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Two Unique Human Decidual Macrophage Populations

Brandy L. Houser,* Tamara Tilburgs,* Jonathan Hill,† Matthew L. Nicotra,* and Jack L. Strominger*

Several important events occur at the maternal–fetal interface, including generation of maternal–fetal tolerance, remodeling of the uterine smooth muscle and its spiral arteries and glands, and placental construction. Fetal-derived extravillous trophoblasts come in direct contact with maternal decidual leukocytes. Macrophages represent ~20% of the leukocytes at this interface. In this study, two distinct subsets of CD14+ decidual macrophages (dMφs) are found to be present in first-trimester decidual tissue, CD11cHI and CD11cLO. Gene expression analysis by RNA microarray revealed that 379 probes were differentially expressed between these two populations. Analysis of the two subsets revealed several clusters of coregulated genes that suggest distinct functions for these subsets in tissue remodeling, growth, and development. CD11cHI dMφs express genes associated with lipid metabolism and inflammation, whereas CD11cLO dMφs express genes associated with extracellular matrix formation, muscle regulation, and tissue growth. The CD11cHI dMφs also differ from CD11cLO dMφs in their ability to process protein Ag and are likely to be the major APCs in the decidua. Moreover, these populations each secrete both proinflammatory and anti-inflammatory cytokines that may contribute to the balance that establishes fetal–maternal tolerance. Thus, they do not fit the conventional M1/M2 categorization. The Journal of Immunology, 2011, 186: 2633–2642.

The maternal–fetal interface in early pregnancy is a highly dynamic tissue where establishment of fetal–maternal tolerance coincides with uterine spiral artery remodeling and placental growth. Decidual leukocytes participate in placental development (1). Specifically, decidual NK cells have been shown to play a role in spiral artery remodeling and trophoblast invasion (1–3), whereas decidual T regulatory cells have been shown to be important for fetus-specific immune tolerance (4, 5). Decidual macrophages, hMφs, comprise the second largest decidual leukocyte population in early pregnancy (10–20%) next to decidual NK cells (60–80%) (6), but their function remains unclear. The large abundance of CD14+ dMφs and the near absence of CD14− dendritic cells (7) suggest that they are the most important professional APCs in the decidua. Therefore, dMφs, which express MHC class II, are likely to play a crucial role in Ag presentation to decidual T cells and may contribute in the establishment of fetal–maternal immune tolerance. In addition to their Ag-presenting function, macrophages may be involved in tissue remodeling through phagocytosis and secretion of extracellular matrix proteins and cytokines. Therefore, macrophages may be critical in the coordination of immune responses and tissue remodeling events that facilitate a successful pregnancy.

Various studies using model systems have categorized macrophages into different subtypes based on cell surface phenotype, cytokine production, and functional properties. Originally, the counterpart to the classical immunostimulatory macrophage was coined as the alternatively activated macrophage (8). Since then other myeloid cell types have emerged including myeloid-derived suppressor cells and tumor-associated macrophages (9, 10). More recently, proinflammatory and anti-inflammatory macrophages induced from peripheral monocytes by different stimuli were named M1 or M2 macrophages to parallel the Th1/Th2 paradigm (11). Despite the nomenclature similarities, no master regulator that directs their formation has been found, emphasizing individual cell plasticity in the myeloid lineage rather than discrete cell types. Although these macrophage model systems have been useful in understanding macrophage functionality, M1/M2 macrophages have yet to be defined in tissue or in specific diseases (12).

Gene expression profiles of CD14+ dMφs compared with maternal peripheral blood (mPB) CD14+ monocytes have demonstrated that dMφs contain transcripts indicative of both an immune regulatory and tissue remodeling phenotype; therefore, dMφs were classified as resembling an M2 phenotype (13). In addition, dMφs have been shown to express receptors important for phagocytosis of degraded extracellular matrix products, (14) as well as in apoptotic cell clearance (15). Furthermore, CD14+ dMφs are not only capable of producing immunomodulatory proteins such as TGF-β, but also have the ability to produce an abundance of proinflammatory IL-6 and IL-8 when stimulated by cell lines expressing the fetal-trophoblast HLA-G homodimer (16).

In this study, we demonstrate that early human decidua tissue contains two distinct subsets of dMφs identified by the level of CD11c expression and termed accordingly as CD11cHI and CD11cLO dMφs. CD11cHI and CD11cLO dMφ populations each produce both proinflammatory and anti-inflammatory cytokines.
and express unique gene signatures that do not allow for their classification as either M1 or M2 macrophages. Rather, these cells are decidual tissue resident macrophages that appear to have distinct functions at the maternal–fetal interface.

Materials and Methods

Blood and tissue samples

First-trimester decidual samples (gestational age: 6–12 wk) were obtained from patients undergoing elective pregnancy termination at a women’s health clinic in Boston, MA. mPB samples were collected from healthy pregnant women (gestational age: 8–12 wk). Leukocytes from mPB were purified using a Ficoll-Hypaque gradient (GE Healthcare) followed by CD14+ selection using magnetic beads (Miltenyi Biotech). Leukocytes from decidual tissue were processed as previously described (16). Released CD11c+CD14+ macrophages were fixed in 1% paraformaldehyde for 10 min at 4°C. Cells were then mounted with Vectashield mounting media (Dako) for 20 min. Primary Abs were applied for 1 h at room temperature in the above fixative, washed in 0.1 M sodium cacodylate buffer, and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB solutions. All specimens were prepared in a routine fixative of 2.5% glutaraldehyde, 2% paraformaldehyde in 0.1 M sodium cacodylate buffer (all from BD Biosciences), and CD206-PE (eBiosciences). Cells were washed and stained for 30 min at 4°C in PBS supplemented with 2% FCS (Invitrogen). A minimum of 50,000 events was collected using the FACSCalibur flow cytometer (Immunochemistry Systems; Becton Dickinson) or the LSRII (BD Biosciences) and data were analyzed using FlowJo software (Tree Star).

Immunohistochemistry, confocal microscopy, and light microscopy

Immunohistochemistry was performed using 4-μm-thick formalin-fixed, paraffin-embedded tissue sections. Slides were soaked in xylene, passed through graded alcohols, and put in distilled water. Slides were then pretreated with 1.0 mM EDTA, pH 8.0 (Zymed, South San Francisco, CA) in a steam pressure cooker (Decloaking Chamber; BioCare Medical, Walnut Creek, CA). All further steps were performed at room temperature in a humidified chamber. Slides were pretreated with peroxidase block (Dako) for 5 min to quench endogenous peroxidase activity, followed by serum-free protein block (Dako) for 20 min. Primary Abs were applied for 1 h (CD14 at 1:100, CD209 at 1:500, all diluted in Dako diluents), then washed in 50 mM Tris-Cl, pH 7.4, and detected with FITC or Cy3 secondary Abs (Invitrogen), diluted 1:200 in DaVinci Green diluents (Biocare Medical). Confocal microscopy was performed using an inverted Zeiss Laser-Scanning Microscope 510 (LSM510) META (Carl Zeiss Microimaging GmbH) with an argon laser emitting at 488 nm and a krypton laser emitting at 568 nm. All images were acquired using a 40× or 63× oil objective in a sequential scan mode. All data were analyzed using LSM (Carl Zeiss Microimaging GmbH), and MoFlo sorted cell populations were stained with May-Grünwald/Giemsa stain buffer (Polysciences), and images were captured using the Olympus BX51 microscope and QImaging microublisher 3.3 RTV system at 60× (Adept Electronic Solutions).

Preparation of labeled RNA and microarray hybridization

Sorted decidual CD11cHI and CD11cLO macrophage populations were washed with PBS and frozen in Stratagenes lysis buffer. Total RNA was isolated according to the manufacturer’s instructions. Affymetrix microarray analysis was performed by GenePattern software (18). Data were analyzed according to the Affymetrix human U133 Plus 2.0 chip protocols. Briefly, each probe was hybridized to a microarray at a concentration of 1 μg/ml of FITC-conjugated 1-μm beads (Sigma-Aldrich) and washed. The arrays were scanned using a GeneArrayer 7000 Fast Real-Time PCR System, and images were captured using SpectraMax Pulse high-throughput microplate spectrophotometer (Molecular Devices). Amplified RNA was reverse transcribed with Stratagene’s AffinityScript Quantitative PCR cDNA Synthesis Kit and according to manufacturer’s protocol. Amplified specific PCR products was detected using the SYBR Green system (Applied Biosystems) in duplicates and normalized to three housekeeping genes: β2-microglobulin, peptidylprolyl isomerase A, and ubiquitin. Relative quantification was performed using the Applied Biosystems 7900 Fast Real-Time PCR system under the following conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. 

Quantitative real-time PCR

Cytokines

MoFlo-sorted CD11cHI and CD11cLO CD14+ macrophages were plated at 10×10⁶ cells/ml in 96-well plate containing 1 μg/ml of FITC-conjugated 1-μm beads (Sigma-Aldrich) and washed with 1× PBS containing LPS (100 ng/ml; Sigma-Aldrich) or LPS and human recombinant IFN-γ (20 ng/ml; PeproTech). All cytokines except TGF-β1 were measured using a multiplex cytokine assay (Bio-Rad) and according to manufacturer’s protocols. TGF-β1 was measured using a sandwich ELISA assay (R&D Systems) and according to manufacturer’s protocol. Subsequent analysis was done using SpectraMax Pulse high-throughput microplate spectrophotometer.

Electron microscopy

Electron microscopy

Preparation of labeled RNA and microarray hybridization

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Electron microscopy

Electron microscopy
Cultures were incubated with anti–CD14-PE, anti–CD45-PercP, and anti–CD11c-APC (BD Biosciences) for 30 min and measured on the FACS-Calibur flow cytometer (Becton Dickinson). Phagocytosis was measured in mean fluorescent units of FITC-labeled beads.

Ag processing assay

Decidual leukocytes were incubated at $3 \times 10^6$ cells/ml for 1 h at 37˚C with the self-quenching molecule DQ-BSA (Invitrogen) or BSA-FITC (Sigma Aldrich) as a control. After incubation, cells were washed with cold buffer on ice to stop the reaction immediately. Cells were then stained with CD14, CD45, and CD11c (as previously described), and analyzed on the FACSCalibur flow cytometer (Becton Dickinson).

Results

dMφs consist of two populations that differentially express CD11c

Tissue-derived macrophages at the maternal–fetal interface are found in several unique microenvironments and are likely to perform multiple disparate functions. To assess this heterogeneity and to distinguish these macrophages from other APCs, such as dendritic cells, these tissue-derived dMφs were characterized by flow cytometry using several conventional cell surface markers. Two distinct CD45⁺CD14⁺ dMφ populations from first-trimester decidual leukocytes were identified that differentially express CD11c at either high (CD11cHI) or intermediate levels (CD11cLO) (Fig. 1A). Analysis of 12 patient samples showed a median of $20 \pm 9.9\%$ (SD) CD11cHI cells, and $68.7 \pm 8.6\%$ CD11cLO cells, and maternal peripheral CD14⁺ monocytes, as assessed by flow cytometry. A paired Student $t$ test was used to determine statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ is. Lines indicate median percentages.

![FIGURE 1. First-trimester dMφs consist of two distinct populations that differentially express CD11c. A, Flow cytometry analysis of gated CD45⁺CD14⁺CD11cHI and CD11cLO dMφs with several distinguishing markers. B, CD45⁺CD14⁺ dMφs that are CD11cHI or CD11cLO are present at a frequency of 20 ± 9.9% and 68.7 ± 8.6%, respectively, as assessed using 12 patients. C, Proportion of cells positive for CD209, CD206, and HLA-DR among CD11cHI dMφs, CD11cLO dMφs, and maternal peripheral CD14⁺ monocytes, as assessed by flow cytometry. A paired Student $t$ test was used to determine statistical significance *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ is. Lines indicate median percentages.](http://www.jimmunol.org/)

Table I. Microarray patient methods

<table>
<thead>
<tr>
<th>Gestational Age</th>
<th>MoFlo Sorting</th>
<th>Microarray</th>
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<td>X</td>
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<td>X</td>
<td>X</td>
<td>x</td>
<td>90:93</td>
</tr>
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</table>

*aCD11cLO only.

*bCD11cHI only.
the common myelomonocytic-derived cell marker CD33, but not
the hematopoietic stem cell marker CD34 or the dendritic cell
markers CD208 (DC-LAMP), CD83, or DEC-205 (data not
shown). To confirm that these dM الص (Fig. 1A) were not derived
from contaminating mPB monocytes, we also analyzed first-tri-
mester mPB CD14+ monocytes. mPB monocytes had near-absent
expression of CD209 and CD206, high level of expression of
HLA-DR (Fig. 1C), and an intermediate expression level of CD11c,
appearing as a single relatively homogenous population (data not
shown), suggesting that neither the CD11cHI nor the CD11cLO dM الص populations are due to peripheral monocyte contamination. More-
over, cellular morphological appearances as analyzed using May-
Gru¨nwald/Geimsa staining of the two populations in comparison
with separated mPB CD14+ monocytes showed that CD11cLO cells
appeared to be a homogeneous population of highly vacuolated
macrophages, whereas CD11cHI cells were not vacuolated and were
more heterogeneous in both size and nuclear phenotype (Supple-
mental Fig. 2B). Thus, CD11cHI and CD11cLO dM ص populations are
not only distinguished by flow cytometric analysis but by their
histological appearance.

**CD11cHI and CD11cLO dM ص have distinct transcriptional
profiles**

To understand what genomic differences distinguish these dM ص subsets, we conducted microarray profiling on highly purified pop-
ulations isolated by flow cytometry. First-trimester decidual tissue
from eight patients was used to isolate CD11cHI and CD11cLO
dM ص (Table I). Unique gene signatures for CD11cHI dM ص and
CD11cLO dM ص were generated based on 1.5-fold differen-
tial expression between the two populations in all 8 patients that were used for the analysis generating 243 specific probes upregulated in the CD11cHI dM ص population and 136 in the CD11cLO dM ص population. Complete unique gene signature for both dM ص populations is presented in Supplemental Fig. 3.

**FIGURE 2.** CD11cHI and CD11cLO dM صs have distinct transcriptional profiles. A volcano plot based on fold change and \( p \) value of genes from the
unique signatures of CD11cHI (right side) and CD11cLO (left side) dM صs in comparison with each other are shown as either red or blue dots, respectively. Genes that are similarly expressed between the two populations are shown as gray dots. Unique gene signatures are based on a \( \geq1.5 \)-fold difference between the two populations in all 8 patients that were used for the analysis generating 243 specific probes upregulated in the CD11cHI dM ص population and 136 in the CD11cLO dM ص population. Complete unique gene signature for both dM ص populations is presented in Supplemental Fig. 3.

**FIGURE 3.** CD11cHI and CD11cLO dM صs have different functional programs. A correlation of coefficients plot for (A) CD11cHI dM ص and (B) CD11cLO
dM ص unique gene signatures, as compared with immune cells in the ImmGen database (18). Genes that correlate will appear as a red box, and those that do
not correlate appear as a blue/black box. C, The correlated gene list that appeared in the labeled red boxes for both dM ص populations and their corre-
spanding gene theme.
CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs were highlighted on the combined data sets and presented as a volcano plot (Fig. 2). A volcano plot is generated based on the mean expression value of an individual probe's fold change, and a p value is calculated on the reproducibility of these changes between the two dMφ populations. Gene signatures were composed of 243 probes upregulated specifically in the CD11c<sup>HI</sup> population (red dots) and 136 probes in the CD11c<sup>LO</sup> population (blue dots). For example, genes encoding cd1c, clec5a (C-type lectin 5a), and ereg are overexpressed in the CD11c<sup>HI</sup> population as compared with the CD11c<sup>LO</sup>. Conversely, CD11c<sup>LO</sup> dMφs overexpress genes encoding dmd (dystrophin), unc5b, and wntb5 (for a complete gene list, see Supplemental Fig. 3). Moreover, Pearson’s correlation matrix imaged as a heat map demonstrates the unique gene differences between these two dMφ populations (Supplemental Fig. 4). We confirmed differential expression of several genes identified by this microarray analysis using quantitative real-time PCR (Supplemental Fig. 5, Supplemental Table 1). These data suggest that CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs have unique and reproducible transcriptomes that could indicate varying functional potential. Raw data files can be found at the National Center for Biotechnology Information Gene Expression Omnibus database under the accession number GSE22342.

**CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs show transcriptional differences indicative of unique functional programs**

To understand what common transcriptional programs are being used by each dMφ population, we cross-referenced the CD11c<sup>HI</sup> and CD11c<sup>LO</sup> gene signatures to the ImmGen database (Fig. 3) (18). The ImmGen database contains microarray data generated from hundreds of unique immune cell types. For a particular pair of genes, the database also indicates whether expression tends to be correlated (i.e., similarly upregulated or downregulated) across multiple immune cell types. Thus, suites of genes involved in common transcriptional programs across immune cells may be identified. For each dMφ subset, we determined the coefficient of correlation from the ImmGen database. Genes that correlate closely with one another appear as red, whereas genes that do not correlate will appear as blue/black. Based on these data sets, CD11c<sup>HI</sup> dMφs upregulate suites of genes involved in invasion, mobility, inflammatory processes including lipid metabolism, and antiapoptotic effects. Conversely, CD11c<sup>LO</sup> dMφs upregulate gene clusters that regulate growth and development, as well extracellular communication including networking (Fig. 3C). Thus, CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs coordinately regulate the expression of genes that are attributable to different functional capabilities.

**Neither dMφ population corresponds to the classical M1 or M2 designation**

Are these CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs unique gene signatures common to other previously reported macrophage-derived gene expression profiles? To answer this question, the CD11c<sup>HI</sup> and CD11c<sup>LO</sup> gene signatures were overlaid on published data sets using GenePattern’s multiplot visualizer tool. These data with overlays determine whether there is any differential enrichment of a particular signature between the two populations. First, comparison with the recently published microarray analysis of dMφs, that were termed “M2,” and maternal peripheral monocytes (13) (record GSEA10612) showed that the unique gene signature for CD11c<sup>LO</sup> dMφs (shown as blue dots) correlated with the published dMφs (Fig. 4A, left side; p < 0.0001). However, the unique gene signature of the CD11c<sup>HI</sup> dMφs (shown as red dots) did not correlate precisely with either dMφs or mPB monocytes but was skewed toward the mPB monocyte genotype (p = 0.002). A second overlay, data comparing in vitro differentiated blood-derived macrophages and peripheral blood monocytes (11) (record GDS2424) also showed that CD11c<sup>LO</sup> dMφs signature tracked with the macrophages, suggesting that this population may have a more terminally differentiated phenotype (Fig. 4B; p < 0.0001). Interestingly, in a third comparison, more stringent unique gene signatures were used that include only probes that have a ≥2-fold expression comparing CD11c<sup>HI</sup> dMφs with CD11c<sup>LO</sup> dMφs. In this comparison, CD11c<sup>HI</sup> dMφs notably shared genes in common with synovial macrophages from rheumatoid arthritis patients and there was relatively little correlation with blood-derived macrophages (Fig. 4C; p < 0.0001; record GSEA10500), possibly sug-

![Figure 4](http://www.jimmunol.org/42211215)
gesting that CD11c\textsuperscript{HI} d\(\text{M}\)s are important mediators of inflammation or tissue remodeling.

Finally, a comparison with in vitro M1 and M2 macrophages derived by cytokine stimulation (11) demonstrated that neither CD11c\textsuperscript{HI} nor CD11c\textsuperscript{LO} d\(\text{M}\)s population precisely correlates with these macrophage transcriptional profiles (Fig. 5A; \(p_{\text{HI}} = 0.01; p_{\text{LO}} = 0.006\)). Moreover, the unique gene signatures for M1 (green) and M2 (purple) M\(\text{Os}\) overlaid with the present data set for d\(\text{M}\)s similarly showed minimal or no correlation (Fig. 5B; \(p_{\text{HI}} = 0.02; p_{\text{LO}} = 0.63\)). Heat maps generated based on selected genes from the M1 and M2 gene signature suggests that CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} d\(\text{M}\)s cannot be distinguished based on this classical categorization of macrophages (Fig. 5C, Supplemental Fig. 6). If there were an enrichment of either M1 or M2 in either d\(\text{M}\) population, then overall more genes would be upregulated (red) in one or the other heat maps. However, because this pattern does not occur, it suggests that neither d\(\text{M}\) population is precisely M1 nor M2.

**CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} d\(\text{M}\)s each secrete both proinflammatory and anti-inflammatory cytokines in vitro**

To examine cytokine secretion by both CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} d\(\text{M}\)s, we cultured purified populations for 16 h in either control media or media that contained 100 ng/ml LPS (L) with or without 20 ng/ml IFN-\(\gamma\) (L/I). Supernatants were analyzed by using either a multiplex assay or standard sandwich ELISA for M1 and M2 cytokines, and were compared with mean expression values that were derived from the normalized RNA microarray data set (Fig. 6). CD11c\textsuperscript{HI} d\(\text{M}\)s constitutively expressed MIP-1\(\beta\), IL-10, IL-6, TNF-\(\alpha\), and TGF-\(\beta\), whereas IL-1\(\beta\), IL-6, IL-10, and TNF-\(\alpha\) were all significantly induced on LPS or LPS/IFN-\(\gamma\) stimulation. CD11c\textsuperscript{LO} d\(\text{M}\)s constitutively expressed similar levels of IL-6, TNF-\(\alpha\), and TGF-\(\beta\), as well as significantly lower levels of IL-10 and MIP-1\(\beta\). The higher level of the immunosuppressive cytokine, IL-10, secreted by the CD11c\textsuperscript{HI} d\(\text{M}\)s on stimulation may be particularly interesting if a natural receptor and ligand were identified that could induce it. These cells could be a major source of IL-10 at the maternal–fetal interface. Thus, both CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} d\(\text{M}\)s constitutively secrete both proinflammatory and anti-inflammatory cytokines.

**CD11c\textsuperscript{HI} d\(\text{M}\)s cells contain neutral lipid bodies and CD11c\textsuperscript{LO} d\(\text{M}\)s have enlarged phagolysosomes**

Microarray results demonstrate that the CD11c\textsuperscript{HI} population up-regulated a series of lipid-associated genes compared with CD11c\textsuperscript{LO} cells (Fig. 3B). To clarify their potential role in lipid metabolism, we sorted d\(\text{M}\)s into CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} populations and analyzed them for the presence of lipids by BODIPY 493/503 \(\text{+ expression}\) (Fig. 7A). Although the CD11c\textsuperscript{LO} cells are highly vacuolated, their vacuoles did not contain BODIPY \(\text{+ lipid bodies}\) (Fig. 7A, panels 5 and 6), whereas \(\sim 35\%\) of the CD11c\textsuperscript{HI} cells contained neutral lipid droplets (Fig. 7B). Furthermore, electron micro-
graphs of sorted CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{fs} confirmed lipid body accumulation in a portion of the CD11c\textsuperscript{HI} population, but not in the phagolysosomes of the CD11c\textsuperscript{LO} macrophages (Fig. 7A, panels 6 and 12). The large vacuoles in the CD11c\textsuperscript{LO} cells contain membranous debris and metal-like particles possibly from phagocytosis. Despite evidence of enlarged phagolysosome compartments in the CD11c\textsuperscript{LO} cells, phagocytic capacity on a per-cell level, as measured by fluorescent bead engulfment assays, was equivalent in the two populations (Fig. 7C). Finally, Ag-processing capacities of these two dM\textsubscript{f} populations were distinct (Fig. 7D). Using the self-quenching DQ-BSA molecule as a substrate, we noted that CD11c\textsuperscript{HI} dM\textsubscript{fs} processed BSA efficiently, suggesting that it is the major APC in the decidua. Moreover, their unique gene signatures indicate divergent functions.

**Discussion**

In this study, two distinct macrophage populations are shown to be present in human first-trimester decidual tissue. The two dM\textsubscript{f} populations can be identified based on high and low expression of CD11c (CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{fs}) and are further distinguished by expression of the phagocytic receptors CD209 (DC-SIGN) and CD206 (MMR) overexpressed on only the CD11c\textsuperscript{LO} dM\textsubscript{f} subset (Fig. 1), as well as by morphological differences (Fig. 7). CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{fs} did not differ in phagocytic capacity; however, only the CD11c\textsuperscript{HI} dM\textsubscript{fs} processed BSA efficiently, suggesting that it is the major APC in the decidua. Moreover, their unique gene signatures indicate divergent functions.

Macrophages have remarkable plasticity that allows them to respond efficiently to varying environmental stimuli, and mounting evidence indicates that initial classification schemes are an oversimplification of a variety of different cells including those that may differentiate in a tissue-specific manner and participate specifically in host defense, wound healing, and/or immune regulation (12). Our own extensive microarray analysis further confirms that dM\textsubscript{f} populations are neither precisely proinflammatory (M1) nor anti-inflammatory (M2). In addition, cytokine analysis showed that both dM\textsubscript{f} populations produce inflammatory cytokines, including TNF-\alpha and IL-1\beta, as well as anti-inflammatory cytokines such as IL-10 and TGF-\beta, although not always to the same extent (Fig. 7). TGF-\beta has been shown to be produced by macrophages after phagocytosis of apoptotic cells in the presence of a proinflammatory environment (19), and is known to drive regulatory T cell and NK cell differentiation (20). CD11c\textsuperscript{HI} dM\textsubscript{fs} constitutively secrete IL-10, and the amount is increased near 4-fold by LPS.

**FIGURE 6.** CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{fs} can each produce both proinflammatory and anti-inflammatory cytokines. Both dM\textsubscript{f} populations were either stimulated with LPS with or without IFN-\gamma, or were left unstimulated for 16 h. Supernatants were collected and measured for cytokine production. All cytokines were measured using a multiplex assay except for TGF-\beta, in which a standard ELISA assay was used. Cytokine mRNA, as measured by microarray analysis and presented as mean expression value, is shown in the right panels of each protein cytokine profile. A nonparametric ANOVA was used for independent group statistical significance, and a paired Student t test was used to measure statistical significance between groups. *p < 0.05; **p < 0.01; ***p < 0.001. CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{fs} are designated as either HI or LO, respectively. L/I, LPS and IFN-\gamma; UNS, unstimulated.
stimulation. Although CD11c<sup>LO</sup> dMΦs secrete no IL-10 before stimulation, after stimulation, IL-10 is slightly increased but only to the low basal levels seen in CD11c<sup>HI</sup> dMΦs. IL-10 is a potent anti-inflammatory cytokine. For example, it aids in the maintenance of tolerance to human gut flora (21), whereas in decidua, it may downregulate the expression of proinflammatory cytokines and protect against inflammation-induced pathology (22).

Interestingly, both dMΦ subsets are also capable of producing proinflammatory cytokines such as TNF-α and IL-1β; therefore, dMΦs may contribute to increased inflammation at the fetal–maternal interface. Both TNF-α and IL-1β have been shown to be important regulators of MCP-1 (23), CSF1, and MIF (24). These cytokines have been shown to be associated with the pathology of pre-eclampsia through enhancement of IL-6 production, which contributes to endothelial dysfunction and macrophage accumulation (25). This finding seems in contrast with the notion that the fetal–maternal interface is an anti-inflammatory environment, but fits more with the hypothesis that immune activation is required to facilitate trophoblast invasion and establishment of fetal–maternal tolerance.

Complete gene analysis in comparison with published data sets shows that neither CD11c<sup>HI</sup> nor CD11c<sup>LO</sup> dMΦs precisely correlate with M1 or M2 blood-derived macrophages (Fig. 6). CD11c<sup>HI</sup> dMΦs do, however, have a propensity to skew with both the monocyte and inflammatory-type macrophage. Furthermore, comparison with synovial fluid macrophages from rheumatoid arthritis patients suggests that these inflammatory-type CD11c<sup>HI</sup> dMΦs are not the same as M1 macrophages, although they share features that are important for in vivo induction of tissue remodeling (Fig. 5C).

Further analysis was carried out using the correlation of coefficients plot to identify clusters of related genes (Fig. 4). Genes associated with lipid metabolism are upregulated in the CD11c<sup>HI</sup> dMΦs, including members of the cd1 family, olr1 (oxidized low-density lipoprotein), isl (lipoprotein lipase), and pparγ (peroxisome proliferation-activated receptor γ). Lipids have been shown to be important mediators in the induction of inflammation (26), as well as in the production of the hormonal response (27). CD1 molecules are important in the presentation of lipid Ag moieties to both T and NK cells (28–30), and may have a large impact on macrophage mediation of inflammation. Specifically, cd1c is significantly upregulated and has been shown to be capable of presenting lipopeptides, specifically N-terminally acylated peptides, to human T cells (28). Approximately 10% of decidual lymphocytes are T cells, and several atypical subsets like TCR<sup>+</sup>CD8<sup>+</sup>, NK T cells, and CD4<sup>+</sup>CD8<sup>-</sup>TCRεβ have been described (31), suggesting that T cell subsets specific for CD1 molecules may be present.

CD11c<sup>HI</sup> dMΦs upregulate genes associated with inflammation including trem1 (triggering receptors expressed by myeloid cell 1) and clec5A. Trem1 is an activating receptor that induces secretion of proinflammatory chemokines and cytokines, thereby amplifying an already present inflammatory stimulus (32). This receptor signals through the adapter molecule DAP12, as does CLEC5A (33). Gene pathway analyses reveal that clec5A is associated with lipid metabolism and inflammation (Fig. 4). CLEC5A has been shown to play a role in osteoclastogenesis (34) and may also play an important role in the initiation of other inflammatory responses. Clec5a, together with trem1, may be key components in inflammatory responses that require lipid-encoding genes expressed by CD11c<sup>HI</sup> dMΦs. These data, together with neutral lipid body accumulation in this subset, suggest an important link among lipids, inflammation, and immune response in first-trimester decidua.

The other subset, CD11c<sup>LO</sup> dMΦs, upregulate a different set of genes that are associated with extracellular matrix formation, networking and extracellular communication, and regulation of...
growth. For example, *dmd* is an important extracellular component, emphasized by mutations that lead to muscular dystrophy. Its importance in muscle cell viability suggests a potential role for CD11c/LO dMΦs in uterine muscle maintenance and growth and/or muscle cell remodeling during spiral artery modifications. Macrophages and macrophage-secreted factors have been shown to play a role in stimulating satellite-compartment muscle stem cells, including uterine muscle stem cells, during muscle cell regeneration in mice (35). Moreover, it is known that the uterus harbors the ability to enlarge to hold the growing fetus during pregnancy, and it is thought that stem cells now play a role in this growth (36). CD11c/LO dMΦs may contribute to the maintenance, differentiation, and fusogenic properties of uterine muscle stem cells (37). Igf1 (insulin-like growth factor 1), a gene important for the development and functional maturation of skeletal tissues and reproductive organs, is also upregulated by the CD11c/LO dMΦs. The Igf1 mouse knockout causes infertility (38) and the underdevelopment of muscle tissue (39). The overexpression of Igf1 by CD11c/LO dMΦs may result in the induction of growth and maturation of muscle stem cells and uterine growth during pregnancy.

Ex vivo culture studies together with phenotypic analysis on second-trimester placental samples further indicate that CD11cHI and CD11c/LO dMΦs are truly independent cell types in the decidua. No evidence of their interconversion was found despite inherent plasticity of macrophages (data not shown). However, these unique subsets of macrophages that have been found to upregulate different genetic programs for distinct functions share several thousand gene transcripts, cytokine secretion profiles, and phenotypic markers. Moreover, based on microarray analysis, both subsets upregulate a variety of genes that are proinflammatory and TGF-β, which has a variety of functions including its role in immune tolerance. The proinflammatory molecules include il-β, c*ox1*, c*ox2*, and c*lgc*. Cyclooxygenase-2, an important isoform that is responsible for the conversion of arachidonic acid into inflammatory mediators such as PGs, has been shown to play a role in preterm labor, suggesting that the inhibition of the formation of PGs could lead to the maintenance of pregnancy (40). C1q is a component of the classical complement cascade, and although most complement is produced by hepatocytes, a growing body of evidence demonstrates the importance of tissue resident macrophages producing complement components locally for regulation of opsonization, phagocytosis, and cytokine production (41). Moreover, C1q is upregulated by macrophages that also produce anti-inflammatory cytokines such as IL-10 and TGF-β (42). Importantly, complement has been shown to play a pivotal role for successful pregnancy outcomes (43), and its production by dMΦs together with inflammatory mediators may be crucial for the induction of tolerance at the maternal–fetal interface.

The early decidua has previously been characterized as a place of immune privilege that contains repressed or suppressed immune cells. However, our data suggest that fetal-placental development may require a necessary state of inflammation. CD11cHI dMΦs may be important in inflammatory processes including lipid metabolism and lipid Ag presentation; CD11c/LO dMΦs may be important in extracellular matrix formation and cell–cell communication, as well as muscle cell development. Together, these dMΦ populations do not fit the conventional M1/M2 paradigm but produce both proinflammatory and anti-inflammatory molecules, thereby contributing to the balance that is necessary for tissue remodeling and growth, as well as for fetal–maternal tolerance.

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Disclosures

The authors have no financial conflicts of interest.

References


Supplemental Figure 1: Patient 5 CD11cHI dMφs correlate with other CD11cHI patient populations. Raw mean expression values for patient 5 alone vs. all other CD11cHI patients reveal a strong correlation as indicated by the straight diagonal line of genes (gray dots). CD11cHI unique gene signature (red dots) incorporated into this analysis shows that patient 5 is not causing gene outliers.

Supplemental Figure 2: CD11cHI and CD11cLO dMφs are morphologically distinct and can be found separately in tissue sections. A) Fresh-fixed paraffin-embedded tissue from gestational time point of 9 weeks stained with anti-CD209-Cy3 (RED) and anti-CD14-alexa488 (GREEN). White arrows emphasize CD14+CD209LO cells (40X objective). The two lower panels are single color controls. (B) May Grünwald-Giemsa staining of patient-paired CD11cHI and CD11cLO dMφs along with separated maternal peripheral CD14+ monocytes (60X objective).

Supplemental Figure 3: Complete list of CD11cLO and CD11cHI decidual macrophage unique gene signatures based on microarray analysis. Complete unique gene signature for CD11cHI and CD11cLO dMφs generated based upon a 1.5 fold or greater gene up regulation as compared to each other. Gene symbol along with the probe ID from the Affymetrix HU 133 2.0 Plus platform is included. Mean expression value with standard deviation (SD) is included for each signature along with the average of the mean expression value of the compared data set. A paired student's t test was used to determine statistical significance.

Supplemental Figure 4: Pearson's correlation plot of CD11cHI and CD11cLO dMφs. Pearson's correlation of coefficients was calculated between each sample and plotted as a heatmap. A value of 1 indicates complete correlation (white boxes). As deviation increases the correlation between samples becomes <1 (blue boxes).

Supplemental Figure 5: Quantitative real-time PCR confirmation of microarray results. (A) Quantitative real-time PCR (qRT-PCR) analysis of a portion of the CD11cHI and CD11cLO dMφs for a six different genes normalized to multiple house keeping genes. Primers that were used can be found in Supplemental Table 1. (B) Microarray results of the same 6 genes for the same set of patients shown as mean expression value.

Supplemental Figure 6: Raw Expression Values of genes for the M1 and M2 signatures for the CD11cHI and CD11cLO dMφs. Raw expression values of each individual patient from the CD11cHI and CD11cLO dMφ for each gene considered part of the M1 or M2 unique gene signatures 11. These raw expression values were utilized to generate the heat maps that are found in Figure 6C.

Supplemental Table 1: Quantitative real-time PCR primers
Supplemental Figure 5

A  Quantitative Real-time PCR

B  Microarray
## Supplemental Table 1: Quantitative real-time PCR primers

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