TGF-β1 SELECTIVELY INDUCES Foxp3 TRANSCRIPTION FACTOR AND REGULATORY FUNCTIONS IN $CD4^+CD25^-$ $CD45RB^{LOW}$ T CELL POPULATION.

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ABSTRACT

$CD4^+$ regulatory T (T_{REG}) cells are important contributors to the induction and maintenance of peripheral tolerance. This heterogeneous population consists of naturally occurring and induced $CD4^+$ T_{REG} cells that share between themselves key immunoregulatory characteristics. Their phenotype and function often relies on the expression of Foxp3 transcription factor and the presence of the immunomodulating cytokine TGF-β1. The inter-dependence between TGF-β1 and Foxp3 in the induction and maintenance of peripheral tolerance is gradually being elucidated. Thus, we investigated the effects of TGF-β1 on induction or maintenance of regulatory functions in $CD4^+CD25^-$ as well as $CD4^+CD25^+$ T cells. TGF-β1 treatment was able, independent from APCs, to promote T_{REG} cell differentiation from non-regulatory $CD4^+CD25^-$ T cells in a concentration-dependent fashion through Foxp3 induction. Next, we investigated the effect of TGF-β1 on purely naive $CD4^+CD25^-$ $CD45RB^{HIGH}$ T cell subset. Fresh or TGF-β1–treated $CD45RB^{HIGH}$ T cells did not display regulatory functions nor Foxp3 expression. In stark contrast, TGF-β1 treatment promoted regulatory activity in the $CD4^+CD25^-$ $CD45RB^{LOW}$ T cells and enhanced Foxp3 expression. Interestingly, fresh $CD45RB^{LOW}$ cells, albeit expressing noticeable levels of Foxp3 mRNA and protein, were unable to suppress effector T (T_{EFF}) cell proliferation. Furthermore, addition of neutralizing anti-IL-10R Ab completely abrogated this suppression, consistent with the ability of TGF-β1 treated $CD45RB^{LOW}$ to synthesize IL-10 mRNA upon re-stimulation *in vitro*. TGF-β1 treatment or blockade did not lead to preferential growth or enhanced function of naturally-occurring $CD4^+CD25^+$ T_{REG} cells, yet it caused a significant increase in Foxp3 expression. Altogether, TGF-β1 preferentially promotes the induction of IL-10 secreting $CD4^+$ regulatory T cells from $CD45RB^{LOW}$ precursors through Foxp3 induction.

ABREGE

Les cellules T régulatrices $CD4^+$ (T_{REG}) jouent un rôle primordial dans l'induction et le maintient de la tolérance périphérique. Cette population hétérogène est composée de cellules T_{REG} naturelles et induites, qui partagent des propriétés immunorégulatrices clés. Leurs phénotype et fonction dépendent de l'expression du facteur de transcription Foxp3 et de la présence de la cytokine immunomodulatrice TGF-β1. L'inter-dépendance du TGF-β1 et de Foxp3 dans l'induction et le maintient de la tolérance périphérique est peu a peu élucidée. Nous avons étudié les effets du TGF-β1 sur l'induction ou le maintient de fonctions régulatrices dans des cellules T $CD4^+CD25^-$ ou $CD4^+$ $CD25^+$. Le traitement au TGF-β1 s'est avéré capable d'induire, proportionnellement a sa concentration, la différentiation de cellules T non régulatrices $CD4^+CD25^-$ en T_{REG}, via l'induction de Foxp3 et indépendamment de la présence de cellules présentatrices de l'antigène. Nous avons ensuite étudié l'effet du TGF-β1 sur une population pure de cellules T naïves $CD4^+CD25^-$ $CD45RB^{HIGH}$. Les cellules T $CD45RB^{HIGH}$, fraîchement isolées ou traitées au TGF-β1, n'ont jamais démontré ni de fonctions régulatrices ni un niveau significatif d'expression de Foxp3. Au contraire, le traitement au TGF-β1 a induit une activité régulatrice parmi les cellules T $CD4^+CD25^-$ $CD45RB^{LOW}$ et a augmenté leur niveau d'expression de Foxp3. De plus, les cellules $CD45RB^{LOW}$ fraîches, bien qu'exprimant des niveaux significatifs d'ARNm et de protéine Foxp3, ont été incapables de supprimer la prolifération de cellules T effectrices. Enfin, l'adjonction d'un anticorps anti-IL-10 a complètement abrogé cette suppression, confirmant ainsi la capacité de cellules $CD45RB^{LOW}$ traitées au TGF-β1 de synthétiser de l'ARNm de l'IL-10 quand restimulées *in vitro*. Ni un traitement au TGF-β1, ni son blocage n'ont conduit à une croissance préférentielle ou une plus grande efficacité des cellules T_{REG} $CD4^+CD25^+$ naturelles, malgré une substantielle augmentation du niveau d'expression de Foxp3.

Ainsi, le TGF-β1 induit la différentiation préférentielle de cellules T régulatrices $CD4^+$ sécrétrices d'IL-10 à partir de précurseurs $CD45RB^{LOW}$, via l'induction de Foxp3.

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STATEMENT OF CONTRIBUTIONS

In accordance with the guidelines of the Faculty of Graduate Studies and Research, McGill University, regarding thesis preparation, I have chosen to present the experimental part of this thesis (chapter II) in the form of a manuscript. A provision in the guidelines reads as follows:

Candidates have the option of including, as part of the thesis, the text of one or more papers submitted or to be submitted, for publication, or the clearly-duplicated text (not the reprints) of one or more published papers.

With respect to the above conditions, I have included as the main body of my thesis (chapter II), the text of a manuscript currently in submission. Chapter II contains its own Abstract, Introduction, Material and Methods, Results, Discussion and Acknowledgements sections.

The experimental work was entirely performed by myself under the supervision of Dr. Piccirillo.

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CHAPTER I

LIST OF ABBREVIATIONS

Ag	Antigen
AICD	Activation induced cell death
APC	Antigen presenting cell
CCR	CC chemokine receptor
CsA	Cyclosporin A
CSIF	Cytokine synthesis inhibitory factor
C-Smad	Common-Smad
CtBP1	C terminal binding protein 1
CTLA-4	Cytotoxic T-lymphocyte associated protein 4
DC	Dendritic cell
dnRII	dominant-negative TGF-βRII
EAE	Experimental autoimmune encephalomyelitis
ER	Estrogen receptor
FcR	Fragment crystallizable
FKH	Forkhead
Fox	Forkhead/Winged-helix box
G-CSF	Granulocyte-colony stimulating factor
GFP	Green fluorescent protein
GITR	Glucocorticoid induced TNF receptor SF18
GM-CSF	Granulocyte, macrophage-colony stimulating factor
GPI	Glycosylphosphatidylinositol
IBD	Inflammatory bowel disease
IDDM	Insulin dependent diabetes mellitus
IFN	Interferon
IL	Interleukin
IL-12Rβ2	Interleukin-12 receptor beta2
IL-2Ra	Interleukin-2 receptor alpha
ILT	Immunoglobulin-like transcript
ILT3/4	Immunoglobulin like transcript 3/4
IP-10	Interferon-inducible protein

IPEX	IDDM, polyendocrinopathy, enteropathy, X-linked
I-Smad	Inhibitory-Smad
iT_{REG}	Induced T regulatory cells
JAK1	Janus kinase-1
LAG-3	Lymphocyte-activation gene 3
LAP	Latency associated peptide
LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LTBP	Latent TGF-β binding protein
LT-α	Lymphotoxin-α
LZ	Leucine zipper
MAP	Mitogen-activated protein kinase kinase
MCP	Membrane co-factor protein
M-CSF	Macrophage-colony stimulating factor
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
NFAT	Nuclear factor of activated T cells
NK	Natural killer
NKT	Natural killer T cell
NOD	Non-obese diabetic
nT_{REG}	Naturally-occurring T regulatory cells
PAF	Platelet-activating factor
PD1	Program death 1
PTP	Protein tyrosine phosphatase
R–Smad	Receptor-Smad
SARA	Smad anchor for receptor activation
SBE	Smad-binding elements
SbE	STAT-binding elements
SCID	Severe combined immunodeficiency
SFK	Src-family kinases
Smad	Sma mothers against decapentaplegic

STAT	Signal transducers and activators of transcription
T1D	Type 1 diabetes
TCR	T cell receptor
T_{EFF}	Effector T cell
TF	Transcription factor
Tg	Transgenic
TGF-βR	Transforming growth factor- β-receptor
TGF-βRIII	Betaglycan
TGF-β	Transforming growth factor- β
T_H1	T helper 1
T_H2	T helper 2
TH3	T helper 3 cell
TLR	Toll-like receptor
TNF	Tumor necrosis factor
TNFR2	Tumor necrosis factor receptor 2
TPRO	T progenitor
TR1	T regulatory 1 cell
T_{REG}	T regulatory cells
TYK2	Tyrosine kinase-2
WT	Wild type

CHAPTER I: INTRODUCTION

1. Notions of immune tolerance

The main role of an immune system is to protect the host from numerous external and internal threats such as pathogenic microorganisms or tumors. In order to mount an appropriate response to these stimuli, fine tuned interactions between various types of leukocytes and a plethora of soluble factors are involved, resulting in the eradication of the threat. Nevertheless, activation is not the sole phenomenon essential to this process. Inhibitory pathways are equally necessary to achieve the balance between the ability to mount non-damaging immune responses against pathogens and to maintain unresponsiveness to self-antigens. As such, the immune system becomes tolerant to specific stimuli. Central or thymic tolerance allows for the survival of functional T lymphocytes, through TCR recognition of host-specific MHC class molecules, in addition to eradication of T cells with high affinity self-peptide recognition (1, 2). T cells bearing a low to medium affinity self-reactive TCR, emerging from the thymus, have the potential to become pro-inflammatory T_{EFF} cells directed against an array of self and non-self antigens. Thus, in order to maintain tolerance in the periphery, a network of inhibitory mechanisms has evolved. Among them, peripheral deletion can be initiated by alloantigen recognition under co-stimulatory blockade. Equally, lymphocytes exposed to alloantigen stimuli can either ignore it or become anergic in their functions afterwards. Regulatory cells represent the main and active form of peripheral tolerance, simultaneously permitting the suppression of autoreactive T cells, the maintenance of normal intestinal immunity towards enteric bacteria, the dampening the anti-pathogen

effector mechanisms from inducing immune pathology, and assistance in T_H1/T_H2 differentiation (3).

2. Regulatory cells.

Currently, the existence of cell-mediated suppression in the regulation of immune responses to self and non-self antigens is well recognized. Numerous leukocytic lineages are endowed with the capacity to control the activation and function of other cells. For instance, immature DC (iDC), in which the presentation of the complete co-stimulatory signal is absent, are unable to fully activate T lymphocytes leading to tolerance (4). *In vitro* allo-stimulation of $CD83^-$ human iDC was accordingly able to induce CTLA-4^{HIGH} T suppressor cells from cord blood $CD4^+$ T cells, and human iDC pulsed with influenza matrix protein induced *in vivo* generation of IL-10 producing $CD8^+$ T_{REG} cells (5). Although B cells are mostly recognized as antibody producing cells, they are also able to function as secondary APC and secrete cytokines. These two characteristics permit specific lymphocytes to acquire regulatory functions. As a result, B_{REG} cells secrete TGF-β and IL-10 which down-regulate pro-inflammatory networks, and as APCs, can either support or dampen T cell responses initiated by DC (6).

2.1. Regulatory T cells.

T cell subsets with regulatory properties have been described among αβ TCR $CD4^+$, $CD8^+$, natural killer (NK) and γδ TCR cells. The mechanism of suppression used by these cells varies (Table 1). $CD8^+$ T_{REG} cells have been shown to block the development of experimental autoimmune encephalomyelitis (EAE) through the diminished activation of T_H1 cells with encephalitogenic properties (7). $CD8^+CD28^-$ T

cells are able to suppress immune responses via cell contact-dependent tolerization of APC by up-regulating immunoglobulin-like transcript (ILT) 3 and 4 on those cells (8). NK-T cells down-regulate T cell activation through immuno-regulatory cytokine secretion. Their introduction in auto-immune-prone animals, such as non-obese diabetic (NOD) mice, has been shown to prevent the onset of diabetes (9). Intra-epithelial γδ T cells play an essential role in intestinal inflammation by down-modulating the αβ T lymphocyte response to antigens (10). Similarly, a skin-resident dendritic cell (DC)-like subset of γδ T cells prevents dermatitis (11).

Cell Type	Mechanism of Suppression	Transcription Factor Expressed	Function
nT_{REG}	- IL-10, TGF-β1 secretion - Cell contact	Constitutive Foxp3	Generation of peripheral tolerance
T_R1	- IL-10 secretion	Inducible Foxp3	Suppression of autoimmunity
T_H3	- TGF-β1 secretion	Foxp3?	Oral tolerance Suppression of autoimmunity
NKT cells	- IL-4, TGF-β1, IL-10 secretion, - Cell-contact	?	Elimination of tumors and intracellular pathogens, suppression of autoimmunity
$CD8^+$	- Cell-contact, - Cytokines? - ILT3/4 induction on DC	Foxp3	Suppression/ regulation of autoimmunity
γδ T cells	- Cell contact ? - Cytokines ?	?	Generation of peripheral tolerance

Table I. General characteristics of the different regulatory T cells subsets.

2.2. Naturally occurring $CD4^+CD25^+Foxp3^+$ T regulatory cells.

Most notably, the best described population of T_{REG} cells belongs to the $CD4^+$ lymphocyte lineage. Beginning with a landmark study in 1995 by the laboratory of S. Sakaguchi, a subset of $CD4^+$ T cells constitutively expressing interleukin (IL)-2α receptor (CD25) was demonstrated to display potent immuno-regulatory functions *in vitro* and *in vivo* (12). Thus, $CD4^+CD25^+$ T cells were described as naturally-occurring or nT_{REG} cells due to their immediate and complete ability to suppress immune responses upon emergence from the thymus (13). nT_{REG} cells consist of an anergic lymphocyte population representing 1-10% of the total $CD4^+$ T cell population in the thymus, peripheral blood, and lymphoid tissues, in both humans and rodents, irrelevant of antigen exposure (13). Depletion of nT_{REG} cells or abrogation of their function in the periphery results in the onset of multi-organ-specific autoimmunity, and also increases tumor immunity, graft rejection, and pathogen clearance (14). Activation of $CD4^+CD25^-$ T (T_{EFF}) cells in the presence of nT_{REG} cells is negatively affected, leading to decreased IL-2 production, IL-2α expression and T cell proliferation (15). For *in vitro* suppression, most studies demonstrate that T_{REG} cells must be in direct contact with their target cells. Production of IL-10, TGF-β, granzyme B, or inhibition of APC function through indolamine 2,3-dioxygenase has been implicated in suppression both *in vitro* and *in vivo;* however, the precise molecular mechanism through which nT_{REG} cells control immune responses are still unknown (16). *Ex vivo*, nT_{REG} cells display a semi-activated phenotype, preferentially expressing surface markers such as CD103, Ly6, galectin-1, OX-40, 4-1BB, CTLA-4, glucocorticoid induced TNF receptor SF18 (GITR), PD1, TNFR2, TGFβR1, neuropilin-1, and LAG-3 (17-20). The discovery of the nT_{REG} specific

transcription factor, Foxp3, whose expression correlates with regulatory properties, allowed for the more detailed detection of regulatory T cell subsets (21). Interestingly, nT_{REG} cell population can also be delimited by the absence of CD45 marker as the majority of T_{REG} cells lose the expression of CD45RB antigen. CD45 is a receptor-like protein tyrosine phosphatase (PTP), responsible for positively modulating the activity of the Src-family kinases (SFK), Lck and Fyn, thus allowing TCR signalling (22). In general, $CD45RB^{HIGH}$ and $CD45RB^{LOW}$ subsets of mouse $CD4^+$ T lymphocytes are considered as naïve and memory T cells respectively, considering that CD45RB antigen is lost upon T cell activation *in vitro* and *in vivo* (23, 24). In a series of adoptive transfer experiments using recipient SCID mice, the $CD4^+CD45RB^{HIGH}$ population was shown to mediate protective T_H1 responses to pathogens despite inducing an auto-immune response. On the other hand, $CD4^+CD45RB^{LOW}$ population prevented the development of auto-immunity and concomitantly exacerbated susceptibility to pathogens (25-27). Subsequently, when $CD45RB^{LOW}$ population was depleted of nT_{REG} cells and the resulting $CD4^+CD25^-CD45RB^{LOW}$ fraction was transferred into SCID mice, the population did not cause autoimmunity and provided a weak protection to pathogenic $CD45RB^{HIGH}$ cell co-transfer (28).

2.3. Induced T regulatory cells.

It has become evident that peripheral mechanisms of T_{REG} generation exist, due to the involution of the thymus with age (29). Indeed, induced $CD4^+CD25^+$ T_{REG} (iT_{REG}) cells can be derived from $CD4^+CD25^-$ T cell fractions and either acquire or expand their suppressive activity as a consequence of *in vivo* or *ex vivo* activation of nT_{REG}-depleted $CD4^+$ T cells under unique stimulatory conditions in the periphery. These iT_{REG} cells can

be generated under various stimulation regimes, including: antigen in the presence of IL-10, TGF-β1, vitamin D3 or dexamethasone, CD40L blockade, allo- or pathogen-derived antigens, non-depleting anti-CD4 and anti-CD8 treatment, bivalent non-mitogenic anti-CD3 therapy, or immature DC populations (30-36). Currently, an array of iT_{REG} cells capable of ensuring the efficient control of peripheral T cell responses at multiple levels has been described and their characterization has been based on their potential to produce certain signature cytokines after antigen challenge *in vitro* and *in vivo*. Some of these iT_{REG} cells include counter-regulatory IFN-γ producing-T_H1 cells and IL-4 producing T_H2 cells, IL-10-producing T_R1 cells, and TGF-β-secreting T_H3 cells induced in the gut following oral ingestion of antigen (Table I) (12, 37-39).

3. Forkhead/Winged-Helix Box (Foxp3) Transcription Factor.

3.1. Functional association between Foxp3 and nT_{REG} cell function.

Given the preponderant role of nT_{REG} cells in preventing the emergence of autoimmune diseases, numerous inbred mice strains that naturally develop autoimmune diseases have been investigated to uncover the genes responsible for the establishment of peripheral tolerance. Among them is a natural mutant mouse, *scurfy*, first identified more than 40 years ago (40-43). *Scurfy* is an X-linked recessive mutation resulting in scaly skin, small, thickened ears, diarrhoea, malabsorption, progressive anemia, thrombocytopenia, leukocytosis, lymphoadenopathy, hepatosplenomegaly and hypogonadism that culminates in death at one month of age (40). The progressive lymphocytic infiltration of multiple organs, as well as increased production of numerous pro-inflammatory cytokines is due to the hyperresponsiveness of $CD4^+$ T cells to TCR

stimulation. A human syndrome that phenotypically resembles *scurfy* in mice, initially recognized in 1982, is termed IPEX (IDDM, enteropathy and endocrinopathy, X-linked) (44, 45). IPEX patients suffer from neonatal onset of IDDM, recurrent infections, enteropathy, thrombocytopenia, anemia, endocrinopathy, eczema and cachexia resulting in fatal failure to thrive (46). In 2001, the *scurfy* mutation was mapped to the Foxp3 gene encoding a transcription factor from the forkhead/winged-helix family (47). Concurrently, FOXP3 mutations in humans were shown to be responsible for IPEX syndrome (44, 48, 49). Unequivocally, $CD4^+$ T cells were the main cause of symptoms; depletion or adoptive transfer of this lymphocyte fraction alleviated or transferred, respectively, the disease in mice (40).

A more stable marker of nT_{REG} cells was deemed desirable for the exclusive identification of the T_{REG} population, since the CD25 receptor could only be used to identify the population in naive $CD4^+$ T cells, being up-regulated upon T cell activation. In 2003, with the almost simultaneous publication of three studies, a direct association between Foxp3 and nT_{REG} cells was made (21, 43, 50). It was demonstrated that Foxp3 is exclusively expressed in the $CD4^+CD25^+$ T cell fraction, that $Foxp3^{-/-}$ or *scurfy* mice do not posses a nT_{REG} population, and that transduction of Foxp3 into murine $CD4^+CD25^-$ non-regulatory cells was sufficient to convert them into a functional suppressor population *in vitro* and *in vivo*.

3.2 Structure of Foxp3.

Forkhead (FKH)/Winged-helix box (Fox) transcription factors are characterized by specific helix-loop-helix DNA binding domains (51). Foxp3 stands apart from other Foxp proteins, which themselves differ from the Fox family, having the FKH domain

atypically at the carboxy-terminal end, and are transcriptional repressors (52). Foxp3 is the smallest member (430 amino acids) due to both the absence of the glutamine-rich sequence at the amino-terminal end and a shorter carboxy-terminal end, adjacent to the FKH domain (Figure A). It has been shown that the FKH domain is required for nuclear localization and DNA binding (53, 54). Foxp3 binds to a DNA consensus sequence that diverges somewhat from the general Fox consensus motif (XYZAAYA, X=A/G, Y=C/T, Z=A/C) (53). Interestingly, the N-terminal domain contains a higher proportion of proline residues, and it has been shown that deletion of the first 200 amino acids is critical for the inhibition of cytokine gene expression (52). Thus, it appears that the amino-terminal half of Foxp3 functions as a transcriptional repressor. Foxp3 also possesses a leucine zipper and C_2H_2 zinc finger domains. It is now known that the leucine zipper domain is required for dimerization of Foxp3, as is the case in the other Foxp proteins (55).

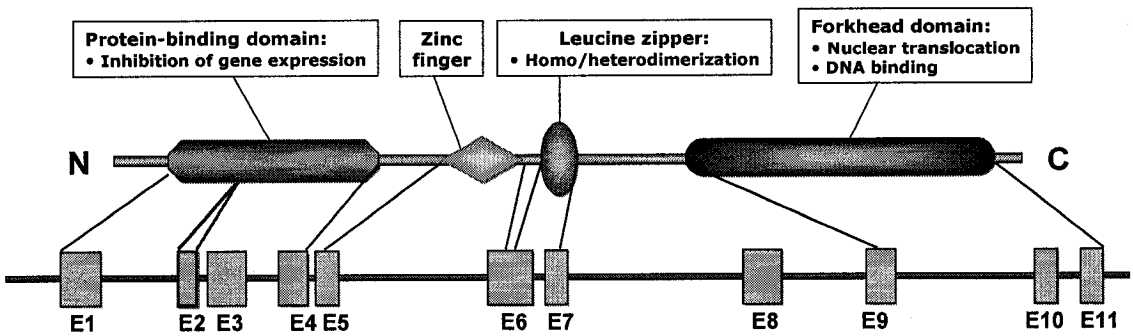

Figure A. Structure of FOXP3 and Foxp3. FOXP3 contains 4 functional domains with different probable functions (color coded). In humans, two isoforms of FOXP3 exist: the first is encoded by 11 exons (in grey) and second isoform, FOXP3Δ2, isoform results from alternate splicing of exon 2 (in red). E, exon.

Murine Foxp3 and human FOXP3 are highly homologous, especially in the FKH domain, containing all of the above mentioned domains. Of highest importance is the identification of a splice variant of FOXP3 that lacks exon 2 (amino acids 70-104) and is expressed at equal levels in human nT_{REG} cells (56, 57). Studies from IPEX patients indicate that mutations in FOXP3 are distributed throughout the gene, with a pattern of clustering among the missence mutations in the regions corresponding to FKH, LZ and the amino terminal domain (52).

3.3. Molecular mechanisms underlying Foxp3 function.

Initial reports established that Foxp3 functions as a transcriptional repressor since its' transient expression reduced transcription of a reporter gene with numerous FKH binding sites (53). Additionally, its' over-expression reduced activation-induced cytokine production and proliferation (58). Motivated by the observations that *scurfy* mice produce high levels of IL-2, and that Foxp3 Tg animals, which over-express Foxp3, produce less IL-2, FKH consensus sites were identified in the murine and human IL-2 promoter (42, 53, 58). Consistently, a sequence of the IL-2 promoter was able to compete with FKH consensus sites for FOXP3 binding (53). Furthermore, forced expression of Foxp3 in $CD4^+$ T cells led to a reduction in IL-2, IL-4 and IFN-γ secretion upon their activation (Figure B) (21, 43, 54, 59). Likewise, Foxp3 Tg animals demonstrate a decrease in $CD8^+$ and $CD4^+$ T cell numbers owing to the inability to produce IL-2, in addition to a delay in cell cycle progression, and thus failure to proliferate (58). This effect was mediated by the ability of Foxp3 to inhibit NF-κB by affecting its transcriptional activity but not DNA-binding activity. Additionally, NFAT-mediated gene expression was blocked by Foxp3 expression. This negative function of Foxp3 depends on physical association with

the Rel homology domain of NF-κB and NFAT (54). Moreover, Foxp3 Tg mice are more prone to undergo apoptosis and display slower cell cycle progression upon TCR triggering (60). Foxp3 might also act as a co-repressor, considering that Foxp1 and Foxp2 associate with transcriptional repressor CtBP1 (52). Although no precise mechanism has yet been described, the finding that the chromatin structure of the IL-2 promoter in nT_{REG} is in a closed configuration, contrary to what is observed in $CD4^+CD25^-$ T cells, supports the previous hypothesis (61). Therefore, it appears that Foxp3 might achieve gene repression in a dual fashion, first by directly binding to FKH sites and displacing NFAT, AP-1, NF-κB or other transcription factors, and second through recruitment of transcriptional co-repressors. The specific genes regulated by Foxp3 have yet to be fully determined.

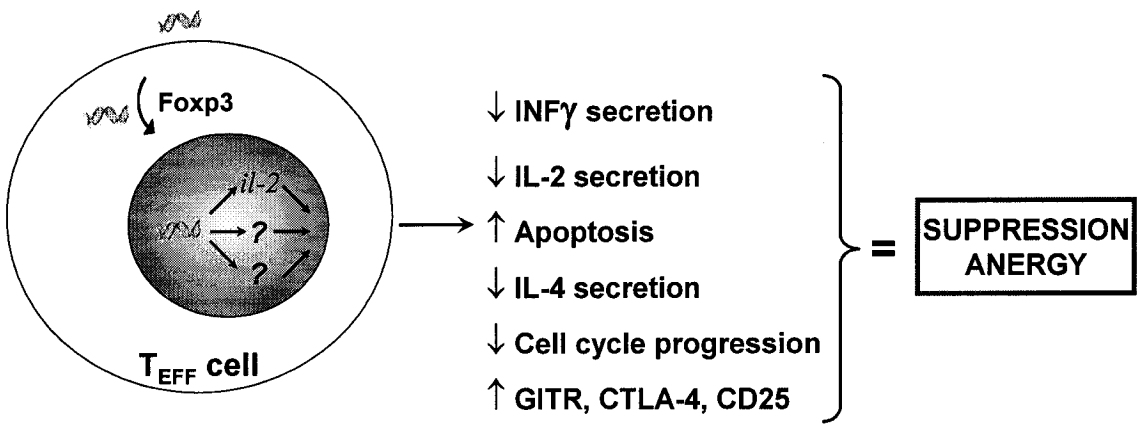

Figure B. Effects of Foxp3 expression in $CD4^+CD25^-$ T_{EFF} cells. Transduction of Foxp3 in non-regulatory T cells leads to expression of genes that induce anergy and suppressive phenotype. ↑, increase; ↓, decrease.

Although initial studies of Foxp3/FOXP3 expression suggest that it is solely expressed in nT_{REG} populations, accumulating evidence points in the converse direction,

particularly in humans (62, 63). Presently, both murine and human nT_{REG} cells have been shown to express high levels of Foxp3/FOXP3 independent of activation status or age, which confers on them their specific suppressor phenotype. Nevertheless, it seems that upon nT_{REG} activation, Foxp3 mRNA levels decrease whereas protein levels increase (50). Consequently, it has been suggested that Foxp3 expression is not as stable as initially thought in the T_{REG} cell pool, and that this TF might possess additional functions in T_{EFF} cell pool associated with cell activation (60). For instance, $CD4^+$ T cells over-expressing Foxp3 under T_H1 or T_H2 driving conditions are able to differentiate into effector cells with concomitant loss of Foxp3 mRNA and protein (60). Nevertheless, in humans, simple activation of $CD4^+CD25^-$ T cells via TCR was shown to lead to expression of FOXP3 and generation of regulatory functions (56, 57, 64). Recently, in a study of the FOXP3 promoter, it was reported that TCR stimulation acts directly on the FOXP3 promoter through binding of NFATc2. In total, six NFAT and AP-1 binding sites were located in the first 500 base pairs of the promoter, in addition to normal TATA, GC and CAAT boxes. Moreover, both T_{REG} and T_{EFF} cells maintained the FOXP3 promoter in the open conformation (65). Consequently, upon TCR triggering, FOXP3 expression was up-regulated in $CD4^+CD25^-$ but not T_{REG} cells, achieving a peak within 48 h post-stimulation and decreasing afterwards, and was abrogated upon addition of cyclosporine A (CsA) (65). These results are contrary to murine $CD4^+CD25^-$ T cell activation experiments where no detectable Foxp3 mRNA or protein levels have been detected, despite the presence of promoter-containing NFAT and AP-1 binding sites (21, 50, 65).

Forced expression of FOXP3 in human non-T_{REG} cells is not completely sufficient to generate fully functional T_{REG} cells, indicating that other not yet described factors,

could be required (21, 50, 56, 64). More specifically, ectopic expression of FOXP3 or exon-2-splice variant in human $CD4^+CD25^-$ T cells initiates a state of hypo-responsiveness and decreased IL-2 production, yet it does not allow the acquisition of significant suppressor activity *in vitro* (56). Thus, from these experiments, it seems that while Foxp3/FOXP3 is a preponderant factor in T_{REG} cell biology it is not the sole TF responsible for direct acquisition of regulatory functions. An important fact to consider is that in both species, activation of T cells leads to Foxp3/FOXP3 expression but the stabilization of mRNA/protein could be differently regulated. In particular, the ability of TGF-β, estrogen, and glucocorticoids to induce Foxp3 expression in the presence of stimulation, with unknown underlying molecular pathways opens the possibility that Foxp3/FOXP3 is under tight transcriptional regulation that differs from translational and post-translational control (66-68).

Overall, it is evident that Foxp3/FOXP3 expression patterns differ between species and also between cell subsets. It remains to be determined which signals lead to induction and up-regulation of Foxp3/FOXP3 versus the maintenance of expression and generation of regulatory functions in different T cell populations, as well as the consequences in humans and rodents.

4. TGF-β: Modulator of Foxp3 expression and T_{REG} cell function.

4. 1. TGF-β expression and cell signalling pathways.

Whereas the involvement of TGF-β and T_{REG} cells in peripheral tolerance is unquestioned (69), the interactions and interdependence between them remain controversial. TGF-β is a homodimeric cytokine present in three isoforms in mammals

(TGF-β1/TGF-β2/TGF-β3) that share redundant, key roles in embryonic development, cell differentiation and proliferation, fibrosis, carcinogenesis, wound healing and immune modulation (70). TGF-β is an ubiquitous molecule, permeating almost all body surfaces. It is found mostly in an inactive latent form, bound to a latency associated peptide (LAP), which in turn, is associated with latent TGF-β binding protein (LTBP) and extracellular matrix. Under specific, activating conditions, TGF-β is secreted by numerous lymphoid cells as active peptide. More specifically, TGF-β1 is the main isoform present in lymphoid organs and serum, having the greatest impact on the immune system, while TGF-β2 and TGF-β3 are mainly expressed in mesenchymal tissues and bones (71). Following its secretion or release from LAP, TGF-β is active for a very short period of time as many ligand traps such as α2-macroglobulin and LAP will inactivate it anew (72). Therefore, upon activation, TGF-β will engage primarily to TGF-βRII homodimer, which has constitutively active serine–threonine kinase activity, subsequently recruiting TGF-βRI (ALK5) homodimer. Whereas TGF-β1 and 3 bind with high affinity to TGF-βRII, TGF-β2 necessitates initial binding with betaglycan (TGF-βRIII) (73). The type I/II receptor complex then auto-transphosphorylates and subsequently phosphorylates receptor (R)–Smad (Smad 2/3) proteins by the activation of kinase domain on the type II receptor. With this, Smad2/3 can associate with common (C)-Smad (Smad4), allowing translocation into the nucleus and weak binding to Smad-binding elements (SBE) (Figure C) (74-76). Usually, TGF-β triggering, via the negative self-inhibitory loop, will induce inhibitory (I)-Smad (Smad7). Smad7 then negatively regulates TGF-β signalling by competing with R-Smad for receptor binding and targets receptors, as well as other Smads, to proteosome degradation (77).

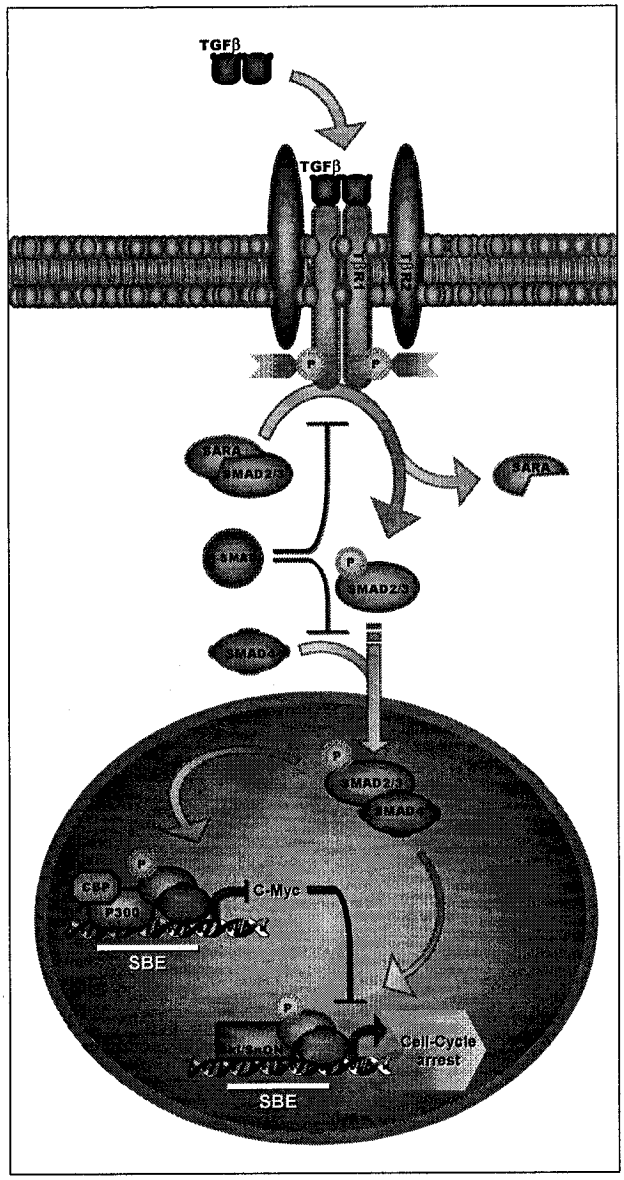

Figure C.

TGF-β signalling pathway.

The control of gene expression by Smads, that is their activation or repression abilities, are mediated by recruitment of other co-activators (CBP/p300) or co-repressors (Sno/Ski) (70). It is worth mentioning that TGF-β signalling pathways are diffuse and diverse, including the Smad-independent MAP kinase pathways and others, depending on cell type and differentiation status (78). However, the diverse and divergent cellular effects of TGF-β ligation rely on similar signalling pathways and the specific integration

of those pathways is cell-dependent, which should be considered when studying the association between TGF-β and heterogeneous T_{REG} cells.

4. 2. TGF-β tolerogenic functions in T cell populations.

4. 2.1. Anti-proliferative and anti-apoptotic functions of TGF-β.

TGF-β1 plays a critical role in the down-regulation of immune responses. It is no surprise that, this cytokine has a potent anti-proliferative effect by suppressing transcription of the T cell growth factor, IL-2 (79). Additionally, TGF-β1 signaling through Smad3 negatively influences cell cycle progression by up-regulating the cyclin-dependent kinase inhibitors p15, p21 and p27 and down-regulating c-myc, cyclin D2 and cyclin E (80). CD28 co-stimulation, which increases IL-2 mRNA stability, diminishes TGF-β anti-proliferative effects, as the residual IL-2 is then able to drive T cell proliferation (81). In TGF-$\beta1^{-/-}$ mice, spontaneous apoptosis occurs among T cell fractions (69). The promotion of T cell survival is associated with decreased levels of c-myc and FasL in TGF-β treated cells (69). TGF-β1 potently inhibits $CD8^+$ T cell proliferation and differentiation; in its presence, $CD8^+$ T cells do not acquire cytotoxic functions due to decreased perforin and FasL expression (82, 83). Moreover, as with macrophages and T_H1 cells, this cytokine will negatively affect IFN-γ production through down-modulation of T-bet (Figure Di) (84).

4.2.2. Influence of TGF-β on T_H1/T_H2 balance.

TGF-β has also been implicated in maintaining the balance between the $CD4^+$ humoral (T_H2) and cell-mediated (T_H1) immune responses. Under normal conditions, epitope recognition by the TCR and co-stimulation leads to T helper cell differentiation into humoral or cell-mediated T_{EFF} cells. Locally secreted cytokines play a dominant role

in this process. In the case of a T_H1 response, IFN-γ secretion by NK cells activates STAT1 transcription factor in differentiating T cells, leading to the expression of T-bet. T-bet induces expression of IL-12Rβ2, making T cells susceptible to autocrine IL-12. IL-12 ligation to its receptor activates STAT4, which in conjunction with T-bet, stabilizes T_H1 phenotype by increasing secretion of IFN-γ and Lymphotoxin-α (LT-α) (85). TGF-β blockade of T_H1 differentiation occurs mainly through the reduction of both T-bet and IL-12Rβ2 expression (70). Consequently, no stabilization of T_H1 phenotype occurs. The generation of T_H2 effector cells relies on IL-4 secretion by DCs or mast cells. Ligation of IL-4 to cognate receptor activates STAT6, leading to the expression of GATA-3 (86). GATA-3, in association with c-MAF transcription factor, initiates epigenetic modifications in the T_H2 gene locus (A 120 kb region encoding IL-4, IL-5 and IL-13 genes) imprinting higher levels of expression of IL-4, IL-5, IL-10 and IL-13 (85). TGF-β has been shown to interfere with GATA-3 expression, resulting in the blockade of humoral differentiation (Figure Di) (87). Significantly, development of T_H2 cells appears to be more sensitive to inhibition by TGF-β than differentiation of T_H1 cells (88). Nevertheless, fully differentiated T_H2 cells are refractory to TGF-β, whereas mature T_H1 cells produce lower than normal levels of IFN-γ upon TGF-β treatment (69). In addition, an interesting regulatory loop exists between IFN-γ and TGF-β, as IFN-γ induces SMAD7 production, an inhibitor of TGF-β signalling (78).

4.2.3. TGF-β immunosuppressive functions in vivo.

The *in vivo* effects of TGF-β are illustrated by the development of a severe autoimmune-like syndrome in TGF-$\beta1^{-/-}$ mice, characterized by the spontaneous and progressive, multi-organ infiltration of mononuclear cells and the presence of pathogenic

anti-nuclear antibodies (89). Crossing TGF-β1$^{-/-}$ mice either to SCID or MHC class II$^{-/-}$ mice results in the absence of autoimmune diseases, directly implicating CD4^{+} T cells as the main target of TGF-β1 (90). Also, in TGF-β1$^{-/-}$ mice, CD8^{+} T cells activate, spontaneously producing effector cytokines (90). This is further corroborated by the observation that disruption of TGF-β1 signalling in T cells by either overexpression of a dominant-negative TGF-βRII (dnRIITg), conditional deletion of the TGF-βRII in hematopoetic progenitors, or inactivation of the gene encoding Smad3, nullifies susceptibility to TGF-β, leading to aberrant T cell responses, and in some instances produces IBD in mice (91, 92).

Taken together, TGF-β plays a crucial role in effector cell biology by affecting T cell proliferation, activation, differentiation and survival. It is plausible that upon self-peptide antigen encounter, in the absence of strong co-stimulation, or during degenerate TCR interactions with MHC-Ag complexes, TGF-β dampens the proliferation of T cells. In addition, during initial host pathogen interactions, TGF-β delays T_H1/T_H2 development until stronger stimuli are able to decisively drive the immune response.

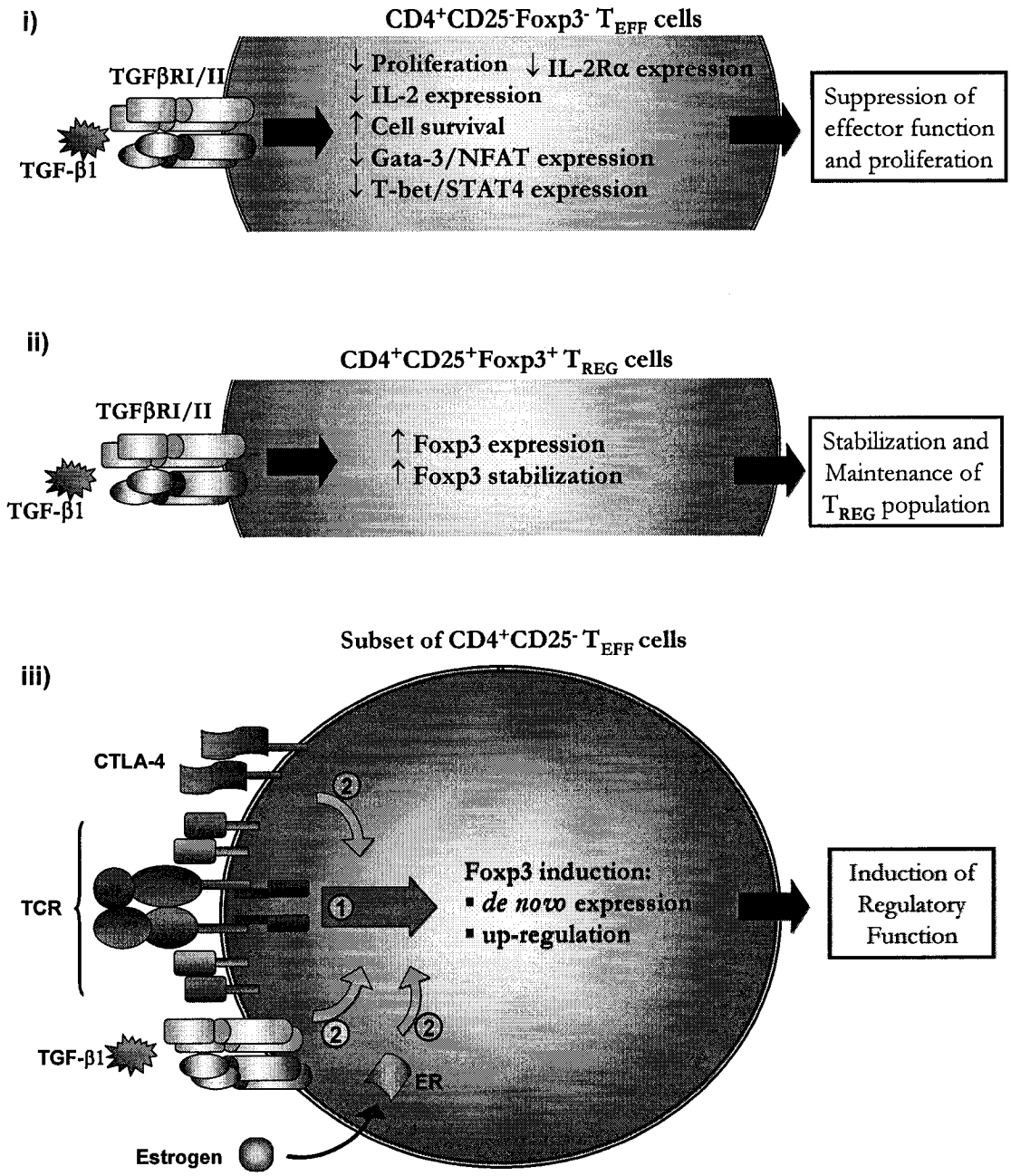

Figure D. Effects of TGF-β1 on different $CD4^+$ T cell populations. Effects of TGF-β1 on **i)** T_{EFF} $CD25^-CD4^+$ T cells **ii)** nT_{REG} $CD25^+CD4^+$ T cells and **iii)** subset of $CD4^+CD25^-$ T_{EFF} cells. In naturally occurring regulatory cells, the main action of TGF-β1 is to maintain a state of regulatory potency, whereas in $CD4^+CD25^-$ T_{EFF} cells, TGF-β1 is immuno-suppressive with the exception of subset of T_{EFF} cells, where it induces Foxp3 expression under specific activating conditions. ↑, up-regulation; ↓, down-regulation; 1,2: order of importance for Foxp3 induction.

4. 3. The role of TGF-β in T_{REG} cell populations.

Presently, accumulating evidence reveals a strong relationship between TGF-β1 and nT_{REG} cells, suggesting the involvement of this immunosuppressive cytokine in $CD4^+CD25^+$ T_{REG} cell-mediated mechanisms of suppression, as well as maintenance and induction of regulatory functions.

4.3.1. TGF-β functions in the suppression mechanism of T_{REG} cells.

One current view implicates TGF-β1 associated with LAP on the surface of T_{REG} cells in a cell contact-dependent mechanism of suppression (93). Numerous studies report that a fraction of $CD4^+CD25^+$ T cells express an inactive form of TGF-β1 complexed to its LAP that is retained as a membrane-bound complex by an undefined surface receptor. This complex is hypothesized to account for the enhanced suppressive capacity of activated $CD4^+CD25^+$ T cells *in vitro*. (93-96). Nonetheless, most murine and human *in vitro* studies conclude that neither secreted nor membrane-bound forms of active or latent TGF-β1 are responsible for contact-dependent suppression mediated by resting or activated $CD4^+CD25^+$ T cells, as it has been demonstrated that WT, TGF-$\beta^{-/-}$, Smad3$^{-/-}$, and dnTRIITg T_{REG} cells remain fully suppressive, and either blockade or addition of TGF-β does not lead to abrogation or enhancement of suppression (97, 98). Moreover, dnRIITg and Smad3$^{-/-}$ T cells, which are resistant to the growth inhibitory effects of exogenous TGF-β1, remain susceptible to suppression by $CD4^+CD25^+$ T cells (98-102). More importantly, $CD4^+CD25^+$ T cells isolated from neonatal TGF-$\beta1^{-/-}$ mice display comparable suppressive activity to WT T_{REG} cells *in vitro*, and in contrast to their $CD4^+CD25^-$ counterparts, are positive for CTLA-4, GITR, CD103 and Foxp3, a phenotype consistent with WT $CD4^+CD25^+$ nT_{REG} cells (98, 103). Interestingly, *in vitro*

$CD25^-LAP^+$ cells were unable to suppress, whereas $CD25^+LAP^-$ T cells were suppressive, further supporting the idea that the sole presence of TGF-β1, either secreted or bound to the cell surface through LAP, is insufficient to mediate suppression (104).

Conflicting results in the literature make it difficult to reach a consolidated view on the role of TGF-β1 in $CD4^+CD25^+$ T_{REG} cell function *in vivo*. Some studies have suggested that production of TGF-β1 by $CD4^+CD25^+$ T_{REG} cells is required to protect SCID mice from IBD induced by $CD4^+CD45RB^{HIGH}$ T_{EFF} cells, as treatment of mice with $CD4^+CD45RB^{HIGH}$ and $CD4^+CD25^+$ T_{REG} cells, along with neutralizing anti-TGF-β antibody reversed suppression (28). Similarly, one recent study reported a requirement for TGF-β1 in $CD4^+CD25^+$ T_{REG} cell-mediated control of $CD8^+$ T cell anti-tumor activity (105). Administration of anti-TGF-β antibody neutralized suppressive activity of $CD4^+CD45RC^{LOW}$ T cells in rat T1D and thyroiditis produced by adult thymectomy and irradiations (106). Further, L. Fahlen *et al.* (2005) has shown that pathogenic dnTBRII T_{EFF} cells do not respond to suppression from WT T_{REG} cells (107). In contrast, Kullberg *et al.* (2005) have shown that $CD4^+CD25^+$ T_{REG} cells from either TGF-$\beta1^{+/+}$ or neonatal TGF-$\beta1^{-/-}$ mice can suppress the incidence and severity of IBD as well as colonic IFN-γ mRNA expression induced by WT $CD4^+CD25^-$ T_{EFF} cells (108). Furthermore, TGF-β-resistant $Smad3^{-/-}$ nT_{REG} cells are equivalent to WT T_{REG} cells in their capacity to suppress disease induced either by WT or $Smad3^{-/-}$ $CD4^+CD25^-$ T_{EFF} cells.

4. 3.2. TGF-β maintains Foxp3 in T_{REG} cell populations.

Another emerging view is that TGF-β1 signalling is necessary for the maintenance of T_{REG} cells through modulation and induction of Foxp3 expression. (Figure Dii) S. Yamagiwa *et al.* (2001) were one of the first groups to show expansion of

regulatory functions in human $CD4^+CD45RA^+RO^-$ T cells, which required the presence of the $CD4^+CD25^+$ T cell subset, through a TGF-β dependent process (109). Similarly, the group of R.A. Flavell has demonstrated that a transient pulse of TGF-β1 in the pancreas of diabetes-prone animals is sufficient to inhibit the development of T1D through preferential expansion of T_{REG} cells, whereas the group of L. Zitvogel demonstrated that tumour cells produce TGF-β, which expands the T_{REG} cell population, and leads to tumour tolerance (110-113). Nevertheless, *in vitro* incubation or pre-incubation of nT_{REG} cells with TGF-β1 does not lead either to preferential growth or increased suppressive functions in this population. The necessary presence of IL-2 for the *in vitro* expansion of anergic T_{REG} cells in these conditions may be masking the selective actions of TGF-β (114). Furthermore, studying the proliferation of nT_{REG} cells in bulk can be misleading due to potential intra-inhibitory effects. Therefore, even though TGF-β1 could selectively promote the growth of T_{REG}, this effect could remain unseen *in vitro* due to suppressive effects between them. Instead, Marie *et al.* (2005) have shown through the use of GFP-Foxp3 knock-in mice crossed to TGF-$\beta1^{-/-}$ mice, that in the absence of this cytokine, the numbers of $CD4^+CD25^+Foxp3^+$ T cells were drastically reduced. Transfer of T_{REG} cells from these animals into TCR β/γ-deficient mice expressing normal levels of TGF-β1, led to an increase in $T_{REG}Foxp3^+$ expressing cells equivalent to WT animals, whereas administration of anti-TGF-β antibody induced a decrease in T_{REG} cells expressing Foxp3 both in WT mice as well as in TGF-$\beta^{-/-}$ mice (115). Most importantly, T_{REG} cells from dnTβRII mice induced colitis when transferred alone into RAG-$1^{-/-}$ mice, accentuating the importance of autocrine TGF-β signalling in the maintenance of this population and potentially showing that without this signal T_{REG} cells, through Foxp3

loss, revert to T_{EFF} cells (107). Therefore, TGF-β, either autocrine or paracrine, is necessary to maintain T_{REG} suppressor phenotype through possible stabilization of Foxp3 expression.

4. 3.3. TGF-β induces iT_{REG} cells via de novo Foxp3 expression.

Yet another function of TGF-β in T cell immunoregulation is to induce regulatory T cells from $CD4^+CD25^-$ T cell precursors through *de novo* Foxp3 expression or up-regulation (Figure Diii). Initial studies have shown that induction of regulatory functions occur in human $CD4^+CD25^-$ T cells upon co-culture with nT_{REG} cells (109, 116, 117). This infectious tolerance depends on nT_{REG}-to-T_{EFF} cell contact, and does not necessarily require the presence of exogenous TGF-β, although it can be abrogated by addition of anti-TGF-β antibody. Subsequently, it was revealed that upon activation and culture of total $CD4^+$ T cells in the presence of TGF-β, the T cell subset expressing the CD25 marker possessed potent suppressive functions, contrary to the $CD25^-$ subset(66). Nevertheless, in these studies it was unclear whether TGF-β interacted primarily with nT_{REG} or T_{EFF} cells. Chen *et al.* (2003) demonstrated that activation of murine $CD4^+CD25^-$ T cells with TGF-β generated regulatory T cells that prevent house dust mite-induced allergic pathogenesis in lungs (66). Interestingly, the induction of suppressive function occurred through up-regulation of Foxp3. Following this work, Y. Wan *et al.* (2005), using mRFP-Foxp3 knock-in mice, demonstrated that in a dose-dependent fashion, TGF-β induced *de novo* expression of Foxp3 in previously non-regulatory $CD4^+CD25^-$ $Foxp3^-$ T cells (118). Another study has suggested that in order to generate iT_{REG}, at least three signals have to be delivered *in vitro* to $CD4^+CD25^-$ T cells, namely through the TGF-β receptor, the TCR as well as CTLA-4 (119). However, the

molecular signalling pathways resulting in up-regulation of Foxp3 are still obscure. The reported mechanism of suppression of those iT_{REG} cells are divergent, relying either on cell contact (109, 120), presence of TGF-β (66, 116, 121) or IL-10 (117), and are probably due to the use of different methodologies and heterogenous $CD4^+CD25^-$ T cell populations.

Overall, TGF-β appears to possess three non-exclusive functions in T_{REG} populations: first in their mechanism of suppression, second in their maintenance through preservation of Foxp3 expression, and third in induction of T_{REG} cells in the periphery through *de novo* Foxp3 production.

5. IL-10: Mediator of T_{REG} function.

As was illustrated earlier, numerous mutually supporting cells types and soluble factors are engaged in maintenance of peripheral tolerance, with $Foxp3^+$ T_{REG} cells and TGF-β cytokine among them. IL-10, known as cytokine synthesis inhibitory factor (CSIF), is also an important contributor to the preservation of peripheral tolerance (122). Significant amounts of evidence demonstrate that IL-10, like TGF-β, is closely involved in nT_{REG} cell function and induction of particular regulatory properties.

5.1. IL-10 expression and cell signalling pathways.

IL-10 is a homodimer of two intertwined polypeptide chains with high structural similarity to IL-19, IL-20, IL-22, Il-24 and IL-26, that can be expressed by a variety of cells in response to an activation stimulus (123). However, recent studies suggest that IL-10 is transcribed to some degree constitutively and subject to control by alteration of post-transcriptional RNA degradation mechanism (123). Upon secretion, IL-10 will bind

to the IL-10 receptor (IL-10R) complex that consists of two ligand-binding subunits (IL-10R-α or IL-10R1) and two accessory signalling subunits (IL-10R-β or IL-10R2) (Figure E). Association of IL-10 to the extra-cellular domain of IL-10R1 activates phosphorylation of the receptor-associated Janus Kinase-1 (JAK1) and Tyrosine Kinase-2 (TYK2), which are constitutively coupled with IL-10R1 and IL-10R2. These kinases next phosphorylate specific tyrosine residues of the IL-10R1 chain permitting signal transducer and activator of transcription-3 (STAT-3) to recognize and temporarily associate with it in order to become phosphorylated. Afterwards, STAT-3 homodimerizes and translocates to the nucleus where it binds with high affinity to the STAT-binding elements (SbE) in the promoters of various IL-10-responsive genes.

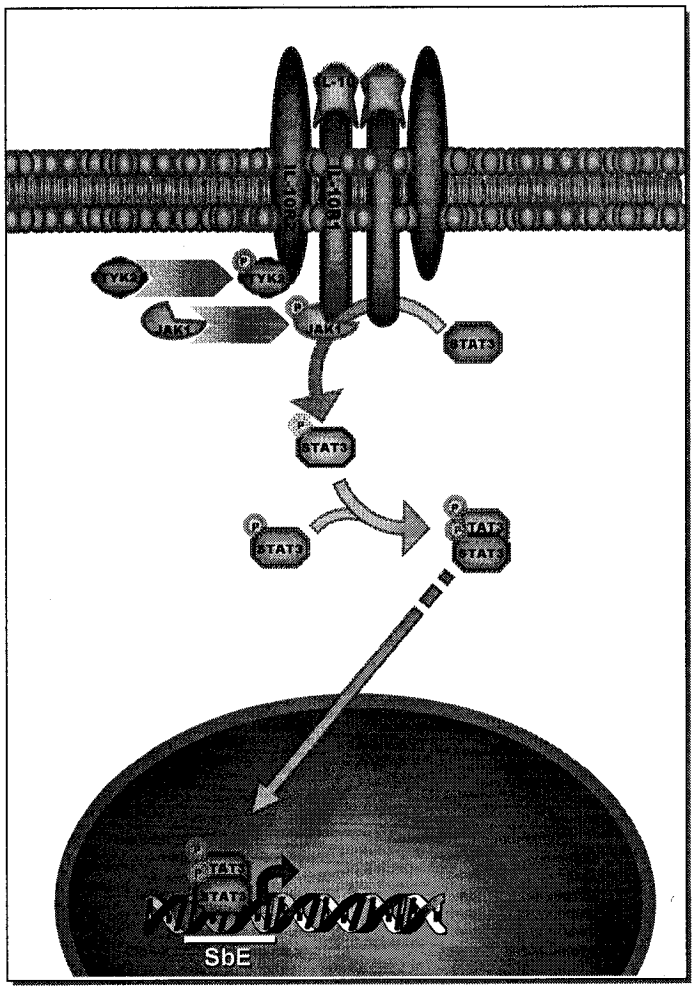

Figure E. IL-10 signalling pathway.

5.2. IL-10 tolerogenic functions in non-T cell populations.

IL-10 potently influences the activities of multiple hematopoietic cells, including macrophages, monocytes, B lymphocytes, DCs and granulocytes, considering that most of those cells are able to express IL-10 receptors (124). The important immunosuppressive role of IL-10 is succinctly illustrated in IL-$10^{-/-}$ mice which develop growth retardation, anaemia and suffer from chronic enterocolitis due to uncontrolled immune responses when stimulated by enteric antigens (125 W., 2001). Importantly, IL-$10^{-/-}$ mice display increased T_H1 responses and, as a consequence, have enhanced clearance of pathogens such as bacteria (92, 126). These mice also show exaggerated

asthmathic and allergic responses, highlighting the function of IL-10 as a modulator of immune responses *in vivo* (127, 128).

Initially, IL-10 was thought to impede presentation of Ag-MHC class I/II complexes on APCs. Presently, it is known to inhibit the production of numerous inflammatory cytokines and chemokines (IL-1α, IL-1β, IL-6, IL-10, IL-12, IL-18, GM-CSF, G-CSF, M-CSF, TNF, LIF, PAF, MCP1, MCP-5, MIP-1α, MIP -1β, MIP -3α, MIP -3β, Rantes, IL-8, IP-10, MIP-2) in macrophages and monocytes (127). Notably, it prevents amplification of pro-inflammatory pathways by inhibiting IL-1/TNF production and ligation to their cognate receptors. Thus, it is able to down-regulate IL-1RI/II expression and enhance production of IL-1R antagonist and soluble TNFR (p55/p75). TLR4 expression, MHC class II presentation and co-stimulation are all unfavorably affected upon macrophage treatment with IL-10. Furthermore, IL-10, contrary to its usual down-modulating role, stimulates phagocytosis via up-regulation of FcγR (CD16 and CD64). Nevertheless, nitric oxide and superoxide ion production are reduced, which culminates in decreased intracellular phagolysosomal killing (129). On the other hand, IL-10 up-regulates expression of CCR1, CCR2 and CCR5 on monocytes, making them more responsive to chemokines (130). IL-10 has also been shown to hinder the production of IL-12 and expression of co-stimulatory molecules by different subsets of DCs (122). IL-10, as one of T_H2 cytokines, has a potent effect on B cells. For example, IL-10 enhances expression of MHC class II molecules and improves the survival of B cells by promoting expression of bcl-2 (122). It also promotes proliferation of B cell precursors and mature cells (122). Another important function involves regulation of isotype switching, as B cells produce large amounts of IgM, IgG1-3 and IgA in the

presence of IL-10 and IgG4 and IgE in presence of IL-4 and IL-10. Likewise, it was shown that IL-10 is a switch factor for IgG1 and IgG3 (122). Overall, IL-10 has anti-inflammatory and suppressive effects on most leukocytes, reducing stimulation by APCs and limiting inflammation in order to terminate and clear infections.

5.3. IL-10 tolerogenic functions in T cell populations.

Most inhibitory effects of IL-10 on $CD4^+$ T cells, such as lower cytokine production and proliferation occur indirectly through a decrease in the antigen-presenting capacity of different types of APCs. Nevertheless, IL-2, TNF-α and IL-5 production in $CD4^+$ T cells is negatively affected by IL-10 depending on culture conditions (122). In contrast, IL-10 enhances cytotoxic properties and proliferation of $CD8^+$ T_{EFF} cells (131).

It is well established that IL-10 participates in T_{REG} cell generation and function as the presence of this cytokine closely correlates with the occurrence of immuno-regulation. As was previously mentioned, IL-10 is directly implicated in nT_{REG} mechanisms of suppression. Initially it was established that the adoptive transfer of $IL\text{-}10^{-/-}$ T cells into $Rag2^{-/-}$ mice was responsible for the induction of colitis (132). Moreover, in a nT_{REG} cell transfer model of colitis, $IL10^{-/-}$ $CD4^+CD45RB^{LOW}$ T cells, enclosing the T_{REG} population, were unable to protect the host from IBD, contrary to WT T cells (133). Further, the inhibition of colitis mediated by WT $CD45RB^{LOW}CD4^+$ T cells relied on IL-10, as treatment with an anti-IL-10 antibody leads to disease development (134).

nT_{REG} cell-secreted IL-10 has also been implicated in susceptibility to infectious diseases, such as leishmaniasis. More specifically, nT_{REG} cells controlled the persistence of the parasite, *Leishmania major* via IL-10-dependent and independent mechanisms, as

mice with impaired IL-10 activity completely eradicated the pathogen but were susceptible to parasite challenge upon re-infection (27). However, the suppressive functions of nT_{REG} cells are not exclusively mediated through IL-10 secretion *in vivo*, considering that other autoimmune diseases are kept under control either by IL-$10^{-/-}$ T_{REG} cells or upon administration of IL-10 blocking antibody (124). As seen in IL-$10^{-/-}$ mice, IL-10 plays a dominant role in intestinal homeostasis, as these mice develop spontaneous ileocolitis beginning two months after birth (125). This might explain the necessity of nT_{REG} cells to utilize IL-10-dependent means of suppression specifically at this anatomical location.

It has been reported that the inclusion of IL-10 during $CD4^+$ T cell activation can result in anergy (135). However, in specific instances, such as the repetitive *in vitro* antigenic stimulation of naïve $CD4^+$ T cells in the presence of IL-10, allows for the post-thymic generation of unique subset of T_{REG} cells called T_R1 (38, 136). T_R1 cells are defined by their ability to produce high levels of IL-10 through which they suppress T cell responses. As well, they can be induced by the treatment with immunosuppressive drug dexamethasone, vitamin D_3, immature DCs, soluble protein and peptide antigens (137-140). Phenotypically, T_R1 cells closely resemble nT_{REG} cells with the expression of CD25, GITR, CTLA-4 and CD103. However, Foxp3 expression seems inducible and does not always correlate with the T_R1 suppressive functions (139). Normally, upon activation, T_R1 cells secrete significant levels of IL-10, TGF-β and IL-5, negligible amounts of IFN-γ and IL-2, and no IL-4. In such cases, IL-10 is detectable within 5 h, reaching highest concentration 24 h post activation (141). Independently of nT_{REG} cells, T_R1 cells are thus well adapted to regulate immune responses via the secretion of IL-10

and TGF-β, suppressing both naive and memory T cell responses *in vivo* and *in vitro* (32, 38). Nonetheless, some confusion regarding the relationship between nT_{REG} and T_R1 cells persists, as the molecular mechanisms governing regulatory functions and means of suppression used by both of these lymphocyte subsets often converge. Consequently, *in vivo*, IL-10 seems to exert the immunosuppressive effects through its secretion by thymus derived nT_{REG} cells or peripherally generated T_R1 cells in parallel with the ability of Il-10 to induce T_R1 cells themselves.

The significance of IL-10, TGF-β and Foxp3 involvement in the generation of peripheral tolerance is comparable to the differences between IL-10-deficient, TGF-β-deficient and Foxp3-deficient mice. Whereas the TGF-$\beta^{-/-}$ and Foxp3$^{-/-}$ mice develop a fatal multi-organ inflammatory disease, IL-$10^{-/-}$ mice do not develop a generalized inflammatory response, which suggests a more restrictive involvement of IL-10 in the generation of the peripheral tolerance (142). TGF-β and Foxp3 play a primordial role in nT_{REG} cell function, preventing the development of generalized immune pathology, hence their requirement in almost all aspects of the nT_{REG} physiology. By contrast, IL-10 may play a more important role in the control of inflammatory responses in tissues, particularly when there is sustained activation of the innate immune response.

CHAPTER II

TGF-β1 modulates Foxp3 expression and regulatory activity in distinct $CD4^+$ T cell subsets.

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Keywords: $CD4^+CD25^+$ regulatory T cells, IL-10, FoxP3, TGF-β1, suppression, tolerance.

Abstract

Although Foxp3 expression is restricted to naturally occurring $CD4^+$ regulatory T cells, very little is known about the various signals that regulate it in T cells. As TGF-β has been reported to modulate Foxp3 expression in T cells, we investigated its effects on the induction or maintenance of regulatory functions in different $CD4^+$ T cell subsets. TGF-β1 priming was able to promote differentiation of regulatory T cells from non-regulatory $CD4^+CD25^-$ T cells in a concentration-dependent fashion through Foxp3 induction. As $CD4^+CD25^-$ T cells remain a highly heterogeneous population with variable degrees of antigen-experience, we then examined the effect of TGF-β1 on naive $CD4^+CD25^-CD45RB^{HIGH}$ T cells. Freshly isolated or TGF-β1 treated $CD4^+CD25^-CD45RB^{HIGH}$ T cells never displayed any regulatory functions or significant Foxp3 expression following TCR activation. In stark contrast, freshly isolated $CD4^+CD25^-CD45RB^{LOW}$ cells, albeit expressing low levels of Foxp3 mRNA and protein, were unable to suppress $CD4^+$ effector T cell proliferation, but acquired regulatory activity and *de novo* Foxp3 expression following TGF-β1 exposure. Furthermore, suppression was IL-10 dependent since anti-IL-10R antibody treatment completely abrogated this suppression, consistent with the ability of TGF-β1 treated $CD4^+CD25^-CD45RB^{LOW}$ to synthesize IL-10 mRNA upon re-stimulation *in vitro*. Lastly, we show that TGF-β1 treatment or blockade did not lead to enhanced expansion or function of naturally occurring $CD4^+CD25^+$ regulatory T cells, although it maintained Foxp3 mRNA and protein expression. Altogether, TGF-β1 promotes the induction of IL-10 secreting $CD4^+$ regulatory T cells from $CD4^+CD25^-CD45RB^{LOW}$ precursors through *de novo* Foxp3 production, and maintains natural T regulatory cell peripheral homeostasis by sustaining Foxp3 expression.

Introduction

Self/non-self discrimination is a key feature of immunological tolerance. Self-tolerance relies on various peripheral mechanisms, including expression of suppressor cytokines, and action of various $CD4^+$ regulatory T (T_{REG}) cells that act at multiple levels of an immune response to inhibit self-reactive T cells that have escaped central tolerance (143). $CD4^+$ T_{REG} cells are potent modulators of T cell-mediated immune responses *in vitro* and *in vivo*, and can be subdivided into two main groups: $CD4^+CD25^+$ naturally-occurring regulatory T (nT_{REG}) cells, which acquire their suppressive functions in thymus, and induced T_{REG} (iT_{REG}) cells that acquire their functions in the periphery (144). nT_{REG} cells represent 5-10% of the peripheral $CD4^+$ T cell pool in healthy rodents and humans, acquire their function during normal thymopoesis, and prevent peripheral autoimmune responses. Functional abrogation of nT_{REG} cells provokes multi-organ autoimmunity, and also increases immunity to tumor, grafts, allergens, and microbial pathogens (3). It has become evident that peripheral mechanisms of T_{REG} cell generation exist, due to decreasing thymic output of T lymphocytes with age, yet equal levels of T_{REG} cells among young and old individuals (29). Indeed, induced $CD4^+CD25^+$ (iT_{REG}) cells can be derived from $CD4^+CD25^-$ T cell fractions and either acquire or expand their suppressive activity as a consequence of *in vivo* or *ex vivo* activation of nT_{REG}-depleted $CD4^+$ T cells under unique stimulatory conditions in the periphery.

Foxp3 is essential for nT_{REG} development and function, and its importance is best illustrated in Foxp3-deficient (-/-) mice and patients suffering from IPEX where mutations or deletions in the *foxp3* gene, halts the development of nT_{REG} cells, and consequentially leads to a fatal autoimmune and inflammatory disease (142, 145). Furthermore, ectopic Foxp3 expression confers a suppressor phenotype in murine $CD4^+CD25^-$ T cells similar to that of nT_{REG}

cells (50). Although most studies support the notion that the thymus represents the major site of nT_{REG} cell development, recent reports suggest that $Foxp3^+$ T cell populations may also be induced in the periphery (66, 121, 146, 147). While Foxp3 expression generally correlates with the suppressive activity of different iT_{REG} cells, a number of exceptions have been documented (140). Currently, the intrinsic and extrinsic factors that induce and maintain Foxp3 expression/function and resulting suppressor activity in normal peripheral $CD4^+$ T cells remain poorly defined.

TGF-β1 is a potent suppressive cytokine critically involved in the induction of tolerance and the regulation of immune responses (69). This is best illustrated by the onset of a severe autoimmune-like syndrome in TGF-$\beta1^{-/-}$ mice characterized by the spontaneous and progressive, multi-organ infiltration of mononuclear cells and pathogenic auto-antibodies (142). Furthermore, disruption of TGF-β1 signaling in T cells by deletion of the TGF-β type II receptor, or inactivation of the receptor-activated *smad3* gene, results in dysregulated T cell responses (91, 92, 102). The mechanism through which TGF-β1 mediates its tolerogenic functions is not totally understood. The potential role of TGF-β1 in the development, differentiation, expansion or suppressive mechanism of nT_{REG} and iT_{REG} cells remains of great importance. TGF-β1 has recently been shown to promote nT_{REG} cell expansion as well as generation of $Foxp3^+$ iT_{REG} cells from $CD4^+CD25^-$ T cells, although the underlying molecular mechanisms remain ill-defined.

Here, we evaluated the effect of TGF-β1 in inducing T_{REG} cells from non-regulatory $CD4^+$ T cell populations, as well as its contribution to the suppressive function and Foxp3 expression in $CD4^+CD25^+$ nT_{REG} population. We confirm that TGF-β1 is able to induce Foxp3 expression and suppressor function in non-regulatory $CD4^+CD25^-$ T cell precursors in a dose-

dependent fashion and in the absence of APC co-stimulatory signals. However, TGF-β1 was unable to induce suppressive functions and Foxp3 expression in activated, naïve $CD4^+CD25^-$ $CD45RB^{HIGH}$ T cells, and its effects were solely restricted to the antigen-experienced $CD4^+CD25^-CD45RB^{LOW}$ T cell subset. We also show that TGF-β1 priming was not required for the acquisition of nT_{REG} cell proliferative and effector functions but could maintain Foxp3 expression in nT_{REG} cells. Finally, the induction of T_{REG} cells by TGF-β1 from antigen-experienced $CD4^+$ T cells resulted in IL-10 expression and anti-IL-10R treatment reversed the suppression mediated by iT_{REG} cells *in vitro*. Thus, TGF-β1 sustains regulatory networks by positively modulating *de novo* Foxp3 expression and induces the post-thymic development of T_{REG} cells.

Materials and Methods

Mice. Foxp3-GFP knock-in (kind gift from A. Rudensky, Univ. Wash), C57BL/6 and OT-I mice (Taconic) were bred and housed in a SPF animal facility at McGill University, and all animal studies were conducted according to institutional norms. Unless specified, mice used in all experiments were 6–10 wk of age.

Reagents. Human rIL-2 was a kind gift from the Surgery Branch (NCI, NIH). Human rTGF-β1 was a kind gift from John Letterio (NCI, NIH). Anti–TGF-β (1D11) and anti-CD3 epsilon (2C11) antibodies were produced in house by supernatant purification on a protein G column. Anti-CD4, anti-FITC and anti-PE magnetic beads were purchased from Miltenyi Biotec. Anti-IL10R Ab (1B1.3a) was a kind gift from Kevin Moore (DNAX). For cell culture, RPMI-1640 was supplemented with 10% heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml), 2 mM L-glutamine, 10 mM Hepes, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate (Invitrogen Life Technologies) and 50 μM 2-ME (Sigma-Aldrich).

Flow cytometric analysis. Single-cell suspensions prepared from lymph nodes (LN) of adult C57BL/6 or Foxp3-GFP knock-in reporter mice were stained and analyzed on FACSCalibur using CellQuest software (BD Biosciences). For direct staining to determine the phenotype of lymphocyte populations, Fc receptors were blocked with anti-CD16/32 Ab (2.4G2; BD PharMingen), and then stained with the following conjugated antibodies: PE- or allophycocyanin- (APC) anti-CD25 (PC61) (eBioscience), PE Cytocrome 7-anti-CD4 (L3T4), TriColor-anti-CD4 (RM4-5) (CalTag Laboratories), PE- or FITC-anti-CD45RB (16A) (eBioscience), PE-anti-CD69 (HI.2F3), PE-anti-CD62L (MEL-14) (BD PharMingen), as well as PE-IgG2a and PE-IgG1 isotype controls. For intra-nuclear Foxp3 staining, cells were stained

with antibodies against surface antigens, then were fixed, permeabilized and stained with PE-Foxp3 (PJK-16s) according to the manufacturer's protocol (eBioscience).

Purification of T cell subsets. Different T cell subsets were isolated on a FACSAria (BD Biosciences) cell sorter as previously described (98). Briefly, LN were collected from adult C57BL/6 or Foxp3-GFP knock-in reporter mice and single cell suspensions were prepared by macerating cells through a sterile wire mesh. The resulting cell suspension was stained with APC-anti-CD25 (10 μg/10^8 cells), PE-anti-CD45RB (3.5 μg/10^8 cells) and PECy7-anti-CD4 (3 μg/10^8 cells) in PBS/2% FCS for 20 min at 4°C, washed, and resuspended in PBS/2% FCS. The purity of the final $CD4^+CD25^-$ $CD45RB^{LOW/HIGH}$ preparation was typically >98%.

In vitro T cell priming assays. To generate iT_{REG} cells *in vitro*, $CD4^+CD25^-$ T cells ($5x10^5$) were cultured in 24-well, flat-bottom microtiter plates in cRPMI-1640, either with plate bound anti-CD3 (10 μg/ml), or soluble anti-CD3 (2 μg/ml) with equal number of APC for 72 h, in the presence or absence of titrated doses of human rTGF-β1 (0.1-10 ng/ml). Irradiated (3,000 Rads), T cell–depleted spleen cells were used as APCs and were prepared as previously described (98). $CD4^+$ or $CD8^+$ responder T cells were prepared from LN of appropriate adult mice, as previously described (17). To measure the effect of TGF-β1 on nT_{REG} cells, $CD4^+CD25^+$ T cells ($5x10^5$) were cultured with 10 μg/ml of plate bound anti-CD3 for 72 h in presence or absence of human rhTGF-β1 (3 ng/ml) and IL-2 (5 ng/ml). In some instances, 10 μg/ml of anti-TGF-β neutralizing Ab was added to the culture.

In vitro proliferation/suppression assays. Proliferation assays were performed by stimulating responding T cells ($2.5x10^4$) in 96 flat-bottom microtiter plates in cRPMI 1640, with plate-bound (10 μg/ml) or with soluble anti-CD3 (2 μg/ml) and irradiated, APC ($1x10^5$) for 72 h at

37°C in 5 % CO_2. For suppression assays, rhTGF-β1 treated or untreated T cells (0.625 to 2.5x10^4) were co-cultured with OT-I transgenic (Tg) $CD8^+$ responder T cells (2.5x10^4), with soluble anti-CD3 (2 μg/ml) and APC (1x10^5) in 96-well plates for 72 h at 37°C / 5 % CO_2. Cell cultures were pulsed with 0.5 μCi ^{3}H-thymidine for the last 6–12 h to determine the extent of suppression. When specified, anti-IL-10R (3.5 μg/ml) was added to the co-cultures.

RT-PCR. Evaluation of Foxp3 mRNA levels in TGF-β1 treated and untreated $CD4^+$ T cells was performed as previously described (50). Briefly, total RNA was extracted from 0.5-1 x 10^6 cells using Trizol reagent (Invitrogen) and reverse transcribed using the Superscript II RNase H reverse-transcriptase (Invitrogen) and pd$(N)_6$ random hexamers (0.05 μg/ul, GE Healthcare). Foxp3 mRNA levels were quantified by RT-PCR using Chromo4 Continuous Fluorescence Detector (Bio-Rad). Foxp3 primers and internal fluorescent TaqMan probes specific were used as previously described (50). 18S rRNA primers and probe were purchased from Applied Biosystems. Relative delta delta Ct method was used to quantify Foxp3 mRNA levels normalized to 18S rRNA. IL-10, TGF-β1 and G3PDH mRNA levels were quantified by semi-quantitative PCR under following conditions: 5 min 95°C fallowed by 25 cycles at 1 min 95°C, 1 min 58°C, 1 min 72°C, 5 min final extension at 72°C. The primers for IL-10, TGF-β1 and G3PDH were obtained from BD Biosciences. PCR products were analyzed on 2 % agarose gels and the intensity of each band was measured by TotalLAB program.

Results

TGF-β1 induces CD4$^+$ T_{REG} cells from CD4$^+$CD25$^-$ T cell precursors.

Some recent reports have suggested that TGF-β1 can promote the development of T_{REG} cells from non-T_{REG} cell fraction through Foxp3 induction (66, 121, 146). As an immediate re-stimulation of T cells leads to activation-induced cell death (AICD), a direct measurement of suppressive activity upon TGF-β1 treatment within 24h was impossible (148). Nevertheless previous studies have revealed that the peak of Foxp3 induction occurs at day 3 post stimulation, which was chosen as the earliest time point for evaluation of suppressive properties (66). In order to assess TGF-β role in inducing T_{REG} functions in non-regulatory CD4$^+$ T cells, CD4$^+$CD25$^-$ T cells were activated in the absence or presence of different concentrations rhTGF-β1 for 3 days, after which the suppressive activity was measured by co-culturing treated cells with CD8$^+$ responding T cells, soluble anti-CD3 (2 μg/ml) and irradiated, T-cell depleted splenocytes as source of APC. While TGF-β1 displayed potent anti-proliferative effects on activated T cells (Fig. 1A), treatment with rhTGF-β1 induced regulatory functions in CD4$^+$CD25$^-$ T cells. *In vitro* activation of CD4$^+$CD25$^-$ T cells in the presence of high concentrations of TGF-β1 (10 ng/ml) induced cell-dose dependent suppression of proliferation attaining 80% suppression at 1:1 T_{REG} to T_{EFF} cell ratio (Fig. 1B, upper panels), in contrast to untreated CD4$^+$CD25$^-$ T cells, which never inhibited the proliferation of CD8$^+$ T cells. This regulatory function decreased concomitantly with lower TGF-β1 priming concentrations such that 20% suppression (at 1:1 T_{REG} to T_{EFF} cell ratio) was achieved at 0.1 ng/ml of TGF-β1 pretreatment. It remained possible that TGF-β1 can mediate this effect indirectly via its action on APC or that APC could compete for the cytokine with responding T cells, thus leading to inferior induction of regulatory phenotype. Therefore, we activated CD4$^+$CD25$^-$ T cells under

plate bound anti-CD3 conditions (APC-free) in the presence of titrated doses of rhTGF-β1 (Fig. 1B, lower panels). Consistent with our previous results, the induction of T_{REG} cells by TGF-β1 was dose dependent as the highest suppression levels were achieved with the highest TGF-β1 doses with suppression decreasing to 57% at 1:1 T_{REG} to T_{EFF} cell ratio at lowest TGF-β1 concentrations. Intriguingly, a higher induction of suppression was achieved in the presence of APC (57% vs 20%) under low-dose TGF-β1 conditions. This data suggests that TGF-β1 can promote the development of T_{REG} cells in the absence of APC-derived signals.

As Foxp3 has been associated with the induction of regulatory function in T cells, and TGF-β1 has been reported to induce its expression, we next compared the Foxp3 mRNA levels in TGF-β1 treated and untreated $CD4^+CD25^-$ T cells. Quantitative RT-PCR analysis revealed that TGF-β1 was able to induce Foxp3 gene expression in treated cells in a dose-dependent manner (Fig. 1C). At low TGF-β1 doses (0.1ng/ml), TGF-β1 induced a two-fold increase in Foxp3 mRNA levels, while at higher doses, a 20 fold higher level of Foxp3 gene expression was observed compared to untreated T cells (Fig. 1C). Notably, the acquisition of suppressive activity in $CD4^+CD25^-$ T cells by TGF-β1 pretreatment was concentration dependent and correlated with greater synthesis of Foxp3 mRNA (Fig. 1C). Collectively, these findings confirm the general observations from previous studies that TGF-β1 induction of suppressive activity in $CD4^+CD25^-$ T cells is associated with Foxp3 up-regulation.

TGF-β1 induces $CD4^+$ T_{REG} cells only from $CD4^+CD25^-CD45RB^{LOW}$ T cells.

Although TGF-β1 is able to induce regulatory activity in $CD4^+$ T cell populations largely devoid of $CD25^+Foxp3^+$ expressing T cells, previous studies do not directly demonstrate that TGF-β1 can drive the differentiation of *bona fide* naïve $CD4^+$ T cells into a

regulatory phenotype. As $CD4^+CD25^-$ T cells remain a considerably heterogeneous population with variable degrees of antigen-experience, we investigated the effect of TGF-β1 priming on a truly naïve subset within $CD4^+CD25^-$ T cells, namely $CD25^-CD45RB^{HIGH}$ T cells. $CD4^+CD25^-CD45RB^{HIGH}$ T cell subset has been previously characterized as potently pathogenic due to its ability to transfer IBD in SCID recipient mice (28). To this end, we FACS sorted $CD4^+CD25^-CD45RB^{HIGH}$ T cells from LN of C57BL/6 mice, tested their suppressive activity directly *ex vivo*, or activated them *in vitro* with plate bound anti-CD3 in the presence or absence of rhTGF-β1 (3 ng/ml) for 3 days, and then assessed their suppressor activity (Fig. 2A). Similarly to freshly isolated $CD25^-CD45RB^{HIGH}$ T cells, TGF-β1 treatment strikingly did not induce any regulatory functions in naïve $CD4^+CD25^-CD45RB^{HIGH}$ T cells, as treated cells never inhibited the proliferative capacity of responder T cells (Fig. 2C).

As $CD4^+CD25^-$ T cells contain approximately 30-40 % of antigen-experienced $CD4^+CD25^-CD45RB^{LOW}$ T cells in adult mice (24), we postulated that this subset might represent the cellular target of TGF-β1 in our system. We first confirmed the antigen-experience of this population by examining the frequency of activated/memory cells. Although the CD69 expression profile was identical in both $CD25^-CD45RB^{HIGH}$ and $CD25^-CD45RB^{LOW}$ T cells, $CD4^+CD25^-CD45RB^{LOW}$ T cells contained approximately 40% of $CD62L^{LOW}$ cells (Fig. 2B). To examine $CD4^+CD25^-CD45RB^{LOW}$ T cells functionally, we FACS sorted $CD25^-CD45RB^{LOW}$ T cells from LN of C57BL/6 mice, tested their suppressive activity directly *ex vivo*, or activated them *in vitro* as described in Figure 1, and then assessed their *in vitro* suppressor activity, relative to $CD4^+CD25^+$ nT_{REG} cells (Fig. 2C). Freshly-isolated $CD4^+CD25^-CD45RB^{LOW}$ T cells did not suppress proliferation of $CD8^+$ T cells compared to nT_{REG} cells, which readily resulted in more than 85 % suppression at a 1:1 T_{REG} to T_{EFF} cell ratio (Fig. 2C).

Most importantly, TGF-β1 pretreated $CD25^{-}CD45RB^{LOW}$ cells were able to suppress proliferation of OT-I cells up to 75% at 1:1 T_{REG} to T_{EFF} cell ratio, in contrast to cells cultured in the absence of TGF-β1 as proliferation levels were equivalent or higher than OT-I cells alone. These results clearly identify the antigen-experienced $CD25^{-}CD45RB^{LOW}$ T cell subset as a cellular target for the regulatory inducing functions of TGF-β1.

TGF-β1 induces Foxp3 mRNA levels in $CD4^{+}CD25^{-}CD45RB^{LOW}$ T cells.

TGF-β1 is characterized by its ability to inhibit the proliferative capacity of responding T cells. To this end, the proliferation of freshly-isolated $CD25^{-}CD45RB^{LOW}$ and $CD25^{-}CD45RB^{HIGH}$ T cells was assessed following anti-CD3 stimulation in the absence or presence of rhTGF-β1. Our results indicate that untreated, $CD25^{-}CD45RB^{LOW}$ cells were hypo-proliferative compared to $CD25^{-}CD45RB^{HIGH}$ cells (Fig. 3A), but upon TGF-β1 priming, the proliferation of both cell subsets was substantially decreased, with $CD25^{-}CD45RB^{HIGH}$ T cells showing a greater susceptibility to the growth inhibitory effects of TGF-β1 than $CD25^{-}CD45RB^{LOW}$ cells (60% versus 44%, respectively, $p<0.02$). In summary, our results show that TGF-β1 can selectively induce the expression of Foxp3 in antigen-experienced $CD25^{-}CD45RB^{LOW}$ T cells and not in naive $CD4^{+}$ T cells.

We next examined Foxp3 mRNA levels in freshly isolated and TGF-β1 primed $CD4^{+}CD25^{-}CD45RB^{LOW}$ and $CD4^{+}CD25^{-}CD45RB^{HIGH}$ T cell subsets relative to $CD4^{+}CD25^{+}$ nT_{REG} cells. Interestingly, $CD4^{+}CD25^{-}CD45RB^{LOW}$ T cells expressed up to a 10 fold increase in Foxp3 mRNA levels than $CD4^{+}CD25^{-}CD45RB^{HIGH}$ T cells directly *ex vivo*, although at significantly lower levels than $CD4^{+}CD25^{+}$ nT_{REG} cells (Fig. 3B). This observation is

consistent with 5-10% of $CD4^+CD25^-CD45RB^{LOW}$ T cells expressing Foxp3 directly *ex vivo* in contrast to naïve T cells (Fig. 2B), albeit not at the same frequency as nT_{REG} cells (data not shown). After 3 days of TGF-β1 priming, no detectable increase in Foxp3 could be observed in naïve T cells, a finding consistent with the apparent absence in regulatory function in these cells following TGF-β1 treatment. In stark contrast, TGF-β1 pre-treatment induced approximately a three-fold increase in Foxp3 mRNA levels in $CD4^+CD25^-CD45RB^{LOW}$ cells compared to untreated cells, and represented a 30 fold increase over $CD4^+CD25^-CD45RB^{HIGH}$ treated T cells ($p<0.002$, Fig. 3B). These levels represented almost 50% of the Foxp3 levels detected in resting $CD4^+CD25^+$ nT_{REG} ($p<0.003$, Fig. 3B).

TGF-β1 induces de novo Foxp3 expression and regulatory function in $CD4^+Foxp3^-CD45RB^{LOW}$ T cell precursors.

As $CD4^+CD25^-CD45RB^{LOW}$ T cells contain a small (~5-10%) endogenous $Foxp3^+$ subset, it remained possible that the TGF-β1 inhibitory functions were induced through preferential expansion/differentiation of these cells, in contrast to *de novo* Foxp3 expression in this subset (63). To discriminate the effects of TGF-β1 on $Foxp3^+$ and $Foxp3^-$ cells within the $CD4^+CD25^-CD45RB^{LOW}$ T cell subset, $CD4^+CD45RB^{LOW}Foxp3^+$, $CD4^+CD45RB^{LOW}Foxp3^-$ and $CD4^+CD45RB^{HIGH}Foxp3^-$ T cells were isolated from Foxp3-GFP knock-in reporter mice, as described (Fig. 4A). First, the proliferation of freshly-isolated $CD25^-CD45RB^{LOW}Foxp3^-$ and $CD25^-CD45RB^{HIGH}$ $Foxp3^-$ T cells was assessed following anti-CD3 stimulation in the absence or presence of rhTGF-β1. The $CD45RB^{LOW}$ subset depleted of $Foxp3^+$ cells were consistently hypoproliferative compared to $Foxp3^-CD45RB^{HIGH}$ cells (Appendix, Fig.1A), but upon TGF-β1 treatment, the proliferation of both cell types was substantially decreased, with $Foxp3^-$ $CD45RB^{High}$ T cells showing a greater susceptibility to the growth inhibitory effects of TGF-β1

than Foxp3$^-$CD45RB^{LOW} cells (59% versus 44%, respectively, $p<0.03$). Moreover, Foxp3$^-$ CD45RB^{LOW} cells, pre-treated for 3 days with TGF-β1, were able to suppress proliferation of OT-I cells up to 55% at 1:1 T_{REG} to T_{EFF} cell ratio contrary to Foxp3$^-$CD45RB^{HIGH} treated cells (Fig. 4B, Appendix Fig. 1B) demonstrating that regulatory functions are induced in populations initially lacking nT_{REG} cells but not from truly naïve T cells. In order to determine whether TGF-β1 induces Foxp3 protein expression, rhTGF-β1 treated and untreated cells were examined for Foxp3/GFP levels by FACS. Our results show that TGF-β1 induces *de novo* expression of Foxp3 only in the activated CD45RB^{LOW} population (5% versus 20%, of rhTGF-β1 treated cells, $p<0.03$) (Fig. 4C). Thus, we show that TGF-β1 priming selectively induces *de novo* Foxp3 protein expression and regulatory functions in CD4$^+$CD25$^-$CD45RB^{LOW} T cells devoid of any Foxp3$^+$ T cells.

TGF-β1 maintains Foxp3 expression in CD4$^+$CD25$^+$ nT_{REG} cells without potentiating their expansion or function.

Recent reports concluded that a latent, membrane-bound form of TGF-β1 on CD4$^+$CD25$^+$ nT_{REG} cells was responsible for their suppressive functions *in vitro* and *in vivo*, albeit never formally demonstrating a direct effect of T_{REG} cell-derived TGF-β1 on responder T cells (94). Furthermore, these studies did not examine the possibility that cell surface TGF-β1, from autocrine or paracrine sources, mediates its effects by actively signaling in CD4$^+$CD25$^+$ T_{REG} cells themselves, possibly maintaining their differentiation, expansion, or suppressive function, as suggested by recent reports (115). To this end, we purified CD4$^+$CD25$^+$ T cells, activated them *in vitro*, and assessed their proliferation and functional activity either following TGF-β1 neutralization or exogenous priming. TGF-β1 treatment did not promote the expansion

of $CD4^+CD25^+$ T cells *in vitro* (Fig. 5A). In addition, neutralization of TGF-β1 or pre-conditioning with rhTGF-β1 *in vitro* failed to abrogate or promote T_{REG} function, respectively, suggesting that autocrine or paracrine sources of TGF-β1 do not modulate the proliferation or suppressor phase of nT_{REG} activity (Fig. 5B). However, when nT_{REG} cells were incubated with rhTGF-β1 for 3 days in the presence of IL-2 (5 ng/ml), an important increase in Foxp3 mRNA levels was detected ($p<0.003$, Fig. 5C). In order to directly investigate the effect of TGF-β1 on Foxp3 protein expression in nT_{REG} cells, $CD4^+Foxp3^+$ cells were sorted from pLN of Foxp3-GFP knock in mice and activated with plate bound anti-CD3 in the presence or absence of rhTGF-β1. Thereafter, Foxp3 levels, as determined by GFP expression, were measured by FACS 24 hrs post-stimulation. We show that in the presence of rhTGF-β1, no significant change in Foxp3 expression was observed, as all cells remained $Foxp3^+$ (Fig. 5D). Interestingly, 10% of $Foxp3^+$ cells lost Foxp3 expression in the absence of exogenous rhTGF-β1 stimulation. Our results show that TGF-β1 does not affect the expansion or function of $CD4^+CD25^+$ nT_{REG} cells, but that TGF-β1 maintains Foxp*3* expression in nT_{REG} cells.

TGF-β1 induced T_{REG} cell effector function is IL-10 dependent.

In recent years, a variety of iT_{REG} cells capable of controlling T cell responses *in vitro* and *in vivo* have been described, and their regulatory functions have largely been attributed to the production of certain signature suppressor cytokines after antigen priming, like TGF-β1 or IL-10 (140, 149). In order to determine whether the *in vitro* suppressive function of TGF-β1-iT_{REG} cells is cytokine dependent, we measured IL-10 and TGF-β1 mRNA levels by RT-PCR in resting or PMA/ionomycin re-stimulated $CD25^-CD45RB^{HIGH}$ or $CD25^-CD45RB^{LOW}$ T cells initially primed in the presence or absence of TGF-β1 stimulation. TGF-β1 gene expression

was not detected in $CD25^{-}CD45RB^{HIGH}$ or $CD25^{-}CD45RB^{LOW}$ T cells, irrespective of stimulation (data not shown). Interestingly, while low levels of IL-10 mRNA were detected in resting TGF-β1 treated $CD25^{-}CD45RB^{LOW}$ cells, significantly higher levels of IL-10 mRNA were detected in reactivated, TGF-β1 treated $CD25^{-}CD45RB^{LOW}$ cells (Fig. 6A). $CD25^{-}CD45RB^{HIGH}$ cells did not express IL-10 in any condition tested (Fig. 6A). These results demonstrate that TCR triggering is required for IL-10 production in TGF-β1 iT_{REG} cells, consistent with the requirement of reactivation of these cells to reveal their regulatory potential.

To further assess the involvement of IL-10 in the suppression function of TGF-□1 iT_{REG} cells, we co-cultured TGF-β1 pre-treated $CD25^{-}CD45RB^{LOW}$ or $CD25^{-}CD45RB^{HIGH}$ cells with responder $CD8^{+}$ T cells, either in the presence or absence of a neutralizing anti-IL-10R blocking Ab. As discussed, TGF-β1 treated or untreated $CD25^{-}CD45RB^{HIGH}$ T cells never displayed any *in vitro* regulatory function under any conditions tested (Fig. 6B, upper panels). In contrast, addition of an anti-IL-10R blocking Ab to TGF-β1 treated $CD25^{-}CD45RB^{LOW}$ T cells almost completely abrogated suppression, reducing suppression levels from 73 % (isotype control) to 17 % (anti-IL-10R Ab) at a 1:1 T_{REG} to T_{EFF} cell ratio (Fig. 6B, lower panels). Similar results were obtained with $Foxp3/GFP^{Negative}$ $CD4^{+}CD25\text{-}CD45RB^{LOW}$ T cells activated in the presence of TGF-β1 (data not shown). These results demonstrate that TGF-β1 iT_{REG} cells produce IL-10 and that their *in vitro* suppressive function is IL-10 dependent.

Discussion

The present study evaluated the ability of TGF-β1 to induce, maintain, or enhance suppressive functions in different $CD4^{+}$ T cell subsets. We show that TCR engagement in conjunction with TGF-β1 stimulation can up-regulate Foxp3 expression and drive the differentiation of T_{REG} cells from peripheral $CD4^{+}CD25^{-}$ T cell progenitors. Strikingly, we

demonstrate that *bona fide* naïve $CD25^{-}CD45RB^{HIGH}$ T cells, known to be potently pro-inflammatory *in vitro* and *in vivo* (28), did not acquire regulatory functions or up-regulate Foxp3 upon TGF-β1 treatment. The effect of TGF-β1 on the fate of T cell activation is determined not only by an essential TCR trigger, but also on the TGF-β1 dose, since the suppressor potency and *de novo* Foxp3 gene expression increases with the priming dose. We also show that this ability to promote *de novo* Foxp3 expression and T_{REG} cell function by TGF-β1 is restricted to an antigen-experienced $Foxp3^{-}CD45RB^{LOW}$ T cells, and not naïve $Foxp3^{-}CD45RB^{HIGH}$, responder T cells. In a similar fashion, TGF-β1 maintains Foxp3 expression in $CD4^{+}CD25^{+}$ nT_{REG} cells, however, without potentiating their expansion or function. Lastly, we show that TGF-β1 drives the expression of IL-10 upon TCR re-activation in primed T cells, and *in vitro* blockade of IL-10 abrogated suppression.

A number of studies show that TGF-β1 is a natural inducer of Foxp3 expression and regulatory activity in nT_{REG} cell-depleted $CD4^{+}$ T cells, namely $CD4^{+}CD25^{-}$ T cells (66, 121, 146). Although these TGF-β1 effects have been substantiated in *in vitro* and *in vivo* models, the authors from these reports fail to demonstrate whether these effects can truly be achieved in truly naïve $CD4^{+}$ T cell populations. Another fundamental difference between these reports and the present study may depend on the time necessary to develop regulatory functions, with naïve T cells requiring prolonged stimulation contrary to memory T cells. Recently human nT_{REG} cells have been shown to derive from rapid turnover of memory populations *in vivo* supporting the claim that the antigen experienced fraction serves as the possible reservoir that is more prone to develop regulatory functions under specific stimulatory conditions (150).The generation of iT_{REG} cells from naïve T cells isolated from Ag-specific TCR transgenic mice on a $RAG2^{-/-}$ background, which do not possess nT_{REG} cells, has been described, however it

remains unclear whether TGF-β1 played any role in this process (147, 151, 152). Other signals such as vitamin D_3 and dexamethasone have been demonstrated to induce IL-10 producing, $Foxp3^-$ iT_{REG} cells suggesting that the induction of Foxp3 is a not a prerequisite for T_{REG} development (140).

Normal, healthy mice harbor a significant frequency of naturally activated T cells, often found within the $CD4^+CD45RB^{LOW}$ pool, which may represent physiological autoreactivity as they are also found in germ-free or antigen-free mice. Several lines of evidence suggest that this pool may represent a reservoir from which T_{REG} cells originate in the periphery (28). Many models show that $CD4^+CD45RB^{LOW}$ cells limit the pathogenicity of the counterpart $CD4^+CD45RB^{HIGH}$ naive T cells as the former include nT_{REG} cells (13, 26). Other studies show that nT_{REG} are not exclusively contained within CD25-expressing $CD4^+$ T cells since nT_{REG} markers like CD103, GITR, CTLA-4, and Foxp3 are also found in the $CD45RB^{LOW}CD25^-$ subpopulation, while being absent in the $CD25^-CD45RB^{HIGH}$ subset (13, 153). Zelenay *et al.* has shown that freshly-isolated $CD4^+CD45RB^{LOW}$ $CD25^-Foxp3^+$ T cells, albeit not directly suppressive *ex vivo*, constitute a reservoir of committed T_{REG} cells that regain CD25 expression upon homeostatic expansion (154). Furthermore, Fontenot *et al.* clearly demonstrated by means of mice containing a GFP-Foxp3 knock-in allele that $CD4^+Foxp3^+CD25^-$ (largely $CD45RB^{LOW}$) and $CD4^+Foxp3^+CD25^+$ possess identical suppressive function *in vitro* and display different gene expression signatures as $CD4^+Foxp3^-CD25^+$ T cells (largely $CD45RB^{HIGH}$) (63). Thus, although the thymic origin of $Foxp3^+CD4^+$ nT_{REG} cells is unquestioned, our results also point to another non-mutually exclusive possibility whereby TGF-β1 may actually serve as T_{REG} differentiating factor for distinct lineage(s) of T_{REG}-like cells found within this subset of $CD4^+Foxp3^-CD25^-$. In this manner, extrinsic signals such as TGF-β1 could possibly favor the

terminal differentiation of pre-existing non-$CD25^+$ thymically derived T_{REG}-like cells in the periphery, and contribute to the peripheral development of those cells.

Our results illustrate that TGF-β1-induced $CD4^+Foxp3^+$ T cells, generated from $CD25^-CD45RB^{LOW}$ precursor T cells, have increased expression of IL-10, and little or no TGF-β1 production upon TCR re-engagement, and the suppressor activity is abrogated with IL-10 neutralization *in vitro*. Chen *et al.* has demonstrated that IL-10 fails to induce Foxp3 in $CD4^+CD25^-$ T cells *in vitro*, indicating that IL-10 is not directly involved in the induction of the T_{REG} phenotype (66). The major inhibitory role of IL-10 is to act on APC function to decrease co-stimulation and secretion of pro-inflammatory cytokines (123, 124). As the action of IL-10 on activated T cells causes re-expression of TGF-βRII, and in turn, promotes TGF-β signaling, it is possible that IL-10 potentiates TGF-β effects (155). TGF-β1-induced Foxp3 can down-regulate Smad7 expression in $CD4^+$ T cells by a direct effect on *smad7* promoter activity, thereby down-modulating the key negative pathway in TGF-β1 signaling (121). Thus, iT_{REG} cells could themselves be more refractive to TGF-β1 inhibitory effects and simultaneously increase the susceptibility of other T cells to this cytokine by secretion of IL-10.

TGF-□1 has also been shown to promote the development, expansion or effector function of $Foxp3^+CD4^+$ nT_{REG} cells, and the apparent expression of a latent, membrane-bound form of TGF-β1 on nT_{REG} cells isolated from inflamed tissues has been at the root of this premise (94, 111, 156). $CD4^+CD25^+$ T cells from mice expressing a dominant negative form of TGFβRII failed to expand *in vivo* and to suppress dextran sulfate sodium induced colitis (107). We recently showed that nT_{REG} cells from either WT or neonatal TGF-$\beta1^{-/-}$ mice are potently suppressive *in vitro*, and can equally suppress the incidence and severity of T cell-induced IBD (108). In this report, we show that exposure of nT_{REG} cells to exogenous TGF-β1 *in vitro* did

not induce the growth of nT_{REG} cells either in the presence or absence of IL-2, an established critical signal for the induction of nT_{REG} cell function *in vitro* and *in vivo*. Our results also show that the suppressive activity was not affected when freshly-isolated $CD4^+CD25^+$ nT_{REG} cells were activated in the presence or absence of exogenous TGF-β1, or anti-TGF-β1 blocking Ab, suggesting that autocrine or paracrine sources of TGF-β1 do not modulate nT_{REG} cell effector function *in vitro*. This is consistent with the observation that TGF-β-resistant $Smad3^{-/-}$ nT_{REG} cells are equivalent to WT nT_{REG} cells in their capacity to suppress IBD (108). However, our results show that Foxp3 gene and protein expression in nT_{REG} cells are significantly increased or maintained following TGF-β1 priming indicating that TGF-β1 may be a stabilizer of Foxp3 expression, and ultimately sustaining nT_{REG} peripheral homeostasis. Consistently, Marie *et al.* showed that TGF-β1 signaling in nT_{REG} cells may promote Foxp3 expression (115). Thus, although TGF-β is dispensable for mediating nT_{REG} activity, long-term TGF-β signaling may be required to sustain regulatory networks by promoting the development of $Foxp3^+T_{REG}$ cells from antigen-experienced $CD4^+$ T cells and bolster Foxp3 expression in $CD4^+$ nT_{REG} cells.

In summary, the present study provides evidence that TGF-β1 plays an essential role in the generation of IL-10-producing, $CD4^+T_{REG}$ cells in the periphery via the induction of Foxp3 in a subset of antigen-experienced $CD4^+$ T cells. In addition, TGF-β1 stabilizes Foxp3 expression in nT_{REG} cells without affecting their expansion or effector function. Our results suggest that TGF-β1 sustain regulatory networks by positively modulating *de novo* Foxp3 expression and inducing the post-thymic development of T_{reg} cells, and maintaining nT_{REG} cell peripheral homeostasis by bolstering Foxp3 expression. The concept that T_{REG} cells can be generated *in vitro* from a large pool of antigen-experienced $CD4^+$ T cells via TGF-β1 leads to the possibility that novel therapeutic strategies may be developed to potentiate the development

or function of these cells in the periphery for the treatment of autoimmune and other chronic inflammatory diseases. Alternatively, manipulation of the TGF-β1/Foxp3 axis may abrogate dominant tolerogenic mechanisms, and consequentially promote immunity to pathogens and cancers.

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Figure 1. TGF-β1 induces CD4$^+$ T_{reg} cells from CD4$^+$CD25$^-$ T cell precursors.

(A) Anti-proliferative effect of TGF-β1 on freshly isolated CD4$^+$CD25$^-$ T cells stimulated with plate bound (5 μg/ml; right panel) or soluble anti-CD3 (2 μg/ml; left panel) with irradiated APC ($2x10^4$) in the absence or presence of increasing concentrations of rhTGF-β1 (0-10 ng/ml) for 3 days. (**B**) Three days post-activation, rhTGF-β1 treated or untreated cells ($1.25\text{-}5x10^4$ cells) were co-cultured with OT-I Tg CD8$^+$ responder T cells ($5x10^4$), irradiated APC ($2x10^5$) and anti-CD3 (2 μg/ml) for 72 h. Cultures were pulsed with 0.5μCi ^{3}H-thymidine for the last 6-12 h of incubation. The data are shown as mean CPM± SD (left panels) or % suppression (right panels). The data are representative of three independent experiments. (**C**) TGF-β1 dose dependent induction of Foxp3 mRNA levels and suppressor function at 1:1 T_{reg} to effector T cell ratio. Relative Foxp3 mRNA levels relative to 18S rRNA were assessed by qRT-PCR in TGF-β1 treated and untreated CD4$^+$CD25$^-$ T cells 3 days post-stimulation.

Figure 1

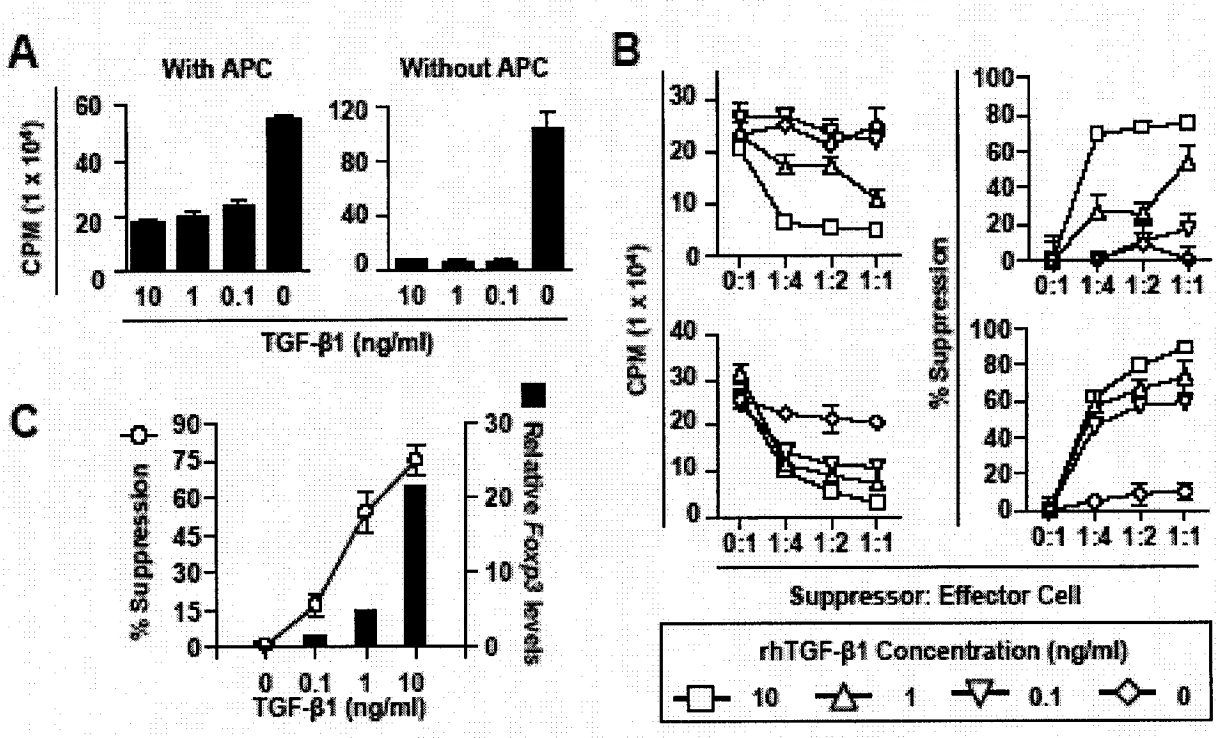

A
With APC
Without APC
CPM (1 x 10⁴)
60
40
20
0
120
80
40
0
10 1 0.1 0
10 1 0.1 0
TGF-β1 (ng/ml)
B
CPM (1 x 10⁴)
30
20
10
0
40
30
20
10
0
0:1 1:4 1:2 1:1
% Suppression
100
80
60
40
20
0
Suppressor: Effector Cell
rhTGF-β1 Concentration (ng/ml)
10
1
0.1
0
C
% Suppression
90
75
60
45
30
15
0
Relative Foxp3 levels
30
20
10
0
0 0.1 1 10
TGF-β1 (ng/ml)

Figure 2. TGF-β1 promotes the development of $CD4^+$ T_{reg} cells uniquely from $CD4^+CD25^-CD45^{Low}$ T cells.

(A) $CD4^+CD25^-CD45RB^{Low}$, $CD4^+CD25^-CD45RB^{High}$ and $CD4^+CD25^+$ populations were FACS sorted from LN of C57BL/6 mice according to the illustrated gates. (**B**) Flow cytometric analysis of CD69, CD62L and Foxp3 expression in $CD45RB^{Low}$ and $CD45RB^{High}$ subsets within $CD4^+CD25^-$ T cells. The regulatory function of $CD4^+CD25^-$ $CD45RB^{High}$ (**C**) and $CD4^+CD25^-CD45RB^{Low}$ (**D**) T cells, relative to $CD4^+CD25^+$ nT_{REG} cells, was measured directly *ex-vivo* (left panel) or after *in vitro* rhTGF-β1 priming (middle and right panels). In brief, specific cell subsets ($5x10^5$) were activated with plate bound anti-CD3 (10 μg/ml) in presence or absence of rhTGF-β1 (3 ng/ml) for 3 days. TGF-β1 treated or untreated cells were then co-cultured at different ratios ($6.25x10^3$ to $2.5x10^4$) with OT-I Tg $CD8^+$ responder T cells ($2.5x10^4$), and stimulated with anti-CD3 (2 μg/ml) in the presence of APC ($1x10^5$). Cultures were pulsed with ^{3}H-thymidine during the last 8h and harvested at 72h. Results of the suppression assay are shown as the mean CPM ± SD (left and middle panels) or % suppression (right panels).

Figure 2

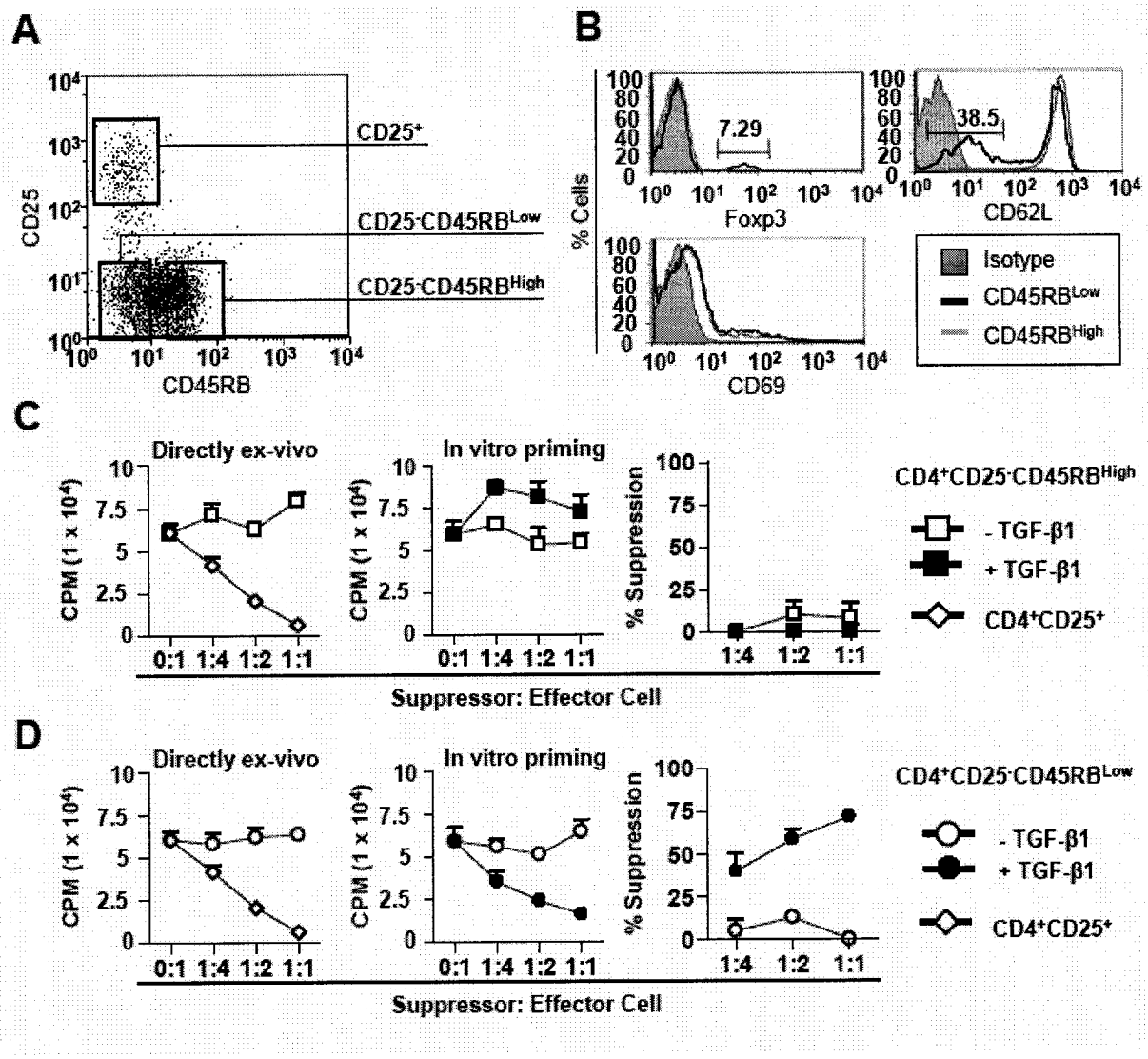

A
CD25
CD45RB
CD25+
CD25-CD45RBLow
CD25-CD45RBHigh
B
% Cells
7.29
Foxp3
38.5
CD62L
CD69
Isotype
CD45RBLow
CD45RBHigh
C
Directly ex-vivo
In vitro priming
CPM (1 x 10⁴)
% Suppression
0:1 1:4 1:2 1:1
1:4 1:2 1:1
CD4+CD25-CD45RBHigh
- TGF-β1
+ TGF-β1
CD4+CD25+
Suppressor: Effector Cell
D
Directly ex-vivo
In vitro priming
CD4+CD25-CD45RBLow
- TGF-β1
+ TGF-β1
CD4+CD25+
Suppressor: Effector Cell

Figure 3. Preferential TGF-β1 effects on proliferation and induction of Foxp3 mRNA levels in $CD4^{+}CD25^{-}CD45RB^{Low}$ T cells.

(A) $CD4^{+}CD25^{-}CD45RB^{Low}$ or $CD4^{+}CD25^{-}CD45RB^{High}$ T cell subsets ($5x10^{5}$) were activated with plate bound anti-CD3 (10 μg/ml) in the absence or presence of rhTGF-β1 (3 ng/ml) for 3 days. Cultures were pulsed with ^{3}H-thymidine during the last 8h and harvested at 72h. Results of the proliferation assay are shown as the mean CPM± SD of triplicate cultures (#, $p<0.02$; *, $p<0.002$). (**B**) Total RNA was isolated from resting or day 3 TGF-β1 stimulated cells ($0.5\text{-}1x10^{6}$), reverse transcribed and relative Foxp3 mRNA levels, normalized to 18S rRNA, were assessed by qRT- PCR. All data are shown as mean of duplicates and are representative of at least three separate experiments (#, $p<0.002$; *, $p<0.003$).

Figure 3

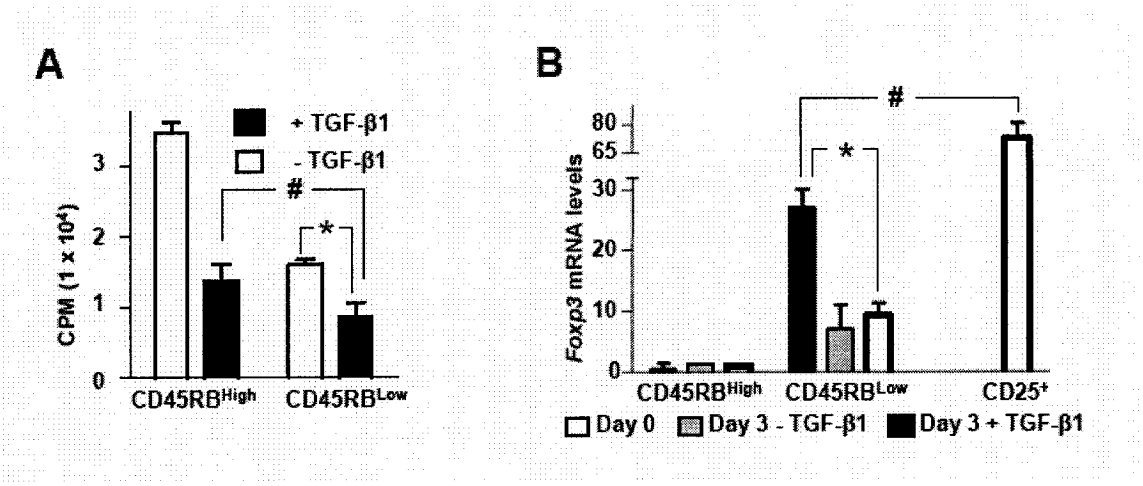

A
+ TGF-β1
- TGF-β1
#
*
CPM (1 x 10⁴)
0
1
2
3
CD45RB^High
CD45RB^Low
B
#
*
Foxp3 mRNA levels
0
10
20
30
65
80
CD45RB^High
CD45RB^Low
CD25+
Day 0
Day 3 - TGF-β1
Day 3 + TGF-β1

Figure 4. TGF-β1 induces de novo Foxp3 expression and regulatory function in $CD4^+Foxp3^-CD45RB^{LOW}$ T cell precursors.

(A) $CD4^+Foxp3^-CD45RB^{Low}$, $CD4^+Foxp3^-CD45RB^{High}$ and $CD4^+Foxp3^+$ populations were FACS sorted from LN of Foxp3/GFP knock-in mice. Gates used are illustrated in the FACS profile shown. Histograms represent Foxp3/GFP profile at Day 0 of the three sorted populations **(B)** $CD4^+Foxp3^-CD45RB^{Low}$ or $CD4^+Foxp3^-CD45RB^{High}$T cells ($5x10^5$) were activated with plate bound anti-CD3 (10 μg/ml) and rhTGF-β1 (3 ng/ml) for 3 days. Treated or untreated cells ($1.25\text{-}5x10^4$ cells) were then co-cultured with OT-I Tg $CD8^+$ responder T cells ($5x10^4$), irradiated APC ($2x10^5$) and anti-CD3 (2 μg/ml). Cultures were pulsed with ^{3}H-thymidine during the last 8h and harvested at 72h. Results of the proliferation assay are shown as % suppression. **(C)** Flow cytometric analysis of Foxp3/GFP expression in initially $Foxp3^-CD45RB^{Low}$ and $Foxp3^-CD45RB^{High}$ subsets treated or not with rhTGF-β1 as described above. The cells were gated on live events based on forward and side scatter and represented ~80% of total acquired cells independent of cell subset or condition tested.

Figure 4

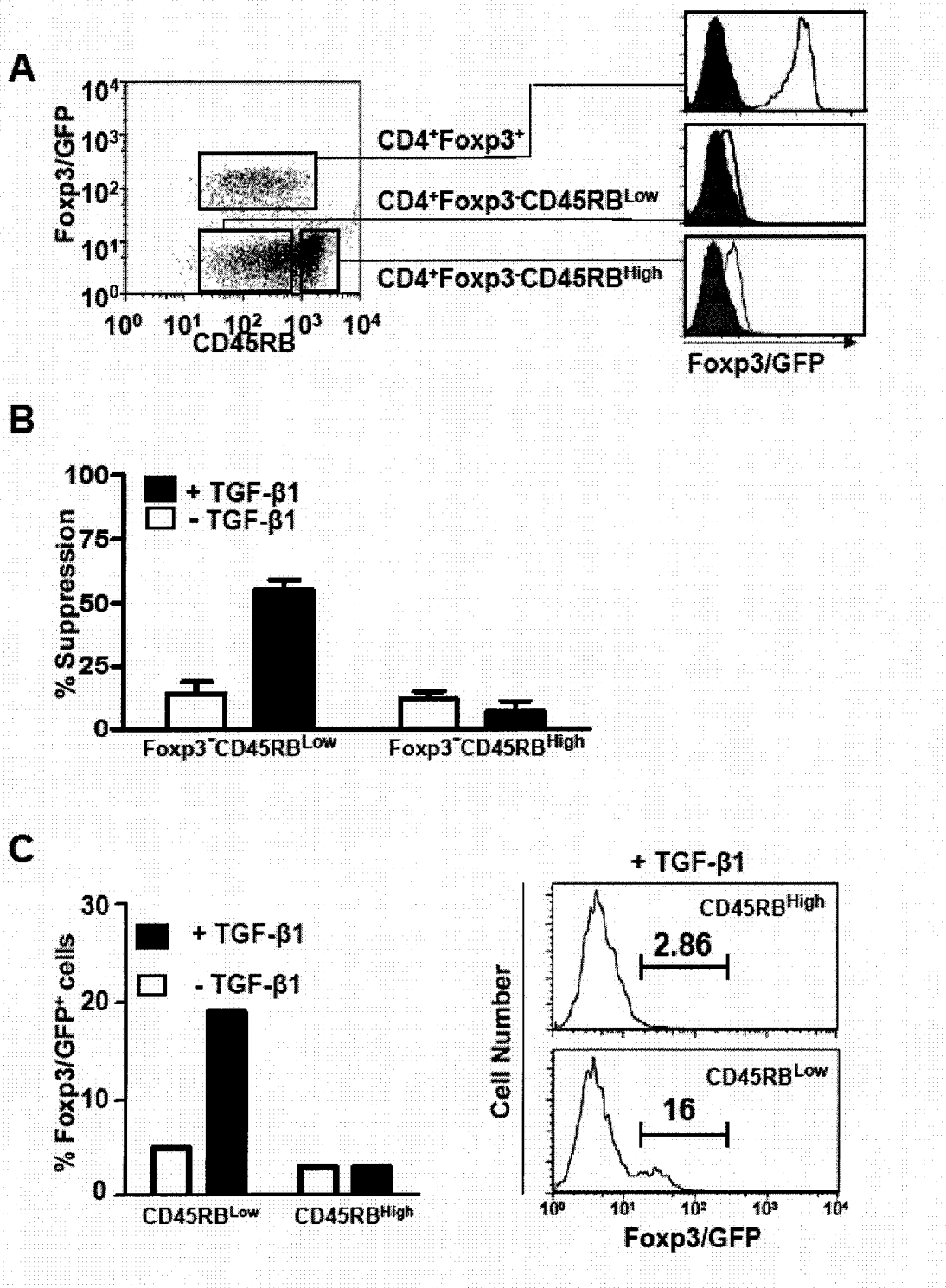

A
Foxp3/GFP
CD45RB
CD4+Foxp3+
CD4+Foxp3-CD45RBLow
CD4+Foxp3-CD45RBHigh
Foxp3/GFP
B
% Suppression
+ TGF-β1
- TGF-β1
Foxp3-CD45RBLow
Foxp3-CD45RBHigh
C
% Foxp3/GFP+ cells
+ TGF-β1
- TGF-β1
CD45RBLow
CD45RBHigh
+ TGF-β1
Cell Number
CD45RBHigh
2.86
CD45RBLow
16
Foxp3/GFP

Figure 5. TGF-β1 maintains Foxp3 expression in $CD4^+CD25^+$ nT_{REG} cells without potentiating their expansion or function.

(A) $CD4^+CD25^+$ nT_{REG} cells ($5x10^5$) were activated with plate bound anti-CD3 (10 μg/ml) in the absence or presence of rhTGF-β1 (3 ng/ml) and IL-2 (5 μg/ml), and proliferation was assessed on day 3 ($p<0.002$). **(B)** $CD4^+CD25^+$ T_{reg} cells ($5x10^5$) were activated with plate bound anti-CD3 (10 μg/ml) and IL-2 (5 μg/ml) alone or with rhTGF-β1 (3 ng/ml) or anti-TGF-β blocking Ab for (10 μg/ml) 3 days. Treated or untreated cells ($1.25\text{-}5x10^4$ cells) were then co-cultured with OT-I Tg $CD8^+$ responder T cells ($5x10^4$), irradiated APC ($2x10^5$) and anti-CD3 (2 μg/ml). Cultures were pulsed with ^{3}H-thymidine during the last 8h and harvested at 72h. Results of the proliferation assay are shown as the mean CPM± SD of triplicate cultures. **(C)** Total RNA was isolated from resting or day 3 TGF-β1 stimulated $CD4^+CD25^+$ nT_{REG} cells ($0.5\text{-}1x10^6$) and relative Foxp3 mRNA levels were measured by qRT-PCR ($p<0.003$). **(D)** FACS analysis of GFP expression of freshly-isolated $CD4^+Foxp3^+$ T cells (Dashed line; $5x10^5$) activated with plate bound anti-CD3 (10 μg/ml) in the absence or presence of rhTGF-β1 (3 ng/ml) for 24 h.

Figure 5

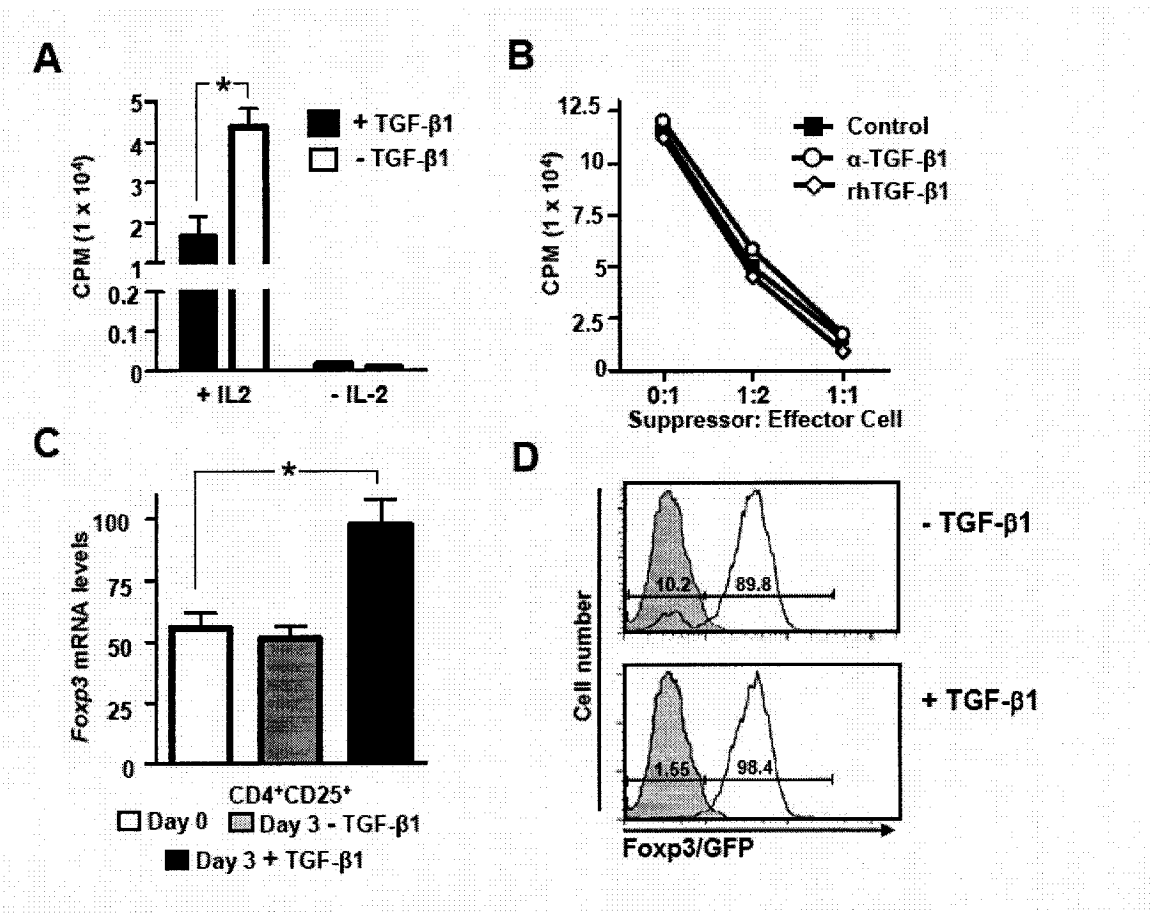
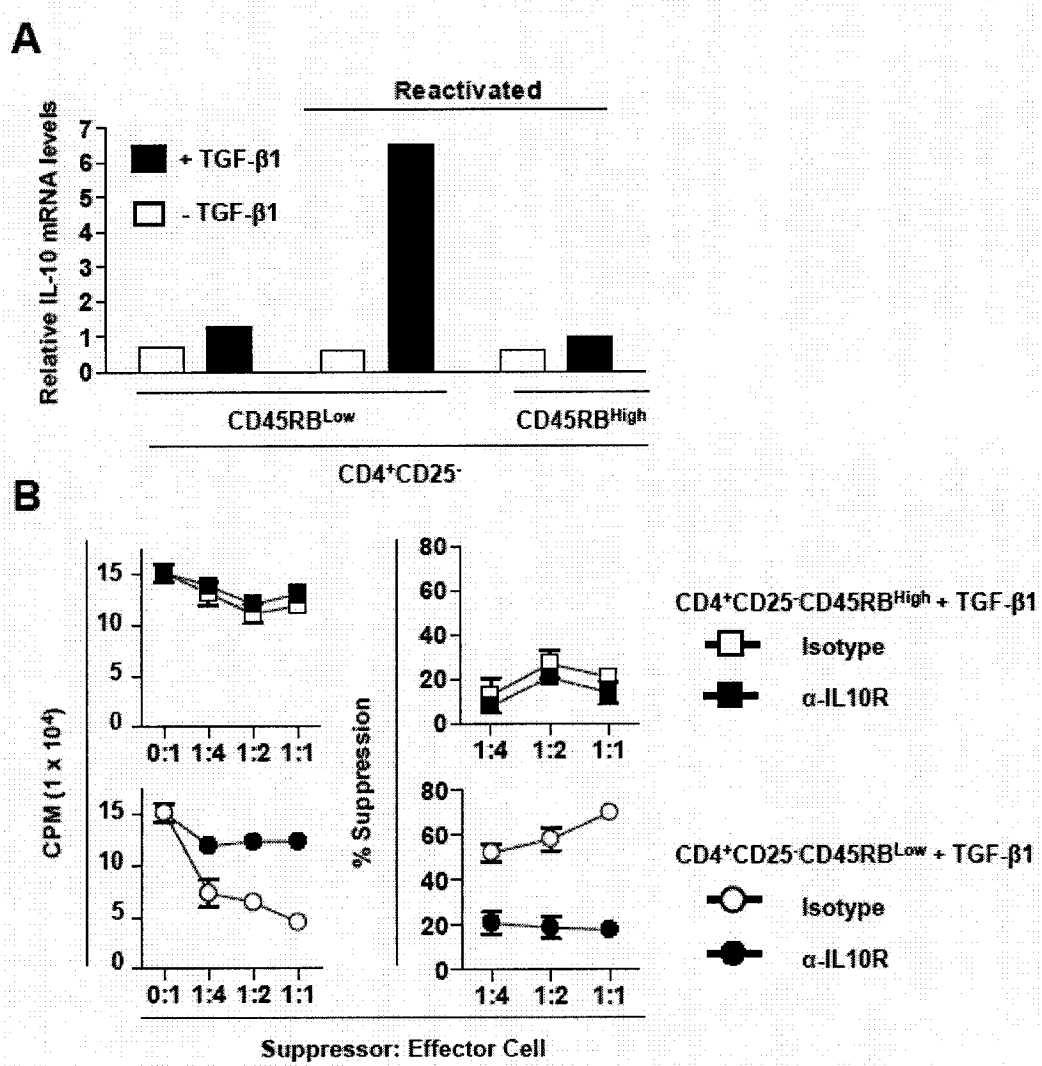

A
CPM (1 x 10⁴)
+ TGF-β1
- TGF-β1
+ IL2
- IL-2
B
CPM (1 x 10⁴)
Control
α-TGF-β1
rhTGF-β1
0:1
1:2
1:1
Suppressor: Effector Cell
C
Foxp3 mRNA levels
CD4⁺CD25⁺
Day 0
Day 3 - TGF-β1
Day 3 + TGF-β1
D
Cell number
- TGF-β1
+ TGF-β1
10.2
89.8
1.55
98.4
Foxp3/GFP

Figure 6. TGF-β1 induced Treg cell effector function is IL-10 dependent.

(**A**) rhTGF-β1 treated or untreated $CD45RB^{Low}$ and $CD45RB^{High}$ T cell subsets were reactivated or not with PMA (10 ng/ml) and ionomycin (250 ng/ml) on day 3 for 6h, and analysis of IL-10 relative to G3PDH gene expression was preformed by RT-PCR on total RNA. Data is presented as IL-10/G3PDH ratio relative to the IL-10/G3PDH ratio of $CD4^{+}CD25^{-}CD45RB^{High}$ untreated cells (set as 1). (**B**) Three days post-activation $CD45RB^{Low}$ and $CD45RB^{High}$ cells were co-cultured at different ratios ($6.25x10^{3}$ to $2.5x10^{4}$) with OT-I Tg $CD8^{+}$ T cells ($2.5x10^{4}$) and stimulated with anti-CD3 (2 μg/ml) in the presence of irradiated APC ($1x10^{5}$) and in the presence or absence of anti-IL-10R or IgG isotype control (3.5 μg/ml). Cultures were pulsed with ^{3}H-thymidine during the last 6h and harvested at 72h. Results of the proliferation assay are shown as the mean CPM± SD of triplicate cultures. The data are representative of three experiments.

Figure 6


A
Reactivated
Relative IL-10 mRNA levels
+ TGF-β1
- TGF-β1
CD45RB^Low
CD45RB^High
CD4+CD25-
B
CPM (1 x 10^4)
% Suppression
0:1 1:4 1:2 1:1
1:4 1:2 1:1
CD4+CD25-CD45RB^High + TGF-β1
Isotype
α-IL10R
CD4+CD25-CD45RB^Low + TGF-β1
Isotype
α-IL10R
Suppressor: Effector Cell

CHAPTER III: General Discussion

Accumulating evidence illustrates a novel role for TGF-β in the preservation of immunological tolerance in the periphery. This is proposed to occur through the maintenance of thymus-derived nT_{REG} cells, as well as through extra-thymic induction of suppressive properties in previously non-regulatory T cells. In order to establish a direct correlation between TGF-β treatment of $CD4^+$ nT_{REG}-depleted or nT_{REG} cell subsets, the induction of Foxp3 and the generation or maintenance of suppressive functions in these populations, was performed the present study. The results of this work shed new light on the unique role of TGF-β by demonstrating that it induces Foxp3 and regulatory functions in heterogeneous $CD4^+$ populations devoid of nT_{REG} cells in a concentration dependant manner. In particular, only a specific subset of $CD4^+$ T cells, namely the antigen-experienced $CD4^+CD25^-CD45RB^{LOW}$ T cells, are susceptible in developing regulatory functions after TGF-β1 exposure, but not the naïve, $CD4^+CD25^-CD45RB^{HIGH}$ T cells. Moreover, a selective *de novo* induction in Foxp3 expression is seen only in the antigen-experienced population. TGF-β1 also affects nT_{REG} cells, which already express high levels of Foxp3. TGF-β1 treatment or blockade of nT_{REG} cells does not enhance the growth or the suppressive activity of this regulatory population. However, the exposure of nT_{REG} cells to TGF-β1 maintains Foxp3 expression as compared with untreated cells. In such a way, a considerable augmentation in Foxp3 mRNA expression levels is detectable between TGF-β1 treated and untreated lymphocytes. Therefore, a novel paradigm of TGF-β tolerogenic functions emerges depending on the specific $CD4^+$ T cell subset. While TGF-β1 has immunosuppressive actions on $CD4^+CD25^-CD45RB^{HIGH}$ cells,

in $CD4^+CD25^-CD45RB^{LOW}$ cells it leads to the *de novo* expression of Foxp3 and generation of IL-10 producing $CD4^+$ T_{REG} cells, whereas it preserves Foxp3 expression in the nT_{REG} cell population.

Several studies have been published demonstrating the generation of $Foxp3^+$ T_{REG} cells from a heterogeneous population of $CD4^+$ cells depleted of nT_{REG} cells (66, 121, 157-159). Furthermore, the generation of the Foxp3-GFP/Foxp3-RFP knock-in animals has shown the direct ability of TGF-β1 to induce *de novo* Foxp3 expression in initially $Foxp3^-CD4^+$ T cells, demonstrating that the induction of regulatory functions in $CD4^+CD25^-$ T cells did not occur solely through preferential expansion of $CD4^+CD25^-$ $Foxp3^+$ cells. Nevertheless, the specific signalling pathway involved in this process remains unknown. As it is demonstrated here, a divergent response is seen upon TGF-β1 treatment of different $CD4^+$ T cell subsets, suggesting a unique TGF-β signalling pathway in each population. In addition, induction of $Foxp3^+$ cells from the specific $CD45RB^{LOW}$ subset of initially $Foxp3^-$ cells and the maintenance of Foxp3 in nT_{REG} cells implies a certain phenotypic similarity. Likewise, healthy mice harbor a significant frequency of naturally activated $CD4^+CD45RB^{LOW}$ T cells, which may represent auto-reactive T cells, as they are also found in germ-free or antigen-free mice (24). Accordingly, the preponderance of medium affinity self-reactive TCR might be over-represented in $CD4^+CD25^-CD45RB^{LOW}$ population and since nT_{REG} cell development occurs through intermediate affinity interactions with self-peptides, both of those populations might share similar characteristics. If this is the case, treatment with TGF-β would allow for the acquisition of suppressive properties in auto-reactive T cell population more easily, increasing the proportion of T_{REG} cells and depleting self-reacting

T cells from the periphery. Conversely, a recent study by Finnson, K.W., *et al.* has shed light on a novel GPI anchored protein, CD109 expressed on activated T cells, keratinocytes, platelets and a sub-population of hematopoietic progenitor cells (160). CD109 is a component of TGF-β receptor system capable of inhibiting TGF-β signalling by modulating the receptor activity and decreasing SMAD2/3 phosphorylation upon receptor ligation. It is probable that expression of this receptor by $CD4^{+}CD25^{-}$ $CD45RB^{HIGH}$ cells might explain their inability to immediately up-regulate Foxp3. Thus, the signalling pathways involved in the induction of Foxp3 could be identical in both populations, providing their ability to respond to TGF-β in a similar fashion.

The results of this study demonstrate that memory $CD4^{+}$ T cells are more prone to develop regulatory functions within 3 days, contrary to naïve $CD4^{+}$ T cells. Nonetheless, the generation of iT_{REG} population from naïve $CD4^{+}CD45RB^{HIGH}$ T cells isolated from Ag-specific TCR transgenic mice on a $RAG^{-/-}$ background, which do not posses nT_{REG} cells, has been described (32). Further, adoptive transfer of TCR transgenic RAG-deficient T cells into cognate ligand-expressing recipients confirmed the acquisition of Foxp3 expression in a subset of transferred cells (147, 151). Since, six NFAT and AP-1 binding sites were located in the first 500 base pairs of the Foxp3 promoter, activation of T cells could have critical effects on induction of Foxp3 (65). For example TCR transgenic $RAG^{-/-}$ T cells clonally activated with their cognate ligand could express Foxp3 more easily than the population of T cells with heterogeneous TCR. A fundamental difference between these reports and the present study may depend on the time necessary to develop regulatory functions, with naïve T cells requiring prolonged stimulation as opposed to memory T cells. The earliest assessment of suppressive

functions upon TGF-β1 treatment could only be performed three days after initial stimulation as rapid and successive re-stimulation of T cells leads to AICD (148). Nonetheless, the study of induction of regulatory functions in $CD4^+CD45RB^{HIGH}$ T cells at time points exceeding three days post-TGF-β triggering could have demonstrated the ability of the naïve population to suppress and remain an interesting experiment to perform. Equally, the delayed acquisition of T_{REG} cell function in naïve population could reflect the additional time this subset requires to mature and acquire memory T cell abilities, such as induction of regulatory functions. Furthermore, in some cases iT_{REG} cells were induced by the treatment with vitamin D_3 and dexamethasone, two drugs recognized as inhibitors of $CD4^+$ T cell function. Additionally, they secreted IL-10 but did not express Foxp3 (140). Thus, treatment of $CD4^+CD45RB^{HIGH}$ population with vitamin D_3 and dexamethasone might gradually induce gene expression that is identical to *de novo* Foxp3 expression in $CD4^+CD25^-CD45RB^{LOW}$ T cells, especially in that Foxp3 and these drugs both negatively regulate T cell functions. Consequently, the present study highlights the temporal and population difference in order to induce regulatory functions upon TGF-β1 treatment.

The action of TGF-β1 on nT_{REG} cells, as it is shown here, consists of maintaining the expression of Foxp3. Thus, in its absence even for 24h, a small fraction of nT_{REG} cells loose the ability to produce Foxp3 suggesting a constant requirement for TGF-β in the maintenance of peripheral tolerance. As such, Tisch *et al.* recently described an age-dependent loss of Foxp3 and TGF-β expression in nT_{REG} cells in NOD mice, linking it to the development of T1D (156). Still, the question remains if the decrease in TGF-β expression was subsequent to Foxp3 decrease or vice-versa. nT_{REG} cells represent a

heterogeneous population, which can be potentially subdivided in several populations. Depending on the expression of TGF-β, nT_{REG} cells could specialize either in the maintenance and the induction of regulatory function or in the down-modulation of T cell responses via cell-contact dependent or independent mechanisms (Figure F). nT_{REG} cells that are unable to produce TGF-β would participate in the down-regulation of immune responses using other means of suppression. It also remains possible that all nT_{REG} cells could make use of TGF-β depending on their maturation status or localization, given that TGF-β availability in the serum is high in the inactive form. Alternatively, during an immune response, inflammatory processes could release TGF-β associated with extracellular matrix, making it available to act on different T cell subsets. For instance, TGF-β can be synthesized directly by macrophages that phagocyte apoptotic cells or from dying T cells (161). Thus, during normal steady state, low levels of TGF-β released from extracellular matrix would be sufficient to maintain Foxp3 levels in nT_{REG} cells. However, during excessive, autoimmune like inflammatory states during which the immune system is over-stimulated, TGF-β released from macrophages and apoptotic cells could additionally drive the immunosuppression as well as induction of regulatory functions. Therefore, some subsets of nT_{REG} cells would require TGF-β in their environment to maintain their phenotype and concomitantly, they could also use it in their mechanism of suppression. Further, the TGF-β1 iT_{REG} cells mediate their function through secretion of IL-10, producing a leukocyte non-specific, immunosuppressive micro-environment. An important fact to note is that $CD4^+$ T cells down-regulate TGF-βRII expression within 24h of activation. This effect on activated cells can be restored by IL-10 treatment, leading to re-expression of TGF-βRII (155). Moreover,

inflammatory signals such as LPS or IFN-γ, induce a down-modulation of TGF-βR expression, thus reducing the responsiveness of cells to this cytokine (155).

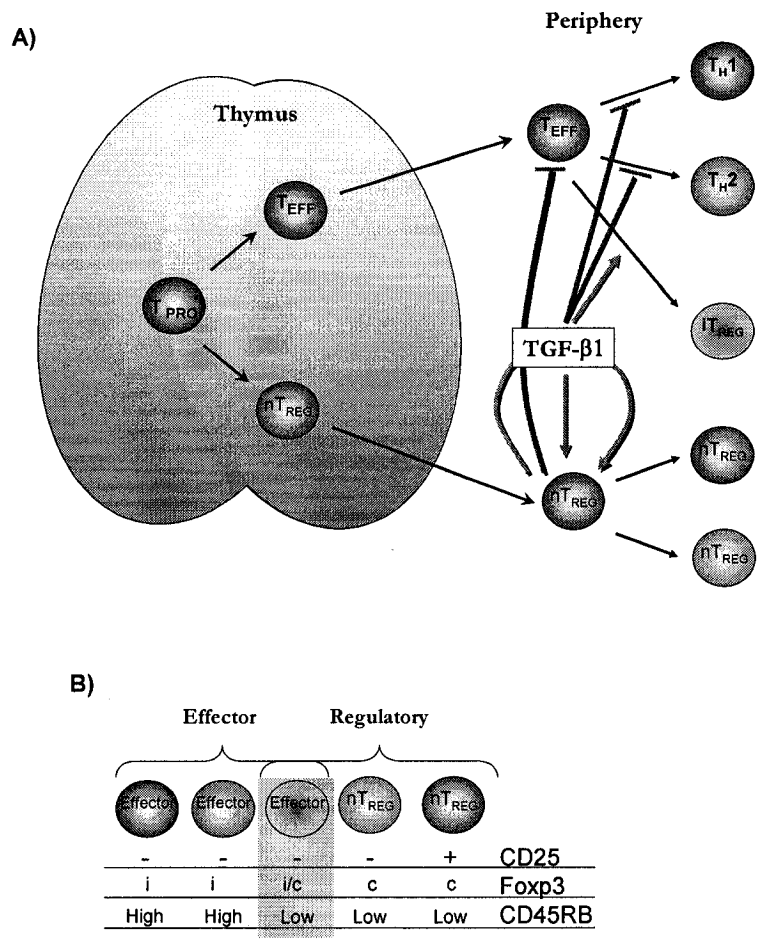


Figure F. Central role of TGF-β1 in CD4$^+$ effector and regulatory T cell biology. **A)** Effector and naturally-occurring T cells develop in the thymus from common T cell progenitors. In the thymus, upon encountering TGF-β1, T_{EFF} cell proliferation and differentiation into T_H1 or T_H2 subset is blocked upon activation. In a small subset of T_{EFF} cells, under specific activating conditions in the presence of TGF-β1, Foxp3 will be expressed *de novo* generating induced T_{REG} cells. Autocrine or paracrine TGF-β1 will maintain natural T_{REG} cell function, whereas TGF-β1 associated on the surface of T_{REG} cells will suppress T_{EFF} cell functions through direct cell contact. **B)** Phenotype of regulatory and T_{EFF} cell subsets. i, inducible; c, constitutive.

Therefore, in such cases, the presence of TGF-β1 would fail to have an impact on those cells. Taking this into consideration, secretion of IL-10 would allow activated T cells to be controlled anew by TGF-β1. Equally, as IL-10 and TGF-β production increases during the resolution of an immune response, iT_{REG} cells might be preferentially active at this stage, after which, they might die or differentiate to full T_{REG} cells.

Another non-exclusive possibility is that T_{REG} cells can mediate suppression of the T_{EFF} population through induction of Foxp3. In this way, the presence of TGF-β and high expression of CTLA-4 by the nT_{REG} cells, two factors involved in Foxp3 induction and mechanism of nT_{REG} suppression, could be easily explained. The ability of soluble TGF-β to generate suppressor activity and Foxp3 induction *in vitro* in the non-T_{REG} population would be an obvious bystander effect. More precisely, cells that are phenotypically most closely related to nT_{REG} cells would become T_{REG} cells, whereas transient expression of Foxp3 in T_{EFF} cells would result in decreased proliferation, as IL-2 secretion would be diminished, and a delay of differentiation would occur as IL-4 and IFN-γ secretion would be decreased, thus leading to overall suppression. Consequently, a delay in division, differentiation and secretion of T_H2/T_H1 cytokines from T_{EFF} cells would occur. This would allow more time for appropriate input signals from innate immune responses to be generated, allowing for overall stabilization of the cytokine environment either in the T_H1 or T_H2 direction. Interestingly, experiments with Foxp3 Tg animals demonstrate that the $CD4^+$ population is delayed in their cell cycle progression and are more susceptible to apoptosis (60). On the other hand, the $CD4^+CD25^-$ $CD45RB^{LOW}$ cells that are phenotypically very similar to T_{REG} cells, express similar genes and are amenable to becoming T_{REG} cells as soon as Foxp3 is induced and

stabilized by CTLA-4 crosslinking and TGF-β stimulation. Under these specific conditions Foxp3 expression would be maintained, allowing this transcription factor to epigenetically modify the cell itself, allowing for the maintenance of regulatory functions.

It seems improbable that the nT_{REG} population in the periphery can be replenished solely by the thymus throughout human life. Due to thymic involution, the reduction in the T_{REG} pool with age should be evident. However, observations indicate the opposite, suggesting that peripheral mechanisms exist to maintain a $CD4^+$ suppressive T cell fraction. Therefore, the total T_{REG} population present in a healthy individual at any given time would consist of a subset of terminally differentiated nT_{REG} cells and a transitional population of induced T_{REG} cells. Equally, Foxp3 has emerged as a dominant molecular switch favoring the development of T_{REG} cells. In support of this, the data presented here indicates that TGF-β1 is able to modulate Foxp3 levels in different T cell populations leading to induction of suppressive functions. More specifically, an emerging trend reveals a selective new role for TGF-β, not only as immuno-suppressor, but also as a Foxp3 stimulation factor that allows the generation and maintenance of T_{REG} cells in the periphery. The ability of TGF-β to generate regulatory functions *in vivo* has not yet been described and remains a complex task to achieve. Nevertheless, its availability in the inactive form in the serum, its high expression in the gut or during resolution of infection, as well as during tumor formation, provide TGF-β a great opportunity to induce regulatory functions. Additionally, the genes affected by TGF-β treatment in the specific cell subtypes should be studied and compared to nT_{REG} and T_R1 cells in order to establish SMAD dependency, and molecular factors other than Foxp3, involved in generation of

regulatory phenotype. For instance, expression patterns between $CD4^+CD25^-$ $CD45RB^{LOW}$ and $CD4^+CD25^-CD45RB^{HIGH}$ cell subsets exposed or not to TGF-β by microarray gene chip assay could lead to discovery of additional genes involved in T_{REG} cell functions. This would also resolve the issue of the relationship between those two cell subtypes.

The possible factors in the etiology of autoimmune diseases could involve defects in T_{REG} cell generation, maintenance or function. Therefore, the concept that T_{REG} cells can be generated *in vitro* from a large pool of antigen-experienced $CD4^+$ T cells via TGF-β1 triggering leads to the possibility that novel therapeutic strategies may be developed to potentiate the development or function of these cells in the periphery for the treatment of autoimmune or other chronic inflammatory diseases. For instance, transient pulse of TGF-β1 in the pancreas of NOD mice was able to prevent the development of T1D (110). On the contrary, blocking TGF-β immunosuppressive functions to enhance the immune response to treat cancer is the major area of research. The role of TGF-β in carcinogenesis has been well established, as tumour cells have been shown to directly synthesize it, as well as it is secreted by host cells in association with tumour growth (113). Moreover, T_{REG} cells can compromise immune response to cancer cell antigens (162, 163). Thus, tumors through TGF-β secretion could divert $CD25^-CD4^+CD45RB^{LOW}$ memory T cells from acquiring effector functions, impelling them to become IL-10 producing, suppressor T cells. Taking into consideration that TGF-β has pluripotent functions in the organism and it is able to directly induce and maintain T_{REG} cells and decrease T cell activation and proliferation, specific abrogation of TGF-β signalling in memory T cell subpopulations would enhance antitumour response. As such, mice with

dnRIITg $CD8^+$ T cells were able to generate tumour-specific cytotoxic T lymphocyte responses (164).

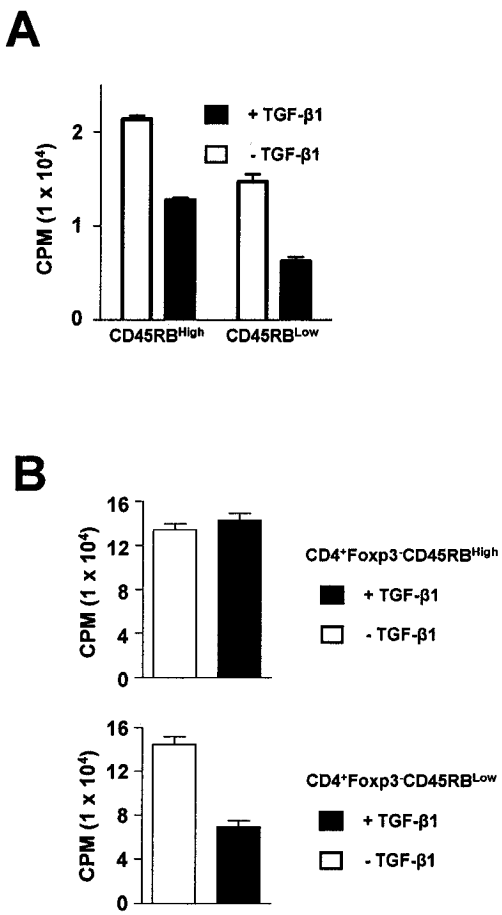
Finally, the present study directly involves TGF-β in maintenance and peripheral generation of T_{REG} cell phenotype. The close examination of such interactions in murine models might be directly transposable to human, endowing us with a better ability to understand the physiology of immune tolerance, to predict pathological states and to ultimately prevent them with appropriate treatment.

APPENDIX

Figure 1. Preferential TGF-β1 effects on proliferation and suppressive functions in $CD4^{+}Foxp3^{-}CD45RB^{Low}$ T cells.

(A) $CD4^{+}Foxp3^{-}CD45RB^{Low}$ or $CD4^{+}Foxp3^{-}CD45RB^{High}$ T cell subsets ($5x10^{5}$) were activated with plate bound anti-CD3 (10 μg/ml) in the absence or presence of rhTGF-β1 (3 ng/ml) for 3 days. Cultures were pulsed with ^{3}H-thymidine during the last 8h and harvested at 72h. Results of the proliferation assay are shown as the mean CPM± SD of triplicate cultures (#, $p<0.02$; *, $p<0.002$). **(B)** The regulatory function of $CD4^{+}Foxp3^{-}CD45RB^{High}$ and $CD4^{+}Foxp3^{-}CD45RB^{Low}$ T cells was measured after *in vitro* rhTGF-β1 priming. In brief, specific cell subsets ($5x10^{5}$) were activated with plate bound anti-CD3 (10 μg/ml) in presence or absence of rhTGF-β1 (3 ng/ml) for 3 days. TGF-β1 treated or untreated cells were then co-cultured at different ratios ($6.25x10^{3}$ to $2.5x10^{4}$) with OT-I Tg $CD8^{+}$ responder T cells ($2.5x10^{4}$), and stimulated with anti-CD3 (2 μg/ml) in the presence of APC ($1x10^{5}$). Cultures were pulsed with ^{3}H-thymidine during the last 8h and harvested at 72h. Results of the suppression assay are shown as the mean CPM ± SD (left and middle panels).

Figure 1


A
+ TGF-β1
- TGF-β1
CPM (1 x 10⁴)
2
1
0
CD45RB^High
CD45RB^Low
B
CD4⁺Foxp3⁻CD45RB^High
+ TGF-β1
- TGF-β1
CPM (1 x 10⁴)
16
12
8
4
0
CD4⁺Foxp3⁻CD45RB^Low
+ TGF-β1
- TGF-β1
CPM (1 x 10⁴)
16
12
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