TNF-α impairs differentiation and function of TGF-β-induced Treg cells in autoimmune diseases through Akt and Smad3 signaling pathway

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Deficiency in the TGF-β-induced regulatory T (iTreg) cell differentiation is associated with compromised immune homeostasis and plays a key role in many autoimmune diseases. Therapeutic intervention to enhance in situ iTreg differentiation has become a promising treatment modality for autoimmune diseases. Here we describe that the development of autoimmune inflammation in experimental autoimmune encephalomyelitis (EAE) is associated with selective impairment of iTreg differentiation largely due to the increased production of TNF-α. The neutralization of TNF-α markedly increases iTreg differentiation, leading to the amelioration of EAE, whereas the depletion of iTreg cells abolishes the therapeutic effect of an anti-TNF-α antibody. The inhibition of iTreg differentiation by TNF-α is mediated through a signaling cascade involving the induction of TNF receptor II (TNFR2) expression and the activation of Akt. The activated Akt in turn interacts with Smad3, resulting in the inhibition of TGF-β-induced Smad3 phosphorylation and consequently the reduction of p-Smad3 results in the decreased binding to the specific binding site of the foxp3 promoter, and finally foxp3 transcription itself. Interestingly, this regulatory pathway is iTreg cell specific as TNF-α does not activate Akt in naturally occurring regulatory T cells, therefore conferring a selective effect of TNF-α and its antagonism on iTreg cells. The study sheds new light on the critical role and underlying mechanism of TNF-α in the regulation of iTreg differentiation and provides a novel rationale for TNF-α antagonistic therapy for autoimmune diseases.

Keywords: tumor necrosis factor alpha (TNF-α), transforming growth factor beta (TGF-β), forkhead box P3 (Foxp3), Smad3, protein kinase B (Akt), autoimmune disease

Introduction
Regulatory T (Treg) cells are characterized by the expression of transcription factor Forkhead box P3 (Foxp3). They play a pivotal role in keeping the inflammatory T cells, e.g. Th1 and Th17 cells, in checking and in maintaining self-tolerance and immune homeostasis (Sakaguchi et al., 2008). Local cytokine milieu in the tissue significantly modulates the balance between inflammatory T cells and Foxp3+ Treg cells (La Cava, 2008). Deficiency in Treg cell numbers and/or function can lead to dominance of inflammatory T cells and progressive breakdown of self-tolerance and perpetuation of inflammation in many human autoimmune diseases, including multiple sclerosis, rheumatoid arthritis (RA) and type I diabetes (T1D) (Buckner, 2010). Rebuilding the balance by boosting the number and/or function of Treg cells have attracted considerable attention as potential treatment of autoimmune diseases (Read et al., 2000; Kohm et al., 2002).

Treg cells comprised a pool of naturally occurring Treg (nTreg) cells and a dynamic population of peripherally induced regulatory T (iTreg) cells (Bluestone and Abbas, 2003). Both populations of Treg cells share the similar phenotypic characteristics but differ in their origin, differentiation, and stability (Curotto de Lafaille and Lafaille, 2009). nTreg cells are generated in the thymus following the stimulation by the high avidity self-antigens (Jordan et al., 2001). IL-2 and other common γ-chain cytokines are also essential for thymic development of nTreg cells (Vang et al., 2008). Although nTreg cells are originally thought to represent a lineage-specific cell type (Rubtsov et al., 2010), recent studies show that nTreg cells can convert to other T cell types, such as Th1, Th2 and Th17 cells, in a pro-inflammatory milieu (Wan and Flavell, 2007; Xu et al., 2007; Zhou et al., 2009).

iTreg cells are induced from naïve conventional CD4+ T cells in periphery under various conditions, including oral tolerance,
tumor microenvironment, and chronic inflammation (Mucida et al., 2005; Liu et al., 2007; Curotto de Lafaille et al., 2008). TGF-β is a key cytokine for iTReg differentiation by inducing phosphorylation of Smad3, which stimulates Foxp3 transcription by binding to the transcription control elements of Foxp3 (Chen et al., 2003; Tone et al., 2008). Mounting evidence suggests that TGF-β-induced iTReg cells are less plastic and more resistant to reprogramming to become inflammatory T cells in inflammatory cytokine milieu (Zheng et al., 2008). Therefore, strategies to promote de novo generation of iTReg cells have become promising therapeutic approaches in the treatment of autoimmune diseases. The significance of iTReg cells in autoimmune diseases stems from mounting evidence that iTReg differentiation is severely impaired in autoimmune diseases (Bluestone et al., 2008). A critical factor that impairs iTReg differentiation at a cellular level is the composition of local cytokines (Wei et al., 2007). However, the key pro-inflammatory cytokine(s) that inhibits the iTReg differentiation in autoimmune disease has not been identified.

TNF-α is a critical cytokine in the initiation and perpetuation of inflammation and is linked to many human autoimmune diseases such as Crohn’s disease, T1D, and RA (Kruglov et al., 2008). TNF-α blocking therapy has become the mainstream treatment for RA patients with proven efficacy, although the mechanism of action is still not completely understood. TNF-α has been shown to inhibit the regulatory function of human CD4⁺CD25⁺iTReg cells in RA patients through the down-regulation of Foxp3 expression (Valencia et al., 2006). Anti-TNF-α therapy also increases the number or the function of Treg cells in patients with Crohn’s disease, T1D, RA, and in an animal model of inflammatory bowel disease (IBD) (Ehrenstein et al., 2004; Nadkarni et al., 2007; Ricciardelli et al., 2008; Boscheri et al., 2010; Ryba et al., 2011). Still, other studies report that TNF-α expands mouse nTReg cells in vitro and even in vivo (Chen et al., 2007; Grinberg-Bleyer et al., 2010). Exactly, how TNF-α regulates T cells in autoimmune diseases has not been delineated.

This study was inspired by our initial observation that TNF-α significantly inhibits iTReg differentiation in vitro. Together with other recently published studies, it prompted us to investigate the effects of TNF-α on nTReg and iTReg cells separately. Through establishing a unique experimental autoimmune encephalomyelitis (EAE) model in chimeric SCID mice, we provide compelling evidence showing that iTReg cells, but not nTReg cells, contribute to the increased total T cell pool in anti-TNF-α therapy. The specific inhibition of iTReg differentiation by TNF-α is mediated by its selective inhibition of TGF-β–Smad3 signaling pathway via the regulation of TNF receptor II (TNFR2)–Akt signaling, which is absent from nTReg cells. The study has important implications in our understanding of the role of TNF-α in inflammation and provides a basis for novel therapeutic strategy for treating autoimmune diseases.

Results

Amelioration of EAE by an anti-TNF-α antibody is associated with increased levels of Treg cells

To elucidate the mechanism by which TNF-α affects T cells in autoimmune diseases, we examined the effect of TNF-α neutralizing antibody on Treg cell development and function in a mouse model of EAE. C57BL/6 mice were induced EAE and injected intraperitoneally with anti-TNF-α or an isotype control antibody every 2 days, starting on the day of immunization. As expected, the neutralization of TNF-α resulted in a substantial delay in the disease onset, a marked reduction in the EAE severity, and a decreased demyelination in the affected spinal cord when compared with control mice (Figure 1A). Consistently, the frequencies of Th1 cells and Th17 cells were significantly reduced both in the spleen and spinal cord of an anti-TNF-α antibody-treated mice (Figure 1A).

The neutralization of TNF-α also had a significant effect on Treg cells. Both the percentages and the number of Treg cells in the lymph node and spleen were markedly elevated following the anti-TNF-α treatment, while the total number of CD4⁺ cells was not significantly changed during the course of EAE (Figure 1B and Supplementary Figure S1A–D). The percentage of Treg cells in the spinal cord was also increased following the anti-TNF-α treatment, despite that the total number of infiltrating inflammatory cells was significantly decreased (Figure 1B and Supplementary Figure S1E). In contrast, the anti-TNF-α treatment did not alter the Treg phenotype (Supplementary Figure S2) or suppressive function as indicated by inhibiting the proliferation of responder cells in vitro (Figure 1C). Importantly, the depletion of CD25⁺ TReg cells by the anti-CD25 antibody treatment partially abolished the treatment efficacy of an anti-TNF-α antibody (Figure 1D), suggesting a role of TReg cells in mediating the anti-TNF-α efficacy on EAE. These results suggest that the mechanism of attenuation of EAE by neutralizing TNF-α depends on the increased levels of Treg cells but not the enhancement of their suppressive function.

Neutralization of TNF-α leads to induction of iTReg cells

To determine whether the elevated levels of Treg cells following the anti-TNF-α treatment is due to an expansion of nTReg cells or an increase of iTReg cells, we established a chimeric mouse model where nTReg and iTReg cells could be distinguished by the expression of either Thy1.1 or Thy1.2. As illustrated in Figure 2A, splenic Thy1.2⁺CD4⁺Foxp3⁺ nTReg cells of Foxp3-gfp.ki mice were mixed with Thy1.1⁺CD4⁺CD25⁻ non-nTReg cells of Thy1.1 congenic mice at a ratio of 1:9 to mimic the physiological ratio between nTReg and non-TReg cells. The cell mixture was adoptively transferred into SCID mice one day before immunization, followed by treatment with anti-TNF-α or an isotype control antibody. Compared with the control antibody-treated mice, the percentages and absolute number of Thy1.1⁺Foxp3⁺ iTReg cells, but not Thy1.2⁺Foxp3⁺ nTReg cells, were markedly increased (~2 folds) in the anti-TNF-α-treated mice (Figure 2B and C, and Supplementary Figure S3). To exclude the possibility that the observed increase in Thy1.1⁺Foxp3⁺ iTReg cells is due to the expansion of a few pre-existing CD25⁻Foxp3⁺ cells in the transferred Thy1.1⁺CD4⁺CD25⁻ population, we carried out a similar mixing experiment by using Thy1.2⁺CD4⁺Foxp3⁻ cells from Foxp3-gfp.ki mice as a source of iTReg cells. The percentage of Thy1.2⁺CD4⁺Foxp3⁺ iTReg cells was also elevated about 2-fold in the periphery of anti-TNF-α-treated mice when compared with control antibody-treated mice (Supplementary Figure S4). A combined analysis of the change of iTReg and nTReg cells revealed that
the increased percentages of total Treg cells after an anti-TNF-α antibody treatment is primarily due to the increased percentages of iTreg cells (Figure 2D).

To further exclude any significant effect of the anti-TNF-α treatment on nTreg cells, we analyzed the transferred Thy1.2+ donor cells in recipient mice. Ten days post-transfer, similar percentages (75%–80%) of Thy1.2+ cells expressed Foxp3 in both groups of mice (Figure 2E). By 18 days post-transfer, although the percentages of Thy1.2+ cells that expressed Foxp3 had decreased, there were still no significant differences between the two groups (57% vs. 65%). Furthermore, the anti-TNF-α treatment did not significantly affect the proliferation of nTreg cells as measured by Ki67 staining (Figure 2F) or suppressive function of nTreg cells (Supplementary Figure S5). In addition, when CD45.2+Foxp3+ nTreg cells from Foxp3-gfp.ki mice were adoptively transferred into CD45.1 wild-type (WT) mice (vs. SCID mice), the anti-TNF-α treatment did not affect their expression of Foxp3 or proliferation (Supplementary Figure S6). At last, the proliferation of iTreg cells was not altered by the anti-TNF-α treatment either, indicating that the enhanced numbers of iTreg cells upon the anti-TNF-α treatment were a result of iTreg differentiation but not expansion (Figure 2G). Together, these results show that the neutralization of TNF-α preferentially promotes the induction of iTreg cells without affecting nTreg cells.

Induction of iTreg cells mediates the efficacy of anti-TNF-α treatment of EAE

To determine the function of de novo induced iTreg cells generated by the anti-TNF-α treatment on EAE, we used chimeric mice deficient in nTreg cells. As illustrated in Figure 3A, CD4+Foxp3− cells of Foxp3-gfp.ki mice were adoptively transferred into SCID mice 1 day before immunization, followed by treatment with anti-TNF-α or an isotype control antibody. By days 12 and 22, both the frequencies and total numbers of iTreg cells were significantly increased in anti-TNF-α-treated mice than in control mice (Figure 3B). The newly generated iTreg cells from the above two groups of mice exhibited comparable suppressive activities in vitro (Supplementary Figure S7). Next, we evaluated the in vivo function of iTreg cells generated...
by the anti-TNF-α treatment. Compared with the control mice, the proliferation of CD4+ effector cells, expression of IFN-γ and EAE development were significantly inhibited in anti-TNF-α-treated mice (Figure 3C–E). Importantly, the depletion of iTreg by an anti-CD25 antibody abolished the effect of the anti-TNF-α treatment (Figure 3D and E), suggesting that iTreg cells are...
functionally required for mediating the effect of an anti-TNF-α antibody on EAE.

To confirm the effect of the anti-TNF-α treatment on iTreg differentiation in immunocompetent mice, we adoptively transferred MOG-specific Thy1.2+CD4+CD25+ cells from 2D2 transgenic mice into Thy1.1 mice and induced EAE in these chimeric mice with anti-TNF-α or control antibody treatment. Similar to that in SCID mice, the level of iTreg cells in anti-TNF-α-treated mice was significantly higher than that in the control mice (Figure 3F). iTreg cells from both group of mice had similar patterns of expression of Treg-related markers (data not shown).

Collectively, these results show that the neutralization of TNF-α stimulates the induction of iTreg cells, leading to amelioration of EAE.

**TNFR2 is required for TNF-α-mediated inhibition of TGF-β-induced iTreg differentiation**

To better understand how TNF-α regulates TGF-β-induced iTreg differentiation, we used an in vitro culture system to induce naïve CD4+CD25+ T cells into iTreg cells by T cell receptor (TCR) stimulation in the presence of TGF-β and mitomycin C-treated APCs derived from EAE mice. When an anti-TNF-α antibody was included in the culture, iTreg differentiation was significantly
enhanced (Figure 4). When recombinant TNF-α was added into respective T cell culture systems, the differentiation of iTreg, but not Th1 or Th17, cells was significantly inhibited (Figure 4B and C). However, iTreg cells generated in the presence of TNF-α exhibited similar suppressive activity as those generated in the absence of exogenous TNF-α (Figure 4D). TNF-α mediated inhibition of iTreg differentiation was not due to increased apoptosis of CD4+ T cells in the culture (Supplementary Figure S8). In addition, TNF-α did not show any significant effect on Foxp3 expression of nTreg cells that were stimulated with anti-CD3 and anti-CD28 antibody either in the presence or absence of TGF-β and high dose IL-2 (Supplementary Figure S9).

We next examined which TNF-α receptor mediates the inhibitory effect of TNF-α on TGF-β-induced iTreg differentiation. Unlike TNFR1 whose expression was relatively low throughout iTreg differentiation, ~30% of T cells began to express TNFR2 at 16 h of iTreg cell induction and ~80% of T cells expressed high levels of TNFR2 at 48 h (Figure 4E). Without TCR stimulation, the TNFR2 expression on Foxp3+ T cells was extremely low and TNF-α alone did not upregulate the TNFR2 expression. Stimulation with anti-CD3 and anti-CD28 antibodies upregulated TNFR2 expression. Neither TGF-β nor TNF-α showed any effect on the expression of TNFR2 in the presence of anti-CD3 and anti-CD28 (Supplementary Figure S10). The addition of anti-TNFR2 blocking antibody into the culture almost completely abolished the inhibitory effect of TNF-α on iTreg differentiation (Figure 4F). The antagonistic effect of anti-TNFR2 in vitro was totally dependent on exogenous TNF-α (data not shown). To determine the requirement of TNFR2 in
anti-TNF-α-mediated iTreg differentiation in vivo, CD4^+CD25^- cells from TNFR2^−/− mice were adoptively transferred into SCID mice followed by immunization and treatment with anti-TNF-α or control antibody. In the absence of TNFR2, the anti-TNF-α treatment failed to enhance the iTreg differentiation as indicated by similar levels of iTreg cells in both groups of mice (Figure 4G). Moreover, the injection of anti-TNFFR2 blocking antibody significantly delayed the onset and ameliorated the severity of EAE (Figure 4H). The percentages of Treg cells in both the lymph node and the CNS were increased in mice treated with the anti-TNFFR2 antibody (Figure 4I). Thus, the TNF-α-mediated inhibition of TGF-β-induced iTreg differentiation is dependent on TNFR2 both in vitro and in vivo.

**TNF-α inhibits foxp3 transcription by inhibiting TGF-β-induced Smad3 phosphorylation.**

We next investigated the molecular mechanism underlying the inhibition of TGF-β-induced iTreg differentiation by TNF-α. Forty-eight hours following the TNF-α treatment, the level of foxp3 transcript was significantly reduced in T cells (Figure 5A). Analysis of the activation state of key transcription factors, which have been shown to play important roles in the direct regulation of foxp3 transcription during iTreg differentiation, showed that TNF-α selectively inhibited the phosphorylation of Smad3 at both 24 and 48 h (Figure 5B). Smad3 is the key transcription factor downstream of TGF-β signaling which is required for mouse iTreg differentiation in vitro and in vivo. We found that TNF-α-mediated inhibition of iTreg differentiation was totally dependent on exogenous TGF-β (Figure 5C). To investigate whether TNF-α indirectly impairs iTreg differentiation by altering the level of TGF-β in the culture, we measured the level of intracellular TGF-β in CD4^-CD25^- T cells at day 3 after iTreg differentiation in the presence or absence of TNF-α. As shown in Figure 5D, there was no change in the level of intracellular TGF-β between the two groups (23.2% vs. 22.0%). Thus, TNF-α impairs iTreg differentiation mainly through the direct blockade of TGF-β signaling rather than through the indirect regulation of TGF-β level in the culture. We analyzed the activation of Smad3, Smad2 and the levels of Smad4 and Smad7 that are known to be involved in TGF-β signaling. In contrast to other Smads, phospho-Smad3 in both the cytoplasm and nucleus was downregulated, suggesting that the TNF-α treatment affects the activation of Smad3 but not the translocation of phospho-Smad3 (Figure 5E). The inhibition of Smad3 phosphorylation by TNF-α was as effective as SIS3 (1 μM), a specific inhibitor of Smad3 phosphorylation (Figure 5F). The overexpression of Smad3 markedly reduced the susceptibility of purified CD4^-Foxp3^- T cells to the inhibition by TNF-α during iTreg differentiation (Figure 5G). Conversely, the expression of a dominant negative Smad3 (Smad3A) in which three serines (S422, S423, S425) (Liu et al., 1997) at the very C terminal were mutated to alanines and therefore cannot be phosphorylated by TGF-β receptor kinases significantly inhibited iTreg differentiation either in the presence or absence of TNF-α (Figure 5G). These results suggest that persistent Smad3 activation induced by TGF-β is essential for sustained Foxp3 expression which is susceptible to modulation by TNF-α.

The core sequence of Smad3-binding element is 5′-GTCT-3′ (Shi et al., 1998). We have scanned the previously reported 1.9-kb promoter and identified three potential Smad3-binding sites which were conserved among several species (Supplementary Figure S11) (Tone et al., 2008). Among them, we focused on the Smad3-binding site located −67 bp upstream of the transcription start site which has also been reported by another group (Samon et al., 2008). To confirm the direct binding of Smad3 to the potential-binding site in the foxp3 promoter, we performed electrophoretic mobility shift assay (EMSA) by using nuclear extracts from primary CD4^- T cells 48 h after iTreg induction. The data showed that Smad3 was biased to bind to the specific-binding site in the foxp3 promoter but not that in the enhancer region (Figure 5H). Complex formation was detected with a WT probe and was inhibited by a competitor probe. A site-specific mutation of the potential Smad3-binding site also abrogated the complex formation, indicating that it is a sequence-specific complex. A supershift EMSA was also performed with an antibody specific to phospho-Smad3 (S423 + S425) and revealed that phospho-Smad3 bound to this region (Figure 5H). In order to directly prove the regulatory role of Smad3 on this Smad3-binding site in the foxp3 promoter, we performed luciferase reporter assay in the mouse lymphoma cell line EL4 cells. Previous report and our data showed no promoter activity in a luciferase reporter plasmid without SV40 enhancer in EL4 cells (data not shown). Indeed, the promoter activity of the 1.9-kb fragments (−1707 to +192) was detected in the presence of SV40 enhancer in EL4 cells stimulated with anti-CD3 and anti-CD28 for 24 h. The overexpression of Smad3 (Smad3) increased the WT foxp3 promoter activity twice over that of the empty vector control group (Vector) (Figure 5I). Mutation of the Smad3-binding site in the foxp3 promoter (Mut) almost completely abrogated the enhancement of Smad3 on the foxp3 promoter activity (Figure 5I). Overall, Smad3 actively regulates the promoter activity of foxp3 by directly binding to the specific site. To evaluate the effect of TNF-α on TGF-β-induced Smad3 binding to the foxp3 promoter and enhancer in physiological condition, a ChIP assay was performed by using nuclear extracts from CD4^- T cells 24 h after iTreg differentiation in the presence or absence of TNF-α. Smad3 selectively bound to the foxp3 promoter region and the binding level was significantly reduced in TNF-α-treated group (Figure 5J). Thus, TNF-α inhibits foxp3 transcription through selective inhibition of TGF-β-induced Smad3 phosphorylation and consequently the reduction of p-Smad3 results in the decreased binding of p-Smad3 to the foxp3 promoter during iTreg differentiation.

**TNF-α inhibits iTreg differentiation through activation of Akt and inhibition of TGF-β-induced Smad3 phosphorylation.**

To evaluate the downstream signaling events triggered by TNF-α in the regulation of TGF-β-induced Smad3 phosphorylation, we investigated NF-κB, p38, Erk and Akt pathways as they can be activated by TNF-α (Wajant et al., 2003). Among the inhibitors tested, only LY294002, a specific PI3K inhibitor, could abolish the inhibition of Foxp3 expression by TNF-α in a dose-dependent manner (Supplementary Figure S12 and Figure 6A). Following the TNF-α treatment, Akt phosphorylation (Ser473) was elevated while TGF-β-induced Smad3 phosphorylation was inhibited, and these effects were abolished by LY294002 treatment (Figure 6B). To directly establish the causal relationship between Akt and TGF-β-induced Smad3 phosphorylation and...
Figure 5 TNF-α inhibits foxp3 transcription by inhibiting TGF-β-induced Smad3 phosphorylation. (A, B, D, E and J) Naïve CD4+Foxp3− T cells were cultured under iTreg differentiation condition in the presence or absence of TNF-α. (A) The level of foxp3 transcript was analyzed by real-time PCR. (B) Immunoblotting analysis of the levels of selected transcription factors. (C) Naïve CD4+Foxp3− T cells were stimulated with anti-CD3 and anti-CD28 in the presence or absence of TGF-β or TNF-α for 3 days. The expression of Foxp3 in CD4+ cells was assayed.
Foxp3 expression, we overexpressed different forms of Akt in purified CD4+Foxp3− T cells by retroviral transduction. The expression of a constitutively activated form of Akt (myr-Akt) led to the inhibition of both TGF-β-induced Smad3 phosphorylation and Foxp3 expression in the absence of TNF-α, which could not be further reduced by the addition of TNF-α (Figure 6C and D). Conversely, expression of a constitutively inactivated form of Akt [Akt (AA)], mutated at phosphorylation sites encompassing Thr308 and Ser473, slightly increased iTreg differentiation in untreated control but markedly restored the inhibition of both TGF-β-induced Smad3 phosphorylation and Foxp3 expression by TNF-α (Figure 6C and D). Akt1 siRNA was used to reduce the endogenous Akt activity in CD4+ T cells. siRNA-treated cells were cultured under iTreg differentiation condition in the presence or absence of TNF-α. The siRNA-induced inhibition of Akt1 expression led to a decreased level of phosphorylated Akt and abrogated the effect of TNF-α on the downregulation of TGF-β-induced phospho-Smad3 at 48 h (Figure 6E). We further investigated whether Akt signaling mediates the inhibition of iTreg differentiation by TNF-α in vivo. CD4+Foxp3− T cells transduced with a control retrovirus or the retrovirus expressing the activated forms of Akt were adoptively transferred into SCID mice followed by induction of EAE and treatment with anti-TNF-α or isotype control antibody. As expected, the anti-TNF-α treatment stimulated iTreg differentiation from CD4+Foxp3− T cells that were transduced with a control retrovirus (Figure 6F). The expression of the activated form of Akt (myr-Akt) in CD4+Foxp3− T cells led to a decreased iTreg differentiation in control antibody-treated mice and this impairment could not be rescued by an anti-TNF-α antibody (Figure 6F).

It has been reported that Akt could physically interact with unphosphorylated Smad3 and inhibit its phosphorylation and nuclear translocation in certain cell lines (Conery et al., 2004; Song et al., 2006). As shown in Figure 6G, Akt exhibited only weak interaction with Smad3 at 24 h during iTreg differentiation. However, the interaction was significantly enhanced by TNF-α treatment. Conversely, the overexpression of a dominant-negative form of Akt [Akt (AA)] in the TNF-α treated T cells significantly decreased the interaction (Figure 6G). Next, to evaluate the effect of interaction between Akt and Smad3 on foxp3 transcription activity, we co-expressed WT or dominant-negative form of Akt with Smad3 in our luciferase reporter assay. As expected, the overexpression of the WT Akt diminished Smad3-driven foxp3 transcription activation (Figure 6H), whereas the overexpression of the inactivated Akt [Akt (AA)], which decreased the interaction of Akt with Smad3, failed to abrogate the effect of Smad3 on the foxp3 promoter activity (Figure 6H). Interestingly, the blockade of Akt signaling by LY294002 did not affect the Foxp3 expression in nTreg cells in the presence or absence of TNF-α (Supplementary Figure S13A). Neither phospho-Akt nor phospho-Smad3 was altered by TNF-α in nTreg cells (Supplementary Figure S13B). In summary, these findings suggest that TNF-α stimulates phosphorylation of Akt, through directly interacting with Smad3, inhibits its phosphorylation and eventually decreases the foxp3 transcription.

Discussion

It is known that nTreg cells in an autoimmune disease setting have limited capacities that are not entirely sufficient to effectively regulate autoimmune inflammation (Haas et al., 2005; Buckner, 2010). Mounting evidence suggests that iTreg cells play a critical role in the control of autoimmune diseases (Curotto de Lafaille et al., 2008). For example, neurons mediate the induction of iTreg cells from encephalitogenic T cells in the CNS and the resulting iTreg cells are capable of suppressing EAE (Liu et al., 2006). iTreg cells are induced by tissue-specific expression of ovalbumin in the pancreas of DO11.10 × RAG−/− mice, which lacks endogenous nTreg cells, leading to protection of mice from autoimmune diabetes (Thompson et al., 2011). In experimental colitis, lack of in situ generated iTreg cells accelerates the disease (Haribhai et al., 2009). The peripheral iTreg differentiation in autoimmune disease is critically dependent upon tissue cytokine milieu. Inflammatory cytokines such as IL-4, IL-6, IL-27 and IFN-γ are shown to impair TGF-β-induced expression of Foxp3, whereas IL-27 is also found to induce iTreg cells in other reports (Bettelli et al., 2006; Mantel et al., 2007; Caretto et al., 2009; Ouaked et al., 2009). Although various cytokines have been shown to affect iTreg differentiation, the key cytokine(s) and the molecular mechanism that regulates iTreg differentiation have yet to be elucidated. It is shown here that the neutralization of TNF-α significantly enhances the iTreg differentiation in both in vitro and in vivo experimental systems. The data described here provide compelling evidence indicating that TNF-α is the key cytokine that critically influences peripheral iTreg differentiation in autoimmune disease.

Our findings provide new insights into the underlying mechanisms by which TNF-α regulate iTreg differentiation and autoimmune inflammation. In this regard, we offer a compelling account of TNF-α-TNFR2-Akt signaling pathway in modulating TGF-β-Smad3 signaling induced iTreg differentiation and reveal a previously undiscovered crosstalk between these two signaling pathways. No matter the extensive understanding of TNFR1 in

(D) Intracellular staining of TGF-β of CD4+ T cells at day 3. (E) Immunoblotting analysis of the levels of Smads in nuclear extracts (NE), cytoplasmic extracts (CE) and total cell lysates (Total) after 48 h. (F) TNF-α or SIS3 was added into the culture at 18 h following iTreg differentiation. The expression of Foxp3 was analyzed at 72 h. (G) The expression of Foxp3 of GFP+ cells which were successfully transduced with retrovirus expressing Smad3 or Smad3A and continuously cultured under iTreg differentiation condition in the presence or absence of TNF-α for 72 h. (H) EMSA assays were performed with the wild-type (WT) or mutant (Mut) probes of Smad3-binding sequences from the foxp3 promoter or enhancer. An unlabeled WT probe was used as a competitor. (I) Luciferase assay of WT or mutant foxp3 reporter plasmids after co-transfection with Smad3 expression plasmid. (J) Binding of Smad3 to specific binding sites of the promoter or enhancer of foxp3 was analyzed by ChIP assay at 24 h. *P < 0.05. Data are representative of three independent experiments (B–H). Error bars indicate the SEM of three independent transfection samples in I and triplicate samples in A and J.
Figure 6 TNF-α inhibits the TGF-β-induced iTreg differentiation through the activation of Akt and inhibition of TGF-β-induced Smad3 phosphorylation. (A) LY294002 was added at different concentrations into the iTreg differentiation culture in the presence or absence of TNF-α at 18 h. The levels of Foxp3 expression in CD4⁺ cells were assayed at 72 h. (B) Immunoblotting analysis of phospho-Akt and phospho-Smad3 in CD4⁺ cells cultured under the iTreg differentiation condition in combined with treatment with LY294002 (2.5 μM) for at 24 h. (C) Activated cells were infected with indicated retrovirus followed by continuously culturing in iTreg differentiation condition in the presence or absence of TNF-α. The successfully transduced GFP⁺ cells were analyzed. (C) The expression of Foxp3 was analyzed at 72 h after transduction. (D) Phospho-Smad3 was analyzed by immunoblotting 24 h after transduction. (E) Immunoblotting analysis of Akt1, phospho-Akt, phospho-Smad3 in CD4⁺ cells. (F) The expression of Foxp3 in successfully transduced CD4⁺GFP⁺ cells was analyzed on day 12 post-immunization. (G) The interaction of Akt with Smad3 was analyzed on the successfully transduced GFP⁺ cells by co-immunoprecipitation.
TNF-α mediated response. the signaling pathways and the biological relevance mediated by TNFR2 are still not clear. To our knowledge, our findings show for the first time that activated Akt signaling play a central role in the TNF-α mediated inhibitory effect on Foxp3 expression of iTreg cells both in vitro and in vivo through inducible TNFR2. The crucial role of Akt signaling in iTreg differentiation is also supported by the previous data showing that inhibition of Akt activation triggered by TCR signaling conferred Foxp3 expression in naive T cells (Sauer et al., 2008). Secondly, Smad3, a key transcription factor, mediates TGF-β signaling and regulates Foxp3 expression in iTreg differentiation. However, the mechanisms involved in regulating Smad3 activity during iTreg differentiation are largely unknown. Our finding revealed a new regulatory mechanism by which physical interaction of activated Akt with Smad3 induced by TNF-α-TNFR2 signaling results in inhibiting TGF-β-induced Smad3 phosphorylation. Our further data showed that although TGF-β-induced Smad3 binding to foxp3 enhancer is initially uninterrupted at the early stage of TNF-α treatment as the reduction of p-Smad3 does not occur early, the binding of Smad3 to the foxp3 promoter is later on reduced due to the decreased level of p-Smad3 in response to TNF-α-TNFR2-Akt signaling. These data reveal that the persistent TGF-β-Smad3 signaling and Smad3 binding to the foxp3 promoter is required for the complete iTreg differentiation. Thirdly, we have provided a large series of data showing that Smad3 drives the foxp3 transcription through direct binding to the foxp3 promoter. However, we did not completely exclude other cofactors that could be involved. In principal, the high-affinity binding of the Smads complex and selectivity of the Smads complex in the recognition of target genes is thought to occur through the incorporation of other cofactors into the Smads complex (Massague and Wotton, 2000). The foxp3 promoter region has been shown to have three NFAT-binding sites (Mantel et al., 2006). Our data showed that NFAT bound to two of the three binding sites (−319 and −372) 48 h after iTreg differentiation (Supplementary Figure S14), indicating that NFAT could potentially cooperate with phospho-Smad3 within the foxp3 promoter region to regulate foxp3 transcription. Overall, we provide a comprehensive set of evidence demonstrating that Akt-Smad3 interaction is the key axis in the signaling cascade that mediates the inhibition of TGF-β-induced iTreg differentiation in response to TNF-α.

Our study sheds new light on the differential effect of TNF-α on iTreg and nTreg cells. We show that neutralization of TNF-α does not affect the number, phenotype, and function of nTreg cells, does not affect the function of existing iTreg cells, but selectively promotes new iTreg differentiation. The different roles of Akt and Smad3 in regulating nTreg and iTreg cells offer an explanation for the selective effect. Akt activation is maintained at low level in nTreg cells (Crellin et al., 2007). Consistently, we show that TNF-α does not induce the up-regulation of Akt signaling in nTreg cells, although 30%-40% of nTreg cells express TNFR2 (Chen et al., 2007). Moreover, the TGF-β-induced activation of Smad3 is dispensable for nTreg cells as the number and function of nTreg cells in Smad3-deficient mice are comparable to those in WT mice (Martinez et al., 2009). In contrast, continued TCR stimulation that result in the sustained activation of Akt signaling attenuated iTreg differentiation (Sauer et al., 2008). TGF-β-Smad3 signaling is required for iTreg differentiation. Thus, the selective inhibition of phospho-Smad3 by activated Akt likely underlies the selectivity of TNF-α on iTreg differentiation. Although it has been reported that TNF-α promotes the expansion of nTreg cells in vitro and in an in vivo model (Chen et al., 2007; Grinberg-Bleyer et al., 2010), we did not observe any significant impairment of nTreg cell expansion in our in vivo system. It is possible that cytokines such as IL-2 may play a more important role in the maintenance and expansion of nTreg cells in our system (Furtado et al., 2002; Duarte et al., 2009).

Discovery of iTreg cells in controlling autoimmune inflammatory responses has led to great enthusiasm for their clinical application for treating autoimmune diseases. The hope is that the impaired iTreg differentiation may be corrected by, for example, transfer of in vitro-generated autologous iTreg cells. Such an approach could be a treatment option for multiple autoimmune diseases including EAE, diabetes, colitis, and lupus (Zheng et al., 2004; Weber et al., 2006; Selvaraj and Geiger, 2008). However, this approach will likely have significant challenges. It is difficult to prepare iTreg cells with a specific antigen specificity, especially when the exact antigen(s) are not known in some autoimmune diseases. Even more importantly, the existing evidence suggests that iTreg cells generated in vitro are phenotypically and functionally unstable (Floess et al., 2007; Chen et al., 2011). In contrast, in vivo-induced iTreg cells do not have these limitations. They are normally antigen specific, therefore are likely more efficacious in treating autoimmune diseases. Furthermore, recent studies show that in vivo differentiated iTreg cells are epigenetically more stable than in vitro differentiated iTreg cells and would lead to a long-lasting therapeutic effect (Floess et al., 2007; Polansky et al., 2008). In this regard, developing strategies to promote in vivo generation of antigen-specific iTreg cells are essential to employ iTreg cells in treating autoimmune diseases. Small molecular weight compounds such as retinoic acid and histone deacetylase inhibitors therapy have been shown to increase the iTreg differentiation in vivo (Benson et al., 2007; Edwards and Pender, 2008).
2011). TNF-α blocking agents are also readily available and may provide an effective way to overcome the impaired peripheral iTreg differentiation in autoimmune diseases. Indeed, the treatment of rheumatoid arthritis with an anti-TNF-α antibody (infliximab) has been shown to stimulate the induction of overall Foxp3+ Treg cells (Nadkarni et al., 2007). In conclusion, our study provides a strong mechanistic explanation for the role of TNF-α blocking treatment for autoimmune diseases.

Materials and methods

Mice

C57BL/6 mice were purchased from the Shanghai Laboratory Animal Center, Chinese Academy of Sciences. Foxp3-gfp.ki mice and MOG-TCR mouse (2D2) were kindly provided by Vijay K. Kuchroo (Harvard Medical School). Thy1.1 mice (B6-PL-Thy1+/Cjl, 000406), CD45.1 mice (B6.SJL-Ptprca Pepcb/Bojl, 002014), SCID mice (B6.CB17-Prkdcscid/Slj, 001913), and Tnfα−/− mice (B6.129S2-Tnfrsf1btm1Wymo/J, 002620) were all purchased from the Jackson Laboratory. All experiments were performed with mice 6–10 weeks old with protocols approved by the Institutional Animal Care and Use Committee.

Induction and treatment of EAE

EAE was induced by MOG (35–55) peptide immunization using a standard protocol without pertussis toxin injection and scored daily (Wang et al., 2006). The various treatment regimens including recombinant mouse anti-TNF-α (eBioscience), anti-TNFFR2 (eBioscience), or type II control antibody (Jackson ImmunoResearch) were intraperitoneally injected into mice every 2 days, starting from the day of immunization (100 μg/mouse). To deplete Treg cells, anti-CD25 (PC61, BD Bioscience, 250 μg/mouse) was intravenously injected into mice at days 0 and 7 after immunization.

In vitro T helper cell differentiation

CD4+ T cells were purified by a CD4 Negative Isolation Kit (Miltenyi Biotec), and different cells were further purified by cell sorting. Cells were cultured in Complete Medium. Purified CD4+CD25− T cells were stimulated with antibodies to CD3 (5 μg/ml) (145-2C11, BD Biosciences) and CD28 (1 μg/ml) (37.51, BD Biosciences) under iTreg (rhTGF-β1, 5 ng/ml, 240-B, R&D Systems), Th1 or Th17 differentiation conditions in the presence or absence of mTNF-α (10 ng/ml, 410-MT-010, R&D Systems). In some experiments, signaling pathway inhibitors including LY294002 (Akt), PD98059 (Erk), SB203580 (p38), SIS3 (Smad3) and Bay-11-7082 (NF-κB) (Sigma-Aldrich) were added into the culture.

Suppression assay

Freshly isolated CFSE-labeled Thy1.1+CD4+CD25− T cells (4 × 104/well for 96 well plate) from naïve Thy1.1 mice were co-cultured with Thy1.2+ Treg cells at the indicated ratio in the presence of mitomycin C (Sigma-Aldrich) treated splenic non-CD4+ cells (8 × 104 cells/well) and anti-CD3 (2 μg/ml) for 3 days. The dilution of CFSE by responder cells was analyzed by flow cytometry.

Western blotting and co-immunoprecipitation

For western blotting, cells were lysed with RIPA buffer containing PMSF and protease inhibitor cocktail. Nuclear and cytoplasmic extracts of CD4T cells were prepared by using NE-PER® Nuclear and Cytoplasmic Extraction Reagents (Thermo) following the manufacturer’s protocol. Antibodies to phospho-Akt (Ser473), Akt, Akt1, phospho-Smad2 (Ser465/467), phospho-Stat1 (Y701), phospho-Stat5 (Y694), phospho-Stat6 (Y641), phospho-p65 (Ser536) (Cell Signaling Technology), phospho-Smad3 (Ser423+Ser425), NFA1 (Abcam), Smad7 (R&D Systems), Tbet (eBioscience), and β-actin (Sigma-Aldrich) were used. For endogenous Akt knockdown experiment, freshly isolated CD4+ cells were transfected by electroporation with Akt1 siRNA (L-040709, Thermo) and differentiated as previously described. For co-immunoprecipitation, Smad3 was immunoprecipitated with an anti-Smad3 antibody (Abcam).

Retroviral transduction

Constructs were generated as previously described (Remy et al., 2004). Retroviral vectors (pMX-ires-gfp) were transiently transfected into the packaging cell line, Plate-E (Cell Biolabs), using Lipofectamine 2000 (Life Technologies). Supernatants were harvested 48 h later. Naïve splenic CD4+ cells were activated with anti-CD3, anti-CD28, and rhTGF-β1 in the presence or absence of TNF-α. After 24 h, these cells were infected with the appropriate supernatants of retrovirus supplemented with rhTGF-β1 or TNF-α in the presence of Polybrene (4 μg/ml, Sigma-Aldrich) and then incubated at 37°C for another 2 days. In some experiments, naïve splenic CD4+ T cells were activated with anti-CD3 and anti-CD28 in the presence of rhTGF-β1. After 24 h, these cells were spin-infected with retrovirus in the presence of Polybrene at 30°C for 2 h and incubated at 37°C overnight. Then these cells were adoptively transferred into naïve SCID mice followed by EAE induction and treatment.

Construction of foxp3 promoter reporter plasmids and luciferase assay

A ~1.9-kb fragment (~1707 to +192 bp) of the foxp3 promoter was cloned into the pGL3-Enhancer vector (Promega). Mutation of the Smad3-binding site in the promoter fragment was generated by using QuikChange® Lightning Site-Directed Mutagenesis Kit (Agilent Technologies). To assay promoter activities, the mouse lymphoma cell line EL4 cells (ATCC) were electroporated (4 × 106 cells per transfection) with the following plasmid combinations: a luciferase reporter plasmid (200 ng), an internal control plasmid phRL-TK (Promega) (20 ng), plus a plasmid expressing Smad3 alone, or with a plasmid expressing different form of Akt. The cells were cultured in the presence or absence of anti-CD3 and anti-CD28. The luciferase activities were analyzed at 24 h. The activities of the firefly luciferase (phL4) were normalized to that of the internal control, Renilla luciferase (phRL-TK) activities.

EMSA assay

EMSA was performed by using LightShift® Chemiluminescent EMSA Kit (Thermo) following the manufacturer’s protocol. Briefly, 10 μg of nuclear extracts were incubated with biotin-labeled or unlabeled oligonucleotides in 20 μl of EMSA reaction buffer [containing 1 μg of poly (dl-dC)] for 30 min at room temperature. For the supershift assay, nuclear extracts in EMSA reaction buffer were incubated for 30 min with anti-Smad3 (Ser423 + Ser425) (Abcam) before the addition of the oligonucleotide probes. Oligonucleotide sequences in the foxp3 promoter and enhancer are as follows: WT Samd3 (in promoter): GAAGAG CGAGGTCTCGGGCTCCACGCCG; Mutant Samd3 (in promoter): GA
Regulation of TGF-β-induced iTreg differentiation by TNF-α in autoimmunity

AGAGCCAGACTGCCG; WT Smad3 (in enhancer): GGGAGCCAGACTGCCG; Mutant Smad3 (in enhancer): GGGAGGCTTGAAGCTTAACAGAC.

Chromatin immunoprecipitation (ChIP)

ChiP assay was performed with Magna ChiP™ A/G Chromatin Immunoprecipitation Kit (Millipore) according to the manufacturer’s instruction using anti-Smad3 (ChiP grade, Abcam) or normal IgG (Abcam). The purified DNA was analyzed by real-time PCR using primers specific for sequences within the Smad3-binding region of the foxp3 promoter or enhancer. The primer pairs were 5′-CCCCCATCGTGAATTAT-3′ and 5′-CCTGCTTGGGGTTGGAACACTG-3′ for the foxp3 promoter; 5′-CTTGCTTGCTTGTGTATAG-3′ and 5′-TGGAGACGAGACGTCCGGCTTCCACGCCG; WT Smad3 (in enhancer): GGGAGCCAGACTGCCG; Mutant Smad3 (in enhancer): GGGAGGCTTGAAGCTTAACAGAC.

Statistical analysis

All data were expressed as the mean ± SD or mean ± SEM and analyzed for statistical significance by the two-tailed Student’s t-test. P < 0.05 is considered statistically significant.

Supplementary material

Supplementary material is available at Journal of Molecular Cell Biology online.

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Conflict of interest: none declared

References


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