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Placental Macrophages Are Impaired in Chorioamnionitis, an Infectious Pathology of the Placenta

Amira Ben Amara,*1 Laurent Gorvel,*1 Karine Baulan,* Justine Derain-Court,* Christophe Buffat,* Christel Vérollet,†‡ Julien Textoris,* Eric Ghigo,* Florence Bretelle,* Isabelle Maridonneau-Parini,†‡ and Jean-Louis Mege*

Pregnancy is dependent on maternal–fetal tolerance that may be compromised because of infections or inflammation of the placenta. In this study, we examined whether the context of placental immune tolerance affected the functions of resident macrophages and if their functions were altered during chorioamnionitis, an infectious pathology of the placenta. Macrophages from at-term placentas expressed CD14, exhibited macrophage microbicidal functions, but were less inflammatory than monocyte-derived macrophages. Moreover, placental macrophages spontaneously matured into multinucleated giant cells (MGCs), a property not exhibited by monocyte-derived macrophages, and we detected MGCs of myeloid origin in placental tissue. Compared with placental macrophages, MGCs exhibited a specific phenotype and gene expression signature, consisting of increased cytoskeleton-associated gene expression along with depressed expression of inflammatory response genes. Furthermore, placental macrophages from patients with chorioamnionitis were unable to form MGCs, but this defect was partially corrected by incubating these placental macrophages with control trophoblast supernatants. MGCs formation likely serves to regulate their inflammatory and cytocidal activities in a context that imposes semiallograft acceptance and defense against pathogens. The Journal of Immunology, 2013, 191: 5501–5514.

The placenta constitutes a complex interface between the mother and the fetus. It is infiltrated early in pregnancy by maternal immune cells, including NK cells, T lymphocytes, and macrophages, which support maternal tolerance to paternal Ags (1). Therefore, the placenta may be a privileged target during infectious disorders. Chorioamnionitis (CA) is an acute inflammation (1). Therefore, the placenta may be a privileged target during infectious disorders. Chorioamnionitis (CA) is an acute inflammation of the membranes and chorion of the placenta and is typically caused by ascending polymicrobial infection in the setting of membrane rupture. The responsible pathogens include Escherichia coli, group B Streptococcus, and, to a lesser extent, Hemophilus sp. and Staphylococcus sp. (2, 3). The pathophysiology of CA remains unclear, although the disease may be a consequence of altered placental immune homeostasis involving placental macrophages (pMΦ), also known as Hofbauer cells.

pMΦ represent 20–30% of the leukocytes present in the first-trimester decidua, and their levels remain high throughout pregnancy (4, 5). Phenotypic characterization of pMΦ has indicated that they express traditional macrophage markers (6). pMΦ eliminate the cell debris produced during implantation (7), and the uptake of apoptotic trophoblasts renders pMΦ unable to produce inflammatory mediators (8). This property, along with the repolarization of placental immune response toward a Th2 profile during pregnancy, may account for the immunosuppressive activities of pMΦ (9–11). In addition, decidual macrophages support the reorganization of uterine wall vasculature, which is essential to the maintenance of placentation and pregnancy (12). The differentiation and functions of pMΦ are influenced by the placental microenvironment and consequently vary according to the pregnancy trimester (9, 13).

A remarkable feature of placental biology is the presence of syncytiotrophoblasts, which result from the fusion of underlying cytotrophoblast cells. This multinuclear syncytiotium lines the villous space and faces toward the maternal blood. Syncytiotrophoblasts regulate the maternal immune response to the fetus and establish an interface between the maternal blood and the embryonic extracellular fluid, facilitating the passive exchange of material between the mother and the embryo (14, 15). This multinucleation process is also observed in cells from the monocyte/macrophage lineage. Indeed, myeloid multinucleated giant cells (MGCs) are classified into several variants, including osteoclasts, Langhans cells, and foreign body–type giant cells (FBGs), each of which is characterized by distinct morphological and functional properties (16). Different stimuli have been reported to induce macrophage fusion (17); for example, monocytes treated with IFN-γ and bacterial supernatants or lectins, such as Con A, differentiate in Langhans-like MGCs (18, 19), whereas IL-4 or IL-13 stimulates the fusion of macrophages into FBG-like MGCs (17). The molecular mechanisms...
behind macrophage multinucleation are poorly understood, although molecules associated with chemotaxis, adhesion, and proteolysis have been shown to be required for inducing macrophage multinucleation (20). It is likely that the tissue microenvironment and the type of stimulus govern the ability of macrophages to fuse into a specific MGC variant.

In this study, we examined whether the placental context affected the functions of resident macrophages and whether the functions of pMΦ were altered during CA, an infectious pathology of the placenta. We found that pMΦ from normal placentas exhibited the typical microbicidal functions of macrophages, were less inflammatory than monocyte-derived macrophages (MDMs), and spontaneously matured into MGCs, a property not exhibited by MDMs. In addition, we detected MGCs of myeloid origin in placental tissue. MGCs exhibited a specific phenotype and gene expression signature compared with pMΦ. Furthermore, pMΦ from CA patients were unable to mature into MGCs, but this defect was partially corrected by incubating pMΦ from CA patients with supernatants from control trophoblasts. Taken together, these results suggest that MGCs regulate the inflammatory and cytokidal activity of pMΦ in a context that imposes constraints such as semiallograft acceptance and defense against pathogens.

Materials and Methods

Placenta collection

Eighteen healthy pregnant control patients and nine CA patients were included in the study after providing written informed consent and after approval was granted from the Comité d’Ethique d’Aix Marseille Université (number 08-012). Placentas from healthy at-term pregnancies and vaginal deliveries as well as pathological placentas were collected in the Gynecology-Obstetrics Department of the Hôpital de la Conception (Marseille, France). CA was suspected according to clinical and biological criteria such as maternal fever (>38.5˚C), vaginal tract colonization with group B Streptococcus, prolonged membrane rupture (>18 h), high C-reactive protein, leukocytosis, and bacteriuria. The diagnosis of CA was confirmed by anatomopathological examination (21), and the CA cases were classified as grade I, grade II, and grade III, respectively, according to the progress of polymorphonuclear neutrophils from the chorion to the amnion (22). Four patients had bacteriological documentation, whereas the cultures of five patients remained negative. The clinical and biological characteristics of the healthy controls and CA patients are presented in Table I.

Immunohistologic analysis

Placentas were excised, and tissue samples were embedded in paraffin. Four-micrometer serial placental sections were deparaffinized in xylene, rehydrated in graded alcohol, and incubated with rabbit anti-CD14 (1/500 dilution; Epitomx) Abs, mouse anti-CD68 (1/2500 dilution; Novocastra), and anti-CD163 (1/250 dilution; Carpinteria) mAbs or isotype controls to ensure specific labeling of macrophages (23, 24). Deparaffinization, Ag retrieval, and immunodetection of CD14, CD68, and CD163 were carried out with an automatic immunostainer (Ventana Discovery) using 3′-diaminobenzidine (Ventana Medical Systems) as a substrate. After wash-

ing, the slides were counterstained with Mayer’s hematoxylin for 10 min, and CD14, CD68, and CD163 were visualized as precipitation products. Indeed, MGCs did not resist the scraping procedure needed to detach intact cells from the plastic substrate. Therefore, they could not be analyzed by flow cytometry (31). Approximately 107 pMΦ or MGCs were scrapped in a buffer (20 mM Tris-HCl, 200 mM NaCl, 1 mM EDTA, and 1% Triton X-100 [pH 7.5]) containing protease inhibitor (Complete, Roche) and phosphatase inhibitor (Phosphostop; Roche) mixtures at 4˚C. The cell lysates were cleared by centrifugation at 10,000 × g for 15 min and stored at −80˚C. The cell lysates were run using the described method. Samples were loaded onto 10% SDS gel, electrophoresed, transferred onto nitrocel-

lulose membranes, and then probed with mouse Abs directed against CD11b, CD11c, CD14, CD68, CD163, or HLA-DR or rabbit anti-tubulin Ab (BD Pharmingen) at 4˚C overnight. After washing, the blots were incubated with HRP-conjugated anti-mouse or anti-rabbit IgG Abs (Pierce), and the reaction was developed using the Immobilon Western Chemilu-

minescent HRP substrate (Millipore). The expression of the indicated Abs was quantified by densitometric scanning and was normalized using anti-

tubulin. The results are expressed in terms of relative intensity, and the values represent the mean ± SEM of three different placentas.
Functional activity of placental cells

The phagocytic activity of pMφ and MGCs was studied as follows. Cells (2 × 10⁵ cells/assay) were incubated with 1.31-μm beads (Kisker Biotech) at a final dilution of 1/1000 for 1 h at 37°C. After extensive washing to remove free beads, the cells were fixed with 5% paraformaldehyde, permeabilized with 0.1% Triton X-100 for 5 min, and labeled with bodipy-phallacidin (Molecular Probes). The uptake of particles was assessed using a TCS SP5 confocal microscope (Leica). pMφ and MGCs also were incubated with opsonized 1-μm latex beads (Sigma-Aldrich) and NBT for 2 h to generate reactive oxygen species (ROS) as described previously (32). After washing, the cells were examined by optical microscopy. The results are expressed as the percentages of cells that ingested particles or generated ROS relative to the total cell numbers.

The capacity of pMφ and MGCs to secrete cytokines was determined by incubating placental cells (5 × 10⁵ cells/assay) with or without 1 μg/ml E. coli LPS (O55:B5; Sigma-Aldrich). After 24 h, culture supernatants were collected and stored at −80°C prior to cytokine detection. The concentrations of TNF and IL-10 in the supernatants were determined using specific immunoassays (R&D Systems), and the results are expressed as the ratio of IL-10 release to TNF release.

NF-κB translocation

The nuclear translocation of NF-κB was analyzed as follows. pMφ and MGCs (2 × 10⁵ cells/assay) were stimulated with 1 μg/ml LPS. After paraformaldehyde fixation and washing, the cells were permeabilized with 0.1% Triton X-100 and incubated with rabbit Abs directed against NF-κB p65 (Cell Signaling Technology) at a 1/100 dilution. After washing, Alexa Fluor 555-conjugated goat Abs directed against rabbit IgG (at a 1/100 dilution) and bodipy-phallacidin at a dilution of 1/250 were added to the macrophages for 30 min. pMφ and MGCs were then washed and incubated with DAPI at a 1/10,000 dilution for 10 min. Coverslips were mounted with Mowiol, and NF-κB nuclear translocation was assessed by epifluorescence microscopy. The NF-κB p65 translocation was quantified as follows. The colocalization of DAPI and NF-κB p65 was determined using the Mander’s ratio, where M1 represent DAPI overlapping on NF-κB. After washing, the cells were examined by optical microscopy. The results are expressed as the percentage of nuclei that showed DAPI overlapping with NF-κB p65 and M2 NF-κB p65 overlapping on DAPI. Unstimulated cells were used to threshold DAPI and NF-κB channels. Three-dimensional representations of NF-κB p65 translocation in CD14+ macrophages and MGCs are shown, where the x- and y-axes represent the cell size and the z-axis represents the fluorescence intensity in merged DAPI (in blue) and NF-κB p65 (in red). The three-dimensional representations were assessed using the ImageJ software surface plot. The colocalization of DAPI and NF-κB in pink. The results are presented as the percentage of ±SEM of Mander’s ratios.

Phosphorylation of signaling molecules

pMφ and MGCs were stimulated with 1 μg/ml LPS, and the phosphorylation of signaling molecules was studied by immunoblotting as described above using Abs at a 1/10000 dilution (Cell Signaling Technology). The activation of the MAPK p38, ERK, and SAPK/JNK was determined using Abs specific for phospho-p38, phospho-ERK, and phospho-SAPK/JNK, respectively. The activation of Stat1 and AKT was assessed using Abs directed against phospho-Stat1 (Tyr701), phospho-Stat1 (Ser727), and phospho-AKT, respectively. The expression of labeled proteins was quantified by densitometric scanning and was normalized using anti-tubulin or, for phospho-ERK or -p38, to the housekeeping gene β-actin (ACTB) and was expressed as the median of fold change = 2^−ΔΔCt, where ΔΔCt = (CtTarget − CtActin)assay − (CtTarget − CtActin)control, as described previously (33). The M1/M2 polarization status of pMφ and MGCs was determined by qRT-PCR as described above. The 9 M1-related and 10 M2-related genes were selected from lists of M1 and M2 genes published elsewhere (35, 36). The results are presented as radar plots.

Statistical analysis

The results are expressed as the means ± SEM and were compared using the nonparametric Mann–Whitney U test. A p value < 0.05 was considered significant. Correlation analyses between the microarray and qRT-PCR data were performed using Spearman rank correlation coefficient.

Results

pMφ mature into MGCs

To understand the role of pMφ in immune responses during pregnancy, we first identified pMφ in placentas by measuring their expression of three macrophage markers, CD14, CD68, and CD163, using immunohistochemistry (Table I). pMφ from third-trimester placentas expressed CD14 in the subchorionic areas. Using serial sections, we found that CD14+ cells also expressed CD68 and CD163, although CD163 staining was more pronounced than that of CD68 (Fig. 1A). We also found that a small number of placental cells contained two nuclei: these cells expressed CD14, CD68, and CD163 as mononucleated pMφ (Fig. 1B).

We next isolated pMφ via CD14+ selection and investigated whether the formation of multinucleated cells could take place in vitro. Isolated pMφ consisted of mononuclear cells with sizes ranging from 10 to 20 μm (Fig. 2A). During culture, spontaneous cell fusion produced MGCs with two to eight nuclei up to 100 μm (Fig. 2B). After 3 d of culture, ~15% of pMφ were MGCs, and this percentage steadily increased, reaching ~90% of MGCs after 12 d (Fig. 2C). The number of MGCs decreased thereafter, and no MGCs were found in culture after 15 d. These results clearly indicated that MGCs were formed by the spontaneous fusion of cultured pMφ.

Because myeloid MGCs are characterized by specific concentrations of F-actin and the organization of their microtubules (17, 30), we examined the cytoskeleton in placental MGCs. In pMφ, F-actin was concentrated in dotted structures with a characteristic vinculin ring called podosomes (Fig. 2D, a–c), and microtubules were organized from the perinuclear centrosome and extended to
the cell periphery (Fig. 2D, d–f). The cytoskeletal organization was clearly different in placental MGCs: these podosomes were reorganized as clusters (Fig. 2E, a–c), rosettes (Fig. 2E, d–f), or belts at the cell periphery (Fig. 2E, g–i). When MGCs contained more than three nuclei, the microtubules did not organize from a single point; rather, they extended from everywhere in the cell or from the nuclear envelope (Fig. 2E, d–f). Taken together, these results showed that pMΦ were able to form MGCs with a specific cytoskeletal organization reminiscent of that found in other myeloid MGCs.

*p = 0.03.

FIGURE 1. Placental macrophages. (A) Serial sections of subchorionic areas from healthy placentas were labeled with anti-CD14, -CD68, and -CD163 mAbs, and macrophages were detected by immuno-histochemistry. Scale bar, 100 µM. (B) Binucleated cells that expressed CD14, CD68, and CD163 are shown.

### Table I. Clinical and biological characteristics of patients with CA

<table>
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<th></th>
<th>Controls (n = 11)</th>
<th>Patients (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal age (y)</td>
<td>29 [24–31]</td>
<td>33 [25–38]</td>
</tr>
<tr>
<td>Positive microbiological vaginal sample (n)</td>
<td>—</td>
<td>Group B <em>Streptococcus</em> (2) <em>E. coli</em> (1)</td>
</tr>
<tr>
<td>Bacteriuria (n)</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>WBCs (10^9/L)</td>
<td>10.0 [9.1–12.8]</td>
<td>17.5 [13.7–21.1]^*</td>
</tr>
<tr>
<td>Lymphocytes (10^9/L)</td>
<td>2.6 [2.3–2.7]</td>
<td>1.9 [1.6–2.0]</td>
</tr>
<tr>
<td>Monocytes (10^9/L)</td>
<td>0.47 [0.38–0.64]</td>
<td>0.86 [0.75–0.91]</td>
</tr>
<tr>
<td>C reactive protein (mg/L)</td>
<td>—</td>
<td>54 [25–78]</td>
</tr>
<tr>
<td>Gestational age (wk)</td>
<td>32</td>
<td>27</td>
</tr>
<tr>
<td>Cesarian section (n)</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Histological CA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Grade I (n)</td>
<td>—</td>
<td>4</td>
</tr>
<tr>
<td>Grade II (n)</td>
<td>—</td>
<td>3</td>
</tr>
<tr>
<td>Grade III (n)</td>
<td>—</td>
<td>2</td>
</tr>
<tr>
<td>Positive culture of placenta (n)</td>
<td>—</td>
<td><em>E. coli</em> (2) <em>Haemophilus influenza</em> (2)</td>
</tr>
</tbody>
</table>

The maternal ages, biological data and placenta weights are given in median and interquartile ranges (in square brackets). *p = 0.03.

pMΦ and MGCs have distinct surface markers

We next compared the phenotype of isolated pMΦ and pMΦ-derived MGCs. Because of the constraints of MGC size and fragility, the phenotypes of the two macrophage subsets were studied using immunoblotting. pMΦ isolated from the placenta and pMΦ-derived MGCs expressed similar levels of CD11b, CD11c, and HLA-DR, whereas CD14 expression was dramatically decreased in MGCs. In contrast, CD68 and CD163 expression was higher in MGCs than in pMΦ (Fig. 3A). Densitometric analyses performed on three different placentas yielded similar results (Fig. 3B). Taken together,
these results showed that the differentiation of pMΦ into MGCs was associated with changes in surface receptor expression.

pMΦ and MGCs are functionally distinct

We further investigated whether pMΦ and MGCs differed in their phagocytic and inflammatory capabilities. pMΦ and MGCs exhibited similar phagocytic activity with >95% of pMΦ and MGCs ingesting beads (Fig. 4A). In addition, most pMΦ and MGCs were positive for NBT reduction, demonstrating that both produced ROS. The inflammatory responses of pMΦ and MGCs were then studied at the transcriptional level using qRT-PCR, and 9 M1- and 10 M2-related genes were assessed. We found that the modulation of M1/M2 genes was similar between pMΦ and MGCs. Interestingly, only 30% of M1 and M2 genes were modulated (Fig. 4B), suggesting that pMΦ and MGCs are not polarized. Using immunoassays, we measured the release of TNF, an inflammatory cytokine, and IL-10, an immunoregulatory cytokine. In the absence of stimulation, MDMs, pMΦ, and MGCs secreted small amounts (<20 pg/ml; n = 5) of TNF and IL-10. However, when stimulated with LPS for 24 h, MDMs and pMΦ secreted different amounts of TNF (750 ± 20 and 250 ± 30 pg/ml, respectively; n = 3), whereas MGCs were incapable of secreting high levels of TNF (<20 pg/ml). LPS-stimulated MDMs and pMΦ released similar amounts of IL-10 (1200 ± 150 and 1200 ± 120 pg/ml, respectively; n = 3), and LPS-stimulated MGCs released smaller amounts of IL-10 (300 ± 50 pg/ml). The TNF/IL-10 imbalance suggested that the inflammatory potential of pMΦ and MGCs was depressed compared with that of MDMs and that among the pMΦ subsets the inflammatory potential of MGCs was dramatically reduced (Fig. 5A). Note that pMΦ, MGCs, and MDMs released similar amounts of IL-6 (>1000 pg/ml), demonstrating that they were fully functional.

We next assessed the inflammatory response of pMΦ and MGCs by measuring NF-κB translocation and MAPK phosphorylation. The nuclear translocation profile of NF-κB differed between LPS-stimulated pMΦ and MGCs. Indeed, NF-κB p65 was transiently translocated to the nucleus in pMΦ but not in LPS-stimulated MGCs (Fig. 5B). A quantitative study revealed that NF-κB p65 translocation was detected after 15 min of stimulation and reached a maximum after 60 min and decreased thereafter in pMΦ. Only a moderate and delayed translocation of NF-κB p65 was observed in MGCs (Fig. 5C, 5D). In pMΦ stimulated with LPS, the phosphorylation of p38...
MAPK reached a maximum level at 15 min poststimulation and was maintained during 120 min, whereas the phosphorylation of p38 was transient in MGCs (Fig. 6A). Densitometric analyses performed on pMw and MGCs from three different placentas led to similar results (Fig. 6B). In contrast, the time course of ERK and SAPK/JNK phosphorylation was similar in LPS-stimulated pMw and MGCs (Fig. 6). Taken together, these results demonstrated that the downmodulation of the inflammatory response in MGCs affected the NF-κB and p38 MAPK pathways and that pMw and MGCs share the microbicidal activities of macrophages but are only weakly inflammatory, which was particularly evident in MGCs.

pMw and MGCs exhibit specific transcriptional signatures

Because pMw and MGCs were functionally distinct, we analyzed their gene expression profiles using whole-genome microarrays and compared them to those of untreated MDMs and MDMs treated with IFN-γ or IL-4. pMw and MGCs exhibited specific upregulation of transcriptional signatures that were distinct from stimulated and unstimulated MDMs, and this specific signature was composed of tissue-specific genes from placental origin (Uniprot Tissue annotation; \( p = 4 \times 10^{-7} \)) (Fig. 7A). Second, we examined whether pMw and MGCs were polarized in a manner similar to MDMs stimulated with IFN-γ (M1 macrophages) or IL-4 (M2a macrophages). For this reason, we selected genes that were modulated by IFN-γ and IL-4 in MDMs and studied their expression in pMw and MGCs. PCA (Fig. 7B) and heat-map analysis (Fig. 7C) clearly showed that neither pMw nor MGCs were polarized into M1 or M2 macrophages, and MGCs did not express the core M1- or M2a-specific genes (Fig. 7C). Third, we compared the signatures of pMw and MGCs (Fig. 7D) and found that 2423 probes (1498 genes) were upregulated and 2704 probes (1948 genes) were downregulated in MGCs compared with pMw. It is also worth noting that 1005 probes (764 genes) upregulated in pMw and 1101 probes (730 genes) upregulated in MGCs were not expressed in MDMs.

We then validated our microarray results by RT-PCR and analyzed the data according to gene functions. First, the modulation of 27 genes was assessed in the same placenta by RT-PCR. We found similar modulation in microarray and RT-PCR (Spearman correlation coefficient = 0.57; \( p = 0.02 \); Supplemental Table I). We also confirmed the modulation of seven genes between placental macrophages and MGC in four independent placentas (Supplemental Fig. 2A). We then performed a functional annotation of the genes specifically upregulated in MGCs and found a functional enrichment in genes involved in cytoskeleton reorganization, using keywords such as “collagen,” “cell leading edge,” “extracellular matrix,” and “focal adhesion” (Table II). Interestingly, the focal adhesion
pathway was affected in MGCs, which exhibited many upregulated genes such as focal adhesion kinase, phosphoinositide 3-kinase, paxillin, vinculin, and myosin L-chain kinase (Supplemental Fig. 1). It is likely that the dramatic reorganization of the actin cytoskeleton that we observed in MGCs was related to modulation of these focal adhesion pathway genes, along with genes involved in the adhesion structures common to focal adhesions and podosomes. In contrast to the enrichment in genes involved in cytoskeleton reorganization, the functional annotation of genes specifically upregulated in MGCs did not reveal keywords such as immune or inflammation.

![Figure 5](http://www.jimmunol.org/)

**FIGURE 5.** Defective inflammatory activity of MGCs. (A) pMφ, MGCs, and MDMs used as controls were stimulated with LPS for 24 h. TNF and IL-10 release was quantified using immunoassays. The results are expressed as the ratio of TNF/IL-10 release and represent the means ± SEM of four different placenta. (B and C) pMφ and MGCs were incubated with fluorescent anti-p65 Abs (red) and DAPI (blue) and stimulated with LPS for different time periods. Representative micrographs of nuclear NF-κB p65 translocation are shown after 60 min (B). Three-dimensional representations of NF-κB p65 translocation in representative pMφ and MGCs are shown. The x- and y-axes represent the cell size, and z-axis represents the fluorescence intensity in merged DAPI (blue) and NF-κB p65 (red) (C). The colocalization of DAPI and NF-κB p65 was quantified using the Mander’s ratio, where M1 represents DAPI overlapping on NF-κB and M1 NF-κB overlapping on DAPI. Unstimulated cells were used to threshold DAPI and NF-κB channels. The results are presented as the percentage ± SEM of Mander’s ratio (D).
inflammatory response. Most immune response genes, including those for inflammatory cytokines, chemokines, and chemokine receptors, TLRs, and different members of the TNF superfamily, were expressed at lower levels in MGCs than in pMϕ (Table III). These results showed that pMϕ and MGCs exhibited specific signatures and that enrichment of cytoskeleton-associated genes and depressed expression of inflammatory/immune response genes could be used to characterize MGCs.

**pMϕ are altered in CA**

We analyzed the distribution of pMϕ in placentas from CA patients using CD14, CD68, and CD163 as macrophage markers. pMϕ from third-trimester placentas expressed CD14, CD68, and CD163 in the subchorionic areas, as demonstrated in serial sections (Fig. 8A). To compare the labeling of pMϕ from CA patients and healthy pregnant women, we systematically quantified positive cells by computerized imaging and examined 30 fields/placenta, including 10 fields each in the subchorionic, intermediate, and subbasal areas. Areas of comparison were strictly similar because the automated system searched identical regions on three serial sections. The results were expressed as the percentage of labeled surface area according to units of villus surface. The numbers of pMϕ were significantly ($p < 0.05$) lower in CA placentas than in placentas from women with normal pregnancies (Fig. 8B). The expression of macrophage markers, such as CD14 and CD68, was similar between placentas from second and third trimesters in controls. Similarly, the expression of CD14 and CD68 was independent of the gestational trimester in patients with CA. In contrast, the expression of CD163 was higher in second-trimester placentas than in third-trimester placentas from both control and CA women (Fig. 9A). The location within the placenta (subchorionic, intermediate, or subbasal region) had no effect on the expression of macrophage markers in either control or CA placentas (Fig. 9B). Interestingly, the severity of the disease had an impact on the pMϕ phenotype, because the level of CD163 expression was significantly ($p < 0.05$) higher in grade III than in grade II CA placentas. The expression of CD163 was also higher ($p < 0.05$) in CA placentas with documented infections compared with CA placentas without documented infections (Fig. 9C).

We then isolated pMϕ from control and CA placentas and analyzed their phenotype by flow cytometry. The expression of CD11b, CD14, CD68, and CD163 was similar between controls and women with CA. In addition, the expression of CD11b and HLA-DR varied among individuals, but no significant differences were found between controls and CA patients (Fig. 8C). To study the polarization status of pMϕ in CA, we selected 9 M1-related genes and 10 M2-related genes and studied their expression by qRT-PCR. Only 3 M1 genes (**EDN1**, **IL15**, and **IL15RA**) were up-regulated in CA patients, whereas the expression of the 6 other M1
genes and 10 M2 genes was similar between CA patients and healthy controls (Supplemental Fig. 2A), emphasizing the absence of macrophage M1/M2 polarization in pMφ. Note that the expression of M1 and M2 genes was similar in CA patients and healthy controls when pMφ were cultured for 9 days: only (CTSC) in the M2 genes was overexpressed in CA patients (see Supplemental Fig. 2A). We also studied the activation status of pMφ in CA: the expression of genes that were found to be upregulated in control pMφ by microarray was analyzed in qRT-PCR; the expression of IL24, IL32, MMP7, INDO, IL10, and PDL1 was similar.
Table II. Functional annotation of upregulated genes in MGCs

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<th>No.</th>
<th>Percentage</th>
<th>p Value</th>
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<td>9.1</td>
<td>1.4 x 10^{-7}</td>
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<tr>
<td>GO_CC Collagen</td>
<td>17</td>
<td>0.9</td>
<td>1.5 x 10^{-5}</td>
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<td>GO_CC Organelle lumen</td>
<td>232</td>
<td>12.3</td>
<td>1.9 x 10^{-3}</td>
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<tr>
<td>GO_CC Organelle envelope</td>
<td>90</td>
<td>4.7</td>
<td>1.2 x 10^{-2}</td>
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<td>GO_CC Cell-leading edge</td>
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<td>GO_BP Nuclear division</td>
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<td>GO_MF Cofactor binding</td>
<td>49</td>
<td>2.6</td>
<td>8.8 x 10^{-3}</td>
</tr>
<tr>
<td>GO_MF Purine nucleotide binding</td>
<td>244</td>
<td>12.9</td>
<td>1.1 x 10^{-2}</td>
</tr>
<tr>
<td>GO_MF NAD or NADH binding</td>
<td>16</td>
<td>0.8</td>
<td>8.6 x 10^{-3}</td>
</tr>
<tr>
<td>KEGG Extracellular matrix receptor interaction</td>
<td>24</td>
<td>1.3</td>
<td>7.7 x 10^{-4}</td>
</tr>
<tr>
<td>KEGG Focal adhesion</td>
<td>40</td>
<td>2.1</td>
<td>2.4 x 10^{-3}</td>
</tr>
<tr>
<td>KEGG Lysosome</td>
<td>26</td>
<td>1.4</td>
<td>9.7 x 10^{-3}</td>
</tr>
<tr>
<td>KEGG Vitamin B₆ metabolism</td>
<td>5</td>
<td>0.3</td>
<td>4.5 x 10^{-2}</td>
</tr>
<tr>
<td>KEGG Pyruvate metabolism</td>
<td>12</td>
<td>0.6</td>
<td>4.1 x 10^{-2}</td>
</tr>
</tbody>
</table>

The genes that were specifically upregulated in MGCs compared with placentae and MDMs were annotated using databases such as GO Cellular Component (GO_CC), GO Biological Process (GO_BP), GO Molecular Function (GO_MF), and KEGG pathways. The number of modulated genes included in the functional annotation is indicated. The percentage corresponds to the ratio of annotated out of the total number of upmodulated genes. The p value was obtained after Benjamini–Hochberg correction.

in CA patients and healthy control. Because the expression of the WNT5A gene was moderately increased in CA patients, we studied the expression of another key gene of the WNT pathway, the LRP6 gene. The expression of LRP6 was significantly (p < 0.05) higher in CA patients than in controls (Supplemental Fig. 2B), suggesting that the inflammatory response was altered in CA patients. To better assess the inflammatory context of CA, we evaluated spontaneous pMϕ production of TNF and IL-10, cytokines representative of the inflammatory/immunoregulatory balance. The production of IL-10 was significantly (p < 0.02) lower in CA patients than in controls, whereas the production of TNF was roughly similar in CA patients and controls (Fig. 8D). To understand the defective production of IL-10 in CA, we investigated the transductional pathway leading to IL-10 production. In pMϕ from healthy controls, Stat-1 was phosphorylated on Tyr701 and Ser727 residues after LPS stimulation. In contrast, Stat-1 was not phosphorylated in CA pMϕ (Supplemental Fig. 3). We also tested the phosphorylation of AKT, which is known to be an inhibitor of the IL-10 pathway. We found that AKT was transiently phosphorylated in pMϕ from healthy controls when they were stimulated with LPS, in CA pMϕ, and AKT phosphorylation was more intense and sustained than in pMϕ from healthy controls (Supplemental Fig. 3). Taken together, these results suggest that the defective IL-10 release by CA pMϕ is associated with the defective phosphorylation of Stat-1 and the sustained activation of AKT.

We further studied the ability of pMϕ from CA patients to differentiate into MGCs and found that these pMϕ were unable to form MGCs. Indeed, after 9 d of culture, the size of the cells from CA patients was dramatically smaller (∼50–70 μm) as compared with the MGCs from healthy women (100 μm), and >98% of the cells contained a unique nucleus (Fig. 8E). The defective generation of MGCs was observed in placentae from second-trimester and third-trimester pregnancies and was independent of the clinical stage of CA. We also found that the placental microenvironment affected the ability of pMϕ to mature into MGCs. To further examine this notion, pMϕ from CA placentae were incubated with supernatants from trophoblasts isolated from healthy women by positive selection using EGFR as a specific marker. Although the majority of pMϕ remained unable to mature into MGCs, ∼10% of the pMϕ recovered the ability to fuse into MGCs (Fig. 8F). Taken together, these results showed that the inflamm-
In this study, we demonstrated the presence of myeloid MGCs in placentas and examined the maturation of pMφ into MGCs, a property of pMφ not reported previously. pMφ were clearly distinct from the MDMs used as controls: pMφ expressed higher levels of CD14 and were less inflammatory than MDMs but were not polarized toward a M2 phenotype. pMφ also spontaneously matured into MGCs, in contrast with MDMs that require cytokines and/or growth factors to mature into MGCs (17). The MGCs derived from pMφ showed a profound reorganization of both F-actin and microtubules, and they formed podosomes organized as clusters or as peripheral rings, which were reminiscent of osteoclast podosome belts (37). The number of nuclei also remained low and randomly distributed throughout the cytoplasm, a distribution that is also observed in osteoclasts and FBGs, whereas the nuclei of Langhans cells are arranged in a circular pattern (17).

MGCs shared the expression of myeloid markers with pMφ but also exhibited phenotypic differences. For example, MGCs...
overexpressed CD68 and downmodulated CD14, which reflected a maturation process associated with MGC formation. The downmodulation of CD14 has also been reported during IL-4–mediated MGC formation (17). Placental MGCs also overexpressed CD163, which deserves specific comment. CD163 is a member of the scavenger receptor superfamily and is mainly expressed by cells of the monocyte/macrophage lineage (38). The expression of CD163 has been detected in CD68+ macrophages from chorionic villi (39) as well as decidual CD14+ macrophages (40). We found that CD163 was coexpressed with CD68 in placental sections. CD163 was also shown to be expressed by M-CSF–differentiated macrophages, which mimic first-trimester pMΦ (41). Furthermore, CD163 has been associated with macrophage polarization (42), and the notion of pMΦ M2 polarization has been largely based on this expression (41, 43).

The phagocytosis-related function of macrophages, which is essential for the removal of cellular debris during pregnancy and consequently for maintaining cellular homeostasis (8), was similar between MGCs and pMΦ. In addition, the differentiation of pMΦ into MGCs did not affect ROS production, whereas the differentiation of pMΦ into MGCs was associated with significant alterations in cytokine production leading to decreased inflammatory potential. pMΦ released TNF and IL-10 in response to LPS, which is consistent with a recent report showing that placental CD14+CD11c+ macrophages secrete both inflammatory and anti-inflammatory cytokines (44). However, pMΦ released IL-10 at a similar level as MDMs but secreted lower amounts of TNF, suggesting a bias toward an anti-inflammatory response. Moreover, MGCs were less inflammatory than pMΦ, as demonstrated by their capacity to release IL-10 but not TNF in response to LPS. This decreased inflammatory activity of MGCs was not due to downregulated CD14 expression, because pMΦ and MGCs produced similar amounts of IL-6 in response to LPS, or due to the skewing of macrophage polarization toward the M2 phenotype. Using a combination of transcriptional biomarkers related to M1 and M2a phenotypes, we found that pMΦ and MGCs were not polarized into well-defined M1 or M2a macrophages. Our results are not completely consistent with previously reported data showing that CD14+ macrophages from first-trimester placentas are polarized toward an M2 profile (45) but are consistent with a recent report showing that CD14+CD11cbright and CD14+CD11clow macrophages from first-trimester placentas were not polarized (44). Placental macrophages may be representative of a population of alternatively (M2b or M2c) activated macrophages. It is also likely that the microenvironments of first-trimester placentas and at-term placentas differentially influence the activation status of pMΦ. Moreover, it is likely that MGC formation is related to defective signaling pathways leading to an inflammatory response, because we found depressed activation of NF-kB p38 MAPK to a lesser extent in MGCs.

To better characterize the activation status of pMΦ and MGCs, we analyzed gene expression signatures using a whole-genome analysis. This analysis revealed that pMΦ exhibited a transcriptional profile distinct from those of circulating monocytes and MDMs, and this result is consistent with previous transcriptional studies conducted with decidual macrophages (41, 45). We also showed that the transcriptomic profile of placental MGCs was distinct from that of pMΦ. The hierarchical clustering and PCA strengthened the finding that pMΦ and MGCs were distinct from polarized macrophages. Moreover, the functional annotation analysis extended the phenotypic and functional characteristics of MGCs, the most striking finding from which was the enriched upregulated gene expression in categories related to membrane activity, cell adhesion, and the extracellular matrix. These upregulated genes included collagen and metalloprotease genes, which contribute to
cell reorganization during the process of MGC formation. Interestingly, two genes, LOXL2 and VEGFC, were upregulated in MGCs. LOXL2 belongs to lysyl oxidase gene family, which is involved in matrix remodeling including collagen in normal and cancerous tissues (46). LOXL2 protein is expressed in chorionic cytotrophoblasts of fetal membranes (47), and its role in the biogenesis of connective tissue makes LOXL2 a candidate fusion effector. Vascular endothelial growth factor (VEGF)-C is a member of the angiogenic factor family and is known to play a role in placenta, in which it may control the migration of trophoblastic giant cells (48). VEGF-C also is expressed in osteoclasts, another variety of giant cells (49). The connection between cytoskeleton and adhesion molecules and the VEGF pathway suggested that tissue reorganization and angiogenesis may be critical properties of MGCs. In contrast to genes involved in cytoskeletal organization, most of the genes involved in the immune/inflammatory response were downmodulated in MGCs, in accordance with our functional studies.

Our study further demonstrated that pMφ are altered in patients with CA, because the expression of CD14 and CD68 was slightly decreased in CA placenta compared with control placentas. A similar decrease in CD68 expression also has been reported in CA placentas (50), whereas two studies using a manual count reported an increase in CD68-positive macrophages in at-term placentas complicated with CA (51, 52). It is likely that the selection of relatively few fields containing at least one CD68+ cell combined with the heterogeneous distribution of cells among the villi account for such discrepancies. In addition, two hypotheses may account for changes in macrophage number in the placenta. First, a reduction in pMφ numbers because of apoptosis is likely because apoptosis has been described in CA placentas (53, 54). Second, pMφ also may be recruited to other sites of fetal inflammation, such as the fetal lungs or membranes, especially in cases of infection of the fetus or amniotic fluid. Supporting this hypothesis, increased fetal lungs or membranes, especially in cases of infection of the fetus or amniotic fluid.

In conclusion, our results show that pMφ exhibit specific properties, including the ability to mature spontaneously into MGCs. We hypothesize that the maturation of pMφ into MGCs represents a novel mechanism for regulating the inflammatory and cytoidal activities of macrophages in a context that imposes contradictory constraints, including semiallograft acceptance and defense against pathogens. Moreover, this cross-talk between pMφ and trophoblasts is significantly affected by placental infections and may shed light on new perspectives for the study of CA.

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Disclosures

The authors have no financial conflicts of interest.

References


