Effects of pro-inflammatory cytokines on iT_{Reg} differentiation and function

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Áhrif bólgumiðlandi boðefna á sérhæfingu og virkni T stýrifrumna

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Ágrip

$CD4^+$ T stýrifrumur (T_{st}) gegna mikilvægu hlutverki í viðhaldi á eðlilegu ónæmissvari og koma í veg fyrir virkjun ónæmiskerfisins gegn eigin vef. T_{st} er skipt í náttúrulegar (nT_{st}) og afleiddar (aT_{st}) og eru flokkarnir óaðgreinanlegir út frá svipgerð en sérhæfing og virkni þeirra er mismunandi. nT_{st} þroskast í hóstakirtli en aT_{st} þrostkast út frá óreyndum T frumum í nærveru TGF-β1 og IL-2. Þær eru taldar gegna hlutverki við bælingu á ónæmissvari, en ekki er vitað á hvaða hátt þær miðla bælingunni. Ljóst er að hlutur ósértæka ónæmiskerfisins í meingerð sjálfsofnæmissjúkdóma er meiri en menn töldu, en þáttur þess í sérhæfingu og virkni $CD4^+$ T_{st} er óljós.

Markmið okkar var að meta áhrif bólgumiðlandi boðefna frá ósértæka ónæmiskerfinu á sérhæfingu og virkni $CD4^+$ aT_{st} úr mönnum.

Tilraunirnar voru framkvæmdar á T frumum úr mönnum. Óreyndar T frumur ($CD4^+CD25^-$) voru einangraðar og virkjaðar með anti-CD3 og ræktaðar með IL-2 og TGF-β með eða án bólgumiðlandi boðefnanna IL-1β og TNFα í fimm daga. Svipgerð aT_{st} var skilgreind sem $CD4^+CD127^-CD25^{high}FoxP3^{hight}$ og metin með flæðifrumusjá. Til að meta getu aT_{st} til að bæla fjölgun heilkjarna blóðfrumna voru aT_{st} ræktaðar með CFSE lituðum heilkjarna blóðfrumum og Epstein-Barr sýktum B frumum húðuðum með súperantigenum.

Flestar $CD4^+$ aT_{st} sérhæfðust í nærveru IL-2 og TGF-β1. Bólgumiðlandi boðefnin IL-1β og TNFα höfðu marktækt bælandi áhrif á sérhæfingu aT_{st} *in vitro*. Sýnt var fram á að aT_{st} höfðu bælivirkni *ex vivo* og hafði bælivirkni þeirra jákvæða fylgni við fjölda aT_{st}. Bælivirkni aT_{st} var hömluð í nærveru IL-1β og TNFα. Niðurstöður okkar sýndu einnig að aT_{st} miðla ekki bælilvirkni sinni í gegnum losun á IL-10 eða IL-35 en hugsanlega gegnum seytun á IL-2.

Abstract

Regulatory $CD4^+$ T cells (T_{Regs}) are one of the key elements of peripheral tolerance and suppression of autoimmune diseases. They are divided into two classes; central natural T_{Regs} (nT_{Regs}) and peripherally induced T_{Regs} (iT_{Regs}) which are phenotypically indistinguishable but differ regarding their differentiation and function. nT_{Regs} develop in the thymus but iT_{Regs} require the presence of IL-2 and TGF-β to be induced. They are thought to have a suppressive function but their mechanism of suppression is unknown. It has been revealed that the innate immune system plays a significant role in the immunopathology of autoimmunity. However, its part in differentiation and function of human $CD4^+$ iT_{Regs} is still unclear.

The aim of this study was to evaluate the role of pro-inflammatory cytokines of the innate immune response on the differentiation and function of human induced T_{Regs}.

All experiments were conducted on human T cells isolated from adult peripheral blood. Naïve T cells ($CD4^+CD25^-$) were isolated and stimulated with anti-CD3 and cultured in the presence of IL-2 and TGF-β with or without the pro-inflammatory cytokines IL-1β and TNFα for five days. The phenotype of iT_{Regs} was defined as $CD4^+CD127^-CD25^{high}FoxP3^{high}$ and they were analysed with flow cytometry. To detect their capacity to suppress PBMC's proliferation, iT_{Regs} were co-cultured with CFSE labelled PBMC's and Epstein-Barr infected B cells pulsed with superantigens.

We found that the highest fraction of *ex vivo* differentiated $CD4^+$ iT_{Regs} was dependent on the presence of both IL-2 and TGF-β1. The pro-inflammatory cytokines IL-1β and TNFα were also demonstrated to significantly inhibit TGF-β1 induced T_{Reg} differentiation *in vitro*. The TGF-β induced T_{Regs} showed strong suppressive function *ex vivo* and their suppressive function correlated positively with their numbers in culture. This suppressive capacity of the iT_{Regs} was contained in the presence of IL-1β and TNFα. Our results showed that the iT_{Regs} did not mediate their suppression through secretion of IL-10 or IL-36 but possibly through IL-2 secretion.

Þakkir

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Abbreviations

Aim-V	Serum free medium
ALK	Activin receptor Like Kinase
APC	Antigen Presenting Cell
APC	Allophycocyanin
BCR	B Cell Receptor
B_{Regs}	Regulatory B Cells
CD	Cluster of Differentiation
CFSE	CarboxyFluorescein diacetate Succinimidyl Ester
CLP	Common Lymphoid Precursor
CTLA-4	Cytotoxic T-Lymphocyte Antigen 4
CXCR3	CXC chemokine receptor 3
DC	Dendritic Cell
DNA	Deoxyribonucleic Acid
EB-Bs	Epstein-Barr infected B cells
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FCS	Fetal Calf Serum
FITC	Fluorescein Isothiocyanate
FoxP3	Forkhead-Winged helix-box protein P3
FSC	Forward Scatter
Gfi-1	Growth Factor Independent 1
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
GVHD	Graft-Versus-Host Disease
Hrs	Hours
HSC	Hematopoietic Stem Cell
IBD	Inflammatory Bowel Disease
ICE	IL-1β-Converting Enzyme
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IL-1RI	Interleukin-1 Receptor 1
IPEX	Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome
IRF4	Interferon Regulatory Factor 4
iT_{Regs}	Induced T regulatory cells
LAG-3	Lymphocyte Activation Gene-3
LAP	Latency Associated Protein
LLC	Large Latent Complex
LTBP	Latent TGF-β Binding Protein
MAPK	Mitogen-Activated Protein Kinase
MHC	Major Histocompatibility Complex
Min	Minutes
miRNA	micro RNA

mL	millilitre
MS	Multiple Sclerosis
mTNFα	Membrane bound form of TNFα
NFAT	Nuclear Factor of Activated T cells
NFκB	Nuclear Factor-κB
ng	nanogram
NK cells	Natural Killer cells
NLR	Nod Like Receptor
nT_{Regs}	Natural T regulatory cells
PAMPs	Pathogen-Associated Molecular Patterns
PBMC	Peripheral Blood Mononuclear Cells
PBS	Phosphate-Buffered Saline
PE	Phycoerythrin
PerCP	Peridinin Chlorophyll Protein
PI	Proliferation Index
PI3K	Phosphatidylinositide 3-Kinase
PRRs	Patter Recognition Receptors
PSGL-1	P Selectin Glycoprotein Ligand 1
RA	Rheumatoid Arthritis
RNA	Ribonucleic Acid
RORγ	RAR-related Orphan Receptor gamma
Rpm	Rounds Per Minute
RT	Room Temperature
SEM	Standard Error of the Mean
SLC	Small Latent Complex
SSC	Side Scatter
STAT	Signal Transducer and Activator of Transcription
sTNFα	Soluble form of TNFα
TACE	Tumour Necrosis factor α Converting Enzyme
T-bet	T-box transcription factor TBX21
T_{conv}	conventional T cell
TCR	T Cell Receptor
TdT	Terminal deoxynucleotidyl Transferase
T_{eff}	T effector cell
TGF-β	Transforming Growth Factor β
TGFβRII	TGF-β Receptor 2
T_H	T helper cell
TLR	Toll Like Receptor
TNFRI	Tumor Necrosis Factor Receptor type 1
TNFRII	Tumor Necrosis Factor Receptor type 2
TNFα	Tumor Necrosis Factor α
T_{Reg}	T Regulatory cell
UP	Unpulsed
µL	microlitre

1 Background

1.1 The immune system

The human body faces many dangers. Among those are pathogens; bacteria, fungi, protozoa, helminths and viruses, who invade and attack. As a protection against those trespassers, the body has developed a complex defence mechanism, our immune system.

The immune system is made up of a variety of cells and molecules. In vertebrates, it is divided in two; the innate and adaptive immune system. The innate immune system is activated when the pathogen enters the body and is the first line of defence. It provides an immediate, nonspecific response against the invader. Cells of the innate system recognise conserved molecular structures, called pathogen-associated molecular patterns (PAMPs) through corresponding patter recognition receptors (PRRs) (reviewed in 1). The PAMPs are present on many infectious microorganisms, but not on the body's own cells. This enables the innate system to successfully distinguish between the pathogen (non-self) from the body's cells and molecules (self). If the innate system fails to eliminate the infection, the adaptive immune system is called into action.

1.2 Adaptive immune system

The adaptive immune system is activated when the innate system fails to eliminate a pathogen and provides a specific response. The response takes days to develop and provides a lifetime of defence against the pathogens that trigger its activation. This is made possible with immunological memory, the most prominent attribute of the adaptive immune system. The adaptive system is constantly developing and depends mostly on highly specific pathogen recognition, provided by lymphocytes.

Lymphocytes are divided into B cells, T cells and natural killer (NK) cells, all developed from the common lymphoid precursor (CLP). However, only T cells (thymus cells) and B cells (bursa-derived cells) are considered to be the main cellular components of the adaptive immune system. They both identify and react to an antigen in a specific manner but differ in their manner of recognition and response. The B cells recognise the antigen through binding of an epitope to the B cell receptor (BCR), an immunoglobulin (Ig) structure bound to the B cell surface. The B cell response is through the secretion of the BCR and is dependent upon help from T cells. When a B cell binds and identifies an antigen, it goes through a process called clonal expansion and develops into a plasma cell. The plasma cell is an activated B cell and divides rapidly and secretes its BCRs. In the soluble form the BCRs are known as antibodies. They are secreted in large quantities and strike the pathogen in an antigen-specific manner.

What distinguishes the adaptive immune response from the innate is the specific recognition and response to a pathogen. This is made possible by the highly variable lymphocyte receptor expression. Each lymphocyte carries antigen receptors which recognise a specific chemical structure. Because of the genetic procedure conducted during lymphocyte development, there are millions of different lymphocytes circulating through the body and are able to recognise and respond to different antigens.

1.3 Human T cells

As all blood cells, the human T cell originates in the bone marrow from a pluripotent hematopoietic stem cell (HSC). The HSC differentiates into myeloid and lymphocyte precursors (reviewed in 2). The lymphocyte precursor is directed to become a T cell through Notch signalling. It has been speculated that the T and B cell precursors can be identified as a $CD34^+$ cells expressing terminal deoxynucleotidyl transferase (TdT) and CD10 (reviewed in 2). The CD34 expression decreases upon further differentiation (3, 4).

The T cell precursor $CD34^+CD1a^-$ travels to the thymus (5) where T cell development takes place, however it has been indicated that the T cell precursor is not committed to the T cell lineage before the cell migrates into the thymus (6). Thymus homing is thought to be regulated by CCR7, CCR9 and P selectin glycoprotein ligand 1 (PSGL-1) in mice (7-10). It has been suggested that CD10 plays a role in thymic homing in humans (11). In thymus, the cells undergo a process of gene rearrangement, proliferation and differentiation affected by various factors, including IL-7 (12). The different stages of T cell development can be tracked by CD3, CD4 and CD8 expression. First, the triple negative $CD4^-CD8^-CD3^-$ cells up-regulate CD4 on the cell surface and become $CD4^+$ immature single positive cells. At this timepoint the cells can still develop into two lineages of T cells, α:β and the γ:δ. The α:β lineage undergoes further development into $CD4^+CD8^+$ double-positive T cells. These thymocytes express low levels of the T cell receptor (TCR) CD3. They live for 3-4 days and are sent to programmed cell death unless rescued by engagement of the TCR in a process called positive selection through binding to self:MHC molecules where self-peptides are presented via protein complex called major histocompatibility complex (MHC). Most of the double positive T cells show no recognition with self-peptide:self-MHC complexes and undergo apoptosis or ‘death by neglect’ because their TCR could not recognize the self MHC molecules. If the TCR engages self MHC molecules the cells receives a survival signal and differentiate into functionally mature class II vs class I recognising $CD4^+$ or $CD8^+$ single positive T cells which express TCRs in high levels. The single-positive T cells undergo further development in the negative selection stage where the cells that recognize self-peptides too strongly undergo apoptosis to eliminate possible self-reactive cells. 98% of lymphocytes entering the thymus are eliminated through apoptosis in the developmental

stages. The remaining 2% leave the thymus and enter the blood stream as naïve T cells (reviewed in 13-15).

1.3.1 Differentiation of T effector cells

Naïve human T cells are primarily located in secondary lymphoid organs (Peyer's patches, lymph nodes and spleen). They are activated when presented with a specific antigen that they recognise by their TCR and a second signal provided by appropriate co-stimulatory molecules. The co-stimulatory signals are delivered by the CD28:B7 family of co-stimulatory molecules. The B7-1 and B7-2 ligands (also called CD80 and CD86) are expressed on antigen presenting cells (APCs) and bind to the CD28 co-stimulatory receptors on the T cell (16, 17). The binding promotes T cell proliferation and differentiation through enhanced IL-2 production and introduce survival signals for the T cells. The antigen is presented to the T cell via MHC on the APC. The $CD8^+$ T cells are presented with antigen through MHC class I. When activated, they differentiate into CD8 cytotoxic T cells and target virus infected cells and tumour cells. The $CD4^+$ T cells recognise MHC class II loaded with the specific antigenic peptides and when activated differentiate into a number of different effector cells.

1.4 Regulatory T cells

1.4.1 From the beginning

In 1976-77 the hypothesis of a suppressor T cell was published in two papers (18, 19). However, it wasn't until 1995 that the phenotype of the T cell subset was presented by Sakaguchi *et al.* (20). They described the suppressive T cells to be $CD4^+$ and to express $CD25^+$, IL-2 receptor alpha chain, and the depletion of this population led to autoimmune disease development in mice. In addition, the deleterious effects could be prevented by administration of $CD4^+CD25^+$ T cells. Six years later, human $CD4^+CD25^+$ T_{Regs} were described (21).

1.4.2 Differentiation of $CD4^+$ regulatory T cells

$CD4^+CD25^+$ T_{Regs} constitute 5-10% of peripheral $CD4^+$ cells in both mice and humans. A part of the population originates in the thymus. This population is currently known as natural regulatory T cells (nT_{Regs}).

The nT_{Regs} arise in the thymus in early stages of human fetal development and mature during the negative selection phase (22). In contrast to other self-reactive T cells which are eliminated at this stage, the nT_{Regs} survive despite their TCRs recognition of self-peptide:MHC complexes. This difference in survival might be explained by the stronger binding of the self-ligands to the TCRs derived from nT_{Regs} than the $CD25^-$ T cells (23). The self-reactive nT_{Regs} express the transcription factor FoxP3 and acquire a selective survival

advantage. The TCR signalling also requires co-stimulation through CD28 receptor for the FoxP3 induction and nT_{Reg} cell lineage commitment. This is supported by the noticeable decrease seen in nT_{Reg} frequencies in CD28-deficient and CD80-86 deficient mice (24, 25). In addition to TCR stimulation, the FoxP3 induction requires numerous additional signalling, including NF-κB (26) and PI3K signalling (27). Upon thymic maturation, the nT_{Regs} are exported to the periphery where they prevent autoimmune responses in collaboration with a T_{Reg} population known as induced regulatory T cells (iT_{Regs}).

iT_{Regs} are developed in the periphery from mature conventional $CD4^+$ T cells. Whereas the nT_{Reg} differentiation involves interactions with self-peptide:MHC complexes, the differentiation of the iT_{Regs} in the periphery likely occurs in response to non-self-antigens (28). These iT_{Regs} have passed the negative selection phase and are therefore not responsive towards self-antigens expressed in the thymus. Besides TCR signalling, other factors appear to effect the iT_{Reg} differentiation. CD28 and cytotoxic T lymphocyte-associated protein-4 (CTLA-4) are homologs and both bind B7. CTLA-4 has been shown to play a role in TGF-β1 mediated FoxP3 induction (29). The role of CD28 in this regard is controversial. Studies have revealed a positive (30) and negative (31) effect of CD28 upon FoxP3 expression. However, the presence of TGF-β1 and IL-2 has numerous times been shown to be essential for the FoxP3 induction by murine iT_{Regs} (32, 33) in the periphery and by human iT_{Regs} *in vitro* (34, 35). In spite of this, it is uncertain how iT_{Regs} are induced and maintained in the periphery *in vivo*.

Although there is a clear distinction between nT_{Regs} and iT_{Regs} differentiation, their phenotype is similar. Both are characterised by low CD127 (IL-7 receptor α chain) surface expression, high CD25 (IL-2 receptor α chain) expression and stable expression of FoxP3. CD127 is down-regulated after human T cell activation. However, unlike memory and effector T cells, its re-expression does not occur in $FoxP3^+$ T cells and they remain $CD127^-$ (36). Although CD25 is considered to be a marker for T_{Regs}, it is also expressed on recently activated non-regulatory T cells. Therefore it is impossible to use as a specific marker. In addition, it has been suggested that, unlike in the mouse, only human T cell subsets expressing the highest levels of CD25 ($CD25^{hi}$) have *in vitro* suppressive activity (21). Stable expression of the transcription factor FoxP3 in mice is found in $CD4^+CD25^+$ T_{Regs} but not in naïve $CD25^-$ or activated $CD4^+$ T cells. Also, FoxP3 expression is known to be vital for the development and function of T_{Regs} in mice and is the most reliable marker known for T_{Regs}. Retroviral transfer of the *FoxP3* gene into $CD4^+CD25^-$ T cells from the periphery bestows regulatory surface phenotype and suppressive function (37, 38). However, FoxP3 can be up-regulated by conventional T (T_{conv}) cells in humans upon TCR stimulation, without conferring regulatory activity (39, 40). Nevertheless, its expression is vital and mutation of the *FoxP3* gene in humans causes deficiency or dysfunction of $CD4^+CD25^+$ T_{Regs} resulting in an

autoimmune disease named immune dysregulation, polyendocrinopathy, enteropathy, X-linked (IPEX) syndrome (41). To this date, no marker has been found to be T_{Reg} specific and therefore, the combination of these markers (CD25, CD127 and FoxP3) is often used to distinguish T_{Regs} from other effector T cells.

Other markers expressed by T_{Regs} are CD28, adhesion molecules CD62L, CTLA-4 and chemokine receptors CCR7, CXCR4 and CCR9 (42). In addition CD103 has been shown to be expressed by $FoxP3^+$ T_{Regs} in humans (43). CD103 is the alpha chain of the adhesion molecule αEβ7 integrin and binds E cadherin on epithelial cells. Its expression by T cells can be induced by TGF-β1 (44) and has been associated with regulatory function (43, 45).

1.4.3 T_{Reg} function

Information regarding the role of T_{Regs} in immune regulation is steadily piling up. However, much remains to be seen regarding their mechanisms of suppression. *In vitro* model systems have pinpointed numerous molecules and processes that participate in the T_{Reg} suppression. Nevertheless, it remains unclear whether any of these studies shed light on how T_{Reg} function *in vivo*.

It has been suggested that human T_{Reg} suppress responder T cells through cytolysis. Human $CD4^+CD25^+FoxP3^+$ T_{Regs} have been shown to express granzyme B as a result of CD3 and CD46 stimulation and terminate target cells through a cell-contact dependent mechanism (46). This has been confirmed in murine studies, where T_{Regs} destroy target cells with granzyme B-dependent mechanisms and those T_{Regs} that are granzyme B deficient show decreased suppressive activity *in vitro* (47). These studies have been confirmed by *in vivo* studies where granzyme B was essential for T_{Regs}-dependent long-lived skin graft tolerance and in T_{Reg} mediated suppression of tumour clearance (48, 49).

Another mechanism that has been proposed is a cytokine-mediated suppression. Several cytokines have been implicated including IL-10 and IL-35. IL-10 is often referred to as an anti-inflammatory cytokine. It is primarily produced and secreted by monocytes, T_H2 cells and T_{Regs}. Even though IL-10 has been found to have no role in human $CD4^+CD25^+$ T_{Reg} function *in vitro* (50, 51), it has been implicated to induce suppression through conversion of conventional T cells to T regulatory cells in humans (51). Also, it has been shown to be essential to keep immune responses in check at environmental interfaces, for example in colon and lungs (52). IL-35 is another candidate for T_{Reg} mediated suppression. IL-35 is a member of the IL-12 heterodimeric cytokine family and is composed of IL-12α (also known as p35) and Epstein-Barr virus-induced gene 3 (Ebi3 or IL-27β) which are encoded by two genes, *IL12A* and *EBI3* (53). FoxP3 depletion has been shown to result in down-regulation of *EBI3* which suggests that *EBI3* is a target gene for FoxP3. Also IL-35 has been shown to be constitutively expressed by mouse $CD4^+CD25^+FoxP3^+$ T_{Regs} and *IL12A* and *EBI3* mRNA

have been observed to be up-regulated in mouse T_{Regs} (54). Also T_{Regs} from *EBI3*$^{-/-}$ and *IL12A*$^{-/-}$ mice have decreased capacity to suppress *in vitro* and were less effective than wild type T_{Regs} to cure inflammatory bowel disease (IBD) in mice (54). Despite the convincing evidence for the role of IL-35 in T_{Regs} in mice, its relevance in human T_{Regs} is controversial. Bardel *et al.* demonstrated that human T cells, including $CD4^+CD25^+FoxP3^+$ T_{Regs} do not express *EBI3* and are therefore unable to express IL-35 (55). Although *IL12A* mRNA was detected in T effector cells and T_{Regs}, *EBI3* mRNA was detected only in T effector cells but not in resting or activated T_{Regs}. A more recent study has revealed a significant up-regulation of *IL12A* and *EBI3* in human T_{Regs} compared with conventional T cells. They also showed that human T_{Regs} not only express IL-35 but also require it for their suppressive function (56). Finally, they presented that the suppression of T_{Regs} led to the conversion of suppressed T_{conv} cells into iTr35 cells, a population of IL-35 induced T_{Regs}.

A limited part of the knowledge of the T_{Reg} function comes from the study of human T_{Regs}. In addition to the mechanisms mentioned above, the study of T_{Reg} function in mice is more comprehensive and can be used to broaden our understanding of the suppressive function of T_{Regs}. CTLA-4 is a well-known molecule expressed on the surface of T cells and restrains their response through binding of B7-1 and B7-2. In mouse models with a selective deletion of CTLA-4, it has been shown to be essential for the suppressive function of T_{Regs} *in vitro* and *in vivo* (57, 58). The basis for this suppressive function has been suggested to be the prevention of B7-1 and B7-2 expression by T_{Regs} which prevents dendritic cells to activate naïve T cells through CD28 and results in inhibition of the immune response (reviewed in 59). Other surface molecules that have been implicated in the T_{Reg} mediated suppression in mice are CD39 and CD73. Both ectoenzymes are highly expressed by T_{Regs}. CD39 hydrolyses extracellular ATP/ADP to AMP. Since extracellular ATP is an indicator of tissue destruction, this process may contribute to the anti-inflammatory mechanism used by $T_{Regs.}$ Also, CD73 dephosphorylates AMP to adenosine which inhibits effector T cell proliferation (60). Another cell surface molecule that plays a potential role in T_{Reg} mediated suppression is LAG-3. It is a CD4 homolog, which binds to MHC class II molecules with high affinity. This binding of a MHC molecule on immature dendritic cells through LAG-3 suppresses dendritic cell maturation and co-stimulatory capacity (61). Finally the secretion of Galectin-1, a β-galactoside binding protein, has been shown to play a role in cell cycle arrest and apoptosis of T_{conv} in mice. It is unclear whether Galectin-1 mediates its effects via contact-dependent mechanisms or as a soluble cytokine. Its blocking has been reported to substantially reduce the inhibitory effects of $CD4^+CD25^+$ T cells in human and mice (62).

It should be noted that none of the aforementioned mechanisms can singly account for T_{Reg} mediated control of immunity. T_{Regs} mediated suppression can involve various

mechanisms and its function depends partly on the nature of their environment and the molecules involved. A few molecules play a key role in the induction and the suppressive function of T_{Regs} and for that reason; their part will be specifically discussed in the following sections.

1.4.3.1 TGF-β1

TGF-β is a pluripotent cytokine and exists in three homologous isoforms, TGF-β1, TGF-β2 and TGF-β3 encoded by different genes with TGF-β1 being the most dominant isoform (63). TGF-β is synthesized as an inactive prepro-TGF-β precursor with a propeptide region and a TGF-β homodimer. The mature TGF-β homodimer is synthesized through proteolytic processes and is non-covalently associated with Latency Associated Protein (LAP) to form a small complex called Small Latent Complex (SLC). The SLC cannot be secreted unless bound to a protein called Latent TGF-β Binding Protein (LTBP) and together they form the Large Latent Complex (LLC) which is important for targeting TGF-β to the extracellular matrix (64). However, TGF-β is inactive in this latent form and needs additional stimuli to be liberated from LAP and LTBP in order to become activated and bind to its receptors. The mechanisms of activation are unclear *in vivo* but can be attained with extreme pH, heat or proteases *in vitro* (64). Activated TGF-β can mediate its biological function as a soluble cytokine or in a cell-cell contact dependent mechanism as a surface-bound molecule. Signalling is mediated through transmembrane serine/threonine kinase receptors, mostly ALK5 (TGFβRI) and TGF-β receptor II (TGFβRII). Active TGF-β1 binds to TGFβRII with high affinity and activates a tetramer complex, consisting of TGFβRII and ALK5 (reviewed in 65). This initiates downstream signalling, where ALK5 phosphorylates Smad proteins 2 and 3 which travel into the nucleus and regulate transcription of target genes (65, 66). Studies have also demonstrated TGF-β signalling through Smad-independent pathways, involving Ras, Rho, MAPK and PI3K (67).

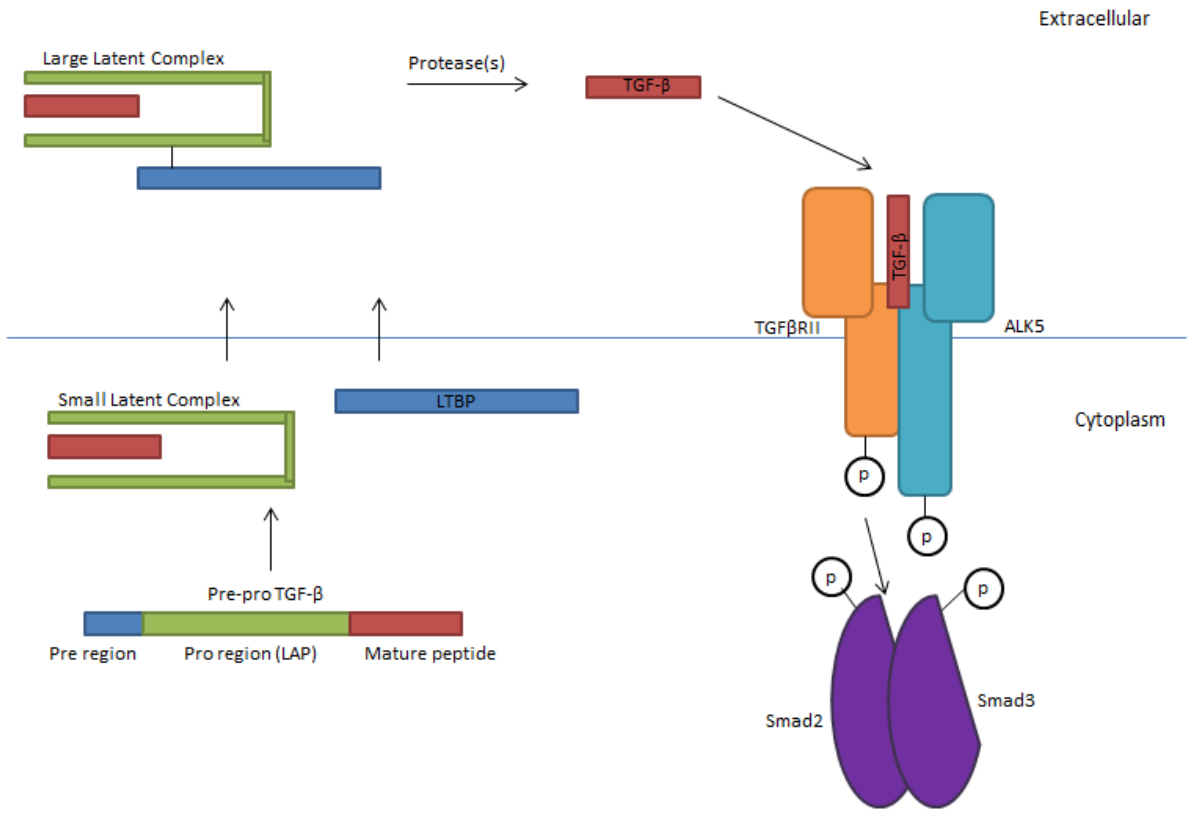

Figure 1: TGF-β induction, activation and signalling pathway.

TGF-β1 plays a significant role in the regulation of the immune system. It keeps in check unnecessary activation of the immune system while encouraging a vigorous response when needed. Among many roles of TGF-β1, it plays a part in the generation of T_{Regs}. Nakamura *et al*. detected in 2001 the presence of TGF-β1 on the surface of $CD25^+$ T cells and showed that neutralizing TGF-β1 antibodies blocked the suppression by T_{Regs} (68). However, this was questioned by a study performed by Piccirillo *et al.* in 2002, where they failed to show a role for TGF-β1 in T_{Reg} suppressive function. Soluble TGFβRII and anti-TGF-β1 were also unable to inhibit suppression (69). More recent studies have shown that TGF-β is essential for the induction of $FoxP3^+$ T_{Regs} *in vitro* and *in vivo* and therefore the induction of peripherally induced T_{Regs} (28, 34). The role of TGF-β1 in this process has been investigated *in vitro* and was shown to reduce the sensitivity to strong stimulation through the TCR (70). This widens the range of TCR stimulation over which conversion to $FoxP3^+$ T_{Regs} can occur and allows for FoxP3 induction at higher concentrations of peptides. TGF-β has also been proposed to regulate iT_{Reg} differentiation through repression of Gfi-1, a transcriptional repressor that inhibits the differentiation of iT_{Regs} and T_H17 cells (71). The transcription factors Smad3 and NFAT have also been proposed to be a part of the mechanism behind TGF-β induction of FoxP3 (reviewed in 72). One study suggests that TGF-β together with TCR signals induces FoxP3 in part by opposing cell cycle-dependent Dnmt1 recruitment to the *FoxP3* locus and

thus opposing its inactivation (73). Consistent with the *in vitro* findings, neutralizing TGF-β was shown to inhibit the differentiation of $FoxP3^+$ iT_{Regs} (74). Despite recent observations, much remains unknown concerning the role of TGF-β in T_{Reg} differentiation and function. Nevertheless, most studies agree that IL-2 is essential for the TGF-β1 induced FoxP3 expression and iT_{Reg} induction (33, 34).

1.4.3.2 IL-2

IL-2 is a pleiotropic cytokine that plays a pivotal role in the immune response. It is best known as a T cell growth factor, inducer of T cell proliferation and modulator of effector cell differentiation. It is known to drive T_{Reg} development but the requirement for IL-2 is different between thymus-derived nT_{Regs} and peripherally induced iT_{Regs}.

$CD4^+CD25^+$ thymocytes from IL-2 deficient mice were found to express normal amounts of FoxP3 mRNA (75). Also, neutralization of IL-2 results in a substantial reduction of FoxP3 expression in the mouse spleen but not in the thymus (76). Therefore, it can be assumed that IL-2 is not necessary for the induction of $FoxP3^+$ nT_{Regs}. In spite of this, IL-2 appears to be vital for iT_{Reg} cell generation and homeostasis. IL-2 neutralization and deletion of IL-2 in T cells has shown it to be required for *in vitro* FoxP3 induction and suppressive function of iT_{Regs} (32). IL-2 mediates its signalling through the transcription factor STAT5 which binds to the *FoxP3* gene and induces FoxP3 expression (reviewed in 77).

Many potential mechanisms behind the suppressive function of T_{Regs} have been previously identified. Additionally, IL-2 has been suggested to affect the suppressive capacity of T_{Regs}. Studies have demonstrated that T_{Regs} inhibit the induction of IL-2 mRNA in responder T cells and thereby mediate suppression (78, 79). What is more, in mice, IL-2 has been shown to be vital for the homeostasis of iT_{Regs} *in vivo* (80) and suppressive function *in vitro* (81). It has been proposed that T_{Regs} mediate their suppressive activity by competing with $FoxP3^-$ T cells for IL-2 and thus inhibiting their proliferation (82). On the other hand, the blocking of IL-2 binding has been shown to have no effect on the function of human T_{Regs} (83).

1.4.3.3 FoxP3

The transcription factor FoxP3 is the most stable marker of T_{Regs} to date. Moreover, it is a significant regulator of their proliferation, differentiation, suppressive function and the repression of alternative T cell differentiation pathways. About 20-30% of FoxP3 dependent-genes are controlled directly by FoxP3. Those include transcription factors and microRNA (miRNAs) and FoxP3 is known to both up-regulate and repress gene expression (84).

NFAT is a transcription factor that regulates T cell activation and anergy by forming a complex with AP-1. In mice, FoxP3 has been shown to mediate T_{Reg} function through NFAT inhibition and is suggested to compete with AP-1 to form NFAT:FoxP3 complexes. This

recruitment of FoxP3 instead of AP-1 results in induction of T cell tolerance instead of T cell activation (85).

MicroRNAs (miRNAs) are newly discovered, small (19~25 nt), noncoding ribonucleic acids (RNAs). They regulate genome expression and are key regulators of various biological processes (86, 87). Changes in the expression of specific miRNAs have been associated with T cell mediated immune responses and $CD4^+$ T cells have been found to express different miRNAs subsets that are linked to cell differentiation, maturation, activation and function (88). The biogenesis of miRNAs is catalysed by the RNases Drosha and Dicer (89). Conditional deletion of Drosha or Dicer has been observed in $FoxP3^+$ regulatory T cell lineages in mice. The knockout mice developed a similar wasting disease to that observed in FoxP3-knockout mice which implicates miRNAs as being essential for T_{Reg} function (90, 91). miR-155 is highly expressed in T_{Regs} and has been identified as a direct target of Foxp3 (89). It has also been observed that fewer T_{Regs} are present in the thymus and the peripheral lymphoid tissues in miR-155 knockout mice (89). The up-regulation of miR-155 through FoxP3 is essential for heightened responsiveness of T_{Regs} to IL-2 (92). miR-146a has recently been shown to be prevalently expressed in T_{Regs} in mice and to be crucial for their suppressor function *in vivo*. It keeps in check undue activation of STAT1 in T_{Regs} and therefore regulates T_H1-mediated pathology and prevents T_{Regs} from changing into IFN-γ-producing T_H1 cells (93).

In addition to the mechanisms mentioned above, FoxP3 prevents differentiation of T_{Reg} precursors into T_{eff} cells. FoxP3 regulates expression of numerous genes in non-regulatory T cells resulting in production of proteins that hinder the cells activation. Simultaneously FoxP3 has been suggested to repress genes that generally promote immune responses in naïve and effector T cells (reviewed in 94). As an example, FoxP3 mediates suppression of IL-17, a cytokine produced by T_H17 cells. The repression results from alteration in the transcriptional regulation of RORγ, a transcription factor that is currently used as a marker for T_H17 cells (95).

Taken together, these studies demonstrate how FoxP3 can affect T_{Reg} differentiation and function in various ways. Recent evidence suggests that the FoxP3 mode of regulation can be tailored to shape a particular immune response, and is not a universal program. The interaction between FoxP3 and the transcription factors IRF4, T-bet and STAT3 is a good example. IRF4 is necessary for differentiation of T_H2 effector cells. If it forms a complex with FoxP3, the involved T_{Regs} are granted an ability to suppress T_H2 responses (96). Likewise, T-bet is essential for T_H1 differentiation. When expressed in T_{Regs}, it enables them to express CXCR3, a regulator of trafficking that is preferentially expressed on T_H1 cells. This enables the T_{Regs} to migrate and accumulate at sites of T_H1 responses (97). STAT3 is required for

stimulation of T_H17 cells. Selective deletion of STAT3 in T_{Regs} has been shown to result in unrestrained T_H17 immune response (98). These studies clearly demonstrate that not only FoxP3, but also the transcription factors involved in T_H cell development are imperative for T_{Reg} function and encourage a tissue-specific response.

1.5 Regulating the regulators – the effects of pro-inflammatory cytokines on T_{Regs}

Cytokines are small soluble molecules that mediate interactions between cells. They are secreted by numerous cells and bind to specific receptors on target cells. Cytokines partake in the innate and adaptive immune response and participate in most immune cell processes including activation, differentiation, migration, development and survival. They are an important factor in the inflammatory response and its resolution and can be grouped into pro-inflammatory (IL-1, IL-6, TNFα) and anti-inflammatory (IL-2, IL-10, TGF-β) cytokines depending on their function (99).

Pro-inflammatory cytokines are not generated constitutively. Their secretion is tightly regulated by the transcription factor NFκB (100) and provoked in the presence of harmful agents or tissue damage.

1.5.1 Regulatory T cells and TNFα

Tumor necrosis factor α or TNFα is a key cytokine in the initiation and coordination of the events involved in inflammation (reviewed in 101). TNFα is generated chiefly by macrophages and monocytes but can be produced by other cell types including T cells, dendritic cells, B cells and NK cells. It is produced as a transmembrane protein (mTNFα) that can be cleaved by TNFα converting enzyme (TACE) to release a soluble form (sTNFα) (102). Both forms of TNFα are biologically active. TNFα binds to two receptors, TNFRI and TNFRII. Both receptors can also be secreted and neutralize the action of TNFα. sTNFα binds preferentially to TNFRI and mTNFα to TNRII. TNFRI is the primary receptor and is constitutively expressed by almost all cell types. It contains an intracellular death domain and generally induces apoptosis through a caspase-3 dependent pathway (reviewed in 101, 103, 104). TNFRII is inducible and its expression is more limited. In contrast to TNFRI binding, TNFRII activation promotes cell survival and proliferation through a NFκB pathway (reviewed in 105).

The success of TNFα antagonist therapy in treatment of rheumatoid arthritis (RA) in humans is evident in a large number of patients (106, 107). TNFα has also been shown to exert its effects in other diseases, including psoriasis, Crohn's disease and multiple sclerosis (108-111). Recent evidence suggests that TNFα is also capable of anti-inflammatory effects. In murine models, exogenous injections of TNFα have been reported to decrease severity of

lupus and type 1 diabetes (112, 113). In humans, a few cases have been reported where anti-TNFα therapy for RA resulted in the induction of lupus-like syndrome. Also, anti-TNFα for treatment of psoriasis caused an exacerbation of the disease in a few cases (114).

The efficacy of anti-TNFα treatment in RA has been associated with restored numbers and function of T_{Regs} (107, 115). Peripheral blood T_{Regs} from RA patients have been reported to have decreased suppressive activity compared to controls (116). This deficiency of T_{Reg} function in RA patients has been associated with diminished FoxP3 expression and increased TNFRII expression (117). Therefore it has been concluded that binding of TNFα to TNFRII on T_{Reg} surface results in decreased FoxP3 expression in RA patients (117). This is supported by a study demonstrating that mTNFα on T_{Regs} is linked to severe RA disease activity (118).

A significant number of RA patients do not respond to anti-TNFα treatment, indicating that TNFα is not consistently associated with reduced T_{Regs} numbers and function (114). Kleijwegt *et al.* have demonstrated positive effects of TNFα on the induction of human T_{Regs} *in vitro* (119). It has also been reported that TNFα expanded and enhanced the suppressive function of murine T_{Regs} (120). In contrast, it has been reported that TNFα negatively regulated the induction of T_{Regs} *in vitro* (107, 121) and Valencia *et al*. demonstrated that TNFα negatively affected the FoxP3 expression and suppressive function of human T_{Regs} *in vitro* (117). In light of these findings, there seems to be some controversy regarding the effects of TNFα upon T_{Regs}. We have previously speculated that these contradictory effects are dose and time dependent (manuscript submitted).

1.5.2 Regulatory T cells and IL-1β

Interleukin (IL)-1β is one of three ligands that make up the IL-1 family. It is produced by a number of cells including monocytes, macrophages, neutrophils and hepatocytes. It is synthesized in the cytoplasm and processed and secreted by a mechanism that involves IL-1β-converting enzyme or ICE, otherwise known as caspase-1 (reviewed in 122). Two signals seem to be necessary for IL-1β to be released from human monocytes. The first is a Toll-like receptor (TLR) mediated induction of transcription and the second signal is an induction of IL-1β processing and secretion which is induced through Nod-like receptors (NLR) (reviewed in 122). IL-1β mediates pro-inflammatory responses through NFκB pathway (123) and initiates the generation of pro-inflammatory mediators such as IL-6 and granulocyte macrophage-colony stimulating factor (GM-CSF) (reviewed in 124). Besides its role in inflammation, IL-1β is involved in cell proliferation, differentiation and apoptosis. It participates in the pathogenesis of inflammatory diseases and a number of autoimmune diseases are associated with high levels of IL-1β (125, 126). In mice, IL-1β is known to enhance proliferation of conventional T cells (127), and enhance expansion of $FoxP3^+$ T_{Regs}

(128). In contrast, IL-1β has been shown to negatively regulate or switch the phenotype of T_{Regs} (129) and $IRAK^{-/-}$ mice (lacking functional IL-1 signalling) have higher numbers of T_{Regs} compared to wild type mice (130). Thus, the role of IL-1β signalling in mice is debatable and few studies have reported its effects in human iT_{Reg} differentiation.

1.6 Other regulators of immune responses

Although $CD4^+CD127^-CD25^+FoxP3^+$ T_{Regs} play a substantial role in the control of immune responses, they are not the only regulators in the immune system. Numerous factors contribute to the regulation of the immune response and it is necessary consider all of them if you want to get a full picture of the process.

Other T cell populations have displayed regulatory function. Among those are inducible $CD8^+$ T cells which do not arise naturally in the thymus but are induced under diverse conditions. They have been shown to play a role in multiple sclerosis (MS) (131) where they eliminate $CD4^+$ pathogenic cells and have been implicated in a therapeutic immune regulation (132). In addition, $CD8^+$ regulatory T cells participate in the anti-tumour response in mouse lungs where their depletion *in vivo* resulted in a decrease in lung tumours but increased subcutaneous tumour growth (reviewed in 133). γδ T cells are a subset of T cells that express a TCR composed of a γ chain and a δ chain. They do not recognize antigens that are associated with MHC molecules like the αβ TCR. Instead they recognise their target antigens directly. These cells reside within the epithelia and contribute to the mucosal tolerance (134). They have also been found to be suppressive and to regulate autoimmunity (reviewed in 135).

T cells are not the only lymphocytes to regulate immune responses. A subset of B cells exhibits regulatory activity in mouse and humans and is referred to as regulatory B cells (B_{Regs}). Their regulatory capacity has been established in a number of mouse models of autoimmune diseases. Those include collagen-induced arthritis, experimental autoimmune encephalitis, diabetes and inflammatory bowel disease (IBD) (reviewed in 136, 137). B_{Regs} act primarily on T-cells, B-cells and NK cells. They regulate through secretion of IL-10, TGF-β and anti-inflammatory antibodies (138-140) and through cell-cell interactions (141). A specific subtype of B_{Regs}, named B10 cells have been characterised in mice as $CD1d^{hi}CD5^+$ B cells that are responsible for most, if not all, IL-10 production by B cells. B10 cells are present in spleen and are able to suppress the proliferation of T cells and T-cell dependent inflammatory responses *in vivo* (141). Another mechanism suggested for B_{Reg} regulation is the generation of regulatory T cells or NK cells. They have been shown to inhibit the progression of colitis in an IBD mouse model through the expansion of NK T cells and T cells (142).

Besides functioning as APC's, the dendritic cells serve other roles, including regulation. They express high levels of CD80 and CD86 and stimulate the differentiation of $CD4^+$ T cells through IL-10 production. IL-10 suppresses the IL-12 production by the dendritic cells and increases the production of IL-4 by T cells or increases the expansion of T_{Regs} (143).

In addition to the regulatory immune cells mentioned above, other factors partake in the regulation of the immune system. These include cytokines, signalling pathways, previously discussed miRNAs, co-stimulation, neutrophils and numerous others that have not been mentioned. Each of these factors contributes to the regulation of the immune system and together with T_{Regs} they coordinate the immune response.

1.7 T_{Regs} in health and diseases

Regulatory T cells have a unique ability to regulate immune responses. This provides them with means to prevent autoimmune diseases, immunopathology and allergy. In addition, they allow protective anti-tumour and anti-pathogen immune responses but inhibit damaging responses to self-antigens. However, T_{Regs} have also been known to suppress antitumor immune responses and favour tumour progression.

Autoimmune diseases are initiated when tolerance breaks down. The importance of T_{Regs} in preventing this breakdown is clear as both human and murine knock-outs of T_{Regs} develop severe autoimmune diseases. This is clearly displayed in the patients with IPEX syndrome, suffering from a genetic deficiency of FoxP3 (41) and the scurfy mouse (144). Defects in regulatory T cells have also been described in patients with MS, psoriasis, type 1 diabetes and rheumatoid arthritis (reviewed in 145).

T_{Regs} regulate allergen-specific responses in numerous ways. They regulate the allergen-induced proliferation and cytokine production of T_H1 and T_H2 cells (reviewed in 146) and suppress the antigen-presenting cells that support the generation of effector T_H2 and T_H1 cells. This is mediated through IL-10 secretion which down-regulates the expression of MHC and co-stimulatory molecules (147). Furthermore, T_{Regs} indirectly suppress the inflammatory functions of mast cells, basophils and eosinophils which are dependent on T_H2 cells for priming and activity (148).

T_{Regs} are present in many solid tumours in humans and have been linked with both a positive and negative outcome in patients (reviewed in 149). Inflammation often develops alongside tumours. T_{Regs} benefit the host by containing the suppression, nevertheless T_{Reg} can also benefit the tumour by suppressing the functions of immune effector cells (reviewed in 150). The accumulation of T_{Regs} in cancer is often associated with poor prognosis (151). This is not surprising as they have been shown to reduce the cytotoxic immune response by suppressing the activity of $CD8^+$ T cells and the NK cells (reviewed in 149, 150). On the other

hand, this is not always the case. The density and infiltration of FoxP3$^+$ T_{Regs} have been positively correlated with improved prognosis and survival (152, 153).

Immunological tolerance is when the immune system does not take a course of action against an antigen. Tolerance exists in three forms, namely central tolerance, peripheral tolerance and acquired tolerance. Here, the focus will be on peripheral and acquired tolerance. Regulatory T cells have a fundamental role in controlling tolerance to self- or foreign antigens. The importance of tolerance is clearly displayed in transplantation. T_{Regs} have been shown to be vital for grafts acceptance. This is confirmed in patients transplanted with lung, liver or kidney grafts (reviewed in 137). T_{Regs} are also responsible for suppression at mucosal sites. In oral tolerance, this is mainly mediated by TGF-β production which regulates immune responses on T cells, B cells, NK cells, macrophages and dendritic cells in an antigen independent manner (reviewed in 154). However, T_{Regs} can have negative effects on the mucosal immunity as TGF-β can lead to differentiation to T_H17 cells and thus suppress the differentiation of T_{Regs} (reviewed in 155). It is clear that T_{Regs} play an essential regulatory role in homeostasis control. They can act in a protective or a harmful manner or both.

1.7.1 Regulatory T cells – clinical applications

After regulatory T cells were established as key molecules in immune homeostasis, they became the focus of intense research for adoptive immunotherapy. T_{Regs} have been tested in mouse models where T_{Reg} transfer were effective in prevention and treatment of inflammatory and autoimmune diseases (20, reviewed in 156). They have also been shown to deliver functional and specific regulation in preclinical animal models *in vivo* (reviewed in 157). Some difficulties must be overcome before T_{Regs} can be used in immunotherapy. T_{Reg} count in circulation is rather low as they represent 5-10% of peripheral blood CD4$^+$ T cells. This entails that after isolation, the population needs to be expanded without losing its regulatory capacities. As the phenotype of T_{Regs} is not clearly distinguished, their isolation can become problematic. Additionally, the optimal culture condition for T_{Regs} with high-dose IL-2 can also give rise to effector T cell populations that produce pro-inflammatory cytokines (reviewed in 157, 158). A possible solution is the addition of rapamycin to the cell culture media which allows the proliferation of T_{Regs} but inhibits the effector T cell proliferation (159).

If these hurdles were to be conquered, the first clinical settings would most likely be graft-versus-host disease (GVHD) and systemic autoimmunity. Among the perks of using T_{Regs} in GVHD is that as they originate in the donor, they have not been subjected to immunosuppressive therapy and therefore show normal replicative potential. GVHD also has a foreseeable timepoint and therapy could thus be specifically timed (reviewed in 157). A published clinical trial expanded CD4$^+$CD25$^+$ T_{Regs} from third party umbilical cord blood *in*

vitro. The cells were infused at various doses and increased frequencies of $CD127^{-}FoxP3^{+}$ T_{Regs} were detected in peripheral blood within one week (160).

Regulatory T cell therapy for autoimmunity would have to involve antigen-specific T cells generated for the treatment. Target antigens would thus have to be identified beforehand. That would include myelin protein in MS and insulin for type 1 diabetes. Many current treatments are thought to involve T_{Regs} in some way. For example, the anti-TNFα therapy in RA patients neutralizes TNFα and has been associated with restored T_{Reg} numbers and function (114).

2 Aims of the study

The aim of this study was to evaluate the role of the pro-inflammatory cytokines of the innate immune response on the differentiation and function of human induced T_{Regs}.

It has been revealed that the innate immune system plays a significant role in the immunopathology of autoimmunity. However, its part in differentiation and function of human $CD4^+$ iT_{Regs} is currently unclear.

2.1.1 Specific aims:

1. **Evaluate the role of IL-2 and TGF-β1 in $CD4^+$ iT_{Regs} differentiation and function *in vitro*.**
2. **Evaluate the role of the pro-inflammatory cytokines IL-1β and TNFα upon induced $CD4^+$ T_{Regs} differentiation and function.**
3. **Evaluate the possible mechanisms involved in the above regulatory pathways.**

3 Materials and methods

All procedures were authorized by the ethical committee of Landspítali University Hospital and The Data Protection Authority.

3.1 Study subjects

The study population consisted of healthy volunteers from the Blood Bank. Volunteers need to fit the Blood Bank criteria; age 18-60 years, weigh more than 50 kg and not receiving any drugs. Adult venous peripheral blood was collected in sodium heparin tubes from healthy volunteers and donated to the study in the form of Buffy Coat. Informed consent was signed by all participants.

3.2 Study design

Peripheral blood mononuclear cells (PBMC's) were isolated from buffy coat using density centrifugation. Naïve $CD4^+CD25^-$ T cells were isolated and subsequently stimulated *in vitro* and phenotyped by flow cytometry. The naïve T cells were stimulated with anti-CD3 and cultured with IL-2 and with or without TGF-β1 and the pro-inflammatory cytokines TNFα and IL-1β for 120 hrs to investigate the iT_{Reg} differentiation. Next, the culture supernatants were harvested and frozen. The T cells were put in a co-culture with CFSE labelled PBMC's and Epstein-Barr infected B cells (EB-Bs) to evaluate the iT_{Reg} suppressive function. After 72 hrs of co-culture, the cells were harvested and analysed using FACS and Modfit LT. The mechanism behind the iT_{Regs} suppressive function was estimated based on cytokine levels in the supernatant. This was performed with enzyme-linked immunosorbent assays (ELISAs) for IL-2, IL-10 and IL-35.

3.3 Isolation of peripheral blood mononuclear cells

One batch of Buffy coat containing 450 ml blood was obtained from the blood bank. The buffy coat is diluted 1:3 in sterile Phosphate Buffer Saline (PBS). Samples were loaded on to 10 mL of Ficoll (Histopaque®-1077) in sterile tubes and centrifuged for 30 min at 1600 rotations per minute (rpm) without brakes at room temperature (RT). PBMC's were collected from the interphase with a Pasteur pipette and transferred to sterile tubes. They were washed with 30 mL sterile PBS for 10 min at 1400 rpm at RT. Supernatant was discarded and cells re-suspended in 30 mL PBS. Cells were counted and their viability obtained using Trypan Blue staining and Countess® Automated Cell Counter (Invitrogen) and calculated as follows:

$$Live\ cell\ concentration/_{mL} \times sample\ (mL) = Total\ number\ of\ cells\ in\ sample$$

After counting, the cells were centrifuged for 5 min at 1300 rpm at RT and supernatant was discarded.

3.4 Isolation of $CD4^+$ T cells

$5x10^7$ cells were re-suspended in 500 µL Isolation Buffer. 25 µL of Flowcomp Human CD4 Antibody (Dynabeads® FlowComp™ Human CD4, Invitrogen) were added, mixed and incubated for 10 min at 2-8°C. Cells were washed with 2 mL Isolation Buffer and centrifuged for 8 min at 350 x g. The supernatant was discarded and 1 mL Isolation Buffer added to the cell pellet and re-suspended. 75 µL FlowComp Dynabeads was added and mixed and incubated for 15 min at RT under rolling. The tube was placed in a magnet (DynaMag™-15 Magnet, Invitrogen) for minimum 1 minute. The supernatant was carefully removed and discarded. Tube was removed from the magnet and at least 1 mL Isolation Buffer added. Bead-bound cells were re-suspended by gentle pipetting 5 times. The tube was placed in the magnet for minimum 1 minute. The supernatant was carefully removed and discarded. Tube was removed from the magnet and the bead-bound cells were carefully re-suspended in 1 mL FlowComp Release Buffer and incubated for 10 min at RT under rolling. The cells were mixed by pipetting 10 times and the tube placed in the magnet for 1 min. The supernatant containing the bead-free cells was transferred to a new tube and again placed in the magnet for 1 min to remove any residual beads. Supernatant was transferred to a new tube and 2 mL Isolation Buffer added, cells counted, followed by centrifugation for 8 min at 350 x g. Supernatant was discarded.

3.5 Depletion of $CD25^+$ cells

Dynabeads (Dynabeads® CD25, Invitrogen) were washed before use. $2.5x10^7$ $CD4^+$ T cells were re-suspended in 1 mL Isolation Buffer. 50 µL of washed Dynabeads CD25 were added and incubated for 30 min at 2-8°C with gentle rotation. The tube was placed in a magnet for 2 min and the supernatant transferred to a new tube. The isolation process resulted in a population of $CD4^+CD25^-$ T cells (Figure 2).

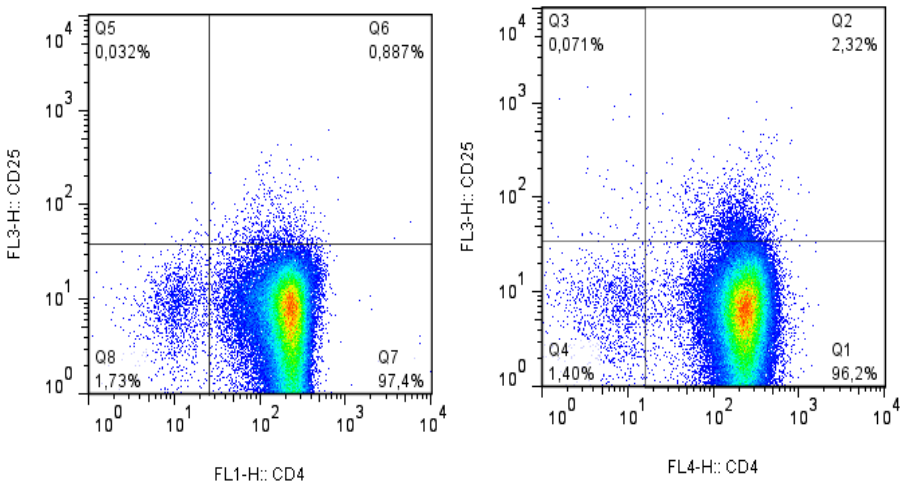

Figure 2: Isolation of naïve CD4$^+$CD25$^-$ T cells. CD4$^+$ positive T cells were isolated and CD25$^+$ cells depleted using Dynabeads (Invitrogen). Results obtained from 2 experiments.

3.6 *In vitro* stimulation of CD4$^+$CD25$^-$ T cells

CD4$^+$CD25$^-$ T cells were stimulated with 1 µg/mL plate-bound anti-CD3ε monoclonal antibody (R&D Systems). CD3 was suspended in sterile PBS Buffer (1 µg/mL) and 100 µL placed in each well in a 96 well U-bottomed cell culture plates (Nunc, Invitrogen). The plate was incubated for 90 min at 37°C. The wells were aspirated and washed with 100 µL PBS. Cells were added and cultured for 120 hrs in the presence of Recombinant Human IL-2 (100 IU, R&D Systems) and/or TGF-β1 (10 ng/mL, R&D Systems). TNFα (50 ng/mL, R&D Systems), IL-1β (10 ng/mL, R&D Systems) were added into selected cultures. After 120 hrs incubation the cells were either harvested and stained for flow cytometer analysis, or washed and used in a suppression assay.

3.7 Cell cultures

CD4$^+$CD25$^-$ T cells were placed in incubators at 37°C in 95% air atmosphere with 5% (v/v CO_2). They were cultured in 96 well U-bottomed cell culture plates (Nunc, Invitrogen).

3.8 Cellular staining

Cells were harvested and stained at different timepoints after stimulation. 100 µL of cell culture was placed in FACS tubes. Gammagard (0.4 mg/mL) was added for 5 min at RT. Cells were stained on the outer surface and intracellular.

3.8.1 Surface staining

FACS tubes were centrifuged for 5 min in 1200 rpm and 4°C and supernatant discarded. 50 µL staining buffer was added to each tube and relevant fluorochrome labelled antibodies added (2 µL of APC-labelled antibodies and 3 µL of FITS-, PE-, and PerCP-labelled antibodies). The tubes were incubated on ice for 20 min. 2 mL of staining buffer was added

to each tube and the tubes were centrifuged for 5 min in 1200 rpm and 4°C and supernatant discarded. Relevant tubes were fixed with 0.5% paraformaldehyde and collected with FACSCalibur flow cytometer (BD Biosciences) within a week. Other tubes were further processed with intracellular staining.

3.8.2 Intracellular staining

Intracellular staining was performed using Foxp3/Transcription Factor Staining Buffer Set (ebioscience). 1 mL Fixation/Permeabilization Concentrate was added to each tube mixed and incubated at 4°C for 40 min in total darkness. Cells were then washed with 2 mL Permeabilization buffer and centrifuged for 10 min at 300 x g and supernatant discarded. 50 µL of Permeabilization buffer was added and 5 µL of intracellular fluorochrome labelled antibodies and isotype controls. The tubes were incubated for 30 min in 4°C. Cells were finally washed two times with 2 mL Permeabilization buffer and collected in flow cytometer staining buffer. Cells were collected with the FACSCalibur within one day.

3.9 Suppression assay

A suppression assay was performed to evaluate the iT_{Reg} suppressive function (Figure 3).

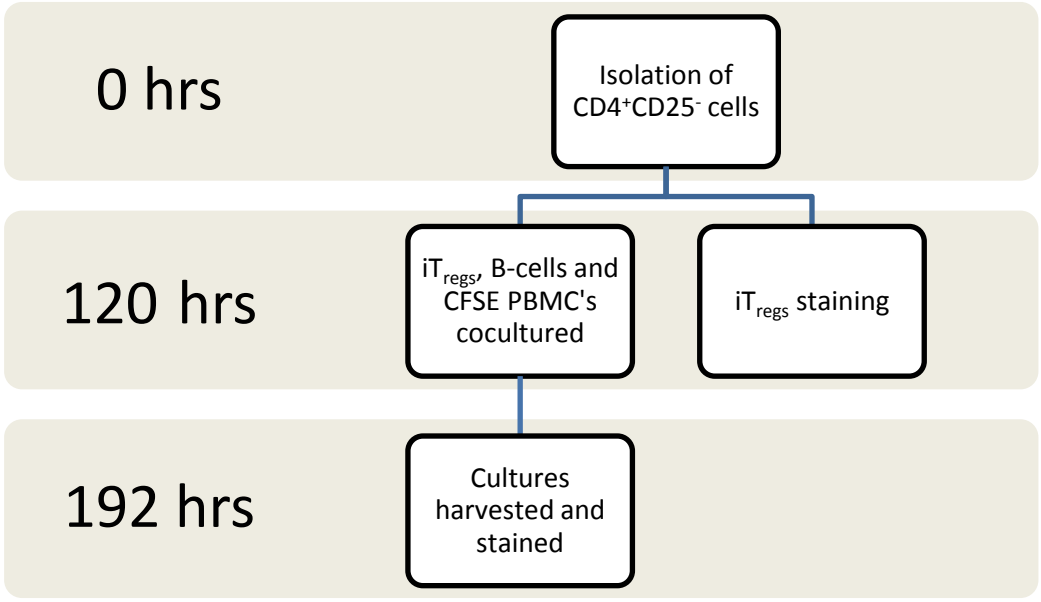

Figure 3: Flow chart of the study design.

3.9.1 B cells pulsed with superantigens

Epstein-Barr infected B cells were cultured in RPMI+10% FCS. B cells were extracted and washed 3 times with sterile PBS and counted. The pellet was re-suspended in AimV+1% FCS in the ratio $10x10^6$/mL and 1 mL placed in tubes. Superantigens (Staphylococcal enterotoxins, SEA, SEB and SEE, 1 µg/mL of each, Toxin Technologies) were added to the

tubes and incubated for 2 hrs at 37°C and mixed every 30 min. Finally, the cells were washed with Aim-V.

3.9.2 CFSE staining

After the PBMCs had been isolated, they were re-suspended in Aim-V in the concentration $10x10^6$/mL. To monitor their proliferation, they were labelled with CFSE (carboxyfluorescein diacetate succinimidyl ester). CFSE is a membrane-permeable fluorescent dye and diffuses into the cell and becomes highly fluorescent. On each cell division cycle, the CFSE is divided in two daughter cells. This enables detection of the cells proliferation and counting of divisions. 5 mM CFSE solution was prepared using CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). 1 µL of the 5 mM solution was added to 1 mL cell culture and mixed rapidly. The tubes were incubated for 5 min in RT and washed 3x with PBS and Aim-V for the final wash. The cells were then re-suspended in AimV in the concentration of $1x10^6$/mL.

3.9.3 iT_{reg} culture with CFSE stained PBMC's and B cells

After 120 hrs culture, the iT_{regs} were collected, counted and washed with PBS. They were then re-suspended in fresh Aim-V and IL-2 (100 IU) in the concentration $1x10^6$/mL.

The B cells (pulsed with superantigens), CFSE stained PBMC's and iT_{regs} were placed in a co-culture. The ratio between PBMC: B cells and between iT_{Regs} and EB-B cells was constant at 10:1 whereas the ratio of iT_{Regs}: PBMCs varied from 1:1 to 1:32 (Figure 4).

	1	2	3	4	5	6	7	8	9	10	11	12
A	Med 1:1 1	Med 1:1 2	TGF-b 1:1 3	TGF-b 1:1 4	a-TNF 1:1 5	a-TNF 1:1 6	TNF-a 1:1 7	TNF-a 1:1 8	IL-1b 1:1 9	IL-1b 1:1 10	11	12
B	Med 1:4 13	Med 1:4 14	TGF-b 1:4 15	TGF-b 1:4 16	a-TNF 1:4 17	a-TNF 1:4 18	TNF-a 1:4 19	TNF-a 1:4 20	IL-1b 1:4 21	IL-1b 1:4 22	23	24
C	Med 1:8 25	Med 1:8 26	TGF-b 1:8 27	TGF-b 1:8 28	a-TNF 1:8 29	a-TNF 1:8 30	TNF-a 1:8 31	TNF-a 1:8 32	IL-1b 1:8 33	IL-1b 1:8 34	35	36
D	Med 1:16 37	Med 1:16 38	TGF-b 1:16 39	TGF-b 1:16 40	a-TNF 1:16 41	a-TNF 1:16 42	TNF-a 1:16 43	TNF-a 1:16 44	IL-1b 1:16 45	IL-1b 1:16 46	47	48
E	Med 1:32 49	Med 1:32 50	TGF-b 1:32 51	TGF-b 1:32 52	a-TNF 1:32 53	a-TNF 1:32 54	TNF-a 1:32 55	TNF-a 1:32 56	IL-1b 1:32 57	IL-1b 1:32 58	59	60
F	61	62	63	64	65	66	67	CFSE- 68	CFSE- 69	CFSE- 70	CFSE- 71	CFSE- 72
G	PBMC +B 73	PBMC +B 74	PBMC +B 75	76	77	78	79	80	81	82	83	84
H	Med UP 85	Med UP 86	TGF-b UP 87	TGF-b UP 88	a-TNF UP 89	a-TNF UP 90	TNF-a UP 91	TNF-a UP 92	IL-1b UP 93	IL-1b UP 94	95	96

Figure 4: Cell culture plate for the suppression assay.

After 72 hrs culture the cells were harvested and stained with surface staining. The cells were collected with the FACSCalibur.

3.10 Cytokines in iT_{reg} culture supernatant

iT_{regs} were isolated and cultured in Aim-V and various cytokines as described above. After 120 hrs of culture, the cells were centrifuged and the supernatant collected and stored at -80°C. The samples were thawed at RT and Enzyme-linked immunosorbent assays (ELISAs) were performed to detect cytokines in the culture supernatant.

3.10.1 Human IL-35 ELISA Ready-Set-Go!

The ELISA was performed with Human IL-35 ELISA Ready-Set-Go! (ebioscience). Capture antibody was diluted 250x in Coating Buffer. Corning Costar 9018 ELISA plate was coated with the antibody 100 µL/well, the plate sealed and incubated overnight at 4°C. The morning after, wells were aspirated and washed 5 times with >250 µL/well Wash Buffer. 5x Assay Diluent was diluted with deionised water and wells blocked with 200 µL/well and incubated at RT for 1 hr. Wells were aspirated and washed 5 times with Wash Buffer. Standards were diluted to 100 IU with 1x Assay Diluent, added to wells and 2-fold serial dilutions performed to make the standard curve. Samples were added 100 µL/wells undiluted and 1:10 dilution in Assay Diluent. The plate was sealed and incubated at RT for 2 hrs. The plate was then aspirated and washed as before. Detection antibody was diluted in 1x Assay diluent and 100 µL/well added to the plate and incubated sealed at RT for 1 hr. The plate was aspirated and washed as before and 100 µL/well of Avidin-HRP diluted in 1x Assay Diluent added. The plate was sealed and incubated at RT for 30 min. The plate was next aspirated and washed 7 times with 1-2 min soaking between washes. 100 µL/well Substrate Solution was added to each well and incubated at RT for 15 min. 50µL of Stop Solution was then added to each well. The plate was read at 450 nm using Finstruments ® Microplate reader. A standard was used to calculate IL-35 levels and the results presented in pg/mL.

3.10.2 Human IL-10 ELISA

Total IL-10 was measured in the supernatant using DuoSet ELISA kit (DY217, R&D systems). Capture anti-IL-10 antibodies were diluted 1:180 in PBS and 100 µL added into wells and incubated overnight at RT. The wells were aspirated and 300 µL/wells of Blocking solution added and incubated for 1 hr at RT. The wells were washed 4 times with Wash Buffer. Standards were diluted to 4 ng/mL with Elisabuffer, added to plate and then diluted by twofold serial dilutions. Samples were diluted 1:50 and 1:100 and added 100 µL/well and incubated for 2 hr at RT. The plate was washed as before. IL-10 Detection antibody was diluted 1:180 in Elisabuffer and 100 µL added into all wells and incubated for 2 hrs at RT. Washed as before. The Streptavidin-HRP was diluted 1:200 in Elisabuffer and 100 µL added

into the wells and incubated for 20 min in the dark at RT. The plate was then washed as before. 100 µL Substrate Solution was added and incubated in the dark at RT until the colour had developed clearly in the highest standard and finally 50 µL of Stop Solution was then added and the plate read immediately at 450 nm. A standard was used to calculate IL-10 levels and the results presented in pg/mL.

3.10.3 Human IL-2 ELISA Set

IL-2 cytokine presence was determined using the Human IL-2 ELISA Set (BD Biosciences). Plate was coated with 100 µL of Capture Antibody diluted in Coating Buffer. The plate was sealed and incubated overnight at 4°C. Wells were aspirated and washed 3 times with ≥300 µL/ well Wash Buffer. Next, the plate was blocked with ≥200 µL/well Assay Diluent and incubated at RT for 1 hr. The plate was washed as before. 500 ng/mL Standards were prepared by dilution in deionised water, added to plate and twofold serial dilution performed. Samples were diluted 1:50 and 1:100 and 100 µL added to all wells. The plate was then incubated for 2 hrs at RT and aspirated and washed 5 times. 100 µL of Working Detector antibody was added and incubated for 1 hr at RT. The plate was next washed 7 times and 100 µL of Substrate Solution added to each well and incubated for 30 min in the dark at RT. Finally 50 µL of Stop Solution was added and the absorbance read at 450 nm within 30 min of stopping reaction. A standard was used to calculate IL-2 levels and the results presented in pg/mL.

3.11 Analysis

The results from the iT_{Reg} differentiation were analysed with FlowJo.

Results from the suppression assay were analysed with Modfit LT software, which determined the proliferation index as the sum of the cells in all generations divided by the computed number of original parent cells theoretically present at the start of the experiment. Results from the ELISAs were analysed with Titri 5.03 and Microsoft Office Excel.

Statistical analyses were performed using GraphPad Prism 5.0 for windows. Results expressed as mean values ± standard error of the mean (SEM). Differences were determined to be significant when $p<0.05$.

4 Results

4.1 Differentiation of iT_{Regs}

4.1.1 T cell phenotype after $CD4^+CD25^-$ isolation

To understand the phenotypical change that the iT_{Regs} undergo during *in vitro* differentiation, we measured the expression of various molecules at the beginning of the culture. In particular the expression of the well-known T_{Reg} markers CD4, CD25, CD127 and FoxP3 was evaluated. After isolation the purity of the $CD4^+CD25^-$ T cells was on the average 94.3%. At the start of culture, 85.8% of the isolated cells displayed $CD127^+$ surface expression and 1.79% were $FoxP3^+$. In addition, 60.5% of the population expressed the naïve T cell marker CD45RA but 35.9% expressed the memory marker CD45RO. Almost all isolated cells expressed the co-stimulatory molecule CD28, 98.2%, and 89.2% expressed the adhesion molecule CD62L. TGF-βRII was expressed on 77.0% of the cells (Figure 5).

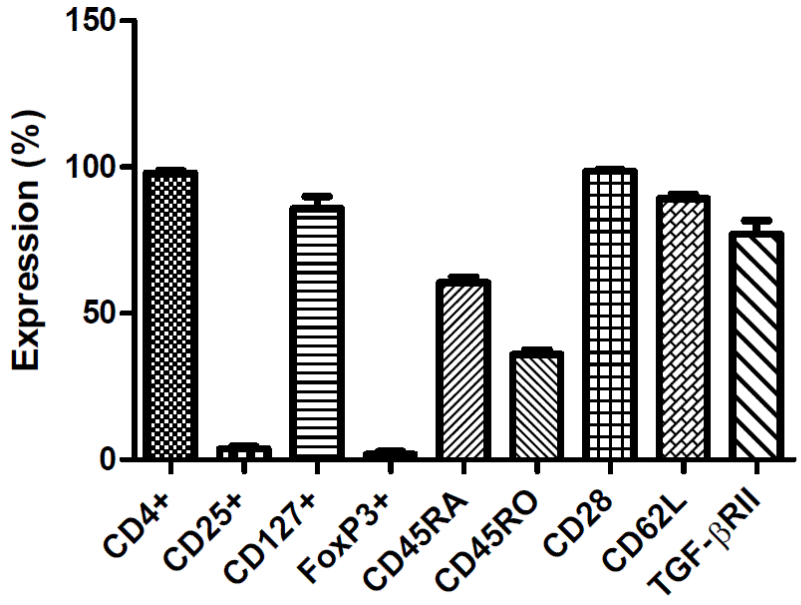

Figure 5: **Expression of various molecules at Day 0.** Isolated $CD4^+CD25^-$ were isolated from healthy human adult peripheral blood. The phenotype was assessed using FACS. Results are shown as mean ± standard error of the mean (SEM), n=4.

4.1.2 IL-1β and TNFα prevent iT_{Reg} differentiation

To examine the success of iT_{Regs} differentiation, the expression of CD4, CD25, CD127 and FoxP3 was investigated after 120 hrs of culture in the presence of IL-2, TGF-β1 and with/without the pro-inflammatory cytokines IL-1β and TNFα. The $CD4^+CD127^-CD25^{high}FoxP3^{high}$ iT_{Regs} population was gated as shown in Figure 6. A well-defined population of $CD25^{high}FoxP3^{high}$ cells emerges in the presence of TGF-β1 but is not present in the culture containing medium only and IL-2 (Figure 6).

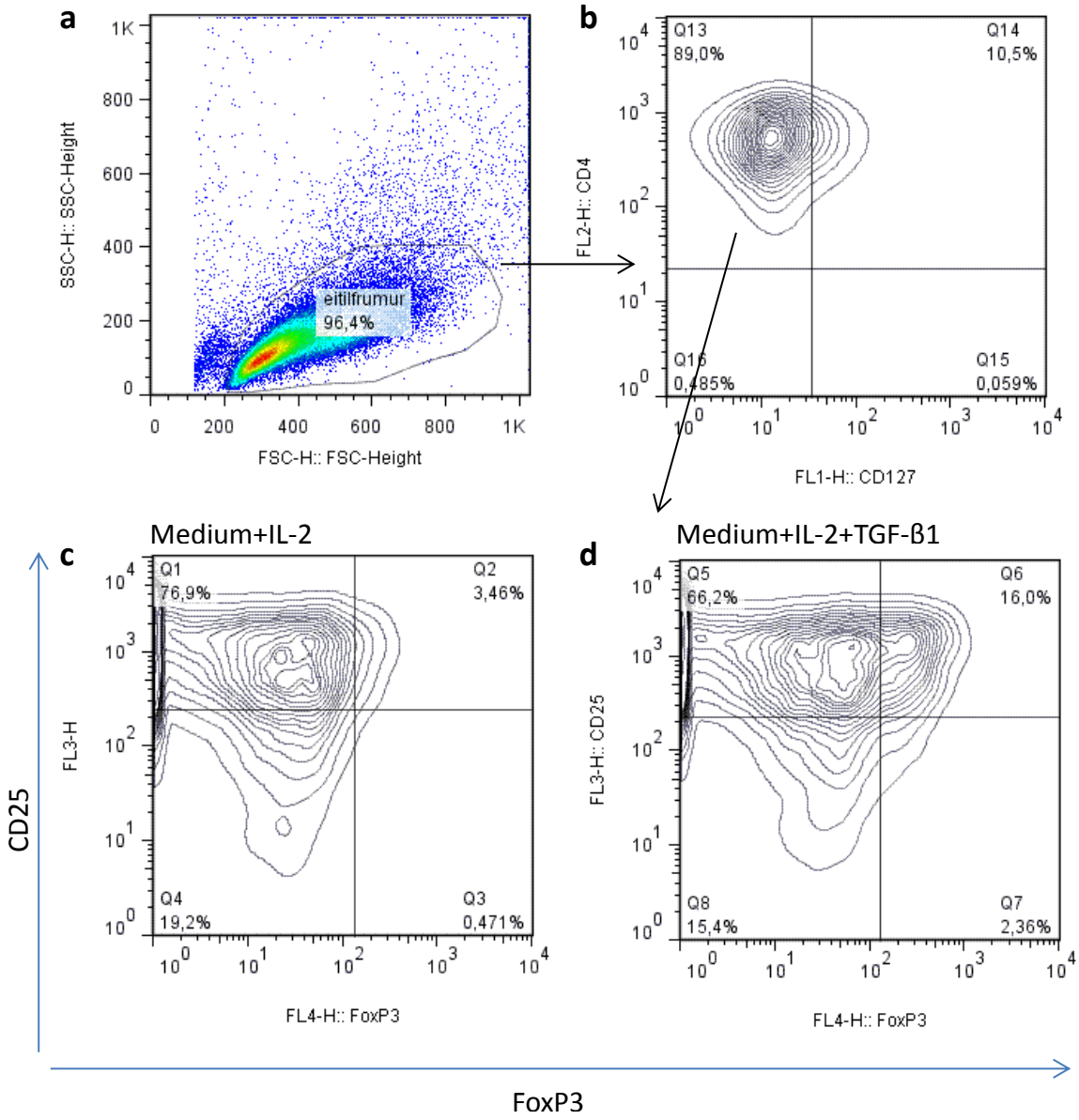

Figure 6: Gating strategy for iT_{Reg} phenotyping. (a) Lymphocytes were gated using forward and side scatter measurements. (b) iT_{Reg} phenotype was further established with $CD127^{-}CD4^{+}$ gating and finally (c-d) $CD25^{high}FoxP3^{high}$ cells were selected as depicted.

The highest fraction of *ex vivo* differentiated $CD4^{+}CD127^{-}CD25^{high}FoxP3^{high}$ iT_{Regs} from naïve human T cells was found to be dependent upon the presence of both IL-2 and TGF-β1 ($p<0.05$) as can be seen in Figure 7. The presence of the pro-inflammatory cytokines IL-1β and TNFα significantly inhibited the differentiation of iT_{Regs}. The presence of TNFα resulted in 68.3% inhibition, whereas IL-1β inhibited the differentiation of 73.5% of the population ($p<0.05$).

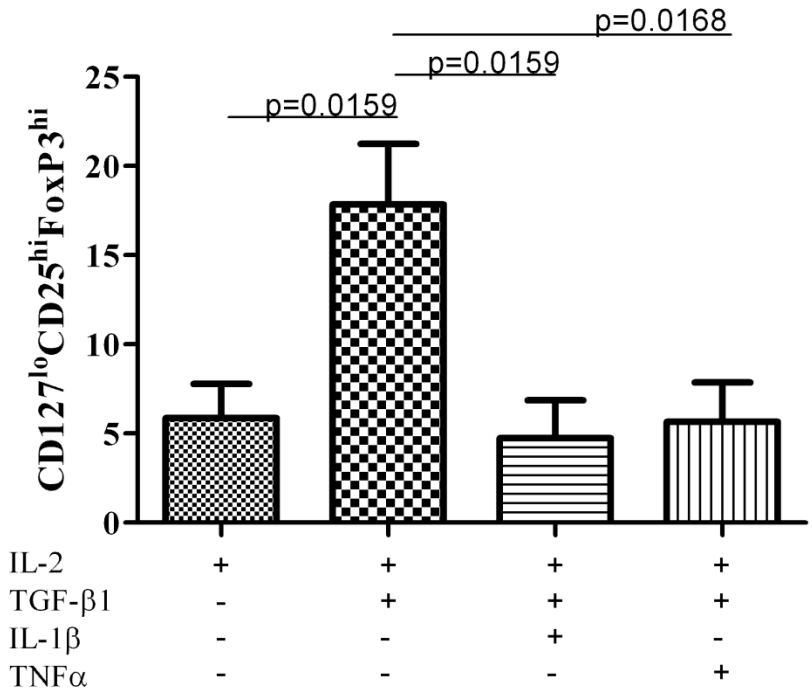

Figure 7: **IL-1β and TNFα prevent iT$_{Reg}$ differentiation.** Adult human peripheral blood CD4$^+$CD25$^-$ T cells were isolated and activated with anti-CD3 (1 µg/mL) for 120 hrs in the presence of IL-2 (100 IU), medium and TGF-β1 (10 ng/mL) with and without TNFα (50 ng/mL) and IL-1β (10 ng/mL). Mean ± SEM, n=5, Mann Whitney *U* test.

4.1.3 TGF-β1 induced the expression of CD103 in iT$_{Regs}$

Next we evaluated the expression of the integrin CD103 on CD4$^+$ iT$_{Regs}$. As shown in Figure 8, the expression of CD103 increased tenfold in the presence of IL-2 and TGF-β1, compared to IL-2 alone (Figure 8).

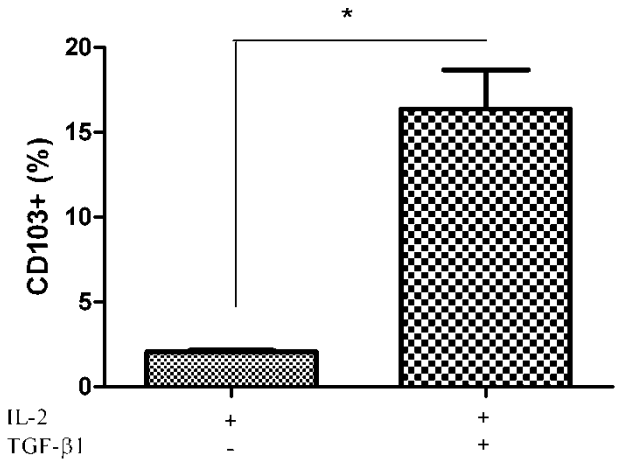

Figure 8: TGF-β1 induced up-regulation of CD103 on iT$_{Regs}$. Adult human peripheral blood CD4$^+$CD25$^-$ T cells were isolated and activated with anti-CD3 (1 µg/mL) for 120 hrs in the presence of medium, IL-2 (100 IU), and with/without TGF-β1 (10 ng/mL). Mean ± SEM, n=4, *p<0.05, Mann Whitney *U* test.

4.2 The suppressive function of *ex vivo* iT_{Regs}

4.2.1 TGF-β1 induced iT_{Regs} contain suppressive function *ex vivo*

In our studies iT_{Regs} definition was based on the current available phenotypic characterization of such cells. However, we could not firmly do so without establishing their suppressive function. Thus, we CFSE labelled PBMC's and co-cultured them for 72 hrs with iT_{Regs} in the ratio 1:1 versus control ($-iT_{Regs}$). As shown in Figure 9, the iT_{Regs} had a significant suppressive effect in our system resulting in a sixfold reduction in PBMC's proliferation in the presence of iT_{Regs} (1:1 ratio, $p<0.05$).

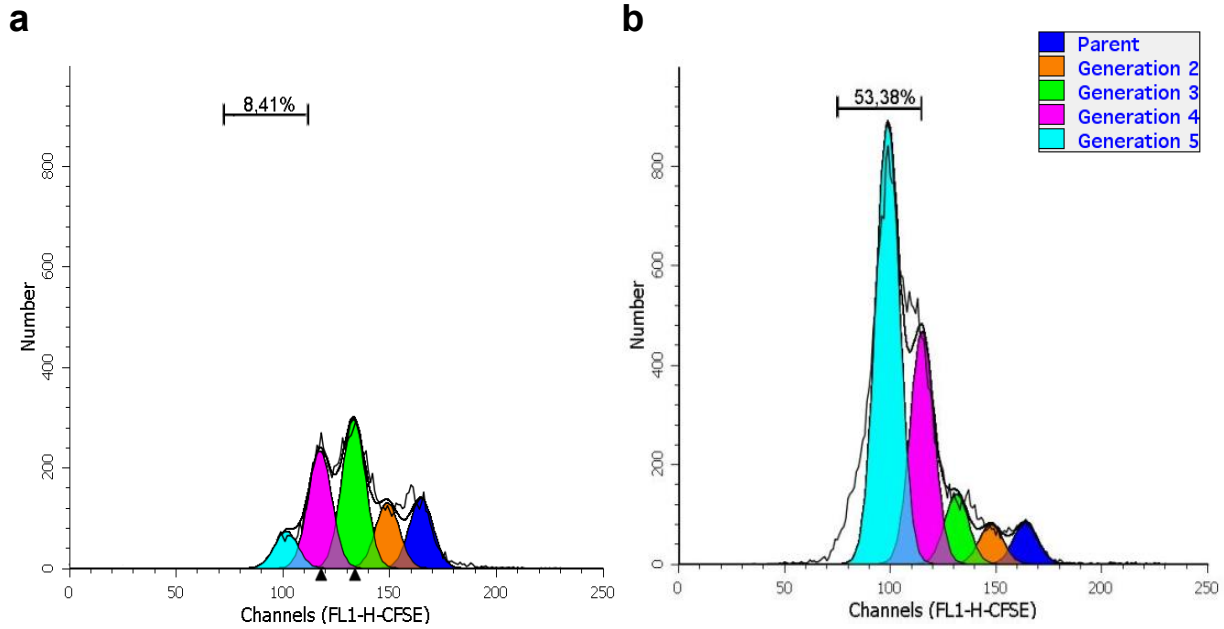

Figure 9: TGF-β1 induced iT_{Regs} contain suppressive function. (a-b) Proliferative response of a PBMC's co-cultured for 72 hrs with iT_{Regs} in the ratio 1:1 versus control ($-iT_{Regs}$) was assessed by CFSE assay. iT_{Regs} were stimulated with anti-CD3 (1 µg/mL) in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/mL) for 120 hrs. EB-B cells pulsed with superantigens were cultured with the PBMC's (1:10) and $T_{Regs.}$ CFSE labelled cells were acquired by BD FACSCalibur, cells were gated on $CD25^{+}$ lymphocytes and percentages of cells in each generation were calculated by Modfit LT software. Parent population is located furthest to the right and the population farthest on the left has undergone most proliferations.

For comparison, unpulsed (UP) Epstein-Barr infected B cells were cultured with CFSE labelled PBMC's for 72 hours. As shown in Figure 10, the unpulsed B cells failed to induce a proper proliferation of the PBMC's (35.9%) compared to the pulsed B cells (58.2%).

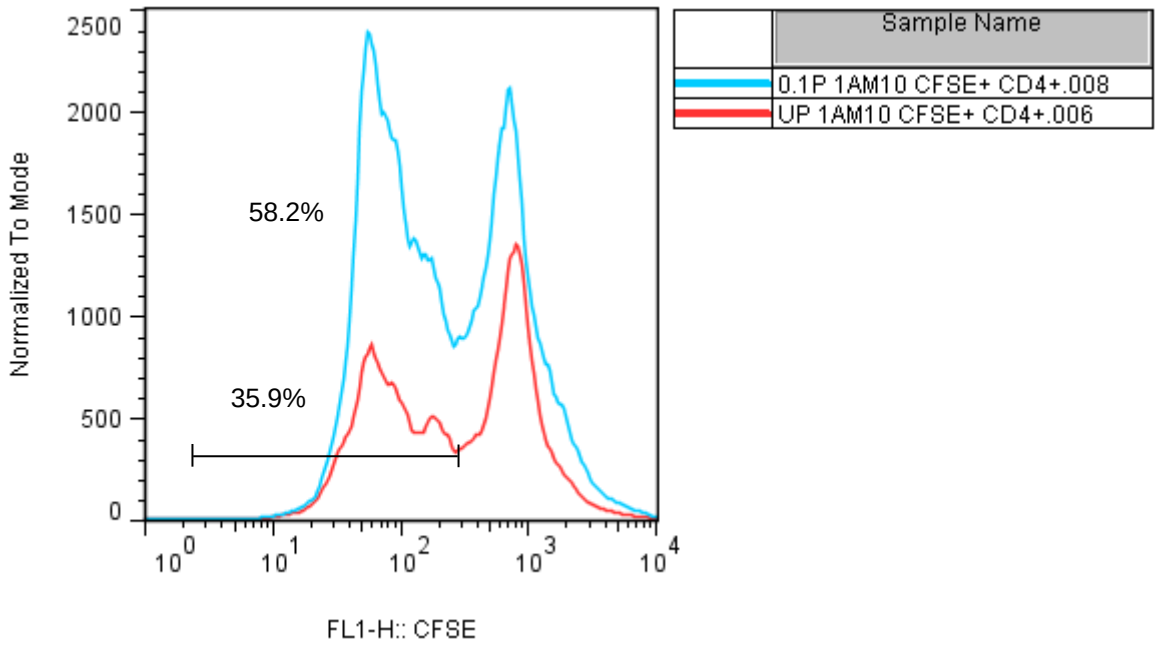

Figure 10: Unpulsed EB-Bs could not induce PBMC's proliferation. Proliferative response of PBMC's co-cultured with EB-B cells for 72 hrs. Unpulsed EB-B cells were cultured with the PBMC's in the ratio 1:10.

4.2.2 The suppressive function of iT_{Regs} correlated positively with their numbers

We further evaluated the suppressive function of the iT_{Regs} and co-cultured them for 72 hrs with CFSE labelled PBMC's as before except that we cultured them in the ratios iT_{Reg}:PBMC, 1:1, 1:4, 1:8, 1:16, 1:32. The suppressive function of *ex vivo* induced $CD4^+$ iT_{Regs} correlated positively with their numbers (Figures 11 and 12).

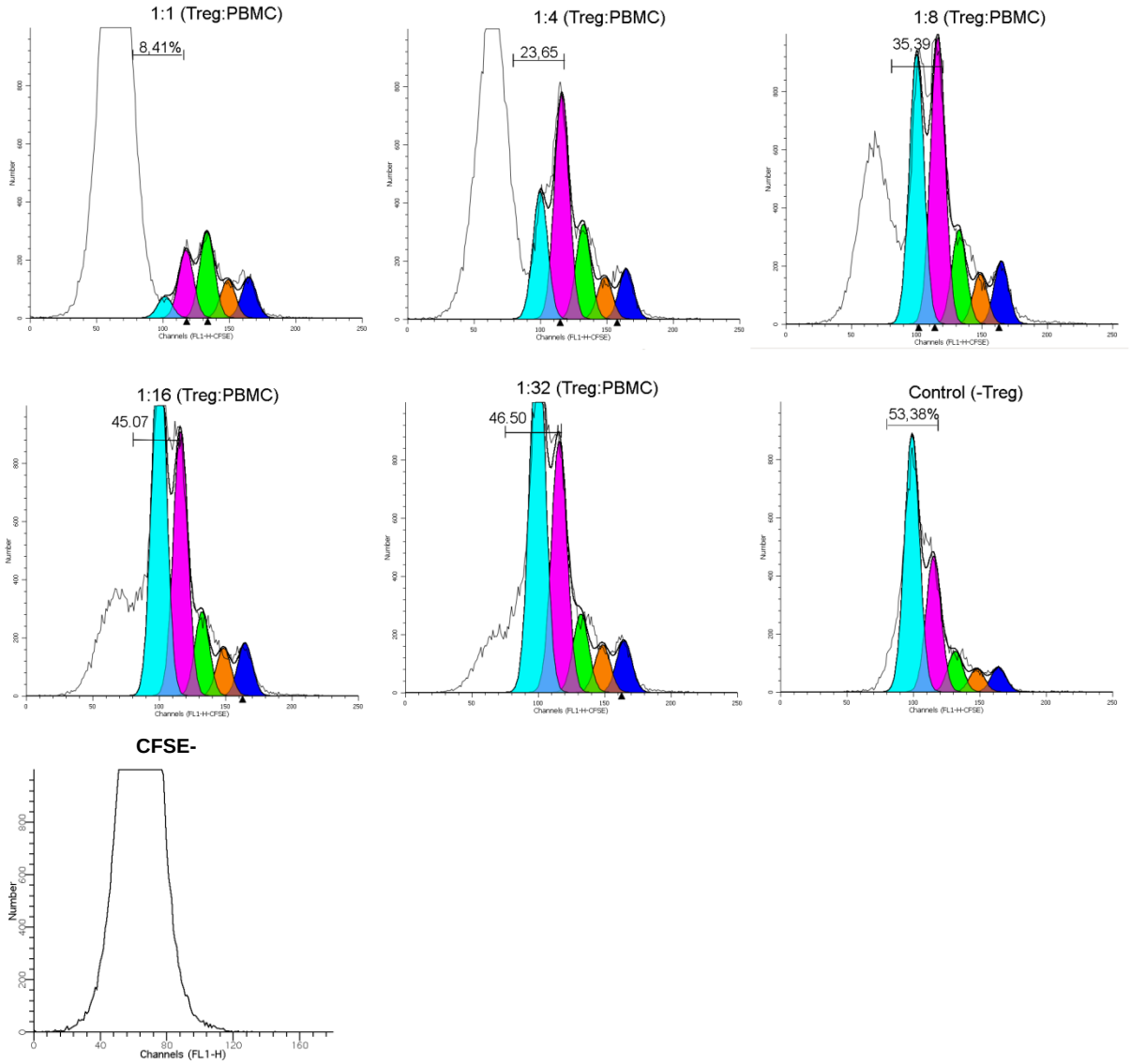

Figure 11: TGF-β1 induced iT_{Reg} and $T_{Responder}$ ratio. Proliferative response of PBMC's co-cultured for 72 hrs with iT_{Regs} in the following ratios; iT_{Reg}:PBMC, 1:1, 1:4, 1:8, 1:16, 1:32. iT_{Regs} were stimulated with anti-CD3 (1 µg/mL) in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/mL) for 120 hrs. EB-B cells pulsed with superantigens were cultured with the PBMC's (1:10) and T_{Regs}. CFSE labelled cells were acquired by BD FACSCalibur, cells were gated on $CD25^+$ lymphocytes.

This correlation between the suppressive function of iT_{Regs} and their numbers in the culture is further summarized in Figure 12. Results are displayed as a Proliferation Index (PI) for the CFSE labelled PBMC's. As shown, a significant difference (p=0.0193) was found between a culture of 1 T_{Reg}:1 PBMC and control.

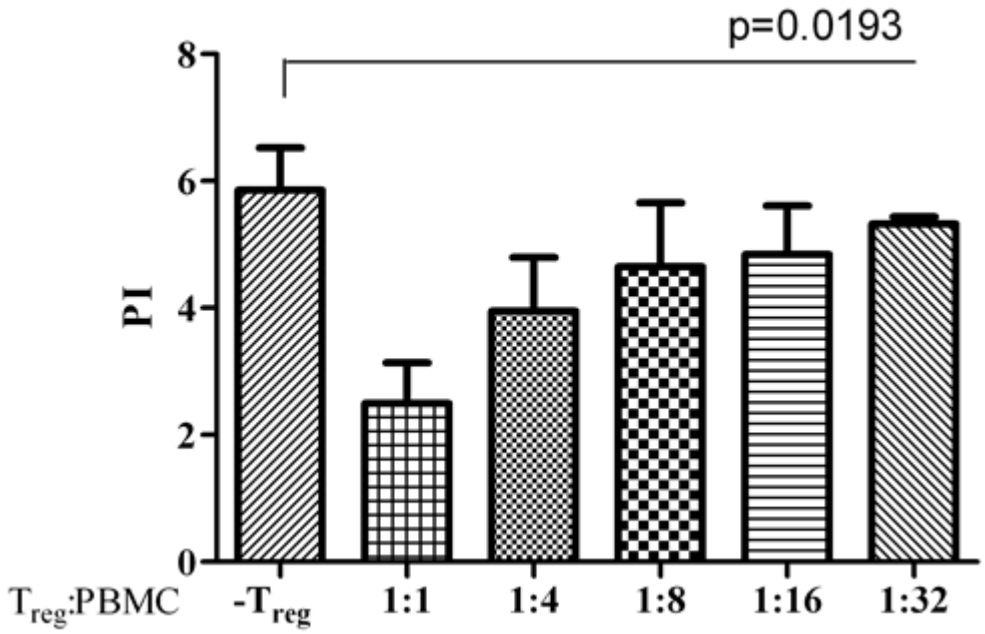

Figure 12: iT_{Reg} suppressive function correlates with iT_{Reg} numbers. Proliferation Index (PI) for PBMC's co-cultured with iT_{Regs} in the following ratios; iT_{Reg}:PBMC, 1:1, 1:4, 1:8, 1:16, 1:32. Results were calculated by Modfit LT, which determined the proliferation index as the sum of the cells in all generation divided by the computed number of original parent cells theoretically present at the start of the experiment. Mean ± SEM, n=3, One-way anova.

4.2.3 IL-1β and TNFα prevent human iT_{Reg} function *in vitro*

As shown above, IL-1β and TNFα significantly inhibited the differentiation of iT_{Reg} *in vitro*. Thus, we also investigated their possible effects upon the iT_{Reg} suppressive function in our model. We observed that both IL-1β and TNFα prevent the suppressive function of the $iT_{Regs.}$ As shown in Figure 12, a significant difference was seen in the PI of the CFSE labelled T cells cultured with the TGF-β1 induced iT_{Regs} cultured in the ratio 1:1. This significance was lost when IL-1β or TNFα were added to the iT_{Reg} culture (Figure 13).

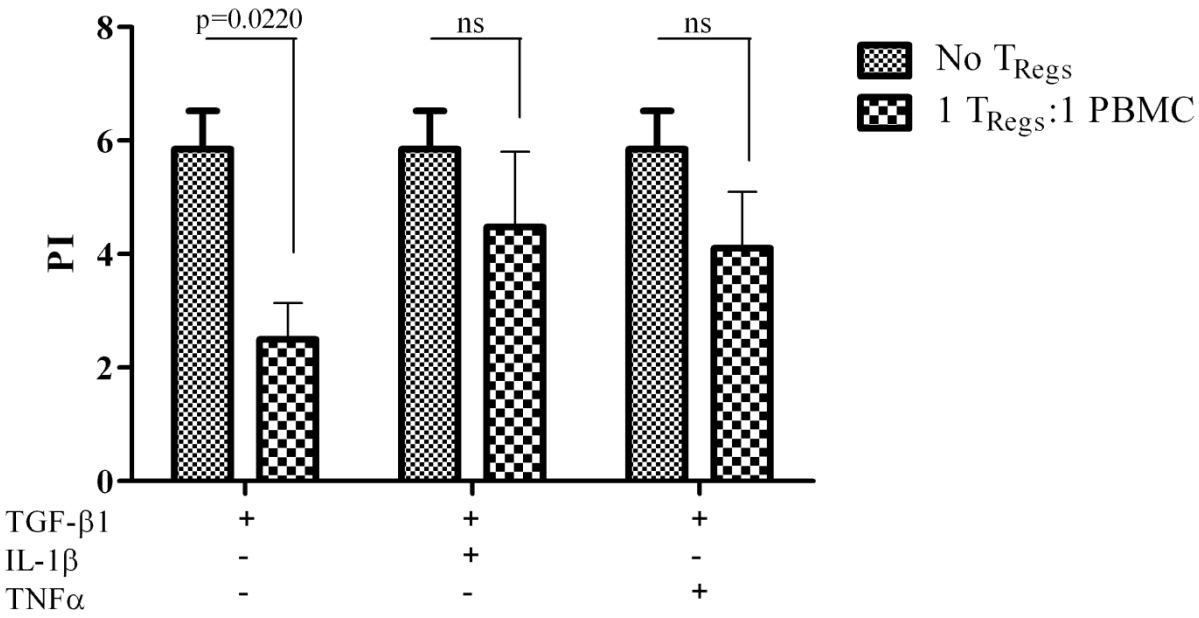

Figure 13: IL-1β and TNFα prevent iT_{Reg} function. PI for adult PBMC's co-cultured with iT_{Regs} in the ratio; iT_{Reg}:PBMC, 1:1. The iT_{Regs} had previously been stimulated with anti-CD3 (1 µg/mL) and cultured in the presence of IL-2 (100 IU), TGF-β1 (10 ng/mL) and with/without IL-1β (10 ng/mL) and TNFα (50 ng/mL) for 120 hrs. Mean ± SEM, n=4, student's *T* test.

4.2.4 $CD4^+$ iT_{Regs} suppressed the proliferation of both $CD4^+$ and $CD8^+$ human T cells

We next wanted to assess if the iT_{Regs} suppressive function was directed at a certain T cell population. During these studies, the potential responder population was additionally labelled with CD4 and CD8 directed antibodies. As shown in Figure 15, the iT_{Regs} suppressed the proliferation of both $CD4^+$ and $CD8^+$ human T cells in our system (Figures 14 and 15).

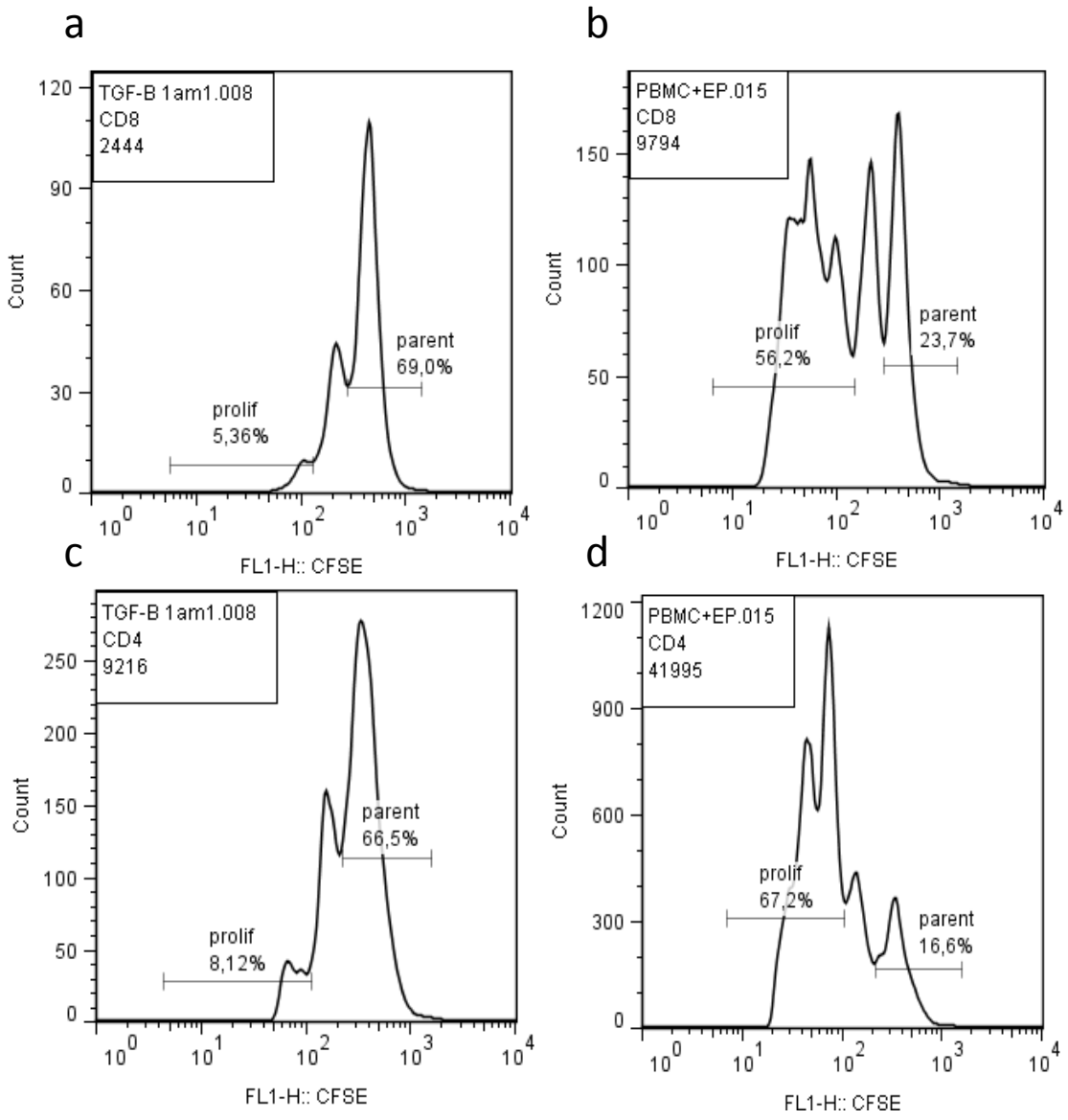

Figure 14: iT_{Regs} suppressed the proliferation of $CD4^+$ and $CD8^+$ T cells. Adult PBMC's were co-cultured with iT_{Regs} in the ratio; iT_{Reg}:PBMC, 1:1. iT_{Regs} had previously been stimulated with anti-CD3 (1 µg/mL) and cultured in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/mL) for 120 hrs. Percentages are shown for the population that has undergone most proliferations (left) and for the parent population (right). (**a**) shows the suppression of proliferation for $CD8^+$ T cells and (**b**) shows the control (without T_{Regs}). (**c**) shows the suppression of proliferation for $CD4^+$ T cells and (**d**) the control (without T_{Regs}).

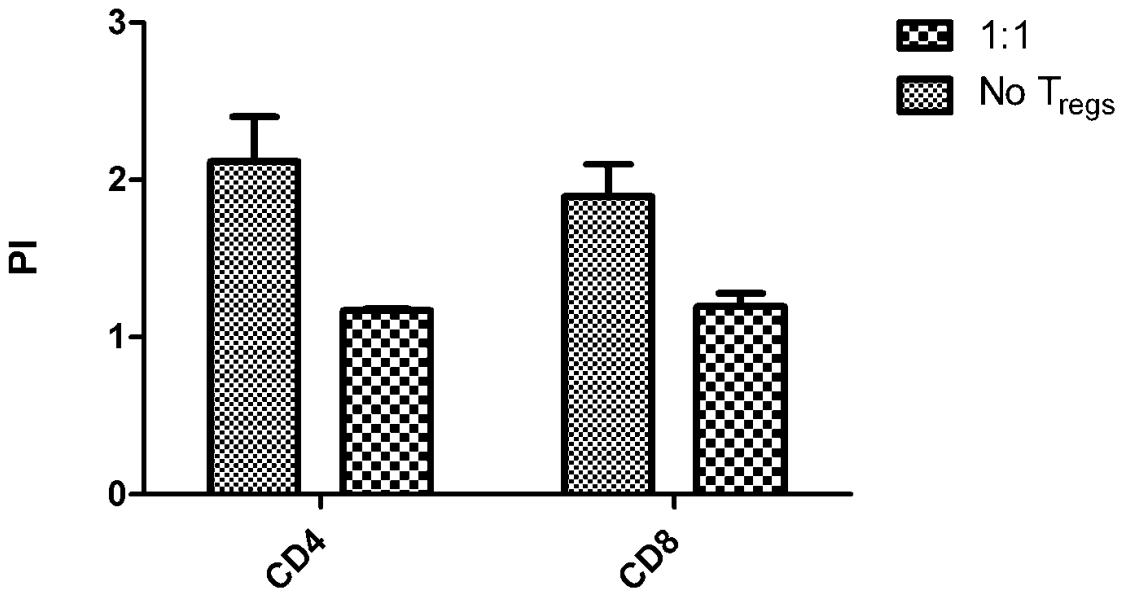

Figure 15: **iT_{Regs} suppressed the proliferation of $CD4^+$ and $CD8^+$ T cells.** PI for adult PBMC's co-cultured with iT_{Regs} in the ratio; iT_{Reg}:PBMC, 1:1. The iT_{Regs} had previously been stimulated with anti-CD3 (1 µg/mL) and cultured in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/mL) for 120 hrs, n=2.

4.3 Cytokine secretion by iT_{Regs}

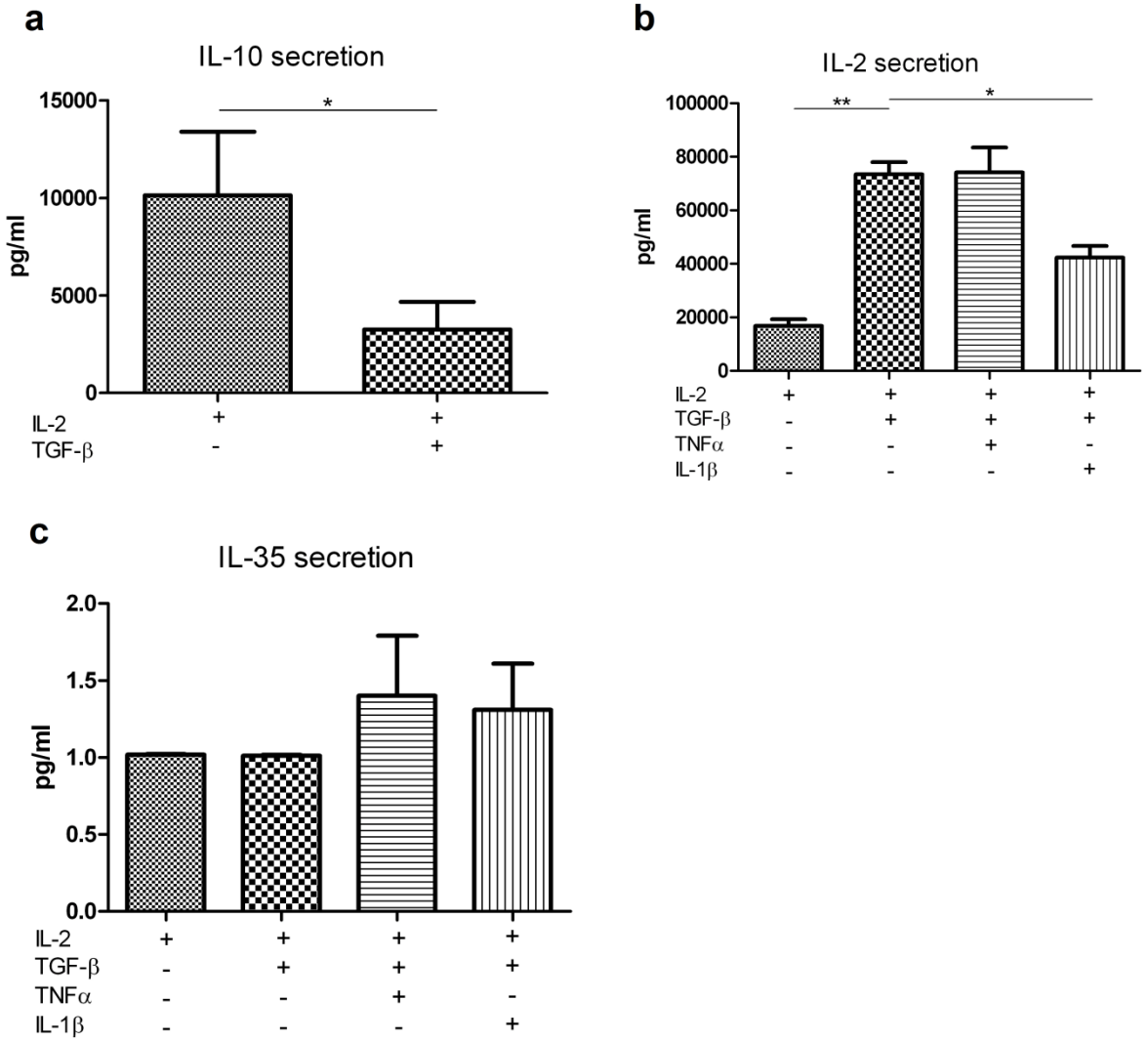

Figure 16: Cytokine secretion by iT_{Regs}. $CD4^+CD25^-$ T cells were isolated from adult human peripheral blood and activated with anti-CD3 (1 µg/mL) in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/mL). Supernatant was harvested after 120 hrs and cytokine secretion was estimated using ELISA. Mean ± SEM, n=3. *p<0.05, **p<0.001, student's *T* test.

T regulatory cells have been shown to mediate their suppressive function through IL-10 secretion. Therefore, we hypothesised that this would also be the case for our iT_{Regs}. We collected supernatant after 120 hrs culture of iT_{Regs} for determining their cytokine secretion. Our results demonstrated that the IL-10 secretion was not increased, but diminished in the presence of TGF-β1 in the culture (Figure 16 (a)).

In addition, differentiated $CD4^+$ iT_{Regs} secreted significantly higher amounts of IL-2 in the presence of TGF-β1 compared to controls. The presence of IL-1β also had a significant negative effect upon IL-2 secretion compared to TGF-β1 alone. However, we found no difference in the IL-2 secretion when TNFα was present in the culture.

Secretion of IL-35 has been suggested as a possible mediator for iT_{Reg} suppression. Although human T_{Regs} do not expressed IL-35, conventional T cells are induced to express IL-35 which enhances suppression. Therefore we measured the presence of IL-35 in the collected supernatant. We found, that negligible amounts of IL-35 were detected in the culture (Figure 16 (c)).

5 Discussion

The aim of this study was to investigate the role of pro-inflammatory cytokines on the differentiation and function of human iT_{Regs}. We have demonstrated that IL-1β and TNFα inhibit TGF-β induced T_{Regs} differentiation *in vitro*. The TGF-β induced T_{Regs} showed strong suppressive function *ex vivo* that had a positive correlation with their numbers in culture. This suppressive capacity of the iT_{Regs} was contained in the presence of IL-1β and TNFα. It was also shown that the iT_{Regs} did not mediate their suppression through secretion of IL-10 or IL-35 but possibly through IL-2 secretion.

5.1 Differentiation of iT_{Regs}

5.1.1 T cell phenotype after $CD4^+CD25^-$ isolation

After the isolation of naïve $CD4^+CD25^-$ T cell population their phenotype was determined. The reason was to gain a better understanding of the phenotypical change that the cells undergo in their differentiation towards becoming iT_{Regs}. The isolation process was successful, resulting in average 94.3% purity of $CD4^+CD25^-$ T cells. Almost 86% of the isolated cells also expressed CD127. This is not surprising as CD127 is commonly expressed by naïve T cells and is down-regulated during activation. CD45RA is a naïve T cell marker and is down-regulated by $iT_{Regs.}$ It was expressed by 60.5% of the cells. This confirms that the majority of the population consists of naïve T cells. FoxP3 expression was found to be 1.79%. This is considerably less than what has been reported, which is 5-10% of $CD4^+$ T cells. However, this is more likely due to the depletion of $CD25^+$ T cells during the isolation process. Finally, it was established that 77% of the isolated cells expressed the TGF-βRII, rendering them responsive towards TGF-β during their differentiation.

5.1.2 IL-1β and TNFα prevent iT_{Reg} differentiation

The aim was to determine the role of IL-2 and TGF-β1 in $CD4^+$ iT_{Regs} differentiation and function *in vitro*. We obtained the highest fraction of *ex vivo* differentiated $CD4^+CD127^-CD25^{high}FoxP3^{high}$ iT_{Regs} in the presence of both IL-2 and TGF-β1 as can be seen in Figure 7. As shown in Figure 6, a well-defined population of $CD4^+CD127^-CD25^{high}FoxP3^{high}$ iT_{Regs} arises in the presence of TGF-β. The iT_{Reg} stimulation triggered the expression of CD25 in more than 80% of cells. This confirms the activation of the iT_{Regs} when compared to the expression of CD25 at the start of culture. In addition, TGF-β was shown to be essential for the induction and its presence resulted in threefold expansion of the iT_{Regs} compared to IL-2 alone and tenfold increase of FoxP3 expression compared to the FoxP3 expression at the start of the culture (Figure 5 and 7). This is in accordance with results previously obtained by Gunnlaugsdottir *et al.* (161) where TGF-β1 induced the differentiation of $CD4^+CD25^+$ T cells

in human umbilical cord blood. This was further established in a more recent study for $CD4^+CD25^{hi}FoxP3^+$T cells from cord blood (162) and has also been confirmed in peripheral blood (32). The presence of IL-2 and TGF-β1 is thus crucial for differentiation of iT_{Regs} *in vitro*.

The pro-inflammatory cytokines IL-1β or TNFα were added to the culture to evaluate their role upon iT_{Reg} differentiation. Both were found to significantly inhibit the differentiation of about 70% of the population. This is in contrast to recent observations where membrane bound TNFα expressed by dendritic cells was crucial for the *in vitro* induction of human T_{Regs} (119). In contrast, others have shown that high dose TNFα (50 ng/mL) significantly inhibits the differentiation of $CD4^+FoxP3^+$ cells after 48 hrs of culture. This effect was dose-dependent as smaller doses of TNFα did not have a significant effect (163, 164). This is in accordance with our results presented here where high dose TNFα (50 ng/mL) and long term cultures inhibited iT_{Regs} differentiation. Valencia *et al.* has also reported that TNFα down-regulates the expression of FoxP3 in human $CD4^+CD25^{hi}$ T_{Regs} (117). In contrast to our results, Geirsdottir revealed that the inhibitory effects of TNFα were lost over time and were not significant after 120 hr culture. However, this study was performed on T cells isolated from cord blood and are not performed using the same cell population. The role of IL-1β signalling during iT_{Regs} differentiation is unclear, and has to our knowledge not been comprehensively studied in humans. Studies in mice have shown that IL-1β enhances expansion of $FoxP3^+$ T cells (128). On the other hand, in humans, IL-1β has been shown to negatively regulate or switch the phenotype of T_{Regs}. IL-1β and IL-2 successfully converted natural human T_{Regs} into T_H17 cells by down-regulating FoxP3 (129). We have not excluded this mechanism in our model and thus it is possible that the inhibitory effects we observe of IL-1β on the iT_{Reg} differentiation are mediated through their conversion to T_H17 cells. However, TGF-β1 is present in our culture and is driving the differentiation of the naïve T cells towards iT_{Reg} phenotype.

5.1.3 TGF-β1 induced the expression of CD103 in iT_{Regs}

Our results regarding the effects of TGF-β1 induction on the expression of CD103 showed tenfold increase in CD103 expression in the presence of IL-2 and TGF-β1 compared to IL-2 alone (Figure 8). Similar observations have been found in human cord blood (162). However, TGF-β1 was not as effective inducer of CD103 in cord blood as in peripheral blood and only approximately 6% expressed CD103 in the presence of TGF-β1 in human umbilical cord blood. The reason behind this difference is unknown considering that both cells were cultured under same stimulatory conditions. The reason could be different methods for gating or more likely, the different cell populations in adult and cord blood.

CD103 is known to be important for T cell trafficking and tissue retention (162). We have demonstrated the up-regulation of CD103 expression in iT_{Regs}, however the role of CD103 in T_{Regs} is still unclear. It is possible that the expression of CD103 on iT_{Regs} contributes to their suppressive function since CD103 expression has been linked to $CD4^+$ T_{Reg} subpopulations. This question can easily be answered by isolating CD103 positive cells before testing the suppressive capacity of the T_{Regs}. In this way, the suppressive function of $CD103^+$ and $CD103^-$ T_{Regs} can be compared and the effects of CD103 expression evaluated.

5.2 The suppressive function of *ex vivo* iT_{Regs}

5.2.1 TGF-β1 induced iT_{Regs} contain suppressive function *ex vivo*

To ensure that our iT_{Reg} population possessed suppressor capacity, we performed an *in vitro* suppression assay. As shown in Figure 9, the iT_{Regs} had a significant suppressive effect in our system resulting in a sixfold reduction in PBMC's proliferation in the presence of iT_{Regs}. A control PBMC's cultured without the presence of iT_{Regs} showed a substantial proliferative response.

The ratio of T_{Reg}:$T_{Responder}$;1:1 cells is high and is unlikely to occur *in vivo*. Therefore, we measured the suppression assay in lower ratios (1:4, 1:8, 1:16 and 1:32). This was also done to confirm that the iT_{Reg} mediated suppression is dose dependent. We observed that the suppressive function of iT_{Regs} correlated positively with their increasing numbers (Figures 11 and 12). We selected the ratios based on previous studies on T_{Reg} suppressive function (56).

Next, we evaluated if the iT_{Regs} preferentially suppressed either $CD4^+$ or $CD8^+$ T cells. As shown in Figures 14 and 15, the iT_{Regs} suppressed the proliferation of both $CD4^+$ and $CD8^+$ human T cells in our system. The ability of T_{Regs} to suppress TCR-induced proliferation of $CD4^+$ and $CD8^+$ T cells *in vitro* has previously been shown in mice (81).

Human $CD4^+CD25^-$ T cells stimulated in the presence of IL-2 and TGF-β1 have been shown before to be capable of inhibiting proliferation of stimulated naïve T cells *in vitro* (165). In this study, the responder cells were CFSE labelled and stimulated with plate-bound anti-CD3 and anti-CD28. This method has been previously used in other models for evaluating T_{Reg} suppressive function. This methodology provides means to focus specifically on the proliferation of the responder population and exclude the potential noise from other responding cells in the culture (reviewed in 158). One limitation is that CFSE at high concentrations can be toxic for cells. However when CFSE labelling is executed optimally this problem is not likely to occur. The first and most basic versions of the suppression assay involved adding ^{3}H-thymidine to a co-culture of T_{Regs} and $T_{Responder}$ cells. In these experiments the ^{3}H-thymidine is incorporated into new strands of chromosomal DNA and the degree of DNA synthesis measured (78, 166). Negative results have been obtained using this model,

where FoxP3$^+$ iT$_{Regs}$ were shown to be unable to confer a suppressor function (40). However, a system of ^{3}H-thymidine incorporation is dependent on the T$_{Regs}$ to be hypo-responsive as has been shown consistently for mouse T$_{Regs}$ (78) *in vitro* and thus the T$_{Regs}$ do not incorporate the ^{3}H-thymidine into their genome. However, in humans, naïve T$_{Regs}$ actively proliferate *in vitro* and differentiate into effector T$_{Regs}$. Thus negative results obtained from this model should be considered with caution since T$_{Regs}$ are not necessarily hypo-responsive *in vitro or in vivo.*

Despite our best efforts, our suppression assay model has a troublesome limitation. This is the failure to isolate the differentiated iT$_{Regs}$ before their co-culture. As seen in Figure 7, no more than 20% of the activated cells confer the T$_{Reg}$ phenotype. Therefore the ideal method would be to isolate these regulatory cells before evaluating their suppression function and discard the remaining cells. However, FoxP3, the only reliable marker for T$_{Regs}$ is expressed intracellular and therefore it is impossible to use as an isolation marker. The most common method is to isolate CD4$^+$CD25$^+$ cells and apply them to the functional studies. However, as seen in Figure 6, this would be futile in our case as the majority of the differentiated T cells expressed CD25 without acquiring the regulatory phenotype.

Our model for the suppression assay is different from the typical models regarding activation of the responder cells. Instead of the more common *in vitro* activation with anti-CD3 and ant-CD28, we opted for a simulation of the *in vivo* process using live APC's to induce the proliferation of the responder cells. The Epstein-Barr transformed B cells have been shown to serve as efficient APC's *in vitro* (167, 168). Strong MHC class II-dependent, but not MHC-restricted T cell proliferation has been observed in response to EB-B cells *in vitro* (169). We coated the EB-B cells with superantigens before they were co-cultured with responder cells. Superantigens circumvent the normal mechanism for T cell activation by specific peptide:MHC complexes (reviewed in 170). The superantigens act as a wedge between the TCR β chain and the MHC class II α chain resulting in a polyclonal activation of T cell populations in the absence of direct contacts between peptide and TCRs. They are thought to mimic the interaction of peptide:MHC complexes with the TCR in terms of affinity and kinetics and achieve similar end results of highly efficient T cell activation as the TCR-peptide:MHC complex (reviewed in 170). To confirm their role in the activation of the responder population, unpulsed (UP) EB-B cells were cultured with CFSE labelled PBMC's (Figure 10). The unpulsed B cells did not induce a proper proliferation of the responder cells compared to pulsed B cells. Therefore, we conclude that our suppression assay model conveyed a significant suppressive function for iT$_{Regs}$ *in vitro*. There is no direct evidence that the *in vitro* functional studies of human T$_{Reg}$ mediated suppression reflect correctly on their function *in vivo*. However, at this time human T$_{Regs}$ suppression assays are limited to the *in*

vitro co-culture system. Therefore, imitating the processes taking place *in vivo* as faithfully as possible provides us with better understanding of the process.

5.2.2 IL-1β and TNFα prevent human iT_{Reg} function *in vitro*

Seeing that IL-1β and TNFα significantly inhibited the differentiation of iT_{Reg} we set out to evaluate their effect on the iT_{Reg} suppressive function as well. Both IL-1β and TNFα were found to prevent the suppressive function of the iT_{Regs} (Figure 13).

The first indication of a role for TNFα and IL-1β in T_{Reg} function is the high expression of their receptors, IL-1RI and TNFRII, on human T_{Regs} compared to naïve or memory T cells (171) and their up-regulation during activation. This is not the first study to analyse the effects of TNFα on the suppressive capabilities of T_{Regs}. TNFα in the same dose (50 ng/mL) has previously been shown to eradicate the ability of human T_{Regs} to suppress the proliferation of T_{eff} cells (117). Exogenous TNFα was shown to inhibit the suppressive capacity of T_{Regs} by signalling through TNFRII and adding of anti-TNFRII also blocked the T_{Reg} suppressive effects. The effects of TNFα on T_{Regs} seem to be somewhat dose-dependent. This is supported by a study where small amounts of TNFα (0 and 5 ng/mL) did not affect T_{Regs} ability to inhibit proliferation of T_{eff} cells (116). However, studies in mice have revealed that TNFα in smaller doses (10 ng/mL) diminishes T_{Reg} ability to inhibit T_{eff} cell proliferation (120). Studies of IL-1β effects on T_{Regs} are harder to come by. IL-1β has been shown to convert human T_{Regs} into T_H17 cells by down-regulating FoxP3 and their suppressor functions (129, 172). In addition, IL-1β is highly unlikely to positively affect T_{Reg} function as human T_{Regs} expressing the IL-1RI did not have higher suppressive capacity compared to IL-$1RI^-$ T_{Regs}. Also activated human T_{Regs} show the capacity to neutralize IL-1β which suggests it plays a physiological role in T_{Reg} function (171).

T_{Regs} from RA patients have been shown to be present in joints but possess abnormal suppressive activity (116, 173, 174). Seeing that pro-inflammatory cytokines are also present in high amounts in the synovial joints, the T_{Regs} are exposed to these cytokines in the synovial fluid. A recent study has shown that the decreased suppressor capacity of T_{Regs} in RA patients was indeed caused by high amounts of TNFα in the joint fluid (173). Nie *et al.* show that the transcriptional activity of FoxP3 and the T_{Reg} suppressor function are regulated by TNFα-dependent dephosphorylation of the FoxP3 DNA-binding domain (173). However, as many studies, Nie *et al.* isolate T_{Regs} from the synovial fluid without distinguishing between thymus-derived natural T_{Regs} and peripheral-induced T_{Regs} (117, 173). Therefore it has not been established whether TNFα affects both populations. As a result, the findings we present here take the next step in determining specifically the effects of pro-inflammatory cytokines on the function of induced T_{Regs}.

5.3 Mechanisms involved in iT_{Regs} regulatory pathways

To determine the iT_{Reg} cytokine secretion, we collected supernatant after 120 hrs culture of iT_{Regs}. Our results demonstrated that IL-10 secretion was not increased, but diminished in the presence of TGF-β1 in the culture (Figure 16). IL-10 has long been considered to contribute to immunosuppression. $CD4^+CD25^+$ T cells from mice have been shown to produce IL-10 *in vivo* (175, 176). Considering that most $CD4^+CD25^+$ T cells in mice are functional T_{Regs} with a suppressive function, it is not surprising that IL-10 secretion has been associated with their suppressor function. Furthermore, $CD4^+CD25^+$ T cells and their secretion of IL-10 can suppress autoimmune disorders that affect the mouse gut (177, 178). However, the role of IL-10 in T_{Reg} regulation has yet to be proven in humans. On the contrary, it has been shown that their inhibition of T cell proliferation *in vitro* could not be abolished by anti-IL-10 (51). We saw a reduction of IL-10 in the presence of TGF-β1 in our iT_{Reg} culture system. This was not consistent for $CD8^+$ iT_{Regs} cultured under the same conditions (unpublished results from Una Bjarnadottir *et al.*). On the contrary, $CD8^+$ iT_{Regs} up-regulated IL-10 secretion in the presence of IL-2 and TGF-β1 compared to IL-2 alone.

Our results show that differentiated $CD4^+$ iT_{Regs} secreted significantly higher amounts of IL-2 in the presence of TGF-β1 (Figure 16). Studies have demonstrated that T_{Regs} mediate suppression by suppressing the induction of IL-2 mRNA in the responder T cell population in mice (78, 166). Likewise, IL-2 has been shown to be essential for T_{Ref} efficient suppressor function *in vitro* (81). The IL-2 production by the responder T cells is crucial for the initiation of the expansion and function of suppressive T_{Regs} (179). The possibility has been raised that T_{Regs} compete with their responder cells for IL-2 consumption, and as a result, inhibit their proliferation. This speculation is valid, seeing that $FoxP3^+$ T_{Regs} express high numbers of the IL-2 receptors and favourably compete for IL-2 which is a vital growth factor for freshly stimulated T cells *in vitro*. On the other hand, the addition of anti-human CD25 that blocks IL-2 binding has no effect on the function of human T_{Regs} (83). This study performed *in vitro* by Tran *et al* was executed in a hybrid system using T_{Regs} from human and responder cells isolated from mice. Thus its reflection on the suppressor function happening in humans *in vivo* is uncertain. Thus we conclude that our results of high IL-2 production in iT_{Reg} culture are supported by other studies. However, we also saw that the presence of IL-1β had a significant negative effect upon IL-2 secretion compared to TGF-β1 alone (Figure 16). In regard to our previous results of IL-1β negative effects on the iT_{Reg} differentiation and function; this presents to us a potential connection between IL-1β effects and IL-2 reduction and the possibility that IL-1β mediated its negative effects through IL-2 inhibition. However, TNFα did not have significant effects upon the IL-2 secretion and it is possible that the pro-inflammatory cytokines do not mediate their negative effects on iT_{Regs} through the same

mechanisms. The same results could not be obtained in $CD8^+$ iT_{Reg} culture, where the presence TGF-β1, TNFα or IL-1β showed no significant effect upon IL-2 secretion (unpublished results from Una Bjarnadottir *et al.*) presenting us with another reason for believing that $CD4^+$ and $CD8^+$ iT_{Regs} do not mediate their effects in a similar manner.

Finally, seeing that convincing evidence exists for the role of IL-35 in T_{Regs} in mice, we evaluated its secretion in the iT_{Reg} culture. We detected negligible amounts of IL-35 in the culture (Figure 16). This is consistent with results obtained by Bardel *et al.* that demonstrated that human $CD4^+CD25^+FoxP3^+$ T_{Regs} are unable to express IL-35 (55). On the other hand, a more recent study revealed a significant up-regulation of *IL12A* and *EBI3* in human T_{Regs} compared with conventional T cells. They also showed that human T_{Regs} not only express IL-35 but also require it for their suppressive function (56). However, their suppression assay is based on the previously criticised method of ^{3}H-thymidine incorporation. Also, even though their results can be regarded as noteworthy, their cell population is attained from cord blood cells and so is not comparable to our iT_{Regs}.

Our findings suggest that iT_{Reg} suppressor function is mediated through IL-2 secretion. Previous results, obtained by Geirsdottir *et al.* have also implied that the negative effects of IL-1β on FoxP3 expression may partially be caused by the reduced expression of TGFβRII and reduced TGF-β signalling. This suggests that the suppressive function of iT_{Regs} is mediated through IL-2 secretion and TGFβRII expression dependent mechanism (163). Furthermore, deficiency in IL-2 related molecules, CD25 and CD122 results in fatal autoimmune or inflammatory disease in mice (180, 181). Although, these results suggest a possible mechanism for suppression, we have yet to verify how our iT_{Regs} suppress their responder population. Further studies would involve collecting and analysing the supernatant of the suppression assay co-culture for cytokine secretion. However, in the co-culture it would be impossible to identify the cell population responsible for the secretion. Another method would be to place the co-culture in a transwell plate where T_{Regs} and $T_{responder}$ cells are separated by a membrane and thus determining if their suppressive function is dependent upon cytokine secretion or is primarily mediated through cell-contact dependent mechanism.

6 Conclusion

Our study of human induced T_{Regs} demonstrates that naïve ($CD4^+CD25^-$) T cells differentiate into $CD4^+CD127^-CD25^{high}FoxP3^{high}$ iT_{Regs} in the presence of IL-2 and TGF-β1 *in vitro*. TGF-β1 was shown to be essential for the induction and its presence resulted in threefold expansion of the iT_{Regs} compared to IL-2 alone. We wanted to detect the role of the innate immune system in the differentiation of T_{Regs}. We discovered that the pro-inflammatory cytokines TNFα and IL-1β suppressed the differentiation of iT_{Regs}. TNFα suppressed 68.3% whereas IL-1β inhibited the differentiation of 73.5% of the population.

CD103 is the alpha chain of the adhesion molecule αEβ7 integrin and is important in T_{Reg} trafficking. TGF-β1 and IL-2 induced the expression of CD103 tenfold compared to IL-2 alone. However the role of CD103 in T_{Regs} function is still unclear.

To confirm the iT_{Reg} identity as regulatory cells we performed a co-culture suppression assay. The iT_{Regs} showed strong suppressive effects on the proliferation of PBMC's. This suppression was dose-dependent as shown by positive correlation of the suppressive function and the $CD4^+$ iT_{Regs} increasing numbers. Seeing that the pro-inflammatory IL-1β and TNFα suppressed iT_{Reg} differentiation, we investigated their possible effects on the iT_{Reg} suppressive function in our model. IL-1β and TNFα were shown to significantly inhibit the iT_{Reg} suppressive function.

Finally, we set out to evaluate possible mechanism for the iT_{Reg} mediated suppressor function. Our results demonstrated that the IL-10 secretion in the iT_{Regs} culture was not increased, but diminished in the presence of TGF-β1 in the culture. In addition, differentiated $CD4^+$ iT_{Regs} secreted significantly higher amounts of IL-2 in the presence of TGF-β1 compared to controls. The presence of IL-1β also had a significant negative effect upon IL-2 secretion compared to TGF-β1 alone. However, we found no difference in the IL-2 secretion when TNFα was present in the culture. Secretion of IL-35 has been suggested as a possible mediator for iT_{Reg} suppression. However, we found negligible amounts of IL-35 in the culture. Thus we suggest that IL-2 secretion is a potential mechanism through which iT_{Reg} mediated their suppressive function *in vitro*.

T_{Reg} have an essential role in regulating immune tolerance and their reduced numbers and function have been implicated in autoimmune diseases. Then again, they also provide unwanted immune suppression in tumours. For that reason, it is necessary to understand the mechanism behind their differentiation and function. We have demonstrated for the first time the effects of IL-1β on T_{Reg} differentiation and function and further established the negative effects of TNFα. As well as increasing our knowledge of the factors involved in regulating

T_{Regs}, these results provide means for developing new treatment options for down-regulating unwanted T_{Reg} differentiation and function.

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Appendix

TNFα and IL-1β have dose dependent effects on differentiation of $CD4^+CD25^{high}CD127^-FoxP3^{high}$ T cells

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Summary sentence: TNFα and IL-1β regulate the IL-2 and TGF-β1 mediated differentiation and suppressive function of human $CD4^+$ iT_{Regs} in a dose and time dependent manner.

Running title: Proinflammatory cytokines, TGFβ1 and T cell differentiation

Key words: T_{Regs}, TGFβ1, IL-2, IL-10

Peripherally induced $CD4^+CD25^{high}CD127^-FoxP3^{high}$ regulatory T cells (iT_{Regs}) are critical for the maintenance of balanced human immune responses. The innate immune system plays a significant role in the pathogenesis of autoimmune diseases. However, its role in the differentiation and function of human $CD4^+$ iT_{Regs} is currently unclear.

In this study we investigated the effect of the proinflammatory cytokines TNFα and IL-1β upon the differentiation and function of human $CD4^+$ iT_{Regs}.

Naïve human $CD4^+CD25^-$ T cells, isolated from neonatal cord blood and healthy adults, were cultured under various stimulatory conditions with/without IL-1β and TNFα. The iT_{Regs} were characterised as $CD4^+/CD25^{high}/CD127^-/FoxP3^{high}$ and their suppressive function evaluated.

TNFα and IL-1β significantly affected the differentiation of human $CD4^+$ iT_{Regs} in a dose and time dependent manner. Low dose and short term conditions promoted their differentiation while high dose and long term conditions were inhibitory. In addition, suppressive function of the $CD4^+$ iT_{Regs} was inhibited by TNFα and IL-1β. This inhibitory effect of TNFα and IL-1β was associated with a significant reduction of the TβRII expression and IL-2 secretion. However, their IL-10 secretion was not affected and IL-35 secretion was not observed in any of the culture conditions tested.

We conclude that the *ex vivo* induction of human $CD4^+$ iT_{Regs} is mediated through TβRII and IL-2 dependent mechanism. The regulatory effect of IL-1β and TNFα on iT_{Reg}

differentiation is dynamic and may to some extent reflect the magnitude and persistence of the initial proinflammatory response.

Introduction

Regulatory T cells (T_{Regs}) are vital for immune suppression and crucial for controlling inflammatory responses, establishing self-tolerance and maintaining immune homeostasis (183).

$CD4^+$ T_{Regs} are divided into two major classes; naturally occurring T_{Regs} (nT_{Regs}) and induced T_{Regs} (iT_{Regs}) that are phenotypically indistinguishable but may differ in functionality and differentiation (184, 185).

Inducible T_{Regs} develop in the periphery from $CD4^+CD25^-$ T cells in the presence of TGF-β1 (34, 186) and IL-2 (77). They are essential for maintaining an effective peripheral immune tolerance and are characterized by high surface expression of the interleukin (IL)-2 α receptor chain (CD25), low expression of interleukin (IL)-7 receptor α (CD127) and stable expression of the transcription factor, forkhead-box protein P3 (FoxP3) (187, 188). FoxP3 has been used as the most specific T_{Regs} marker and is required for normal T_{Regs} function and differentiation (189). Thus, prolonged FoxP3 expression is known to be required to define T_{Regs} indisputably (190). It is well documented that TGF-β1 is essential for T cell homeostasis. This has been established in TGF-β1 knockout mice, which develop multifocal inflammatory autoimmune diseases (191). Furthermore, TGF-β (38, 192), prostaglandin E2 (193) and retinoic acid (194, 195) are the only recognized inducers of FoxP3. Thus, TGF-β1 has a great therapeutic potential, both as the generator of T_{Regs} (196) and possibly also as the main mediator of their immunosuppressive activity (68). Our results have shown that T cells are more responsive towards TGF-β1 mediated suppression during suboptimal stimulatory conditions (161). Accordingly, autoreactive T cells that drive chronic inflammatory responses in autoimmune diseases are probably highly stimulated and therefore unresponsive towards the immune-modifying effects of TGF-β1.

Tumor necrosis factor alpha (TNFα) is a proinflammatory cytokine with a capacity to induce apoptosis. Furthermore, it plays an important role in different biological processes including the induction of other cytokines. TNFα is a key factor in numerous inflammatory diseases. The success of anti-TNF biologicals for the treatment of rheumatoid arthritis (RA) (197), Crohn´s disease and other chronic inflammatory (reviewed in (198)) conditions highlights TNFα as a key mediator of inflammation (199). Membrane bound-TNFα on T_{Regs} has been linked to severe RA disease activity (118) and it has been reported that neutralization of TNFα restored T_{Reg} function and numbers (200). Paradoxically, some researchers have also

shown that TNFα regulates the function and numbers of T_{Regs} (120). Recent study has additionally shown that T_{Reg} in mice and humans are able to shed massive amounts of the TNF receptor type II after few days of activation (199). This can possibly explain the contradictory findings regarding the effect of TNFα on T_{Regs} as described above. However, most studies agree that TNFα plays an important role in T_{Reg} activity, although the exact mechanism is currently unclear.

IL-1β is a proinflammatory cytokine, synthesised as a precursor molecule (pro-IL-1β) by many different cell types (reviewed in ref. (201)). It is involved in the pathogenesis of inflammatory diseases and a number of autoimmune diseases are associated with high levels of IL-1β (125, 126). Additionally, it is an important contributor to the polarization of Th17 cells (129, 173). Despite its possible role in T_{Reg} differentiation in mice (202), the effect of IL-1β on human T_{Regs} remains to be explored. Given the protective role of the proinflammatory cytokines TNFα and IL-1β during infection, and the fact that both cytokines have been reported to antagonise TGF-β1, we hypothesised that these cytokines affect the IL-2 and TGF-β1 mediated induction of iT_{Regs}. Therefore, the aim of the study was to evaluate how stimulatory conditions and proinflammatory cytokines influence the ability of naive human $CD4^+CD25^-$ T cells to differentiate into functional iT_{Regs} ($CD4^+CD25^{high}CD127^-FoxP3^{high}$) from naive T cells.

Materials and Methods

Study Material

Cord blood was obtained after normal deliveries at the Department of Obstetrics & Genecology at Landspitali, University Hospital of Iceland. Mothers with autoimmune disorders were excluded from the study. Adult venous peripheral blood was collected from healthy volunteers. The study was approved by The Ethics Committee of Landspitali University Hospital and The Data Protection Authority.

Isolation of mononuclear cells from blood

Mononuclear cells (obtained from healthy blood donors or from cord blood) were isolated from heparinised blood by density gradient centrifugation over Ficoll-Hypaque (Sigma-Aldrich) at room temperature for 30 minutes. Cord blood was centrifuged at 1200 g and peripheral blood at 450 g.

Isolation of $CD4^+CD25^-$ T cells

$CD4^+$ T cells were isolated with Dynabeads CD4 (Dynal, Invitrogen) according to the manufactures instructions. $CD25^+$ T cells were depleted from the $CD4^+$ T cell population using Dynabeads CD25 (Dynal, Invitrogen). The purity of the isolated $CD4^+CD25^-$ T cells was consistently >95% and their phenotypes, determined by flow cytometry after staining for the surface markers, are listed in table 1.

Table 1: Phenotypic characterisation of $CD4^+CD25^-$ T cells after isolation.

	CD103	CD25	CD28	FoxP3	TβRII	CD127	CD45RA	CD45RO
N (number of analysis)	5.00	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Mean (% of positive cells)	4.22	0.05	85.88	1.89	12.10	0.06	81.60	8.60
Std. Deviation	5.01	0.05	12.49	1.59	4.62	0.06	5.45	15.09
Std. Error	2.24	0.02	6.25	0.71	2.07	0.03	2.34	7.00

T cell cultures

The $CD4^+CD25^-$ T cells were cultured in a serum free medium (AimV, Nunc, Invitrogen) in 96 well U-bottomed cell culture plates (Nunc, Invitrogen) and incubated at 37°C under a 5% CO_2-95% air atmosphere.

Induction of T_{Regs}

T cells ($CD4^+CD25^-$) were stimulated with 1 µg/mL plate-bound anti-CD3ε monoclonal antibody (UCHT) with or without 2 µg/mL soluble anti-CD28 for 24 - 144 hrs in the presence of IL-2 (100 IU) and TGF-β1 (10 ng/mL). TNFα (0.5-50 ng/mL), anti-TNFα (Infliximab, 10µg/mL) and IL-1β (0.1-10 µg/mL) were added into selected cultures (antibodies and

cytokines used are from R&D Systems Inc). Anti-TNFα (Infliximab) was provided by the Department of Rheumatology at Landspitali, University Hospital of Iceland.

Suppression assay

The $CD4^+CD25^-$ T cells were stimulated under iT_{Reg} inducing conditions (anti-CD3 (1 µg/mL), IL-2 (100 IU), TGF-β1 (10 ng/mL)) for 5 days. The cells (termed iT_{Regs} for simplicity) were harvested and co-cultured with CFSE labelled allogeneic PBMC's and Epstein-Barr transformed B cells (EB-B cells) in AimV. The EB-B cells had previously been exposed to superantigens (Staphylococcal enterotoxins, SEA, SEB and SEE, Toxin Technologies, 1 µg/mL of each) for 2 hrs and washed 3 times in PBS. The ratio between PBMC: EB-B cells and between iT_{Regs} and EB-B cells was constant at 10:1 whereas the ratio of iT_{Regs}: PBMCs varied from 1:1 to 1:32. The results were analysed with Modfit LT, which determined the proliferation index as the sum of the cells in all generations divided by the computed number of original parent cells theoretically present at the start of the experiment.

Definition of iT_{Regs}

$CD4^+CD25^{high}CD127^-FoxP3^{high}$ T cells were defined as iT_{Regs}. The gating strategy is shown in figure 1.

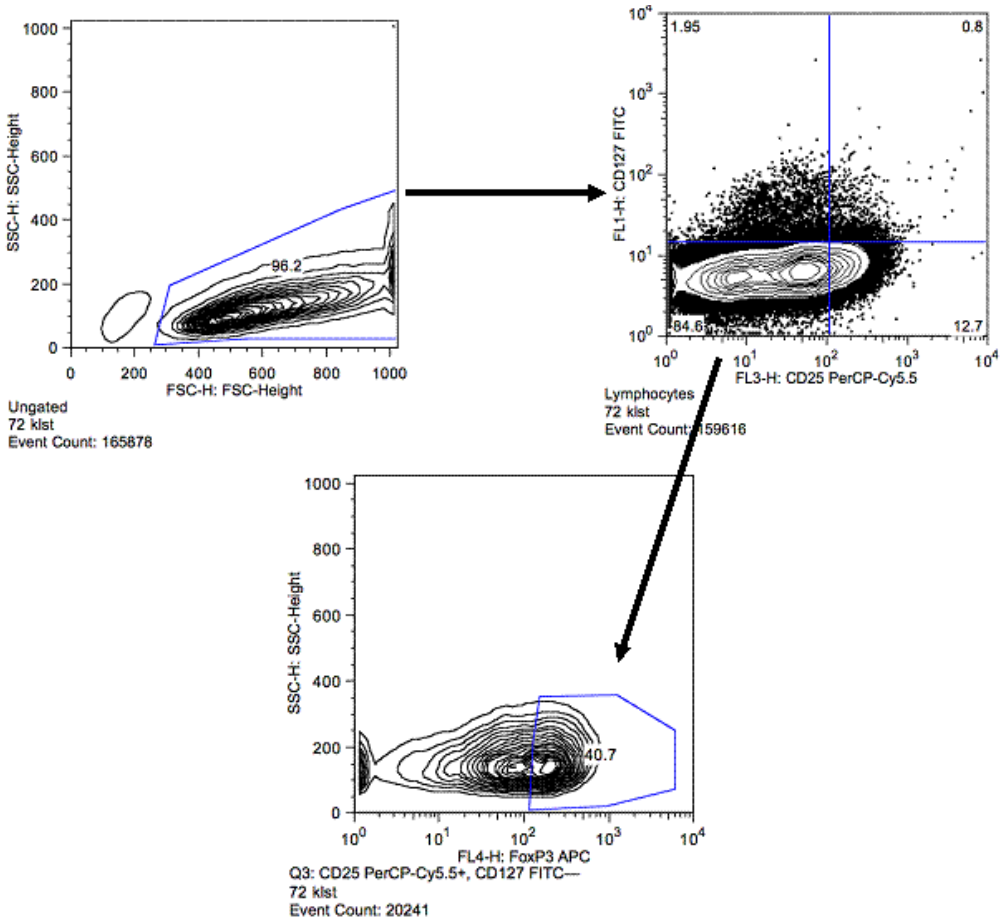

Figure 1: Gating strategy for iT_{Reg} phenotyping. Lymphocytes were gated using forward and side scatter measurements. iT_{Reg} phenotype was further established with $CD127^{-}CD25^{+}$ gating and finally $FoxP3^{high}$ cells were selected as depicted.

Surface markers

The cells were washed with staining buffer (PBS with 2 mM EDTA, 0.5% BSA and 0.1% sodium azide) and incubated for 20 min at 4°C with fluorescent antibodies. After staining the samples were washed and re-suspended in staining buffer. The cells were either fixed in PBS containing 0.5% formalin and stored for up to a week at 4°C or subjected immediately to FACS. A total of 100,000 events were collected in the lymphocyte gate and the data analyzed using either CellQuest (BD Biosciences) or FlowJo (Tree Star Software).

Antibodies and reagents

The following FITC, PE, PerCP, PerCP-Cy5.5 and APC conjugated human antibodies were used for flow cytometric analysis; CD4 (RPA-T4), CD25 (BC96), CD103 (Ber-ACT8), CD45RA (HI100), CD45RO (UCHL1), TβRII (polyclonal goat anti-human), TNFRI (16803), TNFRII (25508), CD127 (eBioRDR5) and FoxP3 (236A/E7). Appropriate isotype controls

were used to set the quadrants and to evaluate background staining. The antibodies were purchased from BD Biosciences, R&D Systems, eBiosciences and Biolegend. Antibodies used for stimulation; anti-CD3 (UCHT1) and anti-CD28 (37407) were purchased from R&D Systems. Recombinant human TGF-β1, IL-2, IL-1β and TNFα were purchased from R&D Systems. SEA, SEB and SEE were purchased from Toxin Technology (Sarasota, FL).

Intracellular straining

The cells were stained for FoxP3 expression as described above. They were fixed and permeabilized with FoxP3 staining set (eBiosciences) and stained with a FoxP3 specific antibody (clone 236A/E7, eBiosciences) or matched isotype control according to the manufacturer's instructions. The cells were analyzed immediately after staining.

Proliferation assay

To assess T cell proliferation, $CD4^+$ T cells were labelled with carboxyfluorescein diacetate succinimidyl ester (CFSE, Invitrogen) prior to stimulation. After washing the cells twice in 10 mL PBS, they were incubated with 0.5 mM CFSE in 1 ml PBS per 10^7 cells at RT for 8 min. To stop the staining reaction, 8 mL FCS were added. The cells were then washed twice in 10 mL AimV medium containing 5% FCS and resuspended in the appropriate volume of medium. The $CD4^+$ T cell proliferation was assessed by flow cytometric analysis of the CFSE dilution.

ELISA

After incubation the cell cultures were centrifuged and cell culture supernatants collected and stored at -80°C. Total TNFα, sTNFRII and IL-10 were measured in the supernatants using Duo Set ELISA kits (DY 210 and DY 726, R&D Systems). IL-2 and IL-35 were measured using Human IL-2 ELISA Set (BD Biosciences) and ELISA Ready-Set-Go! (eBioscience).

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 for windows. Paired student's t-test was used and results expressed as mean values ± standard error of the mean (SEM). For non-parametric t-test the results are expressed as medians and error bars as 25% percentile and 75% percentile. Differences were determined to be significant when $p<0.05$ (two-tailed).

Results

I. FoxP3 expression is increased in response to T cell activation

It is well documented that more naïve T cells are found in cord blood than adult blood due to the limited exposure of cord blood T cells to exogenous antigens. Therefore, the induction of FoxP3 expression was initially evaluated in $CD4^+CD25^-$ T cells isolated from umbilical cord blood. The highest yield of differentiated $CD4^+CD25^{high}CD127^-FoxP3^+$ T cells was obtained when the cells were stimulated with high dose anti-CD3 (10 µg/mL) and anti-CD28 in the presence of TGF-β1 and IL-2 for 72 hrs (84%±4.3, figure 2a). In contrast, low intensity TCR stimulation (anti-CD3=1 µg/mL) with CD28 co-stimulation was not as strong inducer of $CD4^+$ iT_{Regs} differentiation (29.5% reduction, $p<0.001$; Figure 2a). Thus, during our next set of experiments the low dose stimulation model was used to further evaluate possible additional effect of proinflammatory cytokines upon the differentiation of iT_{Regs}. Comparison of $CD4^+CD25^{high}FoxP3^{low}$ ($nonT_{Regs}$) versus the $CD4^+CD25^{high}FoxP3^{high}$ (iT_{Regs}) T cells showed that only the iT_{Regs} population that expanded in the presence of TGF-β1 (% iT_{Regs}: 4.6%±2.7 iT_{Regs} without TGF-β1 vs. 26%±6.8 with TGF-β1; $p<0.001$: Figure 2b). Therefore, the main focus was on the $CD4^+CD25^{high}CD127^-FoxP3^{high}$ population in our remaining studies and they will be defined as $CD4^+FoxP3^+$ cells from now on.

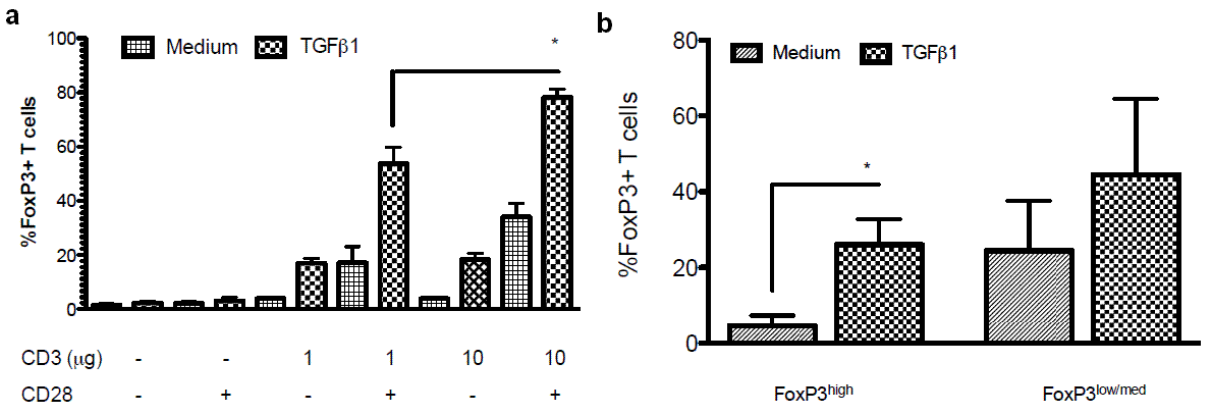

Figure 2: **FoxP3 expression in human umbilical cord blood.** (**a**) Isolated $CD4^+CD25^-$ (1×10^6 cells/mL) from healthy human cord blood were stimulated with or without anti-CD3 (1 and 10 µg/mL), anti-CD28 (1 µg/mL) for 72 hrs. The cells were cultured with IL-2 (100 IU) and either with or without TGF-β1 (10 ng/mL). Mean ± SEM, n=4. *$p<0.05$, paired *t* test. (**b**) Purified $CD4^+CD25^-$ T cells from cord blood were stimulated for 72 hrs and the expression of FoxP3 was evaluated with flow cytometry and gated into $CD4^+CD25^{hi}CD127^-FoxP3^{hi}$ vs. $CD4^+CD25^{hi}CD127^-FoxP3^{low/med}$ according to their fluorescent staining intensity. Mean ± SEM, n=4. *$p<0.001$, paired *t* test.

II. The dual effect of TNFα upon FoxP3 expression

TGF-β1 has preferentially a positive effect upon the differentiation of iT_{Regs} and TNFRII expression has been associated with nT_{Regs}. Therefore, we examined the effect of TNFα upon iT_{Reg} differentiation. As shown in figure 3, low dose TNFα had a significant positive effect upon iT_{Regs} differentiation of $CD4^+CD25^-$ T cells compared with TGF-β1 alone after 72 hrs of *ex vivo* stimulation (54% increase; $p<0.05$). Furthermore, TNFα could not be detected in the culture supernatants in the absence of exogenously added TNFα (up to 72 hrs of culture, data not shown). To evaluate if the magnitude of proinflammatory responses would influence iT_{Reg} differentiation, $CD4^+CD25^-$ T cells were cultured with higher TNFα concentration. In contrast to short term (72 hrs) and low dose TNFα (0.5 ng/ml), short term (48 hrs) stimulation and high TNFα dose (50 ng/mL) had a negative effect on iT_{Regs} differentiation (data not shown). Since CB T cells are immature and known to differ in several ways from their adult counterparts, we also evaluated the effect of long term (120 hrs) high (50 ng/mL) and low (0.5 ng/ml) dose TNFα on adult PB $CD4^+CD25^-$ T cells. Interestingly, the prolonged exposure to a high dose of TNFα significantly inhibited the differentiation of adult PB $CD4^+iT_{Regs}$ ($p<0.05$, figure 3b). However, low dose TNFα did not have negative effects on the differentiation of iT_{Reg} in adult PB (data not shown).

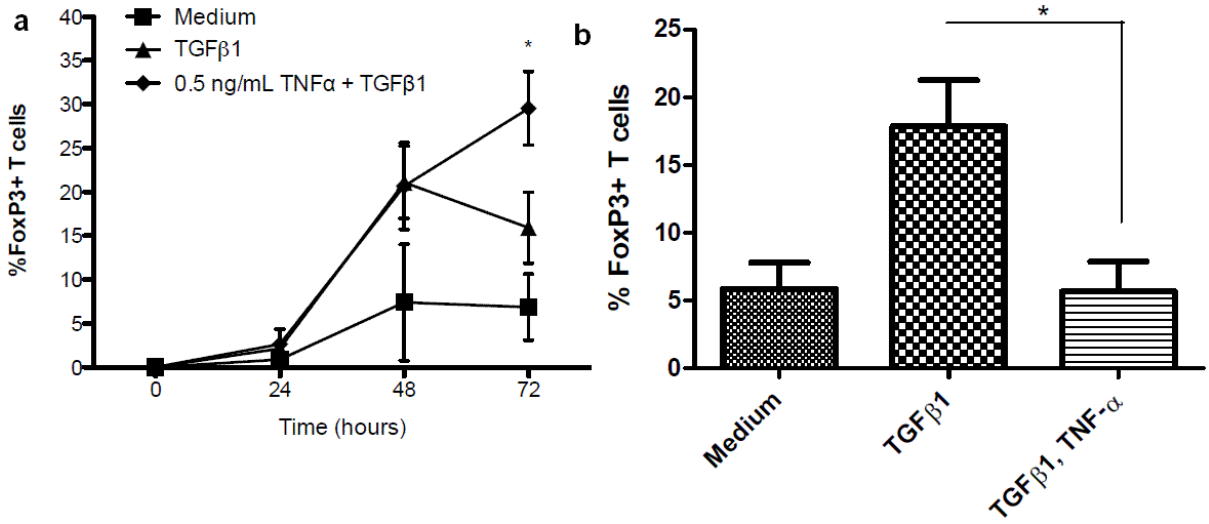

Figure 3: The effect of TNFα on FoxP3 expression. (**a**) Neonatal CB $CD4^+CD25^-$ T cells were isolated and activated with anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) for up to 72 hrs in the presence of IL-2 (medium) with or without TGF-β1 (10 ng/mL) and TNFα (0.5 ng/mL) as shown. Mean ± SEM, n=6. TGF-β1 vs. TGF-β1 + TNFα, *$p<0.05$, paired *t* test. (**b**) Adult PB $CD4^+CD25^-$ T cells were isolated and activated with anti-CD3 (10 µg/mL) for 120 hrs in the presence of IL-2 (100 IU), medium and TGF-β1 (10 ng/mL) with and without TNFα (50 ng/mL). Mean ± SEM, n=5. *$p<0.05$, Mann Whitney *U* test.

III. High consumption of TNFα correlated with upregulation of TNFRII bound receptor

Naive CB $CD4^+CD25^-$ T cells do not express TNFRII. However, when stimulated with anti-CD3/anti-CD28 and IL-2, TNFRII receptor expression is up-regulated after 48 hrs of culture (data not shown). In order to estimate the consumption of TNFα by the T cells, the TNFα in the culture supernatant was measured. High consumption of TNFα was detected in the cultures that reached its maximum effect at 72 hrs. Thus, high dose of exogenously added TNFα (50 ng/mL) had mostly been consumed from the culture medium within 72 hrs (figure 4). The rate of disappearance of TNFα in culture medium correlated negatively with high TNFRII expression per cell (r=-0.759; p=0.002; figure 4). Furthermore, during *ex vivo* induction of iT_{Regs} from $CD4^+CD25^-$ T cells TNFα was negatively correlated with the expression of cell bound TNFRII.

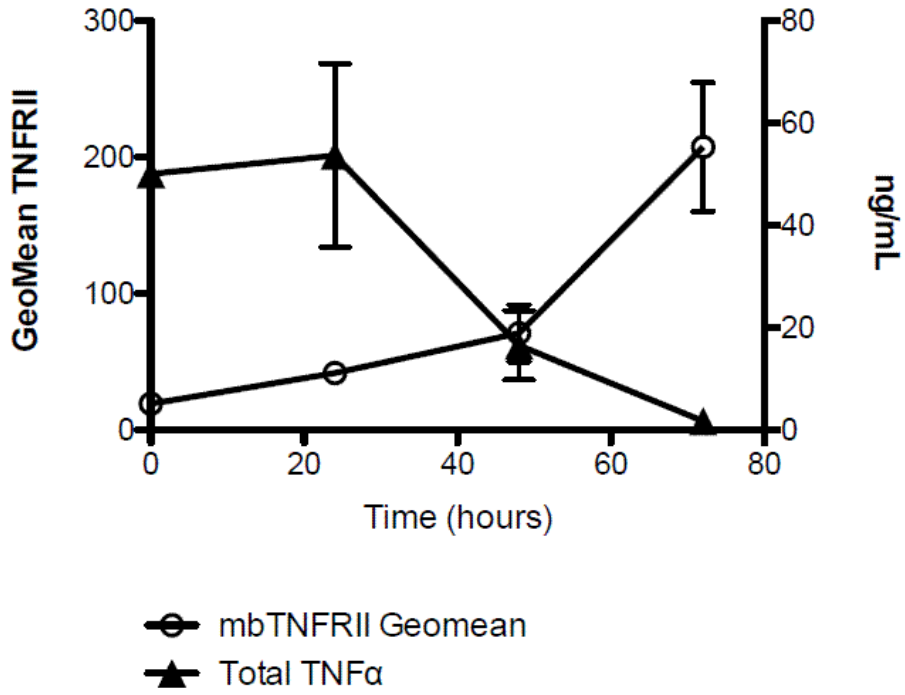

Figure 4: High consumption of TNFα correlated with TNFII expression. Isolated $CD4^+CD25^-$ T cells were stimulated with anti-CD3 (1 µg/mL)/anti-CD28 (1 µg/mL) in the presence of TGF-β1 (10 ng/mL) and TNFα (50 ng/mL). Right y-axis shows total TNFα (ng/mL) measured in culture medium at given time points. Left y-axis shows the intensity of the expressed TNFRII geometrical mean fluorescence intensity) evaluated by flow cytometry. Mean ± SEM.

IV. The effects of IL-1β upon FoxP3 expression

The effect of IL-1β on the differentiation of CB $CD4^+CD25^-$ T cells into $CD4^+FoxP3^+$ T cells was also evaluated. Short term exposure to low dose of IL-1β (0.1 ng/mL) induced a twofold increase of differentiated iT_{Regs} compared to TGF-β1 alone (figure 5, $p<0.05$). In contrast, long term (120 hrs) and high dose of IL-1β (10 ng/mL), significantly decreased the differentiation of iT_{Regs} (73.5% reduction, $p<0.05$; figure 5b). Adult PB CD4+$CD25^-$ T cells responded similarly to prolonged exposure to high dose of IL-1β. As shown in figure 5c, IL-1β significantly reduced the proportion of iT_{Regs} induced from adult T cells. In addition, low dose of IL-1β had similarly positive effects upon iT_{Reg} differentiation in adult PB (data not shown).

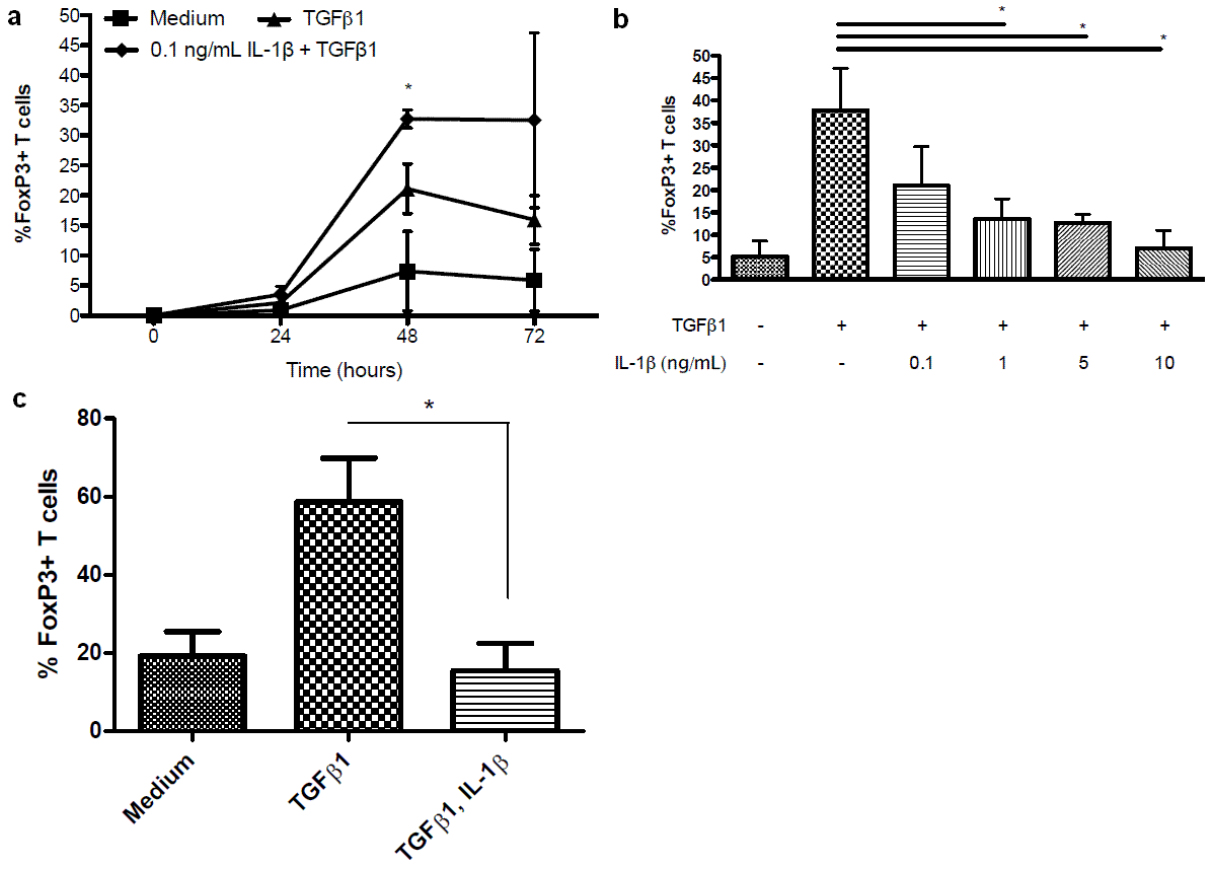

Figure 5: The effect of IL-1β on FoxP3 expression. (**a**) Neonatal CB $CD4^+CD25^-$ T cells were isolated and activated with anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) for up to 72 hrs in the presence of IL-2 (100 IU, medium) with or without TGF-β1 (10 ng/mL) and IL-1β (0.1 µg/mL) as indicated. Mean ± SEM, n=3. TGF-β1 vs. TGF-β1 + IL-1β. *$p<0.05$ (**b**) CB $CD4^+CD25^-$ T cells were isolated and activated with anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) for 120 hrs in the presence of IL-2 (100 IU, medium), with or without TGF-β1 (10 ng/mL) and increasing doses of IL-1β as shown. Mean ± SEM, n=3. TGF-β1 vs. TGF-β1 + IL-1β. *$p<0.05$. (**c**) Adult PB $CD4^+CD25^-$ T cells were isolated and activated with anti-CD3(1 µg/mL) for 120 hrs in the presence of IL-2 (100 IU, medium), with or without TGF-β1 (10 ng/mL), with or without IL-1β (10 ng/mL). Mean ± SEM, n=5. *$p<0.05$.

V. IL-1β inhibits TβRII expression

We next evaluated the effect of IL-1β upon TβRII expression. As shown in figure 6, high dose of IL-1β significantly inhibited the expression of TβRII on $CD4^+FoxP3^+$ T cells following long term (120 hrs) stimulation (46 ± 3.6% reduction, $p<0.05$) compared with cells cultured with TGF-β1 alone.

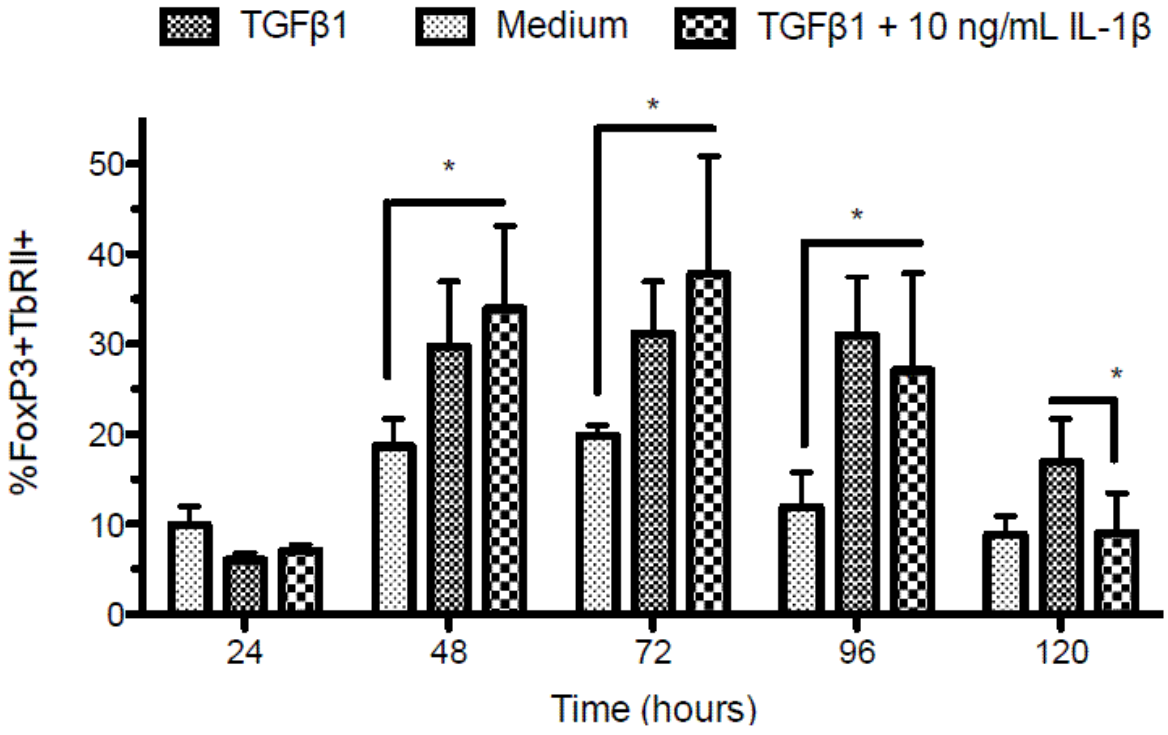

Figure 6: The effect of high dose of IL-1β upon TβRII expression on $CD4^+FoxP3^+$ T cells. CB $CD4^+CD25^-$ T cells were isolated and activated with anti-CD3 (1 µg/mL) and anti-CD28 (1 µg/mL) for up to 120 hrs in the presence of IL-2 (100 IU, medium). TGF-β1 and IL-1β (10 ng/mL) were added into selected cultures as shown. Mean ± SEM, n=3. TGF-β1 vs. TGF-β1 + IL-1β. *$p<0.05$.

VI. Both IL-1β and TNFα inhibited the suppressive function of iT_{Regs}

The functionality of the iT_{Regs} was evaluated using a previously established superantigen driven culture system (185). As shown in figure 7, the iT_{Regs} had a significant suppressive effect resulting in a six fold reduction in PBMC's proliferation in the presence of iT_{Regs} (1:1 ratio, $p<0.05$). Furthermore, both IL-1β and TNFα were found to reduce suppressive function after long term and high dose induction (figure 7d).

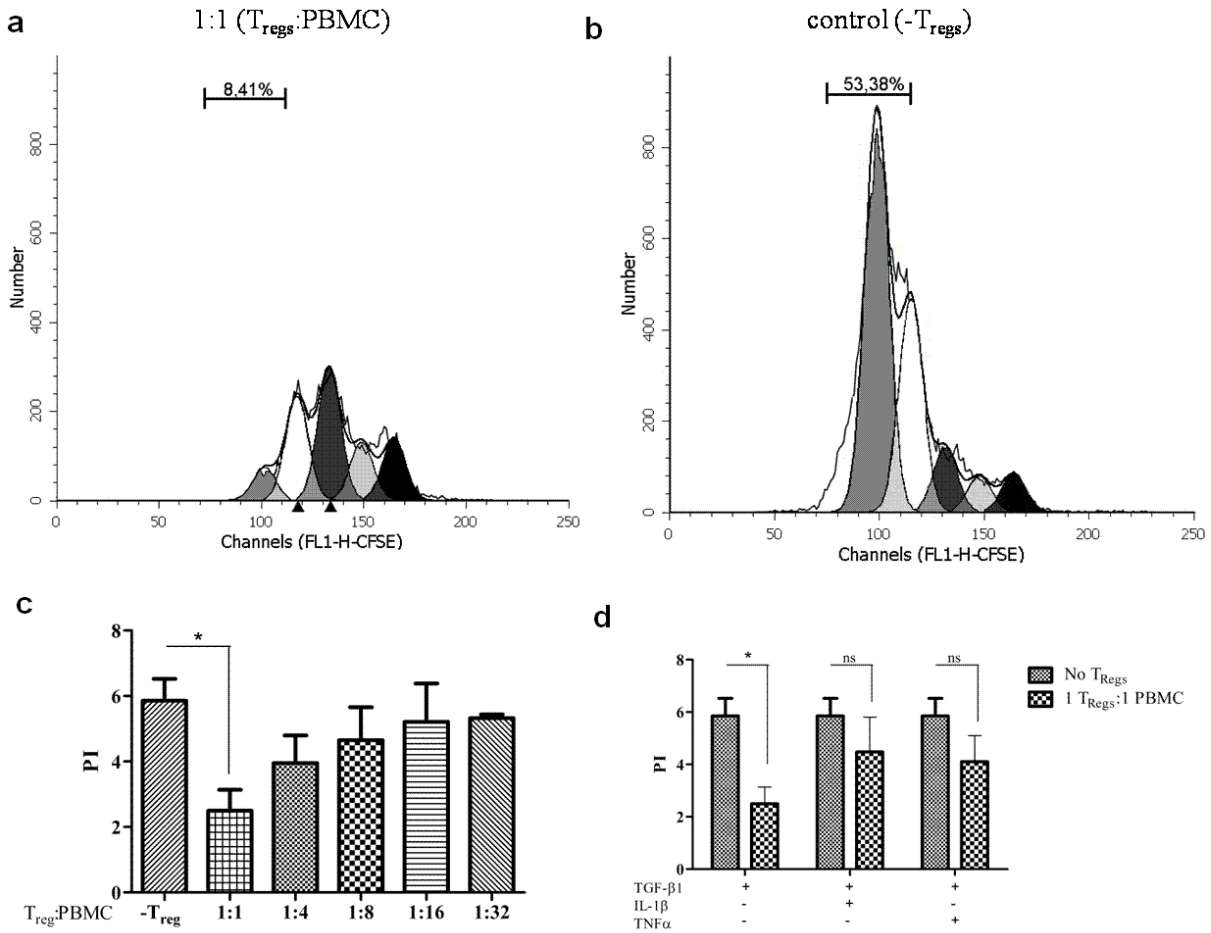

Figure 7: TGF-β1 induced T_{Regs} show suppressive function. (**a-b**) Proliferative response of PBMC's cocultured with $T_{Regs,}$ in the ratio 1:1 versus control (-T_{Regs}), was assessed by CFSE assay. EB-B cells pulsed with superantigens were cultured with the PBMC's (1:10) and T_{Regs}. CFSE labelled cells were acquired by BD FACSCalibur, cells were gated on $CD25^+$ lymphocytes and percentages of cells in each generation were calculated by Modfit LT software. Parent population is located furthest to the right and the population farthest on the left has undergone most proliferations. (**c**) Proliferation index (PI) for the PBMC's co-culture with T_{Regs} in the following ratios; T_{Reg}:PBMC, 1:1, 1:4, 1:8, 1:16, 1:32, calculated by Modfit. Mean ± SEM, n=3. *p<0.05, One way Anova. (**d**) PI for adult PBMC's cocultured with T_{Regs} in the ratio; T_{Reg}:PBMC, 1:1. The T_{Regs} had previously been cultured in the presence of IL-2 (100 IU), IL-2 and TGF-β1 (10 ng/mL), IL-2, TGF-β1 and IL-1β (10 ng/mL) or IL-2, TGF-β1 and TNFα (50 ng/mL) for 120 hrs. Mean ± SEM, n=4. *p<0.05, Mann Whitney *U* test.

VII. Cytokine secretion by iT_{Regs}

The cytokine secretion of iT_{Regs} was evaluated after five days culture. As shown in figure 8, differentiated $CD4^+$ iT_{Regs} secreted significantly lower amounts of IL-10 compared to controls (figure 8). However, they secreted significantly more IL-2 in the presence of TGF-β. IL-1β but not TNFα had significant effects upon IL-2 secretion compared to TGF-β alone. Negligible amounts of IL-35 were detected at all culture conditions tested.

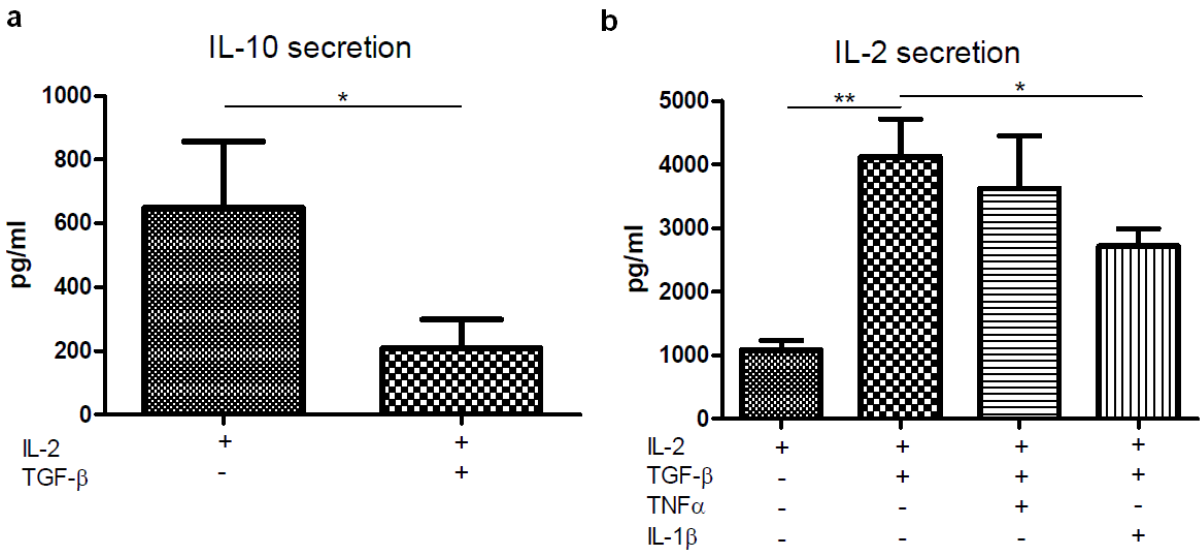

Figure 8: IL-2 and IL-10 secretion by iT_{Regs}. (**a-b**) PB $CD4^{+}CD25^{-}$ T cells were isolated and activated with anti-CD3 (1 µg/mL) in the presence of IL-2 (100 IU), medium and TGF-β1 (10 ng/ml) and with/without IL-1β and TNFα. Supernatant was harvested after 120 hrs and cytokine secretion was estimated with ELISA. Mean ± SEM, n=3. *p<0.05, **p<0.001**,** paired *t* test.

Discussion

In this study we have shown that the proinflammatory cytokines TNFα and IL-1β regulate the IL-2 and TGF-β1 mediated differentiation of human $CD4^+$ iT_{Regs}. The regulatory effects were found to be time and dose dependent. We also demonstrate that adult human $CD4^+CD25^-$ T cells stimulated in the presence of IL-2 and TGF-β1 acquired a regulatory function that was lost if high doses of either TNFα or IL-1β were present during the induction phase. Finally, our results suggest that this could be driven through TβRII expression via IL-2 dependent, but IL-10 and IL-35 independent mechanism.

TNFα has been recognized as a key inducer of pathological immune responses in RA and several other autoimmune disorders. The efficacy of anti-TNFα treatment in RA has been associated with restored numbers and function of T_{Regs} (107, 115). In addition, we have previously demonstrated that the anti-inflammatory effect of anti-TNFα antibody (Infliximab) is in part mediated through TGF-β1 dependent mechanism that is antagonised through TNFα. However, a significant proportion of RA patients do not respond to this treatment and the cases of adverse reaction to this treatment indicate that TNFα is not consistently associated with a reduced numbers or function of T_{Regs} (114). Moreover, the induction of a lupus like syndrome in a few cases suggests that TNFα may promote self tolerance in selected individuals (114). These findings are supported by recent human and murine studies showing that TNFα can either positively or negatively affect T_{Reg} induction and function. Kleijwigt *et al.* demonstrated positive effects of TNFα on the induction of human T_{Regs} *in vitro* (119). They observed that a membrane bound TNFα expressed by dendritic cells (DC's) was critical for the *in vitro* induction of human T_{Regs}. In contrast, Nadkarni *et al.* and Nagar *et al.* have reported that TNFα negatively affects the induction of T_{Regs} *in vitro* (107, 121). Similarly, results regarding the effect of TNFα on T_{Regs} have varied. For example, Valencia *et al.* demonstrated that TNFα negatively affected FoxP3 expression and suppressive function of human T_{Regs} *in vitro* (200). In contrast, several studies suggest that TNFα promotes the function of T_{Regs}. For instance, Chen *et al.* reported that TNFα expanded and enhanced the suppressive function of murine T_{Regs} (120), a process dependent on TNFRII. Furthermore, Grindberg-Bleyer *et al.* demonstrated that TNFα secreted by pathogenic T effector cells enhanced the tolerogenic potential of T_{Regs} that were co-transferred into mice, suffering from autoimmune diabetes (203).

In light of these findings our results are of particular interest as they underline the importance of the dose of the exogenously added TNFα. Thus, in our study, a low dose of

TNFα (0.5 ng/mL) promoted the induction of $CD4^+CD25^{high}CD127^-FoxP3^{high}$, whereas, a high dose (50 ng/mL) reduced their induction. Furthermore, we observed that its positive effect was time-dependent and only present early in the induction phase (figure 4).

Negative effect of high dose TNFα (50 ng/mL) on the differentiation of $CD4^+$ iT_{Regs} has been reported by others (121, 200). In addition, studies using lower dose of TNFα (5-20 ng/mL) did not observe any negative effects upon T_{Reg} differentiation (117, 204). Moreover, it has been demonstrated that high dose of TNFα has antagonistic effect upon the anti-inflammatory role of TGF-β1 on human $CD4^+$ T cells. (205).

The role of IL-1β signalling during iT_{Reg} differentiation is not clear. IL-1β is known to increase proliferation of conventional T cells and studies in mice have also shown that IL-1β enhances expansion of $FoxP3^+$ T cells (202). Conversely, IL-1β has been shown to negatively regulate or switch the phenotype of T_{Regs} (129, 206). In support of this finding, $IRAK^{-/-}$ mice (lacking functional IL-1 signalling) have been reported to have higher T_{Reg} proportions compared with wild type mice (130). This finding suggests that IL-1 signalling negatively regulates T_{Reg} development or maintenance. Similarly, IL-1β, in combination with IL-2 was recently shown to convert natural human T_{Regs} into Th17 lineage cells (129). The effect of IL-1β on differentiating human iT_{Regs}, has however to our knowledge, not been reported previously.

Our findings suggest that exogenously added IL-1β can either enhance or reduce the induction of $FoxP3^{high}$ T_{Regs}, depending on the dose applied. Thus, a low dose of IL-1β (0.1 ng/mL) induced significant differentiation of $CD4^+CD25^{high}CD127^-FoxP3^{high}$ iT_{Regs}, whereas, a high dose of IL-1β (5-10 ng/mL) had a reverse effect. Therefore, our results suggest that both TNFα and IL-1β have a time and dose dependent effect on the IL-2 and TGF-β1 mediated induction of human $CD4^+CD25^{high}CD127^-FoxP3^{high}$ T_{Regs}. Interestingly, the suppressive effects IL-1β on FoxP3 induction were associated with a significant reduction in TβRII expression. These results, therefore, suggest that the negative effects of IL-1β upon FoxP3 expression may partially be caused by the reduced expression of TβRII and consequently reduced TGF-β signalling. These findings indicate that innate immunity may play a major role in defining its final outcome into either a tolerogenic or prolonged inflammatory response.

FoxP3 is the most consistent marker for human as well as murine T_{Regs}. However, the expression of FoxP3 by human T cells is not consistently associated with a suppressive function (190). Furthermore, human $CD4^+CD25^-$ T cells stimulated in the presence of IL-2

and TGF-β1 have either been shown to be suppressive (165) or not, despite expressing high levels of FoxP3 (40). However, our findings clearly demonstrate that $CD4^{+}CD25^{high}CD127^{-}$ $FoxP3^{high}$ human T_{Regs} can be suppressive but this function was lost if high dose TNFα or IL-1β was present during their induction phase.

It has been suggested that T_{Regs} compete with responder cells for IL-2 in mice and thus inhibit their proliferation (82). However, in our model we detected enhanced IL-2 secretion in the presence of TGF-β. Several studies have suggested that iT_{Regs} mediate their suppressive function through the secretion of IL-10 and/or IL-35. However, differentiation of human $CD4^{+}$ iT_{Regs} was associated with a significant reduction of IL-10 production and negligible, amounts of IL-35 were present in all conditions tested. This suggests that the suppressive function is mediated through IL-2 secretion and TβRII expression dependent mechanism. Cytokine dependent mechanisms have been strongly associated with the suppressive function of iT_{Regs} (207). Additionally, our findings suggest that such cytokine dependent mechanisms are the driving force of their suppressive function.

Our study indicates that low levels of TNFα and IL-1β promote the induction of T_{Regs} similar to the low intensity and tolerogenic immune response at mucosal sites. However, during high intensity and prolonged T-cell activation involving the danger signals of the innate immunity, T_{Regs} induction and their function would be suppressed, a condition which would be expected during persistent infection or chronic autoimmune disorders.

Authorship

The study was designed by all authors. Experiments and analysis were performed by S.H. and L.G. and the results were discussed with B.R.L. The manuscript was written by S.H. L.G. and B.G. and edited by B.R.L.

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Disclosures

The authors declare no conflict of interest.

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