Therapeutic effects of TGT-β induced regulatory T cells on the established autoimmune and inflammatory diseases

Song Guo Zheng

▶ **To cite this version:**

Song Guo Zheng. Therapeutic effects of TGT-β induced regulatory T cells on the established autoimmune and inflammatory diseases. Agricultural sciences. Université d'Orléans, 2011. English. NNT : 2011ORLE2061 . tel-00698579

HAL Id: tel-00698579

https://theses.hal.science/tel-00698579

Submitted on 16 May 2012

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

UNIVERSITÉ D'ORLÉANS

ÉCOLE DOCTORALE SCIENCES ET TECHNOLOGIES

Laboratoire d'Immunologie et Embryologie Moléculaires – IEM- UMR6218 - CNRS – Université d'Orléans

THÈSE présentée par :

Song Guo Zheng

soutenue le : **5 décembre 2011**

pour obtenir le grade de : **Docteur de l'université d'Orléans**

Discipline : Science du vivant / Immunologie

Therapeutic effects of TGF-β-induced regulatory T cells on the established autoimmune and inflammatory diseases

THÈSE dirigée par :

Bernhard Ryffel Directeur de Recherche, HDR, CNRS Orléans

RAPPORTEURS :

Nathalie Moiré Docteur UMR Université-INRA 0483, Tours

Julien C. Marie Docteur, Centre de Recherche sur le Cancer, Lyon

---

JURY

Nathalie Moiré	Docteur, UMR Université-INRA 0483, Tours
Julien C. Marie	Docteur, Centre Recherche Cancer, INSERM – CNRS, Lyon
Chantal Pichon	Professeur, Université d'Orléans (Président du jury)
Jacques Van Snick	Professeur, Ludwig Institute for Cancer Res., Bruxelles, Belgique
Catherine Uyttenhove	Professeur, Ludwig Institute for Cancer Res., Bruxelles, Belgique
Makoto Miyara	Docteur, Hôpital Pitié-Salpêtrière, INSERM, Paris

LIST OF CONTENTS

Remerciements or Acknowledgment

I greatly thank Professor Bernhard Ryffel for the opportunity to finalize this thesis project. His enthusiasm, scientific knowledge and inspiration all contribute to the success of this thesis. Although many roads lead to Rome, I have to say I have been taught to use the strictest standard in judging the values of scientific findings. One simply could not wish for a better or friendlier supervisor.

I also offer my sincerest gratitude to other members of my advisor committee, such as Professor Valerie Quesniaux and Professor Francois Erard who have supported me throughout my thesis with his patience and knowledge whilst allowing me the room to work in my own way. I am thankful for other professors including Dr. David A Horwitz and Dr. William Stohl for their supports. Their discussion and constructive suggestions have greatly enhanced the thesis's quality. I also thank the team of Dr Valerie Quesniaux at UMR6218, Molecular Immunology, University and CNRS for the support and the work was supported by the "Fondation pour la Recherche Médicale" (FRM allergy DAL 2007 0822007), the "Fond européen de développement regional" (FEDER Asthme 1575-32168) and Le Studium Orleans,CNRS, Orléans, France.

In the various laboratories and workshops I have been aided in running the equipment and in conducting immunological experiments by Ju-Hua Wang, a super technician. I appreciate other colleagues and collaborators, Dr. David Brand, Dr. Wei Shi, Dr. Pawel Kiela and others for their collaboration and efforts. I also thank Jennifer Palomo, a PhD student, for her selfless assistance. I greatly appreciated the time and attention given to the examination of this work by the thesis committee: Dr. Chantal Pichon, Dr. Nathalie Moiré, Dr. Julien Marie, Dr. Makoto Miyara, Dr. Catherine Uyttenhove, Dr. Jacques Van Snick, and Dr. Francois Erard.

Finally, I would like to thank my wife Julie Wang who gives me a warm family and love and my daughter, Tina Zheng, who is a sophomore in Arcadia High School in California. Her outstanding achievements and leadership ability have always encouraged me to move forward to next step in my scientific career.

I. ABBREVIATIONS

α-SMA	Alpha smooth muscle actin
atRA	Allo-trans retinoid acid
AHR	Airway hyperresponsiveness
BAL	Bronchoalveolar lavage
BFA	Brefeldin A
CFA	Complete Freund's adjuvant
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitations
CHX	Cycloheximide
CKO	Conditional knock-out
CNS	Conserved non-coding DNA sequence
CIA	Collagen-induced arthritis
CII	Collagen II
DC	Dendritic cells
DMSO	Dimethyl sulfoxide
EAE	Experimental autoimmune encephalomyelitis
ELISA	Enzyme-linked immunosorbent assay
GFP	Green fluorescent protein
GVHD	Graft-*vs*-host disease
H&E	Hematoxylin and eosin
HPRT	Hypoxanthine guanine phosphoribosyl transferase
IHC	Immunohistochemistry
i.n.	Intranasal
i.p.	Intraperitoneal
i.v.	Intravenous
iTregs	$CD4^+CD25^+Foxp3^+$ cells generated *ex vivo* with IL-2 and TGF-y
LN	Lymph node
nTregs	Naturally-occurring $CD4^+CD25^+Foxp3^+$ cells
MCh	Methacholine
MS	Multiple sclerosis
IFN	Interferon
KO	Knock out
OVA	Ovalbumin
PAS	Periodic-Acid-Schiff
PBS	Phosphate buffered saline
PMA	Phorbol 12-myristate 13-acetate
RA	Rheumatoid arthritis
SLE	Systemic lupus erythematosus
SMC	Smooth muscle cell
Tregs	Regulatory T cells
TGF-β	Transforming growth factor-β
TβR	TGF-β receptor
TSS	Transcription start site
WT	Wild type

II. ABSTRACT

Therapeutic effects of TGF-β-induced regulatory T cells on the established autoimmune and inflammatory diseases

By

Song Guo Zheng

Advisor of thesis: Professor Bernhard Ryffel

While it has been well recognized that both natural $Foxp3^+$ regulatory T (nTreg) cells and TGF-β-induced Treg (iTreg) cells can prevent autoimmune diseases in animal models, recent studies revealed that injection of nTregs has less therapeutic effects on established autoimmune diseases. It is less clear if iTregs can treat the established autoimmune diseases. We now provide evidence that unlike nTregs, transfer of iTreg cells markedly ameliorate established autoimmune diseases such as allergic asthma, autoimmune arthritis, and chronic GVHD with a lupus like syndrome.

In allergic asthma we observed that adoptive transfer of iTreg significantly suppressed airway and peri-vascular inflammation. iTreg infusion also markedly reduced airway résistance, eosinophil recruitment, mucus hyper-production, airway remodeling and IgE levels. This therapeutic effect was associated with increase of Treg cells ($CD4^+Foxp3^+$) in the draining lymph nodes, and with reduction of Th1, Th2, and Th17 cell responses as compared to untreated and non-Treg cell treated controls.

In collagen-induced arthritis (CIA) both antigen-specific iTregs and expanded nTregs prevented appearance and development of disease. However, only iTregs transfer suppressed established CIA. CIA mice given iTregs have a significantly lower incidence of disease and lower clinic scores than mice given nTregs, Teff cells or no cells. We found while nTregs were converted into Th1/Th17 cells *in vitro* and *in vivo* in the inflammatory milieu, iTregs were resistant to T effector cell conversion in the similar condition. Injection of iTregs to naïve mice displayed similar levels of Foxp3 stability as comparing with nTregs. Of note, the stability of Foxp3 expression was only found in iTreg cells during established CIA. iTregs suppressed Th17 cell differentiation that paralleled with improved clinical scores, collagen II (CII)-specific IgG production and bone erosion. In the chronic GVHD model mimicking lupus the transfer of iTregs to the established lupus disease significantly decreased the levels of anti-dsDNA and proteinuria, and markedly prolonged the survival of lupus. Blocking of TGF-β/TGF-βR pathway using anti-TGF-β antibody or TGF-βRI

(ALK5) inhibitor, or anti-IL-10R antibody almost completely abolished the therapeutic effects of iTregs on lupus-like syndromes, suggesting that TGF-β and/or IL-10 secreted by iTregs play a crucial role in the cell therapy. iTregs can induce the formation of tolerogenic DCs through TGF-β signaling on DCs but not IL-10 signaling. We further observed that DCs isolated from cGVHD with a typical lupus syndrome receiving iTregs but not control cells expressed lower levels of CD80 and CD86 and adoptive transfer of these DCs to another lupus-like disease mouse can suppress the disease development through TGF-β rather than IL-10 signal pathway. We therefore suggest that iTregs are stable and able to target DCs in the inflammatory milieu. These DCs then have become tolerogenic DCs and further suppress disease progression through its direct or indirect effect (inducing new iTregs) in autoimmune and inflammatory disease settings and may result in a long-term protective effect of iTregs in autoimmune diseases. Moreover, we also demonstrated that all-trans retinoic acid (atRA) promotes and sustains the $Foxp3^{+}$ regulatory T cells, and identified that atRA significantly increased histone methylation and acetylation within the promoter and conserved non-coding DNA sequence (CNS) elements at the Foxp3 gene locus and the recruitment of phosphor-RNA polymerase II, while DNA methylation in the CNS3 was not significantly altered. These results will further help to enhance the quantity and quality of development of iTregs and may provide novel insights into clinical cell therapy for patients with autoimmune diseases and those needing organ transplantation.

III. INTRODUCTION, RATIONALE AND OBJECTIVES

III.1 Phenotypic and Functional Characteristic of Regulatory T Cells

It is now well accepted that a cell population called "$CD4^+CD25^+$regulatory or suppressor cells" are critically involved in immune tolerance and homeostasis. In the early 1970s, Gershon and colleagues initially reported that thymocytes from his experimental animal model included a population of suppressor T cells (Gershon and Kondo, 1970). This suggestion was not appreciated until Sakaguchi et al 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 (Sakaguchi et al., 1995).

$CD4^+CD25^+$ 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 Treg cells 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 cells and Tregs. Fortunately, the nuclear transcription factor Foxp3 has been identified as a much more specific marker for Treg cells. Foxp3 is critically involved in the development and function of Treg cells(Fontenot et al., 2003). In mice, the lack of functional Foxp3 expression results in a fatal autoimmune and 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 Treg cells and that the intranuclear location of Foxp3 makes it difficult to use this protein for immunoaffinity-based purification methods although we have recently identified a new technique to improve the isolation of the live Treg cells (Zhou et al., 2010b), $CD4^+CD25^+$ cells are still widely used in the field of the biology of Treg cells without using genetically modified tissues, particular in human studies. Although Foxp3 is considered as a specific marker for Tregs in 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 T effector 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; that 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 activation markers, this aspect may reflect the disparity between these findings. Miyara *et al* have further classified human $FOXP3^+$ cells into three cell subsets: $CD45RA^+FOXP3^{low}$, $CD45RA^-FOXP3^{hi}$ and $CD45RA^-FOXP3^{low}$. Functional assay demonstrated that the $CD45RA^-FOXP3^{low}$ subset contains non suppressor cells, that the $CD45RA^+FOXP3^{low}$ subset contains resting Tregs and that 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 (Miyara et al., 2009).

In addition to Treg frequency, others have also reported that autoimmune disease can alter the functional activity of Tregs. For example, the suppressive activity of $CD4^+CD25^+$ cells isolated from active rheumatoid arthritis patients was significantly decreased (Valencia et al., 2006). It is likely that some intrinsic defect in $CD4^+CD25^+$ cells in active rheumatoid arthritis patients accounts for their decreased functional activity. Similarly, the frequency 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 (Haas et al., 2005; Kumar et al., 2006; Viglietta et al., 2004). These results suggest that the manipulation of nTregs to restore their numbers and function may be therapeutic.

Although most people claim that $CD4^+CD25^+$ in peripheral blood belong to natural Treg cells, we and others would suggest that $CD4^+CD25^+$cells in PBMCs consist of a mixture of both thymic nTregs and those induced in the periphery (induced Tregs, iTregs) (Horwitz et al., 2008; Zhou et al., 2011, Lan Q et al; Zheng S.G.). 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 poor for controlling the disease progression (Zhou et al., 2010a). 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 *et al* have reported that nTreg suppressive activity can be abolished by IL-6 (Pasare and Medzhitov, 2003). Valencia *et al* also revealed that elevated TNF-α may interfere with the suppressive capacity of nTregs (Valencia et al., 2006). There is no question that these pro-inflammatory cytokines are elevated in RA patients (Wakkach et al., 2003). Secondly, Th17 cells may be resistant to the suppressive effects exerted by nTregs. This could explain how nTregs are able to prevent development of disease before Th17 cells become established, while demonstrating ineffective suppression after disease expression is evident. Third, nTregs are inherently unstable and can be converted to Th1, Th2, Th17 and Tfh effector cells when they encounter an inflammatory milieu (Lu et al., 2010b; Tsuji et al., 2009; Wan and Flavell, 2007; Xu et al., 2007; Zhou et al., 2010a).

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 inability 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 suppressing progression of the lupus-like syndromes and CIA. We also documented that 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., 2010a). This indicates that the manipulation of nTregs still holds a promise in the treatment of autoimmune diseases.

III.2 Constitution and Types of Regulatory T Cells

Current studies have demonstrated that Treg cells 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 γδ 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 $CD4^+CD25^+Foxp3^+$ cells (nTregs) described as above, endogenous induced Tregs *in vivo* and those that can be induced *ex vivo* from $CD25^-$ precursors in peripheral lymphoid organs (iTregs) (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 may not be suitable for the treatment of SLE and other autoimmune diseases. TGF-β-induced Tregs will be defined as iTregs in this thesis.

While Yamagiwa *et al* reported that TGF-β promotes endogenous $CD4^+CD25^+$ cells (Yamagiwa et al., 2001), our group first reported that TGF-β does have an ability to induce $CD4^+CD25^-$ cells to become $CD4^+CD25^+$ Treg cells *in vitro* (Zheng et al., 2002). When Foxp3 was identified as Treg marker in Tregs, several groups including us 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).

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 comprised of 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 (Marie et al., 2005), 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). By 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 o r 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; Lu et al., 2010b). 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

Table 1. Differences between nTreg and iTreg cells

		nTreg	iTreg
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 requirement	(-) or TGF- β ? (-) or IL-2?	TGF- β IL-2
	Maintenance requirement	IL-2	IL-2
	Function requirement	TNF- α (+)	TNF- α (-)
Stabilities	IL-6 or others	Convert to Th17, Th1, Th2 and Tfh	No conversion
Phenotype		Helios (+)	Helios (-)
Human		no cytokine production	cytokine production

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 (Lan Q and Zheng SG, unpublished data), but inhibitory CTLA-4 costimulatory molecule and CTLA-4/B7.1 signaling is crucially required for the generation of iTregs (Zheng et al., 2006b). 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 alternate 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* reported that while the TNF-R2 expression is essential for nTregs-mediated suppression of colitis, its expression is not required for iTreg-mediated suppression (Housley et al., 2011). Differing IL-2 and co-stimulatory molecule requirements for Treg cell development, and TNFRII expression requirements for the suppressive function of both nTregs and iTregs suggests 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 rheumatoid arthritis, further studies are required to understand whether this therapy differentially regulates nTregs and/or iTregs development in individual diseases.

As TGF-β either promotes Foxp3$^+$ iTregs, Th9 or 17 cells depending upon other cytokines involved (**Figure 1**), and as nTreg cells 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 become Th17 and other T helper cells (Xu et al., 2007). To demonstrate this, Xu *et al* 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 dominant mice resulted in the failure of Th17 conversion (Lu et al., 2010b; Zheng et al., 2008). 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. In the current study, we will further investigate whether nTregs can be converted into Th17 cells in an *in vivo* model.

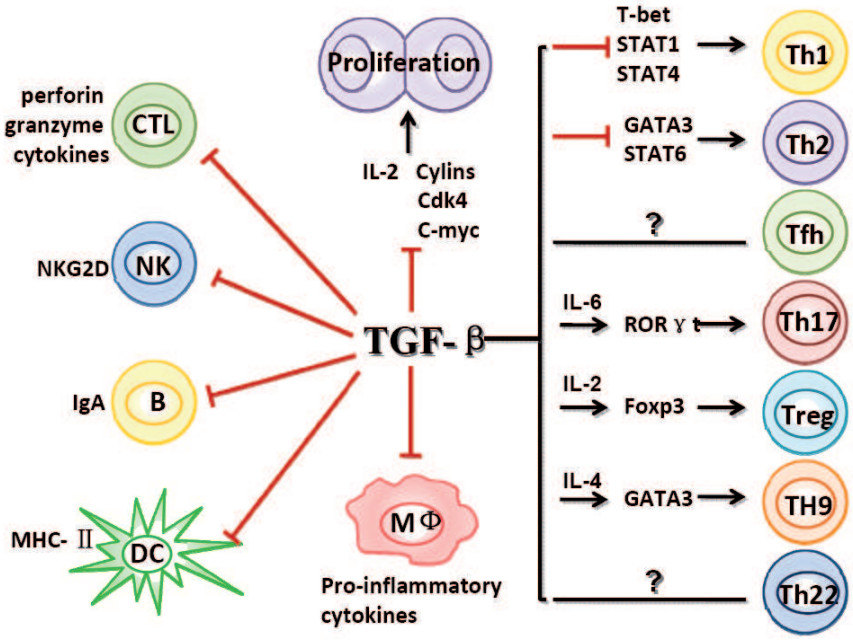

Fig. 1. Multi effects of TGF-β on regulatory and effector T cells. TGF-β inhibits the differentiation, proliferation and function of various immune cells including Th1, Th2 and Tfh cells. TGF-β also promotes iTreg, Th17 and Th9 cell differentiation depending upon the cytokine environment. Additionally,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

In sharp contrast, TGF-β-induced iTregs were found to be completely resistant to the Th17 conversion by IL-6. 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%). 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 alpha-chain (CD126) and beta-chain (CD132) expression on $CD4^+$ cells and these cells expressed significantly lower level of phosphorylated STAT3 expression when stimulated by IL-6 (Zheng et al., 2008). *All-trans* retinoic acid (atRA) has a similar effect on nTreg stability under inflammatory conditions as well (Zhou et al., 2010a). In the current study, we will compare the stabilities and functionalities of both Treg cell subsets in autoimmune inflammatory disease *in vivo*.

Nonetheless, others have reported that TGF-β-induced iTregs were unstable *in vitro* (Floess et al., 2007) and *in vivo* following antigen-stimulation (Chen et al., 2011), 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 and 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 cell 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-induced Tregs 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 Foxp3expression (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.

III.3 Molecular Mechanisms Underlying the iTreg Cell Differentiation

Current studies have demonstrated that several signaling pathways, such as the TGF-β/Smad, IL-2/IL-2R/STAT, T cell receptor (TCR) and costimulatory signaling pathways are crucial for the induction of Foxp3 transcription although the TGF-β receptor (TβR) signaling pathway is considered to be the key one.

As the lack of either TβRI or TβRII will terminate the cellular response to TGF-β (Wrana et al., 1992), it is understandable that transcription factor Foxp3 cannot be induced by TGF-β in TβR deficient T cells (Lu et al., 2010b). In lymphocytes, TGF-β binds to its cognate receptor complex composed of type I (ALK5) and type II receptors. TGF-β type I receptor (TβRI) and type II receptor (TβRII) associate as interdependent components of a heteromeric complex. TβRII is required to activate TβRI in the ligand–receptor complex, and activated TβRI Ser/Thu kinases phosphorylate downstream specific SMAD2 and SMAD3. Upon phosphorylation, these two SMADS bind to their common partner, SMAD4, to formSMAD2/4 and SMAD3/4 complexes. These complexes then translocate to the nucleus and modulate target gene expression (Lagna et al., 1996; Rubtsov and Rudensky, 2007). Unlike Smad2 and Smad4 null mice, Smad3 null mice are viable and survive to adulthood (Datto et al., 1999). Accumulating evidence has revealed that Smad3 is essential for the suppressive effect of TGF-β on IL-2 production and T cell proliferation (McKarns et al., 2004). Smad3 is also required for the suppressive effects of TGF-β on Th2 type cytokine production and Th2 type diseases in the skin (Anthoni et al., 2008).

Recent studies have also begun to explore the roles of Smad pathways in iTreg differentiation. Anthoni and colleagues examined the role of Smad3 in a $Smad3^{-/-}$ murine model of contact hypersensitivity and found that the lack of intact TGF-β signaling via Smad3 resulted in an increased pro-inflammatory, Th2 and Th17 type response in the skin with reduced Foxp3 mRNA in the lymph nodes (Anthoni et al., 2008). These data implicate that the Smad3 may be involved in iTreg cell differentiation. Using an *in vitro* culture system, several groups found that Smad2 or Smad3 plays a significant role in TGF-β-iTreg generation (Jana et al., 2009; Xu et al., 2010).

Tone and colleagues identified an evolutionarily conserved enhancer site in Foxp3 gene that bindsSmad3 and nuclear factor of activated T cells (NFAT), suggesting that TGF-β regulates Foxp3 transcription through a Smad3 dependent pathway. Smad3 and NFAT functioned in a coordinated fashion and were essential for histone acetylation in the enhancer region and induction of Foxp3

transcription(Tone et al., 2008). Xu *et al* also found that retinoic acid (RA) augmentation of TCR- and TGF-β-induced Foxp3 transcription were related processes involving modifications of baseline (TGF-β-induced) phosphorylated Smad3 (pSmad3) binding to a conserved enhancer region (enhancer I). This led to increased histone acetylation in the region of the Smad3 binding site and increased binding of pSmad3 (Xu et al., 2010).

Nonetheless, we and others have recently reported that Smad2 or Smad3 plays a partial role in iTreg differentiation *in vitro* but plays no roles in iTreg differentiation *in vivo* (Lu et al., 2010b; Takimoto et al., 2010). Although it is possible the either Smad2 or Smad3 can compensate for each other, we also observed that TGF-β's downstream non-Smad pathways actually play an important role in iTreg development. TGF-β can activate SMAD-independent pathways such as MAPKs, in T cells(Zhang, 2009). In fact, TGF-β inhibition of IFN-γ induced signaling and Th1 gene expression in $CD4^+$ T cells is Smad3 independent but MAPK dependent mechanism (Park et al., 2007). Among MAPKs, we further observed that ERK and JNK but not P38 activation is involved in TGF-β-mediated Foxp3 induction (Lu et al., 2010b). ERK and JNK may activate AP1 that will coordinate with NTAT to regulate Foxp3 expression. In this project, one of goals is to determine how atRA affects Smad or non-Smad pathways during iTreg cell differentiation.

Although TCR activation is needed for the TGF-β-mediated Foxp3 induction, its role in this process is complicated. Antigen stimulation TCR activates the transcription factor NFAT, a key regulator in T cell activation and anergy. NFAT forms cooperative complexes with the AP-1 family of transcription factors and regulates T cell activation-associated genes. Treg function is mediated by an analogous cooperative complex of NFAT with the forkhead transcription factor Foxp3 (Wu et al., 2006). TCR engagement also activates nuclear factor (NF)-κB (NF-κB) and phosphatidylinositol 3-kinase (PI3K)/Akt/mTOR (mammalian target of rapamycin) axis. Most of these transcription factors conversely play a negative role in the iTreg differentiation although cRel and p65, two out of five NF- κB family members: c-Rel, RelA-p65, RelB, NF-kB1 (p50-p105), and NF-kB2 (p52-p100) play a positive role in iTreg cell differentiation (Ruan et al., 2009). Thus, rapamycin suppresses PI3K/Akt/mTOR axis and promotes iTreg differentiation (Ruan et al., 2009). This is likely that different signaling intensities of TCR activation will result in the expression of different transcription factors and subsequently affect the development of different T effector cell subsets (Zhou et al., 2011).

Many cytokines also affect iTreg differentiation. TGF-β-mediated Foxp3 induction is markedly

reduced in IL-2-deficient T cells and addition of exogenous IL-2 promotes and sustains Foxp3 expression (Davidson et al., 2007; Zheng et al., 2007). In fact, IL-2- and IL-2R-deficient mice have a low frequency of $Foxp3^{+}$cells (Malek and Bayer, 2004). CD28/B7 costimulatory signaling may promote Treg cell production and maintenance through IL-2 production. As IL-4, IL-7, IL-15, and IL-21 also share common γ chain with IL-2R, these cytokines may also affect iTreg differentiation. It has been reported that $IL\text{-}2^{-/-}IL\text{-}15^{-/-}$ double KO mice have a much lower Treg frequency than $IL\text{-}2^{-/-}$ mice, suggesting that IL-15 also affects iTreg cell differentiation and maintenance (Burchill et al., 2007). IL-7 also plays an important role in the maintenance of long-lived memory iTreg phenotype and function (Li et al., 2011). Conversely, IL-4, IL-21, IL-27 as well as IL-6 suppress iTreg cell differentiation.

Signaling downstream of the IL-2R can act through the Janus kinase (JAK)/STAT pathway. STATs comprise a family of several transcription factors that are activated by a variety of cytokines, hormones, and growth factors. STATs are activated through tyrosine phosphorylation, mainly by JAK kinases, which lead to their dimerization, nuclear translocation and regulation of target gene transcriptions. STAT5 molecule is a key component of the IL-2 signaling pathway, the deficiency of which often results in autoimmune pathology due to reduced number of Treg cells (Burchill et al., 2007).

Activated STAT5 is translocated into the nucleus where it binds to highly conserved tandem consensus STAT binding motifs located in the promoter region and/or first intron of the Foxp3 gene and promotes Foxp3 expression (Burchill et al., 2007; Zorn et al., 2006). STAT5a/b is required for optimal induction of Foxp3 *in vitro* and binds directly to the Foxp3 gene (Yao et al., 2007). Conversely, IL-6, IL-21 and IL-27 activate STAT-3 and p-STAT-3 then bind a gene silencer in a second conserved enhancer region (enhancer II) downstream from enhancer I; this leads to a loss of pSmad3-binding to enhancer I and eventually suppresses Foxp3 expression (Xu et al., 2010). IL-4 activates STAT6, and p-STAT6 binds to a promoter just before exon I of the Foxp3 gene and suppresses Foxp3 gene transcription. Retinoic acid can suppress p-STAT6 binding to the Foxp3 promoter and promotes iTreg differentiation. Thus, control of accessibility and binding of different transcription factors provides a common framework for positive and negative regulation of TGF-β-induced Foxp3 transcription.

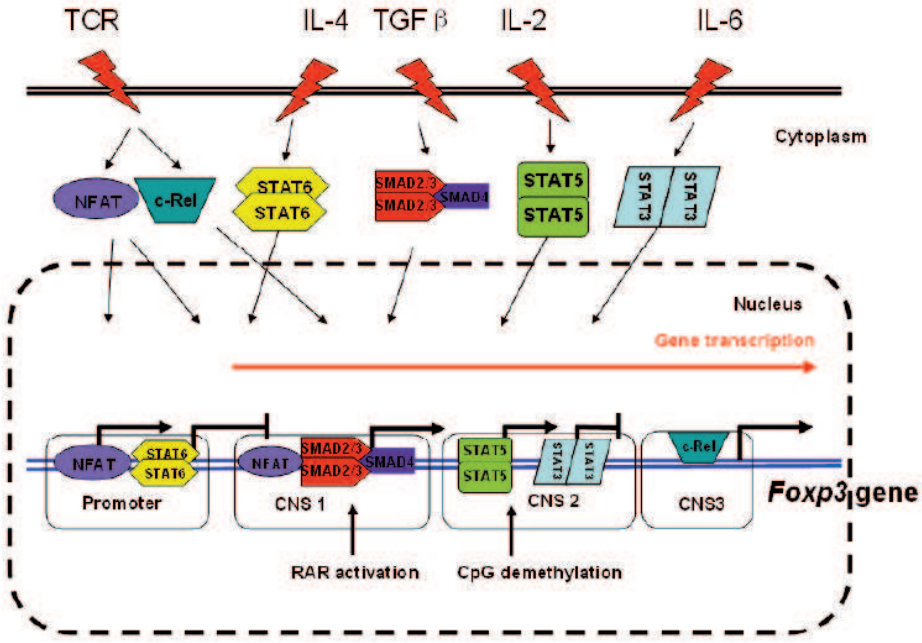

Fig.2. Multi effects of TGF-β, TCR and cytokine signaling pathway-related transcription factors in the regulation of Foxp3 expression and maintenance.

These transcription factors could also work together to regulate Foxp3 expression. Ruan *et al* propose a c-Rel-dependent enhanceosome model, which may apply to explain the regulation of iTreg cell differentiation. They suggest that antigen-presenting cells (APC) carrying specific peptides engage precursor T cells by TCR and CD28, in the presence of TGF-β, ligation of TCR, CD28, and TGF-β receptors leads to the activation of IKKb, which phosphorylates IkBa (inhibitor of kBa), releasing c-Rel and p65. The freed c-Rel-p65 dimer then migrates into the nucleus, binds to the Foxp3 promoter, and induces the formation of a Treg cell-specific multifactorial transcriptional complex including NFAF/AP1/Smad/STAT called ‘‘enhanceosome’’ which comprises transcription factors that bind not only to the promoter but also to the distal enhancers. Current studies have demonstrated that the Foxp3 gene is controlled by a core promoter and at least three distal enhancers

(Mantel et al., 2006; Tone et al., 2008). More recently, researchers have identified that Foxp3 differentiation and maintenance is controlled by three non-coding DNA sequence (CNS1-3) elements (Zheng et al., 2010). While CNS1 containing a TGF-β-NFAT response element and CNS3 containing c-Rel response element are associated with Treg differentiation, CNS2 containing Cbf-β-Runx1, Stat and CpG DNA mostly regulates Foxp3 maintenance. Thus, Treg cell-specific enhanceosome in Foxp3 CNS regions in turn serves as the ‘‘on-and-off’’ switch of the Foxp3 gene and the Treg cell differentiation and maintenance program. A schematic representative of various signaling pathway related transcription factors that regulate Foxp3 transcription and expression has been demonstrated in **Figure 2**.

III.4 Preventive and Therapeutic Roles of Regulatory T Cell Subsets

Both iTregs and nTregs share similar functional characteristics. Adoptive transfer of iTregs generated *ex-vivo* also can prevent the development of autoimmune diseases. For example, Wahl 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 lupus model, our study has demonstrated that iTreg prevented disease development (Zheng et al., 2004b). Weber et al 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 (Weber et al., 2006). Dipaolo et al reported similar preventive role of iTregs in a murine model of autoimmune gastritis (Dipaolo et al., 2007). Similarly, iTreg cells also significantly prevented Th1-mediated colitis on $CD4^+CD62L^+$ T cell transfer *in vivo* (Fantini et al., 2006). Selvaraj et al demonstrated that adoptively transferred iTregs were as potent as natural $Foxp3^+$ Treg in preventing EAE development (Selvaraj and Geiger, 2008). It seems both antigen-specific and non-specific iTregs prevent autoimmune diseases although the former is more efficacious than the latter.

In addition to their use in a preventative 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 graft-*vs*-host disease model characterized by rapid and vigorous formation of SLE-like autoantibodies and the formation of severe immune-complex glomerulonephritis. DBA/2 (D2) mouse T cells induce this syndrome when injected into (DBA/2 x C57BL/6) F1 mice. We found TGF-β-treated DBA/2 T cells not only lost their ability to induce graft-*vs*-host disease 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 (Su et al., 2008). Selvaraj *et al* observed that iTregs were still efficacious in ongoing experimental autoimmune encephalomyelitis (EAE), animal model of multiple sclerosis, and Godebu *et al* also reported that iTregs can revise type I diabetes in animal (Godebu et al., 2008; Selvaraj and Geiger, 2008). Similarly, in autoimmune gastritis model, Nguyen *et al* found that antigen-specific iTregs also still suppressed inflammation and associated pathology when administered late in the process of ongoing disease(Nguyen et al., 2011).

As nTregs seem to be unable to control the progress of established collagen-induced arthritis (CIA), we will plan to conduct a head-to-head comparison of therapeutic effects of antigen specific thymus-derived nTregs and TGF-β-induced iTregs on the established CIA in the current study. 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). We will test the hypothesis that antigen-specific iTregs are superior to nTregs in ameliorating established CIA. We will determine whether iTregs remained stable and fully functional following transfer. The recent studies of Nugyen *et al* indicated that chemokines secreted by antigen-specific TGF-β-induced iTregs regulated T cell trafficking and thereby suppressed 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 all implicate that iTregs may have a therapeutic potential in suppressing the established autoimmune diseases.

III.5 Objective of the PhD Project

Previous studies have revealed that Tregs play an important role in the prevention of autoimmune diseases. However, the adoptive transfer of nTregs to the established autoimmune diseases is less therapeutic and these cells are unstable and can convert to T effector cells and lose the suppressive activity. Interestingly, the iTregs are resistant to T effector cell conversion and stable *in vitro* in the inflammation-like environment. In this study, we will use an *in vivo* model, en established collagen-induced arthritis (CIA), to learn whether both Treg subsets have a different stabilities and functionalities *in vivo*. We will make a head-to-head comparison to determine the preventive and therapeutic roles of both Treg subsets in the ongoing and established CIA. We will learn whether

infusion of iTregs also suppress the ongoing allergic asthma and chronic GVHD with a lupus-like disease in the project.

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 (Andersson et al., 2008; Zheng et al., 2004a). To determine whether infectious tolerance contributes to long-term protective effect of iTregs on autoimmune diseases, we will investigate the interaction of iTregs and DC in the chronic graft-*vs*-host disease with a typical lupus syndrome in the current study. We will test whether infusion of iTregs to lupus mice can induce the formation of tolerogenic DCs. We will further determine underlying mechanisms if it is a case. In this study, we will use DC-specific TβRII conditional KO mice to address this issue. We will also determine molecular mechanisms by which atRA promotes iTreg generation, maintenance and function. These studies will further disclose the mystery of development and function of iTregs in autoimmunity.

IV. ARTICLE 1

Adoptive transfer of TGF-β-induced-regulatory T cells effectively attenuates murine airway allergic inflammation

Song Guo Zheng[1,5] , Wei Xu[2], Qin Lan[1,3], Hui Chen[2], Ning Zhu[4], Xiaohui Zhou[1,3], Julie Wang[1], Huimin Fan[5], David Warburton[2], Dieudonnée Togbe[5], Valerie Quesniaux[5], Wei Shi[2] and Bernhard Ryffel[5]

[1]Division of Rheumatology and Immunology, Department of Medicine, The Keck School of Medicine, University of Southern California, Los Angeles, CA 90033; [2]Developmental Biology and Regenerative Medicine Program, Department of Surgery, The Saban Rrsearch Institute of Children's Hospital Los Angeles, 4650 Sunset Blvd., Los Angeles, CA 90027; [3]Institute of Immunology, Shanghai East Hospital, Shanghai, China; [4]Institute of Immunology, Fujian Medical University, Fuzhou, Fujian Province, China; [5]UMR6218, Molecular Immunology, University and CNRS, 3b rue de la Ferollerie, F-45071 Orleans, France

Manuscript is under review of Journal of Immunology now

Abstract

Both nature (nTreg) and induced-regulatory T lymphocytes (iTreg) are potent regulator of autoimmune and allergic disorders. Defects in Treg cells have been reported in patients with allergic asthma, and therefore replenishment of Treg cells might attenuate asthma. Here we report that adoptive transfer of iTreg cells generated *ex-vivo* with IL-2 and TGF-β effectively attenuated lung and airway allergic inflammation in a murine model of asthma. Immunized mice given $5x10^6$ iTreg cells just before antigen challenge displayed markedly reduced airway résistance, eosinophil recruitment, mucus hyper-production, airway remodeling and IgE levels. This therapeutic effect was associated with increase of Treg cells ($CD4^+Foxp3^+$) in the draining lymph nodes, and with reduction of Th1, Th2, and Th17 cell response as compared to untreated and non-Treg cell treated controls. Therefore, adoptive transfer of iTreg reduces the allergic response, which might be a novel and promising therapeutic approach to treat severe asthma.

Keywords: Asthma, Lung inflammation, T regulatory cells, induced T regulatory cells.

Introduction

Chronic allergic airway inflammation and airway hyperresponsiveness (AHR) are characteristic of atopic asthma pathophysiology. More than 7% of Americans suffer from asthma (Moorman et al., 2007), and annual expenditure for health and lost productivity due to asthma is estimated at nearly $20 billion. The currently available therapeutic approaches for asthma usually include quick symptomatic relief measures directed to relaxation of airway smooth muscle (bronchodilator) and long-term control with suppression of airway inflammation (Fanta, 2009). However, these existing standard asthma therapies have several caveats and remain inadequate. For example, inhaled anti-inflammatory corticosteroids only suppress but do not cure asthmatic inflammation, and long-term use of corticosteroids causes many pleiotropic side effects. Other more recently developed therapies, including inhibitors of leukotriene production and leukotriene receptor blockade, and anti-IgE monoclonal antibody (omalizumab), are used as alternative treatments for persistent asthma. However, limited efficacy, high cost, and lack of responsiveness in some asthma patients are the major drawbacks. Thus, novel and more effective therapeutic approaches for asthma are still needed.

Recent studies have found that immune function dysregulation is one of the key pathogenic mechanisms underlying chronic asthma(Doherty and Broide, 2007). Reduction and/or defects in regulatory T (Treg) cells, which function as negative regulators to suppress excessive immune response and maintain immunological tolerance have been detected in asthma patients(Apostolou and von Boehmer, 2004). Therefore, replenishment of Treg cells is thought to be a promising cell therapeutic approach. However, the use of thymus-derived naturally occurring regulatory T (nTreg) cells has several caveats that may significantly diminish their practical application for asthma treatment. These include limited availability, susceptibility to inflammation-triggered apoptosis, inability in suppressing pro-inflammatory Th17 cells, and self-conversion to Th17 and/or other T effector cells in the milieu of inflammation. In contrast, Treg cells that are induced by TGF-β and IL-2 in combination with low dose antigen exposure have similar phenotypic and functional characteristics to nTreg cells, without the caveats of nTreg cells mentioned above. Herein, we report that adoptive transfer of the induced-Treg (iTreg) cells to Ovalbumin (OVA)-sensitized mice effectively attenuates OVA-induced airway allergic inflammation, airway hyperresponsiveness, and other asthma-like lung pathology by modulating the systemic immune system.

Materials and Methods

Animal care

C57BL/6 mice were purchased from the Jackson Laboratory and bred at the specific pathogen-free animal facility at Keck School of Medicine at University of Southern California. All experiments were approved by the Institutional Animal Care and Use Committee at University of Southern California.

OVA-sensitized mouse asthma model and exogenous cell infusion

6 to 8-week-old female mice weighing 20-25g were used for the experiments. Mice were sensitized by intraperitoneal (i.p.) injections of 25ug OVA (Grade V, Sigma Chemical Co.) mixed with aluminum hydroxide (Pierce) at day 1, and followed by another booster i.p. injection at day 14. These sensitized mice then were challenged with 20μg of OVA or saline control through an intranasal (i.n.) route for three consecutive days (days 25, 26, and 27)to generate an acute allergic asthma model.$5x10^6$ of iTreg cells or control cells generated as described below were intravenously injected into mice on day 22, three days before antigen challenge.

Generation of *in vitro* TGF-β-induced regulatory T (iTreg) cells

Splenic $CD4^+CD25^-CD62L^+CD44^{low}$ naive T cells were isolated by autoMACS (Miltenyi Biotech) from C57BL/6 female mice, which were littermates to the mice used for generating the OVA-asthma model. iTreg cells were then prepared using the methods described in our previous publication(Zheng et al., 2007). Briefly, naïve $CD4^+$ T cells were first isolated by negative selection, in which naïve $CD4^+$ T cells were labeled with FITC-conjugated anti-CD25 mAb and sorted for $CD4^+CD25^-$ T cells. The $CD4^+CD25^-$ T cells were then labeled with PE-conjugated anti-CD62L and Cyc-conjugated CD44, and positively selected by anti-PE magnetic beads ($CD4^+CD62L^+CD44^{low}CD25^-$ cells). These isolated naïve T cells were stimulated with anti-CD3/28 coated beads (1:5 ratio, five cells to one bead) in the presence of IL-2 (40 U/ml) and TGF-β1 (2 ng/ml) for 4-6 days to generate iTreg cells; controls cells were treated with anti-CD3/CD28 beads with IL-2 only. The iTreg cell phenotypes were verified by related molecular marker expression ($CD4^+CD25^+Foxp3^+$) detected by Flow Cytometery. Also the immune suppressive activity of iTreg cells was verified using a standard *in vitro* suppressive assay as previously reported (Zhou et al., 2010b).

Determination of airway hyperresponsiveness (AHR) *in vivo*

On day 28 (24 hours following the last i.n. administration of either normal saline or OVA challenge), the mice were anesthetized with intraperitoneal injections of sodium pentobarbital (90mg/kg). When an appropriate depth of anesthesia was achieved, a tracheostomy was performed, in which a standard 20G × 32 mm Abbocath®-T cannula (Abbott, Sligo, Ireland) was gently inserted into the trachea and secured with suture. The mice were then connected to a computer controlled small animal ventilator (FlexiVent, Scireq, Canada) and ventilated at 150 breath/min with a tidal volume of 10ml/Kg and a positive end-expiratory pressure of 3 cmH_2O. Methacholine (MCh, 40 mg/ml in PBS) was then delivered to the subject by nebulized aerosol. The frequency-independent airway resistance (Raw) in mouse lung was then automatically measured by FlexiVent/SciReq software, and the MCh challenge experiments are repeated at least three times.

Bronchoalveolar lavage (BAL)

The bronchoalveolar lavage was performed three times with 0.8 ml of normal saline in each time, and pooled together. The number of cells in BAL was counted using a hemocytometer. The remaining samples were then centrifuged at 4ºC for 4 min at 400xg to separate cell-free supernatant and cells. An aliquot of supernatant was used to measure BAL protein concentration by Bradford method (Bio-Rad).

Lung histopathology and immunohistochemistry

The lungs were inflated with PBS under 25 cm H_2O, and fixed in 4% paraformaldehyde overnight at 4^0C. The fixed tissues were then processed for paraffin embedding. 5-μm lung tissue sections were stained with Hematoxylin and Eosin (H&E) for lung morphological analyses. Eosinophils in the lung tissue were stained with Discombe's Solution (Discombe, 1946). Periodic-Acid-Schiff (PAS) staining was used to characterize glycoproteins using a kit purchased from Sigma. Briefly, slides were deparaffinized and hydrated, then immersed in Periodic Acid Solution for 5mins. After washing with several changes of distilled water, the slides were incubated in Schiff's reagent for 15mins and counterstained with hematoxylin. The numbers of airways with PAS-positive epithelial cells versus PAS-negative epithelia were then determined in each lung section. Lung inflammatory histopathology was evaluated using a semi-quantitative method reported by other group(Richards et al., 1996). Briefly, at least two sections of each mouse lung were examined using the following criteria. Alveolar inflammation foci were scored 0-5 (0=no foci, 1=≤5, 2=6 -15, 3=16-25, 4=26-35, 5=≥35). The peri-vascular and peribronchiolar inflammation was also scored separately on a 0-5 scale (0=no inflammation, 1=minimal, 2=mild, 3=moderate, 4=marked, 5=severe). Thus, the total inflammation score is the average of alveolar and peri-vascular/bronchiolar inflammation. At least

five mice in each group were selected for this analysis. Immunohistochemical studies were performed using Zymed Histostain-Plus system (Zymed). Alpha smooth muscle actin (α-SMA) antibody was purchased from Sigma (St Louise, MO). Normal serum was used as negative control.

Serum cytokine analysis

Serum was prepared from blood collected after measuring AHR. Samples were frozen at −20°C until analysis. The levels of cytokines (IL-4, IL-5 and IL-13) and IgE in sera were measured using ELISA kits purchasing from Invitrogen or BioLegend, following the manufacturer's instructions.

Intracellular cytokine staining and Flow Cytometry analysis

Lymphocytes in axillary draining lymph nodes and spleen were collected and stained for surface or intracellular markers with combinations of fluorochrome-conjugated mAb specific for CD3, CD4, CD8a, CD62, CD44, IL-17A (BioLegend, San Diego, CA), as well as IL-4, IFNγ (BD Biosciences Pharmingen, San Diego, CA). In the case of intracellular IL-4, IL-17 and IFNγ, cells were stimulated with 0.25 μg/ml PMA and 0.25 μg/ml ionomycin (Calbiochem, La Jolla, CA) for 1 hour, and followed by addition of brefeldin A (5 μg/ml; Calbiochem) and incubation for additional 4 hours prior to processing for intracellular staining. The phenotypes and intracellular cytokine expression were analyzed using a LSRII Flow Cytometry (BD Biosciences, San Diego, CA).

Statistical analysis

The data are presented as the mean. A Student's two-tailed *t* test was used to compare data between the different experimental groups. Differences were considered statistically significant for *P* values<0.05.

Results

Adoptive transfer of *in vitro* TGF-β-induced Treg (iTreg) cells attenuated OVA-induced allergic inflammation in mouse respiratory airways and alveoli.

In OVA-sensitized mice, repeated i.n. OVA challenges at day 25-27 resulted in severe small airway and alveolar inflammation, indicated by excessive inflammatory cell infiltration surrounding small airways and vasculature, as well as alveolar septa (**Fig.3**).

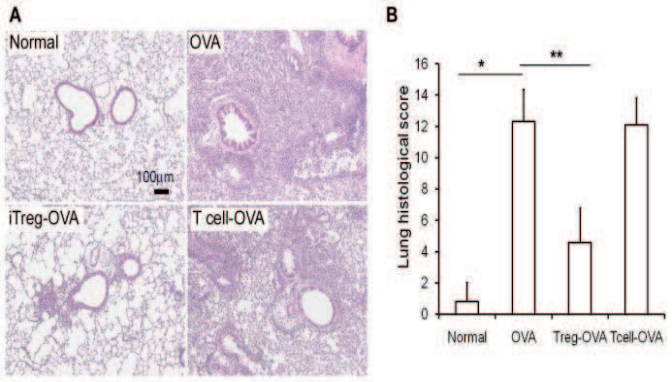

Fig.3. Reduced inflammation in lung tissues by adoptive transferring of iTreg cells prior to OVA challenge. (**a**) Lung tissue sections from the mice with indicated treatments were stained with H&E. Mice with i.n. instillation of saline were used as "Normal" control. iTreg-OVA and T cell-OVA represent mice were treated with adoptive transfer of iTreg cells versus control T cells prior to OVA challenge, respectively. (**b**) The intensities of lung inflammation were graded with scores 0 to 5 (none to severe inflammation, see Materials and Methods for details). *P<0.05, **P<0.01, n=5.

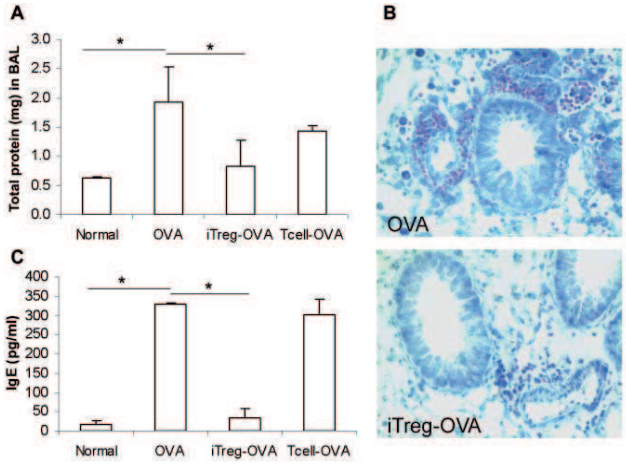

Fig.4. Attenuated allergic inflammation by iTreg cells. (**a**) Total proteins in BAL fluids from mice with different treatments were quantified; (**b**) Eosinophil, detected by Discombe's staining (red intracellular granules), was the major type of cells that were infiltrated in small airways and adjacent vasculature; (**c**) IgE level in serum from different groups of mice was quantified by an ELISA. Increased IgE level in serum by OVA-induced allergic reaction was significantly reduced with iTreg treatment. *P<0.05.

The serum level of IgE was significantly increased, and infiltration of eosinophil around airway was also verified by Discombed staining (**Fig. 4**). Consistent with the lung histological changes, the total amount of proteins in BAL fluid was significantly increased (**Fig. 4**). The number of cells in BAL also increased more than 10-fold than the control groups (data not shown). Moreover, epithelial cell hypertrophy with increased mucin expression in small airways, thickened airway smooth muscle cell (SMC) layer, and resultant smaller lumen with rippled epithelial surface of small airways were also observed in OVA-challenged mouse lungs (**Fig. 5**). Therefore, a typical OVA-allergic airway inflammatory model was verified.

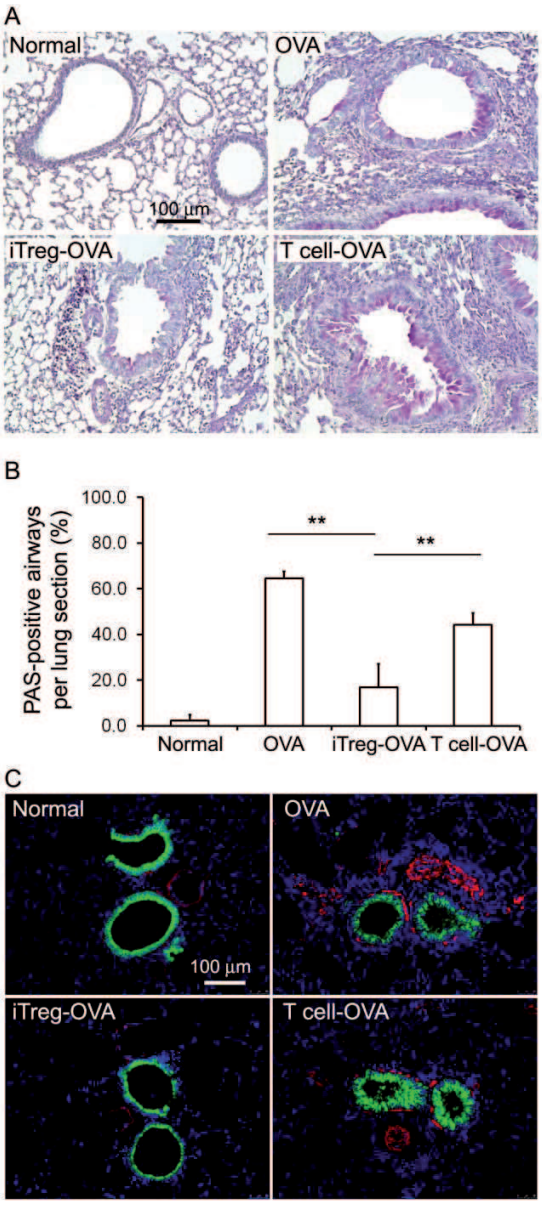

Fig.5. Abnormal airway wall remodeling was subsided with iTreg cell treatment. (**a**) Excessive mucin expression in small airway epithelial cells was detected by PAS staining (red color). (**b**) The numbers of airways with PAS-positive epithelial cells per lung tissue section were quantified in different experimental groups (n=5), suggesting overproduction of mucin. (**c**) Clara cells in small airway epithelia were stained by CCSP immunofluorescence staining (green) and the surrounding airway smooth muscle cells were immunostained by SMA (red). **P<0.01.

Using this established OVA-allergic mouse model, the anti-inflammatory effect of adoptive transfer of iTreg cells was then examined. Three days before OVA challenge (Day 22), a single transfer of $5x10^6$ iTreg cells was given to the mice *via* tail vein injection. T cells cultured without TGF-β addition was used as an additional specificity control. After three-day i.n. OVA challenge, lung specimens were harvested for detailed analyses. iTreg cells, but not control T cells, significantly attenuated OVA-induced allergic inflammation including reduced infiltration of inflammatory cells, particularly eosinophils, in airway and alveolar septa, decreased levels of serum IgE and BAL proteins (**Fig. 3-4**), as well as reduction in the number of cells in BAL by 2-fold ($p<0.05$). Alterations of airway walls subsequent to allergic inflammation, including epithelial hypertrophy and increased mucin production (PAS-positive staining), as well as thickened smooth muscle cells in small airways (**Fig. 5**), were likewise significantly attenuated. These results indicate that adoptive

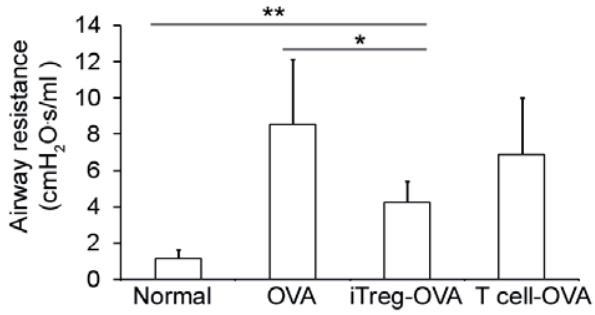

Fig. 6. Adoptive transfer of iTreg cells significantly reduced AHR. Airway resistance was measured upon Mch (40mg/ml) aerosol delivery. Although the airway resistance was still significantly higher in iTreg cell-treated group than that in normal control group, significant reduction of airway resistance was achieved in iTreg cell-treated group compared to non-treated (OVA) or control T cell-treated group. (*P<0.05, **P<0.01).

transfer of iTreg cells prior to OVA allergic challenge can effectively prevent lung inflammation and abnormal airway remodeling.

Adoptive transfer of iTreg cells also effectively inhibited airway hyperresponsiveness (AHR) in OVA-challenged mice.

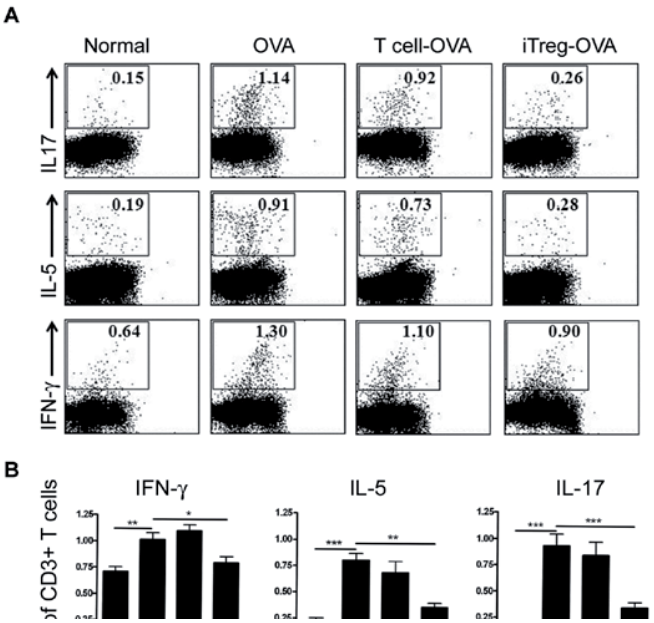

Fig.7. Adoptive transfer of iTreg cells significantly diminished Th1/Th2/Th17 cell frequencies in draining lymph nodes in asthmatic mice. Cells from axillary lymph nodes were isolated and stimulated with PMA and Ionomycin (5 hours), and BFA (4 hours). Intracellular expression of IFN-ϒ, IL-5, and IL-17A in $CD3^+$ T cells were determined by FACS. (**a**) A representative of 9 mice in each group. Cells were gated on $CD3^+$ cells. (**b**) Results were mean ± SEM of values of 9 mice in each group. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

OVA-sensitized mice with repetitive i.n. administration of OVA developed significant AHR to methacholine (MCh) challenge compared to normal control mice (**Fig. 6**). However, adoptive transfer of iTreg cells prior to repetitive challenge of OVA significantly inhibited AHR, although increased AHR was still detected. In contrast, adoptive transfer of T control cells did not significantly reduce AHR, although slight reduction in AHR was detected in some mice. Thus, adoptive transfer of iTreg cells, but not control T cells, prior to allergen exposure can also effectively reduce AHR in addition to the reduction in airway inflammation described above.

Adoptive transfer of iTreg cells modulated systemic immune response to OVA-allergic challenge.

Numerous studies have found that asthmatic inflammation is related to abnormal cellular immunity, including defective Treg cells, inappropriate ratio of Th2 over Th1 cell population, and excessive Th17 cells. Thus, we have evaluated these immune cells and their related cytokine production.

A 2-fold increase of Treg cells ($CD4^+Foxp3^+$) was detected in the draining lymph nodes and the spleen of the mice receiving adoptive transfer of iTreg cells prior to OVA challenge, while there were no significant changes in Treg cells in the comparison to OVA or T control cell-OVA groups. More interestingly, increased Th1, Th2, and Th17 cells caused by repetitive OVA challenge was significantly attenuated by adoptive transfer of iTreg cells prior to OVA administration (**Fig. 7**), but not by control T cells. Consistent with this, serum Th2 cytokines including IL-5 and IL-13 were significantly increased upon OVA challenge (**Fig. 8**). Adoptive transfer of iTreg cells, but not T control cells, was able to partially block these increases in mice. Therefore, adoptive transfer of iTreg cells may modulate both local and systemic immunity, and thus prevent excessive cellular response against OVA-induced allergic reaction and inflammation.

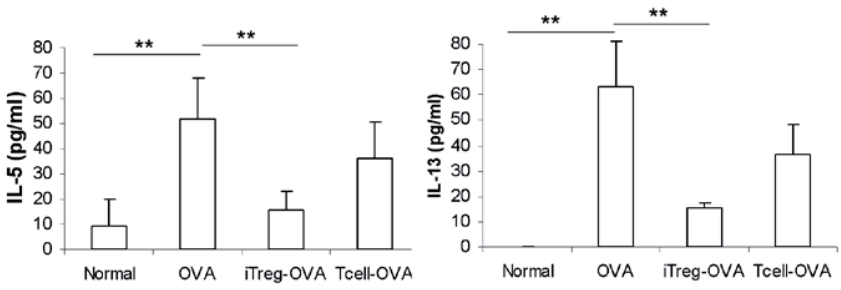

Fig.8. Adoptive transfer of iTreg cells inhibited OVA-induced increase of Th2 cytokines. IL5 and IL13 in mouse serum were quantified by specific ELISA. Significant reduction of IL5 and IL13 in the group receiving iTreg treatment was detected compared to OVA challenge only control group. **P<0.01.

Discussion

Asthma is a chronic inflammatory disorder of the conducting airways with abnormal hyper-responsiveness and airway remodeling, resulting in airflow restriction during breathing (Boxall et al., 2006; Doherty and Broide, 2007). Studies have found that immune function dysregulation is one of the key pathogenic mechanisms underlying airway chronic allergic inflammation, in particular imbalance between Th2 cell and Th1 cell responses (Amin et al., 2000; Romagnani, 2006). More recently, another pivotal subset of $CD4^+$ T cells, Treg cells, were also found to be important in suppressing allergic responses (Bacchetta et al., 2007; Larche, 2007), although defects in Treg cell function have been related to reduced immune tolerance and subsequent autoimmune diseases. The majority of naturally occurring Treg (nTreg) cells in peripheral lymph tissues is derived from the thymus. However, Treg cells can also be induced in the periphery in an antigen-specific, TGF-β-dependent fashion (iTreg), through low dose antigen exposure (Apostolou and von Boehmer, 2004; Zheng et al., 2002).

Allergen specific Treg cell function is defective in patients with allergic diseases including asthma. For example, $CD4^+CD25^+$ T cells from grass pollen-allergic individuals were less able to suppress proliferative responses and IL-5 production by $CD4^+CD25^-$ T cells (Ling et al., 2004). Significant reduction of the $CD4^+CD25^+$ T cell ratio in peripheral blood was detected in patients with persistent or exacerbation of asthma when compared to control groups (Xue et al., 2007). In addition, Treg cells in allergic asthma patients were also decreased in bronchoalveolar lavage (BAL) fluid, possibly due to reduced CCL1-mediated chemotaxis (Hartl et al., 2007; Nguyen et al., 2009). Moreover in mouse asthma models, nTreg cells are present in the lung tissue of sensitized mice and increase upon allergen inhalation. Inhibition of nTreg cells augments respiratory allergen-induced AHR and IgE production, as well as Th2 cytokine levels in BAL fluid (van Oosterhout and Bloksma, 2005). In addition, Th17 cells are a distinct Th population from Th1 and Th2 cells (Infante-Duarte et al., 2000). Th17 cells are involved in many immune disorders including airway hypersensitivity, through production of IL-17, IL-17F, IL-21, and IL-22 that act as pro-inflammatory cytokines (Nakae et al., 2002). Therefore, Th17 cells are considered to be pro-inflammatory effectors. IL-17 mRNA and proteins were reported to be increased in the lungs, sputum, BAL fluids, and sera from asthmatics, and the level of IL-17 correlated with the degree of severity of airway hypersensitivity in asthma patients (Barczyk et al., 2003; Laan et al., 2002; Molet et al., 2001; Wong et al., 2001), suggesting an important pathogenic role of Th17 and IL-17 in asthma pathogenesis and exacerbation.

Although both nTreg and iTreg cells share similar phenotypic characteristics and immune suppressive functions, studies by our group and others have suggested substantive differences regarding their generation, maintenance, and activity. In particular, unlike nTreg development, CTLA-4 (cytotoxic T lymphocyte antigen-4, CD152)-B7, but not CD28-B7 signal, is required for the development of TGF-β-iTreg cells (Zheng et al., 2006b). Moreover, IL-2 is also essential for the generation of TGF-β-induced iTreg cells (Zheng et al., 2007). Most importantly, iTreg cells are resistant to pro-inflammatory cytokine IL-6-induced self-conversion to Th17 cells (O'Connor et al., 2010; Zheng et al., 2008), and also inhibit Th17 cell generation from other T cells (see articles II). Moreover, TGF-β not only stimulates iTreg cell development, but also protects iTreg cells from apoptosis. We have found that more than 50% of T cell receptor-stimulated $CD4^+$ cells are apoptotic, however, only 10% of TGF-β1-treated $CD4^+$ cells are apoptotic as determined by Annexin-V staining (Zheng et al., 2002). Furthermore, iTreg cells survive longer than control cells following adoptive transfer *in vivo* (Zheng et al., 2002). We also have found recently that iTreg cells are resistant to apoptosis in an autoimmune inflammatory milieu (see articles II). In addition, we recently observed that 50% of adoptively transferred $Foxp3^+$ iTreg cells could survive at least one month in recipients *in vivo* (Lu et al.). Conversely, nTreg cells are more plastic and unstable under proinflammatory conditions (Xu et al., 2007; Zheng et al., 2008). These plasticity and instability likely account for the weak protective effect of nTreg cells on asthma and other autoimmune diseases. Due to their stability, it is very likely that iTreg cells, but not nTreg cells, may have a greater advantage in the treatment of established asthma, particularly when in the chronic phase, as well as potentially in other autoimmune and inflammatory diseases.

Although many studies have demonstrated that adoptive transfer of iTregs can control lupus, colitis, gastritis and diabetes in animal models (Fantini et al., 2006; Godebu et al., 2008; Huter et al., 2008; Zheng et al., 2004b), it is less known whether infusion of iTregs can prevent and control the development of asthma. Since immune function dysregulation is one of the key pathogenic mechanisms underlying chronic airway inflammation, selective suppression of such abnormal immune reactions may be an effective approach in the prevention and treatment of asthma. Currently, there is no immune cell therapy available to restore normal immune responses in asthma. Our study has demonstrated that iTreg treatment not only suppresses T effector cells, but also promotes more $CD4^+FoxP3^+$ cells. Our previous studies have demonstrated that injection of iTregs can induce recipient to induce *in vivo* iTregs through a phenomenon called “infectious tolerance” (Zheng et al., 2006a). We believe that infusion iTregs may change the balance of Tregs to T effector cells. Thus, adoptive transfer of anti-inflammatory Treg cells may prove to be a promising cell

therapy for asthma. However, use of thymus-derived naturally occurring regulatory T (nTreg) cells may have several caveats that potentially diminish their practical application for asthma treatment. These include limited availability, susceptibility to inflammation-triggered apoptosis, inability to suppress pro-inflammatory Th17 cells, and self-conversion to Th17 cells in the milieu of inflammation as described above. Moreover, intrinsic defect of $CD4^+CD25^+$ cells in asthma patients also limits the clinical use of these cells as starting population for expansion of nTregs. In contrast, iTreg cells have superior anti-inflammatory activities compared to nTreg cells. Induction of iTreg cells *in vitro* not only avoids systemic application of cytokines and growth factors, but also generates an approach similar to an autograft, by which $CD4^+$ cells from an asthmatic patient can be induced to iTreg *in vitro*, and then could be adoptively transferred back to the same patient to induce immune tolerance to allergens, as well as suppressing abnormal inflammatory responses in the airway without significant side effects. Another advantage is that one can induce sufficient numbers of iTregs for the treatment of patients with asthma and other autoimmune diseases. Therefore, we have tested this approach in the OVA airway allergic mouse model. Adoptive transfer of iTreg cells prior to OVA challenge dramatically attenuated airway allergic inflammation as well as AHR. These experimental results lay the ground work to further explore potential application of *in vitro* TGF-β-induced regulatory T cells as a novel cell therapy to better treat chronic allergic asthma.

V. **ARTICLE 2**

Antigen-specific TGF-β-induced CD4+ regulatory T cells but not expanded nTregs ameliorate established autoimmune arthritis by shifting the balance between Tregs and Th17 cells *in vivo*

Ning Kong[1,2], Qin Lan[1,3], Maogen Chen[1], Julie Wang[1], David A Horwitz[1], Valerie Quesniaux[4], Bernhard Ryffel[4], Zhongmin Liu[3], David Brand[5], Hejian Zou[2], and Song Guo Zheng[1,4]

Running title: iTreg therapy in the established autoimmune arthritis

[1]Division of Rheumatology and Immunology, Department of Medicine, Keck School of Medicine at University of Southern California, USA; [2]Division of Rheumatology, Huashan Hospital at Fudan University, Shanghai, P.R. China; [3]Immune Tolerance Center, Shanghai East Hospital, Tongji University, Shanghai, P.R. China; [4]University of Orléans and Molecular Immunology and Embryology, CNRS UMR6218, Orleans, France;[5]Research Service, Veterans Affairs Medical Center, Memphis; USA

Manuscript has been submitted to PNAS

Abstract

Transferred $CD4^+CD25^+Foxp3^+$ regulatory cells (Tregs) can prevent autoimmune disease, but generally fail to ameliorate established disease. Here we demonstrate that antigen-specific Tregs induced with IL-2 and TGF-β *ex-vivo* (iTregs), but not equivalent expanded thymus-derived nTregs can prevent progression of established collagen-induced arthritis. This was because following transfer, nTregs exhibited decreased Foxp3 and Bcl-2 expression, decreased suppressive activity, and many converted to Th17 cells. By contrast, transferred iTregs were more numerous, retained their suppressive activity in the presence of IL-6 and were resistant to conversion. Remarkably, ten days after transfer iTregs shifted the predominance from Th17 to Treg cells in draining LNs**.** These findings provide evidence that transferred TGF-β-induced iTregs are more stable and functional than nTregs in mice with established autoimmunity. Moreover, iTregs can have tolerogenic effects even in the presence of ongoing inflammation. The therapeutic potential of iTregs in subjects with chronic, immune-mediated inflammatory diseases deserves to be investigated.

Keywords: Collagen-induced arthritis, rheumatoid arthritis, Inflammation, Nature Tregs, induced Treg cells.

Introduction

$CD4^+CD25^+Foxp3^+$regulatory T cells (Tregs) are crucial in maintaining immune homeostasis (Sakaguchi, 2005). Many autoimmune diseases including rheumatoid arthritis (RA) have been reported to have abnormalities in the numbers and/or functions of Tregs (Ehrenstein et al., 2004; Flores-Borja et al., 2008; Heemskerk et al., 2007; van Amelsfort et al., 2004). $CD4^+Foxp3^+$Tregs are heterogeneous and can be divided into three populations: thymus-derived naturally occurring (nTregs), those induced *in vivo*, and those induced *ex-vivo* with IL-2, TGF-β ± retinoic acid or rapamycin (Haxhinasto et al., 2008; Horwitz et al., 2008; Sakaguchi, 2005; Zheng et al., 2002). Although some groups have reported that exogenous polyclonal TGF-β induced Tregs (iTregs) are unstable (Floess et al., 2007), we and others have observed remarkable protective effects of this subset in autoimmune animal models (Huter et al., 2008; Selvaraj and Geiger, 2008; Weber et al., 2006; Zheng et al., 2004b), and that unlike nTregs, these iTregs were resistant to conversion to Th1, Th2, Th17 and Tfh cells under inflammatory conditions (Chai et al., 2002; Lu et al., 2010b; Tsuji et al., 2009; Wan and Flavell, 2007; Xu et al., 2007; Zheng et al., 2008; Zhou et al., 2010a).

Collagen induced arthritis (CIA) has been recognized as a useful animal model for human RA since CIA mimics the symptoms, pathogenesis and progression of RA (Brand et al., 2007). Polyclonal nTregs can alter the development and progress of CIA, but are ineffective in controlling established disease although they became effective after treatment with retinoid acid (Morgan et al., 2003; Zhou et al., 2010a). Since antigen-specific Tregs have more potent protective effects than polyclonal Tregs (Penaranda and Bluestone, 2009), the objective of this study was to compare the relative effectiveness of collagen peptide-specific, IL-2 expanded, nTregs and iTregs induced with IL-2 and TGF-β in mice with established disease. We observed that transferred nTregs failed to suppress established CIA, but iTreg infusion remarkably ameliorated severity and suppressed progression. This was because in these mice with established inflammation, nTregs *in vivo* lost suppressive activity and many converted to Th17 cells *in vivo*. By contrast, $Foxp3^+$ iTregs were stable, more numerous, and had tolerogenic effects.

Materials and Methods

Mice

Eight-week-old female DBA1/J mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Collagen II TcR transgenic Foxp3^{gfp} reporter mice were produced by back-crossing DBA/1J mice with C57BL/6 Foxp3^{gfp} knock-in mice for 11 generations to develop Foxp3 reporter mice on the DBA/1 background, and then intercrossing with CII TcR Tg mice. All mice were housed and treated by National Institutes of Health guidelines for the use of experimental animals with approval of University of Southern California Committee for the Use and Care of Animals (Los Angeles, California).

Induction and assessment of arthritis

Collagen-induced arthritis (CIA) was induced by subcutaneous injection of 50 μl of emulsion containing bovine collagen II and complete Freund's adjuvant (1:1 ratio). For assessment of arthritis, animals were scored for clinical signs every two days as follows: 0: normal joints; 0.5: swelling of one or more digits; 1: erythema and mild swelling of the ankle joint; 2: mild erythema and mild swelling involving the entire paw; 3: erythema and moderate swelling involving the entire paw; 4: erythema and severe swelling involving the entire paw. The clinical scores for each mouse are the sum of the scores for four limbs and the maximal score for each mouse is sixteen.

The generation of CD4$^+$ induced regulatory T cells (iTregs) *ex vivo*

Naïve CD4$^+$CD62L$^+$CD25$^-$CD44^{low} T cells were isolated from spleen cells of DBA/1 CII TcR Tg mice using naïve CD4$^+$ T cell isolation kit (Miltenyi Biotec, Auburn, CA). Cells were cultured in 48-well plates and stimulated with CII peptide (245-270) (50 μg/ml) in the presence of γ–irradiated (30 Gy) APC, IL-2 (R&D systems, Minneapolis, MN) 40 U/ml with (iTregs) or without TGF-β 2ng/ml (R&D systems) (MED) for 4 days. RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES (Invitrogen Life Technologies) and 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT) was used for all cultures. Foxp3 expression was determined by flow cytometry. The suppressive activity of these cells against T cell proliferation was examined with a standard *in vitro* suppressive assay as previously reported (Zheng et al., 2007). 3×10^6 cells were transferred to each DBA/1J mouse on day 0, 14 or 28 after CII/CFA immunization.

Natural regulatory T cell (nTreg) generation

$CD4^+CD25^+$ cells sorted from the thymus in CII TcR Tg mice were expanded with CII peptide (245-270) (50 μg/ml) for 7 days. 300 U/ml IL-2 was renewed every three days. After cultures, cells were harvested and beads were removed. The percentage of $Foxp3^+$ cells was examined by flow cytometry before and after 7 days' expansion. 3×10^6 cells were transferred to DBA/1J mouse on day 0, 14 or 28 after CII/CFA immunization.

Th17 cell differentiation by IL-6 and TGF-β

Naïve $CD4^+$ cells were isolated from splenocytes of normal mice as before and cultured in 96-well plates. Cells were stimulated with 1μg/ml soluble anti-CD3, anti-CD28 and 10μg/ml anti-IFN-γ and anti-IL-4 monoclonal antibodies, irradiated APC (1:1 ratio), 10ng/ml IL-6 with or without 2ng/ml TGF-β for three days. Cells were harvested and stained with anti-IL-17A monoclonal antibody using the intracellularflow cytometry staining protocol as described below.

Proliferation Assay

iTregs generated or nTregs expanded as described above were added to fresh naïve T cells (Treg/T cells=1:4) and were stimulated with anti-CD3 mAb (0.025ug/mL) and irradiated APC (30 Gy, 1:1 ratio) for three days. ^3H was added to cultures for the last 16 to 18 hours and T cell proliferation ([^3H]-thymidine incorporation) was measured by using a scintillation counter.

Histology

For histological examination, mice were anesthetized after the final arthritic index was assessed. One limb from each mouse was removed and preserved in 10% buffered formalin, decalcified, and subsequently trimmed so as to render a longitudinal section through the limb and digits. The specimens were processed, blocked, sectioned, and stained with H&E.

Anti-CII antibodies ELISA

Blood were collected from each mouse on day 14 after adoptive transfer and clotted at room temperature for one hour followed byincubation at 4^0C overnight. Sera were frozen at -20^0C. Anti-CII antibodies were measured by ELISA.

Intracellular Staining for Flow Cytometry

For intracellular staining of cytokines, cultured cells were stimulated with PMA (0.25ug/ml), ionomycin (0.25ug/ml) for five hours and brefeldin A (5ug/ml) for four hours. Cells were then stained with surface markers such as CD4 and CD25 (eBioscience, San Diego, CA) and further

fixed, permeabilized and stained with Foxp3, IL-17A, IFN-γ, IL-4, IL-2 and IL-10 (Biolegend, San Diego, CA).

Real-Time PCR

Total RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA). cDNA was generated using a Omniscript RT kit (Qiagen, Valencia, CA). Foxp3 and Bcl-2 mRNA expression was quantified with ABsolute SYBR Green ROX mix (Thermo, Waltham, MA). Samples were run in triplicate and the relative expression of Foxp3 or Bcl-2 was determined by normalizing the expression of each target to hypoxanthine guanine phosphoribosyl transferase (HPRT). Primer sequences used were as follows: Foxp3 primers: 5'-CCC AGG AAA GAC AGC AAC CTT-3' and 5'-TTC TCA CAA CCA GGC CAC TTG-3'.Bcl-2, 5'-CCT GGC TGT CTC TGA AGA CC-3' and 5'-CTC ACT TGT GGC CCA GGT AT-3'; HPRT 5'-TGA AGA GCT ACT GTA ATG ATC AGT CAA C-3' and 5'-AGC AAG CTT GCA ACC TTA ACC A-3'.

Statistical analysis

Results calculated by using GraphPad Prism 4.0 software (GraphPad Spftware, San Diego, CA) are presented as mean ± SEM. Student *t* test was used to assess statistical significance between two groups, and one-way ANOVA and/or non-parametric tests were used to assess statistical significance among multi-groups. P value<0.05 is considered as statistically significant difference.

Results

Comparable properties of antigen-specific iTregs induced *ex-vivo* and expanded nTregs

We and others previously reported that antigen-specific iTregs can be generated in the presence of IL-2 and TGF-β (Huter et al., 2008; Zheng et al., 2004b). Herein, we used type II collagen (CII)-specific TcR transgenic mice that express an I-A^q-restricted CII (260–267)-specific TCR composed of Vα11.3 and Vβ8.3 for our studies. When naïve $CD4^+CD25^-Foxp3^-CD44^{low}$ cells were stimulated with CII peptide (245-270), few $CD4^+$ cells expressed Foxp3, a key Treg marker. Exogenous TGF-β enabled ~50% of $CD4^+$ cells to express this transcription factor (iTregs) (**Fig.9a,b**). $CD4^+CD25^+$ cells sorted from thymus (nTregs) in CII TcR Tg mice were expanded with CII peptide (245-270) and IL-2 for a week and expressed ~75% of Foxp3. The Foxp3 mRNA and protein expressed by iTregs and expanded nTregs were comparable (Fig.9c, d). Following induction and expansion, these $Foxp3^+$ cells sustained expression of Vβ8.3 as an indication of antigen specificity (Fig.9e). Both antigen-specific iTregs and nTregs exhibited reduced proliferation when re-stimulated with CII peptide (245-270), but

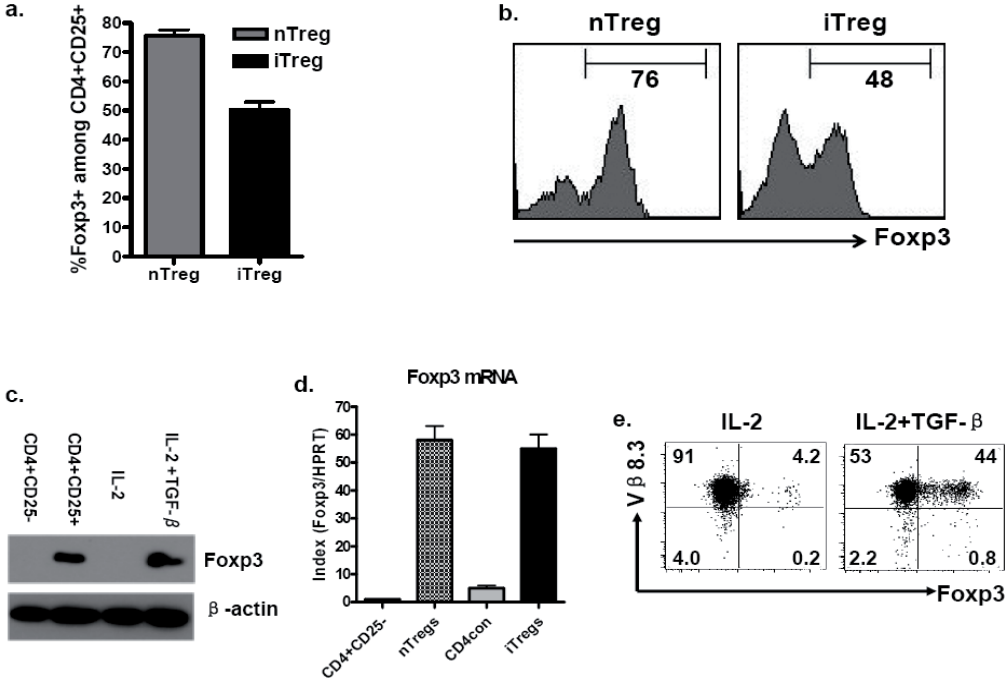

Fig. 9. Differentiation and expansion of antigen-specific iTregs and nTregs. (**a**) Naïve $CD4^+CD25^-CD62L^+CD44^{low}$ cells isolated from collagen-II TcR transgenic $Foxp3^{gfp}$ reporter mice were stimulated with collagen II peptide (245-270, 50 μg/ml) in the presence of IL-2 ± TGF-β for 5 days. $CD4^+CD25$+ cells sorted from CII TcR mice were expanded with CII (245-270) and IL-2 for one week. Intracellular Foxp3 expression among $CD4^+CD25^+$ cells was analyzed by flow cytometry. Values are mean ± s.e.m of five separate experiments. P<0.001 in comparison with no TGF-β treatment. (**b**) Data are representative of five experiments in **a**. Total protein was extracted from various cells and subjected to western blotting (**c**) and Foxp3 mRNA expression was determined by quantitative RT-PCR after normalization to HPRT (**d**). Data are representative of four independent experiments. (**e**) Foxp3 expression on $CD4^+Vβ8.3^+$ cells and data are representative of five experiments in **a**.

proliferation was restored with exogenous IL-2 demonstrating that these cells had become anergic; a

hallmark feature of Tregs (**Fig. 10a**). Accordingly, both subsets also produced little IL-2 or INF-γ, but produced increased levels of IL-10 relative to $CD4^+$control cells (Fig.10b). Both unmanipulated subsets had equivalent suppressive activity (**Fig. 11 a,b**).

Differences between antigen-specific iTregs and nTregs in suppressing T cell proliferation and Th17 differentiation *in vitro*

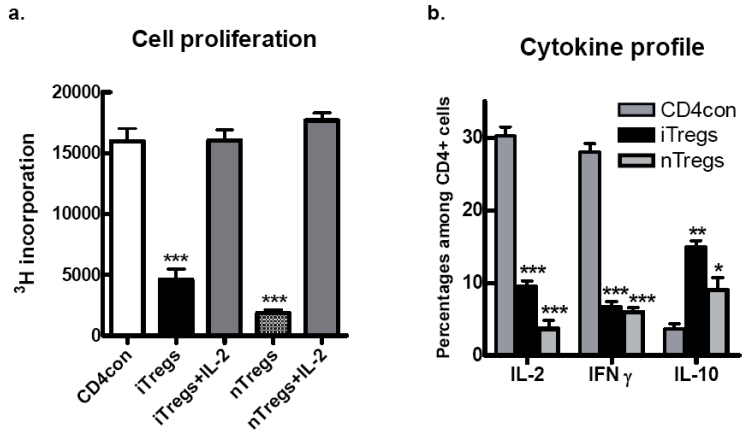

Fig. 10. iTregs displayed anergic status and related cytokine profiles. CD4con and iTregs generated, as well as nTregs expanded as described in s**Fig. 1a** were harvested and restimulated with CII peptide (245-270) (50 μg/ml) in the presence of irradiated APC with or without exogenous IL-2 (20 units/ml) for three days. [^{3}H]-thymidine was added to cultures in the last 18 hours and cell proliferation was measured by using a scintillation counter (**a**). Three days after restimulation, PMA and Ionomycin (last 5 hr), and BFA (last 4 hr) were added to cultures and cytokine production by CD4+ cells were analyzed by flow cytometry (**b**). Data indicate mean ± s.e.m of three independent experiments. ≤**p 0.01, **≤p 0.001, CD4con in comparison to iTregs.

Recent studies have revealed that nTreg's suppressive activity can be abolished with IL-6 (Pasare and Medzhitov, 2003) and this finding was confirmed in the present study. While both nTregs and iTregs displayed excellent suppressive activity against T cell proliferation, only iTregs maintained their suppression in the presence of exogenous IL-6 in vitro (Fig. 11). We conducted two separate proliferation assays using either CFSE labeling to follow proliferation cytometrically (Fig. 11a) or using ^{3}H thymidine incorporation (Fig. 11b) to measure DNA synthesis (Zheng et al., 2007). Both assays revealed similar results.

Given the close relationship between Th17 cells and nTregs, and the critical role played by each in the initiation or prevention of many autoimmune diseases, we next considered examining the effect of each Treg population in controlling Th17 differentiation. In a standard Th17-polarizing culture system, we observed that both IL-6 and TGF-β induced about 25% of TCR-stimulated naïve $CD4^+$ cells to express intracellular IL-17A. The addition of nTregs (in a ratio of 1 nTreg to 4 naïve T cells) actually slightly enhanced $CD4^+$cells expressing IL-17 (Fig. 11c, d) and did not inhibit $CD8^+$cells from producing this cytokine (not shown). Importantly, iTregs markedly suppressed the frequency of $CD4^+IL\text{-}17^+$ (Fig. 11c, d), $CD8^+IL\text{-}17^+$ cells (not shown) and concomitant in vitro production of soluble IL-17 was also reduced (Fig. 11e). These results provide further evidence that relative to

nTregs, iTregs have a superior ability to down-regulate Th17 cell differentiation even in the presence of IL-6.

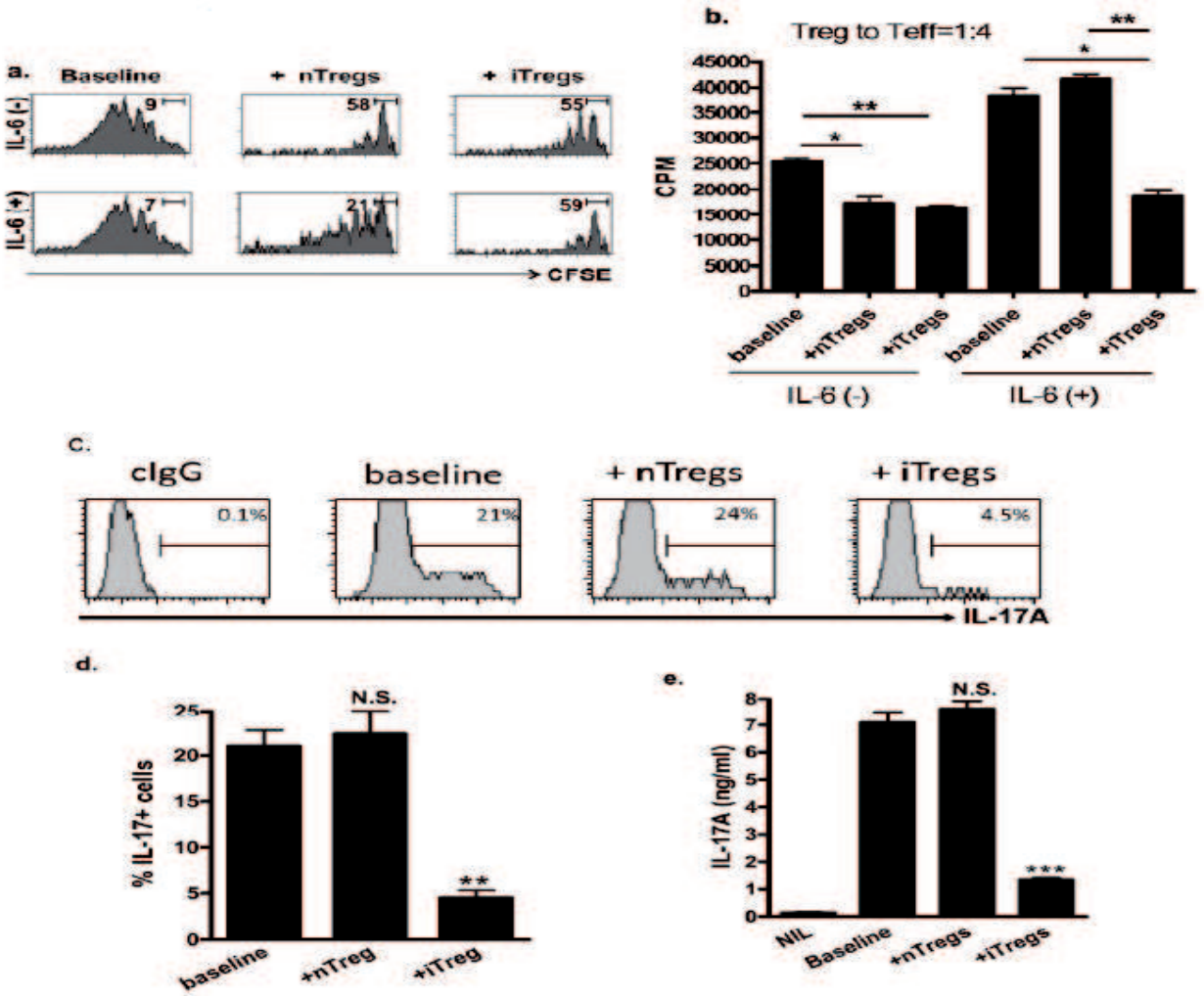

Fig.11. iTregs but not nTregs sustained their suppressive activity in the presence of exogenous IL-6 and suppressed Th17 cell differentiation. (**a**) Thymic $CD4^+CD25^+$ cells (nTregs) sorted from CII TcR transgenic mice were expanded with CII peptide (50 μg/ml) and IL-2 (300 units/ml) for one week, and naive $CD4^+CD25^-$ cells were stimulated with CII peptides, IL-2 andTGF-β for 4 days (iTregs). T responder cells labeled with CFSE were stimulated with anti-CD3 mAb ± exogenous IL-6± Treg subsets (1:4 ratios). Cells were gated on the $CD8^+$ T responder cells and values indicate $CFSE^+$ (undivided $CD8^+$ T cells). (**b**) nTregs and iTregs were generated as above except from CII TcR Tg $Foxp3^{gfp}$ reporter mice. ^{3}H-thymidine was added to cultures at the last 18 hours and incorporation by cycling T cells was measured. (**c,d**) Naïve T cells were polarized for Th17 cells ± CFSE-labeling Treg subsets (1:4 ratios) and percentages of $IL\text{-}17^+CD4^+$ $CFSE^-$ were determined by FACS. (**e**) The soluble IL-17A in supernatants was assayed by an ELISA. Data in **a, c** was representative of four separate experiments and values in **b, d** and **e** were mean ± s.e.m of three independent experiments. **P*<0.05, ***P*<0.01, ****P*<0.001, Tregs in comparison to baseline.

Protective effects of iTregs and nTregs on arthritis when transferred before or shortly after collagen II immunization *in vivo*.

Previous studies revealed that nTregs could limit CIA progression (Morgan et al., 2005; Morgan et al., 2003). To investigate the role of iTregs in this process and compare their functional characteristics with nTregs, we injected 3 x 10^6 iTregs or nTregs at the time of CIA challenge. This dose previously controlled experimental autoimmune encephalomyelitis (EAE) development(Zhou et al., 2010b). CIA incidence and severity was monitored every 3-5 days after cell injection. While $CD4^+$ control cell infusion did not affect incidence or severity, we observed that both nTregs and iTregs markedly decreased the incidence of CIA (Fig. 12a). Among those mice that did eventually develop CIA, nTregs and iTregs similarly delayed the onset of disease and decreased the clinical scores compared to control groups (Fig. 12b). Both Treg subsets also suppressed IgG2a complement-fixing antibodies, and iTregs suppressed total IgG and IgG1 as well (Fig. 12c).

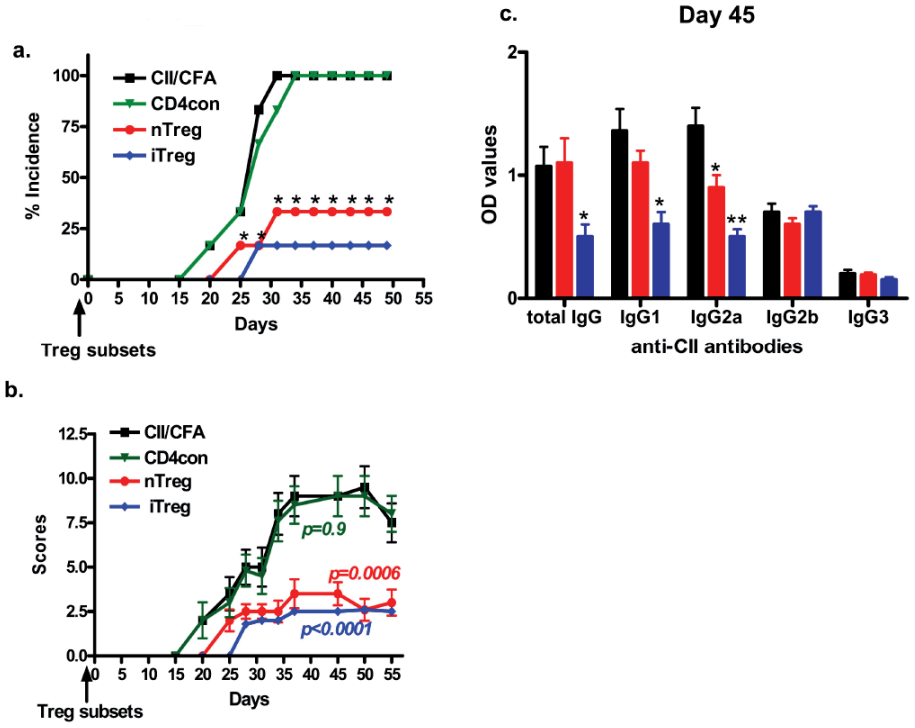

Fig.12. Both nTregs and iTregs significantly prevented the development of autoimmune arthritis. 3x10^6 nTregs or iTregs generated as described in above were transferred IV into DBA/1 mice when mice were immunized with collagen II and CFA. (**a**) Incidence and (**b**) severity of CIA in immunized mice receiving nTregs or iTregs and controls. (**c**) CII-specific IgG subsets in sera harvested day 45 after immunization and were measured by ELISA. At least five mice in each group were included in each experiment and data were combined from two independent experiments and expressed as mean ± s.e.m. *P<0.05, **P<0.01, cell injection group in comparison to CIA model.

We next determined the effect of both Treg subsets on CIA when transferred to mice just prior to disease onset. Since we observed that mice produce substantial levels of pro-inflammatory cytokines

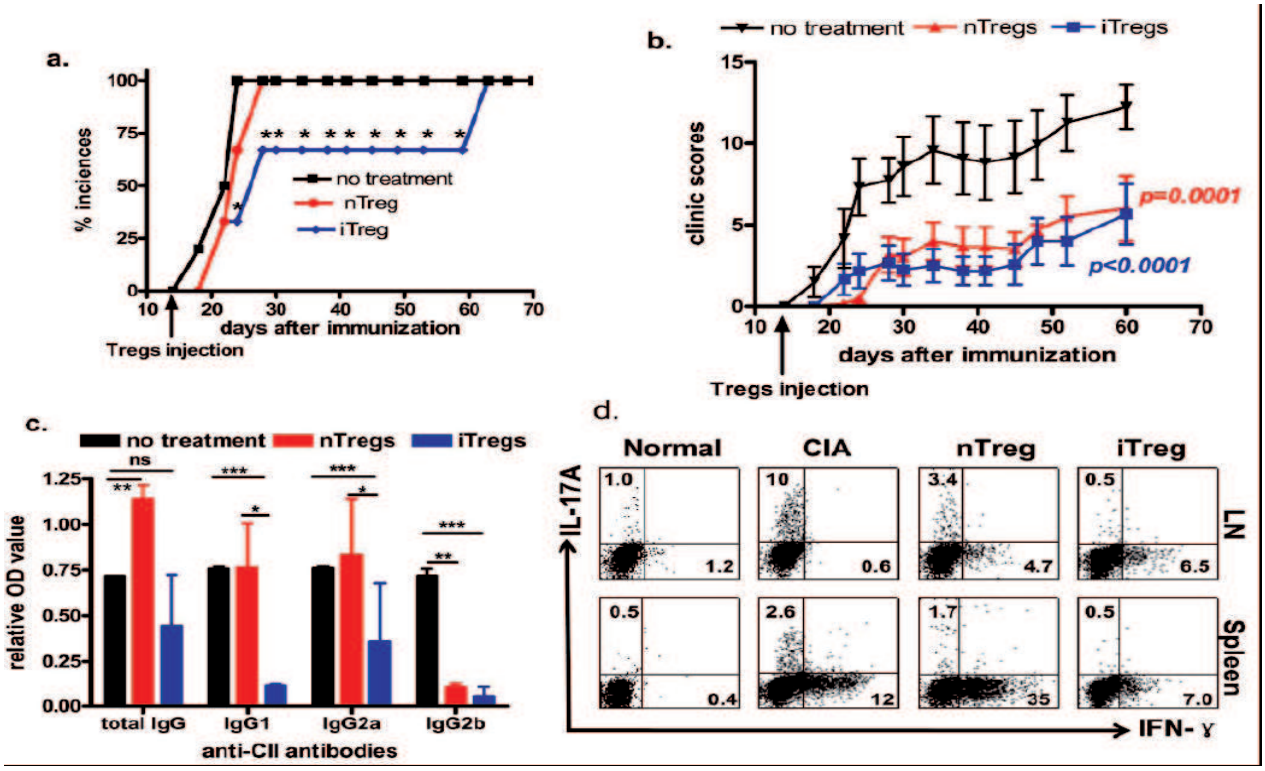

Fig.13. Suppressive effects of nTregs and iTregs on autoimmune arthritis when transferred after immunization but before onset of arthritis. 3x10^6 nTregs or iTregs generated as above were adoptively transferred into DBA/1 mice on day 14 after CII/CFA immunization. The incidence (**a**) and severity (**b**) in each group of mice is shown. (**c**) Sera were collected on day 45 and the anti-CII specific IgG subsets in sera in each group were measured by ELISA. Five mice were included in each group and data were combined from two independent experiments and expressed as mean ± s.e.m.*P<0.05, **P<0.01,***P<0.001. (**d**) Experiments were terminated on day 60. Spleen and popliteal (draining) LN cells were harvested and stimulated with PMA, Ionomycin and BFA. INF-γ and IL-17 production by CD4$^+$ cells were analyzed by flow cytometry. Data are representative of at least five mice per group.*P<0.05, **P<0.01, cell injection group in comparison to control CIA mice.

at day 14 following CIA challenge (not shown), we injected 3x10^{6}Tregs at this time. Twelve days after cell injection, the levels of anti-CII IgG1, IgG2a and IgG2b were significantly lower in the iTreg group compared with controls, while in mice injected with nTregs, only IgG2b was lower (Fig. 13c). At this time the incidence of arthritis had peaked. We observed significantly decreased CIA incidence in mice that received iTregs, but not nTregs (Fig. 13a). Nonetheless, among the mice that did develop disease nTregs or iTregs similarly significantly suppressed the severity of arthritis compared with controls (Fig. 13b). Both Treg types suppressed IL-17$^+$ cells in LNs and spleens in CIA, however, iTregs displayed superior efficacy (**Fig. 13d**). Interestingly, although iTregs decreased splenic INF-γ$^+$ cells, nTregs actually increased them. Thus, as nTregs lose suppressive activity they may develop helper activity since INF-γ promotes B cell differentiation toward plasma

cells (Swanson et al., 2010). The results suggest that iTregs induced *ex-vivo* are at least as effective as nTregs in the prevention of CIA.

Loss of the protective effects of nTregs, but not iTregs, once arthritis was established

To consider clinical relevance, we explored the therapeutic effect of each Treg population on established CIA. Consistent with previous reports (Zhou et al., 2010a), transfer 3×10^6 of nTregs to mice with evident arthritis 28 days after collagen immunization, did not significantly decrease disease severity (Fig. 14a), autoantibody production (Fig. 14b), joint damage, bone erosion, or inflammatory cell infiltration (Fig. 14c). Conversely, injection of iTregs almost completely

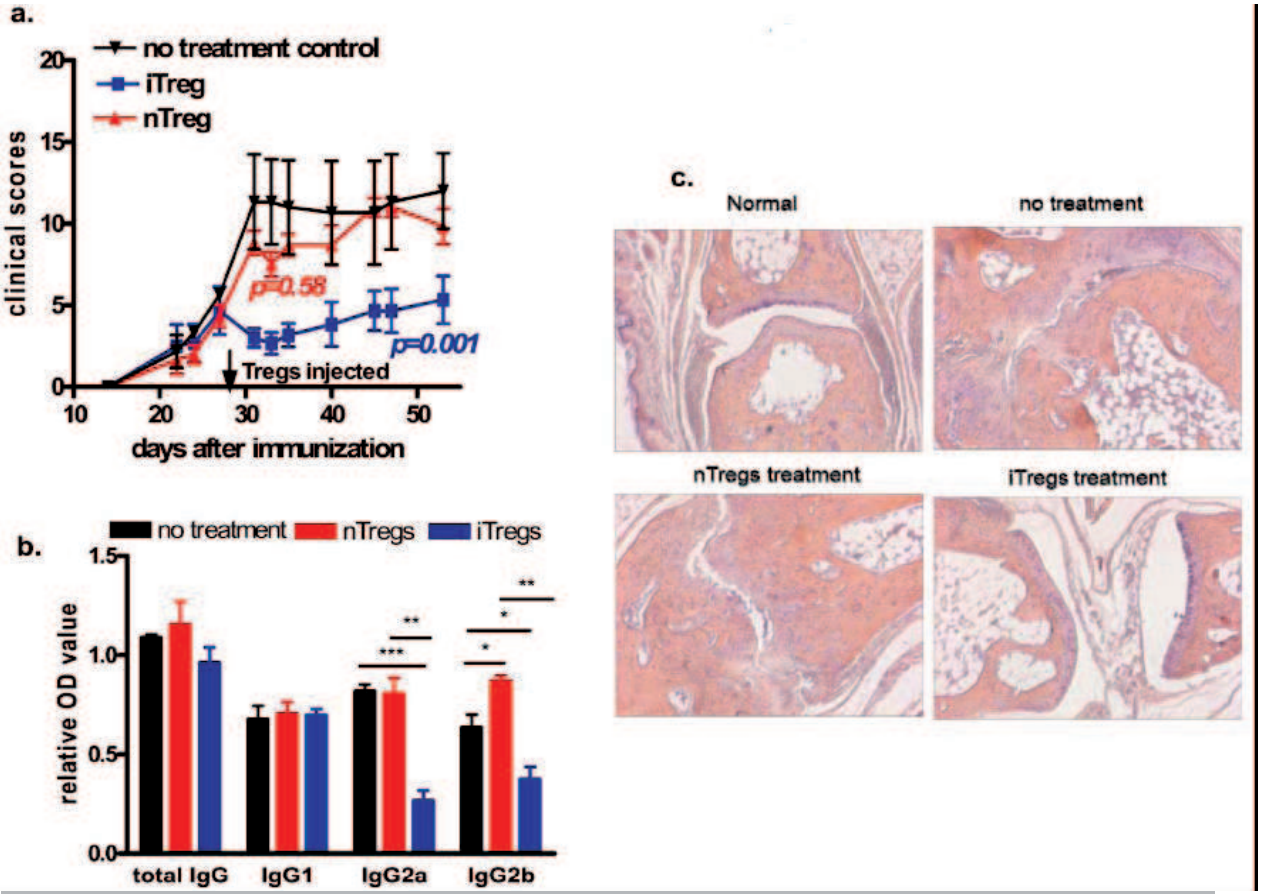

Fig.14. iTregs but not nTregs significantly suppressed the development of established autoimmune arthritis. $3x10^6$ nTregs or iTregs were adoptively transferred to DBA/1 mice that had developed typical arthritis (around day 28 after immunization) and arthritis development was monitored following Treg therapy. (**a**) Clinical scores of each group. (**b**) Anti-CII specific IgG subsets in various groups of mice (± adoptive transfer of Tregs) at two weeks as detected by ELISA. Five mice were included in each group and data were combined from two independent experiments and expressed as mean ± s.e.m. **P*<0.05, ***P*<0.01, ****P*<0.001, cell injection group in comparison to CIA model. (**c**). Histological alteration of the joints of mice from each group. Joints were removed, fixed and conducted with H&E staining. Data are representative of at least five mice per group.

suppressed the progress of disease for two weeks, and afterwards the severity never reached levels observed in controls (Fig. 14a). In addition, iTreg treatment also significantly suppressed IgG2a and IgG2b, but not IgG or IgG1 autoantibody production (Fig. 14b). Moreover, iTreg treatment also markedly reduced articular cartilage and joint pathology, inflammatory cell infiltration, and left the joint space substantially intact (Fig. 14c).

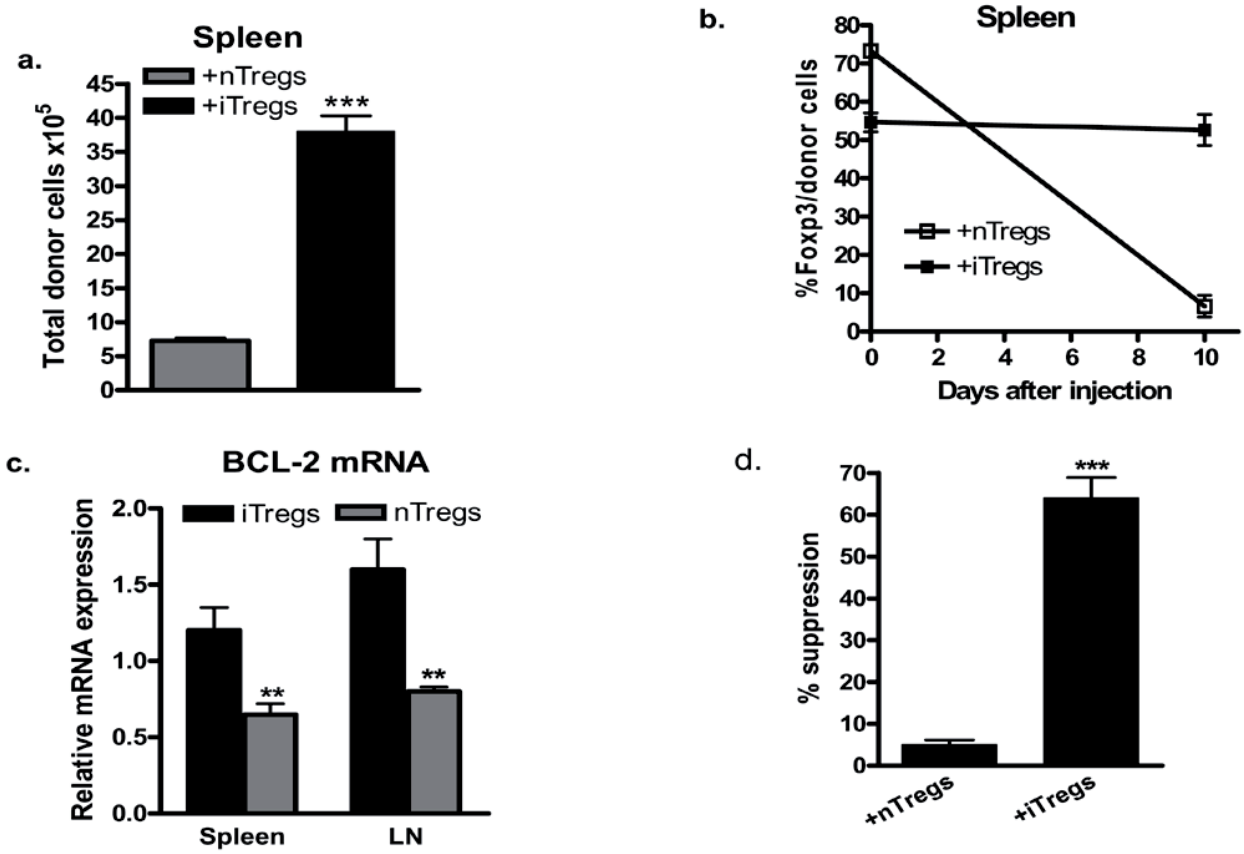

Fig. 15. iTregs but not nTregs maintained their frequency, phenotype and function following adoptive transfer to mice with established arthritis. nTregs or iTregs generated as above were labeled with CFSE. 3x10^6 of these cells were adoptively transferred into mice with evident arthritis. 10 days later, mice were sacrificed, spleen, blood and LN cells were harvested. Foxp3 expression was identified by flow cytometry in either donor (CFSE$^+$) or recipient (CFSE$^-$) cells. (**a**) Total donor iTregs and nTregs in the spleens.Values indicate mean ± s.e.m for five mice per group, ***P<0.001, iTregs in comparison to nTregs. (**b**) Percentages of Foxp3$^+$ cells by donor cells in the spleens before and after cell transfer. (**c**) Donor (CFSE$^+$) cells were sorted at 10 days after infusion and Bcl-2 mRNA expression was determined by quantitative RT-PCR. (**d**) These sorted cells were added to T cells isolated from CIA mice. These cells were then stimulated with anti-CD3 in the presence of irradiated APC. Cell proliferation was determined by [^{3}H]-thymidine incorporation. Suppressive rates of nTregs and iTregs were calculated by the formula: [proliferative value of baseline - sample value/baseline value x100%]. Data in **b-d** were representative of five mice per group. Experiments were repeated with similar results.

Decreased stability of nTregs but not iTregs in mice with established arthritis

Given that nTregs, unlike iTregs, lose suppressive activity in vitro when stimulated with pro-

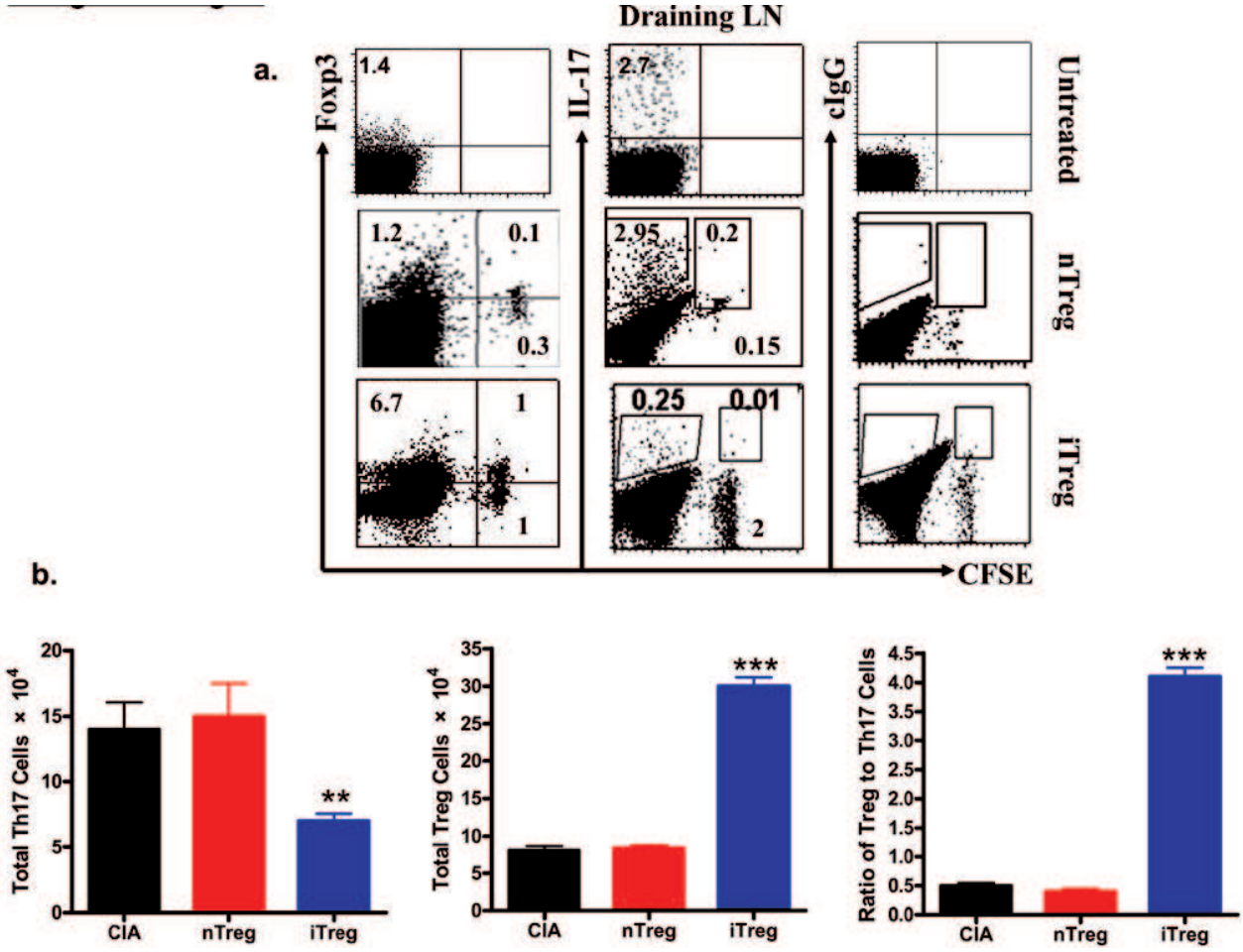

Fig. 16. iTreg but not nTreg were resistant to Th17 cells and increased the ratio of Foxp3⁺ cells to IL-17⁺ cells in draining LNs of recipients. (**a**) 3x10⁶ iTregs and nTregs were labeled CFSE and transferred to DBA/1 mice 28 days after CII/CFA immunization. Mice were sacrificed and IL-17 expression in donor ($CFSE^+$) and recipient ($CFSE^-$) cells in draining LNs were determined by flow cytometry 10 days after cell transfer. (**b**) The ratios of Tregs to Th17 cells in draining LNs in different groups were shown. Data in **a** are representative of five mice per group and values in **b** are mean ± s.e.m of five mice per group. Experiment was repeated with similar results. $^{**}P<0.01$, $^{***}P<0.001$, CIA in comparison to cell treatment group.

inflammatory IL-6, we considered they might be unstable *in vivo*. To address this possibility, we labeled both Treg subsets with CFSE to distinguish them from recipient cells and transferred them to mice with established arthritis. Ten days later the mice were sacrificed and Foxp3 expression in donor cells isolated from spleens and LNs were examined by Flow Cytometry. The percentages and total numbers of donor nTregs surviving in spleens (**Fig. 15a, b**) and LNs (**Fig. 16**) were significantly lower than donor iTregs. Examination of sorted subsets revealed that donor nTregs also expressed significantly lower Bcl-2 mRNA compared to donor iTregs (Fig. 15c). Bcl-2 plays an

important role in the prevention of cell apoptosis (Marsden and Strasser, 2003). Additionally, a significant proportion of donor nTregs but not iTregs lost expression of Foxp3. Although >75% of the $CFSE^+$ nTregs expressed Foxp3 at the time of transfer, 10 days later, <10% of donor nTregs expressed Foxp3 in the spleen and ~25% in draining LN. Conversely, donor iTregs mostly maintained equivalent Foxp3 they expressed at the time of transfer (~50%)(Fig. 15b, 16a). Thus, it was not surprising that donor nTregs recovered in these mice now had markedly less suppressive ability than donor iTregs (Fig. 15d).

We further demonstrated that >50% of donor nTregs in draining LNs had become IL-17^+ cells (Fig. 16a) although few of them converted into IFN-γ^+ and/or IL-4^+ cells (not shown). Fewer nTregs converted to Teff cells in the spleen (**data not shown**). Conversely, donor iTregs were almost completely resistant to Th17 cell conversion in draining LNs (Fig. 16a) as well as Th1/Th2 conversion under similar conditions (not shown).

Transfer of iTregs to CIA mice shifts the predominance from Th17 to Treg cells in draining lymph nodes.

Previous studies reported that the transfer of iTregs can markedly increase recipient $Foxp3^+$ Tregs (Andersson et al., 2008; Zheng et al., 2006a). This was also the case in the present study. As shown in Fig. 16a and b, both percentages and total numbers of $Foxp3^+$ cells in the recipient LNs were markedly increased in the iTreg treatment group relative to untreated CIA. Moreover, the infusion of iTregs to established CIA markedly down-regulated IL-17^+ cell frequencies. FACS analysis revealed that both percentages and total numbers of IL-17^+ cells in the recipient LNs were markedly lower in the iTreg treatment group relative to untreated CIA. Before treatment Th17 cells were twice as numerous as Tregs in draining LN. Ten days later after iTreg treatment, $Foxp3^+$ recipient Tregs were now predominant. They were four times more numerous as Th17 cells. By contrast, total Tregs and Th17 cells in recipients were unchanged in nTreg treated mice **(Fig. 16b**). Thus, treatment with iTregs could markedly alter the balance between recipient Tregs and Th17 cells, and the disease course was changed.

Discussion

The stability and therapeutic effectiveness of $CD4^+$ regulatory cells induced with IL-2 and TGF-β *ex-vivo* is controversial. We and others have reported that these iTregs have protective activities in several experimental models of immune-mediated diseases (Selvaraj and Geiger, 2008; Weber et al., 2006; Zheng et al., 2004b; Zhou et al., 2010b) and are resistant to conversion to Th17 cells (Zheng et al., 2008). By contrast, others have reported that these iTregs were unstable *in vitro* (Floess et al., 2007) and *in vivo* following antigen-stimulation (Chen et al., 2011), and lack protective activity to prevent lethal graft-*versus*-host disease (GVHD) (Floess et al., 2007; Koenecke et al., 2009). To address this controversy, we have conducted a head to head comparison of antigen specific thymus-derived nTregs and TGF-β-induced iTregs. We chose antigen-specific Tregs since these are more protective than polyclonal Tregs in autoimmune diseases (Penaranda and Bluestone, 2009). We chose established autoimmunity because Tregs are generally therapeutic when transferred before the onset of autoimmunity. In the collagen-induced arthritis (CIA) the model we have chosen in this study, polyclonal nTregs can prevent disease but are ineffective in established disease (Morgan et al., 2003; Zhou et al., 2010a). Current study clearly demonstrates that antigen-specific iTregs are superior to nTreg in ameliorating established collagen-induced arthritis. This was because iTregs remained stable and fully functional following transfer. Moreover, these iTregs had tolerogenic effects in draining LN that resulted in a shift from Th17 to Treg predominance.

This striking difference between iTregs and nTregs could not be attributed to differences in the starting populations of the two Treg subsets. Actually, a greater percentage of nTregs expressed Foxp3. They both had equivalent suppressive activities *in vitro*, and both had equivalent therapeutic effects on CIA when transferred before collagen II immunization *in vivo*. Significant differences began to appear when Tregs were injected after CII immunization, but before the onset of arthritis. Here, iTregs were more effective in reducing disease incidence. Consistent with a previous report (Morgan et al., 2003), nTregs also failed to suppress autoantibody production.

There are several possibilities to explain the inability of nTregs to treat CIA and other autoimmune diseases. First, pro-inflammatory cytokines may hamper their suppressive activity. Pasare et al have reported that Treg suppressive activity can be abolished by IL-6 (Pasare and Medzhitov, 2003). Valencia et al also revealed that elevated TNF-α may interfere with the suppressive capacity of nTregs (Valencia et al., 2006). Secondly, Th17 cells may be resistant to nTregs. This may be the reason that nTregs are able to prevent the disease before Th17 cells are established. Third, at least

some nTregs are inherently unstable and can be converted to Th1/Th2/Th17/Tfh effector cells in an inflammatory milieu (Lu et al., 2010b; Tsuji et al., 2009; Wan and Flavell, 2007; Xu et al., 2007; Zhou et al., 2010a).

Several reasons can explain the therapeutic success of iTregs. First, a previous study with polyclonal TGF-β-induced iTregs demonstrated that these iTregs exhibited lower levels of IL-6 receptor and subsequent Stat-3 phosphorylation (Zheng et al., 2008). Thus, they were resistant to IL-6 stimulation and maintained their phenotype and function. Here antigen-specific TGF-β-induced iTregs demonstrated similar IL-6 resistance. Furthermore, these iTregs even suppressed Th17 cell differentiation that is induced by IL-6 and TGF-β. It is understandable that nTregs lack this ability since IL-6 was included in the cultures for Th17 polarization. Secondly, antigen-specific iTregs, but not nTregs were stable in mice with established CIA. Only the former maintained Foxp3 expression and exhibited suppressive activity when recovered from draining LN. Thirdly, studies with Bcl-2 gene expression revealed higher expression in recovered iTregs, suggesting that nTregs were probably more susceptible to apoptosis than iTregs. Previous reports have revealed that TGF-β increases the Bcl-2 expression and decreases T cell apoptosis (Zheng et al., 2002).

The final, and probably most important reason, is that TGF-β-induced iTregs shifted the balance between Treg and Th17 cells in draining LN from Th17 to Treg predominance. This is probably because the transferred Tregs induced immunogenic recipient antigen-presenting cells to become tolerogenic. Thus, ongoing antigen stimulation resulted in Tregs rather than T effector cells (Horwitz et al., 2008). Previously, we reported that a single injection of alloantigen-specific iTregs followed by continuous alloantigen stimulation steadily increased recipient $CD4^+CD25^+Foxp3^+$ Tregs (Zheng et al., 2006a). Here, we demonstrated that mice with CIA had twice as many recipient Th17 cells as Tregs in draining LN. Ten days after iTreg injection, however, recipient $Foxp3^+$ cells were now much more numerous than Th17 cells, and the clinical scores of these animals had decreased. Thus, even an inflammatory environment injected TGF-β-induced iTregs can have tolerogenic effects. The recent studies of Nugyen et al 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).

There are technical reasons that possibly explain why some investigators have generated unstable, ineffective TGF-β-induced iTregs. These groups have used high concentrations of plate-bound anti-CD3 with TGF-β, and stimulated the $CD4^+$ cells for more than 72 hours. Others have demonstrated

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). We have used suboptimal concentrations of anti-CD3 and anti-CD28 coated beads with IL-2 and TGF-β to induce the stable, protective $Foxp3^+$ Treg cells described in this study. It has been claimed that since TGF-β is unable to demethylate Foxp3, the Tregs generated will be unstable (Floess et al., 2007). However, we have recently observed that the methylation status in Foxp3 gene loci does not affect Foxp3 stability (Lu et al.). Others have also observed protective human TGF-β-induced Tregs that exhibit methylated Foxp3 (Hippen et al., 2011).

The findings we have reported with these TGF-β-induced iTregs induced *ex–vivo* may not apply to endogenous iTregs induced *in vivo*. Although sub-optimal TCR stimulation and the presence of TGF-β is also important in the generation of endogenous iTregs, this subset, like nTregs, may be susceptible to Th17 conversion. Because of the documented Teff cell resistance to suppression (Buckner, 2010), it is likely that all endogenous $CD4^+Foxp3^+$ subsets exhibit this characteristic. We suspect that it is the pharmacological concentrations of the IL-2 and TGF-β used that confer resistance. It is for this reason that we distinguish endogenous nTreg and iTreg subsets from iTregs induced *ex-vivo*.

The findings reported in this study must be interpreted with caution. The decrease in arthritis severity was transient, and the clinical scores later increased, although they remained significantly below than that of control mice. Here we induced mouse iTregs and the protocols used must be considerably modified to induce similar human iTregs. Nonetheless, the striking difference between iTregs and nTregs observed in this study suggest that the therapeutic potential of human TGF-β induced iTregs should be fully explored.

VI. ARTICLE 3

Polyclonal CD4$^+$Foxp3$^+$ T cells generated *ex-vivo* with TGF-β suppress the development of lupus *via* induction of tolerogenic dendritic cells

Qin Lan[1], Xiaohui Zhou[1], Julie Wang[1], David Horwitz[1], David Brand[2], Pawel Kiela[3], Valerie Quesniaux[4], Bernhard Ryffel[4] and Song Guo Zheng[1,4]

Running title: iTregs suppress lupus *via* induction of tolerogenic DCs

[1]Division of Rheumatology and Immunology, Department of Medicine, The Keck School of Medicine, University of Southern California, Los Angeles, CA 90033; [2]Research Service, Veterans Affairs Medical Center, Memphis; USA; [3]Departemtn of Immunology, University of Arizona; [4]UMR6218, Molecular Immunology, University and CNRS, 3b rue de la Ferollerie, F-45071 Orleans, France

Manuscript is under preparation now

Abstract

Foxp3^{+} regulatory T cells, dendritic cells and their interaction play a crucial role in the maintenance of immunotolerance. However, the exact mechanism(s) by which these cells interact in controlling immune responses is less understood. Our study demonstrates that CD4^{+}Foxp3^{+} Treg cells generated *ex-vivo* with IL-2 and TGF-β suppressed lupus-like syndromes through TGF-β and IL-10 signaling pathways. iTregs suppress CD80 and CD86 expression on DCs and induce tolerogenic DC formation under both homeostatic and inflammatory conditions through TGF-βR signaling pathways in DCs. Tolerogenic DCs sequentially suppressed immune response through TGF-β but not IL-10. These results demonstrate that interaction between Tregs, TGF-β/IL-10 and DCs sustains the suppressive activity of Treg cells *in vivo*.

Keywords: cGVHD, Lupus, Colitis, T regulatory cells, TGF-β, IL-10, DC, Tolerogenic.

Introduction

$Foxp3^+$ regulatory T (Treg) cells consisting of heterogeneous natural (nTregs) and induced Treg cells (iTregs) play an important role in the maintaining immune tolerance and in preventing autoimmune diseases (Andersson et al., 2008; Horwitz et al., 2008; Zhou et al., 2011). Abnormality in numbers and function of $Foxp3^+$ Treg cells have been reported in many autoimmune disease animal models and in patients, thus, manipulation of Tregs might be therapeutic in these autoimmune and inflammatory diseases.

Dendritic cells (DCs) are specialized antigen-presenting cells (APCs) that initiate and regulate immune responses against foreign as well as self-antigens (Steinman et al., 2003). Different subsets of DCs and their mature status affect the consequences of immune responses. While mature DCs promote adaptive immune response, semi-mature and immature DCs regulate immune tolerance (Horwitz et al., 2008). Several cytokine factors can influence the differentiation and the maturation status of DCs, for example, TGF-β, IL-10, IL-27, Vitamin D3 and IDO play an important role in maintaining the semi-mature tolerogenic phenotype of DCs (Pallotta et al., 2011). Tolerogenic DCs may suppress autoimmunity upon immunization with self-antigens through direct or indirect effect. Tolerogenic DCs that produce significantly low levels of inflammatory cytokines and/or higher amounts of anti-inflammatory cytokines are known to induce and/or expand $Foxp3^+$ Tregs in the periphery, sequentially suppressing immune response through TGF-β and/or IDO-dependent mechanisms (Coombes et al., 2007; Favre et al., 2010; Kaplan et al., 2007).

While many studies have demonstrated that DCs can drive the differentiation of induced Tregs including $Foxp3^+$ induced iTregs and $Foxp3^-$ IL-10-producing Tr1 cells, whether Tregs also drive DCs to become tolerogenic DCs is less studied. We and others have previously reported that Treg cell therapy can sustain a protective effect even after transferred cells have diminished *in vivo* (Selvaraj and Geiger, 2008; Weber et al., 2006; Zheng et al., 2006a). It is likely that "infectious tolerance" (Tregs--tolerogenic DCs--Tregs) contributes to this extended effect exerted by Tregs and that DCs might be a crucial "relay athlete" in this process.

In the current study, we have observed that $CD4^+Foxp3^+$ iTregs generated *ex vivo* with IL-2 and TGF-β suppressed lupus-like syndrome development and progress through TGF-β- and IL-10-dependent mechanism *in vivo*. Co-culture iTregs and DCs *in vitro* or infusion of iTregs to lupus-like syndrome mice can induce $CD11c^+$ cells to become tolerogenic DCs. We further observed that TGF-

β receptor (TβR) signaling on DCs is essential for the induction of the formation of tolerogenic DCs. Tolerogenic DCs then suppress immune responses through TGF-β but not IL-10 signaling pathway and this is accompanied by an increase in $Foxp3^+$ Treg cells in recipients. Together these results have documented the interaction between Tregs, cytokines and DCs in the control of autoimmune response and further provide a new insight into therapeutic manipulation of Tregs in autoimmune and other inflammatory diseases.

Materials and Methods

Mice

Eight-week-old female DBA2, DBA2xC57BL/6 F1, BALB/C, CD45.1 C57BL/6, CD45.2 C57BL/6, C57BL/6 Foxp3^{gfp} knock-in and Rag-1 KO mice were purchased from The Jackson Laboratory (Bar Harbor, ME). DC-specific TGF-β RII conditional KO mice were a gift from Dr. Pawel Kiela at University of Phoenix. These mice (Cre^{+}) were generated by intercrossing of TGF-β RII$^{fl/fl}$ mice and CD11^{cre} mice. All mice were housed and treated by National Institutes of Health guidelines for the use of experimental animals with approval of University of Southern California Committee for the Use and Care of Animals (Los Angeles, California).

The generation of CD4^{+} induced regulatory T cells (iTregs) *ex vivo*

Naïve CD4^{+}CD62L^{+}CD25^{-}CD44^{low} T cells were isolated from spleen cells of DBA2, C57BL/6 or C57BL/6 Foxp3^{gfp} knock-in mice using naïve CD4+ T cell isolation kit (Miltenyi Biotec, Auburn, CA). Cells were cultured in 48-well plates and stimulated with anti-CD3/CD28 coated beads (1 bead to 5 cells, Invitrogen) in the presence of IL-2 (R&D systems, Minneapolis, MN) 40 U/ml with (iTregs) or without TGF-β 2ng/ml (R&D systems) (CD4con) for 4 days. RPMI 1640 medium supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin, 10 mM HEPES (Invitrogen Life Technologies) and 10% heat-inactivated FCS (HyClone Laboratories, Logan, UT) was used for all cultures. Foxp3 expression was determined by flow cytometry. The suppressive activity of these cells against T cell proliferation was examined with a standard *in vitro* suppressive assay as previously reported (Zheng et al., 2007). 5×10^{6} cells were transferred to each D2B6F1 mice.

Co-culture of iTregs or CD4con with DCs

CD11c^{+} cells were isolated from bone marrow or spleens and cultured with GM-CSF (500 u/ml) and IL-4 (500 u/ml) for three days. In some wells, CD4con or iTregs were added to DCs (5:1 ratio) and co-cultures were activated with anti-CD3 (0.5μg/ml) for three days. CD80, CD86, MHC-II expression on CD11c^{+} cells were stained by flow cytometry.

Induction and assessment of cGVHD with a lupus syndrome

As described previously (Shustov et al., 1998), a chronic GVHD with a lupus-like syndrome was induced in D2B6F$_1$ mice by injecting 12×10^{6} D2 CD4^{+} cells through tail vein injection. Other groups received this number of D2 cells plus 5×10^{6} CD4con, iTregs or 5×10^{5} DCs isolated from lupus-like syndrome mice treated with CD4con or iTregs. To determine the suppressive mechanisms of Tregs and tolerogenic DCs *in vivo*, anti-TGF-β1 (2G.7; R&D Systems) (1 mg/mouse) or isotype-

matched IgG1 antibody, anti-IL-10R (250 μg/mouse) or isotype-matched IgG1 antibody and ALK5 inhibitor (LY-364947, Sigma, 0.5 mg/mouse) were i.p. administrated once a week for total four to six weeks. In most experiments, there were five mice per group and experiments have been repeated at least two times. Before transfer and weekly thereafter, the animals were bled and serum IgG and anti-dsDNA autoantibodies were measured by an ELISA (Du Clos et al., 1986). All samples tested for anti-dsDNA were performed at the same time. Serum was diluted 1/400 or 1/800 for anti-DNA and 1/40,000 for measuring IgG. Proteinuria was measured using Albustix reagent strips (Bayer, Elkart, IN). Mice were sacrificed at time points indicated in the different experiments after transfer of parental T cells for assessment of lymphoid hyperplasia and immune complex glomerulonephritis. The total numbers and phenotypes of the spleen cells were determined from single-cell suspensions. The cells were stained with FITC-anti-H-$2^{b,}$ PE-anti-H-2^{d} (BD PharMingen) and single-positive anti-H-2^{d} cells considered to be parental D2 cells. Mice survival was monitored every three days.

Induction and assessment of colitis

0.5 x 10^{6} $CD4^{+}CD45Rb^{high}$ cells sorted from splenocytes in naïve C57BL/6 mice (95-100% purity) were intravenously injected into $Rag1^{-/-}$ mice (C57BL/6). Other groups were also received by $200x10^{3}$ DCs isolated from WT and TGF-βRII DC conditional KO mice that have been previously primed with iTregs or CD4con cells. Body weight was monitored dynamically. Some mice were sacrificed at 4 weeks after T/DC cell transfer. Colon removed for histology, colonic explants culture, mesenteric lymph node cell suspensions cultured in activation plates with anti CD3/CD28 Ab and tissues were measured for quantitative RT-PCR and supernatants analyzed for selected cytokines with xMAP multiplex assay.

Proliferation Assay

iTregs generated or nTregs expanded as described above were added to fresh naïve T cells (Treg/T cells=1:4) and were stimulated with anti-CD3 mAb (0.025ug/mL) and irradiated APC (30 Gy, 1:1 ratio) for three days. In other experiments, T responder cells were stimulated with allogeneic APC or DC. [^{3}H] was added to cultures for the last 16-18 hours and T cell proliferation ([^{3}H]-thymidine incorporation) was measured by using a scintillation counter.

Histology

For histological examination, mice were anesthetized after the final disease index was assessed. Kidney from lupus mice and colon from colitis mice were removed and preserved in 10% buffered

formalin. The specimens were processed, blocked, sectioned, and stained with H&E. Cryostat sections of frozen kidney tissue were examined for deposits of IgG using a standard procedure (Hellmark et al., 1997). Sections were incubated with fluorescence-labeled goat $F(ab')_2$ IgG antiserum to mouse IgG. The sections were read blindly by the same investigator, grading the intensity of fluorescence from 0 to 4+.

Statistical analysis

Results calculated by using GraphPad Prism 4.0 software (GraphPad Spftware, San Diego, CA) are presented as mean ± SEM. Student *t* test was used to assess statistical significance between two groups, and one-way ANOVA and/or non-parametric tests were used to assess statistical significance among multi-groups. *P* value<0.05 is considered as statistically significant difference.

Results:

Polyclonally differentiated iTregs suppressed anti-CD3- and alloantigen-triggered T cell proliferation.

As reported previously, TGF-β is a crucial cytokine that can induce conventional naïve CD4 $^{+}$CD25^{-} cells (non-Tregs) to become iTregs (Zheng et al., 2002). Foxp3, an important transcription factor regulating the development and function of Treg cells(Fontenot et al., 2003), can be induced and mainly expressed on the CD4^{+} and CD25^{+} cell population after TGF-β priming (**Fig. 17a**). These CD4^{+} cells but not CD4^{+} control cells (treated without TGF-β, CD4con) suppressed anti-CD3 stimulated T cell proliferation including CD4^{+} and CD8^{+} cells in CFSE-labeling (**Fig. 17b**) and ^{3}H-thymidine incorporation assays (**Fig. 18a**). Given these cells were produced by polyclonal stimulation and that they displayed the suppressive activity, herein we refer to them as "polyclonally differentiated iTregs or iTreg". Placement of these iTregs in a Transwell assay containing a semi-permeable membrane allows the transport of soluble cytokines but prevents cell-contact abolished the suppressive activity of iTregs. Furthermore, the addition of anti-TGF-β, anti-IL-10 or/and anti-IL-10R antibodies did not significantly abolish the suppressive activity of these cells *in vitro* (not shown), suggesting that cell-contact is needed for the suppressive activity of iTregs at least *in vitro*.

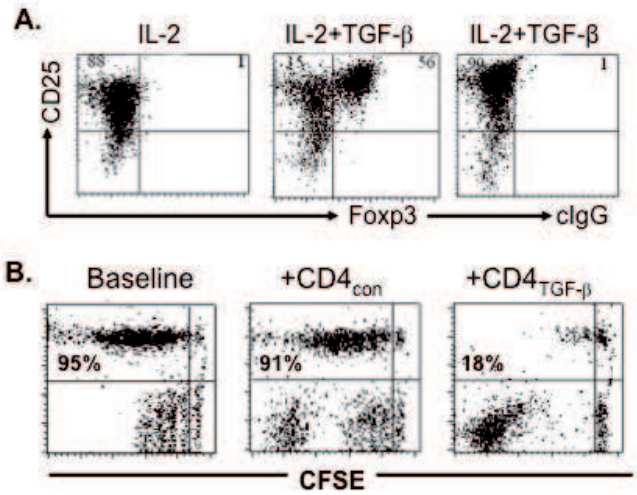

Fig. 17. iTregs expressed Foxp3 and suppressed T cell proliferation. Naive CD4^{+}CD25^{-} cells were stimulated with anti-CD3/CD28 coated beads and IL-2 (CD4con) and TGF-β ($CD4_{TGFβ}$) for 4 days. Foxp3 expression was determined by FACS (**a**). T responder cells labeled with CFSE were stimulated with anti-CD3 mAb±CD4con or$CD4_{TGFβ}$ (1:4 ratios). Cells were gated on the CD8^{+} T responder cells and values indicate CFSE^{+} (undivided CD8^{+} T cells) (**b**). Data was representative of four separate experiments.

It is less known whether polyclonally differentiated iTregs also suppress the antigen-specific immune responses. To address this issue, we performed an immune response assay using alloantigen stimulated T responder cells isolated from DBA/2 mice that were stimulated with γ-irradiated non-T cells isolated from C57BL/6 mice. The CD4con or iTregs generated as described above from DBA/2 were added to some cultures (one $CD4^+CD25^+$ cell to four T responder cells). After three-day cultures, the iTregs but not CD4con cells significantly suppressed alloantigen-stimulated T cell proliferation (**Fig. 17b**). Additionally, when iTregs were prepared from C57BL/6 mice, these cells still suppressed the immune response from T cells isolated from DBA/2 mice (not shown), suggesting that polyclonally differentiated iTregs have developed both antigen-specific and antigen non-specific suppressive roles against T cell immune responses.

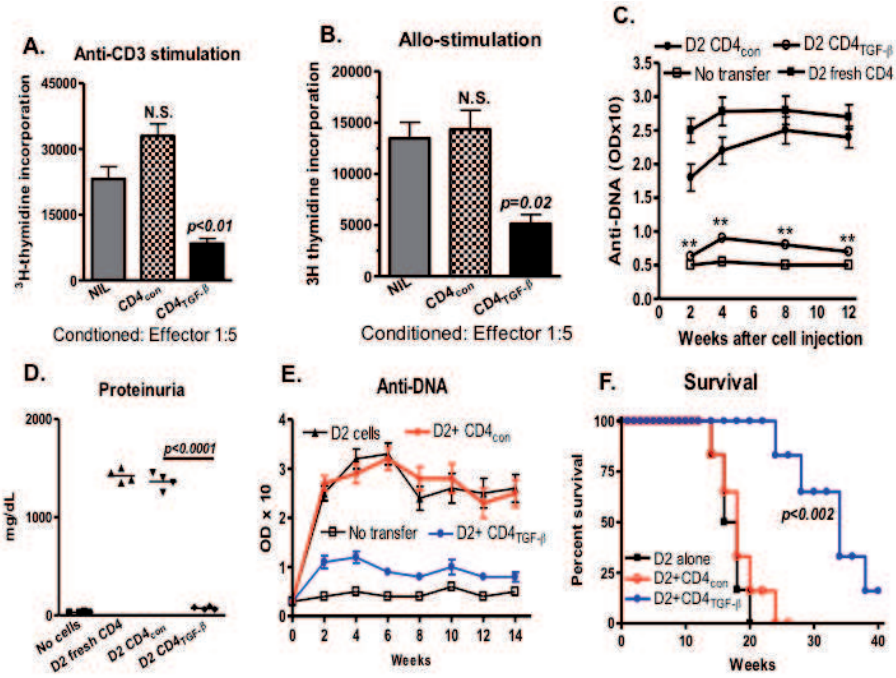

Fig. 18. Polyclonal iTregs suppress anti-CD3 and alloantigen stimulated T cell proliferation and alloantigen-mediated chronic graft-versus-host diseases (cGVHD) with a lupus-like syndrome. iTregs ($CD4_{TGF-\beta}$) or CD4con cells generated as described in methods and materials from were added to CD25-depleted T cells (1 to 5 ratio) in the presence of anti-CD3 and irradiated APC (**a**), or CD25-depleted T cells in the presence of allogeneic APC (**b**) for 3 days. ^{3}H-thymidine was added to cultures for the last 18 hours and incorporation by cycling T cells was measured. Values were mean ± s.e.m of three independent experiments. **P*<0.05, ***P*<0.01, iTregs in comparison to baseline. 12x10^6 fresh CD4+ cells, iTregs or CD4con cells from DBA/2 mice were adoptively transferred into D2B6F1 mice and the levels of anti-DNA (**c**) and proteinuria (**d**) were examined at time indicated. 5x10^6 CD4con or iTregs and 12x10^6 fresh $CD4^+$ cells from DBA/2 mice were co-transferred into D2B6F1 mice, anti-DNA level was dynamically examined (**e**) and mice survival was monitored (**f**). Five mice in each group were included in each experiment and data were combined from two independent experiments. ***P*<0.01, ****P*<0.001, iTregs + D2 cells in comparison to D2 alone.

Polyclonally differentiated iTregs suppressed chronic graft-*vs*-host diseases with a lupus-like syndrome through TGF-βR and IL-10R signaling pathways

Chronic graft-*vs*-host disease (cGVHD) is characterized by a typical lupus-like syndrome including elevated anti-dsDNA, proteinuria and lupus nephritis when parental DBA/2 splenocytes, T or $CD4^+$ cells are adoptively transferred into DBA/1xC57BL/6 F1 mice (Kautz-Neu et al., 2011). The disease is initiated by the activation of the donor cells (D2, $H2^d$) when they encounter with B6 ($H2^b$) antigen. Previous study revealed that antigen-specific iTregs suppressed cGHVD syndromes (Zheng et al., 2004b). In the present study, we will determine whether polyclonally differentiated iTregs also suppress the alloantigen-mediated cGVHD.

As D2 T cells are pathogenic cells and polyclonal iTregs originated from D2 cells, we first asked whether the latter still held a similar pathogenic effect. As shown in **Fig. 18c**, adoptive transfer of $12x10^6$ fresh D2 $CD4^+$ T cells to F1 mice resulted in the elevated anti-DNA production compared to mice that received no cells. The rapid heightened anti-dsDNA titers were observed at 2 weeks and sustained until at least 12 weeks after cell transfer. The infusion of similar doses of CD4con cells had a similar capacity on inducing anti-dsDNA antibody production in F1 mice. In sharp contrast, injection of $12x10^6$ of polyclonally differentiated iTregs did not result in the elicitation of anti-dsDNA antibody. Mice receiving iTregs exhibited anti-DNA levels that were almost comparable to naïve F1 mice without any infusion of D2 cells, indicating that TGF-β priming had changed the behavior of $CD4^+$D2 cells. F1 mice developed markedly high levels of proteinuria at 12 weeks following fresh $CD4^+$D2 or CD4con cells transfer yet iTregs infusion did not result in any significant amounts of proteinuria (**Fig. 18d**), further demonstrating that these TGF-β priming cells can alter their characteristics.

We further determined whether following TGF-β priming these cells can suppress immune responses in the cGVHD with a lupus-like syndrome. To assess this possibility, we co-transferred $12x10^6$ D2 $CD4^+$ cells and either $5x10^6$ iTregs or CD4con cells. We chose this dose of Tregs because a similar dose of antigen-specific Tregs had an ideally protective effect on cGVHD symptoms (Zheng et al., 2004b). As shown in **Fig. 18e**, co-transfer of $CD4^+$D2 cells plus iTregs but not $CD4^+$ control cells markedly suppressed the production of anti-dsDNA antibodies. In sharp contrast, transfer of $CD4^+$D2 cells alone resulted in the death of all mice by 20 weeks post-transfer. Co-transfer of CD4con cells with $CD4^+$D2 cells slightly but not significantly prolonged the survival of mice. Interestingly, co-transfer of $CD4^+$ iTregs with $CD4^+$D2 cells significantly prolonged the survival of cGVHD mice. These results showed that polyclonally differentiated iTregs exhibit

suppressive functionality in both antigen specific and antigen non-specific fashions *in vitro* and *in vivo*.

The mechanism(s) whereby Tregs suppress immune responses is still not yet well defined. Both nTregs and iTregs expressed membrane-bound TGF-β, and secreted active TGF-β and/or IL-10 and this feature is important for their suppression as well as Th17 cell conversion (Xu et al., 2007; Zheng et al., 2008). To determine whether these cytokines are also involved in the suppressive mechanisms of iTregs in cGVHD with a lupus-like syndrome *in vivo*, we used antibodies to neutralize these cytokines and receptor inhibitors in the mouse model. As shown in **Fig. 19A**, co-transfer of iTregs and $CD4^{+}$D2 cells significantly suppressed IgG upregulation (left panel) and prolonged mice survival (right panel), administration of anti-TGF-β antibody not only completely abolished the suppression on IgG upregulation by iTregs, but actually increased the levels of IgG production. Further study will be necessary to determine whether iTregs have changed to a T helper cell phenotype following TGF-β antibody treatment. Anti-TGF-β administration completely reversed the protective effect of iTregs on lupus mice survival, if any, it in fact slightly accelerated the death of lupus mice. This result could not be explained by the effect of antibody on disease self

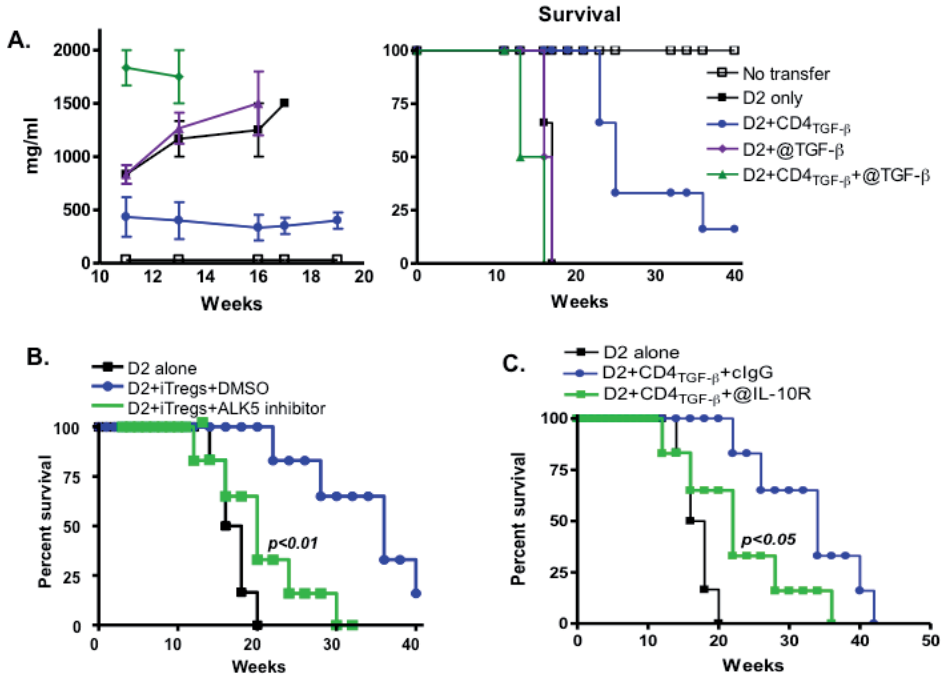

Fig. 19. The suppressive effect of iTregs on cGVHD with a lupus-like syndrome is almost completely dependent upon TGF-β and partially upon IL-10. $12x10^{6}$ fresh D2 $CD4^{+}$ cells or together with $5x10^{6}$ iTregs were transferred into D2B6F1 mice and these mice were also given with anti-TGF-β or control IgG. IgG levels (**a**, left panel) were examined and mice survival (**a**, right panel) was monitored. In other groups, ALK5 inhibitor or control DMSO (**b**), anti-IL-10R or control IgG (**c**) was administrated and mice survival was monitored. Five mice in each group were included in each experiment and data were combined from two independent experiments. $^{*}P<0.05$, $^{**}P<0.01$, anti-TGF-β, anti-IL-10R or ALK5 inhibitor in comparison to control IgG or DMSO.

rather than iTregs because administration of similar doses of antibody alone did not significantly alter the levels of IgG nor did it affect survival.

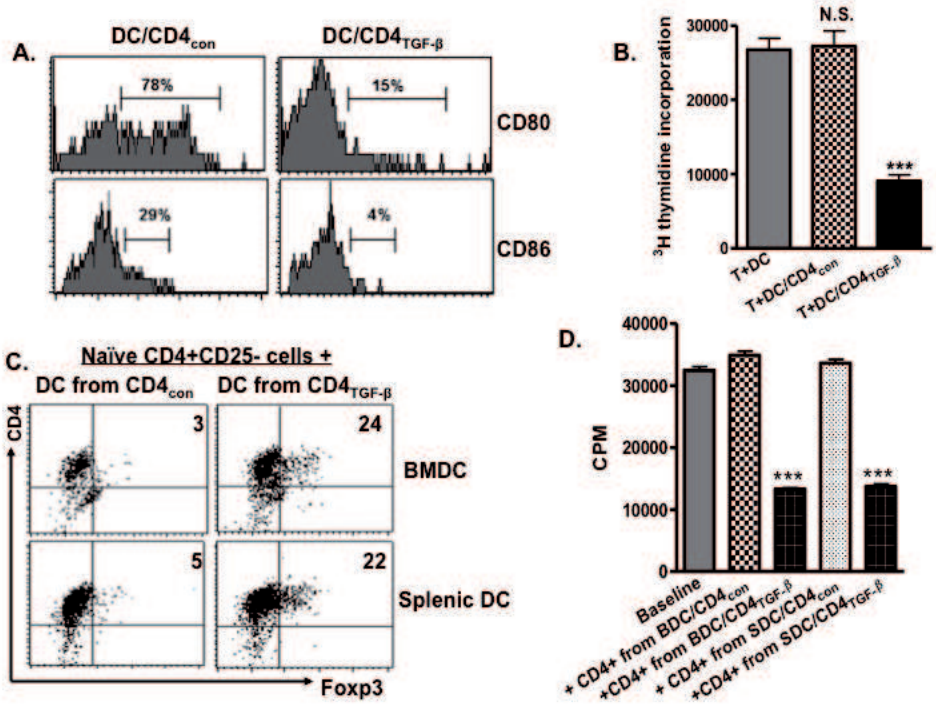

Fig.20. iTregs induce the formation of tolerogenic DCs *in vitro*. CD4con or iTregs generated from CD45.1 B6 mice and CD11c+ cells isolated from B6 mice were co-cultured (5:1 ratio) in the presence of GM-CSF, IL-4 and anti-CD3 for three days. CD80 and CD86 expression on CD11c+ cells were analyzed by flow cytometry (**a**). Data are representative of three separate experiments. When cells were harvested, $CD4^+$ cells were removed by magnetic beads and the remaining $CD11c^+$ cells were added to CD25-depleted T cells (1: 5 ratio) for additional three days. ^3H-thymidine was added to cultures for the last 18 hours and incorporation by cycling T cells was measured (**b**). Values were mean ± s.e.m of three independent experiments. ***P<0.001, DC primed with iTregs in comparison to fresh DC. $CD11c^+$ cells isolated from either bone marrow (BMDC) or spleen and primed with iTreg or CD4con cells were split as described above and added to naïve $CD4^+CD25^-$ cells isolated from CD45.2 B6 mice (1:5 ratio) in the presence of IL-2 (40 units/ml) for three days and Foxp3 expression was measured on gated CD4+CD45.2+ cells using flow cytometry (**c**). Data are representative of three separate experiments. $CD45.2^+CD4^+$ cells primed with DCs were isolated and added to CD25-depleted T cells in the presence of anti-CD3 and APC for an additional three days. ^3H-thymidine was added to cultures at the last 18 hours and incorporation by cycling T cells was measured (**d**). Values were mean ± s.e.m of three independent experiments. ***P<0.001, $CD4^+$ cells primed with DC that had been previously primed with iTregs in comparison to baseline.

To further determine whether the TGF-β signal pathway is crucial for the suppression of iTregs in lupus-like syndrome mice, we also blocked the TGF-β receptor I (ALK5) using ALK5 inhibitor in iTreg-infused lupus mice. We observed that injection of ALK5 inhibitor almost completely abolished the protective effect of iTregs on lupus-like mice survival (**Fig. 19b**). Due to solubility issue, the ALK5 inhibitor must be dissolved in dimethyl sulfoxide (DMSO). To control for any non-specific DMSO toxicity in the immune response, we injected a similar dose of DMSO alone, and this did not change the disease course. Moreover, blockade of IL-10 signaling by infusing anti-IL-10R antibody also significantly altered the survival of lupus-like disease mice treated with iTregs although the effect was less in mice treated with anti-IL-10R than in mice treated with anti-TGF-β antibody or ALK5 inhibitor (**Fig. 19c**). Together, these results suggest that iTregs

can suppress a lupus-like autoimmune response mainly through TGF-β and partially *via* IL-10 signal pathways.

Polyclonally differentiated iTregs induced the formation of tolerogenic dendritic cells through TGF-β signaling pathway in DCs.

Our previous reports demonstrated that iTregs adoptively transferred to recipient mice had a limited lifespan but sustained long-term protective role in prevention of allograft rejection (Zheng et al., 2006a). As iTregs can educate naïve T cells to become a new generation of Foxp3$^+$ Tregs (Zheng et al., 2004a) and DCs may be involved in this propaganda effect (Andersson et al., 2008), herein we have tested the effect of iTregs on DCs maturation and function. When CD11c$^+$ cells isolated from bone-marrow (BM) were co-cultured with CD4con or CD4$^+$ iTregs, iTregs but not CD4con markedly suppressed the up-regulation of CD80 and CD86 expression by DCs (**Fig. 20a**). CD4con or iTregs were induced from CD45.1 C57BL/6 mice. These DCs produced low levels of IL-12 and IL-23 (not shown) and displayed decreased antigen-presenting function. When these DCs that had been previously co-cultured with iTregs were added to T responder cells, the proliferative ability of these T cells was significantly lower compared to T cells stimulated with freshly

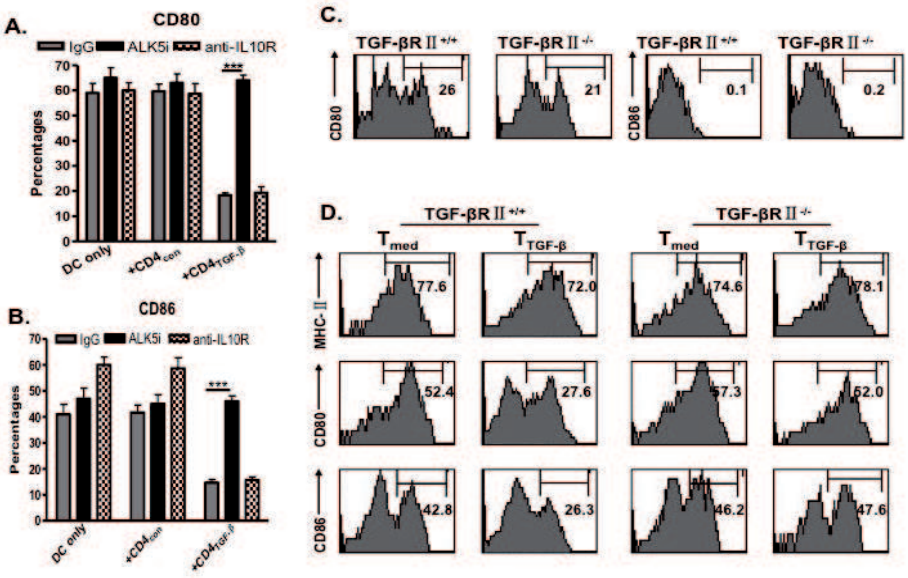

Fig. 21. TGF-β but not IL-10 signaling is required for the formation of tolerogenic DCs induced by iTregs. CD11c$^+$cells aloneor those in combination with CD4con or iTregs (1:5 ratio) were stimulated with GM-CSF, IL-4 and anti-CD3 for three days. In some cultures, anti-IL-10R, control IgG or ALK5 inhibitor was added to cultures. CD80 (**a**) and CD86 (**b**) expression was determined by flow cytometry. Values were mean ± s.e.m of four independent experiments. ***P<0.001, ALK5 inhibitor in comparison to DMSO. (**c**) CD80 and CD86 expression on CD11c$^+$ cells in naïve WT (+/+) and TGF-βRII conditional KO on DC mice (-/-). Data are representative of five mice in each strain. (**d**) Splenic CD11c+ cells isolated from WT and TGF-βRII conditional KO on DC mice were co-cultured with CD4con or iTregs (1:5 ratio) in the presence of GF-CSF, IL-4 and anti-CD3 for three days. MHC-II, CD80 and CD86 expression on CD11c$^+$ cells were analyzed by flow cytometry. Data are representative of five independent experiments.

isolated DCs and DCs that had been previously co-cultured with CD4con cells (**Fig. 20b**). When naïve $CD4^+CD25^-$ cells from CD45.2 C57BL/6 mice were co-cultured with DCs that had been previously treated with CD45.1 iTregs but not CD4con cells in the absence of TGF-β for three days, about 25% of the naïve $CD4^+CD25^-$ cells began expressing CD25 andFoxp3cells (**Fig. 20c**). These cells were gated on CD45.2, thereby excluding the possibility that the $Foxp3^+$ cells were carried over with the initial iTregs. Furthermore, using a T cell suppression assay, we demonstrated that these newly generated $CD4^+CD25^+Foxp3^+$cells developed suppressive capacity. Both BMDC and splenic DCs displayed a similar ability to develop into "tolerogenic DCs" (**Fig. 20d**).

Since both TGF-β and IL-10 are involved in the suppressive activity of iTregs *in vivo* and have a functional capacity to induce tolerogenic DCs (Pallotta et al., 2011), we determined the role of these cytokines in the iTreg-induced formation of tolerogenic DCs. As shown in **Fig. 21a and b**, co-cultures of DCs with iTregs but not CD4con cells suppressed CD80 and CD86 upregulation, but this process was completely abrogated with the addition of ALK5 inhibitor. Surprisingly, addition of anti-IL-10R did not reverse the CD80 and CD86 expression on DCs primed with iTregs. DCs that had been primed with iTregs plus ALK5 but not plus anti-IL-10R antibody mostly restored their antigen-presenting capacity and lost ability to induce other CD4+CD25- cells to become Foxp3+ cells (not shown), indicating that iTregs induce the formation of tolerogenic DCs mainly through TGF-β rather than IL-10 signaling pathway.

We further determined the role of TGF-β signaling pathway on the induction of tolerogenic DCs using mice with a conditioned knockout of TβRII in DCs. The phenotypic features of these mice have been just described by Kiela's group (personal communication). Although these mice eventually developed autoimmunity, between 6-8 weeks of age, the CD80 expression by $CD11c^+$ cells was similar between cre^- (wild type) and cre^+ (conditional DC TβRII KO) mice. CD86 expression on $CD11c^+$ cells was almost undetectable in both cre^- and cre^+ mice (**Fig. 21c**). When $CD11c^+$ cells isolated from cre^- and cre^+ mice were stimulated, we observed that both CD80 and CD86 expression was similarly up-regulated. Interestingly, the addition of iTregs but not CD4con cells, significantly suppressed CD80 and CD86 upregulation in cre^- mice but not in cre^+ mice (**Fig. 21d**). When isolated from cre^- mice, iTregs did not alter the MHC-II expression on DCs, and the functional ability of these DCs to trigger allogeneic immune responses was decreased, however, DCs from cre^+ mice developed potent antigen-stimulating abilities, even after priming with iTregs (data not shown). These results indicate that the TGF-β signaling pathway in DCs is crucial for the formation of tolerogenic DCs induced by iTregs.

Polyclonally differentiated iTregs suppressed the expansion and maturation of DCs *in vivo*

Experiments performed *in vitro* experiments do not necessarily reflect *in vivo* realities. To study this possibility, particularly under inflammatory disease conditions *in vivo*, we conducted the experiments using the cGVHD lupus-like syndrome model. We demonstrated that $CD11c^+$ cells expressed substantial amounts of CD80 and CD86 in F1 mice three weeks after $CD4^+$ D2 cell transfer. Interestingly, co-transfer iTregs and DC cells prevented the upregulation of CD80 and CD86 on $CD11c^+$ cells (**Fig. 22 a**and **b**) as well as B cells (data not shown). Conversely, co-transfer of CD4con cells did not suppress CD80 and CD86 upregulation on $CD11c^+$ and B cells at three weeks after cell transfer.

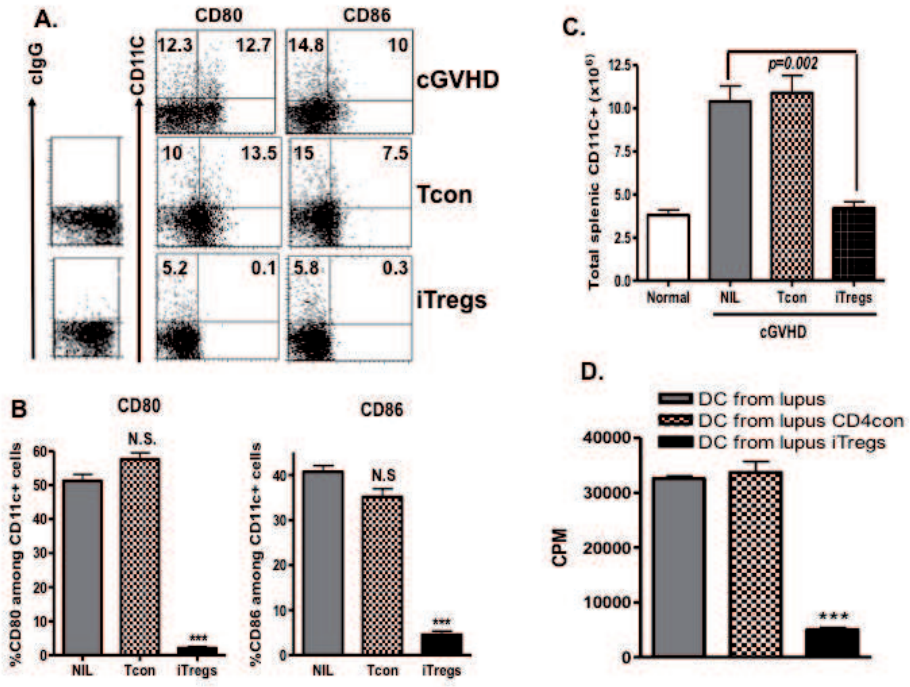

Fig. 22.Adoptive transfer of iTregs to lupus mice suppresses the expansion of DCs and decreases B7 expression by DCs. $12x10^6$ fresh D2 $CD4^+$ cells alone or together with $5x10^6$ CD4con or iTregs were transferred into D2B6F1 mice. Three weeks later, CD80 and CD86 expression in each group of mice was examined on $CD11c^+$ cells (**a, b**) and total splenic $CD11c^+$ cells were counted (**c**). Data are representative or values are mean ± s.e.m of five mice in each experiment and combined with two independent experiments. (**d**) $CD11c^+$ cells were sorted from each group of mice as above and added to cultures containing CD25-depleted T cells isolated from BLAB/C mice for three days. T cell proliferation was determined as above. Values are mean ± s.e.m of three separate experiments.

DCs play an important role in the pathogenesis in SLE (Monrad and Kaplan, 2007). Similarly, F1 mice displayed three-fold expansion of total splenic $CD11c^+$ cells three weeks after D2 cell transfer compared to naïve mice. Co-transfer of CD4con with D2 cells did not alter the total numbers of splenic $CD11c^+$ cells in the spleen. Nonetheless, co-transfer of iTregs with D2 cells almost completely suppressed the expansion of $CD11c^+$ cells in F1 mice (**Fig. 22c**). To determine their functional activity, these splenic $CD11c^+$ cells were further sorted and added to BALB/C naïve T cells for a three day *in vitro* culture, $CD11c^+$ cells sorted from either cGVHD or CD4con cell-

infused cGVHD mice initiated strong alloresponses. In contrast, CD11c$^+$ cells sorted from iTreg-infused cGVHD mice exhibited a reduced ability to stimulate allo T cell proliferation (**Fig. 22d**).

Tolerogenic DCs suppressed cGVHD through TGF-βR but not IL-10R signaling pathway

To further analyze the functional characteristics of tolerogenic DCs in lupus mice treated with iTreg, we have adoptively transferred these DCs to D2B6F1 mice that had received D2 CD4$^+$ T cells. As shown in **Fig. 23a**, compared to D2B6F1 mice which received D2 CD4+ cells alone, infusion of CD11c$^+$ cells with D2 CD4$^+$ cells in lupus mice treated with iTregs to D2B6F1 mice significantly prevented the anti-DNA production in sera at three weeks post transfer. These DCs also suppressed the donor engraftments in recipient spleens (**Fig. 23b**). The sizes of donor engraftments are linked to disease severity of lupus in this model. Importantly, these DCs also markedly prolonged the survival of lupus mice (**Fig. 23c**). In contrast, infusion of similar doses of DCs from CD4con-infused lupus mice did not appear to suppress neither anti-DNA production, donor engraftment expansion nor prolong the survival, exhibiting instead levels in each of these categories that are comparable to D2 CD4$^+$ cell infusion alone (**Fig. 23a-c**). Because tolerogenic DCs can suppress immune responses either directly or indirectly, we also examined the Foxp3$^+$ cell frequency in lupus mice after tolerogenic DC treatment. We observed that infusion of

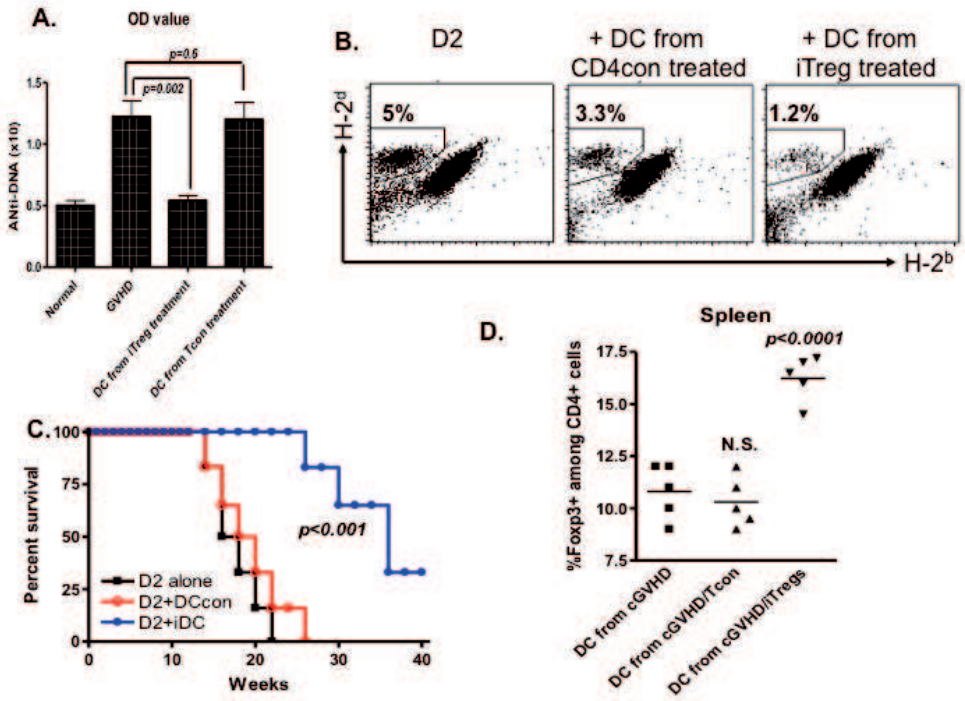

Fig. 23. DCs isolated from lupus mice treated with iTregs but not CD4con cells suppress lupus disease. 12x10^6 fresh D2 CD4$^+$ cells alone or together with 5x10^6 CD4con or iTregs were transferred into D2B6F1 mice. Three weeks later, CD11c$^+$ cells were sorted and 5x10^5 DC and 12x10^6 fresh D2 CD4+ cells were co-transferred into D2B6F1 mice. Anti-DNA levels in sera (**a**) and donor engraftments (H-2^{d+}H-2^{b-} cell population) in the spleens (**b**) were determined in 2 weeks following cell transfer. Mice survival was monitored (**c**) and Foxp3$^+$ cell frequency at one month following cell transfer was determined by flow cytometry.

DCs from iTreg–treated mice markedly increased the frequency of Foxp3$^+$ cells to levels comparable to lupus mice or mice treated with DCs from CD4con-infused mice. Using a standard suppression assay, we also demonstrated that the increased Foxp3$^+$ cell population could be considered as true suppressor cells (not shown).

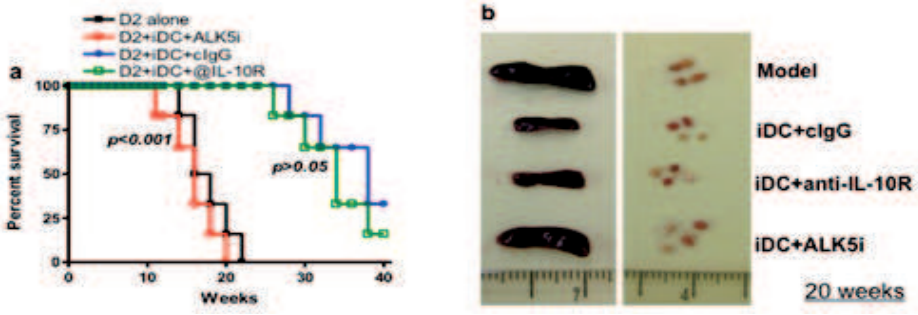

Fig. 24. Tolerogenic DCs suppress lupus through TGF-β but not IL-10 signaling. 5x10^5 CD11c$^+$ cells sorted from lupus mice treated with iTregs or CD4con and 12x10^6 fresh D2 CD4+ cells were co-transferred into D2B6F1 mice. Alk5 inhibitor or DMSO control, anti-IL-10R or control IgG was administrated in some groups. Survival was monitored (**a**) and the sizes of spleens and lymph nodes at 20 weeks following cell transfer were measured (**b**). All experiments were repeated at least twice with similar results.

We next tried to explore the underlying mechanism of how tolerogenic DCs suppress lupus. As in **Fig. 24**, co-transfer of CD11c$^+$ cells (iDC) sorted from lupus mice that received iTregs markedly prolonged survival (**Fig. 24a**) and suppressed splenomegaly and enlarged lymph nodes (**Fig. 24b**). Administration of ALK5 inhibitor with iDC not only completely blocked the suppressive activity of iDC in cGVHD, but also slightly accelerated mice death. Unexpectedly, anti-IL-10R administration did not significantly decrease the mice survival in iDC infusion group. iDC also suppressed IgG deposition in kidney and ALK5 inhibitor but not anti-IL-10R antibody reversed the protective effect of iDC on IgG production and deposition (**Fig. 25**), suggesting iTregs infusion induces the formation of tolerogenic DCs in the context of inflammatory diseases *in vivo* and these DCs suppress T cell-mediated immune responses through TGF-β signaling rather than *via* the IL-10 signaling pathway.

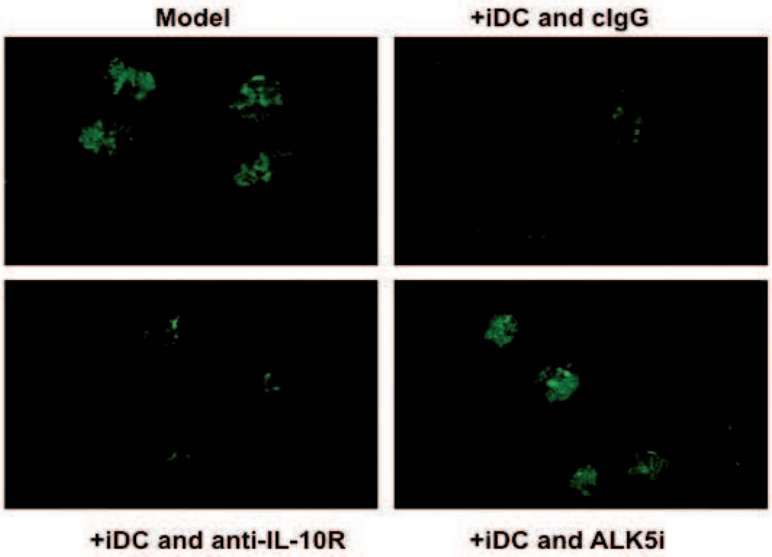

Fig. 25. Tolerogenic DCs suppressed IgG deposit in kidney in cGVHD mice through TβR but not IL-10R signaling.

We conducted another experiment to determine if the TGF-β signaling pathway in DCs is also crucial in the induction of tolerogenic DCs *in vivo*. iTregs were first induced as above except

from Foxp3 GFP knock-in mice (C57BL/6 strain). GFP$^+$ (Foxp3$^+$) cells and control cells (GFP$^-$) were sorted and added to DCs isolated from WT (cre$^-$) andDC conditional TGF-βRII KO (cre$^+$) mice for priming for three days. CD4$^+$CD45Rb^{hi} cells were transferred into Rag-1 KO mice. In other groups, DCs previously primed with iTregs or CD4con cells were recovered and co-transferred into Rag-1 KO mice. The cytokine production in mesenteric lymph nodes was determined using ELISA and quantitative

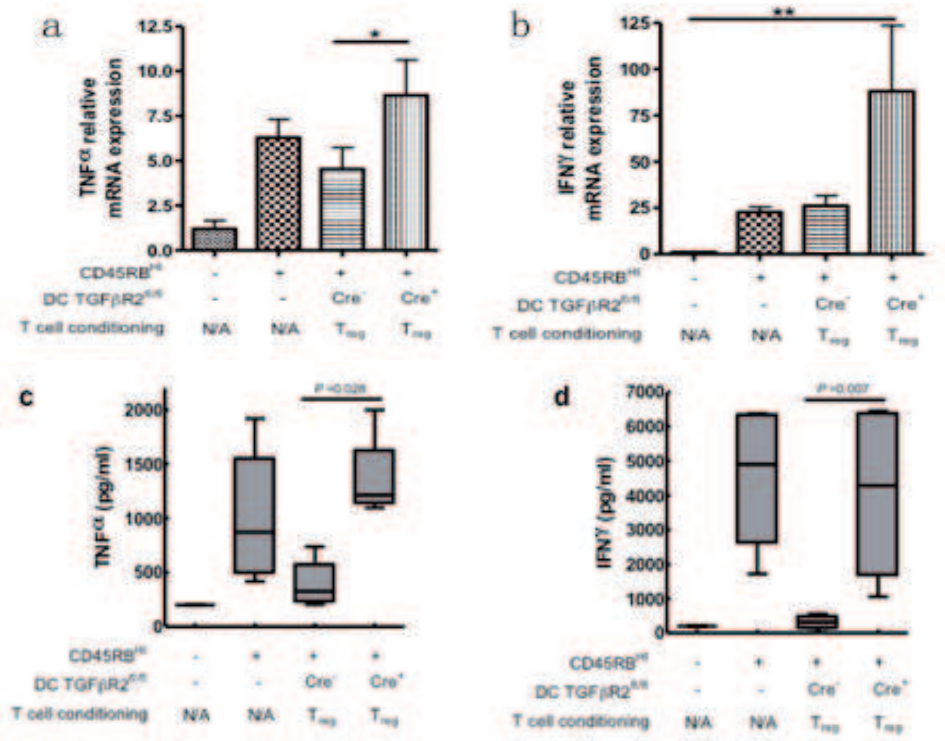

Fig. 26. TGF-β signal in DCs is crucial for the formation of tolerogenic DCs induced by iTregs. iTregs were co-cultured with DCs from wild type (Cre$^-$) and TGF-βRII DC conditional KO mice (Cre$^+$) for three days and these DCs and naïve CD4+CD45RB^{high} cells were co-transferred into Rag-1 KO mice. Mice were sacrificed 4 weeks after cell transfer. MLN cell suspensions cultured in activation plates with anti CD3/CD28 Ab and TNF-α (**a**) and IFN-γ (**b**) mRNA expression was determined by qRT-PCR, and TNF-α (**c**) and IFN-γ (**d**) protein levels were determined by Elisa. All experiments were repeated at least twice with similar results.

RT-PCR. While both IFN-γ and TNF-α protein (**Fig. 24c, d**) and mRNA (**Fig. 26**) were highly elevated by 2 weeks after CD4$^+$CD45Rb^{hi} cell transfer, co-transfer of cre$^-$ DCs primed with iTregs but not cre$^-$ DCs primed with CD4con cells dramatically suppressed IFN-γ and TNF-α production. Nonetheless, co-transfer of cre$^+$ DCs primed with iTregs or CD4con cells did not suppress IFN-γ and TNF-α production, further documenting that iTregs induced the formation of tolerogenic DC *via* TGF-β signaling on DCs.

Discussion

It has been well documented that both nTregs and iTregs suppress the development of autoimmune diseases. However, the mechanisms whereby Treg subsets suppress immune response are still in question. Although cell contact is required for the suppression of immune response of nTregs *in vitro* (Piccirillo et al., 2002), immunosuppressive factors such as TGF-β and/or IL-10 are indeed involved in the suppression of immune response and disease progress by nTregs *in vivo* (Fahlen et al., 2005; Maloy et al., 2003).

Suppressive mechanisms employed by iTregs in immune responses are also less well defined. In the current study, we have demonstrated that TGF-β and TβR signaling are absolutely required and IL-10 plays a partial role in the suppression of lupus, although these soluble factors did not contribute to the suppressive activity of iTregs *in vitro*. These findings are consistent with those for nTregs. It is possible that cell contact may play a dominant role *in vitro* due to the limited mobility of cells in a confined space. However, it is clear that cytokines produced by Treg subsets have a systemic role in the suppression of immune response *in vivo*.

To confirm that TGF-β and its receptor signaling pathway contribute to iTregs-mediated suppression in lupus, we analyzed suppression in the context of either anti-TGF-β antibody or TGF-βR1 (ALK5) inhibitor. Both agents abolished the suppressive function exerted by iTreg treatment to a similar degree. As TGF-β itself plays an important role in the maintenance of immune tolerance (Letterio et al., 1996), we treated lupus mice with anti-TGF-β and ALK5 inhibitor in the absence of iTreg transfer. We found that the doses did not significantly alter the disease course of lupus. Thus, we can rule out that TGF-β and its signaling are related to the suppression of iTreg function.

In general, antigen-specific Tregs have a more potent suppressive ability than non-specific Tregs (Tang and Bluestone, 2008). Another advantage of antigen-specific Tregs is that they can selectively suppress immune responses without comprising other beneficial immune responses, and therefore are especially suitable for providing protection from organ transplantation rejection. Nonetheless, in some autoimmune diseases, such as lupus, the specific antigens are not known or ill–defined and polyclonal Tregs may be suitable for this situation. In the current study, we identified that polyclonal iTregs suppressed anti-CD3 and alloantigen stimulated T cell responses and alloantigen-mediated cGVHD that is comparable to antigen-specific iTregs (Zheng et al., 2004b), implicating that the manipulation of polyclonal iTregs may be therapeutic for these systemic autoimmune diseases that

lack identification of specific antigens. Previous study has also demonstrated that the suppressor effector function of nTregs is antigen nonspecific when they have been activated (Thornton and Shevach, 2000).

DCs play an important role in initiating immune responses and maintaining immune tolerance. DCs are professional antigen presenting cells and are essential mediators of immunity. DCs mature when they encounter antigen and initiate immune response through antigen presentation, co-stimulatory molecule signaling and cytokine production, and sequentially mediate adaptive immune responses. DCs are also crucial for immune tolerance since their ablation has been shown to result in autoimmunity, highlighting the active role that DCs play under steady state conditions in maintaining immune tolerance (Letterio et al., 1996). Immature or semi-mature DCs may mediate the functional activity of tolerogenic DCs.

Consistent with previous reports (Colonna et al., 2004), we have observed tolerogenic DCs induced by iTregs expressed low levels of CD80 and CD86, and produced low levels of IL-12 and IL-23. These DCs displayed a decreased antigen-presenting ability and even educated other conventional $CD4^+CD25^-$ cells to become $Foxp3^+$ regulatory T cells in the absence of exogenous TGF-β. Of note, adoptive transfer of iTregs to lupus mice can still induce the formation of tolerogenic DCs. Previous studies have demonstrated that various anti-inflammatory and immunosuppressive agents such as TGF-β and/or IL-10 potentiate or confer tolerogenicity on DCs (Morelli and Thomson, 2007). In the current study, we have revealed that TGF-β plays a dominant role in the induction of tolerogenic DCs both *in vitro* and *in vivo*.

Ample *in vitro* evidence has been collected with respect to the inhibitory effect of TGF-β on DC activation and maturation. Monocyte-derived DCs expanded in the presence of IL-10, and TGF-β demonstrated reduced IL-12 and IL-23 production and favored T cell anergy and the induction of $Foxp3^+$ regulatory T cells (Torres-Aguilar et al., 2010). Interestingly, the current study reveals that TGF-β rather than IL-10 produced by iTregs is responsible for the formation of tolerogenic DCs *in vitro* and *in vivo*. Moreover, DCs became tolerogenic in lupus mice when they were exposed to iTregs. This is likely that the transferred iTregs produced substantial amounts of active TGF-β and lower levels of IL-10 (Zheng et al., 2002).

The crucial role of TGF-β in the induction of tolerogenic DCs can be further documented using mice with a conditional knockout of TβR2 under a DC-specific promoter, which results in deletion of

TGF-βR signaling in all $CD11c^+$ DCs (DC-TβR2del mice or Cre^+). Others have used conditional knockout mice of TβR1 and this approach also blocked the TGF-β signaling on DCs (Kel et al., 2010). We demonstrated that while iTregs can induce DCs isolated from wild type mice to display the phenotypes of tolerogenic DCs and can suppress colitis initiated by naïve $CD4^+$ cells in Rag-1 KO mice, this did not occur if the DCs were isolated from DC-TβR2del mice.

It is not surprising that adoptive transfer of tolerogenic DCs also suppressed lupus development in the current study. It has been known that tolerogenic DCs can produce TGF-β, IL-10, IL-27, retinoic acid, indoleamine-2,3-dioxygenase and vitamin D (Wakkach et al., 2003). These factors either suppress immune response directly or induce regulatory T cell subsets first and then suppress immune response indirectly. In our study, we have observed that TGF-β but not IL-10 plays an important role in the suppression of lupus disease following adoptive transfer of tolerogenic DCs. It is possible that these tolerogenic DCs mainly produced TGF-β rather than IL-10. This study suggests that tolerogenic DCs may be represented by different subsets that produce different immunosuppressive factors. Although we cannot rule out the direct action of tolerogenic DCs, we believe that these DCs have at least indirectly caused the suppression of lupus since iDCs treatment also increased the frequency of $Foxp3^+$ Tregs in these mice.

It is less well understood why a single injection of iTregs can result in long-term protective effects since these iTregs have a limited lifespan (Selvaraj and Geiger, 2008; Weber et al., 2006; Zheng et al., 2006a; Zheng et al., 2008; Zheng et al., 2004b). It is very likely that infusion of iTregs under autoimmune inflammatory disease conditions and in organ transplantation models can induce the formation of tolerogenic DCs in the recipients, These tolerogenic DCs can then release TGF-β and educate the recipient's conventional T cells to become a new generation of $Foxp3^+$ Tregs in the presence of self or foreign antigens through a mechanism called "infectious tolerance". These new Tregs can continue to maintain immune tolerance and control disease development.

OPEN ACCESS Freely available online

PLoS one

All-Trans Retinoic Acid Promotes TGF-β-Induced Tregs via Histone Modification but Not DNA Demethylation on Foxp3 Gene Locus

Ling Lu[1,4☯], Jilin Ma[5☯], Zhiyuan Li[9], Qin Lan[1,6], Maogen Chen[1], Ya Liu[1], Zanxian Xia[1], Julie Wang[1], Yuanping Han[2], Wei Shi[3], Valerie Quesniaux[7], Bernhard Ryffel[7], David Brand[8], Bin Li[9], Zhongmin Liu[6*], Song Guo Zheng[1,7]

1 Division of Rheumatology, Department of Medicine, Saban Research Institute, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, California, United States of America, **2** Department of Surgery, Saban Research Institute, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, California, United States of America, **3** Developmental Biology and Regenerative Medicine Program, Saban Research Institute, Children's Hospital Los Angeles, Keck School of Medicine, University of Southern California, Los Angeles, California, United States of America, **4** Key Laboratory of Living Donor Liver Transplantation, Nanjing, People's Republic of China, **5** Division of Rheumatology, Immunology and Nephrology, Zhejiang Traditional Chinese Medicine and Western Medicine Hospital, Hangzhou, People's Republic of China, **6** Immune Tolerance Center, Shanghai East Hospital, Tongji University, Shanghai, People's Republic of China, **7** UMR6218, Molecular Immunology, University and Centre National de la Recherche Scientifique, Orleans, France, **8** Research Service, Veterans Affairs Medical Center, Memphis, Tennessee, United States of America, **9** Unit of Molecular Immunology, Institute Pasteur of Shanghai, Chinese Academy of Science, Shanghai, People's Republic of China

Abstract

Background: It has been documented all-trans retinoic acid (atRA) promotes the development of TGF-β-induced $CD4^+Foxp3^+$ regulatory T cells (iTreg) that play a vital role in the prevention of autoimmune responses, however, molecular mechanisms involved remain elusive. Our objective, therefore, was to determine how atRA promotes the differentiation of iTregs.

Methodology/Principal Findings: Addition of atRA to naive $CD4^+CD25^-$ cells stimulated with anti-CD3/CD28 antibodies in the presence of TGF-β not only increased $Foxp3^+$ iTreg differentiation, but maintained Foxp3 expression through apoptosis inhibition. atRA/TGF-β-treated $CD4^+$ cells developed complete anergy and displayed increased suppressive activity. Infusion of atRA/TGF-β-treated $CD4^+$ cells resulted in the greater effects on suppressing symptoms and protecting the survival of chronic GVHD mice with typical lupus-like syndromes than did $CD4^+$ cells treated with TGF-β alone. atRA did not significantly affect the phosphorylation levels of Smad2/3 and still promoted iTreg differentiation in $CD4^+$ cells isolated from *Smad3* KO and *Smad2* conditional KO mice. Conversely, atRA markedly increased ERK1/2 activation, and blockade of ERK1/2 signaling completely abolished the enhanced effects of atRA on Foxp3 expression. Moreover, atRA significantly increased histone methylation and acetylation within the promoter and conserved non-coding DNA sequence (CNS) elements at the Foxp3 gene locus and the recruitment of phosphor-RNA polymerase II, while DNA methylation in the CNS3 was not significantly altered.

Conclusions/Significance: We have identified the cellular and molecular mechanism(s) by which atRA promotes the development and maintenance of iTregs. These results will help to enhance the quantity and quality of development of iTregs and may provide novel insights into clinical cell therapy for patients with autoimmune diseases and those needing organ transplantation.

Citation: Lu L, Ma J, Li Z, Lan Q, Chen M, et al. (2011) *All-Trans* Retinoic Acid Promotes TGF-β-Induced Tregs via Histone Modification but Not DNA Demethylation on Foxp3 Gene Locus. PLoS ONE 6(9): e24590. doi:10.1371/journal.pone.0024590

Editor: Niels Olsen Saraiva Câmara, Universidade de Sao Paulo, Brazil

Received March 11, 2011; **Accepted** August 15, 2011; **Published** September 13, 2011

Copyright: © 2011 Lu et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by National Institutes of Health grants R01 AR059103, R01 AR051558, and R01 HL068597; ACR Within Our Reach; the Arthritis Foundation; an Outstanding Youth Scientist Award from the National Natural Science Foundation of China (30728007); the National Natural Science Foundation of China (30772150, 81001307, and 81100270); and Zhejiang Province National Natural Science Foundation of China (Y2090918). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: zhongmin_liu@sina.com Correction: E-mail:szheng@usc.edu

☯ These authors contributed equally to this work.

Introduction

All-trans-retinoic acid (atRA), a Vitamin A derivative, has profound effects on embryonal morphogenesis, vision, reproduction, cell differentiation, growth, and immune homeostasis [1]. Deficiency of vitamin A leads to exacerbation of experimental colitis [2]. In the immune system, atRA plays important roles in regulating the functions of many different cell types [3]. Vitamin A and its derivatives are capable of ameliorating several models of autoimmunity, including inflammatory bowel disease, rheumatoid arthritis, type I diabetes, and experimental encephalomyelitis [4–5]. In addition to the inhibitory effect of atRA on T effector cell

differentiation and function, atRA has also been shown to be capable of promoting murine $CD4^+Foxp3^+$ Tregs induced by TGF-β from conventional $CD4^+Foxp3^-$ cells, either directly by enhancing TGF-β-driven Smad3 signaling in naive cells and/or indirectly by relieving the production of pro-inflammatory cytokines from murine memory effector cells [6–8]. Such approaches show great promise as these T cells have been shown effective in combating several immune-mediated disorders [9].

$CD4^+CD25^+$ T regulatory (Treg) cells play a critical role in establishing and maintaining self-tolerance. Therefore, enhancing the number and/or function of Tregs represents a potential treatment for patients with autoimmune disorders or those who undergo transplant rejection. atRA can strongly increase TGF-β-induced Foxp3 expression and Treg conversion *in vitro* [6]. Under these conditions, atRA may enhance TGF-β signaling by increasing the expression and phosphorylation of Smad3. On the other hand, it has also been reported that expression of RAR can be increased through TGF-β signaling [10]. Therefore, atRA and TGF-β may cooperatively augment their mutual signaling to further enhance Foxp3 expression. However, the exact roles of atRA in these signaling pathways are less well understood.

We recently reported that while the Smad pathway plays a less important role in the differentiation of $Foxp3^+$ iTregs induced by TGF-β, ERK and JNK kinases which mainly use non-Smad pathways, may play a more significant role in this process [11]. Herein, we further demonstrate that adding atRA to cultures containing TGF-β not only increases Foxp3 expression and maintenance, but also enhances the suppressive activities of these Tregs *in vitro* and *in vivo*. Studies of the underlying mechanism responsible for these observations indicate that atRA upregulates ERK rather than Smad2/3 activation of the TGF-β down-stream signaling pathway. Additionally, Foxp3 induced by a combination of atRA and TGF-β displayed increased Foxp3 binding ability on chromatin compared to that induced by TGF-β alone. We further found that atRA enhances histone methylation and acetylation in Foxp3 promoter and its conserved non-coding DNA sequence elements (CNS2), rather than DNA CpG demethylation of CNS3 in the Foxp3 locus. Thus, atRA improves both the quantity and quality of $Foxp3^+$ iTregs, findings which will be important in the development of superior cell therapies to treat autoimmune diseases and prevent organ transplantation rejection.

Results

atRA directly up-regulates Foxp3 and sustains its expression by $CD4^+$ cells treated with TGF-β

In agreement with previous reports [6], addition of atRA to cultures containing TGF-β significantly enhanced the proportions of $CD4^+CD25^+Foxp3^+$ cells induced from naive $CD4^+CD25^-Foxp3^-$ (or GFP^- cells using WT or Foxp3 GFP knock-in mice). This effect may reflect either direct $Foxp3^+$ cell induction or a secondary effect through suppression of $CD4^+Foxp3^-$ cell expansion [8]. In either case, total Foxp3 protein levels and $Foxp3^+$ cell numbers increased significantly in $CD4^+$ cells treated with the combination of atRA and TGF-β than those treated with TGF-β alone, indicating that atRA has a direct effect on the enhancement of iTreg differentiation, although it may also simultaneously suppress $CD4^+Foxp3^-$ cell expansion (**Figure S1A–D**).

We also examined other phenotypic features related to Treg differentiation. As reported by others [12], addition of atRA to TGF-β markedly increased the expression of CD103, CCR-9, $\alpha_4\beta_7$ by $CD4^+$ cells (**Figure S1E**), we also observed that addition of atRA significantly decreased IL-2 production, increased membrane bound TGF-β expression and slightly increased IL-10 production (**Figure S1E**).

In addition to a direct effect of atRA on $Foxp3^+$ cell differentiation, atRA also maintains Foxp3 expression in TGF-β-primed $CD4^+CD25^+$ cells. As shown in **Figure 1A**, Foxp3 expression in $CD4^+CD25^+$ cells treated with TGF-β appeared on day 1 and peaked on day 3–5. Foxp3 expression gradually declined after 6 days in the culture and remained at lower levels on day 10. Of note, the addition of atRA not only increased, but sustained Foxp3 expression by TGF-β-primed $CD4^+$ cells. We conducted further *in vivo* experiments to determine whether both Treg cell subsets have different fates after cell transfer. iTregs were generated as described above from C57BL/6 Thy1.1 mice and adoptively transferred into syngeneic C57BL/6 Thy1.2 mice. Spleen, blood and lymph node (LN) cells were stained for Foxp3 and Thy1.1 on day 10, 20 and 30 after cell transfer. Thy1.1 expression is used to distinguish the donor cells from recipient cells. While total donor $CD4_{TGF\text{-}\beta}$ cells dramatically declined on day 30, the $Foxp3^+$ cell subset from these cells significantly decreased on day 20 and even more on day 30 after cell transfer in LNs (**Figure 1B-D**), blood and spleen (not shown). In sharp contrast, total donor $CD4_{TGF\text{-}\beta+atRA}$ cell numbers are sustained during 10–30 days after cell transfer. Although the percentages of $Foxp3^+$ population among $CD4_{TGF\text{-}\beta+atRA}$ cells was slightly lower in day 20-30 than in day 0, the $Foxp3^+$ population was still significantly higher in donor $CD4_{TGF\text{-}\beta+atRA}$ cells than in $CD4_{TGF\text{-}\beta}$ cells in LNs (**Figure 1B–C**). This phenonemon was similarly observed in peripheral blood and spleen, excluding the possibility that the re-distribution of donor cells affects the frequency of Treg cells in the different donor cell populations post injection. We further observed that the addition of atRA to TGF-β-treated culture significantly decreases the proportion of Annexin-V^+GFP^+ (apoptotic $Foxp3^+$) cells (**Figure 1E**) and up-regulates the expression of Bcl-2 (an anti-apoptotic gene) (**Figure 1F, left panel**), suggesting that atRA maintains Foxp3 expression through its effect on protecting these cells from apoptosis. Although atRA induces cancer cell apoptosis and contributes to cancer treatment [13], it actually suppresses apoptosis in non-tumor human cells including lymphocytes, eosinophils and neuronal cells [14]. We further demonstrated that atRA/RAR rather than atRA/RXR signal pathway is crucial for the upregulation of Bcl-2 expression since additon of LE540 (an RAR antagonist) rather than of SR11237 (an RXR antagonist) abolished the effect of atRA on Bcl-2 upregualtion (**Figure 1F**, right panel). This is in line with a previous report concluding that the atRA/RAR but not atRA/RXR signal pathway is important for Foxp3 induction [15].

$CD4^+$ cells primed with atRA and TGF-β displayed increased suppressive activities *in vitro* and *in vivo*

Previous reports have demonstrated that TGF-β-primed $CD4^+$ cells are hypoproliferative themselves yet are able to suppress activation and proliferation of other T cells [9,11,16]. As shown in **Figure 2A**, when stimulated with anti-CD3 alone, TGF-β-primed $CD4^+$ cells had a low proliferative ability. These cells still produced variable levels of IL-2 (**Figure S1E**), a possible reason for their incomplete anergy status. Nonetheless, the addition of atRA along with TGF-β significantly decreased IL-2 production and almost completely abolished their proliferation, but this could be restored through the addition of exogenous IL-2 (**Figure 2A**). We next examined their suppressive activity in a standard *in vitro* assay. After four days in culture, almost all CD25 expressing cells were also expressing Foxp3, therefore we could use $CD4^+CD25^+$-based magnetic bead isolation to obtain Foxp3+ cells from cells cultured under the following conditions: TGF-β alone, atRA plus

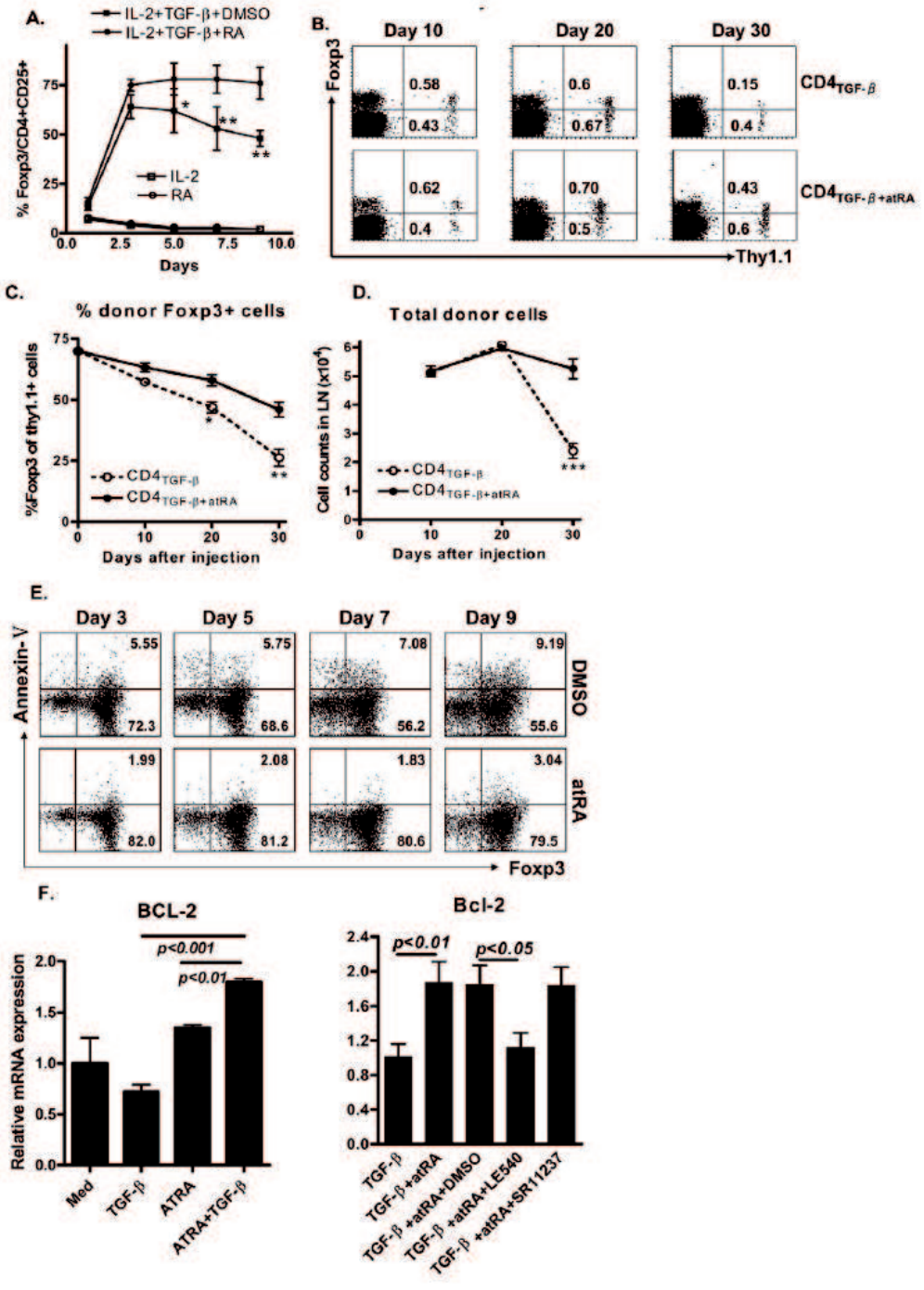
A.
IL-2+TGF-β+DMSO
IL-2+TGF-β+RA
% Foxp3/CD4+CD25+
Days
IL-2
RA
B.
Day 10
Day 20
Day 30
Foxp3
0.58
0.43
0.6
0.67
0.15
0.4
CD4TGF-β
0.62
0.4
0.70
0.5
0.43
0.6
CD4TGF-β+atRA
Thy1.1
C.
% donor Foxp3+ cells
%Foxp3 of thy1.1+ cells
CD4TGF-β
CD4TGF-β+atRA
Days after injection
D.
Total donor cells
Cell counts in LN (x10⁴)
CD4TGF-β
CD4TGF-β+atRA
Days after injection
E.
Day 3
Day 5
Day 7
Day 9
Annexin-V
5.55
72.3
5.75
68.6
7.08
56.2
9.19
55.6
DMSO
1.99
82.0
2.08
81.2
1.83
80.6
3.04
79.5
atRA
Foxp3
F.
BCL-2
p<0.001
p<0.01
Relative mRNA expression
Med
TGF-β
ATRA
ATRA+TGF-β
Bcl-2
p<0.01
p<0.05
TGF-β
TGF-β+atRA
TGF-β +atRA+DMSO
TGF-β +atRA+LE540
TGF-β +atRA+SR11237

Figure 1. atRA sustains Foxp3 expression *via* apoptosis inhibition. (A) Splenic naïve $CD4^+$ cells isolated from $Foxp3^{gfp}$ mice were stimulated as described in the Methods section. Percentages of GFP^+ among $CD4^+$ cells were determined by FACS at the time points indicated. Values were Mean ± SEM of three separate experiments. (B). Splenic naïve $CD4^+$ cells isolated from Thy1.2 C57BL/6 mice were stimulated with anti-CD3/CD28 coated beads (1:5 ratio) with IL-2 and TGF-β ± atRA for 4 days and injected to Thy1.2 syngeneic mice. 10, 20, and 30 days later, mice were sacrificed and Foxp3 expression in the donor cells ($Thy1.1^+$) in spleen, blood and lymph nodes was determined by FACS. Representative Foxp3 expression data from LN cells of one mouse per group (n = 4). The experiment was repeated with similar results. The percentages of $Foxp3^+$ cells in specific donor cell populations (C) and total donor cells (D) in days post injection as indicated are shown. Values were Mean ± SEM of 8 mice in each time point. (D) The experiment was conducted as panel A and the expression of Annexin-V^+ among GFP^+ cells were determined by FACS at the time points indicated. Data is representative of three separate experiments. (F) Total RNA was isolated from different cell groups in panel A and expression of Bcl-2 mRNA was quantitatively determined by qPCR. Data are Mean ± SEM of triplicate wells and representative of three different experiments with similar results. *, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$, $CD4_{TGF\text{-}\beta}$ in comparison to $CD4_{atRA+TGF\text{-}\beta}$.
doi:10.1371/journal.pone.0024590.g001

TGF-β or control (without TGF-β). These cultured $CD4^+$ cells were analyzed in standard suppression assay. As shown in **Figure 2B**, addition of TGF-β-primed $CD4^+CD25^+$ cells to CD25-depleted CD8+ T cells at a ratio of 1:4 significantly suppressed the $CD8^+$ T responder cell proliferation *in vitro*. These cells similarly suppressed $CD4^+$ T responder cell proliferation as well *in vitro* (not shown). Conversely, addition of $CD4^+CD25^+$ effector cells (without TGF-β-primed, CD4con) failed to suppress responder T cell response. Of note, addition of similar doses of $CD4^+CD25^+$ cells treated with both atRA and TGF-β resulted in a greater suppressive activity against the proliferative response of $CD8^+$ T cells (**Figure 2B**) and $CD4^+$ T cells (**Figure 2C**) compared to $CD4^+$ cells treated with TGF-β alone. We also observed differential suppressive capacities *in vitro* using purified GFP^+ Tregs sorted from TGF-β-primed or atRA/TGF-β-primed $CD4^+$ cells using $Foxp3^{gfp}$ knock-in mice (**Figure S2**), indicating the increased suppressive effects of $CD4^+$ iTregs induced by both atRA and TGF-β are not simply due to only enriched proportion of $Foxp3^+$ cell population in this setting.

Suppressive activity *in vitro* does not necessarily reflect a therapeutic effect of Tregs in autoimmune diseases setting *in vivo*. To validate and compare the therapeutic effects of both $CD4^+$ Treg populations generated as above, we have used a rapid-read lupus model as previously reported [9]. In this lupus model, the transfer of parental DBA/2 splenocytes to DBA/2 x C57BL/6 F1 mice caused polyclonal B cell activation and anti-dsDNA autoantibodies in 1–2 weeks, and proteinuria in 8–12 weeks [9,17]. These pathologic effects could be significantly decreased by co-transfer of 5×10^6 TGF-β-primed $CD4^+CD25^+$ cells. Furthermore, the levels of IgG, anti-dsDNA and proteinuria were markedly lower in lupus mice treated with atRA/TGF-β-primed $CD4^+CD25^+$ cells than in lupus mice treated with TGF-β-primed $CD4^+CD25^+$ cells (**Figure 2D**). 3 weeks after cell transfer, LN cells in DBA/2 x C57BL/6 F1 mice resulted in substantial numbers of Th1 cell (**Figure 2E**) but few Th2 and Th17 cells (not shown) compared to F1 mice receiving no cells. Infusion of TGF-β-primed $CD4^+CD25^+$ cells significantly suppressed the Th1 frequency in lupus mice whereas treatment with atRA/TGF-β-primed $CD4^+CD25^+$ cells resulted in the most significant suppression against Th1 cell production in lupus mice (**Figure 2E**). Accordingly, co-injection of TGF-β-primed $CD4^+CD25^+$ cells but not control (CD4con) cells significantly prolonged the survival of lupus mice in a manner consistent with a previous report (**Figure 2F**) [9]. Of note, the protective effect of atRA/TGF-β-primed $CD4^+CD25^+$ cells on lupus survival was even better. While all lupus mice died in 42 weeks following single co-injection of $CD4^+CD25^+$ cells treated with TGF-β, more than 60% of lupus mice still survived at this time point after receiving $CD4^+CD25^+$ cells treated with both atRA and TGF-β. Taken together, these results indicate that addition of atRA to TGF-β strengthens the quantity and quality of induced Tregs and provide a better approach to treatment of autoimmune diseases and other diseases.

The ability of atRA to promote $Foxp3^+$ regulatory T cell differentiation is TGF-β signaling dependent

The inability of atRA alone to induce Foxp3 expression by TCR-stimulated naive $CD4^+CD25^-$ cells (**Figure S1A and C**) indicates that Foxp3 conversion *in vitro* was exclusively dependent on exogenous TGF-β [11,13]. This finding is exemplified best when using TCR-activated naïve $CD4^+$ cells isolated from TGF-β receptor II (TβRII) KO mice. Under these conditions, even a combination of atRA and TGF-β is unable to induce Foxp3 expression (not shown). Using quantitative PCR methods, we now showed that TGF-β increases the TβRII expression by $CD4^+$ T cells (**Figure 3A**). atRA alone was unable to increase TβRI (not shown) and TβRII expression, nor did $CD4^+$ treated with both atRA and TGF-β (**Figure 3A**), indicating the increased binding ability between TGF-β and TβR in $CD4^+$ cells does not appear to contribute to the enhanced effectiveness of atRA to upregulate TGF-β-induced Foxp3 expression.

We next examined whether atRA affects downstream molecules in the TGF-β/TβR pathway, subsequently altering TGF-β's ability to induce Foxp3 expression. The cellular response to TGF-β varies by cell type and by the context of the stimulus. In lymphocytes, TGF-β binds to its cognate receptor complex composed of type I (ALK5) and type II receptors. TβRII is required to activate TβRI in the ligand–receptor complex, and activated TβRI Ser/Thr kinases mainly phosphorylate downstream specific Smad2 and Smad3. We first observed that while TGF-β induces Smad2/3 activation by $CD4^+$ cells, the addition of atRA (**Figure 3B**) slightly increased the level of Smad2/3 phosphorylation 30 min after treatment, with this effect disappearing in 60 min after treatment, suggesting that Smad2/3 early activation probably plays a role in the enhancement of atRA to induce $Foxp3^+$ cell differentiation that is similar with other reports [18,19].

Nevertheless, recent studies have demonstrated that Smad2 or Smad3 plays a redundant role in the differentiation of TGF-β-induced $Foxp3^+$ cells [11,20], we therefore further studied the role of atRA in the promotion of iTreg development using Smad2 or Smad3 KO mice. As shown in **Figure 3C**, addition of atRA significantly increased the proportion of $Foxp3^+$ cells by WT TGF-β-primed $CD4^+$ cells, this phenomenon can be similarly observed in $CD4^+$ cells isolated from Smad3 KO mice although the expression levels and intensities of Foxp3 were somewhat lower compared to WT cells. Because conventional Smad2 KO mice are early embryonic lethal, we generated lymphocyte-targeted Smad2 conditional knock-out (CKO) mice by crossbreeding floxed Smad2 and hCD2-Cre mice [11]. As with the Smad3 KO mice, addition of atRA to the TGF-β treated-$CD4^+$ Smad2 CKO cultures still resulted in upregulated Foxp3 induction. Moreover, addition of SIS3, a specific Smad3 inhibitor [11,21], did not alter the levels of Foxp3 expression in Smad2 CKO $CD4^+$ cells (**Figure 3D**), excluding the possibility that Smad2 and Smad3 compensate for each other in the promotion of $Foxp3^+$ cell differentiation. These

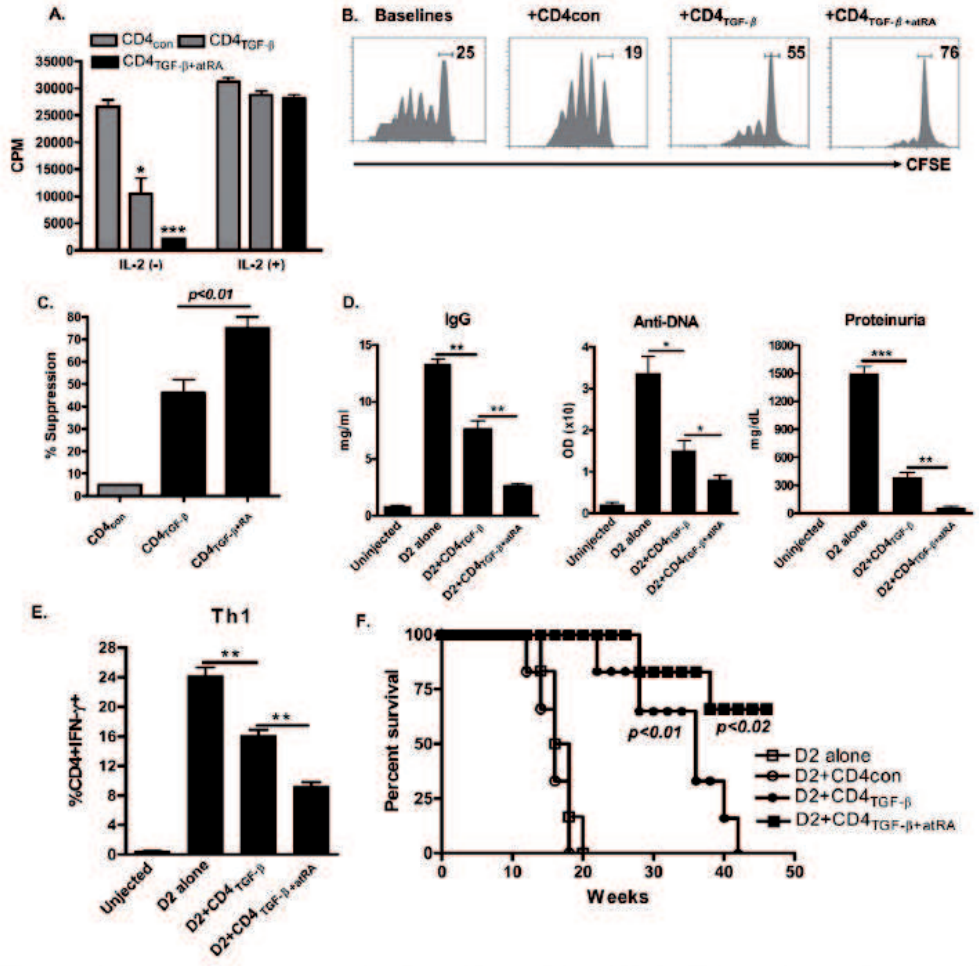

Figure 2. atRA enhances the suppressive activity of TGF-β-iTregs *in vitro* and *in vivo*. (A) CD4+ conditioned cells induced as in Fig. 1 were stimulated with anti-CD3 in the presence of APC for three days and their proliferation were determined by ^{3}H thymidine incorporation. (B, C) CFSE-labeled effector T cells were cultured at a 1:4 ratio with $CD4^+CD25^+$ cells isolated from medium treated $CD4^+$ T cells ($+CD4_{con}$), TGF-β-$CD4^+$ cells ($+CD4_{TGF-β}$), or atRA/TGF-β-$CD4^+$ cells ($+CD4_{TGF-β+atRA}$) or without (Baselines) for 3 days. Representative CFSE plots of the T effector cells are shown as in B, with the percentages of suppression on T effector cells quantified as in C. (D) A chronic GVHD with a lupus-like syndrome was induced in D2B6F1 mice as described previously. 80×10^6 D2 splenocytes were injected into the tail vein (D2 alone). Other groups received this number of D2 cells plus 5×10^6 $CD4_{con}$, $CD4_{TGF-β}$ or $CD4_{TGF-β+atRA}$. The IgG levels and anti-DNA concentration in mice sera four weeks after cells transfer were determined by ELISA, and proteinuria levels eight weeks after cell transfer were measured using Albustix reagent strips (Bayer, Elkart, IN). Values are Mean ± SEM of 6 mice combined with two separated experiments. (E) Similar experiments were conducted as in panel D. Spleen cells from specific groups of mice 3 weeks after cell transfer were harvested and stimulated with PMA (50 ng/ml), Ionomycin (500 ng/ml) for 5 hrs and BFA (5 μg/ml) for 4 hrs. IFN-γf, IL-4 and IL-17 expression on the CD4+ cells was determined by FACS. Data are Mean ± SEM of three separated experiments. (F) The survival of mice conducted in panel A was monitored. $CD4_{con}$ vs $CD4_{TGF-β}$ ($p<0.01$), $CD4_{TGF-β}$ vs $CD4_{TGF-β+atRA}$ ($p<0.02$) (n = 6, each group).
doi:10.1371/journal.pone.0024590.g002

results indicate that atRA promotes TGF-β-induced $Foxp3^+$ cell differentiation via Smad2- or 3-independent signaling pathways.

In addition to Smad signaling, MAPKs including ERK, JNK, and p38 constitute major non-Smad signaling pathways that play a supplemental role in mediating the intracellular responses to TGF-β. As ERK and JNK mainly contribute to iTreg differentiation [11], we examined whether these non-Smad pathway molecules are involved in iTreg promotion by atRA. In a protocol as similarly described for **Figure S1**, we added different MAPK inhibitors (or DMSO control) to cultures of $CD4^+$ T cells incubated with TGF-β. As shown in **Figure 4A**, addition of ERK inhibitors (ERKi) not only completely abolished the increased Foxp3 expression initiated by atRA, but decreased Foxp3 expression levels below that induced by TGF-β itself. We

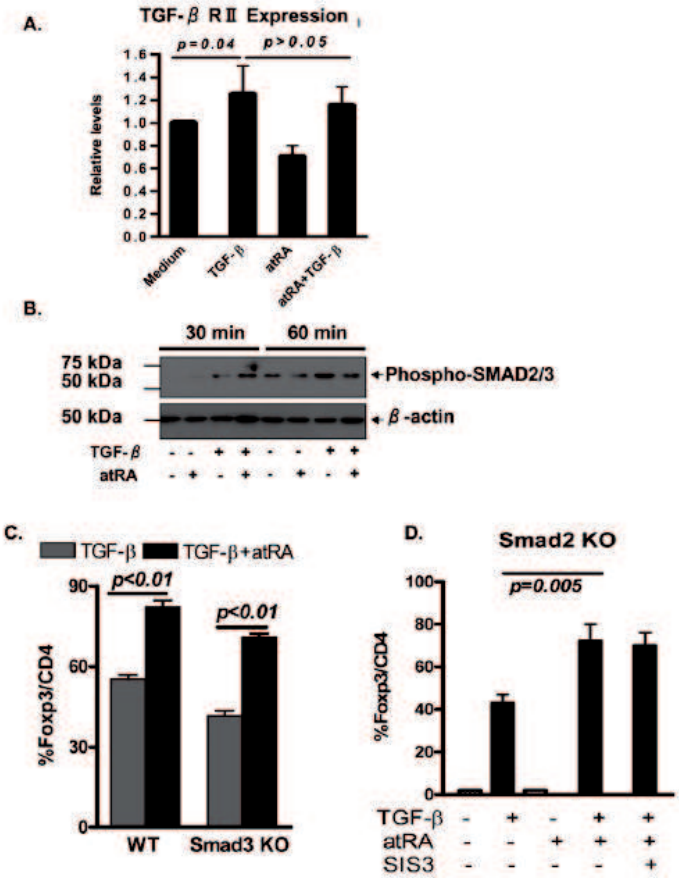

Figure 3. atRA promotes Foxp3 differentiation through Smad independent pathway. (A) Naive CD4$^+$ T cells were activated as in Fig. 1. TβRII expression in different cell groups was analyzed by qPCR. Data are Mean ± SEM of triplicate wells and representative of four separate experiments with similar results. (B) The phosphorylation of Smad2/3 was analyzed by Western blot in different cell groups at 30 min and 60 min. Data are representative of three separate experiments. (C) Naive CD4$^+$ cells isolated from Smad3 KO and wild type mice were TCR stimulated ± atRA ± TGF-β for 4 days and Foxp3 expression was analyzed by FACS. Values are Mean ± SEM of three separate experiments. (D) Naive CD4$^+$ cells were isolated from Smad CKO mice and SIS3 was added to some cultures. Foxp3 analysis was similarly conducted as panel C. Values are Mean ± SEM of three separate experiments.
doi:10.1371/journal.pone.0024590.g003

have revealed that this ERKi did not significantly suppress CD25 expression, T cell activation, as well as cell viability (**Figure S3**), excluding the possibility that ERKi suppresses Foxp3 induction because of its global restraint of TCR-stimulation. Addition of JNKi slightly but not significantly abolished the atRA-regulated Foxp3 increase. However, the addition of p38i had no effect on Foxp3 expression. LE540, an RAR antagonist [22], completely abolished the atRA-regulated Foxp3 increase, providing an ideal positive experimental control and further suggests that an atRA/RAR signal is needed for the atRA-regulated Foxp3 increase [15].

Since different subsets of MAPKs may have distinct roles in atRA-mediated promotion of iTreg differentiation, we next asked whether addition of atRA can alter the expression of activated MAPKs in TCR/TGF-β-primed CD4$^+$ cells. As shown in **Figure 4B and C**, naive CD4$^+$CD25$^-$ cells appeared to express activated P42/44 ERK and p38 by 12 hours after stimulating with anti-CD3/CD28 and IL-2 (med) by Western blotting with phosphospecific antibodies. In agreement with a previous report [11], addition of TGF-β alone significantly increased the activation of ERK but suppressed p38 phosphorylation. Addition of atRA alone to the cultures significantly decreased ERK and p38 activation. Notably, addition of both atRA and TGF-β significantly increased P42/44 ERK early activation and late persistence (even after 5-day culture) (**Figure 4B**) although this combination significantly decreased p38 activation (**Figure 4C**) and had less influence on JNK1 activation (**Figure 4D**). Adding a protein synthesis inhibitor, cycloheximide (CHX), to the cultures for 6 hours (**Figure 4E**) and 12 hours (not shown) did not significantly change ERK activation, suggesting that atRA mainly affects ERK activation rather than ERK synthesis (**Figure 4E**). To explain how atRA sustains the ERK activation (up to five days), we have demonstrated that addition of Bcl-2 inhibitor can suppress the

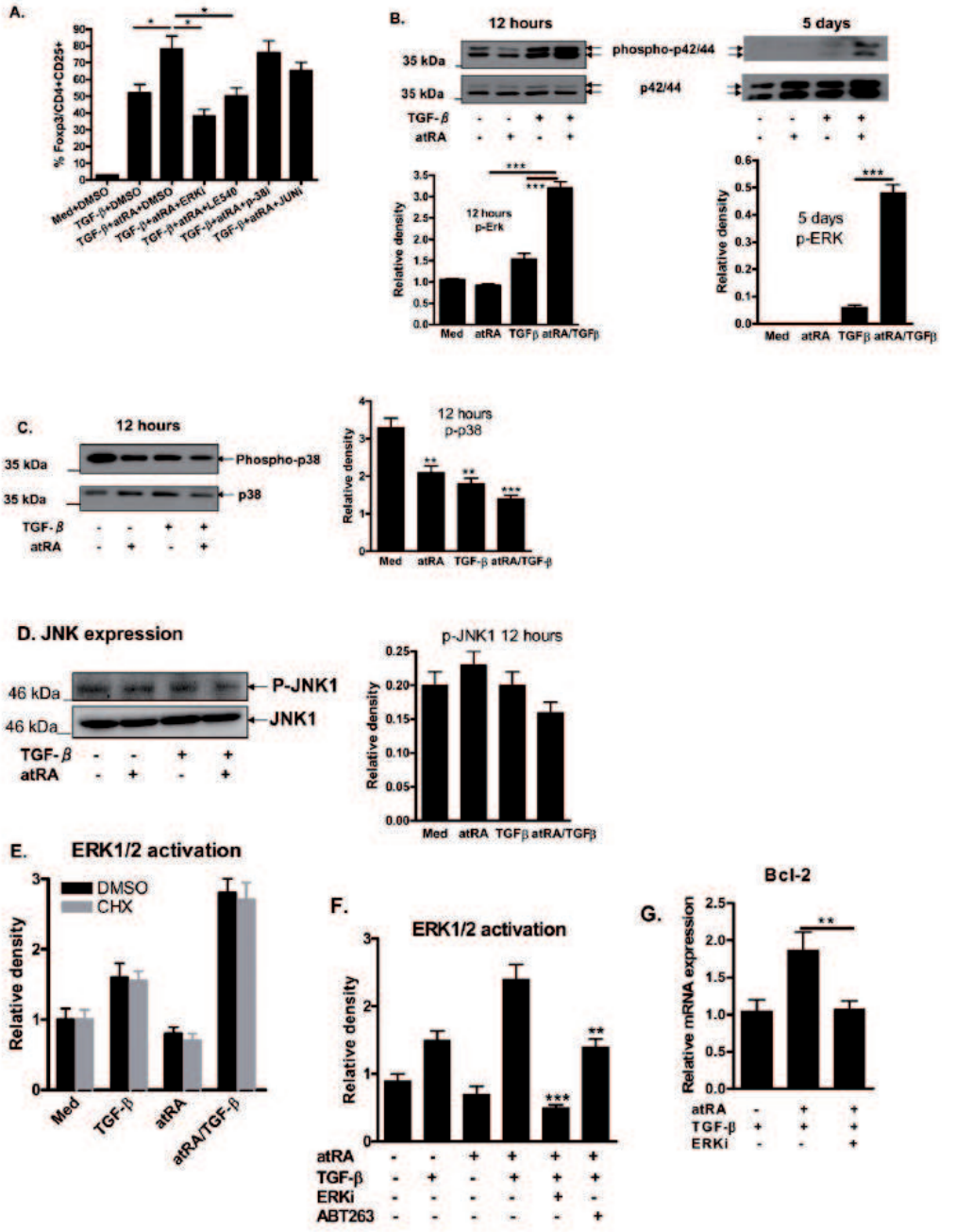
A.
% Foxp3/CD4+CD25+
Med+DMSO
TGF-β+DMSO
TGF-β+atRA+DMSO
TGF-β+atRA+ERKi
TGF-β+atRA+LE540
TGF-β+atRA+p-38i
TGF-β+atRA+JUNi
B.
12 hours
5 days
35 kDa
phospho-p42/44
35 kDa
p42/44
TGF-β
atRA
12 hours p-Erk
5 days p-ERK
Relative density
Med
atRA
TGFβ
atRA/TGFβ
C.
12 hours
35 kDa
Phospho-p38
35 kDa
p38
TGF-β
atRA
12 hours p-p38
Relative density
Med
atRA
TGF-β
atRA/TGF-β
D. JNK expression
46 kDa
P-JNK1
46 kDa
JNK1
TGF-β
atRA
p-JNK1 12 hours
Relative density
Med
atRA
TGFβ
atRA/TGFβ
E.
ERK1/2 activation
DMSO
CHX
Relative density
Med
TGF-β
atRA
atRA/TGF-β
F.
ERK1/2 activation
Relative density
atRA
TGF-β
ERKi
ABT263
G.
Bcl-2
Relative mRNA expression
atRA
TGF-β
ERKi

Figure 4. atRA promotes Foxp3 expression through ERK activation and maintenance. (A) Naïve CD4 T cells were stimulated as in Fig. 1. The different MAPKs inhibitors, DMSO or LE540 were added to some cultures. Values indicate Foxp3 expression and are Mean ± SEM of four separate experiments. (B–D) These cells were stimulated as in panel A for 12 and 120 hrs and ERK, p38 and JNK1 activation was examined by Western blot. The data shown are representative of three independent experiments. The relative density of activated ERK, p-38 and JNK1 to total ERK, p-38 and JNK1 was quantified by Quantity One software and values are Mean ± SEM of three separate experiments. (E) Naïve $CD4^+$ cells were stimulated as in panel B for 6–12 hrs. In some cultures, cycloheximide (20 μg/ml, Sigma, C1988) or control DMSO were added to cultures for 6 and 12 hrs. Values are Mean ± SEM of relative density of ERK1/2 activation for three separate experiments with 6 hrs stimulation. (F) Experiments were similarly conducted as in panel E and ERKi and ABT263 (Bcl-2 inhibitor, 5 μM, Selleck) were added to cultures for 12 hrs (F) to 5 days (not shown) and ERK1/2 activation was similarly determined as panel E. Values are Mean ± SEM of three separate experiments. *$p<0.05$, **$P<0.01$, ***$p<0.001$ (student *t* test).
doi:10.1371/journal.pone.0024590.g004

ERK early activation (**Figure 4F**) and late maintenance (not shown), suggesting that atRA enhances the cell survival of ERK activated CD4+ cells. Interestingly, addition of ERK inhibitor also suppressed the Bcl-2 mRNA up-regulation on CD4+ cells treated by atRA (**Figure 4G**), implicating the interaction of atRA, ERK and Bcl-2 promotes the development and stability of Foxp3+ Treg cells. Previous studies have reported that atRA inhibits ERK activation in some cell types and activates ERK in others [23,24]. These results demonstrate that the effect of atRA on the promotion of TGF-β-induced iTreg differentiation appear to be mainly through ERK activation and p38 inactivation. ERK and Bcl-2 interaction may promote the effect of atRA on iTreg promotion and maintenance.

atRA promotes iTreg development and maintenance through epigenetic modulation of histones rather than through DNA methylation of the Foxp3 gene locus

Given the critical role of atRA in iTreg promotion and maintenance, we sought to explore possible mechanisms for regulation at epigenetic levels. Others have reported that DNA methylation in Foxp3 gene promoter and CpG sites in the +4,201 to +4,500 intronic CpG island in conserved non-coding DNA sequence 3 (CNS3) at the Foxp3 gene locus affects Foxp3 expression and maintenance by Tregs [25,26]. We analyzed the DNA methylation in CpG islands in CNS3 of the Foxp3 locus using a previously described method [27]. Potential CpG methylation sites in CNS3 are outlined in **Figure 5A**. Naïve $CD4^+CD25^-$ cells were stimulated with TCR + IL-2 ± atRA and/or TGF-β for 4 days. Of the eight independent clones we noticed that only 1–3 demethylation sites scattered in these ten CpG sites, which may reflect a fluctuation. Each of the different treatments caused a slight shift in the methylation patterns, but overall they were not significantly different. Therefore, atRA-promoted iTreg development and stability does not appear to be related to the alteration of methylation status of CpG sites in CNS3 in the Foxp3 locus.

In addition to DNA CpG site methylation in Foxp3 locus, we also asked whether atRA affects post-translational modification of histones. As the transcribed regions of active genes are usually associated with modification at histone H3K4 [28], we therefore first examined methylation at this histone in the promoter as well as in the CNS elements at the Foxp3 locus. When CD4+ T cells were cultured with atRA or TGF-β, chromatin immunoprecipitations (ChIPs) revealed a significant enrichment for H3K4me3, one of characteristic epigenetic marks of actively transcribed genes for chromatin. The peak accumulation of H3K4me3 was observed on the CNS2 in CD4+ cells treated with atRA alone and on the promoter in the CD4+ cells treated with both atRA and TGF-β (**Figure 5B**), suggesting that while TGF-β promotes histone H3K4me4 methylation level at promoter region of Foxp3 and facilitates iTreg generation, atRA can dramatically enhance histone methylation level at CNS2 of Foxp3 and at least benefit iTreg maintenance. Previous work had documented that CNS3 facilitates Foxp3 induction whereas CNS2 favors the Foxp3 maintenance [29]. We examined histone H3k4me1, H3K4me2 and H3K9 methylation levels in the promoter and CNS1-3 region of Foxp3 locus in CD4+ cells and their enrichments did not correlate with atRA treatment in CD4+ cells (not shown).

In addition to methylation, lysine acetylation is well known for the epigenetic regulation of gene transcription in immune system [28]. Previous work has demonstrated that atRA increases histone acetylation of the Foxp3 promoter regions in natural Tregs [12]. To address whether atRA also affects histone acetylation of the Foxp3 promoter regions in iTregs, we measured the histone acetylation in the proximal 5′ promoter using chromatin immunoprecipitation (ChIP) in conjugation with qPCR. We examined the H3 N-terminal acetylation around the transcription start site (TSS) of Foxp3 gene. As shown in **Figure 5C**, TGF-β alone slightly increased the H3 acetylation in TSS, while atRA alone had no impact. However, atRA in concert with TGF-β significantly boosted the acetylation in the TSS. Phosphorylation at ser-5 and ser-7 of RNA polymerase II is a marker for active transcription [30]. We found that TGF-β but not atRA increased recruitment of phopshorylated RNA pol II in the TSS. Additionally, atRA marginally stimulated additional recruitment of RNA pol II in the promoter. Together, these results suggest that atRA promotes Foxp3 induction and maintenance through modulating epigenetic settings either in a locus-specific manner or globally on chromatin.

We further evaulated the functional outcome of epigenetic modulation regulated by atRA at the Foxp3 locus. To address this, we examined the extent to which atRA stimulation can modify the level of Foxp3 that is bound to chromatin. As shown in **Figure 5D**, we noted significantly greater amounts of Foxp3 in the nuclear fraction (nucleoplasm) 3 days after TCR stimulation with atRA plus TGF-β. Chromatin-associated Foxp3 was found to significantly increase over time in cultures containing both atRA and TGF-β relative to either alone (**Figure 5D**). These results indicate that atRA plus TGF-β stimulation increases the amount of Foxp3 associated with chromatin in $CD4^+$ T cells. Increased levels of Foxp3 in the chromatin fraction may facilitate its functional activity during an immune response.

Discussion

Emerging evidence indicates that atRA promotes the differentiation of TGF-β-induced $Foxp3^+$ cells [6]. We have confirmed this finding and also demonstrated that $CD4^+$ cells induced by a combination of atRA and TGF-β displayed superior suppressive function *in vitro* and adoptive transfer of these cells resulted in much better suppressive effects on lupus disease development in animal model compared to $CD4^+$ cells treated with TGF-β alone, implicating that a combination of atRA and TGF-β provides an ideal protocol for the preparation of iTreg population and their use in the clinical cell therapy in treating autoimmune disease and organ transplantation settings.

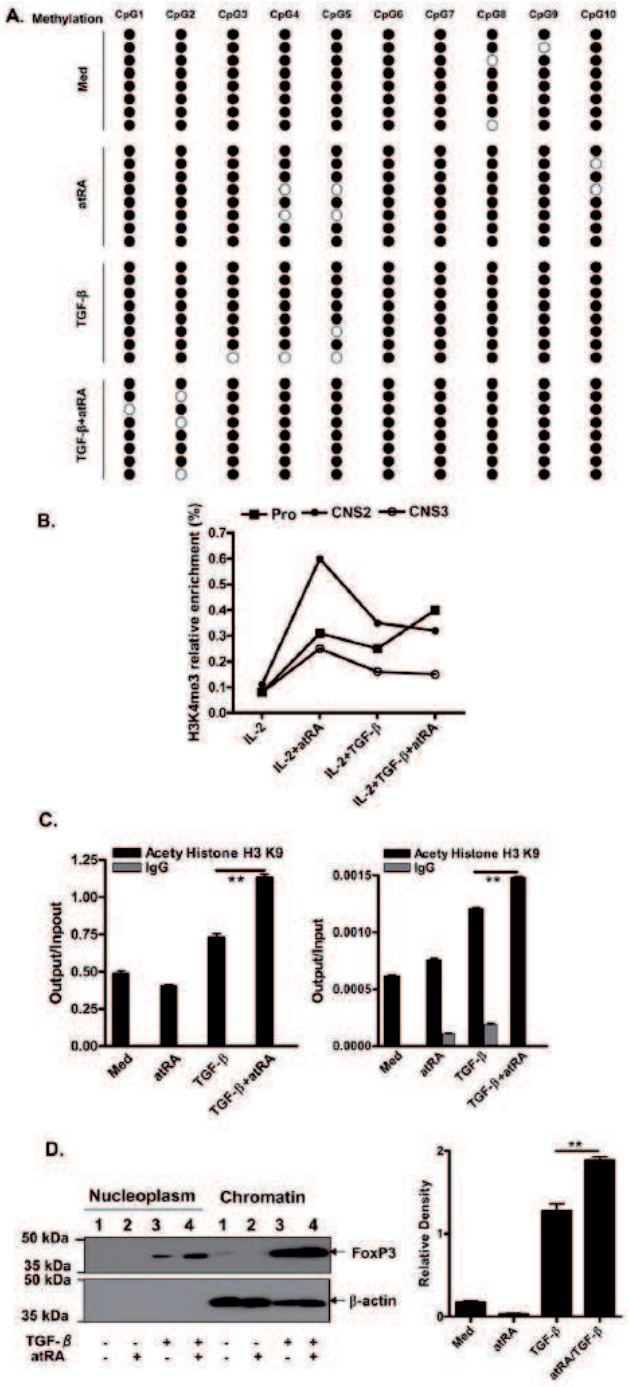
A.
Methylation
CpG1
CpG2
CpG3
CpG4
CpG5
CpG6
CpG7
CpG8
CpG9
CpG10
Med
atRA
TGF-β
TGF-β+atRA
B.
Pro
CNS2
CNS3
H3K4me3 relative enrichment (%)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
IL-2
IL-2+atRA
IL-2+TGF-β
IL-2+TGF-β+atRA
C.
Acety Histone H3 K9
IgG
Output/Input
1.25
1.00
0.75
0.50
0.25
0.00
**
Med
atRA
TGF-β
TGF-β+atRA
Acety Histone H3 K9
IgG
Output/Input
0.0015
0.0010
0.0005
0.0000
**
Med
atRA
TGF-β
TGF-β+atRA
D.
Nucleoplasm
Chromatin
1 2 3 4 1 2 3 4
50 kDa
35 kDa
50 kDa
35 kDa
FoxP3
β-actin
TGF-β
atRA
Relative Density
2
1
0
**
Med
atRA
TGF-β
atRA/TGF-β

Figure 5. atRA and TGF-β affect histone modification not DNA methylation in the Foxp3 gene locus, and also increase the DNA binding ability of Foxp3 protein. (A) Naive CD4+ cells were stimulated as Fig. 1. The DNA methylation status in CNS3 of the Foxp3 gene locus was determined by bisulfite sequencing analysis. Each line represents one DNA strand; open circle, unmethylated CpGs; filled circle, methylated CpGs. (B) Naive CD4+ cells were stimulated as in Panel A. ChIP-qPCR detection of H3K4me3 enrichment at promoter, CNS2 and CNS3 of Foxp3. Data are representative of two similar experiments. (C) A ChIP-qPCR analysis was performed to determine histone acetylation in the Foxp3 promoter. The β-actin gene was included as an internal control. Antibodies for N-terminal histone H3 and ser-5-phopho-RNA pol II were used for immunoprecipitation. The DNA fragments bound to the proteins were determined by qPCR analysis. Results showed are Mean ± SEM of four separate experiments. **$p<0.01$ (student *t* test). (D) Cellular compartments were fractionated into cytoplasmic, nuclear, and chromatin fractions. Equal amounts of proteins were separated by 8% SDS/PAGE, and then transferred to nitrocellulose membrane. Chromatin fractions were immunoblotted successively with antibodies against Foxp3 and β-actin. Relative density of Foxp3 expression was quantified with Quantity One software and values are Mean ± SEM of three separate experiments. **$p<0.01$ (student *t* test).
doi:10.1371/journal.pone.0024590.g005

The addition of atRA not only increased the differentiation of $Foxp3^+$ cells, but also maintained Foxp3 expression by TGF-β-primed $CD4^+$ cells. This finding could be explained by increased induction and/or decreased apoptosis. It has been well documented that activated lymphocytes are prone to activation-induced cell apoptosis. Although TGF-β prevents activated lymphocytes from apoptosis, our data demonstrate that a combination of atRA and TGF-β markedly increased the expression of anti-apoptotic protein such as Bcl-2 in $CD4^+$ cells, and that these increases are accompanied with decreased numbers of Annexin-V^+GFP^+ (apoptotic $Foxp3^+$) cells, an indication that these cells from apoptosis, eventually leading to the maintenance of Foxp3 expression. atRA-mediated upregulation of Bcl-2 seems to be dependent upon the RAR- rather than RXR-signal pathway, and this parallels with the role of atRA/RAR signal pathway in the Foxp3 induction [15]. Another possibility is that atRA can expand the $Foxp3^+$ cells that had been induced by TGF-β. In addition to the direct effect of atRA on promoting iTreg differentiation, atRA also suppresses the expansion of T effector cells and Th17 cell differentiation induced by a combination of IL-6 and TGF-β [6,8]. Cytokines produced by effector T cells will diminish the Treg phenotypes and increase the Treg plasticity [19], although others have reported that atRA can interfere directly with decrease in Treg conversion caused by costimulation and that it can enhance Treg conversion from naive T cells independently of secreted cytokines [31]. Recently, the role of atRA in maintenance of expanded nTregs has also been documented [19].

The addition of atRA to TGF-β endows $CD4^+$ cells with an almost complete anergic status (**Figure 2A**). This effect could be explained by their decreased IL-2 production and/or an increase in IL-10 production. Our data revealed that $CD4^+$ cells treated with TGF-β plus atRA exhibited a lower IL-2 production, a finding which might have contributed to their anergic phenotype. Although IL-10 production by $CD4^+$ cells treated with atRA and TGF-β was not markedly increased, its involvement in the role of atRA in iTreg cell development is still unclear. Another group recently reported that the addition of atRA to TGF-β suppresses the production of IL-10 [32]. Our previous work has demonstrated that TGF-β induces iTreg development through IL-10-independent pathway [33].

One of the interesting findings in the current study is that atRA/TGF-β-induced CD4+ regulatory T cells exhibit an enhanced suppressive T cell response *in vitro* and ameliorated lupus syndromes in a chronic GVHD animal model. These findings can not simply be explained by enhanced Foxp3 expression on $CD4^+$ cells treated with both atRA and TGF-β compared with TGF-β alone since use of purified individual $Foxp3^+$ cells from both groups of cells still displays the similarly functional differences. The remarkable suppressive function of atRA/TGF-β-induced $CD4^+$ regulatory T cells in lupus mice may be related to the alteration of the phenotypes, stability and functionality of these cells *in vivo*. Our findings have demonstrated that iTregs induced with atRA/ TGF-β survived longer and mainained higher Foxp3 expression compared to iTregs induced with TGF-β alone *in vivo*.

We have been focusing on an investigation of the molecular mechanism(s) by which atRA promotes the iTreg development and maintenance. An interrupted TβR signaling pathway would abolish the effect of atRA on the increase of $Foxp3^+$ cell production, and this suggests that atRA should affect TGF-β signaling rather than TGF-β affects atRA signaling. Previous studies have demonstrated quite clearly that the TGF-β signaling pathway is crucial for the induction of $Foxp3^+$ cells [11]. Conversely, the TGF-β-mediated induction is RA receptor α (RAR-α) independent although this receptor is crucial for the effect of atRA on T cell response [12]. In addition, atRA alone without TGF-β is unable to induce $Foxp3^+$ iTregs (**Figure S1A and C**).

The two pathways that can regulate TGF-β signaling include the Smad- and non-Smad pathways. Unlike TGF-β, which can upregulate TβRI and II expression by $CD4^+$ cells, atRA did not increase TβRI and II expression by $CD4^+$ cells (**Figure 3A**), implicating that atRA possibly regulates TGF-β signaling pathway through downstream molecules.

Smad2 and Smad3 are two main targets of activated TβRI and II. Accumulating evidence has revealed that Smad3 is essential for the suppressive effect of TGF-β on IL-2 production, T cell proliferation as well as Th2 type cytokine productions and Th2 type disease in the skin [11]. Although the role of Smad2 or Smad3 in the induction of iTregs is a non-essential one [11,20], it is unclear whether Smad2/3 is involved in the enhanced effects of atRA on $Foxp3^+$ cell differentiation induced by TGF-β. Our data showed that the addition of atRA to TGF-β slightly increased Smad2/3 activation in the 30 min time point but this effect rapidly disappeared by 60 min, implicating that atRA possibly enhances the induction of $Foxp3^+$ cells through the early initiation of Smad2/3 activation. One group has also demonstrated that atRA increases the quantity of $Foxp3^+$ Tregs by enhancing TGF-β-driven Smad3 signaling while another group recently demonstrated that atRA increases histone acetylation in the Smad3 binding sites and binding ability of activated Smad3 [7,34]. We found that even when using either Smad3 KO or Smad2 CKO mice, addition of atRA to TGF-β treatment still increased $Foxp3^+$ cell induction from TCR-activated $CD4^+$ cells although the levels and mean fluorescence intensity (MFI) of Foxp3 were slightly lower than that in WT mice. Another laboratory group has made a similar observation using Smad3 KO mice as well [31]. However, Xu *et al* recently observed that Smad3 is essential for the atRA promotion in Treg differentiation [34]. To explain the difference in the role of Smad3, we would suggest that differences in Treg generation protocols between the two laboratories might contribute to these disparate findings. While Xu *et al* used plate-bound TCR stimulation, our work has used anti-CD3/CD28 coated

beads. The exact role of Smad3 in the process of atRA-driven induced Treg development needs to be further explored with different protocols and other Smad3 knock-out strains.

In the current study, we demonstrate that atRA promotes iTregs differentiation through the MAPK pathway. We observed that the addition of ERK inhibitors completely abolished the enhanced effects of atRA on the promotion of TGF-β-induced Foxp3$^+$ cells. Using cell activation and proliferation experiments, we showed that ERK inhibitors specifically suppressed the increase of Foxp3$^+$ iTreg populations rather than nonspecific suppression of global T cell activation. Consistent with this result, we also observed that the addition of atRA to TGF-β significantly increased ERK activation without enhancing JNK. Conversely, atRA actually suppressed p38 activation. Since p38 activation is associated with T effector cell development and disease pathogenesis [35], atRA may have dual roles in both promoting Treg induction while suppressing T effector cells. The identification of these signaling pathways sheds light on the mechanisms by which atRA promotes the development of the induced Treg subset and will allow for the development of therapeutics that can target specific TGF-β responses in autoimmune and inflammatory diseases.

Our study has also illuminated an intrinsic relationship between Bcl-2 upregulation and ERK activation. ERK activation plays an important role in iTreg induction and promotion of atRA-mediated iTreg differentiation and maintenance. We found that blockade of ERK activation not only suppressed Foxp3 generation, but Bcl-2 upregulation as well. In addition, blockade of Bcl-2 decreases ERK activation, indicating Bcl-2 regulation and interaction of Bcl-2 and ERK may contribute to iTreg maintenance. Future study is needed to determine whether ERK can directly bind Bcl-2 in iTreg cells.

One of the most interesting findings in the current study is that atRA not only promotes iTreg generation and maintenance, but may also alter the binding ability of Foxp3 protein on chromatin. In mammals, epigenetic regulation is mediated by changes in chromatin structure, resulting from either histone modification or DNA methylation [28]. Moreover, we observed that addition of atRA did not alter DNA methylation status in CNS3 sites of the Foxp3 locus in TGF-β-primed CD4$^+$ cells. Although others have claimed that incomplete CpG demethylation in Tregs leads to the loss of Foxp3 expression and suppressive activity [25], the addition of atRA enhances and sustains Foxp3 expression in iTregs, and these cells display an exceptionally strong suppressive activity (**Figure 2B–F**), indicating that CpG DNA demethylation of the Foxp3 locus may not be a crucial factor in Treg stability and functionality.

Histone modification represents another possible mechanism in the regulation of gene expression and chromatin structure [36]. For example, histones can be methylated on lysine and arginine (R) residues, acetylated on lysine (K) residues, phosphorylated on serine and threonine (S/T) residues or ubiquitinated/SUMOylated on lysine residues [37]. Histone methylation is more complex and can be associated with either gene activation or gene repression depending on the methylated residue and the degree of methylation, since lysine residues can be mono-, di- or trimethylated (me1, me2 or me3). In general, the H3K4me3 level indicates gene activation while the H3k27me3 level indicates a repressed gene activity [28]. Our study revealed that atRA or TGF-β treatment can significantly increase the methylation in histone H3K4 in the Foxp3 gene promoter and both atRA and TGF-β treatment upregulated methylation of H3K4 in CNS2 at the Foxp3 locus, indicating that atRA and TGF-β have a synergistic role in the maintenance of Foxp3 but a separate role in Foxp3 induction. Although in an *in vitro* culture, we failed to observe that atRA alone induced Foxp3 expression, others have reported that atRA treatment induced Treg cell-dependent immune tolerance by suppressing both CD4(+) and CD8(+) Teff cells while promoting Treg cell induction and expansion *in vivo* [38]. Future study is needed to determine if atRA also affects the level of histone H3K27me3 modification at the CNS1-3 sites of the Foxp3 locus. Moreover, whether atRA can affect Foxp3 protein acetylation needs to be further addressed since acetylation-mediated Foxp3 protein displayed markedly increased stabilization and functionality [39].

An initial step in the relaxation of chromatin structure is the enzymatic addition of acetyl groups to the positively charged tails of histone H3 and H4 by histone acetyltransferases. Histone acetylation often accompanies gene transcription [40], which is required for the appropriate tissue-specific and context-dependent induction of many genes, and is opposed by the activity of histone deacetylases [41]. We hypothesized that the histone acetylation status of the Foxp3 promoter is responsible for the enhanced effects of atRA on iTreg promotion. Using a ChIP assay, we observed that TGF-β alone can indeed promote Foxp3 acetylation, a finding that is agreement with a previous report [42]. Of note, the histone (H3) acetylation status in iTregs induced by TGF-β and atRA was significantly increased, suggesting that histone/protein deacetylases (HDACs) regulate chromatin remodeling and Foxp3 gene expression and function. It is possible that atRA also affects the histone acetylation in the enhancer region of the Foxp3 gene [34]. Although portions of the total pool of Foxp3 can exist in diverse nuclear sites such as within the nucleoplasm, active and acetylated Foxp3 is preferentially, but not exclusively, bound to chromatin. In fact, histone deactylase inhibitors affect the chromatin binding pattern of Foxp3 [43]. These data suggest that the effect of atRA on Foxp3 mediated regulation of its target gene may be differential and site-dependent, which could explain the differential effect of atRA treatment on the differential induction and function of Foxp3+ Treg cells (**Figure 2**).

In summary, we found that atRA markedly promotes the phenotypic and functional development and maintenance of TGF-β-induced iTregs. Adoptive transfer of these cells to lupus mice highlights their efficient suppressive activity in controlling disease development. We explored the mechanisms involved in the iTreg promotion by atRA, finding that atRA synergizes with TGF-β signaling to induce iTregs mainly via ERK1/2 pathways. Moreover, we observed that atRA significantly increases histone modification including methylation and acetylation but does not affect the DNA demethylation status of CNS3 in the Foxp3 gene locus. We conclude that alterations in the induction and maintenance of Foxp3 gene expression and the conformational changes which promotes its binding on chromatin might both account for increased suppressive activity and stability of iTregs.

Materials and Methods

Mice

C57BL/6 mice were purchased from The Jackson Laboratory. *Smad2*$^{fx/fx}$ and *Smad3* KO mice were provided by Dr. Xiao-Fan Wang at Duke University and Dr. Michael Weinstein at Ohio State University. hCD2-Cre mice were provided by Dr. Dimitris Kioussis at National Institute for Medical Research. *Foxp3 GFP* knock-in mice were a gift from Dr. Talil Chatilla (UCLA). Lymphocyte-specific *Smad2* conditional knockout (CKO) mice were generated by crossing *Smad2*$^{fx/fx}$ with *Smad2*$^{fx/fx}$/hCD2-Cre mice. Mice with genotype of *Smad2*$^{fx/fx}$ were used as a normal control. All animals were treated according to National Institutes

of Health guidelines for the use of experimental animal with the approval of the University of Southern California Committee for the Use and Care of Animals (IACUC #11481).

Cell differentiation and functional assay

Naïve splenic $CD4^+CD25^-CD44^{low}$ cells were stimulated with anti-CD3/CD28 beads (Invitrogen, Carlsbad, CA) at a bead/T cell ratio of 1:5 + IL-2 (20 U/ml, R&D system) ± TGF-β (2 ng/ml, R&D system) for the generation of $Foxp3^+$ Tregs for the numbers of days indicated in the different figure legends. 3 μM SIS3 (Smad3 inhibitor, Calbiochem) was added to cultures one hour before TCR stimulation. An equivalent volume of DMSO was added to cultures as a control. To assess suppressive activities, $CD4^+CD25^+$ cells were isolated using MACS beads from $CD4^+$ conditioned cells after 4-day culture. T cells labeled with CFSE (Invitrogen, Carlsbad, CA) were stimulated with soluble anti-CD3 (0.025 μg/ml) with irradiated non-T cells as APC (1:1). The $CD4^+CD25^+$ cell population generated from different groups of mice were added at a ratio of 1:4 and suppression of cycling CFSE-labeled T cells was assessed as described previously [44]. In other experiments, a [^{3}H] thymidine incorporation assay was also used to evaluate the suppressive activity of iTregs [33]. AIM-V serum-free medium (Invitrogen Life Technologies) supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM HEPES (all obtained from Invitrogen Life Technologies) was used for the generation of $CD4^+$ iTreg or control cells. RPMI 1640 medium supplemented as above with the addition of 10% heat-inactivated FCS (HyClone Laboratories) was used for all other cultures.

Flow cytometry

Anti-TGF-β RII, CD4, CD25, CCR-9,$\alpha_4\beta_7$,CTLA-4, CD28, CD103, CD126, CD127, CD130 and Foxp3 fix/perm buffer set were purchased from Biolegend (San Diego, CA). Anti-TGF-β was a gift from eBioscience. For intracellular/intranuclear staining, cells were first stained with surface antibodies, then were fixed/permeabilized in cytofix/permeabization solution (Biolegend) and stained with anti-CTLA-4 or anti-Foxp3.

Real-Time PCR

Total RNA was extracted with the RNeasy mini kit (Qiagen, Valencia, CA). cDNA was generated using a Omniscript RT kit (Qiagen, Valencia, CA). Foxp3 mRNA expression was quantified with ABsolute SYBR Green ROX mix (Thermo, Waltham, MA). The samples were run in triplicate and the relative expression of Foxp3 was determined by normalizing the expression of each target to hypoxanthine guanine phosphoribosyl transferase (HPRT). Primer sequences were as follows: HPRT 5′-TGA AGA GCT ACT GTA ATG ATC AGT CAA C-3′ and 5′-AGC AAG CTT GCA ACC TTA ACC A-3′; Bcl-2, 5′-CCT GGC TGT CTC TGA AGA CC-3′ and 5′-CTC ACT TGT GGC CCA GGT AT-3′. TβRII, 5′-GGC TCT GGT ACT CTG GGA AA-3′ and 5′-AAT GGG GGC TCG TAA TCC T-3′.

Western blot analysis

Western blot was performed as previously described [44]. Biefly, cells were lysed in buffer containing 25 mM Tris-HCl, 1% deoxycholate, 0.35 M NaCl, phosphatase inhibitor solution (Cayman Chemical), and 1% Triton X-100 (Fischer Scientific). Protein quantity was assayed by bicinchoninic acid (Pierce, Chemical Co.) and 20 μg of protein was loaded per well on a 15% Tris-HCl gel (Bio-Rad). The contents of the gel were transferred in a Trans-Blot semi-dry transfer cell (Bio-Rad) onto nitrocellulose membranes (Amersham Biosciences). The membranes were incubated with different Abs. Ab-bound proteins were detected using an ECL Western blotting analysis system (Amersham Biosciences), and the membranes were exposed to Kodak Biomax XL x-ray film.

Nuclear and DNA preparation

Nuclear extracts were prepared according to the methods of Li *et al* with some modification [45]. Genomic DNA was prepared using the Qiagen Blood & Cell Culture DNA Kit or the Qiagen DNeasy Blood & Tissue Kit when working with smaller cell numbers. DNA concentration was determined with the NanoDrop spectrophotometer and quality was assessed by agarose gel electrophoresis.

Cell lysis, immunoprecipitation, and immunoblotting

Cell lysates were obtained by cell lysis in RIPA buffer (50 mM Tris•HCl, pH 7.4, 0.5% Nonidet P-40, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, with 1 mM PMSF, 1 μg/ml each of Aprotinin, leupeptin and pepstatin, 1 mM Na_3VO_4, and 1 mM NaF), followed by immunoprecipitation with the indicated antibodies, SDS/PAGE, and analyzed by Western blotting with standard procedures. ECL or ECLplus Western blotting detection reagents were used (Amersham Pharmacia Biosciences).

Foxp3 methylation analysis

Foxp3 methylation analysis was conducted with a method as previously reported [26]. In brief, genomic DNA was sonicated to a mean fragment size of 350–400 bp. Four micrograms of each sample was incubated with 200 μL of Protein A–Sepharose 4 Fast Flow beads (GE Healthcare) coated with 80 μg of purified MBD-Fc protein in 2 mL of Ultrafree-MC centrifugal filter devices (Amicon/Millipore) for 3 h at 4°C in buffer containing 300 mM NaCl. Beads were centrifuged to recover unbound DNA fragments (300 mM fraction) and subsequently washed with buffers containing increasing NaCl concentrations (350, 400, 450, and 1000 mM). All fractions were desalted using the MinElute PCR purification kit (Qiagen). The distribution of CpG methylation densities of individual MCIp fractions was controlled by qPCR using primers covering the imprinted SNRPN and a genomic region lacking CpGs (empty 6.2). Fractions containing unmethylated DNA (300~400 mM) or methylated DNA (≥450 mM) were pooled before subsequent labeling.

ChIP (chromatin immuno-precipitation)

ChIP assays were carried out with 5~10 million cells with or without stimulation by using EZ-ChIP (cat. 17–371, Upstate Biotechnology) according to the manufacturer's instructions. After sonication on ice, the chromatin solution was centrifuged for 10 min at 6,000× g. mIgG (Upstate Biotechnology), anti-acetyl histone H3 (Upstate Biotechnology), or anti-mono-methyl-histone-K4 H3 (Cell signaling), or anti-di or tri-methyl-histone-K4 H3 (Abcam), or anti-Foxp3 (e-Bioscience) were used for immunoprecipitation of the supernatant. ChIP analysis was carried out according to the manufacturer's protocol (Upstate/Millipore, Billerica, Massachusetts, United States). Cells ($1\sim5\times10^6$) were fixed with 1% formaldehyde, and chromatin was fragmented by ultrasound. For all ChIP samples, sheared chromatin was predeared by incubation with ProteinA-agarose/salmon sperm DNA (Upstate/Millipore). Subsequently, chromatin was immunoprecipitated by overnight incubation at 4°C with 4-μg antibodies (rabbit isotype, #2027, Santa Cruz Biotechnology, Santa Cruz, California, United States; anti-acetyl-histone H3,

#06–599, Upstate/Millipore; anti–acetyl-histone H4, #06–866, Upstate/Millipore; anti–tri- or di-methyl-K4-histone H3, ab303938, abcam; and anti-mono-methyl-K4-histone H3, #5326, cell signaling.) followed by incubation with Protein A-agarose/salmon sperm DNA for 1 h. Precipitates were defixed and DNA was purified by using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany). The amount of immunoprecipitated DNA was quantified by real-time PCR with LightCycler (Roche Applied Science, Basel, Switzerland) using SYBR Green and the following *Foxp3* primer pair 5′-GAC TCA AGG GGG TCT CA-3′; 5′-TTG GGC TTC ATC GGC AA-3′. For histone methylation level assay at *Foxp3* regions, following primers were used: Promoter forward primer: 5′-CTGAGGTTTGGAGCA-GAAGGA-3′, reverse primer: 5′-TCTGAAGCCTGCCATGTGAA -3′; CNS2 forward primer: 5′-GTTGCCGATGAA GCCCAAT -3′; reverse 5′- ATCTGGGCCCTGTTGTCACA-3′; CNS3 forward primer: 5′- AATGAATG AGACACAGAACTATTAAGATGA -3′; reverse primer: 5′-CAGACGGTGCCACCATGAC -3′.

Statistical analysis

Results were calculated by using GraphPad Prism 4.0 software (GraphPad Spftware, San Diego, CA) are presented as mean ± SEM. Differences in Kaplan-Meier survival curves were analyzed by the log-rank test. Student *t* test was used to assess statistical significance between two groups, and one-way ANOVA and/or non-parametric tests were used to assess statistical significance among multi-groups. Protein quantification in Western Blot was analyzed by Quantity One software (Bio-Rad, Hercules, CA). P value<0.05 is considered as statistically significant difference.

This work was presented in the plenary session of the 2009 annual conference of American College of Rheumatology in Philadelphia.

Supporting Information

Figure S1 atRA enhances Foxp3 expression induced by TGF-β in $CD4^+$ T cells. Naïve $CD4^+$ T cells isolated from C57BL/6 (A) or $Foxp3^{gfp}$ (B) spleen using magnetic beads were stimulated by anti-CD3/CD28 beads ± TGF-β ± atRA for 4 days. Foxp3 expression was analyzed by flow cytometry. Values are Mean ± SEM (A) and representative (B) of five separate experiments. (C) Foxp3 expression by these cells was analyzed by Western Blot (atRA, 0.2 μM). Results are representative of three separate experiments. (D) Absolute numbers of $Foxp3^+$ (GFP^+) cells were presented in different time points of these cells. Mean ± SEM of four independent experiments is shown. (E) Cell phenotypes were analyzed by two different cell subsets. Data is representative of four separate experiments.
(TIF)

Figure S2 Purified $CD4^+Foxp3^+$ cells induced by atRA and TGF-β resulted in increased suppressive activity *in vitro*. Naïve $CD4^+$ T cells isolated from $Foxp3^{gfp}$ spleen using magnetic beads were stimulated by anti-CD3/CD28 beads ± TGF-β ± atRA for 4 days. Foxp3 (GFP) expression by $CD4^+$ cells was analyzed and sorted by flow cytometry after cultures. Freshly sorted GFP^+ (nTregs) and GFP^- (control) cells in $Foxp3^{gfp}$ mice were served as positive or negative controls. These cells were added to anti-CD3-stumlated GFP^- T responder cells in the presence of APC and their suppressive activity was analyzed by thymidine [H^3] incorporation assay as previously described [16]. Mean ± SEM of triplicate experimental data in each group is shown. Data is representative of three separate experiments.
(TIF)

Figure S3 MAPK inhibitors did not affect the cell viability. Naïve CD4 T cells were stimulated with anti-CD3/CD28 coated beads ± TGF-β ± atRA for 4 days. The different MAPKs inhibitors, DMSO or LE540 were added to some cultures. Total viable cell numbers were counted in each well. Values indicate viable cell counts and are Mean ± SEM of four separate experiments.
(TIF)

Author Contributions

Conceived and designed the experiments: SGZ ZML. Performed the experiments: LL JM QL JW YPH DB BL ZYL YL MGC ZXX. Analyzed the data: LL WS BL ZML VQ BR. Wrote the paper: SGZ.

References

1. Giguere V (1994) Retinoic acid receptors and cellular retinoid binding proteins: complex interplay in retinoid signaling. Endocr Rev 15: 61–79.
2. Reifen R, Nur T, Ghebermeskel K, Zaiger G, Urizky R, et al. (2002) Vitamin A deficiency exacerbates inflammation in a rat model of colitis through activation of nuclear factor-kappaB and collagen formation. J Nutr 132: 2743–2747.
3. Winoto A, Littman DR (2002) Nuclear hormone receptors in T lymphocytes. Cell 109: S57–S66.
4. Zunino SJ, Storms DH, Stephensen CB (2007) Diets rich in polyphenols and vitamin A inhibit the development of type I autoimmune diabetes in nonobese diabetic mice. J Nutr 137: 1216–1221.
5. Racke MK, Burnett D, Pak SH, Albert PS, Cannella B, et al. (1995) Retinoid treatment of experimental allergic encephalomyelitis. IL-4 production correlates with improved disease course. J Immunol 154: 450–458.
6. Mucida D, Park Y, Kim G, Turovskaya O, Scott I, et al. (2007) Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science 317: 256–260.
7. Xiao S, Jin H, Korn T, Liu SM, Oukka M, et al. (2008) Retinoic acid increases Foxp3+ regulatory T cells and inhibits development of Th17 cells by enhancing TGF-beta-driven Smad3 signaling and inhibiting IL-6 and IL-23 receptor expression. J Immunol 181: 2277–2284.
8. Hill JA, Hall JA, Sun CM, Cai Q, Ghyselinck N, et al. (2008) Retinoic acid enhances Foxp3 induction indirectly by relieving inhibition from CD4+CD44hi Cells. Immunity 29: 758–770.
9. Zheng SG, Wang JH, Koss MN, Quismorio F Jr., Gray JD, et al. (2004) CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. J Immunol 172: 1531–39.
10. Nugent P, Potchinsky M, Lafferty C, Greene RM (1995) TGF-beta modulates the expression of retinoic acid-induced RAR-beta in primary cultures of embryonic palate cells. Exp Cell Res 220: 495–500.
11. Lu L, Wang J, Zhang F, Chai Y, Brand D, et al. (2010) Role of SMAD and non-SMAD signals in the development of Th17 and regulatory T cells. J Immunol 184: 4295–4306.
12. Kang SG, Lim HW, Andrisani OM, Broxmeyer HE, Kim CH (2007) Vitamin A metabolites induce gut-homing FoxP3+ regulatory T cells. J Immunol 179: 3724–3733.
13. Sakoe Y, Sakoe K, Kirito K, Ozawa K, Komatsu N (2010) FOXO3A as a key molecule for all-trans retinoic acid-induced granulocytic differentiation and apoptosis in acute promyelocytic leukemia. Blood 115: 3787–3795.
14. Ueki S, Mahemuti G, Oyamada H, Kato H, Kihara J, et al. (2008) Retinoic acids are potent inhibitors of spontaneous human eosinophil apoptosis. J Immunol 181: 7689–7698.
15. Schambach F, Schupp M, Lazar MA, Reiner SL (2007) Activation of retinoic acid receptor-alpha favours regulatory T cell induction at the expense of IL-17-secreting T helper cell differentiation. Eur J Immunol 37(9): 2396–2399.
16. Zhou X, Wang J, Shi W, Brand DD, Liu Z, et al. (2010) Isolation of purified and live Foxp3+ regulatory T cells using FACS sorting on scatter plot. J Mol Cell Biol 2(3): 164–169.
17. Rolink AG, Radaszkiewicz T, Pals ST, van der Meer WG, Gleichmann E (1982) Allosuppressor and allohelper T cells in acute and chronic graft-vs-host disease. I. Alloreactive suppressor cells rather than killer T cells appear to be the decisive effector cells in lethal graft-vs.-host disease. J Exp Med 155: 1501–1522.
18. Tone Y, Furuuchi K, Kojima Y, Tykocinski ML, Greene MI, et al. (2008) Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat Immunol 9: 194–202.
19. Zhou X, Kong N, Wang J, Fan H, Zou H, et al. (2010) Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. J Immunol 185: 2675–79.

20. Takimoto T, Wakabayashi Y, Sekiya T, Inoue N, Morita R, et al. (2010) Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. J Immunol 185: 842–855.
21. Jinnin M, Ihn H, Tamaki K (2006) Characterization of SIS3, a novel specific inhibitor of Smad3, and its effect on transforming growth factor-beta1-induced extracellular matrix expression. Mol Pharmacol 69: 597–607.
22. Alique M, Herrero JF, Lucio-Cazana FJ (2007) All-trans retinoic acid induces COX-2 and prostaglandin E2 synthesis in SH-SY5Y human neuroblastoma cells: involvement of retinoic acid receptors and extracellular-regulated kinase 1/2. J Neuroinflammation 4: 1.
23. Zhang H, Rosdahl I (2004) Expression of p27 and MAPK proteins involved in all-trans retinoic acid-induced apoptosis and cell cycle arrest in matched primary and metastatic melanoma cells. Int J Oncol 25: 1241–1248.
24. Komi Y, Sogabe Y, Ishibashi N, Sato Y, Moriwaki H, et al. (2010) Acyclic retinoid inhibits angiogenesis by suppressing the MAPK pathway. Lab Invest 90: 52–60.
25. Floess S, Freyer J, Siewert C, Baron U, Olek S, et al. (2007) Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol 5: e38.
26. Kim HP, Leonard WJ (2007) CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J Exp Med 204(7): 1543–1551.
27. Zhang Q, Wang HY, Liu X, Wasik MA (2007) STAT5A is epigenetically silenced by the tyrosine kinase NPM1-ALK and acts as a tumor suppressor by reciprocally inhibiting NPM1-ALK expression. Nat Med 13: 1341–1348.
28. Wei G, Wei L, Zhu J, Zang C, Hu-Li J, et al. (2009) Global mapping of H3K4me3 and H3K27me3 reveals specificity and plasticity in lineage fate determination of differentiating CD4+ T cells. Immunity 30(1): 155–167.
29. Zheng Y, Josefowicz S, Chaudhry A, Peng XP, Forbush K, et al. (2010) Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature 463(7282): 808–812.
30. Chapman RD, Heidemann M, Albert TK, Mailhammer R, Flatley A, et al. (2007) Transcribing RNA polymerase II is phosphorylated at CTD residue serine-7. Science 318: 1780–1782.
31. Nolting J, Daniel C, Reuter S, Stuelten C, Li P, et al. (2009) Retinoic acid can enhance conversion of naive into regulatory T cells independently of secreted cytokines. J Exp Med 206: 2131–2139.
32. Maynard CL, Harrington RD, Helms WS, Oliver JR, Stephensen CB, et al. (2009) Contrasting roles for all-trans retinoic acid in TGF-beta-mediated induction of Foxp3 and Il10 genes in developing regulatory T cells. J Exp Med 206: 343–357.
33. Lu L, Ma J, Wang X, Wang J, Zhang F, et al. (2009) Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. Eur J Immunol 40: 142–152.
34. Xu L, Kitani A, Stuelten C, McGrady G, Fuss I, et al. (2010) Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. Immunity 33: 313–325.
35. Ono K, Han J (2000) The p38 signal transduction pathway: activation and function. Cell Signal 12(1): 1–13.
36. Jaenisch R, Bird A (2003) Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 33 Suppl: 245–254.
37. Verrier L, Vandromme M, Trouche D (2011) Histone demethylases in chromatin cross-talks. Biol Cell 103(8): 381–401.
38. Van YH, Lee WH, Ortiz S, Lee MH, Qin HJ, et al. (2009) All-trans retinoic acid inhibits type 1 diabetes by T regulatory (Treg)-dependent suppression of interferon-gamma-producing T-cells without affecting Th17 cells. Diabetes 58(1): 146–155.
39. van Loosdregt J, Vercoulen Y, Guichelaar T, Gent YY, Beekman JM, et al. (2010) Regulation of Treg functionality by acetylation-mediated Foxp3 protein stabilization. Blood 115(5): 965–974.
40. Fischle W, Wang Y, Allis CD (2003) Binary switches and modification cassettes in histone biology and beyond. Nature 425: 475–479.
41. Ng HH, Bird A (2000) Histone deacetylases: silencers for hire. Trends Biochem Sci 25: 121–126.
42. Li B, Reynolds JM, Stout RD, Bernlohr DA, Suttles J (2009) Regulation of Th17 differentiation by epidermal fatty acid-binding protein. J Immunol 182: 7625–7633.
43. Samanta A, Li B, Song X, Bembas K, Zhang G, et al. (2008) TGF-beta and IL-6 signals modulate chromatin binding and promoter occupancy by acetylated FOXP3. Proc Natl Acad Sci USA 105: 14023–27.
44. Zheng SG, Wang J, Wang P, Gray JD, Horwitz DA (2007) IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. J Immunol 178: 2018–2027.
45. Li B, Samanta A, Song X, Iacono KT, Bembas K, et al. (2007) FOXP3 interactions with histone acetyltransferase and class II histone deacetylases are required for repression. Proc Natl Acad Sci USA 104: 4571–4576.

VIII. OVERALL DISCUSSION AND PERSPECTIVE

In the current study, we have gained several novel observations: 1). iTregs are equivalent with nTregs in the prevention of autoimmune diseases. 2). Both antigen-specific or polyclonal iTregs suppress autoimmune diseases. 3). iTregs but not nTregs suppressed Th17 cell differentiation *in vitro* and *in vivo* in the presence of IL-6 and other pro-inflammatory cytokines. 4). Unlike nTregs, iTregs are stable in the inflammatory condition *in vivo*. 5). iTregs rather than nTregs suppressed the established CIA. 6). iTregs infusion enhances the $Foxp3^+$ cell frequency and decreases the Th17 cell numbers in disease models. 7). iTregs induce the formation of tolerognic DCs and these DCs then suppress autoimmune diseases through either direct or indirect mechanisms. 8). Both TGF-β and IL-10 are required for the suppressive activity of iTregs *in vivo*, interestingly, only TGF-β is responsible for the induction of tolerogenic DC formation. 9). Tolerogenic DCs suppress disease mainly through TGF-β signaling pathway. 10). atRA promotes and sustains the $Foxp3^+$ regulatory T cells through increasing histone methylation and acetylation within the promoter and conserved non-coding DNA sequence (CNS) elements at the Foxp3 gene locus. Most importantly, we have identified that iTregs possess the different features in the inflammatory conditions and these cells might be superior in the controlling the established autoimmune diseases compared to nTregs. Taken together, these results strongly implicate that the manipulation of iTregs might provide a novel approach in the treatment of patients with autoimmune diseases. It deserves to have a clinical trial to develop human iTreg cell therapy in patients with autoimmune diseases although data generated from animal has to be carefully explained in human.

VIII.1 iTregs are stable and functional in the inflammatory condition

Compelling evidence has now suggested that nTregs are plastic and unstable in the inflammatory condition. Although the significance of this plasticity is not yet established, it is very likely that the plasticity of nTregs decreases the immunosuppressive ability of these cells to suppress immune response and relative diseases. These statements can be proofed by the observations that nTregs had the poorly therapeutic effects on the established autoimmune diseases and Th17-mediated diseases (Huter et al., 2008; Zhou et al., 2010a). It is also less known whether they have become pathogenic and effector cells when nTregs display their plasticity in the inflammatory condition.

Unlike nTregs, iTregs are completely resistant to Th17 cell conversion. We have first reported this observation and the result was further confirmed by other researchers recently (O'Connor et al.,

2010). Although iTregs may also convert to Th1 cells in the certain conditions, this conversion did not affect their suppressive activity (O'Connor et al., 2010).

While most studies demonstrating the different stabilities of Treg cell subsets have been conducted in the experimental systems *in vitro*, we now provided further evidence that iTregs but not nTregs are stable in the established CIA *in vivo*. We clearly demonstrated that most of nTregs have converted to Th17 cells in draining lymph nodes around 10 days after cell transfer although they are less converted to Th17 cells in the spleens. Interestingly, few of nTregs can convert to Th1, Th2 and Tfh cells even in the draining lymph nodes in CIA. These cells are no longer suppressor cells after they are sorted out and functionally assayed. This is curious whether these cells begin to cause diseases. We plan to transfer these cells to Rag-1 KO mice to learn whether these cells can cause colitis and EAE in the future experiments. Unlike nTregs, iTregs seem to be resistant to Th17 cell conversion in the draining lymph nodes and spleens. Similarly, few of iTregs can convert to Th1, Th2 and Tfh cells. When these cells were sorted out, they still suppressed immune responses. These results implicate that iTregs have a more stable feature in the inflammatory condition.

One of interesting findings in the current project is that iTregs are more numerous than nTregs in the established CIA. In addition to nTregs conversion to Th17 cells, we also demonstrated that nTregs predispose to cell apoptosis. Conversely, iTregs seem to be more resistant to cell apoptosis in the inflammatory condition. This provides another advantage for iTreg cell therapy in the autoimmune and inflammatory diseases.

It has to be mentioned that the mechanisms underlying the iTreg cell stability is less known. Although we have observed that iTregs expressed much lower IL-6R and subsequently lower levels of phosphorylated Stat3 after stimulating with IL-6 compared to nTregs, this is possible that iTregs may produce other specific proteins or transcription factors that we have not yet identified contribute to iTreg cell stability. We will focus our study on this aspect in the next 2-3 years.

VIII.2 iTregs but not nTregs ameliorate established autoimmune and Th17-mediated diseases

iTregs are not only resistant to T effector cell conversion, but also suppress the Th17 cell differentiation from naive $CD4^+$ cells when stimulated with anti-CD3/CD28 antibodies in the presence of IL-6 and TGF-β. This finding is novel and interesting. Although this is reasonable that the addition of nTregs to Th17-polarizing cell culture system do not inhibit the Th17 cell

differentiation since exogenous IL-6 can make nTregs themselves to become Th17 cells and addition of exogenous TGF-β promote this Th17 conversion.

How iTregs suppress Th17 cell differentiation in this system is unclear. Several factors can explain this interesting finding. 1). iTregs may produce high level of active TGF-β. Current studies have demonstrated that while low dose of active TGF-β favors Th17 cell differentiation, high level of active TGF-β may suppress Th17 cell production. 2). iTregs may produce more active IL-10. It has been documented that IL-10 may suppress Th17 cell development (Chaudhry et al., 2011). 3). iTregs still produce IL-2 and express T-bet (Th1 transcription factor), which may contribute to the effect of iTregs on Th17 cell suppression and their stability. Study has indicated that IL-2 can restrain the Th17 cell development (Laurence et al., 2007).

The ability of iTregs to suppress Th17 cell development can be further documented in current project in CIA *in vivo*. We demonstrated that iTregs but not nTregs can markedly down-regulate the $CD4^+IL\text{-}17^+$ cells in the draining lymph nodes in the established CIA after cell transfer. Additionally, only iTregs infusion can suppress $CD4^+IFN\text{-}\gamma^+$ cells whereas nTregs infusion conversely promotes $CD4^+IFN\text{-}\gamma^+$ cells at least in the spleens. Similarly, iTregs infusion can also suppress Th17 cell frequency in the ongoing asthma model. It is consistent with previous study that nTregs fails to control Th17-mediated autoimmune diseases (Huter et al., 2008), our data also demonstrated that nTregs has no therapeutic effect on established CIA. However, infusion of iTregs to the established CIA completely prevented the disease progression and significantly ameliorated the severities of CIA that had developed at least in some stages of CIA. Thus, the ability of iTregs to suppress Th17 cell development may provide a rationale that administration of iTregs can control and even treat Th17-mediated diseases.

Of great interest, infusion of iTregs to the established CIA can increase $Foxp3^+$ iTregs in the recipient mice. We believe the increase of $Foxp3^+$ cells in the recipients is due to increase of induced iTregs rather than the expansion of nTregs. Although we have used Helios staining to try to distinguish them, however, this staining is not convinced to make a solid conclusion since Helios is not a specific marker for Tregs. We have conducted more experiments to show this could be an "infectious tolerance" mechanism, where iTregs infusion may dominantly affect DCs to induce the formation of tolerogenic DCs and then induce the development of a new generation of iTregs in the recipients in the presence of appropriate antigen stimulation.

VIII.3 iTregs suppress disease via TGF-β and IL-10 dependent mechanisms

Although many studies have focused on the Treg's development and function, it is still less clear how Tregs suppress immune response and control autoimmunity. In an *in vitro* system, cell contact is always needed for the suppressive activity of iTregs since the neutralization of several soluble cytokines with immunosuppressive activity cannot abolish the suppressive effect but it can if the transwell experiments are set up. In the current project, we also found that iTregs, like nTregs, need cell contact to suppress immune responses. However, it is always said that the result produced from *in vitro* does not necessarily reflect the reality *in vivo*. We have observed this contradiction in the current work. We did find the TGF-β and its receptor signaling pathway plays a dominant role in the suppressing lupus diseases because the neutralization of TGF-β with anti-TGF-β antibody or use of ALK5 inhibitor can completely abolish the protective effect of iTregs on lupus. Additionally, blockade of IL-10R signaling also significantly ablated iTregs effect on lupus treatment. Thus, we believe that iTregs suppress autoimmune disease mainly through the production of TGF-β and IL-10 although other mechanisms may also exist.

VIII.4 iTregs induce the formation of tolerogenic DCs in the inflammatory condition

There are several reasons that promote us to raise a hypothesis that iTregs can act through an "infectious tolerance" mechanism in the controlling autoimmunity. 1). iTregs have a certain life-span, however, the single injection of iTregs can result in a fair long-term protective effect. Thus, the long-term effect could be executed by something else. 2). Using congenic markers (CD45.1 and CD45.2), we have found that injection of iTregs can increase the $Foxp3^+$ iTregs in the recipient in transplantation model (Zheng et al. 2006). Now we have further evidence that this result can be repeated in CIA and lupus models. 3). iTregs suppress autoimmunity mainly through TGF-β and IL-10 production. TGF-β plays a crucial role in the induction of $Foxp3^+$ iTregs and IL-10 promotes this effect. 4). DCs plays an important role in the maintaining immune tolerance in the haemostatic condition. Tolerogenic DCs are responsible for the immune tolerance and TGF-β and/or IL-10 can induce the formation of tolerogenic DCs.

To address this hypothesis, we now have demonstrated that the infusion of iTregs to lupus mice can induce the formation of a typical "tolerogenic DC" subset in lupus mice. These DCs expressed much lower levels of CD80 and CD86, weak ability to initiate the alloantigen responses and even induced other naive $CD4^+CD25^-$ cells to become $Foxp3^+$ Treg cells in the absence of exogenous TGF-β. When adoptive transfer of these DCs to lupus mice, they began to suppress lupus. We therefore

propose that iTregs can educate DCs to become tolerogenic DCs that will educate other T cells to become Foxp3$^+$ iTregs in a circuit or "infectious tolerance" fashion.

As TGF-β can induce Foxp3$^+$ iTregs in the absence of DCs *in vitro*, we also evaluated the role of DCs in the induction of Foxp3$^+$ iTregs *in vivo*. As TβR signaling is crucial for iTregs differentiation, we developed a DCs-specific TβRII condition KO mouse. We have revealed that iTregs will no longer induce the formation of tolerogenic DCs in DCs-specific TβRII KO mouse, suggesting that TβR signaling pathway on DCs is crucial for the induction of tolerogenic DCs and infectious tolerance.

Although both TGF-β and IL-10 are important for the suppressive activity of iTregs, it seems TGF-β plays a dominate role in the induction of tolerogenic DCs, although this conclusion needs to be further decided by using DCs-specific IL-10R condition KO mice. Moreover, tolerogenic DCs suppress lupus through TGF-β but not IL-10, suggesting the tolerogenic DCs formed by infusion of iTregs may be different from that induced by IL-10-priming *in vitro*.

VIII.5 Molecular mechanisms underlying the promotion of iTreg development

Several signaling pathways, such as the TGF-β/Smad, IL-2/IL-2R/STAT, T cell receptor (TCR) and costimulatory signaling pathways are needed for the induction of Foxp3 transcription and TGF-β receptor (TβR) signaling pathway is crucial.

Current studies have begun to look the role of TβR signaling in the regulation of iTregs differentiation. TβR signaling regulates target gene mostly through Smad pathway. Several groups have found that Smad3 plays a significant role in the iTregs differentiation and Smad3-NFAT-AP1 and STAT5 may form a complicated enhanceosome that regulates Foxp3 expression. However, non-Smad3 pathway may also play some roles in the differentiation of iTregs. We previously have reported that ERK and JNK, two MAPKs, are needed for the iTregs differentiation (Lu et al., 2010b).

TGF-β alone never induces 100% of naive CD4$^+$ cells to become Foxp3$^+$ cells. Thus, the discovery of other additives that promote the ability of TGF-β to induce and enhance Foxp3 expression and maintenance will be also an attractive approach for iTreg cell therapy. *All-trans* retinoic acid (atRA), a vitamin A metabolite, could be a proper candidate since atRA can promote iTregs and restrain Th17 cell differentiation (Mucida et al., 2007).

In this study, we further confirmed the ability of atRA to promote iTregs. However, we also observed the new ability of atRA to sustain Foxp3 expression by iTregs. This can be documented *in vitro* and *in vivo*. We also identified the mechanisms by which atRA sustains iTregs through suppressing the apoptosis of iTregs.

We first observed that atRA does not increase TβRI and II expression by $CD4^+$ cells, suggesting atRA affects the downstream of TβR signaling rather than TGF-β and TβR binding ability.

Although some groups reported that Smad3 plays a significant role in the regulation of Foxp3 expression, our study demonstrated that the ability of atRA to increase Foxp3 induction is not associated with Smad3 expression and upregulation because atRA maintains similar functional activity in $CD4^+$ cells isolated from Smad3 KO mice. Conversely, atRA promotes ERK activation and ERK pathway seems to be essential for this process, suggesting that non-Smad pathway plays an important role in atRA-mediated Foxp3 expression.

While others reported that DNA methylation in Foxp3 gene promoter and CpG sites in the +4,201 to +4,500 intronic CpG island in conserved non-coding DNA sequence 3 (CNS3) at the Foxp3 gene locus affects Foxp3 expression and maintenance by Tregs (Floess et al., 2007; Kim and Leonard, 2007). Our study observed that the enhanced effect of atRA on iTregs differentiation is not associated with the DNA demethylation in CpG islands in CNS3. More studies are needed to determine whether DNA CpG methylatonin Foxp3 non-coding region is important for Foxp3 expression and maintenance.

Nonetheless, we did find atRA affects the histone modification. Our study revealed that atRA or TGF-β treatment can significantly increase the methylation in histone H3K4 in the Foxp3 gene promoter and both atRA and TGF-β treatment can upregulate methylation of H3K4 in CNS2 at the Foxp3 locus, indicating that atRA and TGF-β have a synergistic role in the maintenance of Foxp3 but a separate role in Foxp3 induction.

One of the novel observations we made in this study is that atRA promotes the Foxp3 binding in chromatin. We observed that the histone (H3) acetylation status in iTregs induced by TGF-β and atRA is significantly increased, suggesting that histone/protein deacetylases (HDACs) regulate chromatin remodeling and Foxp3 gene expression and function. We conclude that alterations in the induction and maintenance of Foxp3 gene expression and the conformational changes which

promote its binding on chromatin might both account for increased suppressive activity and stability of iTregs induced by TGF-β after adding atRA.

VIII. 6. Future plans

In addition to science, PhD applicant (Song Guo Zheng) who is working as an investigator and director of immune tolerance center at University of Southern California (Los Angeles, US) and an investigator and chair of East Immune Institute at Tongji University Shanghai East Hospital (Shanghai, China) would like to make a further collaboration with investigators from CNRS (Orleans, France) to further study the development and function of regulatory T cells in autoimmunity and organ transplantation after his PhD degree is awarded in CNRS. To facilitate this triangle-side collaboration to pursue this goal, the selected investigators who work in three places will be re-appointed as visiting professors in other institutes and PhD students and postdoctoral fellows can be exchanged to receive further training in the different scientific and cultural environments. Eventually, three sides will collaborate to apply for the international funds and other major funds.

IX. REFERENCES

Allan, S.E., Crome, S.Q., Crellin, N.K., Passerini, L., Steiner, T.S., Bacchetta, R., Roncarolo, M.G., and Levings, M.K. (2007). Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. Int Immunol *19*, 345-354.

Amin, K., Ludviksdottir, D., Janson, C., Nettelbladt, O., Bjornsson, E., Roomans, G.M., Boman, G., Seveus, L., and Venge, P. (2000). Inflammation and structural changes in the airways of patients with atopic and nonatopic asthma. BHR Group. Am J Respir Crit Care Med *162*, 2295-2301.

Andersson, J., Tran, D.Q., Pesu, M., Davidson, T.S., Ramsey, H., O'Shea, J.J., and Shevach, E.M. (2008). CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. J Exp Med *205*, 1975-1981.

Anthoni, M., Fyhrquist-Vanni, N., Wolff, H., Alenius, H., and Lauerma, A. (2008). Transforming growth factor-beta/Smad3 signalling regulates inflammatory responses in a murine model of contact hypersensitivity. Br J Dermatol *159*, 546-554.

Apostolou, I., and von Boehmer, H. (2004). In vivo instruction of suppressor commitment in naive T cells. J Exp Med *199*, 1401-1408.

Bacchetta, R., Gambineri, E., and Roncarolo, M.G. (2007). Role of regulatory T cells and FOXP3 in human diseases. J Allergy Clin Immunol *120*, 227-235; quiz 236-227.

Bagavant, H., and Tung, K.S. (2005). Failure of CD25+ T cells from lupus-prone mice to suppress lupus glomerulonephritis and sialoadenitis. J Immunol *175*, 944-950.

Barczyk, A., Pierzchala, W., and Sozanska, E. (2003). Interleukin-17 in sputum correlates with airway hyperresponsiveness to methacholine. Respir Med *97*, 726-733.

Boxall, C., Holgate, S.T., and Davies, D.E. (2006). The contribution of transforming growth factor-beta and epidermal growth factor signalling to airway remodelling in chronic asthma. Eur Respir J *27*, 208-229.

Brand, D.D., Latham, K.A., and Rosloniec, E.F. (2007). Collagen-induced arthritis. Nat Protoc *2*, 1269-1275.

Buckner, J.H. (2010). Mechanisms of impaired regulation by CD4(+)CD25(+)FOXP3(+) regulatory T cells in human autoimmune diseases. Nat Rev Immunol *10*, 849-859.

Burchill, M.A., Yang, J., Vogtenhuber, C., Blazar, B.R., and Farrar, M.A. (2007). IL-2 receptor beta-dependent STAT5 activation is required for the development of Foxp3+ regulatory T cells. J Immunol *178*, 280-290.

Chai, J.G., Tsang, J.Y., Lechler, R., Simpson, E., Dyson, J., and Scott, D. (2002). CD4+CD25+ T cells as immunoregulatory T cells in vitro. Eur J Immunol *32*, 2365-2375.

Chaudhry, A., Samstein, R.M., Treuting, P., Liang, Y., Pils, M.C., Heinrich, J.M., Jack, R.S., Wunderlich, F.T., Bruning, J.C., Muller, W., and Rudensky, A.Y. (2011). Interleukin-10 signaling in regulatory T cells is required for suppression of Th17 cell-mediated inflammation. Immunity *34*, 566-578.

Chen, Q., Kim, Y.C., Laurence, A., Punkosdy, G.A., and Shevach, E.M. (2011). IL-2 controls the stability of Foxp3 expression in TGF-beta-induced Foxp3+ T cells in vivo. J Immunol *186*, 6329-6337.

Chen, W., Jin, W., Hardegen, N., Lei, K.J., Li, L., Marinos, N., McGrady, G., and Wahl, S.M. (2003). Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. J Exp Med *198*, 1875-1886.

Colonna, M., Trinchieri, G., and Liu, Y.J. (2004). Plasmacytoid dendritic cells in immunity. Nat Immunol *5*, 1219-1226.

Coombes, J.L., Siddiqui, K.R., Arancibia-Carcamo, C.V., Hall, J., Sun, C.M., Belkaid, Y., and Powrie, F. (2007). A functionally specialized population of mucosal CD103+ DCs induces Foxp3+ regulatory T cells via a TGF-beta and retinoic acid-dependent mechanism. J Exp Med *204*, 1757-1764.

Datto, M.B., Frederick, J.P., Pan, L., Borton, A.J., Zhuang, Y., and Wang, X.F. (1999). Targeted disruption of Smad3 reveals an essential role in transforming growth factor beta-mediated signal transduction. Mol Cell Biol *19*, 2495-2504.

Davidson, T.S., DiPaolo, R.J., Andersson, J., and Shevach, E.M. (2007). Cutting Edge: IL-2 is essential for TGF-beta-mediated induction of Foxp3+ T regulatory cells. J Immunol *178*, 4022-4026.

Discombe, G. (1946). Criteria of eosinophilia. Lancet *1*, 195.

Doherty, T., and Broide, D. (2007). Cytokines and growth factors in airway remodeling in asthma. Curr Opin Immunol *19*, 676-680.

Du Clos, T.W., Rubin, R.L., and Tan, E.M. (1986). Monoclonal antibody for DNA measurement in biological fluids. J Immunol Methods *88*, 185-192.

Ehrenstein, M.R., Evans, J.G., Singh, A., Moore, S., Warnes, G., Isenberg, D.A., and Mauri, C. (2004). Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. J Exp Med *200*, 277-285.

Fahlen, L., Read, S., Gorelik, L., Hurst, S.D., Coffman, R.L., Flavell, R.A., and Powrie, F. (2005). T cells that cannot respond to TGF-beta escape control by CD4(+)CD25(+) regulatory T cells. J Exp Med *201*, 737-746.

Fanta, C.H. (2009). Asthma. N Engl J Med *360*, 1002-1014.

Fantini, M.C., Becker, C., Monteleone, G., Pallone, F., Galle, P.R., and Neurath, M.F. (2004). Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. J Immunol *172*, 5149-5153.

Fantini, M.C., Becker, C., Tubbe, I., Nikolaev, A., Lehr, H.A., Galle, P., and Neurath, M.F. (2006). Transforming growth factor beta induced FoxP3+ regulatory T cells suppress Th1 mediated experimental colitis. Gut *55*, 671-680.

Favre, D., Mold, J., Hunt, P.W., Kanwar, B., Loke, P., Seu, L., Barbour, J.D., Lowe, M.M., Jayawardene, A., Aweeka, F.*, et al.* (2010). Tryptophan catabolism by indoleamine 2,3-dioxygenase 1 alters the balance of TH17 to regulatory T cells in HIV disease. Sci Transl Med *2*, 32ra36.

Floess, S., Freyer, J., Siewert, C., Baron, U., Olek, S., Polansky, J., Schlawe, K., Chang, H.D., Bopp, T., Schmitt, E.*, et al.* (2007). Epigenetic control of the foxp3 locus in regulatory T cells. PLoS Biol *5*, e38.

Flores-Borja, F., Jury, E.C., Mauri, C., and Ehrenstein, M.R. (2008). Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. Proc Natl Acad Sci U S A *105*, 19396-19401.

Fontenot, J.D., Gavin, M.A., and Rudensky, A.Y. (2003). Foxp3 programs the development and function of CD4+CD25+ regulatory T cells. Nat Immunol *4*, 330-336.

Gershon, R.K., and Kondo, K. (1970). Cell interactions in the induction of tolerance: the role of thymic lymphocytes. Immunology *18*, 723-737.

Godebu, E., Summers-Torres, D., Lin, M.M., Baaten, B.J., and Bradley, L.M. (2008). Polyclonal adaptive regulatory CD4 cells that can reverse type I diabetes become oligoclonal long-term protective memory cells. J Immunol *181*, 1798-1805.

Haas, J., Hug, A., Viehover, A., Fritzsching, B., Falk, C.S., Filser, A., Vetter, T., Milkova, L., Korporal, M., Fritz, B.*, et al.* (2005). Reduced suppressive effect of CD4+CD25high regulatory T cells on the T cell immune response against myelin oligodendrocyte glycoprotein in patients with multiple sclerosis. Eur J Immunol *35*, 3343-3352.

Hartl, D., Koller, B., Mehlhorn, A.T., Reinhardt, D., Nicolai, T., Schendel, D.J., Griese, M., and Krauss-Etschmann, S. (2007). Quantitative and functional impairment of pulmonary CD4+CD25hi regulatory T cells in pediatric asthma. J Allergy Clin Immunol *119*, 1258-1266.

Haxhinasto, S., Mathis, D., and Benoist, C. (2008). The AKT-mTOR axis regulates de novo differentiation of CD4+Foxp3+ cells. J Exp Med *205*, 565-574.

Heemskerk, M.H., Hagedoorn, R.S., van der Hoorn, M.A., van der Veken, L.T., Hoogeboom, M., Kester, M.G., Willemze, R., and Falkenburg, J.H. (2007). Efficiency of T-cell receptor expression in dual-specific T cells is controlled by the intrinsic qualities of the TCR chains within the TCR-CD3 complex. Blood *109*, 235-243.

Hellmark, T., Niles, J.L., Collins, A.B., McCluskey, R.T., and Brunmark, C. (1997). Comparison of anti-GBM antibodies in sera with or without ANCA. J Am Soc Nephrol *8*, 376-385.

Hippen, K.L., Merkel, S.C., Schirm, D.K., Nelson, C., Tennis, N.C., Riley, J.L., June, C.H., Miller, J.S., Wagner, J.E., and Blazar, B.R. (2011). Generation and large-scale expansion of human inducible regulatory T cells that suppress graft-versus-host disease. Am J Transplant *11*, 1148-1157.

Hoffmann, P., Boeld, T.J., Eder, R., Huehn, J., Floess, S., Wieczorek, G., Olek, S., Dietmaier, W., Andreesen, R., and Edinger, M. (2009). Loss of FOXP3 expression in natural human CD4+CD25+ regulatory T cells upon repetitive in vitro stimulation. Eur J Immunol *39*, 1088-1097.

Horwitz, D.A., Zheng, S.G., and Gray, J.D. (2008). Natural and TGF-beta-induced Foxp3(+)CD4(+) CD25(+) regulatory T cells are not mirror images of each other. Trends Immunol *29*, 429-435.

Horwitz, D.A., Zheng, S.G., Gray, J.D., Wang, J.H., Ohtsuka, K., and Yamagiwa, S. (2004). Regulatory T cells generated ex vivo as an approach for the therapy of autoimmune disease. Semin Immunol *16*, 135-143.

Housley, W.J., Adams, C.O., Nichols, F.C., Puddington, L., Lingenheld, E.G., Zhu, L., Rajan, T.V., and Clark, R.B. (2011). Natural but not inducible regulatory T cells require TNF-alpha signaling for in vivo function. J Immunol *186*, 6779-87

Huter, E.N., Stummvoll, G.H., DiPaolo, R.J., Glass, D.D., and Shevach, E.M. (2008). Cutting edge: antigen-specific TGF beta-induced regulatory T cells suppress Th17-mediated autoimmune disease. J Immunol *181*, 8209-8213.

Infante-Duarte, C., Horton, H.F., Byrne, M.C., and Kamradt, T. (2000). Microbial lipopeptides induce the production of IL-17 in Th cells. J Immunol *165*, 6107-6115.

Jana, S., Jailwala, P., Haribhai, D., Waukau, J., Glisic, S., Grossman, W., Mishra, M., Wen, R., Wang, D., Williams, C.B., and Ghosh, S. (2009). The role of NF-kappaB and Smad3 in TGF-beta-mediated Foxp3 expression. Eur J Immunol *39*, 2571-2583.

Kaplan, D.H., Li, M.O., Jenison, M.C., Shlomchik, W.D., Flavell, R.A., and Shlomchik, M.J. (2007). Autocrine/paracrine TGFbeta1 is required for the development of epidermal Langerhans cells. J Exp Med *204*, 2545-2552.

Kautz-Neu, K., Noordegraaf, M., Dinges, S., Bennett, C.L., John, D., Clausen, B.E., and von Stebut, E. (2011). Langerhans cells are negative regulators of the anti-Leishmania response. J Exp Med *208*, 885-891.

Kel, J.M., Girard-Madoux, M.J., Reizis, B., and Clausen, B.E. (2010). TGF-beta is required to maintain the pool of immature Langerhans cells in the epidermis. J Immunol *185*, 3248-3255.

Kim, H.P., and Leonard, W.J. (2007). CREB/ATF-dependent T cell receptor-induced FoxP3 gene expression: a role for DNA methylation. J Exp Med *204*, 1543-1551.

Koenecke, C., Czeloth, N., Bubke, A., Schmitz, S., Kissenpfennig, A., Malissen, B., Huehn, J., Ganser, A., Forster, R., and Prinz, I. (2009). Alloantigen-specific de novo-induced Foxp3+ Treg revert in vivo and do not protect from experimental GVHD. Eur J Immunol *39*, 3091-3096.

Kumar, M., Putzki, N., Limmroth, V., Remus, R., Lindemann, M., Knop, D., Mueller, N., Hardt, C., Kreuzfelder, E., and Grosse-Wilde, H. (2006). CD4+CD25+FoxP3+ T lymphocytes fail to suppress myelin basic protein-induced proliferation in patients with multiple sclerosis. J Neuroimmunol *180*, 178-184.

Laan, M., Palmberg, L., Larsson, K., and Linden, A. (2002). Free, soluble interleukin-17 protein during severe inflammation in human airways. Eur Respir J *19*, 534-537.

Lagna, G., Hata, A., Hemmati-Brivanlou, A., and Massague, J. (1996). Partnership between DPC4 and SMAD proteins in TGF-beta signalling pathways. Nature *383*, 832-836.

Lan, Q., Fan, H., Quesniaux, V., Ryffel, B., Liu, Z., and Zheng SG. (2011). Induced Foxp3+ regulatory T

cells: a potential new weapon to treat autoimmune and inflammatory diseases? J Mol Cell Biol. 2011 Nov 22. [Epub ahead of print]

Larche, M. (2007). Regulatory T cells in allergy and asthma. Chest *132*, 1007-1014.

Laurence, A., Tato, C.M., Davidson, T.S., Kanno, Y., Chen, Z., Yao, Z., Blank, R.B., Meylan, F., Siegel, R., Hennighausen, L*., et al.* (2007). Interleukin-2 signaling via STAT5 constrains T helper 17 cell generation. Immunity *26*, 371-381.

Letterio, J.J., Geiser, A.G., Kulkarni, A.B., Dang, H., Kong, L., Nakabayashi, T., Mackall, C.L., Gress, R.E., and Roberts, A.B. (1996). Autoimmunity associated with TGF-beta1-deficiency in mice is dependent on MHC class II antigen expression. J Clin Invest *98*, 2109-2119.

Li, C.R., Deiro, M.F., Godebu, E., and Bradley, L.M. (2011). IL-7 uniquely maintains FoxP3(+) adaptive Treg cells that reverse diabetes in NOD mice via integrin-beta7-dependent localization. J Autoimmun. Jul 9. [Epub ahead of print]

Liang, S., Alard, P., Zhao, Y., Parnell, S., Clark, S.L., and Kosiewicz, M.M. (2005). Conversion of CD4+ CD25- cells into CD4+ CD25+ regulatory T cells in vivo requires B7 costimulation, but not the thymus. J Exp Med *201*, 127-137.

Ling, E.M., Smith, T., Nguyen, X.D., Pridgeon, C., Dallman, M., Arbery, J., Carr, V.A., and Robinson, D.S. (2004). Relation of CD4+CD25+ regulatory T-cell suppression of allergen-driven T-cell activation to atopic status and expression of allergic disease. Lancet *363*, 608-615.

Lu, L., Ma, J., Li, Z., Lan, Q., Chen, M., Liu, Y., Xia, Z., Wang, J., Han, Y., Shi, W*., et al.* All-Trans Retinoic Acid Promotes TGF-beta-Induced Tregs via Histone Modification but Not DNA Demethylation on Foxp3 Gene Locus. PLoS One *6*, e24590.

Lu, L., Ma, J., Li, Z., Lan, Q., Chen, M., Liu, Y., Xia, Z., Wang, J., Han, Y., Shi, W*., et al.* (2011). All-Trans Retinoic Acid Promotes TGF-beta-Induced Tregs via Histone Modification but Not DNA Demethylation on Foxp3 Gene Locus. PLoS One *6*, e24590.

Lu, L., Ma, J., Wang, X., Wang, J., Zhang, F., Yu, J., He, G., Xu, B., Brand, D.D., Horwitz, D.A*., et al.* (2010a). Synergistic effect of TGF-beta superfamily members on the induction of Foxp3+ Treg. Eur J Immunol *40*, 142-152.

Lu, L., Wang, J., Zhang, F., Chai, Y., Brand, D., Wang, X., Horwitz, D.A., Shi, W., and Zheng, S.G. (2010b). Role of SMAD and non-SMAD signals in the development of Th17 and regulatory T cells. J Immunol *184*, 4295-4306.

Malek, T.R., and Bayer, A.L. (2004). Tolerance, not immunity, crucially depends on IL-2. Nat Rev Immunol *4*, 665-674.

Maloy, K.J., Salaun, L., Cahill, R., Dougan, G., Saunders, N.J., and Powrie, F. (2003). CD4+CD25+ T(R) cells suppress innate immune pathology through cytokine-dependent mechanisms. J Exp Med *197*, 111-119.
Mantel, P.Y., Ouaked, N., Ruckert, B., Karagiannidis, C., Welz, R., Blaser, K., and Schmidt-Weber, C.B. (2006). Molecular mechanisms underlying FOXP3 induction in human T cells. J Immunol *176*, 3593-3602.

Marie, J.C., Letterio, J.J., Gavin, M., and Rudensky, A.Y. (2005). TGF-beta1 maintains suppressor function and Foxp3 expression in CD4+CD25+ regulatory T cells. J Exp Med *201*, 1061-1067.

Marsden, V.S., and Strasser, A. (2003). Control of apoptosis in the immune system: Bcl-2, BH3-only proteins and more. Annu Rev Immunol *21*, 71-105.

McKarns, S.C., Schwartz, R.H., and Kaminski, N.E. (2004). Smad3 is essential for TGF-beta 1 to suppress IL-2 production and TCR-induced proliferation, but not IL-2-induced proliferation. J Immunol *172*, 4275-4284.

Miyara, M., Amoura, Z., Parizot, C., Badoual, C., Dorgham, K., Trad, S., Nochy, D., Debre, P., Piette, J.C., and Gorochov, G. (2005). Global natural regulatory T cell depletion in active systemic lupus erythematosus. J Immunol *175*, 8392-8400.

Miyara, M., Yoshioka, Y., Kitoh, A., Shima, T., Wing, K., Niwa, A., Parizot, C., Taflin, C., Heike, T., Valeyre, D*., et al.* (2009). Functional delineation and differentiation dynamics of human CD4+ T cells expressing the FoxP3 transcription factor. Immunity *30*, 899-911.

Molet, S., Hamid, Q., Davoine, F., Nutku, E., Taha, R., Page, N., Olivenstein, R., Elias, J., and Chakir, J. (2001). IL-17 is increased in asthmatic airways and induces human bronchial fibroblasts to produce cytokines. J Allergy Clin Immunol *108*, 430-438.

Monrad, S., and Kaplan, M.J. (2007). Dendritic cells and the immunopathogenesis of systemic lupus erythematosus. Immunol Res *37*, 135-145.

Moorman, J.E., Rudd, R.A., Johnson, C.A., King, M., Minor, P., Bailey, C., Scalia, M.R., and Akinbami, L.J. (2007). National surveillance for asthma--United States, 1980-2004. MMWR Surveill Summ *56*, 1-54.

Morelli, A.E., and Thomson, A.W. (2007). Tolerogenic dendritic cells and the quest for transplant tolerance. Nat Rev Immunol *7*, 610-621.

Morgan, M.E., Flierman, R., van Duivenvoorde, L.M., Witteveen, H.J., van Ewijk, W., van Laar, J.M., de Vries, R.R., and Toes, R.E. (2005). Effective treatment of collagen-induced arthritis by adoptive transfer of CD25+ regulatory T cells. Arthritis Rheum *52*, 2212-2221.

Morgan, M.E., Sutmuller, R.P., Witteveen, H.J., van Duivenvoorde, L.M., Zanelli, E., Melief, C.J., Snijders, A., Offringa, R., de Vries, R.R., and Toes, R.E. (2003). CD25+ cell depletion hastens the onset of severe disease in collagen-induced arthritis. Arthritis Rheum *48*, 1452-1460.

Mucida, D., Park, Y., Kim, G., Turovskaya, O., Scott, I., Kronenberg, M., and Cheroutre, H. (2007). Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. Science *317*, 256-260.

Nakae, S., Komiyama, Y., Nambu, A., Sudo, K., Iwase, M., Homma, I., Sekikawa, K., Asano, M., and Iwakura, Y. (2002). Antigen-specific T cell sensitization is impaired in IL-17-deficient mice, causing suppression of allergic cellular and humoral responses. Immunity *17*, 375-387.

Nguyen, K.D., Vanichsarn, C., Fohner, A., and Nadeau, K.C. (2009). Selective deregulation in chemokine signaling pathways of CD4+CD25(hi)CD127(lo)/(-) regulatory T cells in human allergic asthma. J Allergy Clin Immunol *123*, 933-939 e910.

Nguyen, T.L., Sullivan, N.L., Ebel, M., Teague, R.M., and Dipaolo, R.J. (2011). Antigen-Specific TGF-{beta}-Induced Regulatory T Cells Secrete Chemokines, Regulate T Cell Trafficking, and Suppress Ongoing Autoimmunity. J Immunol *187*, 1745-1753.

O'Connor, R.A., Leech, M.D., Suffner, J., Hammerling, G.J., and Anderton, S.M. (2010). Myelin-reactive, TGF-beta-induced regulatory T cells can be programmed to develop Th1-like effector function but remain less proinflammatory than myelin-reactive Th1 effectors and can suppress pathogenic T cell clonal expansion in vivo. J Immunol *185*, 7235-7243.

Pallotta, M.T., Orabona, C., Volpi, C., Vacca, C., Belladonna, M.L., Bianchi, R., Servillo, G., Brunacci, C., Calvitti, M., Bicciato, S.*, et al.* (2011). Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. Nat Immunol *12*, 870-878.

Park, I.K., Letterio, J.J., and Gorham, J.D. (2007). TGF-beta 1 inhibition of IFN-gamma-induced signaling and Th1 gene expression in CD4+ T cells is Smad3 independent but MAP kinase dependent. Mol Immunol *44*, 3283-3290.

Pasare, C., and Medzhitov, R. (2003). Toll pathway-dependent blockade of CD4+CD25+ T cell-mediated suppression by dendritic cells. Science *299*, 1033-1036.

Penaranda, C., and Bluestone, J.A. (2009). Is antigen specificity of autoreactive T cells the key to islet entry? Immunity *31*, 534-536.

Piccirillo, C.A., Letterio, J.J., Thornton, A.M., McHugh, R.S., Mamura, M., Mizuhara, H., and Shevach, E.M. (2002). CD4(+)CD25(+) regulatory T cells can mediate suppressor function in the absence of transforming growth factor beta1 production and responsiveness. J Exp Med *196*, 237-246.

Pot, C., Apetoh, L., and Kuchroo, V.K. (2011). Type 1 regulatory T cells (Tr1) in autoimmunity. Semin Immunol.

Read, S., Greenwald, R., Izcue, A., Robinson, N., Mandelbrot, D., Francisco, L., Sharpe, A.H., and Powrie, F. (2006). Blockade of CTLA-4 on CD4+CD25+ regulatory T cells abrogates their function in vivo. J Immunol *177*, 4376-4383.

Richards, I.M., Kolbasa, K.P., Winterrowd, G.E., Hatfield, C.A., Vonderfecht, S.L., Fidler, S.F., Griffin, R.L., Brashler, J.R., Krzesicki, R.F., Lane, C.L.*, et al.* (1996). Role of intercellular adhesion molecule-1 in antigen-induced lung inflammation in brown Norway rats. Am J Physiol *271*, L267-276.

Romagnani, S. (2006). Regulation of the T cell response. Clin Exp Allergy *36*, 1357-1366.
Ruan, Q., Kameswaran, V., Tone, Y., Li, L., Liou, H.C., Greene, M.I., Tone, M., and Chen, Y.H. (2009). Development of Foxp3(+) regulatory t cells is driven by the c-Rel enhanceosome. Immunity *31*, 932-940.

Rubtsov, Y.P., and Rudensky, A.Y. (2007). TGFbeta signalling in control of T-cell-mediated self-reactivity. Nat Rev Immunol *7*, 443-453.

Sakaguchi, S. (2005). Naturally arising Foxp3-expressing CD25+CD4+ regulatory T cells in immunological tolerance to self and non-self. Nat Immunol *6*, 345-352.

Sakaguchi, S., Sakaguchi, N., Asano, M., Itoh, M., and Toda, M. (1995). Immunologic self-tolerance maintained by activated T cells expressing IL-2 receptor alpha-chains (CD25). Breakdown of a single mechanism of self-tolerance causes various autoimmune diseases. J Immunol *155*, 1151-1164.

Salomon, B., Lenschow, D.J., Rhee, L., Ashourian, N., Singh, B., Sharpe, A., and Bluestone, J.A. (2000). B7/CD28 costimulation is essential for the homeostasis of the CD4+CD25+ immunoregulatory T cells that control autoimmune diabetes. Immunity *12*, 431-440.

Sauer, S., Bruno, L., Hertweck, A., Finlay, D., Leleu, M., Spivakov, M., Knight, Z.A., Cobb, B.S., Cantrell, D., O'Connor, E.*, et al.* (2008). T cell receptor signaling controls Foxp3 expression via PI3K, Akt, and mTOR. Proc Natl Acad Sci U S A *105*, 7797-7802.

Scalapino, K.J., Tang, Q., Bluestone, J.A., Bonyhadi, M.L., and Daikh, D.I. (2006). Suppression of disease in New Zealand Black/New Zealand White lupus-prone mice by adoptive transfer of ex vivo expanded regulatory T cells. J Immunol *177*, 1451-1459.

Seddiki, N., Santner-Nanan, B., Martinson, J., Zaunders, J., Sasson, S., Landay, A., Solomon, M., Selby, W., Alexander, S.I., Nanan, R.*, et al.* (2006). Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. J Exp Med *203*, 1693-1700.

Selvaraj, R.K., and Geiger, T.L. (2008). Mitigation of experimental allergic encephalomyelitis by TGF-beta induced Foxp3+ regulatory T lymphocytes through the induction of anergy and infectious tolerance. J Immunol *180*, 2830-2838.

Serre, K., Benezech, C., Desanti, G., Bobat, S., Toellner, K.M., Bird, R., Chan, S., Kastner, P., Cunningham, A.F., Maclennan, I.C., and Mohr, E. (2011). Helios is associated with CD4 T cells differentiating to T helper 2 and follicular helper T cells in vivo independently of Foxp3 expression. PLoS One *6*, e20731.

Shustov, A., Nguyen, P., Finkelman, F., Elkon, K.B., and Via, C.S. (1998). Differential expression of Fas and Fas ligand in acute and chronic graft-versus-host disease: up-regulation of Fas and Fas ligand requires CD8+ T cell activation and IFN-gamma production. J Immunol *161*, 2848-2855.

So, T., and Croft, M. (2007). Cutting edge: OX40 inhibits TGF-beta- and antigen-driven conversion of naive CD4 T cells into CD25+Foxp3+ T cells. J Immunol *179*, 1427-1430.

Steinman, R.M., Hawiger, D., and Nussenzweig, M.C. (2003). Tolerogenic dendritic cells. Annu Rev Immunol *21*, 685-711.

Su, H., Ye, D.Q., Wang, B.L., Fang, X.H., Chen, J., Wang, Q., Li, W.X., and Zhang, N. (2008). Transforming growth factor-beta1-induced CD4+CD25+ regulatory T cells in vitro reverse and prevent a murine lupus-like syndrome of chronic graft-versus-host disease. Br J Dermatol *158*, 1197-1209.

Swanson, C.L., Wilson, T.J., Strauch, P., Colonna, M., Pelanda, R., and Torres, R.M. (2010). Type I IFN enhances follicular B cell contribution to the T cell-independent antibody response. J Exp Med *207*, 1485-1500.

Takimoto, T., Wakabayashi, Y., Sekiya, T., Inoue, N., Morita, R., Ichiyama, K., Takahashi, R., Asakawa, M., Muto, G., Mori, T.*, et al.* (2010). Smad2 and Smad3 are redundantly essential for the TGF-beta-mediated regulation of regulatory T plasticity and Th1 development. J Immunol *185*, 842-855.

Tang, Q., and Bluestone, J.A. (2008). The Foxp3+ regulatory T cell: a jack of all trades, master of regulation. Nat Immunol *9*, 239-244.

Thornton, A.M., Korty, P.E., Tran, D.Q., Wohlfert, E.A., Murray, P.E., Belkaid, Y., and Shevach, E.M. (2010). Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. J Immunol *184*, 3433-3441.

Thornton, A.M., and Shevach, E.M. (2000). Suppressor effector function of CD4+CD25+ immunoregulatory T cells is antigen nonspecific. J Immunol *164*, 183-190.

Tone, Y., Furuuchi, K., Kojima, Y., Tykocinski, M.L., Greene, M.I., and Tone, M. (2008). Smad3 and NFAT cooperate to induce Foxp3 expression through its enhancer. Nat Immunol *9*, 194-202.

Torres-Aguilar, H., Aguilar-Ruiz, S.R., Gonzalez-Perez, G., Munguia, R., Bajana, S., Meraz-Rios, M.A., and Sanchez-Torres, C. (2010). Tolerogenic dendritic cells generated with different immunosuppressive cytokines induce antigen-specific anergy and regulatory properties in memory CD4+ T cells. J Immunol *184*, 1765-1775.

Tritt, M., Sgouroudis, E., d'Hennezel, E., Albanese, A., and Piccirillo, C.A. (2008). Functional waning of naturally occurring CD4+ regulatory T-cells contributes to the onset of autoimmune diabetes. Diabetes *57*, 113-123.

Tsuji, M., Komatsu, N., Kawamoto, S., Suzuki, K., Kanagawa, O., Honjo, T., Hori, S., and Fagarasan, S. (2009). Preferential generation of follicular B helper T cells from Foxp3+ T cells in gut Peyer's patches. Science *323*, 1488-1492.

Valencia, X., Stephens, G., Goldbach-Mansky, R., Wilson, M., Shevach, E.M., and Lipsky, P.E. (2006). TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. Blood *108*, 253-261.

van Amelsfort, J.M., Jacobs, K.M., Bijlsma, J.W., Lafeber, F.P., and Taams, L.S. (2004). CD4(+)CD25(+) regulatory T cells in rheumatoid arthritis: differences in the presence, phenotype, and function between peripheral blood and synovial fluid. Arthritis Rheum *50*, 2775-2785.

van Oosterhout, A.J., and Bloksma, N. (2005). Regulatory T-lymphocytes in asthma. Eur Respir J *26*, 918-932.

Viglietta, V., Baecher-Allan, C., Weiner, H.L., and Hafler, D.A. (2004). Loss of functional suppression by CD4+CD25+ regulatory T cells in patients with multiple sclerosis. J Exp Med *199*, 971-979.

Wakkach, A., Fournier, N., Brun, V., Breittmayer, J.P., Cottrez, F., and Groux, H. (2003). Characterization of dendritic cells that induce tolerance and T regulatory 1 cell differentiation in vivo. Immunity *18*, 605-617.

Wan, Y.Y., and Flavell, R.A. (2007). Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. Nature *445*, 766-770.

Weber, S.E., Harbertson, J., Godebu, E., Mros, G.A., Padrick, R.C., Carson, B.D., Ziegler, S.F., and Bradley, L.M. (2006). Adaptive islet-specific regulatory CD4 T cells control autoimmune diabetes and mediate the disappearance of pathogenic Th1 cells in vivo. J Immunol *176*, 4730-4739.

Wildin, R.S., Ramsdell, F., Peake, J., Faravelli, F., Casanova, J.L., Buist, N., Levy-Lahad, E., Mazzella, M., Goulet, O., Perroni, L.*, et al.* (2001). X-linked neonatal diabetes mellitus, enteropathy and endocrinopathy syndrome is the human equivalent of mouse scurfy. Nat Genet *27*, 18-20.

Wong, C.K., Ho, C.Y., Ko, F.W., Chan, C.H., Ho, A.S., Hui, D.S., and Lam, C.W. (2001). Proinflammatory cytokines (IL-17, IL-6, IL-18 and IL-12) and Th cytokines (IFN-gamma, IL-4, IL-10 and IL-13) in patients with allergic asthma. Clin Exp Immunol *125*, 177-183.

Wrana, J.L., Attisano, L., Carcamo, J., Zentella, A., Doody, J., Laiho, M., Wang, X.F., and Massague, J. (1992). TGF beta signals through a heteromeric protein kinase receptor complex. Cell *71*, 1003-1014.

Wu, Y., Borde, M., Heissmeyer, V., Feuerer, M., Lapan, A.D., Stroud, J.C., Bates, D.L., Guo, L., Han, A., Ziegler, S.F.*, et al.* (2006). FOXP3 controls regulatory T cell function through cooperation with NFAT. Cell *126*, 375-387.

Xu, L., Kitani, A., Fuss, I., and Strober, W. (2007). Cutting edge: regulatory T cells induce CD4+CD25-Foxp3- T cells or are self-induced to become Th17 cells in the absence of exogenous TGF-beta. J Immunol *178*, 6725-6729.

Xu, L., Kitani, A., Stuelten, C., McGrady, G., Fuss, I., and Strober, W. (2010). Positive and negative transcriptional regulation of the Foxp3 gene is mediated by access and binding of the Smad3 protein to enhancer I. Immunity *33*, 313-325.

Xue, K., Zhou, Y., Xiong, S., Xiong, W., and Tang, T. (2007). Analysis of CD4+ CD25+ regulatory T cells and Foxp3 mRNA in the peripheral blood of patients with asthma. J Huazhong Univ Sci Technolog Med Sci *27*, 31-33.

Yamagiwa, S., Gray, J.D., Hashimoto, S., and Horwitz, D.A. (2001). A role for TGF-beta in the generation and expansion of CD4+CD25+ regulatory T cells from human peripheral blood. J Immunol *166*, 7282-7289.

Yan, B., Ye, S., Chen, G., Kuang, M., Shen, N., and Chen, S. (2008). Dysfunctional CD4+,CD25+ regulatory T cells in untreated active systemic lupus erythematosus secondary to interferon-alpha-producing antigen-presenting cells. Arthritis Rheum *58*, 801-812.

Yao, Z., Kanno, Y., Kerenyi, M., Stephens, G., Durant, L., Watford, W.T., Laurence, A., Robinson, G.W., Shevach, E.M., Moriggl, R.*, et al.* (2007). Nonredundant roles for Stat5a/b in directly regulating Foxp3. Blood *109*, 4368-4375.

Yu, S.L., Wong, C.K., Wong, P.T., Chen, D.P., Szeto, C.C., Li, E.K., and Tam, L.S. (2011). Down-Regulated NOD2 by Immunosuppressants in Peripheral Blood Cells in Patients with SLE Reduces the Muramyl Dipeptide-Induced IL-10 Production. PLoS One *6*, e23855.

Zhang, Y.E. (2009). Non-Smad pathways in TGF-beta signaling. Cell Res *19*, 128-139.

Zheng, S.G. (2011). Emerging topics and new perspectives on regulatory and effector T cells. J Mol Cell Biol. 2011 Dec 2. [Epub ahead of print]

Zheng, S.G., Gray, J.D., Ohtsuka, K., Yamagiwa, S., and Horwitz, D.A. (2002). Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. J Immunol *169*, 4183-4189.

Zheng, S.G., Meng, L., Wang, J.H., Watanabe, M., Barr, M.L., Cramer, D.V., Gray, J.D., and Horwitz, D.A. (2006a). Transfer of regulatory T cells generated ex vivo modifies graft rejection through induction of tolerogenic CD4+CD25+ cells in the recipient. Int Immunol *18*, 279-289.

Zheng, S.G., Wang, J., and Horwitz, D.A. (2008). Cutting edge: Foxp3+CD4+CD25+ regulatory T cells induced by IL-2 and TGF-beta are resistant to Th17 conversion by IL-6. J Immunol *180*, 7112-7116.

Zheng, S.G., Wang, J., Wang, P., Gray, J.D., and Horwitz, D.A. (2007). IL-2 is essential for TGF-beta to convert naive CD4+CD25- cells to CD25+Foxp3+ regulatory T cells and for expansion of these cells. J Immunol *178*, 2018-2027.

Zheng, S.G., Wang, J.H., Gray, J.D., Soucier, H., and Horwitz, D.A. (2004a). Natural and induced CD4+CD25+ cells educate CD4+CD25- cells to develop suppressive activity: the role of IL-2, TGF-beta, and IL-10. J Immunol *172*, 5213-5221.

Zheng, S.G., Wang, J.H., Koss, M.N., Quismorio, F., Jr., Gray, J.D., and Horwitz, D.A. (2004b). CD4+ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-beta suppress a stimulatory graft-versus-host disease with a lupus-like syndrome. J Immunol *172*, 1531-1539.

Zheng, S.G., Wang, J.H., Stohl, W., Kim, K.S., Gray, J.D., and Horwitz, D.A. (2006b). TGF-beta requires CTLA-4 early after T cell activation to induce FoxP3 and generate adaptive CD4+CD25+ regulatory cells. J Immunol *176*, 3321-3329.

Zheng, Y., Josefowicz, S., Chaudhry, A., Peng, X.P., Forbush, K., and Rudensky, A.Y. (2010). Role of conserved non-coding DNA elements in the Foxp3 gene in regulatory T-cell fate. Nature *463*, 808-812.

Zhou, X., Kong, N., Wang, J., Fan, H., Zou, H., Horwitz, D., Brand, D., Liu, Z., and Zheng, S.G. (2010a). Cutting edge: all-trans retinoic acid sustains the stability and function of natural regulatory T cells in an inflammatory milieu. J Immunol *185*, 2675-2679.

Zhou, X., Kong, N., Zou, H., Brand, D., Li, X., Liu, Z., and Zheng, S.G. (2011). Therapeutic potential of TGF-beta-induced CD4(+) Foxp3(+) regulatory T cells in autoimmune diseases. Autoimmunity *44*, 43-50.

Zhou, X., Wang, J., Shi, W., Brand, D.D., Liu, Z., Fan, H., and Zheng, S.G. (2010b). Isolation of purified and live Foxp3+ regulatory T cells using FACS sorting on scatter plot. J Mol Cell Biol *2*, 164-169.

Zorn, E., Nelson, E.A., Mohseni, M., Porcheray, F., Kim, H., Litsa, D., Bellucci, R., Raderschall, E., Canning, C., Soiffer, R.J.*, et al.* (2006). IL-2 regulates FOXP3 expression in human CD4+CD25+ regulatory T cells through a STAT-dependent mechanism and induces the expansion of these cells in vivo. Blood *108*, 1571-1579.

Song Guo Zheng

Effets thérapeutiques des cellules T regulatrices induitent par TGF-β, sur l'autoimmunité et l'inflammation préétablies

Des études récentes ont montré que les cellules nTregs ont moins d'effets thérapeutiques lors de traitements de maladies auto-immunes. L'étude actuelle cherche à déterminer si les iTregs pourraient, être plus efficaces.

Dans l'asthme allergique, nous avons observé que le transfert adoptif de iTreg supprime de façon significative l'inflammation des voies respiratoires et péri-vasculaires, réduit la résistance et le remodelage de ces voies aériennes, le recrutement des éosinophiles, l'hyperproduction de mucus, et les niveaux d'IgE. Cet effet thérapeutique a pu être associé à une augmentation du nombre de Tregs ($CD4^+$ $Foxp3^+$) dans les ganglions lymphatiques drainant, et à la réduction des réponses Th1, Th2, Th17.

Dans l'arthrite induite par le collagène (CIA), les iTregs antigène-spécifiques comme les nTregs proliférant, préviennent le développement de la pathologie. Toutefois, seul le transfert d'iTregs permet de supprimer la CIA lorsque celle-ci est déjà établie. Dans cette situation, les nTregs contrairement aux iTregs, étaient converties en Th17, et perdaient l'expression de Foxp3, cela dans des expériences aussi bien *in vitro* qu'in *vivo*. Les iTregs suppriment la différenciation Th17, ce qui corrèle avec l'amélioration des scores cliniques et des symptômes.

Dans le modèle GVHD chronique, la perfusion d'iTregs diminue les symptômes du lupus. Le blocage de la liaison TGF-β/TGF-βR ou des voies de signalisation de l'IL-10 abolit de façon significative les effets thérapeutiques des iTregs. Celles-ci rendent les DC tolérogéniques, par l'intermédiaire du TGF-β mais pas de l'IL-10. Les DC isolées de souris atteintes de lupus et recevant des iTregs peuvent supprimer la progression de la maladie grâce au TGF-β mais pas à l'IL-10. Ainsi, iTregs ciblent les DC dans le milieu inflammatoire et ces DC devenues tolérogéniques empêchent la progression de maladies auto-immunes grâce à des effets directs ou indirects (induisant par exemple de nouvelles iTregs.

Par ailleurs, nous démontrons que l'acide rétinoïque « all-trans » (atRA) promeut et soutient les cellules Tregs $Foxp3^+$. atRA augmente la méthylation des histones et l'acétylation, dans les locus du gène Foxp3, tandis que la méthylation de l'ADN du gène Foxp3 n'est pas significativement modifiée. Ces résultats peuvent fournir de nouvelles connaissances sur la thérapie cellulaire clinique pour les patients atteints de maladies auto-immunes et pour ceux qui ont besoin de greffes d'organes.

Mots clés : Treg, TGF-b, Autoimmunité, Inflammation, Tolérance

Therapeutic effects of TGF-β-induced regulatory T cells on the established autoimmune and inflammatory diseases

Recent studies revealed that nTregs has less therapeutic effects on established autoimmune diseases. Current study asks if iTregs induced *ex-vivo* with TGF-β can treat the established autoimmune diseases. In allergic asthma we observed that adoptive transfer of iTreg significantly suppressed airway and peri-vascular inflammation, reduced airway résistance, eosinophil recruitment, mucus hyper-production, airway remodeling and IgE levels. This therapeutic effect was associated with increase of Tregs ($CD4^+Foxp3^+$) in the draining LNs, and with reduction of Th1, Th2, and Th17 responses.

In collagen-induced arthritis (CIA) both antigen-specific iTregs and expanded nTregs prevented CIA. However, only iTregs transfer suppressed established CIA. nTregs but not iTregs were converted into Th17 and lost Foxp3 *in vitro* and *in vivo* in established CIA. iTregs suppressed Th17 cell differentiation that paralleled with improved clinical scores and symptoms.

In the chronic GVHD model mimicking lupus the iTregs infusion significantly decreased lupus symptoms. Blocking of TGF-β/TGF-βR or IL-10 signaling pathways significantly abolished the therapeutic effects. iTregs induced the formation of tolerogenic DCs through TGF-β but not IL-10 signaling on DC. DC isolated from lupus mice receiving iTregs can suppress lupus development through TGF-β but not IL-10 signaling. Thus, iTregs target DC in the inflammatory milieu and newly formed tolerogenic DC suppress disease progression through its direct or indirect effect (inducing new iTregs) in autoimmune disease settings.

Moreover, we demonstrated that all-trans retinoic acid (atRA) promotes and sustains the $Foxp3^+$ regulatory T cells. atRA increased histone methylation and acetylation within Foxp3 gene locus, while DNA methylation in Foxp3 gene was not significantly altered. These results may provide novel insights into clinical cell therapy for patients with autoimmune diseases and those needing organ transplantation.

Keywords : Treg, TGF-β, Autoimmunity, Inflammation, Tolerance

Immunologie et Embryologie Moléculaire, IEM, UMR6218

3B, rue de la Ferollerie, 45071 Orléans, France