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The Human Fetal Placenta Promotes Tolerance against the Semiallogeneic Fetus by Inducing Regulatory T Cells and Homeostatic M2 Macrophages

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A successful pregnancy requires that the maternal immune system is instructed to a state of tolerance to avoid rejection of the semiallogeneic fetal–placental unit. Although increasing evidence supports that decidual (uterine) macrophages and regulatory T cells (Tregs) are key regulators of fetal tolerance, it is not known how these tolerogenic leukocytes are induced. In this article, we show that the human fetal placenta itself, mainly through trophoblast cells, is able to induce homeostatic M2 macrophages and Tregs. Placental-derived M-CSF and IL-10 induced macrophages that shared the CD14+CD163+CD206+CD209+ phenotype of decidual macrophages and produced IL-10 and CCL18 but not IL-12 or IL-23. Placental tissue also induced the expansion of CD25highCD127lowFoxp3+ Tregs in parallel with increased IL-10 production, whereas production of IFN-g was limited. Placental-derived factors limited excessive Th cell activation, as shown by decreased HLA-DR expression and reduced secretion of Th1-, Th2-, and Th17-associated cytokines. Thus, our data indicate that the fetal placenta has a central role in promoting the homeostatic environment necessary for successful pregnancy. These findings have implications for immune-mediated pregnancy complications, as well as for our general understanding of tissue-induced tolerance. The Journal of Immunology, 2015, 194: 000–000.

Decidual macrophages are the most abundant APCs throughout pregnancy (5), and they are central in setting the balance between tolerance and proinflammatory responses. Human decidual macrophages have properties predominantly associated with homeostatic M2 macrophages, including expression of the homeostatic scavenger receptor CD163 and the pattern recognition receptors CD206 and CD209, as well as the preferential production of cytokines and chemokines like IL-10, CCL2, and CCL18 (6–11). Decidual macrophages are also functionally regulatory, being able to suppress the production of IFN-g by T cells (12) and to induce Tregs in vitro (13, 14). Tregs are essential to the establishment and maintenance of pregnancy, as shown by murine studies (15, 16). Given their crucial role in both syngeneic and allogeneic pregnancies, Tregs are likely to have a central role in the tolerance of paternal Ags and the general maintenance of a homeostatic environment compatible with fetal survival. In humans, Tregs accumulate in the decidua and show an activated and suppressive phenotype, with high expression of CD25, Foxp3, and CTLA-4 (17, 18).

Although decidual macrophages and Tregs were demonstrated to facilitate pregnancy, the factors that regulate these cells in humans have not been well characterized. The microenvironment has a great influence on leukocyte development; thus, the placenta, being a newly developed and temporary organ closely associated with decidual leukocytes, is a potential candidate for inducing the maternal immune tolerance that is needed for protecting both itself and the fetus. It is noteworthy that, although the placenta is known to be an important source of immune-modulating factors (2), the capability and relative contribution of these factors in the induction of regulatory decidual leukocytes have not been addressed in a physiological setting. In this article, we show that the human fetal placenta itself, particularly through trophoblast cells, is able...
to create a homoeostatic and tolerant environment by producing soluble factors (M-CSF, IL-10, TGF-β, and TRAIL) that induce the polarization of homoeostatic macrophages and the expansion of Tregs, as well as limit excessive Th cell activation.

Materials and Methods

Subjects

First-trimester placental tissues were collected from 45 healthy pregnant women (median age, 25 y; range: 16–42 y) undergoing elective surgical abortions at Linköping University Hospital (Linköping, Sweden) (n = 29) or at the Royal Victoria Infirmary (Newcastle upon Tyne, U.K.) (n = 16). All pregnancies were viable, and the median gestational week was 9 (range 7–11) at the time of sampling. Placental villi were collected from the 35–45% Percoll layer. EVT or CTB cells were isolated from placental chorionic villi, as previously described (19). Briefly, placental villi were enzymatically digested with 0.5 mg DNase I (Sigma-Aldrich), and cell suspensions (digest 1 and 2 combined: EVT; digest 3: CTB) were collected, centrifuged, and cell pellets were resuspended in culture medium (Sigma-Aldrich), and centrifuged, and cell pellets were resuspended in supernatants were removed, combined with newborn calf serum (5% v/v; sciences) and 0.5 mg DNase I (Sigma-Aldrich). At the end of each digest, the purity of isolated CD14+ monocytes and CD4+ cells were >97% and >99%, respectively.

Cell culture

To analyze the effects of placentally derived factors on Th cells and macrophages, CM from placentally explants or CTB, EVT, or HTR-8/SVneo cells was added to Th cell or macrophage cultures at the percentages indicated in the text and figures. Th cells: To analyze the effect of placentally factors in resting, as well as in activated, Th cells, isolated CD4+ T cells were either cultured unstimulated or stimulated with anti-CD3 and anti-CD28 Abs. The 96-well plates (Costar) were precoated with 0.5 μg/ml anti-CD3 and anti-CD28 Abs (low endotoxin; AbD Serotec) for 2 h at 37°C, after which the wells were washed with PBS. For unstimulated cells, plates were coated with PBS only. CD4+ cells were cultured at a density of 50,000 cells/well in 150 μl T cell culture medium, consisting of IMDM (Invitrogen) supplemented with t-glutamine (292 mg/ml; Sigma-Aldrich), sodium bicarbonate (3.024 g/l; Sigma-Aldrich), and streptomycin (50 μg/ml) (Cambrex), 100X nonessential amino acids (10 ml/l); Invitrogen), and 5% heat-inactivated FBS, as well as CM, for 5 d at 37°C and 5% CO2. For blocking experiments, CD4+ T cells were incubated with neutralizing Abs against IL-10R or M-CSF and were added 1 h prior to the addition of CM (for Ab details, see Supplemental Table I).

Macrophages. Macrophages were generated in 24-well plates, as previously described (6), in the presence of 5 ng/ml recombinant human GM-CSF or 50 ng/ml M-CSF (PeproTech) and CM. Blocking experiments were performed with neutralizing Abs against IL-10R or M-CSF and were added 1 h prior to the addition of CM (for Ab details, see Supplemental Table I).

Flow cytometry staining and analysis

Extracellular staining. Cells were resuspended in PBS supplemented with 0.1% FBS (PBS 0.1% FBS) and stained with Abs for extracellular staining and their corresponding isotype controls (for Ab details, see Supplemental Table I) for 30 min at 4°C in the dark. PBS 0.1% FBS was added, followed by centrifugation at 500 × g for 5 min. The cell pellet was resuspended in PBS 0.1% FBS for final flow cytometric analysis. Alternatively, for staining with 7-aminoactinomycin D and Annexin V-PE (BD Biosciences), which were used to assess viability, cells were resuspended and washed in PBS 0.1% FBS–binding buffer (for Ab details, see Supplemental Table I).

Intracellular staining. After extracellular staining, cells were permeabilized, according to the manufacturer’s instructions, using the Foxp3 staining kit (eBioscience), followed by staining with anti-human CTLA-4, Foxp3, T-bet, GATA-3, or Rorγt (for Ab details, see Supplemental Table I) for 30 min at 4°C. After washing, cells were resuspended in PBS 0.1% FBS.

Analysis and gating strategy. Data were acquired using a FACSComp II and analyzed with FACSdiva software version 6.1.2 (BD Biosciences) or BD Biosciences FlowJo software version 10.4.1. Isotype controls were used to set the cut-off for macrophage markers, as well as for some of the CD4 markers (CD25 and the transcription factors Foxp3, T-bet, GATA-3, or Rorγt). The CD25int gate was set according to a slightly lowered expression of CD4 on CD4+ T cells (CD4bb), as previously described (21). The percentage of HLA-DR+ and CD69-expressing cells was set according to the unstimulated control population. The gates for CD39, CTLA-4, and CD127 were set based on the expression in the medium–conditioned supernatant (CD25−) versus the Treg population. Naive (CD45RA+) and memory (CD45R0+) cells were defined based on the discrete CD45RA and CD45R0 populations.

Th cell–suppression assay

To test whether the placental explant CM (PE CM)–induced Tregs were functional, we sorted CD4+CD25+CD161+ Tregs and tested their ability to suppress the proliferation of anti-CD3/CD28–stimulated responder T cells by using the cell division–tracking dye CFSE. Isolated CD4+ T cells were cultured for 5 d in the presence of 6.25% PE CM, with additional stimulation, as described above. On day five, cells were harvested and stained with CD4 PE-Cy7 and CD25 allophycocyanin (BD Biosciences) for subsequent flow cytometry sorting. CD4+CD25+ responder cells and CD4+CD25−CD161+ Tregs were sorted on a FACSArta cell sorter (BD Biosciences). Sorted populations showed purities >98.5% upon realanalysis. The 96-well plates (Costar) were precoated with 0.5 μg/ml anti-CD3 and anti-CD28 Abs (AbD Serotec) for 2 h at 37°C, after which the wells were washed with PBS. CD4+CD25+ responder cells were labeled with 0.1 mM CFSE diluted in DMSO (Sigma–Aldrich) for 5 min at room temperature.

Placental tissues and cells

Placental explants. Immediately after collection, the maternal part of the placenta (also called “decidua”) was removed, and the fetal placental tissue was processed. The fetal placenta (hereafter mostly referred to as “placenta”) was rinsed with sterile saline to remove traces of maternal blood, transported to the laboratory, and washed in sterile PBS. The placental villi were dissected into small pieces (~1–2 mm in diameter) and placed in 24-well plates with culture medium consisting of RPMI 1640 (Life Technologies–Invitrogen, BRL) supplemented with 10% heat-inactivated FBS (PAAs Laboratories) and 1% PEST±t-glutamine (Life Technologies–Invitrogen, BRL). A total of ~50–100 mg of wet tissue was added to each well, with 10 μl culture medium/mg tissue. The placental explants were incubated for ~20–24 h at 37°C and 5% CO2. The conditioned medium (CM) was collected, centrifuged, and stored at aliquots at ~70°C.

Isolation of trophoblast cells. Extravillous trophoblast (EVT) and cytotrophoblast (CTB) cells were isolated from placental chorionic villi, as previously described (19). Briefly, placental villi were enzymatically digested for 3–5 min at 37°C in 0.125% trypsin (BD Biosciences) and 0.5 mg Dnase I (Sigma–Aldrich). At the end of each digest, supernatants were removed, combined with newborn calf serum (5% v/v; Sigma–Aldrich), and centrifuged, and cell pellets were resuspended in culture medium. Cell suspensions (digest 1 and 2 combined; EVT; digest 3: CTB) were layered onto a Percoll (Sigma–Aldrich) gradient (10–70% Percoll) and centrifuged for 30 min (1,200 × g, no brake), after which the EVT or CTB cells were collected from the 35–45% Percoll layer. EVT and CTB cells (1 × 106 cells/ml) were plated in 24-well plates coated with growth factor–reduced Matrigel (BD Biosciences) or fibronecin (Sigma–Aldrich), respectively. Cells were cultured for 24 h at 37°C and 5% CO2, and the CM was collected, centrifuged, and stored at ~80°C. The purity of isolated EVT and CTB cells was confirmed to be >97% by immunostaining for cytokeratin 7 (all trophoblast cells) and HLA-G (EVT), as previously described (19).

HTR-8/SVneo trophoblast cell line. The first-trimester trophoblast cell line HTR-8/SVneo (20), kindly provided by S. Sharma (Department of Pediatrics, Brown University, Providence, RI), was grown in culture medium, consisting of RPMI 1640 supplemented with 5% FBS and 1% PEST/ t-glutamine, to ~70–80% confluence. After three or four passages, adherent cells were removed enzymatically with 0.25% trypsin–EDTA (Sigma–Aldrich). The cells were transferred to 24-well plates at a density of 1 × 105 cells/ml and incubated for 24 h at 37°C and 5% CO2. The CM was collected, centrifuged, and stored at ~70°C.

Isolation of blood cells

PBMCs were isolated on a LymphoPrep gradient (Axis–Shield), according to the manufacturer’s instructions, followed by washing in HBSS (Inviron). Isolated PBMCs were used for isolation of CD14+ monocytes or CD4+ T cells by positive selection using immunomagnetic cell sorting. PBMCs were reconstituted in sterile MACS buffer (PBS supplemented with 2 mM EDTA [Sigma–Aldrich] and 0.5% FBS), and the CD14+ or CD4+ cells were isolated with anti-CD14 or anti-CD4 mAb-coated MicroBeads, according to the manufacturer’s protocol), using MS MACs columns (all from Miltenyi Biotec). The purity of isolated CD14+ monocytes and CD4+ cells were >97% and >99%, respectively.
The cells were washed three times with PBS supplemented with 5% FBS by centrifugation at 300 x g for 5 min. CD4<sup>+</sup>CD25<sup>+</sup> responder cells were plated at 2.5 x 10<sup>4</sup> cells/well, alone or in coculture with CD4<sup>+</sup>CD25<sup>+</sup> Tregs at a ratio of 2:1, and cultured in T cell culture medium for 5 d at 37°C and 5% CO<sub>2</sub>. Cells were harvested, resuspended in PBS 0.1% FBS, and analyzed, as described above, using a FACS Canto II.

**Analysis of cytokines and chemokines with multiplex bead assay**

Multiplex bead assay kits were used, according to the manufacturer’s protocols (Millipore), to analyze CM from placental explants and CTB, EVT, and HTR-8/SVneo cells for the following factors (detection limits are given in parentheses): GM-CSF (1.5 pg/ml), M-CSF (98 pg/ml), IL-10 (0.7 pg/ml), TGF-β1–3 (9.8 pg/ml), IL-1RA (16 pg/ml), IL-1β (3.2 pg/ml), IL-6 (16 pg/ml), TNF (3.2 pg/ml), IL-12p70 (3.0 pg/ml), IL-23 (48 pg/ml), IFN-γ (3.2 pg/ml), IL-13 (16 pg/ml), IL-17 (3.2 pg/ml), IL-2 (3.2 pg/ml), TRAIL (2.4 pg/ml), IL-33 (2.8 pg/ml), LIF (3.6 pg/ml), TSLP (2.4 pg/ml), CCL2 (16 pg/ml), CXCL1-3 (16 pg/ml), CXCL8 (3.2 pg/ml), CXCL10 (16 pg/ml), CXCL11 (11 pg/ml), CCL17 (0.5 pg/ml), CCL20 (9.8 pg/ml), and CCL22 (16 pg/ml). Supernatants from Th cell cultures were analyzed for GM-CSF, IL-2, IL-10, IL-13, IL-17, IFN-γ, and TGF-β1–3, and supernatants from macrophage cultures were analyzed for IL-10, IL-12p70, IL-23, and TGF-β1–3. The analyses were performed using the LumineX 200 IS system (Millipore) and the MasterPlex QT 2010 software (MiraBio). Values below the detection limit were assigned half the value of the detection limit. For all measurements of TGF-β1–3, control medium was analyzed in parallel, and the TGF-β1–3 concentration in the control medium was subtracted from the analyzed samples. All samples were acidified, according to the manufacturer’s instructions, to measure the active form of TGF-β. When analyzing the concentration of cytokines/chemokines produced by Th cells and macrophages exposed to CM, the concentration in the corresponding control CM was subtracted from the concentration measured in the cell supernatants.

**CCL18 ELISA**

Quantification of CCL18 in CM from placental explants, CTB, ETV, and HTR-8/SVneo cells, and macrophages polarized with PE CM was performed with an in-house double-Ab sandwich ELISA (VersaMax; Molecular Devices), as previously described (22). The detection limit was 7.8 pg/ml. For calculating the concentration of CCL18 produced by macrophages, the measured concentration in the corresponding control CM was subtracted from the concentration measured in the cell supernatants.

**Immunohistochemistry**

Formalin-fixed and paraffin-embedded placental tissue sections (4 μm), mounted on Superfrost Plus slides (Thermo Scientific), were deparaffinized by washing three times with Histo-Clear (Histolab), progressively rehydrated from 100 to 50% ethanol, and placed in distilled water. All washing between incubations was performed with PBS-Tween (0.05%, Medicago), with a final wash in distilled water. Ag retrieval was performed for GM-CSF, IL-10, and CD163 by microwave exposure for 20 min in 10 mM Tris–1 mM EDTA (pH 9). Sections were incubated overnight at 4°C with mouse primary mAbs against GM-CSF, M-CSF, IL-10, CD14, or CD163 (for Ab details, see Supplemental Table I), all diluted in PBS containing 3% normal goat serum (Dako) and 1% Triton X-100 (Sigma-Aldrich). After washing, sections were incubated for 30 min with polyclonal goat anti-mouse secondary Abs conjugated with biotin (Dako), diluted 1:300 in PBS containing 3% normal goat serum and 1% Triton X-100. Sections were washed, and endogenous peroxidase activity was blocked by incubating the sections with 3% H<sub>2</sub>O<sub>2</sub> (Sigma-Aldrich) for 20 min. Immunostaining was developed using the VECTASTAIN ABC kit and DAB as substrate (Vector Labs). Slides were mounted with ImmunohistoMount (Sigma-Aldrich). Visualization and photography were performed using an RFCA microscope, DP50 camera, and Studio 3.0.1 software (Olympus).

**Real-time RT-PCR**

Expression of Foxp3 mRNA was analyzed in CD4<sup>+</sup> T cells polarized with PE CM for 5 d, as described above. The PE CM–induced CD4<sup>+</sup> T cells were lysed in RNeasy RLT lysing buffer and frozen at –70°C until total cellular RNA was isolated with an RNeasy Mini Kit, according to the manufacturer’s instructions (both from Qiagen). Quantification of RNA was carried out using an ND-100 NanoDrop (Nanodrop Technologies). The isolated RNA was converted to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer’s protocol. The reverse transcription was performed using an Arktik Thermal cycler (Thermo Scientific). Real-time RT-PCR was performed by mixing 1 μl cDNA with 2x TaqMan Fast Universal MasterMix...
(Applied Biosystems) and primers and probes for Foxp3 (forward primer: 5'-GTGGCCGATGTGAGAA-3'; reverse primer: 5'-GCTGGAATTCGGCCGT-3'; probe: 5'-TGCTGGCA-CAGACCTGCTC-3'). The reactions were performed according to the recommended TaqMan protocol using the 7500 Fast Real-Time PCR System (Applied Biosystems). Samples were run in duplicates, and the RNA content in all samples was normalized to the expression of 18S rRNA. All data were analyzed with the SDS 2.3 version (Applied Biosystems), and quantification was performed using the standard curve method.

Data analysis and statistics

All data were analyzed using GraphPad Prism version 6.0. The majority of the flow cytometry data were normally distributed and, therefore, were analyzed with repeated-measures ANOVA and the Sidak multiple-comparison test or Student paired t test. Data from real-time RT-PCR, multiplex bead assays, and ELISA were analyzed with the Wilcoxon matched-pairs test. Flow cytometry data are expressed as mean and SD, whereas data from the multiplex bead assay, ELISA, and RT-PCR are represented as medians and/or interquartile ranges. The p values \(\leq 0.05\) were considered statistically significant.

Results

Soluble placental factors induce homeostatic M2 macrophages

To assess how decidual macrophages acquire their homeostatic M2 characteristics, we mimicked the decidual macrophage microenvironment by culturing macrophages with CM from first-trimester placental tissue explants. Macrophages were generated from CD14+ monocytes isolated from nonpregnant women. GM-CSF was used as a basic growth factor because it induces M1-like characteristics, making it possible to analyze the potential of placental-derived factors to promote an M2 phenotype in M1-primed macrophages (6). Macrophages generated in the presence of PE CM acquired a phenotype characteristic of decidual macrophages (6), with high expression of CD14, CD163 (scavenger receptor), CD206 (mannose receptor), and CD209 (dendritic cell-specific ICAM-3-grabbing nonintegrin [DC-SIGN]) and reduced expression of ICAM-3 (Fig. 1A, 1B). This phenotype was induced in a dose-dependent manner (Fig. 1B). PE CM (12.5%) also significantly increased the production of the anti-inflammatory

**FIGURE 2.** Soluble factors from placental tissue preferentially induce Foxp3 expression and production of IL-10 in CD4+ T cells. (A) Effect of 6.25 or 12.5% first-trimester PE CM on the polarization of Tregs and Th1, Th2, and Th17 cells. Expression of the transcription factors Foxp3 (Treg), T-bet (Th1), GATA-3 (Th2), and Rorγt (Th17) in the CD4+ T cell population (upper panels). Production of cytokines from the corresponding Th cell subsets (lower panels). (B) Effect of 6.25 and 12.5% PE CM on the activation of CD4+ T cells. (C) Effect of PE CM on the viability of CD4+ T cells. In all experiments, peripheral blood CD4+ T cells (isolated from nonpregnant women) were cultured unstimulated (Unstim) or were stimulated with 0.25 μg/ml anti-CD3 and anti-CD28 Abs (aCD3/CD28) for 5 d in the presence or absence of PE CM. Transcription factors and HLA-DR were analyzed by flow cytometry (all analyses were performed by gating on viable cells). Cytokine production was analyzed with multiplex bead assay. The graphs show the mean + SD (upper panels in (A) and (B) and in (C)) or the median [lower panels in (A) and (B)] of eight individual experiments. *p \(\leq 0.05\), **p \(\leq 0.01\), ***p \(\leq 0.001\). Data were analyzed with: repeated-measures ANOVA and the Sidak multiple-comparison test [upper panels in (A) and (B) and in (C)]; Wilcoxon matched-pairs test [lower panels in (A) and (B)].
cytokine IL-10, whereas it did not affect IL-12 or IL-23 production (associated with an M1 phenotype and Th1 and Th17 induction, respectively) (23) (Fig. 1C). The chemokine CCL18, typically produced by homeostatic M2 macrophages and by human decidual macrophages (8), also was induced by PE CM (Fig. 1C). The production of TGF-β1 did not differ between macrophages stimulated with GM-CSF alone or in combination with PE CM (Fig. 1C), and TGF-β2/TGF-β3 were under the detection limit, regardless of stimulation.

Soluble placental factors preferentially induce Foxp3+ Th cells and IL-10 production

In contrast to other Th subsets, Tregs are enriched in the early human decidua (17, 18). To analyze whether placental-derived factors could be responsible for the unique composition of Th cell subsets, we cultured CD4+ T cells (isolated from nonpregnant women) in the presence of PE CM. To mimic the decidual microenvironment in the resting state, CD4+ T cells were cultured with PE CM without any additional stimulation (“unstimulated”). Unstimulated CD4+ T cells exposed to 6.25 and 12.5% PE CM showed an increased proportion of Foxp3-expressing cells (Tregs) (Fig. 2A, “Unstim”). This increase was paralleled by an increased production of IL-10 but not TGF-β1 (Fig. 2A, “Unstim”). TGF-β2 and TGF-β3 were not detected in unstimulated Th cells, either in the presence or absence of PE CM. Although the proportion of T-bet+ cells (Th1) also was increased in unstimulated cells, this was not mirrored by increased IFN-γ production (Fig. 2A, “Unstim”). The proportion of GATA-3+ (Th2) and Rorgt+ (Th17) CD4+ T cells and the production of IL-13 and IL-17 were not affected by PE CM in unstimulated cultures (Fig. 2A, “Unstim”). Because Foxp3 is known to be transiently induced in activated human CD4+ T cells (24), we tested whether the increased proportion of Foxp3+ cells was a result of increased activation. However, PE CM did not induce expression of the activation markers HLA-DR, CD25, or CD69 or the production of IL-2 and GM-CSF by CD4+ T cells in the absence of TCR stimulation (Fig. 2B, “Unstim”). In addition to the effect on unstimulated Th cells, we aimed to test the ability of placental-derived factors to prevent Th cell activation, represented in this study by anti-CD3/CD28–stimulated Th cells. In contrast to the effect on unstimulated CD4+ T cells, PE CM decreased Th cell activation in anti-CD3/CD28–stimulated cells, as shown by reduced HLA-DR expression (Fig. 2B, “aCD3/CD28”) and reduced secretion of cytokines, including IL-10, TGF-β1, IFN-γ, IL-13, IL-17, and GM-CSF (Fig. 2A, 2B, “aCD3/CD28”). The reduced production of cytokines was not a result of decreased viability, because PE CM did not affect the viability of anti-CD3/CD28–stimulated CD4+ T cells (Fig. 2C, “aCD3/CD28”).

FIGURE 3. Placental soluble factors induce functionally suppressive CD25highFoxp3+ Tregs. (A) Flow cytometry dot plots showing that CD4dimCD25high (left panels), Foxp3+ (middle panels), and CD25highFoxp3+ (right panels) Tregs increased in the CD4+ T cell population when stimulated with 6.25% PE CM (lower panels) compared with medium alone (upper panels) for 5 d. One representative experiment of eight performed is shown. Numbers indicate the percentage of cells in each gate. (B) Real time RT-PCR was used to analyze Foxp3 mRNA expression (normalized to 18S rRNA) in CD4+ T cells cultured for 5 d with or without PE CM. Horizontal lines indicate the median of eight experiments. *p ≤ 0.05, Wilcoxon matched-pairs test. (C) CD25highFoxp3+ Tregs generated in the presence of 6.25% PE CM showed a suppressive and memory phenotype, with low CD127 expression and high levels of the suppressive markers CTLA-4 and CD39 and the memory marker CD45R0. One representative experiment of six performed is shown. Numbers indicate the percentage of cells in each gate. (D) Suppressive effect of PE CM–induced CD4dimCD25high Tregs on the proliferation of anti-CD3/CD28–stimulated and CFSE-labeled CD4+CD25+ responder cells (CD25+). The proliferation of responder cells cultured alone was set to 100%. Data are the mean values of four experiments. *p ≤ 0.05, Student paired t test. (E) Representative graphs showing the proliferation of unstimulated (Unstim) or anti-CD3/CD28–stimulated (aCD3/CD28) CD4+CD25+ responder cells cultured alone or with CD4dimCD25high Tregs. Responder cells in (D) and (E) were cocultured with Tregs (2:1 ratio) for 5 d. In all experiments, CD4+ T cells were isolated from peripheral blood from nonpregnant women.
CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs induced by placental soluble factors are functionally suppressive

Similar to the increased proportion of Foxp3<sup>+</sup> T cells, the proportion of CD4<sup>dim</sup>CD25<sup>high</sup> and CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs were increased in PE CM–exposed CD4<sup>+</sup> T cells compared with unexposed CD4<sup>+</sup> T cells (Fig. 3A). PE CM also increased the expression of Foxp3 at the mRNA level in unstimulated CD4<sup>+</sup> T cells (Fig. 3B). PE CM–induced Tregs were CD127<sup>low</sup> and expressed high levels of the suppressive markers CTLA-4 and CD25, and the majority was memory cells expressing CD45R0 (Fig. 3C), similar to the Tregs in early human decidua (17, 18).

Finally, we tested whether the PE CM–induced Tregs were functional by analyzing their ability to suppress the proliferation of anti-CD3/CD28–stimulated responder T cells. After 5 d of culture with PE CM, CD4<sup>+</sup>CD25<sup>-</sup> responder cells and CD4<sup>dim</sup>CD25<sup>high</sup> Tregs were isolated by FACS. The responder cells were labeled with the cell division–tracking dye CFSE, stimulated with anti-CD3/CD28 Abs, and cocultured with the PE CM–induced Tregs for 5 d at a 2:1 ratio. As seen in Fig. 3D and 3E, PE CM–induced CD4<sup>dim</sup>CD25<sup>high</sup> Tregs significantly suppressed the proliferation of CD4<sup>+</sup>CD25<sup>-</sup> responder cells.

CTB and EVT cells induce homeostatic macrophages and the expansion of Tregs

Next, we tested whether trophoblast cells, the primary cell component of the fetal placenta, could take part in the induction of homeostatic macrophages and Tregs. The purity of the isolated trophoblast cells (CTB: cytokeratin 7<sup>+</sup>HLA-G<sup>+</sup>; EVT: cytokeratin 7<sup>+</sup>HLA-G<sup>+</sup>) was >97% (for details see Materials and Methods). Macrophages cultured with GM-CSF, in combination with 12.5% CM from CTB and EVT cells, acquired the homeostatic phenotype characteristic of decidual macrophages (CD14<sup>+</sup>CD163<sup>+</sup>CD206<sup>+</sup>CD209<sup>-</sup>ICAM-3<sup>-low</sup>) (Fig. 4A), similar to the effect of CM from whole placental tissue (Fig. 1A, 1B). We also tested whether homeostatic macrophages could be induced by the first-trimester trophoblast cell line HTR-8/SVneo, commonly used as a substitute for primary trophoblast cells. As shown in Fig. 4B, HTR-8/SVneo CM induced upregulation of CD14, CD163, and CD206 but failed to induce upregulation of CD209 or downregulation of ICAM-3. Of note, considerably higher concentrations of HTR-8/SVneo CM (50% and 90%) were required for the observed phenotypic changes.

CTB and EVT CM (6.25% and 12.5%) also induced an increased proportion of CD4<sup>dim</sup>CD25<sup>high</sup>, Foxp3<sup>+</sup>, and CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs in the CD4<sup>+</sup> T cell population compared with medium alone (Fig. 4C, Supplemental Fig. 1A, 1B). CM from the HTR-8/SVneo cell line also induced an increased proportion of CD4<sup>dim</sup>CD25<sup>high</sup>, Foxp3<sup>+</sup>, and CD25<sup>high</sup>Foxp3<sup>+</sup> Tregs (Fig. 4D, Supplemental Fig. 1C) but higher concentrations (25% and 50%) were required. We also tested the effect of HTR-8/SVneo CM on Th1, Th2, and Th17 polarization and on Th cell activation. Similar to the effect of PE CM on CD4<sup>+</sup> T cells, HTR-8/SVneo CM preferentially induced the expansion of Foxp3<sup>+</sup> Tregs, but it did not induce an increase in T-bet<sup>-</sup> (Th1), GATA-3<sup>-</sup> (Th2), or Rorγt<sup>-</sup> (Th17) cells in the unstimulated CD4<sup>+</sup> T cell population (Supplemental Fig. 2A, “Unstim”). The levels of IL-10, IFN-γ, IL-13, and IL-17 also were under the detection limit in unstimulated cells exposed to HTR-8/SVneo CM (Supplemental Fig. 2A, “Unstim”). Contrary to the downregulating effect of PE CM on anti-CD3/CD28–stimulated cells, HTR-8/SVneo CM further increased the activated phenotype, with increased expression of HLA-DR, CD25, T-bet, GATA-3, and Rorγt and increased production of IL-10, TGF-β1, IL-17, and IFN-γ (Supplemental Fig. 2, “aCD3/CD28”). In summary, freshly isolated CTB and EVT cells from first-trimester healthy human placenta induce homeostatic macrophages and expand the Treg population, whereas the HTR-8/SVneo cell line only partially induces these regulatory cell types and, in contrast to PE CM, enhances Th cell activation.

M-CSF, IL-10, TGF-β, and TRAIL produced by trophoblast cells promote the polarization of homeostatic macrophages and the expansion of Foxp3<sup>+</sup> Tregs

To identify the factors involved in the induction of homeostatic macrophages and Tregs, we analyzed a panel of soluble factors in PE CM and CM from CTB, EVT, and HTR-8/SVneo cells (Fig. 5; Table I). We showed previously that the decidual macrophage phenotype can be induced in vitro by M-CSF, whereas GM-CSF...
This correlates well with the previously reported localization of TGF-β below the limit of detection.

Representative sections from one individual; 10 individual immunostainings were performed (original magnification x250). Scale bars, 25 μm. <det., below the limit of detection.

counters this effect (6). We also showed that IL-10 enhanced the decidual macrophage phenotype in M-CSF–driven macrophages, as well as restored the M2 phenotype in GM-CSF–driven M1-like macrophages (6). In the current study, we show that the placenta itself is a major source of M-CSF (greatly exceeding the levels of GM-CSF) (Fig. 5, A and Table I) and that M-CSF is expressed in CTB cells and in the syncytiotrophoblast shell surrounding the chorionic villi (Fig. 5E). To evaluate the importance of M-CSF in the induction of decidual macrophages, we used anti–M-CSF–blocking Abs during the polarization process with PE CM. As seen in Fig. 6A, the induction of CD163 was partially reduced when M-CSF was neutralized, whereas CD14, CD206, and CD209 were not affected. IL-10 also was produced by placental tissue and at particularly high levels by CTB and EVT cells, whereas HTR-8/SVneo cells lacked IL-10 production (Fig. 5B, Table I). IL-10 was expressed by CTB cells and in the syncytiotrophoblast shell surrounding the chorionic villi (Fig. 5E). When using blocking Abs against TGF-β1–3 partially reduced the increase of Foxp3+ Tregs induced by PE CM (Fig. 6C). A similar reduction in Foxp3+ cells was observed when CD4+ T cells were cultured in the presence of anti–IL-10R Abs (Fig. 6D). Placental explants and trophoblast cells also produced the apoptosis-inducing factor TRAIL (Fig. 5D, Table I), which was recently localized mainly to the syncytiotrophoblast within the placenta (28). TRAIL was shown to preferentially expand the Treg population and to inhibit expansion of the non-Treg cell pool in a mouse model (29). Because we observed that PE CM significantly reduced the viability of unstimulated CD4+ T cells (Fig. 2C, “Unstim”), we investigated whether this mechanism could apply to the PE CM–induced Treg expansion. Indeed, when using anti–TRAIL–blocking Abs, we observed a significant reduction in the proportion of PE CM–induced Foxp3+ Tregs (Fig. 6E). LIF and CCL18 are other factors proposed to be involved in the generation of inducible Tregs (30, 31). However, neither anti-LIF– nor anti-CCL18–neutralizing Abs had an effect on the expansion of Tregs induced by PE CM (data not shown). In summary, several placental-derived factors seem to act in concert to cause expansion of the Treg pool during early human pregnancy (Fig. 7).

Although there is a lack of knowledge regarding the factors responsible for the specific enrichment of Tregs at the fetal–maternal interface in humans, there are several well-established factors, including TGF-β and IL-10, that generate inducible Foxp3–expressing Tregs in the periphery (25, 26). TGF-β1 and TGF-β2 were detected in PE CM and HTR-8/SVneo CM, whereas only TGF-β2 was detected in CTB and EVT CM (Fig. 5C, Table I). In contrast, TGF-β3 was detected at only low levels in PE CM (Table I). This correlates well with the previously reported localization of TGF-β1–3 in human placental tissue (27). Neutralizing Abs against TGF-β1–3 partially reduced the increased proportion of CD4+Foxp3+ Tregs induced by PE CM (Fig. 6C). A similar reduction in Foxp3+ cells was observed when CD4+ T cells were cultured in the presence of anti–IL-10R Abs (Fig. 6D). Placental explants and trophoblast cells also produced the apoptosis-inducing factor TRAIL (Fig. 5D, Table I), which was recently localized mainly to the syncytiotrophoblast within the placenta (28). TRAIL was shown to preferentially expand the Treg population and to inhibit expansion of the non-Treg cell pool in a mouse model (29). Because we observed that PE CM significantly reduced the viability of unstimulated CD4+ T cells (Fig. 2C, “Unstim”), we investigated whether this mechanism could apply to the PE CM–induced Treg expansion. Indeed, when using anti–TRAIL–blocking Abs, we observed a significant reduction in the proportion of PE CM–induced Foxp3+ Tregs (Fig. 6E). LIF and CCL18 are other factors proposed to be involved in the generation of inducible Tregs (30, 31). However, neither anti-LIF– nor anti-CCL18–neutralizing Abs had an effect on the expansion of Tregs induced by PE CM (data not shown). In summary, several placental-derived factors seem to act in concert to cause expansion of the Treg pool during early human pregnancy (Fig. 7).

Discussion

In this study, we demonstrate that human fetally derived placental tissue promotes the induction of homeostatic macrophages and Tregs, which are essential for fetal tolerance and reproductive success (Fig. 7). The placental-induced macrophages shared the CD14+CD163+CD206+CD209+ICAM-3low phenotype of decidual macrophages (6) and produced IL-10 and CCL18 but not IL-12 and IL-23. We also showed, by blocking experiments, that this phenotype is induced by M-CSF and IL-10, which are primarily
produced by trophoblast cells. Placental tissue also induced the expansion of CD25highCD127loFoxp3+ Tregs in parallel with increased IL-10 production. In addition, expression of the Th1-associated transcription factor T-bet was increased by placental-derived factors, whereas production of IFN-γ was not induced.

In contrast to the initially proposed and still predominant Th2 paradigm (36), the specific polarization of homeostatic M2 macrophages and expansion of Tregs while limiting Th cell activation, indicating that fetal-derived tissue itself is a main inducer of maternal immune cell adaptation. Thus, it seems reasonable that the fetal placenta, as a temporary organ, is the primary trigger of maternal immune cell adaptation. Consequently, although important, the cross-talk between the induced tolerogenic leukocytes might be a secondary mechanism necessary to sustain and enhance fetal tolerance. In addition, our results indicate that trophoblast cells play a major role in this adaptation. Although other placental cells were reported to promote suppressive function in leukocytes (e.g., mesenchymal stem cells) (34, 35), the numerical advantage and anatomical location of trophoblast cells render the fetal placenta, as a temporary organ, is the primary trigger of maternal immune cell adaptation. Importantly, the trophoblast cell line HTR-8/SVneo (20), which is widely used as a substitute for human primary trophoblast cells, only partially induced the regulatory cell types described above, and, contrary to placental tissue, it enhanced Th cell activation (likely due to its lack of IL-10 production). Thus, although useful in many aspects, the results obtained from this cell line should be interpreted with caution.

In vitro studies demonstrated that the cross-talk between decidual cells promotes the development of maternal leukocytes with regulatory properties. This includes induction of Tregs and suppression of T cell activation by decidual macrophages (12, 14) and stromal cells (32, 33), as well as the induction of Tregs through the interaction between decidual macrophages and uterine NK cells (13). However, our novel findings show that placental-derived factors directly promote differentiation of homeostatic M2 macrophages and expansion of Tregs while limiting Th cell activation, indicating that fetal-derived tissue itself is a main inducer of maternal immune cell adaptation. Thus, it seems reasonable that the fetal placenta, as a temporary organ, is the primary trigger of maternal immune cell adaptation. Consequently, although important, the cross-talk between the induced tolerogenic leukocytes might be a secondary mechanism necessary to sustain and enhance fetal tolerance. In addition, our results indicate that trophoblast cells play a major role in this adaptation. Although other placental cells were reported to promote suppressive function in leukocytes (e.g., mesenchymal stem cells) (34, 35), the numerical advantage and anatomical location of trophoblast cells render them the prime candidate for affecting maternal leukocytes in the adjacent decidua. Importantly, the trophoblast cell line HTR-8/SVneo (20), which is widely used as a substitute for human primary trophoblast cells, only partially induced the regulatory cell types described above, and, contrary to placental tissue, it enhanced Th cell activation (likely due to its lack of IL-10 production). Thus, although useful in many aspects, the results obtained from this cell line should be interpreted with caution.

In contrast to the initially proposed and still predominant Th2 paradigm (36), the specific polarization of homeostatic M2 macrophages and the preferential expansion of Tregs, but not of Th2
cells, support the existence of a tolerogenic and homeostatic, rather than a Th2-dominated, uterine environment during human pregnancy. This is in agreement with earlier reports showing that Th2 cytokines (IL-4 and IL-13) induce macrophages distinct from decidual macrophages (CD14<sup>+</sup>CD163<sup>+</sup>) (6), that Tregs but not Th2 cells are enriched in first-trimester human decidua (17), and that high levels of IL-10, in relation to IL-4 (37) and IL-13 (Table I), are present at the fetal–maternal interface. Furthermore, placental-derived soluble factors promoted a general downregulation of Th cell activation and cytokine production in anti-CD3/CD28–stimulated cells. These data indicate a mechanism by which placental tissue prevents excessive T cell activation, irrespective of the type of response (e.g., Th1, Th2, or Th17 associated). Considering that fetal rejection might be caused by activation of placental/fetal-specific T cells, as well as by general T cell activation (e.g., during infections) (38, 39), the ability of the placenta to induce immune cells with a reduced inflammatory potential might be an important contributing mechanism for maintained tissue integrity at the fetal–maternal interface.

Among the factors spontaneously produced by the placental tissue, we identified M-CSF and IL-10 as central for the polarization of homeostatic decidual macrophages. Although we showed previously, using an in vitro model, that these cytokines are the main inducers of decidual macrophages (6), the current study strengthens their relevance in vivo, because the effects of M-CSF and IL-10 were apparent at physiological levels as part of the natural pool of placental-derived cytokines. Of note, these homeostatic M2 characteristics were observed in macrophages generated in the presence of GM-CSF, which alone promotes M1-like macrophages. In addition to the well-known role of M-CSF in macrophage differentiation (40), local M-CSF production was proposed as a general inducer of tissue macrophages with an increased threshold for activation, which may be important for sustaining tissue integrity (41). Similarly, IL-10 is a homeostatic cytokine constitutively produced both at the steady-state and during inflammation to control excessive inflammatory responses (42). The relevance of these cytokines in pregnancy is further supported by their increased levels at the fetal–maternal interface (37, 43), as well as by observations of increased rates of spontaneous abortions in M-CSF–deficient mice (44) and in mice with a local defect in IL-10 production (45). In addition, both M-CSF–deficient mice (46) and IL-10–deficient mice (47, 48) show increased susceptibility to infection-induced fetal loss. Thus, it is likely that placental-derived M-CSF and IL-10 promote macropop-
phases that prevent rejection of the allogeneic fetus by creating a homeostatic microenvironment and, in addition, protect the mother and the fetus against infections without compromising fetal survival. Based on neutralization experiments, the expansion of Tregs appears to be a process whereby several placental factors, including (but likely not restricted to) IL-10, TGF-β, and TRAIL, act in collaboration, rather than being a process driven by one dominating factor. In addition to the factors tested in this study, molecules, such as soluble CD200 and HLA-G, galectin-1, and pregnancy-associated hormones, might influence the generation of Tregs (49–53). Given the importance of Tregs during pregnancy (15, 16), a redundancy in soluble mediators promoting the expansion of Tregs at the fetal–maternal interface is not surprising. Furthermore, these factors may have divergent and complementary effects on Tregs. For instance, TGF-β was described to promote the conversion of non-Tregs into Foxp3-expressing Tregs, rather than to expand an already existing Treg population (25).

In contrast, IL-10 was recently reported to upregulate the anti-apoptotic Bcl-2 specifically in Tregs, but not in conventional T cells, suggesting a mechanism for IL-10–driven maternal Treg expansion during pregnancy (54). Similarly, the apoptosis-inducing factor TRAIL was shown to expand the Treg pool and to inhibit the expansion of non-Tregs (29). Thus, both IL-10 and TRAIL could increase the proportion of Tregs by preferentially promoting the survival of already existing Tregs. Our data showing a specific increase in Foxp3+ Tregs, in parallel with decreased viability of CD4+ T cells after exposure to placental explant CM, is in support of such a mechanism. The importance of TGF-β signaling in maintaining T cell homeostasis was described in a mouse model with T cell–specific deletion of TGF-βRII, in which lethal inflammation developed in association with T cell activation and disrupted Treg induction (55). Similarly, TRAIL was shown to mediate protection against autoimmune diseases in mice by promoting the expansion of Tregs (29, 56). Thus, given the importance of immune tolerance during pregnancy, it is likely that TGF-β and TRAIL, as described for IL-10 (47, 48), contribute to the protection against uncontrolled immune cell activation and fetal loss. In summary, we demonstrated that the fetal placenta itself, particularly through trophoblast cells, is able to create a tolerant uterine environment by the production of soluble mediators (M-CSF, IL-10, TGF-β, and TRAIL) that induce homeostatic macrophages and Tregs and limit excessive Th cell activation (Fig. 7). These findings are relevant for understanding the pathology of immune-associated pregnancy complications, in which both de- cidual macrophages and Tregs have been implicated (1, 2), as well as beyond pregnancy, in areas of cancer, autoimmunity, and transplantation, where similar tolerance and homeostatic mechanisms are over- or underdeveloped.

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Disclosures
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References


