T CELL DIFFERENTIATION

Helios enhances the preferential differentiation of human fetal CD4⁺ naïve T cells into regulatory T cells

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T cell receptor (TCR) stimulation and cytokine cues drive the differentiation of CD4⁺ naïve T cells into effector T cell populations with distinct proinflammatory or regulatory functions. Unlike adult naïve T cells, human fetal naïve CD4⁺ T cells preferentially differentiate into FOXP3⁺ regulatory T (Treg) cells upon TCR activation independent of exogenous cytokine signaling. This cell-intrinsic predisposition for Treg differentiation is implicated in the generation of tolerance in utero; however, the underlying mechanisms remain largely unknown. Here, we identify epigenetic and transcriptional programs shared between fetal naïve T and committed Treg cells that are inactive in adult naïve T cells and show that fetal-derived induced Treg (iTreg) cells retain this transcriptional program. We show that a subset of Treg-specific enhancers is accessible in fetal naïve T cells, including two active super-enhancers at Helios. Helios is expressed in fetal naïve T cells but not in adult naïve T cells, and fetal iTreg cells maintain Helios expression. CRISPR-Cas9 ablation of Helios in fetal naïve T cells impaired their differentiation into iTreg cells upon TCR stimulation, reduced expression of immunosuppressive genes in fetal iTreg cells such as IL10, and increased expression of proinflammatory genes including IFNG. Consequently, Helios knockout fetal iTreg cells had reduced IL-10 and increased IFN-γ cytokine production. Together, our results reveal important roles for Helios in enhancing preferential fetal Treg differentiation and fine-tuning eventual Treg function. The Treg-biased programs identified within fetal naïve T cells could potentially be used to engineer enhanced iTreg populations for adoptive cellular therapies.

INTRODUCTION

The adaptive immune system must generate immunotolerance to prevent or resolve proinflammatory responses that can cause host damage (1–3), while still permitting functional effector responses for host defense against pathogens (4, 5). A primary mechanism that achieves this flexibility is the capacity of CD4⁺ naïve T cells to differentiate into multiple specialized T helper (Th) subsets with either proinflammatory or immunosuppressive functions. The presence of polarizing cytokines within their immediate environment determines the eventual Th1 cell fate by triggering the expression and/or activation of master transcription factors that enact lineage-specific transcriptional programs (6). For example, signaling by transforming growth factor–β (TGF-β) promotes the induction of forkhead box P3 (FOXP3) (7–9), which is the master transcription factor required for the differentiation of naïve T cells into immunosuppressive regulatory T (Treg) cells. Mutations of the FOXP3 gene leading to the absence or dysfunction of Treg cells result in the loss of Treg-mediated immunotolerance and trigger fatal, early-onset multiorgan autoimmunity in both mice and humans (10–15). Autoimmunity resulting from the loss of FOXP3⁺ Treg cell–mediated tolerance in humans, defined as the IPEX (immune dysregulation, polyendocrinopathy, enteropathy, X-linked) syndrome, can manifest within the fetus in utero and result in miscarriage, preterm birth, or death in childhood without hematopoietic stem cell transplant (16–20). The initiation of autoimmunity in IPEX coincides with the emergence of T cells in the second trimester of human development, suggesting that Treg cell–mediated peripheral tolerance is required during fetal development (21, 22). This is supported by the presence of an abundant population of fetal Treg cells in the secondary lymphoid tissues, which comprise a larger percentage of the total CD4⁺ T cell population compared with adults (23–25). However, we previously did not observe a difference in the frequencies of thymic Treg cells in fetal and infant thymus (24), indicating that increased thymic output is not responsible for the increased frequency of fetal Treg cells. Fetal naïve T cells, unlike their adult counterparts, preferentially differentiate into functional Treg cells upon antigen stimulation, which include noninherited maternal alloantigens (i.e., NIMAs) on maternal antigen-presenting cells (24). These findings imply that the abundance of fetal Treg cells observed in fetal lymphoid tissues is due to peripheral conversion from naïve T cells. This propensity for Treg differentiation is retained in vitro, because a high frequency of fetal naïve T cells differentiate into FOXP3⁺ Treg cells upon T cell receptor (TCR) activation even in the absence of exogenous TGF-β (26). The unique capability of fetal naïve T cells to initiate Treg differentiation in the absence of exogenous TGF-β suggests that this ability is cell intrinsic; however, the molecular mechanisms that underlie this predisposition are largely unknown.

Chromatin changes are also implicated in driving the final effector phenotype and function of differentiated T cells, defined by increases in chromatin accessibility of active lineage-specific genes, and the silencing of genes associated with other effector lineages (27, 28). In thymic and peripheral Treg cells, permissive/active histone marks and
DNA demethylation at Treg-associated genes such as IL2RA (i.e., CD25), CTLA4, IKZF2 (i.e., Helios), and IKZF4 (i.e., Eos) (29, 30) must be acquired for commitment to and maintenance of the Treg phenotype (29–32). This Treg-chromatin landscape is acquired within developing thymic Treg precursors before FOXP3 protein expression (30), indicating that a Treg-specific epigenome may be responsible for initiating and promoting the expression of FOXP3. In addition, other key genes associated with the Treg epigenome, such as Helios, are expressed independently of FOXP3 expression (29, 30, 33) and can direct the partial acquisition of the Treg-specific transcriptional signature when overexpressed in FOXP3 CD4+ T cells (34). We therefore hypothesized that fetal naïve T cells might already have a partial Treg-specific epigenetic and transcriptional signature that predisposes them for differentiation toward the Treg cell fate even without exogenous TGF-β signaling.

Here, we interrogated the transcriptional and chromatin landscape of fetal and adult naïve and Treg cells and found that components of the Treg gene regulatory program are activated only in fetal naïve T cells. We then show that the partial Treg-specific gene signature detected at steady state in fetal naïve T cells is retained only in fetal-derived but not adult-derived induced Treg (iTreg) cells. We next identify two Treg-specific superenhancers (SEs) associated with the Helios locus that are active in fetal naïve T cells, in which we subsequently demonstrate the expression of Helios protein. Only iTreg cells generated from fetal naïve T cells in vitro retained Helios expression and were characterized by repression of interleukin-2 (IL-2) production; neither of which were observed in adult iTreg cells. CRISPR (clustered regular interspaced short palindromic repeats)–Cas9 (CRISPR-associated protein 9)–mediated ablation of Helios in fetal naïve T cells impaired their cell-intrinsic ability to differentiate into iTreg cells in the absence of exogenous TGF-β. Analysis of the transcriptome in Helios knockout iTreg cells revealed that Helios enhanced the up-regulation of Treg-specific genes (e.g., IL10) and mediated the repression of proinflammatory genes involved in T effector differentiation and function (e.g., IFNG). Helios ablation in fetal iTreg cells resulted in decreased IL-10 production concurrent with increased interferon-γ (IFN-γ) and IL-2. Given that Helios has been previously characterized to be a gene specific to thymic Treg cells, our data reveal a previously unknown role for Helios as part of a preexisting epigenetic and transcriptional program within human fetal naïve T cells that lowers the threshold for Treg differentiation and functional commitment. Together, we thus identify a TGF-β-independent mechanism unique to fetal naïve T cells that favors their differentiation into Treg cells in vivo from naïve T cells for use in immunotherapy.

RESULTS

Human fetal naïve T cells express a partial Treg transcriptome

Given that fetal naïve T cells preferentially differentiate into Treg cells upon TCR stimulation alone (26), we first asked whether fetal naïve T cells shared elements of their transcriptome with Treg cells that could predispose them toward the Treg lineage. We performed RNA sequencing (RNA-seq) on sorted fetal and adult CD4+ naïve and Treg cells (see fig. S1 for sort gating strategy and purity confirmation). Principal components analysis (PCA) revealed that fetal and adult populations first segregated by cell origin (PC1, fetal or adult) before cell phenotype (PC2, naïve versus Treg; Fig. 1A). PC2 scores for fetal naïve samples were intermediate to adult naïve and Treg samples (Fig. 1A), suggesting intermediate expression of Treg-specific genes in fetal naïve T cells. To test this hypothesis, we first defined a Treg-specific transcriptional signature by identifying genes differentially expressed in both fetal and adult Treg cells relative to adult naïve T cells based on a false discovery rate (FDR) cutoff of <0.05 and log2 fold change (log2FC) increase in expression of 1.5 (fig. S2 and table S1). Fetal naïve T cells had intermediate up-regulation/down-regulation (Fig. 1B) across genes up-regulated/down-regulated in our Treg-specific transcriptional signature. More specifically, relative to adult naïve T cells, fetal naïve T cells had 88 Treg-up-regulated and 42 Treg-down-regulated genes (Fig. 1C). We defined four different clusters within the Treg-specific transcriptome (Fig. 1B and table S1)—two of which corresponded to all Treg-up-regulated genes (clusters 1.1 and 1.2), whereas the other two clusters contained all Treg-down-regulated genes (clusters 1.3 and 1.4). Within Treg-up-regulated genes, fetal naïve T cells did not express canonical Treg genes such as FOXP3, IL2RA, and CTLA4 (cluster 1.1; Fig. 1B). Instead, fetal naïve T cells had increased expression of Treg-up-regulated genes previously associated with Treg function such as CCR4 (35, 36) and KLF10 (37–39). In addition, fetal naïve T cells had increased expression of the transcription factors IKZF2 (Helios) (40–42) and IKZF4 (Eos) (34, 43) (cluster 1.2; Fig. 1, B and C), which are transcribed independently of FOXP3 expression in mice (29, 33, 44). Fetal naïve T cells and both the Treg cell populations shared similar down-regulation of a subset of genes previously characterized as being down-regulated in Treg cells such as TSHZ2 and SERPINB6 (45–47) (cluster 1.3; Fig. 1B). In contrast, genes shared between fetal and adult naïve T cells included known genes contributing to the naïve T cell phenotype such as TCF7 and IL7R (cluster 1.4; Fig. 1B). Together, the presence of a partial Treg-specific signature in fetal naïve T cells could thus potentiate Treg differentiation upon the receipt of TCR signaling, consistent with the lowered threshold and greater propensity for these cells toward Treg differentiation (24, 26).

Fetal-derived iTreg cells maintain differential expression of the partial Treg-specific transcriptome present in fetal naïve T cells

We next asked whether the partial Treg-specific transcriptome detected in fetal naïve T cells would remain differentially expressed in fetal iTreg cells and whether adult iTreg cells also acquire the Treg-specific transcriptome after in vitro differentiation. To assess this, we performed RNA-seq on fetal and adult iTreg cells that underwent differentiation with TCR stimulation in media supplemented with IL-2 alone (IL-2–iTreg) or additionally supplemented with TGF-β (TGF-β–iTreg). PCA revealed that PC1 still segregated iTreg populations by cell origin (fetal or adult), after which populations segregated by the stimulus received during differentiation (absence/presence of TGF-β, PC2; Fig. 2A). This indicated that fetal-derived iTreg cells were still transcriptionally different from adult-derived iTreg cells, although the addition of TGF-β was sufficient to drive differential expression of a shared set of genes. We then evaluated all differentially expressed genes (FDR < 0.05; log2FC > 1.5) across all iTreg populations (Fig. 2B) and defined six clusters (table S2). Both fetal and adult TGF-β–iTreg cells up-regulated two gene clusters (clusters 2.1 and 2.2; Fig. 2B and fig. S3A) and down-regulated one gene cluster (cluster 2.6; Fig. 2B and fig. S3A). Treg-specific genes within clusters 2.1 and 2.2 included genes known to be up-regulated with TGF-β signaling such as FOXP3 and IKZF4 (48), as well genes potentially implicated in Treg differentiation and function such as SEMA4A (49), LTBP1, LTBP4 (50), and...
LGALS3 (51, 52). However, only fetal IL-2– and TGF-β–iTreg cells had increased expression of Treg-specific genes that were up-regulated in ex vivo fetal naïve T cells (Fig. 1C) such as IKZF2, DUSP4, TOX, and RGS1 (cluster 2.3; Fig. 2B and fig. S3A). Decreased transcription of proinflammatory transcripts such as IFNG, IL2, GZMB, and IRF7 (cluster 2.5; Fig. 2B and fig. S3A) and increased expression of genes involved in Treg cell suppressive function such as IL10 (cluster 2.3; Fig. 2B) were only observed in fetal, but not adult, iTreg cells. This suggested that, qualitatively, fetal iTreg cells may have a transcriptome more reflective of ex vivo Treg populations relative to adult iTreg cells. We thus used gene set enrichment analysis (GSEA) to independently evaluate the transcriptomes of fetal- or adult-derived iTreg cells against published gene sets comparing Treg and conventional T cell populations that also underwent TCR- and cytokine-stimulated activation in vitro (fig. S3B and table S3). In comparison with adult iTreg cells, fetal iTreg cells differentiated under both stimulation conditions had enrichment in genes up-regulated in activated Treg populations (Fig. 2B) and had enrichment of the partial Treg-specific signature as defined by clusters 1.2 and 1.3 (Figs. 1B and 2C, left, and fig. S3C). Exogenous TGF-β signaling during Treg differentiation resulted in the enrichment of cluster 1.1 and depletion of cluster 1.4 genes within fetal TGF-β–iTreg but not adult TGF-β–iTreg cells (Fig. 2C, right, and fig. S3D). Our data thus show that differentiating fetal naïve T cells, independent of exogenous TGF-β, retain increased expression of the partial Treg-specific transcriptional signature detected in ex vivo naïve T cells. Furthermore, the expression of these genes does not reach the same levels in adult iTreg cells, suggesting that upstream mechanisms might be responsible for this expression pattern.
Fig. 2. Fetal iTreg cells retain expression of the partial Treg-specific transcriptome detected in fetal naïve T cells in steady state. (A) PCA of RNA-seq data comparing fetal and adult iTreg cells generated in IL-2 alone (IL-2–iTreg) or with added exogenous TGF-β (TGF-β–iTreg). Boxplots show scores for PC1 (bottom) and PC2 (right). (B) Heatmap shows relative expression levels of differentially expressed genes (log₂FC > 1.5; FDR < 0.05) in adult and fetal IL-2–iTreg and TGF-β–iTreg. Clusters are labeled and defined by k-means clustering. Genes associated with Treg or proinflammatory/effector T cell function are labeled. (C) Preranked GSEA was used to assess overrepresentation of predefined activated Treg-associated gene sets (see fig. S3B for details of all gene sets) in fetal (orange) or adult (blue) IL-2–iTreg cells (left) or TGF-β–iTreg cells (right). n = 4 for all conditions. Barplot shows normalized enrichment scores (ES) for gene sets with FDR < 0.05, and arrows denote direction of enrichment in adult or fetal iTreg cells. (D) Preranked GSEA was used to assess overrepresentation of each of the gene clusters identified in Fig. 1B. Barplot shows normalized enrichment scores for all clusters with FDR < 0.05 for fetal (orange) or adult (blue) IL-2–iTreg cells (left) or TGF-β–iTreg cells (right).
for driving the transcriptional differences that favor Treg differentiation in fetal naïve T cells.

**Fetal iTreg cells have increased sensitivity to TGF-β signaling**

In addition to increased expression of genes associated with Treg function, fetal IL-2–iTreg cells strongly up-regulated a gene cluster that contained key genes associated with TGF-β sequestration and downstream signaling—including SMAD1, TGFBR3, LRRC32 (53, 54), and LIN28B (26) (cluster 2.4; Fig. 2B and fig. S4A). Expression of Lin28b protein in fetal naïve T cells contributes to increased expression of TGFBR1, TGFBR3, and SMAD2, as well as increased phosphorylation of SMAD2/3 (26). Glycoprotein A repetitions predominant (GARP) (LRRC32) is expressed highly on the cell surface of activated Treg cells and captures inactive TGF-β bound to the latency-associated peptide (LAP) (53, 55). This reservoir of cell surface–associated TGF-β is implicated in the maintenance of oral tolerance in mice (54) and in the induction of FOXP3 in naïve T cells cocultured with GARP+ Treg cells (53). Here, we demonstrate that fetal but not adult iTreg cells highly expressed GARP (fig. S4B) and LAP in a linear fashion (fig. S4C). Furthermore, fetal iTreg cells have increased transcription of ITGB8 (cluster 3; fig. 2B), the β chain for the integrin αβ3, that processes and releases bioactive TGF-β1 from LAP (54). Because fetal iTreg cells have increased cell surface–associated TGF-β and the machinery to mediate its potential release, we tested whether blockade of TGF-β1 with TGF-β–neutralizing antibodies resulted in decreased fetal Treg differentiation in response to TCR stimulation alone. As hypothesized, fetal Treg induction was blunted in the setting of TGF-β blockage (fig. S4, D and E). However, fetal naïve T cells still retained an increased ability for Treg differentiation over adult naïve T cells, even when exogenous bioactive TGF-β was added (fig. S4, D and E). Hence, although active TGF-β1 biogenesis may contribute to fetal iTreg differentiation in the absence of exogenous TGF-β, additional upstream mechanisms are responsible for driving enhanced fetal Treg differentiation in vitro.

**Fetal naïve T cells share a partial epigenetic landscape with adult Treg cells**

Given that fetal naïve T cells already express a partial Treg-specific signature, we next assessed if we could also detect the presence of permissive epigenetic marks in fetal naïve T cells that could further drive the predisposition toward more robust Treg cell differentiation. To identify these chromatin features, we used assay for transposase-accessible chromatin followed by sequencing (ATAC-seq) and acetylation at Lys37 for histone 3 (H3K27ac) chromatin immunoprecipitation sequencing (ChIP-seq) to compare regions of open and active chromatin in adult Treg cells relative to fetal and adult naïve T cells. SEs and typical transcriptional enhancers (TEs) were classified using the Rank Ordering of Super-enhancers (ROSE) algorithm (56, 57), and PCA was performed across enhancers identified in all samples. Cell origin (fetal versus adult) was the primary source of variance (PC1) in both ATAC-seq (Fig. 3A) and H3K27ac ChIP-seq (Fig. 3B) and, together with cell phenotype (naïve or Treg), largely accounted for differences in the epigenome across all three populations. However, fetal naïve and adult Treg Cell samples clustered together across the second source of variance (PC2) in both datasets (Fig. 3, A and B). This suggested that, in addition to expression of a partial Treg-specific transcriptome, fetal naïve T cells share a small subset of open and active Treg-specific enhancers with adult Treg cells.

To address this possibility, we first independently defined enhanced enhancers differentially enriched for ATAC (fig. S5A) or H3K27ac (fig. S5B) signal in adult Treg cells relative to adult naïve T cells (FDR < 0.05; expression FC > 1.5). Differentially enriched enhancers classified as having both increased H3K27ac and ATAC signals in adult Treg cells were termed Treg-accessible enhancers (fig. S5C and table S4), whereas common enhancers were defined as having no difference in enrichment of both signals (fig. S5C). Treg-inaccessible enhancers with decreased signals were similarly defined (table S5). We then assessed whether any Treg-accessible enhancers were enriched in fetal naïve T cells relative to adult naïve T cells. We found that fetal naïve T cells had increased ATAC and H3K27ac signal at 38.8% (933 of 2405) and 4.6% (110 of 2405) of Treg-accessible enhancers, respectively (Fig. 3C and fig. S5, D and E). These Treg-accessible enhancers (table S4) were annotated to genes previously described to be part of the Treg-specific epigenome, such as IKZF2 (i.e., Helios), IKZF4 (i.e., Eos), and RXRA (i.e., retinoic receptor RXR-α) (29, 30, 32). Similarly, 23.1% (426 of 1837) and 14% (258 of 1837) of Treg-inaccessible enhancers also had decreased ATAC and H3K27ac signal, respectively, in fetal naïve T cells (Fig. 3C and fig. S5, D and E). Together, these data suggest that fetal naïve T cells at steady state are poised for Treg differentiation by the acquisition of a partial Treg epigenomic landscape characterized by increased chromatin accessibility at more than a third of all Treg-accessible enhancers. Given that chromatin accessibility may precede H3K27ac deposition (58), acquisition of the Treg epigenetic signature within fetal naïve T cells could occur in a stepwise fashion where full enhancer activation via H3K27 acetylation is acquired with the triggering of Treg cell differentiation.

In light of this hypothesis, we evaluated transcription factor motif enrichment within all Treg-accessible peaks shared between fetal naïve and committed adult Treg cells (defined in a similar manner as TEs/SEs). Peak calls were used to minimize false positives stemming from the broadness of SE regions. Fetal naïve T cells had minimal enrichment of Treg-accessible H3K27ac peaks but had increased chromatin accessibility at a third of all Treg-accessible ATAC peaks (fig. S6A). These shared Treg-accessible peaks were enriched in binding motifs for the activator protein 1 (AP-1) (fig. S6B) and Runt-related transcription factor 1 (RUNX1) (fig. S6C and table S6), which are downstream of TCR signaling and play critical roles as transcriptional regulators of the FOXP3 locus and as cofactors for FOXP3 (59). We also detected a smaller subset of peaks that had enrichment of binding motifs for signal transducer and activator of transcription 5 (STAT5) (fig. S6D) and SMAD2/3 (fig. S6E). As such, increased chromatin accessibility could potentially synergize with enhancer activation and faster transcription of genes underlying STAT5 and SMAD2/3 binding sites with IL-2 and TGF-β signaling during fetal Treg differentiation. Last, we examined differentially enriched Treg-accessible peaks in fetal naïve T cells for the presence of FOXP3 binding sites previously identified in human Treg cells (60). We show that only 5% (116 of 2213) of shared Treg-accessible peaks with increased ATAC-seq signal have FOXP3 binding sites (fig. S6F). Together, we further illustrate that increased chromatin accessibility within fetal naïve T cells is largely poised to synergize with TCR and cytokine signaling cues and, to a smaller extent, direct binding of FOXP3 to drive their preferential differentiation into Treg cells.

**Fetal naïve T cells have increased open and active chromatin at two Treg-accessible SEs associated with Helios**

SEs are defined by high-density regions of H3K27ac modifications, and they nucleate the assembly of transcription factors to drive expression of genes associated with cell lineage commitment (56, 57, 61).
We saw that highly ranked SEs shared across fetal naïve, adult naïve, and adult T\(_{\text{reg}}\) samples corresponded to genes commonly associated with global T cell development and function such as \(BCL11B\) (62) and \(ETS1\) (63) (fig. S7A and table S7). Because SEs first defined in murine T\(_{\text{reg}}\) cells were shown to have increased accessibility in murine thymic progenitors preceding FOXP3 up-regulation and T\(_{\text{reg}}\) cell...
lineage commitment (30), we asked whether increased accessibility at similar SEs in fetal naïve T cells could contribute to their priming toward TReg differentiation. We identified 121 SEs within all TReg-accessible enhancers, many of which were proximal to canonical TReg genes including FOXP3, IL2RA, CTLA4, TNFRSF4 [i.e., tumor necrosis factor (TNF) receptor superfamily member 4], and IKZF2 (Fig. 3D) as previously described in mice (30). Globally, fetal naïve T cells did not have greater accessible chromatin or H3K27ac enrichment at all TReg-accessible SEs compared with adult naïve T cells (fig. S7B). This suggested that unlike thymic TReg progenitors, fetal naïve T cells might acquire active enhancer marks at the full TReg-accessible SE signature only after TReg differentiation. We next wondered whether any SEs independently classified by ROSE, and preferentially enriched within fetal naïve T cells, were proximal to genes associated with TReg-accessible SEs, because their presence would have been masked by the global analysis. Most genes associated with TReg-accessible SEs did not have enrichment of H3K27ac signal that met the SE cutoff in both fetal and adult naïve T cells (Fig. 3E). One exception was the transcription factor IKZF2 (i.e., Helios), which was unique to fetal naïve T cells (Fig. 3E, left), and previously identified to be one of the first TReg SEs to acquire permissive epigenetic marks in murine thymic TReg progenitors (30). Adult naïve T cells had independent SE classification for one gene, ZC3H12D (Fig. 3E, right), which currently has no reported association with TReg cell function.

Cell-specific SE regions are typically found proximal to genes encoding transcription factors that play key roles in cell identity by controlling the transcription of lineage-specific transcriptional programs (30, 56). We next focused our analysis on evaluating whether any TReg-accessible SEs defined within adult TReg cells had increased enrichment of either ATAC or H3K27ac signal in fetal naïve T cells (Fig. 3C) and were also associated with known transcription factors. Five TReg-accessible SEs associated with four different transcription factors were identified to have increased ATAC signal (Fig. 3F), of which only two TReg-accessible SEs were also differentially enriched for H3K27ac (Fig. 3G). These active, H3K27ac-marked TReg-accessible SEs were located in the intragenic and upstream regions within the Helios locus (Fig. 4A), indicating that active expression of Helios might already be present in fetal naïve T cells. We further observed that Helios was a substantial contributor to the negative directionality of PC2 (Fig. 4B), which drove segregation of TReg cells away from naïve T cells in our RNA-seq dataset (Fig. 1A). Helios was also among the significant TReg-up-regulated genes with increased RNA transcription in fetal naïve T cells (Figs. 1C and 4C). Because enriched permissive epigenetic marks and transcription at the Helios gene locus regulate TReg phenotype and function independent of FOXP3 expression, CCR4 and Eos expression did not demonstrate signal (CCR4; fig. S8C) or H3K27ac signal (Eos; fig. S8D). Relative to FOXP3 expression, CCR4 and Eos expression did not demonstrate a similar shift in expression within fetal naïve T cells from adult naïve T cells when compared with Helios (fig. S8E), which led us to focus on investigating the role of Helios expression in fetal TReg cell differentiation.

The predisposition of fetal naïve T cells toward TReg differentiation is not explained by increased incidence of CD31+ cells in the naïve T cell population or increased proliferative ability

Before further investigations into potential contributions of Helios to TReg differentiation, we sought to address potential confounding factors in our analysis. Previous studies have demonstrated that CD31+ population within the human naïve T cell population is enriched for recent thymic emigrants and have increased TReg differentiation potential (67), making them potential precursors of TReg cells in the periphery. We assessed whether increased CD31+ cell frequency was a contributor to increased TReg differentiation in fetal naïve T cells, because the fraction of the CD31+ population is highest at birth and declines with age (68). Unexpectedly, CD31+ proportions were not different between adult and fetal naïve T cell populations (fig. S9, A and B). In addition, CD31+ naïve T cells isolated from human peripheral blood do not demonstrate increased differentiation in the absence of exogenous TGF-β (66). We thus concluded that the predisposition toward TReg differentiation that we observed within fetal naïve T cells was not attributed to differences in CD31+ proportions.

We further observed that mean CD31 expression levels were reduced within fetal CD31+ naïve T cells (fig. S9C). CD31 is downregulated with TCR signaling (68), and a subset of fetal CD4+ T cells are CD69+ and actively cycling (23). We therefore assessed the expression of CD69 and Ki67, a marker of active proliferation, relative to Helios expression in fetal naïve T cells. Neither fetal nor adult naïve T cells expressed CD69 (fig. S10A). However, as previously characterized (23), a subset of fetal naïve T cells are actively proliferating, whereas adult naïve T cells are mainly Ki67+ (fig. S10B), thus possibly accounting for the reduced CD31 expression in fetal naïve T cells. Most of the Ki67+ population in fetal naïve T cells was also Helios+ (fig. S10C), suggesting that Helios might regulate proliferation. However, with TCR stimulation, both adult and fetal naïve T cells up-regulated Ki67 to a similar extent after 5 days (fig. S10D), indicating that Helios expression does not confer any selective proliferation advantage on fetal naïve T cells during TReg differentiation that may account for their increased TReg differentiation potential.

Fetal naïve T cells do not have increased demethylation at the FOXP3 TReg-specific demethylated region

Because Helios was first identified as a marker of thymic TReg cells (40), we sought to rule out possible contamination of thymic TReg cells by assessing demethylation of the TReg-specific demethylated region (TSDR) at the conserved noncoding sequence 2 region within the FOXP3 gene in our sorted naïve T cell populations (fig. S1). We saw that, as expected, only fetal and adult TReg populations had complete TSDR demethylation, whereas both fetal and adult naïve T cells had a fully methylated TSDR (fig. S11A). This indicated that Helios expression within fetal naïve T cells was cell intrinsic and not due to contamination with thymic TReg cells.

Fetal naïve T cells have increased Helios protein expression at baseline

Using flow cytometry staining, we show that fetal naïve T cells had higher Helios protein expression compared with adult naïve T cells (Fig. 4, D and E). As previously described (40, 66), we identified Helios+ and Helios− FOXP3+ populations in adult TReg cells (Fig. 4F). Fetal TReg cells were all uniformly Helios+, which could indicate that retention of permissive epigenetic marks at Helios TReg-accessible SEs may drive high Helios expression (Fig. 4F). An average of 60% of fetal naïve T cells were Helios+, whereas adult naïve T cells did not express Helios (Fig. 4, F and G). In comparison, we also examined the protein expression of two other TReg-specific genes with increased transcription in fetal naïve T cells with differentially enriched ATAC signal (CCR4; fig. S8C) or H3K27ac signal (Eos; fig. S8D). Relative to FOXP3 expression, CCR4 and Eos expression did not demonstrate a similar shift in expression within fetal naïve T cells from adult naïve T cells when compared with Helios (fig. S8E), which led us to focus on investigating the role of Helios expression in fetal TReg cell differentiation.
Fig. 4. Helios expression is increased in fetal naïve T cells. (A) Tracks show H3K27ac and ATAC signals at two T reg-accessible SEs associated with the Helios (IKZF2) locus. Representative tracks of one replicate shown (H3K27ac ChIP-seq, n = 3; ATAC-seq, n = 2). (B) The top five genes contributing to PC2, which segregates cells by functional subtype (naïve versus T reg) are plotted for both directions: Helios (IKZF2) is highlighted in red. (C) Boxplot shows log2 trimmed mean of M values of normalized RNA-seq reads for Helios in adult naïve, fetal naïve, adult T reg, and fetal T reg cells (n = 4). (D) Helios staining intensity in sorted CD4+CD25+CD27+CD45RA+ adult naive (blue) and fetal naïve (green) and adult CD25 hiCD127 loFOXP3 (orange) and fetal T reg (brown). (E) Boxplot shows quantification of mean fluorescence intensity (MFI) of Helios for adult naïve (n = 8), fetal naïve (n = 10), adult T reg (n = 8), and fetal T reg (n = 10) cells. (F) Flow cytometry analyses of sorted populations in (D). Helios+ and Helios− gates were set on the basis of negative and positive populations in adult T reg samples (bottom left). Adult naïve T cells were universally Helios−. (G) Quantification of Helios+ cells among sorted populations in (F). All statistics were calculated by unpaired two-sided Mann-Whitney test. ***P < 0.001, **P < 0.01, *P < 0.05. All boxplots show median (center line), interquartile range (box), and 10th and 90th percentiles (whiskers).

Fetal naïve T cells up-regulate and maintain Helios expression during iT reg differentiation
Helios is expressed independently of FOXP3 expression (29, 33, 44) and can enhance the acquisition of a T reg-transcriptional signature with the coexpression of FOXP3 (34). As such, Helios expression in fetal naïve T cells might allow them to bypass the need for TGF-β to initiate FOXP3 up-regulation and underlie their preferential differentiation into T reg cells. To assess this, we tracked Helios expression
within fetal and adult naïve T cells during iTreg differentiation with IL-2 alone or with TGF-β added at 1, 3, or 5 days. As previously observed, a higher frequency of fetal naïve T cells differentiated into CD25hiFOXP3hi iTreg cells relative to adult naïve T cells either in the presence or in the absence of exogenous TGF-β (fig. S12, A and B) (24, 25). Fetal naïve T cells highly up-regulated and maintained Helios protein expression during iTreg differentiation, whereas adult naïve T cells did not (fig. S12, C and D). Concurrent up-regulation of both FOXP3 and Helios was observed only in fetal iTreg cells differentiated under both stimulation conditions; even as FOXP3 expression increased with TGF-β stimulation, Helios expression was not up-regulated in adult iTreg cells at any time point (Fig. 5, A and B). Although Helios has been implicated as a marker of activation in proliferating cells (69), we show that both fetal and adult iTreg cells up-regulated Ki67 to the same extent, but only fetal iTreg cells maintained up-regulation of Helios (fig. S12E), thus suggesting that the increase in Helios expression happens de novo in fetal iTreg cells.

![Flow cytometry plots showing the percentage of Helios+FOXP3hi iTreg cells in fetal and adult Treg populations over 1, 3, and 5 days in the absence or presence of TGF-β.](image)

Fig. 5. Helios+ fetal iTreg cells have increased FOXP3 expression. Sorted fetal and adult naïve T cells were stimulated with IL-2 alone or IL-2 + TGF-β at 1, 3, or 5 days and analyzed by flow cytometry. The number of biological replicates for each time point and stimulation condition are specified in table S8. (A) Representative flow cytometry plots show Treg induction in the presence of IL-2 and TGF-β for fetal (top) and adult (bottom) naïve T cells respectively gated on live, CD4+ T cells. (B) Quantification of the percentage of Helios+FOXP3hi iTreg cells gated in (A) for adult and fetal naïve T cells stimulated in the presence or absence of TGF-β. Statistics calculated using two-way ANOVA with Tukey’s honest significant difference posttest, **P < 0.01. Error bars denote means ± SD. (C) Representative flow cytometry plots shown for one fetal sample stimulated with IL-2 and TGF-β at day 1. (D) Quantification of the proportion of fetal Treg cells in the Helios+ or Helios− population as gated in (C) over 1, 3, and 5 days in the absence (left) or presence (right) of TGF-β. (E) Quantification of FOXP3 mean fluorescence intensity for Helios+ and Helios− iTreg cells as gated in (C) across all time points in the absence (left) or presence (right) of TGF-β. Statistics for (D) and (E) were calculated by two-sided Wilcoxon signed-rank test, ***P < 0.001, **P < 0.01. All boxplots show median (center line), interquartile range (box), and 10th and 90th percentiles (whiskers).
The proportions of fetal IL-2–iTreg cells generated across time tracked closely with proportions of adult TGF-β–iTreg cells (fig. S12B), suggesting that Helios expression within fetal naïve T cells could enhance their preferential differentiation into Treg cells independently of exogenous TGF-β. We further examined fetal Helios+ and Helios− populations after Treg differentiation and found that most fetal iTreg cells were within the Helios+ population 24 hours after the initiation of Treg induction (Fig. 5C). The increased frequency of iTreg cells present within the Helios+ over the Helios− population was maintained over time and under both stimulation conditions (Fig. 5D). Helios+ cells also consistently had higher FOXP3 expression (Fig. 5E) relative to Helios− cells, suggesting that Helios expression could potentially drive increased FOXP3 expression in differentiating fetal naïve T cells.

**Fig. 6. CRISPR-Cas9–mediated knockout of Helios in fetal naïve T cells reduces their preferential differentiation into Treg cells**

Given the hypothesized role of Helios in enhancing FOXP3 up-regulation during fetal Treg cell differentiation, we predicted that reduced Helios expression in fetal naïve T cells would subsequently inhibit their cell-intrinsic propensity for Treg differentiation. Using CRISPR-Cas9–mediated editing, we knocked out Helios with two independent guide RNAs (gRNAs) targeting different exons of the gene. Fetal naïve T cells were then assessed for Treg induction post-editing after differentiation in the presence or absence of exogenous TGF-β (Fig. 6A). We first confirmed that both gRNAs were able to successfully disrupt the Helios locus (fig. S13, A to C) and observed specific reduction of Helios protein (Fig. 6B) in comparison with the nontargeting (NT) guide. Both gRNAs resulted in an average of
70% of fetal naïve T cells losing Helios expression (fig. S13D), and the reduction was maintained after 6 days of Treg induction under both stimulation conditions (Fig. 6, B and C). Helios knockout in stimulated fetal naïve T cells reduced subsequent Treg differentiation in the absence of exogenous TGF-β compared with cells that received the NT guide (Fig. 6, D and E), and the reduction in Treg percentage correlated with the extent of knockout generated (fig. S14A). Adult naïve T cells nucleofected with the same guides were used as Treg gating controls (fig. S13E). In contrast, Helios knockout had no effect on fetal iTreg differentiation with addition of exogenous TGF-β (Fig. 6, D and E, and fig. S14B). This indicated that signaling via TGF-β compensated for the loss of Helios-driven Treg differentiation and that Helios and TGF-β may participate in shared signaling pathways. Our data show that Helios expression within fetal naïve T cells plays a role in enhancing preferential Treg differentiation specifically in the absence of exogenous TGF-β. This mechanism present within fetal naïve T cells could lower the threshold required for Treg cell differentiation, thus potentially allowing for the default generation of peripheral Treg-mediated tolerance upon antigen encounter during fetal development.

Helios suppresses IL-2 secretion in fetal iTreg cells
Helios maintains an anergic and nonproliferative state characteristic of the Treg phenotype (73) by mediating the epigenetic silencing of the IL2 locus in Treg cells (74). In contrast to conventional T cells, Treg cells have reduced IL-2 production upon TCR stimulation and depend heavily on paracrine IL-2 for their maintenance (75). Because Helios is highly expressed and maintained in fetal iTreg cells, we investigated whether this led to a corresponding suppression of IL-2 production. As hypothesized, fetal iTreg cells demonstrated less IL-2 production upon restimulation compared with adult iTreg cells (fig. S15A), and suppression of IL-2 was observed regardless of iTreg differentiation conditions (fig. S15B). When delineated on the basis of Helioshi and Helioslo expression (fig. S15C), Helioshi fetal iTreg cells consistently had lower IL-2 production across both stimulation conditions (fig. S15D), indicating that high Helios expression may be associated with greater repression of the IL2 locus. Helios knockout in fetal iTreg cells then resulted in increased IL-2 production in IL-2–iTreg cells (fig. S15, E and F). Furthermore, Helios knockout TGF-β–iTreg cells also produced more IL-2 upon restimulation when compared with the NT control (fig. S15, E and F). This demonstrates that continued Helios expression in fetal naïve T cells not only enhances preferential Treg differentiation but also aids in the repression of IL-2 production in fetal iTreg cells.

Helios knockout results in the down-regulation of genes associated with Treg differentiation and function and the concurrent up-regulation of proinflammatory genes
Helios controls the expression of several key genes involved in Treg suppressive function (76) including GARP. Helios knockout in fetal naïve T cells did not affect FOXP3 or CD25 expression (fig. S14, C and D) but resulted in decreased CTLA-4 expression in fetal iTreg cells (fig. S14E). Helios knockout also resulted in a trend toward down-regulation of GARP and LAP on fetal iTreg cells (fig. S14, F and G). Because the impact of Helios knockout was variable across the conventional Treg markers surveyed and fetal iTreg cells retain expression of a partial Treg-specific transcriptional signature, we wondered whether transcriptional control of other Treg genes by Helios could enhance the conversion of fetal naïve T cells into iTreg cells and influence their subsequent function. Hence, we further assessed the impact of Helios ablation on the fetal iTreg transcriptome by RNA-seq. CRISPR-Cas9 editing was carried out in fetal naïve T cells with Helios gRNA1 (HeliosKO) or the NT control (HeliosWT) before Treg differentiation was induced in the absence or presence of TGF-β (fig. S16A).

PCA revealed that HeliosKO and HeliosWT iTreg cells segregated largely according to whether differentiation occurred in the presence or absence of TGF-β (PC1; fig. S16B). This was not unexpected, because TGF-β signaling is responsible for the up-regulation and repression of a significant subset of Treg-specific genes (Fig. 2, B to D). We also detected a small but distinct segregation of HeliosKO from HeliosWT cells, with PC2 mainly segregating HeliosKO and HeliosWT IL-2–iTreg cells (fig. S15C), whereas PC3 mainly distinguished HeliosKO and HeliosWT TGF-β–iTreg cells (fig. S15D). Because full knockout of Helios expression is not achieved within the total iTreg population with an average of 30% of all cells still retaining Helios expression (fig. S16A), we expected that this would result in a lowered signal-to-noise ratio. We thus used a more generous cutoff, with genes with at least a 10% change in expression (FC > 1.1; FDR < 0.05) were defined to be differentially expressed (fig. S15, E and F).

Given that TGF-β signaling is able to compensate for the defect in Treg induction in HeliosKO iTreg cells (Fig. 6, D and E), we decided to dissect possible pathways controlled by Helios and TGF-β signaling in parallel (Fig. 7A). We first identified differentially expressed genes in both stimulation conditions that would normally be up-regulated with TGF-β signaling within HeliosWT iTreg cells but were down-regulated in HeliosKO cells. This allowed us to detect genes whose expression was potentially enhanced by Helios in a complementary fashion—these genes would show decreased expression in HeliosKO IL-2–iTreg cells but, due to compensation with TGF-β signaling, would have no change in expression in HeliosKO TGF-β–iTreg cells compared with HeliosWT controls (Fig. 7, B and C). Similar cutoffs were used to define genes potentially suppressed by Helios in parallel, which would be up-regulated in HeliosKO IL-2–iTreg cells. We identified 199 down-regulated and 161 up-regulated genes within HeliosKO IL-2–iTreg cells that were not differentially expressed in TGF-β–iTreg cells (Fig. 7B). HeliosKO IL-2–iTreg cells had reduced expression of Treg-specific genes previously identified to be exclusively up-regulated in fetal iTreg cells such as DUSP4, IL10, and ITGA5 (CD103) (Fig. 7B and table S10). Concurrently, HeliosKO IL-2–iTreg cells up-regulated several chemokine and TNF superfamily genes (Fig. 7B and table S10), indicating that Helios may enhance the expression of a subset of Treg genes while simultaneously decreasing expression of proinflammatory genes associated with effector function in the absence of TGF-β signaling.

In addition, synergy between Helios and TGF-β signaling might occur, thus amplifying the expression of Treg-specific genes in an additive manner (Fig. 7A). We thus assessed genes that had decreased expression specifically in HeliosKO TGF-β–iTreg cells (Fig. 7C). Loss of Helios expression resulted in the down-regulation of 351 genes in TGF-β–iTreg cells (Fig. 7C), including SEMA4, PTG51, and RTKN, previously identified in the TGF-β–iTreg gene signature (cluster 2.2; Fig. 2B), as well as TOX1 and RGS1, which are up-regulated in fetal but not adult iTreg cells (cluster 2.3; Fig. 2B and table S10). Conversely, HeliosKO TGF-β–iTreg cells had up-regulation of 251 genes; these comprised chemokine receptor genes, as well as transcription factors involved with T1h1, T1h2, and T1h17 cell differentiation and function such as PRDM1 (Blimp1) (77), GATA3 (78), IKZF1 (Ikarios) (79, 80), and MAF (c-MAF) (81) (Fig. 7C). These data suggest that Helios
performs both parallel and additive roles in enhancing the up-regulation of genes associated with the Treg transcriptional signature and repressing genes that might drive differentiation toward other effector T cell pathways during Treg differentiation.

Last, we identified genes that were either up-regulated or down-regulated in HeliosKO iTreg cells across both induction conditions, implicating possible transcriptional control by Helios independent of TGF-β signaling during Treg differentiation (Fig. 7A). Two hundred genes were down-regulated in HeliosKO iTreg cells, including genes related to Treg phenotype and function such as CTLA4 and LTBP4 (Fig. 7, D and E). Although we did not observe reduced protein expression of FOXP3 in HeliosKO iTreg cells at day 6 of differentiation (Fig. S14C), we observed decreased FOXP3 transcription across both iTreg populations, suggesting that additional posttranscriptional mechanisms probably regulate FOXP3 expression downstream of Helios. HeliosKO iTreg cells also had reduced expression of NFATC4 (nuclear factor of activated T cell 3) and PPARα (peroxisome proliferator-activated receptor α) transcription factors that regulate the repression of proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α (82, 83), as well as the histone H3K27 demethylase KDM6B Junomji domain-containing protein D3 (JMJD3), which suppresses T1/2 and T H 17 programs (Fig. 7, D and E) (84). Loss of Helios expression also led to the up-regulation of 88 genes, including genes attributed to proinflammatory effector T cell function such as IFNG (Fig. 7, D and E). Together, we propose that Helios could potentially play a role in enhancing fetal Treg differentiation through the transcriptional regulation of a key subset of genes that restrict differentiation toward effector T H 1 cell phenotypes while favoring differentiation toward the Treg cell fate.

Helios knockout fetal iTreg cells have decreased IL-10 and increased IFN-γ production

The regulation of cytokine production in Treg cells is important for their suppressive ability; Treg cells must repress secretion of proinflammatory cytokines such as IFN-γ while maintaining production of immunosuppressive cytokines such as IL-10 (85). Given that we detected decreased IL-10 expression in HeliosKO

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**Fig. 7. Ablation of Helios in fetal iTreg results in down-regulation of Treg-specific genes and the concurrent up-regulation of proinflammatory genes.** CRISPR-Cas9 editing was carried out in fetal naïve T cells (n = 6) with Helios gRNA1 (HeliosKO) or the NT control guide (HeliosWT). Edited cells stimulated with αCD3/αCD28/αCD2 tetrastromers in the absence or presence of TGF-β for 6 days, after which changes in their overall transcriptome were assessed by RNA-seq. (A) Schematic showing hypothesized expression levels (dashed lines) of a Treg-specific gene that is up-regulated with IL-2 in the absence or presence of TGF-β signaling, with proposed changes in transcription level given one of the three proposed scenarios of transcriptional control by Helios and TGF-β. Gene expression that is driven by Helios is shown, with the arrows denoting the corresponding decrease in gene transcription of a key subset of genes during Treg differentiation (Fig. 7A). Two hundred genes were down-regulated in HeliosKO iTreg cells, including genes related to Treg phenotype and function such as CTLA4 and LTBP4 (Fig. 7, D and E). Although we did not observe reduced protein expression of FOXP3 in HeliosKO iTreg cells at day 6 of differentiation (Fig. S14C), we observed decreased FOXP3 transcription across both iTreg populations, suggesting that additional posttranscriptional mechanisms probably regulate FOXP3 expression downstream of Helios. HeliosKO iTreg cells also had reduced expression of NFATC4 (nuclear factor of activated T cell 3) and PPARα (peroxisome proliferator-activated receptor α) transcription factors that regulate the repression of proinflammatory cytokines such as IL-2, IFN-γ, and TNF-α (82, 83), as well as the histone H3K27 demethylase KDM6B Junomji domain-containing protein D3 (JMJD3), which suppresses T1/2 and T H 17 programs (Fig. 7, D and E) (84). Loss of Helios expression also led to the up-regulation of 88 genes, including genes attributed to proinflammatory effector T cell function such as IFNG (Fig. 7, D and E). Together, we propose that Helios could potentially play a role in enhancing fetal Treg differentiation through the transcriptional regulation of a key subset of genes that restrict differentiation toward effector T H 1 cell phenotypes while favoring differentiation toward the Treg cell fate.

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IL-2–iTreg cells (Fig. 7B), with a corresponding increase in IFN-γ transcription in both HeliosKO iTreg populations (Fig. 7, D and E), we next validated these observations by assessing IL-10 and IFN-γ in supernatant during iTreg differentiation. We first confirmed that only fetal iTreg cells produced IL-10 during iTreg differentiation in the absence of TGF-β (Fig. 8A), as observed in our transcriptomic analysis of fetal and adult iTreg cells (Fig. 2B). This is further augmented by exogenous TGF-β (Fig. 8A), which may be due to an increased frequency of Treg cell differentiation (fig. S5B). Fetal iTreg cells also produced less IFN-γ than adult iTreg generated under both stimulation conditions (Fig. 8B). Last, both fetal iTreg cell populations had a greater ratio of IL-10 produced over IFN-γ compared with their adult counterparts, and this effect was enhanced in TGF-β–iTreg cells (Fig. 8C). Ablation of Helios with either gRNA1 or gRNA2 then resulted in a reduction of IL-10 produced in HeliosKO IL-2–iTreg cells (Fig. 8D). We observed a small but sustained decrease in IL-10 across HeliosKO TGF-β–iTreg cells that received gRNA1 but did not detect this decrease in gRNA2 (Fig. 8D), which reflected our RNA-seq results showing a minimal decrease in transcription within Helios KO iTreg for Helios knockout to up-regulate TGF-β–iTreg cells, but the effect was less obvious in IL-2–iTreg cells (Fig. 8E). However, across all differentiation conditions, HeliosKO iTreg cells had a decrease in their IL-10–to–IFN-γ ratio (Fig. 8F), especially in TGF-β–iTreg cells. Overall, this suggests a potential role for Helios in regulating the balance of cytokine output from fetal iTreg cells, which could help prevent proinflammatory responses that can be detrimental in utero.

**DISCUSSION**

The predisposition of human fetal naïve T cells toward Treg cell differentiation presents an opportunity to identify potential underlying cell-intrinsic factors that not only enhance our understanding of Treg differentiation and function but also may ultimately be manipulated to improve in vitro iTreg cell generation for cell-based immunotherapies.
We show here that fetal naïve T cells have a partial Treg-specific transcriptome and epigenome and that fetal-derived iTreg cells retain expression of this transcriptome upon differentiation in vitro. Fetal naïve T cells had increased chromatin accessibility at about a third of all defined Treg-accessible enhancers, but only a small percentage were marked by H3K27ac, including two SEs associated with Helios. This suggests that many Treg-specific enhancers are held in a poised (i.e., accessible), but not active, state in quiescent fetal naïve T cells. Hence, the full acquisition of the Treg-specific epigenetic and, subsequently, transcriptional signature might only occur upon TCR activation and/or cytokine signaling that triggers the final commitment to the Treg cell fate. These data thus implicate a broad landscape of Treg-poised chromatin in fetal naïve T cells that contribute to their propensity for Treg differentiation. It is likely that there is also a contribution of additional histone marks to the overall chromatin landscape. For example, because Helios interacts with other histone-modifying proteins such as the nucleosome remodeling and deacetylase (NuRD) corepressor complex (86, 87), sequencing of additional histone marks such as the repressive H3K27me3 mark would allow us to assess possible repression of genes associated with other effector lineages. Future experiments that identify Helios binding sites by Helios ChIP-seq within fetal naïve T cells will be critical to determine whether Helios is required for the acquisition and/or maintenance of active or suppressive epigenetic marks at key Treg-specific genes. These further analyses would reveal a more complete understanding of the contribution of Helios to the fetal epigenome and how it contributes to the overall Treg cell differentiation phenotype.

Our group and others have shown that the human fetal immune system is skewed toward tolerance by the presence of a large Treg cell population (23, 24), which is likely derived from preferential conversion of fetal naïve T cells into Treg cells upon antigen encounter (24). Here, we show that Helios enhances the preferential differentiation and phenotypic commitment of fetal naïve T cells toward the Treg cell fate. Ablation of Helios expression in fetal naïve T cells impaired their cell-intrinsic predisposition for iTreg differentiation, and reduction in Treg proportions was correlated with the extent of Helios knockout. Although the addition of exogenous TGF-β compensates for loss of Helios during Treg generation, the resulting transcriptional landscape in HeliosKO fetal TGF-β-iTreg cells was still affected. Our results suggest that Helios-mediated transcriptional regulation may potentially play a dual role in fetal Treg differentiation—first, by enhancing Treg differentiation through up-regulation of a set of Treg-specific genes even in the absence of TGF-β and, second, by repressing proinflammatory genes and genes that mediate differentiation toward other effector T cell subsets. This is consistent with the established role of Helios in restraining effector T1 and T17 cell programs within committed Treg cells in mice (41, 42). Although global transcriptional differences observed between HeliosKO and HeliosWT iTreg cells were modest, we propose that the observed enhancement of fetal iTreg differentiation is the result of cumulative effects of up-regulation or down-regulation of genes transcriptionally controlled by Helios. Validation of other gene candidates identified here would further clarify the extent of Helios transcriptional control on the human fetal iTreg differentiation. Last, we demonstrate that Helios expression in fetal iTreg cells has functional consequences because the loss of Helios increased the ratio of proinflammatory (IFN-γ) over anti-inflammatory (IL-10) cytokines by these cells. This is consistent with previous observations that Helios deficiency in mouse Treg cells in vivo does not result in overt distortions in FOXP3 expression or Treg proportions at steady state (88), but rather in the loss of immunosuppressive ability and the manifestation of autoimmunity in later life or in response to inflammatory insults (41, 42). Regulation of cytokine and other proinflammatory genes by Helios could then potentially play a role in maintaining the functional stability of fetal iTreg cells in utero to prevent potentially harmful proinflammatory responses.

Given the nature of this study and the exclusive use of primary human cells, there are important limitations to acknowledge in its interpretation. We primarily compared T cells isolated from fetal spleen and adult peripheral blood for our studies, due to practical difficulties in obtaining adult splenic tissue and fetal peripheral blood. Although we cannot fully rule out immunological differences associated with tissue residence, we have taken steps to reduce confounders resulting from this factor by deriving the epigenetic Treg signature from comparisons of adult naïve and adult Treg cells, thus ensuring that only the Treg signature was the main point of comparison between adult and fetal naïve T cells. We were able to additionally rule out contributions from age-specific differences by selecting genes shared between adult and fetal Treg cells in our RNA-seq analysis to ensure that only genes truly contributing to the Treg cell phenotype were included. Gestational age and, on some occasions when it could be determined, sex were the only demographics available for fetal cells. Hence, we were unable to fully rule out any other potential confounders or stratify our samples accordingly. Furthermore, technical variation due to the kinetics of CRISPR-Cas9 editing, together with the inherent biological variability in primary human samples, manifested in the high sample variability that we observed within our editing experiments. We also observed heterogeneity in Helios expression at steady state in fetal naïve T cells, as well as the extent of Helios up-regulation from sample to sample, which could contribute to differences in sensitivity to Helios disruption and subsequent Treg differentiation. Although we focused here on Helios, additional transcription factors unregulated in fetal naïve T cells, such as Eos (IKZF4), could also play complementary or compensatory roles in fetal iTreg differentiation. We envision that future work will aim to use single-cell RNA-seq or ATAC-seq techniques to separate and identify fetal naïve T cell populations that will explain the observed biological heterogeneity. This will allow us to potentially identify subsets that have greater predisposition toward Treg differentiation and to further characterize the underlying factors leading to this phenotype.

Our work here suggests a key role for Helios in establishing early life peripheral tolerance in humans, and there are some indications to support this hypothesis from studies in neonatal mice. When Helios−/− mice were first generated, the authors reported the presence of significant fatality in the first 2 weeks of neonatal life, and a 100% fatality was observed with subsequent crossovers to achieve a full B6 background (88). This timing coincides with the emergence and migration of functional T cells into the secondary lymphoid organs within the neonatal mouse and is developmentally equivalent to when T cells emerge during the second trimester in humans (21). Furthermore, in mice, thymic Treg cell populations generated in an early window from birth to 10 days later are qualitatively different from those generated later in life (89), indicating that Treg cell populations generated in early life are indispensable to the maintenance of lifelong tolerance. However, these studies did not examine the potential contributions of peripheral Treg differentiation from naïve T cells, which is likely an important factor contributing to the dominant
tendency toward tolerance observed in the human fetus. This is particularly significant because there is an increased frequency of rapidly proliferating CD4+ and CD8+CD25- human fetal T cell populations (23) potentially bearing autoreactive TCRs that have escaped thymic deletion as observed in neonatal mice (90). In light of our data presented here, we thus speculate that Helios plays a previously unappreciated role in the generation of peripheral Treg cells in this critical period in early human fetal development where the need for peripheral tolerance is perhaps the most acute.

In vitro human Treg cell differentiation from naive T cells for therapeutic purposes has encountered significant roadblocks due to difficulties in generating pure populations of Treg cells that maintain a stable phenotype over time (70–72). Previous studies have shown that Helios+, but not Helios−, ex vivo Treg cells retain a more highly demethylated TSDR when expanded in vitro (91, 92). We did not observe TSDR demethylation in Helios+ fetal iTreg cells, likely reflecting the inability of in vitro differentiation to capture the conditions or environmental factors that trigger TSDR demethylation. Regardless, we demonstrate that fetal iTreg cells have greater phenotypic resemblance to ex vivo Treg cells in that they retain the expression of Helios, have diminished IL-2 production upon restimulation, and produce IL-10 upon Treg differentiation. These attributes are commonly associated with the Treg cell phenotype but are not acquired in adult human iTreg cell populations. Last, our data show that Helios enhances the expression of genes in parallel with, in addition to, and independently of TGF-β signaling, which favor Treg over effector T17 commitment. This may represent a mechanism by which Helios maintains stable Treg function and identity in vivo (41, 42, 66, 74, 76). Additional studies are thus required to assess this possibility, particularly in proinflammatory environments, and whether manipulation of Helios expression within adult naive T cells can recapture this effect. Further identification of upstream factors in addition to Helios that contribute to the acquisition of the permissive enhancer landscape in fetal naive T cells will likely provide important deeper insight into their predisposition toward Treg differentiation. These findings may then ultimately inform strategies for the generation of stable iTreg cells for use in immunotherapy to establish tolerance in autoimmunity and transplantation.

MATERIALS AND METHODS

Study design

The objective of this study was to determine the molecular mechanisms that underlie preferential Treg cell differentiation in human fetal naive T cells. As such, primary human CD4+ naive T and Treg cells from fetal spleen and adult peripheral blood mononuclear cells (PBMCs) were the primary cell sources used for this study. Transcriptional and epigenetic profiling was carried out using RNA-seq, H3K27ac ChIP-seq, and ATAC-seq. Flow cytometry was used to confirm observations from sequencing datasets, to validate expression levels at baseline, and to assess the activation and subsequent differentiation of fetal and adult naive T cells in vitro. CRISPR-Cas9–mediated knockout was used to confirm the results of the observational studies. The sample size (n = 2 to 6 per experiment) for the sequencing datasets was determined to be the optimal size for statistical analysis and to allow for independent replicates, given the scarcity of cells isolated from each fetal sample. The sample size and experimental replicates for in vitro experiments are subsequently indicated in all accompanying figure legends or supplementary tables. Sample size was determined to be adequate on the basis of the magnitude and consistency of measurable differences between groups. Investigators were not blinded, and samples were equally divided between treatments, i.e., gRNA received. Further details on dataset analysis and experimental technique are detailed in Supplementary Materials and Methods.

Magnetic isolation of CD4+ T cells and fluorescence-activated cell sorting for sequencing and cell culture

Before fluorescence-activated cell sorting (FACS), fetal splenocytes and adult PBMCs were pre-enriched for CD4+ T cells using the EasySep Human CD4+ T Cell Isolation Kit (#17952, STEMCELL Technologies). To obtain sufficient numbers of adult Treg cells for epigenetic analyses, adult PBMCs were also pre-enriched for CD4+CD127low T cells using the Human CD4+CD127low T Cell Pre-enrichment Kit (#19231, STEMCELL Technologies). Enriched cell fractions were incubated in FACS staining buffer [phosphate-buffered saline (PBS) with 2% heat-inactivated fetal bovine serum (HI-FBS) and 2 mM EDTA] with fluorochrome-conjugated anti-human surface monoclonal antibodies (mAbs): CD25 fluorescein isothiocyanate (2A3, BD Biosciences), CD127 phycoerythrin (PE)/BV421 (hIL-7R-M21, BD Biosciences), CD45RA PE-CF594 (HI100, BD Horizon), CD4 PE-Cy7 (SK3, BD Biosciences), and CD27 eFluor780 (O323, Thermo Fisher Scientific). All cells were stained with the live/dead marker (Ghost Dye Violet 510, Tonbo Biosciences) to exclude dead cells. Cells were sorted into supplemented RPMI-1640 [10% HI-FBS, 1-glutamine (300 mg/liter), penicillin (10 U/ml), and streptomycin (10 μg/ml)] based on gating in fig. S1. All sorts were carried out on a BD FACSAria III.

RNA-seq for ex vivo sorted cells

RNA-seq was carried out for 2.5 × 10⁶ cells for four biological replicates of adult and fetal naive and Treg cells. RNA was extracted and purified with the NucleoSpin RNA Kit (MACHERY-NAGEL) and assessed for quality by RNA Pico (Agilent Technologies). Library preparation and sequencing on a HiSeq 3000 were carried out by the Technology Center for Genomics and Bioinformatics (TCGB) core at University of California, Los Angeles.

RNA-seq for adult and fetal iTreg populations

Four biological replicates of sorted adult or fetal naive T cells were stimulated in U-bottomed 96 wells with 5 μl of ImmunoCult Human T cell Activator (CD3/CD28/CD2 tetrancers, STEMCELL Technologies) in 200 μl of culture media [RPMI 1640 supplemented with 10% HI-FBS, L-glutamine (300 mg/liter), penicillin (10 U/ml), streptomycin (10 μg/ml), 10 mM Hepes, 1× minimum essential medium–nonessential amino acids, and β-mercaptoethanol], Exogenous IL-2 (10 ng/ml; PeproTech) and TGF-β (50 ng/ml; PeproTech) were added according to the relevant experimental setup. One hundred microliters of media changes were performed every 2 days starting from the first day of stimulation. At day 6 after induction, RNA from 2.5 × 10⁵ iTreg cells was extracted and purified with the NucleoSpin XS RNA Kit (MACHERY-NAGEL) and assessed for quality by RNA Pico (Agilent Technologies). Ribosomal RNA was removed using the NEBNext Poly(A) mRNA Magnetic Isolation Module (New England Biolabs), and library preparation was carried out using the NEBNext Ultra II Directional RNA Library Prep Kit for Illumina (New England Biolabs). Sequencing on a HiSeq 4000
Flow cytometry staining in sorted naïve and Treg cells
Sorted naïve and Treg cells from fetal and adult samples were incubated in FACS buffer with fluorochrome-conjugated, anti-human surface mAbs. Fixation and permeabilization were performed using the Foxp3/Transcription Factor Staining Buffer Set (Tonbo Biosciences). All cells were stained with a live/dead marker (GhostDye Violet 510, Tonbo Biosciences) to exclude dead cells from analysis. All data were acquired with an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo (BD Biosciences) software. Helios+ and Helios- gates were set on the basis of the clearly positive and negative populations seen in adult Treg samples; all adult naïve T cell samples were observed to be fully within the Helios+ population and were subsequently used to define the Helios- gate for subsequent experiments as a biological negative control.

Treg induction assays
Sorted adult or fetal naïve T cells were differentiated into Treg cells as detailed above according to the relevant experimental setup. For Treg induction with TGF-β blockade, neutralizing anti-TGF-β (2.5 or 0.5 μg/ml; 1D11, R&D Systems) antibody was added to the culture medium where specified. Cells were cultured and harvested after 1, 3, and 5 days for analysis by flow cytometry. Media changes (100 μl) were performed every 2 days starting from the first day of stimulation. Cells were stained, fixed, and permeabilized as detailed in flow cytometry staining. All data were acquired with an LSR II flow cytometer (BD Biosciences) and analyzed with FlowJo (BD Biosciences) software.

CRISPR-Cas9 editing of the Helios locus
CRISPR-Cas9 editing was carried out as previously detailed (93, 94). Cas9 gRNA ribonucleoproteins (RNPs) were assembled before experiments by assembling the gRNAs through incubating 160 μM CRISPR RNA (crRNA) with 160 μM trans-activating crRNA (tracrRNA) (Dharmacon) at a 1:1 ratio for 30 min at 37°C to a final concentration of 80 μM. Assembled gRNAs were then subsequently incubated with 40 μM Cas9-nuclear localization sequence (NLS) (QB3 MacroLab, University of California, Berkeley) at a 1:1 ratio for 15 min at 37°C for a final concentration of 20 μM RNPs. Nucleofection was performed using the Amaxa P3 Primary Cell 96-well Nucleofector Kit and 4D-Nucleofector (Lonza). A total of 1 × 10⁵ infection was performed using the Amaxa P3 Primary Cell 96-well plate precoated with anti-CD3 antibodies (1 μg/ml; HIT3a, BD Biosciences) and supplemented with soluble anti-CD28 antibodies (2 μg/ml; CD28.2, BD Biosciences) and IL-2 (10 ng/ml; PeproTech). Stimulated fetal naïve T cells were washed with PBS and resuspended in 20 μl of P3 solution. Five microliters of the final 20 μM RNP solution was added, along with 1 μl of 100 μM homology directed repair template (HDR) solution. Cells were gently mixed and transferred to the 96-well shuttle device. Cells were electroporated using program EH-115 on the Amava 4D-Nucleofector (Lonza). Eighty microliters of prewarmed culture media was added immediately after nucleofection, and cells were allowed to recover for 15 min at 37°C. Nucleofected cells were then transferred into a 96-well U-bottom plate, and additional culture media were added to a final volume of 200 μl. After 5 hours, cells were spun down, and 150 μl of the culture media was changed to increase cell viability and left to incubate overnight. Cells were stimulated the next day for Treg induction assays as detailed above and harvested at day 6 for analysis.

RNA-seq for HeliosKO and HeliosWT Treg populations
CRISPR-Cas9–mediated Helios knockout in six biological replicates of fetal naïve T cells was performed as described above using Helios gRNA1 or the NT control guide for (all samples paired across nucleasefection conditions). Cells were stimulated for Treg induction assays as detailed above, and 2.5 × 10⁴ iTreg cells were harvested at day 6 for RNA extraction and analysis. RNA extraction, library preparation, and sequencing were carried out as for fetal and adult iTreg populations.

Cytokine bead assays
One hundred microliters of culture supernatant was collected at days 3 and 5 of Treg differentiation with IL-2 only or in the presence of added TGF-β. Supernatants collected at day 5 of Treg differentiation after CRISPR-Cas9–mediated Helios knockout were also analyzed. Supernatants were harvested and stored at −80°C before analysis. IL-10 (#558274) and IFN-γ (#558269) concentrations were measured using the Cytokine Bead Assay Flex Kits (BD Biosciences).

Statistical analyses
All statistical analyses were performed using R (cran.r-project.org, v3.5.2) and Bioconductor (www.bioconductor.org). Tests were specified with each experiment in each figure legend, and P < 0.05 level of confidence was accepted for statistical significance. All statistical tests are nonparametric and two-sided unless mentioned. Paired testing was used when comparisons were made within the same sample to increase resistance toward outlier effects and are specified in the figure legends. Kruskal-Wallis test and Dunn’s posttest with Bonferroni correction were performed using the PMCMRplus (v1.4.1) package. Graphs were made using the ggplot2 (v3.1.0), ggrepel (v0.8.0), and cowplot (v0.9.4) R packages. Heatmaps were generated with the ComplexHeatmap (v1.12.0) R package.

SUPPLEMENTARY MATERIALS
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Materials and Methods
Fig. S1. Gating strategy and purity assessment for sorted naïve and Treg cells.
Fig. S2. Definition of the Treg transcriptional signature.
Fig. S3. Assessing the enrichment of Treg up-regulated or down-regulated genes in fetal and adult Treg populations.
Fig. S4. Fetal iTreg cells have increased sensitivity to TGF-β signaling.
Fig. S5. Identification of Treg-accessible and -inaccessible enhancers.
Fig. S6. Binding motifs for downstream effectors of Treg differentiation are enriched within shared Treg-accessible peaks in fetal naïve T cells.
Fig. S7. The highest ranked SEs shared across all cell populations are associated with T cell development and function.
Fig. S8. Chromatin accessibility and H3K27ac enrichment at the Helios locus in fetal naïve T cells correlate with increased RNA and protein expression.
Fig. S9. Fetal naïve T cells do not have an increased proportion of CD31+ cells relative to adult naïve T cells.
Fig. S10. A fraction of fetal naïve T cells are highly proliferative.
Fig. S11. Fetal naïve T cells do not have demethylation at the FOXP3 conserved noncoding sequence 2 TSDR.
Fig. S12. Fetal naïve T cells up-regulate Helios during Treg induction.
Fig. S13. Validation of CRISPR-Cas9 editing at the Helios locus.
Fig. S14. The effect of CRISPR-Cas9 knockout of Helios on protein expression of Treg functional markers is variable.
Fig. S15. Fetal but not adult, iTreg cells have suppressed IL-2 production after restimulation.
Fig. S16. Helios knockout in fetal iTreg cells result in a subtle shift in the underlying transcriptome.
REFERENCES AND NOTES


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Competing interests: A.M. is a co-founder of Spotlight Therapeutics, A.M., T.L.R., and T.D.B. are co-founders of Arsenal Biosciences. A.M. serves on the scientific advisory board of PACT Pharma, is an advisor to Trizell, and previously served as an advisor to Juno Therapeutics. A.M. has had speaking engagements with or done consulting for Amgen, Thermo Fisher Scientific, Health Advances, Lonza, Bernstein, AbbVie, Genentech, Merck, Illumina, Arcus, the Jackson Laboratory, Nanostring Technologies, GLG, RCM, Analytical, Life Science & Diagnostics Association (ALDA), and Soteria. The Marson laboratory has received sponsored research support from Juno Therapeutics, Epinomics, and Sanofi and a gift from Gilead Biosciences. The other authors declare that they have no competing interests.

Data and materials availability: ATAC-seq, H3K27ac Chip-seq, and all RNA-seq raw and processed files are deposited in the Gene Expression Omnibus database under a superseries entry with the accession number GSE110472. The transcriptional and epigenetic gene lists defined in this manuscript are supplied in supplementary tables as described. All other data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials.

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Helios enhances the preferential differentiation of human fetal CD4⁺ naïve T cells into regulatory T cells
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Helios lights the way to immunotolerance
Fetal naïve T cells are more apt than their adult counterparts to differentiate into regulatory T (T\textsubscript{reg}) cells after stimulation through the T cell receptor. Ng \textit{et al.} investigated the origin of this bias using transcriptional and epigenetic profiling to compare fetal and adult naïve T cells with induced T\textsubscript{reg} cells. Expression of the T\textsubscript{reg}-associated transcription factor Helios by most fetal naïve T cells, but not adult naïve T cells, suggested that Helios might have a key role. CRISPR-Cas9-mediated deletion of Helios in fetal naïve T cells impaired their ability to differentiate into induced T\textsubscript{reg} cells in the absence of exogenous TGF-β. These findings lay the groundwork for development of new translational approaches to coax adult naïve T cells to differentiate into stable T\textsubscript{reg} cells.

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