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

Judit Svensson, Ratnesh Bhai Mehta, Robert Lindau, Elahe Mirrasekhian, Heriberto Rodriguez-Martinez, Göran Berg, Gendie E. Lash, Maria Jenmalm and Jan Ernerudh

Original Publication:

Judit Svensson, Ratnesh Bhai Mehta, Robert Lindau, Elahe Mirrasekhian, Heriberto Rodriguez-Martinez, Göran Berg, Gendie E. Lash, Maria Jenmalm and Jan Ernerudh, The Human Fetal Placenta Promotes Tolerance against the Semiallogeneic Fetus by Inducing Regulatory T Cells and Homeostatic M2 Macrophages, 2015, Journal of Immunology, (194), 4, 1534-1544.
http://dx.doi.org/10.4049/jimmunol.1401536

The Human Fetal Placenta Promotes Tolerance against the Semi-allogeneic Fetus by Inducing Treg Cells and Homeostatic M2 Macrophages

Running title: The human placenta supports fetal tolerance

Judit Svensson-Arvelund[1]*, Ratnesh B. Mehta*, Robert Lindau*, Elahe Mirrasekhian*, Heriberto Rodriguez-Martinez†, Göran Berg*‡, Gendie E. Lash§, Maria C. Jenmalm* and Jan Ernerudh*¶

*Clinical Immunology, †Developmental Biology and ‡Division of Obstetrics and Gynecology, *†‡Department of Clinical and Experimental Medicine, Linköping University, Linköping, Sweden. §Reproductive and Vascular Biology Group, Institute of Cellular Medicine, Newcastle University, Newcastle upon Tyne, UK, ¶Department of Clinical Immunology and Transfusion Medicine, Linköping University, Linköping, Sweden

[1]Corresponding author:

Phone: +46 10 10 32998

Fax: +46 13 132257

E-mail address: judit.svensson@liu.se

ABSTRACT

A successful pregnancy requires that the maternal immune system is instructed to a state of tolerance to avoid rejection of the semi-allogeneic fetal-placental unit. Although increasing evidence supports that decidual (uterine) macrophages and Treg cells are key regulators of fetal tolerance, it is not known how these tolerogenic leukocytes are induced. Here we show that the human fetal placenta itself, mainly through trophoblast cells, is able to induce homeostatic M2 macrophages and Treg cells. 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 and IL-23. Placental tissue also induced the expansion of $CD25^{high}CD127^{low}Foxp3^{+}$ Treg cells in parallel with increased IL-10 production, while production of IFN-γ (Th1), IL-13 (Th2) and IL-17 (Th17) was not induced. The Treg cells expressed the suppressive markers CTLA-4 and CD39, were functionally suppressive and were partly induced by IL-10, TGF-β and TRAIL. Placental-derived factors also 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.

INTRODUCTION

During pregnancy, immune tolerance is naturally induced to avoid rejection of the semi-allogeneic fetal-placental unit. The most prominent changes occur at the fetal-maternal interface, where maternal endometrial leukocytes come into close contact with placental trophoblast cells of paternal origin (1, 2). During pregnancy, the endometrium becomes a specialized tissue (named decidua) with a strikingly high proportion of leukocytes with unique regulatory functions. Two major regulating populations are the decidual macrophages and regulatory T (Treg) cells that play important roles in establishing tolerance and maintaining the homeostatic environment that is crucial for normal fetal development (3, 4).

Decidual macrophages are the most abundant antigen presenting cells throughout pregnancy (5), and they are central in setting the balance between tolerance and pro-inflammatory responses. Human decidual macrophages have properties predominantly associated with homeostatic M2 macrophages, including expression of the homeostatic scavenger receptor CD163, the pattern recognition receptors CD206 and CD209, and 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-γ by T cells (12) and to induce Treg cells *in vitro* (13, 14). Treg cells 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, Treg cells are likely to have a central role in the tolerance of paternal antigens and the general maintenance of a homeostatic environment compatible with fetal survival. In humans, Treg cells accumulate in the decidua, and show an activated and suppressive phenotype, with high expression of CD25, Foxp3 and CTLA-4 (17, 18).

Whereas decidual macrophages and Treg cells have been demonstrated to facilitate pregnancy, the factors that regulate these cells in humans have been poorly characterized. The microenvironment has a great influence on leukocyte development, and 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. Noteworthy, 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 has not been addressed in a physiological setting. Here we show that the human fetal placenta itself, particularly through trophoblast cells, is able to create a homeostatic and tolerant environment by producing soluble factors (M-CSF, IL-10, TGF-β and TRAIL) that induce the polarization of homeostatic macrophages and the expansion of Treg cells and also limit excessive Th cell activation.

MATERIALS AND METHODS

Subjects

First trimester placental tissues were collected from 45 healthy pregnant women (median age 25, range 16-42) undergoing elective surgical abortions at Linköping University Hospital, Sweden (n=29) and at the Royal Victoria Infirmary, Newcastle upon Tyne, UK (n=16). All pregnancies were viable and the median gestational week was 9 (range 7-11) as determined by crown-rump length by using ultrasound. Misoprostol (Cytotec®, Searle) was given to all women prior to surgery. The median number of previous pregnancies was 1 (range 0-12) and the median number of previous births was 1 (range 0-5). For the *in vitro* assays, all blood samples were collected on one or more occasions from 23 healthy non-pregnant female volunteers (median age 27, range 21-44) not taking hormonal contraceptives or any other medication. The time point of sample collection was evenly distributed across the menstrual cycle. All samples were collected after obtaining informed consent (including the parents' consent in the one case where the donor was under the age of 18) and the study was approved by the Local Ethics Committees of Linköping University and Newcastle and North Tyneside.

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 further 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 then dissected into small pieces (~1-2 mm in diameter) and placed in 24-well plates with culture medium consisting of RPMI 1640 (Gibco-Invitrogen, BRL) supplemented with 10% heat-inactivated FBS (PAA Laboratories) and 1% PEST/L-glutamine (Gibco-Invitrogen, BRL). A total of ~50-100 mg of wet tissue was added to each

well, with 10 µl culture medium per mg of tissue. The placental explants were incubated for ~20-24 h at 37° C and 5% CO_2. The conditioned medium (CM) was then collected, centrifuged and stored in 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 3 x 25 min at 37 °C in 0.125% trypsin (BD Biosciences) and 0.5 mg DNase I (Sigma). At the end of each digest, supernatants were removed, combined with newborn calf serum (5% v:v) (Sigma), centrifuged and cell pellets resuspended in culture medium. Cell suspensions (digest 1 and 2 combined: EVT, digest 3: CTB) were layered onto a Percoll (Sigma) gradient (10% -70% Percoll) and centrifuged for 30 min (1200 x *g*, no brake) after which the EVT or CTB cells were collected from the 35-45% Percoll layer. EVT and CTB cells ($1x10^6$ cells/ml) were plated in 24-well plates coated with growth factor-reduced Matrigel (BD Biosciences) or fibronectin (Sigma-Aldrich), respectively. Cells were cultured for 24 h at 37° C and 5% CO_2. The CM was then 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 (Brown University, Providence, RI), was grown in culture medium, consisting of RPMI 1640 supplemented with 5% FBS and 1% PEST/L-glutamine, to ~70-80% confluence. After 3-4 passages, adherent cells were removed enzymatically with 0.25% trypsin-EDTA (Sigma-Aldrich). The cells were then transferred to 24-well plates to a density of $1x10^6$ cells/ml and incubated for 24 h at 37° C and 5% CO_2. The CM was then collected, centrifuged and stored at -70°C.

Isolation of blood cells

PBMC were isolated on a Lymphoprep gradient (Axis-Shield) according to the manufacturer's instructions, followed by washing in HBSS (Invitrogen). Isolated PBMC were used for isolation of $CD14^+$ monocytes or $CD4^+$ T cells by positive selection using immunomagnetic cell sorting. PBMC were resuspended 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 (Miltenyi Biotec) using MS MACS columns (Miltenyi Biotec). The isolated $CD14^+$ monocytes had a purity of >97% and the $CD4^+$ cells >99%.

Cell culture

To analyze the effects of placentally derived factors on Th cells and macrophages, CM from placental explants, CTB, EVT and HTR-8/SVneo cells were added to Th cell or macrophage cultures at the percentages indicated in the text and figures.

Th cells. In order to analyze the effect of placental 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. 96-well plates (Costar) were pre-coated with 0.25 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 per well in 150 µl T cell culture medium, consisting of IMDM (Invitrogen) supplemented with L-glutamine (292 mg/ml; Sigma-Aldrich), sodium bicarbonate (3.024 g/L; Sigma-Aldrich), penicillin (50 IE/ml), streptomycin (50 µg/ml) (Cambrex), 100x nonessential amino acids (10 ml/L; Invitrogen), and 5% heat-inactivated FBS, and the addition of CM, for five days at 37° C and 5% CO_2. For blocking experiments, $CD4^+$ T cells were incubated with neutralizing Abs against IL-10R, TGF-β1-3, LIF, CCL18

or TRAIL and the corresponding isotype controls one hour 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 the addition of CM. Blocking experiments were performed with neutralizing Abs against IL-10R or M-CSF and were added one hour 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 x *g* for 5 min. The cell pellet was resuspended in PBS 0.1 % FBS for final flow cytometric analysis. For staining with 7-Aminoactinomycin D and Annexin V-PE (BD Biosciences), used to assess viability, cells were instead resuspended and washed in Annexin V-binding buffer (BD Biosciences).

Intracellular staining. After extracellular staining, cells were permeabilized according to the manufacturers' 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 FACSCanto II and analyzed with FACSDiva software version 6.1.2 (BD Biosciences) or Kaluza software version 1.1 (Beckman Coulter). 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 and Rorγt). The $CD25^{high}$ gate was set according to a slightly lowered expression of CD4 on $CD4^{+}$ cells ($CD4^{dim}$) 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 resting ($CD25^-$) versus the Treg cell population. Naïve ($CD45RA^+$) 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 Treg cells were functional, we sorted $CD4^{dim}CD25^{high}$ Treg cells 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 five days in the presence of 6.25% PE CM, without additional stimulation, as described above. On day five, cells were harvested and stained with CD4 PE-Cy7 and CD25 APC (BD Biosciences) for subsequent flow cytometry sorting. $CD4^+CD25^-$ responder cells and $CD4^{dim}CD25^{high}$ Treg cells were sorted on a FACSAria cell sorter (BD Biosciences). Sorted populations showed purities of >98.5% upon reanalysis. 96-well plates (Costar) were pre-coated 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. The cells were then washed three times with PBS supplemented with 5% FBS by centrifugation at 300 x *g* for 5 min. $CD4^+CD25^-$ responder cells were plated at 2.5×10^4 cells/well alone or in co-culture with $CD4^{dim}CD25^{high}$ Treg cells at a ratio of 2:1 and cultured in T cell culture medium for five days at 37°C and 5% CO_2. Cells were then harvested, resuspended in PBS 0.1% FBS and analyzed as described above. Cells were analyzed by using FACSCanto 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, CTB, EVT and HTR-8/SVneo cells for the detection of the following factors (detection limits are given in brackets): 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 for IL-10, IL-12p70, IL-23 and TGF-β1-3. The analyses were performed using the Luminex200® IS system (Millipore) and the MasterPlex^{TM} QT 2010 software (MiraiBio). Values below the detection limit were given 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 in order 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, EVT 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 stimulated with CM, the 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 Histoclear (Histolab), and progressively rehydrated from 100% to 50% ethanol and finally placed in distilled water. All washing between incubations was performed with PBS-Tween (0.05%, Medicago) with a final wash in distilled water. Antigen 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). CD14 and CD163 were used to identify placental macrophages (Hofbauer cells). As negative controls, the primary Abs were omitted, and as positive controls, tonsils were immunostained. 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% hydrogen peroxide (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 was carried out by using an RFCA microscope and DP50 camera, and the Studio 3.0.1 software (Olympus).

Real Time RT-PCR

Expression of *Foxp3* mRNA was analyzed in $CD4^+$ T cells polarized with PE CM for five days, as described above. The PE CM-induced $CD4^+$ T cells were lysed in RNeasy RLT lysing buffer (Qiagen) and frozen at -70˚C until total cellular RNA was isolated with an RNeasy Mini Kit according to the manufacturer's instructions (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 of cDNA with 2x TaqMan Fast Universal MasterMix (Applied Biosystems) and primers and probes for *Foxp3* (Forward primer: 5′-GTGGCCCGGATGTGAGAA-3′, Reverse primer: 5′-GCTGCTCCAGAGACTGTACCATCT-3′, Probe: 5′-CCTCAAGCACTGCCAGGCGGAC-3′) or *18s* rRNA (Forward primer: 5′-CGGCTACCACATCCAAGGAA-3′, Reverse primer: 5′-GCTGGAATTACCGCGGCT-3′, Probe: 5′-TGCTGGCACCAGACTTGCCCTC-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 was 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 by using GraphPad Prism version 6.0. The majority of the flow cytometry data was normally distributed and was therefore analyzed with repeated measures ANOVA and Sidak's multiple comparison test or Student's paired t-test. Data from Real

Time RT-PCR, multiplex bead assays and ELISA were analyzed with Wilcoxon matched-pairs test. Flow cytometry data is expressed as mean and SD and for data from the multiplex bead assay, ELISA and RT-PCR, medians and/or interquartile ranges are shown. p values $\leq$ 0.05 were considered statistically significant (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

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 conditioned medium (CM) from first trimester placental tissue explants. Macrophages were generated from $CD14^+$ monocytes isolated from non-pregnant women. GM-CSF was used as a basic growth factor, since 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 placental explant CM (PE CM) acquired a phenotype characteristic for decidual macrophages (6), with high expression of CD14, CD163 (scavenger receptor), CD206 (mannose receptor) and CD209 (DC-SIGN [dendritic cell-specific ICAM-3-grabbing nonintegrin]), and reduced expression of ICAM-3 (Fig. 1, A and B). This phenotype was induced in a dose-dependent manner (Fig. 1B). PE CM (12.5%) also significantly increased the production of the anti-inflammatory cytokine IL-10, while 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), was also 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

Treg cells are, in contrast to other Th subsets, 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 non-pregnant women) in the

presence of PE CM. In order to mimic the decidual microenvironment at 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 (Treg) (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, neither in the presence nor 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 Rorγt^+ (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"). Since 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 and CD69, or 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, here represented 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, IL-2 and GM-CSF (Fig. 2, A and B, "aCD3/CD28"). The reduced production of cytokines was not a result of decreased viability, since PE CM did not affect the viability of anti-CD3/CD28-stimulated $CD4^+$ T cells (Fig. 2C, "aCD3/CD28").

$CD25^{high}Foxp3^{+}$ Treg cells induced by placental soluble factors are functionally suppressive

Similar to the increased proportion of $Foxp3^{+}$ T cells, the proportion of $CD4^{dim}CD25^{high}$ and $CD25^{high}Foxp3^{+}$ Treg cells was increased in PE CM-exposed as compared with unexposed $CD4^{+}$ T cells (Fig. 3A). PE CM also increased the expression of Foxp3 at the mRNA level in unstimulated $CD4^{+}$ T cells (Fig. 3B). The PE CM-induced Treg cells were $CD127^{low}$, expressed high levels of the suppressive markers CTLA-4 and CD39 and the majority were memory cells expressing CD45R0 (Fig. 3C), similar to the Treg cells in early human decidua (17, 18). Finally, we tested whether the PE CM-induced Treg cells were functional, by analyzing their ability to suppress the proliferation of anti-CD3/CD28-stimulated responder T cells. After five days of culture with PE CM, $CD4^{+}CD25^{-}$ responder cells and $CD4^{dim}CD25^{high}$ Treg cells were isolated by FACS. The responder cells were labelled with the cell division-tracking dye CFSE, stimulated with anti-CD3/CD28 Abs and co-cultured with the PE CM-induced Treg cells for five days at the ratio 2:1. As seen in Fig. 3D and E, PE CM-induced $CD4^{dim}CD25^{high}$ Treg cells significantly suppressed the proliferation of $CD4^{+}CD25^{-}$ responder cells.

Cytotrophoblast and extravillous trophoblast cells induce homeostatic macrophages and the expansion of Treg cells

Next we tested whether trophoblast cells, the primary cell component of the fetal placenta, could take part in the induction of homeostatic macrophages and Treg cells. The purity of the isolated trophoblast cells (CTB: Cytokeratin $7^{+}HLA\text{-}G^{-}$; EVT: Cytokeratin $7^{+}HLA\text{-}G^{+}$) 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 homoeostatic phenotype characteristic for decidual macrophages ($CD14^{+}CD163^{+}CD206^{+}CD209^{+}ICAM\text{-}3^{low}$) (Fig.

4A), similar to the effect of CM from whole placental tissue (Fig. 1, A and B). We also tested if 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 and 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 $CD4^{dim}CD25^{high}$, $Foxp3^{+}$ and $CD25^{high}Foxp3^{+}$ Treg cells in the $CD4^{+}$ T cell population, as compared with medium alone (Fig. 4C and Supplemental Fig. 1, A and B). CM from the HTR-8/SVneo cell line also induced an increased proportion of $CD4^{dim}CD25^{high}$, $Foxp3^{+}$ and $CD25^{high}Foxp3^{+}$ Treg cells (Fig. 4D and Supplemental Fig. 1C) but was required at higher concentrations (25% and 50%). 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 $CD4^{+}$ T cells, HTR-8/SVneo CM preferentially induced the expansion of $Foxp3^{+}$ Treg cells, but did not induce an increase in T-bet^{+} (Th1), GATA-3^{+} (Th2) or Rorγt^{+} (Th17) cells in the unstimulated $CD4^{+}$ T cell population (Supplemental Fig. 2A, "unstim"). The levels of IL-10, IFN-γ, IL-13 and IL-17 were under the detection limit in unstimulated cells, also when 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, A and B, "aCD3/CD28"). In summary, freshly isolated CTB and EVT cells from first trimester healthy human placenta induce homeostatic macrophages and

expand the Treg cell population, while 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$^+$ Treg cells

To identify the factors involved in the induction of homeostatic macrophages and Treg cells, we analyzed a panel of soluble factors in PE CM and CM from CTB, EVT and HTR-8/SVneo cells (Fig. 5; a complete list is presented in Table I). We have previously shown that the decidual macrophage phenotype can be induced *in vitro* by M-CSF, while GM-CSF counteracts this effect (6). We also showed that IL-10 not only enhanced the decidual macrophage phenotype in M-CSF-driven macrophages, but also restored the M2 phenotype in GM-CSF-driven M1-like macrophages (6). In the present study, we show that the placenta itself is a major source of M-CSF (greatly exceeding the levels of GM-CSF) (Fig. 5A and Table I) and that M-CSF is located to CTB cells and 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, while CD14, CD206 and CD209 were not affected. IL-10 was also produced by placental tissue and at particularly high levels by CTB and EVT cells, while HTR-8/SVneo cells lacked IL-10 production (Fig. 5B and Table I). IL-10 was expressed by CTB cells and the syncytiotrophoblast, but also by other cells, including placental macrophages (Hofbauer cells) (Fig. 5G). When using blocking Abs against IL-10R, the expression of CD14, CD163, CD206 and CD209 induced by PE CM was significantly reduced in macrophages (Fig. 6B). These results indicate an important role for trophoblast-

derived M-CSF, and in particular IL-10, in the induction of decidual macrophages in early human pregnancy.

Although there is a lack of knowledge regarding the factors responsible for the specific enrichment of Treg cells at the fetal-maternal interface in humans, there are several well-established factors, including TGF-β and IL-10, that generate inducible Foxp3-expressing Treg cells in the periphery (25, 26). TGF-β1 and TGF-β2 were detected in PE CM and HTR-8/SVneo CM, while only TGF-β2 was detected in CTB and EVT CM (Fig. 5C and Table I). In contrast, TGF-β3 was only detected at 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 $CD4^+Foxp3^+$ Treg cells 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 and Table I), which was recently localized mainly to the syncytiotrophoblast within the placenta (28). TRAIL has been shown to preferentially expand the Treg cell population and to inhibit expansion of the non-Treg cell pool in a mouse model (29). Since 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 cell expansion. Indeed, when using anti-TRAIL blocking Abs we observed a significant reduction in the proportion of PE CM-induced $Foxp3^+$ Treg cells (Fig. 6E). Other factors proposed to be involved in the generation of inducible Treg cells are LIF and CCL18 (30, 31). However, neither anti-LIF nor anti-CCL18 neutralizing Abs had an effect on the expansion of Treg cells induced by PE CM (data not shown). In summary, several placental-

derived factors seem to act in concert towards the expansion of the Treg cell pool during early human pregnancy.

DISCUSSION

In this study, we demonstrate that human fetally-derived placental tissue promotes the induction of homeostatic macrophages and Treg cells, which are essential for fetal tolerance and reproductive success (Fig. 7). The placental-induced macrophages shared the $CD14^{+}CD163^{+}CD206^{+}CD209^{+}ICAM\text{-}3^{low}$ 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, primarily produced by trophoblast cells. Placental tissue also induced the expansion of $CD25^{high}CD127^{low}Foxp3^{+}$ Treg cells in parallel with increased IL-10 production. In addition, expression of the Th1-associated transcription factor T-bet was increased by placental-derived factors, while production of IFN-γ was not induced. Expression of GATA-3 and Roryt and production of IL-13 and IL-17, associated with Th2 and Th17 cells respectively, were not induced. The expanded Treg cell population was, like decidual Treg cells (17, 18), predominantly $CD45R0^{+}$, expressed the suppressive markers CTLA-4 and CD39, and was functionally suppressive *in vitro.* The Treg cell expansion was partly mediated by TGF-β, IL-10 and TRAIL produced particularly by trophoblast cells. Additionally, placental-derived factors limited excessive Th cell activation and cytokine production, thus supporting a homeostatic environment compatible with normal fetal development.

In vitro studies have demonstrated that the crosstalk between decidual cells promotes development of maternal leukocytes with regulatory properties. This includes induction of Treg cells and suppression of T cell activation by decidual macrophages (12, 14) and stromal cells (32, 33), as well as the induction of Treg cells 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 Treg cells 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 crosstalk 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 have been reported to promote suppressive function in leukocytes (for instance 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) that 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 their lack of IL-10 production). Thus, although useful in many aspects, 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 Treg cells, but not of Th2 cells, supports the view 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 ($CD14^{low}CD163^{-}$) (6), that Treg cells 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 where 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 not only be caused by activation of placental/fetal-specific T cells but also by general T cell activation, for instance 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 have previously shown by an *in vitro* model that these cytokines are main inducers of decidual macrophages (6), the present study further strengthens their relevance *in vivo*, since 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 has been 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), and 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- (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 macrophages 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 Treg cells appears to be a process where 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 here, molecules such as soluble CD200 and HLA-G, Galectin-1 and pregnancy-associated hormones, might also influence the generation of Treg cells (49-53). Given the importance of Treg cells during pregnancy (15, 16), a redundancy in soluble mediators promoting expansion of Treg cells at the fetal-maternal interface is not surprising. Furthermore, these factors may have divergent and complementary effects on Treg cells. For instance, TGF-β has been described to promote the conversion of non-Treg cells into Foxp3-expressing Treg cells, rather than to expand an already existing Treg cell population (25). In contrast, IL-10 was recently reported to upregulate the anti-apoptotic Bcl-2 specifically in Treg cells but not in conventional T cells, suggesting a mechanism for IL-10-driven maternal Treg cell expansion during pregnancy (54). Similarly, the apoptosis-inducing factor TRAIL was shown to expand the Treg cell pool and to inhibit expansion of non-Treg cells (29). Thus, both IL-10 and TRAIL could increase the proportion of Treg cells by preferentially promoting survival of already existing Treg cells. Our data showing a specific increase in $Foxp3^+$ Treg cells 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 has been described in a mouse model with T cell-specific deletion of the TGF-β receptor II, in which lethal inflammation developed in association with T cell activation and disrupted Treg cell induction (55). Similarly, TRAIL has been shown to mediate protection against autoimmune diseases in mice by promoting the expansion of Treg cells (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 have 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 Treg cells and limit excessive Th cell activation (Fig. 7). These findings are relevant for understanding the pathology of immune-associated pregnancy complications, where both decidual macrophages and Treg cells have been implicated (1, 2), but also beyond pregnancy, in areas of cancer, autoimmunity and transplantation, where similar tolerance and homeostatic mechanisms are over- or under-developed.

ACKNOWLEDGEMENTS

We would like to thank the staff at the Women's clinic at Linköping University Hospital for recruiting patients and helping with samples. We also thank S. Sharma (Department of Pediatrics, Brown University, Providence, RI) for providing the HTR-8/SVneo cell line, P. Cassel for help with multiplexed bead assay, J.E. Andersson for help with ELISA, M. Karlsson for help with IHC, M. Edström for assistance with flow cytometry sorting and B. Innes for help with IHC and isolation of trophoblast cells.

REFERENCES

1. Erlebacher, A. 2013. Immunology of the maternal-fetal interface. *Annu Rev Immunol* 31: 387-411.

2. Svensson-Arvelund, J., J. Ernerudh, E. Buse, J. M. Cline, J. D. Haeger, D. Dixon, U. R. Markert, C. Pfarrer, P. D. Vos, and M. M. Faas. 2014. The Placenta in Toxicology. Part II: Systemic and Local Immune Adaptations in Pregnancy. *Toxicologic pathology* 42: 327-338.

3. Svensson-Arvelund, J., and J. Ernerudh. 2014. The Role of Macrophages in Promoting and Maintaining Homeostasis at the Fetal-Maternal Interface. *Am J Reprod Immunol In Press*.

4. Ernerudh, J., G. Berg, and J. Mjosberg. 2011. Regulatory T helper cells in pregnancy and their roles in systemic versus local immune tolerance. *Am J Reprod Immunol* 66 Suppl 1: 31-43.

5. Bartmann, C., S. E. Segerer, L. Rieger, M. Kapp, M. Sutterlin, and U. Kammerer. 2014. Quantification of the predominant immune cell populations in decidua throughout human pregnancy. *Am J Reprod Immunol* 71: 109-119.

6. Svensson, J., M. C. Jenmalm, A. Matussek, R. Geffers, G. Berg, and J. Ernerudh. 2011. Macrophages at the fetal-maternal interface express markers of alternative activation and are induced by M-CSF and IL-10. *J Immunol* 187: 3671-3682.

7. Lidstrom, C., L. Matthiesen, G. Berg, S. Sharma, J. Ernerudh, and C. Ekerfelt. 2003. Cytokine secretion patterns of NK cells and macrophages in early human pregnancy decidua and blood: implications for suppressor macrophages in decidua. *Am J Reprod Immunol* 50: 444-452.

8. Gustafsson, C., J. Mjosberg, A. Matussek, R. Geffers, L. Matthiesen, G. Berg, S. Sharma, J. Buer, and J. Ernerudh. 2008. Gene expression profiling of human decidual macrophages: evidence for immunosuppressive phenotype. *PLoS ONE* 3: e2078.

9. Laskarin, G., K. Cupurdija, V. S. Tokmadzic, D. Dorcic, J. Dupor, K. Juretic, N. Strbo, T. B. Crncic, F. Marchezi, P. Allavena, A. Mantovani, L. Randic, and D. Rukavina. 2005. The presence of functional mannose receptor on macrophages at the maternal-fetal interface. *Hum Reprod* 20: 1057-1066.

10. Kammerer, U., A. O. Eggert, M. Kapp, A. D. McLellan, T. B. Geijtenbeek, J. Dietl, Y. van Kooyk, and E. Kampgen. 2003. Unique appearance of proliferating antigen-presenting cells expressing DC-SIGN (CD209) in the decidua of early human pregnancy. *Am J Pathol* 162: 887-896.

11. Houser, B. L., T. Tilburgs, J. Hill, M. L. Nicotra, and J. L. Strominger. 2011. Two unique human decidual macrophage populations. *J Immunol* 186: 2633-2642.

12. Sayama, S., T. Nagamatsu, D. J. Schust, N. Itaoka, M. Ichikawa, K. Kawana, T. Yamashita, S. Kozuma, and T. Fujii. 2013. Human decidual macrophages suppress IFN-gamma production by T cells through costimulatory B7-H1:PD-1 signaling in early pregnancy. *J Reprod Immunol* 100: 109-117.

13. Vacca, P., C. Cantoni, M. Vitale, C. Prato, F. Canegallo, D. Fenoglio, N. Ragni, L. Moretta, and M. C. Mingari. 2010. Crosstalk between decidual NK and CD14+ myelomonocytic cells results in induction of Tregs and immunosuppression. *Proc Natl Acad Sci U S A* 107: 11918-11923.

14. Hsu, P., B. Santner-Nanan, J. E. Dahlstrom, M. Fadia, A. Chandra, M. Peek, and R. Nanan. 2012. Altered decidual DC-SIGN+ antigen-presenting cells and impaired regulatory T-cell induction in preeclampsia. *Am J Pathol* 181: 2149-2160.

15. Rowe, J. H., J. M. Ertelt, L. Xin, and S. S. Way. 2012. Pregnancy imprints regulatory memory that sustains anergy to fetal antigen. *Nature* 490: 102-106.

16. Samstein, R. M., S. Z. Josefowicz, A. Arvey, P. M. Treuting, and A. Y. Rudensky. 2012. Extrathymic generation of regulatory T cells in placental mammals mitigates maternal-fetal conflict. *Cell* 150: 29-38.

17. Mjosberg, J., G. Berg, M. C. Jenmalm, and J. Ernerudh. 2010. FOXP3+ regulatory T cells and T helper 1, T helper 2, and T helper 17 cells in human early pregnancy decidua. *Biol Reprod* 82: 698-705.

18. Tilburgs, T., D. L. Roelen, B. J. van der Mast, G. M. de Groot-Swings, C. Kleijburg, S. A. Scherjon, and F. H. Claas. 2008. Evidence for a selective migration of fetus-specific CD4+CD25bright regulatory T cells from the peripheral blood to the decidua in human pregnancy. *J Immunol* 180: 5737-5745.

19. Lash, G. E., K. Naruse, B. A. Innes, S. C. Robson, R. F. Searle, and J. N. Bulmer. 2010. Secretion of angiogenic growth factors by villous cytotrophoblast and extravillous trophoblast in early human pregnancy. *Placenta* 31: 545-548.

20. Graham, C. H., T. S. Hawley, R. G. Hawley, J. R. MacDougall, R. S. Kerbel, N. Khoo, and P. K. Lala. 1993. Establishment and characterization of first trimester human trophoblast cells with extended lifespan. *Exp Cell Res* 206: 204-211.

21. Mjosberg, J., J. Svensson, E. Johansson, L. Hellstrom, R. Casas, M. C. Jenmalm, R. Boij, L. Matthiesen, J. I. Jonsson, G. Berg, and J. Ernerudh. 2009. Systemic reduction of functionally suppressive CD4dimCD25highFoxp3+ Tregs in human second trimester pregnancy is induced by progesterone and 17beta-estradiol. *J Immunol* 183: 759-769.

22. Sandberg, M., A. Frykman, J. Ernerudh, G. Berg, L. Matthiesen, C. Ekerfelt, L. J. Nilsson, and M. C. Jenmalm. 2009. Cord blood cytokines and chemokines and

development of allergic disease. *Pediatric allergy and immunology : official publication of the European Society of Pediatric Allergy and Immunology* 20: 519-527.

23. Wynn, T. A., A. Chawla, and J. W. Pollard. 2013. Macrophage biology in development, homeostasis and disease. *Nature* 496: 445-455.

24. Wang, J., A. Ioan-Facsinay, E. I. van der Voort, T. W. Huizinga, and R. E. Toes. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *Eur J Immunol* 37: 129-138.

25. Chen, W., W. Jin, N. Hardegen, K. J. Lei, L. Li, N. Marinos, G. McGrady, and S. M. Wahl. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *J Exp Med* 198: 1875-1886.

26. Groux, H., M. Bigler, J. E. de Vries, and M. G. Roncarolo. 1996. Interleukin-10 induces a long-term antigen-specific anergic state in human CD4+ T cells. *J Exp Med* 184: 19-29.

27. Simpson, H., S. C. Robson, J. N. Bulmer, A. Barber, and F. Lyall. 2002. Transforming growth factor beta expression in human placenta and placental bed during early pregnancy. *Placenta* 23: 44-58.

28. Stenqvist, A. C., O. Nagaeva, V. Baranov, and L. Mincheva-Nilsson. 2013. Exosomes secreted by human placenta carry functional Fas ligand and TRAIL molecules and convey apoptosis in activated immune cells, suggesting exosome-mediated immune privilege of the fetus. *J Immunol* 191: 5515-5523.

29. Ikeda, T., S. Hirata, S. Fukushima, Y. Matsunaga, T. Ito, M. Uchino, Y. Nishimura, and S. Senju. 2010. Dual effects of TRAIL in suppression of autoimmunity: the inhibition of Th1 cells and the promotion of regulatory T cells. *J Immunol* 185: 5259-5267.

30. Gao, W., L. Thompson, Q. Zhou, P. Putheti, T. M. Fahmy, T. B. Strom, and S. M. Metcalfe. 2009. Treg versus Th17 lymphocyte lineages are cross-regulated by LIF versus IL-6. *Cell Cycle* 8: 1444-1450.

31. Chang, Y., P. de Nadai, I. Azzaoui, O. Morales, N. Delhem, H. Vorng, S. Tomavo, S. Ait Yahia, G. Zhang, B. Wallaert, C. Chenivesse, and A. Tsicopoulos. 2010. The chemokine CCL18 generates adaptive regulatory T cells from memory CD4+ T cells of healthy but not allergic subjects. *FASEB J* 24: 5063-5072.

32. Erkers, T., S. Nava, J. Yosef, O. Ringden, and H. Kaipe. 2013. Decidual stromal cells promote regulatory T cells and suppress alloreactivity in a cell contact-dependent manner. *Stem Cells Dev* 22: 2596-2605.

33. Nagamatsu, T., D. J. Schust, J. Sugimoto, and B. F. Barrier. 2009. Human decidual stromal cells suppress cytokine secretion by allogenic CD4+ T cells via PD-1 ligand interactions. *Hum Reprod* 24: 3160-3171.

34. Abumaree, M. H., M. A. Al Jumah, B. Kalionis, D. Jawdat, A. Al Khaldi, F. M. Abomaray, A. S. Fatani, L. W. Chamley, and B. A. Knawy. 2013. Human placental mesenchymal stem cells (pMSCs) play a role as immune suppressive cells by shifting macrophage differentiation from inflammatory M1 to anti-inflammatory M2 macrophages. *Stem cell reviews* 9: 620-641.

35. Chang, C. J., M. L. Yen, Y. C. Chen, C. C. Chien, H. I. Huang, C. H. Bai, and B. L. Yen. 2006. Placenta-derived multipotent cells exhibit immunosuppressive properties that are enhanced in the presence of interferon-gamma. *Stem cells* 24: 2466-2477.

36. Wegmann, T. G., H. Lin, L. Guilbert, and T. R. Mosmann. 1993. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today* 14: 353-356.

37. Hanna, N., I. Hanna, M. Hleb, E. Wagner, J. Dougherty, D. Balkundi, J. Padbury, and S. Sharma. 2000. Gestational age-dependent expression of IL-10 and its receptor in human placental tissues and isolated cytotrophoblasts. *J Immunol* 164: 5721-5728.

38. Krishnan, L., L. J. Guilbert, T. G. Wegmann, M. Belosevic, and T. R. Mosmann. 1996. T helper 1 response against Leishmania major in pregnant C57BL/6 mice increases implantation failure and fetal resorptions. Correlation with increased IFN-gamma and TNF and reduced IL-10 production by placental cells. *J Immunol* 156: 653-662.

39. Rowe, J. H., J. M. Ertelt, L. Xin, and S. S. Way. 2012. Listeria monocytogenes cytoplasmic entry induces fetal wastage by disrupting maternal Foxp3+ regulatory T cell-sustained fetal tolerance. *PLoS Pathog* 8: e1002873.

40. Wiktor-Jedrzejczak, W., A. Bartocci, A. W. Ferrante, Jr., A. Ahmed-Ansari, K. W. Sell, J. W. Pollard, and E. R. Stanley. 1990. Total absence of colony-stimulating factor 1 in the macrophage-deficient osteopetrotic (op/op) mouse. *Proc Natl Acad Sci U S A* 87: 4828-4832.

41. Hamilton, J. A. 2008. Colony-stimulating factors in inflammation and autoimmunity. *Nat Rev Immunol* 8: 533-544.

42. Mosser, D. M., and X. Zhang. 2008. Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* 226: 205-218.

43. Pampfer, S., E. Daiter, D. Barad, and J. W. Pollard. 1992. Expression of the colony-stimulating factor-1 receptor (c-fms proto-oncogene product) in the human uterus and placenta. *Biol Reprod* 46: 48-57.

44. Pollard, J. W., J. S. Hunt, W. Wiktor-Jedrzejczak, and E. R. Stanley. 1991. A pregnancy defect in the osteopetrotic (op/op) mouse demonstrates the requirement for CSF-1 in female fertility. *Dev Biol* 148: 273-283.

45. Chaouat, G., A. Assal Meliani, J. Martal, R. Raghupathy, J. F. Elliott, T. Mosmann, and T. G. Wegmann. 1995. IL-10 prevents naturally occurring fetal loss in the CBA x DBA/2 mating combination, and local defect in IL-10 production in this abortion-prone combination is corrected by in vivo injection of IFN-tau. *J Immunol* 154: 4261-4268.

46. Qiu, X., L. Zhu, and J. W. Pollard. 2009. Colony-stimulating factor-1-dependent macrophage functions regulate the maternal decidua immune responses against Listeria monocytogenes infections during early gestation in mice. *Infect Immun* 77: 85-97.

47. Thaxton, J. E., R. Romero, and S. Sharma. 2009. TLR9 activation coupled to IL-10 deficiency induces adverse pregnancy outcomes. *J Immunol* 183: 1144-1154.

48. Robertson, S. A., A. S. Care, and R. J. Skinner. 2007. Interleukin 10 regulates inflammatory cytokine synthesis to protect against lipopolysaccharide-induced abortion and fetal growth restriction in mice. *Biol Reprod* 76: 738-748.

49. Blois, S. M., J. M. Ilarregui, M. Tometten, M. Garcia, A. S. Orsal, R. Cordo-Russo, M. A. Toscano, G. A. Bianco, P. Kobelt, B. Handjiski, I. Tirado, U. R. Markert, B. F. Klapp, F. Poirier, J. Szekeres-Bartho, G. A. Rabinovich, and P. C. Arck. 2007. A pivotal role for galectin-1 in fetomaternal tolerance. *Nat Med* 13: 1450-1457.

50. Clark, D. A., A. Keil, Z. Chen, U. Markert, J. Manuel, and R. M. Gorczynski. 2003. Placental trophoblast from successful human pregnancies expresses the tolerance signaling molecule, CD200 (OX-2). *Am J Reprod Immunol* 50: 187-195.

51. Schumacher, A., K. Heinze, J. Witte, E. Poloski, N. Linzke, K. Woidacki, and A. C. Zenclussen. 2013. Human chorionic gonadotropin as a central regulator of pregnancy immune tolerance. *J Immunol* 190: 2650-2658.

52. Schumacher, A., E. Poloski, D. Sporke, and A. C. Zenclussen. 2014. Luteinizing hormone contributes to fetal tolerance by regulating adaptive immune responses. *Am J Reprod Immunol* 71: 434-440.

53. Selmani, Z., A. Naji, I. Zidi, B. Favier, E. Gaiffe, L. Obert, C. Borg, P. Saas, P. Tiberghien, N. Rouas-Freiss, E. D. Carosella, and F. Deschaseaux. 2008. Human leukocyte antigen-G5 secretion by human mesenchymal stem cells is required to suppress T lymphocyte and natural killer function and to induce CD4+CD25highFOXP3+ regulatory T cells. *Stem cells* 26: 212-222.

54. Santner-Nanan, B., K. Straubinger, P. Hsu, G. Parnell, B. Tang, B. Xu, A. Makris, A. Hennessy, M. J. Peek, D. H. Busch, C. P. da Costa, and R. Nanan. 2013. Fetal-maternal alignment of regulatory T cells correlates with IL-10 and Bcl-2 upregulation in pregnancy. *J Immunol* 191: 145-153.

55. Li, M. O., S. Sanjabi, and R. A. Flavell. 2006. Transforming growth factor-beta controls development, homeostasis, and tolerance of T cells by regulatory T cell-dependent and -independent mechanisms. *Immunity* 25: 455-471.

56. Wang, S. H., G. H. Chen, Y. Fan, M. Van Antwerp, and J. R. Baker, Jr. 2009. Tumor necrosis factor-related apoptosis-inducing ligand inhibits experimental autoimmune thyroiditis by the expansion of CD4+CD25+ regulatory T cells. *Endocrinology* 150: 2000-2007.

FOOTNOTES

[1]Corresponding author:

Phone: +46 10 10 32998

Fax: +46 13 132257

E-mail address: judit.svensson@liu.se

[2] Grant support:

This work was supported by Medical Research Council Grant K2013-61X-22310-01-4.

[3] Non-standard abbreviations:

CM, conditioned medium; CTB cells, cytotrophoblast cells; DC-SIGN, dendritic cell-specific ICAM-3–grabbing nonintegrin; EVT cells, extravillous trophoblast cells; PE CM, placental explant CM; Treg cells, regulatory T cells

Table I. *Soluble factors[1] produced by 1st trimester placental tissue and trophoblast cells.*

	Placenta		CTB[2]		EVT[2]		HTR-8[2]	
Macrophage growth factors								
GM-CSF	**29**	(11-134)	**139**	(39-355)	**115**	(22-357)	**4**	(2.6-4.6)
M-CSF	**4,580**	(1,551-10,986)	**1,416**	(480-2,018)	**328**	(208-1,123)	**1,649**	(1,504-2,501)
Cytokines								
Anti-inflammatory								
IL-10	**7.7**	(2.9-63)	**663**	(93-6,000)	**789**	(29-6,000)		<det.
TGF-β1	**573**	(309-813)		<det.		<det.	**580**	(332-714)
TGF-β2	**292**	(145-935)	**165**	(4.9-709)	**4.9**	(4.9-184)	**612**	(390-941)
TGF-β3	**4.9**	(4.9-19)		<det.		<det.		<det.
IL-1RA	**8**	(8-27)	**58**	(26-404)	**53**	(14-410)		<det.
Pro-inflammatory								
IL-1β	**9.7**	(1.6-170)	**168**	(29-1,380)	**568**	(12-961)		<det.
IL-6	**12,410**	(8,418-54,914)	**5,153**	(3,120-20,478)	**4,535**	(131-21,470)	**1,164**	(790-1,621)
TNF	**7.3**	(1.6-59)	**410**	(209-2,552)	**807**	(124-4,016)		<det.
IL-12p70		<det.		<det.		<det.		<det.
IL-23		<det.		<det.		<det.		<det.
Th-related								
IFN-γ		<det.	**3.3**	(1.6-26)	**1.6**	(1.6-5.8)		<det.
IL-13		<det.		<det.		<det.		<det.
IL-17		<det.	**5.3**	(1.6-19)	**1.6**	(1.6-23)		<det.
IL-2		<det.	**3.2**	(1.6-12)		<det.		<det.
Apoptosis-related								
TRAIL	**21**	(7.9-77)	**5.7**	(1.2-25)	**1.2**	(1.2-4.6)		<det.
Others								
IL-33	**6.8**	(1.4-17)		<det.		<det.		<det.
LIF	**23**	(13-62)	**7.7**	(1.8-29)	**1.8**	(1.8-17)		<det.
TSLP	**4**	(1.2-25)	**1.2**	(1.2-7.2)	**1.2**	(1.2-4)		<det.
Chemokines								
Myeloid								
CCL2	**1,701**	(591-8,797)	**13,571**	(2,316-96,913)	**5,091**	(291-49,115)	**18,083**	(17,316-18,377)
CXCL1-3	**870**	(415-11,396)	**6,587**	(3,496-52,852)	**10,308**	(706-46,760)	**433**	(168-636)
CXCL8	**9,094**	(5,292-41,984)	**74,901**	(4,028-373,909)	**75,035**	(11,984-376,163)	**1,201**	(569-1,629)
Homeostatic								
CCL18	**265**	(128-795)	**660**	(32-3,026)	**399**	(67-9,021)		<det.
Th1-associated								
CXCL10	**513**	(146-4,269)	**1,259**	(153-15,339)	**101**	(8-2,200)		<det.
CXCL11	**5.5**	(5.5-28)	**17**	(5.5-171)	**5.5**	(5.5-17)		<det.
Th2-associated								
CCL17	**0.6**	(0.3-1.3)	**1.6**	(0.3-7.7)	**1**	(0.3-11)		<det.
CCL22	**30**	(8-172)	**200**	(90-1,305)	**151**	(43-825)		<det.
Th17-associated								
CCL20	**806**	(428-1,901)	**183**	(136-609)	**30**	(4.9-1,152)		<det.

[1]pg/ml, median (range), <det.= below detection

[2]CTB: Cytotrophoblast cells, EVT: Extravillous trophoblast cells, HTR-8: HTR-8/SVneo trophoblast cell line

FIGURES

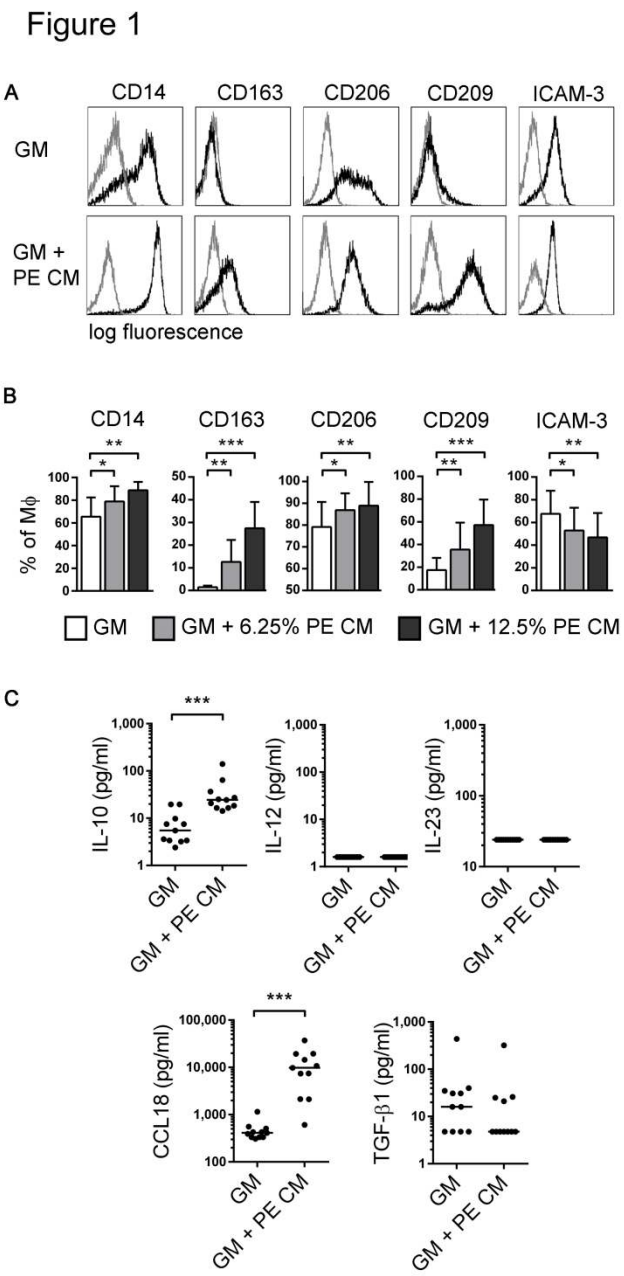

Figure 1

Soluble factors from placental tissue induce macrophages with homeostatic properties. **(A)** Flow cytometry histograms showing that 12.5% placental explant conditioned medium (PE CM) induces the regulatory macrophage markers CD14, CD163 (scavenger receptor), CD206 (mannose receptor) and CD209 (DC-SIGN), and reduces ICAM-3 expression (grey lines denote isotype control-stained cells and black lines denote specific Ab-stained cells).

(B) PE CM induces a homeostatic macrophage phenotype in a dose-dependent manner. **(C)** PE CM (12.5%) induced the production of IL-10 and CCL18 from macrophages, while IL-12, IL-23 and TGF-β1 were not induced. In all experiments, macrophages were generated from $CD14^+$ monocytes (isolated from non-pregnant women) by culturing them for six days in the presence of 5 ng/ml GM-CSF (GM) alone or in combination with PE CM, as indicated. Flow cytometry data was analyzed with repeated measures ANOVA and Sidak's multiple comparison test (B) and cytokine data with Wilcoxon matched-pairs test (C). The figures show representative histograms (**A**) or the mean + SD (**B**) from 12 individual experiments, or the median of 11 experiments (**C**). * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. MΦ: macrophages.

Figure 2

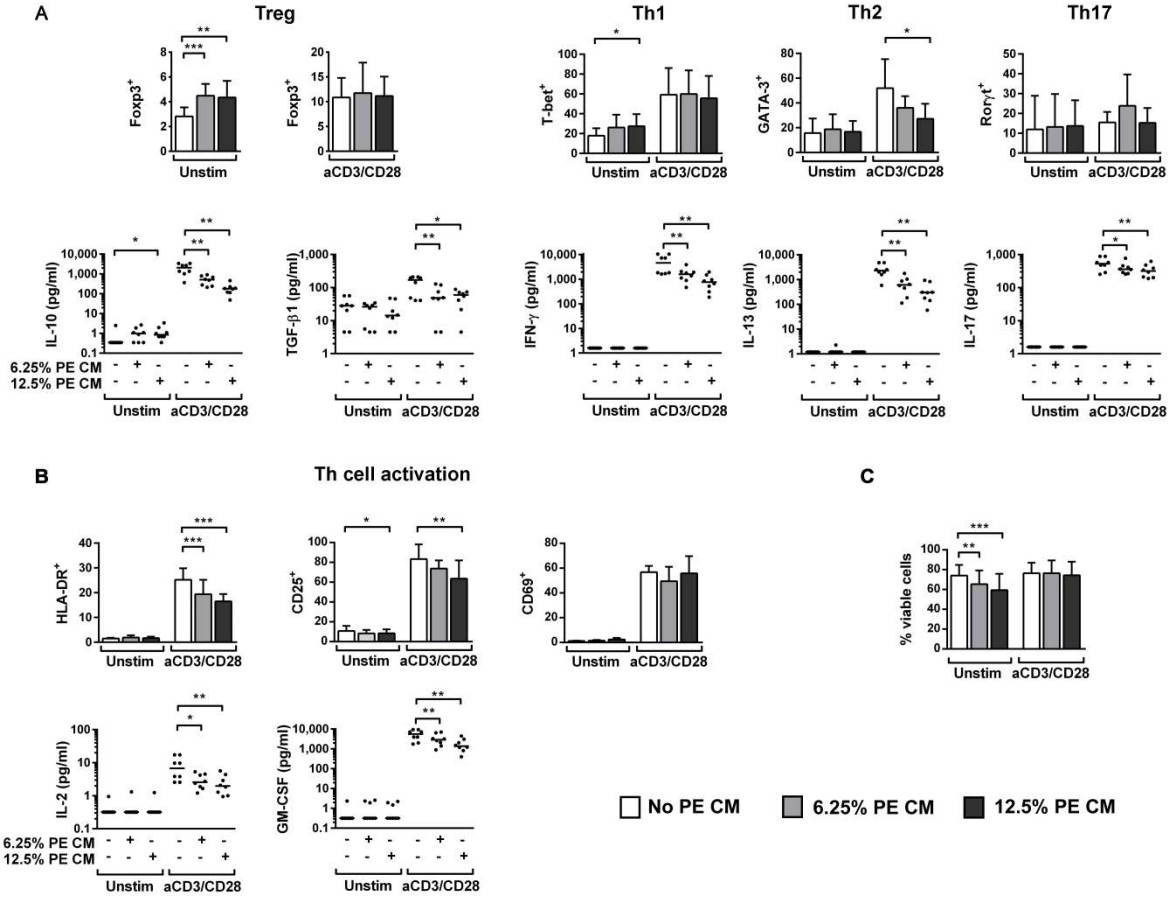

Figure 2

Soluble factors from placental tissue preferentially induce Foxp3 expression and production of IL-10 in CD4⁺ T cells. (A) The graphs show the effect of 6.25% and 12.5% first trimester placental explant conditioned medium (PE CM) on the polarization of Treg, Th1, Th2 and Th17 cells. The upper panel shows the expression of the transcription factors Foxp3 (Treg), T-bet (Th1), GATA-3 (Th2) and Rorγt (Th17) in the $CD4^+$ T cell population and the lower panel shows the production of cytokines from the corresponding Th cell subset. **(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 (**A-C**), peripheral blood $CD4^+$ T cells (isolated from non-pregnant women) were cultured either unstimulated (Unstim) or stimulated with 0.25 µg/ml anti-CD3 and anti-CD28 Abs (aCD3/CD28) for five days in the

presence or absence of PE CM. In all bar graphs **(A-C)**, white bars represent no addition of PE CM, grey bars 6.25% PE CM and black bars 12.5% PE CM. Transcription factors and HLA-DR were analyzed with flow cytometry (all analyses were performed by gating on viable cells). Cytokine production was analyzed with multiplex bead assay. Flow cytometry data was analyzed with repeated measures ANOVA and Sidak's multiple comparison test (**A** and **B**, upper panels; **C**) and cytokine data with Wilcoxon matched-pairs test (**A** and **B**, lower panels). The graphs show the mean + SD (**A** and **B**, upper panels; **C**) or the median (**A** and **B**, lower panels) of eight individual experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 3

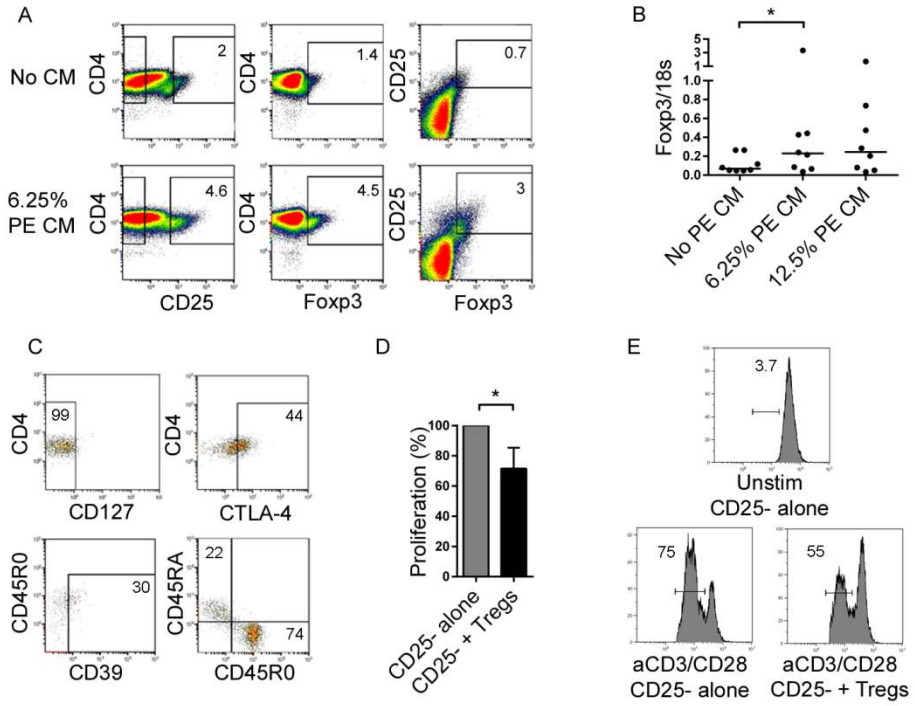

Figure 3

Placental soluble factors induce functionally suppressive CD25^{high}Foxp3^{+} Treg cells. (A) Flow cytometry dot plots showing that CD4^{dim}CD25^{high} (left), Foxp3^{+} (middle) and CD25^{high}Foxp3^{+} (right) Treg cells increased in the CD4^{+} T cell population when stimulated with 6.25% placental explant conditioned medium (PE CM, lower panel) as compared with medium alone (upper panel) for five days. One representative experiment of eight performed is shown. Numbers in the gates indicate 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 five days with or without PE CM. Data was analyzed with Wilcoxon matched-pairs test and bars indicate the median of eight experiments. * $p \leq 0.05$. **(C)** CD25^{high}Foxp3^{+} Treg cells generated in the presence of 6.25% PE CM showed a suppressive and memory phenotype, with low CD127 expression, high levels of the suppressive markers CTLA-4 and CD39 and the memory marker CD45R0. One representative experiment of six

performed is shown. Numbers in the gates indicate percentage of cells in each gate. **(D)** Suppressive effect of PE CM-induced $CD4^{dim}CD25^{high}$ Treg cells on the proliferation of anti-CD3/CD28-stimulated and CFSE-labelled $CD4^{+}CD25^{-}$ responder cells (CD25-). The proliferation of responder cells cultured alone was set to 100%. Data was analyzed with Student's paired t-test and bars indicate the mean values of four experiments, * $p \leq 0.05$. **(E)** Representative histograms showing the proliferation of unstimulated (Unstim) or anti-CD3/CD28-stimulated (aCD3/CD28) $CD4^{+}CD25^{-}$ responder cells cultured alone or with $CD4^{dim}CD25^{high}$ Treg cells. (**D** and **E**) Responder cells were co-cultured with Treg cells (2:1 ratio) for five days. In all experiments, $CD4^{+}$ T cells were isolated from peripheral blood from non-pregnant women.

Figure 4

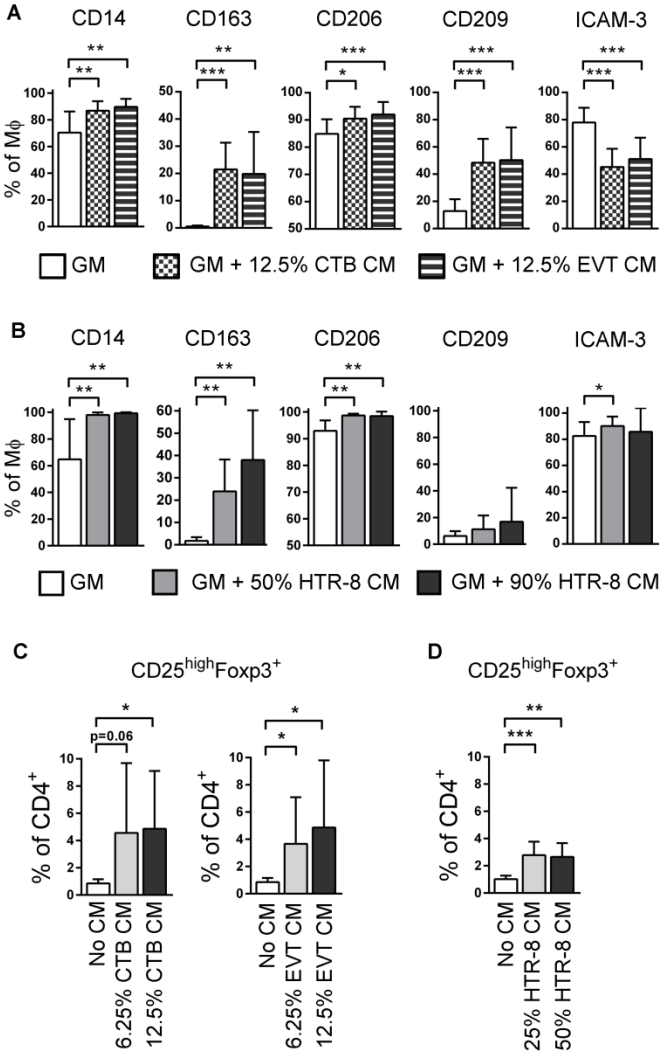

Figure 4

Trophoblast cells contribute to the induction of homeostatic macrophages and the expansion of CD25^{high}Foxp3^{+} Treg cells. (A) Conditioned medium (CM) from isolated first trimester placental cytotrophoblast (CTB) or extravillous trophoblast (EVT) cells induced the regulatory markers CD14, CD163 (scavenger receptor), CD206 (mannose receptor) and CD209 (DC-SIGN), and reduced ICAM-3 expression in macrophages. **(B)** CM from the trophoblast cell line HTR-8/SVneo (HTR-8) partially induced a homeostatic macrophage phenotype with increased CD14, CD163 and CD206 expression but no induction of CD209 or reduction of ICAM-3 expression. **(C)** CTB or EVT CM and **(D)** HTR-8 CM induced an

increased proportion of $CD25^{high}Foxp3^{+}$ Treg cells within the $CD4^{+}$ T cell population. (**A** and **B)** Macrophages were generated from $CD14^{+}$ monocytes (isolated from non-pregnant women) by culturing them for six days with GM-CSF (GM) alone or in combination with CM. (**C** and **D)** $CD4^{+}$ T cells (isolated from non-pregnant women) were cultured for five days with CM at the indicated concentrations. In all experiments cells were analyzed with flow cytometry. Data was analyzed with Student's paired t-test (**A-D**). All graphs show the mean + SD from nine individual experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. MΦ: macrophages.

Figure 5

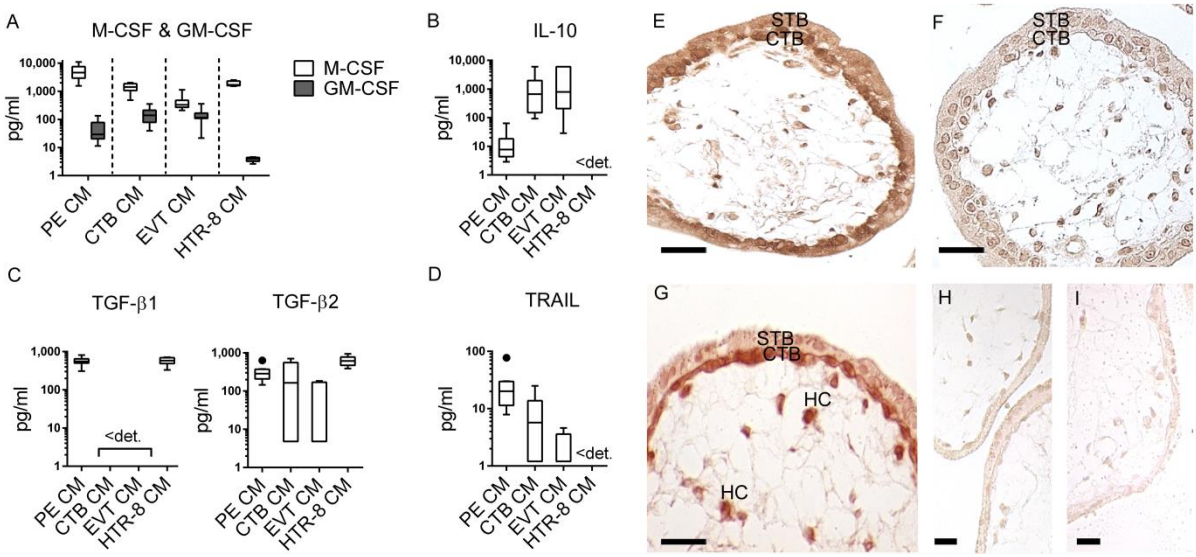

Figure 5

Production and expression of immune modulating factors by placental tissue and trophoblast cells. Multiplex bead assay was used to analyze the concentrations of M-CSF and GM-CSF **(A)**, IL-10 **(B)**, TGF-β **(C)** and TRAIL **(D)** in conditioned medium (CM) from healthy first trimester placental explants (PE CM), isolated cytotrophoblast cells (CTB CM), extravillous trophoblast cells (EVT CM) and the first trimester trophoblast cell line HTR-8/SVneo (HTR-8 CM) (**A-D**). The conditioned medium was collected after 24 h incubation of placental tissue explants (100 mg wet tissue in 1 ml culture medium) or isolated CTB, EVT or HTR-8/SVneo cells ($1x10^6$ cells/ml). PE CM; n=12; CTB and EVT CM; n=7, HTR-8/SVneo CM; n=5. **(E-I)** Immunohistochemistry of formalin-fixed and paraffin-embedded first trimester human placental tissue. M-CSF **(E)**, GM-CSF **(F)** and IL-10 **(G)** were mainly expressed by the syncytiotrophoblast shell (STB) and underlying cytotrophoblast (CTB) cells surrounding the chorionic villi. IL-10 was also strongly expressed by fetal placental macrophages (Hofbauer cells; HC) (**G**). Negative control stainings for M-CSF **(H)** and for GM-CSF and IL-10 **(I)**. Representative sections from one individual; ten individual

immunostainings were performed. Original magnifications: x 250; bars represent 25 µm.

<det.: below detection.

Figure 6

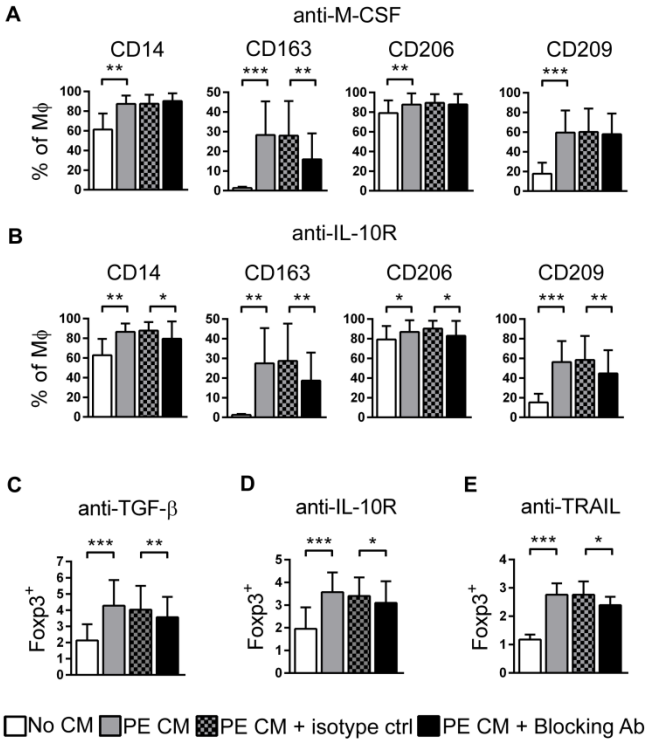

Figure 6

Neutralization of M-CSF, IL-10, TGF-β and TRAIL during the polarization of macrophages or CD4$^+$ T cells with placental-derived soluble factors. (A and B) The graphs show the phenotype of macrophages cultured with 5 ng/ml GM-CSF alone (No CM) or in combination with 12.5% first trimester placental explant conditioned medium (PE CM), and the addition of neutralizing Abs against M-CSF **(A)** or IL-10R **(B)**. M-CSF blocking Abs reversed the induction of CD163 (**A**), while IL-10R blocking Abs inhibited the induction of all regulatory markers (**B**). **(C-E)** The expansion of Foxp3$^+$ cells in CD4$^+$ T cells exposed to 6.25% PE CM was partially reversed by the addition of neutralizing Abs against TGF-β **(C),** IL-10R **(D)** or TRAIL **(E)**. Macrophages (**A** and **B**) generated from CD14$^+$ monocytes were cultured for six days and CD4$^+$ T cells (**C-E**) for five days in the presence or absence of first trimester PE CM and the addition of neutralizing Abs or corresponding isotype controls and were then analyzed with flow cytometry. CD14$^+$ monocytes and CD4$^+$ T cells were isolated

from peripheral blood from non-pregnant women. Data was analyzed with Student's paired t-test and the graphs show the mean + SD from ten (**A and C-E**) or nine (**B**) individual experiments. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Figure 7

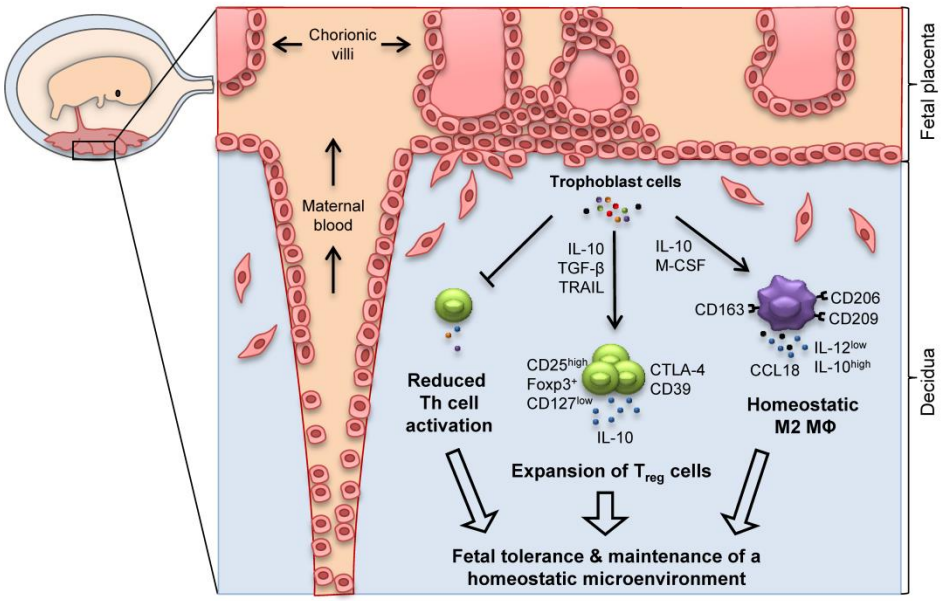

Figure 7

Schematic summary of the main findings in this study. Placental trophoblast cells create a homeostatic and tolerant environment by producing factors, like M-CSF, IL-10, TGF-β and TRAIL, that induce homeostatic macrophages (MΦ) and Treg cells and also limit excessive Th cell activation, thereby supporting normal fetal development.