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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 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 immunoregulatory 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 decidual 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 mPB were then fixed and processed as described in the Flow cytometry section. Leukocytes were subsequently purified using either a Ficoll-Hypaque or a Percoll gradient (GE Healthcare). The Percoll gradient consists of 10 ml of 1.08 g/ml; 12.5 ml of 1.053 g/ml; 20 ml of 1.034 g/ml for density gradient centrifugation (30 min/800 g). Macrophages were collected at the 1.08 and 1.053 g/ml interface, whereas lymphoid cells sediment at 1.034 g/ml. Enriched macrophages were sorted to >90% purity based on CD11c expression. (BD Biosciences) by a MoFlo high-performance cell sorter (Dako) to obtain two sets of macrophages, CD11cHI and CD11cLO.

Flow cytometry

The following fluorescently conjugated mouse anti-human Abs were used for FACS analysis: CD14-FITC, CD14-PE, CD14-APC, CD11c-PECy5.5, CD11c-allophycocyanin, HLA-DR-PE, CD209-PE, CD209-APC, CD208-PE, CD205-PE, CD33-PE, CD14-PE, CD45-PerCp, CD45- allophycocyanin (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 (Immunocytometry 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 hydrated 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 1:50, CD11c 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) (Operon, now Eurofin MWG). MoFlo-sorted CD11cHI and CD11cLO CD14+ macrophages were plated at 1 000 000 cells/ml of FITC-conjugated 1-μm beads (Sigma–Aldrich) in 96-well plates 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

Preparation of labeled RNA and microarray hybridization

Sorted decidual CD11cHI and CD11cLO macrophage populations were washed with PBS and frozen in Stratagene lysis buffer. Total RNA was isolated using Stratagene Absolutely RNA MicroPrep Kit protocol. Purified RNA was subjected to one round of amplification and bio- tinylation using Ambion’s MessageAmp III RNA Amplification Kit. Biotinylated amplified RNA was hybridized to human genome U133 Plus 2.0 chips. Chips were processed at Harvard University’s Microarray Core Facility and according to manufacturer’s protocols. Gene transcript levels were determined using algorithms in the Microarray Suite 5.0 software (Affymetrix). Affymetrix default strategies, e.g. Perfect Match, was assigned a present (expressed) or absent (not expressed) call. Sixteen of 20 samples were of sufficient quality to be fully analyzed (8 patient-paired samples each consisting of CD11cLO and CD11cHI subsets; see Table I). Samples used were considered healthy based on pathological assessment, as well as quantity and quality of RNA and aRNA as determined using the Bioanalyzer (Agilent Technologies). In all but one sample, patient 5, CD11cHI mDNAs with >90% purity were obtained but did not affect the data set (Supplemental Fig. 1).

Quantitative real-time PCR

Amplified RNA was reverse transcribed with Stratagene’s AffinityScript Quantitative PCR cDNA Synthesis Kit and according to manufacturer’s protocol. Amplification of 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 Biosystem’s 2−ΔΔCt method (26) with 7900 Fast Real-Time PCR system with the following conditions: 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 s. Supplemental Table I tabulates all genes and corresponding primer sets used for real-time PCR (Operon, now Eurofin MWG).

Cytokines

Sorted decidual CD11cHI and CD11cLO CD14+ macrophages were plated at 1 000 000 cells/ml of 1 M sodium cacodylate buffer (pH 7.4). Small pieces (1- to 2-mm cubes) of sorted mDNAs were postfixed for at least 2 h at room temperature in the above fixative, washed in 0.1 M cacodylate buffer, and fixed with 1% osmium tetroxide (OsO4)/1.5% potassium ferrocyanide (KFeCN6) for 1 h, washed in water 3×, and incubated in 1% aqueous uranyl acetate [UO2(CH3COO)2·2H2O] for 1 h, followed by two washes in water and subsequent dehydration in grades of alcohol (10 min each: 50, 70, 90%; 20 min: 100%). Samples were then put in propylene oxide for 1 h and infiltrated overnight in a 1:1 mixture of propylene oxide and TAAB Epon (Maricv Canada, St. Laurent, Quebec, Canada). Samples were embedded in TAAB Epon and polymerized at 60°C for 48 h. Ultrathin sections (~6000 nm) were cut on a Reichert Ultracut-S Ultratome (Reichert), picked up on copper grids stained with lead citrate (C6H5O7·Pb), and examined in a JEOL 1200EX Transmission Electron microscope (JEOL) or a Tecnai Spirit BioTWIN (FEI) camera, and images were recorded with an AMT 2k charge-coupled device camera (Advanced Microscopy Techniques).

Boron-dipyrromethene 493/503 staining

Sorted decidual CD11cHI and CD11cLO CD14+ macrophages were fixed in 1% paraformaldehyde for 10 min at 4°C and then washed with 1× PBS containing 2% FBS. Cells were then stained with boron-dipyrromethene 493/503 (BODIPY-493/503) dye at a concentration of 0.1 μg/ml for 20 min at 4°C. Cells were then mounted with Vectashield mounting media (Dako) and coverslipped. Cells were imaged using the inverted Zeiss LSM510 META and analyzed using LSM software.

Phagocytosis assay

Decidual cell leukocyte cultures from a Ficoll-Hypaque gradient were plated at a density of 104 cells/ml for 16 h. After incubation, cultures were divided in half and incubated at either 4°C, as a control, or at 37°C for another 2 h. Then 1 μl/ml of FITC-conjugated 1-μm beads (Sigma–Aldrich) were added to cultures. Beginning at time 0 and collecting every subsequent hour for 3 h, cells were harvested, fixed, and stained at 4°C.
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 (CD11c^{HI}) or intermediate levels (CD11c^{LO}) (Fig. 1A). Analysis of 12 patient samples showed a median of $20 \pm 9.9%$ (SD) CD11c^{HI} cells, and $68.7 \pm 8.6%$ CD11c^{LO} cells comprised the CD45^+CD14^+ dMΦ population (Fig. 1B). These populations uniformly express the macrophage-specific marker CD68, and neither population expresses HLA-G (data not shown).

Further phenotypic analysis showed that CD11c^{HI} dMΦs expressed significantly lower levels of the phagocytic receptors, CD209 (DC-SIGN) and CD206 (mannose receptor), compared with CD11c^{LO} dMΦs (Fig. 1C), and can be distinguished based on these markers in paraffin-embedded sections (Supplemental Fig. 2A). Both dMΦ subsets expressed the MHC class II Ag HLA-DR and

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\*CD11c^{LO} only.

\*CD11c^{HI} 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 dMΦs (Fig. 1A) were not derived from contaminating mPB monocytes, we also analyzed first-trimester 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 CD11c⁺ nor the CD11c⁻ dMΦ populations are due to peripheral monocyte contamination. Moreover, cellular morphological appearances as analyzed using May-Grünwald/Geimsa staining of the two populations in comparison with separated mPB CD14⁺ monocytes showed that CD11c⁻ cells appeared to be a homogeneous population of highly vacuolated macrophages, whereas CD11c⁺ cells were not vacuolated and were more heterogeneous in both size and nuclear phenotype (Supplemental Fig. 2B). Thus, CD11c⁺ and CD11c⁻ dMΦ populations are not only distinguished by flow cytometric analysis but by their histological appearance.

**CD11c⁺ and CD11c⁻ dMΦs have distinct transcriptional profiles**

To understand what genomic differences distinguish these dMΦ subsets, we conducted microarray profiling on highly purified populations isolated by flow cytometry. First-trimester decidual tissue from eight patients was used to isolate CD11c⁺ and CD11c⁻ dMΦs (Table I). Unique gene signatures for CD11c⁺ dMΦs and CD11c⁻ dMΦs were generated based on 1.5-fold difference in all 8 patients that were used for the analysis generating 243 specific probes upregulated in the CD11c⁺ dMΦ population and 136 in the CD11c⁻ dMΦ population. Complete unique gene signature for both dMΦ populations is presented in Supplemental Fig. 3.
ulations from all patients. Genes that did not have a 1.5-fold change in all eight patients were excluded. These commonly differentially expressed probes from CD11c^HI and CD11c^LO 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^HI population (red dots) and 136 probes in the CD11c^LO population (blue dots). For example, genes encoding cd1c, clec5a (C-type lectin 5a), and ereg are overexpressed in the CD11c^HI population as compared with the CD11c^LO. Conversely, CD11c^LO 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^HI and CD11c^LO 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^HI and CD11c^LO 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^HI and CD11c^LO 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^HI dMφs upregulate suites of genes involved in invasion, mobility, inflammatory processes including lipid metabolism, and antiapoptotic effects. Conversely, CD11c^LO dMφs upregulate gene clusters that regulate growth and development, as well extracellular communication including networking (Fig. 3C). Thus, CD11c^HI and CD11c^LO 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^HI and CD11c^LO dMφs unique gene signatures common to other previously reported macrophage-derived gene expression profiles? To answer this question, the CD11c^HI and CD11c^LO 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 GSEA10682) showed that the unique gene signature for CD11c^LO dMφs (shown as blue dots) correlated with the published dMφ (Fig. 4A, left side; p < 0.0001). However, the unique gene signature of the CD11c^HI 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 GDS2429) also showed that CD11c^LO 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^HI dMφs with CD11c^LO dMφs. In this comparison, CD11c^HI 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-
suggesting that CD11c\textsuperscript{HI} dM\textsubscript{f}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} dM\textsubscript{f}s population precisely correlates with these macrophage transcriptional profiles (Fig. 5A; \( p_{HI} = 0.01; p_{LO} = 0.006 \)). Moreover, the unique gene signatures for M1 (green) and M2 (purple) M\textsubscript{f}s overlaid with the present data set for dM\textsubscript{f}s similarly showed minimal or no correlation (Fig. 5B; \( p_{HI} = 0.02; p_{LO} = 0.63 \)). Heat maps generated based on selected genes from the M1 and M2 gene signature suggests that CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{f}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 dM\textsubscript{f} 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 dM\textsubscript{f} population is precisely M1 nor M2.

**CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{f}s each secrete both proinflammatory and anti-inflammatory cytokines in vitro**

To examine cytokine secretion by both CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{f}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} dM\textsubscript{f}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} dM\textsubscript{f}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} dM\textsubscript{f}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} dM\textsubscript{f}s constitutively secrete both proinflammatory and anti-inflammatory cytokines.

**CD11c\textsuperscript{HI} dM\textsubscript{f}s cells contain neutral lipid bodies and CD11c\textsuperscript{LO} dM\textsubscript{f}s have enlarged phagolysosomes**

Microarray results demonstrate that the CD11c\textsuperscript{HI} population upregulated a series of lipid-associated genes compared with CD11c\textsuperscript{LO} cells (Fig. 3B). To clarify their potential role in lipid metabolism, we sorted dM\textsubscript{f}s into CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} populations and analyzed them for the presence of lipids by BODIPY 493/503 + expression (Fig. 7A). Although the CD11c\textsuperscript{LO} cells are highly vacuolated, their vacuoles did not contain BODIPY + 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-

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**FIGURE 5.** Neither CD11c\textsuperscript{HI} nor CD11c\textsuperscript{LO} dM\textsubscript{f}s population can be categorized as strictly M1 or M2. A, Unique gene signatures from the CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{f}s were compared with data sets from M1- and M2-derived M\textsubscript{f}s (11) and are visualized as a volcano plot. The number of gene probes found on either the left or the right side of the volcano plot is shown in the bottom right boxes of each plot. Gray dots indicate the M1/M2 published data set, whereas the red and blue dots still represent CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} unique gene signatures, respectively. \( p_{HI} = 0.01; p_{LO} = 0.006 \). B, Unique gene signatures were generated for M1 and M2 M\textsubscript{f}s based on published signatures and were overlaid onto the CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{f}s complete data set. \( p_{HI} = 0.02; p_{LO} = 0.63 \). C, Heat maps using raw expression values for both the M1 and M2 signatures show cluster analysis for CD11c\textsuperscript{HI} and CD11c\textsuperscript{LO} dM\textsubscript{f}s, as well as indicating upregulation or downregulation of a particular gene in the unique gene signature. The \( p \) values were calculated using the \( \chi^2 \) method in that significance indicates correlation between unique gene data sets and published data.
graphs of sorted CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs confirmed lipid body accumulation in a portion of the CD11c<sup>HI</sup> population, but not in the phagolysosomes of the CD11c<sup>LO</sup> macrophages (Fig. 7, panels 6 and 12). The large vacuoles in the CD11c<sup>LO</sup> cells contain membranous debris and metal-like particles possibly from phagocytosis. Despite evidence of enlarged phagolysosome compartments in the CD11c<sup>LO</sup> 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 dMφ populations were distinct (Fig. 7D). Using the self-quenching DQ-BSA molecule as a substrate, we noted that CD11c<sup>HI</sup> dMφs 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 dMφ populations can be identified based on high and low expression of CD11c (CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs) and are further distinguished by expression of the phagocytic receptors CD209 (DC-SIGN) and CD206 (MMR) overexpressed on only the CD11c<sup>LO</sup> dMφ subset (Fig. 1), as well as by morphological differences (Fig. 7). CD11c<sup>HI</sup> and CD11c<sup>LO</sup> dMφs did not differ in phagocytic capacity; however, only the CD11c<sup>HI</sup> dMφs 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 dMφ populations are neither precisely proinflammatory (M1) nor anti-inflammatory (M2). In addition, cytokine analysis showed that both dMφ populations produce inflammatory cytokines, including TNF-α and IL-1β, as well as anti-inflammatory cytokines such as IL-10 and TGF-β, although not always to the same extent (Fig. 7). TGF-β 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<sup>HI</sup> dMφs constitutively secrete IL-10, and the amount is increased near 4-fold by LPS.
Percentage of CD11c HI cells that are BODIPY +, based on seven patient samples. A, Patient-paired, single-cell images. Confocal images of CD11c HI (panels 1–4) and CD11c LO (panels 5–10) dMØs, which include DAPI, the neutral lipid stain BODIPY495/503, CD14, and differential interference contrast (original magnification ×63). Panels 5 and 11 are May-Grünwald/Giemsa stains (original magnification ×60) and panels 6 and 12 are electron micrographs. B, Percentage of CD11c HI cells that are BODIPY +, based on seven patient samples. C, Mean fluorescent intensity of phagocytic uptake of FITC-labeled latex beads over the course of 4 h. A 4°C control was done in concert to account for beads that could remain attached to the outside of the cell. SD between biological replicates is shown at every time point. D, Ag processing is shown as a percentage of fluorescent intensity using the self-quenching dye DQ-BSA (1 mg/ml) as compared with BSA-FITC (10 mg/ml) over a 1-h incubation period. A paired Student t test for five patients gives a p value <0.001. Error bars represent SD.

stimulation. Although CD11c LO dMØs secrete no IL-10 before stimulation, after stimulation, IL-10 is slightly increased but only to the low basal levels seen in CD11c HI dMØs. 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 dMØ subsets are also capable of producing proinflammatory cytokines such as TNF-α and IL-1β; therefore, dMØs 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 HI nor CD11c LO dMØs precisely correlate with M1 or M2 blood-derived macrophages (Fig. 6). CD11c HI dMØs 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 HI dMØs 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 HI dMØs, including members of the cd1 family, olrl (oxidized low-density lipoprotein), lpl (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). CD11c HI dMØs 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 TCRαβ, NKT cells, and CD4+CD8–TcRαβ have been described (31), suggesting that T cell subsets specific for CD11c HI dMØs may be present.

CD11c HI dMØs 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 HI dMØs. 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 LO dMØs, 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 CD11cLO dMds 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). CD11cLO dMds 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 CD11cLO dMds. The Igf1 mouse knockout causes infertility (38) and the underdevelopment of muscle tissue (39). The overexpression of Igf1 by CD11cLO dMds 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 CD11cLO dMds 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-B, which has a variety of functions including its role in immune tolerance. The proinflammatory molecules include il-1b, cox1, cox2, and c1q. 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-B (42). Importantly, complement has been shown to play a pivotal role for successful pregnancy outcomes (43), and its production by dMds 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 dMds may be important in inflammatory processes including lipid metabolism and lipid Ag presentation; CD11cLO dMds may be important in extracellular matrix formation and cell–cell communication, as well as muscle cell development. Together, these dMds 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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References


