Regulatory T Cells, Transforming Growth Factor- β , and Immune Suppression

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Multiple types of cells and cytokines are found that actively suppress immune responses, a function that is critical to maintain self-tolerance and immune homeostasis. Naturally occurring regulatory T cells (Tregs) and the pleiotropic cytokine transforming growth factor (TGF)- β are the best characterized. Dysregulation of either one leads to various immunopathologies under physiologic conditions, demonstrating their essential roles in immune suppression. Tregs and TGF- β play important roles in the development of lung-related immune disorders, such as asthma and allergy. Understanding the function and regulation of Tregs and TGF- β during immune responses offers therapeutic promise for the control of these diseases. Our laboratory has been interested in understanding the mechanisms of immune suppression, particularly in studying the interrelated functions of Tregs and TGF- β in immune regulation. In this article, we discuss the recent progress that we have made in the relevant areas.

Keywords: T cells, regulatory; transforming growth factor- β ; Foxp3

Active immune suppression is critical for establishing self-tolerance, controlling inflammatory responses, and maintaining immune homeostasis. Naturally occurring regulatory T cells (Tregs), which comprise approximately 10% of peripheral CD4⁺ T cells, are a central component of active immune suppression (1, 2). Foxp3, an X-chromosome-linked transcription factor belonging to the forkhead family, is essential for the development and function of Treg cells (3, 4). *Foxp3* deficiency results in multiorgan autoimmune disorders in humans (IPEX) (5) and mice (Scurfy) (6) due to the ablation of the Treg population.

We have investigated the function and generation of Foxp3 expressing Treg cells. Foxp3 is generally thought to positively control the functions of Treg cells in a binary fashion because Foxp3 expression is sufficient to convey immunosuppressive activity in conventional T cells (3, 4, 7). Thus, current efforts are focusing on associating abnormal numbers of Treg cells with immune disorders. However, the quality of Treg cells is also critical for their function (8). It was found that intra-islet Treg cells expressed lower levels of Foxp3 than Treg cells from other peripheral lymphoid organs in diabetic NOD mice, whereas the frequencies of Foxp3 expressing Treg cells among different compartments were comparable (our observations). Thus, we are interested to know whether one of the quality control mechanisms for Treg cells is through tuning the expression levels of Foxp3. In support of this possibility, emerging evidence associ-

ates decreased Foxp3 expression in Treg cells with various human autoimmune disorders, such as graft-versus-host disease (9), autoimmune myasthenia gravis (10), and multiple sclerosis (11).

To investigate whether lowered Foxp3 expression can be causal for immune disorders, a mouse model in which attenuated expression of endogenous *Foxp3* gene was achieved by a targeted gene knock-in approach in C57BL/6 mice was generated in our laboratory. In these mice, a gene cassette coexpressing luciferase and enhanced green fluorescent protein (GFP), whose translation is under the control of two tandem internal-ribosomal-entry-sites (IRES), was inserted into the 3'-untranslated region (UTR) of the endogenous *Foxp3* locus of C57BL/6 mice to generate Foxp3-IRES-Luciferase-IRES-enhanced GFP (*FILIG*) allele.

Although heterozygous *FILIG* female mice (*FILIG*/+) were fertile and phenotypically normal, hemizygous *FILIG* male mice (*FILIG*/Y) were infertile and runted compared with age-matched wild-type (WT) mice. Over 50% of *FILIG*/Y mice developed scaly skin, and nearly all of them developed eyelid defects resembling blepharitis, a Th2 disorder, around 4 weeks of age. By 3 months of age, all the *FILIG*/Y mice succumbed to an aggressive lymphoproliferative autoimmune syndrome, manifested by extremely enlarged spleens and peripheral lymph nodes, infiltration of leukocytes into nonlymphoid organs, drastically increased levels of autoantibodies in the serum, and activated peripheral CD4⁺ and CD8⁺ T cells. Overall, *FILIG*/Y mice displayed phenotypes reminiscent of *Scurfy* mice (12) and T-cell-specific *Foxp3* knockout mice (3). The transcription of the endogenous *Foxp3* gene was intact in *FILIG* mice, as luciferase expression was detected in the lymphoid and in the nonlymphoid organs from sick *FILIG*/Y mice. By flow-cytometry analysis, GFP expressing cells were detected in CD4⁺ T cells but not other cell types. By intracellular Foxp3 staining, Foxp3 was found to be expressed in GFP⁺ *FILIG* T cells but at five- to tenfold lower levels. Compared with WT Treg cells, the surface expression of "signature genes" for Treg cells, such as CD25, CTLA-4, and GITR (13–15), were decreased in GFP⁺ CD4⁺ T cells from *FILIG*/+ mice. mRNA levels of *Foxp3*, *Cd25*, *Ctla4*, and *Gitr* were also decreased, suggesting that the down-regulation of these genes was at the mRNA level. It was serendipitous to obtain the *FILIG* mice because insertion of luciferase cDNA into 3'-UTR of Foxp3 genes inadvertently introduced four classical adenylate-uridylylate-rich elements (AREs), whose presence in the 3'-UTR of a gene is known to destabilize mRNA (16). Lowered Foxp3 expression in *FILIG* CD4⁺ T cells is therefore likely due to mRNA destabilization caused by the localization of the luciferase sequence in the 3'-UTR of the *Foxp3* mRNA. These results demonstrated that decreased Foxp3 expression altered the properties of Treg cells and resulted in an aggressive lymphoproliferative autoimmune syndrome in hemizygous male mice. Thus, Foxp3 programs the gene expression of Tregs in a tunable and dose-dependent fashion (17).

Further investigation revealed interesting phenotypic changes in the functions of Treg cells, which express decreased Foxp3. Foxp3 is required for the development and maintenance of Treg cells (3). However, decreased Foxp3 expression in *FILIG* T cells did not lead to defective thymic development of Treg cells. In

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addition, by adoptive transfer assays, we found that attenuated Foxp3 expression did not result in intrinsic defects in the homeostatic expansion/maintenance of Treg cells in the periphery. However, in the presence of Treg cells with normal levels Foxp3 expression, Foxp3 low-expressing cells repopulated the periphery poorly. Decreased CD25 expression on GFP⁺ *FILIG* CD4⁺ T cells compared with WT Treg cells could account for this phenomenon because Treg maintenance is dependent on IL-2 signaling (18–20). Extrathymic generation of Foxp3 expressing T cells can be promoted *in vitro* by TGF- β (21–23). TGF- β induced *de novo* Foxp3 expression in GFP⁺ *FILIG* CD4⁺ T cells to a similar extent as in WT Treg cells. Therefore, these results demonstrated that decreased Foxp3 expression did not affect the development, homeostatic expansion/maintenance, or TGF- β -driven *de novo* generation of Foxp3 expressing T cells (17). These findings present the possibility that defective and even ablated Foxp3 expression might not result in the total elimination of the “Treg lineage.” Thus, in *Scurfy* mice and *IPEX* human patients, Foxp3 expressing T-cell subsets are likely to be generated in the thymus and maintained in the periphery. Further studies are warranted to identify and characterize these Treg lineage cells lacking functional Foxp3.

In vitro, hypoproliferative (“anergic”) and immunosuppressive activities are two defining properties for Foxp3-expressing Treg cells that are thought to go hand-in-hand (24). Upon T-cell receptor (TCR) stimulation *in vitro*, although GFP⁺ *FILIG* cells remained anergic, their immunosuppressive activities were greatly impaired (Figures 1a and 1b). Thus, anergy and immunosuppression are two separable properties of Treg cells that are affected differentially by the expression levels of Foxp3 (17). In addition, using a transfer approach, we demonstrated that the intrinsic immunosuppressive activities of Foxp3⁺ *FILIG* CD4⁺ T

cells were also abolished *in vivo* (Figure 1c), although adoptively transferred GFP⁺ *FILIG* cells infiltrated efficiently into lymphoid and nonlymphoid organs. Thus, the suppressive function of Treg requires high-level Foxp3 (17). It remains to be addressed whether *in vitro* anergy is an intrinsic property of Treg lineage totally independent of Foxp3. Despite the fact that multiple molecules, such as CTLA-4 and CD25, have been suggested to contribute to the suppressive activities of Tregs, it remains unsolved whether any of them may be sufficient to render Treg suppressive function. It would be interesting to investigate whether any of these genes are able to reconstitute the Treg function in our mouse model.

The loss of suppressive activity of Treg cells could be sufficient to cause an aggressive lymphoproliferative autoimmune syndrome in *FILIG/Y* mice. However, further analysis of Foxp3⁺ *FILIG* cells revealed that these cells developed effector cell functions that likely contribute to these immune disorders. Based on cytokine production profiles, three types of effector T cells—Th1, Th2, and Th17—have been described (25, 26). Although Treg cells bear self-reactive TCRs (27, 28), GFP⁺ (Foxp3⁺) cells from healthy *FILIG*/+ mice did not exhibit an effector cell phenotype, suggesting that they did not spontaneously activate and convert into effector cells when substantial numbers of WT Treg cells were present (17). A large portion of GFP⁺ cells from diseased *FILIG/Y* mice produced IL-4, whereas the percentages of the cells expressing IL-2, IFN- γ , or IL-17 were only modestly increased compared with WT cells. The percentage of IL-4-producing cells also increased in Foxp3⁺CD4⁺ T cells from *FILIG/Y* mice, consistent with the Th2 disorder observed in these mice (Figure 2). Similarly, upon transfer, GFP⁺ *FILIG* cell preferentially converted into Th2-type effectors, despite the fact that coexisting Foxp3⁺ cells produced large quantities of

Figure 1. Attenuation of Foxp3 expression abrogated the immunosuppressive but not hypoproliferative activities of Treg cells. (a) red fluorescent protein positive (RFP⁺), green fluorescent protein positive (GFP⁺), RFP-CD25⁻, and GFP-CD25⁻ CD4⁺ T cells were sorted from *FIR*/+ (open bars) and *FILIG*/+ (solid bars) mice. Purified cells were activated *in vitro*, and cell proliferation was determined by a T-cell proliferation assay. Data are means $\pm$ SD of triplicates done in one experiment representative of three. (b) Suppression assay performed using sorted RFP⁺CD4⁺ T cells from *FIR*/+ (blue line) or GFP⁺CD4⁺ T cells from *FILIG*/+ (red line) mice as suppressor cells (S) and RFP-CD4⁺ T cells sorted from *FIR*/+ mice as responder cells (R). Data are mean value $\pm$ SD of triplicates done in one experiment representative of three. (c) *Rag1*^{-/-} mice were transferred with RFP⁺CD4⁺ T cells sorted from *FIR*/+ mice alone (black line), or with T-cell mixtures containing one third of RFP⁺CD4⁺ T cells sorted from *FIR*/+ mice (blue line) or GFP⁺CD4⁺ T cells sorted from *FILIG*/+ mice (red line) and two thirds of RFP-CD4⁺ T cells sorted from *FIR*/+ mice. The percentages of body weight changes of the recipient mice were determined weekly for 9 weeks after transfer. Data are means $\pm$ SD of six mice from one experiment representative of two.

IFN- γ and therefore provided Th1-polarizing conditions (17). Thus, Foxp3-expressing cells with decreased Foxp3 expression preferentially converted into Th2-type effector cells *in vivo*, even in the presence of IFN- γ -producing Th1 cells. Upon conversion into Th2-type effectors, GFP⁺ *FILIG* cells potently promoted the Th2 differentiation of conventional T cells *in vivo* and *in vitro* regardless of the Th1 skewing conditions imposed by the cytokine milieu (17). These findings present the possibility that Foxp3-expressing cells might not always suppress but rather can foster the immune responses (e.g., under inflammatory conditions). In fact, TNF- α , a proinflammatory cytokine, has been shown to repress the expression of Foxp3 in human Treg cells (29). It is reasonable to believe that in a highly inflammatory microenvironment, residential Tregs decrease their Foxp3 expression, lose their suppressive function, and might be converted into effector cells to contribute the immune responses. Upon resolution of infection, inflammatory cytokines would subside and Foxp3 levels, and suppressive activity would be resumed.

The findings that attenuated Foxp3 expression subverted Treg suppressive function and converted them into effector cells present more questions. How does Foxp3 wire the gene regulatory circuitry to allow for this tunable regulation? What are the Foxp3-dependent genes of Treg cells that are essential for immune suppression? And upon what stimulation is Foxp3 expression is down- or up-regulated and through what mechanism? These questions need to be further explored for us to gain a better understanding of the biology of Treg and immune suppression.

TGF- β AND IMMUNE SUPPRESSION

Cytokine-mediated immune suppression has also been our long-standing interest. In particular, we are focusing on investigating the function and the regulation of TGF- β in immune responses. TGF- β consists of a family of pleiotropic cytokines that regulate

multiple facets of cellular functions, such as proliferation, differentiation, migration, and survival. TGF- β function in the immune system was first described in 1986 (30, 31). The critical roles of TGF- β in suppressing immune responses were not unraveled until the analysis of TGF- β -deficient mice (32, 33). Later studies evaluating the components of TGF- β signaling networks, including their receptors and intracellular signaling transducers, further confirmed the suppressive role for TGF- β in the immune system (34), revealing its role in regulating the components of adaptive immunity, such as T cells, and innate immunity components, such as natural killer cells (35).

The earlier studies using TGF- β 1^{-/-} mice did not discern whether T cells are direct targets of TGF- β -mediated inhibition because TGF- β 1 modulates the functions of multiple cell types. Several groups including ours have since used transgenic approaches to block TGF- β signaling in T cells by expressing dominant TGF- β receptors (36, 37). These mice displayed much less immune pathology than TGF- β 1^{-/-} mice. This is possibly due to insufficient expression of the transgenes or incomplete inhibition of TGF- β signaling. Subsequent studies demonstrated that deletion of TGF- β RII in bone marrow cells results in an immune pathology similar to the one found in TGF- β 1^{-/-} mice (38). However, the contribution of T cells to such a phenotype remain undetermined. Recently, more definitive studies from our laboratory have uncovered the essential role of TGF- β signaling in controlling the development, homeostasis, and tolerance of T cells through Treg-dependent and Treg-independent (39). Mice with T-cell-specific TGF- β RII deletion (4cre-RII/RII) developed a progressive wasting disease and died by 5 weeks of age. In these mice, leukocytes infiltrated into multiple tissues and nonlymphoid organs, autoantibody levels were elevated, and peripheral T cells displayed activated phenotypes. The numbers of canonical CD1d-dependent natural killer T cells and CD8⁺ T cells were decreased upon TGF- β RII deletion, suggesting a critical role for TGF- β signaling in their development. However, other compartments of T cells seemed to develop normally. Although TGF- β RII^{-/-} Tregs developed normally in the thymus at an even higher percentage than the WT counterparts, they are poorly maintained in the periphery, a finding that is in concert with other groups' results (40, 41). This decrease of Treg cells in the periphery is not due to impaired proliferation but is likely caused by increased apoptosis (39). Together, these studies suggest that TGF- β exerts multifaceted effects on immune cells in a manner dependent on the types of target cells and the microenvironment they are exposed to. The mechanism involved in mediating such diverse functions of TGF- β on the development and maintenance of various T-cell subsets warrants further investigation.

A priori, the T-cell activation phenotype observed in 4cre-RII/RII mice might be attributed to decreased Treg numbers in the periphery. However, using a transfer model, we and others found that the existence of substantial numbers of WT Treg cells did not prevent the spontaneous activation of T cells lacking TGF- β RII (39, 40). This suggests that WT Tregs are not able to suppress T cells that cannot respond to TGF- β , which is consistent with previous reports (42–44). It does not establish a Treg-independent role of TGF- β in controlling T-cell activation because Treg-mediated suppression might be through a TGF- β -dependent mechanism. Compelling evidence to support that TGF- β controls T-cell activation through Treg independent fashion came from the analysis of 4cre-RII/RII mice with OTII TCR transgene on a *Rag1*^{-/-} background, where endogenous Foxp3⁺ Treg cells failed to develop. Substantial portions of T cells from these mice displayed activated phenotype, whereas only a small percentage of these cells produced effector cytokines, which could be due to lack of stimulation from cognate antigens (Figure

Figure 2. Treg cells converted into Th2 effector cells due to decreased Foxp3 expression. The production of IL-2, IL-4, IL-17, and IFN- γ in RFP⁺ and RFP⁻ CD4⁺ T cells from FIR/Y mice and GFP⁺ and GFP⁻ CD4⁺ T cells from FILIG/Y mice.

Figure 3. TGF- β RII-deficient but not wild-type T cells display activated phenotype in the absence of Treg cells. (a) Expression of CD44 and CD62L in splenic RII/RII OT-II and 4cre-RII/RII OT-II T cells, both of which were *Rag1* deficient. These are representative results of five independent experiments. (b) Splenic RII/RII OT-II and 4cre-RII/RII OT-II T cells were stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin for 4 hours and analyzed for the expression of IFN- γ and IL-4 by intracellular cytokine staining.

3). Treg-independent TGF- β -dependent regulation of immune functions is an area that is poorly understood. Future studies are needed to define the mechanisms involved.

T-bet encoded by the *Tbx21* gene is a transcription factor that is critical for IFN- γ production and Th1 differentiation of CD4⁺ T cells (45). We attempted to alleviate/rescue the Th1-type immune disorder observed in 4cre-RII/RII mice by creating 4cre-RII/RII mice deficient in *Tbx21* gene. CD4⁺ T cells from 4cre-RII/RII-*Tbx21*^{-/-} mice remained activated but with much less IFN- γ production. Therefore, TGF- β suppresses T-cell activation through a T-bet-independent mechanism, whereas T-bet

remains essential for IFN- γ expression. In addition, CD4⁺ T-cell numbers were decreased in these mice, likely due to decreased expression of CD122 (IL-2R β) (Figure 4a), a receptor that is important for IL-2 and IL-15 signaling. Further analysis revealed that Th1 skewing conditions preferentially up-regulated CD122 on CD4⁺ T cells *in vitro* in a T-bet-dependent manner (39). Addition of TGF- β inhibited the up-regulation of CD122 on CD4⁺ T cells (Figure 4), suggesting that physiologically TGF- β limits CD4⁺-effector T-cell numbers through controlling IL-2- and IL-15-driven T-cell expansion. Because TGF- β potently inhibits T-bet expression in Th1 cells (46), it remains to be

Figure 4. CD122 expression was inhibited by TGF- β and required T-bet. (a) Naive CD4⁺ T cells isolated from C57BL/6 mice were cultured under Th1 condition in the absence or presence of TGF- β (right) for 4 days, and CD122 expression was compared between different conditions. These are representative results of two independent experiments. (b) Expression of CD122 in splenic CD4⁺ (left) and CD8⁺ (right) T cells of RII/RII (wild-type [WT]), *Tbx21*^{-/-} (T-bet knockout [KO]), 4cre-RII/RII (RII KO), and 4cre-RII/RII *Tbx21*^{-/-} (RII T-bet KO) mice. This is a representative plot of four mice from each group.

addressed whether TGF- β inhibits CD122 expression through T-bet-dependent or independent mechanisms.

Collectively, these studies highlight the multifaceted effects of TGF- β on various immune functions and emphasize the importance of the cellular and environment contexts in directing the discrete roles of TGF- β . Further studies are needed to determine what triggers and mediates TGF- β to perform such a broad spectrum of functions.

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