Functional Characterization and Biomarker Discovery of FOXP3^{+} Regulatory T Cells in Human Health and Disease

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إِهْدَاءْ...

إِلَى أَبِي وَأُمِّي اللّذَيْنِ غَرَسَا فِيْ نَفْسِي الطُّمُوْحَ وَلَمْ يُحَاوِلا يوماً كَبْحَ أَحْلاَمِي ...

إِلَى أَشِقَّائِي وَشَقِيْقَاتِي الَّذِيْنَ كَانُوْا لِيْ نِعْمَ العَوْنِ والسَّنَد ...

إِلَى بَهْجَةِ أَيَّامِي، خُلُوْد وَسَامِي ...

أُهْدِيْكُمْ بَعْضَ مَاصَنَعْتُمْ

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Abstract

$CD4^+FOXP3^+$ Regulatory T cells (Treg) are critical mediators of peripheral tolerance to self and innocuous antigens. In humans, *FOXP3* mutations impair the development of Treg cells and cause the IPEX syndrome characterized by severe autoimmunity and, often, early death. Using a sensitive single-cell cloning strategy, previous work in our lab has revealed a remarkable degree of functional heterogeneity in the human $FOXP3^+$ population whereby approximately 30% of $FOXP3^+$ cells in healthy individuals lack the suppressive capacity expected from Treg cells despite exhibiting the hallmark Treg phenotype. Here we further characterize this heterogeneity by examining the following hypotheses: 1) *FOXP3 regulates Treg phenotype and function through distinct molecular mechanisms*, and 2) *the human $FOXP3^+$ Treg subset comprises a heterogeneous mixture of functionally stable and functionally flexible subpopulations with differential sensitivity to modulatory signals*.

Using primary Treg cells from IPEX patients bearing the pA384T mutation, we demonstrate that this mutation specifically impairs Treg suppressive function while preserving FOXP3's ability to drive the characteristic Treg phenotype and repression of inflammatory cytokine production. This selective defect is related to a diminished interaction of FOXP3 with the histone acetyl transferase TIP60, and can be corrected using small-molecule allosteric modifiers of TIP60 that enhance its interaction with $FOXP3^{A384T}$. These findings highlight a crucial role for the FOXP3-TIP60 interaction in the development of Treg suppressive function.

Moreover, through whole transcriptome analysis and further functional characterization of suppressive and non-suppressive $FOXP3^+$ clones of healthy individuals, we show that the intracellular expression of Helios identifies human Treg cells with maximal suppressive capacity. We further propose two novel surface proteins, namely TIGIT and FCRL3, as reliable markers

that distinguish Treg cell subsets based on Helios expression, and provide a long-sought marker to distinguish human Treg cells from activated effector T cells. Finally, we identify the IL-6 receptor subunit, gp130, as a signalling receptor involved in antagonizing the suppressive capacity of Treg cells, and we demonstrate that inhibition of gp130 improves the suppressive function of human Treg cells.

Overall, these studies identify critical pathways underlying Treg functional heterogeneity and highlight potential strategies for the therapeutic modulation of human Treg activity in disease.

Résumé (translated by my colleague Fernando Alvarez)

Les lymphocytes T régulateurs (Treg), $CD4^{+}Foxp3^{+}$, sont des médiateurs fondamentaux de la tolérance périphérique face aux antigènes du soi et de l'environnement. Chez les humains, des mutations au niveau du gène *FOXP3* affectent le développement des lymphocytes Treg et sont la cause du syndrome IPEX qui est caractérisé par une maladie auto-immune sévère le plus souvent mortelle. Grâce à une stratégie très sensible de clonage de cellules individuelles, de précédents travaux dans notre laboratoire ont révélés l'étendue de l'hétérogénéité fonctionnelle des lymphocytes T $Foxp3^{+}$ chez l'homme; notamment le fait que 30% des cellules T Foxp3 positives d'un individu sain ne possèdent pas la capacité suppressive des Treg, malgré le fait qu'elles exhibent les caractéristiques phénotypiques classiques de cellules Treg. Dans ces travaux, nous examinons plus en profondeur cette hétérogénéité en répondant aux hypothèses suivantes : 1) *Le facteur de transcription Foxp3 régule le phénotype et la fonction des lymphocytes Treg à travers des mécanismes moléculaires distincts* et 2) *La population de lymphocytes Treg chez l'homme est en fait un mélange hétérogène de sous-populations stables et instables qui possèdent différents degrés de sensibilité à des signaux moléculaires*.

En étudiant des lignées primaires de Treg provenant de patient atteints de IPEX, et porteur de la mutation pA384T au niveau du gène *FOXP3*, nous démontrons que cette mutation affecte spécifiquement leur capacité suppressive, tout en préservant l'habileté de Foxp3 à générer les autres caractéristiques phénotypiques des Treg, dont la répression de la production de cytokines inflammatoires. Ce défaut sélectif serait lié à une diminution de l'interaction entre le facteur de transcription Foxp3 et l'histone acétyl-transférase TIP60. Ce défaut peut être corrigé par l'usage de modificateurs allostériques de TIP60 de faible poids moléculaire qui augmentent son temps d'interaction avec le Foxp3 muté, $Foxp3^{A384T}$. Ces résultats exposent le rôle crucial de l'interaction Foxp3-TIP60 dans la fonction suppressive des Treg.

De plus, une analyse complète du transcriptome et une caractérisation fonctionnelle des clones suppressifs et non-suppressifs de Treg d'individus sains ont permis de démontrer que l'expression du facteur de transcription Helios identifie des Treg qui possèdent une plus grande activité suppressive. À travers cette analyse, nous proposons l'usage de deux nouvelles protéines de surface, TIGIT et FCRL3, comme des marqueurs qui distinguent des populations de Treg qui expriment Helios. Ces marqueurs répondent au besoin de la communauté scientifique qui recherchait un moyen de distinguer les lymphocytes Treg humains de lymphocytes T effecteurs activés. Finalement, nous avons identifié une sous-unité du récepteur de l'IL-6, le gp130, comme un récepteur qui envoie un signal antagoniste à l'activité suppressive des Treg. De ce fait, l'inhibition du gp130 améliore la capacité suppressive des Treg.

Ces travaux illustrent certaines des voies de signalisations impliquées dans l'hétérogénéité fonctionnelle des Treg et identifient de potentielles stratégies thérapeutiques pour la modulation de l'activité des lymphocytes Treg humains lors de maladies.

Contributions of Authors

The core of this thesis is a collection of three original studies, one of which has been published, one is submitted and the third is to be submitted soon. I, Khalid Bin Dhuban, under the supervision of Dr. Ciriaco Piccirillo, designed, performed and analyzed the majority of the experiments presented in this thesis. I have also written the three manuscripts presented here. Several co-authors have contributed indispensably to the success of these studied and their contributions are detailed as follows:

Chapter 2: FOXP3-TIP60 interaction is required for the suppressive function of human FOXP3^{+} regulatory T cells

Authors: *Khalid Bin Dhuban[*], Eva d'Hennezel[*], Yan Xiao, Yasuhiro Nagai, Moshe Ben-Shoshan, Hans Ochs, Bruce Mazer, Nicholas Bertos, Morag Park, Troy R. Torgerson, Mark I. Greene, Ciriaco A. Piccirillo, and on behalf of the CIHR/MSSC NET in Clinical Autoimmunity*

In collaboration with Dr. Ciriaco Piccirillo and Dr. Eva d'Hennezel, I was responsible for the experimental design and execution, data analysis and the writing of the manuscript. Dr. Eva d'Hennezel, a former PhD candidate in the laboratory of Dr. Piccirillo initiated the project and contributed significantly towards the experimental design and execution as well as data analysis of the first half of the project. Dr. Moshe Ben-Shoshan, Dr. Troy Torgerson, Dr. Bruce Mazer and Dr. Hans Ochs were involved in the clinical care of the IPEX patients and provided the primary samples used in the study. Dr. Mark Greene, Dr. Yan Xiao and Dr. Yasuhiro carried out the biochemical assessments of FOXP3 interactions and provided the TIP60 modifier used in the

study. Dr. Morag Park and Nicholas Bertos were significantly involved in the microarray analysis performed in the study.

Chapter 3: Co-expression of TIGIT and FCRL3 identifies Helios$^+$ human memory Treg cells

Authors: *Khalid Bin Dhuban, Eva d'Hennezel, Emil Nashi, Amit Bar-Or, Sadiye Rieder, Ethan Shevach, Satoshi Nagata and Ciriaco A. Piccirillo*

In collaboration with Dr. Ciriaco Piccirillo, I was responsible for the experimental design and execution, data analysis and the writing of the manuscript. Dr. Eva d'Hennezel performed the microarray analysis. Dr. Emil Nashi and Dr. Amit bar-Or provided the clinical samples used in the study. Dr. Sadiye Rieder and Dr. Ethan Shevach performed the epigenetic analysis of TSDR methylation. Dr. Satoshi Nagata provided the anti-FCRL3 antibody used in the study.

Chapter 4: Signalling through glycoprotein 130 drives loss of suppressive function in human FOXP3$^+$ regulatory T cells

Authors: *Khalid Bin Dhuban, Sabrina Bartolucci, Eva d'Hennezel, and Ciriaco A. Piccirillo*

In collaboration with Dr. Ciriaco Piccirillo, I was responsible for the project design and execution, data analysis and the writing of the manuscript. Sabrina Bartolucci, an undergraduate student in the laboratory of Dr. Piccirillo provided significant help with the execution and analysis of several experiments examining the effects of IL-6, IL-27 and SC144 on the suppressive function Treg cells *ex vivo*. Dr. Eva d'Hennezel helped with the initial planning and execution of early experiments in the study.

I have also co-authored the following manuscript with Dr. Eva d'Hennezel examining antigen-specific T cell responses in children with food allergy:

- *Khalid Bin Dhuban*, Eva d'Hennezel*, Moshe Ben-Shoshan, Christine McCusker, Ann Clarke, Pierre Fiset, Bruce Mazer, Ciriaco A Piccirillo*. 2013. Altered T helper 17 responses in children with food allergy. *International Archives of Allergy and Immunology*. 162(4): 318-322. * Equal contributors.

In addition, I have co-authored the following study with Dr. Donald Vinh where we described a case of auto-aggression syndrome following autologous hematopoietic stem cell transfer:

- *Donald C. Vinh*, Khalid Bin Dhuban*, Helen Mason, Duncan Lejtenyi, SungMi Jung, Donald C. Sheppard, Damien Faury, Nada Jabado, and Ciriaco A. Piccirillo*. 2012. Acquired Omenn-like syndrome, a novel post-transplant auto-aggression syndrome reversed by rapamycin. *Clinical and Vaccine Immunology.* 19(1): 109–112. * Equal contributors.

In collaboration with Dr. Eva d'Hennezel and Dr. Piccirillo, we wrote the following review article on the immunogenetics of the IPEX syndrome where we discussed the literature of the IPEX syndrome and how we can use the knowledge not only to guide the prognosis and treatment of the IPEX syndrome but also to further our research into the basic biology of human Treg cells. Certain parts of this review article are presented in the introduction (Chapter 1) of this thesis:

- *d'Hennezel E.*, K. Bin Dhuban*, T. Torgerson, and C. Piccirillo.* 2012. The immunogenetics of immune dysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *Journal of medical genetics*. 49:291-302. * Equal contributors.

I have also co-written the following review article with Dr. Piccirillo and former members of his lab on the functional dynamics of murine and human FOXP3^{+} Treg cells. Certain parts of this review article are presented in the introduction (Chapter 1) of this thesis:

- *Bin Dhuban K*, Kornete M*, S Mason E*, Piccirillo CA*. 2014. Functional dynamics of Foxp3^{+} regulatory T cells in mice and humans. *Immunological Rev*iews. 259(1):140-58. * Equal contributors.

With Dr. Piccirillo, I wrote the following review article discussing the current status and challenges of human Treg biology:

- *Khalid Bin Dhuban and Ciriaco A. Piccirillo*. 2014. Markers for Human FOXP3 Regulatory T Cells: Current Status and Implications for Immune Monitoring in Human Disease. *International Trends in Immunity*. 2(4): 162-165.

Also in collaboration with Dr. Piccirillo, I recently wrote the following article reviewing the current knowledge of the IPEX syndrome and how we can use it to better understand human Treg biology. Certain parts of this review article are presented in the introduction (Chapter 1) of this thesis:

- *Khalid Bin Dhuban and Ciriaco A. Piccirillo*. 2015. The immunological and genetic basis of immune dysregulation, polyendocrinopathy, enteropathy, X-linked syndrome. *Current Opinion in Allergy & Clinical Immunology*. 15(6): 525–532.

Finally, with Dr. Piccirillo, I wrote the following article for the Encyclopedia of Life Sciences about the molecular genetics of the IPEX syndrome:

- Khalid Bin Dhuban and Ciriaco Piccirillo. 2015. Molecular Genetics of the IPEX Syndrome. John Wiley & Sons, Ltd. http://www.els.net/. DOI: 10.1002/9780470015902.a0024904

I, Khalid Bin Dhuban, have read, understood and abided by all norms and regulations of academic integrity of McGill University.

Table of contents:

CHAPTER 1

General Introduction

Chapter 1- General Introduction

1. Establishment of immune tolerance to self-antigens

A hallmark feature of the adaptive immune system is its ability to mount specific responses to a diverse array of antigens. For T cells, this antigen-specificity is created by somatic gene rearrangement of the T cell receptor (TCR), creating T cells with a diverse TCR repertoire that recognizes countless unique antigens (reviewed in (1)). Although this process yields a T cell population that can recognize most of the foreign antigens to be encountered throughout an individual's lifetime, it also results in the generation of T cells possessing TCR molecules that recognize self-antigens and thus have the potential of mediating autoimmune responses against the host. Regulatory mechanisms are, therefore, required to eliminate, or control, such potentially auto-reactive T cells in order to maintain self-tolerance

1.1. Central tolerance mechanisms eliminate the majority of autoreactive thymocytes

Starting during T cell development in the thymus, the process of negative selection eliminates the majority of auto-reactive T cells that exhibit a high affinity for self-antigens (reviewed in (2)). During negative selection, tissue-restricted antigens (TRA) are sampled and presented to developing T cells by thymic epithelial cells (TECs) in order to identify and eliminate self-reactive T cells (2). This process is orchestrated by the transcription factor AIRE (Autoimmune Regulator) that is expressed in TECs and induces the expression of TRAs in the thymus. The importance of this process is best evident in cases where mutations in the *AIRE* gene result in a severe systemic autoimmune syndrome known as autoimmune polyendocrinopathy-candidiasis-ectodermal dystrophy (APECED) (3-5).

1.2. Peripheral tolerance mechanisms limit immune response to self and harmless antigens

Although crucial and efficient, thymic negative selection is not sufficient to eliminate all self-recognizing T cells, some of which have been shown to escape this process and circulate in the peripheral blood of even healthy individuals (6, 7). Moreover, there are foreign antigens to which immune tolerance is required and that are not part of the sampled antigens during thymic selection, such as food antigens. Therefore, efficient regulatory mechanisms are required to maintain self-tolerance in the periphery. These include:

1.2.1. Peripheral deletion

A process of peripheral deletion aids in the elimination of T cells that are chronically stimulated by self-antigens in the periphery. Similar to negative selection in the thymus, peripheral deletion is achieved through induction of apoptosis. This process is largely mediated by the Fas (CD95)-FasL (CD178) pathway that is triggered by repeated T cell activation, leading to activation-induced cell death (AICD) (8). The importance of this pathway in immune tolerance is exemplified by the development of progressive lymphadenopathy and splenomegaly in mice lacking Fas or FasL (9). In humans, mutations in these apoptotic genes leads to an autoimmune lymphoproliferative syndrome (ALPS) characterized by polyclonal lymphoproliferation causing severe autoimmunity and increased susceptibility to malignancies (10, 11).

1.2.2. Ignorance:

As thymic negative selection eliminates T cells with strong affinity to self-antigens, it is thought that autoreactive T cells that manage to evade negative selection have a relatively low avidity, requiring a high concentration of their cognate antigens to be activated (12).

Furthermore, naïve T cells usually circulate between blood and the lymphatic system and rarely enter tissues before being activated in the draining lymph nodes (13). Thus, autoreactive T cells are normally physically separated from their cognate antigens and thus ignore them. This ignorance can, however, be broken by tissue injury that results in the release of otherwise hidden self-antigens.

1.2.3. Anergy:

Successful activation of a T cell in the periphery requires the engagement of the TCR as well as costimulatory signals mediated by the ligation of CD28. As costimulatory molecules are upregulated on antigen-presenting cells in response to inflammatory cytokines, expression of costimulatory molecules is low in the resting state and thus a self-reactive T cells is less likely to receive sufficient co-stimulation from antigen-presenting cells. This leads to the induction of T cell anergy which is a long-lived state that is characterized by hypoproliferation and reduced cytokine production upon restimulation (reviewed in (14)).

1.2.4. Inhibitory receptors

While they play an essential role in the T cell activation process, costimulatory molecules on APCs can also provide inhibitory signals that limit T-cell responses and promote tolerance. One such signal involves the inhibitory receptor CTLA-4 which is induced after T-cell activation and binds to the B7 family of costimulatory molecules with high avidity (reviewed in (15)). Engagement of CTLA-4 results in a negative signal that limits cytokine production by T cells and causes cell-cycle arrest. In mice, deficiency of CTLA-4 results in lethal autoimmunity (16, 17). Recently, heterozygous mutations in the *CTLA4* gene have been reported in humans where they cause a complex immune dysregulation syndrome characterized by hyper-activation of effector T cells leading to multiple autoimmune manifestations (18, 19).

Another inhibitory molecule expressed on T cell surface is the programmed death 1 (PD-1) receptor (reviewed in (20)). Engagement of PD-1 on T cells with its ligands PD-L1 and PD-L2 attenuate the activation of the PI3K and Akt pathways downstream of TCR signalling, thus inhibiting the early phase of T cell activation and controlling the expansion of self-reactive T cells (21). The critical role played by PD-1 in immune tolerance is apparent in mice deficient for PD-1 which develop a lupus-like autoimmune disease (22).

Despite the apparently similar consequences of their engagement, the CTLA-4 and PD-1 pathways are thought to play non-redundant roles in the maintenance of tolerance. While the CTLA-4 ligands B7-1 and B7-2 are constitutively expressed in lymph nodes, and thus help control early T cells activation, PD-L1 and PD-L2 are majorly expressed in peripheral tissues and thus regulate activated T cells at later stages of an immune response (reviewed in (23)). Owing to their role as immune checkpoints in the regulation of T cell responses, both CTLA-4 and PD-1 have been successful targets in the immunotherapy of several types of melanoma and non-small cell lung cancers where inhibition of both molecules results in increased anti-tumour immunity (24).

1.2.5. Inhibitory Cytokines

In addition to their role in antigen presentation and control of costimulation, APCs further contribute to the maintenance of tolerance through the production of a number of soluble factors with anti-inflammatory properties such as IL-10, TGF-β, indoleamine 2,3-dioxygenase (IDO) and nitric oxide (reviewed in (25)).

1.2.6. Specialised regulatory cell subsets

Active control of autoimmune responses in the periphery is further mediated by a number of specialized suppressor immune cell subsets that act to inhibit the activation, proliferation and

effector function of autoreactive lymphocytes. Several such subsets have been described including T ($CD4^+$ and $CD8^+$) and B regulatory cells (reviewed in (26-28)). Due to the extreme consequences of their deficiency in mouse and human, the $CD4^+FOXP3^+$ regulatory T cell subset (Treg) is arguably the most crucial regulatory T cell subset and is, therefore, the most characterized.

2. Regulatory T cells: Historical background

The first indication of the presence of a suppressive subset of T cells came from a study by Nishizuka and Sakakura in 1969 where they demonstrated that neonatal thymectomy in normal mice results in distinct outcomes depending on the timing the procedure is performed. Specifically, mice that underwent thymectomy at day 3 developed organ-specific autoimmune responses whereas mice that were subjected to day 1 or day 7 thymectomy did not develop autoimmunity (29). This suggested that auto-reactive T cells exit the thymus into the periphery before day 3, and that a population of suppressive T cells that is crucial for the control of auto-reactive T cells exits the thymus between day 3 and day 7. Moreover, studies by Penhale *et al.* reported that adult thymectomy in rats followed by sub-lethal irradiation resulted in the development of spontaneous thyroiditis and type 1 diabetes in the majority of the animals (30), further demonstrating the existence of a suppressor lymphocyte population that controls autoimmune responses in the periphery. A few years later, Sakaguchi *et al*. showed that splenocytes from adult mice injected into day 3-thymectomized mice within 2 weeks of thymectomy protected against autoimmunity (31). Thus day 3 thymectomy allows the export of auto-reactive T cells but impedes their regulatory counterparts. Although the existence of a suppressor subset was highly suggested by these experiments, progress in their research was significantly hindered by the lack of a marker to isolate them. In 1985, Sakaguchi *et al.* showed

that transfer of $CD5^{High}$-depleted $CD4^+$ T-cell suspensions from normal BALB/c mice into T-cell-deficient BALB/c athymic nude mice caused spontaneous multi-organ autoimmune disease (32). This autoimmunity was preventable by co-transfer of unfractionated $CD4^+$ T cells. In 1993, two group, Powrie et al. and Morrissey et al., independently demonstrated that transfer of normal BALB/c $CD45RB^{High}$, but not $CD45RB^{Low}$ or unfractionated $CD4^+$ T cells, to BALB/c SCID mice induced severe inflammatory bowel disease (IBD) (33, 34), suggesting that the $CD45RB^{Low}$ fraction is enriched for suppressor cells. The quest for a more specific marker of these suppressor cells continued until 1995 when Sakaguchi *et al* identified CD25, the alpha chain of the IL-2 receptor, as a candidate. They showed that $CD25^+$ cells, representing 5-10% of peripheral $CD4^+$ T cells, were present in both the $CD45RB^{Low}$ and $CD5^{High}$ fractions of $CD4^+$ cells (35). Transfer of CD25-depleted $CD4^+$ BALB/c splenocytes into to BALB/c athymic nude mice caused multi-organ autoimmunity with a higher incidence and an increased severity than that caused by the transfer of a similar number of $CD5^{Low}$ or $CD45RB^{High}$ cells. Moreover, co-transfer of $CD25^+CD4^+$ T cells with the CD25-depleted cells prevented inhibited the autoimmunity. Finally, transfer of $CD25^+CD4^+$ T cells from healthy mice early after day-3 thymectomy prevented the development of autoimmunity (35), thus zooming in on what appears to be a highly specialized regulatory T cell subset. A similar population of $CD4^+CD25^+$ T cells was later identified in the peripheral blood of healthy humans (36-41).

In addition to $CD25^+$ cells, other types of $CD4^+$ Treg cells with suppressive ability have been described. These include Tr1 cells which produce high levels of IL-10 and are characterized by their expression of CD49b and LAG-3 (42, 43). Another population termed Th3 was originally observed in mice following oral tolerance induction to myelin basic protein (MBP) (44). Th3 cells produce high levels of TGF-β as a primary mechanism of suppression (45). Unlike the

$CD25^+$ Treg population, which develops in the thymus as a distinct lineage, Tr1 and Th3 cells develop in the periphery from conventional $CD4^+CD25^-$ T cells, and it is not clear if they represent stable regulatory populations.

3. FOXP3 is the master transcriptional regulator of $CD4^+$ regulatory T cells

Despite major advances towards the characterization of $CD4^+CD25^+$ regulatory T cells as a distinct $CD4^+$ T cell subset, a specific marker of this population remained elusive for several years. Although CD25 expression identifies suppressor T cells in the resting state, it is upregulated in all T cells upon activation, precluding its use as a reliable marker. In 2001, Brunkow *et al.* identified mutations in the forkhead-winged helix transcription factor Foxp3 as the underlying cause for spontaneous autoimmunity in the scurfy mouse where an x-linked recessive mutation results in hyperproliferation of $CD4^+$ T cells and multi-organ autoimmunity leading to death 16-25 days after birth (46). The phenotype of the scurfy mouse resembles that of 3-thymectomized mice as well as mice lacking *Ctla-4* or *Tgf-β* suggesting that Foxp3 mutations impair the regulation of T cell responses (46). Studies in parallel have also demonstrated that mutations in the human *FOXP3* gene cause a severe autoimmune syndrome known as immunodysregulation, polyendocrinopathy, enteropathy, X-linked syndrome (IPEX) (47-49). Foxp3 expression is highly specific to αβ T cells, particularly $CD4^+$ T cells, and is not detectable in B cells, γδ T cells, natural killer (NK) cells, macrophages and dendritic cells (DC) (50, 51). A major breakthrough came in 2003 when several groups demonstrated that Foxp3 is specifically expressed in $CD4^+CD25^+$ regulatory T cells, and that ectopic expression of Foxp3 in naive T cells endows them with regulatory T cell phenotypic and functional properties (50-52). Foxp3 has further been shown to be required for regulatory T cell development, survival and function

(50-52). Thus, the discovery of Foxp3 has earned this regulatory T cell subset the status of a *bona fide* $CD4^+$ lineage, now known as $Foxp3^+$ Treg cells.

4. Development of Treg cells

4.1. Thymus-derived Treg cells

A population of $Foxp3^+$ Treg cells develops in the thymus and emerges as a distinct subset with high suppressive capacity. These are termed thymus-derived Treg (tTreg)(53). The thymic events leading to Treg development are not completely elucidated. Research in mouse models has suggested that $CD25^+$ Treg cells appear at the CD4 single positive stage of T cell development. However, their functional maturation and exit from the thymus into the periphery is delayed until day 3 of neonatal life, as evidenced by early studies where day 3 thymectomy results in spontaneous autoimmunity (29). For a developing T cell to become a Treg cell in the thymus, the possession of a self-reactive TCR seems to be required. In these studies Treg cell development in the thymus of TCR-transgenic required the expression of the cognate antigen by a second transgene (54-56). Given that such auto-reactive T cells are supposed to be deleted during the negative selection process, it was suggested that thymic Treg cell development occurs when the cell's TCR avidity for self-antigens is high enough to pass the positive selection process but is sufficiently low to avoid negative selection (54, 56-59).

4.2. Peripherally-induced Treg cells

In addition to thymus-derived Treg cells, it is now clear that Treg cells can also develop in the periphery from naive $FOXP3^-CD4^+$ T cell precursors. These peripherally-induced Treg cells, termed pTreg, develop in a variety of physiological conditions such as lymphopenia or infections

(60); (60-62). Although the precise signalling requirements that lead to the generation of pTreg cells *in vivo* are not clear, TGF-β and retinoic acid are thought to be required (63, 64).

The relative abundance of the two Treg subsets in the healthy immune system, as well as the differences in their role in the maintenance of immune tolerance is not clearly understood, largely due to the lack of specific markers to distinguish tTreg from pTreg. However, studies suggest that both tTreg and pTreg are indispensable for immune tolerance. For instance, in day 3-thymectomized mice, the lack of tTreg cells is not compensated for by pTreg generation *in vivo* (35). On the other hand, by impairing pTreg generation through the deletion of the TGF-β sensitive region of the *Foxp3* enhancer, the Rudensky group has shown that pTreg cells are essential for immune tolerance at mucosal sites (65), and for maternal-fetal tolerance (66).

The quest for a marker that distinguishes tTreg from pTreg cells is still ongoing. The first proposed marker was Helios, a transcription factor of the Ikaros family, which was reported to be specific for tTreg cells in mice (67). However, later studies suggested that Helios can be upregulated in pTreg and iTreg cells under certain conditions of stimulation (68-70), casting doubts over the specificity of Helios as a tTreg marker. More recently, neuropilin 1 (Nrp1) has been proposed as a marker of tTreg cells (71, 72). However, unlike Helios, Nrp1 is not expressed by a significant proportion of thymic Treg cells (73), and it can be also expressed on TGFβ-induced iTreg cells (71). Importantly, Nrp1 is not specifically expressed on human Treg cells, suggesting that it may not be a useful marker to distinguish the two Treg subsets in humans (74).

4.3. *In vitro*-generation of Treg cells

Lastly, Treg cells can be generated *in vitro* from naïve $CD4^{+}FOXP3^{-}$ by TCR stimulation in the presence of exogenous TGF-β and IL-2 (75, 76). These induced Treg cells, termed iTreg, exhibit a similar phenotype to that of tTreg and pTreg and possess a potent suppressive capacity

in mice (77). However, while TGF-β induces FOXP3 expression and Treg phenotype in human naïve conventional $CD4^+$ cells, it does not result in the acquisition of suppressive function (78), indicating that the requirements for the development of suppressive function differ in mouse and humans.

5. Mechanisms of suppression by Treg cells

The ability of Treg cells to suppress a wide range of cell types suggested that they employ a number of different mechanisms to mediate their suppressive functions. Indeed, both contact-dependent and contact-independent mechanisms of suppression have been described that may be differentially required in different contexts with a potentially larger degree of redundancy (Figure 1). Although the importance of some of these mechanisms has been demonstrated both *in vitro* and *in vivo*, it remains difficult to assess the relative importance of one mechanism over the other.

5.1. Direct contact

Early studies examining the requirement for cell-cell contact in the suppressive function of Treg cells using trans-well assays concluded that suppression by Treg cells may be contact-dependant (79, 80). However, later studies have identified several Treg-secreted soluble factors that mediate suppression, some of which require close proximity between Treg cells and their target cells suggesting that Treg cells employ a combination of soluble products as well as membrane-bound molecules to exert their suppressive function. A number of anti-inflammatory cytokines are produced by Treg cells *in vitro* and *in vivo* and have been shown to contribute to the suppressive function of Treg cells.

5.2. Inhibitory cytokines

5.2.1. IL-10

One such regulatory cytokine is IL-10. It is produced by both myeloid and lymphoid cells during an immune response, and signals through the IL-10 receptor complex leading to the activation of Jak1 and Tyk2 (81), and subsequent activation of STAT1, STAT3 and STAT5 transactivation (82). The effects of IL-10 include the inhibition of inflammatory cytokine production in, and the downregulation of co-stimulatory molecules on antigen-presenting cells. Since IL-10 is widely produced by several cell types, the relative importance of Treg-derived IL-10 was unclear until it was demonstrated that Treg-derived IL-10 plays a critical role in Treg suppression of intestinal inflammation in T cell transfer-mediated colitis model, where IL-10 blockade or Treg-specific IL-10 deficiency led to exacerbated inflammation (83). Moreover, the heterogeneous distribution of IL-10-producing Treg cells in various organs suggests that the importance of the IL-10-mediated suppressive mechanism may be dependent on the organ and/or disease model (84). In line with this, Rubstov *et al.* suggested that Treg-derived IL-10 is critical for immune regulation of inflammatory responses at mucosal surfaces but may not be required for regulation of systemic autoimmunity (85).

5.2.2. TGF-β

Another anti-inflammatory cytokine that has been associated with Treg function is TGF-β. It is a pleiotropic cytokine with reported anti-inflammatory effects on cell differentiation, migration and survival (63). Mice deficient for TGF-β present with T cell-mediated autoimmunity in the first few weeks of life (63). A similar outcome has also been observed in mice with T cell-specific abrogation of the TGF-β receptor (86). Moreover, several studies demonstrated a requirement for TGF-β in the control of autoimmunity in a number of mouse models of type1 diabetes (T1D) and colitis (87, 88). Treg cells produce high amounts of TGF-β, both soluble and

membrane-bound. However, blockade of TGF-β only partially abrogates Treg-mediated suppression of T cell proliferation in *in vitro* suppression assays using mouse and human Treg cells (89, 90). Moreover, several studies have reported that TGF-β is not required for the suppressive function of Treg cells (91-94). Thus, while TGF-β seems to be an important regulator of autoimmune responses, it does not seem to play an important role in the suppressive function of $FOXP3^+$ Treg cells.

5.2.3. IL-35

Recent studies have also proposed the production of IL-35 by Treg cells as a mechanism of suppression (95). Comprised of the p35 subunit of the IL-12 complex and the Epstein–Barr virus-induced gene 3 (Ebi3), this cytokine has been shown to be important for controlling inflammatory bowel disease (IBD) in a mouse model with Treg-specific deficiency in one of the IL-35 chains (95). However, these mice did not show spontaneous autoimmunity (95). IL-35 production has not been observed in resting human Treg cells (96). It was rather reported that IL-35 is produced by activated effector T cells and acts to enhance the suppressive function of Treg cells (97). Thus the relevance of IL-35 in Treg biology remains to be further investigated.

5.3. Cytolysis

A more direct mechanism of suppression by Treg cells employs cytotoxic molecules such as Granzyme and perforin to lyse and kill effector cells in target tissues (98). In a study by Grossman *et al.,* activated human Treg cells expressed granzyme A and killed activated target cells in a perforin-dependent fashion (98). Mouse Treg cells have also been shown to express granzyme B upon activation and kill responder cells in a perforin-independent way as deduced from observations that granzyme B deficiency, but not perforin deficiency, rendered mouse Treg

cells less suppressive *in vitro* (99). However, the contribution of Treg-derived granzyme in immune regulation by Treg cells is not clearly established *in vivo*.

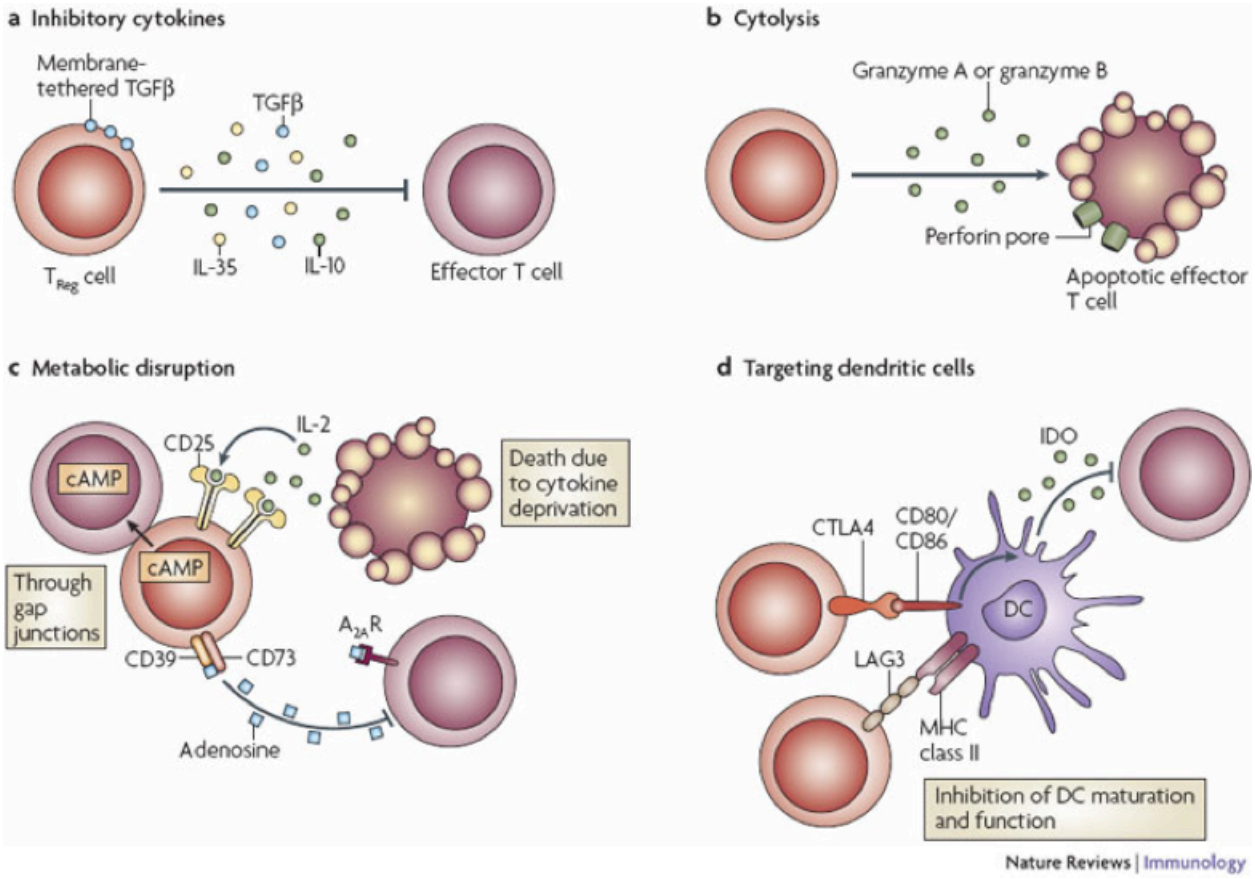

Figure 1. Basic mechanisms of suppression by Treg cells. Vignali *et al.* 2008. *Nat. Rev. Immunol*

5.4. Metabolic disruption

In addition to the aforementioned mechanisms of suppression, Treg cells possess certain features that allow them to suppress effector cells through reduction of metabolically-critical molecules.

5.4.1. IL-2 consumption

IL-2 is a critical growth factor for T cells, and thus it was suggested as a potential target by Treg cells to limit the response of Teff cells. Indeed, early studies have shown that

supplementing a Treg/Tresp suppression co-culture with exogenous IL-2 abolished the suppressive capacity of Treg cells, suggesting that Treg cells, facilitated by their elevated expression of CD25 may suppress target cells by preferential consumption of IL-2 acting as a "cytokine sink" (100, 101). However, this hypothesis has been challenged by later observations that IL-2-deficient and CD25-deficient Treg cells exhibit unaltered suppressive capacity *in vitro* (102). Nonetheless, IL-2 production by Teff cells has been shown to be inhibited by Treg cells during the course of suppression, suggesting that modulation of IL-2 availability may be an important mechanism used by Treg cells to control Teff cell proliferation (91).

5.4.2. CD39/CD73

Another mechanism through which Treg cells can control Teff responses is through the hydrolysis of extracellular ATP which is released by damaged cells and has strong pro-inflammatory effects (103). Through their expression of high levels of the ectoenzymes CD39 and CD73, Treg cells convert ATP into adenosine which exerts potent inhibitory action on activated Teff cells (104-106). CD39-deficient Treg cells show reduced suppressive function both *in vitro* and *in vivo* (106). In humans, CD39 expression by Treg cells was proposed to identify a Treg subset with increased suppressive capacity (107). However, CD39 expression is preferentially expressed by memory Treg cells in humans (104, 107), and it is well-established that memory Treg cells are more potent suppressors than their naïve counterparts (108, 109). Therefore, a direct link between CD39 expression and increased functional potency of human Treg cells remains to be established.

5.5. Targeting antigen presenting cells

In addition to their active suppression of T cell activation and proliferation, Treg cells can also exert their modulatory action on antigen-presenting cells. Targeting of APCs offers an

indirect, yet powerful, means of controlling T cell responses. One mechanism is Treg-mediated modulation of the expression of costimulatory molecules on APCs (110) though CTLA-4. CTLA-4 is an inhibitory receptor expressed constitutively by Treg cells and by all $CD4^+$ T upon activation (40, 41, 111, 112). It has a higher affinity binding to CD80/CD86 than that of CD28, and causes downregulation of co-stimulatory factors on APCs (113-115); (116, 117). Moreover, engagement of CTLA-4 has been recently shown to strip CD80/86 from APCs through transendocytosis (118), thus reducing the immuno-stimulatory potential of APCs. Additionally, engagement of the CTLA-4 pathway can induce the production of indoleamine 2,3-dioxygenase (IDO) from DCs (113, 119). IDO converts the amino acid tryptophan to kynurenine, which is toxic to T cells in proximity (120, 121).

Furthermore, a subset of Treg cells expresses LAG-3, a transmembrane adhesion molecule that binds MHC class II and is thought to inhibit the maturation of DCs (122). LAG-3 deficiency or blockade seems to reduce the suppressive potency of Treg cells *in vitro* (123, 124). However, LAG-3-deficient mice do not develop autoimmunity, suggesting that this mechanism of suppression may not play a major role in Treg function.

As discussed above, Treg cells employ a wide range of molecular mechanisms to control immune responses. It should be noted that the majority of the molecule that have been associated with Treg suppressive function are not uniformly expressed by all Treg cells. This suggests that the Treg subset is highly heterogeneous, and that different inflammatory environments and/or tissues may require different Treg subpopulations. The relative contribution of these multiple suppression mechanisms and the precise requirements that trigger them specifically remain to be investigated.

6. The role of FOXP3 in Treg cells

As discussed above, several lines of evidence clearly demonstrate the vital role played by FOXP3 in driving the transcriptional program of Treg cells. Moreover, Foxp3 is not only required for the development of Treg cells but is also continuously required for the maintenance of Treg function in the periphery as indicated by studies where *Foxp3* was conditionally deleted in fully differentiated peripheral Treg cells. In these experiments, loss of Foxp3 resulted in severe autoimmunity secondary to loss of suppressive function in Treg cells as well as production of otherwise-repressed effector cytokines such as IFNγ, IL-4, and IL-17 (125). Similarly, spontaneous loss of Foxp3 in several lymphopenic animal models results in loss of suppressive function and production of inflammatory cytokines (discussed later in this chapter) (126-128). Major efforts have, therefore, been aimed at elucidating the specifics of how Foxp3 drives the transcriptional program of Treg cells. A number of chromatin immunoprecipitation studies have revealed several hundred genes as direct targets of Foxp3 in mouse and humans (129, 130), and have shown that Foxp3 acts both as a transcriptional repressor of genes such as *Il2, Ifng, IL17 and IL7ra,* as well as a transcriptional activator of genes such as *Il2ra* and *Ctla4*. However, these direct targets only account for less than 50% of the Foxp3-dependent transcriptional program suggested by microarrays, indicating that a significant proportion of the Treg signature is indirectly regulated by Foxp3, through other molecular factors. Indeed, in addition to forming Foxp3/Foxp3 homodimers, Foxp3 interacts with several molecular partners forming higher-order molecular complexes that mediate several aspects of the Foxp3-dependent Treg program. Known molecular partners include NFAT, AML-1/Runx1, NF-kB, ROR-α, as well as a number of histone acetyl transferase (HAT) and histone deacetyl transferase (HDAC)

enzymes. The following section discusses the molecular structure of FOXP3 and some of its known interactions and their downstream effects.

7. FOXP3 structure and molecular interactions in the development of Treg transcriptional signature

The FOXP3 protein is 431 amino acid long, and is comprised of four domains **(Figure 2)**. From the N- to the C terminus, these include: (1) the N-terminal proline-rich repressor (PRR) domain (a.a.70-105); (2) the zinc-finger (ZF) domain (a.a.200–223); (3) the leucine-zipper (LZ) domain (a.a.240-261); and (4) the FKH domain (a.a.338-421).

7.1. N-terminal PRR domain:

The N-terminal PRR has multiple functions. It is specifically required to suppress the NFAT/AP1-driven transcriptional program, which regulates the expression of genes vital to cell survival, pro-inflammatory cytokine production, and the progression of the cell cycle (131). This suppression maintains the anergic state of Treg cells, and prevents the production of pro-inflammatory cytokines, in particular IL-2, in response to TCR stimulation. The N-terminal PRR also directly binds and inhibits the action of ROR-α and -γ transcription factors (132), which are critical for the development of pro-inflammatory Th17 cells (133). IPEX-causing mutations positioned between a.a.1-193, and particularly between a.a.67-132 of FOXP3, disrupt the function of the PRR domain (131). As the interaction of FOXP3 with AP1 requires the integrity of its N-terminal region, it follows that IPEX mutations such as E70H and T108M may affect the maintenance of Treg anergy by FOXP3, while not necessarily disrupting other functions of FOXP3. Consistent with this hypothesis, these mutations have been described in association with milder cases of IPEX (134, 135).

A lysine-rich nuclear export sequence exists in the N-terminal PRR, which makes the FOXP3 protein sensitive to lysine acetylation (136). It has been noted that acetylated FOXP3 is preferentially bound to chromatin, and may demonstrate enhanced promoter occupancy, suggesting that lysine acetylation of FOXP3 may play a role in regulating its function (137). Finally, a sequence in this domain is vital for FOXP3 to bind to, and mediate the function of, histone deacetylases, which alter chromatin. FOXP3 can be co-immunoprecipitated with a number of histone deacetylase molecules (including HDAC 1,7, and 9) (138). The modulation of chromatin configuration alters gene transcription through regulating access to gene promoter regions, and is proposed to be one of the methods by which FOXP3 mediates Treg cell function. This process may regulate access to the promoter region of FOXP3 itself, as well as those of other transcription factors. IPEX mutations located in this region include T108M and P187L. Interestingly, while the former mutation was associated with a milder, late-onset form of IPEX (134), the P187L mutation led to loss of Treg function and disease onset shortly after birth (139, 140).

7.2. ZF domain:

Amino acids 200-223 encode a C2H2 zinc finger. Whilst ZF domains have been shown to play a role in protein-protein interactions, Lopes *et al* have demonstrated that in the case of FOXP3, the role of the ZF in homodimerization is redundant, with the LZ domain being both necessary and sufficient for homodimerization (131). The role of the ZF in normal FOXP3 function is unknown mainly due to the absence of IPEX-causing mutations that affect the ZF domain. However, engineered mutations that destroy the ZF structure do not significantly affect the FOXP3-driven suppression of gene transcription from the *Il2* promoter (131).

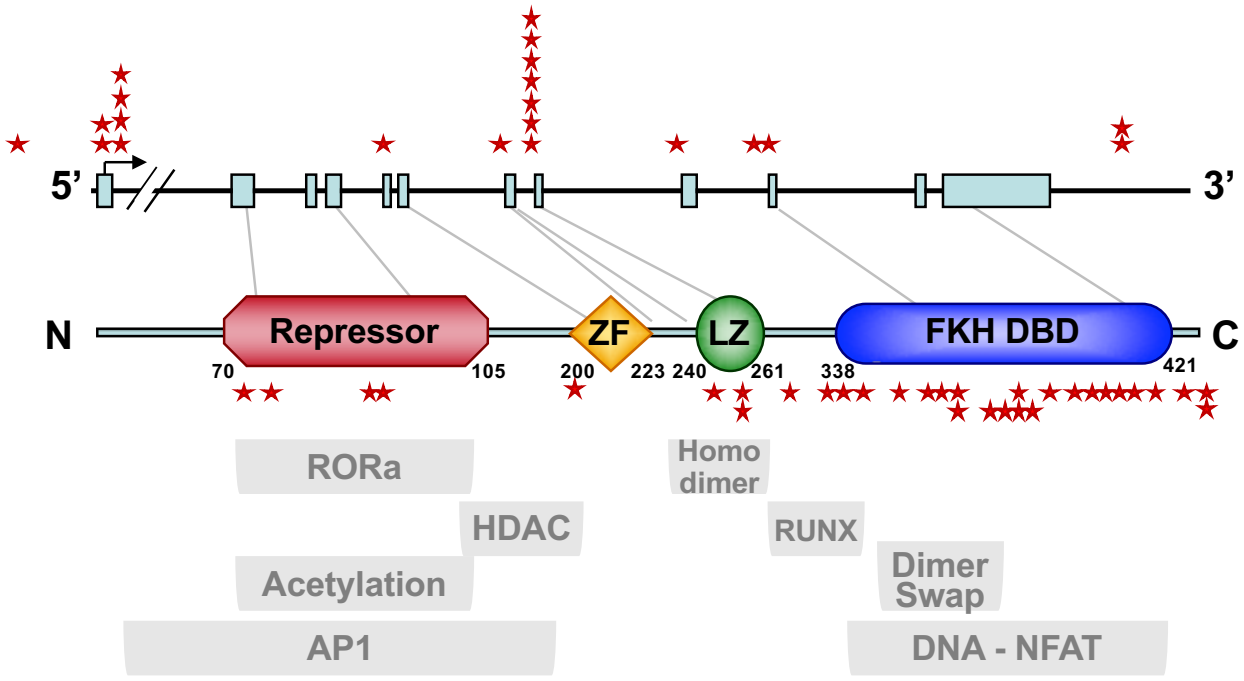

Figure 2. A schematic showing the FOXP3 functional domains and known molecular interactions. The approximate locations of IPEX causing mutations are represented by red stars. Modified from: d'Hennezel and Bin Dhuban *et al.* 2012. *J. Med Genetics*

7.3. LZ domain and LZ-forkhead loop:

The LZ domain spans amino acids 240-261.A naturally-occurring splice variant of FOXP3 lacking exons 2 and 7, which encode the LZ domain, lacks regulatory properties and has been proposed to negatively regulate the function of other FOXP3 isoforms (141). The LZ domain is also the site of a number of other IPEX-causing mutations, the most studied of which is the ΔE251 mutant (140, 142-147). It has been shown that the LZ is required for FOXP3 homo-oligomerization (131, 148), however it is not necessary in order for FOXP3 to form high molecular weight protein complexes (145). Mutations in this domain result in a loss of transcriptional regulation by FOXP3, and a loss of suppressive function in affected Treg cells

(131, 141, 148). Notably, LZ domain mutations result in partial abrogation of the association of FOXP3 with histone acetylase, histone deacetylase and HDAC proteins, and a partial loss in the DNA-binding ability of the resulting protein complex (138). In general, LZ mutations in IPEX are expected to result in severe deficiencies in the regulatory properties of FOXP3.

In between the LZ domain and FKH domain is a region known as the LZ-FKH loop (a.a.261-337). This region of the FOXP3 protein is not a defined functional domain, however it has been shown to be essential for FOXP3 binding to and inhibiting RUNX transcription factors (149). RUNX enhances IL-2 expression in T cells, and the inhibition of this is a mechanism by which Treg cells regulate immune function (150, 151). Disrupting FOXP3/RUNX1 binding prevents the FOXP3-dependent suppression of IL-2 production, while *Runx1*gene silencing in Treg cells impairs their suppressive activity (151), suggesting that Runx1/Foxp3 interactions are essential for Treg function *in vitro*. Mutations in the LZ-FKH loop are associated with relatively mild phenotypes of IPEX (152).

7.4. DNA-binding, FKH domain:

The DNA-binding FKH domain spans a.a.337-421 in the C-terminus of the protein and is highly conserved amongst forkhead transcription factors. The FKH domain mediates FOXP3 binding to DNA *in vitro*, a property essential for all transcriptional functions. The importance of the FKH is exemplified by the observation that a frameshift mutation near the N-terminal of this domain, deleting the FKH domain, is responsible for the phenotype of the scurfy mouse (46). The FKH domain also interacts with the NFAT and NF-κB transcription factors, with engineered mutations in the NFAT-binding residues of the FKH domain leading to a loss in Treg suppression of IL-2 production (153). Thus, loss of the FKH-mediated repression of the

NFAT/AP1 transcriptional repression may lead to a lack of transcriptional repression and production of activation-induced inflammatory cytokines by Treg cells carrying the mutation.

The majority of IPEX-causing mutations identified in the coding regions of FOXP3 are found in the FKH domain, and are expected to have a number of impacts on the function of FOXP3, and Treg cell function. Lopes *et al.* identified several mutations that disrupt the transcriptional repression activity of FOXP3 (e.g. R337P, R347H, F371C and R397W) (131). Another study showed that the F373A mutant also lacked transcriptional repression activity (152). Strikingly, we recently used a single-cell cloning strategy to show that Treg cells from an IPEX patient with the A384T mutation are significantly reduced in their suppressive activity despite the preservation of a normal Treg cell phenotype. (154). Interestingly, the A384T mutation does not affect the binding of FOXP3 to DNA or to any of the major known FOXP3 partners, suggesting that it may alter an as-of-yet unappreciated mechanism of FOXP3 function. In contrast, some FKH mutations do not affect the transcriptional repression of IL-2 by FOXP3 (e.g. I363V, C424Y), or its capacity to drive the suppressive activity of Treg cells (e.g. R347H) (152), although their impact on other FOXP3 functions have not been extensively analyzed

Analysis of the crystal structure of DNA/FOXP3 complexes has also identified a role for the FKH domain in forming a domain-swapped dimer conformation, which is critical to FOXP3 functions (155). Targeted mutations of the dimerization interface of the FKH domain resulted in normal FOXP3 expression and DNA binding, but diminished the expression of some target genes (such as *Il*-2) and abrogated the suppressive function of cells transduced with the mutation (155). Several IPEX mutations directly affect residues that are situated at the domain-swap interface, many of which are associated with severe clinical outcomes (eg; M370L, F367C, F367L, F371C, F373V, F373A, and F374C). The FKH domain also directs the nuclear

localization of FOXP3 through an RKKR nuclear localization motif found between a.a.414-417 (131). However, no IPEX mutation has yet been described in this interval.

As discussed above, different *FOXP3* mutations in IPEX can result in a wide spectrum of clinical severities, likely reflecting the relative importance of the affected molecular interactions. Interestingly, several mutations have been associated with severe clinical presentation despite the expression of normal levels of FOXP3 (156). Therefore, FOXP3 protein detection cannot be used as a reliable diagnosis of IPEX. Experimentally, those mutations that maintain FOXP3 expression provide an excellent platform for the elucidation of critical pathways required for the FOXP3-mediated development of Treg phenotype and function as we will demonstrate in **chapter 2** of this thesis.

8. Regulation of FOXP3 expression

8.1. Epigenetic regulation of FOXP3 expression

Analysis of the Foxp3 locus from various species has identified three highly conserved enhancer regions, now known as conserved non-coding sequences (CNS1, 2 and 3). These regions play a significant role in the regulation of the *Foxp3* gene (157, 158). CNS1 harbours the TGF-β-responsive element and is, therefore, essential for the generation of pTreg and iTreg cells (65). CNS2 contains the Treg-specific demethylated region (TSDR), a CpG island that is completely demethylated in tTregs and pTreg cells (159, 160). In contrast, the TSDR is heavily methylated in conventional $CD4^{+}Foxp3^{-}$ cells as well as in *in vitro*-generated iTreg cells (159). It is thought that TSDR demethylation is a major factor in the stability of FOXP3 expression as TGFβ-induced iTreg cells can lose both FOXP3 expression and suppressive function upon re-stimulation in the absence of TGF-β (159). To further demonstrate the importance of epigenetic

regulation of Foxp3, treatment with the DNA methylase inhibitor Azacytidine enhances the stability of Foxp3 expression in iTreg cells (160, 161). Finally, the CNS3 region contains a c-Rel binding element where c-Rel can bind to the *Foxp3* locus and facilitate its transcription (157). CNS3-deficient mice exhibited reduced frequencies of tTreg cells as well as impaired ability to upregulate Foxp3 in response to TCR stimulation in the presence of TGF-β (65).

8.2. Post-translational modification of FOXP3

In addition to being regulated at the transcription level, FOXP3 undergoes significant post-translational modifications to modulate Treg induction and stability. These include phosphorylation, acetylation as well as ubiquitination. Moreover, FOXP3 expression and maintenance is influenced by several factors in the microenvironment including cytokines, as well as microbial metabolites and TLR ligands (reviewed in (162)).

8.3. Modulation of FOXP3 expression by extrinsic factors

Several soluble factors play important roles in the regulation of FOXP3 expression. These include positive regulators of FOXP3 such as IL-2, TGF- β and retinoic acid. On the other hand, FOXP3 expression is negatively regulated by a number of cytokines such as IL-6, IL-4, TNFα, IL-1β and IL-23 (162), highlighting the dynamic nature of Treg cells in response to various physiological contexts.

8.3.1. IL-2

IL-2 has long been known to be a major growth cytokine for T cell proliferation. It was therefore, expected that IL-2 deficiency would cause impaired T cell proliferation and compromise immune response. However, it was instead observed that IL-2 deficiency causes spontaneous multi-organ T cell-mediated inflammatory disease (reviewed in (163)). A similar

autoimmune syndrome is also observed in mice deficient in CD25 or CD122 (IL-2Rβ), and in humans with CD25 deficiency (164). It is now clear that the autoimmunity in these settings is due to a defective Treg population. Indeed, $Foxp3^+$ Treg cells are diminished in mice lacking either CD25 or IL-2 (165). Furthermore, CD25-deficient mice can be protected from autoimmunity by adoptive transfer of wild-type Treg cells. In addition, T cell-specific abrogation of the IL-2 signalling mediator, STAT5, impairs the development of $Foxp3^+$ Tregs and causes autoimmunity (166, 167). IL-2 signalling plays a major role in the upregulation and maintenance of CD25 and FOXP3 expression in Treg cells during thymic development, as well in the periphery (168-171), thus contributing critically to Treg development and survival. As the *IL-2* gene is a direct target that is negatively regulated by FOXP3, Treg cells do not produce IL-2 themselves and thus require it from other sources. This is likely a mechanism to regulate the expansion of Treg cells given their constitutive and high expression of CD25.

8.3.2. TGF-β

TGF-β is another essential positive regulator of Foxp3. As discussed above, activation of conventional $CD4^+Foxp3^-$ cells in the presence of TGF-β and IL-2 leads to the upregulation of Foxp3 and the generation of functional iTreg cells, at least in mice. *In vivo*, TGF-β signalling has been shown to be critical for the thymic development of Treg cells as well as for the generation of pTreg cells at mucosal sites (172, 173). TGFβ binding to its receptor triggers the activation of the SMAD2 and SMAD3 signalling factors, which directly bind to the TGFβ-responsive element in the CNS1 region of the *Foxp3* locus leading to the expression of Foxp3.

8.3.3. Retinoic Acid

Retinoic acid (RA), a metabolic product or vitamin A secreted is secreted by dendritic cells of the lamina propria and has been shown to synergize with TGF-β in the induction of Foxp3

expression in the gut (64, 174). Mechanistically, RA is thought to enhance the phosphorylation of Smad3 thus potentiating the effects of TGF-β signalling. Moreover, RA was further shown to increase the stability of TGF-β-induced iTreg cells (64, 174). RA can also bind the retinoic Acid receptor (RAR), and the retinoid X receptor (RXR), which subsequently engage with the CNS1 region and upregulate Foxp3 expression (175, 176). As an indirect effect on FOXP3 expression, RA has been shown to inhibit the production of inflammatory cytokines that negatively influence FOXP3 expression, such as IL-6 (177).

8.3.4. IL-6

Initial studies showed that IL-6 inhibits Treg-mediated suppression and causes loss of Foxp3 expression (178, 179). This effect was shown to be mediated by STAT3 activation, which prevents the binding of STAT5 to the *Foxp3* locus (167). Later studies demonstrated that, together with TGF-β, IL-6 inhibits the induction of FOXP3 and development of iTreg cells, and promotes the differentiation of Th17 cells, by favouring the expression of RORγt, the key transcription factor of Th17 (180, 181).

8.3.5. IL-27

IL-27 is a cytokine of the IL-12 family. It is produced by activated APCs and signals through a heterodimeric receptor complex composed of the IL-6 receptor subunit gp130 and the IL-27Ra (182). It results in the activation of both STAT1 and STAT3 and promotes the expression of T-bet and the differentiation of Th1 cells (183). Neufert *et al* showed that IL-27 impairs Foxp3 expression in response to TGF-β (184). This effect was also observed in STAT1-deficient animals, suggesting that the negative effects of IL-27 on FOXP3 expression are STAT1-independent. Indeed, knockdown of STAT3 by Stat3 siRNA significantly neutralized the IL-27 effect and allowed TGF-β-induced Foxp3 expression (185). However, as STAT3 is shared

with the IL-6-signalling pathway, it is difficult to dissociate the effects of the two cytokines.

8.3.6. IL-4

Treg function can also be modulated by IL-4 whose signalling induces GATA-3 expression. GATA-3 can bind to the *Foxp3* promoter region and suppress gene expression (186). In addition, activated STAT6 downstream of IL-4 signalling can bind to the *Foxp3* gene locus and directly prevent *Foxp3* gene activation (187).

Thus while Treg cells represent a distinct lineage of $CD4^+$ cells, their functional commitment and stability can be influenced by multiple factors leading to down-modulation of their suppressive function, and even to loss of Foxp3. The next section highlights recent literature that discusses the concept of lineage commitment of Treg cells.

9. Lineage stability of Treg cells

The question of Treg lineage stability has been the focus of intense research in the last few years. It started with the observation that purified $Foxp3^+$ Treg cells adoptively-transferred into lymphopenic mice can lose Foxp3 expression, downregulate their Treg phenotypic markers including CD25, GITR, and CTLA-4, produce high levels of inflammatory cytokines such as IL-2, IFN-γ and IL-17, and lose the ability to suppress Teff cells *in vitro* (126-128). Interestingly, when co-transferred with $Foxp3^-$ Teff cells, the degree of Foxp3 loss was markedly reduced, suggesting that Teff-derived signals may stabilize Foxp3 expression either directly or indirectly by antagonizing lymphopenia-induced modulatory signals (126, 128). To address this issue in a more physiological lympho-replete environment, a number of fate-mapping mouse models were generated. While the degree of Foxp3 loss in these models was variable (ranging from less than 5% up to 20%) in the steady state and under various challenges, it was significantly lower than that observed in lymphopenic mice (188-190). The origin of these former $Foxp3^+$ cells remains

controversial. While some studies argue that they originate from committed Treg cells that undergo lineage reprogramming (127, 128, 188, 191), others have argued that they originate from a small uncommitted Treg population that expresses low levels of CD25 (126). Moreover, Miyao *et al.* recently suggested that low and transient Foxp3 can be induced in mouse Teff cells upon activation (189). It was suggested that this pool of activated Teff cells, along with a minor subpopulation of uncommitted, peripherally-induced, $Foxp3^+$ Treg cells, constitute the majority of cells that lose Foxp3 in fate-mapping models, suggesting that Treg cells represent a generally stable, but heterogeneous, lineage (189).

In humans, Treg plasticity is difficult to demonstrate as FOXP3 is induced upon Teff activation (78, 192), rendering it challenging to obtain a pure starting population of Treg cells. In some studies, $FOXP3^+$ T cells were found to lose FOXP3 expression after repetitive TCR stimulation *in vitro* (158). However, it is highly likely that the starting population in these studies contained activated Teff cells that out-proliferated their Treg counterparts. One indication of potential signs of human Treg plasticity is the expression of inflammatory cytokines by some $FOXP3^+$ cells (67, 193, 194). However, it is not clear whether these cells are showing early signs of potential lineage instability. Moreover, it has been shown in animal models that some $Foxp3^+$ T cells can exhibit Teff-like features without losing Foxp3 expression. Rather, it was suggested that the upregulation of the transcription factors T-bet, IRF4 and RORγt by $Foxp3^+$ Treg cells may promote their ability to regulate Th1, Th2 and Th17 responses, respectively (195-197), suggesting that the Treg population comprises several subpopulations with different functional specialization.

10. Contribution of FOXP3$^+$ Treg cells to human disease

Numerous mouse models have clearly identified a direct role for FOXP3$^+$ Treg cells in the control of various innate and adaptive responses, and have served as experimental platforms for the validation of novel therapeutic strategies destined to target the development or function of Treg cells for disease prevention or resolution. The data pointing to the relative contribution of FOXP3$^+$ Treg cells in humans have, however, seldom been causal and more frequently correlative in nature.

10.1. IPEX syndrome

The essential contribution of Foxp3$^+$ Treg cells to the maintenance of self-tolerance in humans is best exemplified in the IPEX syndrome caused by loss-of-function, germline mutations in the *FOXP3* gene (47, 48) and impaired Treg development and function (154). To date, more than 60 different *FOXP3* point mutations have been reported, and present with a wide spectrum of clinical manifestations (reviewed in (156)). Abrogative *FOXP3* mutations invariably cause early and severe multiple autoimmune disorders including endocrinopathy, enteropathy, dermatitis and haemolytic anemia, leading to death in infancy if left untreated (156). The inevitable autoimmunity observed in IPEX patients provides compelling evidence of the essential role of FOXP3$^+$ Treg cells in immune self-tolerance in humans. Consequently, it was thought that quantitative and/or qualitative defects in the Treg population may drive the development of common autoimmune diseases in the general population. Indeed, a plethora of studies have attempted to assess the functional status of Treg cells in several autoimmune disorders of humans. However, the bulk of these findings remain inconclusive.

10.2. Type 1 diabetes (T1D)

T1D is one of the most common IPEX-associated disorders. It was, therefore, suspected that

Treg defects could constitute one of the mechanisms leading to the development of T1D. Initial studies relied on the surface expression of CD25 to identify Treg cells and reported a significant numerical reduction in Treg cells in patients with recent or established T1D (198). However, other reports found no difference in the frequency of $CD4^+$ $CD25^{Bright/High}$ (199, 200), or $CD4^+CD25^+FOXP3^+$ cells in the periphery of diabetic subjects (201). Studies in human T1D examining Treg function have also been inconsistent and inconclusive, reporting Treg cells with normal or impaired suppressive ability in T1D patients (199-201). An increased resistance to Treg-mediated suppression has also been suggested by some groups (202, 203).

10.3. Multiple sclerosis (MS)

In MS, studies have reported a normal frequency of $CD4^+CD25^{High}$ Treg cells in the blood of patients at different stages of disease activity (104, 204-210), although an increase in the frequency of Treg cells in the cerebrospinal fluids of MS patients has been documented (207, 209, 210). Functional defects in $CD4^+CD25^{bright/high}$ Treg cells of relapsing-remitting MS patients have been reported (204, 208, 211-213) although such functional defects were not observed by a later study that further purified Treg cells from contaminating activated Teff cells by excluding CD127-expressing cells (208).

10.4. Inflammatory bowel (IBD) disorders

Another clinical hallmark of IPEX is the development of enteritis (156), which suggested that Treg defects could contribute to the development of IBD disorders like Crohn's disease (CD) and ulcerative colitis (UC). Whereas an increase in the frequency of $CD4^+CD25^+FOXP3^+$ T cells has been reported in CD patients, the opposite was observed in UC (214, 215). Furthermore, the frequency of $FOXP3^+$ Treg cells was reported to be reduced in CD patients during relapses but increased during remission (216). No qualitative defects in the suppressive function of Treg cells

of IBD patients have been detected (214, 216-221).

10.5. Rheumatoid arthritis (RA)

In RA, $CD4^{+}CD25^{High}$ cells have been shown to be quantitatively normal in patients with established disease (222-224), while slightly reduced in Treg numbers in earlier stages of the disease (225). Conversely, Han *et al.* reported an increase in $CD4^{+}CD25^{+}FOXP3^{+}$ Treg cell numbers in the periphery of RA patients compared to controls (226). There is, however, an agreement that Treg cells are increased in the synovial fluid of RA patients relative to healthy subjects (224, 227). While a normal suppressive function of peripheral and synovial Treg cells of RA patients has been reported (224, 225, 227), Ehrenstein *et al.* observed a specific defect whereby Treg cells of RA patients are unable to suppress the production of IFNγ and TNFα *in vitro* (228).

10.6. Systemic lupus erythematosus (SLE)

In SLE, a decrease in the percentage of $CD4^{+}CD25^{High}$ cells has been reported by multiple studies (229-233), whereas other studies have observed no quantitative differences between SLE patients and controls when FOXP3 expression was included in the analysis (234). While the suppressive function of Treg cells was found to be impaired by some groups (234-236), others observed no such defects (230, 232).

As discussed above, investigations of the functional status of Treg cells in various autoimmune diseases have not been conclusive. Unlike the IPEX syndrome where *FOXP3* mutations result in global impairment of Treg development and function leading to severe multi-organ autoimmune manifestations, Treg defects in organ-specific autoimmunity may be too subtle to be detected using the common approaches of functional analysis of the global Treg population isolated from peripheral blood. It is possible that such subtle differences may only

manifest at the site of inflammation, and would thus require analysis of tissue-localized Treg cells. However, given the impact of inflammatory cytokines on Treg function, it is difficult to determine whether detectable Treg defects in autoimmune patients are primary causative factors or are rather a result of the associated chronic inflammation. Nonetheless, a deeper understanding of the different factors that modulate Treg function, and the development of specific strategies that enable the potentiation of Treg cells in autoimmune patients will provide a valuable means of controlling autoimmunity in patients regardless of whether Treg defects are primary or secondary to the autoimmune condition.

11. Challenges facing human Treg studies

The analysis of Treg frequency and function in autoimmune patients has further been hindered by several factors. These include differences in the selection of the subject cohorts with respect to patient history, disease stage, as well as technical variations in the methods of Treg isolation (eg; FACS vs. MACS) and assessment of functional readouts (eg; CFSE vs.thymidine incorporation assay). In order to avoid these inter-study variations, normalization of these criteria will be essential to reach significant and clinically relevant biological conclusions.

11.1. Lack of reliable human Treg markers

One major contributor to the inconsistency of studies investigating the functional status of Treg cells in human autoimmunity is the paucity of specific markers that allow the reliable identification and isolation of human Treg cells in immune quiescence and different states of activation (Table 1).

11.1.1. CD25

Most of the earlier studies relied on the co-expression of CD4 and CD25 to identify Treg cells in

PBMCs. However, CD25 is an activation marker that is readily upregulated on conventional $CD4^+$ T cells upon stimulation (237), thus contaminating the $CD25^+$ population with activated Teff cells, a situation likely to occur in patients with high levels of immune activation and inflammation. Restricting the analysis to $CD4^+$ cells expressing high levels of CD25 ($CD25^{High/bright}$: top 1-2% of $CD4^+$ T cells) allows the isolation of a relatively purer population expressing high levels of FOXP3 and a more consistent suppressive ability *ex vivo* (41). However, a substantial proportion of $FOXP3^+$ Treg cells are found within the $CD25^{Low}$ or even $CD25^{Neg}$ populations (238), thus, relying on CD25 expression to identify human Treg cells will exclude the majority of Treg cells. Moreover, there are no established criteria to define the $CD25^{High}$ population, and variations in FOXP3 expression within the $CD25^{High}$ population have been observed in healthy individuals even within the same study (238), which greatly impairs the reproducibility of analyses of patient samples especially in conditions of heightened immune activation.

11.1.2. FOXP3

The expression of FOXP3 is widely accepted as the most specific marker of functional Treg cells. However, it is now clear that a fraction of Teff cells express transient, but high, levels of FOXP3 upon activation, making them indistinguishable from Treg cells in inflammatory conditions (78, 192). Moreover, using a single-cell clonal approach that excludes Teff contaminants (109), analysis of primary $FOXP3^+$ clones from healthy donors revealed a remarkable degree of heterogeneity within this subset. While the majority of $FOXP3^+$ clones exhibit a typical Treg phenotype and potent suppressive abilities, a sizable subset (around 25-30%) of $FOXP3^+$ clones is incapable of suppression despite the maintenance of a Treg surface, anergic, and suppressive phenotypes (109). Importantly, this functional heterogeneity within the

Treg pool has been overlooked by studies assessing the functional status of Treg cells in autoimmune patients using conventional Treg markers.

11.1.3. CD127

Liu *et al.* and others have suggested that the lack of IL-7 receptor alpha chain (CD127) expression on $CD4^+$ cells can be used to identify and sort $FOXP3^+$ Treg cells from $CD25^+$ and $CD25^-$ cells, thus capturing the majority of $FOXP3^+$ cells (238, 239). However, CD127 expression is down-modulated on $CD4^+CD127^+$ upon TCR stimulation *in vitro* (240), and *in vivo* as shown in healthy individuals and in patients with HIV infection (108, 241). Additional markers are still required to exclude contaminating activated Teff cells. One such marker is the expression of L-selectin (CD62L) which is highly expressed on naive T cells and on the majority of $CD4^+CD25^{High}$ Treg cells while down-regulated on activated non- Treg cells (204, 242, 243).

11.1.4. CTLA-4

The majority of human Treg cells constitutively express high levels of intracellular CTLA-4 (111). However, surface CTLA-4 expression is upregulated on all $CD4^+$ T cells upon activation (41, 244), suggesting that CTLA-4 expression cannot distinguish Treg cells from activated Teff during inflammation, and is not suitable for sorting live Treg cells in the resting state due to its intracellular localization.

11.1.5. HLA-DR

HLA-DR is expressed on about 30% of peripheral human Treg cells and has been described as a marker of terminally differentiated Treg cells (245). Functionally, $HLA\text{-}DR^+$ Treg cells are more suppressive than their $HLA\text{-}DR^-$ counterparts, which can express HLA-DR when expanded *in vitro* (245, 246). Although useful for the isolation of a distinct subset of antigen-experienced Treg cells, HLA-DR cannot be used to capture most of the Treg pool, and its

upregulation on activated Teff cells limits its applicability as a Treg marker during immune activation (245).

11.1.6. GARP

GARP (glycoprotein A repetitions predominant) is a recently proposed marker for activated Treg cells, and whose function consists of tethering TGFβ to the cell surface (247). GARP was reported to be differentially expressed on Treg cells upon activation compared with Teff cells (248, 249), and $GARP^{+}FOXP3^{+}$ cells were shown to have an enhanced suppressive ability compared to their $GARP^{-}$ counterparts (248, 250). However, GARP is not expressed on freshly isolated Treg cells, limiting its potential as Treg marker in immune quiescence.

11.1.7. Additional candidates

Several other markers have been described to differentiate Treg from Teff cells, and include CD49d, GITR and TNFR2 (Summarized in Table 1). While many of these markers may be useful in a state of immune quiescence, they are modulated on Teff cells upon activation, limiting their specificity and applicability. Identification of novel specific and stable markers of functional Treg cells is therefore direly needed.

Table 1: Reported human Treg markers. Modified from: Bin Dhuban *et al.* 2014. *Immunological reviews*

Localization	Molecule	Level of expression	Comments
Surface	**CD25**	High	Upregulated on activated Teff cells
	CD127	Low	Down-regulated on activated Teff cells
	GITR	High	Upregulated on activated Teff cells
	GARP	High	Expression limited to activated Treg cells
	CD39	High	Upregulated on activated Teff cells
	CD49d	Low	Upregulated to some degree on activated Treg cells
	TNFR2	High	Upregulated on activated Teff cells
	HLA-DR	High	Expressed on only a fraction of Treg cells; Upregulated on activated Teff cells
	LAP	High	Expression limited to activated Treg cells
	CD121a/CD121b	High	Expression limited to activated Treg cells
Intracellular	**FOXP3**	High	Upregulated on activated Teff cells; Intracellular localization precludes sorting of live cells
	Helios	High	Expressed on the majority of FOXP3+ cells; Intracellular localization precludes sorting of live cells
	CTLA-4	High	Intracellular expression is specific to Treg cells but precludes sorting of live cells; Surface expression is upregulated on activated Teff cells

11.2. Methodological variations in Treg functional assessment

In addition to the challenges caused by the lack of reliable human Treg markers, significant methodological variations are observed among studies that investigated human Treg function in various contexts.

11.2.1. Treg isolation technique: FACS vs. MACS

Protocols for the identification and isolation of $FOXP3^+$ Treg cells invariably call for sorting based on CD25 expression, and the more stringent researchers define Treg cells as those expressing high levels of CD25 ($CD25^{High}$). Therefore, highly sensitive sorting techniques are required in order to ensure the isolation of consistently and uniformly enriched Treg populations, especially for studies that aim to assess the suppressive function of Treg cells *in vitro*. Fluorescence-activated cell sorting (FACS) provides such requirement. However, several studies that compared Treg function in health and disease isolated Treg cells using magnetic-activated cell sorting (MACS) (38, 40, 203, 251, 252). Moreover, despite the increased availability and accessibility of FACS facilities, several groups are still magnetically sorting Treg cells. However, we now know that it is extremely challenging to obtain a highly enriched human Treg population using MACS, and it is even more challenging to obtain comparably enriched Treg populations from different individuals. This technical issue sheds considerable doubt on the validity of early Treg studies that were performed used magnetic sorting. As such, MACS should no longer be accepted for human Treg isolation and FACS needs to be used universally in order to move a step closer to methodological standardization.

11.2.2. Assays for the measurement of suppressive function

Treg function can be evaluated *in vitro* using a co-culture system where effector T cells (Teff) (most often $CD4^+CD25^-$) are activated in the presence of Treg cells at various Treg:Teff ratios in

the presence or absence of antigen-presenting cells (APCs). Several variations of this suppression assay have been used in different studies, mostly manipulating the stimulation method and/or the amount and nature of APCs. Therefore, when evaluating studies where suppression assays are reported, close attention must be paid to the approach used in the assessment of suppressive function as subtle details may significantly influence the readout.

T cell stimulation in suppression assays is often polyclonal, and is performed by TCR activation using anti-CD3 antibodies. Co-stimulation is provided either by the addition of anti-CD28 or by the use of irradiated APCs. Soluble, plate-bound and bead-bound anti-CD3 and anti-CD28 antibodies have all been used in various studies depending on the experimental conditions. It should be noted that the strength of the activation signal greatly influences the suppressive function of Treg cells.

Finally, the functional potency of Treg cells is measured by their ability to inhibit the proliferation of Teff cell in the suppression co-culture. Traditionally, this was achieved by measuring the incorporation of tritiated-thymidine (^{3}H-thymidine) into proliferating cells over the last day of the culture period (36, 38, 40, 41, 238, 239, 251-255). Due to the inability of Treg cells to proliferate when cultured alone *in vitro* in the absence of IL-2, this assessment method assumes that thymidine is incorporated by Teff cells only. However, the presence of Teff cells in the system can provide sufficient amounts of IL-2 to drive Treg proliferation, and therefore, this assessment method cannot reliably distinguish the proliferation of Teff vs. Treg cells. Curiously, several groups around the world are still using this approach to measure Treg function. A more reliable assessment tool is the use of proliferation dyes (e.g. CFSE) to label responding Teff cells and Treg cells prior to their activation (201, 256, 257). These dyes allow the reliable distinction

between Treg and Teff cells and facilitate the accurate assessment of their proliferative responses.

As discussed above, meaningful functional characterization of human Treg function in human autoimmunity will require the identification of novel reliable surface markers for this subsets in humans. Moreover, a deeper understanding of the factors that influence Treg suppressive function is needed in order to design meaningful approaches for the evaluation of Treg function in various disease settings as well as for the development of strategies for the therapeutic application of Treg cells.

12. Therapeutic potential of Treg cells

Owing to their ability to control autoimmune responses, Treg cells present an attractive target for clinical interventions aimed at potentiating immune regulation in autoimmune patients without the risk associated with generalized immunosuppressive regimens. Current strategies for the clinical application of Treg research revolve around two main general approaches: 1) *In vivo* enhancement of Treg activity, and 2) transfer of *ex vivo* expanded Treg cells.

12.1. Strategies for the enhancement of endogenous Treg activity

Specific potentiation of endogenous Treg cells presents an attractive approach for the control of autoimmune responses. A number of strategies have been explored in recent years with promising outcomes.

12.1.1. Low-dose IL-2 therapy

The dependence of Treg cells on IL-2 has inspired the use of IL-2 therapy to preferentially enhance Treg cell expansion *in vivo*. Indeed, two recent clinical trials have shown that treatment with low doses of IL-2 (below those that trigger global expansion of T cells) resulted in

increased Treg frequency and clinical improvement in graft-versus-host disease (GVHD) (258) and hepatitis C virus-induced cryoglobulinaemic vasculitis (259). Importantly, no adverse effects were reported in either trial (258, 259), suggesting that this may be a viable approach to enhance endogenous Treg numbers.

12.1.2. Rapamycin

Rapamycin (Sirolimus) is an immunosuppressive drug that inhibits the mTORC1 pathway, thus inhibiting IL-2 responsiveness in activated T cells (260). Interestingly, rapamycin treatment has been shown to favour the expansion of Treg cells over Teff cells (261-263). Although the mechanisms behind this differential effect of rapamycin are not clear, recent evidence suggest that it may result from rapamycin-mediated increase in Foxo1 and Foxo3a activity, leading to increased Foxp3 expression (264-266). In humans, an increase in Treg frequency *in vivo* was reported in type 1 diabetes patients treated with IL-2 and rapamycin, although clinical efficacy was not observed (267).

12.1.3. Epigenetic modifiers

Other pharmacological approaches are being developed to modify the epigenetic profile of the Foxp3 locus to induce, and stabilize, Foxp3 expression in Teff cells. It started with observations that histone deacetylase (HDAC) inhibitors improve outcome in animal models of transplantation (268). In humans, a recent phase 1/2 clinical trial found that treatment of HSCT recipients with the HDAC inhibitor vorinostat, paired with mycophenolate mofetil and tacrolimus, reduced the incidence of acute GVHD (269), and increased the frequency and suppressive function of Treg cells (270). Although these pharmacological approaches may have positive quantitative and/or qualitative effects on Treg cells, it is, however, difficult to assess the

relative contribution of their Treg-enhancing effects on the overall clinical improvements in patients.

12.2. *Ex vivo* expansion of Treg cells

The therapeutic effects of adoptive Treg transfer in mouse models of autoimmunity have inspired similar strategies in humans where several groups are evaluating the application of Treg cell therapy to control organ-specific autoimmune disorders, as well as to prevent transplant rejection and GVHD (271, 272)(186, 187)(249, 250)(275, 276). In these approaches, Treg cells need to be first isolated from patients, or matching donors, and expanded *ex vivo* using good manufacturing practices (GMP) before being re-infused into patients. Although this approach is in its early stages, a number of phase I clinical trials have shown encouraging results. In 2011, Brunstein *et al.* expanded $CD4^{+}CD25^{+}$ Treg cells isolated from cryopreserved umbilical cord blood (UCB) and infused them into 23 patients who received double UCB transplantation for the treatment of haematological malignancies. In treated patients, Treg infusion resulted in a significant reduction in the incidence of acute GVHD compared to historical controls (273). More recently, Bluestone *et al.* performed a phase I trial evaluating the safety of Treg transfer into type 1 diabetic patients. In this trial 14 diabetic patients received *ex vivo*-expanded, deuterium-labeled $CD4^{+}CD25^{+}CD127^{Low}$ Treg cells. Around 25% of transferred Treg cells were detectable up to a year after treatment (274). Importantly, neither of these clinical trials reported adverse effects (273, 274), demonstrating the safety of this approach. Advanced clinical trials are needed to demonstrate the therapeutic efficacy of these approaches.

Although promising, these approaches are faced with important challenges. As discussed earlier in this chapter, a reliable surface marker of human Treg cells is still lacking. Moreover, it is becoming increasingly evident that human Treg cells are functionally heterogeneous and may

exhibit functional plasticity in response to inflammatory signals. Therefore, identification of reliable and specific markers for human Treg cells, as well as elucidation of factors that modulate Treg function *in vivo* will be needed before these Treg-cell based therapies can be used effectively.

13. Rationale

As discussed above, the importance of the $CD4^+FOXP3^+$ Treg subset in maintaining immune tolerance to self-antigens has been clearly established in several animal models where congenital or acquired Treg deficiency precipitates autoimmunity that can be prevented or remedied by Treg transfer. In humans, the IPEX syndrome presents the most convincing evidence of the indispensable role of Treg cells and for the requirement for the transcription factor FOXP3 in the development and function of Treg cells. However, studies investigating the functional status of Treg cells in human have been inconclusive. This can be attributed to a number of factors that have generally hindered progress towards a better understanding of human Treg cells. First, the signature Treg marker, FOXP3, is expressed intracellularly, which precludes its use for the isolation of live Treg cells for functional assessment. Several surface markers have been found to correlate with FOXP3 expression including high expression of CD25 and low expression of CD127, among others (reviewed in **chapter 1**). While these markers are useful for the identification and isolation of human $FOXP3^+$ cells in resting conditions, they are modulated in Teff cells upon TCR-mediated activation rendering them indistinguishable from Treg cells in inflammatory conditions. As inflammation is a hallmark feature of autoimmune disease, it is thus challenging to distinguish Treg from activated Teff cells in patients. Moreover, even FOXP3 is transiently induced in human Teff cells upon activation (78, 192). Thus reliable comparative analyses of Treg frequency or function in human autoimmunity is not possible using conventional Treg markers, and therefore, there is a pressing need for new specific and reliable surface markers for human Treg cells. Our lab has developed a cloning approach that allows the examination of single *bona fide* human $FOXP3^+$ expressing Treg clones and reliably excludes Teff contaminants (109). This monoclonal approach has revealed a remarkable degree of

functional heterogeneity within the Treg population, whereby approximately one third of $FOXP3^+$ clones lack suppressive function despite expressing high levels of FOXP3 and exhibiting a typical Treg phenotype. These observations demonstrated that FOXP3 expression cannot be used as a reliable indicator of suppressive function in human Treg cells. This also raised a number of questions: Does FOXP3 expression drive Treg phenotype independently of suppressive function? What are the additional molecular requirements for the development of suppressive function in Treg cells? Do non-suppressive $FOXP^+$ cells originate from previously functional Treg cells that lost their suppressive capacity? What are the factors that drive loss of function in committed human Treg cells? Can Treg dysfunction be reversed? The aim of the research presented in this thesis was to address these questions by further characterizing the functional heterogeneity in the human $FOXP3^+$ Treg population in order to identify molecular interactions that are required for the development of human Treg function, as well as factors that modulate Treg function in committed Treg cells. The following general hypotheses will be examined:

1) FOXP3 regulates Treg phenotype and function through distinct molecular mechanisms

2) The human $FOXP3^+$ Treg subset in healthy individuals comprises a heterogeneous mixture of functionally stable and functionally flexible subpopulations with differential sensitivity to modulatory signals.

14. General Objectives

1) Investigation of FOXP3 interactions required for the development of human Treg suppressive function using naturally occurring IPEX-causing *FOXP3* mutations **(Chapter 2, submitted manuscript)**.
2) Identification of novel surface markers that allow reliable distinction between *bona fide* Treg cells and activated Teff cells in humans, and further facilitate the identification of different functional subpopulations of human Treg cells. **(Chapter 3, Published in the journal of Immunology (275))**.
3) Investigation of factors and pathways that modulate the suppressive function of committed human $FOXP3^+$ Treg cells **(Chapter 4, manuscript in preparation).**

CHAPTER 2

Suppression by human FOXP3^{+} regulatory T cells requires FOXP3-TIP60 interactions

CHAPTER 2- Suppression by human FOXP3^{+} regulatory T cells requires FOXP3-TIP60 interactions

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2.1. Bridging statement from Chapter 1 to Chapter 2

Naturally occurring IPEX mutations present unique and highly informative platforms for the study of the role of FOXP3 in human Treg cells. Similar to genetically modified animal models, IPEX allows the examination of human Treg cells with selective alterations in phenotype and function, and thus facilitates the dissection of the various interactions through which FOXP3 orchestrates the Treg lineage. Furthermore, examining the severe impact of *FOXP3* mutations on Treg function should allow a clear distinction between true and extreme functional defects in IPEX patients and subtle functional heterogeneity in the healthy Treg population, and establishes a reference spectrum against which Treg function can be measured in health and disease.

Our lab has previously demonstrated that the $FOXP3^+$ Treg population in healthy individuals contains a significant proportion of non-suppressive Treg cells that otherwise resemble their suppressive counterparts in their expression levels of FOXP3 and other conventional Treg markers (108, 274). These observations suggested that the mechanisms by which FOXP3 drives the development of Treg phenotype can be uncoupled from those controlling the suppressive function. Indeed, in the next chapter we show that a natural IPEX-causing *FOXP3* mutation (p.Ala384Thr) allows the development of a normal Treg phenotype and repression of inflammatory cytokine production but abolishes the suppressive function of primary patient-derived Treg cells. We further investigate the cellular and molecular defects caused by this specific mutation in order to identify novel molecular interactions required for the development of the suppressive function of Treg cells.

2.2. Summary

$CD4^+FOXP3^+$ regulatory T cells (Treg) are critical mediators of immune tolerance and their deficiency due to *FOXP3* mutations in immunodysregulation polyendocrinopathy enteropathy X-linked syndrome (IPEX) patients results in severe autoimmunity. Different *FOXP3* mutations result in a wide range of disease severity, reflecting the relative importance of the affected residues in the integrity of the FOXP3 protein and its various molecular interactions. Here we characterized the cellular and molecular impact of the most common IPEX mutation, p.A384T, on patient-derived Treg cells. We found that the p.A384T mutation abrogates the suppressive capacity of Treg cells while preserving FOXP3's ability to repress inflammatory cytokine production. This selective functional impairment is due in part to a specific disruption of $FOXP3^{A384T}$ binding to the histone acetyl-transferase TIP60 (KAT5), and can be corrected using allosteric modifiers that upon binding to TIP60 further enhance FOXP3-TIP60 binding. These findings reveal the functional impact of TIP60 in FOXP3-driven Treg biology and provide a novel critical target for therapeutic manipulation of Treg activity.

2.3. Results and discussion

$FOXP3^+$ regulatory T cells (Treg) are a $CD4^+$ T cell subset that plays an essential role in the maintenance of immunological tolerance to self and innocuous foreign antigens. Congenital or acquired Treg deficiency in several animal models precipitates autoimmune conditions that can be ameliorated with Treg infusion [1]. An indispensable requirement for Treg development and proper regulatory function is the expression of high levels of FOXP3, a forkhead family transcription factor that orchestrates the Treg-specific genetic program [2]. FOXP3 acts both as a transcriptional activator of Treg-relevant genes such as *IL2RA* and *CTLA4*, and as a repressor of

inflammation-driven genes such as *IFNG* and *IL-2* [3]. While FOXP3 homomeric dimers can mediate transcriptional regulation through direct binding to DNA, FOXP3 has also been shown to interact with several molecular partners forming FOXP3/protein complexes that are required for certain regulatory processes [4,5]. Additionally, the FOXP3 protein undergoes post-translational acetylation by histone acetyltransferase (HAT) enzymes such as TIP60 (KAT5) and p300 (KAT3b), a process that is essential for the stability and transcriptional activity of the FOXP3 homomeric dimer [6,7].

Inherited *FOXP3* mutations in humans cause a severe multi-organ autoimmune condition known as the immunodysregulation, polyendocrinopathy, enteropathy X-linked (IPEX) syndrome. To date, more than 60 different *FOXP3* mutations have been reported in patients, and are mainly found in the forkhead (FKH) domain followed by the N-terminal proline-rich domain[8]. Although the majority of *FOXP3* mutations result in devastating autoimmunity and early death, several mutations have been associated with milder forms of IPEX [8]. This clinical heterogeneity suggests that different *FOXP3* mutations have distinct effects on the cellular functions of Treg cells, likely reflecting the relative importance of the affected residues in the various interactions of FOXP3. Thus studying the molecular and cellular impact of various IPEX-causing mutations in primary Treg cells could reveal critical pathways that are required for specific aspects of FOXP3 function in human Treg cells.

We have previously demonstrated that a missense mutation in the FKH domain of FOXP3 leads to a diminished suppressive function of Treg cells in a severe case of IPEX [9]. This mutation, an Alanine to Threonine substitution at residue 384 of FOXP3 ($FOXP3^{A384T}$), is the most common mutation identified in IPEX [8]. We now show that the $FOXP3^{A384T}$ mutation disrupts specific molecular interactions with TIP60, an effect that can be rescued by a TIP60

allosteric modifier, which enhances FOXP3-TIP60 interactions. Our findings expand our understanding of the molecular partnerships of FOXP3 in human Treg cells and point to a novel avenue for therapeutic modulation of Treg activity.

We first assessed the impact of the A384T mutation on FOXP3 expression levels in two unrelated IPEX patients bearing the same mutation. In both patients, we detected a population of $CD4^+FOXP3^+$ cells, with approximately 50% reduction in FOXP3 MFI compared to age-matched healthy controls (Fig. 1a). These findings indicate that the A384T mutation alters FOXP3 expression levels in Treg cells.

Since FOXP3 is transiently induced in human effector $CD4^+$ cells (Teff) upon activation[10], a reliable distinction between *bona fide* Treg cells and activated Teff cells in inflammatory conditions is not possible, precluding the definitive assessment of Treg function directly *ex vivo*. To circumvent these issues, we examined the phenotype and function of Treg cells at a single-cell level and in a normalized state of immune quiescence after clonal expansion, a strategy that has the potential to uncover Treg-intrinsic functional differences [11,12]. We generated primary Treg and Teff clones from both IPEX patients and respective healthy controls and analyzed their general phenotype, cytokine production and suppressive function. In accordance with our observations in PBMC directly *ex vivo* (Fig. 1a), FOXP3 expression in IPEX-derived Treg clones from both IPEX patients was significantly lower than that observed in control clones (Fig. 1b). Furthermore, IPEX-derived Treg clones expressed CD25 at reduced levels compared to healthy clones (Fig. 1b). In order to determine whether the reduced CD25 expression in $IPEX_{A384T}$ Treg cells reflects a quantitative defect in FOXP3, we calculated the linear correlation between the FOXP3 and CD25 MFIs in both the IPEX and control $FOXP3^+$ clone pools. While FOXP3 and CD25 levels were correlated in both clonal pools, we found that

the slope of the linear regression for the healthy controls is two times greater than that of the IPEX (control slope= 2.245±0.284; IPEX slope= 1.094±0.1262, $p<0.0001$) (Fig. S1). These results suggest that the reduced levels of $FOXP3^{A384T}$ alter its capacity to act as a transcriptional enhancer.

One important role of FOXP3 is to repress inflammatory cytokine production in Treg cells [13,14]. We observed that in PBMCs from IPEX patients, $FOXP3^+$ T cells produce minimal levels of IL-2, IFN-γ or IL-17, similar to healthy controls (Fig. S2). This repression of inflammatory cytokines was also observed in IPEX-derived $FOXP3^+$ clones (Fig. 1c). Furthermore, upon TCR activation in the absence of exogenous IL-2, we found that IPEX-derived $FOXP3^+$ clones are as hypoproliferative as healthy clones, further confirming their inability to endogenously produce IL-2 (Fig. 1d). These observations indicate that the A384T mutation preserves certain aspects of the transcriptional activity of FOXP3, namely the capacity to repress inflammatory cytokine production in Treg cells.

We next assessed the impact of the A384T mutation on the suppressive function of Treg cells. We measured the ability of individual $FOXP3^+$ and $FOXP3^-$ clones from IPEX patients or healthy controls to inhibit the proliferation of allogeneic Teff responder cells isolated from healthy individuals. $FOXP3^+$ clones derived from $IPEX^{A384T}$ patients were severely impaired in their capacity to suppress Teff cells compared to control healthy $FOXP3^+$ clones (Fig. 1e). Notably, the overall suppression by $FOXP3^{A384T}$ clones is not different from that of $FOXP3^-$ clones, which exhibited no significant suppression in either patients or controls. Thus the A384T mutation severely impedes the capacity of FOXP3 to orchestrate the Treg cell suppressive function in IPEX $CD4^+$ T cells.

Although the two patients examined in this study bear the same *FOXP3* mutation, they differed significantly in IPEX severity. Patient #1 presented in the first few weeks of life with severe multi-organ failure, rash, enteritis and endocrine abnormalities, leading to death at the age of 2 months [9], while patient #2 suffered from milder manifestations of atopic dermatitis, neutropenia, diabetes and enteritis [15], and survived into young adulthood, when he underwent bone marrow transplantation at age 19. Despite these differences in disease severity, the p.A384T mutation they carry results in strikingly similar cellular defects in Treg cells. To further demonstrate the direct causality between the A384T mutation and the observed defective Treg function, we next examined the capacity of $FOXP3^{A384T}$ to elicit the Treg cell phenotype and function upon ectopic expression in healthy conventional $CD4^+$ T cells as previously described [16]. The A384T mutation was compared to the R397W mutation, which is known to completely abrogate the DNA-binding capacity of the FKH domain [17,18]. Naïve $CD4^+CD45RA^+CD25^-$ T cells from healthy donors were transduced using a bicistronic lentiviral vector expressing both GFP and FOXP3 (WT, A384T or R397W) (Fig. 2a). We detected high levels of FOXP3 in cells transduced with $FOXP3^{A384T}$, similar to those obtained with the WT FOXP3 construct (Fig. 2b). Investigation of inflammatory cytokine production in FOXP3-transduced cells indicated that both $FOXP3^{WT}$ and $FOXP3^{A384T}$, but not $FOXP3^{R397W}$ or an empty vector, strongly repress the production of IFN-γ, IL-2, and IL-4 in transduced cells (Fig. 2c). Thus, $FOXP3^{A384T}$ preserves the capacity of FOXP3 to repress the inflammatory cytokine profile of transduced cells.

Finally, cells expressing either $FOXP3^{A384T}$ or $FOXP3^{R397W}$ were significantly impaired in their ability to suppress Teff cell proliferation compared to those expressing $FOXP3^{WT}$ (Fig. 2d-e). Collectively, these results show that $FOXP3^{A384T}$ can reprogram T cells into phenotypically Treg-like cells but which are functionally defective and unable to suppress Teff

cell proliferation, confirming our observations *ex vivo* and in primary clones, and further demonstrating the selective impact of the A384T mutation on the suppressive function of Treg cells.

To identify FOPX3-regulated genes specifically required for either Treg suppressive function or Treg phenotype, we assessed the effects of the A384T mutation on FOXP3 transcriptional activity. We compared the gene expression profiles of primary human $CD4^+$ T cells transduced with $FOXP3^{A384T}$ or $FOXP3^{R397W}$, relative to control $FOXP3^{WT}$. The canonical transcriptional program driven by FOXP3 (reference Treg gene signature), obtained by comparing the empty vector and $FOXP3^{WT}$-transduced cells, is most perturbed in cells transduced with $FOXP3^{R397W}$ (Fig. 3a). In accordance with our observed cellular phenotype of primary and FOXP3-overexpressing cell lines, the Treg gene signature of $FOXP3^{A384T}$-transduced cells is partially maintained relative to that of $FOXP3^{WT}$-transduced cells, in both resting and activated conditions (Fig. 3a and 3b). Indeed, over 50% of the genes upregulated in $FOXP3^{WT}$-transduced cells are shared with cells over-expressing $FOXP3^{A384T}$, while less than 10% are shared with cells over-expressing $FOXP3^{R397W}$ (Fig. 3b). Moreover, several previously described FOXP3 target genes (including *STAT6, SELL* and *PIM2)*[19,20] are similarly upregulated in cells expressing the WT or the A384T form of *FOXP3* (Fig. 3b-d), consistent with the notion that the DNA-binding capacity of FOXP3 is better preserved by A384T, unlike R397W. These findings further demonstrate the partial maintenance of certain Treg features by $FOXP3^{A384T}$ despite the significant loss of suppressive capacity.

Part of the transcriptional regulation mediated by FOXP3 requires its binding to a number of molecular partners such as FOXP1, RORα, NFAT, HDAC7 and HDAC9 [5]. We assessed the ability of $FOXP3^{A384T}$ to interact with several of these molecular partners. While it maintains a

normal capacity to bind FOXP1 relative to $FOXP3^{WT}$ (Fig. 4a), $FOXP3^{A384T}$ displays a greatly diminished ability to bind TIP60 (KAT5) (Fig. 4b). TIP60 is a histone acetyltransferase that binds to Foxp3 at the N-terminal and leads to Foxp3 acetylation, a prerequisite to its functional activation and stabilization [7]. The importance of TIP60 in Treg development has been previously demonstrated in knockout mice where Treg-specific TIP60 deficiency impaired Treg development and caused fatal autoimmune manifestations akin to those observed in the scurfy mouse [7]. TIP60 also interact with another histone acetyltransferase, p300, which acetylates TIP60, leading to substrate switching and the dissociation of TIP60 molecules from the TIP60-p300-Foxp3 complex [7]. Curiously, we observed a markedly enhanced interaction between p300 and $FOXP3^{A384T}$ protein (Fig. 4c). One possibility is that p300 binding is increased in compensation for diminished TIP60 binding to $FOXP3^{A384T}$. These observations indicate that the impaired binding of $FOXP3^{A384T}$ to TIP60 can interfere with certain functional properties of FOXP3, and may underlie the diminished suppressive function seen in human Treg cells with the A384T mutation of *FOXP3*.

Our laboratory has developed a series of TIP60-binding small molecules that act on an allosteric cavity in the TIP60 protein [21]. These small allosteric modifiers help stabilize TIP60-Foxp3 interactions by inhibiting the acetylation of TIP60 molecules and thus preventing the release of TIP60 from the TIP60-p300-Foxp3 complex. We sought to examine the ability of one such TIP60 allosteric modifier, SGF003, to enhance the interaction of TIP60 and $FOXP3^{A384T}$. Interestingly, SGF003 treatment led to a marked increase in the binding of TIP60 to both $FOXP3^{WT}$ and $FOXP3^{A384T}$, as early as 30 minutes following the addition of the drug (Fig. 5a and 5b). The increased binding correlated with a significant increase in the suppressive potency of $FOXP3^{A384T}$-transduced cells upon the addition of SGF003 to the co-culture at the time of

activation (Fig. 5c). Notably, SGF003 allowed the rescue of the suppressive function of $FOXP3^{A384T}$-transduced cells to levels comparable to those observed in untreated $FOXP3^{WT}$-transduced cells. SGF003 also enhanced suppression by $FOXP3^{WT}$-transduced cells albeit to a lesser extent, suggesting that potentiation of FOXP3-TIP60 interaction could further boost Treg function in healthy Treg cells. Indeed, treatment of freshly isolated, healthy $CD4^{+}CD25^{+}CD127^{Low}$ Treg cells with SGF003 lead to a significantly enhanced capacity to suppress the proliferation of co-cultured Teff cells (Fig. 5d). Conversely, inhibition of TIP60 interaction with FOXP3 using a TIP60 inhibitor (NU9056) abolishes the suppressive capacity of Treg cells (Fig. 5d). Hence, the FOXP3-TIP60 interaction is indispensable for the suppressive function of Treg cells, and can be enhanced or rescued by small molecule modifiers of TIP60 activity.

Finally, we examined the therapeutic benefit of TIP60 targeting in controlling inflammation *in vivo* in a dextran sodium sulphate (DSS)-induced colitis model. Administration of a homologue of SGF003 (B7A) rescued mice from established colitis as evidenced by weight regain (Fig. 6b), preservation of colon length (Fig. 6c) as well as improved histological scores (Fig. 6d). Compared to the transmural inflammation, goblet cell destruction and extensive thickening of colons in DMSO-treated mice (mean histologic score of 3.2 ± 1.3), B7A therapy preserved colonic histology (mean histologic score of 1.6 ± 1.0, $p<0.01$), with minimal inflammation edema or goblet cell injury. Notably, the beneficial effects of the treatment in this inflammatory condition are apparent despite treating after the onset of the pathology (Fig. 6b), and are associated with a significant increase in the frequency of $Foxp3^{+}$ Treg cells (Fig. 6e). These results further highlight the potential therapeutic application of TIP60 allosteric modifiers for the enhancement of Treg-mediated immune regulation in inflammatory diseases.

The acetylation of the FOXP3 protein mediated by TIP60-p300 cooperation is thought to play an important role in the stability of the FOXP3 protein by preventing its ubiquitination and degradation [4,22,23]. Interestingly, Liu *et al.* have shown that mice with a Treg-specific deletion of p300 ($p300^{fl/fl}$ $Foxp3^{YFP-Cre}$) develop a significantly milder phenotype compared to those with a Treg-specific TIP60-deficiency ($Tip60^{fl/fl}$ $Foxp3^{YFP-Cre}$) despite a significant reduction in Foxp3 acetylation in both models [7,22]. Mice with TIP60-deficient Treg cells succumb to severe autoimmunity very early in life, whereas mice with p300-deficient Treg cells develop normally until 8 weeks of life and only show moderate autoimmune manifestations [7,22]. Thus the severe phenotype associated with TIP60-deficient Treg cells in mice, and the complete abrogation of suppressive function in $FOXP3^{A384T}$ cell observed in the present study suggest that TIP60 plays a critical role in the development of Treg function through mechanisms other than acetylation of FOXP3. One possibility is that TIP60 is recruited by FOXP3 to facilitate the activation of target genes that are critical for the development of the suppressive function of Treg cells. Another possibility is that the molecular partnership between TIP60 and FOXP3 involves non-catalytic functions that are essential to Treg activity. Interestingly, previous studies have demonstrated examples of such non-enzymatic roles of a number of histone deacetylases. For instance, a splice variant HDAC9 that lacks the catalytic domain was found to mediate transcriptional regulation in cardiac myocytes [24,25] through the recruitment of transcriptional co-repressors [25,26]. Similarly, HDAC3 and HDAC5 have been demonstrated to exhibit non-enzymatic functions by participating in the scaffolding of transcriptional regulatory complexes [27,28]. Future studies may reveal novel non-enzymatic roles of TIP60 in Treg biology.

FOXP3 association with TIP60 is mediated by the N-terminal domain of FOXP3, while the forkhead-located A384T mutation lies in the opposite end of the protein. Previous studies

have shown that FOXP3 assembles as an antiparallel homodimer, a configuration that is required for its function [29]. The structural characteristics of this homodimeric FOXP3 render its C-terminal DNA-binding domain spatially adjacent to the N-terminal repressor domain. Thus, specific characteristics of the FOXP3 homodimer may be altered by the A384T mutation, indirectly affecting FOXP3 functional partnerships involving the N-terminal domain while leaving other centrally-located interactions unaffected; this is the case for FOXP3-FOXP1 binding which involves the leucine zipper and zinc finger domains of FOXP3. Such intramolecular interactions between domains and mutations have not been previously described for FOXP3, and could contribute to the complex phenotypes observed in other hypomorphic mutations of FOXP3 [8,30].

In summary, our studies identify FOXP3-TIP60 interaction as a critical requirement for the proper suppressive function of human Treg cells. It further uncovers a mechanism by which a small-molecule modifier of TIP60 can increase human Treg cell function without directly impairing T effector cell responses (Figure 7), providing potential novel therapeutic avenues for targeted therapeutic manipulation of Treg cells. Moreover, this study highlights the significant insights into basic mechanisms of human FOXP3 function that can be gained by studying naturally arising *FOXP3* mutations.

2.4. ACKNOWLEDGMENTS

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Author Contributions: E.H., K.B.D., and C.P. designed the research; E.H., and K.B.D. performed the experiments, analyzed and reported the results; M.B.S., H.O., T.T., and M.G. contributed reagents and samples; E.H., K.B.D., T.T. and C.P. wrote the manuscript; E.H., K.B.D., T.T., C.P. B.D.M., Y.X, Y.N. and M.G. helped write and edit the manuscript. Y.X, Y.N. and M.G. developed SGF003, performed the immunoprecipitation experiments.

Competing Financial Interests: The authors declare no competing financial interests.

2.5. Online Methods

Human subject samples

The two IPEX patients examined both carried the A384T missense mutation in the FKH domain of FOXP3, as determined by genetic sequencing. Patient #1 presented in the first few weeks of life with severe multi-organ failure, rash, enteritis and endocrine abnormalities, leading to death at the age of 2 months [9]. Patient #2 suffered from atopic dermatitis, neutropenia, diabetes and enteritis [15], milder manifestations that allowed him to survive into young adulthood, when he underwent bone marrow transplantation at age 19. Age and sex-matched healthy controls were used in all the analyses of patient-derived primary cells. Informed consents were obtained, and the study was performed in agreement with the ethical review board of McGill University. In both patients, the A384T mutation was identified following sequencing of the *FOXP3* gene (Immunology Diagnostic Laboratory, Seattle Children's Hospital, Seattle, USA).

Multi-parametric flow cytometry and cell sorting

FACS-based phenotype and functional analyses were performed as previously described [32]. All samples were labelled with a fixable viability dye to facilitate live-cell gating (eBioscience). The anti-human FOXP3 antibody clone used was 236A/E7 (eBioscience); all other antibodies were obtained from BDBioscience. Cell sorting was performed using a FACSAriaIIu cell sorter (BDBioscience). Samples were acquired on a FACSCanto or LSRII-Fortessa analyzer (BDBioscience), and analysis was performed using FlowJo software (Treestar).

Generation of $CD4^+$T cell clones

Primary clones were obtained by short-term expansion and subjected to phenotypic and functional assays as previously described [11,32]. Briefly, $CD4^+CD25^{High}$ (top 1%) and $CD4^+CD25^{Neg}$ T cells were isolated from PBMC and dispensed as single cells by FACS sorting.

The cells were activated in the presence of allogeneic irradiated feeders, anti-CD3 and rhIL-2. IL-2 medium was replenished every 2-4 days, and cells were restimulated after 11 days. After 22 days of culture, each clone was subjected to micro-sized functional and phenotypic assays.

FOXP3-GFP expression vectors

cDNAs encoding WT or mutated FOXP3-GFP fusions (provided by T. Torgerson as per Lopes et al., 2006), were subcloned in a modified pLVX-pTight-Puro vector (Clontech), whose promoter was replaced with the EF1α promoter, and pPGK-Puro sequence was replaced with an IRES-GFP sequence. Appropriate cDNAs were inserted upstream of IRES-GFP. Correlation of GFP and FOXP3 expression was confirmed for each vector following primary T cell transduction (see Results).

Lentiviral transduction of primary T cells

Lentiviral pseudo-particles were obtained following 4-plasmid transfection of 293T cells using Lipofectamine (Invitrogen), as previously described [16]. Viral supernatants were collected 48hrs and 72hrs post-transfection and immediately used for transduction of pre-activated primary $CD45RA^{+}CD25^{-}CD4^{+}$ T cells. Infection rates ranged between 10 and 25%. Seven days after transduction, each cell line was FACS-sorted for GFP expression. IL-2 was replenished every 3 days, and cells were expanded for a total of 28 days following initial activation in order to obtain sufficient amounts of cells for analysis. GFP expression was verified, and cells were re-sorted if necessary, prior to analysis.

Intracellular cytokine staining

Cytokine production was assayed by intracellular staining, as previously described [32]. Cells were re-stimulated for 24hrs in the presence of soluble anti-CD3 (clone OKT3, 30 ng/ml), rhIL-2

(200 IU/ml), and in the case of clones or transduced cells, with allogeneic irradiated PBMCs (1:4 T cell:feeder ratio). The cultures were activated with PMA (25 ng/ml), ionomycin (1 μg/ml) (Sigma-Aldrich) and GolgiStop (1:1000) (BDBioscience) for 4hrs, then fixed and permeabilized (eBioscience permeabilization kit), followed by intracellular cytokine staining.

Suppression assays

For T cell clones, suppression assays consisted of co-culturing clone with CFSE-labelled, FACS-sorted, allogeneic $CD4^{+}CD25^{Neg}$ Teff cells obtained from healthy donors (10^4 cells) at a 1:1 clone:Teff cellular ratio, in the presence of TCR stimulation, as previously described. When assessing the suppressive potency of GFP^{+} transduced cells, the CFSE was substituted with the efluor610 Proliferation Dye (eBioscience), and the assay was set with $3x10^4$ responder cells per well and a 1:4 Treg clone:Teff cellular ratio. Proliferation was determined for each co-culture by FACS by measuring the proportion of cells having diluted the proliferation dye after 96hrs of culture, and compared to Teff cells cultured alone.

Proliferation assays

Individual clones ($2x10^3$ cells) were stimulated *in vitro* in triplicates in the presence of TCR stimulation and IL-2, as previously described [32]. Proliferation was assessed by pulsing the cultures with ^{3}H-thymidine (0.5 μCi/ml) for the last 20hrs of the 5 days of culture. ^{3}H-thymidine incorporation in individual wells was then measured using scintillation liquid.

Gene expression analysis

Cells transduced with either WT, A384T or R397W mutated FOXP3 or an empty vector were either left resting or activated for 36 hrs in the presence of 0.5 μg/ml PHA. RNA was isolated using the AllPrep RNA/DNA/Protein kit from Qiagen as per manufacturer's instructions.

Amplification was performed using the Amino Allyl MessageAmp™ II RNA Amplification Kit (Ambion). Samples were hybridized on an Agilent SurePrint G3 Human GE 8x60k chip. Normalization and analysis were performed using the Flex array software.

Cell culture and transfection

293T cells were grown in RPMI-1640 medium supplemented with 10% heat-inactivated FBS and antibiotics (1% penicillin/streptomycin, Invitrogen) at 37°C in a humidified incubator with 5% CO_2 (v/v). Transient transfection was carried out when cells were grown to 80% confluency using a mixture of DNA and Fugene6 (Roche) according to manufacturer's instructions. 24 h after transfection, cells were washed twice with PBS and cell lysates were then prepared for immunoprecipitation and western blot analysis.

Immunoprecipitation

Cells were lysed in modified RIPA buffer (20 mM Tris-Cl, pH 7.5, 2 mM EDTA, 420 mM NaCl, 1% NP40). After centrifugation, the soluble fractions were collected and incubated with anti-HA agarose (Sigma) at 4°C for 2 h. The precipitates were then washed three times with modified RIPA buffer and boiled for 5 min in SDS loading buffer. Samples were analyzed by SDS-PAGE, transferred to nitrocellulose membrane (Millipore), and probed with anti-FLAG HRP (Sigma). Immune complexes were detected using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

Induction of colitis in mice

Freshly prepared 4% DSS (wt/vol) (MP Biomedicals, Irvine, CA) was added daily for 5 days to pH-balanced tap water of study mice (12 mice/group). Colitis was assessed by the daily monitoring of body weight, stool consistency, and fecal blood, as described (de Zoeten EF, Wang L, Sai H, Dillmann WH, Hancock WW. Inhibition of HDAC9 increases T

regulatory cell function and prevents colitis in mice. Gastroenterology. 2010;138:583-94). The effects of TIP60i therapy were assessed by administration of Tip60i (4 mg/kg/d, 7 days, i.p.) or DMSO control, one week after beginning DSS administration.

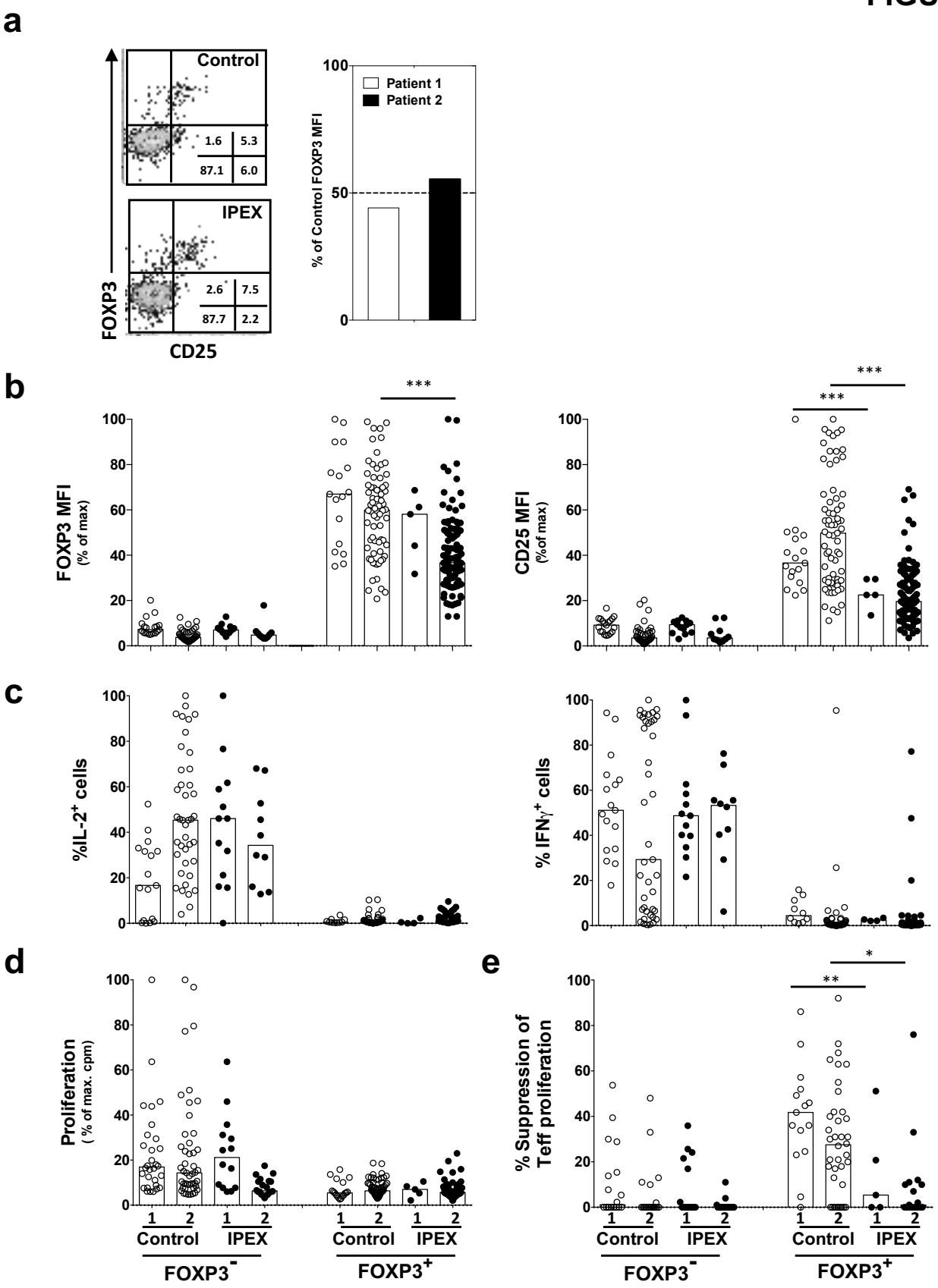
Statistical analysis

Statistical analysis was performed using Prism 5.04 software from GraphPad Software (La Jolla, CA). For all distributions, the D'Agostino and Pearson omnibus test was used to assess normality. Parametric or non-parametric tests were applied consequently. For multiple comparisons, one-way ANOVA was performed, and post-test p values are indicated. For single comparisons, *p* values were calculated by *t* test. Only for comparisons reaching significance (alpha=0.05) is the p value indicated.

2.6. Figures:

Figure 1. Diminished suppressive potency of primary Treg clones from IPEX$_{A384T}$ patients. **a.** FOXP3 and CD25 expression in $CD4^+$ cells of IPEX patients and controls *ex vivo.* **b-e**. Pools of single-cell-derived clones of $FOXP3^+$ and $FOXP3^-$ $CD4^+$ T cells were generated from patients and controls. Shown is **b)** FOXP3 and CD25 expression, **c)** cytokine production, **d)** proliferation of individual T cell clones in the absence of exogenous rhIL-2, and **e)** Suppression of proliferation of allogeneic $CD4^+CD25^-$ T cells. Data is represented as individual clones with line at median ± IQR.

FIGURE 1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5.3
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IPEX
2.6
7.5
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FOXP3
CD25
100
50
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Patient 1
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b
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CD25 MFI
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c
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Proliferation
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Teff proliferation
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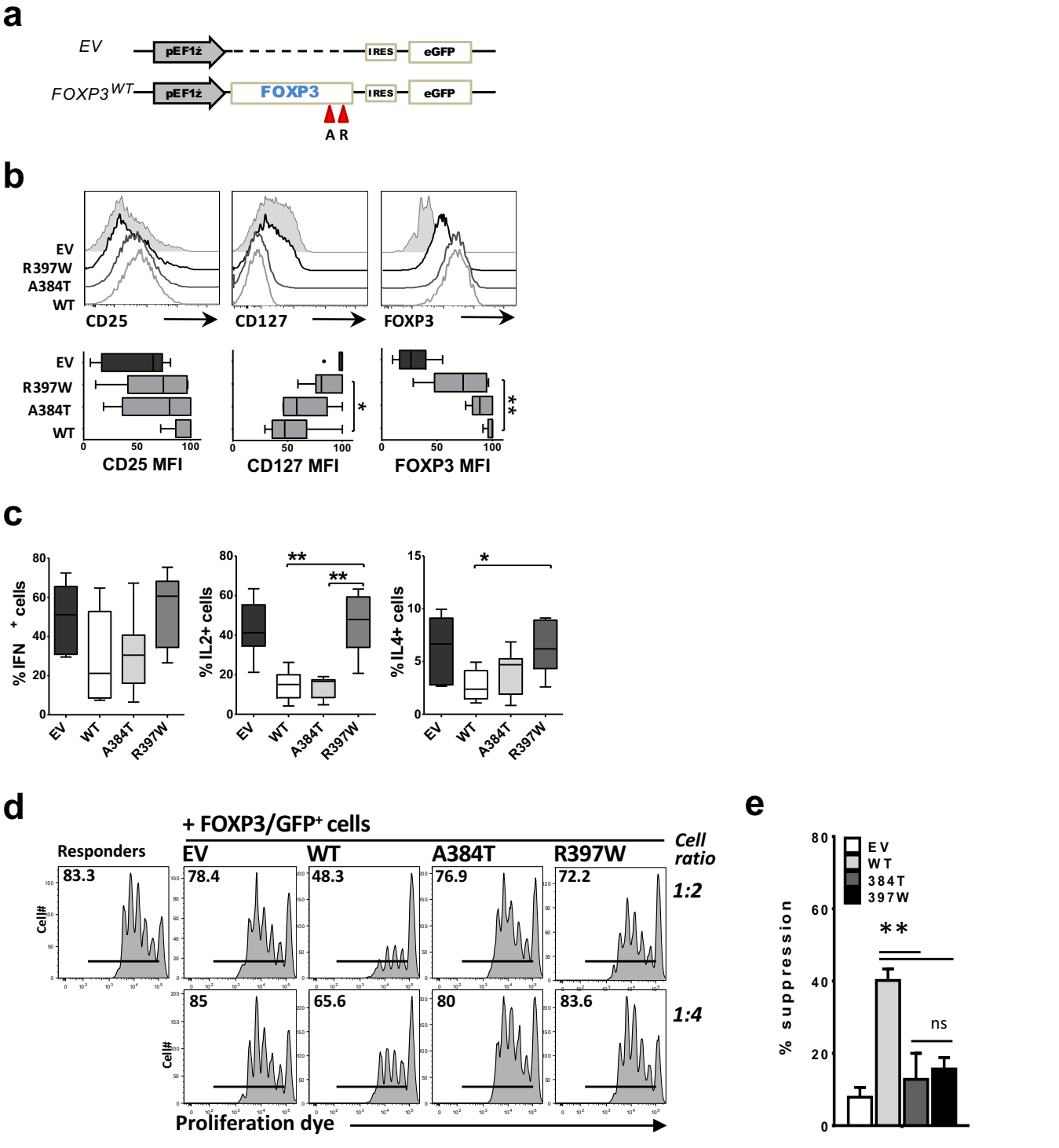
1 2 1 2 1 2 1 2
Control IPEX Control IPEX
FOXP3-
FOXP3+

Figure 2. Impaired development of Treg cell function upon overexpression of FOXP3^{A384T}.

a) Schematic representation of the constructs used in transduction. **b**) Expression levels of FOXP3, CD25 and CD127. **c**) Cytokine production by transduced cells expressing WT or mutated FOXP3. **d-e)** Suppressive function of WT, A384T and R397W FOXP3-transduced cells. Transduced, GFP+ cells were co-cultured with labelled $CD4^{+}CD25^{-}$ Teff cells at a 1:4 ratio. Shown are results from one experiment representative of 11 independent assessments on 7 independent transduction experiments carried out on cells from 4 different donors.

FIGURE 2


a
EV
pEF1ź
IRES
eGFP
FOXP3 WT
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b
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R397W
A384T
WT
CD25
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FOXP3
CD25 MFI
CD127 MFI
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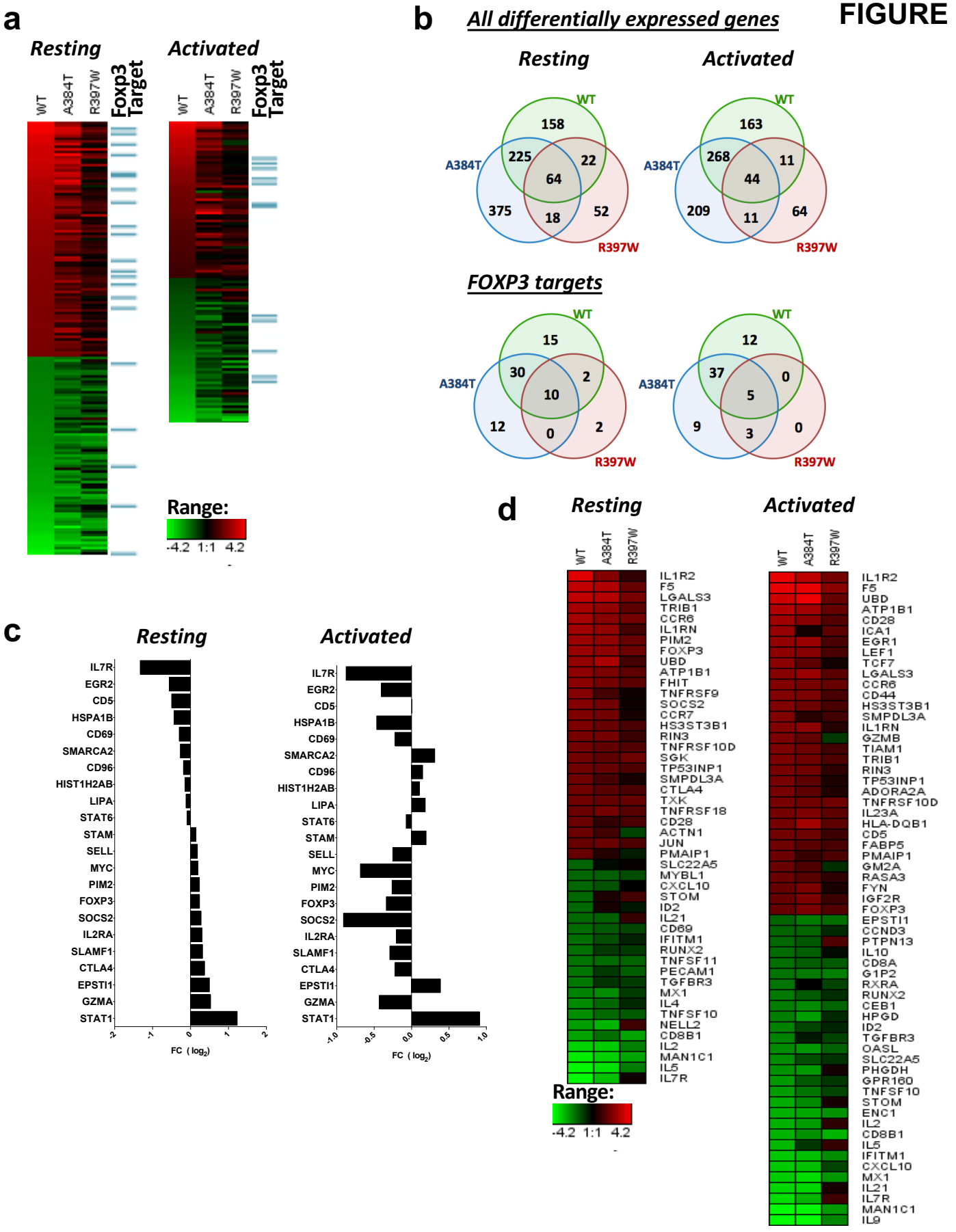
c
% IFN+ cells
% IL2+ cells
% IL4+ cells
d
+ FOXP3/GFP+ cells
Responders
EV
WT
A384T
R397W
Cell ratio
1:2
1:4
Cell#
Proliferation dye
e
% suppression
ns

Figure 3. Alteration of the Treg gene signature by FOXP3^{A384T}. a) Differential alteration of gene signatures in A384T and R397W FOXP3 mutants. Known FOXP3 gene targets (according to chromatin immune-precipitation experiments [19,20] are indicated in blue (right column). **b**) Venn diagram distribution of differentially expressed genes in FOXP3-transduced cells compared to empty vector-transduced cells (upper panel), and distribution of reported FOXP3 target genes (lower panel). **c)** Fold expression differences in known FOXP3 target genes in FOXP3^{A384T} and FOXP3^{WT} cells. **d)** Expression levels of Treg-related genes in WT or mutant FOXP3-transduced resting (left) or activated (right) cells. Treg-related genes were selected based on the proposed list of the Treg signature published by Pfoertner *et al* [31]. The genes differentially expressed in WT vs. empty vector-transduced cells were selected and their expression level is represented for the three FOXP3 variants.

FIGURE 3


a
Resting
Activated
WT
A384T
R397W
Foxp3 Target
Range:
-4.2 1:1 4.2
b
All differentially expressed genes
Resting
Activated
WT
A384T
R397W
158
225
22
64
375
18
52
163
268
11
44
209
11
64
FOXP3 targets
15
30
2
10
12
0
2
12
37
0
5
9
3
0
c
Resting
Activated
IL7R
EGR2
CD5
HSPA1B
CD69
SMARCA2
CD96
HIST1H2AB
LIPA
STAT6
STAM
SELL
MYC
PIM2
FOXP3
SOCS2
IL2RA
SLAMF1
CTLA4
EPSTI1
GZMA
STAT1
FC (log2)
d
Resting
Activated
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F5
LGALS3
TRIB1
CCR6
IL1RN
PIM2
FOXP3
UBD
ATP1B1
FHIT
TNFRSF9
SOCS2
CCR7
HS3ST3B1
RIN3
TNFRSF10D
SGK
TP53INP1
SMPDL3A
CTLA4
TXK
TNFRSF18
CD28
ACTN1
JUN
PMAIP1
SLC22A5
MYBL1
CXCL10
STOM
ID2
IL21
CD69
IFITM1
RUNX2
TNFSF11
PECAM1
TGFBR3
MX1
IL4
TNFSF10
NELL2
CD8B1
IL2
MAN1C1
IL5
IL7R
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LGALS3
CCR6
CD44
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IL1RN
GZMB
TIAM1
TRIB1
RIN3
TP53INP1
ADORA2A
TNFRSF10D
IL23A
HLA-DQB1
CD5
FABP5
PMAIP1
GM2A
RASA3
FYN
IGF2R
FOXP3
EPSTI1
CCND3
PTPN13
IL10
CD8A
G1P2
RXRA
RUNX2
CEB1
HPGD
ID2
TGFBR3
OASL
SLC22A5
PHGDH
GPR160
TNFSF10
STOM
ENC1
IL2
CD8B1
IL5
IFITM1
CXCL10
MX1
IL21
IL7R
MAN1C1
IL9
Range:
-4.2 1:1 4.2

Figure 4. Impaired interaction of FOXP3^{A384T} with TIP60. a-c) 293T cells were transfected with WT or mutant FOXP3 and FLAG-FOXP1, FLAG-TIP60, or FLAG-p300. 24h after transfection, cell lysates were collected and immunoprecipitated, followed by blotting with anti-FLAG or anti-HA HRP. Shown is the binding of FOXP3 mutants to FOXP1 **(a)**, TIP60 **(b)** or p300 **(c)**.

FIGURE 4

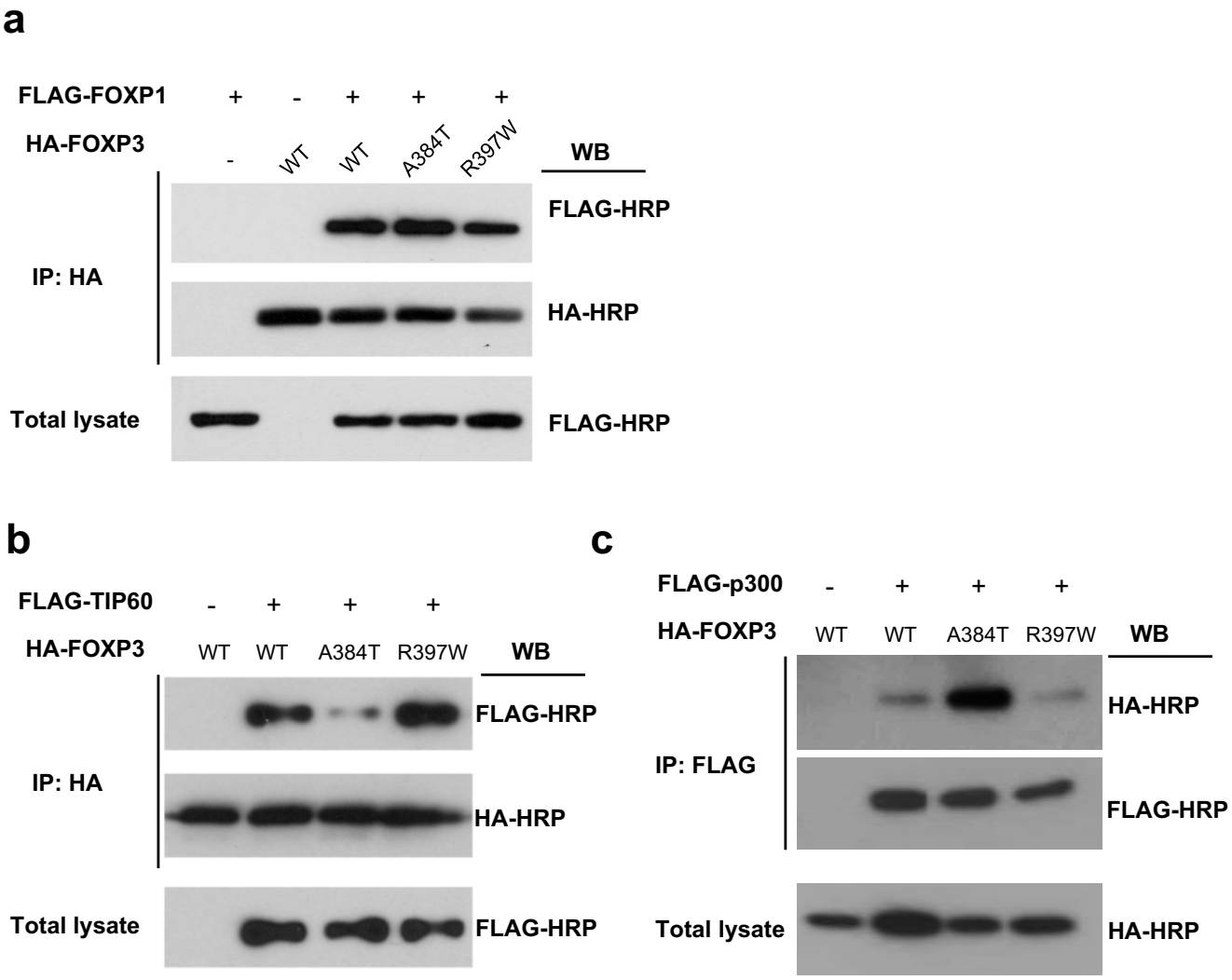

a
FLAG-FOXP1 + - + + +
HA-FOXP3 - WT WT A384T R397W
WB
IP: HA
FLAG-HRP
HA-HRP
Total lysate
FLAG-HRP
b
FLAG-TIP60 - + + +
HA-FOXP3 WT WT A384T R397W
WB
IP: HA
FLAG-HRP
HA-HRP
Total lysate
FLAG-HRP
c
FLAG-p300 - + + +
HA-FOXP3 WT WT A384T R397W
WB
IP: FLAG
HA-HRP
FLAG-HRP
Total lysate
HA-HRP

Figure 5. Allosteric modification of TIP60 by SGF003 restores the suppressive function of FOXP3^{A384T}. a-b) 293T cells were transfected with FOXP3^{WT} **(a)** or FOXP3^{A384T} **(b)** and FLAG-TIP60 in the presence of SGF003. Interaction of FOXP3 with TIP60 was assessed over time by immunoprecipitation. **c)** Effect of SGF003 on the suppressive potency of WT and A384T FOXP3-transduced cells co-cultured with $CD4^+CD25^-$ Teff cells at a 1:4 ratio. **d)** Effect of SGF003, as well as the TIP60 inhibitor NU, on the suppressive potency of freshly sorted healthy $CD4^+CD25^+CD127^{low}$ Treg cells *ex vivo*.

FIGURE 5

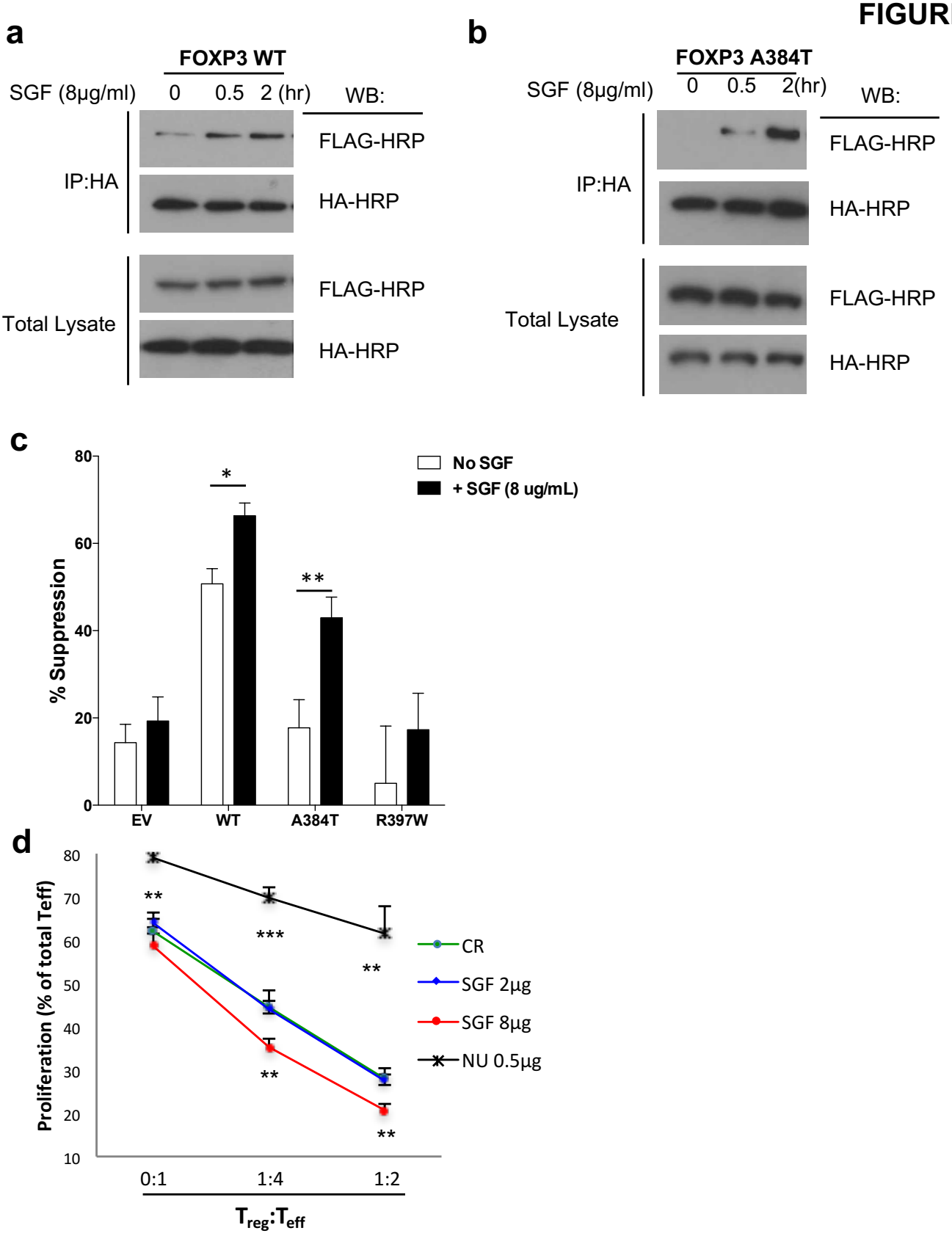

a
FOXP3 WT
SGF (8µg/ml)
0
0.5
2 (hr)
WB:
IP:HA
FLAG-HRP
HA-HRP
Total Lysate
FLAG-HRP
HA-HRP
b
FOXP3 A384T
SGF (8µg/ml)
0
0.5
2(hr)
WB:
IP:HA
FLAG-HRP
HA-HRP
Total Lysate
FLAG-HRP
HA-HRP
c
No SGF
+ SGF (8 ug/mL)
% Suppression
80
60
40
20
0
*
**
EV
WT
A384T
R397W
d
Proliferation (% of total Teff)
80
70
60
50
40
30
20
10
**
***
**
**
**
CR
SGF 2µg
SGF 8µg
NU 0.5µg
0:1
1:4
1:2
Treg:Teff

Figure 6. **Effects of TIP60 modification on murine colitis induced by DSS. (a)** The effect of B7A treatment on the suppressive capacity of murine FOXP3$^+$ Treg cells *in vitro.* **b-e)** One week after beginning DS administration (4%, 5 days), therapy with the TIP60 modifier B7A or DMSO control was begun (i.p.; 4 mg/kg/d; 7 days; 10-12 mice/group). B7A therapy protected from colonic inflammation in DMSO-treated mice. Shown are the protective effects of B7A treatment on body weight **(b)**, colon length **(c)**, gut histology (H&E-stained paraffin sections; bars indicate 200μ) **(d)**, and FOXP3$^+$ Treg expansion **(e)** in B7A-treated compared to DMSO-treated mice.

FIGURE 6

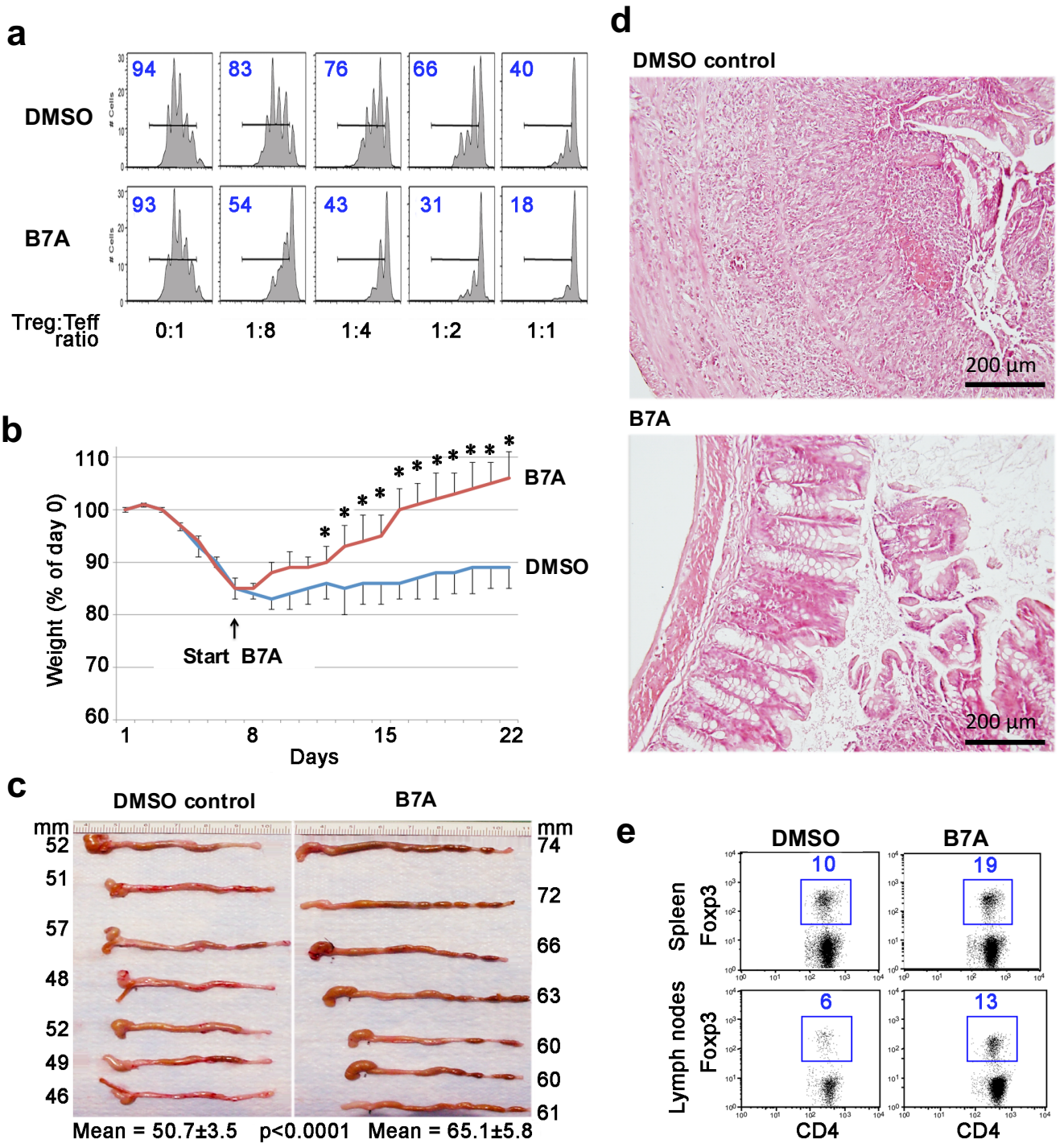
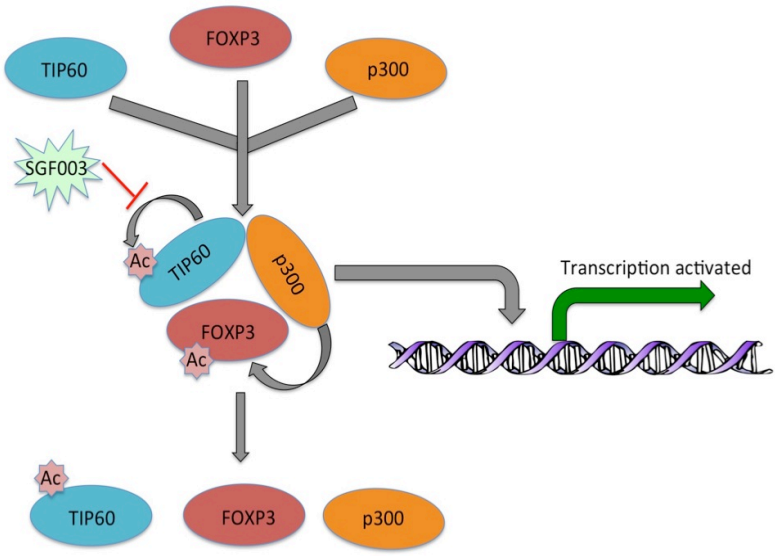

a
DMSO
B7A
94
83
76
66
40
93
54
43
31
18
Treg:Teff ratio
0:1
1:8
1:4
1:2
1:1
b
Weight (% of day 0)
Start B7A
B7A
DMSO
Days
c
DMSO control
B7A
mm
Mean = 50.7±3.5
p<0.0001
Mean = 65.1±5.8
d
DMSO control
B7A
200 µm
e
DMSO
B7A
Spleen
Foxp3
Lymph nodes
CD4

Figure 7. Model of TIP60-p300-FOXP3 interaction in Treg cells. a) FOXP3 interacts with TIP60 and p300 to form a TIP60-p300-FOXP3 complex, in which FOXP3 is acetylated by TIP60 and p300 and subsequently activates transcription of genes required for Treg suppressive function. TIP60 also goes through acetylation in this complex, leading to the dissociation of TIP60 from the TIP60-p300-FOXP3 complex. The presence of an allosteric modifier of TIP60 (SGF003) minimally inhibits auto-acetylation of TIP60 and leads to an increased stable interaction of the TIP60-p300-FOXP3 complex. The stable complex can thus lead to increased transcriptional activity of FOXP3. **b)** In the case of $FOXP3^{A384T}$, interaction of FOXP3 and TIP60 is disrupted and a stable TIP60-p300-FOXP3 complex cannot be formed, leading to decreased transcriptional regulation by FOXP3 affecting the development of the suppressive function of Treg cells. SGF003 stabilizes the TIP60 and $FOXP3^{A384T}$ interaction and restores FOXP3 activity.

FIGURE 7

a


FOXP3
TIP60
p300
SGF003
Ac
TIP60
p300
Transcription activated
FOXP3
Ac
Ac
TIP60
FOXP3
p300

b

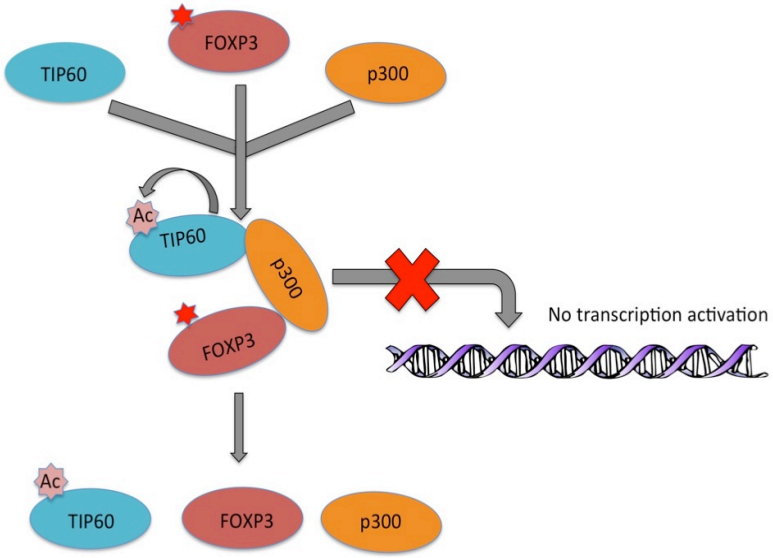

FOXP3
TIP60
p300
Ac
TIP60
p300
No transcription activation
FOXP3
Ac
TIP60
FOXP3
p300

Figure S1. Reduced expression of FOXP3^{A384T} alters its ability to induce CD25 expression. Pools of single-cell-derived clones of FOXP3^{+} and FOXP3^{-} CD4^{+} T cells were generated for patients and controls, and the expression of FOXP3 and CD25 was assessed. Shown is the correlation between FOXP3 and CD25 expression in FOXP3^{+} T cell clones from patient #2 and control. The linear regression is displayed for both the control (left) and IPEX (right) FOXP3^{+} clone pools, with their 95% confidence interval band (dashed lines). Control regression slope = 2.245±0.284; IPEX regression slope = 1.094±0.1262, $p<0.0001$ in comparing the two slopes.

FIGURE S1

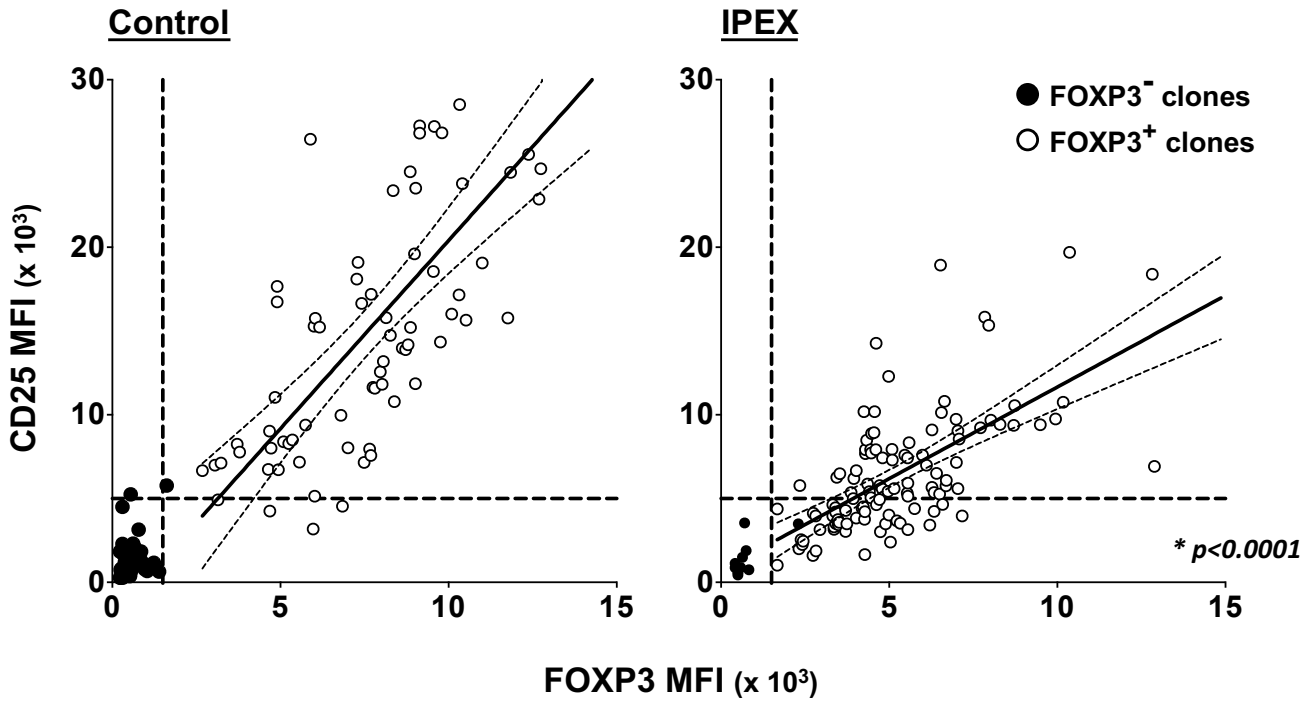
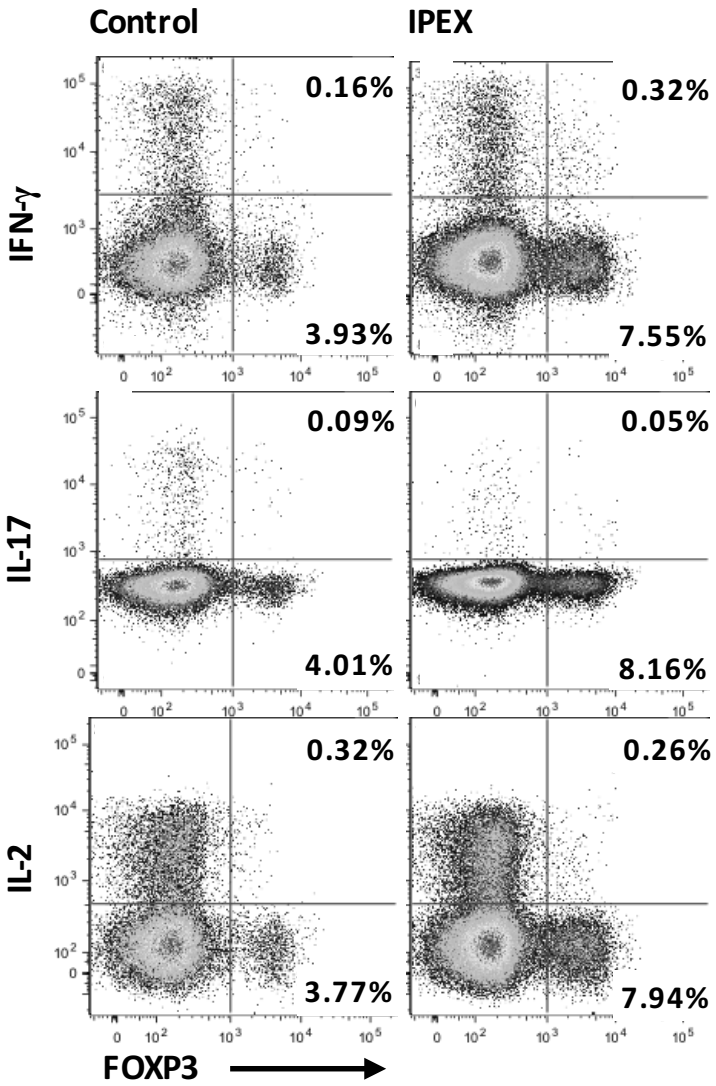

Control
IPEX
FOXP3- clones
FOXP3+ clones
* p<0.0001
CD25 MFI (x 10³)
FOXP3 MFI (x 10³)
0
5
10
15
20
30

Figure S2. Cytokine repression is maintained by FOXP3^{A384T}. Freshly isolated PBMCs from patients or age-matched control donors were stained for the indicated cytokines. Cytokine expression profiles in FOXP3^{+} and FOXP3^{-} CD4^{+} cells are shown.

FIGURE S2


Control
IPEX
IFN-γ
IL-17
IL-2
FOXP3
0.16%
3.93%
0.32%
7.55%
0.09%
4.01%
0.05%
8.16%
0.32%
3.77%
0.26%
7.94%

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CHAPTER 3

Co-expression of TIGIT and FCRL3 identifies Helios^{+} human memory Treg cells

Chapter 3: Co-expression of TIGIT and FCRL3 identifies Helios^{+} human memory Treg cells

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3.1. Bridging statement from Chapter 2 to Chapter 3

As discussed in chapter 1, progress in human Treg research has been significantly hindered by the lack of reliable markers that allow the distinction between *bona fide* Treg cells and activated Teff cells. Moreover, previous studies in our lab have shown that the $FOXP3^+$ population is functionally heterogeneous whereby approximately one third of primary $FOXP3^+$ clones generated from healthy individuals lack the ability to suppress responder cells despite their Treg-like phenotype (108). These observations demonstrated that FOXP3 expression, long thought to be the most specific marker of Treg cells, cannot be used as a reliable indicator of suppressive function in human Treg cells, and that additional factors are required for the development of the Treg suppressive program. Our studies in **chapter 2** have further demonstrated that the mechanisms by which FOXP3 drives the development of Treg phenotype are distinct from those controlling the Treg suppressive function. In the following chapter, we set out to further characterize the functional heterogeneity of human $FOXP3^+$ cells in order to identify novel factors that are required for the manifestation of the characteristic suppressive function of Treg cells, and to discover novel surface markers that better correlate with the suppressive potency of human $FOXP3^+$ cells.

3.2. Abstract

Two distinct subsets of $CD4^+Foxp3^+$ regulatory T cells (Treg) have been described based on the differential expression of Helios, a transcription factor of the Ikaros family. Efforts to understand the origin and biological roles of these Treg populations in regulating immune responses have, however, been hindered by the lack of reliable surface markers to distinguish and isolate them for subsequent functional studies. Using a single-cell cloning strategy coupled with microarray analysis of different Treg functional subsets in humans, we identify the mRNA and protein expression of TIGIT and FCRL3 as a novel surface marker combination that distinguishes $Helios^+FOXP3^+$ from $Helios^-FOXP3^+$ memory cells. Unlike conventional markers that are modulated on conventional T cells (Tconv) upon activation, we show that the TIGIT/FCRL3 combination allows reliable identification of $Helios^+$ Treg cells even in highly activated conditions *in vitro*, and in PBMCs of autoimmune patients. We also demonstrate that the $Helios^-FOXP3^+$ Treg subpopulation harbours a larger proportion of non-suppressive clones compared to the $Helios^+FOXP3^+$ cell subset, which is highly enriched for suppressive clones. Moreover, we find that $Helios^-$ cells are exclusively responsible for the productions of the inflammatory cytokines IFN-γ, IL-2 and IL-17 in $FOXP3^+$ cells *ex vivo*, highlighting important functional differences between $Helios^+$ and $Helios^-$ Treg cells. Thus, we identify novel surface markers for the consistent identification and isolation of $Helios^+$ and $Helios^-$ memory Treg cells in health and disease, and further reveal functional differences between these two populations. These new markers should facilitate further elucidation of the functional roles of Helios-based Treg heterogeneity.

3.3. Introduction

Foxp3$^+$ regulatory T (Treg) cells are critical mediators of immunological self-tolerance. Their absence results in severe multi-organ autoimmunity in humans and mice (1, 2). While the significant contribution of Treg cells in the pathogenesis of autoimmunity has been established based on several animal models (3), investigations on exact pathogenic roles of Treg dysfunction in human autoimmune disorders have resulted in inconclusive findings, mainly due to the lack of specific markers that allow the reliable identification and isolation of a pure Treg population across donors. Most human studies rely on the high expression of CD25 and the low CD127 expression to identify Treg cells (4). However, the expression levels of these two markers are modulated on conventional CD4$^+$ T cells (Tconv) upon activation, making them indistinguishable from Treg cells during immune activation, thereby complicating the interpretation of findings based on these markers. While the expression of FOXP3 can reliably identify human Treg cells in the resting state, its intracellular localization precludes its use for sorting of live cells. Moreover, T cell receptor (TCR)-mediated activation leads to a substantial upregulation of FOXP3 in a fraction of Tconv cells, thus confounding any *ex vivo* Treg phenotypic or functional analysis (5, 6). To circumvent these issues and to characterize *bona fide* Treg cells, we previously used a single-cell cloning approach to dissect the functional heterogeneity within the FOXP3$^+$ population of healthy individuals (7, 8). We observed that the FOXP3$^+$ T cell population, although composed mostly of highly suppressive Treg clones, contains a sizeable subpopulation (approximately 25-30%) of non-suppressive FOXP3$^+$ clones that are indistinguishable from their functional counterparts in terms of the conventional Treg markers(8).

In the present study, we used the same single-cell cloning strategy to identify suppressive and non-suppressive FOXP3$^+$ Treg functional subsets in humans. We further performed microarray

analysis to identify gene products that potentially discriminate these subsets. By comparing the gene expression profiles of these FOXP3$^+$ Treg subsets, we found suppressive clones to have an increased transcription level of the *IKZF2* gene, which encodes the Ikaros family transcription factor, Helios. Helios has been recently proposed as a marker to distinguish thymus-derived Treg cells (tTreg) from peripherally induced ones (iTreg) in mice (9). However, in humans, naïve FOXP3$^+$ cells isolated from healthy blood contain a Helios$^-$ population, suggesting that not all Helios$^-$ FOXP3$^+$ cells are generated in the periphery (10-12). Investigation of the functional relevance of Helios expression in human Treg biology is desired. However, such studies have been hindered by the paucity of surface markers to distinguish them.

Comparing suppressive and non-suppressive clones, we also found an increased expression of the genes encoding two surface proteins: T cell immunoreceptor with Ig and ITIM domains (TIGIT) and Fc receptor-like 3 (FCRL3). TIGIT is an immunoregulatory molecule expressed on memory and activated T cells (13). Functionally, TIGIT has been reported to render dendritic cells (DCs) tolerogenic through interaction with its ligand (CD155) on DCs and induction of IL-10 production (13). TIGIT has also been shown to act as an intrinsic inhibitor of T cell proliferation, similar to the effect of CTLA-4 signaling (14). Recently, Harrison *et al.* showed that TIGIT is transcriptionally targeted by FOXP3 (15), and a role for TIGIT signaling in enhancing Treg-mediated suppression has recently been suggested (16).

FCRL3 (CD307c) is a member of the FCRL family of classical Fc receptor homologues that is expressed on human B cells, some memory T lymphocytes as well as NK cells (17, 18). Although the ligand(s) and physiological function of FCRL3 on T cells are yet to be unraveled, the presence of both ITIM and ITAM motifs in its intracellular domain suggests a role for FCRL3 in the maintenance of homeostatasis via regulation of immune responses (19, 20).

Indeed, some studies have shown a potential role of FCRL3 in regulating B cell differentiation and proliferation initiated by B cell receptor signaling (20, 21). Interestingly, a functional variant in the *FCRL3* promoter has been linked to susceptibility to rheumatoid arthritis (RA), SLE and autoimmune thyroiditis (22), and was associated with increased expression of FCRL3 on T cells of RA patients (23).

Examining the surface expression of TIGIT and FCRL3 on memory FOXP3$^+$ Treg cells, we found a positive correlation of both markers with Helios expression. We demonstrated that the simultaneous use of TIGIT and FCRL3 surface expression allows the consistent discrimination and isolation of live Helios$^+$ and Helios$^-$ memory FOXP3$^+$ populations, each endowed with different functionalities. Notably, we demonstrate here that TIGIT/FCRL3 is a stable marker combination under inflammatory conditions *in vitro*, in PBMCs of autoimmune patients, as well as in the resting state, thus providing a novel approach for further characterization of Treg heterogeneity in health and disease.

3.4. Materials and Methods

Donors and cell isolation.

Peripheral blood mononuclear cells (PBMC) were isolated from buffy coats of healthy donors (n=11; Sanguine Biosciences) using Ficoll-Paque PLUS density gradient (GE Healthcare), and cryopreserved. PBMC samples from untreated patients with relapsing-remitting multiple sclerosis (RRMS; n=6), ulcerative colitis (UC; n=6), Crohn's disease (n=6) and their age and sex-matched healthy controls were obtained from the Montreal Neurological Institute as part of the CIHR NET grant in clinical autoimmunity. PBMC samples from patients with active systemic lupus erythematosus (SLE; SLEDAI-2k score >6) were obtained from the Montreal General Hospital SLE clinic. All samples were collected in accordance with McGill University's research ethics board.

Flow cytometry.

For *ex vivo* flow cytometry analysis, PBMCs were thawed and stained with viability dye (eFluor 780; eBioscience). Purified anti-FcRL3(24) was used where mentioned, followed by staining with F(ab')2 anti-mouse IgG (eFluor 660 or PE; eBioscience) and extensive washing with PBS. Cells were then stained with fluorochrome-conjugated antibodies against CD4 (FITC or V500), CD25 (PE-CF594), CD45RA (Alexa Fluor 700)(BD Biosciences), CD127 (PE), TIGIT (PerCP-eFluor 710), FOXP3 (PE-Cyanine7), CD62L (FITC), HLA-DR (PE-Cy7), CTLA-4 (APC) (eBioscience) and Helios (Pacific Blue)(Biolegend).

For cytokine detection, PBMCs were incubated with PMA (25 ng/ml), ionomycin (1 μg/ml) (both from Sigma-Aldrich) and GolgiStop (eBioscience) for 4hrs, followed by intracellular

cytokine staining with antibodies against IFN-γ (FITC), IL-2 (PerCP-Cy5.5) and IL-17 (APC) (eBioscience).

Flow cytometry analysis was performed on an LSR Fortessa analyzer, and sorting throughout this study was performed on a FACSAria IIu cell sorter (both from BD Biosciences).

Generation of primary $CD4^+$ clones.

Primary $CD4^+$ Treg and Tconv clones were generated from healthy PBMCs by single-cell expansion of FACS-sorted $CD25^{High}$ (top 1%) and $CD25^{Neg}$ cells as described previously (7, 8). The clones were expanded in Xvivo-15 medium (Lonza) supplemented with 5% FBS (Sigma-Aldrich), and were stimulated with soluble anti-CD3 (30 ng/mL; eBioscience), recombinant human IL-2 (200 U/mL; gift of the Surgery Branch, National Cancer Institute, National Institutes of Health) and irradiated human PBMCs as feeders. Fresh medium and IL-2 was replenished on day 5 and every two days thereafter, and clones were passaged as needed. Restimulation was performed on day 11-12, and the clones were further expanded until harvested on day 22-24. Phenotypic and functional assays were performed on the clones in parallel. Clones were generated from 7 different healthy subjects (34-66 $FOXP3^+$ clones/donor).

Suppression assays.

CFSE-based suppression assays were used throughout this study to measure the suppressive potency of Treg cells as we previously described (8). Briefly, responding allogeneic $CD4^+CD25^-$ cells (Tresp) were sorted by FACS, labeled with CFSE proliferation dye (5 μM; Sigma-Aldrich) and plated at 8000 cells/well in U-bottom 96-well plates (Sarstedt) with irradiated PBMCs as feeders (30,000 cells/well). Treg cells were added at different Treg:Tresp ratios and the assays

were stimulated with soluble anti-CD3 (30 ng/mL; eBioscience) for 4 days. Suppression was calculated based on the division index (as calculated by the Flowjo software) of unsuppressed Tresp cells cultured in the absence of Treg cells.

Gene-expression analysis.

Total mRNA was prepared from individual clones representing three populations: 1) suppressive and 2) non-suppressive $FOXP3^{+}$ clones generated from $CD4^{+}CD25^{High}$ cells, and 3) control $FOXP3^{-}$ clones generated from $CD4^{+}CD25^{-}$ cells. The clones were derived from 2 healthy donors, and each group is represented by 2-3 pooled clones per donor. Clone selection was based on a thorough analysis of phenotype and function as we described before (8). Cells were pelleted and resuspended in RLT-Plus lysis buffer (Qiagen), and immediately frozen at -80°C. mRNA reverse-transcription, labeling and hybridization onto an Illumina HumanHT-12_V3 Expression BeadChip™ were performed by the Génome Québec Innovation Centre (Montreal, Canada). Total gene expression data were normalized using the robust-spline (RSN) method, without background correction and with base 2-logarithmic transformation, using the R software. Samples were compared using an F-test (ANOVA) with the RVM-based correction for multiple-hypothesis testing (25), using the R software. To minimize interference from gene expression of the irradiated feeders used to expand the clones, we established a feeder contamination standard curve, by expanding the same clones in the presence of titrated numbers of feeder cells, and processing the resulting samples on the same chip. For each gene expression value, the corresponding contamination contribution was predicted by regression, and a separate, corrected gene expression value was calculated. A gene was considered differentially expressed in two subsets if its corrected fold-change value was ≥ 1.5, and the ANOVA p value was ≤ 0.1.

T cell activation assays.

To investigate the activation-induced modulation of marker expression, $CD4^+CD25^-TIGIT^-FCRL3^-$ cells from healthy PBMCs were FACS-sorted, labeled with CFSE, and activated for 4-6 days *in vitro* with anti-CD3/anti-CD28-coated beads (Miltenyi Biotec) at a ratio of 2 beads: 1 cell. Where indicated, irradiated autologous PBMCs were used as feeders. Protein expression levels of FOXP3, Helios, TIGIT and FCRL3 were measured by flow cytometry over time. In different experiments, whole PBMCs were activated with anti-CD3/anti-CD28-coated beads for 3-5 days hours followed by flow cytometric analysis of marker expression.

Statistical analysis.

Statistical analysis was performed using the GraphPad Prism 5.0 software. One-way ANOVA, followed by Turkey's range test, and the student *t*-test were used where indicated. A p-value of <0.05 was considered significant.

3.5. Results

Lack of suppressive function in human FOXP3$^+$ clones is associated with reduced expression of Helios, TIGIT and FCRL3.

We have recently shown that in about 30% of the FOXP3$^+$ clones derived from healthy individuals, FOXP3 expression does not lead to suppressive function despite the maintenance of a typical Treg phenotype (8) (Figure 1A). In order to further understand this functional heterogeneity, and to identify markers of the different functional subsets of Treg cells, we compared gene expression profiles of FOXP3-positive suppression-positive (FPSP) and FOXP3-positive suppression-negative (FPSN) clones, relative to FOXP3$^-$ control clones (FNSN) (Figure 1 B). As expected, we observed that many common Treg cell phenotypic markers are expressed at comparable levels in both FOXP3$^+$ functional subsets (Figure 1C, D). To further compare FPSP and FPSN clones, we analyzed the Treg-specific demethylated region (TSDR) of the *FOXP3* locus, which is normally demethylated in Treg cells but methylated in Tconv cells (26, 27). Owing to the female origin of the analyzed clones in this study, a Treg clone is expected to be around 50% methylated in its TSDR region due to X-chromosome inactivation (28). Indeed, unlike FNSN clones, which have a 77% methylated TSDR, both FPSP and FPSN clones show only around 54% methylation in their TSDR regions (Figure S1), suggesting that both groups are derived from bona fide Treg cells.

However, several genes were found to be differentially expressed between the two FOXP3$^+$ subsets (Figure 1D and E). One such gene, *IKZF2,* codes for the transcription factor Helios, and was found to be significantly more transcribed in FPSP relative to FPSN clones. (Figure 1E). Moreover, in search for potential extracellular markers of Treg functional subsets, we additionally found the genes coding for the surface receptors TIGIT and FCRL3 to be more

highly expressed in FPSP clones relative to FPSN. The expression of all three genes was lowest in FNSN clones (Figure 1E).

In order to validate the differences observed in gene transcription, we generated primary clones from healthy individuals and examined the expression of FOXP3, Helios, TIGIT and FCRL3 proteins in FPSP, FPSN and FNSN clones. While FOXP3 and TIGIT were expressed equally in FPSP and FPSN clones, both Helios and FCRL3 were significantly lower in FPSN relative to FPSP clones, and lowest in FNSN clones (Figure 2) confirming our observations at the transcription level in regards to Helios and FCRL3, and suggesting a potential role for these markers in the suppressive function of Treg cells.

Differential TIGIT and FCRL3 expression delineates Helios$^+$ and Helios$^-$ memory Treg cells *ex vivo*.

We then examined the protein expression of TIGIT and FCRL3 as well as Helios in CD4$^+$FOXP3$^+$ cells of PBMCs of healthy individuals by flow cytometry. In line with previous studies, Helios protein is expressed in about 70% of CD4$^+$FOXP3$^+$ cells (Figure 3A) (9, 11, 12). In the same experiments, we measured significant levels of TIGIT and FCRL3 protein expression on about 50% of CD4$^+$FOXP3$^+$, with the vast majority of TIGIT and FCRL3-expresing cells exhibiting a memory profile (CD45RA$^-$) (Figure 3A, B). Subsequent analyses are, therefore, restricted to the CD4$^+$CD45RA$^-$ population. Interestingly, both TIGIT and FCRL3 are preferentially expressed on the Helios$^+$ population of memory FOXP3$^+$ cells whereby TIGIT$^+$ and FCRL3$^+$ cells comprise around 80% and 90% Helios$^+$ cells, respectively (Figure 3B). Notably, the combined expression of TIGIT and FCRL3 identifies a memory FOXP3$^+$ population that is even more greatly enriched for Helios$^+$ cells (Median=95.1%; IQR=1.69 %) (Figure 3B, C). Conversely, the lack of both TIGIT and FCRL3 expression on memory FOXP3$^+$

cells denotes a population that is predominantly negative for Helios (Median=17.1%; IQR=5.48%). The ability to discriminate between Helios subsets was precisely reproducible in primary $FOXP3^+$ clones derived from healthy individuals (Figure 3D), demonstrating the reliability of TIGIT and FCRL3 as a novel surface marker combination to stratify Treg populations according to Helios expression *in vitro* and *ex vivo*.

To sort human live Treg cells, most previous studies relied on the high cell surface expression of CD25 (1-2% of $CD4^+$ cells) and the concomitant low expression of CD127. Using this gating strategy, we were able to obtain a population that is enriched for $FOXP3^+$ cells (Median=85.8%; IQR=4.35%) from memory $CD4^+$ cells of healthy PBMC. As expected, the defined $CD4^+CD25^+CD127^{Low}$ cells contained both $Helios^+$ (Median=75.2%; IQR=8.55%) and $Helios^-$ cells with a similar ratio as that in $CD4^+FOXP3^+$ (Figure 4A, B). By further defining $TIGIT^+FCRL3^+$ cells, we consistently obtained a highly enriched $Helios^+$ population (Median=92.2%; IQR=2.25%) from $CD4^+CD25^+CD127^{Low}$ cells. In contrast, a majorly $Helios^-$ population (Median=16.2%; IQR=13.7%) was identified in the $TIGIT^-FCRL3^-$ subpopulation (Figure 4A, B). These results demonstrate that the combined use of TIGIT and FCRL3 achieves the nearly exclusive isolation of live $Helios^+$ and $Helios^-$ Treg subsets without sacrificing cell viability. Furthermore, we assessed the enrichment in FOXP3- and Helios-expressing cells that can be attained by gating on $CD4^+CD45RA^-CD25^+TIGIT^+FCRL3^+$ cells with varying stringencies of the CD25 gate. Interestingly, the same high level of enrichment in $FOXP3^+Helios^+$ cells can be achieved with a CD25 gate encompassing as little as 1% and as much as 20% of memory $CD4^+$ cells (Figure 4C), indicating that TIGIT and FCRL3 can be used to sort memory $Helios^+$ Treg cells with enhanced consistency and recovery when compared to the conventional gating strategies that rely on subjective CD25 gating.

Finally, we compared the expression of a panel of Treg-associated surface molecules on $TIGIT^{+}FCRL3^{+}$ and $TIGIT^{-}FCRL3^{-}$ Treg cells of healthy individuals. Although minor differences in the expression of markers like CD62L, HLA-DR and CTLA-4 can be seen (Figure S2), none of the examined molecules has the ability to define $TIGIT^{+}FCRL3^{+}$ or $TIGIT^{-}FCRL3^{-}$ Treg cells.

TIGIT and FCRL3 reliably identify $Helios^{+}$ Treg cells in activated conditions *in vitro*.

A major drawback of the currently used Treg markers is their modulation on Tconv cells following cell activation, which precludes their reliable use to distinguish Treg and Tconv cells in inflammatory conditions. Thus, we next assessed whether TIGIT and FCRL3 expression could be induced on Tconv following T cell activation. We isolated $CD4^{+}CD45RA^{-}CD25^{-}$ (Tconv) cells and stimulated them with anti-CD3/anti-CD28-coated beads *in vitro*. By 72 hours post-activation, both TIGIT and FOXP3 were upregulated on 40% and 20% of Tconv cells, respectively. However, FCRL3 and Helios, were not upregulated at all on activated Tconv cells even after 5 days in culture (Figure 5A, B). To address the possibility that antigen-presenting cells may be required for the upregulation of Helios and FCLR3 on Tconv cells, we FACS-sorted $CD4^{+}CD25^{-}TIGIT^{-}FCLR3^{-}$ cells and activated them *in vitro* with anti-CD3/anti-CD28-coated beads in the presence of irradiated autologous feeders with or without IL-2. Neither Helios nor FCLR3 was upregulated over the 4-day culture period (Figure 5C). Additionally, we examined the use of TIGIT and FCRL3 on whole PBMC cultures stimulated with anti-CD3/anti-CD28-coated beads. At the peak of activation on day 3, $CD4^{+}CD25^{+}CD127^{Low}TIGIT^{+}FCRL3^{+}$ cells were still majorly $FOXP3^{+}$ (>80%) and highly enriched in $Helios^{+}$ cells (>83%) (Figure 5C). Moreover, we did not observe a significant change in the frequency of $FCRL3^{+}$ or $Helios^{+}$ cells in the $CD4^{+}$ population upon activation of healthy PBMC (Figure S3), further indicating that

both Helios and FCRL3 are not upregulated in Tconv cells upon activation. Finally, we examined the applicability of the TIGIT/FCRL3 combination for flow cytometric analysis of PBMCs derived from patients with various active autoimmune disorders including untreated relapsing-remitting multiple sclerosis (RRMS), ulcerative colitis (UC), Crohn's disease (CD) and active systemic lupus erythematosus (SLE). Similar to our observations in healthy subjects, the memory $CD4^+CD25^+CD127^{Low}$ cell population could be segregated into $Helios^+$ and $Helios^-$ subpopulations by further gating using TIGIT and FCRL3 (Figure S4A). Collectively, these data indicate the stability of this marker combination in inflammatory conditions, and provide a reliable approach for the consistent isolation of Treg cells.

Non-suppressive Treg cells are enriched within the $Helios^-$ subset.

The assessment of the role of Helios expression in the suppressive function of Treg cells has been hindered by the inability to discriminate $Helios^+$ and $Helios^-$ Treg cells *ex vivo.* We exploited the use of our novel combination of surface markers to isolate $Helios^+$ and $Helios^-$ cells directly *ex vivo* for functional assessment. To this end, we co-cultured sorted $CD4^+CD45RA^-CD25^+CD127^{Low}TIGIT^+FCRL3^+$ ($Helios^+$) or $CD4^+CD45RA^-CD25^+CD127^{Low}TIGIT^-FCRL3^-$ ($Helios^-$) cells from healthy PBMCs with CFSE-labeled $CD4^+CD25^-$ responding T cells in the presence of soluble anti-CD3 and irradiated feeder PBMCs. Suppression of proliferation of responding T cells was then measured in a 4-day assay. We show that both $Helios^+$ and $Helios^-$ $FOXP3^+$ cell subsets efficiently suppressed the proliferation of responder T cells, and no significant difference was observed between these subsets (Figure 6A, B). We then assessed whether $FOXP3^+$ Treg cells that differentially express Helios displayed equivalent suppressive activities with prolonged T cell activation and expansion. To this end, we generated individual $FOXP3^+$ clones from healthy PBMCs and assessed suppressive function relative to Helios

expression (Figure 6). Our results show that the Helios$^-$ population contains a significantly higher proportion of non-suppressive clones compared to its Helios$^+$ counterpart, although both populations harbored highly suppressive clones (Figure 6D, E).

Overall, our results show that differential TIGIT and FCRL3 expression on human FOXP3$^+$ Treg cells can efficiently be used to isolate Helios-expressing subsets. Moreover, our data also indicate a greater level of functional heterogeneity within the Helios$^-$ subset of FOXP3$^+$ T cells, as this subset manifests compromised suppressive activity after prolonged cellular expansion.

Helios$^+$ and Helios$^-$ CD4$^+$FOXP3$^+$ cells differ in cytokine production capacity.

The stable expression of FOXP3 in Treg cells results in the transcriptional repression of a number of cytokines such as IFN-γ, IL-17 and IL-2 (29, 30). However, the production of these cytokines has been observed in a small population of memory FOXP3$^+$ cells (31, 32). We analyzed cytokine production in healthy PBMCs *ex vivo*, and found that, compared to FOXP3$^-$ cells, FOXP3$^+$ cells are indeed poor producers of IFN-γ, IL-17 and IL-2 in response to stimulation with PMA and ionomycin (Figure 7B). However, consistent with previous reports (10, 33), we detected a subpopulation of inflammatory cytokine-producing cells within the FOXP3$^+$ population, and these cells were exclusively restricted to the Helios$^-$ subpopulation (Figure 7A, B). Similarly, increased levels of inflammatory cytokines were measured in FACS-sorted TIGIT$^-$FCRL3$^-$, but not TIGIT$^+$FCRL3$^+$, memory Treg cells (CD4$^+$CD45RA$^+$CD25$^+$CD127^{Low}) (Figure 7C). Moreover, analysis of IFN-γ, IL-17 and IL-2 production in CD4$^+$CD45RA$^+$CD25$^+$CD127^{Low} Treg cells of patients of several autoimmune diseases further confirms the restriction of cytokine production to the TIGIT$^-$FCRL3$^-$ fraction of Treg cells (Figure S4B). These data highlight important differences between Helios$^+$ and Helios$^-$ cells, and emphasize the need for further characterization of their functional relevance.

3.6. Discussion

The characterization of the relative function of Helios-defined Treg subsets has been hindered by the lack of reliable surface markers to delineate and isolate them. In this study, we identify the co-expression of TIGIT and FCRL3 as a novel marker combination that allows for a reliable stratification and isolation of human memory FOXP3^{+} Treg cells according to Helios expression. Importantly, we demonstrate the reliability of this marker combination under immune activating conditions *in vitro* and in the periphery of autoimmune patients presenting with various active systemic and organ-specific autoimmune diseases. Moreover, our combined use of TIGIT and FCRL3 has enabled us to highlight significant functional differences between Helios^{+} and Helios^{-} Treg cells, emphasizing the need for further investigation of their respective roles in regulating immune responses.

The modulation of the currently used Treg markers on Tconv upon activation makes it very challenging to identify *bona fide* Treg cells in inflammatory conditions. An important observation in this study is that FCRL3 is selectively expressed by FOXP3^{+} Treg cells, and is not upregulated by Tconv cells upon TCR-mediated stimulation. This is in line with previous observations that reported the lack of FCRL3 upregulation in Treg cells induced from Tconv cells upon activation in the presence of TGF-b (24, 34). Thus, the TIGIT/FCRL3 combination provides an excellent surface marker for Helios^{+} Treg cells in inflammatory conditions. Such marker stability will render comparative functional analysis of Treg function in health and disease more informative. In addition, our observations concerning the upregulation of TIGIT and FOXP3, but not FCRL3 or Helios, on activated Tconv cells indicate that differential expression of these markers can also be used to identify and exclude recently activated Tconv cells, which are expected to exhibit the phenotype TIGIT^{+}FCRL3^{-}FOXP3^{+}Helios^{-} in CD25^{+}CD127^{Low}cells.

The contribution of Helios expression to the suppressive function of Treg cells is poorly defined. While Helios-deficient mice show normal Treg development (9, 35), siRNA-mediated knockdown of Helios expression in human $CD4^+CD25^+$ cells resulted in reduced suppressive function (36). Using our novel marker combination, we find that $Helios^+$ and $Helios^-$ cells exert equal levels of suppression when assessed directly *ex vivo.* This result is in accordance with recent observations by Raffin *et al* that both $Helios^+$ cells and $Helios^-$ cells sorted based on the combined expression of CCR7 and IL-1RI are highly suppressive, although their study reported that $Helios^-$ cells are more suppressive than $Helios^+$ cells (33). It should be noted that the suppression assays in this study used monocytes as feeders and were stimulated with PHA, whereas the assays reported here used irradiated PBMCs and were stimulated with soluble anti-CD3. Interestingly, our analysis of the suppressive capacity of $FOXP3^+$ clones showed that, unlike $Helios^+$ clones that are highly enriched in suppressive clones, $Helios^-$ clones constitute a significantly more heterogeneous mixture of suppressive and non-suppressive clones. Such functional heterogeneity in the $Helios^-$ population may have been masked in our *ex vivo* suppression analysis due to the polyclonal nature of the suppressor population and the functional complementation by the highly suppressive cells within the $Helios^-$ population. Alternatively, the wider functional distribution of $Helios^-$ cells could also be attributed to the possibility that $Helios^-$ cells may be more prone to modulating their suppressive function than $Helios^+$ cells. This could be as a result of prolonged TCR-mediated expansion, or in the presence of certain inflammatory signals, a potential example of which was recently proposed by Raffin *et al.* and involves IL-1β signaling, which causes $Helios^-$ Treg cells to downregulate their suppressive function (33). Such functional diversity within the $Helios^-$ population may suggest a higher plasticity potential in this Treg population in response to the changing microenvironment.

The biological significance of effector cytokine production by FOXP3$^+$ Treg cells is unclear. While some studies on mouse models suggest that upregulation of inflammatory cytokines such as IL-17 and IFN-γ occurs in unstable Treg cells, and precedes loss of FOXP3 and adoption of a Tconv phenotype (37, 38), other studies suggest that some FOXP3$^+$ Treg cells acquire a transient ability to produce IFN-γ and IL-17 to allow efficient homing to Th1 and Th17 inflammation sites, respectively, without loss of suppressive function or down-regulation of FOXP3 (8, 32, 39, 40). We show that the expression of Helios in FOXP3$^+$ cells correlates with a strong repression of effector cytokine production. These findings are in agreement with, and extend, recently reported observations that inflammatory cytokine production in human FOXP3$^+$ cells is restricted to the Helios$^-$ population (10, 12, 33). These results further highlight some potentially important functional differences between Helios$^+$ and Helios$^-$ Treg cells, and warrant more in-depth analysis of the two subsets.

The degree of demethylation in the TSDR region of the *FOXP3* promoter is now used as a measure of stability of FOXP3 expression and suppressive function (26, 27). A number of studies have analyzed this epigenetic parameter in Helios subsets of human Treg cells. Supporting our result, Kim *et al.* reported partial TSDR demethylation (55%) in total FOXP3$^+$Helios$^-$ cells compared to complete demethylation in FOXP3$^+$Helios$^+$ cells, suggesting heterogeneity within the Helios$^-$ subset (10). It should be noted, however, that Helios$^-$ FOXP3$^+$ cells are not restricted to the memory fraction of Treg cells, but are also present in naïve cells in peripheral blood. More recently, Himmel *et al.* analyzed TSDR demethylation in naïve (CD45RA$^+$) FOXP3$^+$ cells and observed comparable demethylation patterns in Helios$^+$ and Helios$^-$ populations (12), suggesting that the methylation observed in Helios$^-$ cells in earlier studies is specific to the memory fraction. Indeed, McClymont *et al.* reported predominant TSDR

methylation in IFN-γ-producing FOXP3^{+} cells, which are majorly restricted to the memory Helios^{-} fraction (41), suggesting that the Helios^{-} population is heterogeneous and encompasses cells with enhanced functional plasticity in order to allow homeostatic adjustments to the needs of the immune response. Due to the lack of reliable markers for Helios subsets of Treg cells, all these epigenetic analyses were performed on fixed cells, and thus further delineation of the functional significance of Helios expression in human Treg cells was not previously possible.

The functional role of TIGIT in Treg biology has only started to be unravelled. A recent study by Joller *et al.* found that TIGIT signalling enhanced Treg-mediated suppression of Th1 and Th17, but not of Th2, responses. Such selectivity is achieved through the production of fibrinogen-like protein 2 (Fgl2), which prevents the suppression of Th2 responses (16). Other reported TIGIT-mediated regulatory functions include its active tolerogenization of dendritic cells through binding to CD155 and induction of IL-10 secretion (13), as well as its intrinsic inhibition of T cell activation and proliferation (14).

Two independent studies have previously examined the association between the expression of FCRL3 on Treg cells and their suppressive function. While Swainson *et al.* reported that FCRL3 expression identified Treg population with a reduced suppressive potency (34), Nagata *et al.* observed a comparable suppressive capacity in the FCRL3^{+} and FCRL3^{-} fractions of CD25^{+}CD127^{Low} Treg cells (24). Further investigation is warranted to elucidate the potential molecular link between the expression of these markers and Helios expression, and to determine the precise function of FCRL3 and TIGIT in Treg cells.

In conclusion, we identified TIGIT and FCRL3 expression as a novel surface marker combination that allows the isolation of live Helios^{+} and Helios^{-} memory Treg cells. Our data further revealed phenotypic and functional differences between human Helios^{+} and Helios^{-} Treg

cells. The novel surface markers presented here will facilitate in-depth investigations into the functional heterogeneity of these Treg subsets and may provide important clues to allow directed manipulation of specific Treg populations.

3.7. Acknowledgments.

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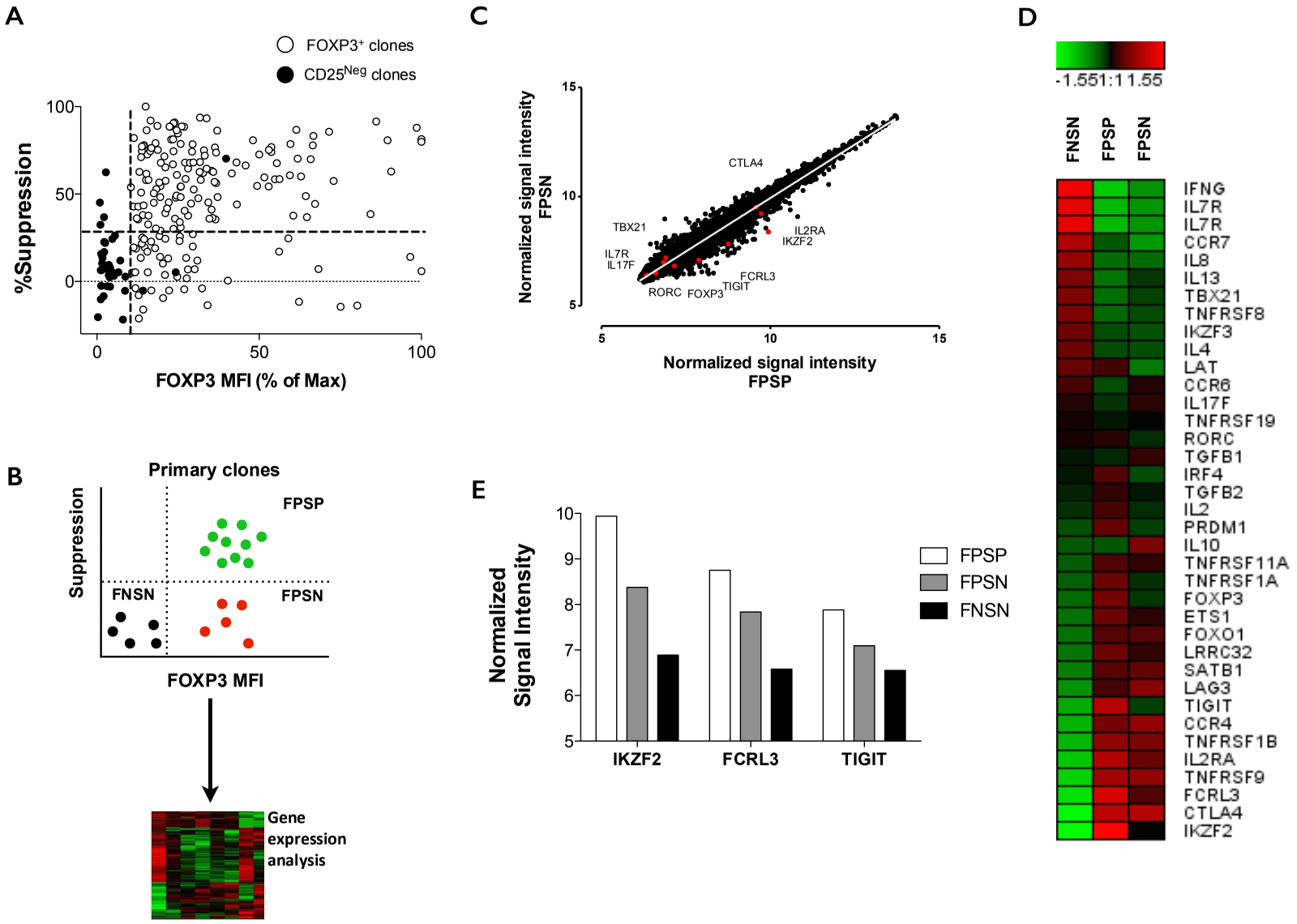
Conflict-of-interest disclosure: The authors declare no competing financial interests.

3.8. Figures:

Figure 1. Gene expression analysis of suppressive vs. non-suppressive FOXP3^{+} clones. Illumina BeadChip analysis of total mRNA prepared from individual clones representing three populations: 1) suppressive and 2) non-suppressive FOXP3^{+} clones generated from CD4^{+}CD25^{High} cells, and 3) control FOXP3^{-} clones generated from CD4^{+}CD25^{-} cells. **A)** Correlation of suppressive potency with FOXP3 expression levels in primary FOXP3^{+} and FOXP3^{-} (CD25^{Neg}) clones generated from 3 healthy donors. The dotted lines represent the cut-offs for FOXP3 MFI (x-axis) and % suppression (y-axis) determined through the overall FOXP3 MFI and suppressive capacity of the control FOXP3^{-} clones generated from CD4^{+}CD25^{-} cells. **B)** Schematic illustrating the process of selection of representative clones. **C)** Scatter plot showing normalized gene expression level in suppressive vs. non-suppressive clones in the resting state. **D)** Heatmap comparing variations in the expression of selected Treg- and Tconv-associated genes relative to the median mRNA levels across the three subsets **E)** Relative mRNA expression levels of TIGIT, FCRL3 and Helios in the three functional categories. Representative clones were derived from two healthy donors, and 2-3 similar clones/subset/donor were pooled to obtain sufficient mRNA.

Figure 1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FOXP3+ clones
CD25Neg clones
%Suppression
FOXP3 MFI (% of Max)
B
Primary clones
Suppression
FPSP
FNSN
FPSN
FOXP3 MFI
Gene expression analysis
C
Normalized signal intensity FPSN
Normalized signal intensity FPSP
CTLA4
TBX21
IL7R
IL17F
IL2RA
IKZF2
FCRL3
RORC
FOXP3
TIGIT
D
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IFNG
IL7R
IL7R
CCR7
IL8
IL13
TBX21
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LAT
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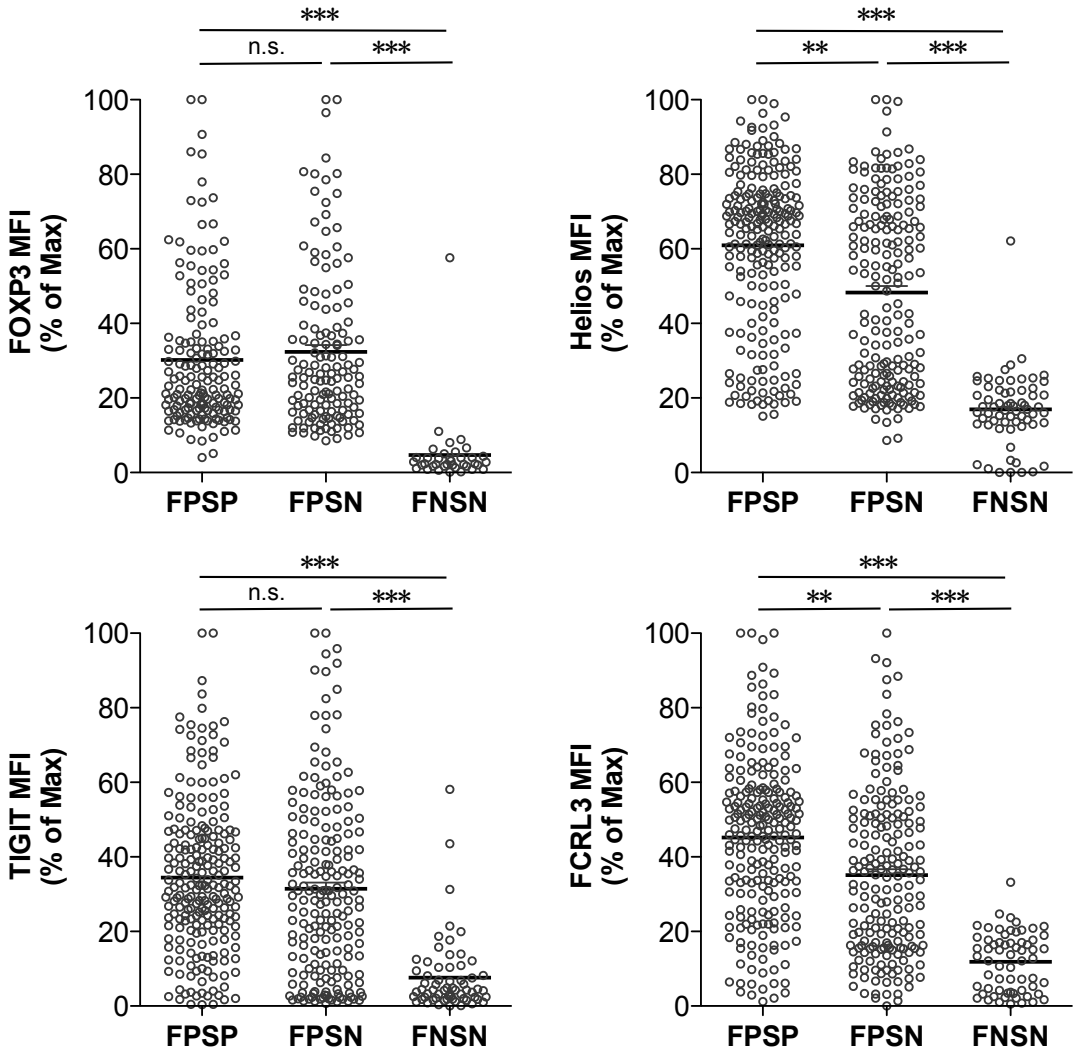
E
Normalized Signal Intensity
FPSP
FPSN
FNSN
IKZF2
FCRL3
TIGIT

Figure 2. FPSN clones comprise majorly Helios$^-$ clones and display a reduced FCRL3 expression. Primary Treg and Tconv clones were generated by single-cell cloning of FACS-sorted CD25^{High} and CD25^{Neg} cells of 4 healthy individuals. Marker expression analysis was performed immediately after the harvest on day 22-24, and 4-day CFSE-based suppression assays were carried out using allogeneic CD4$^+$CD25$^-$ cells as responders at a 1:1 Treg:Tresp ratio in the presence of irradiated PBMCs and anti-CD3 (30 ng/mL). Suppression was measured relative to the division index of the unsuppressed Tresp-alone control. Shown are the expression levels of FOXP3, Helios, TIGIT and FCRL3 in FPSP, FPSN and FNSN clones immediately after harvest. Statistical analysis was done with the one-way ANOVA followed by Tukey's post-test (p-value: * $\leq$ 0.05; ** $\leq$ 0.01; *** $\leq$ 0.001).

Figure 2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n.s.
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FOXP3 MFI (% of Max)
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Helios MFI (% of Max)
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TIGIT MFI (% of Max)
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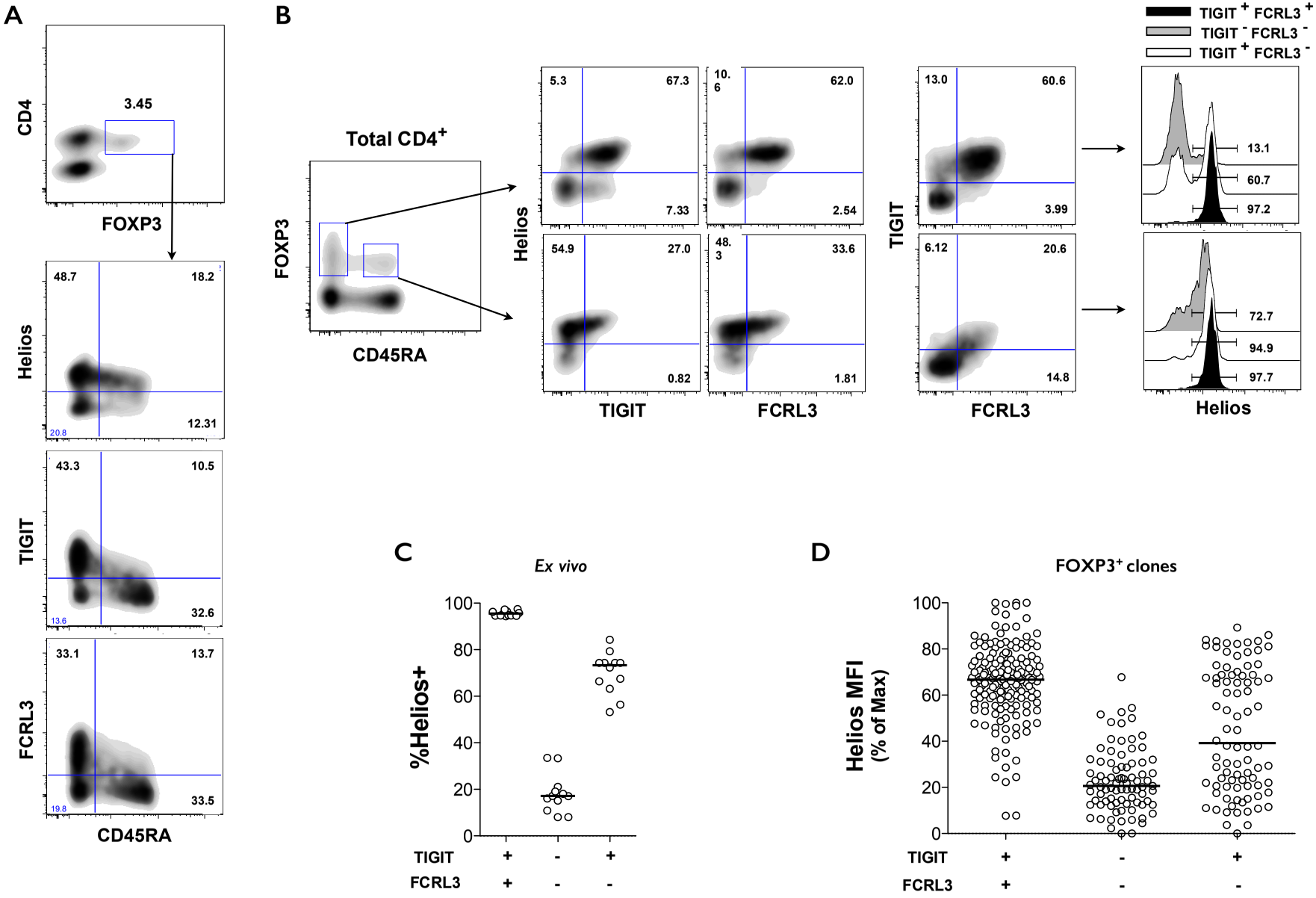
FCRL3 MFI (% of Max)

Figure 3. The combined expression of TIGIT and FCRL3 discriminates between Helios$^+$ and Helios$^-$ subsets in memory FOXP3$^+$ cells. A-C) PBMCs from healthy subjects were analyzed *ex vivo* by flow cytometry. **A)** The expression of TIGIT, FCRL3 and Helios on naïve and memory CD4$^+$FOXP3$^+$ populations. **B)** Representative plots showing the correlation of TIGIT and FCRL3 expression with Helios expression in naïve vs. memory CD4$^+$FOXP3$^+$ cells. **C)** Combined analysis of 11 healthy individuals showing the application of TIGIT and FCRL3 in the identification Helios$^+$ and Helios$^-$ subsets within memory FOXP3$^+$ cells. **D)** The applicability of TIGIT/FCRL3 combination in discriminating Helios subsets in FOXP3$^+$ clones generated from 7 healthy donors (n=299 clones).

Figure 3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TIGIT
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FCRL3
33.1
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19.8
33.5
CD45RA
B
Total CD4+
FOXP3
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Helios
5.3
67.3
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Helios
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Figure 4. TIGIT/FCRL3 combination provides a reliable surface marker for the isolation of Helios^{+} and Helios^{-} memory Treg cells. PBMCs from 11 healthy subjects were analyzed *ex vivo* by flow cytometry. **A)** Representative FACS plots showing the application of the TIGIT/FCRL3 marker combination in distinguishing Helios subsets within memory CD4^{+}CD25^{+}CD127^{Low} cells. **B)** The identification of Helios subsets by TIGIT/FcRL3 surface markers is precisely reproducible in healthy samples with a wide range of Helios expression. Shown is the frequency of Helios^{+} (top) and FOXP3^{+} (bottom) cells in Treg populations gated using the conventional markers (CD25^{+}CD127Low; referred to as 'Total') compared to further gating using different combinations of TIGIT and FCRL3. **C)** The TIGIT/FCRL3 combination allows the identification of consistently enriched FOXP3^{+}Helios^{+} populations with less stringent gating on CD25. Shown is the frequency of FOXP3^{+} and Helios^{+} cells in TIGIT^{+}FCRL3^{+} cells obtained from variably stringent CD25^{+} gates on memory CD4^{+} cells.

Figure 4

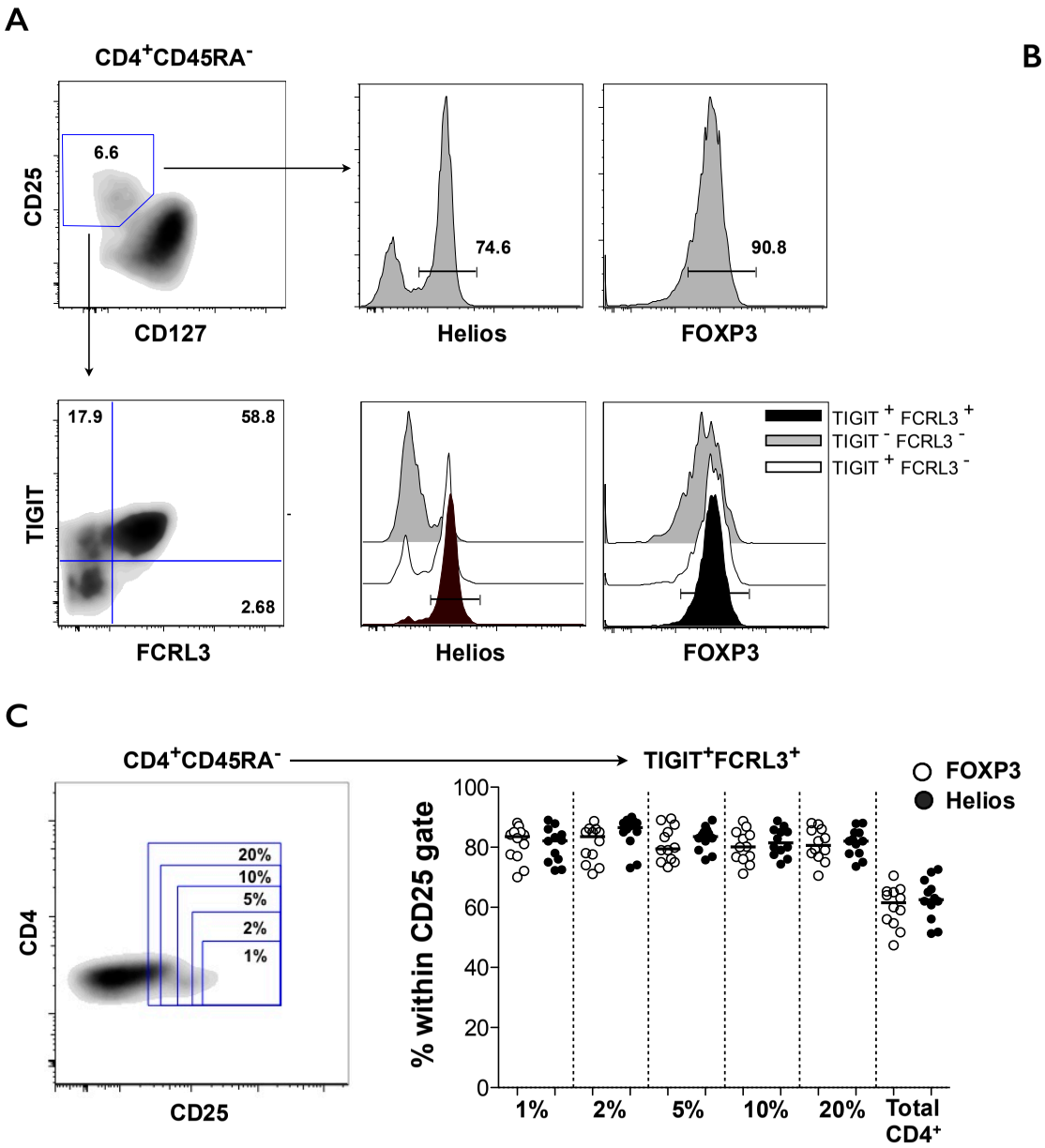

A
CD4+CD45RA-
CD25
6.6
CD127
Helios
74.6
FOXP3
90.8
TIGIT
17.9
58.8
2.68
FCRL3
TIGIT + FCRL3 +
TIGIT - FCRL3 -
TIGIT + FCRL3 -
B
CD4+CD45RA-CD25+CD127Low
% Helios+ cells
% FOXP3+ cells
Total
TIGIT+
FCRL3+
TIGIT+FCRL3+
TIGIT-
FCRL3-
TIGIT-FCRL3-
TIGIT+FCRL3-
C
CD4+CD45RA-
TIGIT+FCRL3+
CD4
CD25
20%
10%
5%
2%
1%
% within CD25 gate
FOXP3
Helios
Total CD4+

Figure 5. The TIGIT/FCRL3 marker expression can reliably identify Helios^{+} and Helios^{-} Treg cell subsets in inflammatory contexts. A) FACS-sorted TIGIT^{-}FCRL3^{-} memory CD4^{+}CD25^{-} cells from PBMCs of a healthy donor were labeled with CFSE, and activated *in vitro* with anti-CD3/anti-CD28-coated beads at a ratio of 2 beads: 1 cell for 5 days. Shown are representative FACS plots, and **B)** the kinetics of activation-induced marker upregulation in 3 separate experiments on 3 healthy individuals. **C)** Total CD4^{+}CD25^{-}TIGIT^{-}FCRL3^{-} cells from PBMCs activated *in vitro* with anti-CD3/anti-CD28-coated beads at a ratio of 2 beads: 1 cell with or without rhIL-2 in the presence of irradiated autologous feeders for 4 days. Shown is the expression of FCRL3 and Helios on the activated CD4^{+}CD25^{-}TIGIT^{-}FCRL3^{-} cells compared to FACS-sorted CD4^{+}CD25^{+}CD127^{Low}TIGIT^{+}FCRL3^{+} plated in parallel. **D)** Whole PBMCs from a healthy individual were stimulated with anti-CD3/anti-CD28-coated beads at a ratio of 2 beads: 1 cell for 72 Hrs. Representative FACS plots from similar experiments on 3 healthy donors are shown.

Figure 5

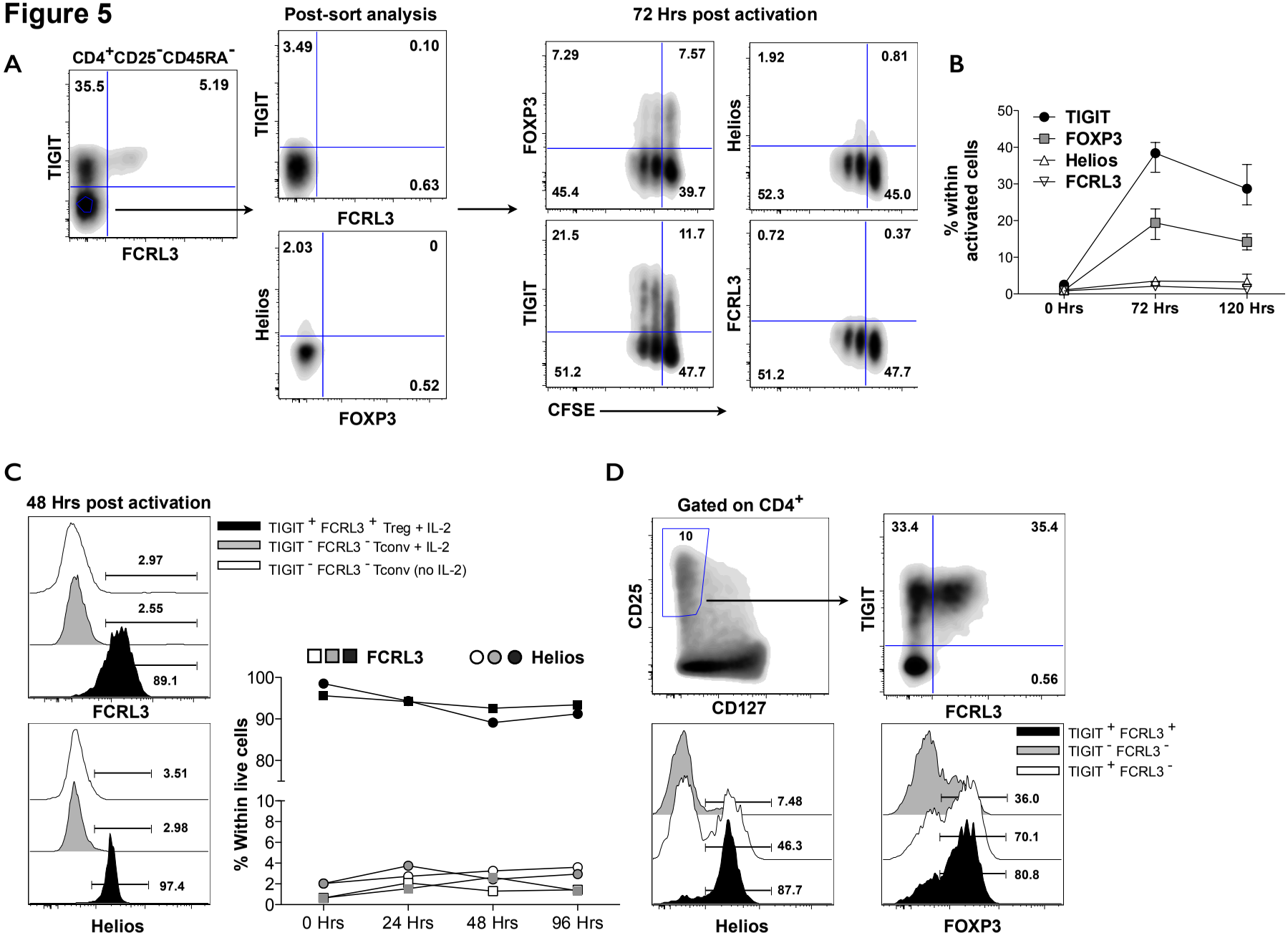

A
CD4+CD25-CD45RA-
35.5
5.19
TIGIT
FCRL3
Post-sort analysis
3.49
0.10
0.63
2.03
0
0.52
Helios
FOXP3
72 Hrs post activation
7.29
7.57
45.4
39.7
1.92
0.81
52.3
45.0
21.5
11.7
51.2
47.7
0.72
0.37
51.2
47.7
CFSE
B
% within activated cells
TIGIT
FOXP3
Helios
FCRL3
0 Hrs
72 Hrs
120 Hrs
C
48 Hrs post activation
TIGIT + FCRL3 + Treg + IL-2
TIGIT - FCRL3 - Tconv + IL-2
TIGIT - FCRL3 - Tconv (no IL-2)
2.97
2.55
89.1
3.51
2.98
97.4
FCRL3
Helios
% Within live cells
0 Hrs
24 Hrs
48 Hrs
96 Hrs
D
Gated on CD4+
10
CD25
CD127
33.4
35.4
0.56
TIGIT
FCRL3
7.48
46.3
87.7
36.0
70.1
80.8
TIGIT + FCRL3 +
TIGIT - FCRL3 -
TIGIT + FCRL3 -
Helios
FOXP3

Figure 6. An increased frequency of non-suppressive clones is found within the FOXP3^{+}Helios^{-} subset. The suppressive potency of Helios^{+} and Helios^{-} Treg cells was compared *ex vivo* **(A-B)** and in primary clones **(C-E)**. For *ex vivo* suppression assays, CD4^{+}CD25^{+}CD127^{Low}TIGIT^{+}FCRL3^{+} and CD4^{+}CD25^{+}CD127^{Low}TIGIT^{-}FCRL3^{-} cells were FACS-sorted and tested for the capacity to suppress the proliferation of CFSE-labeled CD4^{+}CD25^{-} Tresp cells stimulated with soluble anti-CD3 and irradiated PBMCs for 4 days. **A)** Representative CFSE dilution histograms showing suppression of Tresp cells. **B)** Combined suppression analysis from 3 different experiments using cells from 3 different healthy individuals. (**C-E)** Primary Treg and Tconv clones were generated by single-cell cloning of FACS-sorted CD25^{High} and CD25^{Neg} cells of 7 healthy individuals. Marker expression was performed immediately after the harvest on day 22-24, and 4-day CFSE-based suppression assays were carried out using allogeneic CD4^{+}CD25^{-} cells as responders at a 1:1 Treg:Tresp ratio in the presence of irradiated PBMCs and anti-CD3 (30 ng/mL). **C)** The expression of CD25, FOXP3 and Helios in representative clones at harvest. **D-E)** Suppressive potency of FOXP3^{+}Helios^{+} (n=196 clones), FOXP3^{+}Helios^{-} (n=103 clones) and FOXP3^{-} (n=59 clones). Suppression was measured relative to the division index of the unsuppressed Tresp-alone control. Statistical analysis was done with the one-way ANOVA followed by Tukey's post-test (p-value: * $\leq$ 0.05; ** $\leq$ 0.01; *** $\leq$ 0.001).

Figure 6

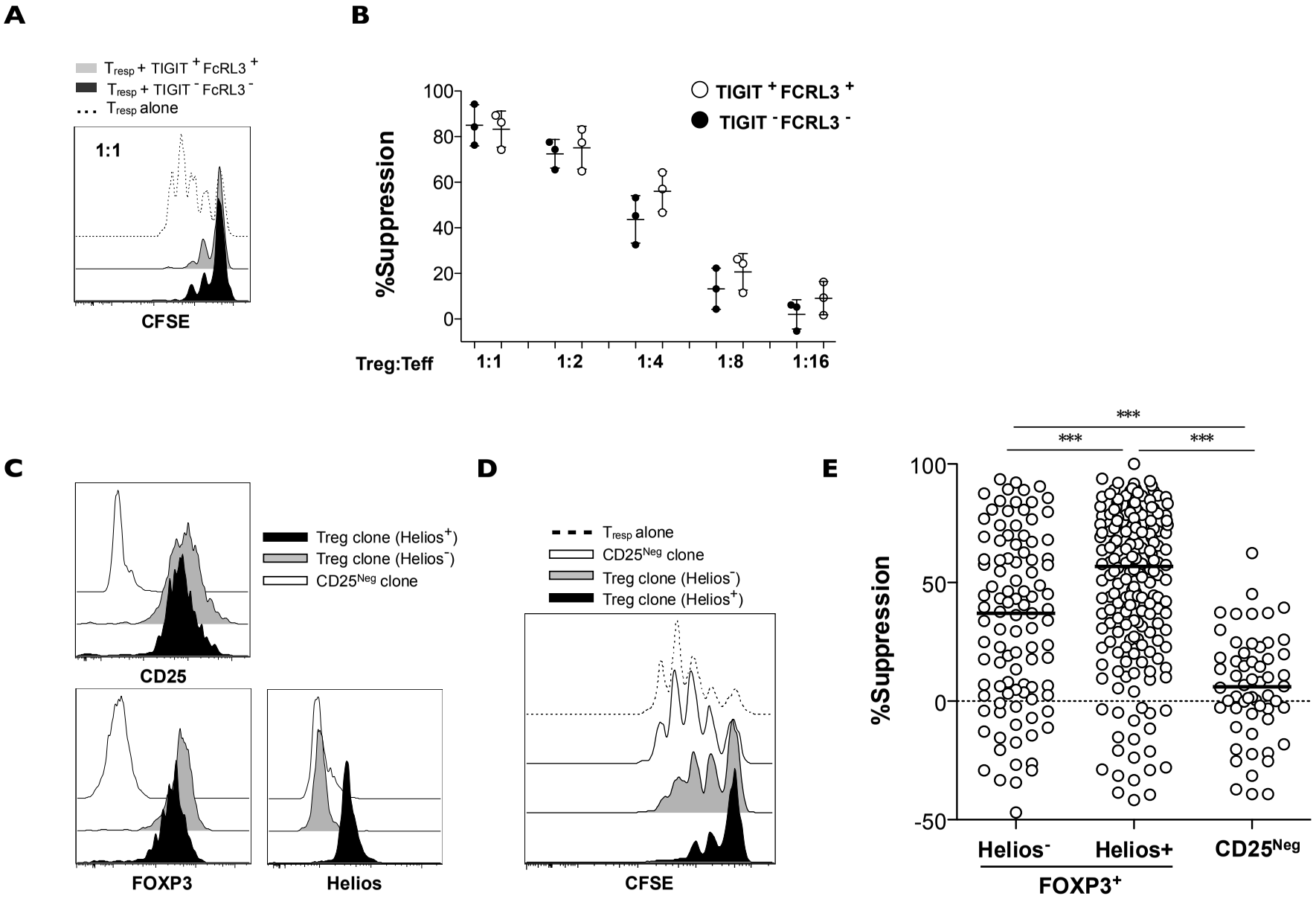

A
Tresp + TIGIT + FcRL3 +
Tresp + TIGIT - FcRL3 -
Tresp alone
1:1
CFSE
B
TIGIT + FCRL3 +
TIGIT - FCRL3 -
%Suppression
100
80
60
40
20
0
Treg:Teff
1:1
1:2
1:4
1:8
1:16
C
Treg clone (Helios+)
Treg clone (Helios-)
CD25Neg clone
CD25
FOXP3
Helios
D
Tresp alone
CD25Neg clone
Treg clone (Helios-)
Treg clone (Helios+)
CFSE
E
***
***
***
%Suppression
100
50
0
-50
Helios-
Helios+
CD25Neg
FOXP3+

Figure 7. Inflammatory cytokine production in FOXP3^{+} cells is restricted to the Helios^{-} fraction. A) PBMCs were incubated *ex vivo* with PMA (25 ng/ml), ionomycin (1 μg/ml) and GolgiStop for 4hrs, followed by intracellular cytokine staining. Shown are **A)** representative flow cytometry plots, and **B)** the frequency of cytokine-producing cells in the indicated subsets analyzed from 11 different healthy samples. **C)** Cytokine production in healthy FACS-sorted TIGIT^{+}FCRL3^{+} vs. TIGIT^{-}FCRL3^{-} Treg cells (CD4^{+}CD45RA^{-}CD25^{+}CD127Low). Statistical analysis was done with the one-way ANOVA followed by Tukey's post-test (A), or with the student *t*-test (B).

Figure 7

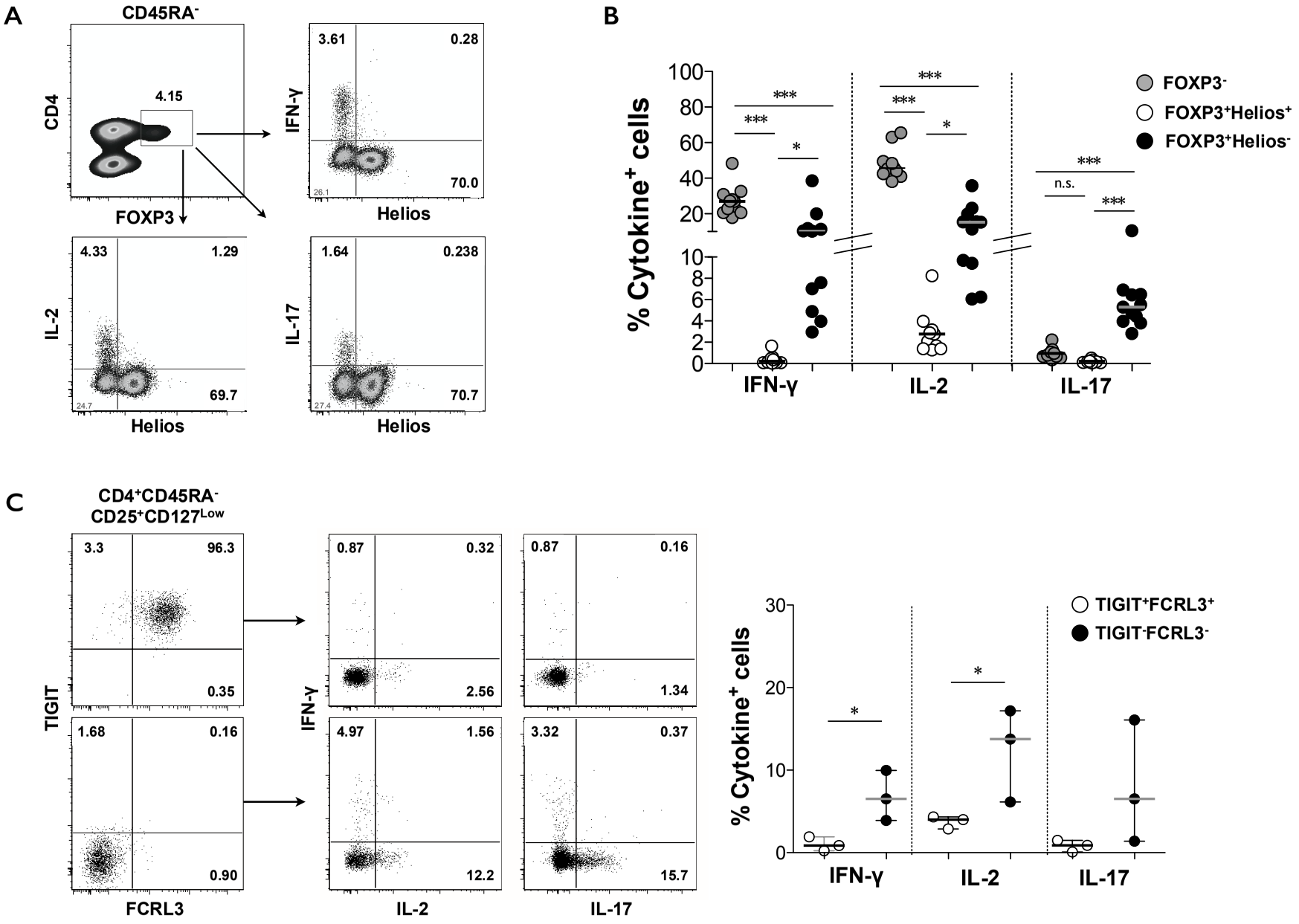

A
CD45RA-
CD4
4.15
FOXP3
IFN-γ
3.61
0.28
70.0
Helios
IL-2
4.33
1.29
69.7
Helios
IL-17
1.64
0.238
70.7
Helios
B
% Cytokine+ cells
FOXP3-
FOXP3+Helios+
FOXP3+Helios-
IFN-γ
IL-2
IL-17
n.s.
C
CD4+CD45RA-
CD25+CD127Low
TIGIT
3.3
96.3
0.35
FCRL3
1.68
0.16
0.90
IFN-γ
0.87
0.32
2.56
IL-2
4.97
1.56
12.2
IL-17
0.87
0.16
1.34
3.32
0.37
15.7
% Cytokine+ cells
TIGIT+FCRL3+
TIGIT-FCRL3-
IFN-γ
IL-2
IL-17

Figure S1. FPSP and FPSN clones have equally demethylated TSDR regions. Genomic DNA was extracted from female-derived primary FPSP, FPSN and FNSN clones and analyzed for TSDR methylation. Shown is the mean + SEM of % methylation of 10 cytosine guanine dinucleotides (CpGs) of the human Treg specific demethylation region (TSDR). n= 2-4 clones per group. Statistical analysis was done with the one-way ANOVA followed by Tukey's post-test.

Figure S1

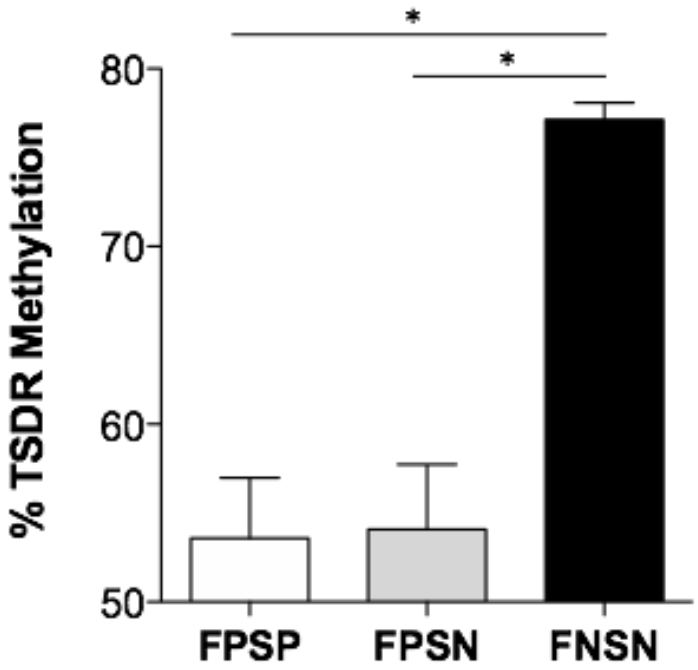


Figure S2. Analysis of the expression of Treg-associated molecules on TIGIT^{+}FCRL3^{+} vs. TIGIT^{-}FCRL3^{-} Treg cells. PBMCs from healthy subjects were analyzed *ex vivo* by flow cytometry. Shown are representative FACS plots as well as the combined analysis of marker expression on Treg subsets of 11 healthy donors. Statistical analysis was done with the one-way ANOVA followed by Tukey's post-test

Figure S2

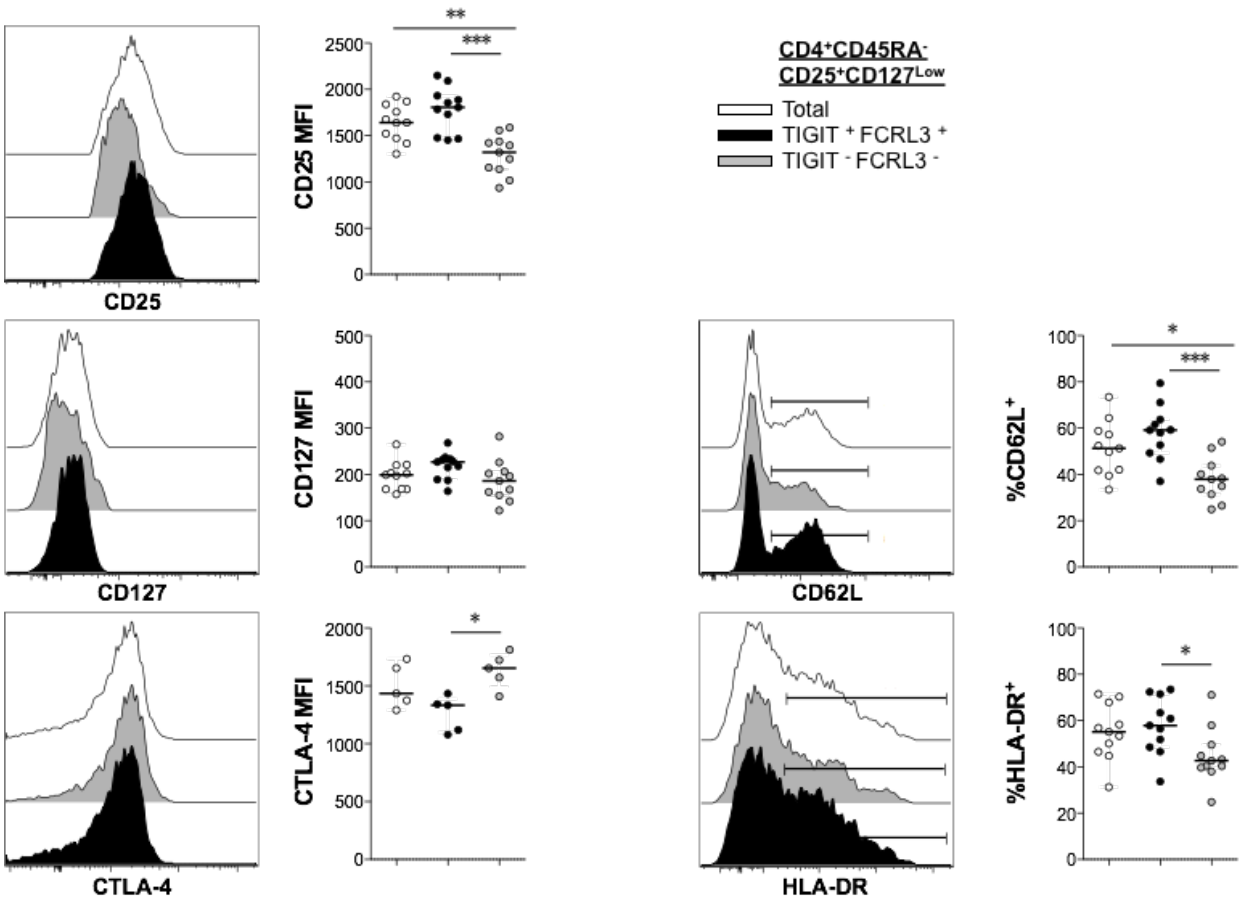
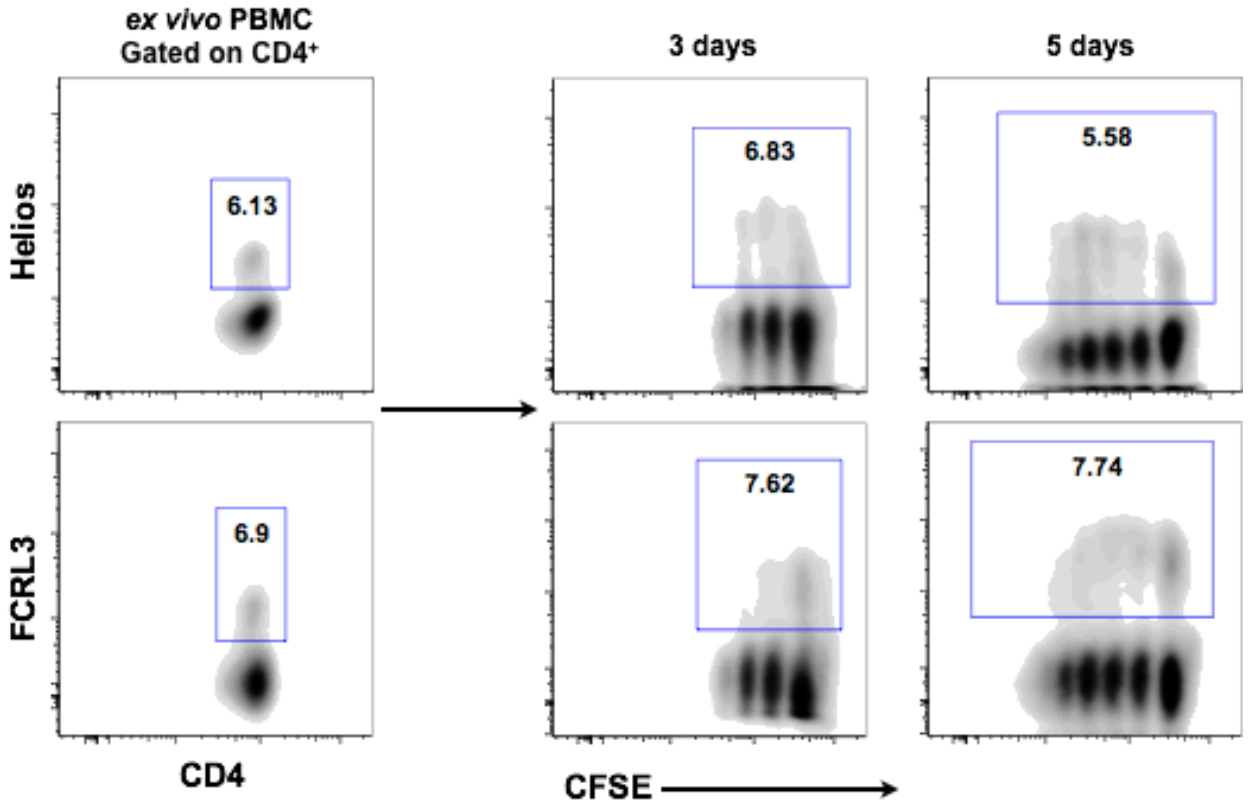

CD4+CD45RA-
CD25+CD127Low
Total
TIGIT + FCRL3 +
TIGIT - FCRL3 -
CD25
CD25 MFI
CD127
CD127 MFI
CTLA-4
CTLA-4 MFI
CD62L
%CD62L+
HLA-DR
%HLA-DR+

Figure S3. Helios and FCRL3 are not induced on Tconv upon *in vitro* activation of PBMC. Whole PBMC from 3 healthy individuals were labeled with CFSE and stimulated with anti-CD3/anti-CD28-coated beads at a ratio of 2 beads: 1 cell for 5 days. Shown is the expression of Helios and FCRL3 on $CD4^+$ cells at day 0, 3 and 5.

Figure S3


ex vivo PBMC
Gated on CD4+
3 days
5 days
Helios
6.13
6.83
5.58
FCRL3
6.9
7.62
7.74
CD4
CFSE

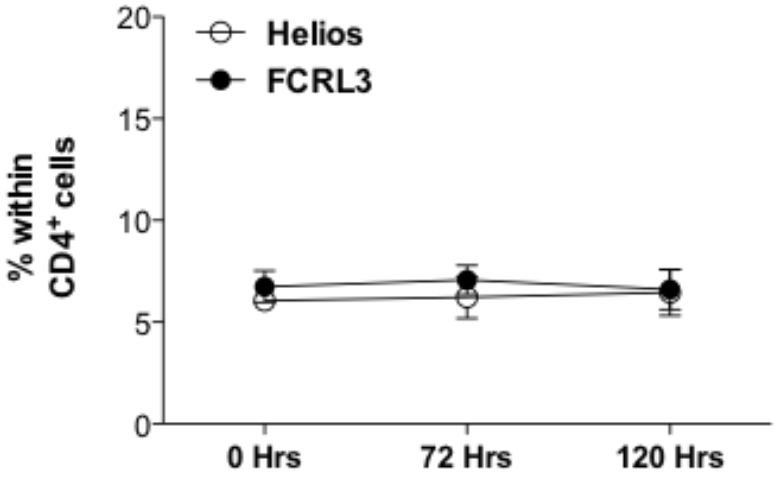
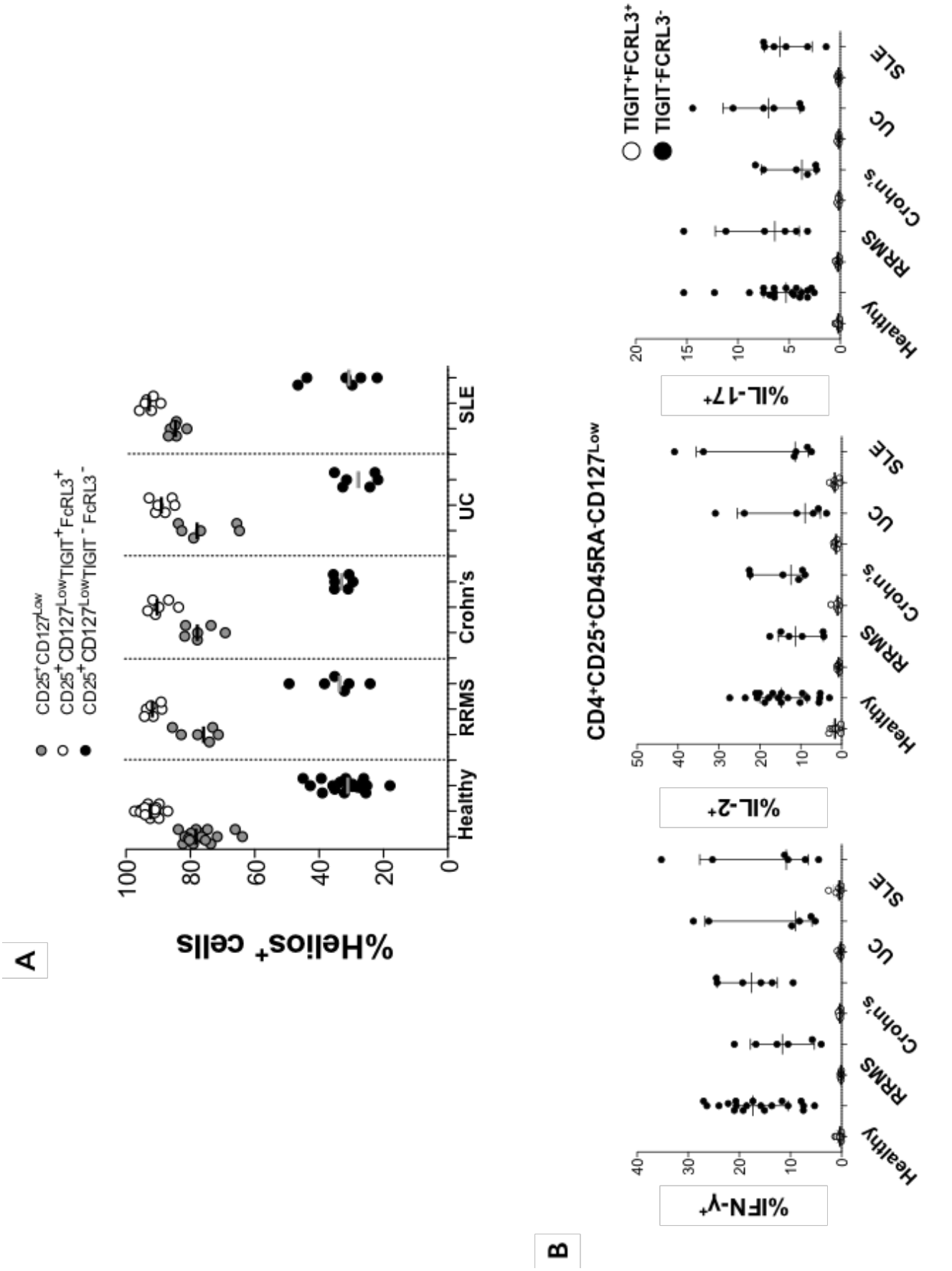

Helios
FCRL3
% within CD4+ cells
20
15
10
5
0
0 Hrs
72 Hrs
120 Hrs

Figure S4. Phenotype and cytokine production in TIGIT^{+}FCRL3^{+} vs. TIGIT^{-}FCRL3^{-} Treg cells of healthy and autoimmune donors. PBMCs from untreated patients with relapsing-remitting multiple sclerosis (n=6), Crohn's disease (n=6), ulcerative colitis (n=6), and treated systemic lupus erythematosus (n=6; SLEDAI-2k >6) and their age and sex-matched healthy controls (n=19) were analyzed by flow cytometry directly *ex vivo* **(A)** or after incubation with PMA (25 ng/ml), ionomycin (1 μg/ml) and GolgiStop for 4hrs **(B)**. Shown is **A)** the percentage of Helios-expressing cells in TIGIT/FCRL3-identified Treg subsets, and **B)** the frequency of cytokine-producing cells in the indicated subsets.

Figure S4


A
%Helios+ cells
CD25+CD127Low
CD25+CD127Low TIGIT+ FcRL3+
CD25+CD127Low TIGIT- FcRL3-
Healthy
RRMS
Crohn's
UC
SLE
B
CD4+CD25+CD45RA-CD127Low
%IFN-γ+
%IL-2+
%IL-17+
TIGIT+FCRL3+
TIGIT-FCRL3-

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CHAPTER 4

Signalling through gp130 Drives Loss of Suppressive Function in Human FOXP3^{+} Regulatory T Cells

Chapter 4-Signalling through gp130 Drives Loss of Suppressive Function in Human FOXP3^{+} Regulatory T Cells

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4.1. Bridging statement from Chapter 3 to Chapter 4

In the previous chapter, we have highlighted the functional heterogeneity of the human FOXP3$^+$ Treg population and identified novel surface markers that will significantly improve our ability to identify functional Treg cells in health and disease. We have shown that while the majority of FOXP3$^+$ cells are potent suppressors, a substantial proportion of human FOXP3$^+$ cells in healthy individuals fail to suppress the proliferation of responder T cells despite expressing a Treg-like phenotype. Using gene expression analysis and epigenetic profiling, we have further demonstrated that these non-suppressive FOXP3$^+$ cells highly resemble their functional counterparts indicating that they likely originate from previously functional Treg cells. Given the critical role played by Treg cells in the maintenance of self-tolerance, and the potential implication of Treg dysfunction in the pathogenesis and progression of autoimmune disorders, we sought in the following chapter to identify factors that drive loss of Treg function in humans. Identification of an 'Achilles' heel' of human Treg cells could lead to the discovery of novel and specific therapeutic targets that allow the modulation of Treg function in various disease settings.

4.2. Abstract:

The $CD4^+FOXP3^+$ regulatory T cell (Treg) subset is an indispensable mediator of immune tolerance to self and innocuous antigens. While high and stable expression of the transcription factor FOXP3 is considered a hallmark feature of Treg cells, our previous studies of human Treg cells have demonstrated that the $FOXP3^+$ Treg subset is functionally heterogeneous in that approximately one third of $FOXP3^+$ cells in healthy individuals have a diminished capacity to suppress the proliferation of responder cells. Notably, these non-suppressive Treg cells are indistinguishable from functionally suppressive Treg cells using the conventional markers of human Treg cells. In this study we investigate potential factors that underlie loss of suppressive function in human Treg cells. We show that high expression of the IL-6 family cytokine receptor subunit gp130 identifies Treg cells with reduced diminished suppressive capacity *ex vivo* and in primary $FOXP3^+$ clones. We demonstrate that 2 gp130-signalling cytokines, IL-6 and IL-27, impair the suppressive capacity of human Treg cells. Finally, we show that SC144, a small molecule inhibitor of gp130 signalling, significantly enhances the suppressive function of human Treg cells. These results highlight the role of gp130 in regulating the function of human Treg cells and propose that modulation of gp130 function can serve as a potential avenue for the therapeutic manipulation of human Treg function.

4.3. Introduction:

$CD4^+FOXP3^+$ regulatory T cells (Treg) play an essential role in the maintenance of tolerance to self and harmless antigens. Congenital or acquired deficiency in Treg cells results in severe autoimmunity in several animal models as well as in humans, and adoptive transfer of Treg cells controls autoimmunity in several animal models (1, 2). Several studies have examined potential defects in the Treg population as underlying, or contributory, factors in human organ-specific autoimmunity (reviewed in (1)). While several groups reported numerical and functional defects in the Treg compartment in several autoimmune diseases such as multiple sclerosis, type 1 diabetes, rheumatoid arthritis and systemic lupus erythematosus, others have observed normal Treg frequency and function in these diseases (reviewed in (1)). In addition to potential disease heterogeneity and methodological variations that may have contributed to the variable findings in these studies, a significantly more influential limitation is the lack of reliable markers of human Treg cells (1). While a number of surface markers allow the isolation of highly enriched Treg cells in resting conditions, most of the conventional human Treg markers are modulated on effector T cells (Teff) upon TCR-mediated activation, thus leading to the inclusion of activated Teff contaminants in populations isolated using conventional Treg markers (1). This results in considerable variability in the purity of Treg cells isolated from healthy individuals and autoimmune patients with high levels of inflammation, thus influencing the interpretation of results. Furthermore, human FOXP3 is transiently upregulated in Teff cells upon TCR-mediated activation without endowing them with suppressive function (3, 4), thus further blurring the distinction between Treg and activated Teff cells in humans.

Using a sensitive single-cell cloning strategy that allows the discrimination between activated Teff contaminants and *bona fide* FOXP3-expressing Treg cells, we have recently shown that the human $FOXP3^+$ Treg population is functionally heterogeneous, containing a

sizeable proportion of cells with an impaired capacity to suppress the proliferation of Teff cells despite exhibiting the hallmark surface phenotype of functional Treg cells (5, 6). We have further demonstrated that this FOXP3-positive suppression-negative (FPSN) subpopulation, resembles its FOXP3-positive suppression-positive (FPSP) counterpart in the demethylation status of the Treg-specific demethylated region (TSDR) of the *FOXP3* locus, as well as in the global gene expression profile (6). These findings show that these non-suppressive $FOXP3^+$ cells likely originate from previously functional Treg cells. There are currently no markers that identify these dysfunctional $FOXP3^+$ cells and their prevalence and potential role in autoimmunity remains unexplored. This study aims to investigate factors that drive loss of suppressive function in committed human Treg cells, and to identify surface markers associated with human Treg dysfunction.

Several inflammatory mediators have been shown to modulate the function of Treg cells, including inflammatory cytokines such IL-1β, TNF-α and IL-6, as well as several TLR ligands and microbial metabolites (Reviewed in (7)). The effects of IL-6 on Treg function have been particularly widely studied. IL-6 plays a critical role in regulating the balance between T helper 17 (Th17) cells and Treg cells, by favouring the differentiation of Th17 cells over Treg cells in the presence of TGF-β (8, 9). Exogenous IL-6 has also been shown to inhibit Treg-mediated suppression in mice (10-12) and humans (13). Clinically, elevated concentrations of IL-6 have been detected in the sera and urine of SLE patients, and were found to correlate with disease severity (14). IL-6 is also highly elevated in the synovia of rheumatoid arthritis (RA) patients (15) and in the intestinal mucosa of inflammatory bowel disease (IBD) patients (16). Blockade of IL-6 using tocilizumab, an approved treatment for RA and other autoimmune disorders, has been

shown to correlate with increased frequency of Treg cells, although Treg function has not been assessed in these settings (17-20).

IL-6 signals through a receptor complex comprised of IL-6R (CD126) and gp130 (CD130) (21). IL-6R is expressed on some T cells, B cells, neutrophils and monocytes. However, cell-surface expression of IL-6R is not necessary for the cell to respond to IL-6, as it has been shown that soluble IL-6R (sIL-6R) is produced in humans by alternative splicing (22, 23), can bind IL-6, and the IL-6/sIL-6R complex can then interact with gp130 on the cell surface in a process termed trans-signalling (24, 25).

Glycoprotein 130 (gp130) is a surface receptor that constitutes parts of the receptor complex for several cytokines, including IL-6, IL-27, IL-11, leukemia inhibitory factor (LIF), Oncostatin M (OSM), Ciliary Neurotrophic Factor (CNTF), cardiotrophin 1 (CT-1), and cardiotrophin-like cytokine (CLC)(26). Gp130 is ubiquitously expressed on hematopoietic and non-hematopoietic cells. Deletion of mice gp130 is embryonically lethal due to defects in cardiac development as well as impaired hematopoiesis (27). The role of gp130 in immune development is best illustrated in studies where postnatal conditional abrogation of gp130 in hematopoietic cells results in impaired lymphocyte development (28).

IL-27 is a cytokine of the IL-12 family. It is a heterodimer composed of the IL-27p28 and the Epstein-Barr virus induced 3 (Ebi3) subunits, and is produced by activated antigen-presenting cells such as dendritic cells and macrophages (29). IL-27 signals through the IL-27 receptor complex comprised the IL-27RA (WSX-1) and gp130 (29). Both pro- and anti-inflammatory roles have been described for IL-27. As a pro-inflammatory cytokine, IL-27 has been shown to induce the production of IFN-γ and favour the differentiation of Th1 cells in a STAT1-dependent manner (30-32). Furthermore, IL-27 impairs TGFβ-induced generation of

Treg cells (33). On the other hand, IL-27 has been shown to increase the production of IL-10 by effector $CD4^+$ and Tr1 cells (34-36), and to attenuate Th17-mediated inflammation in the EAE model (37, 38). The effects of IL-27 on natural Treg function and particularly on human Treg cells is largely unexplored.

In this study we aimed to further characterize the functional heterogeneity in the human $FOXP3^+$ Treg population by investigating factors that drive loss of suppressive function in human Treg cells. We found that expression of gp130 identifies Treg cells with reduced suppressive function. Furthermore, we show that IL-6 and IL-27, both signalling through gp130, impair the suppressive capacity of Treg cells. Finally, inhibition of gp130 signalling using a small molecule inhibitor (SC144) significantly improves Treg suppressive function. These results highlight the important role of gp130-signalling in modulating the suppressive function of human Treg cells and present a novel avenue for the therapeutic modulation of Treg function.

4.4. Materials and Methods:

Donors and cell isolation.

Peripheral blood mononuclear cells (PBMC) were purified from buffy coats of healthy donors (Sanguine Biosciences) using Ficoll-Paque PLUS density gradient (GE Healthcare), and were cryopreserved.

Reagents:

PBMCs were thawed and stained with viability dye (eFluor 780; eBioscience). Antibodies against CD4 (FITC or V500), CD25 (APC), CD45RA (Alexa Fluor 700)(BD Biosciences), CD127 (PE-eFluor 610), FOXP3 (PE), TIGIT (PerCP-eFluor710) (eBioscience), Helios (Pacific Blue; Biolegend). Purified anti-FCRL3 antibody was provided by Dr. Satoshi Nagata (39), and was detected with F(ab')2 anti-mouse IgG (PE-Cy7; eBioscience). Flow cytometry analysis was performed on an LSR Fortessa analyzer, and sorting throughout this study was performed on a FACSAria IIu cell sorter (both from BD Biosciences). SC144 (Sigma Aldrich), recombinant human IL-6, IL-27, CLC, IL-11 (R&D systems) and LIF (Peprotech) were added to suppression assays where indicated at the time of activation.

Generation of primary $CD4^+$ clones:

Primary $CD4^+$ clones were generated from healthy donors by single-cell sorting of $CD25^{High}$ and $CD25^{Neg}$ cells as described previously (5, 6). The clones were stimulated with soluble anti-CD3 (30 ng/mL; eBioscience), recombinant human IL-2 (200 U/mL) and irradiated human PBMCs as feeders, and propagated in Xvivo-15 medium (Lonza) supplemented with 5% FBS (Sigma-Aldrich). Fresh medium and IL-2 was added on day 5 and every two days thereafter, and clones were passaged as required. Clones were re-stimulated on day 11-12 and

further expanded until harvested on day 22-24. Phenotypic analysis and functional assessment of suppressive function were performed in parallel.

Suppression assays:

Suppression assays were performed as previously described (5). Briefly, responding allogeneic $CD4^+CD25^-$ cells (Teff) were FACS-sorted, stained with the CFSE proliferation dye (5 μM; Sigma-Aldrich) and plated at 8000 cells/well in U-bottom 96-well plates (Sarstedt) with irradiated PBMCs as feeders (30,000 cells/well). Treg cells were added to the culture at a ratio of 1:1 and the assays were stimulated with soluble anti-CD3 (30 ng/mL; eBioscience) for 4 days. Where indicated, suppression assays were performed in the presence or absence of irradiated feeders and stimulated with anti-CD3/anti-CD28-coated beads at 1:2 beads/cells. Suppression was estimated based on the division index of Teff cells cultured in the absence of Treg cells.

Where indicated, recombinant human IL-6, IL-11, IL-27, CLC (all at 1-100ng/mL) or LIF (100-1000 ng/mL) were added to the suppression assay at the time of activation. Treatment with SC144 was performed at the time of activation by supplementing the co-culture with a final concentration of 0.001-10μmol/L of SC144.

Statistical analysis:

Statistical analysis was performed using the GraphPad Prism 6.0 software. One-way ANOVA, followed by multiple-comparison testing, and the student *t*-test were used where indicated. A p-value of <0.05 was considered significant.

4.5. Results:

Loss of suppression in Treg clones is driven by APC-derived factors

Although stable FOXP3 expression is considered a specific feature of Treg cells, it is now established that human $CD4^+$ Teff cells can express FOXP3 upon activation, making them indistinguishable from Treg cells in inflammatory contexts (3, 4). This activation-induced FOXP3 expression is transient and usually subsides within a few days of activation, providing the basis of our single cell cloning approach whereby we expand clones generated from single $CD4^+CD25^{High}$ and $CD4^+CD25^{Neg}$ cells and analyze their phenotypic and functional profile at the end of the activation cycle, thus allowing activation-induced FOXP3 expression to subside resulting in a normalized state of immune quiescence. At the time of harvest, only clones expressing stable levels of FOXP3 (i.e. Treg clones) maintain their high FOXP3 levels, thus reliably eliminating contaminating Teff cells. Using this approach, we have previously shown that Treg clones in healthy individuals harbour a population that is functionally impaired despite exhibiting the prototypic phenotype of Treg cells including high and stable expression of FOXP3 (Figure 1A-B) (5, 6). These observations demonstrated the heterogeneous nature of the human Treg population, and suggested the existence of different subpopulations within the $FOXP3^+$ subset with variable suppressive potencies.

All of our previous assessments of the suppressive capacity of $FOXP3^+$ clones were performed in the conventional way where irradiated PBMCs are used as antigen-presenting cells (APC) to provide co-stimulation in the suppression assay (6, 40). APCs are a major source of several inflammatory cytokines, some of which have been shown to alter the function of mouse and human Treg cells (7). Therefore, we asked whether the loss of suppressive function observed in FPSN clones is driven by inflammatory factors provided by the APCs. To assess this

hypothesis, we examined the suppressive capacity of FOXP3$^+$ Treg clones in the presence or absence of irradiated APCs. Interestingly, the lack of APCs in the co-culture almost completely rescued the FPSN clones and allowed them to exhibit a high capacity of suppression (Figure 1C). The absence of APCs had no significant effect on the suppressive potency of the already suppressive FPSP clones, nor did it affect the lack of suppression in control FOXP3-positive, suppression-negative (FNSN) clones (Figure 1C). To determine the nature of the APC-derived factors driving the observed Treg dysfunction, we examined the suppressive capacity of Treg clones in the presence of purified supernatant of activated APCs. APC-derived supernatant was sufficient to cause a significant reduction in the suppressive potency of FNSN clones whose suppressive capacity was restored in the absence of APCs (Figure 1C). These data demonstrate that the lack of suppressive function associated with FPSN clones is largely caused by soluble APC-derived factors, and can be reversed.

Elevated gp130 expression in non-suppressive FOXP3$^+$ clones

We have previously performed a whole-genome expression analysis on FPSP, FPSN and FNSN clones in order to identify gene products that distinguish human Treg from activated Teff cells and further identify the different functional subpopulations of Treg cells (6). Examining the expression levels of inflammatory cytokine receptors on the three populations, we observed an increased level of transcription of the IL-6 receptor subunit (IL-6R) on both FOXP3$^+$ Treg subpopulations relative to FOXP3$^-$ controls in resting and activated conditions (Figure 2A). Although we did not observe a significant difference in IL-6R expression between FPSP and FPSN clones, we were prompted to further investigate the IL-6 pathway in the Treg clones due to the well-established role of IL-6 as a major antagonist of Treg function (10-13, 41). To that end, we generated FOXP3$^+$ and FOXP3$^-$ clones from CD4$^+$CD25^{High} and CD4$^+$CD25$^-$ cells,

respectively, and identified FPSP, FPSN and FNSN clones by examining their suppressive capacity in the presence of irradiated APCs. In parallel, we examined the expression of IL-6R on the three subsets by flow cytometry prior to activation. IL-6R was expressed by most examined clones and we found no significant differences among the three subsets in the expression levels of the IL-6R protein (Figure 2B). Given the possibility of IL-6 trans-signalling in cells that do not express IL-6R (24, 25), we examined the expression levels of the other subunit of the IL-6 receptor complex, gp130, whose surface expression is required for IL-6 signalling. Interestingly, despite its mRNA being equally expressed by the three populations (Figure 2C), gp130 protein was significantly elevated on the surface of FPSN clones compared to FPSP and FNSN clones (Figure 2D), suggesting a potential role for this cytokine receptor in the functional impairment of the FPSN subset.

Preferential expression of gp130 on naïve CD4$^+$ cells

We next characterized the *ex vivo* expression of gp130 on total CD4$^+$ cells and in Treg cells specifically in healthy individuals. We found that gp130 is preferentially expressed on naïve (CD45RA$^+$) CD4$^+$ T cells, while the memory (CD45RA$^-$) compartment contains a minor population of gp130^{High} and a major population that is gp130^{Low} (Figure 3A). A similar pattern of gp130 expression was observed in naïve vs.memory Treg (CD4$^+$CD25$^+$CD127^{Low}) cells (Figure 3B). This suggests a potential role for gp130 signalling in the differentiation of naive CD4$^+$ T cells. We recently reported that Helios expression is associated with enhanced suppressive capacity and maximal repression of inflammatory cytokine production by human Treg cells (6). We further identified two surface proteins, TIGIT and FCRL3, as a reliable marker combination that distinguishes Helios$^+$ from Helios$^-$ Treg cells. Here we sought to investigate whether gp130 can be used in conjunction with these novel surface markers to allow the differential

identification and isolation of suppressive and non-suppressive Treg cells. Since both TIGIT and FCRL3 are preferentially expressed on memory Treg cells (6) while gp130 is highly expressed on naïve Treg cells, we analyzed the expression of these markers on naïve and memory Treg subpopulations further distinguished based on gp130 expression (Figure 3B). In accordance with previous reports, we found FOXP3 expression to be lower in naïve relative to memory Treg cells (42) (Figure 3B). Within the memory Treg subset, we did not observe a significant difference in FOXP3, Helios or TIGIT protein expression levels between $gp130^{High}$ and $gp130^{Low}$ (Figure 3B). However, the frequency of $FCRL3^{+}$ cells is significantly reduced within the memory $gp130^{High}$ Treg cells (Figure 3B), suggesting that FCRL3 could be used in conjunction with gp130 to identify maximally suppressive Treg cells. It remains to be assessed whether the expression of TIGIT, FCRL3 or Helios could alter the susceptibility of Treg cells to inflammatory mediators and renders them differentially resistant to the modulatory effects of gp130 signalling.

Ex vivo gp130 expression identifies Treg cells with reduced suppressive capacity

We next sought to examine the correlation between gp130 expression and suppressive function of Treg cells directly *ex vivo*. To that end, we FACS-sorted the following 3 subpopulations of $CD4^{+}CD25^{High}CD127^{Low}$ Treg cells: 1) $CD45RA^{-}gp130^{Low}$, 2) $CD45RA^{-}gp130^{High}$ and 3) $CD45RA^{+}gp130^{High}$. Given the high expression of gp130 on all naïve $CD4^{+}$ T cells, the fourth subpopulation $CD45RA^{+}gp130^{Low}$ is almost nonexistent and, therefore, we did not include it in the analysis (Figure 4A). Within the memory Treg population, we found that the suppressive potency of memory $gp130^{High}$ cells was significantly lower than that of $gp130^{Low}$ cells, and was comparable to that of naïve Treg cells which have previously been reported to have a greatly reduced suppressive capacity in comparison with memory Treg cells (Figure 4B)(42). These results confirm our findings in Treg clones that gp130 identifies Treg cells with

reduced suppressive capacity, and suggest that gp130 likely transmits inflammatory signals that dampen the suppressive capability of Treg cells.

IL-6 and IL-27 drive loss of suppressive function in Treg cells

Several cytokines utilize gp130 as part of their receptor complexes, activating various downstream signalling pathways and driving different biological processes (43). Here we sought to identify gp130-signalling cytokines that alter the suppressive function of Treg cells. We generated Treg clones and assessed their capacity to suppress the proliferation of Teff cells in the presence of the gp130-signalling cytokines IL-11, LIF or CLC, IL-6 and IL-27. We first analyzed the effects of these cytokines on the proliferative capacity of responder Teff activated in the absence of Treg cells. We observed that IL-6 on its own significantly increases the proliferation of Teff cells (Figure 5A). We next examined the suppressive potency of Treg clones in the presence of exogenous cytokines. While the suppressive potency of Treg clones was not altered upon the addition of exogenous IL-11, LIF or CLC, both IL-6 and IL-27 markedly decreased Treg-mediated suppression (Figure 5B). However, while IL-27 exhibits a significantly higher modulatory effect on $gp130^{High}$ relative to $gp130^{Low}$ clones, IL-6 alters the suppressive function of $gp130^{High}$ and $gp130^{Low}$ clones to a similar extent, suggesting that IL-6 may additionally alter Treg-mediated suppression through the enhancement of Teff proliferation (Figure 5A).

We next assessed the impact of exogenous IL-6 and IL-27 on the suppressive function of Treg cells *ex vivo*. To that end, we isolated $CD4^{+}CD25^{+}CD127^{low}$ Treg cells and measured their capacity to suppress the proliferation of $CD4^{+}CD25^{-}$ Teff cells in the presence of titrated amounts of IL-6 or IL-27. In accordance with our previous observations (Figure 5A), IL-6 treatment caused a significant increase in the proliferation of Teff cell in the absence of Treg

cells (Figure 6A), thus supporting the possibility that IL-6 may inhibit Treg-mediated suppression through its additional effects on Teff cells. Both IL-6 and IL-27 significantly reduced the suppressive function of Treg cells in a dose-dependent manner (Figure 6B-C). These data suggest that while both IL-6 and IL-27 inhibit the suppressive function of human Treg cells, IL-27 likely acts directly on Treg cells while IL-6 could also indirectly impair Treg function through enhancement of Teff cell proliferation.

Inhibition of gp130 signalling enhances the suppressive function of Treg cells

Given the shared requirement for gp130 in the signalling of IL-6 and IL-27, we hypothesized that blockade of gp130 signalling could prevent IL-6 and IL-27 signalling events that antagonize Treg function, and may therefore enhance the suppressive capacity of Treg cell. Xu *et* al. recently described a small molecule inhibitor, SC144, that binds to, phosphorylates, and deglycosylates, gp130 leading to its internalization and degradation (44). We assessed the suppressive function of $CD4^{+}CD25^{+}CD127^{low}$ Treg cells *ex vivo* in the presence titrated doses of SC144. We indeed observed a significant increase in the suppressive potency of Treg cells upon the addition of SC144 (Figure 7). We are currently evaluating the effects of SC144 on naïve vs.memory Treg cells as well as in memory Treg cell with differential gp130 expression. Our observations thus far implicate gp130 signalling in the negative regulation of Treg function, and highlight this specific pathway as a potential target for the therapeutic modulation of human Treg activity.

4.6. Discussion:

Our previous investigations of human FOXP3$^+$ Treg clones revealed a remarkable degree of functional heterogeneity within the FOXP3$^+$ Treg subset whereby approximately one third of FOXP3$^+$ clones have a significantly reduced, and even diminished, capacity to suppress responder T cells. Notably, these non-suppressive Treg cells are indistinguishable from functionally suppressive Treg cells using the conventional markers of human Treg cells. In this study we set out to investigate potential factors that underlie such loss of function, and to identify biomarkers that identify FOXP3$^+$ cells that lack suppressive function. We demonstrated that APC-derived factors are responsible for the loss of Treg function and identified gp130 as a surface receptor that is highly expressed in non-suppressive FOXP3$^+$ clones. We further showed that exogenous IL-6 and IL-27 negatively modulate Treg function, while a small molecule-mediated inhibition of gp130 signalling enhances the suppressive function of Treg cells. These results highlight the role of gp130 in regulating the function of human Treg cells and propose modulation of gp130 function as a potential avenue for the therapeutic modulation of human Treg function.

Previous studies have associated IL-27 with both pro-inflammatory and anti-inflammatory roles in several animal model. The pro-inflammatory effect of IL-27 include its ability to induce the production of T-bet and IFN-γ (30-32). Harker *et al.* recently described a critical role for IL-27 in mediating CD4+ T cell responses against chronic Lymphocytic Choriomeningitis Virus (LCMV) infection (45). Moreover, in a model of helminth-induced inflammatory bowel disease (IBD), *Il27ra* deficiency impaired Th1 responses in the intestine resulting in inefficient worm expulsion and delayed onset of colitis (46). IL-27 has also been shown to inhibit the TGFβ-mediated induction of Treg cells (33), and transgenic mice overexpressing IL-27 succumbed to spontaneous inflammation associated with a severe

diminishment of their Treg pool (47). Furthermore, Cox *et al.* reported that adoptive transfer of *Il27ra*$^{-/-}$ Treg cells into lymphopenic mice resulted in attenuated colitis that was attributed to increased induction of peripheral Treg cells (48). In contrast, a recent study by Do *et al.* reported that *Il27ra*$^{-/-}$ Treg cells are defective in their suppressive capacity and are unable to suppress inflammation in a colitis model (49). The authors further reported that stimulation of Treg cells in the presence of IL-27 substantially improved the suppressive function of Treg cells *in vitro* and *in vivo* (49). The contrasting results reported by Do *et al.* and Cox *et al.*, despite using the same colitis model, could be a result of the different mouse genetic backgrounds used in the two studies, C57BL/6 and BALB/c, respectively (48, 49). Furthermore, it is becoming increasingly appreciated that differences in the composition of commensal microbiota have a significant influence on immune responses and disease outcome even within animals of the same strains (50), and therefore the influence of microbial composition in animal models on these seemingly contrasting findings cannot be ruled out.

It should be noted that the majority of the studies that examined the *in vivo* effects of IL-27 have used *Il27ra*$^{-/-}$ mice. However, IL-27 may not be the only ligand for IL-27RA. Indeed, a recent study by Wang *et al.* has demonstrated that IL-35 also signals through a receptor complex involving the IL-27RA (51). IL-35 is an anti-inflammatory cytokine expressed by a suppressive subset of mouse T cells termed iTr35 (52, 53). Interestingly, IL-35 also shares the Ebi3 with IL-27 (52, 53). Thus, the shared nature of the IL-27RA complicates the interpretation of studies that relied on *Il27ra*$^{-/-}$ mice to examine the role of IL-27 *in vivo*.

Interestingly, Do *et al.* have also examined the effects of IL-27 on the suppressive function of human Treg cells and reported that IL-27 significantly improves their suppressive function (49). While this is in contrast to our findings showing a negative impact of IL-27 on

human Treg function, there are important differences in the assessment of Treg function between the two studies. While we added IL-27 at the time of activation of the suppression co-culture, Do *et al.* activated Treg cells separately in the presence or absence of IL-27 for three days prior to co-culturing with Teff cells. Furthermore, Do *et al.* did not report details on the levels of activation achieved in their suppression assays to allow a clear estimation of the quality and magnitude of the modulation of Treg activity achieved by IL-27 (49). Importantly, we observed no effect of IL-27 on Teff cells cultured alone as a control, suggesting that our observations of reduced Treg function in the presence of IL-27 are due to its direct action on Treg cells. Further studies are underway to confirm the inhibitory effects of IL-27 on human Treg cells by examining the expression of the IL-27Ra subunit, and assessing the impact of its blockade, on human Treg cells.

The mechanism through which IL-27 acts on Treg cells is unclear. However, some insight can be gained from previous studies where IL-6 was shown to inhibit Treg function through the activation of the STAT3 pathway and overcoming the FOXP3-mediated inhibition of RORγt (54). IL-27 signalling through gp130 has been also been shown to activate STAT3 (55, 56) and, therefore, it is possible that the mechanism of inhibition of Treg function is shared between IL-6 and IL-27. Additionally, IL-27 activates STAT1 leading to the inhibition of IL-2 production by T cells through the activation of the suppressor of cytokine signalling 3 (SOCS3) (47). Furthermore, IL-27 was found to interfere with T cell responsiveness to IL-2 through a mechanism that does not involve altering the expression of CD25. Given the vital role played by IL-2 in the survival and function of Treg cells, this latter effect of IL-27 likely plays a significant role in modulating Treg function. Further investigation of the signalling events in human Treg

cells in response to IL-27 is required to elucidate specific mechanisms through which it inhibits Treg-mediated suppression.

An interesting observation in our study is the restoration of suppressive capacity in non-suppressive FOXP3$^+$ clones upon the elimination of APCs from the co-culture. This indicates that modulation of Treg function is largely mediated by local inflammatory factors, and is reversible, thus highlighting the resilient yet adaptable nature of human Treg cells in response to cues in the microenvironment. Our data suggest that a significant proportion of FOXP3$^+$ Treg cells in healthy individuals exhibit this functional adaptability. It is likely that other pathways than the gp130 pathway described here contribute to the modulation human Treg cells. The significance of this heterogeneity in the immune system is not clear. However, one potential advantage of having subpopulations of Treg cells with differential responsiveness to inflammatory mediators would be the facilitation of a rapid down-modulation of Treg suppressive function in response to an infection. This would allow a partial relief of Treg activity and an enhanced initiation of an effective protective response. It would, therefore, be critical that modulated Treg cells can regain their suppressive capacity upon pathogen clearance. Whether deregulated and chronic production of inflammatory mediators can cause permanent loss of Treg function and contribute to autoimmunity remains to be investigated. There is, however, mounting evidence in mouse models indicating that Treg cells can, under inflammatory conditions such as lymphopenia or chronic infections, lose their Foxp3 expression and suppressive and differentiate into effector T-like cells with inflammatory potential (57-59). It is not clear if these former Foxp3$^+$ can regain Foxp3 expression and suppressive capacity in homeostatic conditions *in vivo*. Unlike the normal heterogeneity observed in healthy individuals where FOXP3 expression is maintained in non-suppressive Treg cells allowing them to restore their suppressive capacity,

potential Foxp3 loss in a highly inflammatory environment may cause long-lasting quantitative and qualitative defects in the Treg population.

Our study also highlights the importance of considering the influence of the inflammatory milieu on Treg cells when assessing Treg function in human autoimmune diseases. Although intrinsic defects in Treg function may be a possible underlying cause of organ-specific autoimmunity, it is highly likely that Treg dysfunction could be a consequence of functional modulation by extrinsic factors that are abundant *in situ* in inflammatory conditions. Monitoring Treg cells with an increased susceptibility to functional modulation, $gp130^{high}$ for instance, in autoimmune patients could provide valuable insight into the functional status of the Treg population in these patients. Moreover, regardless of whether Treg dysfunction is causative or secondary in organ-specific autoimmunity, identification of pathways that interfere with Treg function is highly needed in order to design strategies through which Treg function can be specifically enhanced in patients to allow a better control of autoimmune responses.

4.7. Acknowledgments:

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4.8. Figures:

Figure 1. Loss of suppressive function in FOXP3$^+$ Treg cells is driven by APC-derived factors. Primary FOXP3$^+$ clones were generated from healthy donors as described above, and were analyzed for their ability to suppress allogeneic CD4$^+$CD25$^-$ Teff cells in a 1:1 Treg:Teff suppression assay in the presence or absence of irradiated PBMCs (APCs). **A)** Representative FACS plots showing CD25 and FOXP3 expression in representative FPSP, FPSN and FNSN clones and their suppressive function in the presence or absence of APCs. **B)** FOXP3 expression in FPSP, FPSN and FNSN clones before, or 48 hours after re-stimulation with anti-CD3 and irradiated APCs. **C)** Suppressive potency of FOXP3$^+$ and FOXP3$^-$ clones in the presence or absence of APCs. Shown are the suppression values for 61 FPSP, 31 FPSN and 9 FNSN clones from one representative experiment of 3 different experiments where clones were generated from 3 different healthy individuals.

Figure 1

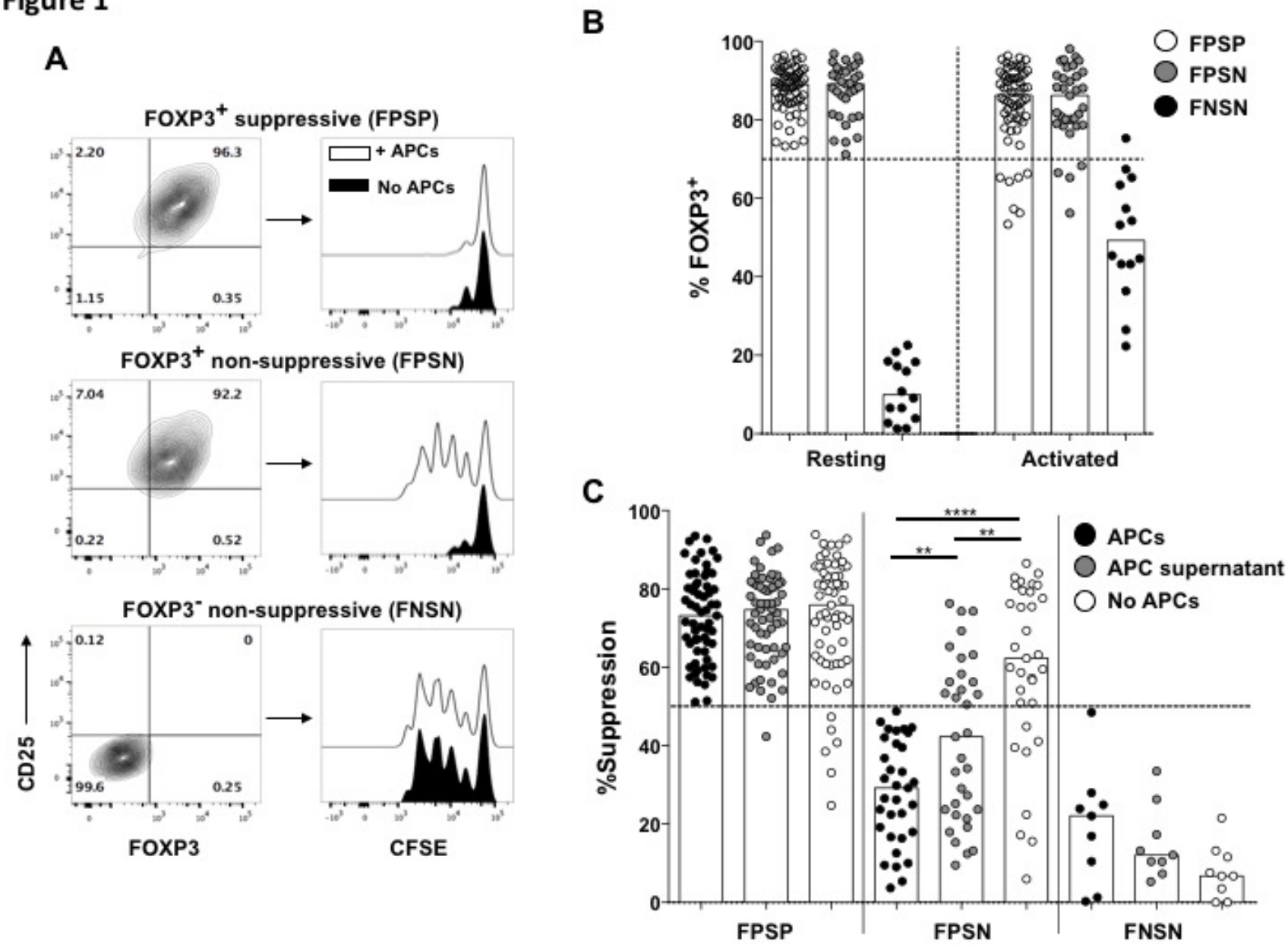

A
FOXP3+ suppressive (FPSP)
2.20
96.3
1.15
0.35
+ APCs
No APCs
FOXP3+ non-suppressive (FPSN)
7.04
92.2
0.22
0.52
FOXP3- non-suppressive (FNSN)
0.12
0
99.6
0.25
CD25
FOXP3
CFSE
B
% FOXP3+
100
80
60
40
20
0
Resting
Activated
FPSP
FPSN
FNSN
C
%Suppression
100
80
60
40
20
0
****
**
**
APCs
APC supernatant
No APCs
FPSP
FPSN
FNSN

Figure 2. Elevated gp130 expression in non-suppressive FOXP3$^+$ clones. Primary FOXP3$^+$ clones were generated from healthy donors as described above. FPSP, FPSN and FNSN clones were identified based on their ability to suppress allogeneic CD4$^+$CD25$^-$ Teff cells in a 1:1 Treg:Teff suppression assay in the presence of irradiated APCs. Shown are the relative mRNA expression levels of the IL-6 receptor chains IL-6R **(A)** and gp130 (IL-6ST) **(C)** in FPSP, FPSN and FNSN clones as measured at the resting state at harvest or 24 hours after activation (6). Panels **B** and **D** show the protein expression levels of IL-6R **(B)** and gp130 **(D)** on FPSP, FPSN and FNSN clones at the time of harvest**.** Shown are the results from one representative experiment of 6 different experiments where clones were generated from 6 different healthy individuals.

Figure 2

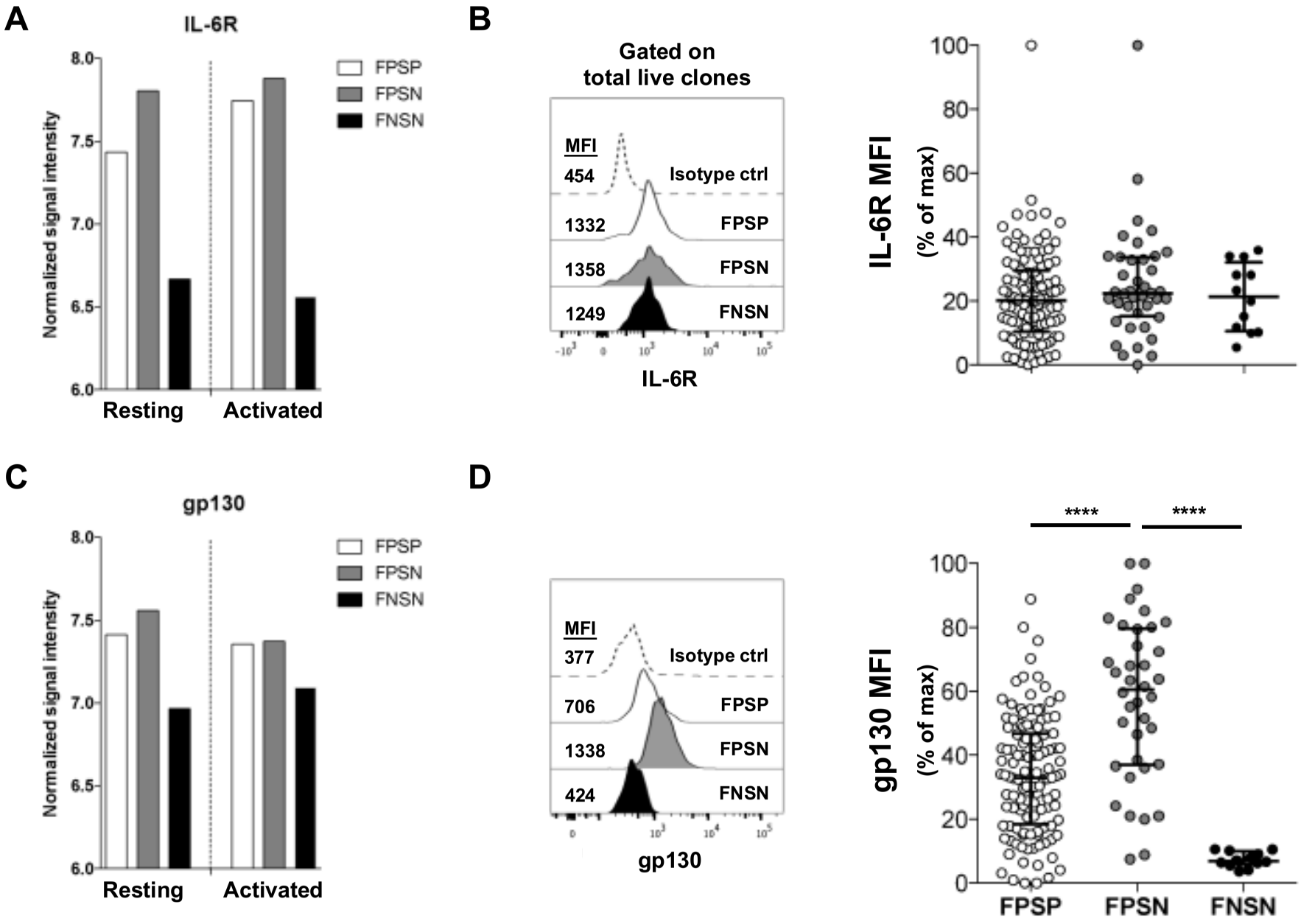

A
IL-6R
Normalized signal intensity
8.0
7.5
7.0
6.5
6.0
Resting
Activated
FPSP
FPSN
FNSN
B
Gated on
total live clones
MFI
454
Isotype ctrl
1332
FPSP
1358
FPSN
1249
FNSN
IL-6R
IL-6R MFI
(% of max)
100
80
60
40
20
0
C
gp130
Normalized signal intensity
8.0
7.5
7.0
6.5
6.0
Resting
Activated
FPSP
FPSN
FNSN
D
MFI
377
Isotype ctrl
706
FPSP
1338
FPSN
424
FNSN
gp130
gp130 MFI
(% of max)
****
****
100
80
60
40
20
0
FPSP
FPSN
FNSN

Figure 3. gp130 is preferentially expressed on naïve CD4$^+$ cells. PBMCs were isolated from healthy individuals and analyzed *ex vivo* by flow cytometry for the expression of the indicated markers. **A)** The expression of gp130 on naïve vs.memory CD4$^+$ T cells. **B)** The expression of gp130 on naïve vs.memory Treg cells and its correlation with the expression of FOXP3, Helios, TIGIT and FCRL3. Shown are the combined results from 7 different healthy individuals.

Figure 3

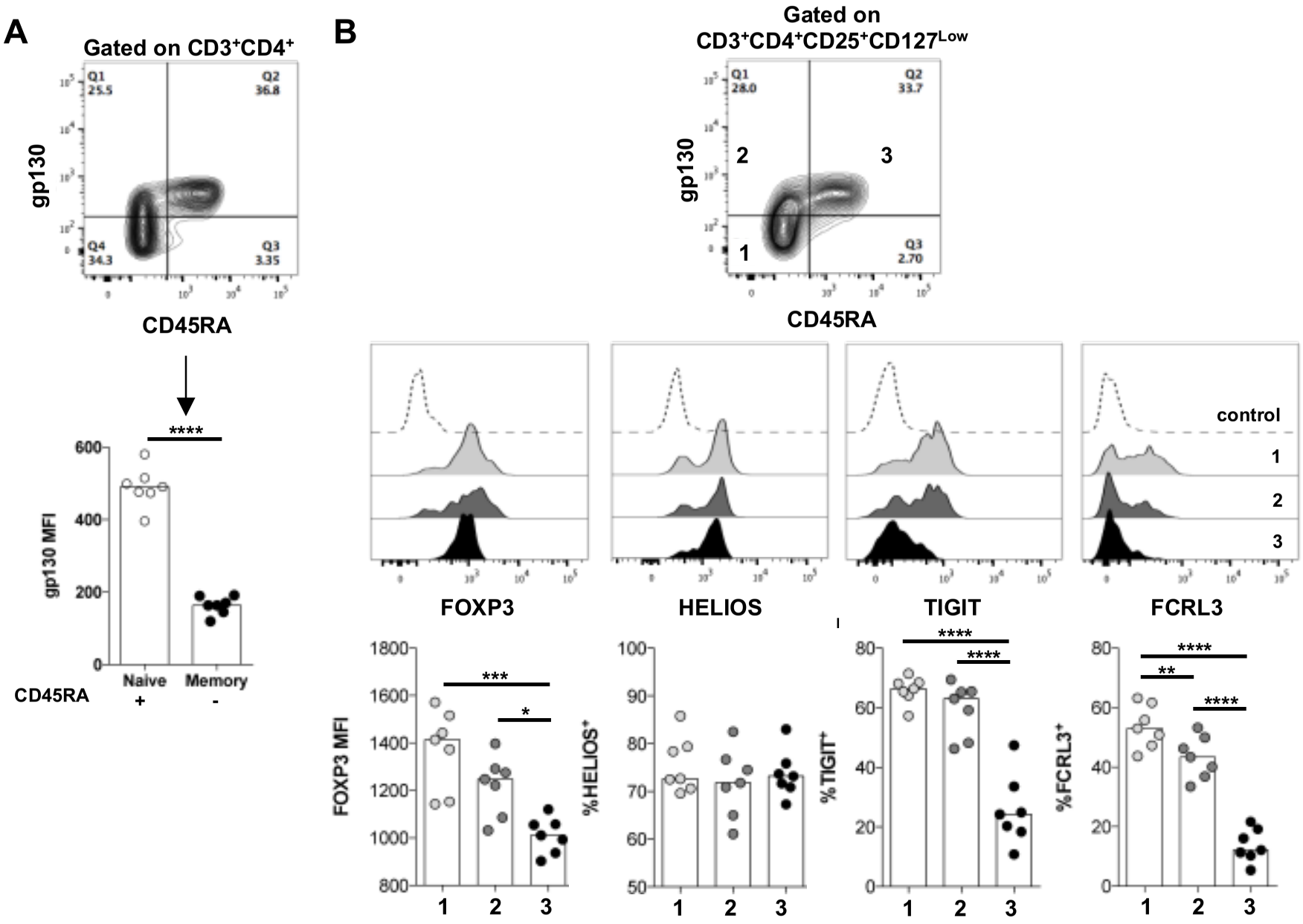

A
Gated on CD3+CD4+
Q1 25.5
Q2 36.8
Q4 34.3
Q3 3.35
gp130
CD45RA
gp130 MFI
****
600
400
200
0
Naive +
Memory -
CD45RA
B
Gated on CD3+CD4+CD25+CD127Low
Q1 28.0
Q2 33.7
Q3 2.70
2
3
1
gp130
CD45RA
control
1
2
3
FOXP3
HELIOS
TIGIT
FCRL3
FOXP3 MFI
%HELIOS+
%TIGIT+
%FCRL3+
***
*
****
****
****
**
****
1
2
3

Figure 4. High gp130 expression identifies Treg cells with reduced suppressive capacity *ex vivo*. Naïve and memory Treg cells ($CD4^{+}CD25^{High}CD127^{Low}$) cells were FACS-sorted according to their gp130 expression levels and co-cultured with CFSE-labeled, FACS-sorted $CD4^{+}CD25^{-}$ Teff cells in the presence of anti-CD3 + irradiated APCs for 96 hours. Shown are representative FACS plots and the percentage of suppression of Teff cells at multiple Treg:Teff ratios in 2 different experiments on 2 different healthy donors.

Figure 4

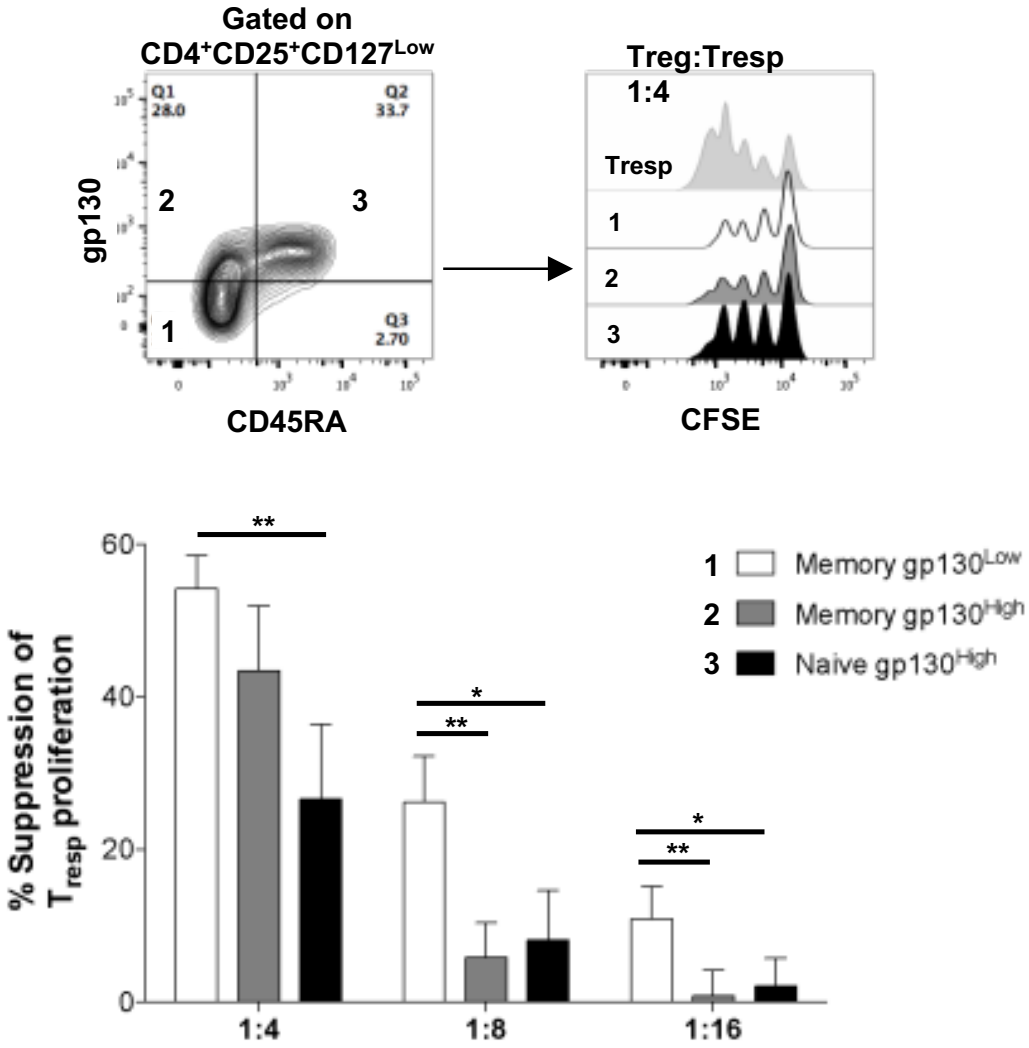

Gated on
CD4+CD25+CD127Low
Q1
28.0
Q2
33.7
Q3
2.70
1
2
3
gp130
CD45RA
Treg:Tresp
1:4
Tresp
1
2
3
CFSE
% Suppression of
Tresp proliferation
60
40
20
0
1:4
1:8
1:16
**
*
**
*
**
1 Memory gp130Low
2 Memory gp130High
3 Naive gp130High

Figure 5. IL-27 and IL-6 drive loss of suppression in FOXP3^{+} clones. FOXP3+ clones were generated from healthy donors and assessed for their ability to suppress allogeneic CD4+CD25- Teff cells in a 1:1 Treg:Teff suppression assay in the absence of irradiated APCs. **A)** The effects IL-6 (50 ng/mL), IL-11 (100 ng/mL), IL-27 (20 ng/mL), LIF (100 ng/mL) and CLC (1 ug/mL) on the proliferative response of Teff cells activated in the absence of clones. **B)** The effects of the indicated cytokines on the suppressive potency of gp130^{High} (gp130 MFI>600) vs.gp130^{Low} (gp130 MFI<500) FOXP3^{+} clones identified based on their gp130 expression levels before activation. Shown are the results from one representative experiment of 3 different experiments where clones were generated from 3 different healthy individuals.

Figure 5

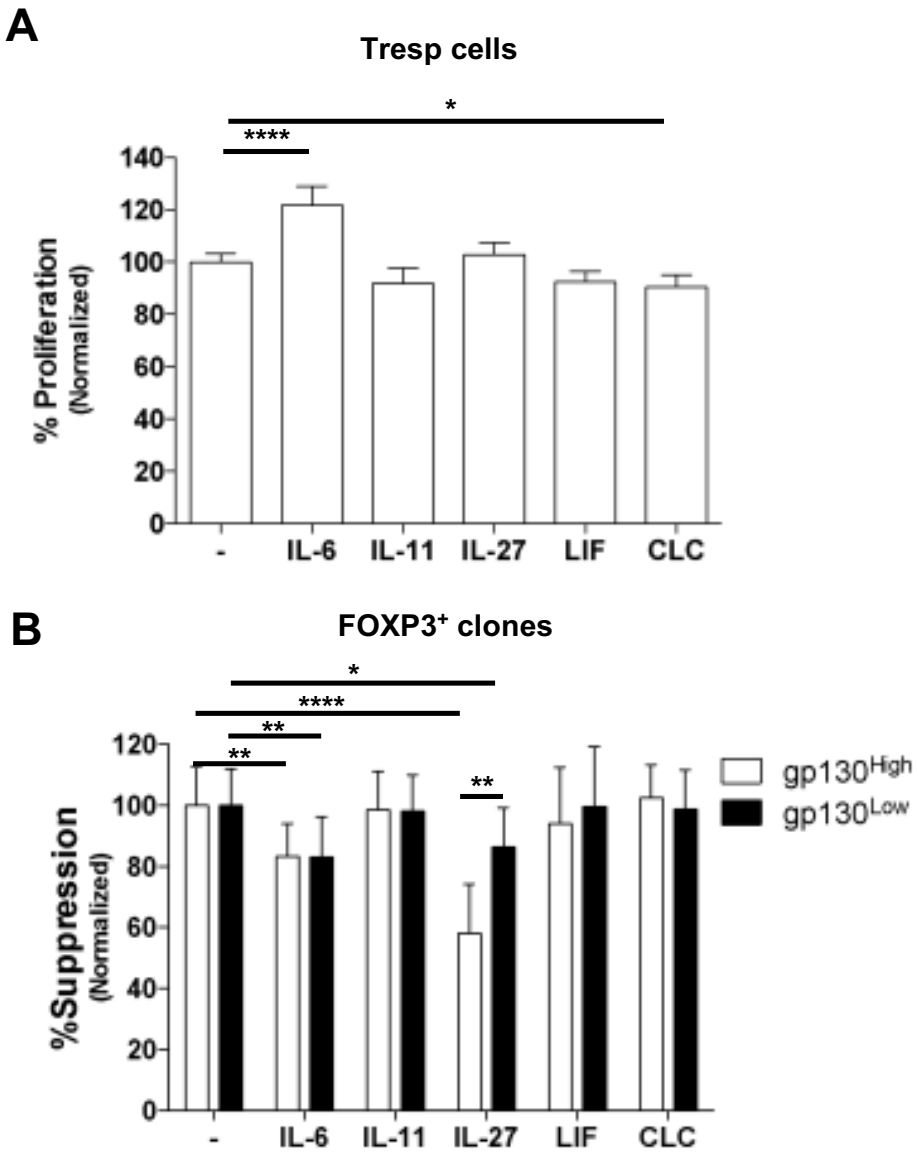

A
Tresp cells
*
****
% Proliferation (Normalized)
140
120
100
80
60
40
20
0
-
IL-6
IL-11
IL-27
LIF
CLC
B
FOXP3+ clones
*
****
**
**
**
%Suppression (Normalized)
120
100
80
60
40
20
0
gp130High
gp130Low
-
IL-6
IL-11
IL-27
LIF
CLC

Figure 6. IL-27 and IL-6 drive loss of suppression in Treg cells *ex vivo*. Treg cells ($CD4^{+}CD25^{High}CD127^{Low}$) cells were FACS-sorted and co-cultured with CFSE-labeled, FACS-sorted $CD4^{+}CD25^{-}$ Teff cells in the presence of anti-CD3 + irradiated APCs and exogenous IL-6 or IL-27 for 96 hours. **A)** The impact of IL-6 and IL-27 on the proliferative response of Teff cells activated in the absence of Treg cells. **B-C)** The effect of IL-6 **(B)** and IL-27 (**C)** on the suppressive capacity of Treg cells. Data are from 3 different experiments performed on cells isolated from 3 different healthy individuals.

Figure 6

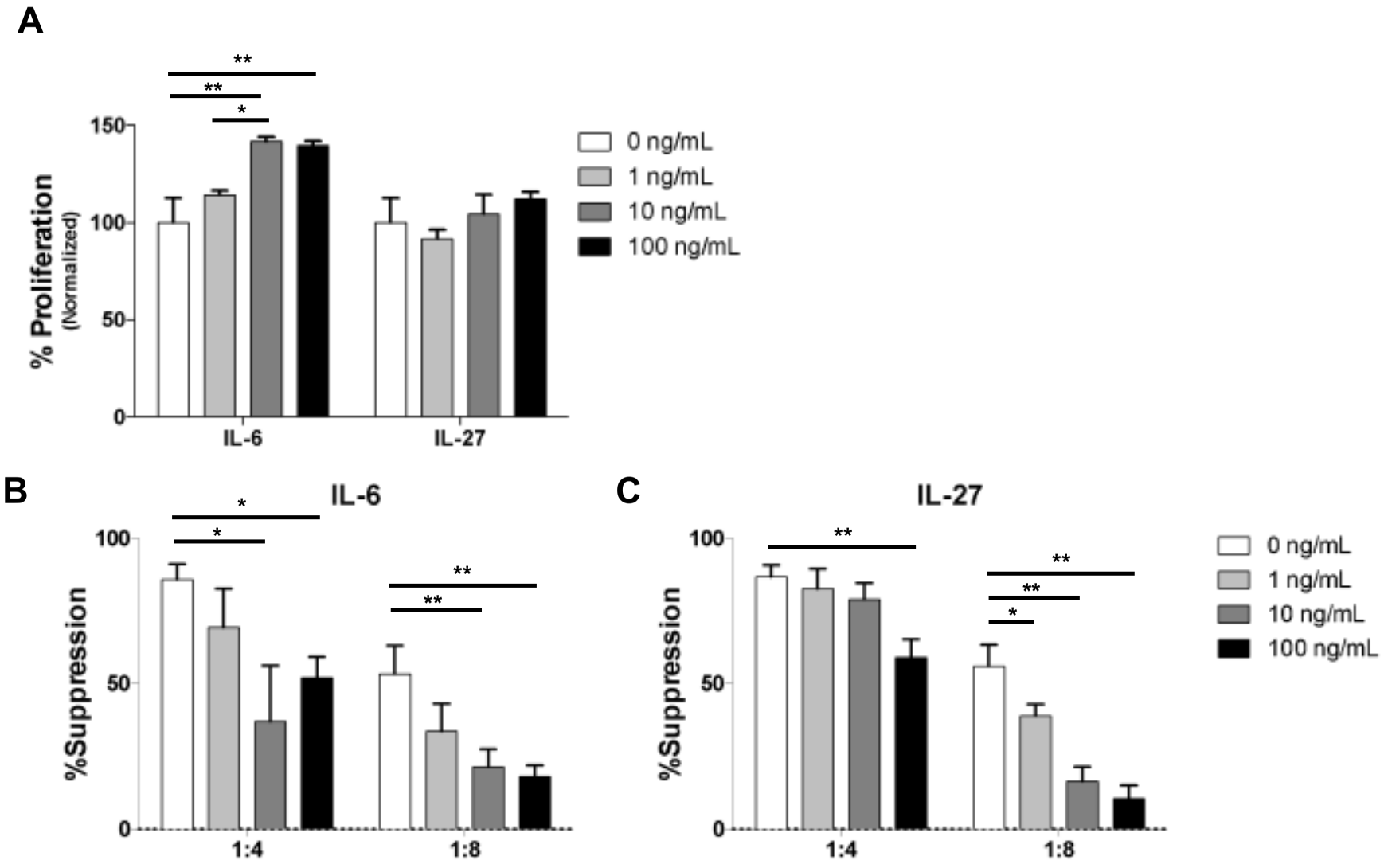
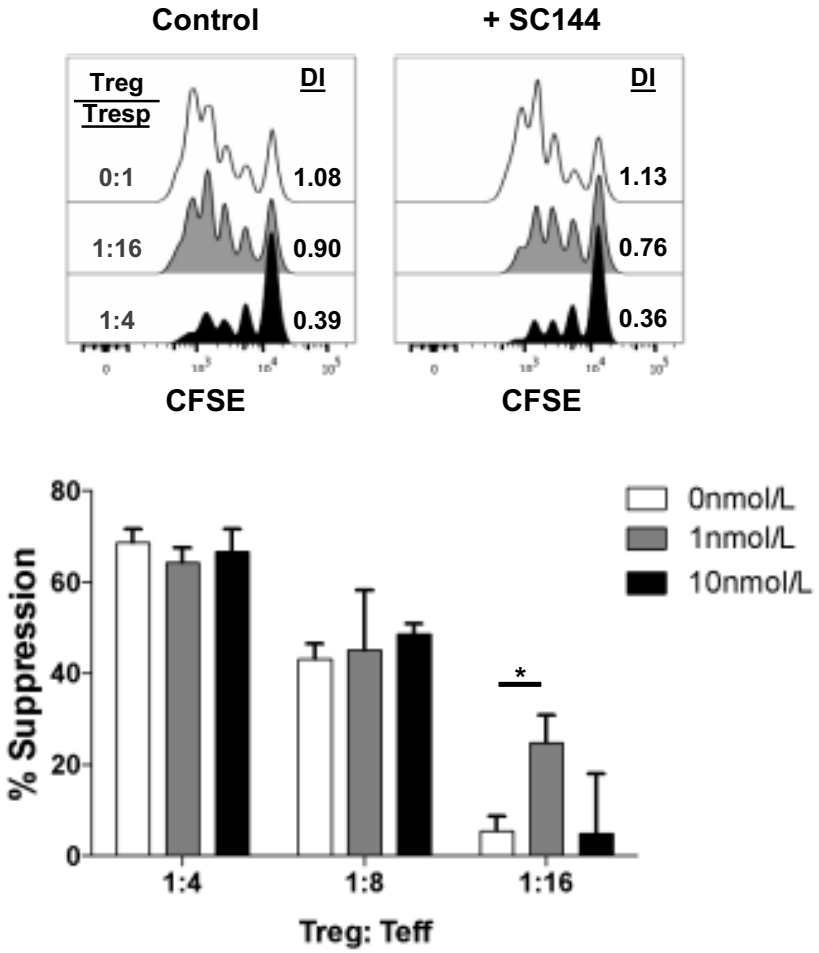

A
**
**
*
150
100
50
0
% Proliferation
(Normalized)
IL-6
IL-27
0 ng/mL
1 ng/mL
10 ng/mL
100 ng/mL
B
IL-6
*
*
**
**
100
50
0
%Suppression
1:4
1:8
C
IL-27
**
**
**
*
100
50
0
%Suppression
1:4
1:8
0 ng/mL
1 ng/mL
10 ng/mL
100 ng/mL

Figure 7. Impact of gp130 inhibition on the suppressive function of Treg cells. Treg cells ($CD4^{+}CD25^{High}CD127^{Low}$) cells were FACS-sorted and co-cultured with CFSE-labeled, FACS-sorted $CD4^{+}CD25^{-}$ Teff cells in the presence of anti-CD3 + irradiated APCs and titrated amounts of SC144 for 96 hours. Shown is the impact of SC144 treatment on the suppressive capacity of Treg cells at different Treg:Teff ratios. Data are from 3 different experiments performed on cells isolated from 3 different healthy individuals.

Figure 7


Control
+ SC144
Treg
Tresp
DI
0:1
1.08
1:16
0.90
1:4
0.39
1.13
0.76
0.36
CFSE
CFSE
0nmol/L
1nmol/L
10nmol/L
% Suppression
80
60
40
20
0
*
1:4
1:8
1:16
Treg: Teff

4.9. References:

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CHAPTER 5

General Discussion and Conclusions

CHAPTER 5- General Discussion and Conclusions

Over the past decade and a half, the FOXP3$^+$ regulatory T cell population has been the focus of intense research that clearly demonstrated their indispensability to the balanced function of the immune system in several animal models. In these models Treg deficiency or Treg depletion precipitate autoimmunity, while Treg infusion protects susceptible mice and even ameliorates established autoimmunity (276). Studies in mice have greatly benefited from genetic tools that allow the manipulation and reliable tracking of Treg cells *in vivo*, thus allowing for clear demonstrations of the critical role played by this subset in immune tolerance. In contrast, the human Treg field has faced significant challenges that hindered its progress. First, FOXP3, thought to be the most specific marker for Treg cells, is expressed intracellularly and thus its detection requires fixation and permeabilization of Treg cells. While this issue was easily circumvented in mice by developing reporter animals that allow the isolation of live Foxp3$^+$ Treg cells, FOXP3 expression cannot be used as a marker for the isolation of live human Treg cells for functional assessment. Therefore, surface markers of Treg cells were required. Indeed, several such markers have been described including high expression of CD25 concomitantly with low expression of CD127, as well as other surface markers that identify subpopulations of FOXP3$^+$ cells (reviewed in chapter 1 and (28)). While a number of these markers correlate very well with FOXP3 in resting conditions, TCR-mediated activation modulates the expression of all previously reported markers on Teff cells making them indistinguishable from Treg cells in inflammatory conditions (28). Human Treg studies are further complicated by the fact that FOXP3 is highly upregulated in human Teff cells upon activation without endowing them with suppressive function (78, 192). Thus, dominant inflammation in autoimmune diseases makes it very challenging to distinguish Treg from activated Teff cells in patients, shedding considerable

doubt on the validity of studies that used conventional Treg markers to isolate and functionally compare Treg cells from autoimmune patients and healthy controls. To circumvent this limitation, we have optimized a single-cell cloning approach that allows the reliable discrimination between *bona fide* human FOXP3^{+} Treg cells and activated Teff cells (109, 275). Empowered by the ability of this approach to allow the unbiased comparative assessment of Treg function in healthy and autoimmune conditions, we set out to investigate the impact of IPEX-causing mutations on the development of various aspects of Treg cell phenotype and function. IPEX provides the most compelling evidence of the critical role of Treg cells in the maintenance of self-tolerance in humans, where *FOXP3* mutations result in severe multi-organ autoimmunity and often-early death. Experimentally, IPEX provides a unique platform where naturally-occurring *FOXP3* mutations simulate the extreme settings in genetically-manipulated animal models such as the scurfy mouse, thus revealing key interactions of FOXP3 that are required for different aspects of Treg phenotype and function. Despite the rarity of IPEX samples, our work in **chapter 2** has demonstrated the valuable insight into human Treg biology that can be gained through investigation of natural IPEX mutations. Indeed, in this particular study we were able to characterize the cellular Treg defects caused by a specific IPEX mutation, and to identify the underlying molecular defects thus highlighting a critical pathway through which FOXP3 drives suppressive function in Treg cells.

5.1. Uncoupling the mechanisms governing phenotype and suppressive function in Treg cells

An interesting observation in our study in **chapter 2** was that the A384T mutation in FOXP3 selectively abrogates the suppressive function of Treg cells with minimal impact on the FOXP3-regulated Treg phenotype and repression of inflammatory cytokine production. These

studies thus suggest that FOXP3 regulates Treg phenotype and function through distinct molecular mechanisms, allowing cells to develop a characteristic Treg phenotypic profile independently of suppressive function. Interestingly, previous studies in mice where the *Foxp3* gene was mutated to partially disrupt the Foxp3 protein have showed that Foxp3 disruption allowed the development of Treg cells that exhibit the hallmark Treg phenotype and cytokine repression but lack suppressive capacity (277, 278). Although these studies have concluded that Foxp3 is not required for the development of Treg phenotype, the Foxp3 protein in these models was not completely abrogated. It is likely that the residual Foxp3 protein, particularly from the N-terminal domain, was sufficient to regulate the transcription of genes responsible for Treg phenotype and cytokine repression but was too excessively disrupted to orchestrate the genetic program responsible for the development of suppressive function in these models. These studies further support our conclusions that FOXP3 independently regulates Treg phenotype and function. A possible advantage of this dissociation of Treg phenotype and function could be the enhancement of Treg functional flexibility, whereby Treg cells can transiently adjust their suppressive function in response to local inflammatory signals. In this model, the hallmark Treg phenotype provides the basic identifiers of $FOXP3^+$ Treg cells and endows them with the essential receptors to survive, home to inflammatory sites and maintain their lineage identity. On the other hand, Treg cells need to adapt to the microenvironment by adjusting their suppressive potency to allow the initiation and progression of protective immune responses. As such, possessing the capacity to modulate their suppressive function while maintaining their lineage identity could be an important feature that allows Treg cells to act as immune rheostats and maintain the fine balance between protective immune responses and self-tolerance.

5.2. Transient flexibility of Treg cells

The stability of the Treg lineage has been questioned over the past few years. It was triggered by the observation that Foxp3$^+$ Treg cells transferred into lymphopenic mice can lose their Foxp3 expression and suppressive function, and additionally produce high levels of inflammatory cytokines such as IL-2, IFN-γ and IL-17 assuming a Teff-like phenotype (126-128). Depending on the tissue examined, these exFoxp3$^+$ cells constitute up to 90% of transferred Treg cells. However, the degree of Foxp3 loss was significantly lower (ranging from less than 5% up to 20%) in later studies using different fate-mapping mouse models in the steady state and under various challenges (188-190), supporting the notion of Treg stability. While some studies propose that exFoxp3$^+$ originate from committed Foxp3$^+$ cells that undergo lineage switching or reprogramming in response to homeostatic signals (127, 128, 191, 279), others have argued that they originate from a small population of uncommitted CD25^{Low} Treg cells that out-proliferate their Treg counterparts (126, 189). In humans, some studies on have suggested that FOXP3$^+$ Treg cells can lose FOXP3 expression after repetitive TCR stimulation *in vitro* (158). However, due to the intracellular localization of FOXP3, as well as phenomenon of activation-induced FOXP3 expression, it is very likely that the examined Treg population in these studies contained activated Teff cells that could have influenced the study outcome.

Theoretically, the notion of Treg lineage plasticity could pose a major challenge to ongoing attempts of cellular therapy where Treg cells are expanded *ex vivo* and re-infused into patients to control autoimmune responses. If a significant fraction of the transferred Treg cells succumb to inflammatory signals in patients and convert to Teff cells with a pathogenic potential, the outcome could be detrimental. Furthermore, Treg cells are enriched for self-recognizing T cell receptors (280, 281), suggesting that Treg plasticity in recipient patients could

result in disastrous autoimmune responses. Interestingly, however, despite the high degree of Foxp3 loss observed in Treg cells injected into lymphopenic mice, these former Treg cells do not cause disease in host mice, unlike Teff cells transferred in parallel which cause severe colitis (126, 128). In humans, a recent phase I clinical trials that examined the safety of Treg adoptive immunotherapy in type 1 diabetic patients observed no adverse events (274). Similarly, no severe side effects were reported in an earlier Treg therapy trial that assessed the safety of Treg therapy in the prevention of graft versus host disease following umbilical cord blood transplantation in patients suffering from haematological malignancies (273). Although the stability and fitness of the transferred Treg populations in these early clinical trials remain to be properly assessed, these trials suggest that potential Treg plasticity will likely not pose a significant threat to the promise of therapeutic strategies involving the transfer of Treg cells. Finally, it should be noted that Treg plasticity studies that demonstrated high levels of Foxp3 loss in mice were performed in highly contrived lymphopenic environments (126-128). From fate-mapping studies, it seems that the magnitude of Foxp3 loss may not be large enough to warrant concerns regarding the lineage stability of Treg cells.

To reconcile the seemingly contentious findings regarding Treg plasticity, a "transient flexibility model" was proposed by Liston and Piccirillo (282). This model suggests that, while the Treg population is stable in the steady state, the increase in inflammatory factors during infections increases the flexibility of Treg cells and allow their reprogramming into ex-Treg cells in order to increases the rate of clearance. Once Teff cells are abundant, spontaneous reversion of ex-Treg cells occurs, reducing the risk of autoimmunity. In support of the transient flexibility potential of Treg cells, our studies in **chapter 4** have demonstrated that the lack of suppressive function in non-suppressive $FOXP3^+$ clones is not a permanent fate, and that it is rather

transiently caused by local APC-derived factors. Importantly, the majority of these non-suppressive FOXP3$^+$ clones maintain their Treg phenotype and cytokine repression capabilities, further supporting the transient nature of their dysfunction. In this study, we have identified IL-6 and IL-27 and their shared signalling receptor subunit, gp130, as likely culprits driving the modulation of suppressive function in responsive Treg cells. However, several other factors have been previously shown to modulate Treg function. These include other inflammatory cytokines, TLR ligands, microbial metabolites and complement proteins (reviewed in (162)). Our study further suggests the existence of multiple Treg subpopulations with differential abilities to respond to specific local signals and undergo transient functional flexibility in order to allow the initiation and progression of protective responses. It is possible that some inflammatory factors could target pathways such as those involving the FOXP3-TIP60 interaction, thus resulting in specific disruption of suppressive function without causing loss of FOXP3. On the other hand, some inflammatory factors may alter molecular interactions upstream of FOXP3, thus destabilizing FOXP3 expression and potentially causing lineage conversion. It is, therefore, plausible that such inflammatory signals, when dysregulated, could cause permanent Treg dysfunction and allow the initiation and progression of autoimmune responses.

5.3. The role of Helios in Treg cells

In search of key markers that better correlate with Treg suppressive function, we have shown in chapter 3 that Helios expression identifies human Treg cells with higher suppressive capacity, and that lack of Helios expression in Treg cells is associated with production of high levels of inflammatory cytokines, suggesting an important role for Helios expression in enhancing the functional potency and stability of human Treg cells. We have further proposed a novel surface marker combination that demarcates Helios$^+$ and Helios$^-$ human Treg cells, thus

providing the necessary tools for further investigation of the role of Helios in human Treg biology. Importantly, our studies provide a long-sought surface marker combination that allows the distinction between human Treg cells and activated Teff cells, thus allowing the identification and monitoring of *bona fide* Treg cells in highly inflammatory conditions.

Following the publication of our manuscript, two recent studies investigated the functional role of Helios in murine Treg cells. Kim *et al*. and Sebastian *et al.* reported that Treg-specific deletion of Helios does not seem to have an early detectable impact on mice (283, 284). However, at 5-6 months of age, mice start to develop symptoms of systemic autoimmunity characterized by splenomegaly, lymphadenopathy, increased size and number of lymphoid follicles and germinal centers, as well as lymphocyte infiltration in non-lymphoid tissues such as the liver, kidney and salivary gland (283, 284). Interestingly, Sebastian *et al.* also observed that Helios-deficient Treg cells were particularly defective in controlling T follicular helper cell function, suggesting that loss of Helios significantly alters the development of follicular Treg cells (283). Moreover, compared to WT Treg cells, Helios-deficient Treg cells exhibited a markedly reduced survivability and stability in reconstituted lymphopenic mice (283). Finally, early viral challenge with LCMV accelerated the autoimmune phenotype in Helios-deficient mice suggesting that Helios deficiency alters the stability of Treg cells in inflammatory settings (284). Mechanistically, this study suggested that the role of Helios in Treg cells involves its binding to the *STAT5b* gene and enhancing the activation of the IL-2Rα–STAT5 pathway, leading to elevated levels of Foxp3 and increased suppressive potency of Treg cells (284). These studies are in accordance with our findings correlating Helios expression in human Treg cells with increased suppressive potency and functional robustness. Interestingly, we and others have shown that TCR-mediated activation of human $CD4^{+}CD25^{-}$ cells, which results in the

upregulation of FOXP3, does not induce Helios expression (67, 275, 285), even in the presence of TGF-ß (67). This raises the possibility that the lack of Helios expression in activated human Teff cells is preventing them from exhibiting suppressive function following FOXP3 induction. In other words, is Helios expression required for the early stages of development of suppressive function in human Treg cells? Moreover, an important question that needs to be addressed relates to the regulatory mechanisms controlling Helios expression in Treg cells. Our discovery of TIGIT and FCRL3 as a novel surface marker combination that distinguishes Helios$^+$ and Helios$^-$ human Treg cells should significantly facilitate such studies. We are currently investigating potential molecular and functional association between Helios, TIGIT and FCRL3.

5.4. Investigation of the role of FCRL3 in Treg cells

The biological function of FCRL3 in the immune system is largely unknown. It is one of six members of the FCRL family of classical Fc receptor homologues that are expressed mainly on B cells. Interestingly, FCRL3 is the only member of this family expressed in cells other than B cells, namely on NK cells, and some CD8$^+$ cells (286-288). Among CD4$^+$ cells, FCRL3 is mainly expressed on FOXP3$^+$ Treg cells (275, 288). FCRL3 is also the only member of the FCRL family whose natural ligand remains elusive. Interestingly, the FCRL3 gene is missing in mice, suggesting that the existence of FCRL3 in humans may be the result of an evolutionary adaptation of the human immune system (287). A potentially important functional role of FCRL3 in immune regulation is suggested by several genome-wide association studies that strongly link single nucleotide polymorphisms in the *FCRL3* gene to multiple autoimmune disorders (287, 289-294). Interestingly, FCRL3 harbours both activating (ITAM) and inhibitory (ITIM) motifs in its intracellular domain indicating the possibility of a dual regulatory role for FCRL3. Indeed, studies on B cells lines have indicated that FCRL3 ligation could inhibit tyrosine

phosphorylation and calcium mobilization downstream of B cell receptor signalling (295). On the other hand, FCRL3 ligation augmented B cell proliferation in response T cell-independent TLR-9 signalling (296). These studies suggest that FCRL3 may utilize its ITIM and ITAM motifs to differentially regulate innate and adaptive immune responses. Significantly less is known about the role of FCRL3 on T cells. Two previous studies have concluded that FCRL3 expression on Treg cells identifies a population that is markedly hypoproliferative even in the presence of high amount of IL-2 (288, 297). Notably, in both studies Treg cells were activated in the absence of APCs. While our ongoing work confirms these previous findings in the absence of APCs, we observed that $FCRL3^+$ Treg cells can proliferate as well as their $FCRL3^-$ counterparts in the presence of APCs (data not shown), suggesting that FCRL3 could deliver an inhibitory signal to $FCRL3^+$ Treg cells unless bound to an extrinsic ITAM-triggering ligand provided by the APCs. We can thus speculate that FCRL3 expression by Treg cells could represent a mechanism by which Treg cells regulate their own activation and expansion. Interestingly, we were able to recapitulate the anti-proliferative effects of FCRL3 in T cells transduced with the full FCRL3 protein but not with a truncated FCRL3 that lack the intracellular domain (data not shown). We are currently dissecting these signalling events by overexpressing FCRL3 constructs that lack specific ITIM and ITAM tyrosine residues. Furthermore, we are in the process of identifying cell subsets that express the natural ligand(s) for FCRL3 using an FCLR-Fc fusion protein. Additionally, we have established a collaboration to specifically identify FCRL3 ligand(s) using expression cloning of human cDNA libraries. These studies will further our understanding of the regulatory mechanisms governing Treg cells, and may provide additional targets for controlling Treg responses.

5.5. Enhancing Treg function in human autoimmunity

The compelling association between *FOXP3* mutations and the development of autoimmunity in IPEX patients has suggested that Treg defects may also play a primary role in the pathogenesis of common organ-specific autoimmune disorders. However, as discussed in **chapter 1**, studies that evaluated the functional status of Treg cells in autoimmune patients have resulted in inconclusive findings. In addition to the previously discussed lack of reliable human Treg markers, most of these studies have examined Treg cells isolated from peripheral blood after disease onset. In these settings, it is not clear whether peripheral Treg cells can reflect the functional status of Treg cells at the site of inflammation. Moreover, we now know that the function of Treg cells can be significantly modulated by inflammatory factors. Since the vast majority of Treg analyses in human autoimmunity have been performed after disease onset, it is quite challenging to distinguish primary causality from secondary effects in studies where Treg defects have been reported. Therefore, pre-disease onset analysis of Treg function in predisposed patients could provide more informative insight into the functional status of Treg cells during the primary immune events leading up to disease. One disease that offers such a possibility is type 1 diabetes for a number of factors including its early age of onset and its high sibling relative risk (298).

On the other hand, a number of observations suggest that global Treg defects should not be expected when analyzing the function of Treg cells in autoimmune conditions. First, most of these disorders affect specific organs or tissues, whereas the general Treg defects in IPEX patients lead to severe, systemic and multi-organ autoimmunity. Moreover, while IPEX develops neonatally, the age of onset for most autoimmune diseases varies significantly with some disorders manifesting during childhood while others may be delayed until early adulthood or

even middle age. Therefore, it is unlikely that a global Treg defect would be underlying common autoimmune diseases. Rather, it is possible that certain inflammatory events in susceptible autoimmune patients can cause specific modulation of the function of subpopulations of responsive Treg cells in these patients, thus contributing to the onset and/or progression of immune responses against self-antigens without causing severe and systemic autoimmunity. A number of genetic and environmental factors could interact to play a role in the differential susceptibility in autoimmune individuals. For instance, several GWAS studies on multiple autoimmune diseases have a suggested an association between susceptibility and a number of inherent genetic variability in Treg-relevant genes such as *IL2RA*, *IL7RA* and *PTPN22* (299). Moreover, accumulating literature in the past few years has established a significant link between the composition of the commensal microbiota and susceptibility to, and severity of, autoimmune diseases in several models (reviewed in (300)). Furthermore, a number of viral infections have been associated with onset of autoimmunity (reviewed in (301)). Inflammatory responses to such infections can potentially lead to the modulation of subpopulations of Treg cells in specific tissues leading to break-down in self-tolerance. Indeed, the importance of environmental components in driving autoimmunity is interestingly illustrated in the two IPEX patients analyzed in **chapter 2**. Despite carrying the same *FOXP3* mutation (p.A384T), the two patients had markedly contrasting disease severity. The first patient presented in the first few days of life with severe multi-organ failure, rash, enteritis and endocrine abnormalities, leading to death at the age of 2 months (154). The second, unrelated, patient suffered from milder manifestations that included atopic dermatitis, neutropenia, diabetes and enteritis (302). He survived with intermittent immunosuppressive regimens into young adulthood when he underwent bone marrow transplantation at the age of 19. Our analysis of Treg function has clearly demonstrated

that this mutation equally abrogated the suppressive capacity of Treg cells isolated from both patients, suggesting that the discrepant disease presentations did not result from a differential impact of this mutation on Treg cell function in the two patients. Furthermore, the family of the first patient later had another son who, unfortunately, succumbed to IPEX in an identical disease course to that observed in his sibling. These observations demonstrate that, even in the presence of a deleterious *FOXP3* mutation that impairs Treg function, factors beyond genetics contribute significantly to the magnitude of immune dysregulation in patients.

Regardless of whether Treg defects are causative of autoimmune disorders or secondary to such conditions, strategies that allow the stabilization and potentiation of endogenous Treg function are likely to be beneficial in controlling autoimmunity. A great advantage of such targeted approaches would be their potential of reducing the need for wide-spectrum immunosuppressive agents that exert global effects on the adaptive immune system and leave the patient with increased risk of infections and malignancies. In this thesis, we have proposed two such targeted strategies: The first involves the enhancement of FOXP3-TIP60 interaction using a small molecule allosteric modifier of TIP60 (**chapter 2**). In the second approach (**chapter 4**), we have proposed the use of a small molecule inhibitor of gp130 signalling as a potential approach to increase the functional potency of the human Treg subset. Further studies are needed to examine the safety and efficacy of both strategies as stabilizers and functional enhancers of Treg cells in humans.

5.6. Concluding remarks

There is an expression in Arabic that literally translates to ‘holding the stick from the middle’. It means to be in control of a situation and to be equidistant from all potential players. That is how I view Treg cells. They are at the nexus of inflammation and immunosuppression.

Their deficiency results in autoimmunity while their overabundance facilitates infections and cancer growth. This regulatory role requires a high degree of functional flexibility that we are only beginning to appreciate. I, therefore, believe that furthering our understanding of human Treg biology, and developing reliable strategies to assess and modulate Treg function in various disease setting should provide powerful, yet targeted, means to improve various aspects of human health.

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Appendix 1: List of Abbreviations

A384T	alanine to threonin mutation at residue 384
Ag	Antigen
AICD	Activation-induced cell death
AIRE	autoimmune regulator
AML	acute myeloid leukemia
AMP	Adenosine monophosphate
ANOVA	analysis of variance
AP1	Adaptor protein 1
APC	antigen-presenting cells
APECED	autoimmune polyendocrinopathy, candidiasis and ectodermal dystrophy
ATP	Adenosine tri-phosphate
Aza	Azacytidine
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CFSE	Carboxyfluorescein succinimidyl ester
ChIP	Chromatin immunoprecipitation
CLC	Cardiotrophin-like cytokine
CNS	Conserved non-coding sequences
CNTF	Ciliary neurotrophic factor
CpG	Cytosine-phospho-guanine motif
CSF	cerebrospinal fluid
CTLA-4	cytotoxic T-lymphocyte-associated protein 4
DC	dendritic cell
DNA	deoxyribonucleic acid
DNMT	DNA (cytosine-5-)-methyltransferase
EAE	experimental autoimmune encephalitis
Ebi3	Epstein-Barr virus induced gene 3
EV	empty vector
FACS	fluorescence assisted cell sorting
FasL	Fas ligand
FBS	Fetal bovine serum
FCRL3	Fc receptor-like 3
FKH	forkhead domain
FNSN	FOXP3 negative non-suppressive
FOXP3	Forkhead winged helix protein 3
FOXP1	Forkhead winged helix protein 1
FPSN	FOXP3 positive non-suppressive
GARP	Glycoprotein A repetitions predominant
GFP	green fluorescent protein

GITR	tumor necrosis factor receptor superfamily, member 18
Gp130	Glycoprotein 130
GVHD	graft versus host disease
GWAS	genome wide association studies
HAT	histone acetyl transferase
HDAC	Histone deacetylate
HLA	human leukocyte antigen
HRP	Horseradish peroxidase
HSCT	hematopoietic stem cell transplantation
IBD	inflammatory bowel disease
IDO	Indoleamine 2, 3-dioxygenase
IFN	interferon
IKZF	Ikaros zing-finger family
IL	interleukin
IL-2R	interleukin 2 receptor
IL-6R	interleukin 6 receptor
IL6ST	interleukin 6 signal transducer
IL-7R	interleukin 7 receptor
IL-27R	interleukin 27 receptor
IPEX	Immuno-deficiency, Polyendocrinopathy, entheropathy, X-linked
IQR	Inter-quartile range
IRES	Internal ribosome entry sequence
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibition motif
iTreg	*in vitro* induced regulatory T cells
JAK	Janus Kinase
LAG	lymphocyte-activation gene
LAP	latency-associated peptide
LCMV	Lymphocytic Choriomeningitis Virus
LIF	Leukemia inhibitory factor
Log	logarithm
LZ	leucine zipper domain
mAb	monoclonal antibody
MACS	magnetic bead assisted cell sorting
MBP	myelin binding protein
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MHCII	major histocompatibility complex class2
miRNA	micro RNA
mRNA	messenger RNA

MS	multiple sclerosis
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
NFAT	nuclear factor of activation in T cells
NFkB	nuclear factor kappa B
NK	natural killer
N-terminal	amino-terminal
nTreg	naturally-occurring regulatory T cells
OSM	Oncostatin M
PBMC	peripheral blood mononuclear cells
PD1	programmed cell death 1
Pi3K	Phosphatidylinositol 3-kinases
PIM2	proviral integration site 2
PMA	Phorbol 12-myristate 13-acetate
PTPN22	Protein tyrosine phosphatase, non-receptor type 22
R397W	Arginine to tryptophan mutation at residue 397
RA	retinoic acid
RA	rheumatoid arthritis
RAR	retinoic acid receptor
rhIL-2	recombinant human IL-2
FPSP	FOXP3 positive suppressive
RNAi	RNA interference
ROR	RAR-related orphan receptor
RRMS	Relapsing-Remitting Multiple Sclerosis
RUNX	runt-related transcription factor
SCID	severe combined immune deficiency
SD	standard deviation
SELL	selectin L
SLE	systemic lupus erythematosus
SLEDAI-2k	Systemic lupus erythematosus disease activity index 2000
SMAD	*sma*ll body size and mothers against decapentaplegic homolog
SNP	single nucleotide polymorphism
STAT	signal-transducer and activator of transcription protein
T1D	type-1-diabetes
T-Bet	T-cell-specific T-box transcription factor
TCR	T-cell receptor
TEC	thymic epithelial cells
Teff	effector T cells
TGFβ	transforming growth factor beta

TIGIT	T cell immunoreceptor with Ig and ITIM domains
TIP60	Tat-interactive protein-60
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
Tr1	type-1 regulatory T cells
Treg	regulatory T cells
TSA	tissue-specific antigen
TSDR	Treg-specific demethylated region
UC	Ulcerative Colitis
WT	Wild type
ZF	Zing finger