinase regulates T cell interferon-gamma production and host resistance to Toxoplasma gondii. *Journal of Experimental Medicine* 205: 2803-12

37. Watford WT, Wang CC, Tsatsanis C, Mielke LA, Eliopoulos AG, Daskalakis C, Charles N, Odom S, Rivera J, O'Shea J, Tsichlis PN. 2010. Ablation of Tumor Progression Locus 2 Promotes a Type 2 Th Cell Response in Ovalbumin-Immunized Mice. *Journal of Immunology* 184: 105-13

38. Acuff NV, Li X, Kirkland R, Nagy T, Watford WT. 2015. Tumor progression locus 2 differentially regulates IFNgamma and IL-17 production by effector CD4+ T cells in a T cell transfer model of colitis. *PLoS One* 10: e0119885

39. Moss RB, Moll T, El-Kalay M, Kohne C, Hoo WS, Encinas J, Carlo DJ. 2004. Th1/Th2 cells in inflammatory disease states: therapeutic implications. *Expert Opinion on Biological Therapy* 4: 1887-96

40. Sakaguchi S, Fukuma K, Kuribayashi K, Masuda T. 1985. Organ-specific autoimmune diseases induced in mice by elimination of T cell subset. I. Evidence for the active participation of T cells in natural self-tolerance; deficit of a T cell subset as a possible cause of autoimmune disease. *J Exp Med* 161: 72-87

41. Powrie F, Leach MW, Mauze S, Caddle LB, Coffman RL. 1993. Phenotypically Distinct Subsets of Cd4(+) T-Cells Induce or Protect from Chronic Intestinal Inflammation in C - B-17 Scid Mice. *International Immunology* 5: 1461-71

42. Morrissey PJ, Charrier K, Braddy S, Liggitt D, Watson JD. 1993. Cd4+ T-Cells That Express High-Levels of Cd45rb Induce Wasting Disease When Transferred into Congenic Severe Combined Immunodeficient Mice - Disease Development Is Prevented by Cotransfer of Purified Cd4+ T-Cells. *Journal of Experimental Medicine* 178: 237-44

43. Sakaguchi S, Sakaguchi N, Asano M, Itoh M, Toda M. 1995. Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. *J Immunol* 155: 1151-64

44. Bennett CL, Christie J, Ramsdell F, Brunkow ME, Ferguson PJ, Whitesell L, Kelly TE, Saulsbury FT, Chance PF, Ochs HD. 2001. The immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) is caused by mutations of FOXP3. *Nature genetics* 27: 20-1

45. Fontenot JD, Rasmussen JP, Williams LM, Dooley JL, Farr AG, Rudensky AY. 2005. Regulatory T cell lineage specification by the forkhead transcription factor foxp3. *Immunity* 22: 329-41

46. Hori S, Takahashi T, Sakaguchi S. 2003. Control of autoimmunity by naturally arising regulatory CD4+ T cells. *Adv Immunol* 81: 331-71

47. Josefowicz SZ, Rudensky A. 2009. Control of regulatory T cell lineage commitment and maintenance. *Immunity* 30: 616-25

48. Salomon B, Lenschow DJ, Rhee L, Ashourian N, Singh B, Sharpe A, Bluestone JA. 2000. B7/CD28 costimulation is essential for the homeostasis of the CD4(+)CD25(+) immunoregulatory T cells that control autoimmune diabetes. *Immunity* 12: 431-40

49. Zhang R, Huynh A, Whitcher G, Chang J, Maltzman JS, Turka LA. 2013. An obligate cell-intrinsic function for CD28 in Tregs. *J Clin Invest* 123: 580-93

50. Chen W, Jin W, Hardegen N, Lei KJ, Li L, Marinos N, McGrady G, Wahl SM. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875-86

51. Davidson TS, DiPaolo RJ, Andersson J, Shevach EM. 2007. Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. *J Immunol* 178: 4022-6

52. Shevach EM, DiPaolo RA, Andersson J, Zhao DM, Stephens GL, Thornton AM. 2006. The lifestyle of naturally occurring CD4(+)CD25(+)Foxp3(+) regulatory T cells. *Immunological Reviews* 212: 60-73

53. Burchill MA, Yang J, Vogtenhuber C, Blazar BR, Farrar MA. 2007. IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. *J Immunol* 178: 280-90

54. Fontenot JD, Rasmussen JP, Gavin MA, Rudensky AY. 2005. A function for interleukin 2 in Foxp3-expressing regulatory T cells. *Nat Immunol* 6: 1142-51

55. Soper DM, Kasprowicz DJ, Ziegler SF. 2007. IL-2Rbeta links IL-2R signaling with Foxp3 expression. *Eur J Immunol* 37: 1817-26

56. Yao Z, Kanno Y, Kerenyi M, Stephens G, Durant L, Watford WT, Laurence A, Robinson GW, Shevach EM, Moriggl R, Hennighausen L, Wu C, O'Shea JJ. 2007. Nonredundant roles for Stat5a/b in directly regulating Foxp3. *Blood* 109: 4368-75

57. Setoguchi R, Hori S, Takahashi T, Sakaguchi S. 2005. Homeostatic maintenance of natural Foxp3(+) CD25(+) CD4(+) regulatory T cells by interleukin (IL)-2 and induction of autoimmune disease by IL-2 neutralization. *J Exp Med* 201: 723-35

58. Curotto de Lafaille MA, Lino AC, Kutchukhidze N, Lafaille JJ. 2004. CD25- T cells generate CD25+Foxp3+ regulatory T cells by peripheral expansion. *J Immunol* 173: 7259-68

59. D'Cruz LM, Klein L. 2005. Development and function of agonist-induced CD25+Foxp3+ regulatory T cells in the absence of interleukin 2 signaling. *Nat Immunol* 6: 1152-9

60. Zheng Y, Rudensky AY. 2007. Foxp3 in control of the regulatory T cell lineage. *Nat Immunol* 8: 457-62

61. Li MO, Flavell RA. 2008. Contextual regulation of inflammation: a duet by transforming growth factor-beta and interleukin-10. *Immunity* 28: 468-76

62. Chen Q, Kim YC, Laurence A, Punkosdy GA, Shevach EM. 2011. IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. *J Immunol* 186: 6329-37

63. Li MO, Sanjabi S, Flavell RA. 2006. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 25: 455-71

64. Fahlen L, Read S, Gorelik L, Hurst SD, Coffman RL, Flavell RA, Powrie F. 2005. T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. *Journal of Experimental Medicine* 201: 737-46

65. Liu Y, Zhang P, Li J, Kulkarni AB, Perruche S, Chen W. 2008. A critical function for TGF-beta signaling in the development of natural CD4+CD25+Foxp3+ regulatory T cells. *Nat Immunol* 9: 632-40

66. Sun CM, Hall JA, Blank RB, Bouladoux N, Oukka M, Mora JR, Belkaid Y. 2007. Small intestine lamina propria dendritic cells promote de novo generation of Foxp3 T reg cells via retinoic acid. *Journal of Experimental Medicine* 204: 1775-85

67. Benson MJ, Erickson LD, Gleeson MW, Noelle RJ. 2007. Affinity of antigen encounter and other early B-cell signals determine B-cell fate. *Current Opinion in Immunology* 19: 275-80

68. Atarashi K, Tanoue T, Shima T, Imaoka A, Kuwahara T, Momose Y, Cheng G, Yamasaki S, Saito T, Ohba Y, Taniguchi T, Takeda K, Hori S, Ivanov, II, Umesaki Y, Itoh K, Honda K. 2011. Induction of colonic regulatory T cells by indigenous Clostridium species. *Science* 331: 337-41

69. Arpaia N, Campbell C, Fan X, Dikiy S, van der Veeken J, deRoos P, Liu H, Cross JR, Pfeffer K, Coffer PJ, Rudensky AY. 2013. Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. *Nature* 504: 451-5

70. Atarashi K, Tanoue T, Oshima K, Suda W, Nagano Y, Nishikawa H, Fukuda S, Saito T, Narushima S, Hase K, Kim S, Fritz JV, Wilmes P, Ueha S, Matsushima K, Ohno H, Olle B, Sakaguchi S, Taniguchi T, Morita H, Hattori M, Honda K. 2013. Treg induction by a

rationally selected mixture of Clostridia strains from the human microbiota. *Nature* 500: 232-6

71. Mucida D, Pino-Lagos K, Kim G, Nowak E, Benson MJ, Kronenberg M, Noelle RJ, Cheroutre H. 2009. Retinoic acid can directly promote TGF-beta-mediated Foxp3(+) Treg cell conversion of naive T cells. *Immunity* 30: 471-2; author reply 2-3

72. Benson MJ, Pino-Lagos K, Rosemblatt M, Noelle RJ. 2007. All-trans retinoic acid mediates enhanced T reg cell growth, differentiation, and gut homing in the face of high levels of co-stimulation. *J Exp Med* 204: 1765-74

73. Xiao S, Jin H, Korn T, Liu SM, Oukka M, Lim B, Kuchroo VK. 2008. Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. *J Immunol* 181: 2277-84

74. Nolting J, Daniel C, Reuter S, Stuelten C, Li P, Sucov H, Kim BG, Letterio JJ, Kretschmer K, Kim HJ, von Boehmer H. 2009. Retinoic acid can enhance conversion of naive into regulatory T cells independently of secreted cytokines. *J Exp Med* 206: 2131-9

75. Smith PM, Howitt MR, Panikov N, Michaud M, Gallini CA, Bohlooly YM, Glickman JN, Garrett WS. 2013. The microbial metabolites, short-chain fatty acids, regulate colonic Treg cell homeostasis. *Science* 341: 569-73

76. Izcue A, Coombes JL, Powrie F. 2006. Regulatory T cells suppress systemic and mucosal immune activation to control intestinal inflammation. *Immunol Rev* 212: 256-71

77. Uhlig HH, Coombes J, Mottet C, Izcue A, Thompson C, Fanger A, Tannapfel A, Fontenot JD, Ramsdell F, Powrie F. 2006. Characterization of Foxp3+CD4+CD25+ and IL-10-secreting CD4+CD25+ T cells during cure of colitis. *J Immunol* 177: 5852-60

78. Mottet C, Uhlig HH, Powrie F. 2003. Cutting edge: cure of colitis by CD4+CD25+ regulatory T cells. *J Immunol* 170: 3939-43

79. Makita S, Kanai T, Nemoto Y, Totsuka T, Okamoto R, Tsuchiya K, Yamamoto M, Kiyono H, Watanabe M. 2007. Intestinal lamina propria retaining CD4(+)CD25(+) regulatory T cells is a suppressive site of intestinal inflammation. *Journal of Immunology* 178: 4937-46

80. Mikami Y, Kanai T, Okazawa A, Ono Y, Hayashi A, Sujino T, Kamada N, Matsuoka K, Hisamatsu T, Okamoto S, Takaishi H, Inoue N, Hibi T. 2010. Competition Between Colitogenic T Helper 1 and 17 CD4(+) T Cells Contributes to the Amelioration of Colitis. *Digestive Diseases and Sciences* 55: 2700-

81. Ostanin DV, Bao J, Koboziev I, Gray L, Robinson-Jackson SA, Kosloski-Davidson M, Price VH, Grisham MB. 2009. T cell transfer model of chronic colitis: concepts, considerations, and tricks of the trade. *Am J Physiol Gastrointest Liver Physiol* 296: G135-46

82. Asseman C, Read S, Powrie F. 2003. Colitogenic Th1 cells are present in the antigen-experienced T cell pool in normal mice: control by CD4+ regulatory T cells and IL-10. *J Immunol* 171: 971-8

83. Read S, Malmstrom V, Powrie F. 2000. Cytotoxic T lymphocyte-associated antigen 4 plays an essential role in the function of CD25(+)CD4(+) regulatory cells that control intestinal inflammation. *Journal of Experimental Medicine* 192: 295-302

84. Maloy KJ, Salaun L, Cahill R, Dougan G, Saunders NJ, Powrie F. 2003. CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. *J Exp Med* 197: 111-9

85. Sojka DK, Hughson A, Fowell DJ. 2009. CTLA-4 is required by CD4(+)CD25(+) Treg to control CD4(+) T-cell lymphopenia-induced proliferation. *European Journal of Immunology* 39: 1544-51

86. Asseman C, Mauze S, Leach MW, Coffman RL, Powrie F. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *Journal of Experimental Medicine* 190: 995-1003

87. Annacker O, Pimenta-Araujo R, Burlen-Defranoux O, Barbosa TC, Cumano A, Bandeira A. 2001. CD25+ CD4+ T cells regulate the expansion of peripheral CD4 T cells through the production of IL-10. *J Immunol* 166: 3008-18

88. Murai M, Turovskaya O, Kim G, Madan R, Karp CL, Cheroutre H, Kronenberg M. 2009. Interleukin 10 acts on regulatory T cells to maintain expression of the transcription factor Foxp3 and suppressive function in mice with colitis. *Nat Immunol* 10: 1178-84

89. Vignali DAA, Collison LW, Workman CJ. 2008. How regulatory T cells work. *Nature Reviews Immunology* 8: 523-32

90. Sakaguchi S, Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T. 2009. Regulatory T cells: how do they suppress immune responses? *International Immunology* 21: 1105-11

91. Collison LW, Workman CJ, Kuo TT, Boyd K, Wang Y, Vignali KM, Cross R, Sehy D, Blumberg RS, Vignali DA. 2007. The inhibitory cytokine IL-35 contributes to regulatory T-cell function. *Nature* 450: 566-9

92. Saraiva M, O'garra A. 2010. The regulation of IL-10 production by immune cells. *Nature Reviews Immunology* 10: 170-81

93. Moore KW, de Waal Malefyt R, Coffman RL, O'Garra A. 2001. Interleukin-10 and the interleukin-10 receptor. *Annu Rev Immunol* 19: 683-765

94. Hawrylowicz CM, O'Garra A. 2005. Potential role of interleukin-10-secreting regulatory T cells in allergy and asthma. *Nat Rev Immunol* 5: 271-83

95. O'garra A, Barrat FJ, Castro AG, Vicari A, Hawrylowicz C. 2008. Strategies for use of IL-10 or its antagonists in human disease. *Immunological Reviews* 223: 114-31

96. Rubtsov YP, Rasmussen JP, Chi EY, Fontenot J, Castelli L, Ye X, Treuting P, Siewe L, Roers A, Henderson WR, Muller W, Rudensky AY. 2008. Reaulatory T cell-derived interleukin-10 limits inflammation at environmental interfaces. *Immunity* 28: 546-58

97. Marie JC, Letterio JJ, Gavin M, Rudensky AY. 2005. TGF-beta 1 maintains suppressor function and Foxp3 expression in CD4(+)CD25(+) regulatory T cells. *Journal of Experimental Medicine* 201: 1061-7

98. Yoshimura A, Muto G. 2011. TGF-beta function in immune suppression. *Curr Top Microbiol Immunol* 350: 127-47

99. Fox JM, Sage LK, Huang L, Barber J, Klonowski KD, Mellor AL, Tompkins SM, Tripp RA. 2013. Inhibition of indoleamine 2,3-dioxygenase enhances the T-cell response to influenza virus infection. *Journal of General Virology* 94: 1451-61

100. Sugiyama Y, Kakoi K, Kimura A, Takada I, Kashiwagi I, Wakabayashi Y, Morita R, Nomura M, Yoshimura A. 2012. Smad2 and Smad3 are redundantly essential for the suppression of iNOS synthesis in macrophages by regulating IRF3 and STAT1 pathways. *International Immunology* 24: 253-65

101. Powrie F, Carlino J, Leach MW, Mauze S, Coffman RL. 1996. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *J Exp Med* 183: 2669-74

102. Liu JQ, Liu ZZ, Zhang XJ, Shi Y, Talebian F, Carl JW, Yu C, Shi FD, Whitacre CC, Trgovcich J, Bai XF. 2012. Increased Th17 and Regulatory T Cell Responses in EBV-Induced Gene 3-Deficient Mice Lead to Marginally Enhanced Development of Autoimmune Encephalomyelitis. *Journal of Immunology* 188: 3099-106

103. Wirtz S, Billmeier U, Mchedlidze T, Blumberg RS, Neurath MF. 2011. Interleukin-35 Mediates Mucosal Immune Responses That Protect Against T-Cell-Dependent Colitis. *Gastroenterology* 141: 1875-86

104. Vignali DA, Kuchroo VK. 2012. IL-12 family cytokines: immunological playmakers. *Nat Immunol* 13: 722-8

105. Minami Y, Kono T, Miyazaki T, Taniguchi T. 1993. The IL-2 receptor complex: its structure, function, and target genes. *Annu Rev Immunol* 11: 245-68

106. Bachmann MF, Oxenius A. 2007. Interleukin 2: from immunostimulation to immunoregulation and back again. *Embo Reports* 8: 1142-8

107. Thornton AM, Shevach EM. 1998. CD4+CD25+ immunoregulatory T cells suppress polyclonal T cell activation in vitro by inhibiting interleukin 2 production. *J Exp Med* 188: 287-96

108. de la Rosa M, Rutz S, Dorninger H, Scheffold A. 2004. Interleukin-2 is essential for CD4(+)CD25(+) regulatory T cell function. *European Journal of Immunology* 34: 2480-8

109. Walker LS. 2013. Treg and CTLA-4: two intertwining pathways to immune tolerance. *J Autoimmun* 45: 49-57

110. Cederbom L, Hall H, Ivars F. 2000. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *Eur J Immunol* 30: 1538-43

111. Tivol EA, Borriello F, Schweitzer AN, Lynch WP, Bluestone JA, Sharpe AH. 1995. Loss of CTLA-4 leads to massive lymphoproliferation and fatal multiorgan tissue destruction, revealing a critical negative regulatory role of CTLA-4. *Immunity* 3: 541-7

112. Waterhouse P, Penninger JM, Timms E, Wakeham A, Shahinian A, Lee KP, Thompson CB, Griesser H, Mak TW. 1995. Lymphoproliferative disorders with early lethality in mice deficient in Ctla-4. *Science* 270: 985-8

113. Karandikar NJ, Vanderlugt CL, Walunas TL, Miller SD, Bluestone JA. 1996. CTLA-4: a negative regulator of autoimmune disease. *J Exp Med* 184: 783-8

114. Takahashi T, Tagami T, Yamazaki S, Uede T, Shimizu J, Sakaguchi N, Mak TW, Sakaguchi S. 2000. Immunologic self-tolerance maintained by CD25(+)CD4(+) regulatory T cells constitutively expressing cytotoxic T lymphocyte-associated antigen 4. *J Exp Med* 192: 303-10

115. Liu Z, Geboes K, Hellings P, Maerten P, Heremans H, Vandenberghe P, Boon L, van Kooten P, Rutgeerts P, Ceuppens JL. 2001. B7 interactions with CD28 and CTLA-4 control tolerance or induction of mucosal inflammation in chronic experimental colitis. *J Immunol* 167: 1830-8

116. Keir ME, Butte MJ, Freeman GJ, Sharpe AH. 2008. PD-1 and its ligands in tolerance and immunity. *Annu Rev Immunol* 26: 677-704

117. Buchbinder EI, Desai A. 2016. CTLA-4 and PD-1 Pathways: Similarities, Differences, and Implications of Their Inhibition. *Am J Clin Oncol* 39: 98-106

118. Francisco LM, Salinas VH, Brown KE, Vanguri VK, Freeman GJ, Kuchroo VK, Sharpe AH. 2009. PD-L1 regulates the development, maintenance, and function of induced regulatory T cells. *J Exp Med* 206: 3015-29

119. Wang L, Pino-Lagos K, de Vries VC, Guleria I, Sayegh MH, Noelle RJ. 2008. Programmed death 1 ligand signaling regulates the generation of adaptive Foxp3+CD4+ regulatory T cells. *Proc Natl Acad Sci U S A* 105: 9331-6

120. Wang WS, Lau R, Yu DH, Zhu WW, Korman A, Weber J. 2009. PD1 blockade reverses the suppression of melanoma antigen-specific CTL by CD4(+)CD25(Hi) regulatory T cells. *International Immunology* 21: 1065-77

121. Wang C, Thudium KB, Han M, Wang XT, Huang H, Feingersh D, Garcia C, Wu Y, Kuhne M, Srinivasan M, Singh S, Wong S, Garner N, Leblanc H, Bunch RT, Blanset D, Selby MJ, Korman AJ. 2014. In vitro characterization of the anti-PD-1 antibody nivolumab, BMS-936558, and in vivo toxicology in non-human primates. *Cancer Immunol Res* 2: 846-56

122. Lieberman J. 2003. The ABCs of granule-mediated cytotoxicity: new weapons in the arsenal. *Nat Rev Immunol* 3: 361-70

123. Grossman WJ, Verbsky JW, Barchet W, Colonna M, Atkinson JP, Ley TJ. 2004. Human T regulatory cells can use the perforin pathway to cause autologous target cell death. *Immunity* 21: 589-601

124. Gondek DC, Lu LF, Quezada SA, Sakaguchi S, Noelle RJ. 2005. Cutting edge: Contact-mediated suppression by CD4(+)-CD25(+) regulatory cells involves a granzyme B-dependent, perforin-independent mechanism. *Journal of Immunology* 174: 1783-6

125. Zhao DM, Thornton AM, DiPaolo RJ, Shevach EM. 2006. Activated CD4+CD25+ T cells selectively kill B lymphocytes. *Blood* 107: 3925-32

126. Huehn J, Polansky JK, Hamann A. 2009. Epigenetic control of FOXP3 expression: the key to a stable regulatory T-cell lineage? *Nat Rev Immunol* 9: 83-9

127. Turner MS, Kane LP, Morel PA. 2009. Dominant role of antigen dose in CD4+Foxp3+ regulatory T cell induction and expansion. *Journal of Immunology* 183: 4895-903

128. Gottschalk RA, Corse E, Allison JP. 2010. TCR ligand density and affinity determine peripheral induction of Foxp3 in vivo. *J Exp Med* 207: 1701-11

129. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, Rudensky AY. 2010. Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. *Nature* 463: 808-12

130. Kim HP, Leonard WJ. 2007. CREB/ATF-Dependent T-cell receptor-induced FoxP3 gene expression: A role for DNA methylation. *Cytokine* 39: 20-1

131. Mantel PY, Ouaked N, Ruckert B, Karagiannidis C, Welz R, Blaser K, Schmidt-Weber CB. 2006. Molecular mechanisms underlying FOXP3 induction in human T cells. *J Immunol* 176: 3593-602

132. Gupta S, Manicassamy S, Vasu C, Kumar A, Shang W, Sun Z. 2008. Differential requirement of PKC-theta in the development and function of natural regulatory T cells. *Mol Immunol* 46: 213-24

133. Wang H, Zhao L, Sun Z, Sun L, Zhang B, Zhao Y. 2006. A potential side effect of cyclosporin A: inhibition of CD4(+)CD25(+) regulatory T cells in mice. *Transplantation* 82: 1484-92

134. Gabrysova L, Christensen JR, Wu X, Kissenpfennig A, Malissen B, O'Garra A. 2011. Integrated T-cell receptor and costimulatory signals determine TGF-beta-dependent differentiation and maintenance of Foxp3+ regulatory T cells. *Eur J Immunol* 41: 1242-8

135. Liu H, Yao S, Dann SM, Qin H, Elson CO, Cong Y. 2013. ERK differentially regulates Th17- and Treg-cell development and contributes to the pathogenesis of colitis. *Eur J Immunol* 43: 1716-26

136. Ma J, Ding Y, Fang X, Wang R, Sun Z. 2012. Protein kinase C-theta inhibits inducible regulatory T cell differentiation via an AKT-Foxo1/3a-dependent pathway. *J Immunol* 188: 5337-47

137. Sauer S, Bruno L, Hertweck A, Finlay D, Leleu M, Spivakov M, Knight ZA, Cobb BS, Cantrell D, O'Connor E, Shokat KM, Fisher AG, Merkenschlager M. 2008. T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. *Proc Natl Acad Sci U S A* 105: 7797-802

138. Delgoffe GM, Kole TP, Zheng Y, Zarek PE, Matthews KL, Xiao B, Worley PF, Kozma SC, Powell JD. 2009. The mTOR kinase differentially regulates effector and regulatory T cell lineage commitment. *Immunity* 30: 832-44

139. Zeng H, Yang K, Cloer C, Neale G, Vogel P, Chi H. 2013. mTORC1 couples immune signals and metabolic programming to establish T(reg)-cell function. *Nature* 499: 485-90

140. Haxhinasto S, Mathis D, Benoist C. 2008. The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. *J Exp Med* 205: 565-74

141. Zeng H, Chi H. 2015. Metabolic control of regulatory T cell development and function. *Trends Immunol* 36: 3-12

142. Hall JP, Kurdi Y, Hsu S, Cuozzo J, Liu J, Telliez JB, Seidl KJ, Winkler A, Hu Y, Green N, Askew GR, Tam S, Clark JD, Lin LL. 2007. Pharmacologic inhibition of Tpl2 blocks inflammatory responses in primary human monocytes, synoviocytes, and blood*. *Journal of Biological Chemistry* 282: 33295-304

143. Fantini MC, Becker C, Monteleone G, Pallone F, Galle PR, Neurath MF. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *J Immunol* 172: 5149-53

144. Davidson A, Diamond B. 2001. Autoimmune diseases. *N Engl J Med* 345: 340-50

145. Kamradt T, Mitchison NA. 2001. Tolerance and autoimmunity. *N Engl J Med* 344: 655-64

146. Smilek DE, Ehlers MR, Nepom GT. 2014. Restoring the balance: immunotherapeutic combinations for autoimmune disease. *Dis Model Mech* 7: 503-13

147. Kontzias A, Kotlyar A, Laurence A, Changelian P, O'Shea JJ. 2012. Jakinibs: a new class of kinase inhibitors in cancer and autoimmune disease. *Curr Opin Pharmacol* 12: 464-70

148. Boland BS, Sandborn WJ, Chang JT. 2014. Update on Janus kinase antagonists in inflammatory bowel disease. *Gastroenterol Clin North Am* 43: 603-17

149. Sandborn WJ, Ghosh S, Panes J, Vranic I, Wang W, Niezychowski W, Study AI. 2014. A phase 2 study of tofacitinib, an oral Janus kinase inhibitor, in patients with Crohn's disease. *Clin Gastroenterol Hepatol* 12: 1485-93 e2

150. Iwata S, Tanaka Y. 2016. Progress in understanding the safety and efficacy of Janus kinase inhibitors for treatment of rheumatoid arthritis. *Expert Rev Clin Immunol*: 1-11

151. Norman P. 2014. Selective JAK inhibitors in development for rheumatoid arthritis. *Expert Opin Investig Drugs* 23: 1067-77

152. Hahn BH, McMahon MA, Wilkinson A, Wallace WD, Daikh DI, Fitzgerald JD, Karpouzas GA, Merrill JT, Wallace DJ, Yazdany J, Ramsey-Goldman R, Singh K, Khalighi M, Choi SI, Gogia M, Kafaja S, Kamgar M, Lau C, Martin WJ, Parikh S, Peng J, Rastogi A, Chen W, Grossman JM, American College of R. 2012. American College of

Rheumatology guidelines for screening, treatment, and management of lupus nephritis. *Arthritis Care Res (Hoboken)* 64: 797-808

153. Singh JA, Saag KG, Bridges SL, Jr., Akl EA, Bannuru RR, Sullivan MC, Vaysbrot E, McNaughton C, Osani M, Shmerling RH, Curtis JR, Furst DE, Parks D, Kavanaugh A, O'Dell J, King C, Leong A, Matteson EL, Schousboe JT, Drevlow B, Ginsberg S, Grober J, St Clair EW, Tindall E, Miller AS, McAlindon T, American College of R. 2016. 2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis. *Arthritis Care Res (Hoboken)* 68: 1-25

154. Calabrese LH, Calabrese C, Kirchner E. 2016. The 2015 American College of Rheumatology Guideline for the Treatment of Rheumatoid Arthritis Should Include New Standards for Hepatitis B Screening: Comment on the Article by Singh et al. *Arthritis Care Res (Hoboken)* 68: 723-4

155. Steward-Tharp SM, Song YJ, Siegel RM, O'Shea JJ. 2010. New insights into T cell biology and T cell-directed therapy for autoimmunity, inflammation, and immunosuppression. *Year in Immunology 2* 1183: 123-48

156. Dejaco C, Duftner C, Grubeck-Loebenstein B, Schirmer M. 2006. Imbalance of regulatory T cells in human autoimmune diseases. *Immunology* 117: 289-300

157. Bluestone JA, Buckner JH, Fitch M, Gitelman SE, Gupta S, Hellerstein MK, Herold KC, Lares A, Lee MR, Li K, Liu W, Long SA, Masiello LM, Nguyen V, Putnam AL, Rieck M, Sayre PH, Tang Q. 2015. Type 1 diabetes immunotherapy using polyclonal regulatory T cells. *Science translational medicine* 7: 315ra189

158. Tang Q, Henriksen KJ, Bi M, Finger EB, Szot G, Ye J, Masteller EL, McDevitt H, Bonyhadi M, Bluestone JA. 2004. In vitro-expanded antigen-specific regulatory T cells suppress autoimmune diabetes. *J Exp Med* 199: 1455-65

159. Powrie F, Read S, Mottet C, Uhlig H, Maloy K. 2003. Control of immune pathology by regulatory T cells. *Novartis Found Symp* 252: 92-8; discussion 8-105, 6-14

160. Tang QZ, Bluestone JA. 2006. Regulatory T-cell physiology and application to treat autoimmunity. *Immunological Reviews* 212: 217-37

161. Riley JL, June CH, Blazar BR. 2009. Human T Regulatory Cell Therapy: Take a Billion or So and Call Me in the Morning. *Immunity* 30: 656-65

162. Spence A, Klementowicz JE, Bluestone JA, Tang Q. 2015. Targeting Treg signaling for the treatment of autoimmune diseases. *Curr Opin Immunol* 37: 11-20

163. Hancock WW, Akimova T, Beier UH, Liu Y, Wang L. 2012. HDAC inhibitor therapy in autoimmunity and transplantation. *Ann Rheum Dis* 71 Suppl 2: i46-54

164. DuPage M, Chopra G, Quiros J, Rosenthal WL, Morar MM, Holohan D, Zhang R, Turka L, Marson A, Bluestone JA. 2015. The Chromatin-Modifying Enzyme Ezh2 Is Critical for the Maintenance of Regulatory T Cell Identity after Activation. *Immunity* 42: 227-38

165. Lopez-Pelaez M, Soria-Castro I, Bosca L, Fernandez M, Alemany S. 2011. Cot/tpl2 activity is required for TLR-induced activation of the Akt p70 S6k pathway in macrophages: Implications for NO synthase 2 expression. *Immunology* 135: 105-

166. Xing Y, Hogquist KA. 2012. T-Cell Tolerance: Central and Peripheral. *Cold Spring Harbor Perspectives in Biology* 4

167. Fontenot JD, Rudensky AY. 2005. A well adapted regulatory contrivance: regulatory T cell development and the forkhead family transcription factor Foxp3. *Nat Immunol* 6: 331-7

168. Wildin RS, Ramsdell F, Peake J, Faravelli F, Casanova JL, Buist N, Levy-Lahad E, Mazzella M, Goulet O, Perroni L, Bricarelli FD, Byrne G, McEuen M, Proll S, Appleby M, Brunkow ME. 2001. X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. *Nat Genet* 27: 18-20

169. Sakaguchi S. 2004. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. *Annu Rev Immunol* 22: 531-62

170. Bluestone JA, Abbas AK. 2003. Natural versus adaptive regulatory T cells. *Nat Rev Immunol* 3: 253-7

171. Tang Q, Lee K. 2012. Regulatory T-cell therapy for transplantation: how many cells do we need? *Curr Opin Organ Transplant* 17: 349-54

172. Tang Q, Bluestone JA, Kang SM. 2012. CD4(+)Foxp3(+) regulatory T cell therapy in transplantation. *J Mol Cell Biol* 4: 11-21

173. Croft M, Benedict CA, Ware CF. 2013. Clinical targeting of the TNF and TNFR superfamilies. *Nat Rev Drug Discov* 12: 147-68

174. Hall JP, Kurdi Y, Hsu S, Cuozzo J, Liu J, Telliez JB, Seidl KJ, Winkler A, Hu Y, Green N, Askew GR, Tam S, Clark JD, Lin LL. 2007. Pharmacologic inhibition of tpl2 blocks inflammatory responses in primary human monocytes, synoviocytes, and blood. *J Biol Chem* 282: 33295-304

175. Belich MP, Salmeron A, Johnston LH, Ley SC. 1999. TPL-2 kinase regulates the proteolysis of the NF-kappaB-inhibitory protein NF-kappaB1 p105. *Nature* 397: 363-8

176. Serebrennikova OB, Tsatsanis C, Mao C, Gounaris E, Ren W, Siracusa LD, Eliopoulos AG, Khazaie K, Tsichlis PN. 2012. Tpl2 ablation promotes intestinal inflammation and tumorigenesis in Apcmin mice by inhibiting IL-10 secretion and regulatory T-cell generation. *Proc Natl Acad Sci U S A* 109: E1082-91

177. Guo J, Zhang J, Zhang X, Zhang Z, Wei X, Zhou X. 2014. Constitutive activation of MEK1 promotes Treg cell instability in vivo. *J Biol Chem* 289: 35139-48

178. Thorstenson KM, Khoruts A. 2001. Generation of anergic and potentially immunoregulatory CD25+CD4 T cells in vivo after induction of peripheral tolerance with intravenous or oral antigen. *J Immunol* 167: 188-95

179. Thornton AM, Korty PE, Tran DQ, Wohlfert EA, Murray PE, Belkaid Y, Shevach EM. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *J Immunol* 184: 3433-41

180. Belkaid Y, Oldenhove G. 2008. Tuning microenvironments: induction of regulatory T cells by dendritic cells. *Immunity* 29: 362-71

181. Herman AE, Freeman GJ, Mathis D, Benoist C. 2004. CD4+CD25+ T regulatory cells dependent on ICOS promote regulation of effector cells in the prediabetic lesion. *J Exp Med* 199: 1479-89

182. Wing K, Onishi Y, Prieto-Martin P, Yamaguchi T, Miyara M, Fehervari Z, Nomura T, Sakaguchi S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science* 322: 271-5

183. Kanamaru F, Youngnak P, Hashiguchi M, Nishioka T, Takahashi T, Sakaguchi S, Ishikawa I, Azuma M. 2004. Costimulation via glucocorticoid-induced TNF receptor in

both conventional and CD25(+) regulatory CD4(+) T cells. *Journal of Immunology* 172: 7306-14

184. Zheng SG, Wang JH, Stohl W, Kim KS, Gray JD, Horwitz DA. 2006. TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. *J Immunol* 176: 3321-9

185. Verhagen J, Gabrysova L, Shepard ER, Wraith DC. 2014. CTLA-4 Modulates the Differentiation of Inducible Foxp3(+) Treg Cells but IL-10 Mediates Their Function in Experimental Autoimmune Encephalomyelitis. *Plos One* 9

186. Tsatsanis C, Patriotis C, Bear SE, Tsichlis PN. 1998. The Tpl-2 protooncoprotein activates the nuclear factor of activated T cells and induces interleukin 2 expression in T cell lines. *Proc Natl Acad Sci U S A* 95: 3827-32

187. Procaccini C, De Rosa V, Galgani M, Abanni L, Cali G, Porcellini A, Carbone F, Fontana S, Horvath TL, La Cava A, Matarese G. 2010. An oscillatory switch in mTOR kinase activity sets regulatory T cell responsiveness. *Immunity* 33: 929-41

188. Josefowicz SZ, Niec RE, Kim HY, Treuting P, Chinen T, Zheng Y, Umetsu DT, Rudensky AY. 2012. Extrathymically generated regulatory T cells control mucosal T(H)2 inflammation. *Nature* 482: 395-U1510

189. Piccirillo CA, Shevach EM. 2004. Naturally-occurring CD4+CD25+ immunoregulatory T cells: central players in the arena of peripheral tolerance. *Semin Immunol* 16: 81-8

190. Sriskantharajah S, Guckel E, Tsakiri N, Kierdorf K, Brender C, Ben-Addi A, Veldhoen M, Tsichlis PN, Stockinger B, O'Garra A, Prinz M, Kollias G, Ley SC. 2014. Regulation of experimental autoimmune encephalomyelitis by TPL-2 kinase. *J Immunol* 192: 3518-29

191. Haxhinasto S, Mathis D, Benoist C. 2008. The AKT-mTOR axis regulates de novo differentiation of CD4(+)Foxp3(+) cells. *Journal of Experimental Medicine* 205: 565-74

192. Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, Strober W. 2010. Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. *Immunity* 33: 313-25

193. Hanauer SB. 2006. Inflammatory bowel disease: epidemiology, pathogenesis, and therapeutic opportunities. *Inflamm Bowel Dis* 12 Suppl 1: S3-9

194. O'Shea JJ, Ma A, Lipsky P. 2002. Cytokines and autoimmunity. *Nat Rev Immunol* 2: 37-45

195. Zanin-Zhorov A, Ding Y, Kumari S, Attur M, Hippen KL, Brown M, Blazar BR, Abramson SB, Lafaille JJ, Dustin ML. 2010. Protein kinase C-theta mediates negative feedback on regulatory T cell function. *Science* 328: 372-6

196. Williams LM, Rudensky AY. 2007. Maintenance of the Foxp3-dependent developmental program in mature regulatory T cells requires continued expression of Foxp3. *Nat Immunol* 8: 277-84

197. Sands BE, Kaplan GG. 2007. The role of TNFalpha in ulcerative colitis. *J Clin Pharmacol* 47: 930-41

198. Yamamoto M, Yoshizaki K, Kishimoto T, Ito H. 2000. IL-6 is required for the development of Th1 cell-mediated murine colitis. *Journal of Immunology* 164: 4878-82

199. Ito R, Shin-Ya M, Kishida T, Urano A, Takada R, Sakagami J, Imanishi J, Kita M, Ueda Y, Iwakura Y, Kataoka K, Okanoue T, Mazda O. 2006. Interferon-gamma is causatively involved in experimental inflammatory bowel disease in mice. *Clinical and Experimental Immunology* 146: 330-8

200. Khan WI, Motomura Y, Wang H, El-Sharkawy RT, Verdu EF, Verma-Gandhu M, Rollins BJ, Collins SM. 2006. Critical role of MCP-1 in the pathogenesis of experimental colitis in the context of immune and enterochromaffin cells. *American Journal of Physiology-Gastrointestinal and Liver Physiology* 291: G803-G11

201. Read S, Greenwald R, Izcue A, Robinson N, Mandelbrot D, Francisco L, Sharpe AH, Powrie F. 2006. Blockade of CTLA-4 on CD4(+) CD25(+) regulatory T cells abrogates their function in vivo. *Journal of Immunology* 177: 4376-83

202. Das S, Cho J, Lambertz I, Kelliher MA, Eliopoulos AG, Du K, Tsichlis PN. 2005. Tpl2/cot signals activate ERK, JNK, and NF-kappaB in a cell-type and stimulus-specific manner. *J Biol Chem* 280: 23748-57

203. Collison LW, Pillai MR, Chaturvedi V, Vignali DAA. 2009. Regulatory T Cell Suppression Is Potentiated by Target T Cells in a Cell Contact, IL-35-and IL-10-Dependent Manner. *Journal of Immunology* 182: 6121-8

204. Min B, Yamane H, Hu-Li J, Paul WE. 2005. Spontaneous and homeostatic proliferation of CD4 T cells are regulated by different mechanisms. *J Immunol* 174: 6039-44

205. Lawrenz M, Visekruna A, Kuhl A, Schmidt N, Kaufmann SH, Steinhoff U. 2012. Genetic and pharmacological targeting of TPL-2 kinase ameliorates experimental colitis: a potential target for the treatment of Crohn's disease? *Mucosal Immunol* 5: 129-39

206. Li X, Acuff NV, Peeks AR, Kirkland R, Wyatt KD, Nagy T, Watford WT. 2016. Tumor Progression Locus 2 (Tpl2) Activates the MTOR Pathway, Inhibits FoxP3 Expression and Limits Treg Immunosuppressive Functions. *J Biol Chem*

207. Hu Y, Green N, Gavrin LK, Janz K, Kaila N, Li HQ, Thomason JR, Cuozzo JW, Hall JP, Hsu S, Nickerson-Nutter C, Telliez JB, Lin LL, Tam S. 2006. Inhibition of Tpl2 kinase

and TNFalpha production with quinoline-3-carbonitriles for the treatment of rheumatoid arthritis. *Bioorg Med Chem Lett* 16: 6067-72