The immune compartment at the maternal-fetal interface throughout human pregnancy

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Chapter 02

Three types of functional regulatory T cells control T cell responses at the human maternal-fetal interface

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Summary

During pregnancy, maternal regulatory T cells (Tregs) are important in establishing immune tolerance to invading fetal extravillous trophoblasts (EVTs). $CD25^{HI}FOXP3+$ Tregs are found at high levels in decidual tissues and have been shown to suppress fetus-specific and non-specific responses. However, limited data are available on additional decidual Treg types and the mechanisms by which they are induced. This study investigated three distinct decidual CD4+ Treg types in healthy pregnancies with a regulatory phenotype and the ability to suppress T cell responses: $CD25^{HI}FOXP3+$, $PD1^{HI}IL\text{-}10+$, and $TIGIT+ FOXP3^{dim}$. Moreover, co-culture of HLA-G+ EVTs or decidual macrophages with blood CD4+ T cells directly increased the proportions of $CD25^{HI}FOXP3+$ Tregs compared to T cells cultured alone. EVTs also increased $PD1^{HI}$ Tregs that could be inhibited by HLA-C and CD3 antibodies, suggesting an antigen-specific induction. The presence of distinct Treg types may allow for the modulation of a variety of inflammatory responses in the placenta.

Introduction

During pregnancy, CD4+CD25^{HI}FOXP3+ regulatory T cells (Tregs) are found at high levels in decidual tissue and have the ability to suppress fetus-specific and non-specific responses (1, 2). Most interestingly, HLA-C mismatched pregnancies (where the fetus and extravillous trophoblasts (EVTs) express an HLA-C allotype that the mother does not have) had increased levels of functional CD4+CD25^{HI} Tregs in decidua, compared to HLA-C matched pregnancies (3). Furthermore, *in vitro* co-culture of naive CD4+ T cells with EVTs directly increased the proportion of CD4+FOXP3+ Tregs, compared to CD4+ T cells cultured alone (4, 5, 6). This suggests that maternal T cells may specifically recognize fetal HLA-C, but its expression on EVTs promotes immune tolerance. The importance of maternal immune tolerance for fetal HLA-C is further illustrated by a recent study suggesting that HLA-C antibodies may contribute to the etiology of miscarriage (7). The proportion of circulating FOXP3+ Tregs was shown to be diminished in maternal blood obtained after spontaneous preterm birth (SPTB) (8, 9, 10), preeclampsia (PE) (11, 12) and in decidual tissue obtained after recurrent spontaneous miscarriage (13, 14). Furthermore, clonally expanded CD4+ CD25^{HI}CD127–CD45RA– Treg populations were observed in healthy term pregnancy decidua, and failure of this clonal expansion may be related to development of preeclampsia (15). The importance of Tregs was also demonstrated in murine pregnancy models (16, 17, 18, 19). Depletion of CD25+ Tregs during allogeneic matings, but not syngeneic matings, resulted in an increased resorption rate (16). Besides highlighting the role for Tregs, this also demonstrated that in the absence of Tregs, effector cells cause immunologic rejection of allogeneic fetal or placental tissues. A more recent murine study demonstrated that FOXP3+ Tregs with specificity to paternal antigens were generated extrathymically and accumulated in the placenta. In this study, females with impaired ability for extrathymic Treg induction showed increased fetal resorption rates and had increased influx of immune cells to the placenta in allogeneic matings, but not syngeneic matings (20). Other pathways and molecules have been demonstrated to play a role in Treg induction during pregnancy, including the blockade of the PD1-PDL1 pathway that led to reduced decidual CD25+FOXP3+ Treg numbers and increased embryo resorption in mice, which could be abrogated by adoptive CD25+FOXP3+ Treg transfer (21). This demonstrates the importance of PD1-PDL1 for decidual Treg induction. However, specific factors that contribute to diminished decidual Treg numbers or function during pregnancy complications have not been identified in human pregnancy.

Thus far, research on the role of Tregs in human pregnancy has mainly focused on FOXP3+ Tregs (22, 13, 14, 8, 9, 23, 25, 10, 2,15), while other types of FOXP3- Tregs have not been studied in as much detail. Most importantly, only a handful of studies provide functional analysis of peripheral blood (11, 12) and decidual Tregs (2, 3) during human pregnancy. FOXP3, in combination with high expression of CD25 and HELIOS and the absence of

CD127 expression, primarily identifies natural Tregs (nTregs), although whether HELIOS is a defining marker for human nTregs remains controversial. The nTregs are generated in the thymus, are specific for self-antigens, and are responsible for preventing anti-self (autoimmune) responses (26, 27). In contrast, induced Tregs (iTregs) are generated in the periphery and can be specific for a large variety of antigens, including allo-antigens and viral-antigens (28, 29, 30). A well-characterized type of iTregs are Tr1 cells that secrete high levels of IL-10; express PD1; co-express CD49b and LAG3, but do not express FOXP3; and are important in the control of alloimmune responses (31, 32). Other iTregs include TIGIT+ cells that modulate antigen-presenting cells (APC) through interaction with CD155 on APCs and Tr35 cells that function through secretion of IL-35, an immune suppressive cytokine (33, 34). A large variety of other markers have been used to identify distinct iTreg populations (including but not limited to FOXP3, CD25, GITR, TIM3, CD39, LRRC32 (also known as GARP), LAP, and CCR8) (35, 36, 37, 38). None of these markers are truly specific for iTregs, as they can also be expressed on activated T cells. Thus, to identify iTregs, functional assays are required to demonstrate their capacity to suppress immune responses such as proliferation, cytokine secretion, and cytotoxicity (39, 30). In this study, we provide extensive phenotypic and functional characterization of three types of decidual CD4+ Tregs in uncomplicated human pregnancies and investigate the ability of HLA-G+ HLA-C+ EVTs and decidual macrophages, the main APCs at the maternal-fetal interface, to increase Treg proportions.

Results

Distinct CD4+ T cell types with a regulatory phenotype are present in decidual tissue

FACS analysis on freshly isolated peripheral blood CD4+ T cells (CD4+ pTs) and decidual CD4+ T cells (CD4+ dTs) isolated from first-trimester decidua (gestational age 6–12 weeks) and term placenta decidua basalis (d.basalis) and decidua parietalis (d.parietalis) (gestational age > 37 weeks) was performed to determine cell surface expression of CD45, CD4, CD25, PD1, TIGIT, CD127, CD45RA, CD49b, and LAG3 and intracellular expression of FOXP3 and HELIOS. A clear population of activated nTregs was identified in all tissues based on the high expression of CD25, FOXP3, and HELIOS and the lack of CD45RA and CD127 (Fig. 1; Fig. S1A-S1D) (26). While the percentage of FOXP3+ and HELIOS+ cells within this $CD25^{HI}$ population significantly decreased in term pregnancy decidua, the proportion of HELIOS+ cells within $CD25^{HI}$FOXP3+ cells remained relatively stable (Fig. 1D, right panel). A second T cell population was identified based on the high expression of PD1, the lack of FOXP3 and HELIOS, and low CD25. LAG3 and CD49b were not expressed by CD4+ dTs. While both $CD25^{HI}$ and $PD1^{HI}$ cells co-expressed high levels of TIGIT, a third population of CD4+ dTs also expressed high levels of TIGIT and low levels of FOXP3, HELIOS, PD1, and CD25 (Fig. 1; Fig. S1A-S1D). FOXP3 expression in TIGIT+ cells was significantly lower than in $CD25^{HI}$ cells (Fig. S1E). The t-Distributed Stochastic Neighbor Embedding (t-SNE) analysis confirmed the separation of these three T cell

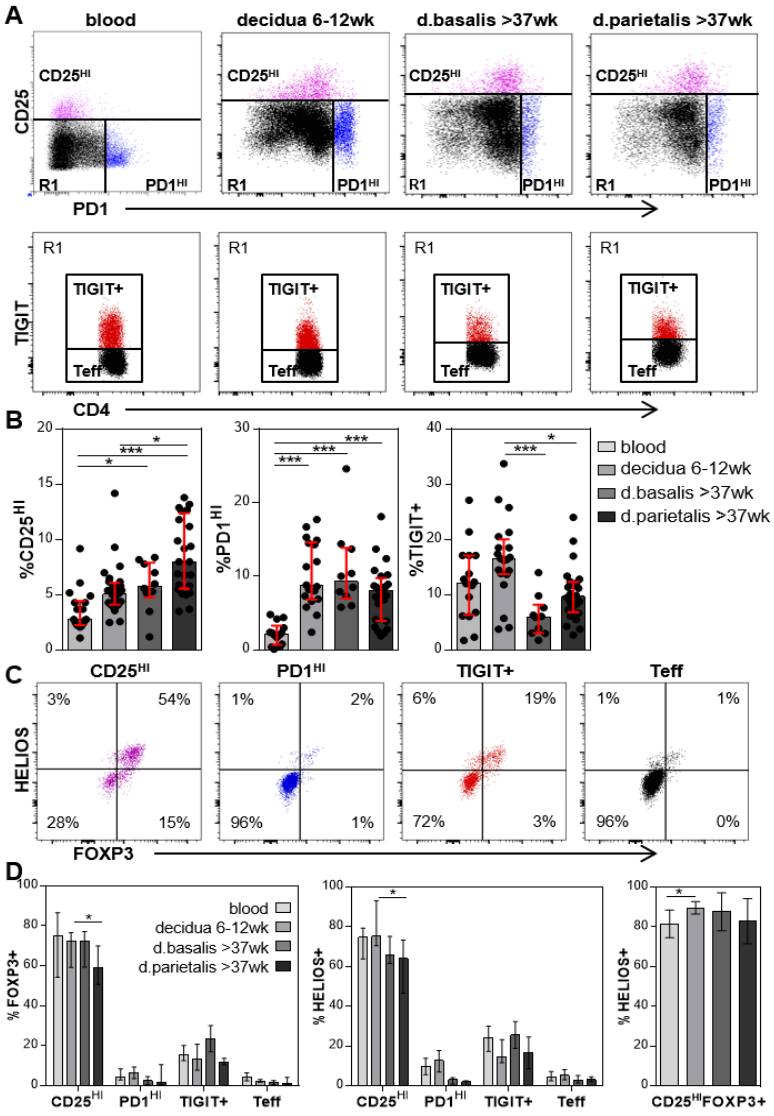

Figure 1. Three distinct CD4+ T cell populations express Treg-associated markers.
(A and B) Representative FACS plots (A) and percentages (B) of CD25^{HI}, PD1^{HI}, and TIGIT+ cells within CD4+ T cells in blood, decidua 6–12 weeks, d.basalis >37 weeks, and d.parietalis >37 weeks. (C and D) Representative FACS plots (C) and percentages (D) of FOXP3+ and HELIOS+ cells within the four CD4+ T cell types and HELIOS+ cells within CD25^{HI}FOXP3+ cells in blood, decidua 6–12 weeks, d.basalis >37 weeks, and d.parietalis >37 weeks (n = 8–10). Bars represent median and interquartile range; *p < 0.05, **p < 0.01 and ***p < 0.001.
See also Figures S1-S3.

populations (Fig. S2) that hereafter will be named (1) $CD25^{HI}$FOXP3+, (2) $PD1^{HI}$, and (3) TIGIT+. These three CD4+ T cell types were purified by FACS sort and analyzed for their ability to produce the pro- and anti-inflammatory cytokines IFNɣ, IL-2, and IL-10 upon phorbol 12-myristate 13-acetate (PMA)/Ionomycin stimulation. $PD1^{HI}$ T cells from all tissues expressed the highest levels of IL-10 and coexpressed IFNɣ (Fig. S3), suggesting a resemblance to Tr1 cells (31). $CD25^{HI}$FOXP3+ cells of all tissue compartments expressed the lowest levels of IL-10, IFNɣ, and IL-2, whereas TIGIT+ cells expressed high levels of IFNɣ and IL-2 and low levels of IL-10. A limitation of this study is the lack of clinical information on the blood and tissues used for experiments that may have impacts on Treg phenotypes and contribute to the observed variation in Treg proportions. However, a previous report did not find an influence of clinical variables such as mode of delivery and fetal sex on the presence of CD4+ CD25dim activated T cells and CD4+$CD25^{HI}$FOXP3+ Tregs in decidual tissues of term pregnancy (3).

$CD25^{HI}$FOXP3+, $PD1^{HI}$, and TIGIT+ suppress proliferation of CD4+ and CD8+ Teff cells

To determine the capacity of $CD25^{HI}$FOXP3+, $PD1^{HI}$, and TIGIT+ T cells to suppress proliferation of effector T cells (Teffs), carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4+ or CD8+ Teffs were stimulated with anti-CD3 and -CD28 beads in the presence or absence of sample-matched Tregs for 4 days in a 1:2 Treg to Teff ratio. CFSE profiles were analyzed to determine the percentage of undivided cell (generation 0) and the average number of divisions per cell (division index) within the CD4+ and CD8+ Teffs cultured with and without Tregs (Fig. 2A). All three CD4+ Treg types in decidual samples of 6–12 weeks suppressed proliferation of CD4+ Teffs. This is illustrated by the significant increase in the percentage of undivided cells (Fig. 2B), and a significant decrease of the division index (Fig. 2E) upon co-culture of Tregs and Teffs, compared to stimulated Teff cultured alone. Additionally, purified $PD1^{dim}$ cells from 6-12 week decidual tissues did not suppress proliferation of CD4+ T cells (Fig. S4A). $CD25^{HI}$ FOXP3+ T cells also suppressed proliferation of sample-matched CD8+ Teffs, whereas $PD1^{HI}$ and TIGIT+ cells did not consistently reduce CD8+ Teff proliferation (Fig. 2C and 2F). Analysis of the three T cell populations from term placenta d.parietalis demonstrated that these cells had a reduced capacity to suppress CD4+ Teff proliferation, compared to their first-trimester counterparts (Fig. 2D and 2G), whereas peripheral blood $CD25^{HI}$ and $PD1^{HI}$, but not TIGIT+ cells, suppressed proliferation of CD8+ Teffs (Fig. S4B-S4D). To determine if IL-10 secretion by $PD1^{HI}$ Tregs is their predominant mechanism to suppress proliferation of CD4+ Teffs, as was previously shown for Tr1 cells (31, 32), anti-IL-10R blocking antibodies or immunoglobulin G (IgG) control antibodies were added to anti-CD3 and -CD28 stimulated CFSE labeled CD4+ Teffs cultured alone or with sample-matched $CD25^{HI}$ or $PD1^{HI}$ cells. Indeed, addition of IL-10R antibodies abrogated the suppressive capacity of $PD1^{HI}$ cells but not of the $CD25^{HI}$ cells that were added in parallel control cultures (Fig. 2H).

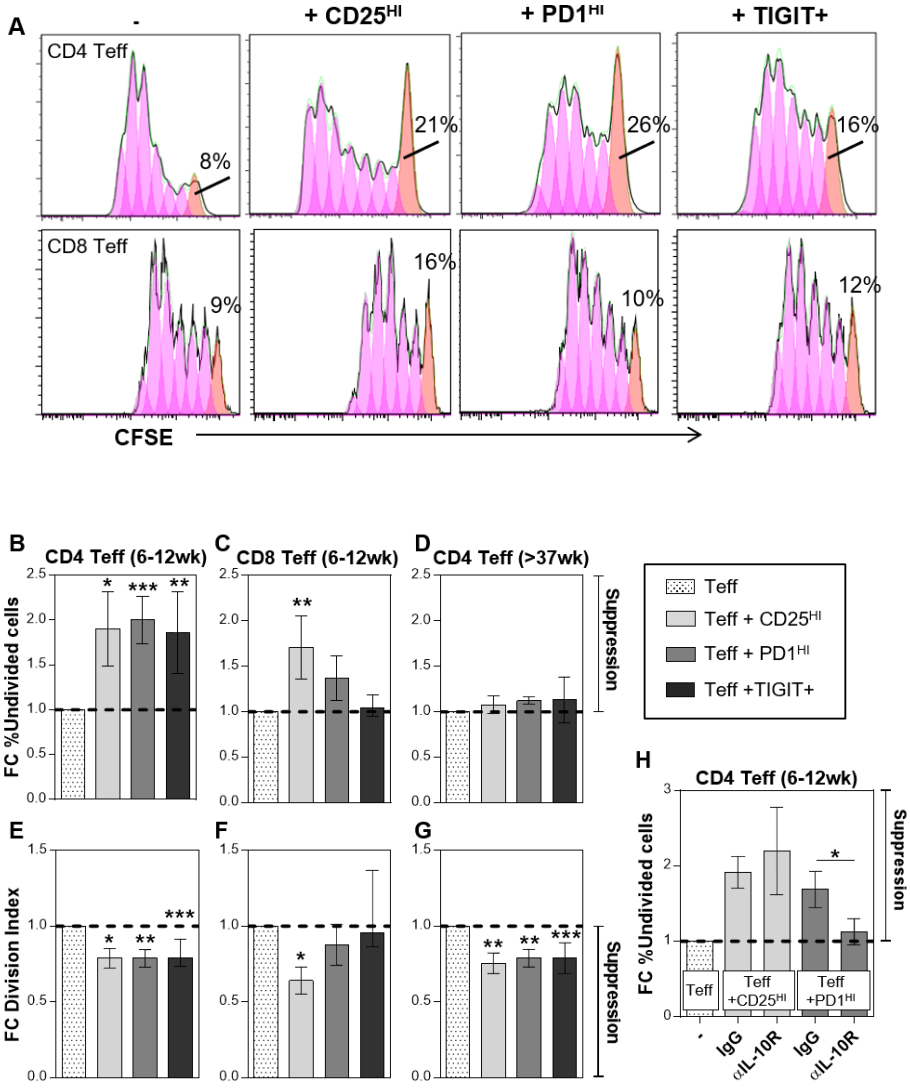

Figure 2. Decidual Tregs suppress proliferation of Teffs.

(A) CFSE dilution of 6-12-week decidual CD4+ Teffs (upper panel) and CD8+ Teffs (lower panel) stimulated with anti-CD3/28 and co-cultured with or without CD25HI, PD1HI, or TIGIT+ Tregs during 4 days in a 1:2, Treg: Teff ratio. Percentages of the undivided Teffs are shown. (B–G) Fold change (FC) in the percentage of undivided cells (B–D) and in the division index (E–G) after the addition of CD25^{HI}, PD1^{HI}, or TIGIT+ Tregs, compared to CD4+ Teffs 6–12 weeks (B and E), CD8+ Teffs 6–12 weeks (C and F), and CD4+ Teffs >37 weeks (D and G) cultured alone. (H) Addition of IL-10R blocking antibodies to Teffs +PD1^{HI} but not Teffs + CD25^{HI} cultures inhibit suppression of proliferation, measured by FC in percentage of undivided cells, compared to isotype control cultures. Bars represent median and interquartile range; In (B)–(G): n = 7–11; (H): n = 3–4; *p < 0.05, **p < 0.01 and ***p < 0.001; values > 1 for FC % undivided cells and < 1 for FC division index represent suppression of proliferation. Division index reflects the number of divisions per cell as calculated in FlowJO v7.6.5. See also Figure S4.

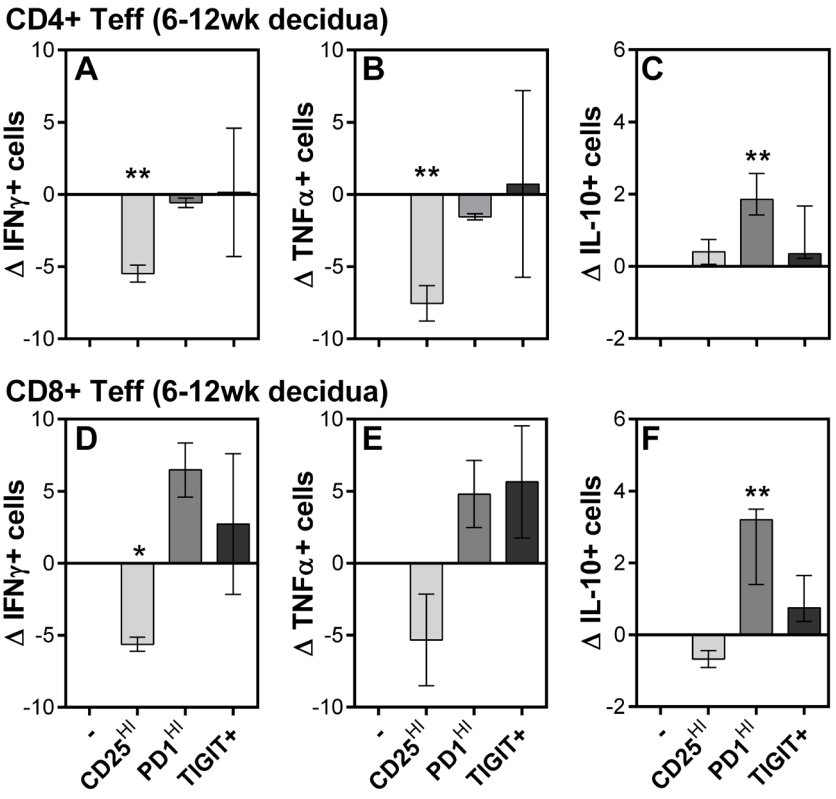

Figure 3. Decidual Tregs influence cytokine production by CD4+ and CD8+ Teffs.
(A–F) Graphs depict absolute change (D) in percentage of IFN-ɣ+ (A and D), TNF-α+ (B and E), and IL-10+ (C and F) decidual CD4+ Teffs (top panels) and decidual CD8+ Teffs (bottom panels) upon addition of sample-matched $CD25^{HI}$, $PD1^{HI}$, or TIGIT+ T cells, compared to CD4+ Teffs and CD8+ Teffs cultured alone. Bars represent median and interquartile range; n = 4–5; *p < 0.05, **p < 0.01. See also Figure S5.

$CD25^{HI}$FOXP3+ and $PD1^{HI}$ Tregs influence cytokine production of CD4+ and CD8+ Teffs

Next, the capacity of decidual $CD25^{HI}$FOXP3+, $PD1^{HI}$, and TIGIT+ Tregs to influence production of pro- and anti-inflammatory cytokines by CD4+ and CD8+ Teffs was investigated. CFSE-labeled CD4+ Teffs and CD45-Alexa700 labeled CD8+ Teffs were cultured with or without sample matched Tregs in a 1:1:1 CD4 Teff: CD8 Teff: Treg ratio and stimulated with anti-CD3 and -CD28. At day three, the cells were restimulated with PMA and Ionomycin for 6h in the presence of GolgiStop for the last 4h. CD4+ Teffs, CD8+ Teffs, and Tregs were identified (Fig. S5A) and analyzed for the presence of intracellular cytokines IFN-ɣ, TNF-α, and IL-10 (Fig. S5B). Addition of $CD25^{HI}$ cells significantly decreased the percentage of IFN-ɣ+ and TNF-α+ CD4+ and IFN-ɣ+ CD8+ Teffs (Fig. 3A, 3B, and 3D). Addition of $PD1^{HI}$ cells significantly increased the percentage of IL-10+ CD4+ and CD8+ Teffs (Fig. 3C and 3F) but also increased the percentage of IFN-ɣ + CD8+ Teffs (Fig. 3D).

TIGIT+ cells did not consistently change the production of cytokines by CD4+ or CD8+ Teffs (Fig. 3). Analysis of cytokine production by Tregs themselves confirmed the overall low expression of cytokines by $CD25^{HI}$ cells and high expression of IL-10 by $PD1^{HI}$ cells (Fig. S5C). Thus, besides suppressing Teff proliferation, both $CD25^{HI}$ and $PD1^{HI}$ Tregs also modulated the production of cytokines by Teffs.

$CD25^{HI}$FOXP3+, $PD1^{HI}$, and TIGIT+ Tregs from decidua and blood express distinct transcriptional profiles

To further investigate the molecular mechanisms the three Treg types may utilize to modulate T cell proliferation and cytokine production, RNA was isolated from $CD25^{HI}$ FOXP3+, $PD1^{HI}$, and TIGIT+ Tregs purified from peripheral blood, first-trimester decidua (6–12 weeks), and term pregnancy d.parietalis (>37 weeks). The BioMark Fluidigm 96x96 QPCR chip was used to detect gene expression. Sixty-six primer pairs had detectable CT values and melting curves (gene and primer list are included in Table S1). CT values were normalized against GAPDH (ΔCT), and the fold change (FC) was calculated relative to the median ΔCT of the blood CD4+ Teffs. Expression levels of CD25, FOXP3, PD1, and TIGIT confirmed the purity of the Treg fractions (Fig. S6A). K-means cluster analysis of gene expression in the four T cell populations visualized two separate gene clusters in blood and decidua. Cluster I identified a set of genes, including CD25, FOXP3, TIGIT, CD39, LRRC32 (GARP), ST2, BATF, and CCR8, which are highly expressed by $CD25^{HI}$ FOXP3+, low in $PD1^{HI}$, and intermediate in TIGIT+Tregs (Fig. 4A). Cluster II identified a set of genes, including PD1, IFNɣ, IL-10, CCR5, and CXCR3, that are upregulated by $PD1^{HI}$ Tregs (Fig. 4B). Heatmaps depicting the differentially expressed genes identified in these clusters are shown (Fig. 4C-4E). These data further support that $CD25^{HI}$ FOXP3+, $PD1^{HI}$, and TIGIT+ are separate Treg types that may utilize distinct molecular mechanisms of immune modulation. Additional K-means cluster analysis revealed several key regulatory genes that differ throughout gestation and between decidual and blood Tregs (Fig. S6B and S6C). Decidual $CD25^{HI}$FOXP3+ Tregs show increased expression of CCR5, ST2, CD25, BATF, IL10, GITR, LRRC32 (GARP), and CCR8, compared to blood $CD25^{HI}$FOXP3+ Tregs, while decidual $PD1^{HI}$ Tregs had increased expression of IL-10, IFNɣ, and CCR5, compared to blood $PD1^{HI}$ Tregs. Interestingly, first trimester decidual TIGIT+ Tregs increased expression of IL-10, IFNɣ, LRRC32, and GZMA, while term pregnancy TIGIT+ Tregs had increased expression of, for example, VEGFA, IFNɣ, GITR, and CD39 (Fig. S6B and S6C). Flow cytometric analysis of freshly isolated Tregs from all tissues confirmed differential protein expression of key mRNAs identified here (Fig. S7). The increased expression of a variety of cytokines (e.g., IL-10, IFNɣ), chemokines (e.g., CCR5, CCR8), activation markers (e.g., HLA-DR, GZMA), and co-inhibitory genes and molecules (e.g., CD39, TIM3, LRRC32) on decidual Tregs may suggest increased Treg activation and suppressive function in decidual tissue to regulate inflammatory responses at the maternal fetal interface.

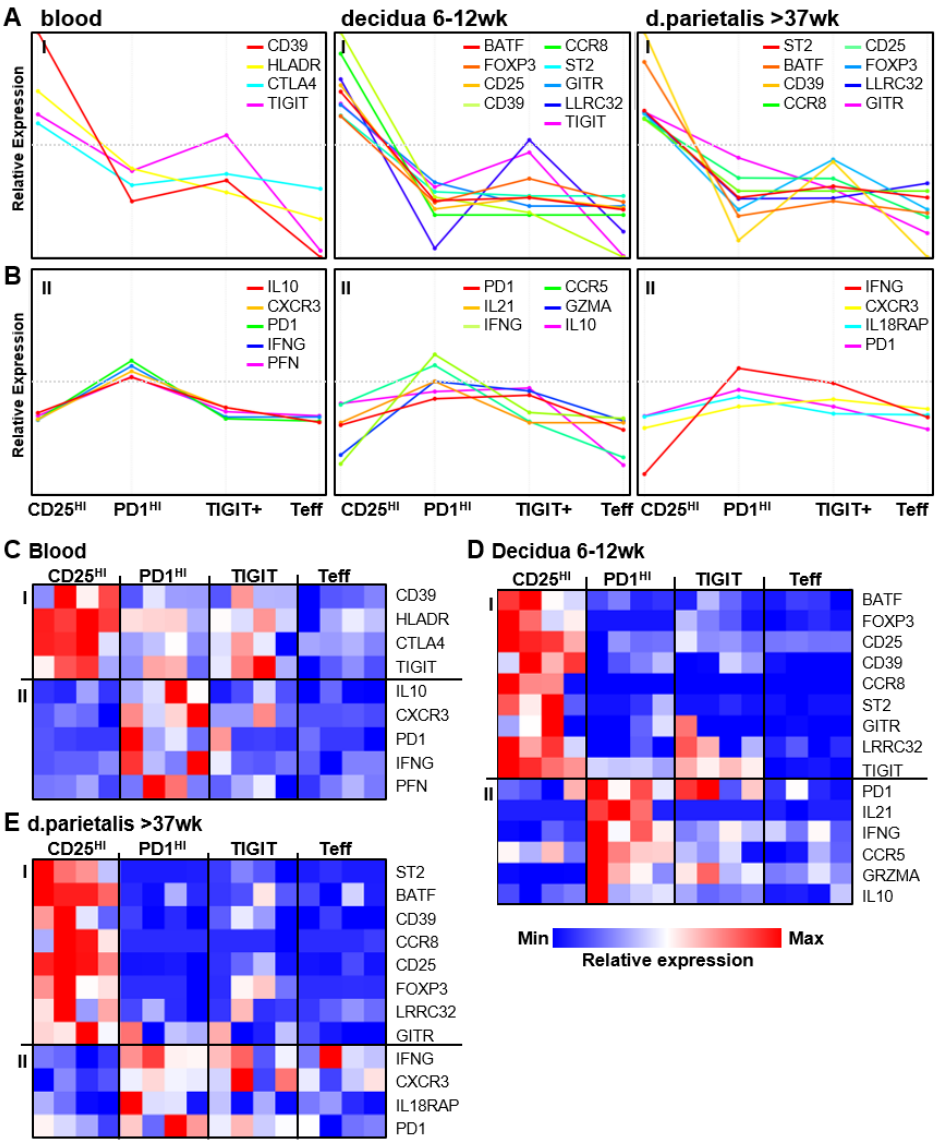

Figure 4. Gene expression profiles of CD4+ Tregs and Teffs.

(A and B) K-means clustering identified two clusters of genes (A and B) that significantly correlate their mRNA expression pattern among CD25^{HI}, PD1^{HI}, TIGIT+, and Teffs. Blood (left panels), decidua 6–12 weeks (middle panels), and d.parietalis >37 weeks (right panels) are depicted. (C–E) Heatmaps depict differentially expressed genes identified in these clusters in blood (C), decidua 6–12 weeks (D), and d.parietalis >37 weeks (E). n = 4 for all T cell types. See also Figures S6 and S7.

EVTs and decidual macrophages increase Treg proportions during co-culture

Previous studies demonstrated that EVTs directly increased FOXP3 levels in CD4+ $CD25^{HI}$ Tregs during co-culture (5, 6) and that decidual macrophages favored Treg differentiation (39). To investigate the capacity of EVTs and decidual macrophages to increase the three Treg types, EVTs and decidual macrophages isolated from first-trimester placental tissue were cocultured with naive CD4+ pTs for 3 days as described previously (6). Co-culture of EVTs or decidual macrophages with CD4+ pTs significantly increased the proportion of FOXP3+ and HELIOS+ Tregs (Fig. 5A). EVTs, but not decidual macrophages, also increased the proportion of $PD1^{HI}$ Tregs, while neither EVTs nor decidual macrophages changed the TIGIT+ population (Fig. 5A). To determine whether EVTs and decidual macrophages increased Treg proportions through cell-cell contact or by secretion of soluble factors, EVTs or decidual macrophages and CD4+ pTs were co-cultured in a transwell system. Separation of EVTs or decidual macrophages from CD4+ pTs by a transwell membrane resulted in a small but not significant decrease in the induction of FOXP3+ and HELIOS+ cells by EVTs and decidual macrophages (Fig. 5B and 5C), suggesting that both cell-cell contact and soluble factors may play a role in the induction of FOXP3+ cells by EVTs and decidual macrophages. In contrast, when EVTs and CD4+ pTs were separated by a transwell membrane, the increase in the proportion of $PD1^{HI}$ cells was abrogated, demonstrating that cell-cell contact is required here (Fig. 5B). To further investigate the mechanisms by which EVTs and decidual macrophages increase Treg proportions, additional cell cultures were established where EVTs or decidual macrophages were co-incubated with CD4+ pTs in the presence of IgG controls and blocking antibodies for a panel of co-inhibitory molecules and anti-inflammatory cytokines. The increase in FOXP3+ cells was not reversed by addition of any of the blocking antibodies tested in EVT co-cultures (TCR coreceptor CD3; HLA-C; HLA-G receptor ILT2; HLA-G; coinhibitory molecule PDL1; and the immune modulatory cytokine TGFβ) (Fig. 5D) and decidual macrophage co-cultures (ILT2, HLA-DR, PDL1, TGFβ, IL-10 receptor (IL-10R)) (Fig. 5E). Interestingly, addition of HLA-C and CD3 antibodies significantly inhibited the induction of $PD1^{HI}$ cells by EVTs (Fig. 5F), while blocking HLA-G interactions resulted in a small but not significant reduction. Blocking ILT2, PDL1, and TGFβ in the EVTs and CD4+ T cell co-cultures did not significantly affect induction of $PD1^{HI}$ cells by EVTs (Fig. 5F). The question of whether placental viral infections alter Treg induction or Treg stability and thereby exacerbate placental inflammation is clinically important. EVTs were infected with human cytomegaloviruses (HCMVs), the most common pathogen to infect the placenta and a major cause of congenital disease (41). Interestingly, no differences were observed in the capacity of healthy or HCMV-infected EVTs to increase $CD25^{HI}$FOXP3+ and $PD1^{HI}$ Treg proportions (Fig. 5G). Collectively, these results suggest that Tregs can locally be induced by EVTs and decidual macrophages and that antigen-specificity may be involved.

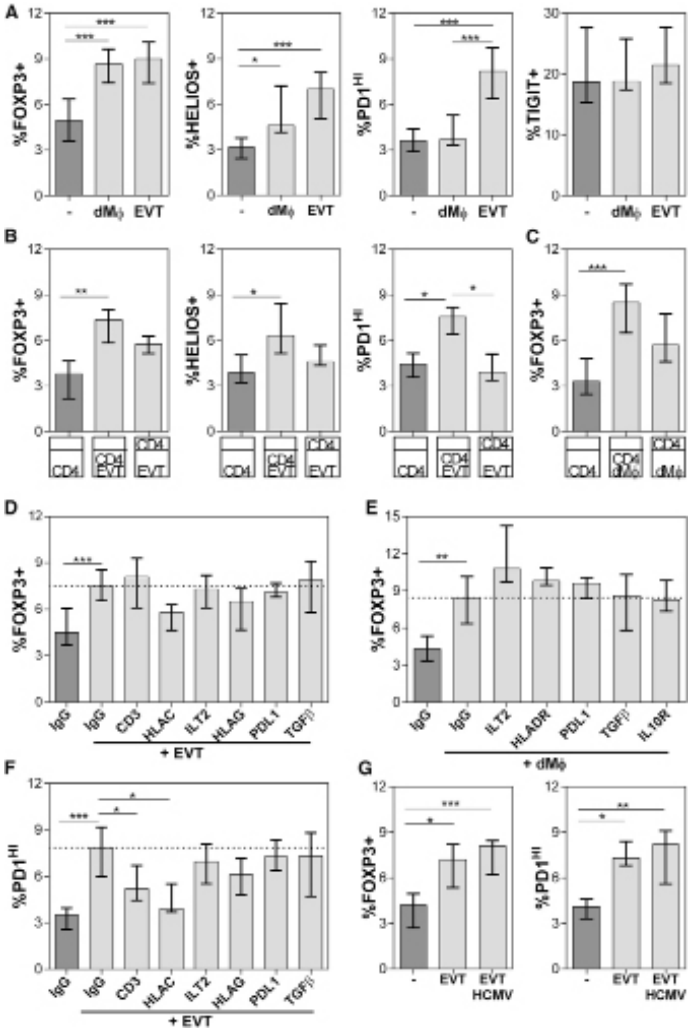

Figure 5. EVTs and decidual macrophages increase Treg proportions.

(A) CD4+ T cells were cultured alone or in the presence of EVTs or decidual macrophages for 3 days. Cells were analyzed for the percentage of FOXP3+, HELIOS+, PD1^{HI}, and TIGIT+ cells as described in Figures S1A and S1B. (B) CD4+ T cells were cultured alone, with EVTs, or with EVTs separated by a transwell membrane for 3 days and analyzed for the percentage of FOXP3+, HELIOS+, and PD1^{HI} cells. (C) CD4+ T cells were cultured alone, with decidual macrophages, or with decidual macrophages separated by a transwell membrane for 3 days and analyzed for the percentage of FOXP3+ cells. (D–F) CD4+ T cells were cultured alone or with EVTs or decidual macrophages in the presence of IgG, or blocking antibodies for CD3, HLA-C, ILT2, HLA-G, PDL1, TGFb, and IL-10R and analyzed for the percentage of FOXP3+ (D and E) and PD1^{HI} (F) cells. (G) CD4+ T cells were cultured alone or with EVTs or HCMV-infected EVTs and analyzed for the percentage of FOXP3+ and PD1^{HI} cells. Bars represent median and interquartile range; n = 8–14; *p < 0.05; **p < 0.01; ***p < 0.005.

Discussion

Modulation of co-inhibitory molecules and Tregs function has exceptional therapeutic potential for treatment of a wide variety of inflammatory disorders, including cancer, chronic infection, autoimmune disease, and pregnancy complications. This study has refined our view of Treg populations at the maternal-fetal interface by presenting phenotypic and functional data of three Treg populations, $CD25^{HI}$ FOXP3+, $PD1^{HI}$FOXP3-IL-10+, and TIGIT+$FOXP3^{dim}$ Tregs, found in decidual tissues of human first-trimester and term pregnancy. Functional suppression assays confirmed that decidual $CD25^{HI}$FOXP3+ Tregs suppress proliferation and production of IFNɣ and TNFα by CD4+ and CD8+ Teffs. Decidual $PD1^{HI}$ Tregs were shown to suppress proliferation of CD4+ (but not CD8+) Teffs in an IL-10-dependent manner. $PD1^{HI}$ Tregs also increased expression of IL-10 in Teffs, possibly resulting in a positive feedback loop sustaining Teff suppression while inducing additional IL-10 secreting Tregs. Decidual TIGIT+ cells significantly inhibited CD4+ T cell proliferation but did not influence CD8+ Teff proliferation or cytokine production, suggesting TIGIT+ Tregs only have limited capacity to suppress T cells, but their ability to suppress other cell types (e. g. APCs) was not investigated here. Further analysis of gene and protein expression of all Treg types in blood and decidua clearly separated the three Treg types and revealed that many immune regulatory molecules (e. g. CTLA4, ST2, LRRC32, GITR, IFNɣ, and IL-10) have increased expression in decidua compared to blood. Thus, decidual Tregs are highly activated and have the potential to influence immune responses through a variety of molecular pathways and cellular targets. Of importance here is further investigation into the contribution of decidual Treg types on the modulation of decidual CD8+ effector-memory T cells, which were shown to have signatures of T cell activation and dysfunction (42).

Most interesting here is the discovery of the role of HLA-C in the induction of $PD1^{HI}$ Tregs by EVT. EVTs, but not decidual macrophages, have the capacity to directly increase $PD1^{HI}$ Tregs through cell-cell contact. Blocking of the TCR co-receptor CD3 or HLA-C in these EVT and CD4 T cell co-cultures significantly reduced this increase, suggesting that antigen-specificity may be involved. Although it is well established that CD4+ T cells recognize major histocompatibility complex (MHC) class II molecules, MHC class I restricted CD4+ T cells have been reported (42). Decidual $PD1^{HI}$ Tregs somewhat resemble Tr1 cells that have been extensively characterized in murine tissues (44) and human peripheral blood (31, 45). Their similarities include the high expression of PD1, IL-10, IFNɣ, and granzymes and the lack of FOXP3 expression. In contrast to Tr1 cells that were shown to depend on the ILT2-HLA-G pathway for their induction (46), induction of $PD1^{HI}$ cells by EVTs induction was not dependent on the HLA-G receptor ILT2, while directly blocking HLA-G during the EVT and CD4 T cell co-culture resulted in a small but not significant decrease of $PD1^{HI}$ Tregs induction. Tr1 cells were also shown to be induced by DC-10 through IL-10 secretion and interaction of HLA-G and ILT4 (47). Thus, other

decidual cell types such as HLA-G+ DC-10, that are found in decidual tissues may contribute to induction of the PD1^{HI} Tregs described here (48). The main mechanisms of Tr1-mediated suppression are the secretion of IL-10 and killing of APCs by granzyme B (49). The expression of perforin and granzymes in first-trimester decidua PD1^{HI} Tregs may suggest they can also diminish APC activity using this pathway. Tr1 cells were identified in HLA-mismatched fetal liver hematopoietic stem cell transplant (HSCT) patients and were shown to suppress allogeneic responses in transplant patients and prevent autoimmune responses (50, 32). While the specificity of decidual PD1^{HI} Tregs remains unknown, the requirement for direct cell-cell contact with EVTs for their induction, as well as the role for the TCR co-receptor CD3, may provoke speculation that decidual PD1^{HI}IL-10 Tregs have specificity for fetal allo-antigens expressed by EVTs (e.g., HLA-C). In comparison with decidual PD1^{HI} Tregs, decidual CD25^{HI}FOXP3+ Tregs had an increased capacity to suppress proliferation and a more potent capacity to suppress IFNɣ and TNFα production by CD4+ and CD8+ Teffs. Thus, the mechanisms of suppression utilized by CD25^{HI}FOXP3+ Tregs and PD1^{HI} Tregs as well as the effects on their cellular targets are inherently different. The increased levels of co-inhibitory proteins and/or mRNAs (e.g., CTLA-4, GITR, CD39, ST2, LRRC32) expressed by decidual CD25^{HI}FOXP3+ Tregs compared to blood CD25^{HI} FOXP3+ Tregs suggests increased Treg activation and suppressive function in decidual tissue to regulate inflammation at the maternal-fetal interface. Furthermore, it demonstrates that decidual Tregs may utilize distinct molecular mechanisms of immune modulation against a variety of cellular targets beyond the inhibition of CD4+ and CD8+ Teff responses.

The question of whether decidual Tregs are nTregs generated in the thymus with specificity for self-antigens or iTregs generated in the periphery with specificity for paternal antigens is immunologically interesting and of clinical relevance. It was previously reported that HELIOS is a marker of thymic-derived Tregs, while HELIOSneg Tregs were induced from FOXP3neg T cells in the periphery (51). HELIOS and FOXP3 double-positive Tregs had increased suppressive capacity, more stable FOXP3 expression, and dissimilar TCR repertoire compared to HELIOS^{neg}FOXP3+ Tregs (52, 53). Instability of HELIOS-deficient Tregs was also associated with conversion to a T-effector phenotype and enhanced antitumor immunity (54), and HELIOS was shown to control certain aspects of Treg- suppressive function, differentiation, and survival (52). However, the lack of HELIOS expression does not exclusively identify human iTregs (55). Here, we demonstrate that human decidual CD25^{HI}FOXP3+ Tregs have a high expression of HELIOS, which reduces in term pregnancy decidua as well as in cases of miscarriage, as was shown previously (13). Furthermore, we demonstrate that co-culture of CD4+ T cells with EVTs or decidual macrophages both significantly increased the expression of FOXP3 and HELIOS, advocating for either a local expansion of FOXP3+ and HELIOS+ nTregs or a possible *de novo* induction of FOXP3+ and HELIOS+ iTregs. The increased suppressive capacity of first-trimester CD25^{HI} Tregs compared to term pregnancy CD25^{HI} cells aligns with the observation that HELIOS

expression increases and/or stabilizes Treg function. Clonally expanded CD4+ $CD25^{HI}$CD127–CD45RA– Treg populations were observed in term pregnancy decidua (15), and preferential recruitment of fetus-specific $CD25^{HI}$FOXP3+ Tregs from the maternal peripheral blood to the maternal-fetal interface has been suggested to occur in human pregnancy (2). Furthermore, CD4+$CD25^{HI}$ Tregs had increased suppressive function when they were isolated from decidual tissue with a maternal-fetal HLA-C mismatch compared to an HLA-C match (2). In neither of these studies was HELIOS expression investigated, but an explanation to align these observations may be that $CD25^{HI}$FOXP3+ Tregs are a mixed population of self-specific nTregs and fetus-specific iTregs. Another explanation may suggest that an increased induction of other iTreg types (e.g., $PD1^{HI}$ Tregs) induced in response to fetal allo-antigens may indirectly increase or enhance $CD25^{HI}$FOXP3+ Tregs stability and/or function through secretion of immune-suppressive IL-10 and/or other factors. Because of the intracellular nature of HELIOS and the lack of other markers to distinguish $CD25^{HI}$FOXP3+ nTregs from iTregs, it is technically not possible to investigate functional differences between these cells from human tissues. A further focus on the mechanisms by which both $CD25^{HI}$FOXP3+ and $PD1^{HI}$IL-10 Tregs are induced by EVTs and decidual macrophages at the maternal-fetal interface as well as the effects of their mutual interactions will contribute to answering burning questions on their origin and antigen specificity.

In contrast to our expectations, infection of EVTs with HCMVs did not diminish the ability of EVTs to increase $CD25^{HI}$FOXP3+ and $PD1^{HI}$ Tregs, suggesting that HCMV infection does not alter the capacity of EVTs to promote immune tolerance. This is in line with a previous observation that decidual natural killer (NK) cells degranulated in response to HCMV-infected maternal decidual stromal cells, but not in response to HCMV-infected EVTs, suggesting that immune tolerance is maintained at the expense of efficient clearance of HCMV infection (41). Relevance of the distinct Treg populations should be addressed by studying their presence and functionality in cases of pregnancy complications. Decreased proportions of decidual CD4+$CD25^{HI}$ FOXP3+ and HELIOS+ Tregs were associated with spontaneous recurrent miscarriage (13,14) and preeclampsia (11, 12, 56). The detailed phenotypic characterization, combined with gene expression and extensive functional analysis of multiple Treg populations as presented here, provides a strong platform for guiding analysis of altered Tregs in clinical conditions for which no systematic functional Treg characterization has been performed.

This study provides strong evidence that multiple types of decidual Tregs, including nTregs and iTregs, play a key role in maintaining maternal-fetal immune tolerance during pregnancy. Moreover, the decidual micro environment contains cell types, particularly EVTs and decidual macrophages, which have mechanisms to stabilize and expand Treg populations. Of importance here is the data demonstrating that blocking the TCR co-receptor CD3 as well as HLA-C during EVT-CD4 T cell co-cultures inhibited the increase in $PD1^{HI}$ Tregs, suggesting antigen-specificity. Further, characterization of decidual Tregs

and Teffs in placental materials obtained after spontaneous preterm birth, preeclampsia, and intrauterine infections will accelerate discovery of therapeutic targets to prevent and cure these severe pregnancy complications, as is underway for treatment of many types of cancer and autoimmune diseases.

Materials and Methods

Discarded human placental and decidual materials (gestational age 6-12 weeks) were obtained from women undergoing elective pregnancy termination at a local reproductive health clinic. Term placental tissues (gestational age > 37 weeks) were obtained from healthy women after uncomplicated pregnancy at term delivered by elective cesarean section or uncomplicated spontaneous vaginal delivery at Tufts Medical Center. All tissues were visually inspected for signs of excessive inflammation (including discoloration, large infarctions and foul odor) and only healthy tissues were used for further processing. Peripheral blood leukocytes were isolated from discarded leukopacks from healthy volunteer blood donors at the Massachusetts General Hospital in Boston, MA.
All human tissue used for this research was de-identified, discarded clinical material. No clinical information including the fetal sex and sex of blood donors was available for analysis. The Committee on the Use of Human Subjects (the Harvard IRB) determined that this use of placental and decidual material is Not Human Subjects Research. Term placental tissue was collected under a protocol approved by Tufts Health Sciences IRB. All procedures to process these human blood and tissues materials are described in method details.

Isolation of T cells and decidual macrophages

The procedures to isolate lymphocytes and EVT have recently been described (6) and are also described in detail hereafter. To isolate 1st trimester decidual lymphocytes, villous and decidual tissues from elective pregnancy terminations were macroscopically identified and separated. Decidua parietalis from term pregnancy was collected by removing the amnion and delicately scraping the decidua parietalis from the chorion. Decidua basalis was macroscopically dissected from the maternal side of the placenta. Collected decidual tissues were washed with PBS, minced and thereafter digested with 0.1% collagenase type IV and 0.01% DNase I (Sigma-Aldrich) gently shaking in a water bath for 75 min at 37°C. After digestion, released lymphocytes from 1st trimester and term placenta decidua were washed with RPMI 1640 (Life technologies) containing 10% FBS (Atlanta Biologicals) for 8 min at 1800 rpm and filtered through 100 mm, 70 mm and 40 mm sieves (BD, Labware; NJ). Lymphocytes were dissolved in 20 mL 1.023 g/ml Percoll (GE Healthcare) and layered on a Percoll gradient (10 mL 1.080 g/ml; 15ml 1.053 g/ml) for density gradient centrifugation (25min, 2000rpm). Decidual lymphocytes were isolated from the 1.080 – 1.053 g/ml interface, and decidual macrophages were isolated from the 1.053 – 1.023 g/ml interface. Cells were washed twice with RPMI and directly stained

for flow cytometric analysis on a BD LSR-II or for FACS sort on a BD FACS ARIA-II. Peripheral blood CD4+ T cells were isolated using a RosetteSep human CD4+ enrichment cocktail (Stem Cell Technologies) followed by Ficoll (GE Healthcare) density gradient centrifugation (20 min, 2000 rpm).

Flow Cytometry

Antibodies used for flow cytometry are listed in the Key Resources Table. For surface staining, cells were stained for 30 min on ice in PBS 1% NCS. For intracellular staining, cells were fixed and permeabilized using the eBioscience FOXP3 staining kit (eBiosciences). For detection of intracellular cytokines, CD4+ T cells were stimulated for 6 hours with phorbol 12-myristate 13-acetate (PMA; 1 mg/ml; Sigma) and Ionomycin (1 mg/ml; Sigma), and Golgistop was added for the last 4 hours (1 ml/ml; BD Biosciences). Cells were fixed and permeabilized using the BD CytoFix/CytoPerm kit (BV Biosciences). Acquisition and analysis was performed on a LSR-II (BD) using FACS Diva software. t-SNE analysis was performed using FlowJo 10 software.

Suppression of T cell proliferation assay

Purified decidual or peripheral blood CD4+ T cells were sorted on a BD FACS Aria into four fractions (CD4+CD25^{HI}, CD4+PD1^{HI}, CD4+TIGIT+ and CD4+ Teff) according to the gating strategy described in Figure S1. Decidual CD8+ T cells were obtained from the same decidual sample. For CFSE labeling CD4+ Teff and CD8+ cells were resuspended in PBS at a concentration of 0.2 -1.0 x106 cells per ml. CFSE (Invitrogen) was added in a 1:2500 dilution and cells were incubated for 5 min in a water bath at 37^{o}C. Cells were washed with X-VIVO 10 supplemented with 50U IL-2 and 5% human AB serum (Corning) for 8 min at 1800 rpm. Cells were resuspended at 0.4 x106 cells per ml in X-VIVO 10 supplemented with 50U IL-2 and 5% human AB serum. Treg were cultured with the CFSE-labeled Teff in a 1:2 ratio of 20.000 Treg: 40.000 Teff cells, with the addition of Dynabeads Human T-Activator CD3/CD28 (1 ml/ml). After four days, cells were collected and stained for CD4-PerCP, CD8-Alexa700 and CD45-PacificOrange. CFSE dilution of CD4+Teff and CD8+ T cells was analyzed using the proliferation analysis tool of FlowJo v7.6.5 software. The percentage of undivided cells (generation 0) was calculated based on the total number of cells. The division index was calculated by FlowJo software and reflects the average number of divisions per cell. Suppression of T cell cytokine production assay Purified decidual CD4+CD25^{HI}, CD4+PD1^{HI}, CD4+TIGIT+, CD4+ Teff and CD8+ T cells were obtained as described above (Fig. S1). CD4+ Teff cells were labeled with CFSE (Invitrogen) and CD8+ cells were labeled with CD45-Alexa700. Treg were cultured with CD4+ and CD8+ Teff in a 1:1:1 ratio (20.000 Treg: 20.000 CFSE+ CD4+ Teff: 20.000 Alexa 700+ CD8+ Teff). Control cultures without Treg contained CD4+ and CD8+ Teff in a 1:1 ratio (30.000 CFSE+ CD4+ Teff: 30.000 Alexa700+ CD8+ Teff). CD3/CD28 Dynabeads (1 ml/ml) were added to all cultures and after three days, cells were re-stimulated with 2.5

ng/mL PMA in combination with 0.1 mg/mL ionomycin for 6h in the presence of 1 mg/mL GolgiStop (BD Bioscience). Cells were collected and stained for cell surface expression of CD4-PerCP, CD8-Alexa700, CD45-Pacific Orange and intracellular expression of IL-10, IFNg and TNFa upon fixation and permeabilization (CytoFix/Cyto PermTM Plus kit (BD)). Acquisition was performed on an LSR-II (BD) using FACS Diva software for analysis following the gating strategy shown in (Fig. S4).

RNA isolation and QPCR chip analysis

Purified CD4+ T cells from four samples of blood, decidua 6-12wk and d.parietalis >37wk (Fig. S1A) were resorted into four types CD4+CD25^{HI}, CD4+PD1^{HI}, CD4+TIGIT+ and CD4+ Teff (Fig. S1B) and collected directly into 600 mL Trizol reagent (Life technologies) supplemented with 0.5 mL glycogen (20mg/ml; Affymetrix) and stored at 80°C until RNA isolation. Total RNA was isolated using the RNAeasy Micro Kit (QIAGEN) per manufacturer's instruction. RNA was analyzed on a Nanodrop to determine RNA yield and integrity. RNA quality of all samples was further confirmed by performing a QPCR analysis for GAPDH and FOXP3 expression. In short: RNA was reverse transcribed with Stratagene's AffinityScript QPCR. cDNA Synthesis Kit and amplification of specific PCR products for FOXP3 and GAPDH were detected using the PerfeCTa SYBR Green Super Mix with Low ROX (QuantaBio) in duplicates. Subsequently, high quality samples were run in duplicate on the BioMark Fluidigm QPCR 96.96 chip. 1.25ml DNA was pre-amplified in a 96-well plate using the Fluidigm PreAmp Master mix combined with 500nM forward and reverse primer of each primer pair (2min at 95°C, 10 thermal cycles of 15sec at 95°C and 4min at 60°C)(primers are listed in Table S1). Exonuclease treatment to remove unincorporated primers was carried out using the Exonuclease I at 40U/ml (New England BioLabs). 2 ml of the Exonuclease I dilution was added to each pre-amplification reaction and incubated in a thermal cycler for 30min at 37°C and 15min 80°C. A 5-fold dilution was prepared in TE buffer (10mM Tris-HCl, 1.0mM EDTA, TEKnova, PN T0224). Sample pre-mix for the 48 samples was prepared using 2ml of the prepared cDNA with 20X DNA binding dye sample loading reagent (Fluidigm) and 2X SsoFast EvaGreen supermix with low ROX (Bio-Rad). The assay mix was prepared in a separate 96-well plate consisting of 2X assay loading reagent, 1X DNA suspension buffer and 100mM of mixed forward and reverse primers. Chips were primed, loaded with both assay and sample mix and run on BioMark readout instruments as described by the manufacturer, at Harvard University's core facility. Biomark data were processed using Fluidigm Biomark software. Quality control was based on EvaGreen reagent allowing for detection of unspecific PCR product and formation of primer dimers using the BioMark software. Samples of failed reactions were automatically excluded by the software. The resulting values for 66 primers of 48 samples were normalized by subtracting CT values for GAPDH (ΔCT) and subsequent conversion into fold-change values relative to the median peripheral blood Teff (2ΔΔCT). K-means cluster analysis was performed using the

fold-change values of the 66 genes in Express Cluster V1.3 in Genepattern https://cloud.genepattern.org/gp. Differential expression of significantly correlating genes was based on K-means with a minimum of a 1.5-fold change.

Isolation of EVT

Isolation of EVT and co-culture with CD4+ T cells was performed as described previously (6) and are also described in detail hereafter. 1st trimester villous tissue was gently scraped from the basal membrane and the tissue was digested for 8 min at 37°C with a trypsin (0.2%) EDTA (0.02%) solution. Trypsin was quenched with DMEM/F12 medium containing 10% Newborn Calf Serum (NCS) and1%Pen/Strep (all from GIBCO) and filtered over a gauze mesh. Filtrate was washed once and layered on Ficoll (GE Healthcare) for density gradient centrifugation (20 min, 2000 rpm). Cells were collected, washed once and incubated for 20 min at 37°C in a 30mm tissue culture dish for removal of macrophages. To establish untouched (free of antibody staining) EVT cultures, for each sample the percentage CD45-HLA-G+ EVT was determined by FACS analysis for EGFR1, HLA-G and CD45. Samples with > 8% CD45-HLA-G+ cells were stained for CD45 and sorted for viable CD45- large trophoblast cells. 50.000 CD45-HLA-G+ EVT (calculated based on percentage HLA-G+ cells and total cell number) were plated in 48 well cell culture plates (Costar) pre-coated with fibronectin (100 mL 20ng/ml 45min, BD), in DMEM/F12 (GIBCO) supplemented with 10% NCS, pen/strep and glutamine, insulin, transferrin, selenium (GIBCO), 5ng/ml EGF (Peprotech) and 400 units human gonadotropic hormone (Sigma). Trophoblasts were incubated for 2h at 37°C and thereafter washed 3 times to remove all non-adherent VT. Cultures resulted in 50%–80% HLA-G+ EVT.

Co-culture of EVT, decidual macrophages and CD4+ T cells

50.000 EVT or decidual macrophages were co-incubated with 100.000 CD4+ pT from unrelated blood donors in X-Vivo10 medium (Lonza), supplemented with 5% human AB serum and 50 units/ml IL-2 (Biolegend). For blocking experiments, LEAF-purified anti- HLA-G (MoAb 87G; 20 mg/ml), anti-ILT2 (MoAb GHI/75; 20 mg/ml), anti-PDL1 (MoAb 29E.2A3; 20 mg/ml), anti-TGFβ (MoAb 19D8; 25 mg/ml), anti-IL-10R (MoAb 3F9; 20 mg/ml), anti-HLA-DR (MoAb L243; 20 mg/ml), purified anti-HLA-C (MoAb DT-9; 10 mg/ml) and IgG controls (all from Biolegend), or anti-CD3 (MoAb SPV-T3b; 25 mg/ml) (ThermoFisher Scientific) were added 30 min before addition of CD4+ T cells. For transwell assays, 100.000 EVT or decidual macrophages were plated in 24 well cell culture plates pre-coated with fibronectin, non-adherent EVT were removed and 200.000 CD4+ pT were added directly to the EVT and decidual macrophage cultures or were added on top of the membrane after insertion of a 0.4 mM trans well membrane (Costar). CD4+ pT were harvested after 60 hours and analyzed by Flow cytometry for CD45, CD4, CD25, PD1, TIGIT and intracellular FOXP3 and HELIOS.

HCMV Infection of EVT

High titer virus stocks of HCMV-AD169-GFP (IE-1-GFP) (a gift from Prof. Donald Coen at Harvard Medical School) was obtained by infecting Human Foreskin Fibroblasts (HFF) (ATCC) and collecting supernatants after 7 days. Supernatants were aliquoted and snap frozen in liquid nitrogen until use. Primary EVT were infected with HCMV at an MOI of 2-4 for 12 hours (41). Infected cells were imaged in a Nikon EclipseTi fluorescence microscope at 20x magnification. HCMV infection of EVT reached > 80% at day 2, and no cytopathic effects on EVT were visible after HCMV (41). For co-culture assays, HCMV infected cells were washed twice after 12 hours of infection and 100.000 CD4+ pT were added. CD4+ pT were harvested after 48 hours and analyzed by Flow cytometry for CD45, CD4, CD25, PD1, TIGIT and intracellular FOXP3.

Quantification and statistical analysis

All data was analyzed using GraphPad Prism version 6.07 software. To determine differences between 2 paired groups a non-parametric Wilcoxon Signed Rank test was performed (Fig. S1E). To determine differences among more than 2 unpaired groups, a nonparametric Kruskal-Wallis test with Dunn's multiple comparison post-test was performed (Fig. 1, 2, 3, and 5; Fig. S3, S4, and S7). For the Dunn's post-test the mean ranks were compared to control column A (Fig. 2B 2G and 3; Fig. S4 and S7), column B (Fig. 5D-5F) or with the mean rank of every column (Fig. 1, 5A-5C, and 5G; Fig. S3). P values < 0.05 were considered to denote significant differences. *$p < 0.05$; **$p < 0.01$; *** $p < 0.005$ are indicated within each figure. Sample size indicates biological replicates of individual placental of blood cell isolates and are indicated indicted in each figure legend. Sample sizes were not determined beforehand.

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Supplemental Information

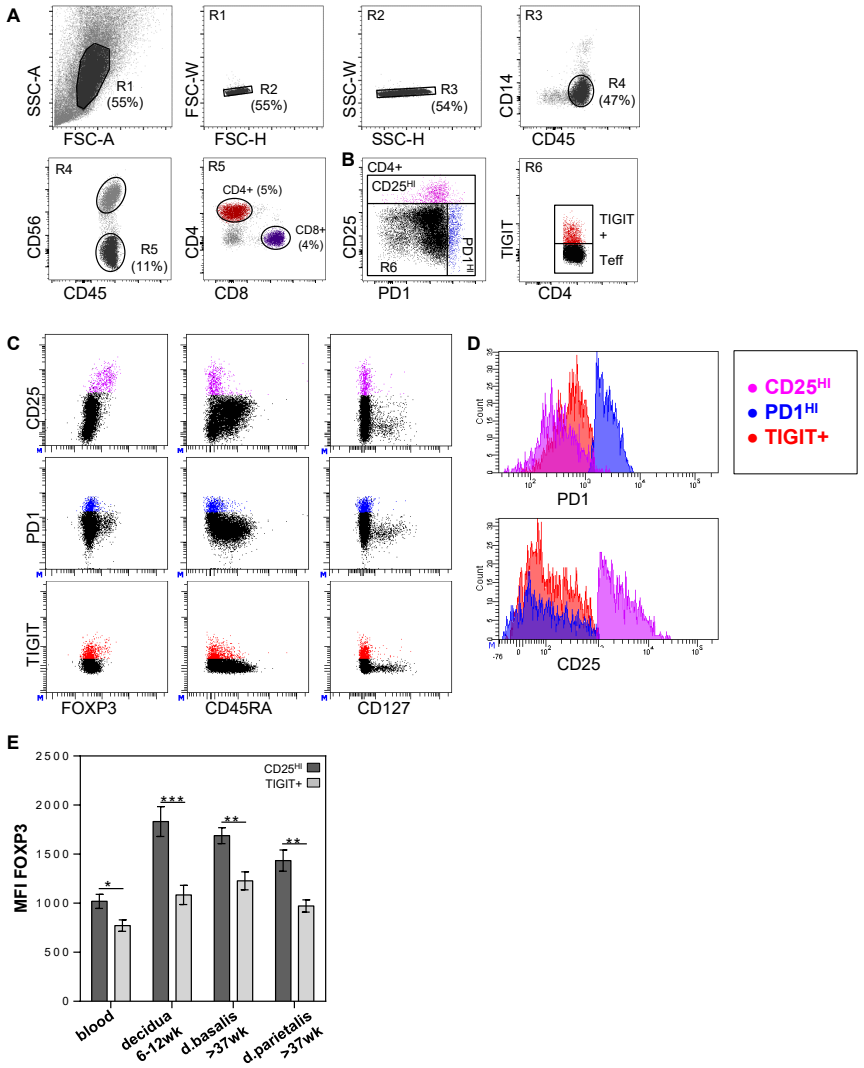

Figure S1. FACS gating strategy.
Related to Figure 1. A) CD4+ and CD8+ T cells were identified by selecting live cells (R1), duplicates were excluded (R2, R3), and CD45+CD56−CD3+CD4+ (CD4+) and CD45+CD56−CD3+ CD8+ (CD8+) T cells were selected. B) Based on CD25, PD1 and TIGIT expression, CD4+ T cells were further separated into $CD25^{HI}$, $PD1^{HI}$, TIGIT+ and Teff cells. Representative FACS plots of C) FOXP3, CD45RA and CD127 expression and D) PD1 and CD25 expression in $CD25^{HI}$, $PD1^{HI}$ and TIGIT+ cells in a 6-12wk decidual sample. E) Graph depicts the expression of FOXP3 (MFI) within $CD25^{HI}$ and TIGIT+ cells in blood, decidua 6-12wk, decidua basalis >37wk and d.parietalis >37wk. n=8-10. Bars represent median and interquartile range; n= 8-10; *P<0.05, **P<0.01, ***P<0.001.

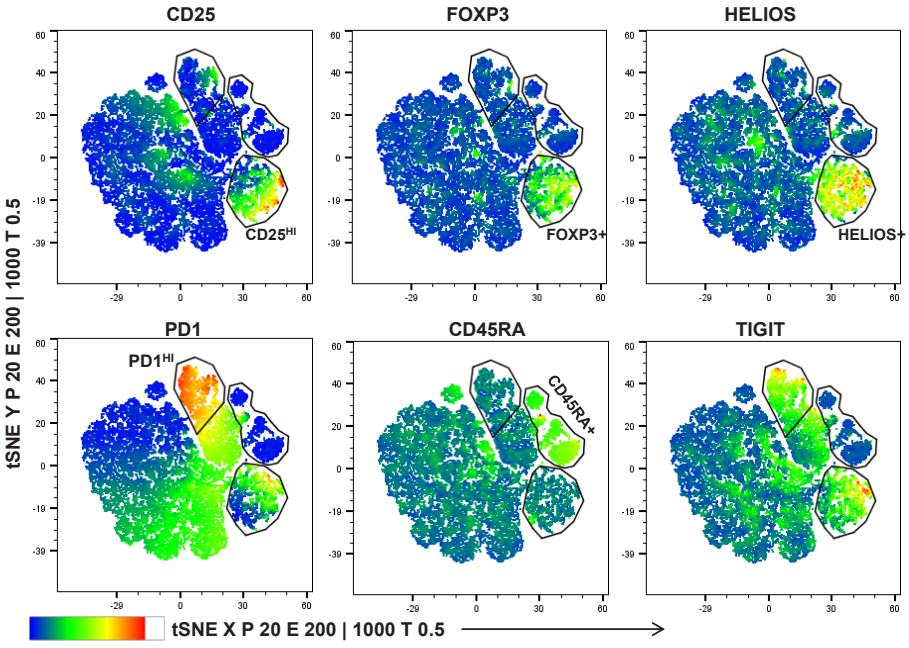

Figure S2. tSNE analysis of decidual CD4+ T cells.
Related to Figure 1. Representative tSNE plots of one decidual CD4+ T cell sample (6-12wk), highlighting CD25, FOXP3, HELIOS, PD1, CD45RA and TIGIT. Three separate CD4+ T cell populations are identified i) $CD25^{HI}$FOXP3+HELIOS+, ii) $PD1^{HI}$ and iii) CD45RA+. TIGIT is co-expressed by $CD25^{HI}$ and $PD1^{HI}$ cells but not by CD45RA+ cells. TIGIT is also expressed by a large proportion of the remaining CD4+ T cells, these TIGIT+ cells are clearly identified in Fig 1B bottom panels.

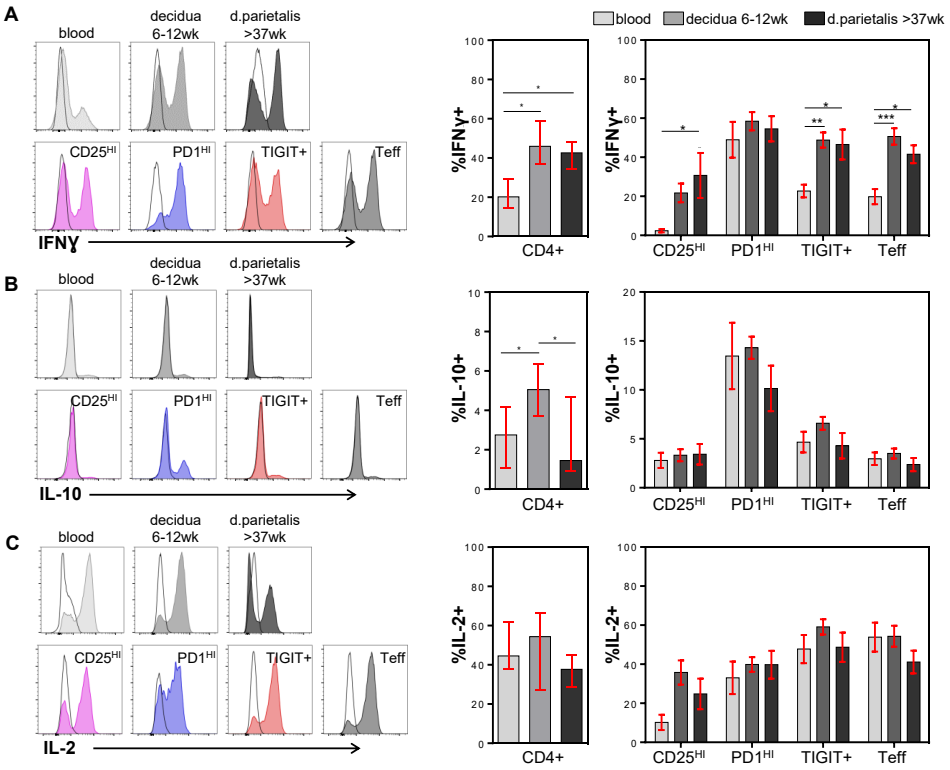

Figure S3. IFNγ+, IL-2+ and IL-10+ expression by Treg.

Related to Figure 1. Representative histograms of A) IFN, B) IL-10 and C) IL-2 expression by CD4+ T cells in blood, decidua 6-12wk and d.parietalis >37wk and in the four T cell subpopulations (CD25^{HI}, PD1^{HI}, TIGIT+ and Teff) in decidua 6-12wk. Graphs depict percentage of A) IFN+, B) IL-10+ and C) IL-2+ cells within CD4+ T cells (left) and the four T cell subtypes (right). Bars represent median and interquartile range; n= 8-12; *P<0.05, **P<0.01, ***P<0.001. Kruskal-Wallis with Dunn's post-test comparing the mean ranks to the mean rank of every other column (column A,B,C) within each of the T cell populations.

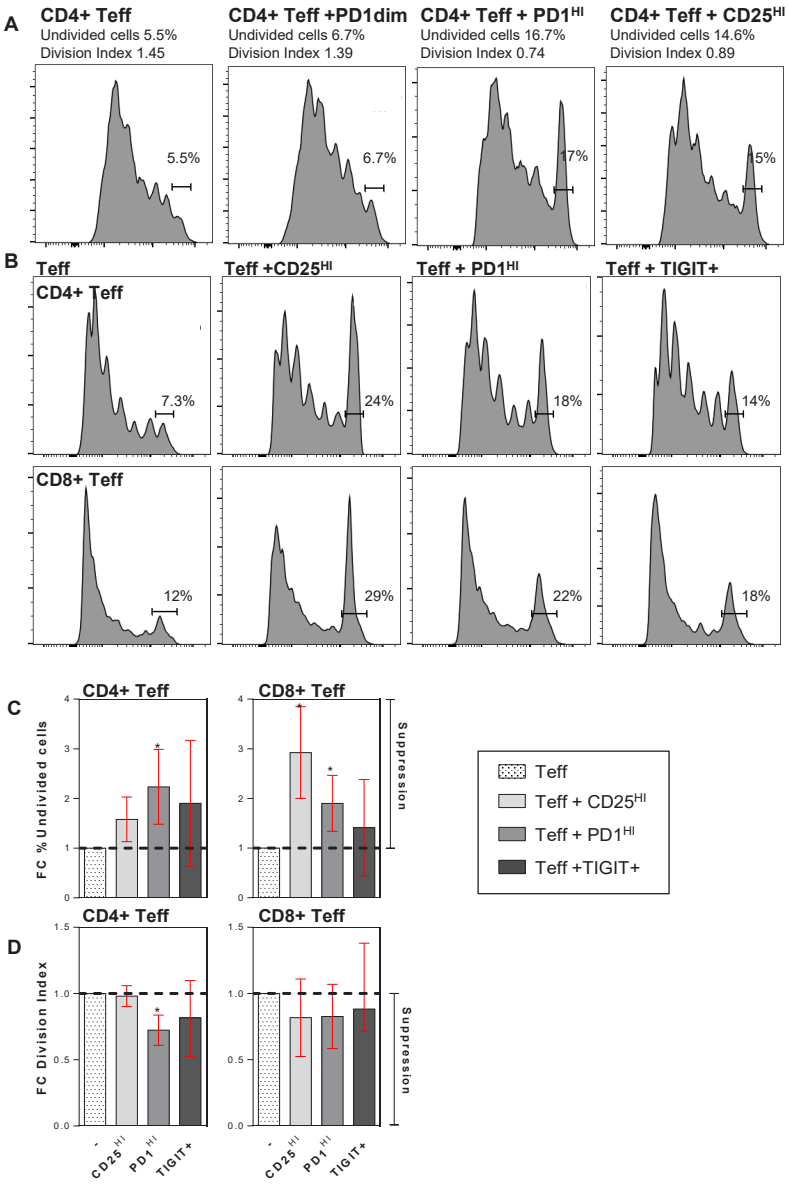

Figure S4. Treg suppression of T cell proliferation.

Related to Figure 2. A) CFSE dilution of 6-12wk decidual CD4+ Teff cells stimulated with anti-CD3/28 and co-cultured with or without PD1^{dim}, PD1^{HI} or CD25^{HI} cells and B) CFSE dilution of peripheral blood CD4+ Teff cells (upper panels) and CD8+ Teff cells (bottom panels) stimulated with anti-CD3/28 and co-cultured with or without CD25^{HI}, PD1^{HI} or TIGIT+ cells. Cells were cultured for four days in a 1:2, Treg:Teff ratio. Percentages of the undivided Teff cells are shown. C) Fold Change (FC) in percentage undivided peripheral blood Teff cells and D) FC in division index after addition of CD25^{HI}, PD1^{HI} or TIGIT+ Treg, compared to CD4+ Teff cells (left panels) and CD8+ Teff cells (right panels) cultured alone. Bars represent median and interquartile range; n=5-7; *P<0.05, Kruskal-Wallis with Dunn's post-test comparing columns B-D to column A was performed. Values >1 for FC %undivided cells and <1 for FC division index represent suppression of proliferation. Division index reflects the number of divisions per cell as calculated in FlowJO v7.6.5.

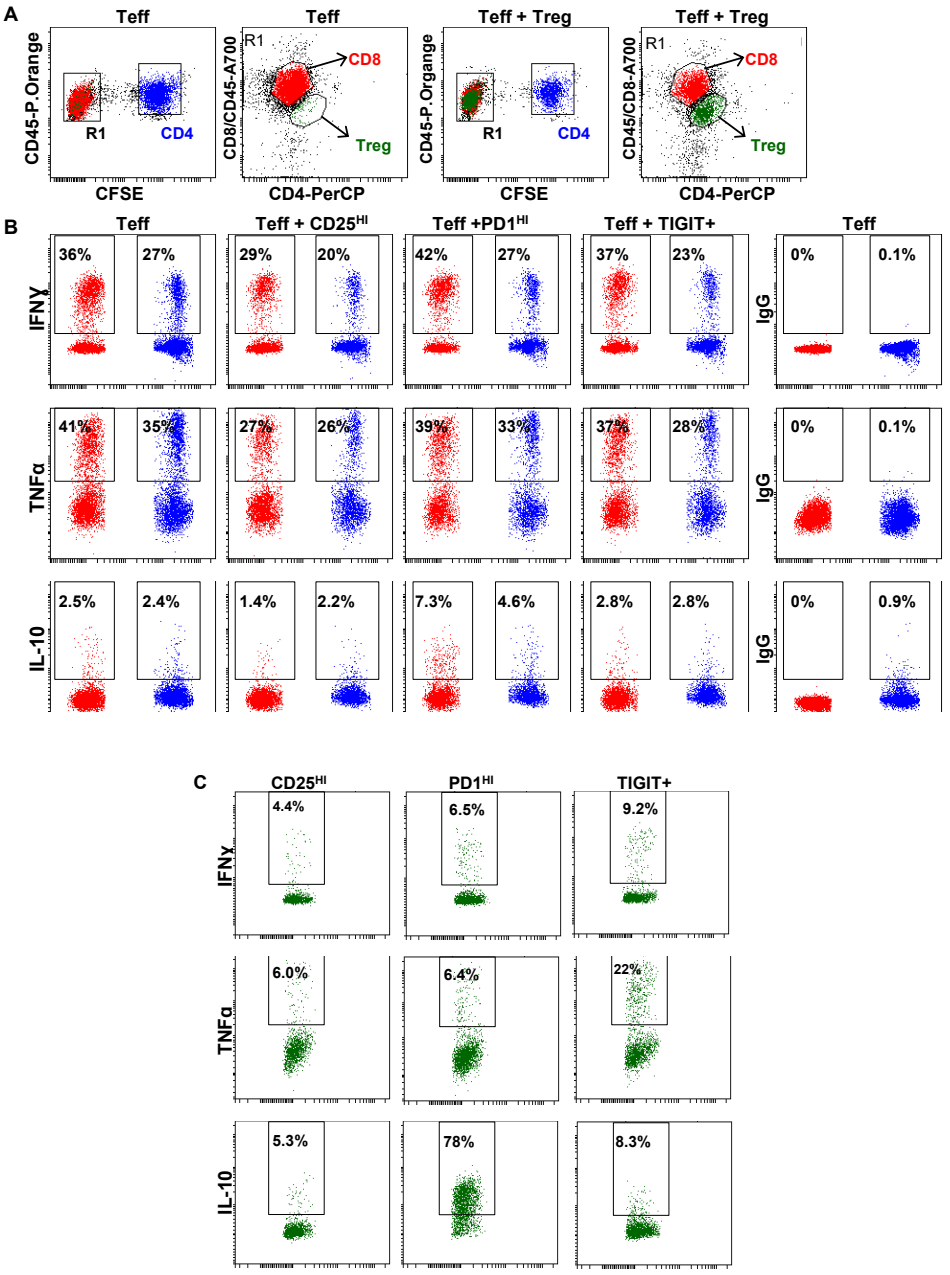

Figure S5. Decidual Treg influence cytokine production by CD4+ and CD8+ Teff cells.
Related to Figure 3. A) Gating strategy identifies CD45+CFSE+CD4+ T cells (Blue), CD45+CFSE-CD8+ T cells (Red) and CD45+CFSE-CD8- Treg (green). B) Representative FACS plots of IFNɣ , TNF-α and IL-10 expression in decidual CD4+ Teff (Blue) and decidual CD8+ Teff (Red) upon addition of $CD25^{HI}$, $PD1^{HI}$ or TIGIT+ Treg compared to CD4+ Teff and CD8+ Teff cultured alone and IgG controls. C) Representative FACS plots of IFNɣ , TNF-α, IL-10 expression in decidual $CD25^{HI}$, $PD1^{HI}$ or TIGIT+ Treg in the same co-cultures.

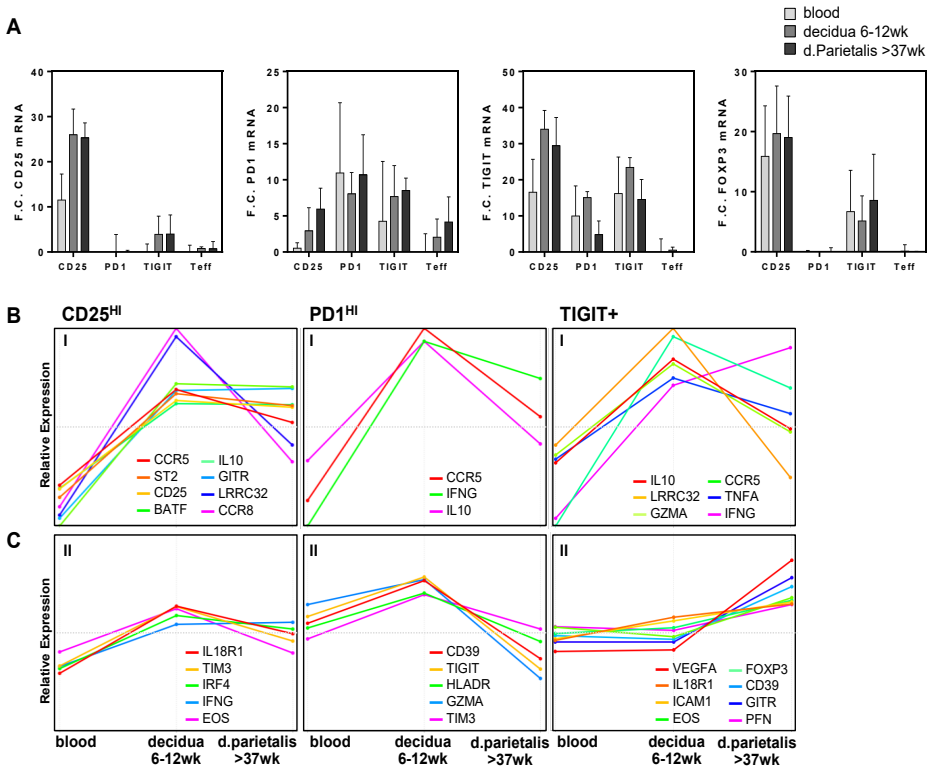

Figure S6. Gene expression profiles of CD4+ Treg.
Related to Figure 4. A) FC mRNA values of CD25, PD1, TIGIT and FOXP3 (n=4). Bars represent median and inter-quartile range. (B and C) K-means clustering identified two clusters of genes that significantly correlate their mRNA expression pattern in blood, decidua 6-12wk and d.parietalis >37wk. $CD25^{HI}$ (left panels), $PD1^{HI}$ (middle panels) and TIGIT+ (right panels) are depicted.

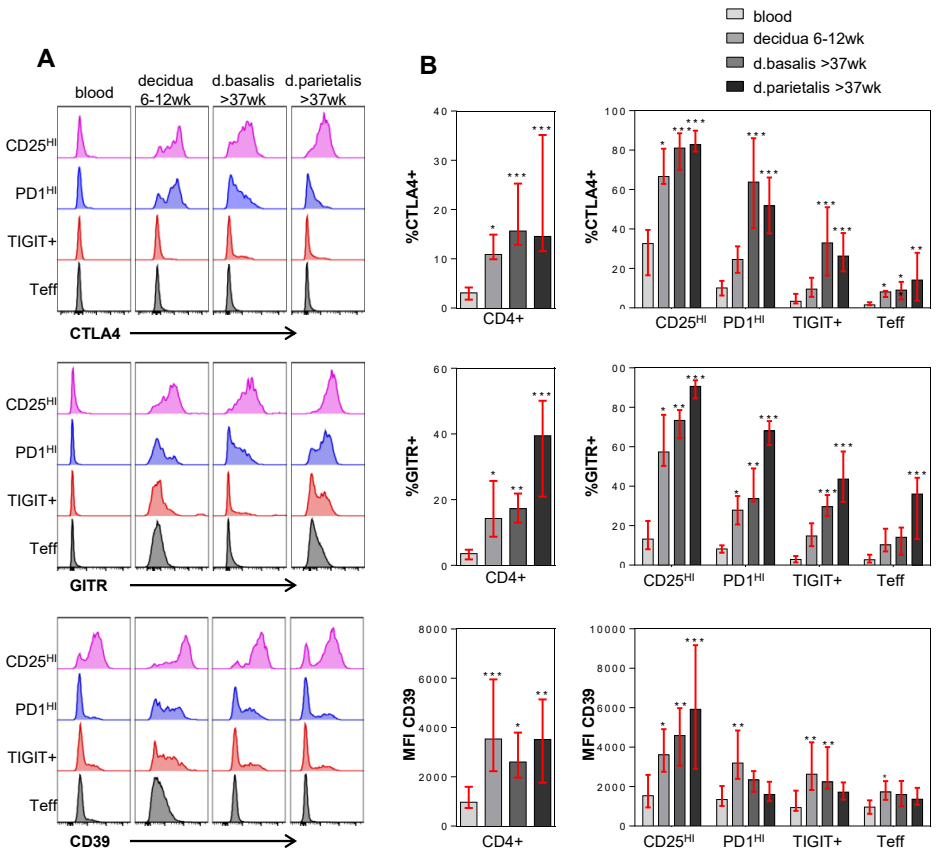

Figure S7. Expression of CTLA-4, GITR and CD39 by Treg.

Related to Figure 4. A) Representative histograms of CTLA-4 (top panels), GITR (middle panels) and CD39 (bottom panels) within CD25^{HI}, PD1^{HI}, TIGIT+ and Teff cells of blood, decidua 6-12wk, d.basalis >37wk and d.parietalis >37wk. B) Graphs depict percentage of CTLA-4+ (top panels), percentage of GITR+ (middle panels) and Mean Fluorescence Intensity (MFI) of CD39 (bottom panels) within the total CD4+ cell population and within CD25HI, PD1^{HI}, TIGIT+ and Teff cells of blood, decidua 6-12wk, d.basalis >37wk and d.parietalis >37wk. (n= 8-12) Bars represent median and interquartile range. *P<0.05, **P<0.01 and ***P<0.001. Kruskal-Wallis with Dunn's post-test comparing all decidual fractions (columns B-D) to blood (column A) was performed.