The role of Mxi1-SRβ in the generation and homeostasis of Foxp3+ T regulatory cells

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April, 2017

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master of Science

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Abstract (English)

CD4+Foxp3+ T regulatory (Treg) cells are critical mediators of self-tolerance and immunological homeostasis. For this reason, Treg cells must be able to quickly sense and adapt to their dynamic surroundings. We used a poly-ribosome-associated microarray technique to measure the relative abundance of mRNAs being actively translated into protein in activated Treg compared to T effector (Teff) cells. Mxi1, an anti-proliferative transcription factor previously linked to the transforming growth factor beta (TGFβ) signalling pathway, was found to be translated to higher levels (3 fold) in activated Treg compared to Teff cells. Since TGFβ signalling plays a fundamental role in the generation of Treg cells in the periphery, we hypothesize that Mxi1 plays a vital role in the generation of regulatory T cells. Mxi1-SRβ expression was the predominant isoform expressed in activated Treg cells, and its expression was induced by TGFβ. Teff cells overexpressing Mxi1-SRβ were less proliferative, produced less of the inflammatory cytokines IFN-γ, TNF-α as well as IL-2, and had a greater propensity to up-regulate Foxp3 following exposure to TGFβ. Ectopic Mxi1-SRβ expression also caused both Teff cells and pTreg cells to up-regulate the TGFβ-dependent, Th17-specific transcription factor RORγt. Finally, Mxi1-SRβ abrogated the pathogenic potential of diabetogenic CD4+ T cells in a mouse model of type 1 diabetes as a result of decreased Teff cell proliferation and increased Treg cell frequency. Together, these findings indicate that Mxi1 could be involved in the generation of Treg cells through the TGFβ pathway in the periphery as well as mediating overall Treg cell homeostasis by regulating Treg cell proliferation.

Résumé (Français)

Les lymphocytes T régulateurs (Treg) CD4+Foxp3+ sont des médiateurs essentiels de la tolérance au soi et du maintien de l'homéostasie. Pour cette raison, les cellules Treg doivent posséder la capacité de reconnaitre et de s'adapter à leur environnement. Nous avons analysé par une technique de micro-puce à ARN l'ensemble des gènes associés aux poly-ribosomes chez des Tregs et des cellules T effectrices (Teff) de manière à comparer l'abondance relative d'ARN messagers (ARNm) en phase d'être traduites en protéines. Nous avons découvert que l'ARNm du facteur de transcription Mxi1, dont la protéine est associée à des propriétés antiprolifératives et lié à la signalisation du facteur de croissance transformant beta (TGFβ), était 3 fois plus abondant dans les cellules Treg que dans les Teff. Puisque le signal induit par le TGFβ joue un rôle essentiel dans la génération et le maintien des cellules Treg dans la périphérie, nous avons alors émis l'hypothèse que Mxi1 joue un rôle vital dans ce processus. En effet, nous avons découvert que l'isoforme beta (SRβ), mais pas alpha (SRα), joue un rôle important dans l'induction du facteur de transcription Foxp3. Les cellules Teff qui sur-expriment Mxi1-SRβ par l'entremise d'une transformation génétique prolifèrent moins, produisent moins des cytokines pro-inflammatoires IFNγ, TNFα et d'IL-2 et possèdent une plus grande propension à exprimer Foxp3 lorsqu'exposé au TGFβ. De plus, les cellules dont nous avons induits la surexpression de Mxi1 montraient une plus grande propension à exprimer le facteur de transcription RORγT, associés à des cellules Th17. Finalement, la surexpression de Mxi1-SRβ permet de bloquer le potentiel diabétogénique des lymphocytes T CD4+ dans un modèle murin de diabète de type 1 en diminuant leur prolifération et augmentant le ratio de Treg. Ces résultats démontrent que Mxi1 est impliqué

dans la génération et la prolifération des cellules Treg grâce à son effet sur la signalisation du TGFβ et contribue ainsi à l'état d'homéostasie immunologique.

Acknowledgments

I would first like to sincerely thank my supervisor, Dr. Ciro Piccirillo of McGill University for giving me the opportunity to work in such an outstanding laboratory. The door to his office was always open and he was always able to give me guidance and support in my work. He also challenged me to think critically of my work, and allowed me to take control of this project, which has helped build my potential as a scientific researcher.

I would next like to thank Dr. Bruce Mazer and Dr. Woong-Kyung Suh of my advisory committee. Both Dr. Mazer and Dr. Suh provided me with invaluable insight for my project and helped guide me in my research.

I would also like to thank Helen Mason, our research technician, and Marie-Helene Lacombe from the immunophenotyping core facility for enabling me to conduct meaningful and relevant research.

Finally, I would like to express my profound gratitude to my lab colleagues, who made my time in the lab so enjoyable and positive. They provided me with endless ideas about my project, and endless good times throughout my degree at McGill University.

Authors contributions

C.P. conceived and supervised the project. C.P. and N.P. planned experiments. N.P. performed all of the experiments, including constructing and validating retroviral vectors and developing a flow cytometric approach to study Mxi1. N.P. analyzed data and wrote this thesis.

Scientific statement of integrity

I am familiar with the McGill University Academic Integrity Policy and I understand the potential consequences should my thesis be found to contain plagiarized content or violate this policy in any other way.

Abbreviations list

Abbreviation	Full name
Treg	T regulatory
DN	Double negative
DP	Double positive
TCR	T cell receptor
CD	Cluster of differentiation
MHC	Major histocompatibility complex
LCK	Lymphocyte-specific protein-tyrosine kinase
ITAM	Immunoreceptor tyrosine-based activation motifs
NFAT	Nuclear factor of activated T cells
MAPK	Mitogen activated protein kinase
SP	Single positive
CTL	Cytotoxic T lymphocyte
Th	T helper
IL-2	Interleukin 2
IFNγ	Interferon gamma
TNFα	Tumor necrosis factor alpha
STAT	Signal transducer and activator of transcription
Runx	Runt-related transcription factor
T-bet/ tbx21	T-box transcription factor
Gata-3	Gata-3 transcription factor
IgE	Immunoglobulin E
RORγt	RAR-related orphan receptor gamma
TGFβ	Transforming growth factor beta
SOCS	Suppressor of cytokine signalling
Foxp3	Forkhead-box p3
IPEX	immunodysregulation, polyendocrinopathy, enteropathy X-linked
CNS	Conserved non-coding sequence
CTLA-4	Cytotoxic T lymphocyte-associated antigen 4
GITR	Glucocorticoid-induced TNF receptor
LAG3	Lymphocyte antigen gene3
Tconv	T conventional
Teff	T effector
C-Rel	C-Rel transcription factor
tTreg	Thymic-derived Treg
GALT	Gut-associated lymphoid tissue
MLN	Mesenteric lymph node
pTreg	Peripherally-induced Treg
NK	Natural killer
DC	Dendritic cell
AMP	Adenosine monophosphate
IBD	Irritable bowel disease
PI3	phosphatidylinositol 3
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells

RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
LAP	Latency-associated protein
SLC	Small latent complex
LTBP	latent TGFβ-binding protein
ALK	Activin-like kinase
TGFβR	Transforming growth factor receptor
AICD	Activation-induced cell death
Smad	Smad
Fas	Fas
APC	Antigen-presenting cell
PKC	Protein kinase C
TSDR	Treg-specific demethylated region
GFP	Green fluorescent protein
DNA	Deoxyribonucleic acid
TIGIT	T cell immunoreceptor with Ig ITIM domains
FCRL3	Fc receptor-like protein 3
HDM	House dust mite
miR	Micro RNA
Polysome	Poly-ribosome
Mxi1	Max-interactor 1
Mad	Max-dimerization
Myc	Myc
SID	Sin3 interaction domain
HDAC	Histone deacetylase
IRES	Internal ribosomal entry site
PMA	Phorbitol 12-myristate 13-acetate
MACS	Magnet-associated cell sorting
FACS	Fluorescence associated cell sorting
CFSE	Carboxyfluorescein succinmidyl ester
mAB	Murine antibody
MSCV	Murine stem cell virus
PCR	Polymerase chain reaction
T1D	Type 1 diabetes
EV	Empty vector
WT	Wild type
D.I.	Division index
NOD	Non-obese diabetic
Atg	Autophagy
mTOR	Mammalian target of rapamycin

Tables and Figures list

Background

1.1 Introduction

The immune system is under tight regulation to ensure temporally and spatially appropriate responses. CD4+ T helper cells are critical in controlling the progression of immune responses through their distinct functional abilities. There are numerous specific subsets of T helper cells, each with a defined role dependent on the particular pathogen and location of insult. Among these, T regulatory (Treg) cells are unique in that they suppress inflammatory responses unlike other T helper subsets that function to eliminate the threat. Treg cells are responsible for self-tolerance and inhibiting excessive damage to healthy tissues following pathogen clearance. For this reason, Treg cells must be able to sense their surroundings and alter their gene expression promptly to ensure immunological homeostasis is maintained, as dysregulation of the Treg cell population can lead to severe autoimmune disease and death. We have sought to investigate the function of a gene found to be tightly regulated post-transcriptionally in Treg cells.

1.2 T cell development

Mature T cell generation begins in the bone marrow, where common lymphoid progenitor cells exit this primary lymphoid organ and traffic to the thymus. Upon entering the thymus, the common lymphoid progenitor cell begins its differentiation process in the thymic cortex and is not expressing the main markers of T cells. These are the CD3, CD4 and CD8 molecules. This cell is termed a double negative (DN) cell, as it is negative for the expression of both the CD4 and CD8 co-receptors (1). DN T cells go through 4 stages as negative expressers of these 2 markers, with each stage being divided according to the expression of the CD44 and CD25 receptors. DN T cells give rise to either $\alpha\beta$ or $\gamma\delta$ T cells, where they express either the α and β or

the γ and δ chains of the TCR, respectively (2). Those destined to become αβ T cells express the pre-TCR α chain at the DN3 stage. This α chain is encoded by a non-rearranging locus and pairs with the β chain, which has undergone somatic rearrangement generating an amino acid sequence unique to that developing T cell. This pre-TCR complex associates with the CD3 collection of proteins, which are responsible for proximal signal transduction. Active signalling through the β chain, known as β selection, is essential to inhibit further mutations leading to DN3 developmental arrest. Following β selection and emergence form late DN3 and DN4, T cells undergo recombination at the TCR-α locus producing a mature αβ antigen receptor. It is at this step that T cells being to express the co-receptors CD8 and CD4. They express both proteins and become double positive (DP) T cells (3,4).

DP T cells migrate to the thymic medulla where they are exposed to peptides associated with major histocompatibility complexes (MHC) on antigen presenting cells, where MHC class I present peptides to CD8-associated TCRs and MHC class II to CD4. Only certain mature single positive T cells are successfully selected for at this step of maturation and enter the peripheral lymphatic system. There are 4 processes that select for mature CD4+ and CD8+ T cells: death by neglect, negative selection, positive selection and lineage specific development. The majority of T cells react so poorly to presented peptides that the intracellular signalling is insufficient to maintain the viability of those cells, and the result is death by neglect. Contrarily, some TCRs react very strongly to self-peptide. Emergence of these clones of T cells would lead to autoimmunity directed at the host. For this reason, these cells go through a process of negative selection, where strong reactivity to self initiates programmed cell death and elimination of that T cell clone. In between death by neglect resulting from a lack of reactivity and negative selection from an overly

strong reactivity are T cells that react moderately to self-peptide. These T cell clones are positively selected and this leads to lineage-specific development of either CD4+ or CD8+ mature T cells (1,5).

An integral component of thymocyte development is that of signal transduction following TCR-antigen recognition. The production of intracellular messenger proteins is critical for the generation of life or death signals, which mediate the survival of a mature T cell. Moreover, signal transduction is important for the maintenance of expression of the CD4 or CD8 co-receptor molecules on DP T cells (1). The first step of receptor-antigen signalling is ligand recognition by the TCR, which activates the lymphocytes-specific protein-tyrosine kinase (LCK) associated with the CD3 portion of the TCR complex as well as the intracellular tails of CD4 and CD8. LCK phosphorylates the immunoreceptor tyrosine-based activation motifs (ITAM) of CD3, and this leads to the recruitment of the SYK-family kinase ZAP70. Activated LCK phosphorylates ZAP70 leading to the phosphorylation of several other enzymes and adapters ultimately resulting in the nuclear translocation of the nuclear factor of activated T cells (NFAT) and the activation of mitogen-activated protein kinase (MAPK) pathways. Together these signals contribute to the regulation of gene expression required for the function of mature T cells (6).

The final step in the production of mature single positive (SP) T cells is the loss of expression of one co-receptor molecule, the CD4 on cytotoxic T lymphocytes (CTL), or the CD8 on T helper (Th) cells. This is achieved in two ways, with the first being silencing of one co-receptor following the initial interaction of TCR-ligand with the co-receptor molecule, where the optimal interaction is favored. The second model of maturation involves the random silencing of one co-receptor molecule, followed by testing of the resulting TCR-ligand with the co-receptor.

No matter the method, emerging from the thymus are mature SP T cells committed to either the T helper (CD4+) or cytotoxic T cell (CD8+) lineage (7).

1.3 T helper cell differentiation

Mature CD4+ T helper (Th) cells have various roles and functions, and these functions are achieved by recognition of their cognate antigen presented by professional antigen-presenting cells (APC) and differentiation into specialized Th cells with distinct phenotypes. The decision of Th cells to enter a given lineage fate depends on a number of factors including extracellular environmental cues as well as intracellular signalling.

1.3.1 Th1 cell differentiation and function

Among the first Th cell lineage to be described was the Th1 cell. Th1 cells are the key players in protection against intracellular bacterial infections (8). The hallmark transcription factor distinguishing the Th1 cell is T-bet. Th1 cells are functionally distinct from other Th cell lineages in that they characteristically produce the pro-inflammatory cytokines interleukin (IL)-2, interferon-gamma (IFNγ) and tumor necrosis factor-alpha (TNFα). The release of these cytokines results in the activation of macrophages and leads to cell-mediated immunity as well as phagocyte-dependent protective responses (8).

Upon antigen recognition and TCR signalling, *tbx21,* the gene encoding the T-bet protein, is expressed and this is enhanced by the activation of the signal transducer and activator of transcription (STAT) 1 transcription factor. STAT1 expression is under the regulation of a positive feedback loop in response to autocrine and paracrine IFNγ. In turn, T-bet promotes the expression of the IL-12 receptor β chain, increasing the cells responsiveness to IL-12 (9). Activation of naïve CD4+ T cells in the presence of IL-12 skews the cells towards a Th1 phenotype.

The main role of IL-12 signalling during T cell activation is the transcription of the signal transducer of activated T cells 4 (STAT4). STAT4 enables the expression of multiple Th1-specific genes such as *ifnγ.* STAT4 also interacts with T-bet to induce the expression of other important Th1-genes (10). One of the targets of T-bet is the gene encoding Runx3. Together, T-bet and Runx3 bind the enhancer and promoter region of the *ifnγ* gene, further propagating it's expression. Additionally, T-bet and Runx3 contribute to the differentiation towards a Th1 phenotype by binding a silencer in the *il4* gene, resulting in the suppression of expression of Th2-specific genes. Finally, one of the most important functions of T-bet is to counteract GATA-3, the master regulator of the Th2 cell lineage (11).

1.3.2 Th2 cell differentiation and function

Like the Th1 cell lineage, CD4+ T cells can become activated and differentiate towards a Th2 phenotype. The main function of the Th2 cell type is to protect the host from helminths infecting cutaneous and mucosal sites. Moreover, Th2 cells are implicated in humoral immunity and contribute to allergy pathophysiology such as asthma (12). The key transcription factor of Th2 cells is GATA-3, where activation of naïve T cells in the presence of certain cytokines leads to the upregulation of GATA-3 expression and results in differentiation of naïve T cells to effector Th2 cells. Th2 cells characteristically secrete IL-4, IL-5 and IL-13. In certain instances, they can also produce IL-6, IL-9 and IL-10. The effects of Th2 cells secreting these cytokines is the induction of strong IgE antibody responses and eosinophil accumulation. Moreover, cytokine release by Th2 cells inhibits phagocytic cells and therefore the response is termed phagocyte-independent inflammation (13).

In-vitro studies of T cell polarization assessed the ability of cytokines to push naïve T cells towards a given lineage. It was demonstrated that the addition of IL-4 to a culture of naïve TCR-activated T cells could induce them to proliferate and differentiate towards Th2 cells in the absence of IL-2 (14,15). However, it has been proven that IL-2 is important for Th2 cell differentiation, and it was later shown that IL-2 signalling in TCR-activated T cells was necessary for the production of IL-4 by T cells (16,17). One of the key downstream molecules during IL-2 signalling is STAT5. It has been demonstrated that robust STAT5 activation is required for the induction of IL-4 and differentiation of naïve T cells to Th2 cells. Weak STAT5 signalling diminished the conversion towards a Th2 phenotype (18). As previously mentioned, the integral Th2 transcription factor is GATA-3. IL-4 signalling has been shown to be vital for the induction of GATA-3 expression in activated T cells. Where IL-2 was essential for the upregulation of IL-4 production, it is not required for GATA-3 expression, instead it is IL-4 signalling that is. Importantly, although STAT5 activation by IL-2 signalling and GATA-3 expression via IL-4 signalling are independent pathways in Th2 cell differentiation, they do work in unison to enhance the cell fate decision towards a Th2 phenotype (19).

1.3.3 Th17 cell differentiation and function

Despite the long time dominance of the Th1/Th2 paradigm, where CD4+ Th cells differentiated into either one of these two lineages, other phenotypes have been discovered and described. This has given rise to another pro-inflammatory CD4+ Th cell fate, one that is characterized by the production of IL-17A and IL-17F; the Th17 cell. Th17 cells protect the host from extracellular pathogens, particularly at the mucosal level, such as in the gut (20). In addition to IL-17, Th17 cells can produce IL-21, IL-22 as well as IFNγ in certain instances, as will be

discussed. The key transcription factor of the Th17 cell is RORγt, and research with inhibitors has shown that an inverse agonist targeting RORγt inhibits Th17 differentiation as well as IL-17 production in CD4+ T cells *in-vivo* (21).

Like both Th1 and Th2 cells, TCR signalling is required for a naïve CD4+ T cell to be able to differentiate into a Th17 cell. A result of TCR activation is the production of intercellular calcium and the activation of the transcription factor nuclear factor of activated T cells (NFAT). The proximal promoter of *Il7A* in humans contains two binding sites for NFAT and are important for the regulation of IL-17 production (22).

As previously discussed, cytokines produced by innate immune cells, such as IL-12 and IL-4, are important mediators of CD4+ T cell differentiation towards the Th1 and Th2 cell phenotypes, respectively. In line with this, activated dendritic cells (DC) produce IL-6, a potent pro-inflammatory cytokine. IL-6 has been shown to be vital for the differentiation of Th17 cells, where $IL6^{-/-}$ mice have reduced Th17 cells (23). Moreover, IL-21, which is produced by some Th17 cells, also contributes to Th17 cell differentiation and importantly, the maintenance of the Th17 cell phenotype (24). Interestingly, though it was previously hypothesized that IL-23 was a key inducer of Th17 differentiation, recent research has proved that naïve CD4+ T cells don't express the IL-23 receptor (IL-23R). Instead of being a factor that pushes naïve Th cells towards the Th17 phenotype, IL-23 is now viewed as being an important mediator of pathogenicity of IL-17 cells *in-vivo* (25,26).

An indispensable factor that induces Th17 cell differentiation in combination with IL-6 is transforming growth factor beta (TGFβ). Without the downstream signalling of TGFβ, IL-6 can not push naïve Th cells towards the Th17 phenotype. IL-6 and IL-21 activate STAT3, which stimulates

the expression of suppressor of cytokine signalling (SOCS)3, a negative regulator of STAT-dependent cytokine production and a negative regulator of Th17 cells. TGFβ is critical in Th17 differentiation because it inhibits the IL-6-mediated expression of SOCS3 in T cells, thus restoring and enhancing STAT3 signalling and ultimately enabling naïve CD4+ T cells to continue to the Th17 phenotype (27).

Although IFNγ was described above as being expressed by Th1 cells, that doesn't suggest that it is only expressed by such cells. Recent studies have shown that Th17 cells are capable of producing both IL-17 and IFNγ, and that these cells also express the Th1-delineating transcription factor T-bet (28). The phenotypes that Th cells take on are environment-dependent, and we are discovering the subtleties that allow Th cells to differentiate towards a given phenotype. Moreover, this data supports the possibility that terminally-differentiated Th cells may be able to re-program to begin to show phenotypes of another Th cell lineage.

1.3.4 T regulatory cell differentiation and function

Contrary to the three T cell lineages described above, which exert pro-inflammatory effector functions to protect the host from invading pathogens, there exists a lineage of CD4+ T cells which possesses potent anti-inflammatory capabilities. T regulatory (Treg) cells are a subset of CD4+ Th cells whose role is contrary to combating pathogens. Instead, Treg cells are responsible for regulating immune homeostasis to prevent prolonged inflammation, inhibit autoimmune disease and ensure self tolerance (29). Briefly, there exists two distinct subsets of Treg cells, those derived in the thymus and those derived in the periphery through genetic reprogramming. The master regulator transcription factor required for differentiation and conferring the suppressive capacity of all Treg cells is Forkhead Box p3 (Foxp3), a gene located

on the X chromosome. Foxp3 regulates the transcription of more than 700 genes and enables Treg cells to regulate immune responses in a variety of ways including the release of suppressive cytokines like IL-10 and TGFβ (30–32). The factors regulating Treg cell differentiation and function are distinct from other T cell subsets, and an in depth description of the Treg cell subset is discussed below.

CD4+ T cell subset	Master Regulator Transcription factor	Differentiating cytokines	Effector cytokines produced
Th1	T-bet	IL-12	IFNγ, TNFα, IL-2
Th2	Gata-3	IL-4	IL-4, IL-5
Th17	RORγt	TGFβ, IL-6	IL-17
Treg	Foxp3	TGFβ, IL-2	IL-10, TGFβ

Table 1. List of differentiating cytokines, master regulator transcription factor and characteristic effector cytokines expressed by the Th1, Th2, Th17 and Treg subsets of CD4+ T cells.

1.4 Discovery and early characteristics of Treg cells

Elaborating on the discovery of suppressive CD4+ T cells, murine studies gave rise to the discovery of the fundamental gene for Treg cell differentiation and suppressive function. The Scurfy mouse was first described in 1959 and the phenotype wasn't described until 1991 as a mouse who developed lethal lymphoreticular disease, a disease that was dependent on the development of T cells (29). Moreover, in 1969, it was demonstrated that thymectomy of neonatal mice at day 3 of age lead to the destruction of the ovaries. While this was first believed

to be due to a lack of hormone production, it was later found to be an autoimmune process, as inflammation and tissue destruction was found in other organs as well (33). It was in 2000 that the gene responsible for the scurfy phenotype was discovered. The mutation leading to Scurfy in mice is found in the *foxp3* locus of the genome and the result of this mutation is an autoimmune-like lymphoproliferative disease (30). Similarly, in humans, mutations in the Foxp3 gene leads to a lethal disease called immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX). The discovery that mutations in the Foxp3 gene lead to the rare IPEX was made in 2001 and manifestations include but aren't limited to autoimmune enteropathy, thyroiditis, dermatitis and type 1 diabetes (34).

1.4.1 Functional role of Foxp3 in Treg cells

In the early 2000's, Sakaguchi's group investigated the functional role of Foxp3 in Treg cells and the resulting effects on the overall function of Treg cells. They first showed that there was a subset of CD4+ T cells that expressed CD25 as well as CD45RO, which is an isoform of the leukocyte common antigen and is expressed by memory cells, and that these cells were refractory to proliferate following TCR stimulation. Following antigen recognition via the TCR, provided the adequate costimulatory signals, CD4+Foxp3- T conventional (Tconv) cells will proceed to expand and proliferate to generate large numbers of TCR-restricted cells. These CD25+CD45RO+ T cells were different in that they did not proliferate under these *in-vitro* conditions. Moreover, when co-cultured with CD4+CD25- T cells, the CD25+CD45RO+ T cells suppressed the proliferation of the CD25-T cells (35).

They next demonstrated that these CD25+CD45RO+ T cells preferentially expressed the transcription factor Foxp3. They measured Foxp3 mRNA levels in T cell subsets as well as CD8 T

cells and B cells. Foxp3 was expressed very minimally in CD8 T cells and B cells, but was expressed in CD4 T cells. Notably, Foxp3 mRNA levels were 60-fold greater in the CD25+ subset compared to the CD25-. Moreover, when comparing the CD45RO+ subset to the CD45RO- subset within the CD25+ T cells, Foxp3 mRNA was transcribed 12-fold more in the RO+ subset. This indicated that Foxp3 correlated with the suppressive phenotype in T cells, and that it was the subset expressing Foxp3 that possessed regulatory functionality (35).

To test whether Foxp3 was able to induce the suppressive phenotype of regulatory cells, naïve CD4+CD25- Tconv cells were transduced with a Foxp3-encoding virus. Interestingly, the transduced cells did take on the regulatory phenotype, where they were hyporesponsive following TCR activation, and they did not produce pro-inflammatory cytokines like IL-2, IFNγ or IL-4, like normal CD4+ T effector (Teff) cells do. Moreover, the Foxp3 transduced cells expressed higher levels of CTLA-4, GITR and CD25 than the untransduced cells (35). These findings indicate that Foxp3 provides Treg cells with the ability to suppress Teff cell proliferation, as well as having direct effects on the differentiation of T cells towards the Treg phenotype by inducing the expression of various Treg-relevant surface markers. More recent research has shown that Foxp3 has approximately 700 target genes, with some of these genes being transcriptionally suppressed, like the IL-2 gene, whereas some are transcriptionally activated, such as cytotoxic T lymphocyte-associated antigen 4 (CTLA-4) and Foxp3 itself (31).

1.4.2 Markers of Treg cells

In addition to Foxp3, Treg cells, which account for 5-10% of CD4+ T cells in humans, constitutively express the IL-2 receptor α chain (CD25). IL-2 is a cytokine produced by activated immune cells, particularly T cells and dendritic cells. It signals through the STAT5 signalling

pathway following binding to its cognate receptor CD25. IL-2 signalling causes activated cells to proliferate during an immune response to create a huge number of activated and in the case of T cells, clonally-specific cells. For Treg cells, IL-2 signalling is required because the STAT5 protein binds and both induces as well as stabilizes the expression of the Foxp3 gene (36). In addition to Foxp3 and CD25, Treg cells are characterized by their expression of CD45RO, CTLA-4, glucocorticoid-induced TNF receptor (GITR) and lymphocyte activation gene 3 (LAG-3), with Foxp3 regulating the expression of many of these Treg-specific markers (37). Although Foxp3 is the master switch for all Treg cells, the timing of Foxp3 induction can vary during T cell development, and can even occur in fully developed Teff cells in the periphery.

1.5 The Development of thymic-derived Treg cells

There are two distinct pathways that give rise to the generation of Foxp3+ Treg cells, with the first being in the thymus. Treg cells coming from the thymus are termed thymic-derived Treg (tTreg) cells. As stated above, T cell progenitor cells migrate to the thymus from the bone marrow, where they proceed to go through the process of positive or negative selection, where T cells with strong reactivity to self are negatively selected, and only those that have weak self-reactivity are allowed to be positively selected and exit the thymus. There is a model of Treg development that suggests that it is during this selection process that tTreg cells arise. Mouse studies showed that TCR specificity towards self-antigens was important for the development of tTreg cells (38). This means that instead of the T cells with high reactivity to self being clonally deleted, they may turn into Treg cells. A study that supported this hypothesis consisted of using mice with a limited TCR repertoire. In these mice it was found that the repertoires of Treg cells had very little overlap with that of Teff cells. Moreover, Teff cells that were engineered to have

a TCR specificity from the Treg repertoire were more proliferative in lymphopenic hosts, supporting the notion that the Treg cell repertoire was highly biased towards self antigen (39,40).

Treg cells originating from the thymus are much more stable than those arising in the periphery, an this is in part due to the difference in methylation states of a specific region of the Foxp3 gene between tTreg and pTreg cells. Unlike pTreg cells, whose Foxp3 gene is heavily methylated at the Treg-specific demethylated region (TSDR), tTreg cells have a hypomethylated TSDR enabling easy access for the transcriptional machinery to produce *foxp3* mRNA and ultimately Foxp3 protein. This epigenetic state in tTreg cells ensures stability of Foxp3 expression, which is required for Treg cell homeostasis and suppressive function (41).

Another finding supporting the hypothesis that self-reactivity drives Treg development in the thymus comes from a mouse model that uses the expression of the green fluorescent protein (GFP) to identify TCR activation. In this model, GFP was found to be greater in thymic CD4+ T cells that expressed Foxp3 compared to those that did not express Foxp3, consistent with the idea that increased self-reactivity leads to Treg development (42).

The result of Treg cell development based on self-reactivity in the thymus is the production of a pool of Treg cells that may have a memory of the self antigens presented in the thymus. Interestingly, unlike conventional memory cells, which are able to persist in the absence of cognate antigen, the maintenance of the memory Treg cell pool from the thymus appears to depend on the access to antigen in peripheral tissue. A recent study showed that activation of Treg cells in the periphery by antigen lead to their proliferation and persistence in the tissue where the antigen originated from. This enables Treg cells to persist and respond to self antigens that are released after injury, inhibiting the induction of autoimmunity (43).

1.5.1 The role of NF-κB signalling in tTreg cell development

Antigen recognition and activation through the TCR activates various downstream pathways, each having potentially different genetic targets and cellular effects. Among these pathways, the nuclear factor κB (NF-κB) has been shown to be an important pathway for Treg cell development in the thymus. This was first demonstrated by murine studies using mice lacking factors involved in the NF-κB pathway. These mice had a dramatic decrease in frequency of tTreg cells (44). Moreover, overexpression of NF-κB resulted in the development of Treg cells in the thymus even in the absence of TCR reactivity to self (45). These data demonstrate that NF-κB signalling is both necessary and sufficient for efficient Treg development in the thymus. Mechanistically, it is c-Rel, a member of the NF-κB family of transcription factors, that binds to conserved non-coding sequence (CNS3) of the *foxp3* gene and causes its transcription. This has been shown to occur as early as the CD4+CD8+DP phase of thymocytes, identifying NF-κB as one of the pioneer transcription factors in turning on Foxp3 transcription and inducing Treg development in the thymus (46).

1.5.2 Markers of tTreg cells

Treg cells emerging from the thymus or being generated in the periphery by genetic reprogramming of Foxp3- Teff cells are both suppressive cells, however their distinct functions have been argued over the years. It was initially believed that tTreg cells were biased to self whereas pTreg cells were biased to pathogenic entities, such as commensal bacteria in the gut. However, these findings have been contradictory, with some researchers claiming that it's in fact tTreg cells that harbor the majority of TCRs specific for commensal bacteria (47). Of particular interest, identifying markers of these distinct Treg populations is a hot topic of research in recent

years, as finding ways to isolate the more stable tTreg cells for clinical use is a highly desired ability.

In 2010, *Thornton et al* identified a group of genes in the Ikaros family of transcription factors made up of 5 DNA-binding proteins each containing 6 highly conserved C2H2 zinc fingers. 4 of these are important for DNA binding, and the other 2 are responsible for homo and heterodimerization. Eos and Pegasus, 2 of the 5 members, are widely expressed across most tissues of the body. Contrarily, Ikaros, Aiolos and Helios are restricted to the hematopoietic system. Ikaros is expressed in all hematopoietic cells, whereas Aiolos is found in lymphocyte populations like T and B cells. Helios, however, is only found in cells of the T cell lineage (48,49). Studies inducing mutations in members of this family such as Ikaros and Aiolos have proven the importance of this family in Immune cell generation and function, as null mutations lead to the absence of certain compartments of immunity, such as NK cells and B cells in the context of non-functional Ikaros (50). Until 2010, the function of these proteins had only partially been studied. Thornton demonstrated that Helios, one the Ikaros transcription factors, was preferentially expressed in Treg cells and that Helios expression was confined to the thymic-derived Treg cells (51). This was the first discovery of a reliable marker to distinguish Treg cells emerging from the thymus, who express the transcription factor Helios, from Treg cells arising in the periphery and do not express Helios. This claim remains controversial to this day, where some researchers believe that not all tTreg cells express Helios (52). This topic is under fierce research among Treg biologists, and for good reason, as it is yet to be determined what induces Helios expression in Treg cells. Nonetheless, Helios was the first reliable marker of tTreg cells, and remains a reliable marker for this Treg population.

Recently, another hallmark marker of highly suppressive Treg cells has been identified by our lab. *Bin Dhuban et al, 2015* demonstrated that the Helios+ Treg cells described above also co-express two surface markers, TIGIT and FCRL3, and that this co-expression alongside Foxp3 is unique to these Treg cells. This finding was ground breaking for Treg research, as it now enabled the isolation of highly suppressive, most likely thymic-derived, Treg cells for experimental use and even potential clinical uses (53). TIGIT is a co-inhibitory molecule that inhibits autoimmunity through binding CD155 on DCs and preventing the production of pro-inflammatory cytokines like IL-12, while stimulating the expression of IL-10 (54). FCRL3 is expressed on many immune cells including B cells and dendritic cells, and its ligand is yet to be identified. Research is ongoing to further describe the role of FCRL3 in Treg cell generation and function.

1.6 Treg cell induction in the periphery

In addition to thymic-derived Treg cells, who stably express Foxp3 when they exit the thymus, conventional CD4+Foxp3- T cells can experience certain microenvironments in the periphery, and receive signals that cause Foxp3 to be turned on. These newly generated Treg cells are termed peripherally-induced Treg (pTreg) cells. In the early 2000's, *Chen et al* were among the first to describe that naïve CD4+CD25- T cells could be isolated from the periphery and exposed to certain cytokines *in-vitro* causing them to begin expressing the Treg master regulator, Foxp3. At this point it had been suggested that TGFβ was capable of inducing Treg cells in humans, but *Chen et al, 2000* were the first to show this effect in naïve Teff cells, and also to demonstrate a mechanism for this TGFβ-mediated induction of Foxp3. They achieved this by TCR-activating isolated CD4+CD25- T cells in the presence of TGFβ. They found that these conditions were capable of converting CD25- T cells into suppressive CD25+ T cells, shown by their

expression of the Treg marker CTLA-4. They also demonstrated that these induced suppressive cells secreted active TGFβ, a hallmark Treg cytokine, and that Foxp3 expression was induced. Finally, this group proved the finding that these *in-vitro* induced Treg cells were capable of suppressing immune responses *in-vivo*. They performed this using an inducible model of asthma using house dust mite (HDM). Adoptive transfer of *in-vitro* TGFβ-mediated Foxp3-induced Treg cells reduced inflammation as shown by a reduced infiltration of pro-inflammatory cells to the lung of HDM-challenged mice and reduced Th1 and Th2-related cytokine production (55).

Induced Treg cells have been shown to be just as efficient at suppressing inflammation as tTreg cells, but it has been demonstrated that their mechanisms of suppression are different. For example, in a study where *in-vitro* antigen-specific induced Treg (iTreg) cells were adoptively transferred into recipient mice who experienced spontaneous autoimmune gastritis, the suppression elicited by the iTregs was shown to be at the level of Teff cell priming by DCs. Moreover, it has also been demonstrated that tTreg cells impede trafficking of inflammatory cells to a site of inflammation, whereas pTreg cells suppress T cell priming, but don't have as much of an effect on T cell trafficking (56).

The suppressive ability of iTreg cells has been further demonstrated by infusing the Scurfy mouse with iTreg cells. It was shown that iTreg cells were capable of reducing the autoimmunity that the Scurfy mouse experienced. As previously stated, pTreg cells are less stable than tTreg cells. This is for a number of reasons, with one of the main ones being that the TSDR of pTreg cells has heavier methylation than tTreg cells. This makes pTreg cells more likely to lose Foxp3 expression under certain conditions. One of the key cytokines involved in Foxp3 maintenance and Treg homeostasis is IL-2. Treg cells express CD25 constitutively because they require IL-2 in

order to maintain expression of Foxp3. Studies have been conducted where antigen-specific iTreg cells are re-stimulated with their cognate antigen in the absence of TGFβ, and the result was an abrupt loss of Foxp3 expression. Contrarily, Foxp3 expression was maintained for 2 weeks when these Treg cells were activated in the presence of IL-2. This same type of experiment was performed *in-vivo* where iTreg cells were transferred and those that were re-stimulated with antigen rapidly lost Foxp3 expression compared to those that were not activated and maintained Foxp3 expression for a month. Interestingly, the iTreg cells that lost Foxp3 expression did not become pathogenic Teff cells (56). Since it was shown that the Scurfy mouse was able to receive iTreg cells and these Treg cells were able to sustain in the highly inflammatory environment, it was hypothesized that there was something beneficial for the Treg cells in this pro-inflammatory setting. To test this question, mice who had been administered iTreg cells were immunized with IL-2 complexes, since IL-2 is released during an immune response to induce T cell proliferation. The IL-2 complexes caused the iTreg cells to proliferate more and also express higher levels of Foxp3. Most notably, some mice received IL-2 as well as antigens to activate the antigen-specific iTreg cells. When assessing the TSDR of these iTreg cells, it was found that their TSDR was largely demethylated. Moreover, when IL-2 was neutralized using anti-IL-2 complexes, Foxp3 expression was quickly lost in iTreg cells in this setting and they were also less suppressive (56,57). Take together, these findings suggest that IL-2 is a critical cytokine for the maintenance of Foxp3 expression and overall pTreg cell homeostasis.

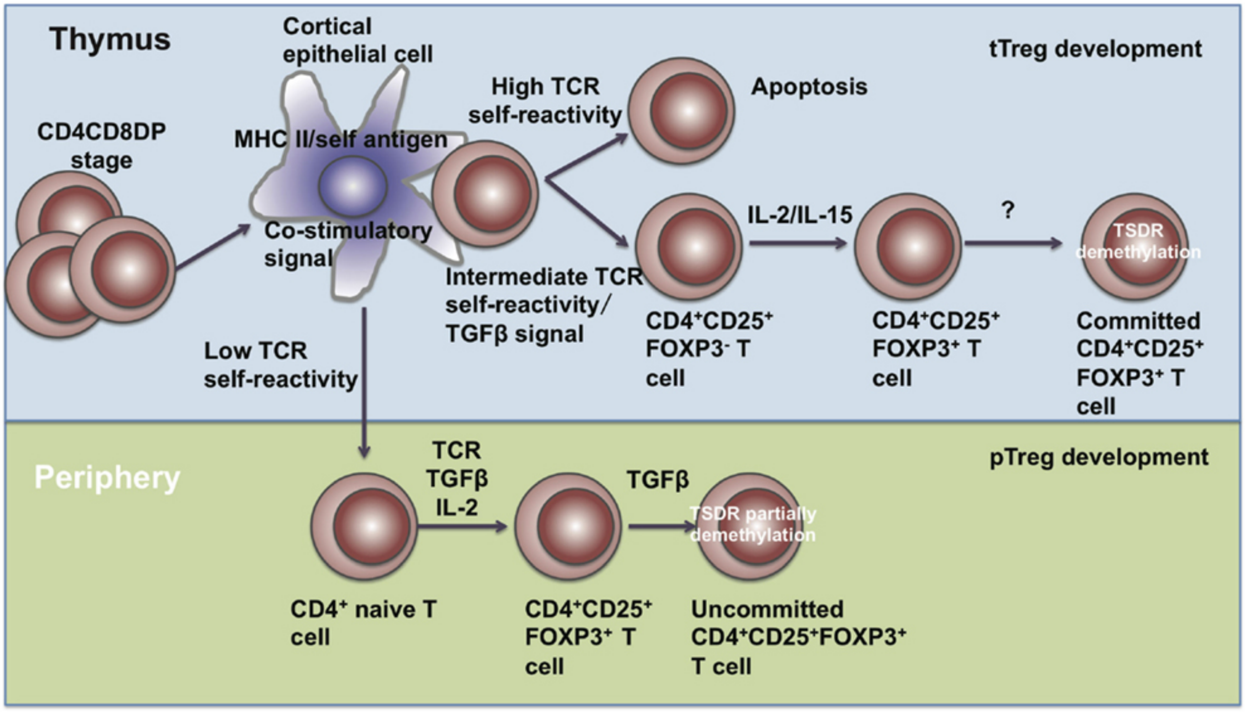
It has been extensively shown that Teff cells require IL-2, TGFβ and TCR stimulation in order to turn on the expression of Foxp3 and become pTreg cells (55–57). A remaining question was where in the body did Teff cells experience these signals. Treg cells accumulate during

inflammation at the local site of immune challenge and have been shown to play important roles in the resolution of immune response and even in tissue remodelling during inflammation (58). The Bluestone group asked where these Treg cells originated from. One option is that tTreg cells migrate to the site of inflammation. However, another possibility is that local Teff cells encounter the required factors to convert to Foxp3+ pTreg cells. *Yadav et al, 2013* showed that macrophages play an important role in pTreg generation in the lung mucosa by expressing TGFβ. Moreover, they found that Treg cells accumulated in the muscle during inflammation, and that these Treg cells could be arising *de-novo*. They hypothesized that pTreg cells are vital in controlling local inflammation, and that specific environments produce the proper signals to generate pTreg cells (58). The gut is one of the main sites of pTreg induction. A study using TCR-transgenic CD4+Foxp3- T cells adoptively transferred into mice followed by administration of cognate antigen resulted in significant accumulation of pTreg cells in the gut compared to other sites. Sequencing of the TCRs of these pTreg cells revealed that their TCR repertoire was biased towards bacterial and food antigens. This suggested that pTreg cells are implicated in tolerance to gut flora and ingested foods. However, it has also been reported that the TCRs of colonic Treg cells are shared between tTreg and pTreg cells, indicating that the gut is also populated largely by tTreg cells. Nonetheless, the gut is a site that presumably produces increased amounts of factors like TGFβ and leads to the induction of peripherally-induced Treg cells important for tolerance to non-self antigens (59).

1.6.1 The TGFβ signalling pathway during Foxp3 induction

TGFβ1 is a multifunctional protein depending on the nature of the responding cell and environmental setting. In the context of pTreg cells, TGFβ is critical for the induction of Foxp3 in Teff cells and mediates the conversion from Teff to pTreg cells (55). TGFβ belongs to a superfamily

of TGFβ proteins, all which carry out their function through type 1 and 2 transmembrane serine/ threonine kinase receptors. The family of type 1 receptors are called the activin-like kinase (ALK) receptors, with five existing. TGFβ interacts primarily with ALK5, and also with TGFβ receptor II (TGFbRII) (91). Active TGFβ first binds the TGFβRII, and its affinity for this receptor is enhanced by the betaglycan TGFbRIII. TGFβ signalling is initiated when an active TGFβ dimer binds the TGFβRII as well as the ALK5 tetramer (92). The TGFβ-receptor binding signal is relayed to the nucleus by Smad proteins, with 8 existing in vertebrates. These 8 Smad proteins are categorized into three groups. The first group of Smads are those that associate with the ALK5 and termed R-Smads. R-Smad1, 2, 3, 5 and 8 are make up this group. The second group consists of only Smad4, also termed as Co-Smad4 because it is the common Smad protein. Lastly, there are two inhibitory Smads; I-Smad6 and 7. R-Smads are sequestered in the cytoplasm when TGFβ is not present and signalling is not initiated. Upon ligation of TGFB with ALK5, R-Smad2 and 3 are phosphorylated and translocate to the nucleus with the help of Co-Smad4, however the latter is nor required for this translocation step. Once in the nucleus, phosphorylated R-Smad2 and 3 bind to the Smad binding element found in certain genes, such as the CNS1 of Foxp3 (93,94). Smad complexes regulate gene expression by interacting with coactivators that contain histone acetyl tranferases (HAT) or histone deacetylases, which activate of suppress transcription, respectively. In the case of *foxp3,* binding of phosphorylated R-Smad3 to CNS1 leads to acetylation and expression of the Foxp3. Ultimately, Foxp3 enables the conversion from Teff to suppressive pTreg cells through the induction of Treg-specific proteins (95).



Nie et al, 2015. Front Immunol. (60)

Figure B1. Treg cells develop in the thymus as well as the periphery through distinct mechanisms.

1.7 Role of the conserved non-coding sequences 1, 2 and 3 of the *foxp3* gene in Treg cell development and function

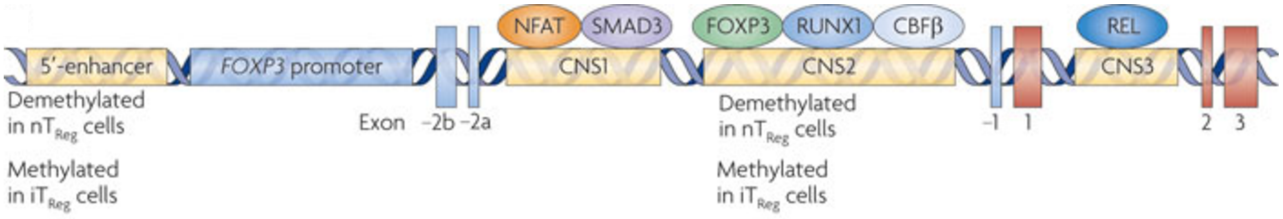
CNS3 was the first non-coding sequence of the *foxp3* gene to be identified. Murine as well as *in-silico* studies revealed that a sequence of CNS3 could be bound by the c-Rel protein. c-Rel is a nuclear protein that plays an important role in chromatin remodeling and affects gene expression through causing remodelling at the promoter of a gene (46,61). Mouse studies using c-Rel knockout mice demonstrated that in the absence of c-Rel, thymocytes and peripheral T cells showed a severe impairment in Foxp3 expression. It was also shown that the absence of CNS1 had little effect on the generation of Treg cells from the thymus. This indicates that CNS3 plays

an important role in the generation of tTreg cells, where its absence results in drastically diminished tTreg frequencies (46).

Although CNS1 was dispensable for tTreg development, it has been shown to be vital for the generation of pTreg cells. *In-vitro* and *in-vivo* studies have revealed that in mice deficient in CNS1, the induction of Foxp3 from Tconv cells is impeded. In particular, these results were found in the gut-associated lymphoid tissues (GALT). Upon further analysis of areas such as the mesenteric lymph node (MLN), a key site of TGFβ-mediated Foxp3 induction, it was found that compared to the spleen or non gut-draining lymph nodes, where pTreg numbers were almost unaffected, areas like the MLN harbored far less pTreg cells in mice lacking the CNS1 region of the *foxp3* gene. This can be explained since CNS1 is bound by the Smad3 protein following TGFβ signalling (62). These findings support the claim that unlike tTregs, who rely on CNS3, in order for Tconv cells to turn on the Foxp3 in sites like the MLN, the CNS1 region of the *foxp3* gene is required (46).

CNS2 is unique compared to CNS1 and CNS3 in that its chromatin is relatively non-permissive, with regions of heavy methylation, at least in Foxp3- Tconv cells as well as *in-vitro* induced Treg cells. This led researchers to hypothesize that CNS plays a role in not all Treg cells, but those that are mature. Murine studies using mice lacking the CNS2 portion of the *foxp3* gene revealed that Treg cell numbers were in fact unchanged in young mice. Strikingly, it was only in mice older than 6 months who possessed decreased numbers of Treg cells. Further studies illustrated that CNS2 was required for the maintenance of Foxp3 expression in mature Treg cells. CNS1 and CNS3 were dispensable for the process of Foxp3 maintenance, where CNS2 was not (46). These studies outlined the three unique roles played by the three conserved non-coding

regions of the *foxp3* gene in Treg cell generation and maintenance. They are vital to ensure proper expression of the *foxp3* gene, which produces the Foxp3 protein that is essential for the suppressive functionality of Treg cells (63).



Müller et al, 2010. Nat Rev Immunol. (64)

Figure B2. Regulation of Foxp3 expression by transcription factors associated with the conserved non-coding sequences of the *foxp3* gene.

1.8 Mechanisms of Treg cell-mediated immunosuppression

Treg cells are capable of suppressing the pro-inflammatory action of a number of immune cells including, but not limited to, B cells, natural killer (NK) cells, CD4+ T cells, CD8+ T cells as well as macrophages and DCs. The mechanisms by which Treg cells inhibit each cell population can be different and varies depending on the inflammatory microenvironment (65–69).

Treg cells suppress other CD4+ T cell subsets via direct cell-cell contact and the release of cytokines as well as indirectly through the inhibition of antigen-presenting cell function. Upon cell-cell contact, Treg cells can directly impede the proliferation and IL-2 production of TCR-activated Tconv cells. For this, Treg cells must also be activated through their TCR, however costimulation through the CD28 co-receptor is inessential in Treg cells (70). Antigen-experienced

Treg cells release cytokines such as IL-10 and TGFβ as well as other factors like granzymes and perforins that lyse Tconv cells. Moreover, Treg cells can deliver negative signals to Tconv cells such as causing the upregulation of cyclic AMP leading to inhibition of proliferation and IL-2 production (70). Treg cells also express the CD39 and CD73 molecules which directly interact with responding Tconv cells. These are examples of Treg cells causing direct suppression of Tconv cell expansion by cell-cell contact. *In-vitro* experiments pioneered the notion of direct cell-cell contact mechanisms of suppression by Treg cells, where Treg cells failed to suppress Tconv cell proliferation when the two populations were separated by a semi-permeable membrane (70–72).

1.8.1 Treg cells suppress inflammation by secreting IL-10

The integral anti-inflammatory cytokines secreted by Treg cells are IL-10 and TGFβ. IL-10 is a pleiotropic cytokine encoded by the *il-10* gene consisting of 5 exons located on the first chromosome. Although it is one of the two defining cytokines secreted by Treg cells, the immunosuppressive factor is also secreted by other immune cells such as the recently described Foxp3- Tr1 cell; another anti-inflammatory T cell population, as well as B cells, macrophages, DCs, CD8+ T cells and others (73–76). In each of these different cell types, the signals that induce IL-10 production are different. In Macrophages and DCs, IL-10 production is triggered following the Toll-like receptors recognizing and binding to pathogen associated molecular patterns present on foreign cells like bacteria (76). Interestingly, the exact environmental triggers that stimulate IL-10 production by Treg cells remain elusive. *In-vitro* activation of Treg cells fails to stimulate IL-10 production with the exception of Treg cells isolated from the gut, which are able to secrete the interleukin following *ex-vivo* activation (77). That said, *in-vivo* studies have proven that IL-10

expression is critical for Treg-mediated immune suppression, where disruption in IL-10 signalling leads to unwanted inflammatory conditions like irritable bowl disease (IBD) (78). IL-10 can act on other T cell populations directly upon ligation with its IL-10 receptor (IL-10R) on responding T cells. This event affects the CD28 costimulatory signalling pathway. Specifically, IL-10 inhibits the phosphorylation of a tyrosine residue on the CD28 receptor. This phosphorylation is the initial step of CD28 signalling, and its blockade inhibits the phosphatidylinositol 3 (PI3)-kinase p85 from binding CD28, blocking the costimulatory pathway (79). This process ultimately blocks T cell activation and proliferation because when a T cell recognizes and binds its cognate MHC-bound antigen, it requires the CD28 costimulatory signal for it to become activated. Antigen recognition in the absence of CD28 costimulation leads to a cellular state called T cell anergy, a state of functional inactivation. Thus, IL-10 is capable of directly rendering potentially potent pro-inflammatory T cells unresponsive and functionally inert (79).

On the other hand, IL-10 secreted by Treg cells can act on the antigen presenting cells, halting the progression of an immune response by limiting T cell activation. Macrophages and DCs express the IL-10R. It was shown that IL-10 was responsible for inhibiting the expression of costimulatory molecules on the surface of macrophages, leading to a dramatic decrease in the activation and proliferation of T cells when co-cultured with the macrophages (80). Likewise, there are findings demonstrating that DCs that are matured *in-vitro* in the presence of IL-10 struggle to promote a Th1 response *in-vivo*. This could be explained by a decreased expression of CD80, an important costimulatory molecule expressed by DCs used to activate T cells (81,82).

IL-10 expression is regulated transcriptionally by the transcription factors Specific protein 1 (sp1) and sp3. These along with nuclear factor kappa beta (NF-κB), STAT3 and interferon-

regulatory factor 1 (IRF1) have been found to bind the *il-10* gene and transactivate it in macrophages (83,84). Moreover, *il-10* mRNA stability plays a role in the overall levels of IL-10 protein within the cell. This means that cells potentially transcribe the *il-10* gene into messenger RNA, however translational control of gene expression ultimately regulates whether the *il-10* gene is completely expressed into a functional protein (85). This could potentially provide the Treg cells with a functional advantage to be more prepared to react to their given inflammatory environment and influence the progression of an immune response.

1.8.2 Production of the suppressive Treg cell cytokine; TGFβ

Transforming growth factor beta (TGFβ) is another critical cytokine involved in Treg cell development, maintenance and suppressive function. The protein is expressed from the *tgfβ* gene located on chromosome 19 in humans and 7 in mice is one of the most important soluble factors in Treg cell function. There exist three isoforms of the TGFβ protein, β1, β2 and β3, with β1 being the predominant isoform expressed in the immune system, however all three have similar effects *in-vitro* (86). Mature TGFβ arises from an immature precursor complex, called pre-pro TGFβ. The pre region of pre-pro TGFβ contains a signal sequence, causing pre-pro TGFβ to be cleaved at the pro region by a furin-like peptidase in the Golgi apparatus (87). Once this N terminal pro-peptide has been removed, the remaining peptide in homodimer form is called latency-associated protein (LAP). LAP non-covalently associates with a molecule of mature TGFβ and together these molecules form the small latent complex (SLC), or latent TGFβ. SLC can be secreted in this form, or it can interact with the latent TGFβ-binding protein (LTBP), which aides in shuttling TGFβ into the extracellular matrix. TGFβ must dissociate from the LAP or LTBP in order for it to be able to bind its receptor; the TGFβ receptor (88). Dissociation of TGFβ from the

LAP or LTBP can be achieved easily *in-vitro* using extreme pH, temperature as well as different proteases, however *in-vivo* the mechanisms which activate TGFβ are less clear. It has been proposed that through proteases such as transglutaminases, or by conformational changes in LAP via thrombospondin activity, latent TGFβ is activated and becomes functional (89,90).

1.8.3 Treg cells suppress inflammation through TGFβ secretion

Although TGFβ1 is produced by many leukocytes, including macrophages, DCs, B cells and CD8 T cells, the predominant producer of TGFβ are Treg cells. As its name suggests, TGFβ was first described as a growth factor for transformed (tumor) cells. In 1986, *Kehrl et al* examined the effects of TGFβ on the immune system and revealed that it had the opposite effects on T and B cells. The cytokine inhibited the proliferation of T and B lymphocytes *in-vitro*, as shown by a sever decrease in DNA production in cells cultured in the presence of TGFβ. Moreover, they showed that activated T cells up-regulated TGFβ mRNA production, indicating that T cells were the source of this anti-proliferative cytokine (96). However, it wasn't until *in-vivo* studies using mice deficient in the TGFβ gene that the importance of TGFβ production by Treg cells was described. Mice that were deficient in the TGFβ gene experienced sever inflammation after only 2 weeks of age, and displayed symptoms of wasting syndrome. These mice died by the age of 3-4 weeks, and were found to have massive infiltration of lymphocytes in major organs including the heart and lungs. This was one of the first studies to show that TGFβ was a critical mediator of immune homeostasis, where its absence lead to autoimmunity and early death (97). One of the ways that TGFβ inhibits inflammation is by suppressing the production of the the pro-inflammatory cytokine IL-2, which is pre-dominantly produced by activated T cells. It was shown that TGFβ carried out this function through direct inhibition of the IL-2 promoter. Moreover, the Smad-

binding element is located upstream of the IL-2 promoter. This element enables the Smad complex to bind and turn of transcription of the IL-2 gene. Additionally, the absence of Smad3 abrogated the blockade of IL-2 production resulting from TGFβ administration (98,99). However, since the addition of IL-2 to cultures with TGFβ-mediated inhibition of IL-2 production was unable to completely restore the pro-proliferative effect of IL-2, it meant that there were other mechanisms that TGFβ was inhibiting inflammation.

TGFβ plays a number of other roles in inhibiting immune responses. It not only discourages the production of IL-2 by T cells leading to decreased proliferation of Teff cells, but it also plays a role in T cell differentiation and survival. As previously described here, naïve T cells differentiate into T cell lineages following activation, depending on their environmental cues. The presence of cytokines like IFNγ and IL-12 promote Th1 differentiation, whereas IL-4 promotes the Th2 lineage. Many groups have shown that TGFβ inhibits the differentiation of these activated T cells into the various effector phenotypes like Th1 and Th2 cells (100). The mechanisms behind this inhibition is a bit foggy, but what is known about it is that TGFβ suppresses Teff cell differentiation through inhibition of expression of the key transcription factors required for the lineage commitment from a naïve CD4+ T cell to a Th1, Th2 or other Th lineage (101,102).

In addition to the suppression of CD4+ T cell differentiation and effector function, TGFβ also impedes the inflammatory mechanisms of CD8+ CTLs. Studies have shown that TGFβ inhibits the production of perforins and IFNγ by CTLs, and also that Treg-derived TGFβ is important for the inhibition of exocytosis of granules from CTLs, reducing their cytolytic potential (103).

To further elucidate the suppressive role of TGFβ on T cells, research has focused on blocking TGFβ signalling in T cells and observing the physiological effects *in-vivo*. For this, one

group created a mouse model where a mutated form of the TGFβRII was downstream of the CD4 promoter. This produced a CD4+ T cell-specific knockout of TGFβ signalling. Mice with this mutation developed sever autoimmunity resulting from an excessive differentiation of CD4+ T cells to the Th1 lineage. Moreover, these cells were hyperproliferative contributing to the increased immunopathology observed in these animals. However, these mice experienced significantly less immunopathology than complete body knockout of TGFβ (104). This study provides an example of the homeostatic role that TGFβ plays in the immune system, ensuring that pro-inflammatory responses don't progress out of control and result in unwanted autoimmunity.

TGFβ also plays a vital role in regulating cell survival. Although the above described roles of TGFβ have been related to reducing inflammation by inhibiting the proliferation and differentiation of inflammatory cells, TGFβ is also critical in regulating the survival of these inflammatory cells during the initiation of immune responses. When T cells recognize and interact with their specific antigen in the presence of CD28 costimulation, these cells proliferate and expand to great numbers to eliminate the foreign pathogen. TGFβ is important in this step of an adaptive immune response by inhibiting apoptosis of the proliferating T cells. Studies have shown that in mice deficient in TGFβ, peripheral T cells undergo spontaneous apoptosis (105). Moreover, TGFβ interact with IL-2 to block activation-induced cell death (AICD) of recently activated and proliferating T cells. One of the mechanisms by which TGFβ protects T cells from apoptosis is by blocking the Fas pathway, which is one of the two major pathways of apoptosis (106). These findings demonstrate that TGFβ can also promote T cell survival and effector function by impeding them from dying.

Finally, an indirect way that TGFβ inhibits inflammation is through the promotion of Treg cell generation as well as maintenance of Treg cells. As mentioned, Treg cells function to suppress immune responses. As previously discussed, TGFβ plays an integral role in promoting Treg cell generation, particularly in areas of the body rich in TGFβ, such as the gut (107), and is another way in which TGFβ has anti-inflammatory functionality (108).

1.8.4 Treg cells suppress inflammation by interacting with antigen-presenting cells

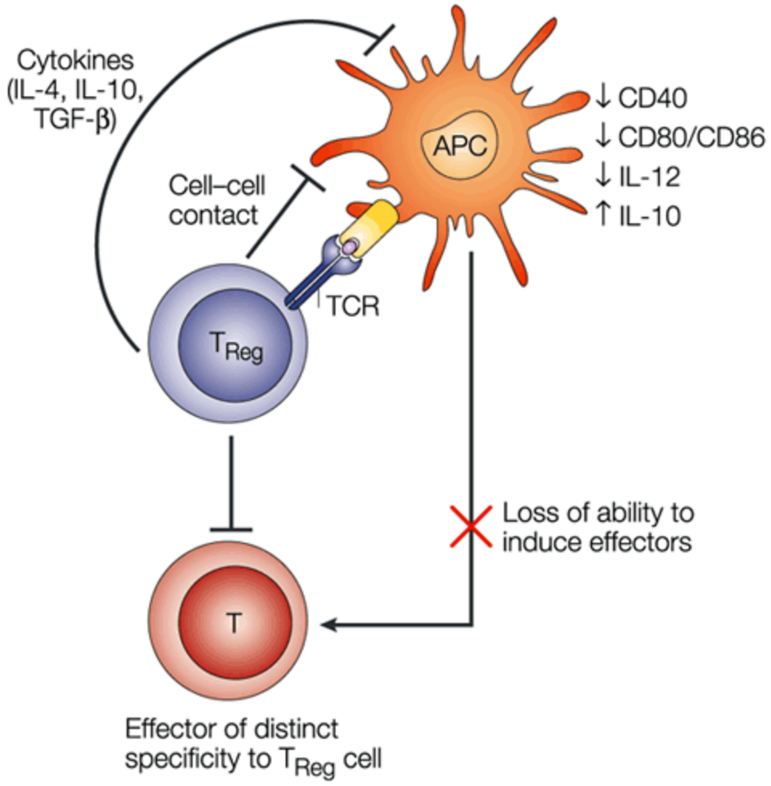
In addition to cell-cell contact, Treg cells can inhibit Teff cell activation and proliferation indirectly by interacting with the professional antigen-presenting cells (APC); dendritic cells. Tregs can down-regulate the expression of the B7 costimulatory molecules, CD80 and CD86, expressed on APCs. The B7 molecules ligate the CD28 co-receptor on T cells, an essential step in TCR activation. As previously mentioned, Treg cells express the CTLA-4 protein on their surface. CTLA-4 also ligates the CD80 and CD86 molecules on APCs, and the result of this interaction is reduced expression of the B7 molecules as well as block binding by CD28 on T cells seeking costimulation following antigen encounter. This phenomenon impedes the APCs from providing the costimulatory signals that responding Tconv cells require for activation and expansion (109). Notably, CTLA-4 has a higher affinity for the B7 molecules than does CD28, providing Treg cells with a competitive advantage to bind CD80 or CD86 over CD28. Studies using CTLA-4-deficient mice or blocking CTLA-4 have proven that Treg cells rely on this suppressive mechanism to inhibit T cell activation, where the absence of the protein led to autoimmunity (110).

Although CTLA-4 mimics the interacting ability of CD28 with the B7 molecules, the two surface proteins are very different. Unlike CD28 which spends most of its time on the surface of cells, CTLA-4 is highly endocytic, meaning it is most commonly found within intracellular vesicles.

Moreover, it is always present as a homodimer, does not undergo conformational change upon binding, and has a cytoplasmic tail that has no enzymatic function (111,112). The intrinsic signalling events that take place following ligation of CTLA-4 with the B7 molecules on APCs is unclear, and studies have suggested various downstream effects of the interaction, including disrupting Zap70 microclusters, interaction of the cytoplasmic tail of CTLA-4 with protein kinase C (PKC) as well as affecting T cell motility, where CTLA-4 increased T cell motility, reducing their interaction time with APCs (113–115). Although the cytoplasmic tail of CTLA-4 has been shown to be dispensable for Treg-mediated suppression (116), other studies have shown that mutated cytoplasmic tails inhibiting PKC interaction impairs suppression, suggesting that the cytoplasmic region of CTLA-4 is essential (114). Findings have lead to the conclusion that CTLA-4 cytoplasmic tails are involved in suppression by controlling the quantity, timing and localization of CTLA-4 molecules on the surface of Treg cells. The intracellular portion of CTLA-4 contains a tyrosine-based YVKM motif mediating endocytosis of the ligand. This has been suggested to be vital for controlling the turnover of CTLA-4 molecules, since sufficient CD28 signalling is important for Treg homeostasis. Moreover, having a pool of CTLA-4 molecules within the cell is advantageous when controlling T cell activation upon TCR ligation. This enables Treg cells to quickly block costimulation and activation of the TCR-ligated T cells (117).

Another molecule expressed by Treg cells mediating T cell suppression at the level of antigen presentation is Lag-3. Lag-3 is highly homologous to the CD4 co-receptor, but shares only 20% of the amino acids with CD4. Lag-3 binds the MHCII molecule, and much like CTLA-4 with the B7 molecules, it binds with higher affinity than its competitor (118,119). Lag-3 suppresses T cell activation and proliferation by blocking the availability of peptide-bound MHCII molecules from

TCR-specific CD4 T cells. It has been shown that Lag-3 is important for the maintenance of a tolerogenic state of CD8 T cells to self (120). Together, these are examples of how Treg cells are capable of suppressing an immune response on different levels, be it at the level of direct inhibition of pro-inflammatory T cells via suppressive cytokines, or upstream at the level of antigen presentation and T cell activation by APCs.



Von Herrath et al, 2003. Nat Rev Immunol. (121)

Figure B3. Treg cells suppress inflammation through cell-cell contact and soluble mechanisms.

1.9 Treg cells are plastic and prone to genetic reprogramming

Recently, a breadth of research has begun to explore the possibility of CD4+ T cells being more plastic than previously thought. Many researchers believe that T cells differentiate into a given lineage, and that cell remains committed to that lineage for its lifespan. However, recent findings demonstrate that CD4+ T cells are able to take on different phenotypes, with Treg cells being a prime example. Like in Treg cells, TGFβ plays a critical role in the differentiation of Th17 cells in the mucosa (122). There is therefore a dynamic balance between Th17 cells and Treg cells in a given locale, and this distribution depends on the environmental cues. It has been shown that Foxp3 is able to bind RORγt and suppress its transcription, demonstrating the counterbalance between the two populations of T cells. Moreover, mouse studies illustrated that IL-17-producing cells coming from Foxp3+ Treg cells could be found when in the presence of TGFβ (123). Other studies proved that this IL-17 secretion is dependent on the expression of RORγt expression by the Treg cells, where they can co-express the two lineage specific transcription factors. IL-6 and IL-1β, two hallmark pro-inflammatory cytokines, are critical for the genetic reprogramming of Treg cells towards Th17 cells. This indicates that Treg cells are sensitive to their microenvironment, and certain inflammatory signals can cause them to begin to express transcription factors characteristic of other T helper cell lineages. This raises the question of whether Treg cells lose Foxp3 in favour of becoming pathogenic cells to migrate to a site of inflammation, where they can then re-convert to suppressive Treg cells and inhibit or resolve inflammation. This area of research is still controversial and research is ongoing to continue to uncover the dynamic roles that Treg cells play, however one thing is certain, Treg cells are highly sensitive to their environment, and can alter their gene signature accordingly (124).

1.10 Environmental sensing and post-transcriptional regulation of gene expression in Treg cells

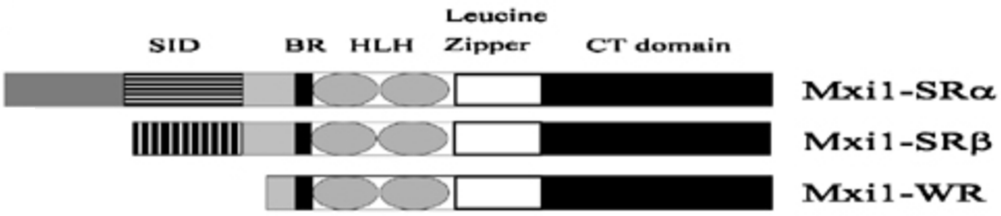
T cells are capable of sensing their surroundings and altering their gene expression program accordingly. For example, the mammalian target of rapamycin (mTOR) is a protein that integrates cues such as nutrient availability and directs T cell fates. Active mTOR promotes the pro-inflammatory cell types Th1, Th2 and Th17, while disfavouring Foxp3+ Treg cell differentiation. Blockade of the mTOR pathway restores Treg cell generation and impedes inflammatory cell differentiation (125). The necessity of quickly adapting to a given stimulus indicates that Treg cells must have distinct post-transcriptional mechanisms to ensure timely gene expression of specific genes. Micro (mi) RNAs are important mediators of post-transcriptional regulation of gene expression. It has been shown that mice with a Foxp3+ Treg cell-specific deletion of the important miRNA processing protein, Dicer, develop severe autoimmunity from loss of suppressive Treg cell function (126). Moreover, Treg cells can also induce the expression of miRNAs that promote their own maintenance. Foxp3 induces the expression of miR-155 which leads to enhanced Treg sensitivity to IL-2. This is achieved by miR-155 inhibiting the expression of SOCS1, an inhibitor of STAT5, which induces the expression of Foxp3 in response to IL-2 (127,128). Finally, Treg cells also regulate their functional mechanisms post-transcriptionally. IL-10 expression is regulated by the microRNA miR466l, which stabilizes the IL-10 mRNA and ensures its translation by inhibiting deadenylation (129). With increasing evidence that gene expression is controlled post-transcriptionally, our lab sought to investigate distinct mechanisms of post-transcriptional and translational regulation of gene expression in Treg cells. Transmitting an external signal into the nucleus, transcribing mRNA of a given set of genes, translating the mRNA into a polypeptide and folding of the protein is a lengthy process.

This could leave Treg cells at a disadvantage when they must quickly react to a given signal and promptly alter their gene expression. For this reason, we applied a genome-wide assessment of preferentially translated mRNAs in Foxp3+ Treg cells compared to Teff cells, termed a poly-ribosome (polysome) microarray. This technique isolates cytosolic mRNAs, leaving behind any mRNAs found in the nucleus that aren't ready to be translated. Moreover, cytosolic mRNAs are assessed for the presence of ribosomes actively bound to them, indicating that they are being translated into protein. We applied this method to CD4+Foxp3+ Treg cells in a resting or *in-vitro* activated state and compared their collection of preferentially-translated genes to resting or activated Teff cells. We found that activated Treg cells had subsets of genes that were preferentially translated, and that these genes could be clustered based on their physiological function. For example, certain genes involved in chromatin modification, cell cycle and protein catabolic processes were found to have higher levels of mRNAs being translated into protein in activated Treg cells. The advantage of this approach over conventional microarrays is that it separates cytosolic mRNAs that are bound by ribosomes from mRNAs either in the nucleus or not being translated, providing a more precise indication of gene expression. Among the genes found to be preferentially translated in activated Treg cells was Max-interactor 1 (Mxi1), a protein involved in cell cycle (130).

1.11 Mxi1 is a transcriptional repressor

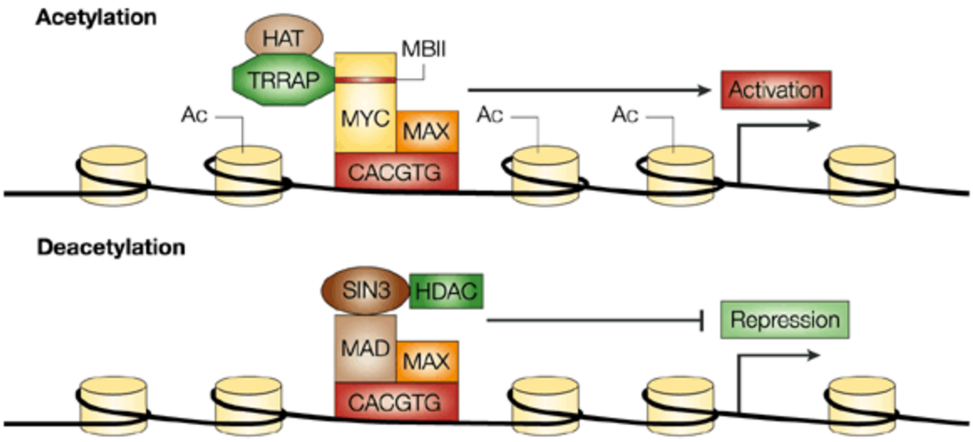
The *mxi1* gene encodes the Mad2 protein, also termed Mxi1, which belongs to the Myc-Mad-Max network. Mad proteins are basic helix-loop-helix leucine zipper (bHLHLzip) transcription factors that influence cell proliferation and differentiation (131). As a result of alternate splicing, there are two similar variants of the Mxi1 protein, the SRα and SRβ isoforms.

These proteins differ in their first exon and have similar function (132). Mxi1 dimerizes with Max via the bHLH region, and this heterodimer binds to the consensus E box element with the sequence CACGTG found on various genes (133). Mxi1 competes with the oncogenic protein Myc for interaction with Max and subsequently E boxes. Myc-Max binds the E box sequence resulting in expression of the target gene, progression of the cell cycle and proliferation. Contrarily, Mxi1-Max heterodimers repress transcription of the same target genes resulting in reduced proliferation and increased differentiation (131). *Delpuech et al, 2007* showed that the mechanism by which Mxi1 achieves this repression involves the N terminal region of Mad proteins, which contains the mSin3 interaction domain (SID) (134). mSin3 is a homolog of the *Saccharomyces cerevisiae* transcriptional co-repressor Sin3p and exists in two variants, A and B (131). mSin3A interacts with histone deacetylase 1 (HDAC1) resulting in transcriptional repression. As their name suggests, HDACs deacetylate the surrounding histones, causing the chromatin to become more compacted and blocking expression of the associated genes (135). Therefore, mSin3A binds the SID motif of Mxi1-SRα and SRβ, which are capable of binding DNA at E boxes with the help of Max, and the result is the recruitment of HDAC1 leading to histone deacetylation and gene silencing (134). There is another splice variant of Mxi1, termed Mxi1-WR, which lacks the SID motif responsible for the suppressive function of Mxi1-SRα and SRβ and therefore is not associated with transcriptional repression (136). Importantly, it has been demonstrated that prolonged exposure of epithelial cells to TGFβ lead to them expressing the Mxi1 protein (137). Since Treg cells rely on the TGFβ pathway for development, maintenance and suppression, this raises the possibility that Mxi1 could be involved in Treg cell biology through the TGFβ pathway.



Dugast-Darzacq et al, 2004. Oncogene. (136)

Figure B4. Mxi1 is alternatively spliced to create three unique mRNAs and proteins. Mxi1-SRα and SRβ contain SID motifs allowing for the recruitment of histone deacetylases. Mxi1-WR lacks this motif and repressive functionality.



Pelengaris et al, 2002. Nat Rev Cancer. (138)

Figure B5. The Myc-Mad-Max transcription factor network regulates transcription of numerous genes through the interaction with E-box sites and recruitment of histone modifiers.

Project Rationale

Our lab has demonstrated that mRNA signatures that correspond with CD4+ T cell proteomes are regulated at the translational level. *Bjur et al, 2013* identified clusters of genes that were preferentially translated in activated Foxp3+ Treg cells compared to Foxp3- Teff cells. Among these genes, Mxi1 was one that was translated 3 fold more in the Treg subset compared to the Teff subset of cells following activation. Mxi1 has been shown to be expressed in response to TGFβ in epithelial cells, and TGFβ is essential for Foxp3 induction and maintenance in Treg cells. This suggests that Mxi1 could be an important factor in Treg cell biology. The majority of research conducted on Mxi1 has been involved in cancer progression and blockade, as Mxi1 competes with the oncogene Myc. We are the first to explore the role of Mxi1 in immune cells, particularly, in the generation and homeostasis of CD4+Foxp3+ Treg cells.

Hypothesis

We Hypothesize that Mxi1-SRβ plays an important role in the generation of CD4+Foxp3+ Treg cells, and could also be implicated in the overall homeostasis of the Treg cell pool.

Specific Objectives

We will address the following questions regarding the role of Mxi1 in the generation and homeostasis of Foxp3+ Treg cells.

1. Assess the expression levels of Mxi1 in CD4+ T cell subsets *ex-vivo* and under specific activating and environmental conditions.
2. Determine the functional role of Mxi1-SRβ in CD4+ T cells *in-vitro* using a retroviral expression system to overexpress the Mxi1-SRβ isoform in CD4+ T cells.
3. Determine the functional role of Mxi1-SRβ in CD4+ T cell subsets *in-vivo* by adoptively transferring Mxi1-SRβ-overexpressing CD4+ T cells into recipient mice.

Materials and Methods

Mice

C57BL/6, WT Ly5.2.C57BL/6.Foxp3^{eGFP}, congenic Ly5.1.C57BL/6.TCR$\beta^{-/-}$ and BDC2.5.Foxp3-IRES-GFP were obtained from The Jackson laboratory. NOD.TCR$\alpha^{-/-}$ were a generous gift from C. Benoist (Harvard University). All mice were maintained in SPF conditions at RI-MUHC and used between the ages of 8 and 12 weeks of age.

Antibodies and Flow Cytometry

Single cell suspensions prepared from different organs were stained and acquired on BD Fortessa or Fortessa X20 and analyzed with FlowJo software. Surface phenotyping was performed with the following fluorochrome-conjugated or biotinylated Abs: anti-mCD4 (GK1.1), anti-mCD45.2 (104) from eBioscience, anti-mVβ4 (KT4) and anti-hCD8 (RPA-T8) from BD. The expression of Mxi1-SRα (ARP36812_P050) (Avivasysbio, San Diego, CA), Mxi1-SRβ (AF4185) (R&D systems, Burlington, ON), Foxp3 (FJK-16s), Helios (B56) (BD, San Jose, CA) was determined by intracellular staining performed according to the manufacturers' protocols. To assess cytokine production, T cells were re-stimulated for 3.5 hours at 37°C with PMA (20ng/ml), ionomycin (1nM) (Sigma-Aldrich, Oakville, ON) and BD GolgiStop^{TM} (1:1000) and then stained intracellularly with IL-2 (JES6-5H4), TNFα (MP6-XT22), IFNγ (XMG1.2) from eBiosience.

Cell Purification

For *in-vitro* assays, $CD4^+$ T cells were purified from splenocytes and LN cells based on CD4 expression (L3T4) (Miltenyi Biotech, San Diego, CA) using the autoMACS cell sorter (Miltenyi Biotech, San Diego, CA) from C57BL/6 or congenic Ly5.2.$Foxp3^{eGFP}$ mice. Specifically, $CD4^+$ T cells (>90% purity) were obtained by positive selection. Irradiated T-depleted feeder cells were isolated from splenocytes and LN cells based on CD4 expression and separated by negative selection using the autoMACS cell sorter (Miltenyi Biotech, San Diego, CA). For adoptive transfer studies and *in-vitro* suppression assays, T cell subsets were purified from splenocytes and LN cells using a FACSAria cell sorter (>98% purity). $CD4^+Foxp3^-$ Teff and $CD4^+Foxp3^+$ Treg cells were isolated from congenic Ly5.2.$Foxp3^{eGFP}$ mice, $CD4^+Foxp3^-$hCD8t$^+$ T cells were isolated from congenic Ly5.2.$Foxp3^{eGFP}$ mice and BDC2.5.Foxp3-IRES-GFP mice.

***In-vitro* proliferation assays**

In-vitro proliferation assays were performed by culturing 5×10^4 CFSE (1:10,000) or v450 (1:1000) proliferation dye-labelled CD4+ T cells in 96-well flat-bottom microtiter plates in complete RPMI 1640 (Gibco, Waltham, MA) supplemented with 10% heat-inactivated FBS, with 2×10^5 irradiated T-depleted feeder cells and 1μg/ml of soluble anti-CD3 mAb at 37°C for 72 hours.

Retroviral overexpression studies

Mxi1-SRβ overexpression experiments were preformed by viral transduction with MSCV-Mxi1-SRβ-IRES-hCD8t retrovirus particles. Briefly, Mxi1-SRβ was removed from the parent vector by PCR, and cloned into the MSCV-IRES-hCD8t retroviral vector using NotI-HF and XhoI restriction

sites, a generous gift from Dr. Connie Krawczyk (McGill University). The Mxi1-SRβ sequence was confirmed by Sanger sequencing. Viral particles were produced by transfection of 293T cells with MSCV-Mxi1-SRβ-IRES-hCD8t vector and pCL-Eco retrovirus packaging vector (addgene) and Lipofectamine 3000, according to the manufacturer's protocol.

Adoptive T cell transfers

FACS-sorted $CD4^+Foxp3^-$, $CD4^+Foxp3^+$ and $CD4^+Foxp3^-hCD8t^+$ T cells from congenic $Ly5.2.Foxp3^{eGFP}$ donor mice were transferred intravenously (i.v.) into $Ly5.1.C57BL/6.TCR\beta^{-/-}$ recipient mice at the indicated numbers. 3×10^5 FACS-sorted $CD4^+Foxp3^-hCD8t^+$ T cells from BDC2.5.Foxp3-IRES-GFP donor mice were transferred i.v. into $NOD.TCR\alpha^{-/-}$ recipient mice. Blood glucose levels were determined each day thereafter with Hemoglucotest Kits (Roche Diagnostics, Montreal, QC) and T1D was diagnosed when 2 consecutive values > 20 mM.

Statistical Analysis

Results are expressed as Mean ± SD. To determine whether results were statistically significant, the Student's *t* test or, in some group comparisons, 2-way ANOVA analyses were performed. For p values: *<0.05, **<0.01, ***<0.001, ****<0.0001.

Results

Activated and memory CD4+Foxp3+Treg cells express high levels of Mxi1-SRβ.

In order to examine differential gene expression at the post-transcriptional and translational level, we applied a genome-wide polysomal microarray approach comparing translation of mRNA into protein in resting and activated Foxp3-Teff and Foxp3+Treg cells. We found that certain clusters of genes were preferentially translated in activated Treg cells compared to activated Teff cells (130). Max-interactor 1 (Mxi1) was among these genes that were highly translated in activated Treg cells (Fig 1A). We sought to determine whether the same preferential expression of Mxi1 could be observed at the protein level on a per cell basis. We developed a multiparametric flow cytometric approach to measure Mxi1-SRα and SRβ isoform expression in lymphocytes by using two polyclonal antibodies. One antibody recognized the unique N terminus of the SRα isoform, and the second recognized the shared middle region of both the SRα and SRβ isoforms (supplemental Fig 1A). This approach allowed us to examine the differential expression of the Mxi1-SRa and Mxi1-SRβ in CD4+ T cell subsets by flow cytometry (Fig 1B). We sought to determine whether the same preferential expression of Mxi1 at the translational level was observed at the protein level. For this we assessed Mxi1 expression in memory and naïve Treg cells. Memory T cells have experienced their cognate antigen and undergone clonal expansion, therefore allowing us to determine the expression profile of Mxi1 within previously-activated T cells. In the MLN, Mxi1-SRβ, but not SRα was found to be expressed at a significantly greater level in memory Treg cells compared to naïve Treg cells ($p<0.05$) (Fig 1C).

This lead us to measure the expression profile of Mxi1 in *in-vitro* activated Treg cells. Throughout a 96 hour activation assay, both Mxi1-SRα and SRβ expression peaked between 48 and 72 hours post-activation. In concurrence with the increased expression in memory Treg cells we saw, it was the Mxi1-SRβ isoform that was preferentially expressed in *in-vitro*-activated Treg cells to a significant level ($p<0.01$ at 48 hours and $p<0.05$ at 72 hours post-activation). These results suggest that Mxi1-SRα and Mxi1-SRβ could have mutually exclusive roles in Treg cell biology, with the SRβ isoform being highly expressed in activated Foxp3+Treg cells.

Mxi1-SRβ expression is increased in proliferating CD4+ T cells.

One of the results of T cell activation from antigen experience is clonal expansion. Since we found Mxi1-SRβ to be highly expressed in activated Treg cells, and Mxi1-SRβ has been previously described as an anti-proliferative transcription factor (139), we asked whether Mxi1-SRβ was linked to T cell proliferation following activation. For this we assessed Mxi1-SRβ expression in both resting and proliferating CD4+ T cells using KI67 as a marker of proliferation. Indeed, compared to resting CD4+ T cells, both proliferating Foxp3-Teff and Foxp3+Treg cells expressed significantly greater levels of Mxi1-SRβ protein directly *ex-vivo* ($p<0.001$). There was no significant difference in Mxi1-SRβ expression between proliferating Teff and proliferating Treg cells.

The primary effector function of Treg cells is to suppress the expansion of pro-inflammatory Foxp3-Teff cells. To achieve this, Treg cells must be in the activated state and proliferating. We asked whether Mxi1-SRβ expression is elevated in proliferating Treg cells during

suppression. To this end, we performed an *in-vitro* suppression assay with FACS-sorted Foxp3-Teff cells labeled with CFSE proliferation dye as responders, T-depleted irradiated lymphocytes as feeder cells for costimulation and Foxp3+Treg cells in varying ratios of Treg:Teff from 1:1 to 1:8. At a ratio of 1:1 Treg:Teff, Teff proliferation was suppressed almost completely. As the number of Treg cells diminished, the suppression diminished accordingly (Fig 2B left panel). When gating on the responding Teff cells and Treg cells in culture and assessing Mxi1-SRβ expression within these populations, the expression of Mxi1-SRβ increased in both Teff and Treg cells as the number of Treg cells decreased. Moreover, Mxi1-SRβ remained significantly higher in the Treg cells than Teff cells at most ratios (Fig 2B, bottom panel). When correlating the Mxi1-SRβ expression within the Treg cell population at each Treg:Teff ratio with the level of Treg cell proliferation (measured by KI67), it was evident that Mxi1-SRβ expression followed the level of proliferation of the Treg cells.

In order to better assess the link between Mxi1-SRβ expression and cellular proliferation, we applied an *in-vivo* model of T cell activation resulting in cellular expansion. FACS-sorted donor GFP-Teff cells from WT Ly5.2.C57BL/6.Foxp3^{eGFP} mice were adoptively transferred into congenic Ly5.1.C57BL/6.TCRβ mice intravenously. The result of this T cell transfer is T cell activation and expansion resulting from the lymphopenic environment within the recipient animals. 14 days post-transfer, the WT cells were harvested from the spleen, PLN, MLN and colon of the recipients, and Mxi1-SRβ expression was assessed alongside proliferation (KI67). Compared to the resting (KI67-) Foxp3-Teff cells, Mxi1-SRβ expression was increased in the proliferating Teff cells. Moreover, in this mouse model, some Foxp3-Teff cells encountered certain microenvironments, in the MLN and colon, forcing them to turn on Foxp3 expression and become peripherally-

induced Treg (pTreg) cells. Notably, within this subset of proliferating pTreg cells, Mxi1-SRβ expression was greater than in resting pTreg cells (data not shown) and, to a significant degree, greater than in resting Foxp3-Teff cells (Fig 2C). Together these data suggest that Mxi1-SRβ is not only closely linked to cellular activation and proliferation of T cells, but that it also may play an additional role in the generation of pTreg cells.

Mxi1-SRβ overexpression reduces the production of pro-inflammatory cytokines in CD4+Foxp3-Teff cells in-vitro and in-vivo.

One of the main effector functions of CD4+ Teff cells during an immune response is the production and secretion of pro-inflammatory cytokines, such as IL-2, TNFα and IFNγ. In order to produce these proteins, T cells must be actively proliferating. Given our data revealing the link between Mxi1-SRβ expression and proliferation, we asked whether Mxi1-SRβ overexpression in Foxp3-Teff cells could impede the production of pro-inflammatory cytokines. To test this, we cloned the Mxi1-SRβ cDNA into a murine stem cell virus (MSCV) retroviral overexpression system to force the ectopic expression of Mxi1-SRβ protein in target cells. CD4+ T cells were activated plate-bound with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml), transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus and kept in culture for 6 days. Cells were re-stimulated with PMA and ionomycin in the presence of GolgiStop^{TM} for 3.5 hours at 37°C. Compared to the empty vector (EV) transduced cells, a significantly smaller frequency of Mxi1-SRβ overexpressing cells produced IL-2 ($p<0.0001$), TNFα ($p<0.01$) and IFNγ ($p<0.01$) (Fig 3A, bottom panel). Moreover, a significantly

smaller total number of cells were found to be producing the pro-inflammatory cytokines (Fig 3A, right panels).

With this we used the *in-vivo* model described above to further test the effects of Mxi1-SRβ overexpression on the production of pro-inflammatory cytokines by CD4+ T cells. Mxi1-SRβ overexpressing WT GFP-T cells were FACS-sorted 7 days post-transduction and adoptively transferred into congenic Ly5.1.C57BL/6.TCR$\beta^{-/-}$ mice intravenously. 14 days post-transfer, a smaller frequency of cells overexpressing Mxi1-SRβ were producing TNFα ($p<0.001$) and IFNγ ($p<0.05$) in the colon; a homing site for Teff cells in this model (Fig 3B middle panels). There was also a significant reduction in the amount of TNFα ($p<0.01$) and IFNγ ($p<0.001$) being produced per cell in cells receiving the Mxi1-SRβ virus compared to the EV (Fig 3B, bottom panels). These findings indicate that increased Mxi1-SRβ expression hinders the pro-inflammatory phenotype of Foxp3-Teff cells.

Mxi1-SRβ expression is increased in peripherally-induced CD4+Foxp3+ Treg cells in-vitro and in-vivo.

With Mxi1-SRβ being expressed to a greater extent in activated total Treg cells, and overexpression studies indicating that Foxp3- T cells become less inflammatory with increased Mxi1-SRβ expression, we asked whether Mxi1-SRβ was implicated in the induction of pTreg cells. We performed an *in-vitro* Foxp3 induction assay using FACS-sorted GFP-T cells from C57BL/6.Foxp3^{eGFP} mice. Foxp3 was induced with the addition of TGFβ (5ng/ml) for 4 days. Compared to cells that did not up-regulate Foxp3 following the addition of TGFβ, induced Treg

cells expressed significantly more Mxi1-SRβ ($p<0.0001$) (Fig 4A, bottom panel). We then tested this question *in-vivo,* by adoptively transferring FACS-sorted GFP- T cells from WT Ly5.2.C57BL/6.Foxp3^{eGFP} mice into congenic Ly5.1.C57BL/6.TCRβ$^{-/-}$ lymphopenic mice. As previously described, some Foxp3- T cells experience microenvironments that favour the induction of Foxp3 expression. Particularly, the MLN is a site with high levels of TGFβ where a large frequency of Foxp3 induction occurs, and these pTreg cells traffic to the colon (data not shown). CD4+ T cells that induced Foxp3 in this system expressed more Mxi1-SRβ in all organs assessed compared to CD4+ T cells that did not (Fig 4B, bottom panels).

To determine whether TGFβ signalling is contributing to the increase in Mxi1-SRβ expression in pTreg cells, we pulsed total CD4+ T cells with TGFβ (0.1ng/ml) for 24 hours and measured Mxi1-SRβ expression. TGFβ administration resulted in a decrease in Mxi1-SRβ expression in Foxp3- Teff cells between 0 and 24 hours. Conversely, TGFβ pulsing significantly increased the expression of Mxi1-SRβ in Treg cells compared to Teff cells at 24 hours (Fig 4C, right panel). Together, these data demonstrate that Mxi1-SRβ expression is increased in pTreg cells and TGFβ signalling contributes to this increase.

Mxi1-SRβ overexpression impedes cellular proliferation of CD4+ T cells in-vitro and in-vivo.

In order to further investigate the relationship between Mxi1-SRβ and cellular proliferation of CD4+ T cells, we performed proliferation studies with Mxi1-SRβ-overexpressing CD4+ T cells to determine whether Mxi1-SRβ was functioning to inhibit proliferation. To this end, we isolated total CD4+ T cells from C57BL/6 mice and labelled the cells with CFSE proliferation

dye. The cells were activated and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t or EV as previously described and their proliferation was tracked for 96 hours. The division index (D.I.), indicating the average number of divisions each cell in the culture went through, was calculated after 48 hours and each 24 hours thereafter. For each time point assessed, the D.I. of CD4+ T cells overexpressing Mxi1-SRβ was significantly less than the cells transduced with EV ($p<0.001$ at 48 hours, $p<0.01$ at 72 hours, $p<0.001$ at 96 hours) (Fig 5A, right panel). In addition, WT Foxp3-hCD8t+ T cells were FACS-sorted and adoptively transferred into congenic C57BL/6.TCRb$^{-/-}$ mice, and proliferation was assessed by KI67 staining 14 days post-transfer. In accordance with the previous *in-vitro* studies, CD4+ T cells overexpressing Mxi1-SRβ were less proliferative than CD4+ T cells transduced with EV in all organs assessed ($p<0.05$ in spleen, $p<0.001$ in PLN, $p<0.0001$ in MLN) (Fig 5B, bottom panels). Thus, Mxi1-SRβ functions as an inhibitor or CD4+ T cell proliferation.

Mxi1-SRβ overexpression enhances the generation of peripherally-induced Foxp3+ Treg cells in-vitro and in-vivo.

Since we found that Mxi1-SRβ hinders the pro-inflammatory nature of Foxp3- Teff cells, and it is expressed to a greater extent in Foxp3+ Treg cells, we tested whether Mxi1-SRβ overexpression could enhance the conversion of Foxp3- Teff cells to Foxp3+ Treg cells *in-vitro* and *in-vivo.* To this end, we first performed an *in-vitro* TGFβ-mediated Foxp3 induction on FACS-sorted GFP- T cells from C57BL/6.Foxp3^{eGFP} mice. GFP- T cells were activated and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus or EV, and at 24 hours, TGFβ (5ng/ml) was administered to the

culture. Foxp3+ frequency was assessed 3 days post- TGFβ addition. There was a stark difference in the frequency of Foxp3+ Treg cells between the EV (34.9%) and the Mxi1-SRβ (69.7%) transduced Teff cells ($p<0.001$) (Fig 6A, bottom panel). Moreover, in our *in-vivo* model with adoptively transferred WT CD4+Foxp3-hCD8t+ T cells in congenic Ly5.1.C57BL/6.TCRβ$^{-/-}$ lymphopenic mice, there was a significant increase in the frequency of Foxp3+ T cells in the MLN ($p<0.01$) and colon ($p<0.05$) in Mxi1-SRβ overexpressing cells after 14 days *in-vivo* (Fig 6B, bottom panel). These findings indicate that Mxi1-SRβ is potentially involved in the conversion of conventional Foxp3- Teff cells into Foxp3+ Treg cells.

RORγt-expressing CD4+ Teff and Treg cells express high levels of Mxi1-SRβ.

With evidence that TGFβ induces the expression of Mxi1-SRβ in Treg cells, we asked whether TH17 cells also have increased expression of Mxi1-SRβ. Since, much like Treg cells, Th17 cells rely on TGFβ signalling for differentiation, we measured the expression of Mxi1-SRβ in RORγt+Foxp3- Th17 cells *ex-vivo* by flow cytometry. We found that the RORγt+Foxp3- cells expressed greater levels of Mxi1-SRβ protein compared to the RORγt-Foxp3- Teff cells. We also measured the expression of Mxi1-SRβ in CD4+ T cells co-expressing RORγt and Foxp3. These are Treg cells who are undergoing genetic reprogramming to express the TH17-specific transcription factor RORγt, or vice versa. Interestingly, we found that the RORγt+Foxp3+ Treg cells expressed significantly more Mxi1-SRβ ($p<0.001$) than the RORγt-Foxp3+ Treg cells. These findings suggest that Mxi1-SRβ expression is elevated not only in Treg cells, but in other TGFβ-relevent CD4+ cell types (Fig 7A, right panel).

Mxi1-SRβ overexpression enhances the expression of RORγt in both CD4+ Teff and Treg cells in-vivo.

After discovering that Mxi1-SRβ is also expressed at high levels in RORγt+ compared to RORγt- T cells, we tested whether ectopic expression of Mxi1-SRβ could help promote Th17 cell differentiation *in-vivo*. For this we used the adoptive transfer of Mxi1-SRβ-overexpressing CD4+GFP- T cells method described above, and 14 days post-transfer we harvested the spleen, mesenteric lymph node and colon of recipient mice. Compared to the empty vector transduced Teff cells, Teff cells transduced with Mxi1-SRβ experienced greater differentiation towards the Th17 cell type in all 3 organs. The colon harbored the greatest difference in the frequency of RORγt+ Teff cells between emtpy vector and Mxi1-SRβ-transduced T cells. Moreover, greater than 50% of the peripherally-induced Treg cells found in the colon had also up-regulated RORγt following Mxi1-SRβ overexpression compared to under 20% for the empty vector controls (Fig 8A, middle right panels). Finally, both the absolute numbers of RORγt+Foxp3- Teff cells as well as RORγt+Foxp3+ Treg cells in the colon had increased significantly ($p<0.05$, $p<0.01$, respectively) following Mxi1-SRβ overexpression compared to their empty vector controls (Figure 8A, bottom right panels). These findings suggest that Mxi1-SRβ plays a role in TGFβ-mediated cellular differentiation in both Treg and Th17 cell types.

Mxi1-SRβ overexpression delays Type 1 diabetes onset in-vivo.

Finally, with the above findings indicating that Mxi1-SRβ not only reduces the pro-inflammatory potential of Foxp3- Teff cells, but enhances the conversion of these cells to Foxp3+ Treg cells, we asked whether Mxi1-SRβ overexpression could delay the onset of T1 diabetes in mice. For this we overexpressed Mxi1-SRβ in CD4+ T cells from NOD.BDC2.5 mice, a transgenic who's TCR repertoire is restricted to the chromogranin A protein expressed in the β islets of the pancreas, and adoptively transferred FACS-sorted CD4+Foxp3-hCD8t+ Teff cells into lymphopenic NOD.TCRa$^{-/-}$ mice. The blood glucose of the recipient mice was measured each day following transfer. 83.3% of the mice receiving EV-transduced Teff cells developed diabetes within 10 days post-transfer. Conversely, only 14.3% of the mice receiving Mxi1-SRβ overexpressing Teff cells developed diabetes within the 17 day time frame, and the mouse that did develop disease developed it at day 16 (Fig 9A, bottom panels). When analyzing the total recovery of transferred cells, we found that significantly less cells accumulated in the pancreas of mice receiving Mxi1-SRβ overexpressing Teff cells compared to EV-transduced cells, indicating that, like previously shown, Mxi1-SRβ hindered the proliferative capacity of the Teff cells (Fig 9B, right panel). An assessment of pro-inflammatory cytokine production by transferred cells within the pancreas of recipient mice revealed that there were significantly less cells producing TNFα ($p<0.01$) and IFNγ ($p<0.01$) when overexpressing Mxi1-SRβ compared to EV-transduced cells (Fig 9C). Interestingly, in a system that does not normally harbour a high frequency of pTreg cells, mice receiving Mxi1-SRβ overexpressing Foxp3- Teff cells had a greater frequency of Foxp3+ pTreg cells than mice

receiving EV-transduced cells ($p<0.05$) (Fig 9D). Together, these results demonstrate that Mxi1-SRβ controls cellular expansion and effector function, as well as promotes the generation of Foxp3+ pTreg cells *in-vivo*.

Discussion

The ability of immune cells to quickly sense and react to external stimuli is a necessity to ensure robust and appropriate protection against infiltrating pathogens. For Teff cells, this means upregulating the production of pro-inflammatory proteins like IFNγ and TNFα. However, unlike Teff cells, Treg cells function to actively suppress immune responses, be it to self or following pathogen clearance to restore immunological homeostasis. For this, Treg cells sense incoming signals in the local milieu and once activated, respond by producing anti-inflammatory mediators like IL-10, TGFβ and various surface proteins. We used a genome-wide microarray analysis of cytosolic mRNAs bound by ribosomes, indicating active translation into protein, to study differential gene expression between *in-vitro* activated Teff and Treg cells. This revealed that activated Treg cells preferentially translate clusters of genes compared to Teff cells. Among these genes, Mxi1 was translated significantly more (3-fold) in activated Treg cells compared to activated Teff cells. We demonstrated for the first time that the expression of clusters of functionally-related genes in activated Treg cells is regulated at the post-transcriptional level. Unlike the central dogma, where differential gene expression is assessed at the transcriptional level with the production of *de-novo* mRNAs, we suggest that Treg cells have a stock of mature mRNAs that are readily available for translation if that protein is required at a given time during an immune response.

The Mxi1 protein exists in three different forms: the SRα isoform, SRβ isoform and WR isoform. The SRα and SRα isoforms each contain the SID, which recruits histone deacetylases and represses transcription. The WR isoform lacks the SID, and therefore lacks the strong repressive functionality. For this reason we began by analyzing expression of the Mxi1-SRα and

SRβ isoforms in Treg cells under homeostatic conditions. Upon analyzing the expression of Mxi1 in resting Treg cells taken directly *ex-vivo* we found that Mxi1-SRβ was preferentially expressed in the subset of Treg cells displaying a memory phenotype ($CD62L^{lo}CD44^{hi}$), but not Mxi1-SRα. A memory phenotype demonstrates a terminally-differentiated cell that has previously been activated during an immune response. This indicates that only the SRβ isoform is involved with the production of terminally-differentiated Treg cells, and its expression remains elevated in these cells. These findings corroborate with previous research conducted looking at the involvement of Mxi1 in the generation of terminally-differentiated cells, and take it a step further in identifying the unique isoform contributing to terminal differentiation. The beginnings of Mxi1 research linked terminal differentiation with Mad levels in various cell types including adipocytes, epithelial cells, myeloid cells and even neurons. These studies showed that unlike the competing Max-interactor; Myc, which was found at elevated levels in proliferating cells, Mxi1 was expressed to a greater extent in differentiating cells (140). In line with this, we showed that the terminally-differentiated Treg cells of the memory subset expressed increased levels of Mxi1, and further described this expression to be exclusive to the SRβ isoform.

The microarray data suggesting that Mxi1 is preferentially translated in activated Treg cells identified the Mxi1 gene only, not the particular isoforms of Mxi1 that are highly expressed in the TCR-activated Treg cells compared to Teff cells. To determine whether one Mxi1 isoform protein was found at greater levels in Treg cells, we activated CD4+ T cells *in-vitro* and monitored Mxi1-SRα and SRβ in the Foxp3- and Foxp3+ subsets. We demonstrated that it was the Mxi1-SRβ isoform that was preferentially expressed in activated Treg cells, as the Foxp3+ subset displayed significantly greater Mxi1-SRβ protein levels than the Foxp3- subset. Moreover, Mxi1-SRα was

found at similar levels in both the Foxp3- and Foxp3+ T cell subsets. These findings suggest that the microarray data showing increased translation of Mxi1 mRNA into protein could be attributed to spliced mRNA generating the Mxi1-SRβ isoform of the Mxi1 gene. Additionally, we demonstrated that during an *in-vitro* suppression assay using purified Treg and Teff cells, the expression level of Mxi1-SRβ increased as the number of Treg cells decreased. Moreover, we showed that the proliferation of these Treg cells correlated positively with Mxi1-SRβ expression. One possible explanation for why Mxi1-SRβ expression was increased as the number of Treg cells decreased, and correlated very well with the proliferative rate of the Treg cells, is provided by early research conducted on Mxi1 and its link to cellular proliferation and differentiation. *Zervos et al, 1973* demonstrated that proteins belonging to the *mad* family genes, Mxi1 being one of these, are expressed during proliferation. They suggested that Mad proteins are differentially regulated to antagonize or modulate the effects of Myc during periods of cellular proliferation (141). Indeed, it can be seen in our data that this is occurring. As the number of Treg cells decreases in an *in-vitro* suppression assay, the Treg cells are forced to proliferate at a greater rate in an attempt to suppress increased Teff proliferation. Therefore, the transcriptionally-suppressive transcription factor, Mxi1-SRβ, is called upon to impede the cellular proteins driving proliferation. Myc is a positive regulator of cell cycle and proliferation, and is more than likely expressed in the proliferating Treg cells. Mxi1-SRβ expression is thus increased in an attempt to counteract the proliferative effects of Myc.

Another possible explanation for why Mxi1-SRβ was expressed to a greater extent in activated Treg cells is that the pool of Treg cells is kept low early during inflammation to enable pro-inflammatory T cells to properly expand and eliminate a pathogen. The *in-vitro* suppression

assay also supports this possibility. The pool of Treg cells is smaller early during an immune response, and therefore the expression of Mxi1-SRβ is elevated following activation to prevent them from expanding prematurely and limiting the ability of the pro-inflammatory T cells to clear the pathogen. During the course of the immune response IL-2 is produced to a high level by activated Teff cells (142). Treg cells require IL-2 to proliferate, and perhaps this increase in available IL-2 causes Mxi1-SRβ expression to fall and allows Treg cells to proliferate. The end result is the suppression of Teff cell proliferation by activated Treg cells, which have now accumulated to larger proportions due to a decrease in Mxi1-SRβ expression.

We performed a preliminary assessment of the effects of IL-2 on Mxi1-SRβ expression in Treg cells (Data not shown). We activated Treg cells in the presence of increasing levels of exogenous IL-2 and measured Mxi1-SRβ expression 24 hours post-activation. We found that as the concentration of IL-2 increased, the expression of Mxi-SRβ decreased. These findings support the possibility that Mxi1-SRβ plays a role in the population size of Treg cells through controlling their proliferation. As previously mentioned, IL-2 is required for Treg cell expansion, and in our findings using Mxi1-SRβ overexpression, Mxi1-SRβ impedes the proliferation of Teff cells *in-vitro* and *in-vivo*. Therefore, as the concentration of IL-2 increases during the course of an immune response, Mxi1-SRβ expression decreases allowing Treg cells to expand and regulate the expansion of the Teff cells. The initial microarray data also supports this interpretation. In this assay, purified Treg and Teff cells were cultured in the presence of IL-2. Since Treg cells require IL-2 for Foxp3 expression maintenance as well as proliferation, they take up the surrounding IL-2 but cannot make their own, so the overall concentration of IL-2 decreases with time and Mxi1-SRβ expression increases. When the purified Teff cells are activated, they also use up the

exogenous IL-2. However, Teff cells produce and secrete IL-2 following activation, so the concentration of IL-2 is elevated compared to the purified Treg cells and this drives the expression of Mxi1-SRβ down in the Teff cells.

The majority of the literature surrounding Mxi1 is involved in tumorigenesis and cancer progression (143,144). Mutations in the Mxi1 gene lead to tumor formation due to increased heterodimerization of Myc-Max complexes, driving proliferation. Moreover, Myc expression has been proven to be essential for proper T cell priming by DCs (145). The hallmark function of Treg cells is to provide anti-inflammatory signals to surrounding immune cells and inhibit inflammation. Thus, Treg cells don't express the pro-inflammatory cytokines such as IFNγ and TNFα to the same extent as other T helper cells like Th1 cells. In our overexpression system, where we overexpressed the Mxi1-SRβ protein in Teff cells, we demonstrated that a significantly smaller proportion of these cells expressed pro-inflammatory cytokines like IFNγ, TNFα and IL-2, and the level of expression on a per-cell basis was also significantly diminished compared to empty vector controls. These results were demonstrated both *in-vitro* and *in-vivo*, and they suggest that Mxi1-SRβ could be contributing to the anti-inflammatory nature of Treg cells. Ectopic expression of the protein could force conventional, highly inflammatory, CD4+ T cells to become less inflammatory, much like the phenotype of Treg cells. We also demonstrated that overexpression of the Mxi1-SRβ isoform in Teff cells also significantly decreased their proliferative rate both *in-vitro* and *in-vivo*. This could also be contributing to the decrease in cytokine production we saw since T cells need to be actively proliferating in order to produce cytokines (146). Mxi1-SRβ could be suppressing the expression of the pro-inflammatory cytokines assessed here at the transcriptional level, if these genes possess an E-box in their promoter

regions. On the other hand, the reduction in cytokine production could be an indirect result of increased Mxi1-SRβ levels in these Teff cells. To elaborate, maybe since the Teff cells are less proliferative, they are unable to produce cytokines to the same extent as those transduced with the empty vector control, which proliferate normally. Whether a direct effect on the expression of pro-inflammatory cytokine production or resulting from an overall decreased proliferative capacity of Teff cells overexpressing Mxi1-SRβ, we demonstrated that Mxi1-SRβ overexpression inhibited the production of pro-inflammatory cytokines.

Interestingly, we found that Mxi1-SRβ was expressed more in peripherally-induced Treg cells than in thymic-derived Treg cells. Our microarray study that first described Mxi1-SRβ to be preferentially-translated in Treg cells was done primarily using thymic-derived Treg cells as they were taken from mouse spleen which comprises primarily tTreg cell. When comparing the Mxi1-SRβ protein levels between pTreg cells and tTreg cells, it was the pTreg cells that expressed more of the protein. The initial poly-ribosomal microarray demonstrated that tTreg cells harbored a pool of Mxi1 mRNA that were readily translated to protein following activation. However, we investigated the possibility that Mxi1-SRβ also played an important role in the generation of peripherally-induced Treg cells.

Mxi1 has been described to be expressed in cells undergoing terminal differentiation (140). Since we found that Mxi1-SRβ was expressed greater in pTreg cells compared to tTreg cells, we hypothesized that Mxi1-SRβ could be involved in the differentiation of Teff cells to pTreg cells. Since tTreg cells are stable expressers of Foxp3, and have a stable Treg phenotype, it could be possible that these non-differentiating cells don't express as much Mxi1-SRβ, as they are already programmed to the Treg lineage. Overexpressing Mxi1-SRβ prior to performing an *in-vitro* Foxp3

induction assay resulted in a drastic increase in the frequency of Teff cells converting to Foxp3+ iTreg cells. We tested whether overexpressed Mxi1-SRβ lead to a direct increase in the level of expression of Foxp3 in the Teff cells converting to Treg cells, however this was not the case, as both the empty vector and Mxi1-SRβ-transduced iTreg cells expressed similar levels of Foxp3. Moreover, we examined the expression of various Treg cell markers to see if Mxi1-SRβ was inducing the expression of these markers (not shown here). Again, the overexpression did not lead to increased expression of any of the Treg markers tested. This indicates that Mxi1-SRβ could be playing an indirect role in enhancing the *in-vitro* induction of Foxp3 in Teff cells. We tracked the proliferation of the cells during this *in-vitro* induction assay, and found that the Mxi1-SRβ transduced Teff cells that converted to Treg cells were less affected by the anti-proliferative nature of Mxi1 compared to the Teff cells that did not up-regulate Foxp3. This means that the increased frequency of induced Treg cells could be due to a more drastic proliferative deficiency in the Teff cells compared to the iTreg cells.

Interestingly, we also found that TGFβ could stimulate the expression of Mxi1-SRβ in tTreg cells. The previous findings of increased Foxp3 induction following Mxi1-SRβ overexpression suggested that the increase in iTreg cells was an indirect result of a proliferative advantage of the induced Treg cells over the Teff cells. However, when pulsed with TGFβ for 24 hours, Treg cells up-regulated the expression of Mxi1-SRβ, whereas Teff down-regulated the protein. This suggests that Mxi1-SRβ could play a distinct role in Treg cells compared to Teff cells following TGFβ exposure. TGFβ is the critical cytokine for Foxp3 induction and maintenance of expression in Treg cells. The pool of Treg cells assessed for this experiment was taken from the spleen, meaning it consists of both tTreg cells and pTreg cells. Therefore, there are two

possibilities here, the first being that Mxi1-SRβ expression could be linked to the maintenance of Foxp3 expression in tTreg cells. The second possibility is that it is the pTreg cells that are accounting for this increased Mxi1-SRβ expression following TGFβ stimulation. This would suggest that perhaps the Mxi1-SRβ is involved in the pathway of TGFβ-mediated induction of Foxp3. In order to further delineate which of these two possible scenarios are occurring, the experiment would need to be repeated using Helios expression as an indicator of tTreg cells, so that the level of Mxi1-SRβ expression could be measured in tTreg cells and pTreg cells independently. The findings from this experiment also support the possibility that it is not just a proliferative advantage of iTreg cells that leads to an increased frequency of Foxp3+ iTreg cells following Mxi1-SRβ overexpression. Perhaps Mxi1-SRβ is involved in the signalling pathway of TGFβ during Foxp3 induction. Since Mxi1-SRβ is a transcriptional repressor, presumably, it would enhance Treg induction by inhibiting the expression of certain genes that would counteract the upregulation of Foxp3 in Teff cells.

It has previously been demonstrated that prolonged TGFβ exposure induced Mxi1 expression in epithelial cells, and that this expression accompanied decreased cell growth (137). This study assessed Mxi1 expression by Western blot, and stained for all isoforms of the Mxi1 protein in epithelial cells. Here we measured the expression of only the Mxi1-SRβ isoform in Treg cells by flow cytometry, so it is fair to say that the role of Mxi1-SRβ could be cell type specific, where in CD4+ T cells it may aid in the conversion of Teff cells to Treg cells through the TGFβ signalling pathway. Another valuable avenue of research to further uncover the link between TGFβ, Mxi1-SRβ and Treg cells would be to measure the amount of phosphorylated Smad proteins in Mxi1-SRβ-overexpressing T cells following TGFβ stimulation. This would illustrate

whether the increased availability of Mxi1-SRβ protein would enhance the TGFβ signalling pathway, and support the idea that Mxi1-SRβ plays a direct role in inducing Foxp3 expression through TGFβ.

Although we proved that Mxi1-SRβ impeded the expression of pro-inflammatory cytokines like IFNγ and TNFα as well as enhanced the conversion of Teff cells to Treg cells, we also demonstrated that Mxi1-SRβ promoted the Th17 cell lineage. Th17 cells are closely related to Treg cells, since they share the necessity for TGFβ for their differentiation (27,55). Our data show that CD4+RORγt+Th17 cells have a greater expression of Mxi1-SRβ than CD4+RORγt-T cells. Moreover, Mxi1-SRβ overexpression significantly enhanced the generation of RORγt expressing Teff and Treg cells. This seems contradictory to much of our data, since Th17 cells are highly inflammatory. However, these findings provide further support that Mxi1-SRβ is an important factor for TGFβ signalling in T cells, and that it could be enhancing Treg and Th17 cell differentiation at a molecular level. TGFβ promotes the generation of Th17 cells through the inhibition of SOCS3. SOCS3 is a negative regulator of STAT3 signalling, which is essential for Th17 differentiation. *Jiang et al, 2008* demonstrated that the expression of Mad1, a protein belonging to the same family as Mxi1, is induced by STAT3. STAT3-dependent Mad1 expression was impeded by SOCS3, however when the SOCS3 binding site on a protein upstream in the signalling pathway; granulocyte-colony stimulating factor (G-CSF), was mutated, Mad1 expression increased (147). Therefore, this supports the possibility that TGFβ down-regulates the expression of SOCS3 during Th17 differentiation through Mxi1-SRβ. In our system, ectopic expression of Mxi1-SRβ promoted the expression of the master regulator of Th17 cells. This could be due to an increased suppression of the SOCS3 gene by Mxi1-SRβ. To further test this, we would need to

measure expression of SOCS3 in this same setting to see whether Mxi1-SRβ is in fact impeding SOCS3 expression, allowing for increased Th17 differentiation. Nonetheless, these findings support the hypothesis that Mxi1-SRβ plays a key role in TGFβ signalling in T cells, where it promotes both the Treg and Th17 cell types in distinct ways.

An important cellular process that has been demonstrated to be critical for Treg cell homeostasis and maintenance of self-tolerance is that of macroautophagy (referred to here as autophagy). It has been shown that loss of important autophagy genes like Atg5, 7 and 12 lead to loss of Foxp3 expression as well as Treg cell numbers, and leads to autoimmunity (148). Autophagy is a process of catabolic targeting of cellular components for lysosomal degradation and recycling in response to environmental stimuli. It has context and cell-specific functions in T cells. For example, following TCR-activation, CD4+ T cells up-regulate autophagy genes to drive clonal expansion, whereas in CD8+ T cells, autophagy is critical for memory-cell formation (149,150). In Treg cells, it has been illustrated that autophagy serves as a signal-dependent regulator of cell survival and homeostasis by inhibiting excessive apoptosis and metabolism (151). Specifically, *Wei et al, 2016* reported that Atg7 and 5 deletion in Treg cells lead to loss of tolerance and enhanced tumor clearance in mouse studies. Treg cells deficient in these essential autophagy genes had increased mTORC1 activity as well as c-Myc expression. Excessive c-Myc expression lead to increased proliferation of the dysregulated Treg cells. This becomes important for us because it suggests that perhaps these autophagy-deficient Treg cells have impaired Mxi1-SRβ expression. Mxi1 inhibits the expression of c-Myc, so in the absence of autophagy, Mxi1-SRβ expression could be reduced, allowing c-Myc to be expressed to higher-than-normal levels and leading to Treg cell dysregulation (152).

In this study, the researchers also reported that autophagy-deficient Treg cells had an increased expression of IFNγ and were more inflammatory. However, to a lesser extent was IL-17 found in these Atg7 null Treg cells. These findings support our findings, where Mxi1-SRβ reduced the expression of IFNγ in Teff cells, but enhanced the differentiation of Th17 cells. Perhaps the increased IFNγ expression in the mutant Treg cells is in part due to loss of Mxi1-SRβ expression, however since IL-17 could be dependent on Mxi1-SRβ expression as well, it does not experience an enhanced expression in the absence of Mxi1-SRβ. In combination with this study, our *in-vivo* model of type 1 diabetes using Mxi1-SRβ-overexpressing Teff cells supports the hypothesis that Mxi1-SRβ is involved in the regulation of Teff and Treg cell homeostasis. We adoptively transferred Mxi1-SRβ overexpressing Teff cells genetically designed to have a TCR-specificity for the beta islet cells of the pancreas. The finding that Teff cells with elevated levels of Mxi1-SRβ failed to induce T1D could be explained using the above findings where autophagy regulates T cell homeostasis. Perhaps the increased Mxi1-SRβ levels promoted autophagy in the transferred Teff cells and inhibited them from becoming highly inflammatory by limiting the expression of IFNγ. Moreover, it is possible that, in this setting, increased autophagy promoted the maintenance of pTreg cells, and these Treg cells were able to suppress the onset of T1D. Finally, it is likely that elevated levels of Mxi1-SRβ inhibited the expression of c-Myc, ultimately impeding the proliferation of the Teff cells. It would be worthwhile to compare the expression of certain essential autophagy genes like Atg5 or 7 in Mxi1-SRβ-overexpressing T cells to test whether there is a link between environmental cues, Mxi1-SRβ, autophagy and overall T cell homeostasis.

An important note to make is that in order to confirm our findings described through the use of ectopic Mxi1-SRβ expression, we should complement these by knocking down the Mxi1-SRβ isoform. Overexpression studies allowed us to test whether excess Mxi1-SRβ protein could enhance Treg generation, however in order to truly test whether Mxi1-SRβ is essential for processes like Foxp3 induction and Treg cell homeostasis, we would need to knockout Mxi1-SRβ from Teff cells and perform the same experiments above. We expect that in the absence of the Mxi1-SRβ protein, Teff cells would be less prone to up-regulate Foxp3 under polarizing conditions, and would become hyper-inflammatory due to the lack of transcriptional and proliferative control from Mxi1.

In summary, our study is the first to uncover the relationship between Mxi1-SRβ and Treg cells. The role of Mxi1-SRβ in Treg cells is reliant on the external environment, and we have shown that it is capable of enhancing the generation of peripherally induced Treg cells when TGFβ is available. Moreover, we have demonstrated that Mxi1-SRβ regulates the proliferative and inflammatory ability of CD4+ T cells, and this regulation could be linked to homeostatic processes like autophagy. Further research focusing on the the molecular pathways that Mxi1-SRβ participates in will clarify the dynamic mechanisms of Mxi1-SRβ-mediated regulation of gene expression in Treg cells as well as CD4+ T cells as other CD4+ T cell subsets. The therapeutic implications of Mxi1-SRβ-mediated Treg cell generation and homeostasis are such that molecular intervention of Mxi1-SRβ could enhance tumor clearance by CD4+ and CD8+ T cells. Contrarily, promoting Mxi1-SRβ expression could prevent autoimmune disease progression.

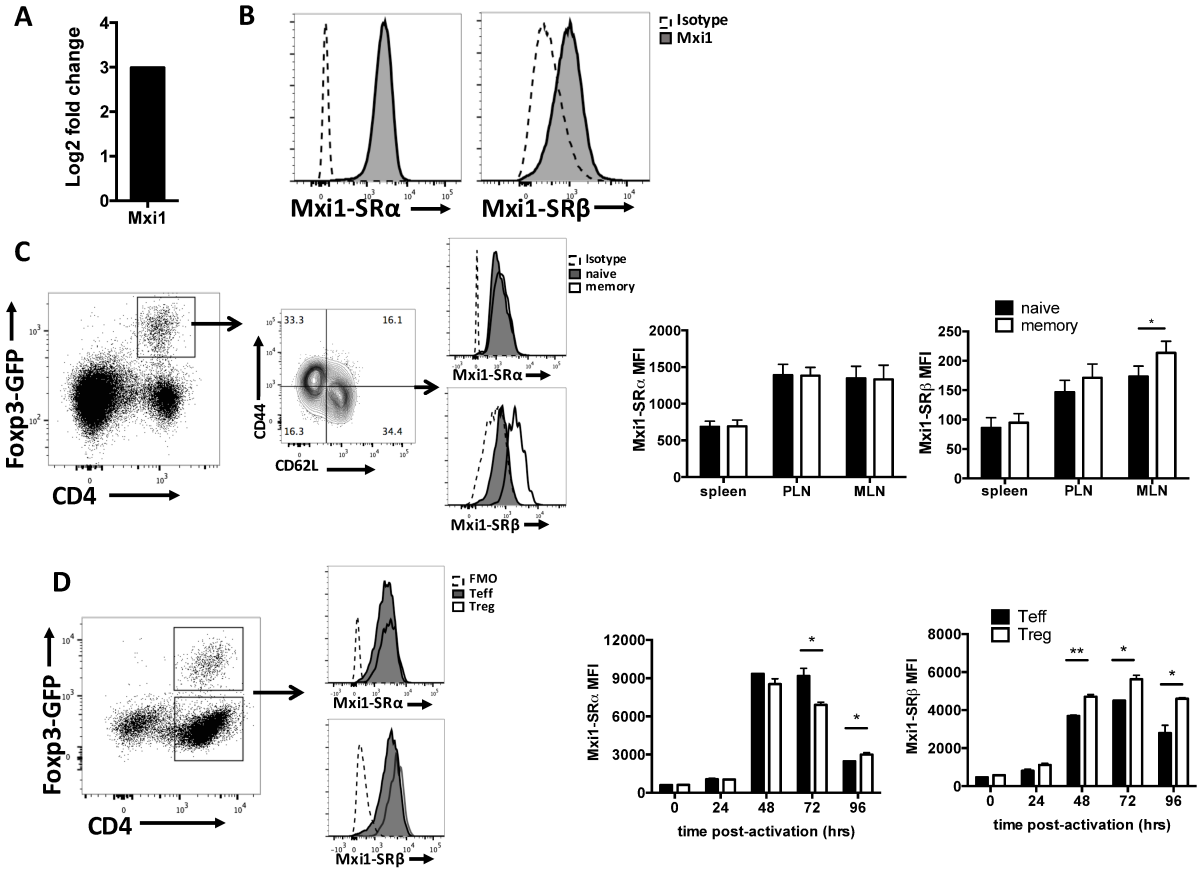
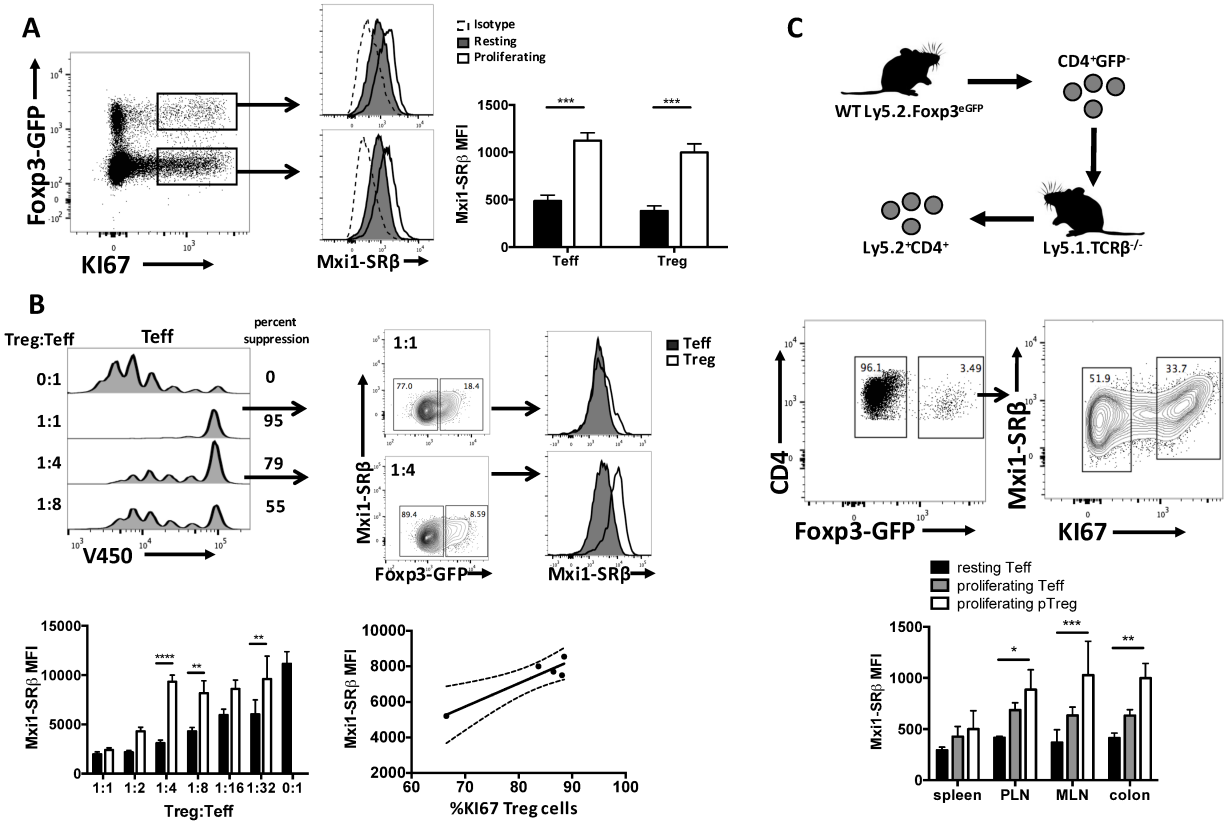

A
Log2 fold change
Mxi1
B
Isotype
Mxi1
Mxi1-SRα
Mxi1-SRβ
C
Foxp3-GFP
CD4
CD44
CD62L
33.3
16.1
16.3
34.4
Isotype
naive
memory
Mxi1-SRα
Mxi1-SRβ
Mxi1-SRα MFI
Mxi1-SRβ MFI
spleen
PLN
MLN
naive
memory
D
FMO
Teff
Treg
time post-activation (hrs)

Figure 1. Activated and memory CD4+Foxp3+ Treg cells express high levels of Mxi1-SRβ. **A.** FACS-sorted CD4+GFP-Teff and CD4+GFP+Treg cells from pooled splenocytes and LN cells of C57BL/6.Foxp3^{eGFP} mice were activated plate-bound with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml). Total cytosolic mRNA was harvested and fractionated based on their association with poly-ribosome complexes using a sucrose gradient. A whole genome microarray was performed to identify differential mRNA translation between Teff and Treg cells. Results are expressed as the Log2 fold change in translational events between Treg and Teff cells. **B.** *Ex-vivo* stain of pooled splenocytes and LN cells isolated from 8-12 week old C57BL/6.Foxp3^{eGFP} mice with Mxi1-SRα and Mxi1-SRβ antibodies and detection by flow cytometry. **C.** Lymphocytes were harvested from the spleen, PLN and MLN of C57BL/6.Foxp3^{eGFP} mice. Cells were stained for memory (CD44^{hi}CD62L^{lo}) and naïve (CD44^{lo}CD62L^{hi}) phenotypes, and Mxi1-SRα and SRβ expression was measured in memory vs naive Treg cell subsets. **D.** Lymphocytes from pooled splenocytes and LN cells were isolated from C57BL/6.Foxp3^{eGFP} mice and activated with T-depleted irradiated feeder cells and anti-CD3 (1ug/ml). Bar graphs represent the median fluorescence intensity of Mxi1-SRα and SRβ in Teff and Treg cells between 0-96 hours of activation. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.


A
Foxp3-GFP
KI67
Mxi1-SRβ
Isotype
Resting
Proliferating
Mxi1-SRβ MFI
Teff
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B
Treg:Teff
Teff
percent suppression
0:1
1:1
1:4
1:8
0
95
79
55
V450
1:1
1:4
Foxp3-GFP
Mxi1-SRβ
Teff
Treg
Mxi1-SRβ MFI
1:1 1:2 1:4 1:8 1:16 1:32 0:1
Treg:Teff
%KI67 Treg cells
C
WT Ly5.2.Foxp3eGFP
CD4+GFP-
Ly5.2+CD4+
Ly5.1.TCRβ-/-
CD4
Foxp3-GFP
Mxi1-SRβ
KI67
resting Teff
proliferating Teff
proliferating pTreg
spleen
PLN
MLN
colon

Figure 2. Mxi1-SRβ expression is increased in proliferating CD4+ T cells. **A.** Lymphocytes were isolated from pooled splenocytes and LN cells of C57BL/6.Foxp3^{eGFP} mice and Mxi1-SRβ expression was measured in proliferating (KI67^{+}) and resting (KI67^{-}) CD4+ T cells. **B.** *In-vitro* suppression assay with 5 x 10^4 CD4+GFP- Teff cells and a decreasing ratio of CD4+GFP+ Treg:CD4^{+}Foxp3^{-} Teff cells from 1:1 to 1:8 in the presence of 2 x 10^5 T-depleted irradiated feeder cells all from C57BL/6.Foxp3^{eGFP} mice and anti-CD3 (1ug/ml). Mxi1-SRβ expression and proliferation (KI67) was assessed in all ratios. **C.** FACS-sorted donor CD4^{+}GFP^{-} Teff cells isolated from pooled splenocytes and LN cells of WT (Ly5.2) C57BL/6.Foxp3^{eGFP} mice were adoptively transferred into Ly5.1.C57BL/6.TCRb$^{-/-}$ mice. WT cells were harvested 14 days post-transfer and Mxi1-SRβ expression was assessed in proliferating (KI67^{+}) and resting (KI67^{-}) Teff and peripherally-induced Treg (pTreg) cells. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

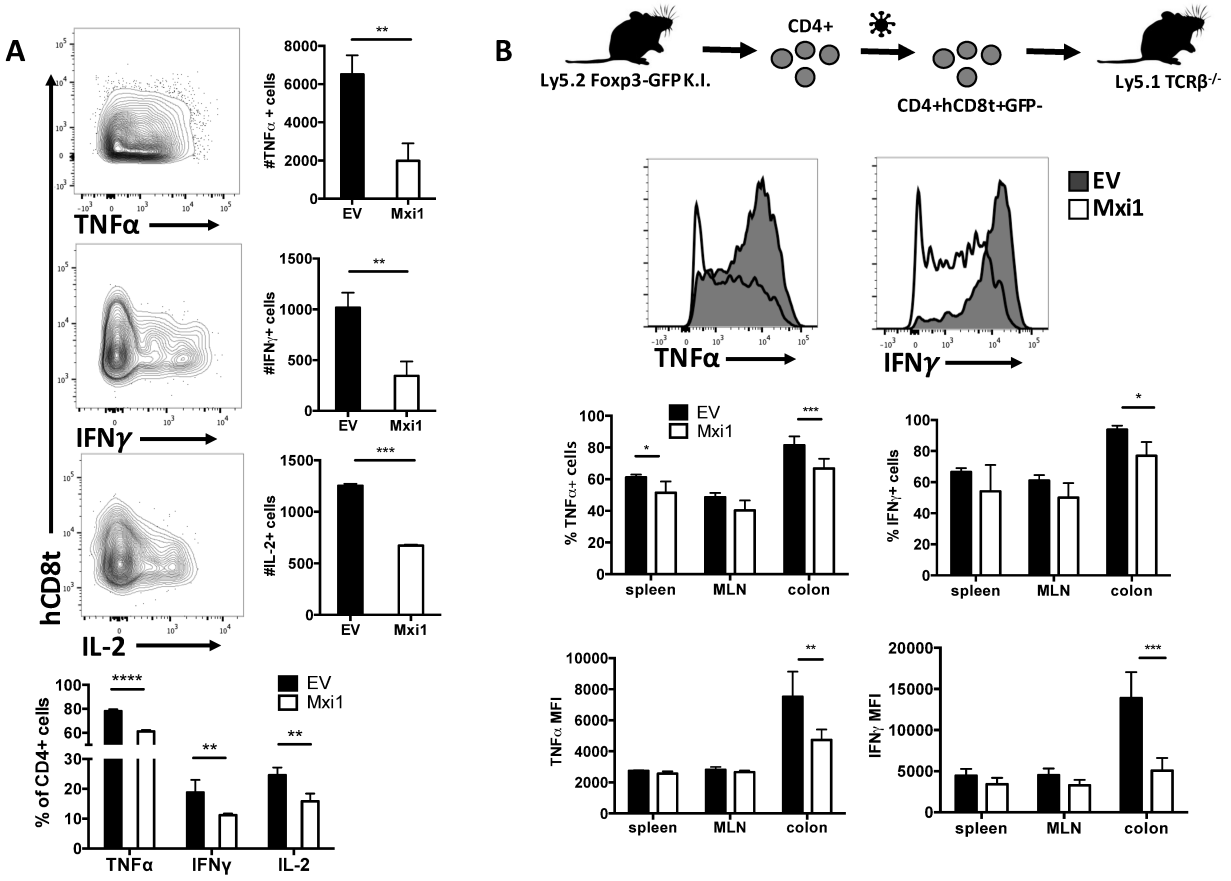
A
TNFα
IFNγ
IL-2
hCD8t
#TNFα + cells
#IFNγ+ cells
#IL-2+ cells
EV
Mxi1
% of CD4+ cells
TNFα
IFNγ
IL-2
B
Ly5.2 Foxp3-GFP K.I.
CD4+
CD4+hCD8t+GFP-
Ly5.1 TCRβ-/-
EV
Mxi1
% TNFα+ cells
% IFNγ+ cells
spleen
MLN
colon
TNFα MFI
IFNγ MFI

Figure 3. Mxi1-SRβ overexpression reduces the production of pro-inflammatory cytokines in CD4+Foxp3- Teff cells *in-vitro* and *in-vivo*. **A.** autoMACS-sorted CD4+ T cells isolated from pooled splenocytes and LN cells of C57BL/6.Foxp3^{eGFP} mice were activated plate-bound with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml) and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles at 24 hours post-activation. 5 days post-transduction, cells were re-stimulated with PMA and ionomycin in the presence of Golgi stop for 3.5 hours. TNFα, IFNγ and IL-2 expression was measured by flow cytometry. **B.** autoMACS-sorted CD4+ T cells from WT (Ly5.2) C57BL/6.Foxp3^{eGFP} mice were activated and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles as previously described and expanded *in-vitro* for 7 days. FACS-sorted CD4+GFP-hCD8t+ Teff cells were adoptively transferred into Ly5.1.C57BL/6.TCRb$^{-/-}$ mice. 14 days post-transfer, WT cells were harvested from spleen, MLN and colon. TNFα and IFNγ expression was assessed by flow cytometry. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

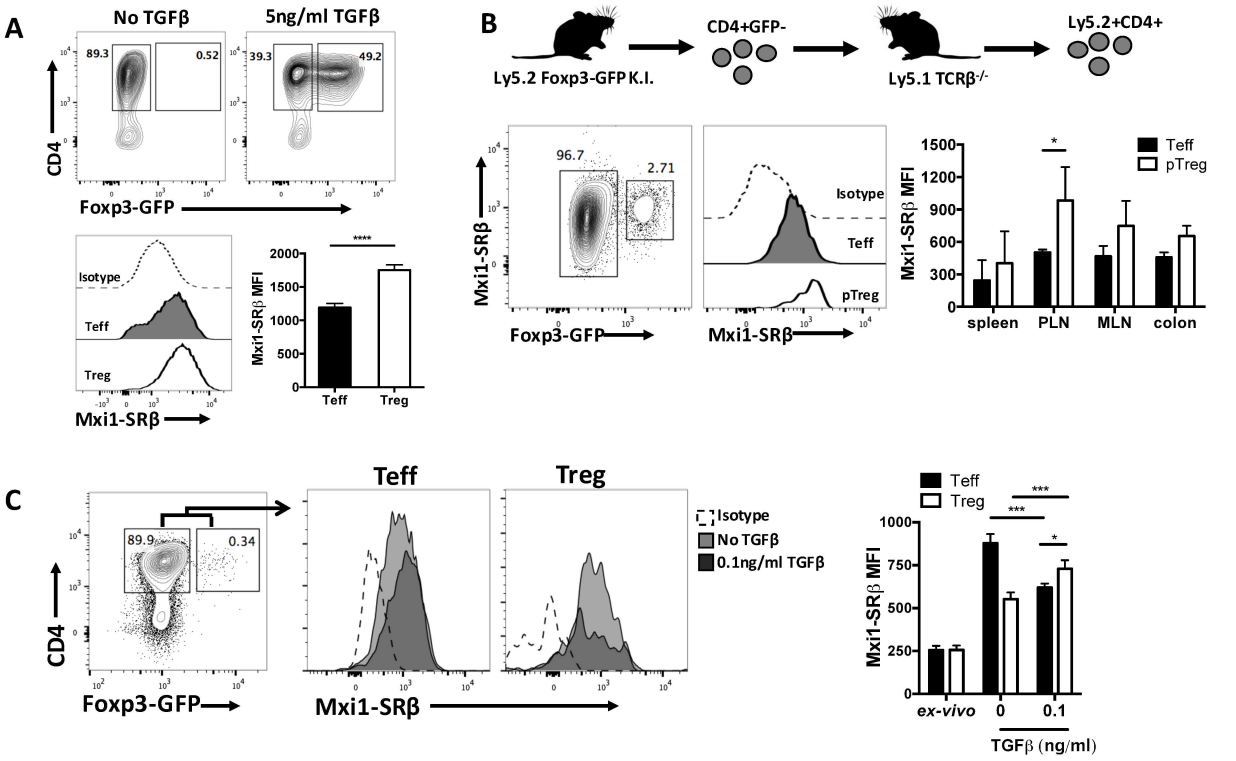
A
No TGFβ
5ng/ml TGFβ
89.3
0.52
39.3
49.2
CD4
Foxp3-GFP
Isotype
Teff
Treg
Mxi1-SRβ
Mxi1-SRβ MFI
****
Teff
Treg
B
Ly5.2 Foxp3-GFP K.I.
CD4+GFP-
Ly5.1 TCRβ-/-
Ly5.2+CD4+
96.7
2.71
Foxp3-GFP
Mxi1-SRβ
Isotype
Teff
pTreg
Mxi1-SRβ MFI
*
Teff
pTreg
spleen
PLN
MLN
colon
C
89.9
0.34
CD4
Foxp3-GFP
Teff
Treg
Isotype
No TGFβ
0.1ng/ml TGFβ
Mxi1-SRβ
Teff
Treg
***
***
*
Mxi1-SRβ MFI
ex-vivo
0
0.1
TGFβ (ng/ml)

Figure 4. Mxi1-SRβ expression is increased in peripherally-induced CD4+Foxp3+ Treg cells *in-vitro* and *in-vivo*. **A.** *in-vitro* TGFβ-mediated Foxp3 induction assay. FACS-sorted CD4+GFP- Teff cells were activated plate-bound with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml) in the presence of TGFβ (5ng/ml) for 96 hours. Mxi1-SRβ expression was measured by flow cytometry. **B.** FACS-sorted donor $CD4^{+}GFP^{-}$ Teff and CD4+GFP+Treg cells isolated from pooled splenocytes and LN cells of WT (Ly5.2) C57BL/6.$Foxp3^{eGFP}$ mice were adoptively transferred into Ly5.1.C57BL/6.$TCRb^{-/-}$ mice. 14 days port-transfer, WT cells were harvested from spleen, PLN, MLN and colon. Mxi1-SRβ expression was measured in all organs and cell subsets by flow cytometry. **C.** autoMACS-sorted CD4+ T cells isolated from pooled splenocytes and LN cells of C57BL/6.$Foxp3^{eGFP}$ mice were activated plate-bound with anti-CD3 (5ug/ml) and anti-CD28 (2ug/ml) in the presence of TGFβ (0.1ng/ml) for 24 hours. Mxi1-SRβ expression was measured by flow cytometry. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

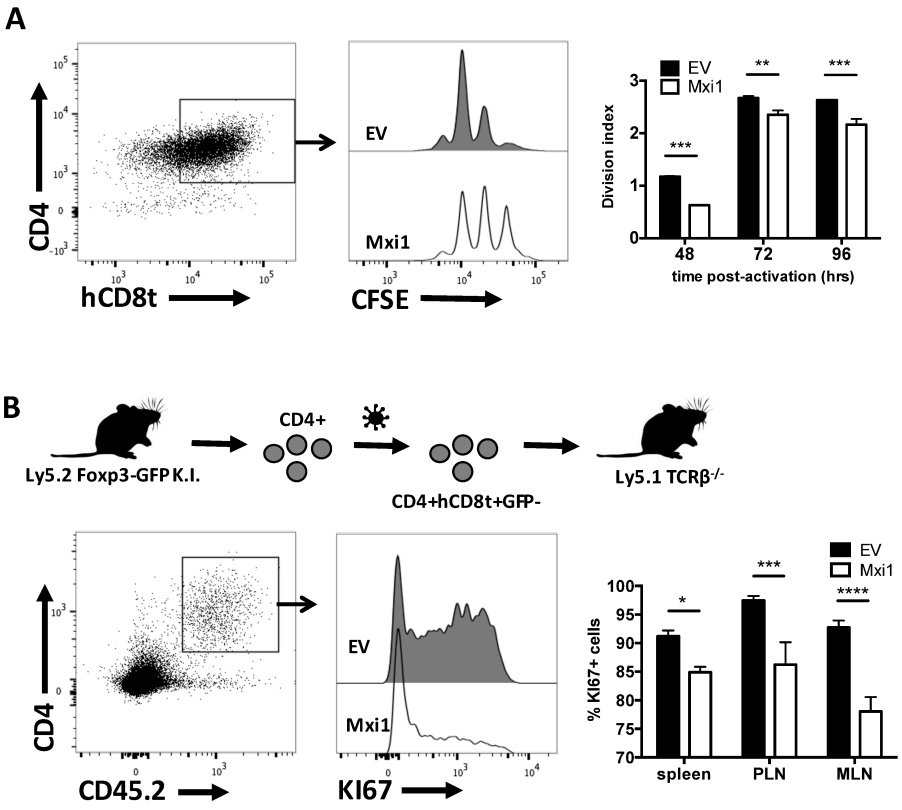
A
CD4
hCD8t
EV
Mxi1
CFSE
EV
Mxi1
Division index
48
72
96
time post-activation (hrs)
B
Ly5.2 Foxp3-GFP K.I.
CD4+
CD4+hCD8t+GFP-
Ly5.1 TCRβ-/-
CD4
CD45.2
EV
Mxi1
KI67
% KI67+ cells
spleen
PLN
MLN

Figure 5. Mxi1-SRβ overexpression impedes cellular proliferation of CD4+ T cells *in-vitro* and *in-vivo*. **A.** autoMACS-sorted CD4+ T cells were isolated form pooled splenocytes and LN cells of C57BL/6.Foxp3^{eGFP} mice. Cells were labeled with CFSE proliferation dye, activated plate-bound and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles as previously described. Proliferation was assessed by flow cytometry every 24 hours following 48 hours of activation. **B.** autoMACS-sorted CD4+ T cells from WT (Ly5.2) C57BL/6.Foxp3^{eGFP} mice were activated and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles as previously described and expanded *in-vitro* for 7 days. FACS-sorted CD4+GFP-hCD8t+ Teff cells were adoptively transferred into Ly5.1.C57BL/6.TCRb$^{-/-}$ mice. 14 days post-transfer, WT cells were harvested from spleen, PLN and MLN. Proliferation was assessed by intracellular KI67 staining. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

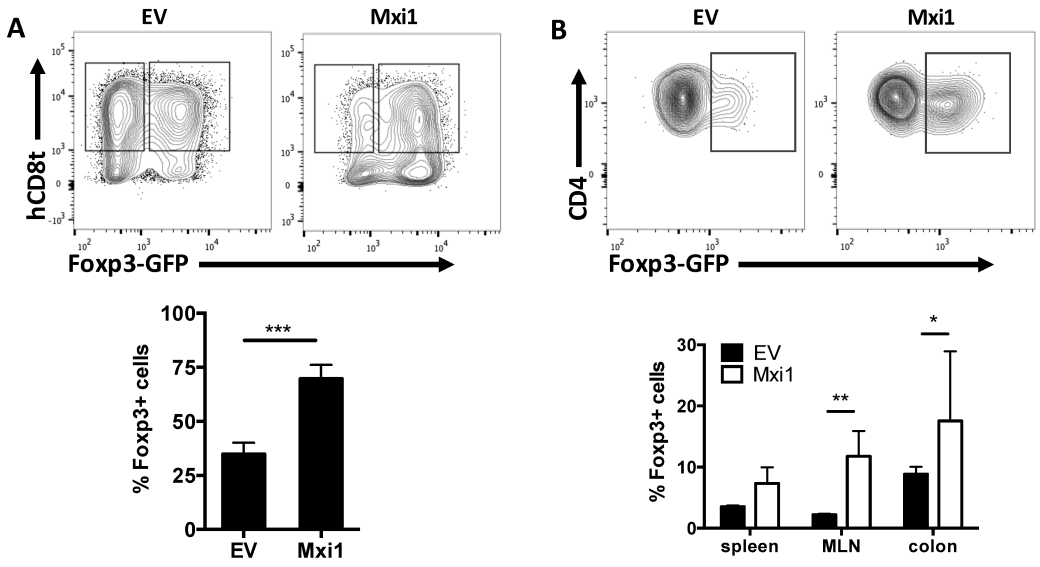
A
EV
Mxi1
hCD8t
Foxp3-GFP
B
EV
Mxi1
CD4
Foxp3-GFP
% Foxp3+ cells
***
EV
Mxi1
% Foxp3+ cells
EV
Mxi1
**
*
spleen
MLN
colon

Figure 6. Mxi1-SRβ overexpression enhances the generation of peripherally-induced Foxp3+ Treg cells *in-vitro* and *in-vivo*. **A.** FACS-sorted CD4+GFP- T cells were isolated from pooled splenocytes and LN cells of C57BL/6.Foxp3^{eGFP} mice. Cells were activated plate-bound and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles as previously described. At 24 hours, TGFβ (5ng/ml) was added to culture to induce Foxp3 expression. **B.** autoMACS-sorted CD4+ T cells from WT (Ly5.2) C57BL/6.Foxp3^{eGFP} mice were activated and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles as previously described and expanded *in-vitro* for 7 days. FACS-sorted CD4+GFP-hCD8t+ Teff cells were adoptively transferred into Ly5.1.C57BL/6.TCRb$^{-/-}$ mice. 14 days post-transfer, WT cells were harvested from spleen, MLN and colon. Foxp3 expression was assessed by flow cytometry. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

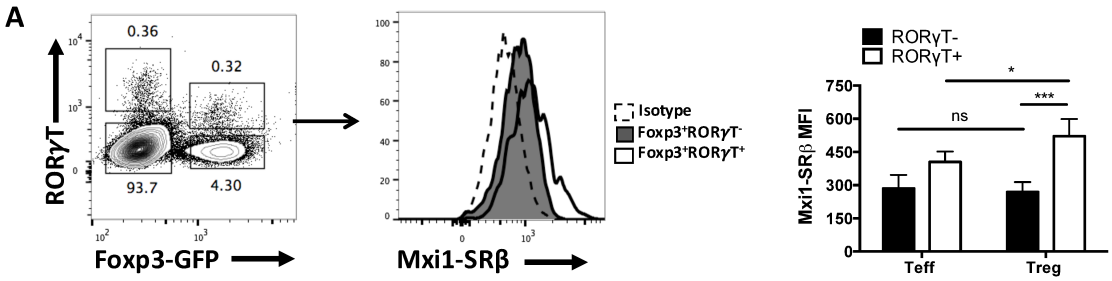
A
0.36
0.32
93.7
4.30
RORγT
Foxp3-GFP
Mxi1-SRβ
Isotype
Foxp3+RORγT-
Foxp3+RORγT+
RORγT-
RORγT+
Mxi1-SRβ MFI
ns
*
***
Teff
Treg

Figure 7. RORγt-expressing CD4+ Teff and Treg cells express high levels of Mxi1-SRβ. **A**. autoMACS-sorted CD4+ T cells isolated from pooled splenocytes and LN cells of WT (Ly5.2) C57BL/6.Foxp3^{eGFP} mice were stained *ex-vivo* for RORγt and Mxi1-SRβ. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

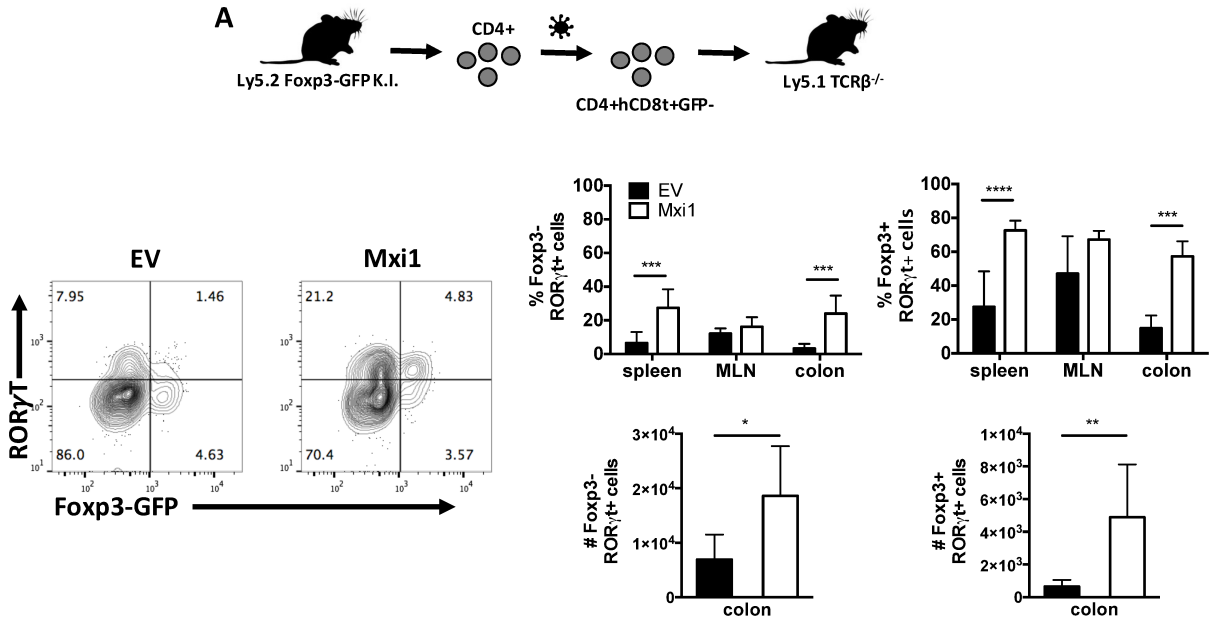
A
Ly5.2 Foxp3-GFP K.I.
CD4+
CD4+hCD8t+GFP-
Ly5.1 TCRβ-/-
EV
Mxi1
7.95
1.46
86.0
4.63
21.2
4.83
70.4
3.57
RORγT
Foxp3-GFP
EV
Mxi1
% Foxp3- RORγt+ cells
% Foxp3+ RORγt+ cells
# Foxp3- RORγt+ cells
# Foxp3+ RORγt+ cells
spleen
MLN
colon
***
****
*
**

Figure 8. Mxi1-SRβ overexpression enhances the expression of RORγt in both CD4+ Teff and Treg cells *in-vivo*. **A**. autoMACS-sorted CD4+ T cells from WT (Ly5.2) C57BL/6.Foxp3^{eGFP} mice were activated as previously described and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles and expanded *in-vitro* for 7 days. FACS-sorted CD4+GFP-hCD8t+ Teff cells were adoptively transferred into Ly5.1.C57BL/6.TCRb$^{-/-}$ mice. 14 days post-transfer, WT cells were harvested from spleen, MLN and colon. RORγt and Mxi1-SRβ expression were assessed by flow cytometry. Similar results were obtained in two independent experiments. Results represent the mean ± SD.

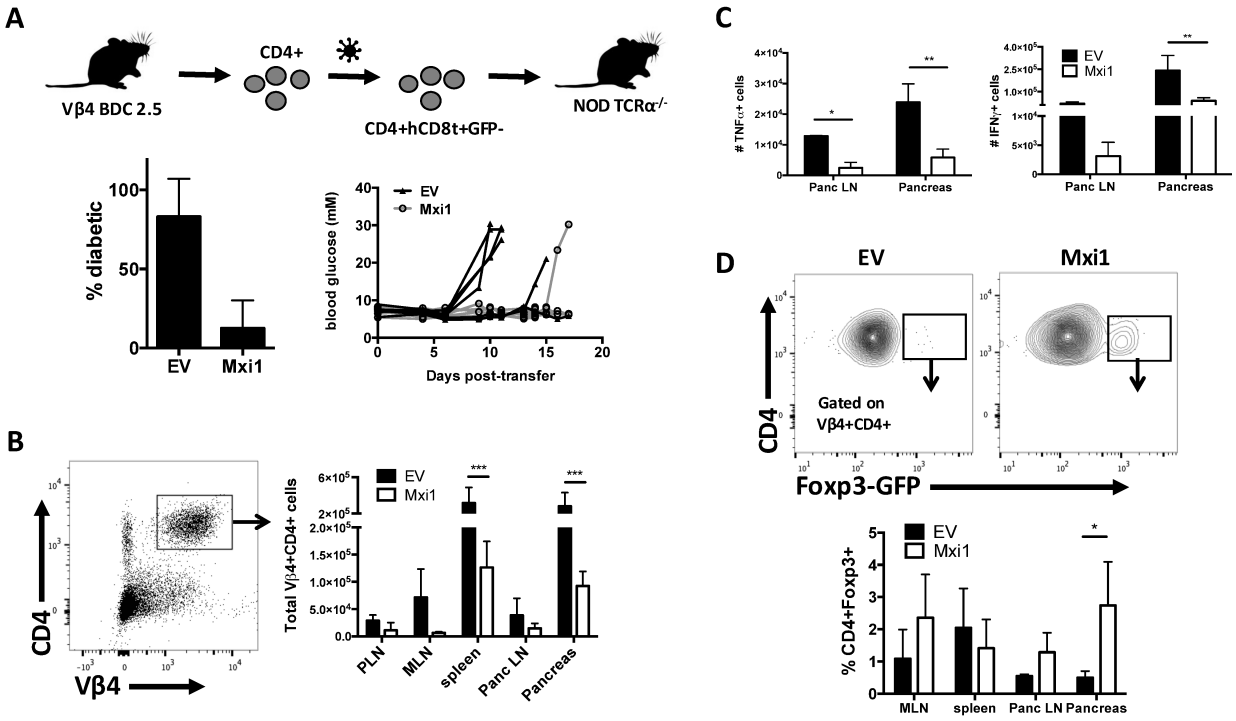
A
Vβ4 BDC 2.5
CD4+
CD4+hCD8t+GFP-
NOD TCRα-/-
% diabetic
EV
Mxi1
blood glucose (mM)
Days post-transfer
B
CD4
Vβ4
Total Vβ4+CD4+ cells
PLN
MLN
spleen
Panc LN
Pancreas
C
TNFα+ cells
IFNγ+ cells
D
EV
Mxi1
Gated on Vβ4+CD4+
Foxp3-GFP
% CD4+Foxp3+

Figure 9. Mxi1-SRβ overexpression delays Type 1 diabetes onset *in-vivo*. **A.** autoMACS-sorted CD4+ T cells isolated from pooled splenocytes and LN cells of Vb4.NOD.BDC2.5 mice were activated and transduced with MSCV-Mxi1-SRβ-IRES-hCD8t virus particles as previously described and expanded *in-vitro* for 7 days. FACS-sorted CD4+GFP-hCD8t+ Teff cells were adoptively transferred into NOD.TCRa^{-}/$^{-}$ mice. Blood glucose was monitored each day thereafter and T1D was diagnosed following 2 consecutive readings >20mM. **B.** Representative FACS plot and bar graphs depicting recovered Vb4+ cells from recipient mice. **C**. Recovered Vb4+ cells were re-stimulated *in-vitro* with PMA and ionomycin in the presence of GolgiStop^{TM} for 3.5 hours. TNF and IFNγ expression was measured by flow cytometry. **D.** Representative FACS plots and bar graphs depicting Foxp3 induction in recovered Vb4+ cells. Similar results were obtained in 3 independent experiments. Results represent the mean ± SD.

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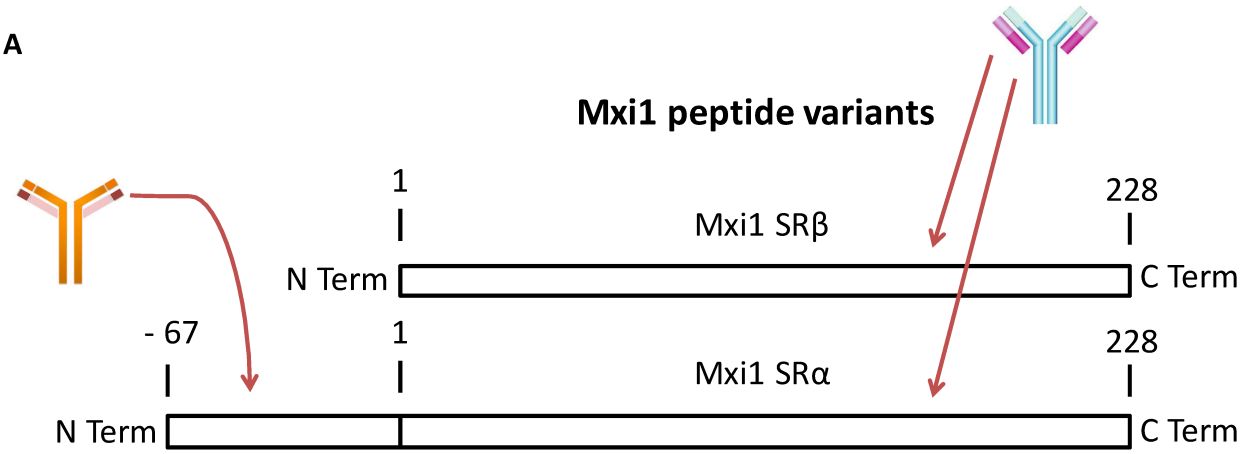
Supplemental Figures



Supplemental Figure 1. Graphic depicting the development of a flow cytometric staining approach to distinguish the Mxi1-SRα isoform from the SRβ isoform.