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Review

Induced Foxp3$^+$ regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases?

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Foxp3$^+$ T regulatory cells (Tregs) consisting of natural and induced Treg subsets play a crucial role in the maintenance of immune homeostasis against self-antigen. The actions designed to correct defects in numbers or functions of Tregs may be therapeutic in the treatment of autoimmune diseases. While recent studies demonstrated that natural Tregs are instable and dysfunctional in the inflammatory condition, induced Tregs (iTregs) may have a different feature. Here we review the progress of iTregs, particularly focus on their stability and function in the established autoimmune diseases. The advantage of iTregs as therapeutics used under inflammatory conditions is highlighted. Proper generation and manipulation of iTregs used for cellular therapy may provide a promise for the treatment of many autoimmune and inflammatory diseases.

Keywords: autoimmune and inflammatory diseases, immunoregulation, regulatory T cells, TGF-β, Foxp3, Th17 cells, atRA

Introduction

It is now well accepted that a cell population called 'CD4$^+$CD25$^+$ regulatory or suppressor cells' is critically involved in immune tolerance and homeostasis. Gershon and Kondo (1970) initially reported that thymocytes from their experimental animal model included a population of suppressor T cells. This suggestion was not appreciated until Sakaguchi et al. (1995) found that a population of CD4$^+$CD25$^+$ cells did indeed possess immunosuppressive activity that is now referred to as regulatory T cells or natural regulatory T cells, nTregs.

CD4$^+$CD25$^+$ cell populations also exist in humans, although only the CD4$^+$CD25^{bright} cell population appears to display an immune suppressive effect. A better approach for the identification of human Foxp3$^+$ T regulatory cells (Tregs) is to target the CD4$^+$CD25$^+$CD127$^{-/low}$ population (Seddiki et al., 2006).

CD25 is also an activation marker for lymphocytes. Thus, the utility of CD25 expression as a Treg marker is limited since it does not discriminate between activated T effector (Teff) cells and Tregs. Fortunately, the nuclear transcription factor Foxp3 has been identified as a much more specific marker for Tregs. Foxp3 is critically involved in the development and function of Tregs (Fontenot et al., 2003). In mice, the lack of functional Foxp3 expression results in a fatal lymphoproliferative disorder known as scurfy and mutations of the human FOXP3 gene results in a human syndrome known as IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked), which is characterized by autoimmune disease expression in multiple endocrine organs (Wildin et al., 2001).

Despite the fact that Foxp3-GFP 'knock-in' studies clearly demonstrate that there is a very broad spectrum of CD25 expression on Tregs and that the intranuclear location of Foxp3 makes it difficult to use this protein for immunoaffinity-based purification methods although we have identified a new technique to improve the isolation of the live Tregs (Zhou et al., 2010a), CD4$^+$CD25$^+$ cells are still widely used in the field of the biology of Tregs without using genetically modified tissues, particular in human studies. Although Foxp3 is considered as a specific marker for Tregs in the mouse, this may not be the case for human Tregs. Recent data demonstrate that FOXP3 (FOXP3 for human cells and Foxp3 for mouse cells) may be upregulated in rapidly proliferating human T cells and might be viewed as an activation marker for human T cells (Allan et al., 2007). More studies are needed to determine how FOXP3 might also be expressed on rapidly proliferating human Teff cells and more specific molecular markers to identify human Tregs are also desirable.

Many studies have revealed that the numbers of CD4$^+$CD25$^+$ cells and CD4$^+$FOXP3$^+$ cells in patients with various autoimmune diseases are diminished and that this Treg deficit is associated

with disease activity (Tritt et al., 2008). This peripheral Treg deficit in patients with autoimmune diseases is not resultant from their redistribution to different organs (Miyara et al., 2005). Diminishment of Tregs in the face of autoimmunity is not a universal finding. Other groups have actually observed the converse; and the numbers of human $CD4^+CD25^+$ cells can be increased under these circumstances (Yan et al., 2008). Since CD25 and FOXP3 can also be classified as activated makers, this aspect may reflect the disparity between these findings. Miyara et al. (2009) have further classified human $FOXP3^+$ cells into three cell subsets: $CD45RA^+FOXP3^{low}$, $CD45RA^-FOXP3^{hi}$, and $CD45RA^-FOXP3^{low}$. A functional assay demonstrated that the $CD45RA^-FOXP3^{low}$ subset contains non-suppressor cells, the $CD45RA^+FOXP3^{low}$ subset contains resting Tregs, and active Tregs are found in the $CD45RA^-FOXP3^{hi}$ subset. Using these criteria, they found that Treg cell numbers were indeed diminished in patients with active autoimmune disease.

In addition to Treg frequency, others have also reported that autoimmune diseases can alter the functional activity of Tregs. For example, the suppressive activity of $CD4^+CD25^+$ cells isolated from active rheumatoid arthritis (RA) patients was significantly decreased (Valencia et al., 2006). It is likely that some intrinsic defect in $CD4^+CD25^+$ cells in active RA patients accounts for their decreased functional activity. Similarly, the number of $CD4^+CD25^+$ cells in patients with multiple sclerosis (MS) is unaltered; however, the functional activity of these cells to suppress T cell immune responses including antigen-specific or non-specific stimulation is also decreased (Viglietta et al., 2004; Haas et al., 2005; Kumar et al., 2006). These results suggest that the manipulation of nTregs to restore their numbers and function may be therapeutic.

Although most people claim that $CD4^+CD25^+$ cells in peripheral blood belong to nTregs, we and others would suggest that $CD4^+CD25^+$ cells in PBMCs consist of a mixture of both thymic nTregs and periphery induced Tregs (iTregs) (Horwitz et al., 2008; Zhou et al., 2011). There is no specific marker that can distinguish nTregs from iTregs so far. Although Shevach's group recently reported that Helios, an Ikaros family transcription factor, may be helpful for distinguishing nTregs from iTregs (Thornton et al., 2010), others reported that Helios is also highly expressed on Th2 and T follicular helper cells and may be associated with the differentiation of these cells (Serre et al., 2011).

It has been well known that the adoptive transfer of nTregs can prevent the appearance and development of autoimmune diseases in many animal models. Conversely, there are also considerable numbers of studies demonstrating that the therapeutic effect of nTregs on established diseases is fairly unsatisfactory. For example, the efficacy of adoptive transfer of nTregs to established collagen-induced arthritis (CIA) is low for controlling the disease progression (Zhou et al., 2010b). Injection of nTregs to established lupus had mild protective effects and it failed to suppress lupus glomerulonephritis and sialoadenitis (Bagavant and Tung, 2005; Scalapino et al., 2006). Moreover, adoptive transfer of nTregs was unable to suppress other Th17-mediated autoimmune disease (Huter et al., 2008).

There are several possibilities that could explain the inability of nTregs to treat CIA and other autoimmune diseases. First, pro-inflammatory cytokines may hamper their suppressive activity. Pasare and Medzhitov (2003) have reported that Treg suppressive activity can be abolished by interleukin-6 (IL-6). Valencia et al. (2006) also revealed that elevated tumor necrosis factor-alpha (TNF-α) may interfere with the suppressive capacity of nTregs. There is no question that these pro-inflammatory cytokines are elevated in RA patients (Salliot et al., 2011). Second, Th17 cells may be resistant to the suppressive effects exerted by nTregs. This could explain why nTregs are able to prevent development of disease before Th17 cells become established, while demonstrating ineffective suppression after disease symptoms are evident. Third, nTregs are inherently unstable and can be converted to Th1, Th2, Th17, and Tfh effector cells when they encounter an inflammatory milieu (Wan and Flavell, 2007; Xu et al., 2007; Tsuji et al., 2009; Lu et al., 2010b; Zhou et al., 2010b).

There are still other reasons that could hamper the utilization of nTregs as therapeutics. First, the intranuclear location of Foxp3 makes it difficult to purify nTregs for functional study. Second, nTregs constitute only 1%–2% of human $CD4^+$ T cells. nTregs must be expanded *ex vivo* to gain sufficient numbers for therapy. Although several groups have claimed that expansion *in vitro* can overcome this problem (Hippen et al., 2011), other laboratories have reported that repeated expansion alters Treg phenotype and function (Hoffmann et al., 2009). Third, the expansion of nTregs from patients with RA and MS for therapeutic purposes may be problematic due to potential other intrinsic defects in RA and MS Tregs. nTreg instability, Teff cell resistance and the influence of an inflammatory milieu may individually or collectively account for the failure of nTregs to control established autoimmune diseases.

Of interest, the plasticity of nTregs under inflammatory conditions could be fixed with cytokines or other compounds. Our group recently reported that while nTregs become Th17 cells in the presence of IL-6, these cells also lost their suppressive role in the progression of the lupus-like syndromes and CIA. And the pretreatment of nTregs with IL-2 combined with TGF-β, or *all-trans* retinoic acid (atRA), a vitamin A metabolite, can render these nTregs resistant to Teff cell conversion and allow them to begin to suppress lupus and CIA progression (Zheng et al., 2008; Zhou et al., 2010b). This indicates that the manipulation of nTregs still holds a promise in the treatment of autoimmune diseases.

iTregs and their development

Current studies have demonstrated that Tregs are a heterogeneous set of cells that consist of $CD4^+CD25^+Foxp3^+$ cells, IL-10-producing $CD4^+$ Tr1 cells, TGF-β-producing Th3 cells, $CD8^+$ cells, NK T cells, $CD4^-CD8^-$ T cells, and $\gamma\delta$ T cells (Horwitz et al., 2004; Tang and Bluestone, 2008). $CD4^+$ Treg subsets can be further classified into three main populations: thymus-derived naturally occurring nTregs, endogenous iTregs *in vivo*, and iTregs induced *ex vivo* from $CD25^-$ precursors (Zheng et al., 2002). Although IL-10-induced Tr1 cells represent another cell population of iTregs, they do not express Foxp3 and produce considerable levels of IL-10 (Pot et al., 2011). As IL-10 may promote autoimmune response through stimulating B

cell activation and its level is highly increased in active systemic lupus erythematosus (SLE) patients (Yu et al., 2011), Tr1 cells may not be suitable for the treatment of SLE and other autoimmune diseases. TGF-β-iTregs will be defined as iTregs in this review.

While Yamagiwa et al. (2001) reported that TGF-β promotes endogenous $CD4^+CD25^+$ cells, our group first reported that TGF-β does have an ability to induce $CD4^+CD25^-$ cells to become $CD4^+CD25^+$ Tregs *in vitro* (Zheng et al., 2002). When Foxp3 was identified as Treg marker, several groups immediately found that TGF-β can induce Foxp3 expression in iTregs (Chen et al., 2003; Fantini et al., 2004; Zheng et al., 2004a). Additionally, other studies have also clearly demonstrated the capacity of $Foxp3^+$ Tregs *in vivo* through TGF-β-dependent mechanism (Liang et al., 2005; Chen and Konkel, 2010).

Phenotypically, both nTregs and iTregs express similar molecules such as CD25, CTLA-4, GITR, CCR4, CD62L, and Foxp3, and express $CD45RB^{low}$ in mice and CD45RO in humans. $CD4^+CD25^+Foxp3^+$ cells in the periphery have been considered as a mixed population comprising nTregs and iTregs. Although Helios might possibly help to distinguish nTregs from iTregs (Thornton et al., 2010), more specific molecular markers are needed to distinguish both Treg cell populations.

Although both nTreg and iTreg subsets share similar phenotypes and display comparable suppressive activity, several factors distinctly affect their development, stability and function (Table 1). First, nTregs develop in the thymus through recognition of self-antigens. A high and medium affinity cognate interaction between self-peptide:MHC complex and T cell receptor is required for this process. They also require CD28 co-stimulation because they do not develop in CD28 deficient mice (Salomon et al., 2000). Although IL-2 and TGF-β play an important role in the maintenance of the pool size of nTregs, both cytokines are redundant for their development since both IL-2 and TGF-β knock-out mice contain $CD4^+CD25^+Foxp3^+$ regulatory T cells in the thymus (Piccirillo et al., 2002). In contrast, the generation of iTregs is dependent upon the presence of both TGF-β and TGF-β receptor signals since the absence of TGF-β or TGF-β receptors, or blocking the TGF-β receptor signal prevents the induction of Foxp3 expression and the subsequent functional suppressive capacity (Lu et al., 2010a, b). Similarly, IL-2 plays an essential role in the differentiation of $Foxp3^+$ iTregs. TGF-β fails to induce $Foxp3^+$ iTregs from naïve $CD4^+CD25^-$ precursor cells in IL-2 deficient mice (Zheng et al., 2007). The conversion of $CD4^+CD25^-$ cells in the periphery to $CD25^+$ iTregs requires a suboptimal TCR stimulation and thus environmental antigens may sufficiently trigger iTreg development. The absence of CD28 co-stimulatory molecules does not affect the differentiation of iTregs (our unpublished data), but inhibitory CTLA-4 co-stimulation and CTLA-4/B7.1 signaling is crucially required for the generation of iTregs (Zheng et al., 2006). This conclusion is further documented by an observation that the blocking of CTLA-4/B7.1 signal abolished the capacity of TGF-β to induce iTregs in wild type mice (Read et al., 2006). OX40/OX40L, an alternative CD28/B7-independent co-stimulatory pathway, also negatively regulates the development and function of both nTregs and iTregs. While stimulation of mature nTregs by OX40 results in the loss of suppression of T cell proliferation and cytokine production, the generation of iTregs is completely abolished by OX40 although OX40 does not affect the generation of nTregs (So and Croft, 2007).

Recently, Housley et al. (2011) reported that while the TNF-R2 expression is essential for nTregs-mediated suppression of colitis, its expression is not required for iTreg-mediated suppression. While cell-contact is essential for the suppressive activity of nTregs, soluble cytokines may play a dominant role in iTregs' suppression. Recent study has also demonstrated that HOZOT cells, an activated cytotoxic Treg cell line, can penetrate into target cells to exert their functional role via formation cell-in cell structures called emperipolesis (Takeuchi et al., 2010). It remains to be determined whether both nTregs and iTregs also need emperipolesis to affect target cells. Differing IL-2 and co-stimulatory molecule requirements for Treg development, and TNFRII expression requirements for the suppressive function of both nTregs and iTregs suggest that nTregs and iTregs are possibly heterogeneous populations and that integration of both Treg subsets is required for the maintenance of normal immune homeostasis. It is also likely that both nTreg and iTreg subsets can either act in concert or separately on different targets. In addition, as anti-TNF-α therapy has been widely used in treating patient with RA, further studies are required to understand whether this therapy differentially regulates nTreg and/or iTreg development in individual diseases.

Table 1 Differences between nTregs and iTregs.

	nTregs	iTregs
Origin	Thymus	Periphery
TCR stimulation		
Antigen	Self	Self and environmental
Affinity	High to medium	Suboptimal
Co-stimulations		
Essential	CD28	CTLA-4
OX40/OX40L	Function deficient	Generation deficient
Cytokines		
Generation essential	(−) or TGF-β? (−) or IL-2?	TGF-β IL-2
Maintenance essential	IL-2	IL-2
Function essential	TNF-α (+)	TNF-α (−)
Stabilities		
IL-6 or others	Conversion to Th17, Th1, Th2, and Tfh	No conversion, possibly to Th1
Phenotype	Helios (+)	Helios (−)
Human	No cytokine production	Cytokine production

iTregs and their stability in the inflammatory condition

As TGF-β either promotes $Foxp3^+$ iTregs, Th9 or 17 cells depending upon other cytokines involved (Figure 1), and as nTregs express a membrane-bound form of TGF-β and this TGF-β has functional activities, it is reasonable to assume that IL-6 can convert nTregs to Th17 and other T helper cells (Xu et al., 2007). To demonstrate this, they used purified nTregs from Foxp3 GFP knock-in mice to exclude the possibility that $CD4^+CD25^+Foxp3^-$ non-Tregs made this conversion. We used both wild type and Foxp3 GFP knock-in mice to confirm this observation (Zheng et al., 2008). Endogenous TGF-β produced by nTregs is critically required for this conversion since blocking TGF-β receptor I signal or using nTregs from TGF-β receptor II

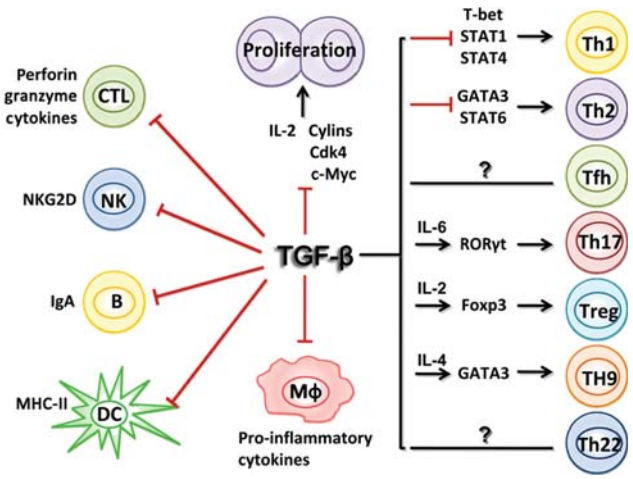

Figure 1 Multi-effects of TGF-β on regulatory and effector T cells. TGF-β inhibits the differentiation, proliferation, and function of various immune cells, including Th1, Th2, and Tfh cells. TGF-β also promotes iTreg, Th17, and Th9 cell differentiation depending upon the cytokine environment. Additionally, TGF-β inhibits maturation and function of other immune cells such as $CD8^+$ CTLs, NK cells, DCs, and macrophages.

dominant mice resulted in the failure of Th17 conversion (Zheng et al., 2008; Lu et al., 2010b). Moreover, activation of nTregs with IL-6 resulted in decreased Foxp3 expression and suppressive activity both *in vitro* and *in vivo*. Furthermore, adoptive transfer experiments revealed that nTregs treated with IL-6 *ex vivo* lost their ability to protect mice from a lupus-like disease (Zheng et al., 2008). Thus, in an IL-6 rich inflammatory milieu, nTregs may be unstable and lose the functional activity. Moreover, recently it has been demonstrated that nTregs can be converted into Th17 cells in an *in vivo* model (our unpublished data).

In sharp contrast, we first reported that TGF-β-induced iTregs were completely resistant to the Th17 conversion by IL-6 (Zheng et al., 2008). Another group has recently confirmed this finding (O'Connor et al., 2010). Although they found that iTregs could convert into Th1 cells, interestingly, this conversion did not significantly affect their suppressive activity. This difference cannot be explained by insufficient production of TGF-β by iTregs since both nTregs and iTregs expressed similar levels of membrane-bound TGF-β (20%–25%) and secreted similar levels of active TGF-β (about 40 ng/ml). Furthermore, the resistance of iTregs to Th17 conversion also may not be explained by alterations in TCR stimulation since anti-CD3/CD28 activated nTregs can still differentiate into Th17 cells upon IL-6 stimulation. To account for this difference between nTregs and iTregs, we found that the combination of IL-2 and TGF-β down-regulated IL-6 receptor expression and function in activated T cells. We have observed that both cytokines markedly decreased IL-6 receptor α-chain (CD126) and β-chain (CD132) expression on $CD4^+$ cells and these cells expressed significantly lower level of phosphorylated STAT3 when stimulated by IL-6 (Zheng et al., 2008). atRA has a similar effect on nTreg stability under inflammatory conditions as well (Zhou et al., 2010b). We further observed the differences of stability of both Treg cell subsets *in vivo*. About 50% of nTregs converted to Th17 cells in draining LNs 10 days after cell transfer to established CIA. Conversely, iTregs were completely resistant to Th1, Th2, and Th17 cell conversion. When these cells were sorted for *in vitro* analysis, nTregs mostly lost suppressive activity, whereas the functional activity of iTregs was mostly intact (our unpublished data). These results indicate iTregs are stable and functional in the inflammatory condition and may have a greater advantage to treat autoimmune and inflammatory diseases compared with nTregs.

Nonetheless, others have also reported that TGF-β-induced iTregs were unstable *in vitro* (Floess et al., 2007) and *in vivo* (Chen et al., 2011) following antigen-stimulation, and lack protective activity to prevent lethal graft versus host disease (GVHD) (Floess et al., 2007; Koenecke et al., 2009). It has been claimed that the Foxp3 promoter on TGF-β-induced iTregs but not nTregs is methylated, which accounts for their instability (Floess et al., 2007). However, we have recently observed that the methylation status in Foxp3 gene loci does not affect Foxp3 stability. Moreover, addition of atRA to TGF-β promoted iTreg stability and maintenance *in vitro* and *in vivo* and this effect is unrelated to CpG methylation in Foxp3 promoter but related to acetylation of Foxp3 histone (Lu et al., 2011). Others have also observed protective human TGF-β-iTregs that exhibit methylated Foxp3 (Hippen et al., 2011). To explain these controversial results, we consider the technical reasons are possibly responsible for the generation of unstable, ineffective TGF-β-induced iTregs in these groups. They have used high concentrations of plate-bound anti-CD3 with TGF-β, whereas our group has used suboptimal concentrations of anti-CD3 and anti-CD28 coated beads with IL-2 and TGF-β. It has been known that strong, sustained TCR stimulation activates the mTOR/Akt signaling pathway which facilitates Teff cell differentiation and inhibits Foxp3 expression and Treg differentiation (Sauer et al., 2008). Treg generation is best established with suboptimal TCR stimulation that facilitates Foxp3 expression (Horwitz et al., 2008).

These studies also raise the possibility that nTregs and iTregs may have distinct roles in the adaptive immune response. In response to microbial infections nTregs could possibly serve as a first line of host defense by differentiation to IL-17 producing cells which contribute to neutrophil mobilization and have other pro-inflammatory effects. After eradication of invading pathogens, the late appearance of TGF-β-induced iTregs would not only terminate the antigen-specific response, but also prevent the emergence of non-specifically stimulated or cross-reactive self-reactive T cells. Accordingly, failure of this mechanism could result in an immune-mediated disease.

Preventive roles of iTregs in the autoimmune diseases

iTregs and nTregs also share similar functional characteristics. Adoptive transfer of iTregs generated *ex vivo* also can prevent the development of autoimmune diseases. For example, Wahl's group has demonstrated that adoptive transfer of TGF-β-converted/induced iTregs prevented house dust mite-induced allergic pathogenesis and inflammation in lungs in an asthmatic mouse model (Chen et al., 2003). In a lupus model, our study has demonstrated that iTreg prevented disease development (Zheng et al., 2004b). Weber et al. (2006) observed that injection of murine islet-specific

$CD4^+$ iTregs prevented spontaneous development of type 1 diabetes and inhibited development of pancreatic infiltrates and disease onset orchestrated by Th1 effectors in NOD mice. Dipaolo et al. (2007) have also reported similar preventive role of iTregs in a murine model of autoimmune gastritis. Similarly, iTregs also significantly prevented Th1 mediated colitis on $CD4^+CD62L^+$ T cell transfer *in vivo* (Fantini et al., 2006). Selvaraj and Geiger (2008) demonstrated that adoptively transferred iTregs were as potent as natural $Foxp3^+$ Tregs in preventing experimental autoimmune encephalomyelitis (EAE) development. It seems both antigen-specific and non-specific iTregs prevent autoimmune diseases although the former is more efficacious than the latter.

Therapeutic roles of iTregs in the autoimmune diseases

In addition to their use in a preventive role, adoptive transfer of these cells to ongoing diseases still suppressed disease development in a lupus-like syndrome model (Zheng et al., 2004b). We have developed a chronic GVHD model characterized by rapid and vigorous formation of SLE-like autoantibodies and the formation of severe immune-complex glomerulonephritis. DBA/2 mouse T cells induce this syndrome when injected into (DBA/2 × C57BL/6) F1 mice. We found TGF-β-treated DBA/2T cells not only lost their ability to induce GVHD but also prevented other parental T cells from inducing lymphoid hyperplasia, B cell activation, and an immune-complex glomerulonephritis. Moreover, a single transfer of TGF-β-conditioned T cells to animals that had already developed anti-dsDNA Abs decreased the antibody titer, suppressed proteinuria, and doubled survival (Zheng et al., 2004b). This result was further confirmed by a study from Su et al. (2008). Selvaraj and Geiger (2008) observed that iTregs were still efficacious in ongoing EAE, animal model of MS, and Godebu et al. (2008) also reported that iTregs can revise type I diabetes in animal. Similarly, in autoimmune gastritis model, Nguyen et al. (2011) found that antigen-specific iTregs also still suppressed inflammation and associated pathology when administered late in the process of ongoing disease.

Recently, we conducted a head-to-head comparison of therapeutic effects of antigen-specific thymus-derived nTregs and TGF-β-induced iTregs on the established CIA. We chose antigen-specific Tregs since these are more protective than polyclonal Tregs in autoimmune diseases (Penaranda and Bluestone, 2009). It has been known that polyclonal nTregs can prevent disease but are ineffective in established CIA disease (Morgan et al., 2003; Zhou et al., 2010a, b). Our data clearly demonstrated that antigen-specific iTregs are superior to nTreg in ameliorating established CIA. This was because iTregs remained stable and fully functional following transfer. Moreover, these iTreg had tolerogenic effects in draining LN that resulted in a shift from Th17 to Treg predominance (our unpublished data). The recent studies of Nugyen et al. (2011) indicate that chemokines secreted by antigen-specific TGF-β-induced iTregs regulate T cell trafficking and thereby suppress ongoing autoimmune. They reported that these iTregs were therapeutic in an ongoing autoimmune gastritis model (Nguyen et al., 2011). Others have also reported that only TGF-β-induced iTregs but not nTregs suppressed Th17-mediated diseases (Huter et al., 2008). These studies suggest that iTregs may have a therapeutic potential in suppressing the established autoimmune diseases.

To explain the long-term effect of single infusion of iTregs on autoimmune diseases, although others have claimed that long-term survival of selected antigen-specific iTregs can account for this phenomenon (Godebu et al., 2008), 'infectious tolerance' may be another main reason for immune tolerance effect of iTregs (Zheng et al., 2004a; Andersson et al., 2008). We recently have observed that injected iTregs suppressed DC accumulation and maturation in spleen and lymph nodes, and induced tolerogenic or regulatory DC formation. Moreover, injection of DCs isolated from lupus mice that had received iTregs but not control cells were able to prevent other parental T cells from inducing typical lupus syndromes in (DBA/2 × C57BL/6) F1 mice (our unpublished data).

Development and function of iTregs in humans

While the ability of TGF-β to induce $Foxp3^+$ iTregs is well recognized in mouse cells, it is arguable that whether this cytokine can induce human iTregs. Unlike Foxp3 expression in mouse cells, FOXP3 expression in human T cells may be also an activation marker, for example, conventional anti-CD3 stimulation of human $CD4^+$ cells in the absence of TGF-β results in a small transient expression of FOXP3 (Morgan et al., 2005; Allan et al., 2007) although not all groups support this observation (Tran et al., 2007). Both Shevach's group (Tran et al., 2007) and our group (Zheng et al., 2004a) consistently observed TGF-β markedly increased FOXP3 expression in human $CD4^+$ T cells. It is notable these TGF-β induced $CD4^+FOXP3^+$ cells in human are not anergic cells. They produce ample IL-2 and other cytokines when re-stimulated with anti-CD3/CD28 beads. Addition of these cells to other T cells fails to suppress the T responder cell proliferation *in vitro*. Therefore, they conclude that TGF-β is unable to induce human T cells to develop the suppressive activity despite their high level of FOXP3 expression.

Nevertheless, Yamagiwa et al. (2001) have previously observed TGF-β can indeed promote the induction of human $CD4^+$ Tregs. When naïve human $CD4^+$ cells were stimulated with alloantigen plus TGF-β for 1 week, the TGF-β-treated but not control cells (without TGF-β) prevented $CD8^+$ T cells from proliferating in response to alloantigens and from becoming cytotoxic effector cells. Moreover, these regulatory cells exerted their suppressive activities in remarkably low numbers and maintained these effects even after they were expanded. We subsequently also reported that with a low-dose staphylococcal enterotoxin B stimulation, TGF-β did convert human $CD4^+CD25^-$ cells to become Th3-like suppressor cells. Similarly, these cells also had potent suppressive activity since adding as few as 1% of these TGF-β-primed $CD4^+$ T cells to fresh $CD4^+$ cells and B cells markedly suppressed IgG production. The inhibitory effect was mediated by TGF-β and was also partially contact dependent (Zheng et al., 2002).

Indeed, polyclonally activated human $CD4^+$ T cells primed with TGF-β were less suppressive both *in vitro* and *in vivo*. We have used a xenograft model in which human PBMCs were adoptively transferred into SCID common-γ chain knock-out mice to develop the syndrome of xeno GVHD. We have observed that

co-transfer of TGF-β-induced human $CD4^+$ T cells was less able to suppress the expansion and cytokine production by human T cells in these mice. Interestingly, co-transfer of $CD4^+$ T cells generated with TGF-β plus atRA can prolong the survival of diseased mice, indicating that with the proper modification, TGF-β is still able to induce human iTreg cell differentiation (Lu et al., 2010c). More recently, two groups have also demonstrated that the combination of both TGF-β and rapamycin is able to induce human iTregs (Hippen et al., 2011; Qian et al., 2011). In these studies, atRA or rapamycin alone was unable to induce human iTregs, raising a possibility that TGF-β is still a key factor mediating the differentiation of iTregs in human although the underlying mechanisms of the difference in the development of mouse and human iTregs need to be further studied.

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