EXPRESSION PATTERNS AND ROLES OF THE IKAROS FAMILY OF TRANSCRIPTION FACTORS IN HUMAN REGULATORY T CELL DEVELOPMENT AND FUNCTION.

By

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Abstract

Regulatory T cells (Tregs) are a small subset of immune cells that are responsible for downregulating the immune response and maintaining immune tolerance. Tregs are generally defined by a high expression of the transcription factor FOXP3. Dysfunction of Treg activity or number is the basis of many inflammatory diseases. Thus, there has been much research on understanding Treg development in the thymus and activity in the periphery and Tregs are being studied as a potential cellular therapy. One form of Treg therapy is generating engineered Tregs (eTregs), which involves expressing Treg genes in conventional T cells through retroviral or lentiviral transduction. The work described here investigates the role of the Ikaros family of transcription factors in eTreg function and thymic Treg development. The Ikaros family members, namely Helios and Eos, have been implicated as critical mediators of Treg induction and function. In the first study, we hypothesized that ectopic expression of Helios with FOXP3 is required for optimal engineered Treg immunosuppression. In the second study, we hypothesized that Ikaros family member expression correlates with Treg marker expression and defines points of Treg lineage commitment within CD4+ mature single positive (MSP) Tregs in the thymus.

In the first study, we generated eTregs by retrovirally transducing total human T cells with combinations of FOXP3, Helios (Hel-FL) and Δ3B Helios (Hel-Δ3B), a relevant splice variant of Helios. FOXP3+Hel-FL eTregs were the only eTregs able to delay disease in a xenogenic Graft versus Host Disease model. *In vitro*, FOXP3+Hel-FL CD4+ eTregs suppressed T cell proliferation more effectively than FOXP3 and FOXP3+Hel-Δ3B CD4+ eTregs. However, both FOXP3+Hel-FL CD8+ eTregs and FOXP3+Hel-Δ3B CD8+ eTregs were more effective than FOXP3 alone. RNA Sequencing of the CD4+ and CD8+ eTregs demonstrated that the addition of Hel-FL to FOXP3 in eTregs changed gene expression in cellular pathways and the

Treg signature compared to FOXP3 alone or FOXP3+Hel-Δ3B. Thus, overexpression of Hel-FL with FOXP3 in eTregs changed gene expression in Tconvs and mediated immunosuppression *in vivo* and *in vitro*. Additionally, there is a functional difference between the endogenous splice variants of Helios in mediating CD4+ and CD8+ T cell immunosuppression.

In the second study, we used novel CD4+ mature single positive (MSP) thymocyte populations that our laboratory previously defined to track CD4+ CD25+ FOXP3+ human Treg development from human thymus samples. We then characterized protein expression of Ikaros family members and Treg markers of Tregs from each of these populations. We found that a majority of Tregs can be found in the distinct MSP6 population and these Tregs have heterogenous expression of Helios and CD39 and CD127. Within the MSP1-MSP5 populations expression of Ikaros family members transiently changed and Helios and Eos correlated with the percent of Tregs within each population. Thus, using Ikaros family members and Treg markers within subsets of CD4+ thymocytes, we were able to more precisely determine where thymic Tregs originate from and critical points of Treg development. Overall, the results of this research provide further insight into the role of the Ikaros family members in Treg function and development and can be used to improve current Treg therapy.

Supplemental Information:

S1. RNA Sequencing Raw CPMs: Spreadsheet containing raw counts per million (CPMs) from RNA Sequencing of eTregs described in Chapter 2. FL= FOXP3+Hel-FL, d3B= FOXP3+Hel-Δ3B.

S2. Gene Fold Changes: Spreadsheet of top 2000 genes changed when comparing RNA Sequencing of eTregs described in Chapter 2. FL= FOXP3+Hel-FL, d3B= FOXP3+Hel-Δ3B.

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Chapter 1 : Introduction

Parts of this chapter were previously published as an open access article (CC-BY) and reprinted here in part with adaptations. [1] Seng, A. and N. Dunavin, Mesenchymal stromal cell infusions for acute graft-versus-host disease: Rationale, data, and unanswered questions. ADVANCES IN CELL AND GENE THERAPY, 2018. 1(2): p. e14. [2] Seng, A. and T.M. Yankee, The Role of the Ikaros Family of Transcription Factors in Regulatory T cell Development and Function. J Clin Cell Immunol, 2017. 8(2).

Regulatory T cells (Tregs) are a subset of immune cells that are critical for suppressing the immune response and promoting tolerance. There was much debate from the 1970s until the late 1990s whether or not there was a specific group of T cells that was solely responsible for suppressing immune activity. It became clear through neonatal thymectomy experiments in mice and rats that there was indeed a subset of T cells responsible for protecting against the autoimmune diseases caused by neonatal thymectomy [3, 4]. Further phenotyping experiments began to slowly determine surface markers that helped defined this group of suppressor cells in mice. These markers included $CD4^{+}$, $CD45RC^{lo}$, $CD45RB^{lo}$ and $CD5^{hi}$ [5-9]. In 1995, Sakaguchi et al. determined that CD25, the alpha chain of the IL-2 receptor, was key in identifying suppressor T cells [10]. They demonstrated that transferring splenocytes that were depleted of CD25+ CD4+ T cells into T cell-deficient mice caused autoimmune disease and restoring this subset of T cells inhibited disease [10]. It was these key experiments that confirmed the identity of Tregs and led to the wide acceptance of Tregs as a novel subset of T cells.

With surface markers available to identify Tregs and *in vitro* experiments to test Treg function, much progress has been made in investigating Treg biology. CD4+ CD25+ FOXP3hi

Tregs represent 3-5% of $CD4^+$ T cells in the blood in both mice and humans [11, 12]. FOXP3 has been shown to be a critical regulator of Treg development and activity [13-15]. Mutations in FOXP3 in both mice and humans result in Treg dysfunction and development of multiple autoimmune diseases, known as immunodysregulation polyendocrinopathy enteropathy X-linked (IPEX) syndrome in humans [16-18]. In addition, Tregs also express high levels of CD25, GITR, and CTLA-4, and low levels of CD127 [10, 19-21]. In humans, some activated T cells transiently upregulate CD25 and low levels of FOXP3 [22], making it difficult to isolate pure Treg populations. A majority of Tregs are thymus-derived Tregs (tTregs), generated during T cell development [23-25]. Naïve T cells in the periphery can also differentiate into peripherally-derived Tregs (pTregs) upon antigen stimulation of naïve T cells in the presence of tolerogenic dendritic cells and TGF-β-secreting T cells [26-28]. Within these major groups of Tregs exists multiple smaller subsets that differ in surface markers, transcription factor expression, cytokine secretion and immunosuppressive activity [29, 30]. Additionally, *in vitro*-induced Tregs (iTregs) can be generated by stimulating $CD4^+$ T cells with various cytokines and drugs such as TGFβ, rapamycin and retinoic acid [31]. Finally, engineered Tregs (eTregs) can be produced by transducing $CD4^+$ conventional T cells (Tconvs) with genes known to convey Treg activity such as FOXP3 [32]. Tregs can directly or indirectly suppress immune activity via a variety of actions. Mechanisms of Treg-mediated immune suppression include inhibiting Tconv proliferation and activation, secretion of immunosuppressive cytokine, killing of reactive immune cells and induction of anergy [33, 34].

Tregs play a major role in human health and disease. Impaired Treg numbers or function has been linked to overactive immune responses, which contributes to diseases such as autoimmunity, allergy, and graft-versus-host disease (GVHD) [8-10]. Conversely, tumors often

contain numerous Tregs that suppress anti-tumor immunity [11]. Thus, the ability to manipulate the number or function of Tregs could have profound therapeutic effects. In pre-clinical studies intravenous injection of *ex vivo* expanded Tregs has been used to treat murine models of multiple sclerosis, inflammatory bowel disease, and GVHD [12-14]. Treg adoptive transfer is currently being tested in clinical trials to treat GVHD, type I diabetes and transplant rejection, as well as other inflammatory diseases [35-39]. These trials have demonstrated the safety of Treg adoptive transfer therapy in humans and have had moderate success in ameliorating disease. While Treg therapy is promising, there are still major obstacles to overcome, including obtaining sufficient Treg numbers, ensuring Treg purity and maintaining Treg persistence *in vivo*. Gaining a better understanding of Treg biology will help address these limitations.

One aspect of Treg biology that could offer insight into Treg expansion and potency is the role of the transcription factor Helios in Treg function. Helios is a member of the Ikaros family of transcription factors that is highly expressed in 70% of human Tregs [25]. All T cells in both mice and humans express two isoforms of Helios, a full length form (Hel-FL) and shorter isoform lacking half of the 3rd exon (Hel-Δ3B) [40, 41]. Treg specific knock-out of total Helios in mice results in development of spontaneous autoimmunity at 6-8 months of age, decreased Treg immunosuppressive function, secretion of pro-inflammatory cytokines, and survival [42, 43]. In humans, knock down of Helios in Tregs via siRNA resulted in reduced suppressive activity and reduced FOXP3 expression [44]. Helios-positive (Helios+) Tregs more effectively suppress cytokine secretion by pro-inflammatory T cells *in vitro* compared with their Helios- counterparts in both mice and humans [25, 45-48]. Thus, Helios is a key mediator of Treg activity in both mice and humans. However, how Helios functions to enhance Treg function has

not been delineated. The aim of the work described in this dissertation was to determine the effect of endogenous Helios splice variants on human Treg development and function.

Origin of Tregs

Thymic Treg development

Approximately 70% of circulating Tregs are generated in the thymus during T cell development [23-25]. The mechanisms for human Treg commitment in the thymus are largely unknown as well as when this commitment step takes place. Understanding Treg development is crucial, as inherent Treg dysfunction or reduced Treg numbers is the basis for many autoimmune diseases including inflammatory bowel disease (IBD), Type I diabetes and myasthenia gravis [49-55]. Additionally, there are currently no accepted markers that can be used to distinguish human tTreg from pTregs in the periphery. Characterization of the developmental pathway leading to tTregs could also lead to the identification of such markers.

The complete mechanism of tTreg commitment in the thymus is unclear, but T cell receptor (TCR) signal strength, co-stimulation and cytokine stimulation are major mediators [56, 57]. In mice, it has been established that Treg development requires a strong interaction of the TCR with its cognate self-antigen presented by thymic epithelial cells (TECs) or antigen presenting cells (APCs) [58, 59]. TCR signaling in human Treg development can only be indirectly tested, but expression of positive selection markers, including CD69 and CD27, are upregulated on developing Tregs [60-64]. Another indication of TCR engagement in human Treg development is the expression of CTLA-4 in DP and CD4+ SP tTregs [63, 65, 66]. Additionally, patients deficient in ZAP-70, a key regulator of T cell signaling, have reduced numbers of developing Tregs in the thymus [67]. Expression of FOXP3 can be induced by TCR engagement

as a result of binding sites for NFAT and AP-1 in the FOXP3 promoter, which are both downstream targets of TCR signaling [68]. In addition to TCR signaling, various co-stimulation pathways are upregulated in Treg development through interaction with TECs or dendritic cells (DCs) including JAK3/STAT-5, Notch, CD80/CD86, ICOS/ICOSL and CD40/CD40L [69-71]. Finally, IL-2 and IL-15 stimulation is important in maintaining tTreg proliferation and survival during development [72, 73]. DCs and other thymocytes are likely the sources of IL-2 and B cells, macrophages and TECs have been identified as the primary sources of IL-15 in the thymus [72].

With the limited Treg markers that are available, namely FOXP3 and CD25, it has thus far been concluded that human Treg lineage commitment can occur at $CD4^+$ $CD8^+$ double positive (DP) and $CD4^+$ single positive (SP) developmental stages [56]. In general, commitment of FOXP3+ DP cells to the Treg lineage is thought to be driven by interaction of progenitor Tregs with cortical TECs and macrophages via TCR followed by stimulation by IL-2 and IL-15 [63, 70, 72]. FOXP3+ DP cells can differentiate into FOXP3+ CD4+ SP tTregs or FOXP3+ CD8+ SP tTregs. CD103+ FOXP3+ DP cells are likely the subset that differentiates into CD8+ SP tTregs as CD4+ SP tTregs do not express high levels of CD103. Alternatively, mature FOXP3- CD25- CD4+ SP in the medulla can acquire FOXP3 and CD25 expression through various combinations of TCR engagement with mTECs or APCs presenting tissue-specific self-antigens, activation of co-stimulatory pathways and IL-2 and IL-15 stimulation [69, 72, 74, 75]. The exact path that thymocytes follow to Treg lineage commitment can be further elucidated using other Treg markers.

Our laboratory defined novel subsets of human thymocytes using multi-parameter flow cytometry with the goal of identifying the sites at which the major T cell developmental

checkpoints occur [2, 76, 77]. Human thymocytes were isolated from discarded thymus from pediatric cardiac surgery patients. The first novel subset is the immature single positive (ISP) $CD4^+$ population. Consistent with previous reports [78-82], we found that in about half of patients, $CD4^-CD8^-$ double negative (DN) thymocytes differentiate into ISP $CD4^+$ thymocytes before expressing CD8 to become $CD4^+CD8^+$ double positive (DP) thymocytes [76]. In the other half of patients, CD4 and CD8 are expressed simultaneously, resulting in few ISP $CD4^+$ cells. In a few rare cases, an ISP $CD8^+$ population is present where CD8 is expressed prior to CD4. In some cells, expression of TCRβ occurs during the ISP $CD4^+$ stages, which a critical step in DN to DP transition. Other cells do not express TCRβ until the early DP stages. After β selection, we found that DP thymocytes decrease their CD4 expression to become transitional single positive (TSP) $CD8^+$ thymocytes [77]. Positive selection occurs in the TSP $CD8^+$ developmental stage. In addition, the initial steps towards CD4/CD8 lineage commitment begin in the TSP $CD8^+$ stage. Cells destined to become mature $CD8^+$ thymocytes continue to down-regulate CD4 and remain $CD8^+$. Cells destined to become mature $CD4^+$ thymocytes express CD4 to return to the $CD4^+CD8^+$ DP population before down-regulating CD8 [77].

To identify where Tregs emerge within our newly defined subsets of T cell development, we initially analyzed CD25 and FOXP3 expression. These markers were first detected in post-selection DP thymocytes that are newly committed to the CD4+ T cell lineage. Additionally, CD25hi FOXP3hi cells were found in the mature $CD4^+$ SP population. Because all tTregs express Helios, we added the analysis of Helios expression to FOXP3 and CD25 to facilitate the identification of developing Tregs [77]. Among mature $CD3^{hi}CD4^+$ thymocytes, Helios was only highly expressed in cells that also expressed FOXP3 and CD25. Helios+ FOXP3+ CD25+ thymocytes could also be found within the DP population, specifically among cells that had

already undergone positive selection. These cells were between the TSP $CD8^+$ thymocyte stage and the mature SP $CD4^+$ stage, indicating that these cells are in the process of committing to the SP $CD4^+$ lineage [77]. Thus, using this combination of markers, we could trace to the developmental stages in which commitment to the $CD4^+$ T cell lineage occurs. Independently of FOXP3 and CD25, Helios was also expressed in subsets of TSP $CD8^+$ thymocytes [77]. At this stage, Helios might mark the earliest Treg population or thymocytes undergoing negative selection [83]. My dissertation work further expands on this research by correlating expression of Treg markers to Ikaros family members during the development of mature SP $CD4^+$ thymocytes.

Differentiation of peripheral Tregs

In addition to tTregs that develop in the thymus, naïve CD4+ CD25- FOXP3- T cells can differentiate into peripherally-derived Tregs (pTreg) cells upon recognition of cognate antigen under tolerogenic conditions [26]. It is difficult to study human pTreg generation as there are no accepted markers that distinguish pTregs from tTregs. However, in mice, lineage tracing experiments can be used to track Foxp3- CD4+ cells that upregulate Foxp3 upon differentiation into pTregs [84]. Through these studies, the types of antigens and the conditions needed to promote pTreg differentiation have been established. Analysis of the TCR specificity of murine pTregs revealed that generally recognize non-self antigen [84-91]. Chronic exposure of mice to low levels of foreign peptides via oral or intranasal routes can also increase the number of pTregs [27, 92-95]. Thus, the source of non-self antigens can come from environmental exposure to food or allergens. An additional source of pTreg cognate antigen is commensal bacteria [90, 96, 97]. Lathrop et al. [90] reported that murine pTregs isolated from the colon had a different TCR repertoire compared to other Tregs isolated from the periphery. Most of these TCRs recognized

antigens derived from commensal bacteria. Additionally, a specialized subset of CD103+ DCs in the mesenteric lymph nodes of mice was identified to be critical in pTreg generation in the gut [98]. Analysis of Treg TCR repertoire from both the thymus and peripheral blood in humans has revealed that CD25+ CD4+ Tregs have a similar diversity as CD25- CD4+ Tconvs [99]. Additionally, Tregs targeting allergens have been isolated from human peripheral blood and a commensal bacteria-derived molecule was able to induce human Tregs *in vitro* [100, 101]. Thus, pTregs that recognize non-self antigens could potentially exist in humans as well as mice. Naïve CD4+ T cells can differentiate into pTregs through recognition of cognate non-self antigen under non-inflammatory conditions.

Various soluble mediators of pTreg differentiation have also been identified. In both mouse and humans, the secretion of the cytokine TGFβ by dendritic cells is critical for pTreg differentiation [28, 102-105]. In mice, CD8+ CD205+ splenic DCs in peripheral lymphoid tissues and CD103+ DCs in the mesenteric lymph nodes are the primary sources of TGFβ during pTreg generation [98, 104, 106, 107]. TGFβ promotes survival of pTregs and directly drives FOXP3 expression in naïve CD4+ Tregs through downstream recruitment of the transcription factors Smad3 and NFAT to the FOXP3 promoter [89, 108]. Additionally, TGFβ maintains Treg phenotypic stability by opposing recruitment of DNA methyltransferase 1 (Dnmt1) to the conserved non-coding sequence 2 (CNS2) region of the FOXP3 gene locus [109]. CNS2 contains the Treg-specific demethylated region (TSDR) which needs to remain unmethylated to enhance FOXP3 expression [110, 111]. In the gut, retinoic acid secretion by DCs is required in addition to TGFβ to convert naïve CD4+ T cells to pTregs in response to commensal bacteria antigens [98]. Retinoic acid directly induces FOXP3 expression in pTregs and also blocks conversion of naïve CD4+ T cells to other Th subsets [112-115]. IL-2 is another cytokine required for

differentiation of naïve CD4+ T cells to pTreg. IL-2 not only supports pTreg growth and survival but directly opposes signals for Type 17 helper (Th17) cell differentiation [116, 117]. Naïve CD4+ T cell conversion to a Th17 cell requires TCR stimulation in the presence of TGFβ, similar to pTreg differentiation, and differs in the additional requirement of IL-6 [118]. IL-2 activation of STAT5 directly constrains Th17 differentiation [116]. The combination of TGFβ, retinoic acid and IL-2 is able to convert naïve human CD4+ T cells into functional *in vitro* induced Tregs (iTregs) [119, 120]. Thus, TGFβ, retinoic acid and IL-2 are critical for inducing and promoting the survival of pTregs.

In addition to TCR stimulation and cytokines, direct interaction of naïve CD4+ T cells with costimulatory molecules on APCs is important in pTreg differentiation. The signals that promote a pTreg phenotype would normally be inhibitory to a Tconv. For example, CTLA-4 is normally expressed on activated Tconv and binds CD80 or CD86 on APCs which leads to downregulation of pro-inflammatory pathways [121, 122]. Conversely, CTLA-4 is required for TGFβ-mediated pTreg differentiation and CTLA-4/CD80 interaction after TCR activation aids in FOXP3 expression after TGFβ stimulation [123]. Another critical costimulatory pathway involved in pTreg differentiation is CD28 activation. In the differentiation of naïve CD4+ T cells into pro-inflammatory Tconv, CD28 binding to B7 proteins on APCs is one of the primary "second signal" interactions required for the T cell to differentiate and become fully activated [124, 125]. In pTreg differentiation, CD28 activation is detrimental to pTreg generation [104, 126]. Overall, pTregs make up a unique subset of Tregs that have a TCR repertoire that recognizes non-self antigens and differentiate under specific conditions in the periphery.

Treg subsets

Upon stimulation, naïve CD4+ T cells have the potential to differentiate into multiple helper T (Th) cell subsets depending on co-stimulatory signals and cytokine stimulation. Each Th subset has a specific lineage defining transcription factor that drives specific functions and cytokine secretion [127]. Activated T cells then proliferate, migrate to distant sites depending on chemokine receptor expression and mediate an immune response. Activated T cells have the potential to differentiate into memory T cells which become re-activated upon subsequent exposure to the same cognate antigen [128]. While Tregs are often thought as one small subset of CD4+ T cells, they are a very heterogenous population. Under various conditions, Tregs can adopt different patterns of gene expression that modulate function and localization. Similar to Tconvs, naïve Tregs can become memory Tregs following activation [129-133]. Additionally, there are Treg subsets that express similar transcription factors and cytokines as other Th subsets [29, 134-138]. There are also groups of CD4+ T cells that do not express FOXP3 but are able to suppress via suppressive cytokine secretion [139-141]. Finally, there are also CD8+ Tregs that are primarily thymus-derived [43, 56, 142, 143]. The following sections will describe each of these Treg subsets in depth.

Naïve vs Memory Tregs

In both mice and humans, after TCR stimulation, naïve T cells become activated and lose CD45RA expression and gain CD45RO expression, a marker of effector or memory T cells depending on the co-expression of other surface markers [144]. Multiple studies have identified and characterized CD45RA+ and CD45RO+ Tregs in humans and termed these populations as naïve and memory Tregs [139-141]. Naïve Tregs are primarily derived from the thymus as the

majority of these cells express CD31, a marker of recent thymic emigration [132]. A majority of Tregs in humans are CD45RO+ [145]. Upon activation, CD45RA+ Tregs lose CD45RA expression and gain CD45RO+ expression [132, 133]. There is also evidence that FOXP3- Tconvs can also gain a memory Treg phenotype [132]. Both populations suppress responder T cell proliferation to a similar degree *in vitro* but also differ in immunosuppressive mechanisms. Naïve Tregs expressed higher levels of CXCR4 which localizes these cells to the bone marrow. On the other hand, memory Tregs have higher CCR4 and cutaneous lymphocyte antigen (CLA) expression which drives migration primarily to the skin [132]. Another difference between naïve and memory Tregs is their proliferative potential. Under homeostatic conditions, memory Tregs undergo a higher rate of proliferation than naïve Tregs [132, 133]. During co-culture with Tconvs in a suppression assay, naïve Tregs are activated and proliferate whereas memory Tregs undergo a higher rate of cell death, likely due to activation induced cell death [133]. This proliferation of memory Tregs is thought to be the primary source of new Tregs in humans as the thymus involutes during aging. In fact, older adults have a higher percentage of memory Tregs compared to naïve Tregs [132]. Thus, upon activation, naïve Tregs from the thymus differentiate into memory Tregs resulting in a change in gene expression and function.

Thelper subsets

Under different inflammatory conditions, naïve CD4+ Tconvs differentiate into various Thelper (Th) subsets with specialized functions [127]. The most common subsets are Type 1 helper (Th1), Type 2 helper (Th2), Type 17 helper (Th17) cells and T follicular helper (Tfh) cells and each has a lineage specific transcription factor that drives subset-specific function. Th1 cells express Tbet and target intracellular pathogens [146, 147], Th2 cells express GATA3 and are

responsible for killing parasites and mediate allergic reactions [146, 147], Th17 cells express RORγt and are specialized to clear fungal infections [147, 148] and Tfh cells express Bcl6 and mediate B cell maturation in germinal centers [149]. The Thelper subsets also express specific chemokine receptors and cytokines to localize cells to specific tissues and mediate a specialized immune response [150-156]. Duhen et al. [29] stained human Tregs for the chemokine receptors that distinguish the different Thelper subsets and FACS sorted the Tregs based on the pattern of chemokine receptor expression. Interestingly, there was a corresponding Treg for each Th population based on chemokine receptor expression. Specifically, Th1, Th2, Th17 and Th22-like Tregs were identified. Furthermore, these Th-like Treg subsets co-expressed FOXP3 with each Th lineage-specific transcription factor and had similar cytokine secretion profiles. Despite secretion of pro-inflammatory cytokines, each Treg subset was able to suppress proliferation of responder Tconvs *in vitro* [29]. Additional studies have identified Tregs that specifically target Tfh cells and express similar chemokine receptors and transcription factors [134, 157]. While production of pro-inflammatory cytokines does not affect *in vitro* suppression by these Treg subsets, they could affect Treg function *in vivo*. For example, a higher level of IL-17 producing Tregs have been identified in the mucosal tissue of patients with Crohn's disease compared to healthy patients [158]. While these IL-17-producing Tregs remain suppressive *in vitro*, IL-17 has been demonstrated to drive Crohn's disease pathogenesis, though it is unclear whether or not these Tregs are beneficial or detrimental to disease [158, 159]. Thus, under different inflammatory conditions, Tregs can also acquire specialized functions to better target different Th CD4+ subsets and mediate downregulation of inflammation. However, the secretion of pro-inflammatory cytokines by these Treg subsets can also exacerbate disease.

Tr1 and Th3 cells

In addition to Treg subsets that mimic Tconv subsets, there are unique subpopulations of Tregs that can develop from naïve CD4+ T cells in the periphery under specific cytokine stimulation. Interestingly, these subsets are able to suppress inflammation despite a lack of FOXP3 expression. Type 1 regulatory (Tr1) cells develop through TCR stimulation in the presence of high levels of IL-10 and Th3 cells develop in the presence of TGFβ [160, 161]. DCs are the primary source of IL-10 and TGFβ in the generation of Tr1 and Th3 cells respectively [162, 163]. Tr1 cells were first identified and characterized in OVA-specific TCR transgenic mice through repeated stimulation of T cells with OVA in the presence of IL-10 [140]. Th3 cells were identified in mice after induction of oral tolerance to myelin basic protein (MBP). In this model, Th3 cells were able to suppress MBP-specific Th1 cells via production of TGFβ [141]. Both Tr1 cells and Th3 cells upregulate expression of CTLA-4 and CD25, classical markers of Tregs [139, 160, 161]. Expression of integrin α4β1mediates migration of Tr1 cells to the endothelium of inflamed peripheral tissue, whereas Th3 cells express integrin α4β7, also known as CD103, and localize to mucosal tissues [139]. Tr1 cells primarily mediate immunosuppression via secretion of IL-10, a generally inhibitory cytokine. Th3 regulate the immune response through secretion of TGFβ which can drive conversion of naïve T cells into FOXP3+ pTregs as described previously, a phenomenon known as infectious tolerance or the bystander effect [164]. Both cells can also alter DC stimulation of T cells, indirectly reducing inflammation [139, 164]. Tr1 cells have been identified in humans and tested as a potential cell therapy in inflammatory bowel disease [165, 166]. Th3 cells have yet to be identified in humans but human iTregs can be induced by stimulation of naïve CD4+ T cells in the presence of TGFβ in addition to IL-2 and

retinoic acid [119, 120]. Thus, Tr1 and Th3 cells can develop in the periphery aid in immune regulation along with tTregs and FOXP3+ pTregs.

CD8+ Tregs

Treg research has primarily been focused on CD4+ Tregs but CD8+ Tregs also exist and were first identified in 1970 by Gershon and Kondo [167]. CD8+ Tregs have been difficult to study until recently due to a lack of defined markers to distinguish them from conventional CD8+ T cells. CD8+ Tregs in mice are CD8+, Ly49+, CD122+ and CD44+ [143, 168] and in humans, the predominant CD8+ Treg subset that has been described is CD8+, CD28- [169, 170]. Additional markers of human CD8+ Tregs include upregulation of CD25, CD122, CD45RO and/or CCR7[171]. In addition to these surface markers, FOXP3+ and FOXP3- CD8+ Tregs also exist. As previously described, FOXP3+ CD8+ Tregs develop from DP thymocytes. The TCR's on most CD8+ Tregs are restricted to a non-classical MHC Ib molecule called Qa-1 in mice [172, 173] and HLA-E in humans [174-176]. This section will focus on the Qa-1/HLA-E restricted FOXP3+ CD8+ Tregs, which are the most well characterized CD8+ Treg population. Under homeostatic conditions, Qa-1/HLA-E presents peptides derived from the signal sequence of classical MHCI [177]. During stress or under abnormal antigen processing conditions such as cancer, loading of MHCI peptide onto Qa-1/HLA-E is disrupted and presentation of a different peptide results in CD8+ Treg stimulation via TCR [178-183]. The Qa-1/HLA-E-peptide complex can bind TCR or NKG2A/CD94, a set of receptors expressed by natural killer (NK) cells [184, 185]. Qa-1 knock out mouse experiments demonstrated the importance of Qa-1-peptide-TCR interaction in CD8+ Treg development, as these mice developed severe autoimmune disease [168, 186, 187]. Additionally, in humans, CD8+ Tregs have been identified in the tumor

microenvironment and act as mediators of tumor immune evasion [188-190]. CD8+ Tregs regulate the immune response through direct mechanisms such as cytolysis of CD4+ T cells, cell-cell contact via inhibitory co-receptors such as CTLA-4, and indirect actions such as modifying DC expression of costimulatory molecules and inhibitory cytokine secretion [168, 191, 192]. CD8+ Tregs are primarily found in mucosal tissues and rarely found in the blood [56]. Thus, CD8+ Tregs as well as CD4+ Tregs play a major role in immune regulation and human disease.

Treg Phenotype Stability

With the ability of Treg subsets to acquire the ability to secrete pro-inflammatory cytokines under certain inflammatory conditions, the question has arisen if Tregs can lose their immunosuppressive capacity. The primary regulators of Treg function are FOXP3 expression and epigenetic modifications within the Treg genome, which will be discussed in detail in later sections. If any of these factors are lost, Tregs have a higher potential of converting into a pro-inflammatory phenotype. There has been much debate over the stability of the Treg phenotype. This question has primarily been addressed in Treg lineage tracing assays in mice. These assays use FOXP3-GFP mice that express a FOXP3 driven Cre that cleaves the stop codon out of a Rosa-LSL-YFP or a ROSA-LSL-RFP reporter. Thus, any cells that express FOXP3 would be GFP+ and YFP+/RFP+ but upon loss of FOXP3, cells would be GFP- YFP+/RFP+. One experiment by Zhou et al. [193], determined that a small subset of CD4+ FOXP3+ T cells lost FOXP3 expression under lymphopenic or inflammatory conditions and these cells were termed “exTregs.” Furthermore, when these exTregs were transferred into mice, they caused autoimmunity [193]. Interestingly, exTregs were able to regain FOXP3 expression after

inflammation was resolved or IL-2 was administered [194]. However, another experiment by Rubtsov et al. [195] that integrated GFP into the FOXP3 locus rather than using FOXP3-GFP expressed on an artificial chromosome and used a conditional Cre that was induced by tamoxifen exposure. They found that FOXP3+ Tregs were stable and 96% of the cells maintained FOXP3 expression under lymphopenic and inflammatory conditions [195].

To reconcile these differences, Miyao et al. [196] repeated these experiments and also integrated GFP into the chromosome and used a ROSA-LSL-RFP reporter. They found that there was indeed a group of GFP- RFP+ cells that lost FOXP3 expression. However, they determined that these cells were actually FOXP3- cells that transiently upregulated FOXP3 and then lost FOXP3 expression. These cells were RFPlo, CD25lo and GFP+ prior to losing FOXP3 expression [196]. The differences in these experiments may have been due to the timing of the FOXP3 labeling as Zhou et al. and Miyao et al. labeled FOXP3+ cells from the start and Miyao et al. labeled FOXP3 at a certain timepoint with the addition of tamoxifen. It is likely that more exTregs were observed in the experiments performed by Zhou et al. and Miyao et al. because there was a longer time frame to label the cells expressing low levels of FOXP3 or unstably expressing FOXP3. On the other hand, the cells that were GFP+ in Rubtsov et al. were likely committed Tregs that were stably expressing FOXP3. The idea that not all FOXP3+ cells are committed to a Treg phenotype is termed the "heterogeneity model" [197, 198]. This is evident in humans as Tconvs can acquire transient low FOXP3+ expression following stimulation [133, 199, 200]. Despite the debate over the origin of the exTregs, it clear that in mice, there is a subset of FOXP3+ cells that lose FOXP3 expression under lymphopenic and inflammatory conditions. Furthermore, these exTregs are able to cause disease [193]. In humans, CD4+ CD25hi CD127lo FOXP3+ Tregs can lose FOXP3 expression after multiple TCR stimulations *in vitro* and memory

Tregs have been shown to lose FOXP3 expression after long term expansion *in vitro* [133, 201, 202]. The potential of Tregs converting into autoreactive Tconvs is of great concern especially in the field of Treg immunotherapy. Thus, it is critical to elucidate the factors required to identify uncommitted FOXP3+ cells and maintaining Treg stability.

Mechanisms of Treg-mediated immunosuppression

Tregs mediate immune homeostasis through suppression of multiple aspects of immune activity including Tconv proliferation and cytokine secretion, expression of costimulatory molecules and antigen presentation by APCs [33]. Different Treg functions have generally been elucidated via *in vitro* suppression assays where Tregs are co-cultured with responder Tconvs and proliferation of Tconvs is assayed following stimulation. However, the context of Treg function has proven to be important as mechanisms that were identified as not critical for *in vitro* suppression have been shown to be necessary in various murine models of inflammatory diseases. This section will focus on the direct and indirect mechanisms of Treg-mediated immune suppression that target Tconv and APCs that have been identified in *in vitro* and *in vivo* assays.

Treg suppression of Tconv

Tregs can directly suppress proliferation and function of Tconvs following stimulation *in vitro*. The primary effect of Treg suppression is a reduction of Tconv IL-2 production, a key T cell growth factor as well as other pro-inflammatory cytokines such as IFNγ [203-207]. Reduction of IL-2 mRNA in Tconv after co-culture with Tregs *in vitro* has been observed in both murine and human cells [203-206]. How Tregs mediate reduce IL-2 production by responder T

cells is largely unknown, but *in vitro,* cell-cell contact is required [204, 206, 208]. Schmidt et al. [209] demonstrated that following co-culture of Tconv and pre-activated Tregs *in vitro,* there was a decrease in calcium influx following TCR stimulation in Tconvs. This decrease in calcium influx reduced downstream NFAT and NFκB activity and directly inhibited cytokine transcription. The exact mechanism of Treg-mediated reduction in Tconv calcium signaling has yet to be elucidated. Tregs can also compete with Tconvs for IL-2 via high expression of the IL-2 receptor alpha chain (CD25) and expression of the other IL-2 receptor components [205, 210]. Pandiyan et al. [211] demonstrated that Treg-mediated cytokine starvation led to apoptosis of Tconv. This mechanism of Treg suppression has been controversial as multiple studies did not observe apoptosis in Tconvs following Treg co-culture in both human and murine cells [207, 212, 213]. Additionally, blocking CD25 on human Tregs did not reduce Treg-mediated suppression of mouse Tconvs and adding IL-2 only partially reduced Tconvs suppression by Tregs [207, 214]. While Tregs likely compete with Tconvs for IL-2, this is not likely the primary mechanism of Treg immunosuppression. Thus, changing the cytokine milieu is a critical mechanism of Treg activity.

In addition to affecting availability of cytokines, Tregs can also block Tconv activity through secretion of inhibitory cytokines. The primary Treg inhibitory cytokines that have been identified are TGFβ, IL-10 and IL-35. TGFβ predominately acts to convert Tconv to Tregs in the presence of DCs as previously described [214, 215]. TGFβ can also act to suppress proliferation and cytokine secretion in Tconvs [216]. The importance of TGFβ in Treg-mediated suppression is controversial. The role of TGFβ in maintaining tolerance is evident in the fact that TGFβ or TGFβ receptor KO mice develop autoimmunity [217, 218]. However, TGFβ-deficient Tregs can still suppress Tconv proliferation *in vitro* [219, 220]. Interestingly, TGFβ production by Tregs is

necessary to suppress colitis *in vivo* [219, 220].Thus, the importance of TGFβ in suppressing inflammation may depend on the disease. IL-10 is a predominately inhibitory cytokine but can be pro-inflammatory at different concentrations and cell contexts [221, 222]. Tconvs treated with IL-10 *in vitro* have reduced cytokine secretion [222] and naïve Tconv can be converted to IL-10 secreting Tr1 cells in the presence of IL-10 during TCR stimulation [165]. IL-10 is specifically important in preventing gut inflammation as IL-10 KO mice spontaneously develop colitis [223]. Treg production of IL-10 is important in the suppression of multiple CD4+ Th subsets including Th17 cells and Th1 cells [224, 225]. IL-35 is a cytokine that is made up of Epstein-Barr virus induced gene 3 (*Ebi3*) and *IL12a* [226]. IL-35 KO Tregs have reduced suppression *in vitro* and *in vivo* in a murine colitis model [227]. Human Tregs do not normally make a high level of IL-35 but IL-35 production can be induced by iTregs *in vitro* [203]. Treg secretion of inhibitory cytokines plays a key role in inhibiting Tconv proliferation and activation.

While many Treg functions are mediated through soluble mediators, Tregs can also directly interact with Tconv through cell-cell contact. One inhibitory surface molecule that mediates direct contact between Tregs and Tconvs is Galectin-1, a β-galactoside-binding protein [228]. Galectin-1 is upregulated on Tregs upon TCR stimulation and binds ligand on Tconvs which leads to cell cycle arrest, apoptosis and reduced cytokine secretion. Blocking Galectin-1 reduces Treg suppression in both human and mice [228]. In addition to direct inhibition through surface molecules, both human and murine Tregs can directly kill Tconvs via perforin activity and granzyme secretion [229, 230]. In murine tumor models, approximately 5-30% of Tregs expressed granzyme B and these Tregs could kill NK and CD8+ T cells [231]. Thus, direct contact of Tregs and Tconvs is critical in some mechanisms of Treg suppression.

Treg suppression of APCs

Tregs can indirectly suppress Tconvs through interactions with APCs. Tregs have several surface molecules that interact with APCs and reduce APC activation of Tconv. This section will focus on the activity of CTLA-4, LAG-3 and Nrp-1. CTLA-4 deficiency or blockade in mice leads to autoimmunity that is reversed upon transfer of wild-type Tregs [20, 232]. Human Tregs require CTLA-4 to suppress APC-mediated Tconv proliferation *in vitro* [209]. Binding of CTLA-4 to CD80/CD86 on DCs leads to downregulation of these costimulatory molecules. The lack of costimulation following CTLA-4 binding of DCs inhibits Tconv activation both *in vitro* and *in vivo* [233-235]. CTLA-4 stimulation also leads to indoleamine 2,3-dioxygenase (IDO) secretion by DCs which acts to suppress Tconv proliferation [236, 237]. LAG-3 is another inhibitory surface protein that is upregulated on Tregs. Binding of LAG-3 to MHCII on DCs inhibits DC activation and maturation leading to decreased antigen presentation [238, 239]. Treg expression of neuropilin-1 (Nrp-1) promotes Treg interaction with immature DCs, allowing for prolonged suppression and reduces antigen presentation by APCs [240, 241]. Blocking Nrp-1 *in vitro* reduces suppression when Tconvs are stimulation with low amounts of antigen [240]. Direct interaction of Tregs with APCs plays a major role in reducing Tconv activation via TCR-MHCII stimulation and co-stimulation.

In addition to inhibitory surface molecules, Treg TCR's can also recognize cognate peptide presented on MHCII on APCs. The role of antigen specificity in Treg function is under constant debate. Polyclonal Tregs can suppress a murine model of experimental autoimmune encephalitis (EAE) and OVA-specific diabetes by blocking proliferation of effector T cells in lymph nodes [242, 243]. It is thought that polyclonal Tregs do not require TCR stimulation to mediate suppression. However, there is evidence that antigen-specific Tregs are more potent than

polyclonal Tregs at suppressing autoimmunity *in vivo* [244, 245]. Multiple studies used two photon laser scanning to investigate interaction of antigen-specific Tregs with APCs and Tconvs [246, 247]. These studies demonstrated that antigen-specific Tregs had longer interactions with naïve antigen-loaded DCs and reduced antigen-specific Tconv-DC interactions. These interactions led to reduced cytokine production by Tconvs. Interestingly, there was no evidence of direct Treg-Teff interactions, indicating that *in vivo*, Tregs primarily target DCs. A recent publication by Akkaya et al. [248] expanded on these observations and demonstrated that antigen-specific Tregs were binding DCs via TCR-MHCII binding and removing MHCII-peptide complexes through a process known as trogocytosis. This novel Treg mechanism reduces APC presentation of antigens to Tconv and subsequent activation. Thus, while polyclonal Tregs are able to inhibit the immune response *in vitro* and *in vivo*, antigen-specific Tregs have a unique set of immunosuppressive properties that make them more potent than polyclonal Tregs.

Finally, Tregs also use soluble mediators to suppress APCs. IL-10 not only directly reduces Tconv proliferation and cytokine production but also acts to downregulate costimulatory molecule expression on APCs [165]. Additionally, 50% of human Tregs express a pair of surface ectonucleases, CD39 and CD73, that together cleave extracellular ATP into adenosine [249-252]. Adenosine binds the A2A on DCs and decreases costimulation by APCs [253, 254]. CD39-deficient Tregs have reduced suppressive capacity [252]. Tregs utilize multiple soluble molecules to inhibit APC stimulation and activation of Tconvs.

FOXP3 and Tregs

A hallmark of Tregs is high expression of the transcription factor FOXP3. FOXP3 is necessary for Treg-mediated immune homeostasis, as mice and humans deficient in FOXP3

develop severe autoimmune diseases[17, 18]. Ectopic expression of FOXP3 in both murine and human $CD4^+$ Tconvs conveys a Treg phenotype including reduced cytokine production and slowed proliferation. Furthermore, these FOXP3 overexpressing cells were able to suppress responder T cell activity *in vitro* and *in vivo* [14, 32, 255]. Thus, FOXP3 not only defines Tregs in both mice and humans but is the key transcription factor in driving Treg activity.

Structure and downstream signaling

FOXP3 is a member of the subfamily P of the FOX protein family [256]. Members of the FOX protein family all have a forkhead/winged helix DNA binding region [257]. Other structural features of FOXP3 include a central domain in the C terminus, which contains a C2H2 zinc finger and leucine zipper, and a repressor domain in the N-terminus [258, 259]. FOXP3 primarily mediates transcriptional regulation through interaction with other transcription factors. The forkhead domain of FOXP3 binds the target sequence of AP-1, a downstream transcription factor that is activated with TCR signaling. Consequently, binding of FOXP3 to this sequence blocks AP-1-NFAT interaction and inhibits T cell activation [260]. FOXP3 alters T cell transcription through formation of oligomers with other transcription factors via the zinc finger and leucine zipper domains [261]. For example, FOXP3 binding of DNA with NFAT is required for repression of *Il2*, *Ctla-4* and *Cd25* transcripts and Treg suppressive activity [260]. Binding of FOXP3 via the leucine zipper to the AML1/Runx1 complex is also critical for suppression of IL-2 production and suppression by Tregs [262]. FOXP3 is also able to remodel chromatin and gene accessibility through binding of HAT/HDAC complexes via the repressor domain in the N-terminus [263]. For example, Foxp3 gets acetylated by the HAT TIP60 which increases binding of Foxp3 to the *Il2* promoter and increases repression of *Il2* [264]. In Tregs with a Th phenotype,

FOXP3 is able to directly inhibit subset-specific transcription factors and reduce pro-inflammatory activity [262, 265]. While the exact mechanism of FOXP3 activity have yet to be elucidated, it is clear that FOXP3 alters existing transcriptional machinery in a T cell to promote a Treg phenotype.

Induction of FOXP3 in Tregs

As described in the "Origin of Tregs" section above, multiple factors are critical for inducing FOXP3 expression in Tregs. These factors promote binding of transcription factors to one or more of the conserved non-coding sequences (CNSs) in the *Foxp3* locus. Four FOXP3 CNSs have been identified and each one is specific to different aspects of Treg function such as tTreg development, pTreg differentiation or maintenance of Treg stability [57]. The most critical factor in promoting FOXP3 expression in Tregs is IL-2 signaling via STAT5. A deficiency of IL-2 or IL-2 signaling in both mice and humans leads to a significant decrease Treg numbers and increase in autoimmunity [262, 266-271]. STAT5 directly binds to CNS2 which maintains Treg phenotype and heritability of FOXP3 expression [272]. As previously described, TGFβ directly recruits Smad3 and NFAT to CNS1 [89, 108]. TGFβ also blocks demethylation of the TSDR in CNS2 which is required to maintain stable FOXP3 expression [109]. Binding of Treg TCR to cognate peptide on MHCII initiates FOXP3 expression in developing Tregs [273, 274]. TCR signaling activates multiple transcription factors involved in binding of *Foxp3* CNSs such as NFAT, NFκB and AP-1 [68]. In addition to initiating FOXP3 expression, maintenance of a high level of FOXP3 has been demonstrated to be critical to retaining a Treg phenotype. Mice altered to have low levels of Foxp3 or induced loss of Foxp3 had a loss of Treg suppressive activity and an acquisition of effector T cell function [275, 276]. Thus, multiple factors not only initiate

FOXP3 in Tregs but must maintain FOXP3 expression in order for Tregs to maintain a suppressive phenotype.

Role of FOXP3 in Treg function

It is evident that FOXP3 is the key mediator of Treg function as knock out of FOXP3 in Tregs blocks suppressive activity and overexpression of FOXP3 in Tconv conveys expression of canonical Treg genes [193, 277]. All the effects of FOXP3 expression in Tregs are unknown but knock-down and knock-out studies have revealed a few functions. Specific downstream effects of FOXP3 activity have been previously described including inducing expression of Treg markers such as CD25 and CTLA-4 and repression of IL-2 [260, 278, 279]. FOXP3 directly binds to 20-30% of the genes it regulates and acts as both a transcriptional repressor or enhancer [280-282]. Interestingly, through RNA sequencing and microarray assay analysis, it has been demonstrated that FOXP3 overexpression in Tconvs is not enough to completely recapitulate a Treg transcriptional signature and part of the signature can be induced in the absence of FOXP3 [283, 284]. Thus, while FOXP3 is the hallmark transcription factor in Tregs, other factors are required to completely convey a Treg phenotype.

The Ikaros Family of Transcription Factors and Tregs

In addition to FOXP3, members of the Ikaros family of transcription factors are highly expressed in Tregs [25, 46, 285]. The five members of the Ikaros family are Ikaros, Helios, Aiolos, Eos and Pegasus. Helios and Eos are highly expressed in a majority of Tregs [25, 285]. While the expression of Helios and Eos is well characterized, the downstream signaling and Treg

functions mediated by these transcription factors are less clear. This section will summarize what is known about all of the Ikaros family members in Treg development and function.

Structure and downstream signaling

Each Ikaros family member has four DNA-binding zinc finger motifs near the N-terminus and two C-terminal zinc fingers that mediate protein-protein interactions [17]. Each family member can homodimerize or heterodimerize via the C-terminal zinc fingers in every possible combination [17-21]. Furthermore, each member can undergo alternative splicing that eliminates one or more of the N-terminal zinc fingers [22-24]. Deletion of more than two zinc fingers results in a dominant negative form of the protein that can dimerize with other family members but cannot bind DNA [17]. Thus, changes in one Ikaros family member often leads to changes in downstream signaling of all the family members.

The two Ikaros family members that have been extensively studied in Tregs are Helios and Eos. Both Helios and Eos have been shown to be highly expressed in a majority of Tregs in both mice and humans [46, 285]. Moreover, ectopic expression of these transcription factors in mouse Tconvs has been demonstrated to convey part of the Treg signature [277] . Thus, these transcription factors play a major role in mediating the Treg phenotype. Downstream signaling of Helios and Eos in Tregs has yet to be fully elucidated but recent work in murine Tregs has shed light on the topic. Kim et al. [43] carried out ChIP-Seq analysis on Helios-bound sites in both CD4+ and CD8+ murine Tregs. These data revealed that Helios bound primarily promoter regions of genes involved in apoptosis and survival, cell cycle progression and autophagy. Importantly, the IL-2Rα-STAT5 pathway was a major target in the Helios gene network [43]. As previously described, the IL-2Rα-STAT5 pathway is crucial for maintaining Treg FOXP3

expression and survival [286]. While Helios does not directly interact with Foxp3, Eos is able to bind Foxp3 in murine Tregs and mediate downstream activity [285]. Eos interaction with Foxp3 facilitates repression of pro-inflammatory genes such as *Il2* or *Ifng.* Knocking down Eos in Tregs reduced Foxp3-mediated suppression of IL-2 production in murine Tregs [285]. Additionally, Eos recruits a co-repressor, C terminal binding protein 1 (CtBP1), to promoters and together, Eos and CtBP1 mediate histone modifications and methylation of genes that are silenced in Tregs [285]. Thus, while Helios primarily maintains high FOXP3 expression and improved survival in murine Tregs through STAT5 signaling, Eos aids in the gene silencing activity of FOXP3 and directly silences genes through mediation of epigenetic modifications.

Expression of Ikaros family members during Treg development

During our analysis of human T cell development, we tracked the expression patterns of Ikaros family members. Using intracellular staining and flow cytometry, we showed that protein levels of Ikaros, Aiolos, and Helios increase when thymocytes undergo β selection, but the increase in Helios expression is greater than Ikaros and Aiolos. Further, the increase observed for Ikaros and Helios was transient while Aiolos levels remained elevated as thymocytes continued to mature. Similarly, the protein levels of Ikaros, Helios, and Aiolos increased when thymocytes underwent positive selection. Again, the spike in Ikaros and Helios expression was transient while Aiolos levels remained elevated in subsequent thymocyte populations[77].

These data indicate that the ratio of Ikaros family members changes at β selection and positive selection, suggesting that the nature of the dimers likely changes. The significance of changing the dimer composition as cells progress through T cell development is unknown, but it is likely to influence the transcriptional activity of the complex [287].

Role of Ikaros family members in Treg function

Most Tregs express Helios, including all thymic $CD4^+$ Tregs and approximately 70% of circulating Tregs [25], and Tregs that lack Helios express Aiolos [46]. In both mice and humans, $Helios^+$ Tregs more effectively suppress cytokine production by Tconv cells [45, 48], while $Helios^-$ Tregs secrete more pro-inflammatory cytokines, including IFNγ, IL-2, and IL-17 [46]. Besides differences in functionality between Helios+ and Helios- Tregs, Helios+ Tregs are more stable under inflammatory conditions than Helios- Tregs [25, 47, 288, 289]. Knock-down of Helios in murine Tregs decreased immunosuppressive function and survival *in vitro* and increased pathology in a murine colitis model [290]. Conversely, overexpression of Helios in murine Tregs improved secretion of immunosuppressive cytokines and suppression of T cell proliferation *in vitro* [291]. Experiments with Treg-specific Helios knockout mice have further elucidated Helios' role in Treg function *in vivo*. These mice developed spontaneous autoimmune disease at 6-8 months of age, which was attributed to increased numbers of activated CD4+ and CD8+ T cells and germinal center B cells [43]. Of the CD4+ T cells, T follicular helper (Tfh) cells and Type I helper T (Th1) cells were the most affected [42, 43]. Both CD4+ and CD8+ Tregs isolated from these mice demonstrated reduced suppression in an *in vitro* suppression assay in *Rag2-/-* mice [43]. Additionally, both these Treg subsets demonstrated increased apoptosis and reduced survival *in vivo* under inflammatory conditions [42, 43]. CD4+ Helios deficient Tregs also had increased IFNγ production when stimulated *in vitro*. Thus, Helios plays a major role in suppressing a pro-inflammatory phenotype in Tregs and promoting Treg survival. While it is clear that Helios expression maintains a suppressive phenotype in human Tregs, ectopic of Helios in Jurkat cells, a human T cell line, actually reduces survival [44]. Thus, there may be differences between the role of Helios in murine versus human Tregs.

Eos is also expressed in Tregs and is often co-expressed with Helios [46]. As previously described, Eos directly binds FOXP3 and is necessary for FOXP3-mediated gene repression [285, 292]. Eos can also inhibit expression of non-Treg genes such as IL-2 [277, 285, 288]. Tregs can convert into Tconv cells under inflammatory conditions and this process requires downregulation of Eos [288]. Additionally, knock-down of Eos via siRNA in Tregs results in reduced immunosuppressive activity both *in vitro* and *in vivo* in a murine colitis model [285]. Interestingly, Tregs isolated from Eos knock out mice did not differ from wild-type Tregs in immunosuppressive activity or cytokine secretion, indicating a redundant role for Eos in Tregs [293]. These differences in results were attributed to the possibility the siRNA knock down of Eos may allow for expression of dominant-negative isoforms of Eos resulting in differing downstream effects compared to the global mouse knock out. Thus, the Ikaros family members can control the stability and phenotype of Tregs and mediate very specific functions in Tregs.

Treg therapy and engineered Tregs

The immunosuppressive properties of Tregs have led to much research into utilization of Tregs as therapy for a variety of inflammatory diseases. In general, Tregs are isolated from patients or a third party, non-immunogenic source, such as umbilical cord blood (UCB), expanded ex vivo and transfused back into patients. Trials for multiple diseases such as GVHD, IBD and Type I diabetes have shown that Treg infusions are safe, but only moderately successful [35-39]. A major challenge is expanding Tregs to numbers required to treat disease. Another difficulty of Treg therapy is isolating a pure population of Tregs. Tregs are commonly isolated by selecting $CD4^+$ and $CD25^+$ T cells but these markers are also expressed by activated Tconvs [22]. This leads to potential contamination of Tregs with Tconvs that could exacerbate disease.

Another limitation of Treg therapy is stability of phenotype as Tregs can convert to a Tconvs and lose immunosuppressive activity [198].

Given these challenges in utilizing expanded Tregs from blood for treatment of inflammatory disease, alternative approaches have been investigated. Because Tregs represent rare populations *in vivo,* attempts have been made to produce them in culture [23]. For example, *in vitro*-induced Tregs (iTregs) can be generated by stimulating $CD4^+$ T cells with various cytokines and drugs such as TGFβ, rapamycin and retinoic acid [31]. However, current iTregs do not retain FOXP3 expression and do not effectively treat a humanized model of GVHD [31]. tTregs from discarded human thymus have also been explored as a source of Tregs for therapy. Larger numbers of Tregs could be isolated from the thymus and they remained stable under inflammatory conditions, unlike Tregs from peripheral blood. Furthermore, expanded tTregs delayed GVHD in a xenogeneic murine GVHD model more effectively than Tregs from peripheral blood [289]. These data indicate that Treg therapy has great potential, but there is much room for improvement.

Another approach to overcome the limitations of Treg therapy is the generation of engineered Tregs (eTregs). eTregs are created by expressing known Treg genes via retroviral or lentiviral transduction of CD4+ T cells isolated from peripheral blood. Total CD4+ T cells make up about 4-20% of total leukocytes and can be isolated in greater numbers and expanded more quickly than naturally occurring Tregs. Co-expression of Treg genes with a transduction marker allows for purification of transduced cells and ensures homogeneity [294]. Additionally, an inducible caspase can be included in the DNA construct that can act as a suicide gene should the engineered Tregs result in any unwanted side effects [295]. Constitutively expressing Treg transcription factors should stabilize the Treg phenotype by out-competing any other

transcription factors that could convert the Treg into a Tconv. Finally, engineered Tregs can be designed to express receptors that can target them to a specific tissue, resulting in localized immunosuppression. While the advantages of engineered Tregs are clear, the Treg genes necessary to create an optimal engineered Treg are still undefined.

As previously described, enforced FOXP3 expression in Tconvs is able to convey immunosuppressive function in both human and murine T cells [14, 32]. These FOXP3 expressing eTregs were able to reduce proliferation of responder cells and delay disease in murine colitis and GVHD models [14, 32, 255]. However, when compared to endogenous Tregs in a murine arthritis model, FOXP3 eTregs were not as effective at reducing symptoms [296]. This is expected as Hill et al. [277] demonstrated that ectopic Foxp3 expression in murine Tconv only partially conveys a Treg gene signature. Another characteristic of Tregs is the secretion of the immunosuppressive cytokine IL-10 [165]. Ectopic expression of IL-10 in Tconvs ameliorated disease in murine IBD models but not as efficiently as naturally occurring Tregs [297]. Other Treg mechanisms that could be incorporated into eTregs are killing of immune cells via perforin or granzyme B and contact-dependent regulation of immune cells via molecules such as CTLA-4 or LAG-3 [33].

My dissertation work addressed whether Helios expression improves the function of FOXP3-transduced eTregs. In addition to FOXP3, the Ikaros transcription factor Helios is highly expressed in about 70% of FOXP3+ Tregs [25]. tTregs have been demonstrated to suppress more effectively than pTregs and tTregs express higher levels of Helios than pTregs [11, 21]. Helios+ Tregs can more effectively suppress Tconv cytokine secretion and are more stable under inflammatory conditions than Helios- Tregs [9, 20, 21]. Knock-down of Helios in Tregs decreased immunosuppressive function *in vitro* and *in vivo* in a murine IBD model [23].

Furthermore, enforced Helios expression in Tregs has been demonstrated to improve Treg function [24]. Finally, ectopic expression of both Helios and Foxp3 in murine CD4+ Tconvs increased the Treg signature index compared to Tconvs expressing Foxp3 alone [298]. Thus, the hypothesis of my thesis was that ectopic expression of Helios with FOXP3 in eTregs would result in improved immunosuppression and stability of FOXP3 transduced eTregs.

Graft versus Host Disease (GVHD)

GVHD is the most common disease being studied in the Treg therapy field and the work in this dissertation used a xenogeneic graft versus host disease model. Allogeneic hematopoietic stem cell transplants (HSCT) have become routine treatment for patients suffering from hematological malignancies such as leukemia [299]. A significant complication of HSCT is graft-versus-host disease (GVHD), which affects approximately 50% of HSCT patients and is lethal in approximately half of the patients who suffer from GVHD [300]. GVHD occurs when donor-derived T cells present in the graft attack recipient's organs. Acute GVHD (aGVHD) and chronic GVHD (cGVHD) are defined by different clinical symptoms caused by an underlying difference in immunopathology and timing of disease onset [301]. aGVHD presents as rapid systemic inflammation and multi-organ dysfunction driven primarily by activated T cells while cGVHD presents as a late-onset autoimmune-like disease mediated by both T cells and B cells [301]. This section will focus on the pathogenesis and pre-clinical studies of aGVHD. The only successful therapy includes highly immunosuppressive drugs, which increase the risk of infection and are highly toxic [302, 303]. Because these drugs are unsuccessful in half of GVHD patients [304], there is a critical need for improved GVHD therapies with minimal toxicity that promote long-term remission.

Disease Pathogenesis

It is difficult to fully match major and minor antigens for bone marrow donors and recipients. Thus, many HSCT patients receive grafts from partially matched donors and alloreactivity is kept under control with immunosuppressants. This immunosuppression is ineffective in 30-50% of patients who develop GVHD. Acute GVHD develops when donor immune cells are transferred along with donor stem cells. The presence of donor immune cells is required for proper engraftment of stem cells, prevention of infection and a graft-versus-tumor effect to help clear cancerous cells [305-307]. In GVHD, donor T cells are activated by APCs presenting host antigens following tissue damage caused by the conditioning regimen. The activated T cells then secrete inflammatory mediators that recruit and activate other arms of the immune system which results in a systemic inflammatory response [308]. Symptoms of disease include multi-organ failure, predominantly in the gut and the liver, and skin pathologies [309].

Murine models of GVHD

Acute GVHD models have been developed in mice, canines, pigs, and non-human primates. The most common models are the major histocompatibility complex (MHC)-mismatch mouse model and the xenogeneic mouse model (xenoGVHD) [1, 301]. Both models are lethal, and acute GVHD progression is tracked over time using a clinical scoring system described by Cooke and colleagues [310]. Briefly, five clinical signs are assessed on a scale of 0, 1, or 2 at several time points: weight loss, posture, fur texture, skin integrity and activity. Most studies aim to cure GVHD in these models but often a delay in disease progression is observed rather than

total prevention of disease due to the robust immune response to mismatched allografts and xenografts.

In the MHC-mismatch model, lethally irradiated mice receive splenic T cells and T cell-depleted bone marrow cells from MHC-mismatched mice. The mice engraft with donor hematopoietic cells and transplanted CD4+ and CD8+ T cells target allogeneic tissue antigens leading to the disease manifestations. Mice will develop acute GVHD by 10-20 days. C57BL/6 ($H\text{-}2^b$)→ BALB/c ($H\text{-}2^d$) is the most commonly used MHC-mismatch model [311]. The other major acute GVHD murine model is the xenoGVHD model, which involves sublethal irradiation of NOD-SCID IL-2Rγnull (NSG) mice followed by IV injection of human peripheral blood mononuclear cells (PBMCs), causing death from acute GVHD in 30-50 days [312, 313]. In this model, human T cells, primarily CD4+ T cells, are activated by human antigen presenting cells (APCs) among the PBMCs [314]. These T cells then expand and traffic to target organs such as the lung, liver, kidney and skin [315, 316].

As with all murine models, there are limitations with the xenoGVHD and MHC-mismatch models [317]. The radiation dose, mouse strain, anatomical sites of T cell transfer, and microbial environment can all affect disease severity and lead to experimental variability. Other factors that differ from standard clinical practice include the use of irradiation as the only form of conditioning and the lack of GVHD prophylaxis in control groups [301, 318]. Large animal acute GVHD models in canines, pigs, and non-human primates may offer more human-like conditions to study acute GVHD but required specialized expertise [319-321]. Despite these limitations, insights from animal studies have led to what is now the standard of care for the treatment and prevention of acute GVHD.

Treg Therapy in GVHD

Pre-clinical studies of Treg therapy for prevention of disease in murine models of GVHD has yielded promising results. Multiple studies have demonstrated that murine Treg infusion is able to control GVHD in a murine MHC-mismatch model [322-324]. Furthermore, Treg treatment did not prevent a graft-versus-tumor affect in murine tumor models [322-324]. Infusion of human Tregs in the xenoGVHD model were also able to prevent disease mediated by human T cells. These studies tested human Tregs from a variety of sources including peripheral blood, umbilical cord blood, discarded human thymus and *in vitro* induced Tregs [289, 325-329]. These studies have revealed mechanisms of Treg-mediated immunosuppression that are critical for delaying disease in the xenoGVHD model. Hahn et al. [330] demonstrated that administration of soluble Glycoprotein A repetitions predominant (GARP) improved human Treg prevention of xenoGVHD. Conversely, blocking GARP/TGFβ1 interaction with monoclonal antibodies was able to inhibit Treg-mediated immunosuppression in xenoGVHD mice [331] . Additionally, Bacher et al. [332] found that IFNα was able to reverse the protective effect of Tregs in xenoGVHD by suppressing cAMP in Tregs. Arginine methylation of FOXP3 in Tregs was also found to be critical in maintaining suppressive function of Tregs in xenoGVHD (Kagoya et al. 2018). Thus, both murine and human Treg infusions are able to prevent disease in two murine GVHD models and these pre-clinical studies provide insight into the mechanisms necessary for Treg-mediated suppression of GVHD.

There are currently ten phase I or II clinical trials in the recruiting or ongoing utilizing Tregs in GVHD prevention or treatment. Five phase I clinical trials studying Treg treatment or prevention of GVHD have been completed and results published. These studies have demonstrated that Treg infusions derived from autologous T cells, allogeneic T cells or UCB are

safe and non-toxic. The first in-human trial with adoptive Treg transfer as a treatment for acute and chronic GVHD (aGVHD and cGVHD) was carried out by Trzonkowski et al. in 2009 [333]. Unfortunately, due to limited patient numbers and complications in Treg production, nothing could be concluded about safety. Brunstein et al. [37] carried out the first clinical trial with UCB-derived Tregs and treated 23 patients with aGVHD. This treatment resulted in a decrease in grade II-IV aGVHD incidence compared to historical controls. However, there was an issue with expanding enough Tregs for treatment, and six out of 13 Treg products did not reach the target cell levels [37]. Martelli et al. [334] also had promising results after infusing 43 patients with high risk leukemia with freshly isolated donor-derived Tregs. This was the first study that examined Treg therapy in patients that also received donor Tconv in addition to a HSCT. There was a complete absence of GVHD in patients who received $2x10^6$ Tregs along with improved reconstitution of the immune system and reduced leukemia relapse. Theil et al. [335] tested Treg therapy in five patients with cGVHD. They combined Treg infusion with low dose IL-2 to improved Treg survival which was well tolerated by the patients. Moreover, all the patients experienced improved disease symptoms or at least stability of symptoms. The low dose IL-2 did increase T cell activation but did not negatively affect the benefits of the Treg infusion. Finally, the ALT-TEN trial carried out by Bacchetta et al. [336] used Tr1 cells rather than FOPX3+ CD4+Tregs to treat patients who underwent chemotherapy and T cell-depleted HSCT due to a high risk/advanced hematological malignancy. Unfortunately, few conclusions could be drawn from this study as only four patients survived and the purity of the Tr1 cell product was low. In conclusion, it is clear there is potential for Treg therapy as a treatment for GVHD but there are many obstacles, including improving Treg cell number and purity, that still need to be overcome.

Chapter 2 : Ectopic expression of the two major isoforms of Helios with FOXP3 play different roles in mediating engineered regulatory T cell immunosuppression

Abstract

Regulatory T cells (Tregs) are a subset of immune cells that suppress the immune response. Treg therapy for inflammatory diseases is being tested in the clinic with moderate success. However, it is difficult to isolate and expand Tregs to sufficient numbers. Engineered Tregs (eTregs) can be generated in larger quantities by genetically manipulating conventional T cells to express FOXP3. These eTregs can suppress *in vitro* and *in vivo* but not as effectively as endogenous Tregs. We hypothesized that ectopic expression of the transcription factor Helios along with FOXP3 is required for optimal eTreg immunosuppression. To test this, we generated eTregs by retrovirally transducing total human T cells with combinations of FOXP3, Helios (Hel-FL) and Δ3B Helios (Hel-Δ3B), a relevant splice variant of Helios. FOXP3+Hel-FL eTregs were the only eTregs able to delay disease in a xenogenic Graft versus Host Disease model. *In vitro*, FOXP3+Hel-FL CD4+ eTregs suppressed T cell proliferation more effectively than FOXP3 and FOXP3+Hel-Δ3B CD4+ eTregs. However, both FOXP3+Hel-FL CD8+ eTregs and FOXP3+Hel-Δ3B CD8+ eTregs were more effective than FOXP3 alone. RNA Sequencing of the CD4+ and CD8+ eTregs demonstrated that the addition of Hel-FL to FOXP3 in eTregs changed gene expression in cellular pathways and the Treg signature compared to FOXP3 alone or FOXP3+Hel-Δ3B. Thus, overexpression of Hel-FL with FOXP3 in eTregs changed gene expression in Tconvs and mediated immunosuppression *in vivo* and *in vivo*. Additionally, there is a functional difference between the endogenous splice variants of Helios in mediating CD4+ and CD8+ T cell immunosuppression.

Introduction

Tregs are a subset of T cells that promote immune tolerance and suppress the immune response. Tregs represent 3-5% of $CD4^+$ T cells in the blood and are characterized by the expression of the FOXP3 transcription factor, high CD25 and low CD127 expression. A majority of Tregs are thymus-derived Tregs (tTregs), generated in the thymus during T cell development. Naïve T cells can also differentiate into peripherally-derived Tregs (pTregs). Tregs mediate immune homeostasis through suppression of immune activity [33]. Tregs down-regulate the immune response via a variety of mechanisms such as inhibiting pro-inflammatory conventional T cells (Tconv) proliferation and activation, secretion of immunosuppressive cytokine, killing of reactive immune cells and induction of anergy.

The immunosuppressive properties of Tregs have led to much research into utilization of Tregs as a cellular therapeutic. The most numerous clinical trials are aimed at preventing Graft versus Host Disease (GVHD) in which Tregs are isolated from leukopheresed blood and co-infused with hematopoietic stem cells [337]. Alternatively, Tregs can be expanded from cord blood [329]. Other clinical trials are testing Tregs as treatment for inflammatory bowel disease, type I diabetes and transplant rejection [35-39]. These trials have shown that Treg infusions are safe, but only moderately successful. A major challenge is expanding Tregs to numbers required to treat. For example, in GVHD one trial, six out of 13 Treg products did not reach the target cell levels [37]. Another major challenge of Treg therapy is isolating a pure population of Tregs. Tregs are commonly isolated by selecting $CD4^+$ $CD25^+$ T cells, but these markers are also expressed by activated Tconvs [22]. This leads to potential contamination of Tregs with Tconvs that could exacerbate disease. Another limitation of Treg therapy is stability of phenotype as Tregs can convert to a Tconvs and lose immunosuppressive activity[198].

Given these challenges in utilizing expanded Tregs from blood, alternative approaches are being investigated. Because Tregs represent rare populations *in vitro*, attempts have been made to produce them in culture [23]. For example, *in vitro*-induced Tregs (iTregs) can be generated by stimulating $CD4^+$ T cells with various cytokines and drugs such as TGFβ, rapamycin and retinoic acid [31]. However, the iTregs that have been generated up until now do not retain FOXP3 expression and do not effectively treat a humanized model of GVHD [31]. tTregs from discarded human thymus have also been explored as a source of Tregs for therapy. Larger numbers of Tregs can be isolated from the thymus and they remain stable under inflammatory conditions, unlike Tregs from peripheral blood. Furthermore, expanded tTregs delayed GVHD in a xenogeneic murine GVHD model more effectively than Tregs from peripheral blood [289]. These data indicate that Treg therapy has great potential, but there is much room for improvement.

Engineered Tregs (eTregs) are generated by transducing CD4+ Tconvs with genes known to convey Treg activity such as FOXP3. The same T cell transduction technology is currently being used to express chimeric antigen receptors in T cells for cancer immunotherapy and has been proven to be safe [338]. eTregs provide solutions to many of the limitations of endogenous Tregs. Total CD4+ T cells can be isolated in greater numbers and expanded more quickly than naturally occurring Tregs. Co-expression of Treg genes with a transduction marker allows for purification of transduced cells and ensures homogeneity of the cell population [294]. Constitutively expressing Treg transcription factors stabilizes Treg phenotype [296]. Finally, eTregs can be modified to express receptors, such as chimeric antigen receptors, that can target them to a specific tissue resulting in localized immunosuppression [296]. While the advantages

of eTregs are clear, the Treg genes necessary to create an optimal engineered Treg are still undefined.

High expression of the transcription factor FOXP3 is a hallmark of naturally occurring Tregs. FOXP3 is necessary for Treg-mediated immune homeostasis, as mice and humans deficient in FOXP3 develop severe autoimmune diseases [17, 18]. Enforced FOXP3 expression in human CD4+ T cells mediates suppression of Tconv proliferation *in vitro* [32]. FOXP3-transduced cells can reduce symptoms in murine colitis and GVHD models [14, 255]. However, in a study using a murine arthritis model, FOXP3-transduced cells were not as effective as endogenous Tregs at reducing joint destruction or decreasing the number of pathogenic Th17 cells in the joint [296]. Additionally, microarray data has shown that FOXP3 is not sufficient to convey complete Treg gene regulation in murine T cells [277]. These observations indicate that FOXP3-expressing eTregs show promise, but expression of additional genes are required to completely mimic endogenous Treg function.

In addition to FOXP3, the transcription factor Helios, a member of the Ikaros family, is highly expressed in about 70% of $FOXP3^{+}$ Tregs [25]. Treg-specific knock out mice develop spontaneous autoimmune disease at 6-8 months of age and Tregs from these mice have reduced suppressive activity and survival [42, 43]. In humans, FOXP3+ Helios+ Tregs more effectively suppress Tconv cytokine secretion and are more stable under inflammatory conditions than FOXP3+ Helios- Tregs [25, 288, 289]. It has been demonstrated that co-expression of Helios and Foxp3 in murine CD4+ Tconvs increases Treg transcriptional signature index compared to Foxp3 alone [298]. For these reasons, we hypothesized that ectopic expression of the Helios with FOXP3 is required for optimal eTreg immunosuppression. Here, we report that through dual retroviral transduction, total human T cells can be genetically modified to express high levels of

FOXP3 and Helios. We generated eTregs that co-expressed FOXP3 with the following two endogenous splice variants of Helios found in human Tregs [40, 41]: full length Helios (Hel-FL) and a shorter form, Δ3B Helios (Hel-Δ3B). FOXP3+Hel-FL eTregs were the most effective at immunosuppression *in vivo* in a xenogeneic GVHD model and in an *in vitro* suppression assay. Adding Hel-FL to FOXP3 in eTregs was able to convey immunosuppressive properties to both CD4+ and CD8+ human Tregs and these eTregs had differential gene expression and enrichment of cellular pathways at a transcriptional level compared to FOXP3 and FOXP3+ Hel-Δ3B eTregs. Thus, we were able to improve current eTreg production and generate both CD4+ and CD8+ eTregs by ectopically expressing FOXP3 and Hel-FL.

Materials and Methods

Isolation of Human Peripheral Blood Mononuclear Cells (PBMC)

All studies involving human subjects were conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. Blood was collected from healthy adult volunteer donors under signed informed consent with approval from the Institutional Review Board of the University of Kansas Medical Center. Blood was collected in heparin tubes and PBMC were isolated via Ficoll-Paque (GE Healthcare, Chicago, IL) density centrifugation with SepMate tubes (STEMCELL Technologies Inc., Vancouver, BC).

Construction and production of retroviral vectors

Retroviral constructs were generated to express cDNA for FOXP3, full length Helios (Hel-FL) or a short isoform of Helios (Hel-Δ3B). The NCBI Reference Sequences for FOXP3, Hel-FL and Hel-Δ3B are NM_014009.3, NM_016260.2 and NM_001079526.1, respectively. The SFG retroviral vector, RDF and pEQPAM3 retroviral packaging plasmids were generously donated by Dr. Malcom Brenner at Baylor College of Medicine. Genes were cloned into the SFG vector via Gibson Assembly [339]. The expression of cDNA is driven by a strong retroviral promoter in the 5' LTR. Multiple genes of interest were expressed by linking them in frame with a picornavirus 2A ribosomal skip peptide, which ensures equivalent expression of multiple proteins [292]. The FOXP3 construct contains truncated CD19 (ΔCD19) cDNA and the Hel-FL and Hel- Δ3B contains truncated CD34 (ΔCD34) cDNA which act as transduction markers and allowed for purification of transduced cells via antibody-bound magnetic beads. The NCBI Reference Sequences for CD19 and CD34 are NM_001178098.1 and NM_001025109.1 respectively. The truncated ΔCD19 and ΔCD34 sequences only contain the signal peptide,

extracellular and transmembrane regions of the protein. ΔCD19 and ΔCD34 alone vectors were generated as negative controls. FOXP3, ΔCD19 and ΔCD34 were codon optimized with the Invitrogen GeneArt Gene Synthesis service (Thermo Fisher Scientific, Waltham, MA) prior to being cloned into the SFG vector. Hel-FL and Hel-Δ3B gene sequences were not altered prior to cloning. Viral particles were generated by transfecting HEK 293T cells with SFG vectors containing the genes of interest and the retroviral packaging vectors. Transfection was carried out with Fugene HD Transfection Reagent (Promega, Madison, WI). Viral supernatants were collected 2 and 3 days after transfection and stored at -80 C until use.

Activation and Transduction of Human T cells

Human T cells were activated in complete media containing Aim V medium (Thermo Fisher Scientific, Waltham, MA) and 2% human AB serum (Bio-Techne, Minneapolis, MN). 3 x 10^6 PBMCs at 10^6/mL were stimulated with plate bound anti-CD3 (2 µg/mL OKT3; Bio X Cell, West Lebanon, NH) and anti-CD28 (2 µg/mL 9.3; West Lebanon, NH). After 2 days of activation, complete medium was supplemented with 200 U/mL of recombinant human IL-2 (rhIL-2) (PeproTech, Rocky Hill, NJ). Cells were passed every 2-3 days at 1-2 x 10^6 cells/ml in complete medium supplemented with rhIL-2 at 200 U/mL. Activated T cells were transduced with viral supernatants containing ΔCD34 vectors 5-6 days post activation. Non-tissue culture 6 well plates were coated in Retronectin (Takara Bio Inc., Kusatsu, Shiga Prefecture, Japan) at 20 µg/mL in PBS overnight at 4 °C or 2 hours at room temperature. 1.5 mL of viral supernatant per well was bound to Retronectin coated plates by centrifuging plates for 2 hours at 2000 xg at 32 °C. Viral supernatant was removed and 1.5 mL of viral supernatant along with 2.5-3 x 10^6 T cells were added to each well. T cells were in complete medium with rhIL-2 at 200 U/mL at 10^6

cells/mL. Transduced cells are positively selected 2 days post transduction with anti-human CD34 CELLection magnetic beads (Thermo Fisher Scientific, Waltham, MA). Beads were removed with a magnet 2 days later and cells were transduced with viral supernatants containing ΔCD19 vectors as previously described. Transduced cells were positively collected 2-3 days post transduction with CELLection Biotin Binder Kit beads (Thermo Fisher Scientific, Waltham, MA) coated with biotinylated anti-human CD19 (HIB19, Biolegend, San Diego, CA). Beads were removed 2 days later and used in assays up to 10 days post the last transduction. The resulting cell strains were ΔCD19+ΔCD34, FOXP3-ΔCD19+ΔCD34, FOXP3-ΔCD19+ Hel-FL-ΔCD34 and FOXP3-ΔCD19+ Hel-Δ3B-ΔCD34.

Real time PCR

Helios splice variants were detected in transduced cell strains using real time PCR. RNA was isolated from cells using the Qiagen RNeasy Mini Kit (Qiagen, Germany). RNA was converted to cDNA using the Taqman High Capacity RNA to cDNA kit (Thermo Fisher Scientific, Waltham, MA). Real time PCR was performed using the following primers for Helios: F 5' TGATGGCTATATAACGTGTGACAA 3', R 5' CTCACACTTGAAGGCCCTAATC 3'. Splice variants were visualized using gel electrophoresis.

Mice and Xenogeneic Murine GVHD Model

All animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. NOD-SCID IL-2Rγnull (NSG) mice were purchased from Jackson Laboratories and bred at the University of Kansas

Medical Center. Mice were maintained under specific pathogen-free conditions using sterile food, water, bedding, and caging.

NSG mice (8-12 weeks old) of both sexes received 1.5 Gy of whole body irradiation. The next day, the mice were anesthetized via 2% inhaled isoflurane and injected retro-orbitally with 10^7 human PBMC alone, 10^7 PBMCs with 5 X 10^6 eTregs or PBS alone. Cells were re-suspended in 100 μl of sterile PBS. Mice were examined for signs of GVHD, using the GVHD scoring system established by Cooke et al. [310]. Five categories were assessed on a scale of 0, 1, or 2 for each. Weight loss: 0= <10% change, 1= 10-25% change, 2= >25% change, Posture: 0= normal, 1= hunching at rest, 2=hunching impairs movement, Fur texture: 0= normal, 1= mild to moderate ruffling, 2= severe ruffling, Skin integrity: 0=normal, 1=scaling of paws/tail, 2= obvious areas of denuded skin, Activity: 0=normal, 1=mild to moderately decreased, 2=stationary unless stimulated. Mice were sacrificed when they reached a GVHD score of ≥7 or lived until 42 days. The researcher assessing score was blinded to the treatment of each mouse. Serum was collected to be analyzed for cytokines, the spleen was collected for flow cytometry and samples from the ear, lung, liver and kidney were frozen for digital PCR.

Flow Cytometry

Culture cells or single cell suspensions of spleens from mice were stained with various combinations of the following anti-human antibodies: CD3 APC-Cy7, CD4 PE-Cy7, CD4 eFluor-610, CD8 Alexa Fluor 488, CD8 Brilliant Violet 785, CD19 Brilliant Violet 421, CD34 Brilliant Violet 605, CD39 Brilliant Violet 510, CD25 PerCPCy-5.5, CD127 Brilliant Violet 650, CD73 APC-Cy7, CCR4 PE-Cy7, GITR PE-Cy5, CTLA-4 PE, CD62L AF700 (BioLegend, San Diego, CA). Intracellular transcription factor staining was done using the eBioscience™ FOXP3

Staining Buffer kit (Thermo Fisher Scientific, Waltham, MA) with anti-human FOXP3 PE, anti-human Helios Alexa Fluor 647, anti-Helios Brilliant Violet 421(BioLegend, San Diego, CA). Samples were run on a Becton-Dickson LSRII (Becton-Dickson, Franklin Lakes, NJ) or Attune NxT (Thermo Fisher Scientific, Waltham, MA).

Cytometric Bead Array

Serum samples from mice were analyzed for cytokines using the 13-plex CD8/NK Legendplex Kit (Biolegend, San Diego, CA).

Digital PCR

Genomic DNA was isolated from mouse tissue samples with a Qiagen DNeasy Kit. Digital PCR reactions were prepared according to the protocol for EVA Green binding dyes for the BioRad LX200 digital PCR machine (Biorad, Hercules, CA). 0.5 μL of the restriction enzyme HindIII was added to each reaction to digest the genomic DNA. The following primers were used to detect human CD3 epsilon genomic DNA (NCBI Reference Sequence: NG_007383.1): Forward Primer: 5’ AGGCTGCCTTAACTCCCAAG 3’, Reverse Primer: 5’ GCCCTACCAGCTGTGGAAAC 3’. The following primers were used to detect the codon optimized CD19 present in the transduced eTregs: Forward Primer: 5’ CTTCAACGTGTCCCAGCAGA 3’, Reverse Primer: 5’ GATCCTTCCACGTTCACGGT 3’. Both these primers will yield a single band of 105 bp. Digital PCR reaction conditions were as follows: Lid at 105 °C, 95 °C for 10 min (1 cycle); 95 °C for 30 s, ramp 2 °C/s and 55 °C for 1 min, ramp 2 °C/s (40 cycles); 72 °C for 10 min, 12 °C hold.

Activation Induced Cell Death Assay

Cells were resuspended at a concentration of 10^6 cells/mL in complete Aim V media. $2x10^5$ cells from each cell strain were stimulated with plate-bound anti-CD3 (10 µg/mL OKT3; Bio X Cell, West Lebanon, NH) and anti-CD28 (1 µg/mL 9.3; West Lebanon, NH) for 2, 4 or 6 days. Cells were collected at each time point and stained with Zombie Green Fixable Viability Dye and Annexin V PE (Biolegend, San Diego, CA) and assessed for cell death via flow cytometry.

Intracellular Cytokine Staining

Cells were resuspended at a concentration of 10^6 cells/mL in complete Aim V media. $2x10^5$ cells from each cell strain were stimulated with plate-bound anti-CD3 (10 µg/mL OKT3; Bio X Cell, West Lebanon, NH) and anti-CD28 (1 µg/mL 9.3; West Lebanon, NH) for 6 hours in the presence of Golgi Stop (Becton-Dickson, Franklin Lakes, NJ) and Brefeldin A (Sigma Aldrich, St. Louis, MO). Cells were stained with the following extracellular antibodies: anti-human CD4 PE-eFluor610 (Becton-Dickson, Franklin Lakes, NJ), anti-human CD8 Brilliant Violet 785 and anti-human CD19 APC-Cy7 (Biolegend, San Diego, CA). Afterwards, cells were fixed with 2% paraformaldehyde (Sigma Aldrich, St. Louis, MO) and permeabilized with permeabilization buffer from the eBioscience™ FOXP3 Staining Buffer kit (Thermo Fisher Scientific, Waltham, MA) and stained with the following antibodies: anti-human IL-2 FITC, anti-human IFNγ Pacific Blue, anti-human IL-10 Alexa Fluor 647 or anti-human IL-21 Alexa Fluor 647, anti-human IL-4 PE-Cyanine-7, anti-human IL-17A Brilliant Violet 605 (Biolegend, San Diego, CA).

Suppression Assay

Autologous target T cells were labeled with the eBioscience™ Cell Proliferation Dye eFluor670 from (Thermo Fisher Scientific, Waltham, MA) and co-cultured with each eTreg cell strain at a 1:1 ratio with no stimulation or stimulation with anti-CD3 and anti-CD28 coated DYNAL™ Dynabeads™, Human T-Activator (Thermo Fisher Scientific, Waltham, MA) at 1:10 bead: target cell ratio. The final concentration of cells was at $5x10^5$ cells/ml. After 96 hours, target cell proliferation was assayed via flow cytometry. Cells were also stained with Zombie Green Fixable Viability Die and anti-human CD4 PE-Cy7, anti-human CD8 PE, anti-human CD19 APC-Cy7, anti-human CD25 PerCPCy5.5 (Biolegend, San Diego, CA).

RNA Sequencing

FOXP3-ΔCD19+ΔCD34, FOXP3-ΔCD19+ Hel-FL-ΔCD34 and FOXP3-ΔCD19+ Hel-Δ3B-ΔCD34 cells were generated from PBMCs isolated from three different healthy human donors. Cells were collected Day 5 after the second transduction and stained with anti-human CD4 Pacific Blue and anti-CD8 Alexa Fluor 488 (Biolegend, San Diego). CD4+ and CD8+ cells were isolated via flow cytometry assisted cell sorting on a BD FACS Aria III. RNA was isolated using a Qiagen RNeasy Mini Kit (Qiagen, Germany). TruSeq stranded mRNA sequencing libraries were performed using the Illumina TruSeq Sample preparation kits and NuGEN sample preparation kit and paired end RNA sequencing data was generated using an Illumina NovaSeq 6000 Sequencing System (Illumina, San Diego, CA).

Adaptor removal was performed by cutadapt [340]. After adaptor removal, QC was done with fastqc (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). Samples were then aligned to human genome (hg38) with RSEM [341] and bowtie2 [342], after which transcript

counts were obtained. Using the Bioconductor package “edgeR” [343], we first normalized the data by library size and then filtered out genes that have low expression. Genes were retained if their cpm (count per million) value was larger than 1 for at least two samples out of the 18 total considered in this study. After filtering low/non-expressed, 13,955 remained for subsequent statistical analysis (**S1)**. Next, we performed a series of differential expression analyses, comparing gene expression between different pairs of cell types. There were a total of six comparisons being performed: FOXP3 CD4 vs FOXP3 Helios-FL CD4, FOXP3 CD4 vs FOXP3 Helios-d3B CD4, FOXP3 CD8 vs FOXP3 Helios-FL CD8, FOXP3 CD8 vs FOXP3 Helios-d3B CD8, FOXP3 Helios-FL CD4 vs FOXP3 Helios-d3B CD4, FOXP3 Helios-FL CD8 vs FOXP3 Helios-d3B CD8. For each comparison, a volcano plot depicting the -log(p-value) as a function of log-fold change in expression, was generated.

We further filtered genes with differential expression by taking 2,000 genes with the lowest false discovery rate (FDR) (**S2**). We then restricted this gene list to genes with an expression change that was the same direction in all 3 donors. We made these filtered lists for each donor and comparison and used the CPMs to carry out gene set enrichment analysis (GSEA) with the GSEA v3.0 software from the Broad Institute. We looked at enrichment in the KEGG pathway gene sets (c2.cp.kegg.v6.2.symbols.gmt) to identify and visualized significantly enriched pathways in different eTreg cell strain comparisons [344, 345].

In order to examine the Treg related gene expression, we generated heatmaps based the cpm value from count data. More specifically, we compiled two lists of Treg genes based on comparisons of Tconvs vs Tregs generated by Miyara et al., Mold et al. and Bonacci et al. [133, 346, 347], referred to as the “up gene list “ and the “down gene list”. On the basis of the differential expression results generated for gene expression comparisons of specific cell types,

we first subset the genes that had a nominal, uncorrected p value < 0.05 based on either up or down Treg gene list. Then, we filtered genes that only show expected expression pattern, aka, all three subjects are up regulated in an up_gene comparison or vice versa, so that we kept up regulated genes in an up_gene heatmaps and kept down regulated genes in down_gene heatmaps. Further, we merged two comparisons of heatmaps into one. The two heatmaps being merged have difference in the type of cell strain and share same cell type (CD4 or CD8) and gene list (up or down gene list). The merged heatmap contains all genes in either of the two comparisons. For each cell in the heatmap, we calculated the difference of cpm values between two strains of cell for one subject and divided by average cpm value of that gene in all three subjects.

Data/Statistical Analysis

Data are stored on a secure server at the University of Kansas Medical Center. Data were collected and analyzed with GraphPad Prism 7 (GraphPad Software, [La Jolla, CA]). Data reported at each time point for GVHD score and weight loss were an average of the scores and weights of the mice left alive and the last scores and weights of any deceased mice in each group. Mann-Whitney tests were done to compare GVHD scores at each time point. The log-rank (Mantel-Cox) test was used for analysis of Kaplan-Meier curves. Differences between groups were compared via Mann Whitney tests. Differences between groups with data normalized to a control were compared using the Wilcoxon test. $p \leq 0.05$ was considered to be significant. Results show mean ± standard error mean unless otherwise indicated.

Results

Dual retroviral transduction can convey FOXP3 and Helios expression in human T cells

In order to generate human eTregs that express both FOXP3 and Helios, we developed a dual transduction protocol. This was required as transduction of human T cells with a vector that contained both Helios and FOXP3 had downregulation of FOXP3 expression and the truncated ΔCD19 transduction marker (**Figure 2-1A-B**). There were multiple Helios binding sites present in the cDNA construct containing Helios and FOXP3 (data not shown) and Helios can downregulate gene expression upon binding [40]. With the dual transduction protocol, Helios-overexpressing cells did not downregulate expression of the truncated ΔCD34 expression until about four days post-transduction (data not shown). We could use magnetic bead separation to purify Helios+ ΔCD34+ cells two days post-transduction and removed the magnetic beads. Then, we transduced these cells with the SFG-FOXP3-ΔCD19 retroviral vector and repeated magnetic bead purification to obtain human eTregs that highly expressed Helios and FOXP3 (**Figure 2-2B**). In contrast to previously published eTreg studies, we transduced total human T cells rather than purified CD4+ T cells [32]. It has been demonstrated that Helios is required to mediate CD8 Treg function [43]. Thus, we hypothesized that co-expression of FOXP3 and Helios could convey immunosuppressive function to human CD8+ T cells. Additionally, transduction of total T cells would reduce purification steps required to generate these eTregs in a clinical setting. Both CD4+ and CD8+ T cells within the total T cell population expressed high levels of Helios and FOXP3 (**Figure 2-2B**). We also chose to investigate the two different endogenous splice variants of Helios, Hel-FL and Hel-Δ3B, in eTreg function. RT-PCR gel electrophoresis showed that the overexpressed isoform of Helios was the predominant splice variant of Helios expressed

in each eTreg (**Figure 2-2D**). Thus, we generated eTregs strains that overexpress FOXP3, FOXP3+Hel-FL and FOXP3+ Hel-Δ3B and an empty vector control, ΔCD19+ΔCD34.

FOXP3+Hel-FL eTregs delay disease in vivo in a xenoGVHD model

In order to assess the suppressive capacity of each eTreg cell strain *in vivo*, we utilized a xenogeneic Graft versus Host (xenoGVHD) disease model in which sublethally irradiated NSG mice were intravenously injected with human PBMCs without or with each eTreg cell strain or empty vector control cells. Mice treated with FOXP3+Hel-FL eTregs had significantly delayed GVHD progression compared to mice with PBMCs only (**Figure 2-3A**). Additionally, FOXP3+Hel-FL eTregs significantly improved survival based on a log-rank test compared to mice with PBMCs only (**Figure 2-3B**). Mice treated with FOXP3+Hel-FL eTregs had a median survival of 36 days compared to mice with PBMCs only, which had a median survival of 21 days. Interestingly, mice treated with FOXP3 or FOXP3+Hel-Δ3B eTregs did not significantly delay GVHD with median survivals of 27.5 days and 29 days respectively.

Next, irradiated NSG mice were injected with human PBMCs without or with each eTreg cell strain or empty vector control cells and euthanized at an early timepoint of 12 days to assess mice at different stages of disease. As observed in the long term xenoGVHD experiment, mice treated with FOXP3+Hel-FL had the lowest average GVHD score at this time point with a mean score of 0.6 ±0.4 compared to 2.8 ±0.86 for mice injected with PBMCs only (**Figure 2-4A**). Spleens from each mouse were processed into a single cell suspension and analyzed via flow cytometry. The number of total splenocytes was not significantly affected by eTreg treatment, but all three eTreg cell strains significantly decreased the percent of CD3+ CD19- non-transduced T cells in the spleen (**Figure 2-4B-C**). Each eTreg also increased the percent of

FOXP3+ CD25hi and FOXP3+ Helios+ Tregs in the CD3+ CD19- non-transduced T cell population (**Figure 2-4D**). However, even though all three eTreg cell strains conveyed these immunosuppressive effects in the spleen, FOXP3+Hel-FL eTregs were still the most effective at delaying GVHD (**Figure 2-4A**).

Serum cytokines from these early time point mice were analyzed via cytometric bead array and revealed many differences between the treatment groups. Compared to the empty vector control treated mice, all three eTreg strains decreased multiple pro-inflammatory proteins in the serum, including IL-4, TNFα, sFas, sFasL, granzymes A and B, perforin and granylysin (**Figure 2-5**). Interestingly, both FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs decreased IL-6 in the serum and FOXP3 and FOXP3+Hel-FL eTregs decreased IFNγ (**Figure 2-5**). Thus, FOXP3+Hel-FL eTregs could effectively delay disease and improve survival in a xenoGVHD model, while FOXP3 and FOXP3+Hel-Δ3B eTregs could not, but the mechanism was unclear.

Hel-FL and Hel-Δ3B co-expression with FOXP3 differentially regulate CD4+ and CD8+ eTreg suppression

The ability of each eTreg strain to suppress T cell proliferation was also tested *in vitro*. In addition to total eTregs, CD4+ and CD8+ T cells were purified via magnetic bead separation and assayed separately. Freshly isolated human T cells were labeled with a proliferation dye and co-cultured with each eTreg cell strain, total, CD4+ or CD8+, at a 1:1 ratio. Cells were stimulated with anti-CD3 and anti-CD28 coated beads for four days before being assayed via flow cytometry. In agreement with the *in vivo* data, FOXP3+Hel-FL total eTregs were the most effective at suppression compared with FOXP3 and FOXP3+Hel-Δ3B total eTregs, with a mean percent suppression of 46.21 ±12.54% vs 16.47 ±4.526% vs 21.67 ±8.658%, respectively

(**Figure 2-6A**). The same was true for CD4+ eTregs with FOXP3+Hel-FL suppressing proliferation at a mean of 40.82 ±10.36% compared to 21.19 ±8.968% and 19.06 ±8.968% suppression by FOXP3 and FOXP3+Hel-Δ3B CD4+ eTregs respectively (**Figure 2-6A)**. However, both FOXP+Hel-FL and FOXP3+Hel-Δ3B overexpression in CD8+ eTregs was able to most effectively mediate suppression of T cell proliferation with a percent suppression of 45.85 ±7.794% and 48.30 ±10.88%, respectively, compared to FOXP3 alone, which had a percent suppression of 21.68 ±11.01% (**Figure 2-6A**). In conclusion, CD4+ T cells transduced with FOXP3 and Hel-FL could were the most effective at suppressing T cells *in vitro* compared to FOXP3 and FOXP3+Hel-Δ3B. Both FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD8+ eTregs were more effective than FOXP3 CD8+ eTregs alone at suppressing T cell proliferation.

Ectopic overexpression of FOXP3 without and with Helios reduces T cell survival in vitro and in vivo

Previous studies reported that overexpression of FOXP3 in primary human T cells reduced proliferation *in vitro* [32], and ectopic expression of Helios in Jurkat cells, a human T cell line, also resulted in reduced survival *in vitro* [44]. Thus, we analyzed the proliferation and survival of each eTreg cell strain. We observed that overexpression of FOXP3 in human T cells reduced proliferation over time and the addition of either isoform of Helios with FOXP3 expression further reduced proliferation (**Figure 2-7A**). There was also an increase in activation-induced cell death in all three eTreg cell strains, with more death observed in both the Helios-expressing eTregs (**Figure 2-7C**). This decreased survival in all eTreg strains was observed *in vivo* in the xenoGVHD mice euthanized at an early time point. Flow cytometry of the spleen revealed a decrease in percent of CD3+ CD19+ transduced eTregs compared to empty vector

controls (**Figure 2-7B**). Digital drop PCR for copies of CD3 and the codon-optimized CD19, which is only present in the transduced cells, was performed on genomic DNA from lung, liver and spleen of xenoGVHD euthanized at an early timepoint. All eTreg treated mice had a reduced CD19/CD3 copy number ratio compared to empty vector control mice indicating a decrease of transduced cells detected in those tissues (**Figure 2-7D**). Thus, overexpression of FOXP3 without or with either isoform of Helios reduces proliferation and survival of human T cells *in vitro* and *in vivo*.

FOXP3 overexpression without and with Helios affects human T cell expression of Treg markers and cytokine production

Overexpression of FOXP3 in primary human T cells has been shown to mediate expression of Treg markers; specifically, increased expression of CD25, GITR, CTLA-4 and decreased expression of CD127 [32]. We used multi-parameter flow cytometry to analyze expression of the following Treg markers on all three eTreg cell strains: CD25, CD127, CD73, CD39, CTLA-4, GITR, CCR4 and CD62L. Human T cell surface expression of CD39, CTLA-4, GITR and CD62L was not significantly affected by FOXP3 overexpression without and with either isoform of Helios compared to empty vector control cells (data not shown). All three eTreg cell strains had significantly decreased CD127 expression on CD4+ eTregs and increased CCR4 expression on both CD4+ and CD8 eTregs (**Figure 2-8A**). CD25 was significantly increased for both CD4+ and CD8+ eTregs with FOXP3+Hel-FL and only CD8+ eTregs with FOXP3+Hel-Δ3B (Figure 2-8A).

Cytokine production by each eTreg cell strain was assessed via stimulation with plate bound anti-CD3 and anti-CD28 in the presence of Golgi transport inhibitors, Brefeldin A and

monensin. In agreement with previously published data, FOXP3 overexpression reduced production of IL-2, IFNγ and IL-4 by CD4+ human T cells [32]. FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs also had a similar reduction in IL-2, IFNγ and IL-4 production by CD4+ T cells (**Figure 2-8B**). FOXP3 ectopic expression without and with either isoform of Helios also reduced IL-2 production by CD8+ (**Figure 2-8C**). In summary, transduced human T cells expressing FOXP3, FOXP3+Hel-FL or FOXP3+Hel-Δ3B upregulate certain Treg surface markers and have reduced pro-inflammatory cytokine secretion.

Hel-FL and Hel-Δ3B co-expression with FOXP3 have different effects on the enrichment of genes in cellular pathways and Treg transcriptional signature

Despite the differences in immunosuppression observed between FOXP3+Hel-FL compared to FOXP3 and FOXP3+Hel-Δ3B *in vitro* and *in vivo*, there were no obvious differences between these cell strains with regards to proliferation and survival, Treg markers and cytokine secretion (**Figures 2-7 and 2-8**). Thus, we utilized RNA sequencing (RNA Seq) to determine if there were any differences between these eTreg cell strains at a transcriptional level. Three different healthy donor T cells were transduced with FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B. Then CD4+ and CD8+ T cells from each eTreg cell strain were separated via fluorescence-activated cell sorting (FACS). RNA was extracted from each sample and analyzed via RNA Seq.

Comparison of gene expression fold change revealed that adding either isoform of Helios to FOXP3-overexpressing CD4+ and CD8+ eTregs did change gene expression compared to FOXP3 alone (**Figure 2-9A**). We then carried out Gene Set Enrichment Analysis (GSEA) pathway analysis using GSEA v3.0 from the Broad Institute and the Kyoto Encyclopedia of

Genes and Genomes (KEGG) database. Both FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD4+ and CD8+ eTregs had changes in pathway enrichment when compared to FOXP3 alone (**Figure 2-9B**). Notably, there were more increases in pathway enrichment in the FOXP3+Hel-Δ3B vs FOXP3 comparison compared to the FOXP3+Hel-FL vs FOXP3 comparison in both CD4+ and CD8+ eTregs.

Expectedly, there were fewer differences in gene expression when comparing FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs (**Figure 2-10A**). Despite the small differences in gene expression, FOXP3+Hel-FL had different enriched KEGG pathways when compared to FOXP3+Hel-Δ3B in both CD4+ and CD8+ eTregs (**Figure 2-10B**). Three of these enriched pathways were the same in CD4+ and CD8+ FOXP3+Hel-FL eTregs: p53 signaling pathway, cell adhesion molecules and cytokine-cytokine receptor interaction. Interestingly, some of the genes that were changed in these two common pathways did differ between CD4+ and CD8+ eTregs (**Figure 2-10C**).

In order to determine changes in Treg-related genes in the eTreg cell strains, we generated lists of genes based on comparisons of Tconvs vs Tregs generated by Miyara et al., Mold et al. and Bonacci et al. [133, 346, 347]. We compiled genes that were either up-regulated or down-regulated in Tregs compared to Tconvs, named the “TREG UP” and “TREG DOWN” gene lists respectively. We then analyzed whether adding Hel-FL or Hel-Δ3B to FOXP3-overexpressing eTregs led to up-regulation of the TREG UP genes and down-regulation of the TREG DOWN genes, indicating an increase in Treg signature. Interestingly, FOXP3+Hel-Δ3B had more genes in the Treg signature that were differentially expressed than FOXP3+Hel-FL when compared to FOXP3. This was true for both CD4+ and CD8+ eTregs. Additionally, there

were many Treg genes that were only differentially expressed in either FOXP3+Hel-FL or FOXP3+Hel-Δ3B when compared to FOXP3 in both CD4+ and CD8+ eTregs.

In summary, Hel-FL or Hel- Δ3B co-expression with FOXP3 in CD4+ and CD8+ eTregs changed gene expression when compared to FOXP3 and this led to changes in gene enrichment of cellular pathways. Hel-Δ3B had more instances of increased enrichment of pathways than Hel-FL when co-expressed with FOXP3 in CD4+ and CD8+ eTregs. Additionally, there were differences in gene expression and pathway enrichment when directly comparing FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs and some gene changes were different between CD4+ vs CD8+ eTregs from the same eTreg cell strain. Finally, FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD4+ and CD8+ eTregs had increased changes in Treg signature genes compared to FOXP3 alone. Some of these genes were unique to either FOXP3+Hel-FL or FOXP3+Hel-Δ3B. FOXP3+Hel-Δ3B had a greater number of Treg signature genes that were differentially expressed.

Figure 2-1: Helios overexpression downregulates expression of cDNA on the same vector. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors. T cells were activated with anti-CD3 and anti-CD28 antibody stimulation, cultured in IL-2 containing media and transduced with retroviral particles containing cDNA for genes of interest. The cDNA on these cells were expressed on a single SFG retroviral vector. Transduced cells were purified with antibody-coated magnetic bead particle separation specific for the transduction marker ΔCD19. **A)** Representative dot plots of CD19 and FOXP3 expression for FOXP3, FOXP3-Hel-FL and FOXP3-Hel-Δ3B eTregs **B)** Graphs summarize the geometric mean fluorescence intensity (GMFI) of FOXP3 and CD19 of the indicated eTreg population normalized to empty vector control cells. N=2.

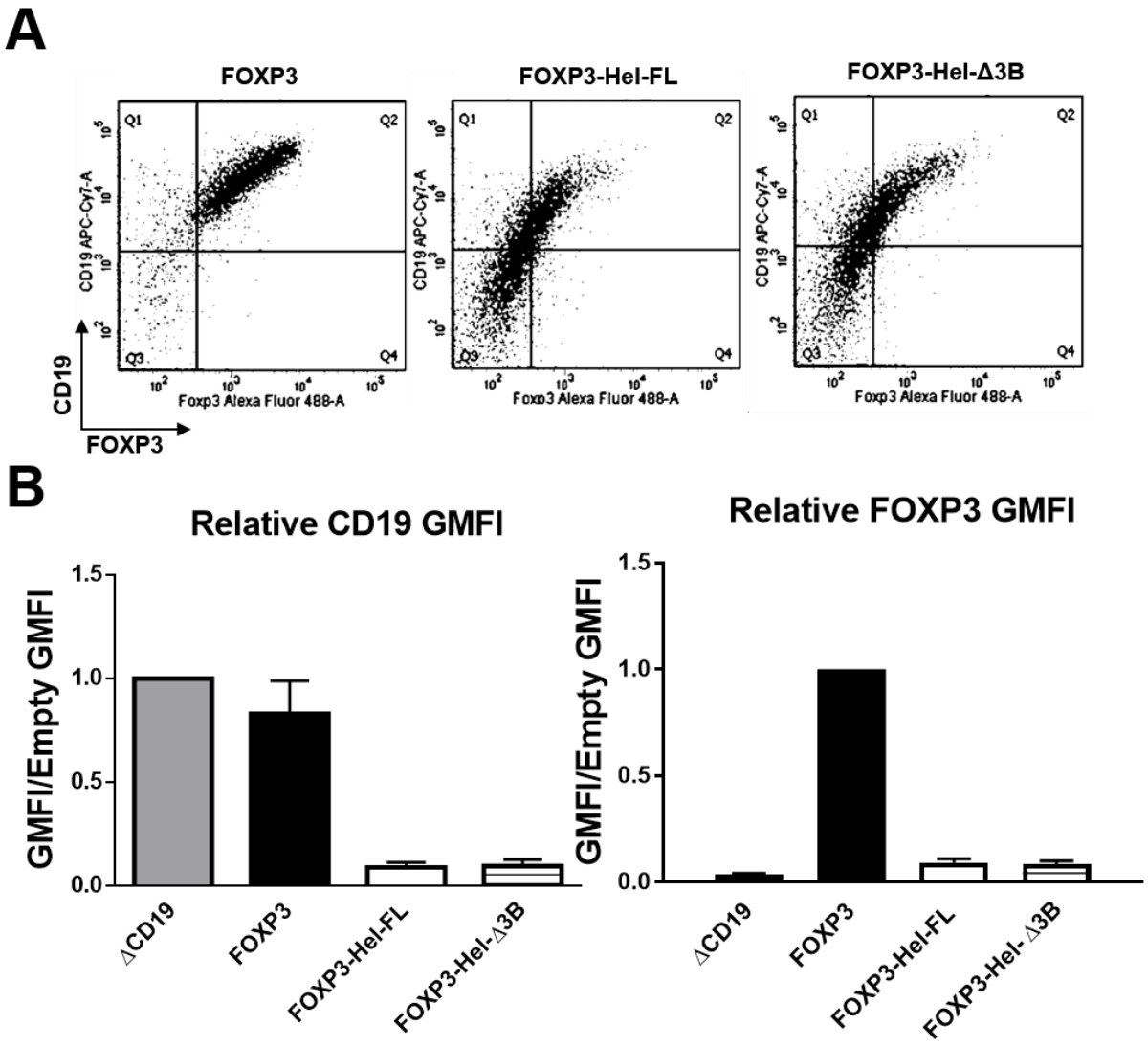

A
FOXP3
FOXP3-Hel-FL
FOXP3-Hel-Δ3B
CD19 APC-Cy7-A
Foxp3 Alexa Fluor 488-A
Q1
Q2
Q3
Q4
CD19
FOXP3
B
Relative CD19 GMFI
Relative FOXP3 GMFI
GMFI/Empty GMFI
1.5
1.0
0.5
0.0
ΔCD19
FOXP3
FOXP3-Hel-FL
FOXP3-Hel-Δ3B

Figure 2-2: Retroviral transduced human T cells express FOXP3 and/or Helios. Peripheral blood mononuclear cells (PBMCs) were isolated from healthy blood donors. T cells were activated with anti-CD3 and anti-CD28 antibody stimulation, cultured in IL-2 containing media and transduced with retroviral particles containing cDNA for genes of interest. Transduced cells were purified with antibody-coated magnetic bead particle separation specific for transduction surface markers. **A)** Illustration of SFG retroviral vector containing genes of interest and transduction surface markers. Helios and FOXP3 protein expression in **B)** CD4+ eTregs and **C)** CD8+ eTregs were assessed via intracellular transcription factor staining and flow cytometry. Dot plots are representative figures and graphs summarize the geometric mean fluorescence intensity (GMFI) of FOXP3 and Helios of the population positive for the protein of interest, N=8-9 and 5 different donors. **D)** Representative figure of Helios mRNA expression assessed via RT-PCR and visualized via gel electrophoresis.

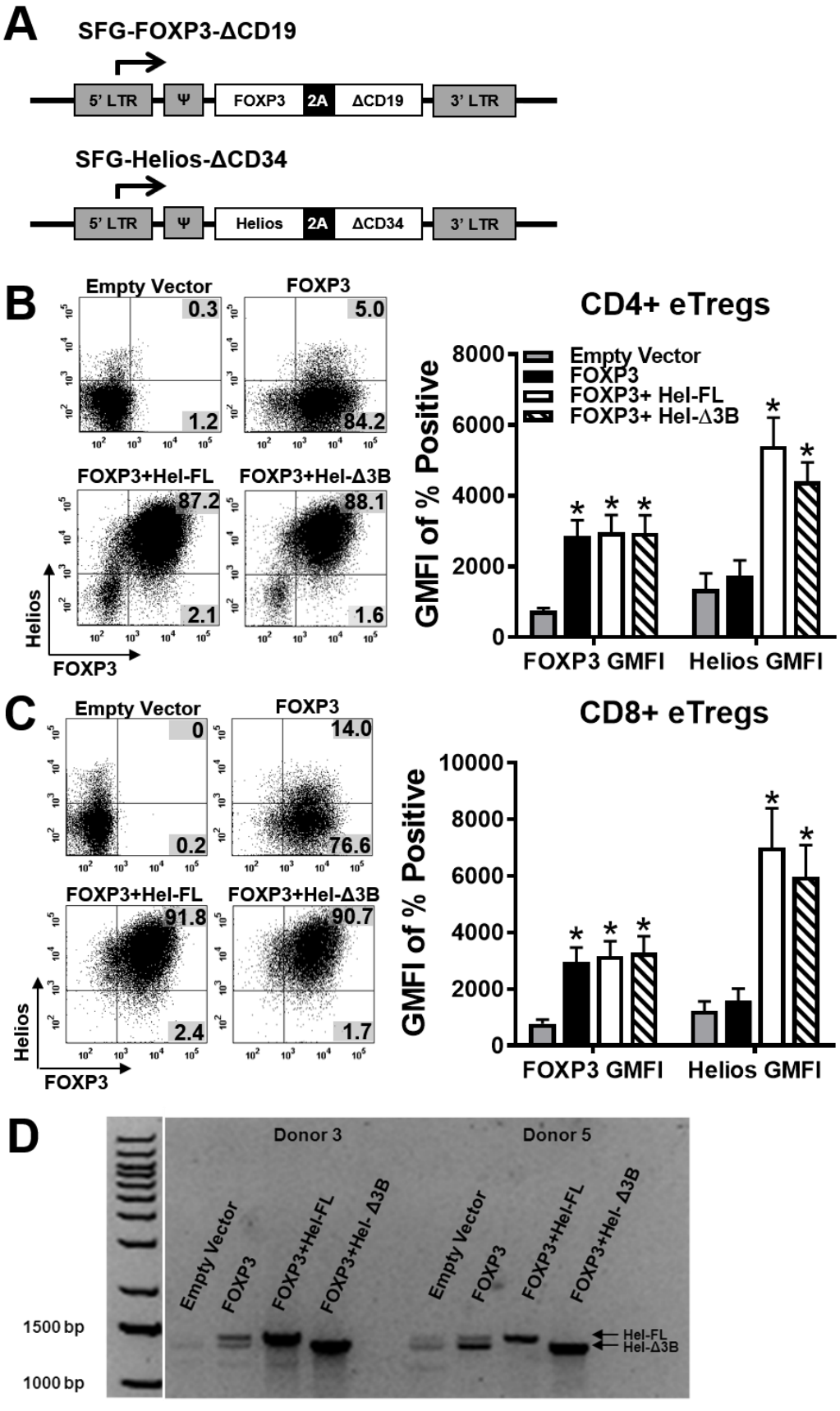

A
SFG-FOXP3-ΔCD19
5' LTR
Ψ
FOXP3
2A
ΔCD19
3' LTR
SFG-Helios-ΔCD34
5' LTR
Ψ
Helios
2A
ΔCD34
3' LTR
B
Empty Vector
FOXP3
FOXP3+Hel-FL
FOXP3+Hel-Δ3B
0.3
5.0
1.2
84.2
87.2
88.1
2.1
1.6
Helios
FOXP3
CD4+ eTregs
GMFI of % Positive
Empty Vector
FOXP3
FOXP3+ Hel-FL
FOXP3+ Hel-Δ3B
FOXP3 GMFI
Helios GMFI
C
Empty Vector
FOXP3
FOXP3+Hel-FL
FOXP3+Hel-Δ3B
0
14.0
0.2
76.6
91.8
90.7
2.4
1.7
Helios
FOXP3
CD8+ eTregs
GMFI of % Positive
FOXP3 GMFI
Helios GMFI
D
Donor 3
Donor 5
Empty Vector
FOXP3
FOXP3+Hel-FL
FOXP3+Hel- Δ3B
1500 bp
1000 bp
Hel-FL
Hel-Δ3B

Figure 2-3: FOXP3 + Hel-FL eTregs delay disease progression in a xenogeneic GVHD murine model. 8-12 week old NSG mice were sub-lethally irradiated. The next day, the mice were injected retro-orbitally with 10^7 human PBMCs alone or with $5x10^6$ empty vector control cells or eTregs. **A)** GVHD score was monitored until day of sacrifice. *= $p<0.05$ compared to PBMCs only based on a one-tailed Mann-Whitney test for each time point. **B)** Kaplan-Meier curve of survival. Death was marked when GVHD score was ≥7. *= $p<0.05$ compared to PBMCs only as determined by log-rank test.

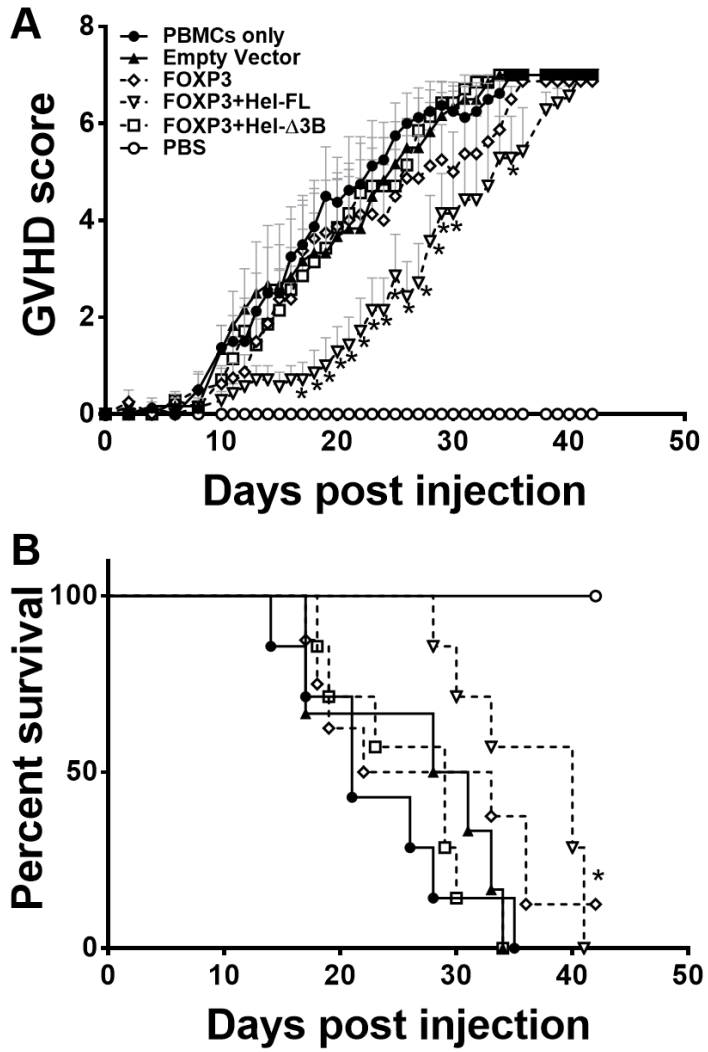

A
GVHD score
PBMCs only
Empty Vector
FOXP3
FOXP3+Hel-FL
FOXP3+Hel-Δ3B
PBS
Days post injection
B
Percent survival
Days post injection

Figure 2-4: FOXP3+Helios-FL overexpression in T cells reduces inflammation in xenoGVHD mice at an early timepoint. 8-12 week old NSG mice were sub-lethally irradiated. The next day, the mice were injected retro-orbitally with 10^7 human PBMCs alone or with $5x10^6$ empty vector control cells or eTregs. **A)** GVHD score was monitored until day of sacrifice. Mice were all euthanized at an early time point of 12 days and spleens were isolated, processed into a single cell suspension, labeled with antibodies for flow cytometry and analyzed for **B)** number of total splenocytes, **C)** percent of CD3+ CD19- responder T cells and **D)** percent of Tregs within the CD19- responder T cell population. Tregs were defined as CD3+ CD4+ CD25+ FOXP3+ or CD3+ CD4+ CD25+ FOXP3+ Helios+. N=3-5 for each group and *= $p<0.05$ compared to PBMCs only based on a one-tailed Mann-Whitney test.

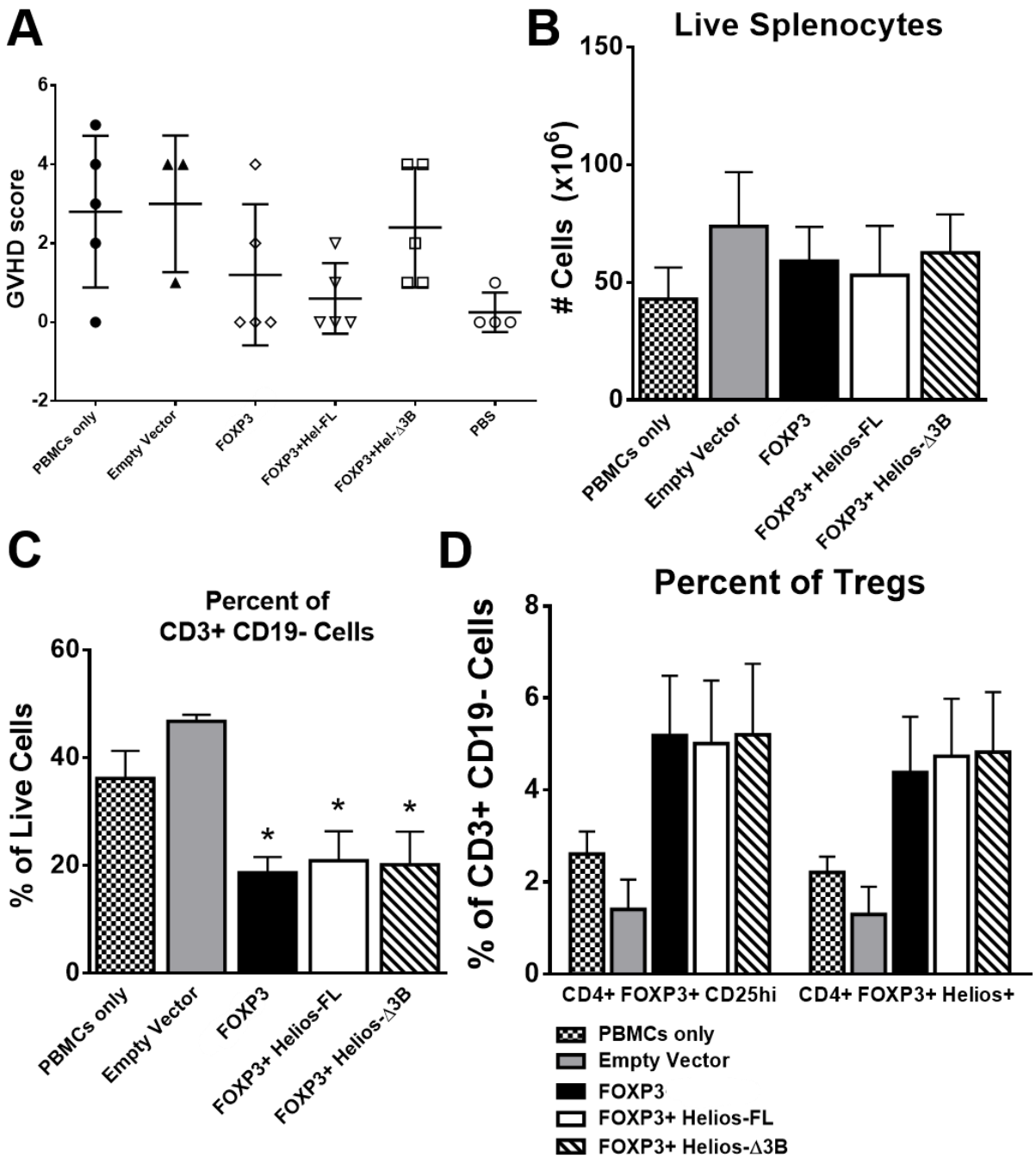

A
GVHD score
6
4
2
0
-2
PBMCs only
Empty Vector
FOXP3
FOXP3+Hel-FL
FOXP3+Hel-Δ3B
PBS
B
Live Splenocytes
Cells (x10^6)
150
100
50
0
PBMCs only
Empty Vector
FOXP3
FOXP3+ Helios-FL
FOXP3+ Helios-Δ3B
C
Percent of
CD3+ CD19- Cells
% of Live Cells
60
40
20
0
*
*
*
PBMCs only
Empty Vector
FOXP3
FOXP3+ Helios-FL
FOXP3+ Helios-Δ3B
D
Percent of Tregs
% of CD3+ CD19- Cells
8
6
4
2
0
CD4+ FOXP3+ CD25hi
CD4+ FOXP3+ Helios+
PBMCs only
Empty Vector
FOXP3
FOXP3+ Helios-FL
FOXP3+ Helios-Δ3B

Figure 2-5: eTreg treatment of xenoGVHD mice reduces pro-inflammatory proteins in the serum. 8-12 week old NSG mice were sub-lethally irradiated. The next day, the mice were injected retro-orbitally with 10^7 human PBMCs alone or with $5x10^6$ empty vector control cells or eTregs. Mice were all euthanized at an early time point of 12 days and serum was isolated from blood recovered post-mortem. Soluble proteins were quantified using cytometric bead array. N=3-5 for each group and *= $p<0.05$ compared to PBMCs only based on a one-tailed Mann-Whitney test.

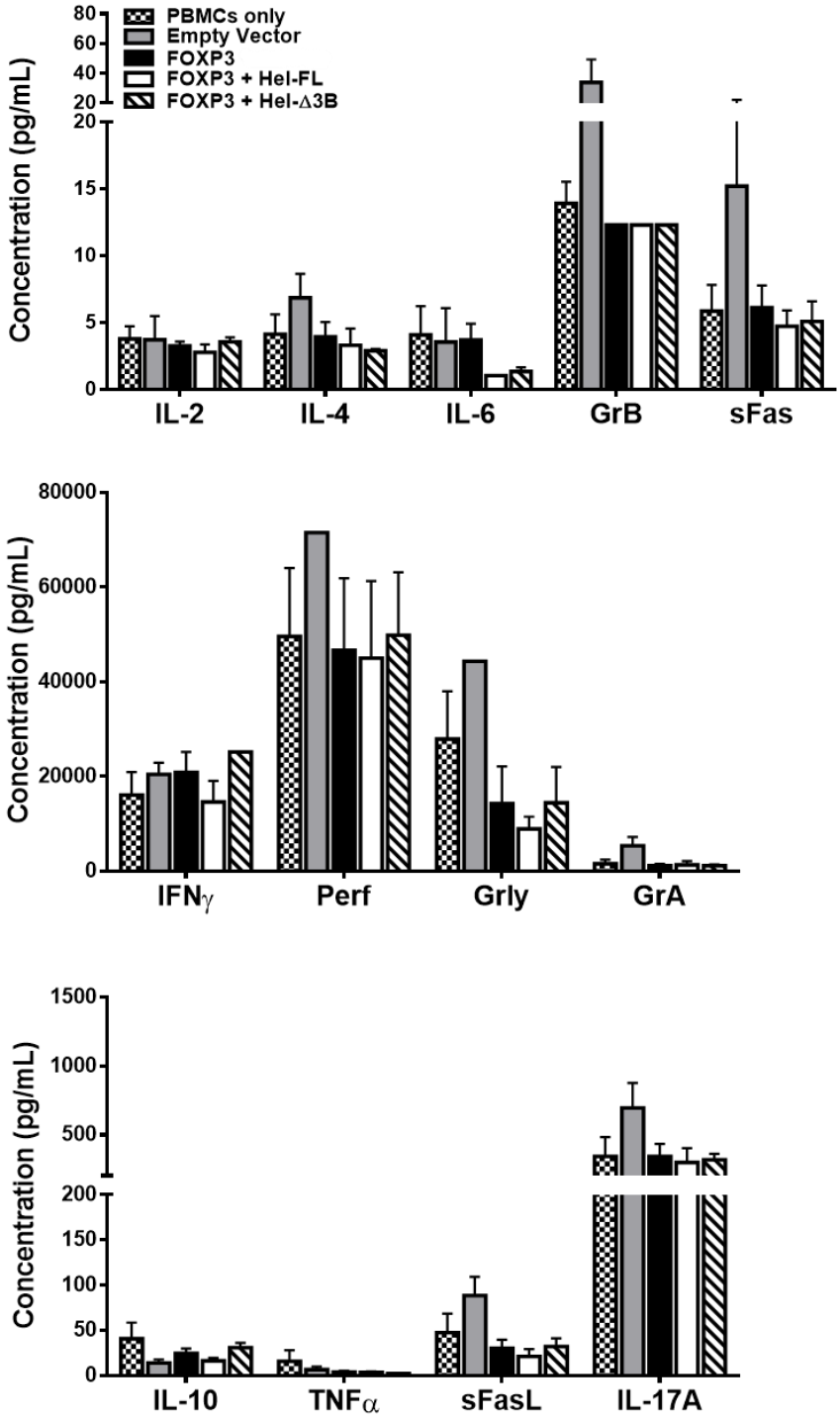

PBMCs only
Empty Vector
FOXP3
FOXP3 + Hel-FL
FOXP3 + Hel-Δ3B
Concentration (pg/mL)
80
60
40
20
20
15
10
5
0
IL-2
IL-4
IL-6
GrB
sFas
Concentration (pg/mL)
80000
60000
40000
20000
0
IFNγ
Perf
Grly
GrA
Concentration (pg/mL)
1500
1000
500
200
150
100
50
0
IL-10
TNFα
sFasL
IL-17A

Figure 2-6: FOXP3+Hel-FL and FOXP3+Hel-Δ3B differentially mediate CD4+ and CD8+ eTreg suppression. Labeled autologous target Tconv cells were co-cultured with each eTreg cell strain or empty vector control cells with no stimulation or stimulation with anti-CD3 and anti-CD28 coated beads. CD4+ and CD8+ eTregs and empty vector cells were purified via antibody coated magnetic bead separation. Cells were plated at effector to target ratios of 1:1. After 96 hours, target cell proliferation was assayed via flow cytometry. **A)** Percent suppression for each eTreg cell strain. N=5-7 for each group with 4 different donors. Percent suppression is calculated by the following equation: [(percent responder proliferation alone)-(percent responder proliferation with transduced cells)]/ (percent responder proliferation alone) x 100. *= $p<0.05$ compared to empty vector control based on a one-tailed Wilcoxon test. **B)** Representative dot plots of responder cell proliferation 96 hours after co-culture with eTregs or empty vector control.

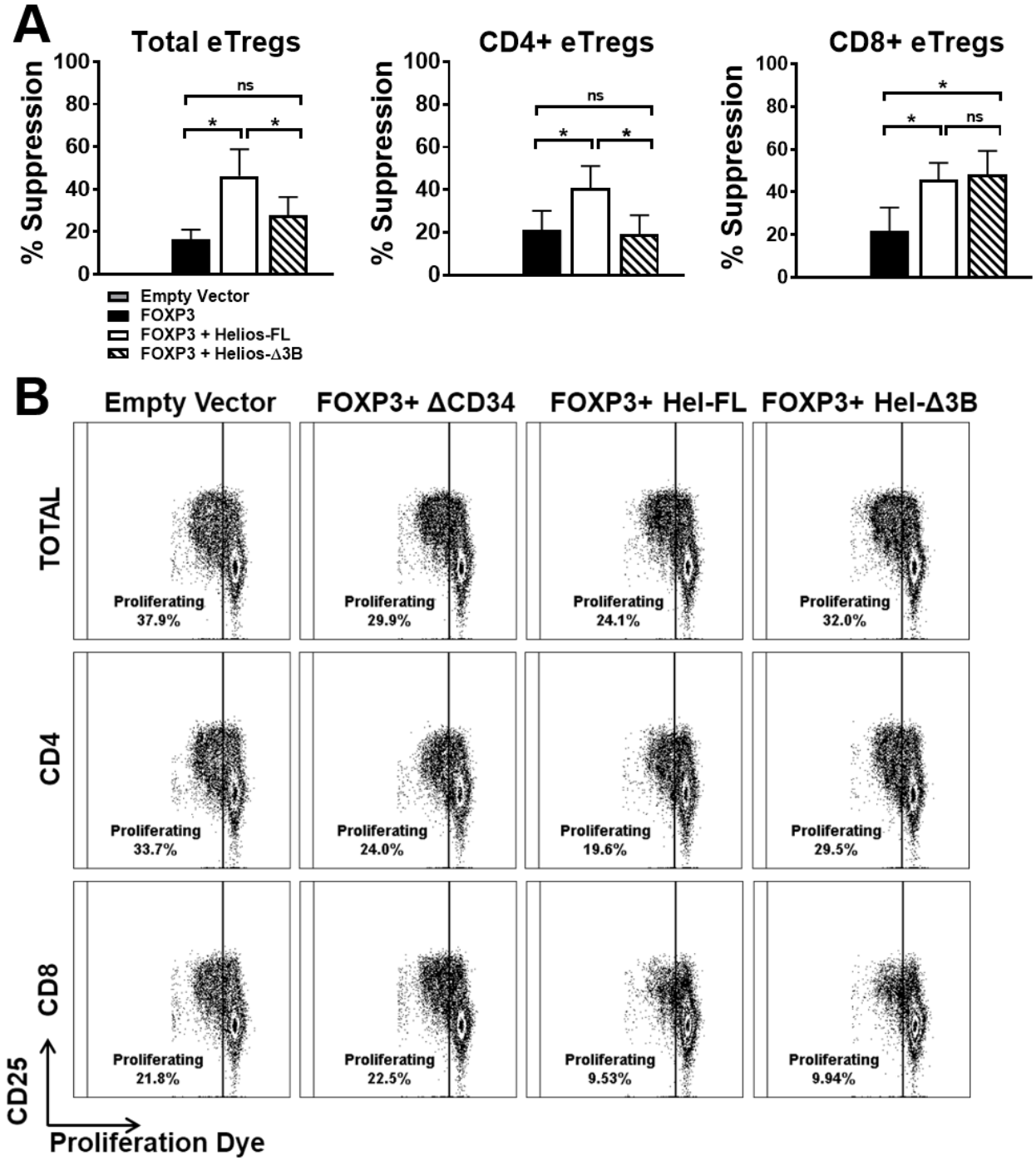

A
Total eTregs
CD4+ eTregs
CD8+ eTregs
% Suppression
100
80
60
40
20
0
ns
*
*
Empty Vector
FOXP3
FOXP3 + Helios-FL
FOXP3 + Helios-Δ3B
B
Empty Vector
FOXP3+ ΔCD34
FOXP3+ Hel-FL
FOXP3+ Hel-Δ3B
TOTAL
CD4
CD8
Proliferating 37.9%
Proliferating 29.9%
Proliferating 24.1%
Proliferating 32.0%
Proliferating 33.7%
Proliferating 24.0%
Proliferating 19.6%
Proliferating 29.5%
Proliferating 21.8%
Proliferating 22.5%
Proliferating 9.53%
Proliferating 9.94%
CD25
Proliferation Dye

Figure 2-7: FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs have reduced proliferation and survival. A) Cell counts of eTregs growing in IL-2 supplemented media were collected over 9 days. N=4 for each group from 4 different donors. *= $p<0.05$ compared to empty vector control based on a one-tailed Mann-Whitney test for each time point. **B)** Frequency of transduced eTregs in the spleens of xenoGVHD mice sacrificed day 12 post-injection via flow cytometry. Mice were treated as previously described. Transduced eTregs and empty vector control cells were identified as CD3+ CD19+ via flow cytometry. **C)** eTregs were stimulated for 2, 4 and 6 days with anti-CD3 and anti-CD28 plate bound antibody. Numbers of live cells per µL were assessed via flow cytometry. Live cells were defined as Zombie Green and Annexin V negative cells. N=5-6 from 4-6 different donors for each group. *= $p<0.05$ compared to empty vector control based on a one-tailed Mann-Whitney test. **D)** Copy number of CD19/Copy number of CD3 to detect transduced eTregs or empty vector control cells in tissues from xenoGVHD mice sacrificed day 12 post-injection via flow cytometry. Genomic DNA was isolated from each tissue and digital drop PCR was used to quantify CD19 copy numbers and CD3 copy numbers. Primers for CD19 were specific for codon optimized CD19 that was only expressed in eTregs or empty vector control cells.

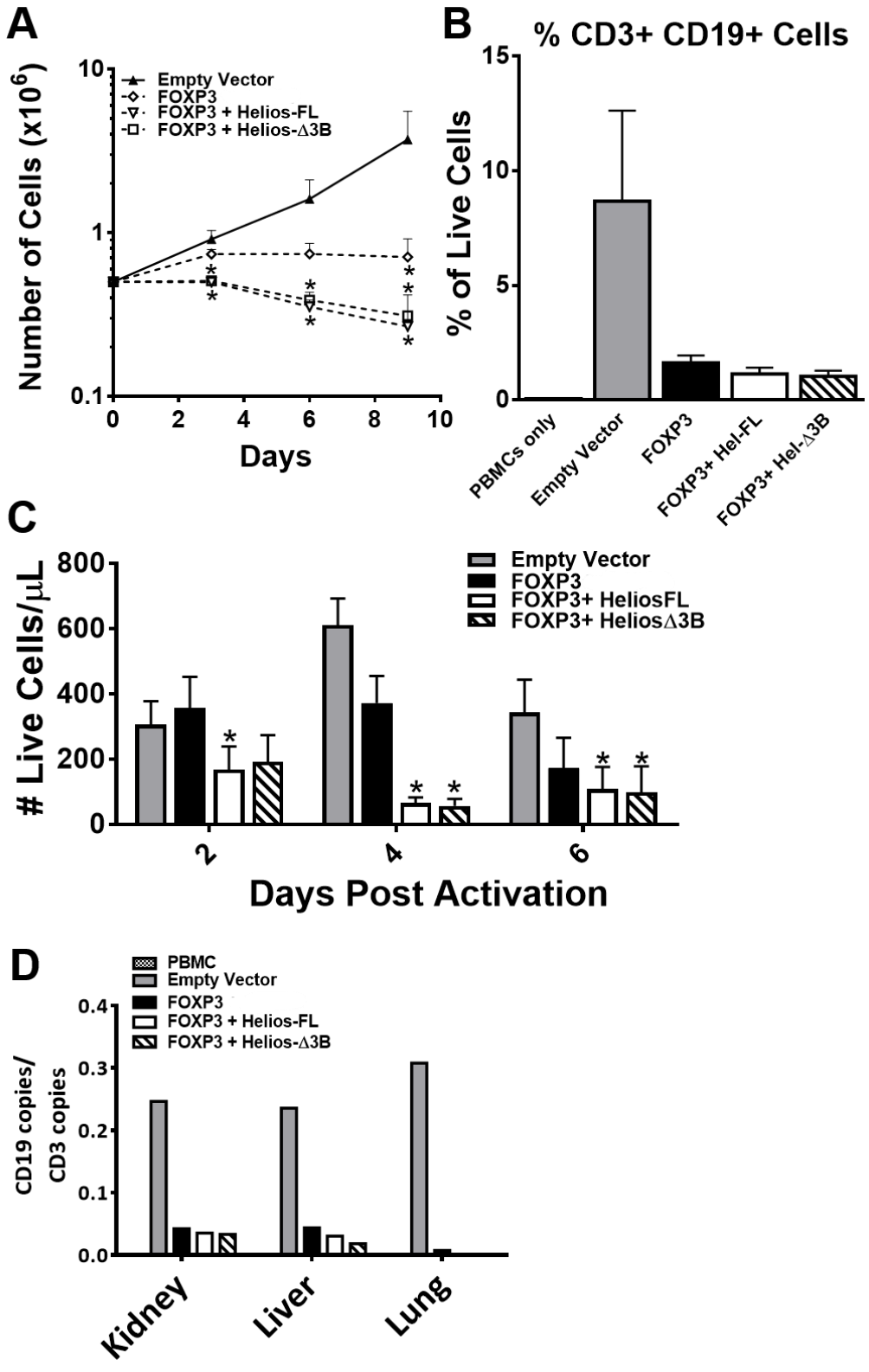

A
Number of Cells (x10^6)
Empty Vector
FOXP3
FOXP3 + Helios-FL
FOXP3 + Helios-Δ3B
Days
B
% CD3+ CD19+ Cells
% of Live Cells
PBMCs only
Empty Vector
FOXP3
FOXP3+ Hel-FL
FOXP3+ Hel-Δ3B
C
Live Cells/μL
Empty Vector
FOXP3
FOXP3+ HeliosFL
FOXP3+ HeliosΔ3B
Days Post Activation
D
PBMC
Empty Vector
FOXP3
FOXP3 + Helios-FL
FOXP3 + Helios-Δ3B
CD19 copies/ CD3 copies
Kidney
Liver
Lung

Figure 2-8: FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs express regulatory T cell markers and have reduced cytokine production. A) Expression of Treg markers by CD4+ and CD8+ eTregs. Marker expression was assessed via flow cytometry and plotted as geometric mean fluorescence intensity (GMFI) of the population positive for the marker. N=3-7 and 5 different donors. *= $p<0.05$ compared to empty vector control based on one-tailed Mann-Whitney test. **B)** Cytokine production by CD4+ and CD8+ eTregs. eTregs were stimulated for 6 hours with anti-CD3 and anti-CD28 plate bound antibody and Brefeldin A and Golgi Stop. Cells were assessed for cytokine production via intracellular cytokine staining and flow cytometry. Values normalized to empty vector control and N=4-9 with 4-6 different donors for each group. *= $p<0.05$ compared to empty vector control based on one-tailed Mann-Whitney test.

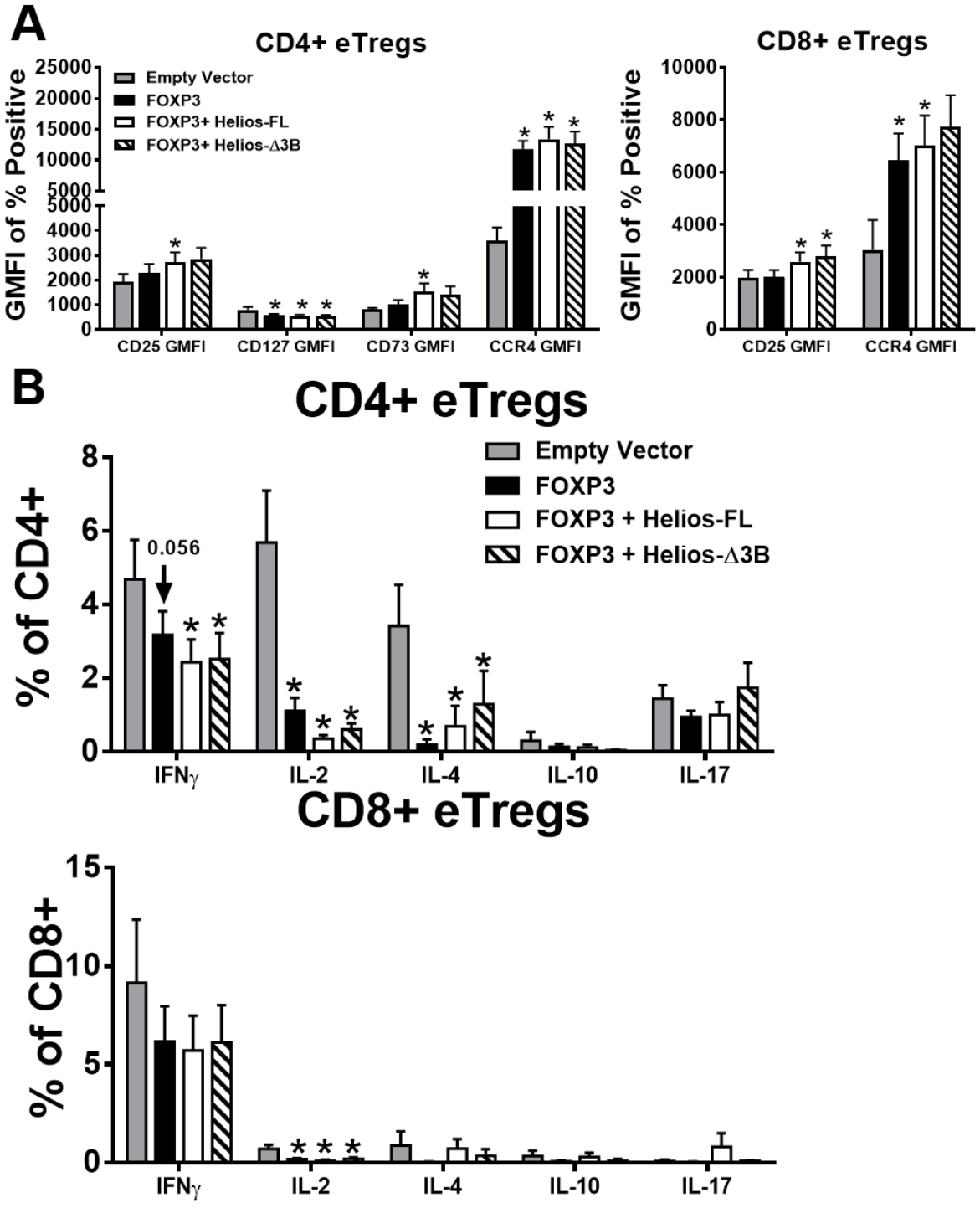

A
CD4+ eTregs
GMFI of % Positive
Empty Vector
FOXP3
FOXP3+ Helios-FL
FOXP3+ Helios-Δ3B
CD25 GMFI
CD127 GMFI
CD73 GMFI
CCR4 GMFI
CD8+ eTregs
B
CD4+ eTregs
% of CD4+
0.056
IFNγ
IL-2
IL-4
IL-10
IL-17
CD8+ eTregs
% of CD8+

Figure 2-9: Hel-FL or Hel-Δ3B co-expression with FOXP3 alters gene expression and pathway enrichment in CD4+ or CD8+ eTregs compared to FOXP3 alone. All comparisons in this figure use FOXP3 eTregs as the baseline for comparison of CD4+ or CD8+ eTregs as indicated. **A)** Volcano plots depicting -$\log_{10}$p-value versus log Fold Change (FC). Within the volcano plots, genes were colored if they had a nominal, uncorrected p value less than 0.05. Blue color denotes down regulation while red color represents up regulation. The two vertical lines represent logFC = -2 and logFC=2. The horizontal line presents -log10(0.05). **B)** Summary of normalized enrichment scores (NES) of KEGG pathways with $p<0.05$ that were enriched in the comparison of two eTreg cell strains indicated following gene set enrichment analysis (GSEA). Blue bars are pathways enriched in the baseline eTregs and red bars are pathways enriched in eTregs being compared.

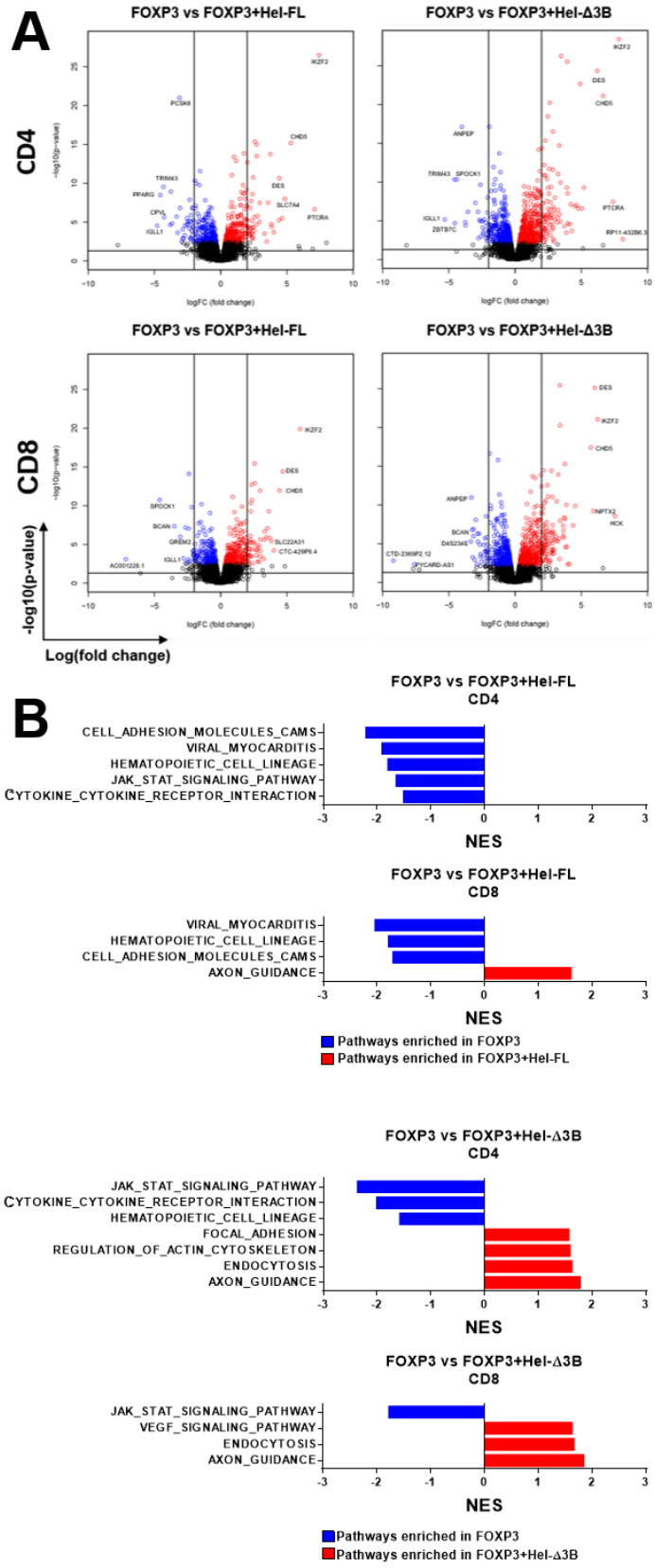

A
FOXP3 vs FOXP3+Hel-FL
FOXP3 vs FOXP3+Hel-Δ3B
CD4
CD8
-log10(p-value)
logFC (fold change)
Log(fold change)
B
FOXP3 vs FOXP3+Hel-FL
CD4
CELL_ADHESION_MOLECULES_CAMS
VIRAL_MYOCARDITIS
HEMATOPOIETIC_CELL_LINEAGE
JAK_STAT_SIGNALING_PATHWAY
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
NES
CD8
AXON_GUIDANCE
Pathways enriched in FOXP3
Pathways enriched in FOXP3+Hel-FL
FOXP3 vs FOXP3+Hel-Δ3B
FOCAL_ADHESION
REGULATION_OF_ACTIN_CYTOSKELETON
ENDOCYTOSIS
VEGF_SIGNALING_PATHWAY
Pathways enriched in FOXP3
Pathways enriched in FOXP3+Hel-Δ3B

Figure 2-10: FOXP3+Hel-Δ3B mediates different gene transcription and pathway enrichment in CD4+ and CD8+ eTregs compared to FOXP3+Hel-FL. All comparisons in this figure use FOXP3+Hel-FL eTregs as the baseline for comparison, either CD4+ or CD8+ as indicated. **A)** Volcano plots depicting -$\log_{10}$p-value versus log Fold Change (FC). Within the volcano plots, genes were colored if they had a nominal, uncorrected p value less than 0.05. Blue color denotes down regulation while red color represents up regulation. The two vertical lines represent logFC = -2 and logFC=2. The horizontal line presents -log10(0.05). **B)** Summary of normalized enrichment scores (NES) of KEGG pathways with $p<0.05$ that were enriched in comparisons of two eTreg cell strains following gene set enrichment analysis (GSEA). Blue bars are pathways enriched in the baseline eTregs and red bars are pathways enriched in eTregs being compared. **C)** Heatmaps of genes altered in common pathways enriched in CD4+ and CD8+ eTregs in each of the three donors for the FOXP3-Hel-FL (FL1-FL3) vs FOXP3+Hel-Δ3B (Δ3B1-Δ3B1) comparison.

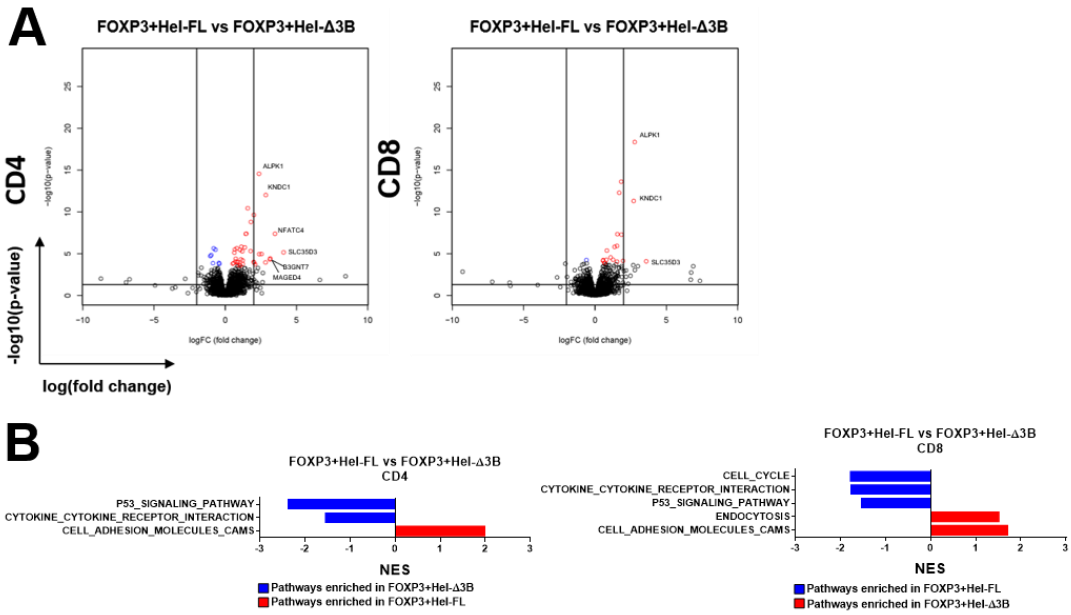
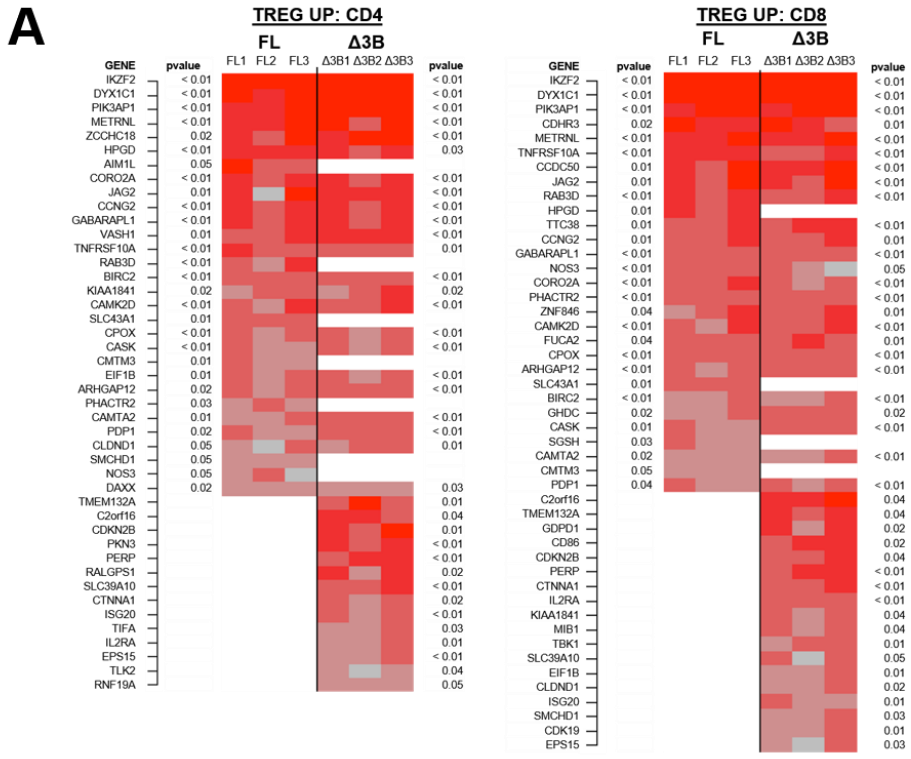

A
FOXP3+Hel-FL vs FOXP3+Hel-Δ3B
FOXP3+Hel-FL vs FOXP3+Hel-Δ3B
CD4
CD8
-log10(p-value)
log(fold change)
logFC (fold change)
ALPK1
KNDC1
NFATC4
SLC35D3
B3GNT7
MAGED4
B
FOXP3+Hel-FL vs FOXP3+Hel-Δ3B
CD4
P53_SIGNALING_PATHWAY
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
CELL_ADHESION_MOLECULES_CAMS
NES
Pathways enriched in FOXP3+Hel-Δ3B
Pathways enriched in FOXP3+Hel-FL
FOXP3+Hel-FL vs FOXP3+Hel-Δ3B
CD8
CELL_CYCLE
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION
P53_SIGNALING_PATHWAY
ENDOCYTOSIS
CELL_ADHESION_MOLECULES_CAMS
NES
Pathways enriched in FOXP3+Hel-FL
Pathways enriched in FOXP3+Hel-Δ3B
C
P53 SIGNALING PATHWAY
CD4
GENE
PERP
CASP9
CD82
CDK6
SERPINE1
CCNG1
PPM2B
FAS
BBC3
ATM
CDKN2A
BAX
SESN1
ZMAT3
TNFRSF10B
MDM2
CDKN1A
EI24
GADD45A
CD8
GENE
TP73
THBS1
PERP
CASP9
CDK6
BAX
PPM2B
CDKN1A
CCNG1
PPM1D
MDM2
EI24
GADD45B
TNFRSF10B
FAS
SESN1
GADD45G
GADD45A
SFN
CYTOKINE-CYTOKINE RECEPTOR INTERACTION
CD4
GENE
CXCL16
IL1R1
IL1R2
KIT
TPO
PDGFB
PDGFRB
ACVR2B
IL2RA
TGFB3
BMPR2
FLT4
TNFRSF13C
BMPR1A
TNFRSF4
CCR8
TNFRSF11A
IL21R
IL4R
TNFSF12
TGFBR1
FAS
CSF1
EDA2R
TNFSF8
IL12RB1
LTB
IL18R1
TNFRSF10B
CXCR3
CD70
TNFSF9
TNFRSF10D
CCL25
LTA
TNFSF13
CCR5
CXCR6
TNFSF15
CD8
GENE
IL1R1
IL17RB
CXCL16
ACVR2B
IL2RA
BMPR2
KIT
TNFRSF11A
ACVR1
LIF
IL21R
TNFSF12
CCR2
TNF
TNFRSF25
TNFSF8
TNFSF14
TNFRSF10A
CD70
TNFRSF10B
CSF1
FAS
CCR1
IL12RB1
LTA
CXCR3
CCR5
TNFRSF10D
IL18RAP
CXCR6
IL18R1
TNFSF15
CELL ADHESION MOLECULES CAMs
CD4
GENE
PDCD1LG2
PVRL1
PTPRF
SDC4
HLA-DRB5
HLA-DRB1
HLA-DQA
CD86
NLGN2
MADCAM1
PECAM1
MPZ
L1CAM
PVR
HLA-DRA
MPZL1
ITGA4
ITGAE
CD8
GENE
CD86
PTPRF
L1CAM
NEGR1
SDC4
HLA-DRB5
HLA-DRB1
PECAM1
ALCAM
PDCD1LG2
HLA-DQA
HLA-DRA
ITGA4
NLGN3
MPZL1

Figure 2-11: Hel-FL or Hel-Δ3B co-expression with FOXP3 mediate different gene transcription of Treg signature genes in CD4+ and CD8+ eTregs. Heatmaps comparing expression of Treg signature genes that are **A)** upregulated in Tregs (TREG UP) or **B)** downregulated in Tregs (TREG DOWN) compared to Tconv. Each heatmap shows differential expression of genes in each of the three donors for FOXP3 vs FOXP3+ Hel-FL (FL, donors FL1-FL3) and FOXP3 vs FOXP3+ Hel-Δ3B (Δ3B, donors Δ3B1-Δ3B1) in both CD4+ and CD8+ eTregs as indicated. For each cell in the heatmap, the difference of cpm values between two strains of cell for one subject was calculated and divided by average cpm value of that gene in all three subjects.


A
TREG UP: CD4
FL
Δ3B
GENE
pvalue
FL1 FL2 FL3 Δ3B1 Δ3B2 Δ3B3
pvalue
IKZF2 < 0.01 < 0.01
DYX1C1 < 0.01 < 0.01
PIK3AP1 < 0.01 < 0.01
METRNL < 0.01 < 0.01
ZCCHC18 0.02 < 0.01
HPGD < 0.01 0.03
AIM1L 0.05
CORO2A < 0.01 < 0.01
JAG2 0.01 < 0.01
CCNG2 < 0.01 < 0.01
GABARAPL1 < 0.01 < 0.01
VASH1 0.01 < 0.01
TNFRSF10A < 0.01 0.01
RAB3D < 0.01
BIRC2 < 0.01 < 0.01
KIAA1841 0.02 0.02
CAMK2D < 0.01 < 0.01
SLC43A1 0.01
CPOX < 0.01 < 0.01
CASK < 0.01 < 0.01
CMTM3 0.01
EIF1B 0.01 < 0.01
ARHGAP12 0.02 < 0.01
PHACTR2 0.03
CAMTA2 0.01 < 0.01
PDP1 0.02 < 0.01
CLDND1 0.05 0.01
SMCHD1 0.05
NOS3 0.05
DAXX 0.02 0.03
TMEM132A 0.01
C2orf16 0.04
CDKN2B 0.01
PKN3 < 0.01
PERP < 0.01
RALGPS1 0.02
SLC39A10 < 0.01
CTNNA1 0.02
ISG20 < 0.01
TIFA 0.03
IL2RA 0.01
EPS15 < 0.01
TLK2 0.04
RNF19A 0.05
TREG UP: CD8
FL
Δ3B
GENE
pvalue
FL1 FL2 FL3 Δ3B1 Δ3B2 Δ3B3
pvalue
IKZF2 < 0.01 < 0.01
DYX1C1 < 0.01 < 0.01
PIK3AP1 < 0.01 < 0.01
CDHR3 0.02 0.01
METRNL < 0.01 < 0.01
TNFRSF10A < 0.01 < 0.01
CCDC50 0.01 < 0.01
JAG2 0.01 < 0.01
RAB3D < 0.01 < 0.01
HPGD 0.01
TTC38 0.01 < 0.01
CCNG2 0.01 0.01
GABARAPL1 < 0.01 < 0.01
NOS3 < 0.01 0.05
CORO2A < 0.01 < 0.01
PHACTR2 < 0.01 < 0.01
ZNF846 0.04 0.01
CAMK2D < 0.01 < 0.01
FUCA2 0.04 0.01
CPOX < 0.01 < 0.01
ARHGAP12 < 0.01 < 0.01
SLC43A1 0.01
BIRC2 < 0.01 < 0.01
GHDC 0.02 0.02
CASK 0.01 < 0.01
SGSH 0.03
CAMTA2 0.02 < 0.01
CMTM3 0.05
PDP1 0.04 < 0.01
C2orf16 0.04
TMEM132A 0.04
GDPD1 0.02
CD86 0.02
CDKN2B 0.04
PERP < 0.01
CTNNA1 < 0.01
IL2RA < 0.01
KIAA1841 0.04
MIB1 0.04
TBK1 0.01
SLC39A10 0.05
EIF1B 0.01
CLDND1 0.02
ISG20 0.01
SMCHD1 0.03
CDK19 0.01
EPS15 0.03
B
TREG DOWN: CD4
FL
Δ3B
GENE
pvalue
FL1 FL2 FL3 Δ3B1 Δ3B2 Δ3B3
pvalue
SECTM1 < 0.01 < 0.01
DNAJC6 < 0.01 < 0.01
C11orf74 0.03
NELL2 < 0.01 < 0.01
PTPRK < 0.01
STX3 < 0.01 < 0.01
TOP1MT < 0.01 0.05
HYI < 0.01 < 0.01
ZNF629 0.01
APP 0.01
ACVR2A < 0.01 0.01
PDE4D < 0.01 < 0.01
LGALS3BP < 0.01 < 0.01
OCRL 0.04
SSBP2 < 0.01 0.01
PIK3CB 0.01 < 0.01
CTDSP2 < 0.01 < 0.01
TMEM71 0.01 0.03
DUSP7 < 0.01 < 0.01
RGL4 0.01 < 0.01
ASB13 0.03 0.03
TSEN2 0.04 0.04
OXNAD1 0.01 0.01
VGLL4 0.01 0.01
SNTB1 0.01
STX17 0.03
SLC20A1 0.02 < 0.01
BHLHE40 0.03 0.01
ATP1A1 < 0.01 < 0.01
PRMT5 0.03 0.03
SPIN1 0.02 0.04
BAG3 0.05
CAD 0.04
NOSIP 0.04 0.01
PRKCA 0.04 0.01
TPM2 0.02
EMR1 0.01
PANK1 < 0.01
GBP4 < 0.01
GIMAP5 < 0.01
GIMAP8 < 0.01
STXBP1 < 0.01
PDIA5 0.04
POU6F1 < 0.01
GOLGA7B 0.01
IFITM3 < 0.01
CDC14A < 0.01
GTPBP8 0.02
FASTKD1 < 0.01
FAM65B < 0.01
LRRN3 < 0.01
IFITM2 < 0.01
TFPT 0.05
MSH6 0.02
EIF4G1
TREG DOWN: CD8
FL
Δ3B
GENE
pvalue
FL1 FL2 FL3 Δ3B1 Δ3B2 Δ3B3
pvalue
SECTM1 < 0.01
AOAH < 0.01 0.04
NELL2 < 0.01 < 0.01
ZNF629 < 0.01 < 0.01
FRY 0.04
DNAJC6 < 0.01 < 0.01
AK5 < 0.01
ACVR2A < 0.01
APP 0.01
PDE4D < 0.01 < 0.01
HYI 0.01 < 0.01
SSBP2 < 0.01 < 0.01
TMEM71 < 0.01 < 0.01
STX3 < 0.01 0.02
FZD6 < 0.01
LGALS3BP < 0.01 < 0.01
CTDSP2 < 0.01 < 0.01
OXNAD1 < 0.01 < 0.01
GBP4 < 0.01 < 0.01
PIK3CB 0.01 < 0.01
PDIA5 0.03 0.01
SNTB1 < 0.01
TOP1MT 0.04
AKR1B1 0.01
DUSP7 0.01 0.03
LRRN3 0.01 < 0.01
VGLL4 0.01 < 0.01
SLC20A1 0.01 < 0.01
CDC14A 0.01 < 0.01
BHLHE40 0.02 < 0.01
IFITM2 0.01 < 0.01
STX17 0.04
TPCN1 0.02
SPIN1 0.01 < 0.01
NOSIP 0.01 < 0.01
ATP1A1 0.01 < 0.01
PRKCA 0.02 < 0.01
FAM65B 0.04 < 0.01
STIM2 0.01 0.02
SGSM3 0.04 0.02
C19orf60 0.05
GIMAP5 < 0.01
GIMAP8 < 0.01
STXBP1 < 0.01
S100B 0.01
GOLGA7B < 0.01
IFITM3 < 0.01
PANK1 0.05
RGL4 < 0.01
ACSL6 0.01
TMEM229B 0.04
GTPBP8 0.03
SNX18 0.01
TFPT 0.01
SPTBN1 0.01
TNFAIP8 0.03
UBTF 0.04

Discussion

Here we described a dual retroviral transduction system that was able to overexpress FOXP3+Hel-FL in total human T cells and convert these T cells into CD4+ and CD8+ eTregs with immunosuppressive properties both *in vitro* and *in vivo*. Additionally, we are the first to describe differential roles for the two endogenous isoforms of Helios in mediating suppressive function in CD4+ and CD8+. Finally, we provided transcriptional profiling of human eTregs that expressed FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B and compared these profiles to KEGG pathways and published Treg signatures. Together, these findings not only provide insight into the role of Helios and FOXP3 co-expression in Treg function but improve current human eTreg generation protocols and increase the potential for eTregs to be used in the clinic.

Helios has been described as a key Treg transcription factor for many years but its function in Tregs is still being defined. Experiments using Treg-specific Helios knock out mice have demonstrated that Helios plays a major role in mediating both CD4+ and CD8+ Treg function and survival [42, 43]. The Helios+ subset of human CD4+ Tregs have improved stability in pro-inflammatory environments compared to Helios- CD4+ Tregs [42, 43]. A subset of Helios+ CD8+ Tregs have also been defined and have been shown to target T follicular helper cells [192]. Our work demonstrated that co-expression of FOXP3 with Hel-FL in total human cells was able to more effectively delay disease in a xenoGVHD model compared to FOXP3 alone. Both CD4+ and CD8+ FOXP3+Hel-FL eTregs had the most suppressive capacity *in vitro* compared to FOXP3 alone. However, there were no obvious differences in survival, Treg marker expression or cytokine production (**Figures 2-7 and 2-8**). There was a change in FOXP3+Hel-FL eTregs in expression of genes compared to FOXP3 alone in immune pathways, such as cell adhesion molecules and JAK/STAT signaling, and Treg-related genes. This change in

transcription was expected as ectopic expression of Helios and FOXP3 separately and together in mouse Tconvs mediated expression of different Treg signature genes [277, 298]. Further studies will be needed to investigate the roles of the genes altered by Hel-FL expression in Treg function.

An unexpected result was the differences between FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTreg function. To the best of our knowledge, there have been no studies comparing the function of Hel-FL and Hel-Δ3B in primary human T cells. We demonstrated that FOXP3+Hel-FL overexpression improves CD4+ eTreg suppressive activity, FOXP3+Hel-Δ3B overexpression does not. Interestingly, FOXP3+Hel-Δ3B does improve suppressive activity of CD8+ eTregs to a similar degree as FOXP3+Hel-FL eTregs. Both Hel-FL and Hel-Δ3B co-expression with FOXP3 changed gene expression when compared to FOXP3 alone and there were changes that were unique to each isoform of Helios.

Correlation of the gene changes in FOXP3+Hel-FL and FOXP3+ Hel-Δ3B with our functional studies could reveal more about the molecular mechanisms required to convey immunosuppressive properties to CD4+ and CD8+ T cells. For example, our RNASeq and GSEA data showed that FOXP3+Hel-FL CD4+ eTregs had increased gene enrichment in p53 signaling and cytokine-cytokine receptor interaction and decreased gene enrichment in cell adhesion molecules (CAMs) when compared to FOXP3+Hel-Δ3B CD4+ eTregs (**Figure 2-10B**). p53 signaling is important for CD4+ Treg induction in mice [348]. The different cytokine receptors that were upregulated on FOXP3+Hel-FL CD4+ eTregs were chemokine receptors such as CCR5 and CXCR6 which have been demonstrated to be expressed on endogenous human Tregs [349] and these receptors drive immune cell trafficking to sites of inflammation [350-353]. Finally, the only CAM that has been extensively studied in Treg function is ICAM-1

which is not differentially expressed in FOXP3-Hel-FL vs FOXP3-Hel-Δ3B CD4+ eTregs (**Figure 2-10C**) [354, 355]. The differences we observed in CAM expression between FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD4+ eTregs could be linked to T cell immunosuppressive function, though further studies are needed. Thus, the changes we found in these three KEGG pathways could explain why FOXP3+Hel-FL CD4+ eTregs were more effective at suppressing *in vivo* and *in vitro* than FOXP3+Hel-Δ3B CD4+ eTregs.

The same three pathways were also changed in FOXP3+Hel-Δ3B CD8+ compared to FOXP3+Hel-FL CD8+ eTregs but these two eTreg cell strains suppress equally well. Further examination revealed there were differences in the specific genes that were changed in these three pathways when comparing CD4+ and CD8+ FOXP3+Hel-Δ3B eTregs (**Figure 2-10C**). Thus, the specific gene expression differences in these pathways that were unique to the CD4+ eTregs could identify the genes important in mediating T cell suppressive activity. Alternatively, CD8+ eTregs might not require these three pathways to suppress. Additionally, we found that both CD4+ and CD8+ FOXP3+Hel-Δ3B had a higher Treg signature compared to FOXP3+Hel-FL eTregs based on the number of genes that were differentially expressed in our Treg signature gene lists. However, based on the functional differences between FOXP3+Hel-FL and FOXP3+Hel- Δ3B CD4+ eTregs, it is likely the genes that are differentially expressed between these two eTreg cell strains that are critical to CD4+ T cell immunosuppressive function rather than the number of genes changed. Similarly, the gene expression differences between the FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD8+ eTregs may not be critical to CD8+ T cell immunosuppression as these two cell strains suppress at a similar level.

These findings indicate that the endogenous isoforms of Helios play different roles in CD4+ vs CD8+ T cells. Hel-Δ3B lacks half an exon in a zinc finger domain, which would affect

its ability to bind DNA. Thus, differences between the effect of FOXP3+Hel-Δ3B overexpression in CD4+ vs CD8+ T cells likely arises from epigenetic differences between the cell subsets and promoter accessibility. Another example of Ikaros family members playing different roles in CD4+ and CD8+ T cells is the critical role of Ikaros in CD8+ T cell development but not CD4+ development [356]. Investigating the differences between FOXP3, FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD4+ and CD8+ eTregs could help define which signaling pathways are critical for CD4+ and CD8+ Treg function. Further studies are required to elucidate the roles of these Helios splice variants in general T cell development and function.

Overall, we generated a novel protocol to genetically manipulate human T cells to express high levels of FOXP3+Hel-FL, which results in immunosuppressive CD4+ and CD8+ eTregs. FOXP3+Hel-FL eTregs are more effective than FOXP3 eTregs at suppressing both *in vivo* and *in vitro* and have changes in gene expression that affect immune pathway and Treg signature genes. We also discovered that Hel-FL and Hel-Δ3B affect CD4+ and CD8+ T cells differently when co-expressed with FOXP3. These new findings define new roles for endogenous Helios splice variants in both CD4+ and CD8+ Tregs and provide an improved human eTreg protocol that could be used to treat a variety inflammatory disease.

Chapter 3 : Expression profiling of Ikaros family members and Treg markers in thymic CD4+ regulatory T cell development in the CD4+ mature single positive stages

Abstract

Regulatory T (Tregs) are a subset of immune cells that maintain homeostasis by promoting immune tolerance and suppressing the immune response via a variety of mechanisms such as secreting cytokines, killing reactive immune cells, and inducing anergy. Tregs have proven to be difficult to study as there are no definitive Treg surface markers. Analysis of the expression of members of the Ikaros family of transcription factors during Treg development can aid in defining Treg surface markers. Here, we used novel CD4+ mature single positive (MSP) thymocyte populations that our laboratory has previously defined to track CD4+ CD25+ FOXP3+ human Treg development from human thymus samples. We then characterized protein expression of Ikaros family members and Treg markers of Tregs from each of these populations. We found that a majority of Tregs can be found in the distinct MSP6 population and these Tregs have heterogenous expression of Helios and CD39 and CD127. Within the MSP1-MSP5 populations expression of Ikaros family members transiently changed and Helios and Eos correlated with the percent of Tregs within each population. Thus, using Ikaros family members and Treg markers within subsets of CD4+ thymocytes, we were able to more precisely determine where thymic Tregs originate from and critical points of Treg development.

Introduction

Regulatory T (Treg) cells are approximately 3-5% of $CD4^+$ T cells and function to promote immune tolerance and maintain immunologic homeostasis. Detailed mechanisms by which Tregs down-regulate immune responses vary and have been reviewed elsewhere [34], but include secreting IL-10 and TGFβ, which suppress the proliferation and activation of pro-inflammatory conventional T (Tconv) cells [33]. Tregs can also convert ATP into the immunosuppressive molecule adenosine and modulate metabolic activity [252]. Beyond their activities on other T cells, Tregs can directly interact with dendritic cells and downregulate co-stimulation of Tconv cells [357]. Tregs can also suppress macrophages and B cells[358-360].

Impaired Treg numbers or function has been linked to overactive immune responses, which contributes to diseases such as autoimmunity, allergy, and graft-versus-host disease [361-363]. Conversely, tumors often contain numerous Tregs that suppress anti-tumor immunity [364]. Thus, the ability to manipulate the number or function of Tregs would have profound therapeutic effects. For example, injection of Tregs has been successfully used in murine models of multiple sclerosis, inflammatory bowel disease, and graft-versus-host disease [289, 365, 366]. However, translating these results into the clinic has been challenging, despite many attempts.

One challenge faced in Treg therapy has been to accurately identify this small cell population. Tregs are characterized by high expression of the FOXP3 transcription factor. In addition, Tregs express CD25, GITR, and CTLA-4, but low levels of CD127. In humans, these markers can also define activated T cells [22], making the isolation of a pure Treg population nearly impossible. Further, Tregs can differentiate into pro-inflammatory $CD4^+$ T cells under the appropriate conditions [198], so a pure Treg population could become a mixed population due to the plasticity of the differentiation state.

A potential solution to the problem of obtaining a pure, stable Treg population is to isolate Tregs from the thymus instead of peripheral blood. Dijke, et. al. [289] showed that thymic-derived Tregs were more effective than Tregs obtained from peripheral blood in preventing graft-versus-host disease in a murine model of the disease. The most likely reason for the difference in efficacy is the stability of thymic Treg function, even in the presence of pro-inflammatory cytokines. These data demonstrate the value in identifying the characteristics that define thymic Tregs and explain the stability of this population.

One defining feature of thymic Tregs is their high expression of Helios, a member of the Ikaros family transcription factors. In addition, Tregs can express Ikaros, Aiolos, and Eos. Each Ikaros family member has four DNA-binding zinc finger motifs near the N-terminus and two C-terminal zinc fingers that mediate protein-protein interactions [367]. Each family member can homodimerize or heterodimerize via the C-terminal zinc fingers in every possible combination [287, 367-370]. To further complicate this family of proteins, each member can undergo alternative splicing that eliminates one or more of the N-terminal zinc fingers [371-373]. Deletion of more than two zinc fingers results in a dominant negative form of the protein that can dimerize with other family members but cannot bind DNA [367]. Examining the entire Ikaros family in Tregs offers a tool analyze Treg development and function. Here, we described protein expression of the Ikaros family in human CD4+ CD25+ FOXP3+ Tregs as they progress through the CD4+ mature single positive (MSP) stages that we have previously described [77]. We also describe the expression of the Treg markers CD39 and CD127 in each of these subsets.

Methods

Human thymocytes

Human thymus samples were obtained, following informed consent of the parent or guardian, from children (0–18 years) who underwent corrective surgery for congenital cardiac defects at Children's Mercy Hospital (Kansas City, MO). Tissue samples were de-identified and void of any clinical data. Samples were obtained in compliance with the Institutional Review Boards at our institutions. Representative figures are from one thymus and multiple thymi from different subjects were collected to ensure reproducibility and numbers are indicated in the figure legends.

Flow cytometry

The anti-human antibodies, anti-CD3−APC-Cy7, anti-CD7-FITC, anti-CD8α-Brilliant Violet (BV) 785, anti-CD25-PerCP-Cy5.5, anti-CD38-Alexa Fluor (AF) 700, anti-CD44-PE-Cy7, anti-CD45RO-PE-Cy5, anti-FOXP3-AF647, anti-Helios-BV421, anti-CD39-BV510, anti-CD127-BV650, Armenian hamster IgG-BV421 control and mouse IgG1κ-PE control were purchased from Biolegend (San Diego, CA). Anti-CD4-PE-eFlour 610, was purchased from ThermoFisher Scientific (Waltham, MA), and anti-Eos-PE, anti-Ikaros-PE, anti-Aiolos-PE and mouse IgG1κ-PE control were purchased from BD Biosciences (San Jose, CA).

Single-cell suspensions of human thymocytes were labeled on their surface as previously described [374]. For intracellular staining, surface-labelled cells were fixed and permeabilized using the Foxp3/Transcription Factor Staining Buffer Set from ThermoFisher Scientific (Waltham, MA), according to the manufacturer's instructions. Cells were analyzed using a BD

LSR II (BD Biosciences) and data were analysed using BD FACSdiva software (BD Biosciences) or FlowJo (TreeStar Inc., Ashland, OR).

Statistical analysis

For comparisons across groups, the paired t-test analysis and multiple comparisons analysis with Tukey post hoc test were performed using Graphpad Prism 6 (GraphPad Software Inc., La Jolla, CA), and significance was defined as $p< 0.05$ and standard error of mean (SEM) was reported.

Results

CD4+ CD25+ FOXP3+ Tregs are most frequent in the CD4+ MSP3-MSP5 and MSP6 populations.

Current thymic Treg studies use CD4+ CD25+ expression to isolate thymic Tregs that have about 50% FOXP3 expression initially and about 80% FOXP3 expression after culture [289]. We first determined where these CD4+ CD25+ FOXP3+ were most abundant within the CD4+ MSP stages that we defined in our previous publication [77]. Briefly, these populations were defined as CD4+ single positive (SP) thymocytes that have high CD3 expression, indicating they are undergoing positive selection [375, 376]. 6 distinct populations, MSP1-MSP6, fit these criteria and were defined based on their expression of CD7, CD44, CD38 and CD45RO (**Figure 3-1**). The sequence of these stages was determined based on CD38 and CD45RO expression as we previously observed that CD38 and CD45RO decreases as CD3hi populations mature based on surface activation marker expression [77]. MSP6 cells did not fit these criteria as these cells had increased expression of maturation and activation markers, low CD38 expression but high CD45RO. Thus, the MSP6 population was excluded from the MSP developmental stages and will be referred to as a separate thymocyte population in this study. Upon analysis of the percent of each MSP population that expressed CD25 and FOXP3, the highest percentages of Tregs was found in the MSP3-MSP5 and MSP6 populations, 8.343 ± 2.518%, 3.485 ± 0.6756%, 4.630 ± 0.5003% and 50.95 ± 5.503% respectively (**Figure 3-2**). The high percentage of CD4+ CD25+ FOXP3+ T cells in the MSP6 population was unexpected as MSP6 cells only consist of 2.038 ± 0.6633% of CD4+ CD3hi CD7hi thymocytes.

Ikaros family member expression changes in CD4+ CD25+ FOXP3+ Tregs as they develop through the CD4+ MSP1-MSP5 developmental stages and have altered expression in the MSP6 population.

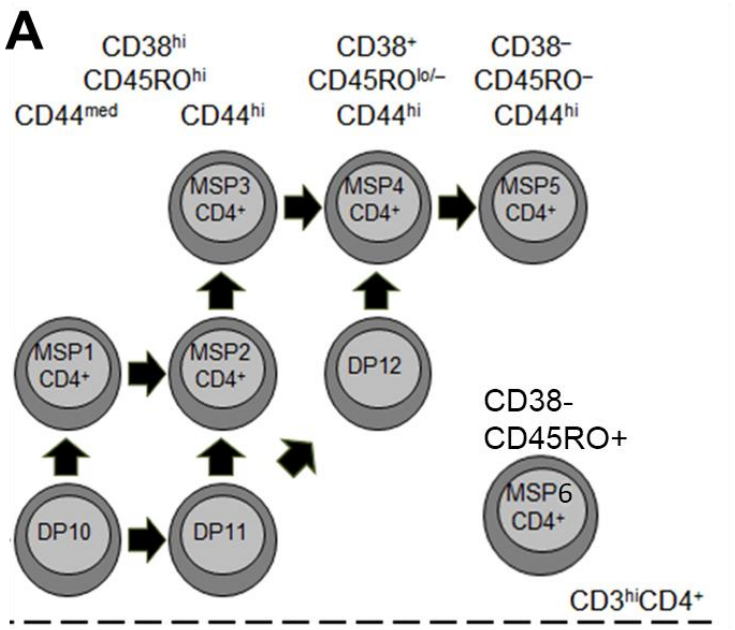
Next, we examined the intracellular protein expression levels of Helios, Eos, Aiolos and Ikaros in CD4+ CD25+ FOXP3+ Tregs within each MSP population. As the Tregs progress through the MSP1-MSP5 populations, Helios, Eos and Aiolos increase and then decrease (**Figure 3-3**). Ikaros levels slightly increase and then slightly decrease as Tregs move from MSP1 to MSP5 stages. In the MSP6 population, Helios, Aiolos and Ikaros are relatively unchanged in Tregs compared to Tregs in the MSP5 population. Interestingly, the decrease in Helios GMFI in the MSP6 Tregs is due to a split into Helios- and Helios+ populations rather than an overall decrease in expression of the whole population. Eos is increased in MSP6 Tregs compared to MSP5 Tregs. Thus, in the Tregs within the MSP1-5 populations, Helios, Eos and Aiolos expression changes in similar pattern while Ikaros gradually declines. The Tregs in the MSP6 population have similar expression of Helios, Aiolos and Ikaros as MSP5 but an increase in Eos.

The Treg markers CD39 and CD127 have the highest expression on CD4+ CD25+ FOXP3+ Tregs in the MSP6 population.

We chose to examine two markers of Tregs, CD39 and CD127. It has been reported that CD39+ Tregs and CD127- Tregs could be found within human CD4+ SP thymocyte population [62, 63, 377, 378]. CD39+ Tregs were found within all the MSP1-MSP5 populations ranging from 6.375 ± 4.725% on MSP1 Tregs to 21.65 ± 8.362% on MSP5 Tregs (**Figure 3-4A**). CD39 expression only slightly increased as Tregs progressed through these populations with average

CD39 GMFI of 381.0 ± 135.8 on MSP1 Tregs and 425.8 ± 49.36 on MSP5 Tregs (**Figure 3-4B-C**). CD127 was not completely absent on MSP1-MSP5 Tregs with a small percentage of CD127+ cells in each subset ranging from 16.73 ± 0.8616% on MSP4 Tregs to 38.93 ± 7.005% on MSP5 Tregs (**Figure 3-4A**). There was a slight increase, decrease and then another increase in CD127 expression as Tregs progressed from MSP1 to MSP5 stages with an average GMFI of 764.3 ± 112.2 on MSP1 Tregs and 425.8 ± 269.2 on MSP5 (**Figure 3-4B-C**). Interestingly, CD39 and CD127 had very high expression on MSP6 Tregs with an average CD39 GMFI of 6155 ± 3127 and average CD127 GMFI of 1547 ± 197.2 (**Figure 3-4B-C**). Further evaluation of CD39 and CD127 expression on the MSP6 Tregs reveal distinct populations of CD39+ CD127+ and CD39- CD127+ (**Figure 3-4D**). Thus, CD39 and CD127 expression increased on Tregs from MSP1 to MSP5 stages and had the highest expression on MSP6 Tregs. The MSP6 Tregs had discrete populations based on CD39 and CD127 expression.

Figure 3-1: Description and flow cytometry gating scheme of CD4+ MSP populations. A) Summary of markers defining CD4+ MSP1-MSP6 populations. Modified and printed with permissions from Mitchell, J.L., A. Seng, and T.M. Yankee, *Expression patterns of Ikaros family members during positive selection and lineage commitment of human thymocytes.* Immunology, 2016. **149**(4): p. 400-412. **B)** Flow cytometry gating strategy for CD4+ MSP1-MSP6 populations.


A
CD38hi
CD45ROhi
CD44med
CD44hi
CD38+
CD45RO lo/–
CD44hi
CD38–
CD45RO–
CD44hi
MSP3 CD4+
MSP4 CD4+
MSP5 CD4+
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DP10
DP11
CD3hiCD4+

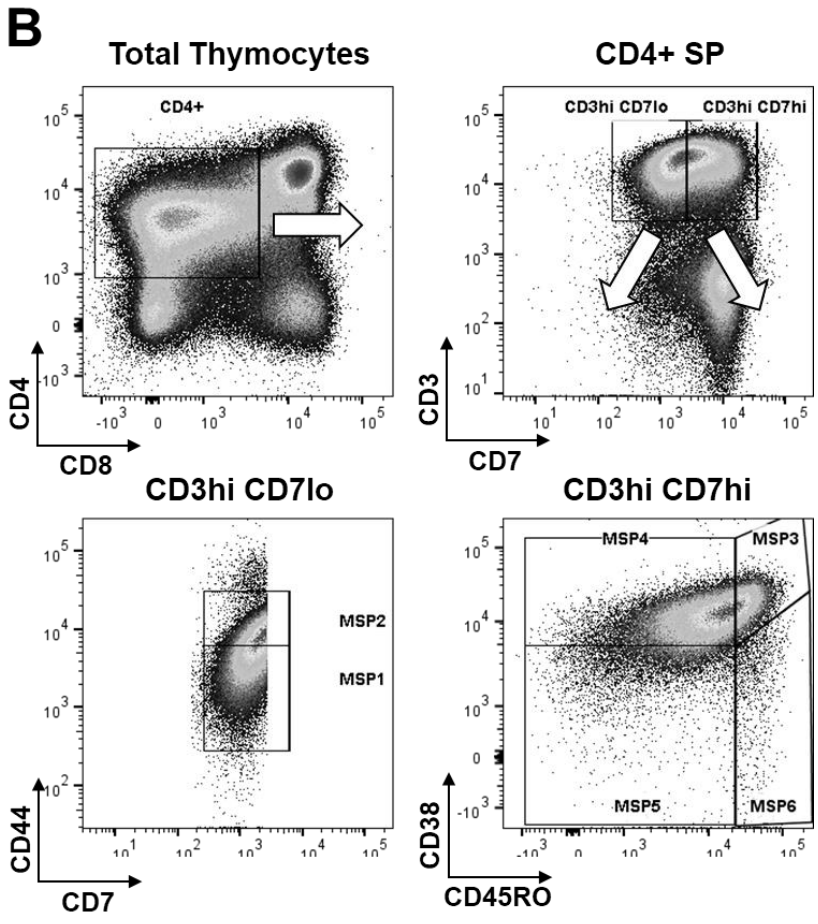

B
Total Thymocytes
CD4+
CD4
CD8
CD4+ SP
CD3hi CD7lo
CD3hi CD7hi
CD3
CD7
CD3hi CD7lo
MSP2
MSP1
CD44
CD7
CD3hi CD7hi
MSP4
MSP3
MSP5
MSP6
CD38
CD45RO

Figure 3-2: Frequency of CD4+ CD25+ FOXP3+ Tregs are highest in CD4+ MSP3-MSP5 and MSP6 populations. Thymocytes were labeled with fluorophore-labeled antibodies extracellularly and intracellularly and analyzed via flow cytometry. **A)** Summary of percent of CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population. N=4. **B)** Representative contour plot of CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population.

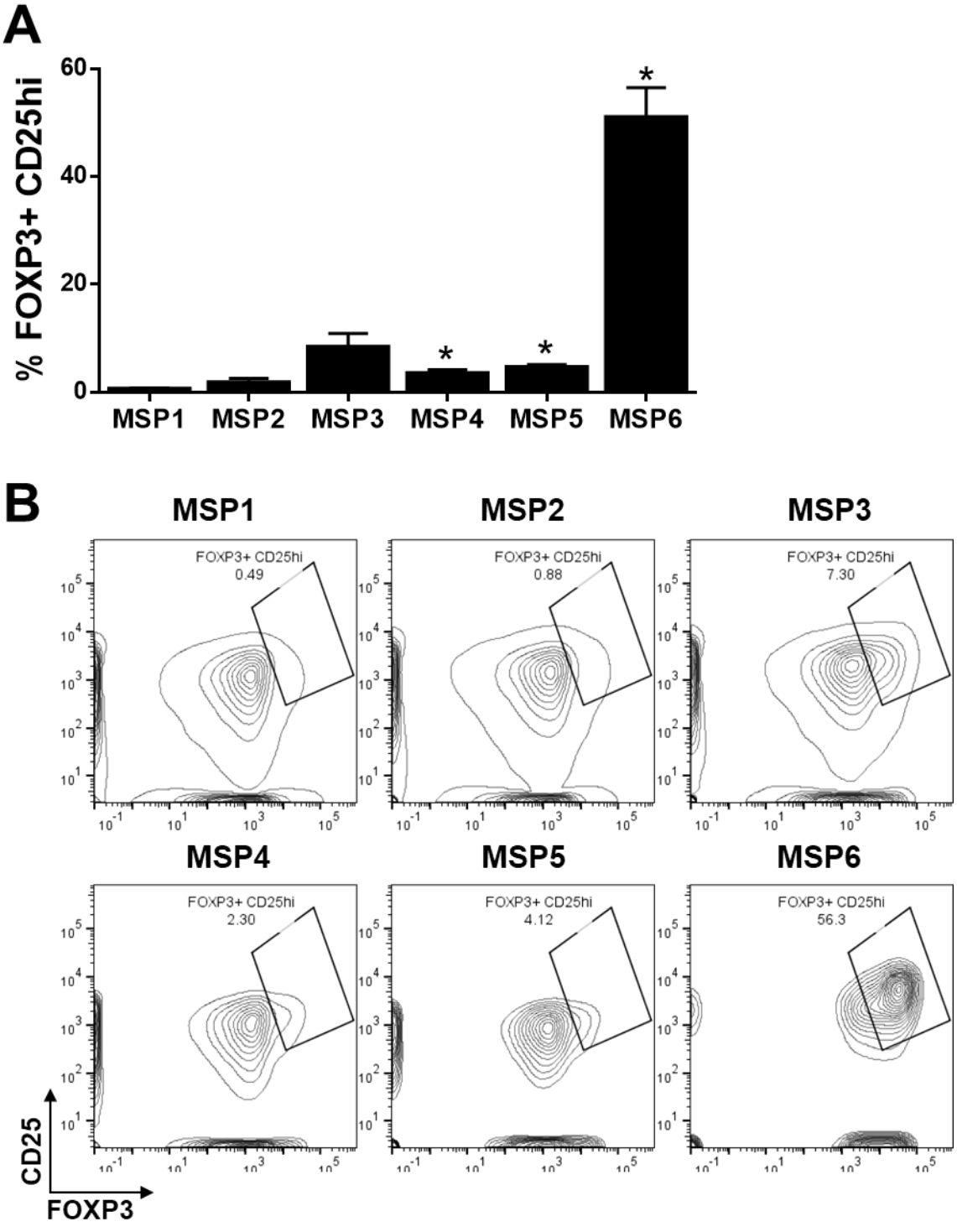

A
% FOXP3+ CD25hi
60
40
20
0
*
*
*
MSP1
MSP2
MSP3
MSP4
MSP5
MSP6
B
MSP1
MSP2
MSP3
FOXP3+ CD25hi
0.49
FOXP3+ CD25hi
0.88
FOXP3+ CD25hi
7.30
MSP4
MSP5
MSP6
FOXP3+ CD25hi
2.30
FOXP3+ CD25hi
4.12
FOXP3+ CD25hi
56.3
CD25
FOXP3

Figure 3-3: Expression of Ikaros family members change in CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population. Surface stained thymocytes were intracellularly stained with anti-Helios, anti-Aiolos, anti-Eos or anti-Ikaros antibodies. **A)** Summary of the geometric mean fluorescence (GMFI) of Helios, Eos, Aiolos or Ikaros in the CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population. N=4. $p<0.05$ of sample compared to MSP1 following paired t-test analysis and multiple comparisons analysis with Tukey post hoc test. **B)** Representative histogram of Helios, Eos, Aiolos or Ikaros expression in the CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population. The Y-axis of each histogram is on the modal setting which scales all channels as a percentage of the maximum count. Black lined graphs represent the thymocytes stained with the antibody of interest and gray shaded graphs represent the appropriate IgG isotype control for each antibody.

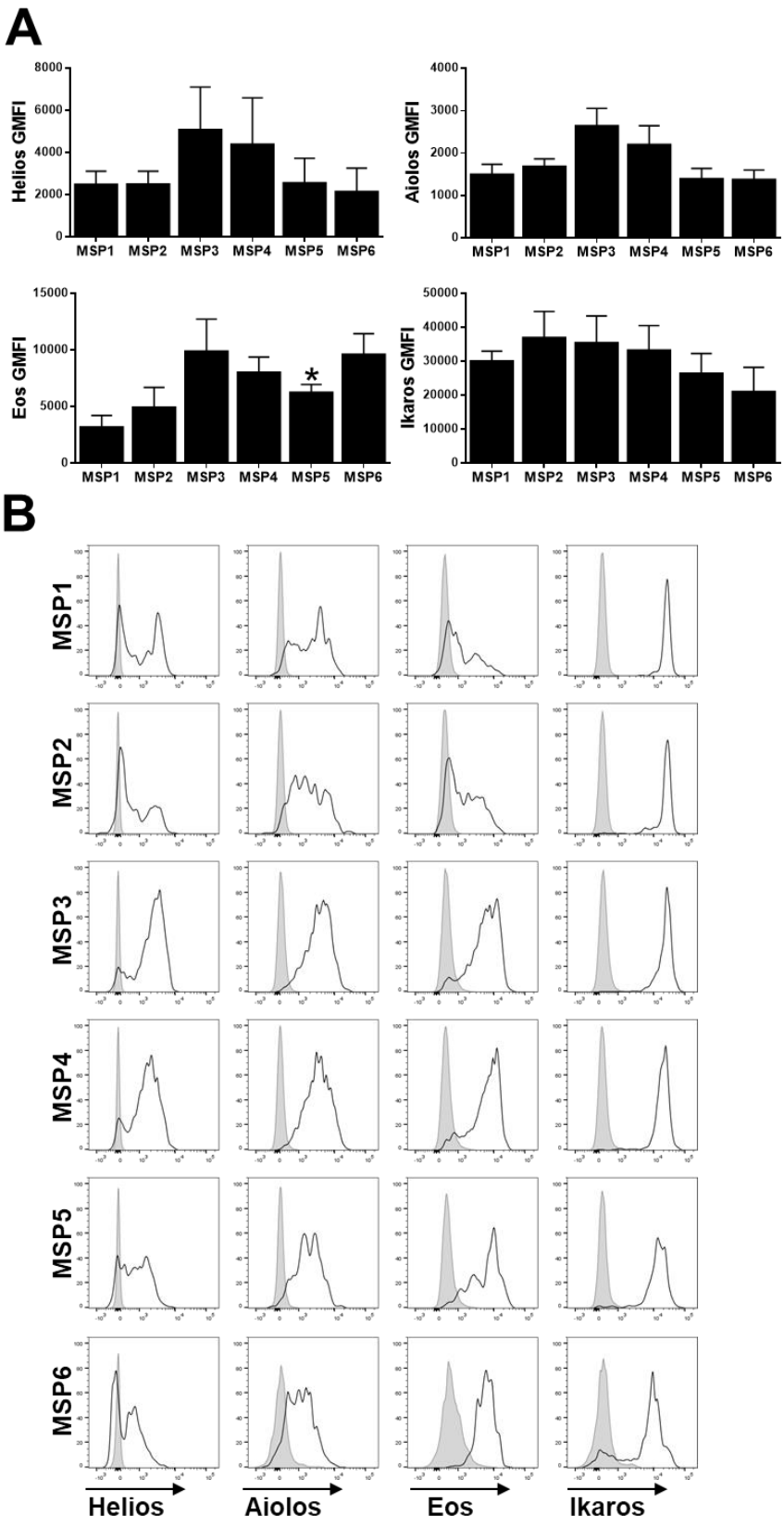
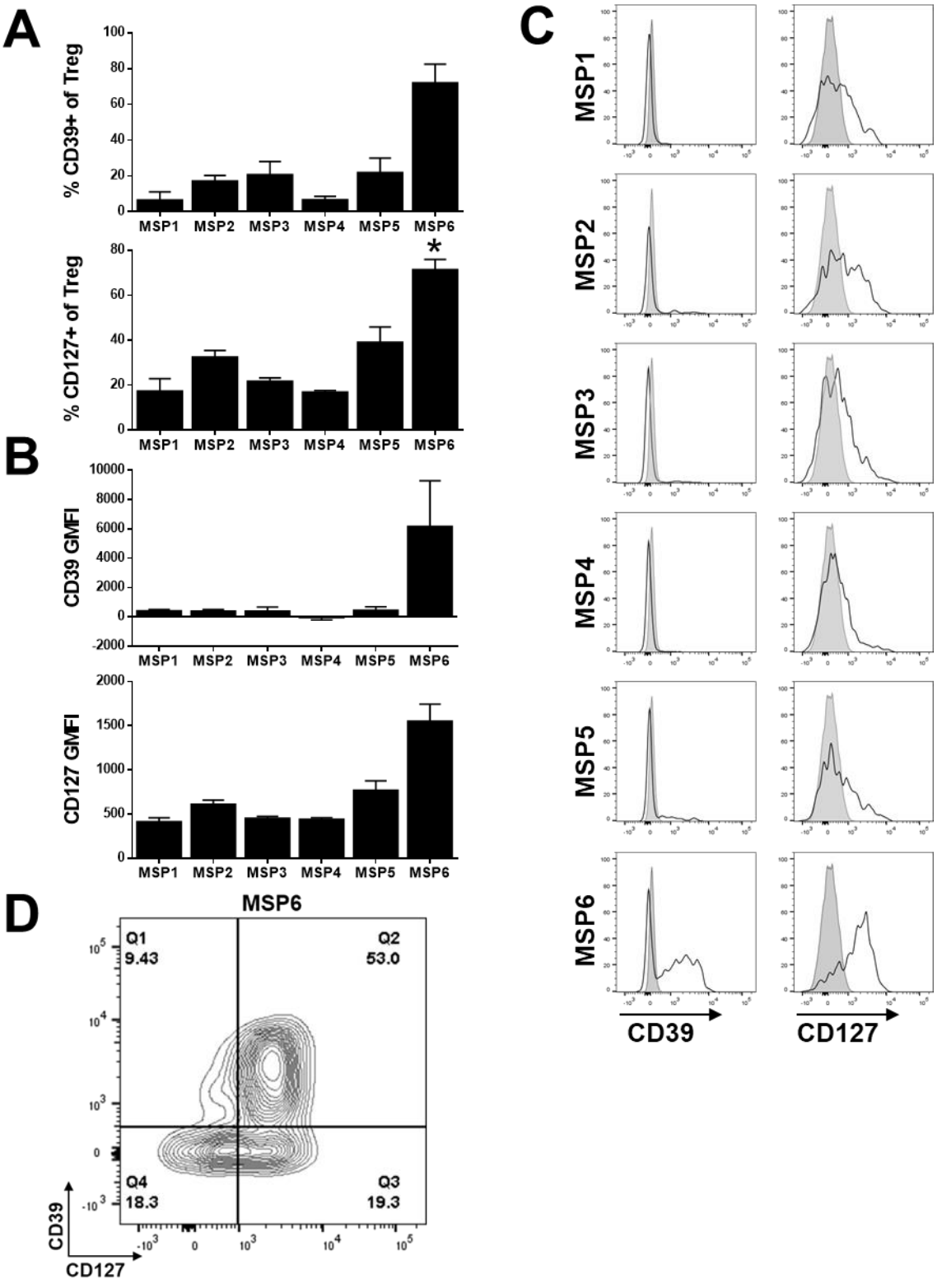

A
Helios GMFI
Aiolos GMFI
Eos GMFI
Ikaros GMFI
MSP1
MSP2
MSP3
MSP4
MSP5
MSP6
*
B
MSP1
MSP2
MSP3
MSP4
MSP5
MSP6
Helios
Aiolos
Eos
Ikaros

Figure 3-4: Expression of the Treg markers CD39 and CD127 is the highest in the MSP6 population. Surface stained thymocytes were analyzed via flow cytometry. Summary of the **A)** Percent positive and **B)** geometric mean fluorescence (GMFI) of CD39 and CD127 in the CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population. N=4. **C)** Representative histogram of CD39 or CD127 expression on the CD4+ CD25+ FOXP3+ Tregs in each CD4+ MSP population. The Y-axis of each histogram is on the modal setting which scales all channels as a percentage of the maximum count. Black lined graphs represent the thymocytes stained with the antibody of interest and gray shaded graphs represents unstained thymocytes. **D**) Representative contour plot of CD39 vs CD127 expression on the CD4+ CD25+ FOXP3+ Tregs in the CD4+ MSP6 population.


A
% CD39+ of Treg
MSP1 MSP2 MSP3 MSP4 MSP5 MSP6
% CD127+ of Treg
*
B
CD39 GMFI
CD127 GMFI
C
MSP1
MSP2
MSP3
MSP4
MSP5
MSP6
CD39
CD127
D
MSP6
Q1
9.43
Q2
53.0
Q4
18.3
Q3
19.3
CD39
CD127

Discussion

The data presented here characterized expression patterns of Ikaros family members and Treg markers on CD4+ CD25+ FOXP3+ human Tregs found within novel CD4+ CD3hi MSP subsets we previously described [77] and a small MSP6 population that doesn't fit within these developmental stages. We report that CD4+ CD25+ FOXP3+ T cells are most abundant in the MSP3-5 and MSP6 populations. There is a transient increase in Helios, Eos and Aiolos and slight decrease in Ikaros expression as the Tregs move through the MSP1-MSP5 stages. There is low expression of both the Treg markers CD39 and CD127. The MSP6 Treg population had distinct Ikaros family and CD39 and CD127 expression compared to the MSP1-MSP5 thymocyte populations. This is interesting as the majority of the CD4+ CD3hi CD25+ FOXP3+ T cells are derived from the MSP6 population.

Within the MSP1-MSP5 populations, the transient increase in Helios and Aiolos and the slight decrease in Ikaros in Tregs was similar to the changes we previously reported for the overall MSP populations and not unique to the Tregs. The transient increase in Eos was a novel finding and this transient increase is also found int the overall MSP populations as well (data not shown). MSP6 Tregs had high Eos expression relative to the MSP1-MSP5 Treg populations and had two distinct Helios+ and Helios- populations. The changes in the Ikaros family members in the Tregs could be significant as both Helios and Eos play major roles in inducing and maintaining Treg phenotype. Helios maintains FOXP3 and CD25 expression via the IL-2Rα-STAT5 pathway [43]. Eos directly binds FOXP3 which leads to downstream suppression of genes such as *Il2* and *Ifng* which conveys an immunosuppressive phenotype [285]. Thus, the increase of Helios in Tregs in the MSP3 and MSP4 populations could explain the increase CD25+ FOXP3+ cells in these populations. Additionally, increased expression of Eos in MSP3

and MSP4 Tregs could be important in Treg development. Overall, further studies of the role of Helios and Eos in Treg development could aid in defining Treg lineage commitment.

We found distinct expression of the Treg markers CD39 and CD127 in the MSP1-MSP5 and MSP6 Treg populations. In the periphery, CD39 is upregulated upon Treg activation and acts with CD73 to convert ATP into the immunosuppressive molecule adenosine [249-252]. CD127 is downregulated on Tregs and is inversely related to Treg immunosuppressive capacity [21, 379]. CD39+ Tregs were most abundant in MSP6 population and were likely the CD39+ Tregs detected in the CD4+ SP thymocytes by Nunes-Cabaço et al. [63]. Nunes-Cabaço et al. reported lower expression of CD127 of FOXP3 CD4+ SP thymocytes compared to the corresponding FOXP3- population. However, while MSP1-MSP5 Tregs had relatively low expression of CD127, we found high expression of CD127 on the MSP6 Tregs. Additionally, comparing CD39 and CD127 expression on MSP6 revealed distinct CD39- CD127-, CD39+ CD127-, CD39+ CD127+ and CD39- CD127+ populations. Thus, CD39 expression on CD4+ CD3hi MSP thymocytes was as expected however we did not find low CD127 expression on the MSP6 subset.

The MSP6 subset is clearly a distinct subset of CD4+ CD3hi thymocytes compared to the MSP1-MSP5 populations which appear to follow a clear developmental sequence. We hypothesize that the MSP6 subset is made up of re-circulating T cells. These cells are CD45RO+ which is a memory T cell marker and indicates that these cells have been previously activated [144]. This previous activation could explain the distinct populations of CD39 and CD127 expressing CD25+ FOXP3+ T cells. In humans, FOXP3 and CD25 not only define CD4+ Tregs but can also be transiently upregulated by Tconvs [133, 199, 200]. Thus, the CD25+ FOXP3+ that express the expected CD39 and CD127 expression pattern, CD39+ CD127-/lo or CD39-

CD127-/lo are likely the true Tregs and the CD39+ C127+ and CD39 and CD127- MSP6 CD25+ FOXP3+ could be activated CD4+ T cells or peripherally induced Tregs that do not have a complete Treg phenotype. They are likely peripherally induced Tregs as these cells make up a large proportion of cells that are defined as Tregs in thymic Treg studies and these cells are able to suppress *in vivo* and *in vitro*. Further functional studies and analysis of Treg markers are required to determine the nature of this diverse MSP6 population that has a high percentage of FOXP3 CD25+ T cells.

In conclusion, Treg development can be traced through the novel CD4+ MSP1-MSP5 populations that we recently defined. Helios and Eos upregulation during the MSP3 stage likely increases FOXP3 and CD25 expression and drives Treg development in the MSP stages. CD4+ CD25+ FOXP3+ Tregs isolated from the thymus are predominantly in the MSP6 subset and it should be noted that these Tregs have a heterogenous cell population based on CD39 and CD127 expression.

Chapter 4 : Treatment of Graft Versus Host Disease with Wharton's Jelly Mesenchymal Stem Cells

Abstract

The objective of this study was to determine if Wharton's Jelly Mesenchymal Stem Cells (WJMSC) could effectively prevent disease in a xenogeneic murine graft versus host disease (xenoGVHD) model. In the xenoGVHD model, mice were injected with human peripheral blood mononuclear cells (PBMC) alone or with varying doses of WJMSC. Upon reaching an endpoint disease score, mice were euthanized. T cells were then isolated from spleens and analyzed via flow cytometry. Results from this study demonstrated that administration of fewer than $2x10^6$ WJMSC can be safely administered to mice via retroorbital injection. In addition, treatment of xenoGVHD mice with of $\geq 10^6$ WJMSC improved survival in and delayed disease progression. Further, administration of $\geq 10^6$ WJMSC increased percent of effector T cells and reduced percent of naïve T cells isolated from xenoGVHD mouse spleens. In conclusion, these data suggest that there is potential for utilization of WJMSC as graft versus host disease (GVHD) prophylaxis. This study was not conducted in compliance with Good Laboratory Practice (GLP) regulations; however, laboratory Standard Operating Procedures (SOP) were followed.

Introduction

Graft versus host disease (GVHD) is a common complication in allogeneic hematopoietic stem cell transplant (HSCT) that results in about 15-30% of the deaths following transplantation[300]. GVHD manifests when donor immune cells, primarily T cells, attack recipient organs. Current GVHD treatments are insufficient, with only about 50% of patients experiencing a sustained response[304]. Mesenchymal stem cells (MSC) from human bone marrow have been investigated as treatment of GVHD with promising results[380]. However, bone marrow-derived MSCs are painful to collect and are difficult to expand to therapeutic doses[380]. MSCs can also be isolated from Wharton's Jelly, the embryonic mucosal tissue between the amniotic epithelium and the umbilical vessels, and these MSCs are able to proliferate at a higher rate than bone marrow derived MSCs[381, 382]. The efficacy of Wharton's Jelly mesenchymal stem cells (WJMSC) at treating GVHD has yet to be determined. This project will determine if WJMSC can effectively prevent GVHD in a xenogeneic mouse model.

Methods

Isolation of Wharton's Jelly Mesenchymal Stem Cells

WJMSC were isolated from human umbilical cord segments following an Investigational Review Board-approved informed consent process. The tissue was thoroughly washed with PBS containing antibiotics to remove traces of blood and minimize potential bacterial contamination. The 2 arteries and 1 vein were removed and a final phosphate buffered saline (PBS) wash was conducted. The tissue was then minced into 2-3mm pieces and incubated in xeno-free, serum-free media, to allow the cells to migrate out of the tissue and attach to the tissue culture dish. Explanted cells were then expanded through a total of 5 passages to generate approximately 2 x 10^6 WJMSC for *in vivo* and *in vitro* studies.

Recovered cells from passage 5 were centrifuged and resuspended at a concentration of 6 x 10^6 WJMSC/mL in 70% Plasmalyte A, 5% human serum albumin (has) and 10% dimethylsulfoxide (DMSO) (v/v) then frozen using a controlled rate freezer to assure consistency. Long-term storage was at ≤ -150°C. For administration, cells were removed from liquid nitrogen storage and placed at 37°C with intermittent gentle agitation until the cell suspension was fully thawed. The cells were then gently mixed and can be stored at 4°C for up to 6 hours prior to administration.

Isolation of Human Peripheral Blood Mononuclear Cells (PBMC)

Blood was collected from five healthy adult volunteer donors under signed informed consent with approval from the Institutional Review Board of the University of Kansas Medical Center. Blood was collected in heparin tubes and PBMC were isolated via Ficoll-Paque (GE Healthcare [United Kingdom]) density centrifugation with SepMate tubes (STEMCELL Technologies Inc. [Vancouver, BC]). PBMC were always used fresh and never frozen.

Mice and Xenogeneic Murine GVHD Model

All animal studies were performed in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals. NOD-SCID IL-2Rγnull (NSG) mice were purchased from Jackson Laboratories and bred at the University of Kansas Medical Center. Mice were maintained under specific pathogen-free conditions using sterile food, water, bedding, and caging. NSG mice (8-12 weeks old) received 1.5 Gy of whole body irradiation. The next day, the mice were anesthetized via 2% inhaled isoflurane and injected retro-orbitally with 10^7 human PBMC alone or with one of the following doses of WJMSC: 10^7, $2x10^6$, $1.5x10^6$, $1x10^6$, $5x10^5$, $1.25x10^5$, $3.125\ x10^4$. Cells were re-suspended in 100 ul of sterile PBS (Thermo Fischer [Waltham, MA]). Mice were examined for signs of GVHD, using the following GVHD scoring system[310]. Five categories were assessed on a scale of 0, 1, or 2 for each. Weight loss: 0= <10% change, 1= 10-25% change, 2= >25% change, Posture: 0= normal, 1= hunching at rest, 2=hunching impairs movement, Fur texture: 0= normal, 1= mild to moderate ruffling, 2= severe ruffling, Skin integrity: 0=normal, 1=scaling of paws/tail, 2= obvious areas of denuded skin, Activity: 0=normal, 1=mild to moderately decreased, 2=stationary unless stimulated. Mice were sacrificed when they reach a GVHD score of ≥6 or live longer than 42 days. The researcher assessing score was blinded to the treatment of each mouse for 20 out 44 mice assessed.

Flow Cytometry

Spleens from mice were processed into a single cell suspension and stained with the following antibodies (BioLegend [San Diego, CA])): anti-human CD3 APC-Cy7, anti-human CD4 Pacific Blue, anti-human CD8 Brilliant Violet 785, anti-human CD25 PerCPCy5.5, anti-human CD127

Brilliant Violet 650, anti-human CCR-7 PE-Cy7, anti-human CD45RA Alexa Fluor 700. Samples were run on a Becton-Dickson LSRII (Becton-Dickson [Franklin Lakes, NJ])).

Data/Statistical Analysis

Data is stored on a secure server at the University of Kansas Medical Center. Data was collected and analyzed with GraphPad Prism 6 (GraphPad Software, [La Jolla, CA]). Data reported at each time point for GVHD score and weight loss were an average of the scores and weights of the mice left alive and the last scores and weights of any deceased mice in each group. Two-way ANOVA was done to compare GVHD scores and weight loss at various time points. The log-rank (Mantel-Cox) test was used for analysis of Kaplan-Meier curves. Differences between two groups with unmatched data were compared using an unpaired t-test. $p \leq 0.05$ was considered to be significant. Results show mean ± standard error mean unless otherwise indicated.

Results

Administration of fewer than $2x10^6$ WJMSC to mice is safe.

To identify the maximum number of cells we can safely inject into mice, mice were injected with 10^7 PBMC and varying numbers of WJMSC, as shown in **Table 4-1**. All mice injected with 10^7 WJMSC died immediately after administration. Additionally, two out of three mice receiving $2x10^6$ WJMSC died immediately. All mice that received fewer than $2x10^6$ survived the injection of cells. Thus, administration of fewer than $2x10^6$ WJMSC to mice via retro-orbital injection is safe.

Administration of 10^6 WJMSC or greater improved survival and delayed GVHD disease progression in a xenoGVHD model.

To test the immunosuppressive capacity of WJMSC *in vivo*, the same mice described in Table 1 were evaluated for GVHD onset and progression. Each group of mice receiving WJMSC was compared to the control mice receiving PBMC alone. None of the groups were statistically different. However, the comparison of GVHD scores between mice receiving 10^6 WJMSC and the control group resulted in a probability of the two lines being identical being less than 20% (Data not shown). By contrast, the GVHD scores between mice receiving $5x10^5$ and control mice were nearly identical (p=0.62, data not shown). Based on this difference, the data were reanalyzed by combining groups of mice that received $\geq 10^6$ WJMSC and mice that received $<10^6$ WJMSC and comparing them to mice that received PBMCs alone. Of the mice that survived initial injection of WJMSC, dosing $\geq 10^6$ WJMSC significantly improved survival with median survival time equal to 30 ± days compared to 23± days for PBMCs alone (p= 0.0417) and 20 ± days for $<10^6$ WJMSC, (**Fig. 4-1A**). Administration of doses $\geq 10^6$ WJMSC also significantly delayed GVHD disease progression (p= 0.0194) and decreased weight loss over time (p=0.1108)

compared to PBMCs alone. $<10^6$ WJMSC did not significantly change GVHD score (p=0.5249) or weight loss (p=0.5393) compared to PBMCs alone. (**Figs. 4-1B and 4-1C**). These data suggest that administration of $\geq 10^6$ WJMSC can improve survival and reduce disease in a xenoGVHD model.

Administration of 10^6 WJMSC or greater increased percent of effector T cells and reduced percent of naïve T cells isolated from xenoGVHD mouse spleens.

To determine the potential mechanism of immunosuppression by WJMSC, cells were isolated from spleens of euthanized mice that had reached a GVHD score of 6 or greater and phenotype was analyzed via flow cytometry. The percentage of $CD4^+$ T cells that were effector memory T cells, defined as $CCR7^-CD45RA^-$, trended higher in mice that received $\geq 10^6$ WJMSC compared to mice that received PBMCs only; 40.98% ± 11.4% (n=9) versus 32.63% ± 19,6% (n=9) (*p*=0. 0.0560, **Fig. 4-2A**). The percentage of effector memory $CD8^+$ T cells trended higher in mice that received $\geq 10^6$ WJMSC compared to PBMCs alone; 28.43% ± 5.8% (n=9) versus 23.25% ± 15.1% (n=4) (*p*=0.2186, **Fig. 4-2B**). Interestingly, this population of effector memory $CD8^+$ T cells was significantly increased in mice that received $<10^6$ WJMSC compared to PBMCs alone; 40.54% ± 12.36% (n=7) (*p*=0.0001, **Fig. 4-2B**). The percentages $CD4^+$ T cells that were regulatory T cells (Tregs), defined as CD4+ CD25hi CD127lo, were nearly identical between the three groups; 4.81% ± 4.98% (n=9) of $CD4^+$ T cells from mice receiving $\geq 10^6$ WJMSC, 5,27% ± 5.4% of $CD4^+$ T cells from mice receiving $<10^6$ WJMSC (n=7) and 3.9% ± 2.7% (n=4) of $CD4^+$ T cells from mice receiving PBMCs only (**Fig. 4-2A**). Thus, administration of $\geq 10^6$ WJMSC mediated an increase in the percent of effector $CD4^+$ T cells and any dose of WJMSC caused an increase in effector memory $CD8^+$ T cells.

Table 4-1: WJMSC dosage and survival of mice

Table 1: WJMSC Dosage			
# of WJMSC	**# of PBMC**	**# of mice**	**# of mice that survived initial injection**
0	10^7	7	7
3.125×10^4	10^7	3	3
1.25×10^5	10^7	3	3
5×10^5	10^7	7	7
10^6	10^7	6	5
1.5×10^6	10^7	6	6
2×10^6	10^7	3	1
10^7	10^7	3	0

Figure 4-1: Administration of 10^6 WJMSCs or greater improved survival in and delayed GVHD disease progression in a xenoGVHD model. 8-12 week-old NSG mice were sub-lethally irradiated. The next day, the mice were injected retro-orbitally with 10^7 human PBMCs alone or with the doses of WJMSC listed in table 1. Mice that survived injection from 4 separate experiments were pooled into three groups: PBMCs alone (n=7), PBMCs+ $<10^6$ WJMSCs (n=13) and PBMCs+ $\geq 10^6$ WJMSCs (n=11). **A)** Kaplan-Meier curve of survival; log-rank test was performed. Death was marked when GVHD score was ≥6. **B)** GVHD score and **C)** weight loss were monitored until day of sacrifice. *Curves were significantly different between PBMCs only vs PBMCs+ $\geq 10^6$ with $p<0.05$ as determined by two-way ANOVA.

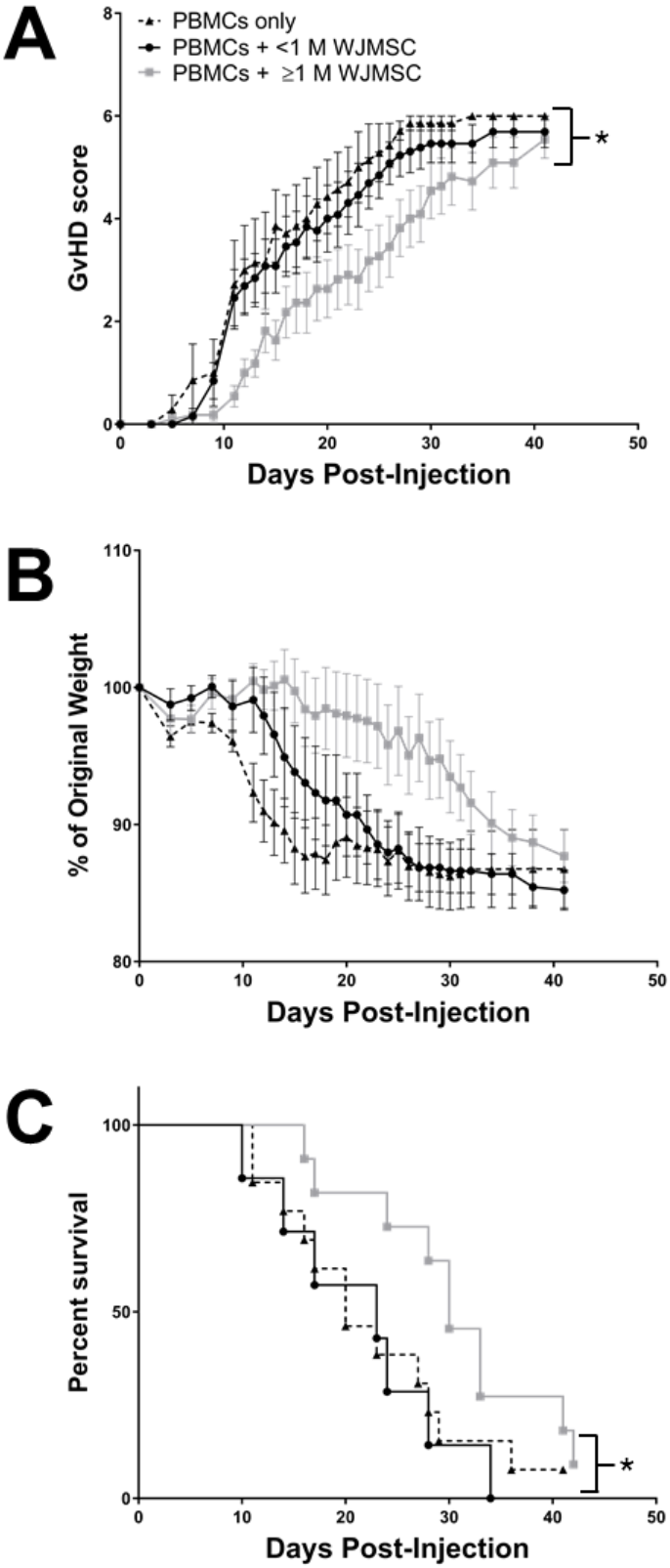
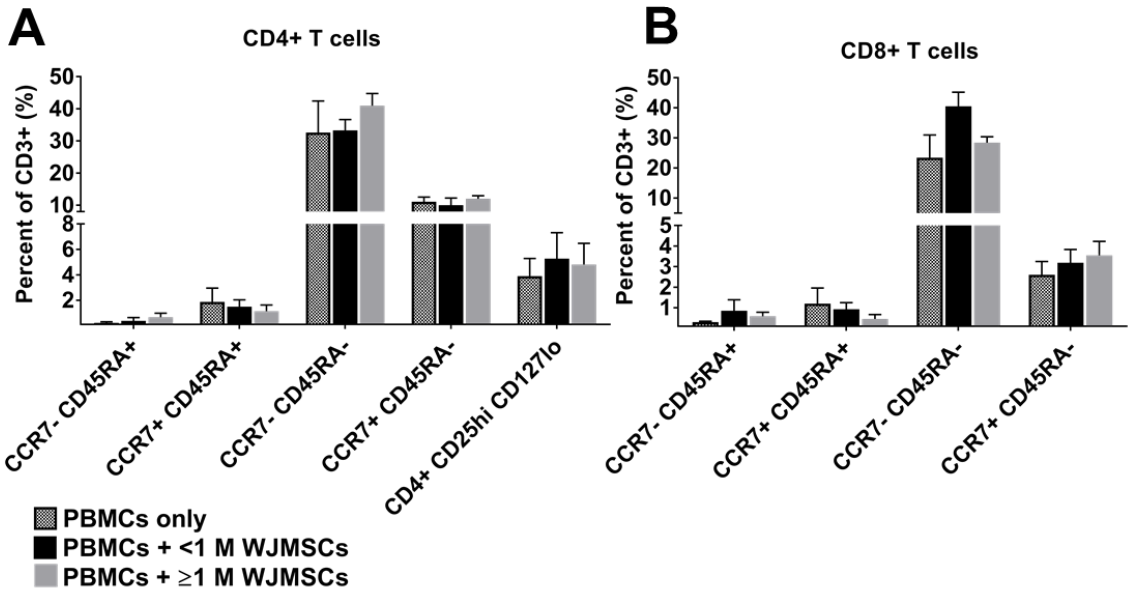

A
PBMCs only
PBMCs + <1 M WJMSC
PBMCs + ≥1 M WJMSC
GvHD score
Days Post-Injection
*
B
% of Original Weight
Days Post-Injection
C
Percent survival
Days Post-Injection
*

Figure 4-2: Administration of ≥10^6 WJMSCs or greater increased percent of effector T cells and reduced percent of naïve T cells isolated from xenoGVHD mouse spleens. Spleens from euthanized mice were processed into a single cell suspension and analyzed via flow cytometry. The mean and standard deviation of the percentages of CD4+ T cell subsets (*A*) and CD8+ T cell subsets (*B*) from mice receiving PBMCs only (n=4), PBMCs± <10^6 WJMSCs (n=7) and PBMCs+ ≥10^6 WJMSCs (n=9) group were calculated.


A
CD4+ T cells
Percent of CD3+ (%)
50
40
30
20
10
8
6
4
2
CCR7- CD45RA+
CCR7+ CD45RA+
CCR7- CD45RA-
CCR7+ CD45RA-
CD4+ CD25hi CD127lo
B
CD8+ T cells
Percent of CD3+ (%)
50
40
30
20
10
5
4
3
2
1
CCR7- CD45RA+
CCR7+ CD45RA+
CCR7- CD45RA-
CCR7+ CD45RA-
PBMCs only
PBMCs + <1 M WJMSCs
PBMCs + ≥1 M WJMSCs

Discussion

In this small pilot study, we demonstrated that WJMSC can delay GVHD disease progression and improve survival in a xenogeneic murine GVHD model. Furthermore, this is the first study to analyze the phenotype of human T cells isolated from xenoGVHD mice that have been treated with WJMSC. There was an increase in the proportion of T cells that were effector cells in mice treated with $\geq 10^6$ WJMSC.

Injection $\geq 2x10^6$ WJMSC may be lethal to mice because these cells have been shown to occlude vasculature and can cause pulmonary emboli leading to immediate death[383]. We administered the PBMC and WJMSC via retro-orbital injection which leads to more rapid distribution of cells than intravenous injection via tail vein. This increase in infusion rate leads to increased risk of pulmonary emboli, explaining the increased frequency of immediate death observed in this study. Our data indicate that no mortality was observed at WJMSC doses below 2×10^6 cells.

It was expected that WJMSC delay disease in a xenoGVHD model given the ability of WJMSC to suppress T cell proliferation *in vitro* [384]. Furthermore, WJMSC have been shown to attenuate disease in a murine model of asthma, a murine model of colitis and a rat model of autoimmune encephalitis[385-387]. Though the WJMSC did not entirely prevent disease, there was a delay in disease progression as measured by survival, GVHD score and rate of weight loss. Of these parameters, GVHD score and survival reached statistical significance. Additionally, the WJMSC were not primed as described in Polchert et al. [388]; priming may have resulted in improved outcome. Overall, these data demonstrate that there is potential for utilization of WJMSC as GVHD prophylaxis.

The increase in effector memory $CD4^+$ and $CD8^+$ T cells in xenoGVHD mice treated with $<10^6$ and $\geq 10^6$ WJMSC was unexpected. Given the immunosuppressive properties of WJMSC *in vitro*[384], we hypothesized that there would be reduced differentiation of T cells when co-injected with WJMSC *in vivo*. However, T cell phenotype after co-culture with WJMSC has not been studied extensively, thus it is not clear how T cells respond to WJMSC *in vivo*. It is difficult to define the exact mechanism of WJMSC-mediated immunosuppression in this experiment as cells were analyzed when animals reached the endpoint of disease. It is possible that WJMSC may have allowed animals to live long enough for T cells to differentiate explaining the increase in effector memory T cells in the $\geq 10^6$ WJMSC group versus the $<10^6$ WJMSC group. Future experiments will be needed to analyze cells from treatment and control groups at the same time point in the middle of disease progression to gain a better idea of how WJMSC alter PBMC-mediate disease progression versus PBMC alone. We have demonstrated that $\geq 10^6$ WJMSC correlates with an increase in effector memory T cell ratio in a xenoGVHD model compared to PBMCs alone.

In conclusion, these data demonstrate that administration of fewer than $2x10^6$ WJMSC is safe. In addition, treatment of xenoGVHD mice with of $\geq 10^6$ WJMSC improved survival in and delayed GVHD disease progression. Further, administration of $\geq 10^6$ WJMSC increased percent of effector T cells and reduced percent of naïve T cells isolated from xenoGVHD mouse spleens.

Chapter 5 : Conclusions

This work provides further insight into the role of Ikaros family members in human Treg function and development. In Chapter II, we co-expressed FOXP3 with Hel-FL or with Hel-Δ3B in total human T cells to generate eTregs. We demonstrated that co-expression of FOXP3 and Hel-FL is not only able to improve CD4+ eTreg function, but also generate CD8+ eTregs compared to FOXP3 alone. Furthermore, we showed that Hel-Δ3B co-expression with FOXP3 doesn't change CD4+ eTreg function but improves CD8+ eTregs suppression compared to FOXP3 alone. This work not only improves existing eTreg technology, it offers a more detailed look at the role of Helios and its endogenous splice variants in mediating T cell immunosuppression. In Chapter III, we used multiparameter flow cytometry to trace Treg development in the human thymus and describe changes in the Ikaros family members and Treg markers during this development. Using subsets of CD4+ MSP populations we previously defined [77], we were able to determine which population contained the highest percentage of CD4+ CD25+ FOXP3+ Tregs. We then defined Ikaros protein expression and Treg marker expression within Tregs from these populations. Interestingly, the small MSP6 population, which did not fit normal CD4+ T cell development, contained the highest number of Tregs which had subpopulations based on Ikaros family and Treg marker expression. These data provide additional detail to human Treg development and the thymic Treg phenotype in general. Overall, these studies highlight the critical roles that the Ikaros family of transcription members in Treg function and development and emphasize the need the further studies to fully characterize the role of Ikaros family splicing and downstream gene regulation in Tregs.

Dual expression of FOXP3 and Hel-FL in human total T cells conveys immunosuppressive function

With the discovery of Tregs, there has been much effort to utilize Tregs as a cellular therapy for inflammatory diseases such as GVHD, transplant rejection and type I diabetes [35-39]. Barriers to adoptive Treg transfer include difficulties in generating sufficient numbers to treat, obtaining pure populations and survival of Tregs *in vitro*. To overcome these limitations, there have been attempts to artificially generate Tregs by ectopically expressing FOXP3 in both mouse and human CD4+ Tconv [14, 32, 255]; we have termed these cells "engineered Tregs" or "eTregs." FOXP3 eTregs express Treg markers, suppress T cell proliferation *in vivo* and *in vitro* [14, 32, 255]. However, when directly compared to endogenous Tregs, FOXP3 eTregs are not as effective at suppressing and do not completely recapitulate Treg immunosuppressive capacity [296]. This is expected as RNA Sequencing of FOXP3 transduced murine CD4+ Tconvs revealed that FOXP3+ expression does not completely convey the Treg gene signature [277]. The same study demonstrated that ectopic expression of Helios, another Treg transcription factor, regulated different Treg genes compared to FOXP3 [277]. Furthermore, Helios+ Tregs in humans have improved stability of the Treg phenotype under inflammatory conditions compared to Helios- Tregs [9, 20, 21]. Thus, we hypothesized that the addition of Helios to FOXP3 in eTregs would improve the immunosuppressive capacity of eTregs both *in vivo* and *in vitro*.

We incorporated many novel aspects into our generation of eTregs. First, we chose to retrovirally transduce both CD4+ and CD8+ human T cells, whereas existing eTreg work has only transduced CD4+ T cells. We decided to include CD8+ T cells because Helios has been implicated as a critical mediator of CD8+ Treg function [43, 191]. Additionally, removing the CD4+ purification step would facilitate translation of this process into the clinic. Second, we

implemented a two-step transduction protocol, as Helios downregulated expression of transduction markers and cDNA on the same plasmid in about four days with our retroviral transduction system. Finally, we investigated the role of the shorter Hel-Δ3B isoform of Helios in addition to the full length Hel-FL isoform to determine if there were any functional differences between these two splice variants.

Our work revealed that FOXP3+Hel-FL eTregs had the most robust immunosuppressive ability both *in vivo* and *in vitro*. In the xenoGVHD model, FOXP3+Hel-FL eTregs were the only cells that were able to significantly delay disease. *In vitro*, both CD4+ and CD8+ FOXP3+Hel-FL eTregs had increased suppression compared to FOXP3 eTregs. In comparing FOXP3 and FOXP3+Hel-FL eTregs, the increased immunosuppression by FOXP3+Hel-FL eTregs can be correlated with gene expression changes in KEGG pathways and Treg transcriptional signature in both CD4+ and CD8+ cells. These genes need to be further investigated as potential mediators of T cell suppressive activity. Additionally, we know that the effects of Helios we observed are independent of its effects on FOXP3 transcription because FOXP3 is overexpressed in these cells. Thus, additional work is required to determine what downstream signals are affected by Helios and FOXP3 co-expression and how these signals mediate improved eTreg function. These results improve our understanding of Helios in Treg function.

Functional differences between endogenous splice variants of Helios

Little is known about the roles of the two Helios splice variants expressed in human T cells. Hel-Δ3B is lacking half of exon 3 which would affect its DNA-binding domain [40]. Hel-FL and Hel-Δ3B have been overexpressed in 293T HEK T cells or COS7 cells to assess suppression of transcription via a *Hes1* promoter-driven luciferase assay [40, 389]. *Hes1* contains

Helios DNA binding domains. These assays yielded conflicting results as both demonstrated that Hel-FL suppressed *Hes1* promoter activity but one study found that Hel-Δ3B did not suppress activity [40] and the other found Hel-Δ3B suppressed activity as well as Hel-FL[389]. These discrepancies may be due to differences in the cell lines used and they do indicate a potential difference in Hel-FL and Hel-Δ3B transcriptional activity. In regards to the differences in these Helios isoforms in T cells, only Hel-FL has been studied in Jurkat cells, a human T cell line, and there have not been any studies in primary human T cells. Ectopic of Hel-FL in Jurkats by Getnet et al. resulted in increased apoptosis and they concluded that Helios alone was insufficient to convey Treg properties [44].

Given the lack of data comparing Hel-FL to Hel-Δ3B, it was unexpected that FOXP3+ Hel-Δ3B eTregs did not perform equally as well as FOXP3+Hel-FL eTregs *in vivo*. *In vitro*, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs were similar in proliferation, survival, Treg marker expression and cytokine secretion. Despite these similarities, FOXP3+Hel-FL and FOXP3+Hel-Δ3B eTregs did differ in *in vitro* suppression of T cell proliferation. FOXP3+Hel-FL CD4+ eTregs had improved suppression compared to CD4+ FOXP3+Hel-Δ3B eTregs. Interestingly, FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD8+ eTregs had similar suppressive capacity and both were better than FOXP3 only CD8+ eTregs. These data not only demonstrated a functional difference between Hel-FL and Hel-Δ3B, but this difference was specific to CD4+ T cells. RNA Sequencing data revealed that FOXP3+Hel-FL CD4+ eTregs had differences in gene transcription of KEGG pathways and Treg-related genes compared to FOXP3+Hel-Δ3B CD4+ eTregs. Specifically, p53 signaling was significantly downregulated in FOXP3+Hel-Δ3B CD4+ eTregs (**Figure 2-10B**) and p53 has been demonstrated to be critical in CD4+ Treg induction in mice [348]. Additionally, chemokine receptors were upregulated in the cytokine-cytokine

receptor interaction pathway for FOXP3-Hel-FL CD4+ eTregs (**Figure 2-10B**) which could mediate increased migration to sites of inflammation. In regards to the Treg signature genes, based on functional data, it is likely that gene differences that were unique to the FOXP3+Hel-FL vs FOXP3 or FOXP3+Hel-Δ3B vs FOXP3 comparisons are key mediators of T cell suppression. There were also changes in KEGG pathways and Treg signature genes when comparing FOXP3+Hel-FL and FOXP3+Hel-Δ3B CD8+ eTregs. However, based on the function of these eTregs, these changes may not be as critical in mediating CD8+ T cell immunosuppressive function. Overall, these data have shown that Hel-FL and Hel-Δ3B co-expression with FOXP3 differentially mediate immunosuppression in human CD4+ and CD8+ T cells and these differences may be due to changes in downstream gene expression. Whether these transcriptional changes are directly due to Helios activity or indirectly due to Helios interaction with other proteins requires further research.

Correlating the functional and transcriptional differences between FOXP3+Hel-FL eTregs and FOXP3+Hel-Δ3B eTregs can provide abundant information about T cell-mediated immunosuppression. It is unclear why FOXP3+Hel-FL eTregs were more effective *in vivo* than FOXP3+Hel-Δ3B eTregs. One reason could be a larger of number of eTregs were present in mice treated with FOXP3+Hel-FL eTregs because both CD4+ and CD8+ Tregs were highly suppressive whereas there were only suppressive CD8+ eTregs present in FOXP3+Hel-Δ3B eTregs treated mice. Another possibility is CD8+ eTregs might be insufficient to delay disease in a xenoGVHD model. Thus, further investigation of the differences between FOXP3+Hel-FL eTregs and FOXP3+Hel-Δ3B eTregs will not only determine differences in downstream gene signaling but differences in CD4+ and CD8+ eTreg function and requirements for preventing disease in the xenoGVHD model.

Expression of Ikaros family members during CD4+ Treg development through CD4+ MSP1 to MSP5 stages

Treg development in humans has been difficult to characterize due to limitations of *in vitro* assays and many differences between murine and human T cell development. Thus far, it has been determined that TCR stimulation of FOXP3+ DP thymocytes, followed by stimulation by IL-2 and IL-15 is one pathway to Treg lineage commitment [63, 70, 72]. Additionally, FOXP3 and CD25 expression can be induced in mature FOXP3- CD25- CD4+ SP through a combination of TCR engagement, IL-2 and IL-15 stimulation and co-stimulation [69, 72, 74, 75]. When Treg lineage occurs during the different stages of T cell development has been determined by tracing CD4+ CD25+ FOXP3+ T cells. Human Treg lineage commitment can occur at $CD4^+$ $CD8^+$ double positive (DP) or the $CD4^+$ single positive (SP) developmental stages [56]. FOXP3+ CD4+ SP tTregs or FOXP3+ CD8+ SP tTregs can develop from FOXP3+ DP thymocytes.

Our work provides more details on the path that thymocytes follow to Treg lineage commitment by examining the novel CD4+ MSP1-MSP5 subsets of T cell development previously defined by our laboratory [77] as well as investigating a new MSP6 subset. Upon determining the frequency of CD4+ CD25+ FOXP3+ within the CD4+ MSP1-MSP5 subsets, we found that the MSP3-MSP5 cells had the highest frequency of Tregs. Interestingly, there was an increase in Helios and Eos in these Tregs at the MSP3 stage. These two members of the Ikaros family are critical for induction of FOXP3 and CD25 and Treg function [43, 285]. Thus, their upregulation and increase in Treg frequency in the MSP population are likely correlated and indicate a role in these two factors in Treg commitment and development in the thymus. Functional assays in the future will be required to determine the maturity of the these developing Tregs and if relative expression of Ikaros proteins affects their activity. Overall, examining

Ikaros family members in relation to Treg abundance at different stages at Treg development could help precisely define points of Treg commitment.

CD4+ MSP6 thymocytes represent a high percentage of CD4+ CD3hi CD25+ FOXP3+ Tregs in the human thymus

When the CD4+ MSP1-MSP5 populations were defined, a small subset of CD4+ CD3hi CD38lo CD45RO+ cells, referred to as MSP6, were excluded from the developmental sequence because their expression of CD38 and CD45RO did not correlate with mature thymocytes. However, when we analyzed these cells for the presence of CD4+ CD25+ FOXP3+ Tregs, we were surprised to find that approximately 50% of MSP6 cells were Tregs. Upon further evaluation of Ikaros family members and the Treg markers, CD39 and CD127, we hypothesized that these Tregs were likely re-circulating Tregs from the periphery. Within this population, the certain subsets express markers that are consistent with thymic Tregs such as Helios+, CD127lo, CD39+. However, other subsets express combinations of these markers that would not be expected in thymic Tregs. Thus, it is likely that some of these Tregs are FOXP3- Tconvs that were peripherally induced to express FOXP3 or pTregs. pTregs lack the epigenetic modifications present in tTregs and thus do not always have the same marker expression as tTregs [198]. These are likely pTregs and not activated Tconvs that transiently upregulated FOXP3 and CD25 because studies have shown that the CD4+ CD25+ FOXP3+ cells isolated from human thymus are able to suppress and majority of these cells would come from the MSP6 population [289]. In conclusion, within our novel T cell development subsets, we identified markers that could help identify re-circulating Tregs in the thymus. Further functional and phenotypical analysis of these Tregs will be required to support this hypothesis.

Significance and Future Directions

The results from these studies clarify the roles of the Ikaros family of transcription factors in Treg biology and provide information that can further Treg immunotherapy. We are the first to describe a method that co-expresses both FOXP3 and Hel-FL in human total T cells and effectively mediates immunosuppression by both CD4+ and CD8+ T cells. Future work can be done to determine if survival of these cells could be improved or if multiple dosages would improve delay of GVHD. Furthermore, these eTregs could be tested in the treatment of other inflammatory diseases such as diabetes or transplant rejection. In addition to a novel eTreg protocol, our RNA Sequencing data provides the first transcriptional profile of CD4+ and CD8+ human T cells ectopically expressing both FOXP3 and Helios. Further evaluation of the transcriptional changes between these cells can provide a more detailed look into the role of Helios in mediating immunosuppression with FOXP3 in both CD4+ and CD8+ T cells. We are also the first group to describe a functional difference between the endogenous Helios splice variants Hel-FL and Hel-Δ3B in primary human T cells. Given the differences we see in overexpressing these Helios isoforms, it would be interesting to define the endogenous splicing of Helios in different Treg and T cell subsets in both healthy patients and disease states. Finally, the expression profiling of Ikaros family members in Tregs found in the CD4+ MSP1-MSP5 populations may have defined a more precise stage of Treg lineage commitment. Similar studies could be applied to earlier stages of T cell development to determine other points of Treg commitment. Additionally, phenotyping of Tregs found in the MSP6 population could have revealed new markers for re-circulating Tregs in the thymus and provided more details on thymic Tregs that are currently being used in clinical trials [390]. Overall, the data from this work will

significantly impact studies investigating the Ikaros family in Treg-mediated immunosuppression and development and Treg therapy in general.

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