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Human Tregs at the maternal-fetal interface show site-specific adaptation reminiscent of tumor Tregs

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Brief Summary: Human regulatory T cells at the maternal-fetal interface show uterine site-specific functional adaptation with late-stage effector differentiation, chronic activation, Th1 polarization, and tumor-infiltrating-Treg-like features.
Abstract

Regulatory T cells (Tregs) are crucial for maintaining maternal immune-tolerance against the semi-allogeneic fetus. We investigated the elusive transcriptional profile and functional adaptation of human uterine Tregs (uTregs) during pregnancy. Uterine biopsies, from placental bed (=maternal-fetal interface) and incision site (=control), and blood were obtained from women with uneventful pregnancies undergoing Caesarean section. Tregs and CD4+ non-Tregs were isolated for transcriptomic profiling by Cel-Seq2. Results were validated on protein and single cell level by flow cytometry. Placental bed uterine Tregs (uTregs) showed elevated expression of Treg signature markers, including FOXP3, CTLA-4 and TIGIT. Their transcriptional profile was indicative of late-stage effector Treg differentiation and chronic activation, with increased expression of immune checkpoints GITR, TNFR2, OX-40, 4-1BB, genes associated with suppressive capacity (HAVCR2, IL10, LAYN, PDCD1), and transcription factors MAF, PRDM1, BATF, and VDR. uTregs mirrored non-Treg Th1 polarization and tissue-residency. The particular transcriptional signature of placental bed uTregs overlapped strongly with that of tumor-infiltrating Tregs, and was remarkably pronounced at the placental bed compared to uterine control site. Concluding, human uTregs acquire a differentiated effector Treg profile similar to tumor-infiltrating Tregs, specifically at the maternal-fetal interface. This introduces the novel concept of site-specific transcriptional adaptation of Tregs within one organ.
Introduction

In the past decade, T cells have been identified in various human and murine non-lymphoid tissues. (1, 2) These tissue-resident memory T cells (TRM) do not recirculate, serve as first-line responders to infections, and are characterized by expression of signature molecules such as CD69, which prevents their tissue egress.(1, 3–8) TRM adapt to tissue environments by acquiring a specialized functional phenotype that depends on micro-environmental cues.(9, 10) Also regulatory T cells (Tregs), critical gatekeepers of immune homeostasis,(11) have been recently identified in murine and human tissues.(12–16) Like TRM, Tregs can become resident and gain a polarized phenotype, with functional specialization depending on the tissue or organ, which is controlled on a transcriptional level.(14–22) Although increasing evidence in mice supports functional adaptation of Tregs to non-lymphoid tissue environments,(23) studies in humans are still scarce.(14, 17) However, transcriptional adaptation of Tregs has gained special interest in the tumor environment, due to the important therapeutic implications.(24) Tumor-infiltrating Tregs (TITR) display a unique and specialized transcriptional signature,(25) associated with activation and functional specialization, including increased suppressive capacity.(25–27) Tissue and tumor Tregs undergo differentiation reminiscent of effector Tregs, with potent suppressive capacity, and are characterized by expression of CD45RO, and increased CD25, CTLA-4, and HLA-DR.(28–31) Furthermore, effector Tregs (in tissues and tumors) express high levels of immune checkpoint molecules OX40, 4-1BB, GITR, TIGIT, ICOS and transcription factors such as BLIMP-1 (encoded by PRDM1) and BATF.(19, 25–27, 31–34). Effector Tregs can mirror effector T helper (Th) cell polarization, by acquiring co-expression of FOXP3 with chemokine receptors and transcription factors associated with Th1 (CXCR3, T-bet), Th2 (GATA3, IRF4) or Th17 (RORγt, STAT3) differentiation.(19, 35–38). This specific polarization is associated with an enhanced suppressive efficacy towards the matching T effector response.(31, 36, 45, 37–44) Since most of these insights have been generated in mice, it is still largely unknown whether these principles also apply to human tissue Tregs.
As recently highlighted,(46) one of the most interesting, but yet elusive tissue sites for Treg function in humans is the maternal-fetal interface. Pregnancy is a mystifying biological process when viewed from an immunological perspective, posing a unique challenge to the maternal immune system.(47, 48) While peripheral immunity against pathogens needs to remain intact, the semi-allogeneic fetus and placenta, which may harbor foreign paternal antigens, need to be tolerated.(49) The maternal immune response is therefore delicately balanced, and requires tight regulation especially locally at the maternal-fetal interface, which is underlined by the fact that human decidual T cells can recognize and actively respond to fetal cord blood cells.(47–51) Maternal Tregs are consequently indispensable for successful embryo implantation and pregnancy outcome, and contribute to maternal-fetal tolerance on multiple levels.(47, 52, 53) Depletion of murine maternal Tregs causes pregnancy loss due to immunological rejection of the fetus.(53, 54) In humans, maternal Tregs are abundantly present in the gravid uterus,(55–62) and normal human pregnancy is characterized by increased numbers of Tregs in the periphery and at the maternal–fetal interface.(56, 61, 63, 64) In patients with preeclampsia, a severe hypertensive pregnancy disorder, and patients with recurrent miscarriages, Treg numbers are reduced both at the maternal-fetal interface and in the periphery,(57, 65–70) implying that also in humans local presence of Tregs in the pregnant uterus is required for successful pregnancy outcome.

Previous studies investigating the maternal, uterine immune system in humans have been limited by the practical challenge of acquiring biopsy material of the uterine wall and have made use of the thin superficial decidual layer attached to the delivered placenta, which is heavily contaminated by fetal immune cells and may not be representative of the maternal Treg status during pregnancy. Moreover, the functional and transcriptomic profile of human uterine Tregs (uTregs) from the maternal-fetal interface and its relation to Tregs from other human tissues remains to be elucidated. Here, we investigated functional adaptation and specialization of highly purified human, exclusively maternal, resident uTregs in myometrial biopsies from the maternal-fetal interface. We performed transcriptomic profiling and functional in vitro assays, as well as flow cytometry to study their phenotypic heterogeneity on protein level in single cell resolution. To identify tissue (site)-specific functional adaptation, we compared these uTregs to uTregs from a distant
uterine control site and maternal peripheral blood Tregs, in addition to tissue- and site-matched resident CD4\(^+\) non-Treg T cells. Lastly, we compared the specific profile of functional adaptation of uTregs to known Treg signatures from other human and murine tissue sites, including tumors.

**Results**

*Uterine Tregs are bona fide suppressive Tregs*

The frequency of CD25\(^{hi}\)FOXP3\(^+\) Tregs within the CD4\(^+\) T cell population was similar between blood and uterine tissue and ranged from 2.5 to 13.5\% (Figure S1A). For transcriptional profiling, the CD3\(^+\)CD4\(^+\)CD25\(^{hi}\)CD127\(^-\) population (Tregs) and CD3\(^+\)CD4\(^+\)CD25\(^-\)CD45RA\(^-\) memory T cells (Tconv) were FACS sorted from peripheral blood and myometrial biopsies from 5 women with uncomplicated pregnancies undergoing Caesarean section. In myometrium, Tconv were selected for CD69 positivity. The sorting strategy is shown in Figure S1B. Confirming the maternal origin of the sorted cells, the female-specific gene *XIST* was highly expressed in all samples, whereas transcripts of the male-specific gene *SRY* were undetectable in all samples, including pregnancies with male offspring (Figure S1C). Principal component analysis (PCA) of transcriptomic profiles showed that uTregs from the maternal-fetal interface are clearly distinct from blood-derived Tregs (bTregs), and that also uterine T conv (uTconv) and blood-derived Tconv (bTconv) clearly cluster apart (Figure 1A). Notably, PC1, mounting the difference between the cell sources, accounted for >60\% of the variance, whereas PC2, explaining variance between Treg and Tconv populations, accounted for only 11\% of the variance. To assess whether the sorted population of uTregs were bona fide Tregs, we analyzed enrichment of a published core Treg gene signature (71) in uTregs compared to uTconv and bTregs by gene set enrichment analysis (GSEA). Expression of Treg core signature genes was not only enriched compared to uTconv, but, remarkably, also more pronounced in uTregs than in bTregs, indicating that uTregs are bona fide Tregs with enhanced expression of Treg core
signature genes (Figure 1B-C). Indeed, expression of many of the published Treg markers (71) was higher in uTreg than bTreg (Figure 1D). Higher expression of the Treg-identifying molecules FOXP3 and CTLA-4 in uTregs than bTregs was confirmed on protein level (Figure 1E-G). Also TIGIT, a key checkpoint molecule associated with specialized suppressive function, (72) was highly expressed in uTregs, with the majority uTregs being positive for TIGIT (Figure 1H). Consistently, GSEA showed significant enrichment of a previously identified TIGIT+ Treg signature (Figure 1I). (72) Suppression assays, although technically challenging due to low cell numbers, confirmed the suppressive potential of uTregs on proliferation and cytokine production of healthy donor peripheral blood-derived CD4+ T cells (Figure 2 and Figure S1D-E). 2 out of 4 uTreg donors showed particularly high suppressive capacity of uTregs on cytokine production of IL-2, IL-10, IFNγ and TNFα, already at a 1:8 (Treg:Tconv) ratio, compared to bTregs. These results confirm that the sorted uTregs are bona fide functional Tregs, with enhanced expression of Treg signature genes.

**The uTreg signature indicates an activated and effector Treg profile**

To investigate the functional adaptation of uTregs to the specific environment of the maternal-fetal interface, we determined both their functional differentiation and (T helper) polarization, both of which may be influenced by the tissue environment. (12, 13, 20, 33, 73–75) To identify the uTreg-specific transcriptional signature, we assessed their differential gene expression with both bTregs and uTconv. A large number of genes were differentially expressed between uTregs and bTregs (Figure 3A), with significant upregulation of 1966 genes and downregulation of 1997 genes in uTregs (padj<0.05 and |Log2FC|>0.05). To isolate the uTreg specific signature, we also compared gene expression between uTreg and uTconv, yielding 465 upregulated genes, including the Treg-identifying genes FOXP3, IL2RA, CTLA4, TIGIT and IKZF2, and 103 downregulated genes in uTregs (padj<0.05 and |Log2FC|>0.5; Figure 3B) 236 genes were specifically upregulated (225 after removal of duplicate genes) and 23 genes specifically downregulated in uTregs compared to both bTregs and uTconv (Figure 3C and table S4 and S5). Among the downregulated genes were ITGA6, IL7R, CCR7, TTC39C, PLAC8, ATF7IP2, ABLIM1, MGAT4A, PRKCB, GIMAPs as well as transcription factors TCF7, LEF1, and SATB1, indicating late-stage
differentiation of Tregs. The 225 upregulated genes were involved in cytokine signaling, TNF receptor signaling, and glycolysis (Figure 3D). Selected genes from the top 5 pathways included those related to Treg activation and effector differentiation, such as immune checkpoints of the TNF receptor superfamily (TNFRSF13B (TACI), TNFRSF18 (GITR), TNFRSF1B (TNFR2), TNFRSF4 (OX40), TNFRSF8 (CD30), TNFRSF9 (4-1BB)) and HLA-DR, CD80, and LRRC32 (GARP). Also genes associated with suppressive capacity (CTLA4, ENTPD1, HAVCR2, IL10, IL2RA, LAG3, LAYN, LGALS1, PDCD1, and TOX2) were highly expressed in uTregs (Figure 3E). Furthermore, cytokine receptors of the IL-1 and IL-2 family (IL1R1, IL1R2, IL1RAP, IL1RN, IL2RA, IL2RB) and specific chemokine receptors (CCR1, CXCR6) showed increased and specific expression in uTregs (Figure 3E). Transcription factors specifically upregulated in uTregs included BATF, CEBPB, ETS2, ETV7, HES1, IKZF4, MAF, NFIL3, PRDM1, VDR, and ZBTB32 among others (Figure 3E). This transcriptomic profile, and especially high expression of BATF, PRDM1, and immune checkpoint molecules, reflects previously identified crucial signatures of effector Treg differentiation and function, especially in tissues. We confirmed upregulation of immune checkpoints associated with effector Treg differentiation/chronic stimulation GITR, OX-40, 4-1BB, and PD-1, HLA-DR, and ICOS in uTregs even compared to uTconv on protein level (Figure 3F). Since increased expression of many of these genes pointed towards an activated phenotype, we confirmed this by demonstrating significant enrichment of published gene sets of in vitro activated Tregs, in uTregs (Figure S2, table S3). Taken together, uTregs at the maternal-fetal interface have a highly differentiated transcriptional signature suggestive of a specialized function with high suppressive capacity and high responsiveness to environmental cues, which is reflective of late-stage effector differentiation and chronic activation.

uTregs have a tissue-resident phenotype and share transcriptional specialization with uTconv

To examine whether uTregs at the maternal-fetal interface represent a resident population or rather transiently infiltrating cells, we assessed the expression of tissue-residency related markers and gene signatures. uTregs had a significantly higher gene and protein expression of key residency molecule CD69.
than bTregs and bTconv, similar to uTconv (Figure 4A-B). Expression analysis and GSEA with published human TRM signatures showed a pattern of upregulated and downregulated genes as previously described for human TRM in general (Figure 4B), and specifically in CD4+ (and CD8+) TRM from lung and skin (Figure 4B), (4, 14, 86) confirming the tissue-resident profile in uTregs as compared to bTregs. Next, we identified the shared tissue-specific adaptation of uTregs and uTconv to the maternal-fetal interface. A large proportion of upregulated and downregulated genes was shared between uTregs and uTconv compared to their counterparts from blood (Figure S3A; 1032 genes up and 1348 down; padj<0.05 and |Log2FC|>0.05), which again suggests that the specific tissue environment at the maternal-interface accounts for a significant part of their adapted transcriptional profile. Shared upregulated genes were involved in cytokine signaling (Figure S3B), highlighting the integration of a spectrum of microenvironmental cues, while shared downregulated genes were reflective of ribosomal processes involved in RNA translation (Figure S3C). Taken together, uTregs have a TRM signature which reflects a shared adaptation to the tissue environment of the maternal-fetal interface between uTregs and uTconv.

**uTregs mirror uTconv Th1 polarization with a predominance of T-bet+CXCR3+ Tregs**

Effector Tregs can acquire different T helper phenotypes with coexpression of FOXP3 and lineage-defining transcription factors T-bet (TBX21, Th1), GATA3 (Th2), RORγt (RORC, Th17), as well as lineage-associated cytokine and chemokine receptors. (35) We investigated whether uTregs and uTconv underwent a, possibly shared, T helper polarization. uTregs showed significantly increased expression of Th1-related TBX21 compared to bTreg, which mirrored the increased expression of TBX21 in uTconv (Figure 5A). Th2-related GATA3 and Th17-related RORC were not significantly differentially expressed between uTreg and bTreg (and uTconv and bTconv), although RORC showed a trend towards downregulation, which was confirmed on protein level (Figure 5A-B). Increased expression of T-bet was also confirmed on protein level, with 6-87% (median 22%) of uTregs showing positivity for T-bet (Figure 5C-D). Also the Th1-related cytokine receptor IL18R1 was increased in both uTregs and uTconv compared to blood T cells on gene and protein level (Figure 5E). Investigation of chemokine receptor expression, related to both T helper
polarization and tissue-specific homing. (87, 88) showed that chemokine receptors associated with naive Tregs and lymphoid tissue environments CCR7 and CXCR5 were downregulated in uTregs compared to bTregs, on gene and protein level (Figure 5F-G). Chemokine receptors upregulated in uTregs included CCR2, CCR5, CXCR3, CXCR4, CCR1, and CXCR6 (Figure 5F and H), which largely mirrored expression by uTconv. CCR1 and CXCR6 were specifically upregulated in uTregs, both previously identified as part of the conserved murine tissue Treg signature. (18) The Th1-associated CXCR3 (36, 89) and Th1/inflammation-associated CCR5 (89–92) had significantly higher gene and protein expression in uTregs and uTconv compared to their counterparts from blood (Figure 5F and H). Although the variable percentage of T-bet+ Tregs suggests heterogeneity in uTreg subspecialization, virtually all uTregs (and uTconv) were positive for CXCR3 (84-100%, median 93%), and the majority expressed CCR5 (22-83%, median 62%) (Figure 5I). Consistent with these findings, a previously published gene signature of T-bet+CXCR3+ Tregs from the pancreas of prediabetic mice was highly enriched in uTregs compared to bTregs (Figure 5J). (38) In conclusion, uTregs at the maternal-fetal interface show Th1 polarization mirroring uTconv, with high expression of Th1-related markers T-bet and CXCR3. Furthermore, uTregs express an array of chemokine receptors, some of which uTreg-specific and others shared with uTconv, with which they can integrate a variety of locally produced signals. uTreg and uTconv cells may therefore rely on both unique and shared cues to guide their migration to and retention at the uterine maternal-fetal interface.

The uTreg signature at the maternal-fetal interface overlaps with tumor-infiltrating Treg signatures

We questioned whether the highly differentiated uTregs from the maternal-fetal interface would resemble Tregs from other human and murine tissue sites or would show a uniquely adapted profile. Well-studied murine tissue Treg populations include Tregs from visceral adipose tissue (VAT), muscle, and intestines. (12, 16, 18, 73, 93) Each population displays a tissue-specific phenotype with expression of certain 1) transcription factors, 2) chemokine receptors and 3) preference towards a T helper (Th) lineage differentiation when compared to spleen Tregs. (12, 13, 16, 18, 73) A murine PAN-tissue signature, shared by VAT, muscle and intestinal Tregs, was also identified. (18) GSEA in Figure 6A shows that the shared
murine PAN-tissue Treg signature was also strongly enriched in uTregs, again highlighting its generalized expression in tissue Tregs, apparently even conserved across species. Overlaying significantly upregulated genes in uTreg (versus bTreg) with murine tissue-specific or tissue-shared Treg signatures,(18) yielded a large amount of shared genes between uTregs and murine VAT-, colon- and muscle-derived Tregs (Figure 6B, numbers in each field represent overlap of the specific field with significantly upregulated genes in uTreg). 59 genes were shared among all 3 murine tissues and uTregs, including IL1RL1 (receptor for IL-33, ST2), AREG, IL10, IRF4, GZMB, TNFRSF9, BHLHE40, NR4A1, NR4A3, and CCR2, many of which have been described as crucial regulators for effector and/or tissue Treg function (Figure 6C).(12, 32, 40, 78–80, 94) 12 of the 59 genes were even part of the uTreg-specific core signature as defined in Figure 3 (CCR1, CXCR6, ELL2, FGL2, GEM, IL10, LAPT4M, SNX9, TNFRSF8, NFIL3, NR4A3, and PRDM1). This indicates that uTreg display features of tissue adaptation, which are highly conserved across tissues and species.

The investigation of human tissue-derived Tregs has proven challenging, and only limited data are available. To assess how uTregs compare to other human tissue Tregs, we analyzed enrichment of three previously published gene sets of significantly upregulated genes in healthy skin, colon and lung Treg compared to blood Treg (table S3).(14, 17) All three signatures were significantly enriched in uTregs compared to bTregs, indicating that the tissue profile of uTregs shows similarities with human Tregs from various tissue sites (Figure S4).

Human Tregs infiltrating the unique tissue-environment of tumors (TITR) have been studied more extensively. Comparison of genes significantly upregulated in uTregs versus bTregs with seven recently published gene signatures of TITR infiltrating a variety of human tumors (table S3),(25, 95–100) yielded a remarkable overlap with each of the TITR signatures with up to 65% of genes shared with uTregs (table S5). Of the 41 genes that were shared among ≥4 of the 7 TITR signatures (table S6), a notable 31 were also part of the 225 genes in the uTreg core signature. Figure 6D shows the number of genes shared between the uTreg core signature and each of the TITR signatures and healthy tissue-derived Treg signatures. Remarkably, 93 (41.3%) of the 225 core uTreg genes were overlapping with specifically upregulated genes
from HCC-infiltrating Tregs, (98) 54 with the unique TITR signature identified by De Simone et al, (25) 49 with breast cancer TITR genes, (95) and 40 with OX-40+ Treg from cirrhotic/tumor liver tissue (Figure 6D). (99) Importantly, the 225 uTreg core signature genes showed less overlap with healthy tissue-derived Treg specific signatures from human healthy colon, lung and skin. The genes that were most often shared between uTregs and TITR were IL1R2 (7/7), TNFRSF1B, CTSC, DPYSL2, LAPTMB4B (6/7), TNFRSF4, TNFRSF18, LAYN, IL2RA, ENTPD1, NCF4, SDC4 and CRADD (5/7) (Figure 6E), whereas with healthy tissue-Treg signatures PDGFA was most often shared (3/3) (Figure 6F).

GSEA showed that also many of the non-overlapping genes from the published TITR signatures were significantly enriched in uTreg compared to bTreg (Figure 7A). Genes in the leading edge of ≥3/7 tumor-specific GSEA analyses that were highly expressed in uTregs compared to bTregs, but not part of the uTreg core (mostly because their high expression was shared with uTconv), are shown in Figure 7B. These included CREB3L2 (6/7) EBI3, GCNT1, ICOS (5/7), ACTA2, ARHGEF12, BCL2L1, CCND2, PRDX3, SLAMF1 (4/7), CXCR3, CD7, CAECAM1, CD79B, and MICAL2 (3/7), amongst others. Remarkably, genes specifically upregulated in breast cancer-infiltrating Tregs compared to Tregs from normal breast parenchyma or significantly upregulated in colon cancer Tregs compared to healthy colon Tregs showed a particularly high enrichment in uTregs, suggesting that uTregs are not just similar to Tregs from breast or colon tissue, but specifically to the highly differentiated/activated Tregs from the tumor environment (Figure 7C). (26, 100) By overlapping these cancer-versus-healthy tissue Treg signatures with significantly upregulated genes in uTregs (versus bTregs), we identified 12 ‘cancer-specific’ genes expressed by uTregs (Figure 7D): CD80, IL1R2, LAYN, MYO7A, TNFRSF4, TNS3, TRAF3, VDR, DUSP4, HSPA1A, HSPA1B, and IFI6. The first 8 of these were also part of the uTreg-specific core signature, again highlighting the specificity c.q. importance of receptors IL1R2, LAYN, TNFRSF4, CD80 and transcription factor VDR for human Tregs in a tumor(-like) microenvironment. Also tumor-specific downregulated genes were shared with the uTreg core signature: CCR7, PLAC8, and TCF7. In conclusion, these results indicate that uTreg from the maternal-fetal interface have a transcriptional core signature which is shared specifically with the specialized transcriptional profile of tumor-infiltrating Tregs.
Next we wondered whether uTreg would be merely adapted to the microenvironment in uterine tissue, or specifically adapted to the tissue site at the maternal-interface. To investigate this site-specific adaption within one organ, we compared uTregs from the maternal-fetal interface, i.e. placental bed (pb\textsuperscript{uTregs}), to uTregs from a distant uterine site, i.e. the incision site made during Caesarean section (inc\textsuperscript{uTregs}). Confirmation of Treg identity and TRM signature for inc\textsuperscript{uTregs} is shown in Figure S5A-F. The differentially expressed genes between inc\textsuperscript{uTregs} and bTregs were similar to those between pb\textsuperscript{uTregs} and bTregs (Figure 8A). Also PCA showed that gene expression profiles of pb\textsuperscript{uTregs} and inc\textsuperscript{uTregs} were rather similar, compared to bTregs (Figure 8B). However, direct comparison of pb\textsuperscript{uTregs} and inc\textsuperscript{uTregs} revealed a substantial difference between the two populations (Figure 8C). First, protein expression of the core Treg transcription factor FOXP3 was lower in inc\textsuperscript{uTregs} than pb\textsuperscript{uTregs}, comparable to bTregs (Figure 8D). This was not due to inc\textsuperscript{uTreg} contamination with bTregs, since expression of CD69 was similar between pb\textsuperscript{uTregs} and inc\textsuperscript{uTregs} (Figure S5D). Protein expression of other core Treg markers CTLA-4 and TIGIT was also lower in inc\textsuperscript{uTregs} than pb\textsuperscript{uTregs} (Figure 8D). This indicates that pb\textsuperscript{uTregs}, derived from the maternal-fetal interface, have a more pronounced expression of Treg signature markers, suggesting enhanced activation/differentiation in comparison with their uterine counterparts from the incision site. Differential gene expression analysis revealed 558 upregulated and 125 downregulated genes in pb\textsuperscript{uTregs} versus inc\textsuperscript{uTregs} (Figure 8E).

The heatmap in Figure 9A shows a selection of previously highlighted genes in this manuscript that proved to be differentially expressed between pb\textsuperscript{uTregs} and inc\textsuperscript{uTregs}. These results suggest that Tregs cannot only adapt to the microenvironment within a certain tissue, but will specifically adapt to the environmental cues at a certain tissue site. Pathway analysis showed that upregulated genes pb\textsuperscript{uTregs} versus inc\textsuperscript{uTregs} were related to PD-1 signaling, cytokine signaling, TCR signaling, and T helper cell differentiation (Figure S5G). Indeed, PD-1 was higher expressed in pb\textsuperscript{uTregs} and inc\textsuperscript{uTregs} on gene and protein level (Figure 9A-B), and GSEA showed enrichment of a TCR-activated Treg signature in pb\textsuperscript{uTregs} compared to inc\textsuperscript{uTregs} (Figure
Furthermore, pbuTreg-specific core genes associated with effector Treg differentiation including TNFRSF4 (OX-40 protein, Figure 9C) and transcription factors BATF, MAF, PRDM1, and VDR, among others, were significantly higher expressed in pbuTreg than in incuTreg (Figure 9A), again suggesting that pbuTreg show more pronounced differentiation towards an effector Treg phenotype. Since pbuTregs appeared to be especially differentiated at the maternal-fetal interface, we assessed whether the TITR-like profile of pbuTregs was also more pronounced than in incuTregs. Remarkably, 5 out of 7 tested published TITR signatures were significantly enriched in pbuTregs compared to incuTregs (P<0.05; Figure 9D). More specifically, GSEA with signatures differentiating between TITR and their counterparts from a matched healthy tissue site, showed significant enrichment in pbuTregs compared to incuTregs (Figure 9E). CCR8 and ICOS, which were present in in 6 out of 7 TITR signatures, as well as TNFRSF18 (GITR), were significantly higher expressed in pbuTregs than in incuTregs and bTregs on protein level (Figure 9F). CCR8 has been shown to be highly enriched in tumor Treg cells and associated with a poor prognosis in several cancers. Thus, pbuTregs at the maternal-fetal interface specifically acquire a highly differentiated effector profile similar to tumor-infiltrating Tregs, which is more pronounced even compared to a uterine tissue site distant from the maternal-fetal interface.

Discussion

Here, we demonstrate for the first time that, in pregnancy, human uterine Tregs have a highly differentiated transcriptional profile, which is specifically enriched at the maternal-fetal interface and is reminiscent of the specialized profile of tumor-infiltrating Tregs. With these findings we answer a long-standing question on how Tregs are functionally specialized at the maternal-fetal interface to modulate local effector T cell responses, preventing an allo-reaction against the fetus. Moreover, we introduce the novel concept of site-specific adaptation of Tregs within one organ or tissue. This again substantiates the notion that Tregs are capable of adapting their transcriptional program driven by micro-environmental cues.
We have demonstrated that uTregs at the maternal-fetal interface display a highly activated and late-stage differentiated effector profile, (32, 34, 76, 77, 79) with high BATF and PRDM1, low SATB1, increased expression of molecules associated with suppressive capacity and abundant expression of TNFR superfamily members. Also others found that non-lymphoid-tissue Tregs display an activated phenotype compared to lymphoid-organ and circulating Tregs.(12, 20, 73) Both BATF and the TNFRSF-NF-κB signaling axis are crucial in the survival of Tregs and maintenance of a stable effector Treg phenotype, especially in tissues.(15, 28, 32, 33, 78, 79, 101) It is now recognized that Tregs adapt to their tissue environments, with common adaptations across many tissues, such as increased expression of IL10, IL1RL1 (encoding ST2, an IL-33 receptor subunit), AREG (encoding amphiregulin), CTLA4, TIGIT, BATF and IRF4, and low expression of LEF1 and TCF7, but, importantly, also tissue-specific signatures.(12, 13, 15, 16, 18) These tissue-specific signatures counter the notion that tissue Tregs merely have a more activated, effector or memory state. Rather, they have a specialized adapted program,(93) likely matching the specific requirements of a certain tissue site.(10, 13, 18, 102, 103)

To our knowledge, the concept of site-specific transcriptional adaptation of Tregs within one tissue or organ is novel, taking into account that tumors represent a completely altered tissue and not a different site within the same organ. We show that uTregs display features suggestive of a high responsiveness to micro-environmental cues, such as a range of TNF receptor superfamily members and chemokine receptors. With such a matrix of options to detect signals from the microenvironment, Tregs are likely able to adjust not only to the tissue or organ of their residence, but even to specific sites within that tissue, based on micro-environmental cues. Most likely, implantation of the placenta, i.e. the multitude of signals produced by myometrium-invading trophoblast,(104) are the primary cues effectuating micro-environmental changes at the maternal-fetal interface. Trophoblast attracts Tregs to the maternal-fetal interface by production of hCG and CXCL16, the ligand for CXCR6.(105, 106) Moreover, in vitro co-culture of HLA-G+ extravillous trophoblast with CD4+ T cells increased Treg numbers and FOXP3 expression level,(107, 108) indicating that Tregs may also be locally induced or expanded by trophoblast. Thus, it is likely that signals produced
by invading trophoblast at the maternal-fetal interface account for at least some of the site-specific transcriptional adaptations in uTregs.

The T helper response at the maternal-fetal interface was previously suggested to be skewed away from a pro-inflammatory Th1 response, to prevent a pathogenic allo-reaction against the fetus, resulting in a Th2 dominant response during the second trimester. However, during the third trimester, a pro-inflammatory Th1 response may be essential for initiation of labor.(reviewed in(48)) In line with this, our findings indicate that the T helper response in the uterus at term is dominated by Th1 polarization, although well-controlled. uTregs at the maternal-fetal interface appear to be specifically equipped to effectively suppress Th1 responses. Although we observed heterogeneity of T-bet protein expression in uTreg, CXCR3 expression was remarkably homogeneous, with 84-100% of uTregs being CXCR3+. CXCR3 (and T-bet) expressing Tregs are especially adept to suppress Th1 responses.(36, 38, 45, 89) Furthermore, the majority of uTregs expressed TIGIT, OX-40 and/or CCR5. Tregs expressing TIGIT preferentially inhibit Th1 and Th17 responses,(72) a subpopulation of OX-40 expressing Tregs can differentiate into Th1-suppressing Tregs,(109) and also CCR5 expression on Tregs has been associated with more effective suppression of Th1 responses.(90) Thus, the necessary pro-inflammatory Th1 response at the maternal-fetal interface at term appears to be controlled by specifically differentiated and Th1-polarized Tregs. So far, Th1-like Tregs have been described mainly in inflammatory environments, such as infections, autoimmune diseases and transplantation reactions,(45, 89, 110–112) whereas tissue-resident Tregs were mostly characterized as being Th2-skewed (VAT, muscle)(13, 18) or Th17 skewed (intestines).(73) DiSpirito et al. however recently also identified a subset of T-bet expressing Tregs in muscle and colon,(18) indicating that they can be present also in steady-state tissues.

We are the first to study exclusively maternal, myometrial tissue-resident Tregs from the maternal-fetal interface. Previous studies of uterine Tregs had to resort to the use of more easily accessible decidua, due to the difficulty of acquiring human myometrium. Since decidual tissue is of fetal origin, it may not only be contaminated with fetal immune cells, but it also does not allow for studying the unique, and specifically maternal, uterine environment underlying the placenta, in which the complex process of spiral artery
remodeling takes place. The only publications that we know of investigating FOXP3 expression in actual human placental bed biopsies demonstrated that the percentage of FOXP3⁺ T cells was significantly decreased in patients with pre-eclampsia, and FOXP3 mRNA expression was reduced in endometrial biopsies of infertile women, highlighting the importance of functional Tregs for a healthy pregnancy. (57, 113) From human decidual data, it is known that the frequency of clonally expanded populations of effector Treg cells is increased in decidua of 3rd trimester cases compared to 1st trimester cases. (114) Decidual Tregs display a more pronounced suppressive phenotype than in blood, with increased expression of FOXP3, CTLA-4, CD25, HLA-DR, ICOS, GITR, and OX-40, which recapitulates our findings. (58, 59, 63, 115) Recently, three types of functional regulatory T cells were identified at the human maternal-fetal interface, of which the CD25⁺FOXP3⁺ population matches the here studied population. (108) These Tregs effectively suppressed CD4⁺ and CD8⁺ T cell proliferation and IFNγ and TNFα production. Transcripts identified by qPCR array as specific for this subset were IL2RA, FOXP3, TIGIT, ENTPD1, LRRC32, IL1RL1, BATF, and CCR8, as well as increased expression of CCR5, IL10, and GITR compared to blood Tregs. (108) which confirms our findings of an activated Treg phenotype at the maternal-fetal interface. A study investigating chemokine receptor expression of CXCR3, CCR4, and CCR6 in decidual Tregs by flow cytometry, showed that CCR6⁺CXCR3⁺ Th1 cells were increased, CCR6⁺CCR4⁺ Th17 cells were nearly absent, whereas CCR4⁺ Th2 frequencies were similar in blood and decidua. (58) which is also in line with our findings. Taken together, this indicates that the here identified activated phenotype of myometrial uTregs has overlapping characteristics with decidual Tregs.

We observed that uTregs from the maternal-fetal interface display a peculiar differentiated effector phenotype similar to TITR, defined by high expression of IL1R2, LAYN, CD80, VDR, and TNFRSF4, amongst others, with specific enrichment of TITR signatures compared to Treg signatures from matched, unaffected tissue sites. This observation may be explained by recent insights on the similarity of the immune environment at the maternal-fetal interface and tumors. (48) Both the receptivity of the myometrium towards implantation of the blastocyst and invasiveness of the trophoblast show striking similarities with implantation of tumor metastases in healthy tissues. (116, 117) Tumor cells can modulate their immune
environment into an anti-inflammatory milieu and can recruit and/or induce suppressor cells among which high numbers of Tregs.(118, 119) Just as in tumors, a tolerogenic mode of antigen presentation with indirect allore cognition of low levels of antigens predominates at the maternal-fetal interface.(120) Also others reported striking similarities between early Treg responses to embryo and tumor implantation.(54) These findings imply that the micro-environment at the maternal-fetal interface may be a unique mammalian tissue site that under challenged, but physiological conditions resembles a tumor micro-environment: an actively remodeling tissue site distinct from a steady-state tissue, with low-grade inflammation, and newly infiltrating/invading cells. These dynamic characteristics may account for the unique transcriptional adaptation of Tregs.

Although we observed global changes in gene expression patterns in uTregs, flow cytometry revealed an expression gradient of many markers across the uTreg population, suggesting that uTregs consist of a heterogenous population with various stages of differentiation and possibly sub-specialization. Single cell sequencing techniques and mass cytometry are indeed starting to reveal the heterogeneity of Treg populations in tissues and tumors.(15, 27, 97, 98, 121–123)

A unique strength of our study is that we compared the transcriptomic profile of a highly specific and highly purified maternal Treg cell subset from myometrial biopsies, not only to their counterpart in blood, but also to a tissue and site-specific Treg control population and matched Tconv. We validated our key findings on protein level in single cell resolution by flow cytometry. We only studied term pregnancies, due to the practical limitation of delivery of the infant and placenta. It would therefore be interesting to investigate term-dependent changes in uTreg profiles in future studies. Although protocols for tissue digestion may induce transcriptional changes,(124) many of the uTreg specific genes were previously found not to be affected by a tissue digestion protocol similar to but harsher than the one used here.(17)

Our findings have important implications. TITR are currently under heavy investigation as targets in cancer immunotherapy. However, we demonstrate that signatures identified in TITR are not as unique as previously assumed, and that they may be shared by Tregs with specialized functions in other human tissues that may still be unknown. On the other hand, our results may lead to new targets for cancer immunotherapy,
since profiling of Tregs in a variety of tissues under physiological, but not necessarily steady-state conditions, may help to identify truly TITR-specific expression patterns. Moreover, increased understanding of immunoregulatory mechanisms at the maternal-fetal interface during healthy pregnancy gives not only unique insights into human immunobiology of pregnancy, but also aids to elucidate the pathological changes in Tregs in pregnancy disorders such as preeclampsia, fetal growth restriction or recurrent miscarriage, as many studies have pointed towards a role for Treg defects of deficiency in these disorders. (65–67, 105, 114, 125, 126) Lastly, functional adaptation of human Tregs to different tissues and specific tissue sites is still largely unexplored. The receptivity of Tregs to their environmental stimuli and subsequent sub-specialization may be exploited for therapeutic purposes.

In conclusion, we have shown that human Tregs show functional adaptation with tumor-infiltrating-like features specifically at the maternal-fetal interface, which introduces the novel concept of tissue site-specific transcriptional adaptation of human Tregs.

**Methods**

*Study design: Participants and biopsies*

This study is part of the Spiral Artery Remodeling (SPAR) cohort study, which is an on-going effort to investigate the adaptation of the uterus to placental development by obtaining site-specific uterine biopsy samples in women undergoing Caesarean section. Detailed description of the study set-up and protocol was previously published. (127) For this analysis, we included 20 women who delivered by elective Caesarean section, i.e. without any contractions or other signs of labour such as rupture of membranes, after an uneventful pregnancy and without any major underlying pathology, N=5 of which were included for transcriptomics of T cell populations, N=4 for suppression assays, and N=11 for flow cytometry. Baseline characteristics are provided in table S1. One tube of sodium-heparin blood was taken from each donor.
before Caesarean section. After delivery of the neonate and placenta, the placental bed was manually located and two biopsies of the central placental bed from the inner uterine myometrial wall were obtained as previously described.(127) Additionally, biopsies were taken from the incision site when the placenta was not situated on this part of the uterine wall.

**Lymphocyte isolation**

Peripheral blood mononuclear cells (PBMC) were isolated from blood diluted 1:1 with basic medium (RPMI 1640 (Gibco) with Penicillin/Streptomycin (Gibco), L-glutamine (Gibco)), by ficoll-density centrifugation (GE Healthcare-Biosciences, AB). PBMC were washed in basic medium with 2% fetal calf serum (FCS, Biowest) and PBS or staining buffer consisting of cold PBS supplemented with 2% FCS and 0.1% sodium-azide (Severn Biotech Ltd.). The biopsy samples were collected in basic medium supplemented with 10% FCS and minced into pieces of 1 mm³ in PBS (Gibco). The biopsies were enzymatically digested with 1 mg/mL collagenase IV (Sigma) in medium for 60 minutes at 37°C in a tube shaker under constant agitation at 120 rpm. To dissolve the remaining biopsy pieces after digestion and remove any remaining lumps, the biopsies were pipetted up and down multiple times and poured over a 100 µm Cell Strainer (BD Falcon). Cells were subsequently washed in staining buffer and filtered through a 70 µm cell strainer and prepared for flow cytometry or flow cytometry-assisted cell sorting.

**Flow cytometry**

For flow cytometric experiments without restimulation, PBMC and uterine cells were first incubated in Fixable viability dye eFluor506 (eBioscience) 1:300 in PBS for 20 min at 4°C, and washed in PBS. For surface staining cells were incubated with the antibodies shown in table S2 for 20 minutes in staining buffer at 4°C, and subsequently washed in the same buffer. Cells were permeabilized with 1 part fixation/permeabilization concentrate and 3 parts fixation/permeabilization diluent (eBioscience) for 30 minutes at 4°C and subsequently incubated overnight with intracellular antibodies (table S2) in 10x diluted Permeabilization buffer (Perm, eBioscience) 4°C. The next day, cells were washed with Perm and measured
on the LSR Fortessa (BD). For intracellular cytokine measurement, PBMC and uterine cells were first incubated with surface staining, washed, and then restimulated with 20 ng/ml phorbol 12-myristate 13-acetate (PMA, Sigma) and 1 μg/ml ionomycin (Sigma) for 4 hours with addition of Monensin (Golgistop, BD Bioscience) during the last 3.5 hours at 37°C. Afterwards, cells were stained with the viability dye, permeabilized, intracellularly stained and measured as described above.

**Flow cytometry-assisted cell sorting**

Cells were incubated with surface antibodies (table S2) for 20 minutes in staining buffer at 4°C, washed in the same buffer and filtered through a 50 µm cell strainer (Filcon, BD). For suppression assays, cells of the CD3+CD4+CD25+CD127- cell population (Tregs) and CD3+CD4+CD25- cell population (Tconv) were directly sorted into tubes with 500 µL FCS on a FACSArray™ III (BD). For RNA sequencing, 2000 cells of the CD3+CD4+CD25+CD127- cell population (Tregs) and CD3+CD4+CD25-CD45RA- (CD69+ from biopsies, CD69- from blood) cell population (Tconv) were sorted into Eppendorfs containing 125 µL PBS. After sorting, 375 µL Trizol LS (Thermo Fisher Scientific) was added to each vial and vials were stored at -80°C until RNA isolation.

**Suppression assays and cytokine measurement**

After sorting, peripheral blood and uterine Tregs and Tconv were washed in PBS and resuspended in basic medium with 10% human AB serum (Sanquin). Previously isolated and frozen healthy donor (HC) PBMC were labelled with 2 µM CellTrace Violet (ThermoFisher) as described previously.(128) Treg or Tconv populations were added to 15,000 HC PBMC at different ratios and cells were co-incubated for 4 days at 37°C. Supernatants were collected for cytokine measurement by multiplex assay before cells were stained with surface antibodies for CD3, CD4 and CD8 as described above and measured on a FACS Canto (BD).
Whole transcriptome sequencing

For RNA isolation, the vials were thawed at room temperature and 100 µL chloroform was added to each vial. The vials were shaken well and spun down at 12000g for 15 minutes at 4°C. The aqueous phase was transferred into a new tube and RNA was mixed with 1µl of GlycoBlue (Invitrogen) and precipitated with 250 µL isopropanol. Cells were incubated at -20°C for one hour and subsequently spun down at 12000g for 10 minutes. The supernatant was carefully discarded and the RNA pellet was washed twice with 375 µL 75% ethanol. Vials were stored at -80°C until library preparation. Low input RNA sequencing libraries from biological sorted cell population replicates were prepared using the Cel-Seq2 Sample Preparation Protocol(129) and sequenced as 2 x 75bp paired-end on a NextSeq 500 (Utrecht Sequencing Facility). The reads were demultiplexed and aligned to human cDNA reference using the BWA (0.7.13).(130) Multiple reads mapping to the same gene with the same unique molecular identifier (UMI, 6bp long) were counted as a single read.

Data analysis and statistics

RNA sequencing data were normalized per million reads per sample. Differentially expressed genes between the cell populations were identified using the DESeq2 package in R 3.5.1 (CRAN), with correction for donor batch (design=~Donor+Cellpop) and input of all genes. Genes with false discovery rate (FDR) adjusted p value (padj)<0.05 and log2(Fold Change)>0.5 or <-0.05 (further annotated as |Log2FC|>0.5) were considered differentially expressed. Principal component analysis (PCA) was performed in DESeq2 based on the constructed model including donor correction. Pathway enrichment analysis was conducted in Toppgene Suite publicly available online portal and pathways with Bonferroni-corrected p-values<0.05 were considered statistically significant.(131) For heatmap analysis, gene expression was mean-centered and scaled per gene and hierarchical clustering was performed with Ward’s method and Euclidian distance. Gene set enrichment analysis (GSEA(132)) was conducted with Broad Institute software, by 1000 random permutations of the phenotypic subgroups to establish a null distribution of enrichment score, against which a normalized enrichment scores and multiple testing FDR-corrected q values were calculated. Gene sets
with an FDR<0.05 were considered significantly enriched. Gene sets were either obtained from provided data in publications or by analyzing raw data using GEO2R (NCBI tool).(133) An overview of used signatures is provided in table S3. For flow cytometric data, median fluorescent intensities (MFI) and percentages of positive cells were analyzed in FlowJo (LLC). For graphic representation, data were analyzed in GraphPad Prism (GraphPad Software). To assess significant differences on protein level between groups, Two-way ANOVA with Tukey post hoc test was used and multiplicity-adjusted p values<0.05 were considered statistically significant.

The datasets generated for this study have been submitted to a public repository on GitHub (https://github.com/JudithWienke/Human-uterine-Tregs). The raw data files could not be submitted due to GDPR constraints, but any additional required data can be requested with the corresponding author.

**Study approval**

All patients received study information and signed informed consent prior to participation. This study was reviewed and approved by the local Institutional Ethical Review Board of the University Medical Center Utrecht (16-198).
Author contributions

LB recruited and included patients, and collected clinical data. JW, LB, RS and LvdB performed all wet-lab experiments. MM performed the RNA sequencing and helped with data-analysis. JW performed all data-analyses and wrote the manuscript. PN consulted on biopsy preparation, tissue integrity and uterine T cell distribution and phenotype. BvR and FvW supervised JW, LB, LvdB and RS, and were closely involved in setting up the study protocol, collection of data, data analysis and writing of the manuscript. All authors critically revised the manuscript. The authors have declared that no conflict of interest exists. All authors declare no other support from any organization for the submitted work than the grants reported in the funding section; no financial relationships with any organizations that might have an interest in the submitted work, no other relationships or activities that could appear to have influenced the submitted work.

Acknowledgements

We thank Michal Mokry, Noortje van Dunen and Nico Lansu for their help with RNA sequencing. We thank the multiplex core facility, and especially Jeroen van Velzen and Pien van der Burght for their advice and (both practical and moral) support during FACS sorting. We thank Tatjana Vogelvang for her help in the collection of samples at the Diakonessenhuis in Utrecht and Arie Franx for his involvement in establishing the SPAR study initiative at the Wilhelmina Children’s Hospital. FW was supported by a VIDI grant (91714332) from the Netherlands Organization for Scientific Research (NWO, ZonMW).
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Figure 1. Tregs at the maternal-fetal interface are bona fide Tregs. (A) Principal component analysis of bTregs, bTconv, uTregs and uTconv (all n=5). (B+C) Gene set enrichment analysis (GSEA) with published Treg signature gene set (71) comparing uTreg and uTconv (B) and uTreg and bTreg (C) (all n=5). NES = normalized enrichment score, FDR = False Discovery Rate adjusted P value. (D) Heatmap of genes in leading edge of GSEA analysis comparing enrichment of published Treg signature genes in uTregs and bTregs. Expression values were mean-centered and scaled per gene. (E) Representative gating strategy of bTregs, uTregs and uTconv out of 5 experiments. (F) Representative expression of CTLA-4 in uTregs out of 6 experiments. (G) Ex vivo protein expression of core Treg molecules FOXP3 (n=10) and CTLA4 (n=6) measured by flow cytometry. (H) Ex vivo protein expression of Treg signature molecule TIGIT (n=6) measured by flow cytometry. (G+H) Multiplicity adjusted P value of Two-way ANOVA with Tukey post hoc test. MFI = median fluorescent intensity. (I) GSEA of TIGIT+ Treg signature (n=5). NES = normalized enrichment score, FDR = False Discovery Rate adjusted P value. uTreg = uterine Treg, bTreg = blood Treg, uTconv = uterine CD4+ conventional T cells (non-Tregs), bTconv = blood CD4+ conventional T cells (non-Tregs).
Figure 2. Tregs at the maternal-fetal interface are bona fide Tregs with suppressive capacity (A-D) Suppression assay assessing cytokine production of IL-2 (A), IFNγ (B), TNFα (C), and IL-10 (D) by anti-CD3 stimulated (or unstimulated) healthy CD4+ T cells in the supernatant by multiplex immunoassay after 4 days of coculture with healthy donor bTregs, maternal bTregs, or uTregs at a 1:8, 1:4 and 1:2 ratio. Data represent median with interquartile range. N=4 donors, but not every condition could be measured for each donor due to limited availability of material. Therefore, some conditions contain data from 3 donors. uTreg = uterine Treg, bTreg = blood Treg, HC = healthy non-pregnant control.
Figure 3. The uTreg core signature. (A+B) Volcano plot of differential gene expression between uTregs and bTregs (A) or uTregs and uTconv (B) (all n=5). (C) Venn diagrams yielding genes specifically upregulated (padj<0.05 and Log2FC>0.05, upper panel) or downregulated (padj<0.05 and Log2FC<-0.5, lower panel) in uTreg compared to bTreg and uTconv. (D) Pathway analysis (ToppGene pathways) of 236 genes specifically upregulated in uTregs. P-values<0.05 after Bonferroni correction were considered significant. (E) Heatmap showing gene expression of genes in top 5 pathways and selected downregulated genes in the uTreg core signature, related to Treg activation or effector differentiation (upper panel), cytokine signaling (middle panel; including downregulated CCR7 and IL7R) and transcription factors (lower panel). Expression values were mean-centered and scaled per gene. (F) Protein expression of GITR (TNFRSF18), OX-40 (TNFRSF4), 4-1BB (TNFRSF9), PD-1 (PDCD1), HLA-DR and ICOS. uTregs were gated as CD3+CD4+CD25hiFOXP3+ cells. Multiplicity adjusted P value of Two-way ANOVA with Tukey post hoc test. N=6 each. MFI = median fluorescent intensity; NS = Not significant.
Figure 4. Tregs at the maternal-fetal interface have a tissue-resident profile. (A) Gene and protein expression of CD69 in sorted T cell populations. DE = differentially expressed genes with padj<0.05. Boxplots with median; box indicates 25th to 75th percentiles, whiskers indicate min and max values (n=5). FACS data: Representative plot of 5 experiments, uTregs were gated as CD3⁺CD4⁺CD25hiCD127⁻. MFI = median fluorescent intensity. Two-way ANOVA with Tukey post hoc test. (B) Heatmap with a published human core tissue-resident signature (4) in uTreg compared to bTreg. Expression values were mean-centered and scaled per gene. (C) Gene set enrichment analysis (GSEA) with published genes identifying human lung CD4⁺ and CD8⁺ TRM compared to blood memory cells; left panel(86)) and genes upregulated/downregulated in skin CD4⁺ TRM compared to blood CD4⁺ T cells (right panel(14), in uTregs vs bTregs. NES = normalized enrichment score. FDR = False Discovery Rate adjusted P value.
Figure 5. uTreg and uTconv polarization at the maternal-fetal interface. (A) Gene expression of lineage-defining transcription factors *TBX21* (T-beta), *GATA3* (GATA-3), and *RORC* (RORγt). P values from differential gene expression analysis. DE = differentially expressed padj<0.05. Boxplots with median; box indicates 25th to 75th percentiles, whiskers indicate min and max values (all n=5). (B-D) Protein expression of RORγt (B) and T-beta (C-D). uTregs were gated as CD3+CD4+CD25hiFOXP3+ cells. MFI = median fluorescent intensity. Multiplicity adjusted P values of two-way ANOVA with Tukey posthoc test. (n=5) (E) Gene and protein expression of *IL18R1* (IL-18R1). Gene expression: Boxplots with median; box indicates 25th to 75th percentiles, whiskers indicate min and max values (n=5). DE = differentially expressed padj<0.05. Protein expression: uTregs were gated as CD3+CD4+CD25hiFOXP3+ cells. Multiplicity adjusted P values of two-way ANOVA with Tukey posthoc test. MFI = median fluorescent intensity. (n=5) (F) Heatmap showing gene expression of chemokine receptors. Expression values were mean-centered and scaled per gene. DE = differentially expressed padj<0.05. (G-I) Protein expression of chemokine receptors downregulated (G) and upregulated (H+I) in uTregs. uTregs were gated as CD3+CD4+CD25hiFOXP3+ cells. P values of two-way ANOVA with Tukey posthoc test. MFI = median fluorescent intensity. (n=5) (J) Gene set enrichment analysis with published gene set of CXCR3+T-beta+ Tregs from the pancreas of prediabetic mice,(38) comparing uTregs and bTregs. NES = normalized enrichment score, FDR = False Discovery Rate adjusted P value.
Figure 6. uTregs share their transcriptional signature with tissue- and tumor-infiltrating Tregs. (A) Gene set enrichment analysis with a published murine PAN-tissue gene signature, (18) comparing uTregs and bTregs. NES = normalized enrichment score, FDR = False Discovery Rate adjusted P value. (B) Venn diagram showing the numbers of genes upregulated in uTregs compared to bTregs (padj<0.05) (and genes in the uTreg core signature in parentheses), which are represented in tissue-specific and tissue-shared published murine gene signatures. (18) VAT = Visceral adipose tissue. (C) Heatmap showing the expression of the 59 genes that were part of the murine PAN-tissue signature and upregulated in uTregs compared to bTregs (padj<0.05). (18) Expression values were mean-centered and scaled per gene. (D) The number of genes shared between the uTreg core signature and published human TITR signatures or healthy tissue Treg signatures. (14, 17, 25, 95–100) Numbers behind bars indicate the number of shared genes out of the total number of genes in the specific signature. (E) The genes that were most often shared between the uTreg core signature and human TITR signatures (shared in >3/7 signatures). (F) The genes that were most often shared between the uTreg core signature and human healthy tissue Treg signatures (shared in >1/3 signatures).
Figure 7. uTregs have a functional profile similar to tumor-infiltrating Tregs. (A) Gene set enrichment analysis (GSEA) with published TITR-specific signatures in uTregs vs bTregs. (25, 95–100) NES = normalized enrichment score, FDR = False Discovery Rate adjusted P value. (B) Heatmap showing expression of genes in the leading edge of >2/7 GSEA analyses from figure 7A, which were not represented in the uTreg core signature. Expression values were mean-centered and scaled per gene. (C) GSEA with published gene signatures specific to Tregs from tumor-tissue compared to the healthy tissue counterpart in uTregs vs bTregs. (26, 100) NES = normalized enrichment score, FDR = False Discovery Rate adjusted P value. (D) Venn diagrams showing shared genes between uTregs and genes specifically upregulated in Tregs from tumor-tissue compared to the healthy tissue counterpart. (26, 100)
Figure 8. uTregs show site-specific adaptation to the maternal-fetal interface. (A) Venn diagrams of genes upregulated (left panel) and downregulated (right panel) in both incuTregs and pbuTregs compared to bTregs. (B) PCA of bTregs (n=5), pbuTregs (n=5) and incuTregs (n=4). (C) PCA of pbuTregs (n=5) and incuTregs (n=4). (D) Protein expression of FOXP3, CTLA-4, and TIGIT. uTregs were gated as CD3+CD4+CD25hiFOXP3+ cells. Multiplicity adjusted P value of Two-way ANOVA with Tukey post hoc test. Left upper p-value: blood vs placental bed; right upper p-value: placental bed vs incision site; lower p-value: blood vs incision site. MFI = median fluorescent intensity; NS = Not significant. (E) Volcanoplot of differentially expressed genes between pbuTregs and incuTregs. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.
Figure 9. uTregs show site-specific adaptation to the maternal-fetal interface. (A) Heatmap with previously highlighted genes in this manuscript which were differentially expressed between \( p_u \)Turegs and \( i_u \)Turegs. Expression values were mean-centered and scaled per gene. (B+C) Protein expression of PD-1 (B) and OX-40 (C). uTregs were gated as CD3\(^+\)CD4\(^+\)CD25\(^{hi}\)FOXP3\(^+\) cells. Multiplicity adjusted p value of Two-way ANOVA with Tukey post hoc test. Left upper p-value: blood vs placental bed; right upper p-value: placental bed vs incision site; lower p-value: blood vs incision site. MFI = median fluorescent intensity; NS = Not significant. (n=6) (D) Gene set enrichment analysis (GSEA) with published TITR-specific signatures in \( p_u \)Turegs vs \( i_u \)Turegs. (25, 95–100) NES = normalized enrichment score. FDR = False Discovery Rate adjusted P value. (E) GSEA with published gene signatures specific to Tregs from tumor-tissue compared to the healthy tissue counterpart in \( p_u \)Turegs vs \( i_u \)Turegs. (26, 100) (F) Protein expression of CCR8 (n=5), ICOS (n=6) and GITR (n=6). uTregs were gated as CD3\(^+\)CD4\(^+\)CD25\(^{hi}\)FOXP3\(^+\) cells. Multiplicity adjusted P value of Two-way ANOVA with Tukey post hoc test for protein. Left upper p-value: blood vs placental bed; right upper p-value: placental bed vs incision site; lower p-value: blood vs incision site. MFI = median fluorescent intensity; NS = Not significant. ****P<0.0001, ***P<0.001, **P<0.01, *P<0.05.