Baby's First Macrophage: Temporal Regulation of Hofbauer Cell Phenotype Influences Ligand-Mediated Innate Immune Responses across Gestation

Dominika Swieboda, *Emory University*
Erica Johnson, *Emory University*
Jacob Beaver, *Emory University*
Lisa Haddad, *Emory University*
Elizabeth Ann L Enninga, *Mayo Clinic, Rochester*
Matthew Hathcock, *Mayo Clinic*
Sarah Cordes, *Emory University*
Valerie Jean, *Emory University*
Ivy Lane, *Emory University*
Ioanna Skountzou, *Emory University*

*Only first 10 authors above; see publication for full author list.*

**Journal Title:** JOURNAL OF IMMUNOLOGY

**Volume:** Volume 204, Number 9

**Publisher:** AMER ASSOC IMMUNOLOGISTS | 2020-05-01, Pages 2380-2391

**Type of Work:** Article | Final Publisher PDF

**Publisher DOI:** 10.4049/jimmunol.1901185

**Permanent URL:** https://pid.emory.edu/ark:/25593/vvjg4

Final published version: [http://dx.doi.org/10.4049/jimmunol.1901185](http://dx.doi.org/10.4049/jimmunol.1901185)

**Copyright information:**

This is an Open Access work distributed under the terms of the Creative Commons Attribution 4.0 International License ([https://creativecommons.org/licenses/by/4.0/rdf](https://creativecommons.org/licenses/by/4.0/rdf)).

*Accessed October 1, 2023 11:46 PM EDT*
Baby's First Macrophage: Temporal Regulation of Hofbauer Cell Phenotype Influences Ligand-Mediated Innate Immune Responses across Gestation

Dominika Swieboda, Erica L. Johnson, Jacob Beaver, Lisa Haddad, Elizabeth Ann L. Enninga, Matthew Hathcock, Sarah Cordes, Valerie Jean, Ivy Lane, Ioanna Skountzou and Rana Chakraborty

*J Immunol* 2020; 204:2380-2391; Prepublished online 25 March 2020;
doi: 10.4049/jimmunol.1901185
http://www.jimmunol.org/content/204/9/2380

Supplementary Material http://www.jimmunol.org/content/suppl/2020/03/24/jimmunol.1901185.DCSupplemental

References This article cites 51 articles, 5 of which you can access for free at: http://www.jimmunol.org/content/204/9/2380.full#ref-list-1

Why *The JI*? Submit online.

- Rapid Reviews! 30 days* from submission to initial decision
- No Triage! Every submission reviewed by practicing scientists
- Fast Publication! 4 weeks from acceptance to publication

*average

Subscription Information about subscribing to *The Journal of Immunology* is online at: http://jimmunol.org/subscription

Permissions Submit copyright permission requests at: http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts Receive free email-alerts when new articles cite this article. Sign up at: http://jimmunol.org/alerts
Baby’s First Macrophage: Temporal Regulation of Hofbauer Cell Phenotype Influences Ligand-Mediated Innate Immune Responses across Gestation

Dominika Swieboda,*† Erica L. Johnson,* Jacob Beaver,† Lisa Haddad,‡ Elizabeth Ann L. Enninga,§ Matthew Hathcock,¶ Sarah Cordes,‡ Valerie Jean,‡ Ivy Lane,† Ioanna Skountzou,‡ and Rana Chakraborty*,§,†,‡

The importance of fetal placental macrophages (Hofbauer cell [HCs]) is underscored by their appearance 18 d postconception and maintenance through term; however, how human HCs evolve during healthy pregnancy and how microenvironment and ontogeny impact phenotype and function remain unknown. In this study, we comprehensively classify human HCs ex vivo, interrogate phenotypic plasticity, and characterize antiviral immune responses through gestation. Activated HCs were abundant in early pregnancy and decreased by term; molecular signatures emphasize inflammatory phenotypes early in gestation. Frequency of HCs with regulatory phenotypes remained high through term. Furthermore, term HCs exhibited blunted responses to stimulation, indicating reduced plasticity. IFN-α is a key placental IFN that appeared less protective than IFN-γ, suggesting a potential weakness in antiviral immunity. Ligand-specific responses were temporally regulated: we noted an absence of inflammatory mediators and reduced antiviral gene transcription following RIG-I activation at term despite all HCs producing inflammatory mediators following IFN-γ plus LPS stimulation. Collectively, we demonstrate sequential, evolving immunity as part of the natural history of HCs through gestation. The Journal of Immunology, 2020, 204: 2380–2391.

Macrophages are a heterogeneous population of immune cells whose functions include Ag presentation, phagocytosis, cytokine secretion, and coordination of downstream innate and adaptive immunity. These cells orchestrate immune response generation and tissue homeostasis and repair by constantly changing their functional state in response to the local tissue microenvironment (1). Broadly classified as M1 or M2, macrophage differentiation follows Th cell paradigms (2, 3). This simplification only partially reflects characteristics of macrophage phenotypic intermediates (4). Classically activated M1 macrophages secrete proinflammatory cytokines, mediate pathogen resistance, and contribute to tissue destruction. Alternatively activated macrophages are subdivided by distinct surface molecule expression, cytokine secretion, and effector function (5, 6). M2a macrophages are activated by IL-4 plus IL-13 and involved in tissue repair and immunoregulation; M2b responses support humoral and allergic reactions and are induced by immune complexes; M2c macrophages, which arise from IL-10 or glucocorticoid stimulation, suppress inflammation and remodel extracellular environments (5).

Placental macrophages, Hofbauer cells (HCS), were first described toward the end of the 19th century (7) as large, pleomorphic, highly vacuolated cells of fetal origin (8–11). Found in chorionic villi as early as 18 d postconception (9), they are maintained through birth, albeit with reduced numbers (12). Although their ontogeny has not been fully characterized, studies suggest that the earliest HCs derive from mesenchymal progenitor cells, followed by progressive recruitment of in situ differentiated yolk sac–, fetal liver–, and bone marrow–derived monocytes (Fig. 1) (13). They preferentially localize near fetal vessels and trophoblasts, suggesting roles in placental development, immunity, and homeostasis (14). Term HCs are a mixture of M2 subtypes, with heterogeneous surface marker expression, cytokine secretion, and function (14–17). They express the three FcγRs (18) and the pan-macrophage marker CD68 (19) and can be stimulated by glucocorticoids (20) and IL-10 (21) to express CD163, CD206, and CD209 (21). Unstimulated HCs constitutively express IL-10 and TGF-β (22). Variations in shape, vesiculation (13), surface molecule expression, effector function (23),...
and motility (12) are all consistent with plasticity and a wide range of roles in placental homeostasis. Furthermore, term HCs are capable of robust proinflammatory responses to bacterial LPS (24) and viruses (25–28). However, there are few studies describing HC phenotype and function through the natural time-course of a healthy pregnancy.

In eutherian mammals, the placenta alters local and systemic maternal immunity to limit infection and sustain a healthy pregnancy (29–32). Immunologic homeostasis at the maternal–fetal interface can be characterized by three distinct phases (33, 34). Implantation and placentaation during the first and early second trimester resemble an “open wound,” which requires a strong inflammatory response (35, 36). Next, the mother, placenta, and fetus reach symbiosis, with induction of an anti-inflammatory/tolerogenic state (37). Parturition necessitates recrudescence of the inflammatory process, delivery, and placental rejection (38, 39).

It is unknown how human HCs evolve during gestation and how the combined influences of microenvironment and ontogeny impact phenotype and function. The only multidimensional analysis of HC diversity through gestation was performed by Reyes et al. (17). In this study of rhesus macaques, HCs exhibited plasticity and pleomorphism through gestation. We undertook a comprehensive classification of human HCs ex vivo, interrogated plasticity following in vitro stimulation, and characterized antiviral immune responses through gestation.

Materials and Methods

Ethics statement

Second trimester human placentae were obtained from a free-standing clinic in Atlanta, GA, from consenting donors who elected to terminate pregnancies prior to 21 wk and 6 d of gestation. Human term placentae (>37 wk gestation) were collected from hepatitis B, HIV-1 seronegative women (>18 yr of age) immediately after elective caesarean section without labor from Emory Midtown Hospital, Atlanta, GA. This study was approved by the Emory University Institutional Review Board (IRB 00021775). Written informed consent was acquired from all donors before sample collection. Samples were deidentified before primary HC isolation.

Placental dissection and Hofbauer isolation

HCs were isolated from membrane-free villous placenta as previously described (22). On average, the purity was >95%. After isolation, HCs were cultured in complete RPMI medium consisting of 1× RPMI (Corning Cellgro), 10% FBS (Optima; Atlanta Biologics), 2 mM l-glutamine (Corning Cellgro), 1 mM sodium pyruvate (Corning Cellgro), 1× antibiotics (penicillin, streptomycin, amphotericin B; Corning Cellgro) at 37°C and 5% CO2. HCs were treated with the following as indicated, following resuspension per the manufacturer’s instructions: 50 ng/ml LPS (00-4976) (Thermo Fisher Scientific); 100 IU/ml IFN-α A/D (PHC4044); 20 ng/ml IFN-γ (PHC4031); 20 ng/ml IL-4 (PHC0044); 20 ng/ml IL-13 (PHC0134) (Life Technologies); 100 ng/ml IL-29 (ab50932), 20 ng/ml IL-10 (ab6613) (Abcam); 50 µg/ml heat-agglutinated IgG (HAGG) produced from IgG human serum (L4506) (Sigma-Aldrich) as previously described (40); and 20 ng/ml IL-1β (78034.1) (Stecmill Technologies); 5′-ppp-dsRNA (tirl-3prna), control for 5′-ppp-dsRNA (tirl-3prna) (InvivoGen).

FACS

HCs (250,000 per sample) were blocked for 10 min on ice with 0.25 µl/sample Human TruStain FCx (BioLegend) in FACS buffer (1× PBS, 0.1% BSA, 1 mM EDTA) and live/dead stained for 10 min on ice with Calcein Violet 450AM (Life Technologies). HCs were stained for surface markers for 20 min on ice using 0.25 µl/sample of the following anti-human Abs in FACS buffer CD209 (E9A8), HLA-DR (L243) CD163 (GH161), and CD68 (Y182A), (BioLegend); CD14 (61D3) (Invitrogen); hMMR (FAB25342T) (R&D Systems); CD86 (2331[FUN-1]) and CD80 (L307.4) (BD Biosciences). FACS samples resuspended in FACS buffer were run on a CytoFLEX flow cytometer following calibration using 6 Peak Rainbow Calibration Particles (BioLegend) and analyzed using FlowJo software. Compensation values were calculated using UltraComp eBeads (Life Technologies) and gating strategy as in Supplemental Fig. 1.

RNA isolation and RT-PCR

Directly isolated, treated, and control HCs (100,000 cells per condition) were lysed in RNA Lysis Buffer (Zymo Research). Total RNA was isolated from cells using the Quick-RNA MiniPrep Kit (Zymo Research). Purified RNA was reverse transcribed using random primers with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). HC gene expression was quantified by RT-PCR using Quantitect SYBR Green PCR Kits (Qiagen) using specific primers for host genes. CT values were normalized to the reference gene β-actin and represented as indicated. HC gene expression was quantified by RT-PCR using Quantitect SYBR Green PCR Kits (Qiagen) using specific primers as follows: B-actin (forward [FWD] 5′-GGC CTA CTC CCA AAG TCC AC-3′, reverse [REV] 5′-GGT AAG GCC TGG CTT CCT CCA CC-3′), PTTL (FWD 5′-GCC ACA AAA AAT CAC AAG CCA-3′, REV 5′-CCA TGG TGT GAA CCG CCG-3′), IFITM1 (FWD 5′-GCC TTC ATA GTC GCC TCC-3′, REV 5′-AGA TTA CGA ACT CAA GAC TCA-3′), OAS1 (FWD 5′-GGA CCC CAG GCA AGA TTT G-3′), ARG2 (FWD 5′-GCC GAG TGG ATC TCA CCA C-3′, REV 5′-GAG AAT CCT GGC ACA TCG GGA A-3′), STAT6 (FWD 5′-CGA GGT GAT ATC TTC CTT-3′, REV 5′-GCA GGA GTT TCT AAT CCG TG-3′), STATA (FWD 5′-CCA CGG GAC CCT CTG G-3′, REV 5′-GTT CCG GGG AGT CAT GCA G-3′), STAT3 (FWD 5′-ACC AGC AGT ATC GTC GCT C-3′, REV 5′-GCC ACA ATC GCA GCA TCT-3′), IFNA (FWD 5′-GAG TAC TTT GTC GAT GTG A-3′, REV 5′-TGG TAT CTG TCT TGA CAA C-3′), IFNB (FWD 5′-GTC TCC TCC AAA TTG CTC TC-3′, REV 5′-ACA GGA GGT TCT GAC ACT GA-3′), MDA5 (FWD 5′-GAG ATG GAG AAT AAC TCA TCA G-3′, REV 5′-CTC TTC TTC ATG ACG ACT CTC-3′), MX1 (FWD 5′-CAA TCA GGC TGC TGA CAT TG-3′, REV 5′-GTC CCT CTG CTT GTG GAT G-3′), RIG-I (FWD 5′-ATC CCA GTG TAT GAA CAG CAG-3′, REV 5′-GCC TGT AAC TCT ATC CCC ATC-3′), and VIPERIN (FWD 5′-CCA GTG CAA CTA AAA ATG CCG C-3′, REV 5′-CCG TCT TGA AGA AAT GOC TCC TC-3′). RT-PCR was performed in 96-well plates and run on a Thermo Fisher QuantStudio 5.

Luminex

Cytokine concentrations in the supernatants of treated HCs (500,000 cells per condition) and accompanying controls were assessed using a human cytokine 25-plex panel (Invitrogen) per the manufacturers’ instructions. Plates were read on a Luminex 100 Analyzer.

Statistical analysis

Baseline expression of phenotypic markers was analyzed using linear regression and the resulting graph showing 95% confidence bands of the best-fit line. R2 (goodness-of-fit) is reported in Fig. 2, and deviation of the slope from zero is in the Results. Differences in canonical polarization at baseline were analyzed using two-way ANOVA, with significance set at p < 0.05. Phenotypic markers, cytokine production, and gene expression were analyzed using multiple one-sample t tests comparing untreated and treated values. Analyses were corrected for multiple comparisons by controlling the false discovery rate. Discovery was determined using the two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli, with Q = 5%, without assuming consistent SD (41). Changes in canonical polarization after treatments were analyzed with two-way ANOVA with correction for multiple comparisons by controlling the false discovery rate, as above. Outlier identification was performed using the ROUT method, with Q = 5%.

All statistical analysis was performed using GraphPad Prism 8.1.2 software. In each of the main and supplemental figure legends, n represents the number of placental donors from which HCs were derived. Further experimental statistical details are described in the figure legends.

Results

Early/midgestation is associated with an abundance of activated HCs, whereas term cells are less galvanizable

To determine if HC phenotype changes with gestational age, we employed FACS to characterize isolated, CD14+ early/midgestation (12–24 wk gestational age) and term (≥37 wk) HCs ex vivo using established macrophage surface markers (5, 6) (Fig. 1).
The percentage of HCs expressing each marker is visualized on a per donor basis. We noted a higher frequency of activated CD68\(^+\), CD80\(^+\), or CD86\(^+\) HCs in early/midgestation (Fig. 2Ai–iii, Supplemental Table I). An average of 75% of HCs isolated from placentae between 12 and 22 wk gestation were CD68\(^+\), compared with 40% at term (Fig. 1Ai, Supplemental Table I); linear regression revealed 63% of variation in CD68 expression can be explained by gestational age, with a significantly (\(p = 0.0001\)) nonzero slope of 2% fewer CD68\(^+\) cells per week of advanced gestation. Furthermore, 96% of early/midgestation HCs were CD80\(^+\); at term, CD80\(^+\) HC frequency varied, with groups of high- and low-expressing donors (Fig. 2Aii, Supplemental Table I). As such, only 50% of variation in CD80 can be attributed to advancing gestational age; however, advancing gestational age is associated with a 1.8% loss in CD80\(^+\) cell per week (\(p = 0.0014\)). CD86 expression was limited to 13% of early/midgestation HCs and decreased 4-fold by term to an average of 3% (Fig. 2Aiii, Supplemental Table I); 56% of variation in CD86 can be attributed and decreased 4-fold by term to an average of 3% (Fig. 2Aiii, Supplemental Table I). Median fluorescence intensity (MFI) was analyzed as a measurement of molecular density and was not associated with gestational age for CD68, CD80, CD86, or HLA-DR (Supplemental Table I). Therefore, although there were fewer activated HCs at term, these expressed a phenotype comparable to activated HCs in early/midgestation.

The frequency of CD209\(^+\) HCs was reduced from 99% in early/midgestation to 79% at term, with 50% of variation dependent on advancing gestational age, and a loss of 0.5% of CD86\(^+\) cells per week (\(p = 0.0005\)). HLA-DR\(^+\) HC frequency showed donor variability and no association with gestational age (Fig. 2Aiv, Supplemental Table I). Median fluorescence intensity (MFI) was 3.2-fold higher in early/midgestation (\(p = 0.0006\)) and CD163\(^+\) and CD206\(^+\) cells were constant in frequency to advancing gestational age, with a loss of 0.5% of CD86\(^+\) cells per week (\(p = 0.0005\)). The T cell chemokine RANTES was 3.2-fold higher in early/midgestation (\(q = 0.0008\)) and 11.4% at term had a non-M1–M2 macrophage subtype.

Finally, protein analysis was performed on supernatants collected from HCs after 48 h of culture to determine if phenotypic variations potentiated functional diversity (Supplemental Table III). We observed negligible production of monocyte growth factor GM-CSF, eosinophil growth factor IL-5, and T cell growth factors IL-2, or IL-15; inflammatory cytokines IFN-\(\gamma\), IL-12, and IL-17A; anti-inflammatory cytokine IL-13; and chemokines eotaxin and MIG. One donor (15.4 wk) was excluded from evaluation following outlier analysis because of suspected abnormal clinical characteristics. Although the data are preliminary, we observed some trends of gestationally dependent cytokine production when comparing average cytokine concentrations produced by HCs isolated at early/midgestation and term. Early/midgestation HCs produced 1.7-fold more IFN-\(\alpha\) than term HCs (\(q = 0.01\)). Additionally, inflammatory IL-1\(\beta\) and IL-6 were 2.5-fold higher at early/midgestation. Anti-inflammatory IL-1RA was 3.2-fold higher in early/midgestation (\(q = 0.0008\)) and IL-4 was 2-fold higher (\(q = 0.04\)). The T cell chemokine RANTES was 6.8-fold higher at early/midgestation (\(q = 0.0005\) and IP-10 trended higher as well. Production of monocyte chemoattractant MCP-1 and proinflammatory granulocyte chemoattractants MIP-1\(\alpha\) and MIP-1\(\beta\) was 6.2-fold (\(q = 0.0006\)), 3.5-fold (\(q = 0.03\)), and 3-fold (\(q = 0.008\)) higher, respectively, at early/midgestation. Finally, neutrophil chemoattractant IL-8 was produced in increasing quantities as gestation progressed, with term cells producing 20% more than early/midgestation HCs (\(q = 0.02\)).

Early/midgestation HCs exhibited greater plasticity compared with term HCs

Functional plasticity is an essential feature of macrophages that allows tailored stimuli-dependent responses, as well as education of downstream adaptive immune responses. To investigate whether gestational age impacts plasticity, we treated HCs in vitro with stimuli chosen to recapitulate macrophage polarization into the

---

**FIGURE 1.** HC phenotypic heterogeneity likely stems from a combination of different origins and changing microenvironment at the fetal–maternal interface.
FIGURE 2. Temporal kinetics of human HCs reveal that early/midgestation has an abundance of activated M2A and M2C macrophages, whereas term HCs are less galvanized and predominantly of the M2C-phenotype. Human placental macrophages were isolated from freshly obtained tissue samples. (A) Individual donors are distinguished by color. CD14+ live macrophages populations were costained for activation markers (Ai) CD68, (Aii) CD80, (Aiii) CD86, MHC class II cell surface receptor (Aiv) HLA-DR, and regulatory markers (Av) CD163, (Avi) CD206, and (Avii) CD209, followed by representative flow plots of average of samples <16 wk, 16–24 wk, and at term. Linear regression was performed to analyze the natural progression of phenotypes occurring through gestation. (B) Relative expression of (Bi) ArgI and ArgII and (Bii) iNOS was determined by qRT-PCR of HCs. (Figure legend continues)
canonical macrophage subtypes: classically activated M1, which are functionally proinflammatory and antimicrobial, as well as subclasses of alternatively activated M2 macrophages, which are broadly anti-inflammatory. M2A macrophages enhance endocytic activity and promote cell growth and tissue repair. M2B macrophages regulate the breadth and depth of immune responses and inflammatory reactions with production of both pro- and anti-inflammatory cytokines. Finally, M2C macrophages play crucial roles in the phagocytosis of apoptotic cells. Although these treatments were not designed to recapitulate conditions observed during normal or pathogenic pregnancies, evaluation of HC responses to conventional macrophage stimuli provides valuable insight into HC macrophage biology.

We modeled M1 polarization using IFN-γ plus LPS (43). In early/midgestation, HLA-DR+ HCs was 2.4-fold more common 24 h posttreatment (HPT) (q = 0.01) and continued rising through 48 HPT. CD163+ HCs decreased 47% (q = 0.0002) (Fig. 3Ai, Supplemental Table II). iNOS and Arg1 relative expression was analyzed 24 HPT to evaluate transcriptional regulation of differentiation. iNOS transcription rose 200-fold in early/midgestation (Fig. 3Bi), suggesting potential for antimicrobial activity; Arg1 was unaltered. Ex vivo HC M2-preference was expected based on previous reports (15–17); IFN-γ plus LPS induced an M1-skew in some donors (Fig. 3Bii) (44). Subtype analysis revealed a decrease in M2A-type macrophages at 24 HPT, whereas M2C-type and non-M1/M2 subtypes modestly increased (Fig. 3C). As different STAT analogs are upregulated during macrophage subtype differentiation and cytokine-activated initiation, we evaluated STAT1, STAT3, STAT5A, and STAT6 transcription (45). Following IFN-γ plus LPS, <1% of early/midgestation HCs were M1-like, despite a 10-fold increase in STAT1, a subset downstream of IFN-γ signaling (Fig. 3D). M2A-associated STAT6, inflammatory signaling–associated STAT5A, and M2C-associated STAT3 remained unchanged. IFN-γ plus LPS influenced the secretion of early/midgestation HCs compared with untreated control; growth factors GM-CSF, IL-7, and IL-15 increased (all q < 0.01) (Fig. 3Ei, Supplemental Table III). Secretion of inflammatory IL-1β expanded by 69-fold (q = 0.01), IL-12 by 2735-fold (q = 0.08), TNF-α by 14,538-fold (q = 0.004), and IL-6 beyond the range of our assay (Fig. 3Eii, Supplemental Table III). IL-1RA, IL-4, IL-10, and IL-13 were boosted by 1.8-fold (q = 0.05), 2.6-fold (q = 0.01), 576-fold (q = 0.05), and 3.9-fold (q = 0.01), respectively (Fig. 3Eiii, Supplemental Table III). Proinflammatory chemokines MIP-1α and MIP-1β rose 5-fold and 2.1-fold, respectively (both q < 0.01). T cell chemoattractant RANTES increased 2.3-fold (q = 0.04). Absence of increases in neutrophil chemoattractant IL-8 suggest a protective mechanism against deleterious inflammatory effects at the maternal–fetal interface (Fig. 3Eiv, Supplemental Table III).

HLA-DR+ HCs at term were 2.5-fold more frequent after 48 h of IFN-γ plus LPS stimulation (Fig. 3Ai, Supplemental Table II); a 2.7-fold CD80 upregulation was similarly delayed (q = 0.005) (Fig. 3Aii, Supplemental Table II). We noted a 2.4-fold reduction in CD163+ HCs also 48 HPT (q = 0.00002). Both Arg1 and iNOS transcription expanded 10-fold (Fig. 3Bi), maintaining a neutral M1/M2 ratio (Fig. 3Bii). M2C-type macrophages at baseline constituted 72.8% of term HCs but were 13% 48 HPT with IFN-γ plus LPS (Fig. 3C). M2A-type cell frequency increased 4.8-fold by 48 HPT (q = 0.0007). Two percent of HCs were categorized as M1 after 48 HPT. Freshly isolated term HCs had little transcription of STAT1 but 24 HPT with IFN-γ plus LPS increased STAT1 100-fold; STAT5A increased 3-fold; STAT3 and STAT6 remained unchanged (Fig. 3D). IFN-γ plus LPS-stimulated term HCs secreted similar cytokines to early/midgestation HCs: growth factors GM-CSF and IL-7 were increased 19-fold (q = 0.02) and 7.3-fold (q = 0.02), respectively, over untreated controls (Fig. 3Ei, Supplemental Table III). Inflammatory cytokines increased but did not reach statistical significance (Fig. 3Eii, Supplemental Table III). IL-4 expanded 5.7-fold (q = 0.017) (Fig. 3Eiii, Supplemental Table III). Proinflammatory chemokines MIP-1α and MIP-1β were elevated 34-fold and 5.3-fold, respectively, over untreated controls (Fig. 3Ev, Supplemental Table III). Early/midgestation HCs appeared primed for a fast and robust response, as evidenced by the timing of phenotypic changes, and changes in secreted proteins. Term HCs produced some of the same cytokines after extended stimulation, equally supporting the recruitment of monocytes and T cells while maintaining a baseline M1/M2 ratio.

We used IL-4 plus IL-13 to model M2A-type polarization (43). In early/midgestation, we noted a 1.9-fold loss of CD68+ cells (q = 0.00003) and a 4.6-fold expansion of CD86+ cells 48 HPT (Fig. 4Ai, Supplemental Table II). We detected a 2.8-fold increase in HLA-DR density 24 HPT (q = 0.004). Expression of CD80, CD206, and CD86 increased 48 HPT (Fig. 4Aii, Supplemental Table II). Arg1 transcription was reduced 24 HPT, whereas iNOS increased 10-fold (Fig. 4Bi); increased iNOS transcription reduced M2 dominance (Fig. 4Bii). IL-4 plus IL-13 treatment reduced M2A-like cells. At baseline, these cells made up 40.4% of HCs, but after 24 HPT, this decreased to 10.4% (q < 0.0001) (Fig. 4C); we noted a greater frequency of M2C-type HCs. STAT1 transcription was upregulated 10-fold, STAT6 was also elevated, and no changes were noted in STAT3 or STAT5A transcription (Fig. 4D).

At term, we observed a 1.7-fold loss of CD68+ HCs (q = 0.002) and CD86+ HC frequency increased 9.7-fold 48 HPT (q = 0.0005) (Fig. 4Ai, Supplemental Table II). Furthermore, we noted a 1.9-fold increase in CD209+ HCs (q = 0.05) (Fig. 4Aii, Supplemental Table II). CD206 and CD209 expression increased 24 HPT. At 48 HPT, CD80 expression increased 2.6-fold (q = 0.006) and CD86 rose 1.6-fold (q = 0.0001). We observed no changes in Arg1 or iNOS transcription (Fig. 4Bi), and therefore no change in M1/M2 ratio (Fig. 4Bii). As expected following IL-4 plus IL-13, the M2A subtype increased 3.2-fold from baseline 24 HPT (Fig. 4C). M2C-type HCs decreased to 42.5% 24 HPT and rebounded 48 HPT, suggesting a strong M2C preference at term. STAT1, STAT3, and STAT5A transcription were increased; confoundingly, STAT6 transcription, which is associated with IL-4 plus IL-13 signaling, was reduced (Fig. 4D). IL-4 plus IL-13–induced phenotypic changes occurred faster and more robustly at term than in early/midgestation HCs. Additionally, IL-4 plus IL-13 treatment predictable increased M2A-like macrophages at term, not in early/midgestation.

We modeled M2B-type polarization using IL-1β treatment and HAGG (40). At early/midgestation, no changes were observed at the population level or with surface molecule density (Supplemental Table II). No changes were observed in Arg1 or iNOS transcription (Fig. 5Bi), and M1/M2 ratio was maintained (Fig. 5Bii). We noted a 4-fold reduction (q = 0.005) in M2A-like cells through 48 HPT, with a 1.6-fold increase (q = 0.006) in
M2C-type cells (Fig. 5C). STAT1 and STAT6 transcription was minimally increased, whereas STAT3 and STAT5A remained constant (Fig. 5D).

In term HCs, we observed a 1.5-fold decrease in CD163+ HC frequency 48 HPT (Fig. 5Ai, Supplemental Table II) and no changes in surface molecule density (Supplemental Table II). No significant changes in ArgI or iNOS transcription (Fig. 5Bi) or M1/M2 ratio were detected (Fig. 5Bii). We observed opposite polarization patterns at term compared with early/midgestation HCs; M2C-like cells reduced to 32.5% 24 HPT (Fig. 5C), and M2A-like cells expanded 4-fold. We found a 100-fold increase in STAT1 and STAT5A transcription, 10-fold STAT3 increase, and maintenance in STAT6 (Fig. 5D). In sum, both early/midgestation and term HCs exhibited resistance to immune complex–mediated activation.

Finally, M2C-polarization studies were conducted using IL-10 stimulation (43). CD86+ early/midgestation HCs increased 4.7-fold by 48 HPT (Fig. 6Ai, Supplemental Table II). We observed a delayed 1.9-fold increase in CD163+ cell frequency (q = 0.009), as well as a 1.7-fold increase of CD163 MFI (q = 0.00004) (Fig. 6Aii, Supplemental Table II). HLA-DR density reduced 2.6-fold 24 HPT (q = 0.00002). ArgI and iNOS transcription was unaffected (Fig. 6Bi), and the baseline M1/M2 ratio was maintained (Fig. 6Bii). The proportion of M2C-like cells following IL-10 treatment reached 76.8% 24 HPT (q = 0.001) (Fig. 6C). STAT6 transcription expanded 10-fold; transcription of the remaining STATs was unchanged (Fig. 6D).

In term HCs, we observed a delayed 2.8-fold increase in CD163+ cell frequency (q = 0.03) and a 2.6-fold reduction in CD86+ HCs (q < 0.00001) (Fig. 6Ai, Supplemental Table II). No additional phenotypic changes were observed (Supplemental Table II). Decreased ArgI and decreased iNOS transcription (Fig. 6Bi) maintained M1/M2 neutrality (Fig. 6Bii). Confounding, M2C HCs were reduced from 72.8 to 29% 24 HPT (q, 0.0001) (Fig. 6C). This was accompanied by a 4.0-fold increase in M2A-like cells (q = 0.0006). STAT1, STAT3, and STAT5A transcription increased ~10-fold, and STAT6 slightly decreased (Fig. 6D). Both early/midgestation and term HCs progressed deeper into a regulatory phenotype following IL-10 exposure.

FIGURE 3. IFN-γ + LPS costimulation reveals early/midgestation HCs demonstrate greater plasticity than term macrophages. HCs were stimulated with IFN-γ + LPS simultaneously to induce a M1 phenotype. (A) Mean log10 (fold change) ± SEM of (Ai) percentage of positive and (Aii) MFI of positive population with values meeting thresholds q < 0.05 and fold change of ±1.5, followed by representative flow plots therein. (Bi) Relative expression of ArgI and iNOS was determined by qRT-PCR using the ΔΔCt method. (Bii) M1/M2 ratio as ΔCt-ARGI/ΔCt-iNOS visualized as median and quartiles. (C) HCs were grouped into canonical macrophage subtypes via FACS as in Supplemental Fig. 1. (D) Relative expression of STAT1, STAT3, STAT5A, and STAT6 ex vivo and after stimulation was determined by qRT-PCR using the ΔCt method. (E) Quantification of (Ei) growth factors, (Eii) inflammatory cytokines, (Eiii) anti-inflammatory cytokines, (Eiv) IL-2R, and (Ev) chemokines following 48 h of in vitro culture. Open circles indicate control untreated cells and closed circles indicate IFN-γ + LPS-stimulated cells, visualized as mean ± SEM. Changes over untreated were analyzed via multiple one-sample t tests, with discovery determined using the Two-stage linear step-up procedure of Benjamini, Krieger, and Yekutieli. *q < 0.05, **q < 0.01. n.d., not detected.
Placental IFN-ι may be less protective than IFN-α

Our data suggests HCs differentially respond to polarizing stimuli with advancing gestational age. Therefore, we investigated whether this translated to differential responses to antiviral IFNs. We treated HCs with IFN-α or IFN-ι, which is prevalent at the placenta and implicated in local viral resistance (46). Following IFN-α stimulation of early/midgestation HCs, CD80 expression increased 1.7-fold 48 HPT (\(q = 0.003\)) (Fig. 7Ai, Supplemental Table II). IFN-ι induced no significant marker changes (Supplemental Table II). M2C-like cells increased 1.7-fold (\(q > 0.0001\)) 24 HPT, and M2A-like cells were lost (Fig. 7B, 7C) following both IFN-α and IFN-ι stimulation. At term, neither IFN-α nor IFN-ι influenced HC phenotype (Supplemental Table II). Following stimulation of term HCs with IFN-α or IFN-ι, M2C-like cells were lost and M2A-like cells increased 4-fold (\(q = 0.005\)) (Fig. 7C). Non-M1/M2 subtype increased among term but not early/midgestation HCs.

Key IFN-stimulated gene (ISG) expression is pivotal to protection from viral infection. To determine antiviral mediator activation following IFN stimulation, we evaluated ISG transcription via qRT-PCR at short and prolonged exposures (Fig. 7E). IFN-α rapidly induced vigorous transcription of IFIT1, IFITM1, OAS1, and Viperin in both early/midgestation and term HCs. Importantly, IFN-ι did not induce ISG transcription as quickly or robustly at all timepoints; in both early/midgestation and term HCs, a longer IFN-ι exposure period was required to reach the ISG induction levels noted three HPT with IFN-α. Early/midgestation and term HCs responded similarly to IFN-α and IFN-ι stimulation. Both populations remained phenotypically stable through 48 HPT, and both upregulated key ISGs to similar levels.

RIG-I agonism reveals that viral pattern recognition receptor responses may be temporally regulated

We observed differential responses to macrophage-polarizing stimuli across gestation; however, we noted similar responses to antiviral IFNs throughout gestation. We further evaluated these functional discrepancies by interrogating the RIG-I pattern recognition receptor (PRR) pathway. Early/midgestation HCs increased frequency of CD86+ HCs 48 HPT (\(q = 0.003\)) (Fig. 8A, Supplemental Table II). IFN-α and IFN-β transcription increased 43-fold (\(q = 0.09\)) and 75-fold (\(q = 0.09\)), respectively (Fig. 8Ei). MDA5 and RIG-I transcription rose 7- and 8-fold, respectively (Fig. 8Eii). Together, these suggest a capacity for antiviral responses following both active infection and...
replication of virus. Directly antiviral MX1 and Viperin were 9- and 49-fold more abundant after RIG-I agonism (Fig. 8Eiii). Although these findings are preliminary because of limited sample size, we observed increases in IFN-α (Fig. 8Fi), IFN-γ, IL-1β, IL-12, and TNF-α over untreated controls in early/midgestation (Supplemental Table III). Finally, we observed induction of T cell chemoattractants RANTES and IP-10 (Fig. 8Fiv, Supplemental Table III).

Term HCs were phenotypically stable after RIG-I activation (Supplemental Table II) but increased iNOS 3.3-fold 24 HPT (Fig. 8B). We observed a loss of M2C-like cells and a 4-fold M2A-like cell increase (q = 0.005) (Fig. 8C). STAT1 rose 300-fold, STAT3 30-fold, and STAT5A 22-fold (Fig. 8D). STAT6 transcription was unchanged. IFN-α transcription was unchanged, and IFN-β was only 10-fold higher (Fig. 8Ei). MDA5 and RIG-I both increased 3-fold but less than at early/midgestation (Fig. 8Eii). Finally, ISG MX1 was upregulated 6-fold and Viperin 8-fold (Fig. 8Eiii), similarly to early/midgestation.

The RIG-I pathway exemplified the greatest difference between early/midgestation and term HCs. Early/midgestation HCs upregulated activation molecules, antimicrobial-associated enzymes, and inflammatory transcription factors; this all cumulated in production of inflammatory mediators that was not seen in term HCs. Enhanced IFN, PRR-associated, and VIPERIN gene transcription at early/midgestation underscores the robustness of the RIG-I–induced response.

**Discussion**

HCs are an abundant immune cell population in the human placenta and present throughout gestation. They may be the “first” fetal immune cell. However, there are very few studies identifying changes to phenotype and function during healthy pregnancy. We address this gap demonstrating dynamic immunophenotypes ex vivo across gestation. Activated HCs were present from early pregnancy in high numbers. Although their activated cell frequency decreased by term, phenotype essentially remained unchanged. Cytokine quantification and enzyme expression underscored inflammatory phenotypes early in gestation and suggest recruitment of labor-promoting neutrophils at term (47). HCs expressing tolerogenic markers were highest at midgestation, accompanied by an M2A–to-M2C-like shift. Chemokine levels may reflect monocytic recruitment midgestation, the time when fetal liver and bone marrow–derived monocytes populate the placenta. Our data suggest the existence of two subgroups at term: CD80hi, iNOSi+, and M2B-like HCs and a subset echoing regulatory phenotypes observed midgestation. Phenotypic discrepancies may prepare for parturition and placental rejection, which
has been previously reported to occur with a resurgence of inflammation and increases in iNOS in labored trophoblasts (42).

The presence of M2B-like macrophages in some donors at term suggests a role for this subtype in initiating or regulating labor-associated inflammation; M2B macrophages have been reported to regulate the depth and breadth of inflammatory reactions (47). In summary, HCs appear to be a heterogenous population of M2-like macrophages, with regulatory and anti-inflammatory functions critical to maternal–fetal homeostasis and fetal development. Their differentiation may be etiologically determined and only further influenced by currently undefined local immune signals (13). Our study suggests that HC phenotypes are temporally regulated through gestation, and these changes impact susceptibility to vertically transmissible infections.

**FIGURE 6.** Early/midgestation HCs treated with IL-10 progress toward a term HC phenotype. HCs were stimulated with IL-10 to induce an M2C phenotype. (A) Mean log₁₀ (fold change) ± SEM of (Al) percentage of positive and (Aii) MFI of positive population with values meeting thresholds \( q < 0.05 \) and fold change of \( \pm 1.5 \), followed by representative flow plots therein. (Bi) Relative expression of ArgI and iNOS was determined by qRT-PCR using the \( \Delta\Delta Ct \) method. (Bii) M1/M2 ratio calculated as \( \Delta Ct\text{-ArgI}/\Delta Ct\text{-iNOS} \) visualized as median and quartiles. (C) HCs were grouped into canonical macrophage subtypes via FACS as in Supplemental Fig. 1. (D) Relative expression of STAT1, STAT3, STAT5A, and STAT6 ex vivo and after stimulation was determined by qRT-PCR using the \( \Delta Ct \) method. **\( q < 0.01 \), ***\( q < 0.001 \), ****\( q < 0.0001 \).

**FIGURE 7.** Placental IFN-\( \lambda 1 \) has reduced antiviral effects, as compared with IFN-\( \alpha \). HCs were stimulated with (A, B, and D) IFN-\( \alpha \) or (C and D) IFN-\( \lambda 1 \). (A) Mean log₁₀ (fold change) ± SEM of MFI of positive population with values meeting thresholds \( q < 0.05 \) and fold change of \( \pm 1.5 \), followed by representative flow plots therein. (B and C) HCs were grouped into canonical macrophage subtypes via FACS as in Supplemental Fig. 1. (D) Relative expression of ISGs IFIT1, IFITM1, OAS1, and VIPERIN at early (three HPT) and late (24 HPT) timepoints was determined by qRT-PCR using the \( \Delta\Delta Ct \) method visualized as mean ± SEM. *\( q < 0.05 \), **\( q < 0.01 \).
We interrogated HC functional plasticity through gestation via in vitro macrophage-polarizing stimuli. HC plasticity was reduced at term; phenotypic changes were less marked and delayed. Following stimulation with IFN-γ plus LPS, which may occur during maternal bacterial infection, early/midgestation HCs demonstrated elevated iNOS and STAT1 transcription, whereas term HCs maintained their M1/M2 ratio. Phenotypic changes and pyrogen production (24) were comparable but delayed at term. This difference in response may be due to no ex vivo STAT1 transcription at term; in contrast, early/midgestation HCs exhibited transcription of all measured STATs, including STAT1. Importantly, we did not observe any neutrophil-chemotactic IL-8; recruitment of these strongly inflammatory cells to such an immunoregulated organ may result in preterm labor or miscarriage. All HCs responded similarly to IL-4 plus IL-13 stimulation, consistent with M2 immunodominance-like phenotypes (14–17). It follows that these HCs are resilient to phenotypic changes induced by immune complexes in the presence of inflammatory cytokines and that changes in STAT gene transcription alone may not be sufficient to produce phenotypic changes in our experimental timeline. Because transcytosis of maternal IgG is a key function of the placenta, our data suggest there may be mechanisms to prevent aberrant immune complex activation of HCs during this process, potentially through FcγRIIb-mediated inhibition (48). HCs have previously been reported to express FcγRIIb and endocytosed IgG colocalizes with FcγRIIb in endothelial cell endosomes. Early/midgestation HC stimulation with IL-10 promoted a term HC–like phenotype, with increased expression of regulatory markers and loss of activation.

We evaluated how IFN responses differ from viral PRR activation. Term HC phenotype was stable following IFN stimulation; IFN-α activated early/midgestation HCs. Sensitivity to IFN-α was greater than to IFN-λ1, as IFN-α induced transcription of ISGs faster and more robustly. IFN-λ1 is a key IFN produced by placental syncytiotrophoblasts, and the reduced antiviral state following stimulation with this IFN may highlight a gap in placental antiviral immunity. RIG-I initiates antiviral responses to viral RNA (49), and stimulation with RIG-I agonist mimics activation of this PRR following select viral infections. RIG-I–induced ISG
transcription was stronger at early/midgestation. Early/midgestation HCs quickly adopted classically activated signatures and some donors produced inflammatory cytokines whereas term HCs were unaffected despite transcriptional adaptations. Kim et al. (50) demonstrated pro-M1 gene hypermethylation, and Blumenstein et al. (51, 52) suggested suppression of cytokine signaling (SOCS) protein is differentially regulated with the onset of labor at term. As such, genomic modifications or inhibitory proteins may contribute to plasticity variations. Inflammatory mediators can damage the villous cell barrier or induce preterm labor; (53) therefore, this response may be protective or pathogenic. Additional studies are needed to define HC function in healthy pregnancies, and those confounded by infectious and noninfectious complications.

Tissue-specific immune responses among privileged sites are well documented (54). We demonstrate temporal control of immune responses as part of the natural order of placental matura-
tion. We demonstrate changing HC phenotype ex vivo and evolving responses to select stimuli. Understanding how HCs maintain pregnancy is of utmost importance to guide development of therapies to offset aberrant HC phenotype-mediated complications during pregnancy.

A deficiency in the study is lack of labored tissues at term and preterm, which would allow for analysis of mechanisms responsible for placental rejection, guidance on pathogenic changes, and potential therapeutic targets. Conclusions in this study and in similar studies are limited by incomplete patient histories: pla-
centae from elective terminations are considered “normal and healthy”; however, no information was available to investigators regarding why pregnancy was terminated. This study would have benefited from a greater sample size with a greater range of gestational ages, especially in the first trimester; limited donors may not reflect the “true” situation of a specific time interval.

Future studies should prioritize collecting a greater number from a breadth of gestational ages and consider donor demographics. Studies detailing HCs isolated from pathogen-premature deliveries could identify important changes leading to parturition. Analyzing HCs from labored placentae may elucidate the M2B-like phenotype of placental macrophages: the Hofbauer cell. Possible implications for a fetal macrophage. Placenta 8: 65–76.


Bright, N. A., C. D. Ockleford, and M. Anwar. 1994. Ontogeny and distribution of Fc gamma receptors in the human placenta. Transport or immune surveil-


Johnsson, E. L., and R. Chakraborty. 2012. Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by in-


Dekel, N., Y. Gnaisky, I. Granot, and G. Mor. 2010. Inflammation and im-

delphia, p. 545–565.


