Growth Factors, Cytokines, and Cell Cycle Molecules

Altered Decidual DC-SIGN⁺ Antigen-Presenting Cells and Impaired Regulatory T-Cell Induction in Preeclampsia

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Regulatory T (Treg) cell expansion is required for tolerance of the semi-allogeneic fetus in healthy pregnancy and impaired in preeclampsia in humans. However, the reasons remain unknown. Herein, we show that expansion of CD4⁺Helios[−]Foxp3⁺ adaptive Treg (iTreg) cells, rather than CD4⁺Helios⁺Foxp3⁺ natural Treg cells, accounts for this expansion in healthy pregnancy. This expansion is even more pronounced in the decidua, where there is an overrepresentation of iTreg cells. In preeclampsia, however, there is impaired systemic iTreg cell expansion, associated with a lack of iTreg cell overrepresentation in the decidua. Because decidual antigen-presenting cells (APCs) may be important for iTreg cell induction, we studied decidual CD14⁺ APCs using immunohistochemistry and flow cytometry. We show that decidual CD14⁺DC-SIGN⁺ APCs are closely associated with Foxp3⁺ Treg cells. Furthermore, CD14⁺DC-SIGN⁺ cells display a distinct phenotype compared with their CD14⁺DC-SIGN[−] counterparts. In particular, they have increased expression of tolerogenic molecules, HLA-G, and immunoglobulin-like transcript 4. *In vitro*, CD14⁺DC-SIGN⁺ APCs from healthy pregnant women induced iTreg cells significantly more efficiently than CD14⁺DC-SIGN[−] APCs. Conversely, in preeclampsia, both CD14⁺DC-SIGN⁺ and CD14⁺DC-SIGN[−] APCs induced iTreg cells poorly. These results suggest that decidual CD14⁺DC-SIGN⁺ APCs may play important roles in iTreg cell induction, a process that is defective in preeclampsia and likely contributes to its pathogenesis. (*Am J Pathol* 2012, 181:2149–2160; <http://dx.doi.org/10.1016/j.ajpath.2012.08.032>)

Maternal immune tolerance of the semi-allogeneic fetus is a requirement for healthy pregnancy. Disturbance of this immune tolerance may play a role in preeclampsia,¹ which affects 2% to 7% of human pregnancies and causes significant maternal and fetal morbidity.² Interestingly, preeclampsia is more common in primiparous women³ and long-term sperm exposure before conception is protective,⁴ whereas change of partner in a subsequent pregnancy increases the risk of preeclampsia.⁵ These clinical observations seem to imply the involvement of the adaptive immune system with alloantigen specificity and immunological memory.

Indeed, the expansion of Foxp3⁺ regulatory T (Treg) cells, which are essential for maternal-fetal immune tolerance in healthy pregnancy,^{6,7} is impaired in preeclampsia,⁸ implicating altered Treg cell homeostasis in the pathogenesis of preeclampsia. More important, Treg cells may be further classified into Helios⁺ natural Treg (nTreg) cells and Helios[−] adaptive Treg (iTreg) cells⁹; nTreg cells are generated in the thymus, and iTreg cells are derived from naive CD4⁺ T cells in the periphery.¹⁰ Although nTreg cells are important for maintaining tolerance to self-antigens, murine studies have shown that iTreg cells play important roles at mucosal surfaces, and in cancers, chronic infection, and inflammation, where they mediate tolerance to foreign and self-antigens.¹¹ Because maternal recognition of fetal alloantigen is largely restricted to indirect antigen presentation by maternal antigen-presenting cells (APCs),¹² we suggest

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that, in pregnancy, induction of iTreg cells by tolerogenic APCs at the maternal-fetal interface may be important for immune tolerance toward the allogeneic fetus.

Many studies have reported the role of APCs, including dendritic cells (DCs)^{13,14} and macrophages,^{15,16} in Treg cell induction. In pregnancy, DCs may also play a role within the decidual microenvironment in shaping the immune response.¹⁷ More specifically, in the murine model, galectin-1, expressed by endometrial stromal cells, skews DC differentiation toward a tolerogenic phenotype, facilitating the expansion of Treg cells.¹⁸ In humans, decidual CD14⁺ APCs induced Treg cells *in vitro*, dependent on interferon- γ -induced up-regulation of indoleamine 2,3-dioxygenase.¹⁹ Of particular interest, a subset of decidual CD14⁺ APCs expressing DC-SIGN (DC-specific intercellular adhesion molecule 1-grabbing nonintegrin) is unique to the pregnant endometrium.²⁰ We, therefore, sought to determine the phenotype of these cells and whether they may play a role in tolerance induction.

Our results indicate that iTreg cell expansion occurs in healthy pregnancy but not in preeclampsia. Furthermore, this expansion may originate from the decidua, where distinct populations of CD14⁺DC-SIGN⁺ APCs are especially proficient in iTreg cell induction in healthy pregnancy. This process is impaired in preeclampsia, in association with defective iTreg cell expansion.

Materials and Methods

Patients

Two cohorts of patients were recruited: one was from Nepean Hospital (Sydney, Australia), and the other was from the Canberra Hospital (Canberra, Australia). Overall, 17 non-pregnant healthy female controls, 32 term healthy pregnant women, and 21 preeclamptic women in the third trimester of pregnancy were recruited from Nepean Hospital, where phenotypical analysis by flow cytometry and functional studies were performed. All peripheral blood samples were drawn before the onset of labor. Twenty term healthy pregnant and 20 preeclamptic women were recruited from Canberra Hospital, where immunohistochemistry (IHC) on the placenta of these women was performed. Preeclampsia was defined per the International Society for the Study of Hypertension in Pregnancy criteria as onset of high blood pressure ($>140/90$) and proteinuria (>0.3 g/24 hours) after 20 weeks of gestation.²¹ Patients with other pregnancy-related complications, such as gestational diabetes, chorioamnionitis, placenta previa, chronic villitis, or underlying autoimmune problems, were excluded from the study. Patient demographics from both cohorts are summarized in Supplemental Table S1 (available at <http://ajp.amjpathol.org>).

Mononuclear Cell Isolation

Decidual mononuclear cells (MNCs) were obtained as previously described.²² Briefly, decidua basalis was carefully dissected from the maternal surface of freshly delivered placenta. This was washed with PBS until the

eluent was macroscopically clear of blood. The tissue was then minced by a pair of fine scissors to 1- to 2-mm pieces. A cocktail of collagenase (300 U/mL), hyaluronidase (2 mg/mL), and DNase (50 μ g/mL) (Sigma-Aldrich, St. Louis, MO), added to RPMI 1640 medium containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 5% fetal calf serum, was used for enzymatic digestion of decidual tissue. This mixture, 10 mL, was used per 1 g of wet tissue weight, incubated at 37°C with gentle rotation. A regimen of three times 20 minutes pulsed digestion, followed by 1-hour digestion, was observed. At the end of each period of digestion, supernatant containing released cells was removed. This was then washed once in PBS. At the end of the digestion, all washed supernatants were combined and filtered through a 70- μ m sieve. The MNCs were subsequently isolated using Ficoll-Hypaque (Amersham Pharmacia, Piscataway, NJ) density gradient centrifugation. Peripheral blood MNCs were isolated using Ficoll-Hypaque density gradient centrifugation. All samples were frozen at -196°C and subsequently thawed for analysis by flow cytometry.

Monoclonal Antibodies and Flow Cytometry

Antibodies used for flow cytometry are listed in Supplemental Table S2 (available at <http://ajp.amjpathol.org>). Before staining of decidual MNCs, samples were incubated with human IgG (200 μ g/100 μ L of cell suspension) at room temperature for 10 minutes to prevent non-specific antibody binding of Fc receptors by tissue macrophages. Surface staining was performed as previously described.⁸ Intracellular staining for Foxp3 was performed using the anti-human staining set and protocol (Biolegend, San Diego, CA). Dead cells were excluded using the blue or aqua live/dead fixable dead cell stain kit (Invitrogen, Carlsbad, CA). Data were acquired on LSRII (BD Pharmingen, San Jose, CA) and analyzed by FlowJo (Treestar, San Carlos, CA).

IHC and Cell Counting

Placental bed biopsy specimens were taken from the maternal surface of the placentas and then formalin fixed and processed into paraffin blocks using routine laboratory methods. Sequential double IHC staining was performed to examine the colocation of Foxp3 and DC-SIGN using the Leica Bond fully automated system (Vision Biosystems, Mt. Waverley, Australia), following a standard protocol. Briefly, tissue sections were cut (4 μ m thick) and dried in an oven at 60°C for 1 hour. Foxp3 (ab10563; Abcam, Cambridge, UK), 1:200 dilution for 20 minutes using Bond Epitope Retrieval Solution (BERS) 2 from Leica Microsystems (Sydney, Australia) (catalogue no. AR9640), pH 8.9 to 9.1, was applied to the tissue section, followed by a labeled streptavidin-biotin horseradish peroxidase detection system, with the 3,3'-diaminobenzidine (DAB) chromogen (Leica Bond Polymer Refine Detection Kit). DC-SIGN (BD Pharmingen, 1:500 dilution), for 30 minutes using BERS 1, pH 6, was then applied, and a labeled streptavidin-biotin alkaline phosphatase detec-

tion system with a red chromogen (Leica Bond Polymer Refine Detection Kit) was used. The sections were counterstained with hematoxylin to allow visualization of the nuclei, coverslipped, and viewed. The number of DC-SIGN⁺ and Foxp3⁺ cells was counted across 10 high-power fields (original magnification, $\times 40$). All sections were counted by two independent assessors (P.H. and M.F.) with a high intraclass correlation coefficient of 0.963 ($P < 0.001$). Sequential double staining was also performed to examine the co-expression of DC-SIGN and HLA-G in the decidua, following a similar protocol previously described. HLA-G (MEM-G/1; Abcam, 1:50 dilution for 21 minutes using BERS 2, pH 8.9 to 9.1) was applied as the primary antibody, followed by DC-SIGN (1:500 dilution for 20 minutes using BERS 1, pH 6) using a labeled streptavidin-biotin alkaline phosphatase detection system with a blue chromogen (Vector Alkaline Phosphatase Substrate Kit III). The sections were counterstained with light neutral red to allow visualization of the nuclei. All antibodies used are included in [Supplemental Table S2](#) (available at <http://ajp.amjpathol.org>).

In Vitro T-Cell Differentiation Assay

Freshly obtained decidual MNCs were used for isolation of CD14⁺DC-SIGN[−] and CD14⁺DC-SIGN⁺ APCs. All samples were initially incubated with human IgG to block the FcR receptor to prevent non-specific binding. To exclude contamination by decidual T cells, anti-CD3 microbead-labeled decidual MNCs were passed through the magnetic column. The CD3-negative fraction was then labeled with phosphatidylethanolamine anti-DC-SIGN antibody (DCN46), which has previously not interfered with DC-SIGN function.²³ This was followed by labeling with anti-phosphatidylethanolamine microbeads and positive selection by magnetic cell sorting. The DC-SIGN[−]negative fraction was then positively selected by anti-CD14 microbeads. Purities of approximately 80% were routinely achieved. Allogeneic CD4⁺CD25[−] T cells were isolated from peripheral blood MNCs of consented donors by magnetic cell sorting (Miltenyi Biotec, Bergisch Gladbach, Germany). Isolated decidual DC-SIGN⁺ or CD14⁺DC-SIGN[−] APCs were cultured with allogeneic carboxyfluorescein succinimidyl ester (CFSE)-labeled CD4⁺CD25[−] T cells in an APC responder ratio of 1:5 in RPMI 1640 medium containing 2 mmol/L L-glutamine, 100 U/mL penicillin, 100 μ g/mL streptomycin, and 10% fetal calf serum. Cultures were incubated at 37°C for 10 days, and cells were fed with fresh medium on day 5. In all cases, no additional cytokines were added to the culture. At day 10, cultured cells were collected for fluorescence-activated cell sorting (FACS) analysis or suppression assay.

Suppression Assay

Decidual APC-primed, non-CFSE-labeled CD4⁺ T cells were washed and then cultured with autologous CFSE-labeled CD4⁺CD25[−] T cells at a ratio of 1:1. As a negative control, fresh autologous non-CFSE-labeled CD4⁺CD25[−] T cells were cultured with autologous

CFSE-labeled CD4⁺CD25[−] T cells at a ratio of 1:1. Stimulation was achieved with MicroBead-coated biotinylated anti-CD3 and anti-CD28 (Miltenyi Biotec). Cells were cultured in medium for 80 to 90 hours and subsequently harvested for flow cytometry. The percentage of proliferation was determined by gating on dividing cells based on CFSE expression. The percentage of suppression was determined as the difference in proliferation between the negative control and the test sample, expressed as a percentage of the former.

Statistics

An unpaired *t*-test was used for comparisons between independent samples. A paired *t*-test was used for paired peripheral blood and decidual samples. A Pearson's correlation was used for all correlation data. $P < 0.05$ was considered statistically significant.

Study Approval

The research was conducted according to protocols and was reviewed and approved by the Human Research Ethics Committee of Nepean Hospital and the ACT Health Human Research Ethics Committee.

Results

Expansion of Peripheral Blood

CD4⁺Helios[−]Foxp3⁺ iTreg Cells Seen in Healthy Pregnancy Is Impaired in Preeclampsia

Researchers have previously shown an increased percentage of peripheral blood Treg cells in healthy pregnancy compared with preeclampsia.^{8,24} In this study, we sought to further delineate the Treg cell population using the marker Helios, an Ikaros transcription factor family member, to differentiate between nTreg (Helios⁺) and iTreg (Helios[−]) cells ([Figure 1A](#); see also [Supplemental Figure S1](#) at <http://ajp.amjpathol.org> for gating strategies). There was a significant increase of peripheral blood CD4⁺Helios[−]Foxp3⁺ iTreg cells in healthy pregnancy compared with non-pregnant ($P < 0.0001$) and preeclamptic ($P < 0.0001$) women ([Figure 1A](#)). Surprisingly, we found that the percentage of CD4⁺Helios⁺Foxp3⁺ nTreg cells in healthy pregnancy was not significantly increased ([Figure 1A](#)), suggesting that, at least at term, the expansion of the Treg cell population in healthy human pregnancy was primarily due to expansion of iTreg rather than nTreg cells. This was reflected in the increased proportion of Helios[−] iTreg cells within the Treg cell population (defined as CD4⁺CD25⁺Foxp3⁺, CD4⁺Foxp3⁺, or CD4⁺CD25⁺CD127^{lo}) ([Figure 1, B and C](#)) in healthy pregnancy, compared with preeclamptic or non-pregnant women ([Figure 1D](#)). No significant association between iTreg cell percentage and maternal age, gestation, parity, or infant birth weight was found (see [Supplemental Table S3](#) at <http://ajp.amjpathol.org>). The difference in iTreg cell percentage between normal pregnancy and preeclampsia remained significant after con-

Figure 1. Distribution of nTreg and iTreg cells in the peripheral blood of non-pregnant (NP; $n = 17$), healthy pregnant (P; $n = 32$), and preeclamptic (PE; $n = 21$) women. **A:** Representative flow cytometry plot showing the nTreg and iTreg populations within CD3⁺CD4⁺ T cells (middle panel). The iTreg and nTreg cell frequencies for NP, P, and PE are shown on the left and right panels, respectively. **B:** Gating strategies for Tregs are shown within CD3⁺CD4⁺ T cells using CD25⁺Foxp3⁺, Foxp3⁺, and CD25⁺CD127^{lo}, as indicated. **C:** The gated population in **B** was then assessed for Helios expression. **D:** Comparison of Treg cell composition in NP, P, and PE based on % Helios⁻ in the three differentially defined Treg cell populations, as indicated. Unpaired *t*-test, * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

trolling for these variables using univariate analysis ($F = 18.4$, $P < 0.0001$). These results indicated that there was significant peripheral induction of iTreg cells in healthy pregnancy, which is impaired in preeclampsia.

Overrepresentation of iTreg Cells in the Decidua in Healthy Pregnancy Is Absent in Preeclampsia

In mice, the expansion of Treg cells in pregnancy was at least partially alloantigen dependent.²⁵ If this was also true in humans, then a higher percentage of iTreg cells at the maternal-fetal interface, where abundant fetal alloantigens could drive iTreg cell induction, might be expected. We, therefore, examined the composition of Treg cells in the decidua in healthy pregnancy and preeclampsia (Figure 2A; see also Supplemental Figure S1 at <http://ajp.amjpathol.org>). Pairwise comparisons were made between the peripheral blood and the corresponding decidua. This showed a higher percentage of Foxp3⁺ Treg cells in the decidua com-

pared with peripheral blood, in both healthy pregnancy and preeclampsia (Figure 2B). No significant difference was found in the percentage of decidual Foxp3⁺ Treg cells between healthy pregnancy and preeclampsia (Figure 2B); however, decidual Treg cell composition differed significantly. There was an overrepresentation of Helios⁻ iTreg cells in the decidua in healthy pregnancy (Figure 2C). This was not the case in the preeclamptic decidua, where the percentage of Helios⁻ iTreg cells was essentially the same as in the peripheral blood and significantly lower than in healthy pregnancy ($P < 0.001$) (Figure 2C). Together, these results indicated that, in healthy pregnancy, there was significant local induction of iTreg cells, either within the decidua or in local draining lymph nodes. This local induction of iTreg cells was impaired in preeclampsia, likely leading to a reduced systemic Treg cell pool. In contrast, the high percentage of Helios⁺ nTreg cells in the preeclamptic decidua suggested recruitment of these cells from peripheral blood, leading to a comparable total percentage of Foxp3⁺ Treg cells to healthy pregnancy.

Figure 2. Comparison of Treg cell composition between paired maternal blood (MB) and decidua (DT) in healthy pregnancy ($n = 15$) and preeclampsia ($n = 11$). **A:** Representative flow cytometry plots showing the Foxp3⁺ population within CD3⁺CD4⁺ T cells in MB and DT for both healthy pregnant and preeclamptic women (top panel). The composition of the respective Foxp3⁺ Treg cells was then assessed based on Helios expression (bottom panel). **B:** Pairwise comparisons of MB and DT Treg cell frequencies in healthy pregnant (left panel) and preeclamptic (middle panel) women. A comparison of DT Treg cell frequency between healthy pregnancy (P) and preeclampsia (PE) is shown (right panel). **C:** Pairwise comparison of Treg cell composition (% Helios⁻ iTreg within Foxp3⁺ cells) between MB and DT in healthy pregnancy (left panel) and preeclamptic (middle panel) women. A comparison of this Treg cell composition between P and PE within DT is shown (right panel). Comparisons made between MB and DT samples and between P and PE samples were calculated using paired and unpaired *t*-tests, respectively. ** $P < 0.01$, *** $P < 0.001$.

Figure 3. IHC showing DC-SIGN⁺ APCs and their relationship with Foxp3⁺ Treg cells in the decidua. **A:** Representative image taken at a magnification of $\times 630$ of decidual DC-SIGN⁺ APCs (left panel, stained in pink). Scale bar = 20 μ m. **Right panel,** numerical comparison of DC-SIGN⁺ cells per 10 high-power fields (HPFs) in healthy pregnancy (P; $n = 20$) and preeclampsia (PE; $n = 20$). Bars represent the mean $\pm$ SEM. Unpaired t -test, *** $P < 0.001$. **B:** Representative image showing close proximity of Foxp3⁺ Treg cells (stained in brown, arrows) and DC-SIGN⁺ APCs (stained in pink) in healthy pregnancy (left panel) and preeclampsia (right panel). **C:** Numerical correlation between Foxp3⁺ Treg cells and DC-SIGN⁺ APCs in healthy pregnancy (left panel, $n = 20$) and preeclampsia (right panel, $n = 20$). Pearson's correlation, r and P values as indicated.

Decidual DC-SIGN⁺ APCs and Their Close Proximity to Treg Cells

Because APCs played an important role in extrathymic Treg cell induction, we examined decidual APCs using IHC. In particular, DC-SIGN, a marker of immature DCs,²⁶ was used. DC-SIGN⁺ cells were seen scattered throughout the decidua, in both healthy pregnant and preeclamptic samples. Their morphological characteristics mainly resembled DCs, which formed networks among large decidual cells (Figure 3A). The mean number of DC-SIGN⁺ APCs was significantly higher in preeclampsia compared with healthy pregnancy ($P < 0.001$) (Figure 3A). To determine the relationship between decidual Treg cells and DC-SIGN⁺ APCs, sequential double staining was used with the combination of DC-SIGN and Foxp3. Most Foxp3⁺ cells were closely associated with DC-SIGN⁺ APCs in both healthy pregnancy and preeclampsia (Figure 3B). Although one previous study demon-

strated contact between natural killer cells and DC-SIGN⁺ APCs in the decidua,²⁰ this contact has not been demonstrated for decidual Treg cells. Such proximity suggested significant interaction between these cells, which may be important for the maintenance of immune tolerance. Indeed, Treg cells exerted part of their suppressive function through modulation of APCs.²⁷ Conversely, Treg cells could also be induced by APCs from naïve CD4⁺ T cells.²⁸

There was a strong correlation between the number of Foxp3⁺ cells and DC-SIGN⁺ cells in healthy pregnancy ($r = 0.825$, $P < 0.0001$), but not in preeclampsia ($r = 0.308$, $P = 0.187$) (Figure 3C). This was confirmed by flow cytometry (see Supplemental Table S4 at <http://ajp.amjpathol.org>). Although no causal relationship could be drawn from these data, they suggested that there was a disturbance of DC-SIGN⁺ APC/Treg cell interaction in preeclampsia.

Decidual CD14⁺DC-SIGN⁺ APCs Are Phenotypically Distinct and Differ between Healthy Pregnancy and Preeclampsia

Because Treg cells showed special affinity for decidual DC-SIGN⁺ APCs, we examined the phenotype of these APCs in more detail using multicolor flow cytometry, comparing DC-SIGN⁺ with DC-SIGN[−] cells (Figure 4A). DC-SIGN⁺ cells were all CD14⁺, although their CD14 mean fluorescence intensity (MFI) was modestly, but significantly, lower than DC-SIGN[−] APCs (see Supplemental Figure S2 at <http://ajp.amjpathol.org>). Compared with decidual DC-SIGN[−] APCs, DC-SIGN⁺ APCs had a higher expression of HLA-DR and costimulatory molecules, CD80 and CD86 (Figure 4B). Furthermore, these DC-SIGN⁺ APCs expressed the chemokine receptor CCR7 (see Supplemental Figure S2 at <http://ajp.amjpathol.org>), suggesting that they might be capable of migration and antigen presentation in local draining lymph nodes.²⁹

Based on their relationship with decidual Treg cells, we hypothesized that these DC-SIGN⁺ APCs would possess tolerogenic properties. Because some subsets of macrophages and DCs expressed the tolerogenic molecule, HLA-G,^{30,31} we looked for HLA-G expression in decidual APCs. We found that approximately 25% of CD14⁺ cells themselves expressed HLA-G in healthy pregnancy (Figure 4C), which was somewhat surprising, because HLA-G was generally thought to be expressed only by extravillous trophoblasts in the decidua.³² We further showed that DC-SIGN⁺ APCs have a much higher intensity ($P < 0.0001$) (Figure 4D) and percentage ($P < 0.0001$) (Figure 4E) of HLA-G expression compared with DC-SIGN[−] APCs. The expression of HLA-G by DC-SIGN⁺ APCs was confirmed by IHC, where colocalization of DC-SIGN and HLA-G was observed in the decidua (Figure 4F). All CD14⁺ APCs expressed immunoglobulin-like transcript 4 (ILT4), a known receptor for HLA-G³³; however, DC-SIGN⁺ APCs expressed significantly higher levels of ILT4 compared with DC-SIGN[−] APCs ($P < 0.01$) (Figure 4B).

Figure 4. The unique phenotype of decidual DC-SIGN⁺ APCs in healthy pregnancy. **A:** Decidual leukocytes were selected from live cells based on forward scatter (FSC) and sideward scatter (SSC; **top left panel**). CD14⁺ APCs were then selected as shown (**top right panel**). A proportion of CD14⁺ APCs are DC-SIGN⁺ (**bottom right panel**), and gating was based on isotype control (**bottom left panel**). **B:** CD14⁺DC-SIGN⁺ APCs express higher levels of CD80, CD86, HLA-DR, and ILT4, as shown by flow cytometry dot plots (**left panel**) and MFI (**middle panel**). This is statistically significant, as shown by the bar graphs in the **right panel** ($n = 15$). **C:** HLA-G expression of decidual CD14⁺ APCs is demonstrated (**right panel**) with gating based on isotype control (**left panel**). **D:** Representative histogram showing fluorescence intensity of HLA-G expression in CD14⁺DC-SIGN⁺ and CD14⁺DC-SIGN⁻ APCs (**left panel**) and comparison of MFI ($n = 15$) between these cells. **E:** Percentage of HLA-G⁺ cells in CD14⁺DC-SIGN⁺ APCs (**middle top panel**) and CD14⁺DC-SIGN⁻ APCs (**middle bottom panel**). A significantly higher percentage of CD14⁺DC-SIGN⁺ APCs are HLA-G⁺ (**right panel**, $n = 15$). Paired *t*-test, ** $P < 0.01$, *** $P < 0.001$. **F:** IHC showing colocalization of HLA-G (brown) and DC-SIGN (blue) in the decidua of a healthy pregnant woman. The large brown cells are HLA-G⁺ extravillous trophoblasts (EVTs). Original magnification, $\times 630$. Scale bar = 20 μm .

We did not find a significant difference in the percentage of DC-SIGN⁺ cells within CD14⁺ APCs between healthy pregnancy and preeclampsia (see [Supplemental Figure S2](#) at <http://ajp.amjpathol.org>). However, preeclamptic CD14⁺DC-SIGN⁺ APCs showed a signifi-

cantly decreased percentage ($P < 0.0001$) ([Figure 5A](#)) and intensity ($P < 0.05$) ([Figure 5B](#)) of HLA-G expression compared with their counterparts in healthy pregnancy. This was also true of ILT4 expression ($P < 0.05$) ([Figure 5C](#)). No significant differences were observed for CD80

Figure 5. Differential expression of HLA-G and ILT4 by DC-SIGN⁺ APCs in healthy pregnancy and preeclampsia. **A:** HLA-G expression by CD14⁺ APCs (**middle panel**) in healthy pregnancy (P; **left panel**) and preeclampsia (PE; **right panel**). Gating based on isotype control (**top panel**). A comparison of the percentage of CD14⁺DC-SIGN⁺ APCs expressing HLA-G in healthy pregnancy and preeclampsia is shown in the **bottom panel** and the dot plot. **B:** HLA-G MFI of CD14⁺DC-SIGN⁺ APCs in healthy pregnancy (fragmented line) compared with preeclampsia (solid line), as shown by a representative histogram (**left panel**) and a summary dot plot (**right panel**). **C:** Comparison of ILT4 expression (MFI) by CD14⁺DC-SIGN⁺ APCs between healthy pregnancy (fragmented line) and preeclampsia (solid line), as shown by a representative histogram (**left panel**) and a summary dot plot (**right panel**). Unpaired *t*-test, **P* < 0.05, ****P* < 0.001.

and CD86 expression in these cells between preeclampsia and healthy pregnancy. Together, these data suggested that CD14⁺DC-SIGN⁺ APCs were most likely activated DCs, capable of antigen presentation in the decidua and probably also in draining lymph nodes. Paradoxically, they express HLA-G and ILT4, which could skew the immune response toward tolerance induction.^{34,35} Their phenotype was altered in preeclampsia, with decreased HLA-G and ILT4 expression, which might contribute to the impaired tolerance induction in this disease.

iTreg Cell Induction by Decidual CD14⁺ APCs

To test whether decidual CD14⁺ APCs induced iTreg cells *in vitro*, we isolated CD14⁺DC-SIGN⁺ and CD14⁺DC-SIGN⁻ APCs from term decidua in healthy pregnancy (see [Supplemental Figure S3](http://ajp.amjpathol.org) at <http://ajp.amjpathol.org>). These isolated APCs were cultured with purified allogeneic CFSE-labeled CD4⁺CD25⁻ T cells for 10 days. The starting population of CD4⁺CD25⁻ T cells contained ≤1% CD25⁺CD127^{lo} or Foxp3⁺ Treg cells (see [Supplemental Figure S3](http://ajp.amjpathol.org) at <http://ajp.amjpathol.org>). The degree of alloproliferation and the percentage of CD4⁺Foxp3⁺ T cells were then measured by flow cytometry. The induced cells were gated based on their Foxp3 and CFSE expression and the various populations analyzed, according to [Figure 6A](#). Notably, Foxp3⁺ Treg cells were also CD127^{lo} and largely Helios⁻, consistent with an iTreg cell phenotype^{9,36} ([Figure 6B](#)). This was in contrast with the small amount of Foxp3⁺ cells in the starting population, which were mainly Helios⁺ nTreg cells (see [Supplemental Figure S3](http://ajp.amjpathol.org) at <http://ajp.amjpathol.org>). In healthy pregnancy, the percentage of Foxp3⁺ cells within proliferating cells was significantly higher in cells co-cultured with CD14⁺DC-SIGN⁺ APCs compared with those co-cultured with CD14⁺DC-SIGN⁻ APCs ([Figure 6, C and D](#)). This was also true of the total percentage of Foxp3⁺ cells (data not shown). The same result was obtained in an experiment using FACS-sorted decidual APCs and naïve CD4⁺CD25⁻CD127^{hi} T cells (see [Supplemental Figure S4](http://ajp.amjpathol.org) at <http://ajp.amjpathol.org>). Interestingly, despite the higher expression of HLA-DR and costimulatory molecules in CD14⁺DC-SIGN⁺ APCs, they induced less alloproliferation compared with CD14⁺DC-SIGN⁻ APCs in healthy pregnancy (data not shown). Because Foxp3 might be expressed by activated conventional T cells with no suppressive activity,³⁷ we analyzed whether the presence of induced Foxp3⁺ cells in these cultures affected the proliferation of non-Treg cells (Foxp3⁻). We found that in healthy pregnant women, the percentage of proliferation in non-Treg cells (Foxp3⁻) inversely correlated with the percentage of Foxp3⁺ Treg cells ([Figure 6E](#)). This suggested that the induced Foxp3⁺ cells were functional Treg cells and not activated non-Treg cells, because they exhibited dose-dependent suppressive characteristics. These findings were supported by suppression assays, which showed that, after 10 days, CD14⁺DC-SIGN⁺ APC-primed T cells remained more suppressive than CD14⁺DC-SIGN⁻ APC-primed T cells in healthy pregnancy ([Figure 6, F and G](#)).

Finally, we hypothesized that because CD14⁺DC-SIGN⁺ APCs in preeclampsia have reduced expression of tolerogenic molecules HLA-G and ILT4, they might have a decreased ability to induce iTreg cells *in vitro*. Indeed, preeclamptic CD14⁺DC-SIGN⁺ induced significantly fewer Foxp3⁺ cells than their counterparts in healthy pregnancy (Figure 6, C and D), although most of these Foxp3⁺ cells were also Helios⁺ (see Supplemental Figure S3 at <http://ajp.amjpathol.org>), consistent with iTreg cells. No difference in iTreg cell induction capacity was observed between CD14⁺DC-SIGN⁺ and CD14⁺DC-SIGN⁻ APCs in preeclampsia. Together, these data suggested that in healthy pregnancy, a specialized population of CD14⁺DC-SIGN⁺ APCs were par-

ticularly proficient in iTreg cell induction. This process was impaired in preeclampsia and explains the impaired iTreg cell expansion.

Discussion

Although preeclampsia may be caused by a deficit in maternal immune tolerance toward the fetus, the exact mechanism remains elusive. The evidence presented in this study implicates an important role of decidual CD14⁺DC-SIGN⁺ APCs in local iTreg cell induction in healthy pregnancy, leading to systemic expansion of iTreg cells. This process is defective in preeclampsia, resulting in poor iTreg cell induction and likely compensatory nTreg cell recruitment to the inflamed preeclamptic decidua to control the local alloresponse. Indeed, increased type 1 helper T effector T cells³⁸ and activated CD8⁺ T cells³⁹ are found in preeclampsia, likely leading to a local alloresponse. This proposed model is summarized in Figure 7 and represents a new concept for the establishment of immune tolerance in human pregnancy. Furthermore, it is a novel disease model of preeclampsia and reinforces the involvement of both the innate and adaptive immune system in its pathogenesis.

Although the expansion of Treg cells in pregnancy is well documented, the factors regulating the Treg cell compartment have been unclear. In the murine model, uterine Treg cell accumulation occurs during estrus,⁴⁰ in keeping with the potentiating effect of estrogen on Treg cells, independent of alloantigen.⁴¹ However, evidence also indicates the role of alloantigen in driving Treg cell expansion in mice.²⁵ In humans, the role of alloantigen in Treg cell expansion has been much more difficult to dissect. In this study, we show that in healthy pregnancy, the systemic expansion of Treg cells is largely due to the induction of iTreg cells. This iTreg cell expansion is reflected by an increase in Helios⁺ cell percentage within the CD4⁺Foxp3⁺ T-cell population. Although recent work has shown that in healthy non-pregnant individuals, ap-

Figure 6. *In vitro* Treg cell induction by decidual CD14⁺ APCs. CFSE-labeled CD4⁺CD25⁻ allogeneic T cells are cultured with either CD14⁺DC-SIGN⁺ or CD14⁺DC-SIGN⁻ APCs. Cells are analyzed on day 10 by flow cytometry, gating on live CD3⁺CD4⁺ T cells. **A:** A representative flow cytometry plot showing the gating strategy of various populations. The % Foxp3⁺ cells within proliferating cells is calculated as follows: a/(a + c). The % proliferating Foxp3⁺ cells is calculated as follows: c/(c + d). **B:** Representative plots showing the phenotype of induced Foxp3⁺ cells. **C:** Representative plots showing the % Foxp3⁺ cells within proliferating cells induced by DC-SIGN⁺ and DC-SIGN⁻ APCs in healthy pregnancy and preeclampsia. **D:** Summary bar graph (mean ± SEM) showing the % of Foxp3⁺ cells within proliferating cells induced under various conditions in healthy pregnancy (P) and preeclampsia (PE). A paired *t*-test is used for comparison between DC-SIGN⁺ and DC-SIGN⁻ cultures, and an unpaired *t*-test is used between P and PE. Data show five independent experiments. **P* < 0.05, ***P* < 0.01. **E:** Scatterplot showing the relationship between % proliferating Foxp3⁺ cells and % Foxp3⁺ cells within proliferating cells. **F:** Representative plots showing proliferation of CFSE-labeled autologous CD4⁺CD25⁻ T cells in the presence of T cells induced by DC-SIGN⁺ (middle panel) and DC-SIGN⁻ (right panel) APCs compared with negative control (left panel). **G:** Summary bar graph (mean ± SEM) showing suppressive capacity of CD4⁺ T cells induced by DC-SIGN⁺ and DC-SIGN⁻ APCs in healthy pregnancy. Data show three independent experiments. Paired *t*-test, **P* < 0.05.

Figure 7. Proposed model of tolerance induction in healthy pregnancy and immunopathogenesis in preeclampsia. In healthy pregnancy, decidual $CD14^{+}DC-SIGN^{+}$ APCs express high levels of HLA-G and ILT4, perhaps under the influence of IL-10. These APCs promote the conversion of naive T cells into iTreg cells, which, in turn, suppress T-effector cells and alloresponse. In preeclampsia, decidual $CD14^{+}DC-SIGN^{+}$ APCs express less HLA-G and ILT4, possibly because of a decreased level of IL-10. These APCs induce fewer iTreg cells, resulting in ineffective control of alloresponse, which is partially rescued by recruitment of nTreg cells from peripheral blood.

proximately 50% of $CD4^{+}Helios^{-}Foxp3^{+}$ cells are likely activated conventional T cells,⁴² in pregnancy, isolated Treg cells actually have higher suppressive capacity compared with those in preeclampsia.⁴³ This implies that expanded iTreg cells in pregnancy are likely functional suppressive Treg cells.

The fact that $Helios^{-}$ iTreg cells are overrepresented in the decidua compared with the peripheral blood would suggest that they are locally induced, probably in the decidua or local draining lymph nodes. On the other hand, the significant presence of nTreg cells in the decidua also implicates recruitment of these cells from peripheral blood. Thus, we propose that in human pregnancy, nTreg and iTreg cells may act synergistically to maintain tolerance toward the fetus. It is possible that iTreg cell expansion might even be more pronounced earlier in pregnancy because Treg cell expansion peaks in the second trimester and slowly decreases thereafter.⁴⁴ This will have to be examined in further longitudinal studies.

The decidua is the mucous membrane of the pregnant uterus, and iTreg cells are important for mucosal immune tolerance. Prime examples for this are the gut and lung, where iTreg cells play an important role in controlling murine experimental colitis⁴⁵ and allergic airway inflammation,⁴⁶ respectively. Interestingly, a distinct population of $CD103^{+}$ DCs in the gut appears to be specialized in iTreg cell induction.⁴⁷ This is analogous to our finding that a unique population of $CD14^{+}DC-SIGN^{+}$ APCs is particularly efficient in iTreg cell induction. These $CD14^{+}DC-SIGN^{+}$ APCs are only found during pregnancy and not in the non-pregnant endometrium,²⁰ implicating their unique role in pregnancy.

Although previous studies have explored the overall phenotype and gene expression of decidual $CD14^{+}$ APCs,^{48–50} in this study, we compared and contrasted the differences between $CD14^{+}DC-SIGN^{+}$ and $CD14^{+}DC-SIGN^{-}$ APCs. We show that, although these $CD14^{+}DC-SIGN^{+}$ APCs possess better T-cell stimulatory capacity (higher expression of CD80, CD86, and HLA-DR) compared with their $DC-SIGN^{-}$ counterparts, they also express higher levels of tolerogenic molecules HLA-G and ILT4. We suggest that these cells most likely represent a form of mucosal DCs that have acquired tolerogenic properties in the process of differentiation

due to favorable environmental factors. In line with this, we present new evidence that these $CD14^{+}DC-SIGN^{+}$ APCs are closely associated with decidual Treg cells *in situ* and function to induce iTreg cells *in vitro*. Notably, a previous study has shown that the percentage of decidual $DC-SIGN^{+}$ APCs peaks in the second trimester,⁴⁸ corresponding with the peak of Treg cell expansion in pregnancy. How these $CD14^{+}DC-SIGN^{+}$ APCs function to induce Treg cells is unclear, although we suggest that their expression of HLA-G and ILT4 may play a role. Indeed, indirect evidence shows a correlation between HLA-G expression and Treg cells in other contexts, such as transplantation³¹ and cancer.⁵¹ Furthermore, transfection of APCs with HLA-G1 also leads to the induction of immunosuppressive T cells *in vitro*.⁵² ILT4, on the other hand, has played a role in Treg cell induction by IL-10-treated DCs.⁵³ Further studies are required to elucidate such mechanisms.

Intriguingly, we found that, in preeclampsia, the physiological iTreg cell induction observed in healthy pregnancy is compromised. Our data suggest that the lack of systemic Treg cell expansion previously reported in preeclampsia is largely due to impaired iTreg cell induction, whereas the nTreg cell population in preeclampsia is comparable to healthy pregnancy. Indeed, a recent study demonstrated that extrathymic iTreg cell generation is crucial for allogeneic murine pregnancy and that absence of iTreg cells leads to increased fetal loss and defective placental spiral artery formation.⁵⁴ We suggest that this is echoed in the human disease of preeclampsia, in which we demonstrate an impairment of iTreg cell generation, which likely leads to poor placentation and the subsequent manifestations of preeclampsia.

A previous study has shown that decidual $CD14^{+}$ APCs in preeclampsia have an altered phenotype compared with their counterparts in healthy pregnancy, with a decreased $CD163/CD14$ ratio, suggestive of reduced alternatively activated (M2), tolerogenic macrophages.⁵⁵ Herein, we provide new evidence that, in preeclampsia, $CD14^{+}DC-SIGN^{+}$ APCs have reduced HLA-G and ILT4 expression, associated with impaired iTreg cell induction capacity *in vitro*. Although the role of transforming growth factor- β in iTreg cell induction was not examined in this study, we have previously shown that endoglin, a transforming growth fac-

tor- β inhibitor, is increased in preeclampsia.⁸ Because transforming growth factor- β is important for iTreg cell induction,⁵⁶ increased endoglin may also contribute to impaired iTreg induction in preeclampsia *in vivo*. However, our current results indicate that even when removed from the *in vivo* environment, isolated CD14⁺DC-SIGN⁺ APCs from preeclamptic patients had reduced iTreg cell induction capacity *in vitro*. This implies that, in preeclampsia, these cells have an intrinsic defect in iTreg cell induction, independent of endoglin. This is in keeping with the observation that in the preeclamptic decidua, the overrepresentation of iTreg cells (ie, % Helios⁺) seen in healthy pregnancy is absent, suggestive of poor local iTreg cell induction. The resulting nTreg cell predominance also raises the possibility that in preeclampsia, increased compensatory recruitment of nTreg cells to the decidua occurs to control local inflammation. This is consistent with evidence showing that Treg cells are attracted to sites of inflammation.^{57,58}

The reason for the reduced HLA-G and ILT4 expression in preeclamptic CD14⁺DC-SIGN⁺ APCs is unclear; however, we speculate that the reduced peripheral blood and placental IL-10 level in preeclampsia^{55,59,60} may contribute. Indeed, IL-10 arrests DC maturation⁶¹ and promotes HLA-G⁶² and ILT4 expression^{53,63} in maturing monocytes *in vitro*. Thus, it is conceivable that a reduced placental IL-10 concentration can lead to reduced HLA-G and ILT4 expression in preeclampsia.

Our findings may also explain the predominance of primiparous women and the partner specificity in preeclampsia, because a previous healthy pregnancy could promote the formation of alloantigen-specific iTreg cells, which could dampen the alloresponse of subsequent pregnancies with the same partner. Nevertheless, we did not find a significant correlation between parity and iTreg cell percentage in healthy pregnancy, perhaps because pregnancy-induced iTreg cells only persist for a limited period, because these cells may be less stable than nTreg cells.⁶⁴ Indeed, this may explain why the protective effect of the same partner in subsequent pregnancies is influenced by birth interval.⁶⁵ Further longitudinal studies are needed to explore the longevity and stability of iTreg cells in humans.

In conclusion, we have shown that iTreg cell expansion accounts for much of the Treg cell expansion in healthy human pregnancy. Furthermore, a unique population of CD14⁺DC-SIGN⁺ APCs is particularly capable of iTreg cell induction and may be important for this expansion. In addition, this is a physiologically relevant process, which is impaired in preeclampsia. These findings reinforce the collaborative involvement of both the innate and the adaptive immune system in the physiological process of pregnancy and the pathogenesis of preeclampsia.

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