Placental macrophage (Hofbauer cell) response to viral and bacterial ligands is influenced by fetal sex

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Article

Keywords: Hofbauer cells, placental macrophages, PAMPs, infection, fetal sex

Posted Date: April 11th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1522078/v1

Abstract

Bacterial and viral infections of the placenta are associated with inflammation and adverse pregnancy outcomes. Hofbauer cells (HBCs) are specialised fetal-origin macrophages in the placental villi and are proposed to protect the fetus from vertical transmission of pathogens; however, they are poorly understood. Here, we have performed quantitative proteomics on term HBCs under resting conditions and following exposure to bacterial and viral pathogen associated molecular patterns (PAMPs), and investigated the contribution of fetal sex to these responses. Resting HBCs expressed a plethora of proteins pertinent to macrophage function, including chemokines, cytokines, Toll-like receptors, and classical and non-classical major histocompatibility complex class I and II molecules. HBCs mounted divergent responses to bacterial versus viral PAMPs but exhibited protein expression changes suggestive of a switch towards a more pro-inflammatory phenotype. A comparison between male and female HBCs, showed that the latter mounted a much stronger and wider response. Sexual dimorphism in HBCs was primarily associated with lipid metabolism in males and cytoskeleton organisation in females. We provide a novel and comprehensive understanding regarding the phenotype of term placental macrophages and their sex-dependent responses to infectious triggers.

Introduction

Placental macrophages, termed Hofbauer cells (HBCs), are the only immune cell subset found in human placental villous tissue (Castellucci, Zaccheo, & Pescetto, 1980). These fetal-origin macrophages are present throughout gestation (Castellucci et al., 1987) and contribute to immune protection of the fetus during pregnancy. HBCs also help maintain homeostatic conditions in the placenta by regulating processes such as angiogenesis (Loegl et al., 2016), vasculogenesis (Seval, Korgun, & Demir, 2007) and tissue remodelling (Khan, Katabuchi, Araki, Nishimura, & Okamura, 2000), consistent with their characterisation as anti-inflammatory M2-like macrophages (Loegl et al., 2016; Schliefsteiner et al., 2017).

HBCs are highly plastic, allowing them to adapt their phenotype in response to environmental signals. In pregnancy complications, such as preeclampsia and chorioamnionitis, HBCs change in their number and phenotype (Przybyl et al., 2016; Tang et al., 2013; Vinnars, Rindsjo, Ghazi, Sundberg, & Papadogiannakis, 2010; Yang et al., 2017). HBCs respond to, and are targets for, several pathogens (reviewed in Fakonti, Pantazi, Bokun, and Holder (2022)). HBCs release proinflammatory cytokines, activate NFκB signalling, and upregulate M1 proinflammatory immunophenotype-associated genes upon encounter with whole bacteria or bacterial pathogen associated molecular patterns (PAMPs) *in vitro* (Abrahams, Tang, Mor, & Guller, 2020; Mezouar et al., 2019; Sutton et al., 2019). Moreover, HBCs fight bacterial infection by releasing extracellular traps - protrusions containing metalloproteases (Doster, Sutton, Rogers, Aronoff, & Gaddy, 2018)- as well as undergoing pyroptosis, a form of inflammatory programmed cell death involving inflammasome-associated caspase-1 (Abrahams et al., 2020). First trimester HBCs have the capacity to proliferate *in situ* in response to bacterial ligands, phagocytosing *Escherichia coli* ligands and microspheres (Thomas et al., 2021). HBCs also respond to a range of viruses. For example, Hendrix *et al.*,

demonstrated that HBCs infected with γ-herpesvirus, MHV-68, *in vitro* release the proinflammatory cytokine interleukin (IL)-1β, activating endothelial cells towards a pro-neutrophilic response (Hendrix et al., 2020).

In contrast to these studies of HBC infection-control, several other studies suggest that HBCs either do not respond, or respond in favour of pathogen dissemination. For example, Schliefsteiner, Ibesich, and Wadsack (2020) demonstrated that HBCs resist bacterial cues and do not change their M2-like phenotype, despite producing tumour necrosis factor-α (TNF-α) and interferon γ (IFN-γ) in response to bacterial PAMPs. HBCs have also been reported to act as reservoirs for replication and transmission of respiratory syncytial virus *in vitro* (Bokun et al., 2019). Furthermore, HBC co-infection with cytomegalovirus has been shown to enhance the replication of HIV-1 (Johnson et al., 2018).

A recent study profiled the gene expression of placental macrophages from first-trimester placentas along with maternal blood and immune cells from the decidua, using spatiotemporal single-cell RNA sequencing (scRNAseq), developing a single-cell atlas of the maternal-fetal interface containing information on cell location and cell-cell interactions in the decidua and placenta (Vento-Tormo et al., 2018). But despite numerous studies looking at HBCs in placental samples from infected mothers, or the *in vitro* response of HBCs to pathogens, a comprehensive understanding of the functional role of HBCs during infection is still lacking. Most studies have looked at a small number of specific surface proteins, or cytokine release, rather than an untargeted approach. Finally, although the influence of sex in immunological responses is increasingly acknowledged (Klein & Flanagan, 2016), and sex differences in placental genes, proteins, and function have been reported (Allard, Giraud, Segura, & Sebire, 2019; Rosenfeld, 2015; Sun et al., 2020), there is a dearth of knowledge regarding the potential association between fetal sex and HBC phenotype and function.

In this study, we sought to comprehensively characterise term HBC phenotype and responses to pathogens, and the contribution of fetal sex to these responses. Utilising quantitative proteomics, we found that term HBCs contain a number of immune-related proteins including cytokines, chemokines, Toll-like receptors (TLRs) and scavenger receptors. We show that HBCs mount divergent responses to bacterial versus viral pathogen-associated patterns (PAMPs), but exhibit phenotypic changes suggestive of an M2-like to M1-like switch in response to both. Finally, we provide the first clear picture of sex-dependent differences in HBC phenotypes and responses, primarily related to lipid metabolism in males, and cytoskeleton organisation in females. These findings provide a novel understanding into the phenotype of term placental macrophages, and their sex-dependent responses to infectious triggers.

Results

Patient information

A total of ten placentas were included in this study; with a 50:50 balance of male and female fetal sex (Supplemental Table 1). All subjects were White and non-Hispanic, and all deliveries were elective caesarean sections, without labor, with a median gestational age at delivery of 39weeks + 1day.

Proteomic profile of term placental macrophages

A total of 5892 protein IDs were identified in our quantitative proteomic analysis of HBCs (Supplementary file 1). Functional enrichment analysis of these proteins revealed their involvement in metabolic processes, viral processes, antigen processing and presentation, membrane disassembly, cell cycle phase transition, cytokine production, and gene expression amongst other biological processes (Figure 1A and Supplementary file 1). Interrogation of their molecular function found that these proteins were primarily RNA-, protein-, cell adhesion molecule-, and enzyme-binding. Cellular component analysis revealed the association of these proteins with membrane-bound vesicles, extracellular region parts, cell junctions, and the cytosol (Supplementary file 1). HBCs expressed a range of proteins pertinent to macrophage function (Figure 1B), such as chemokines (CCL8, CXCL8, CXCL3, CCL20, CXCL1, CXCL5, CCL3, CXCL10, CXCL2, CXCL11, CCL4, CCL2) and chemokine receptors (CCR1, CCR7), cytokines (IL16, IL18, IL6, IL4I1, IL1B, IL1A, IL36γ, TNF-α, IK) and cytokine receptors (CD40, IL3RA, IL17RA, IL10Rβ), as well as Toll-like receptors 2, 3, 7 and 8 (TLR2, TLR3, TLR7, TLR8). Term HBCs were positive for classical major histocompatibility complex (MHC) class I (HLA-A, HLA-B, HLA-C), non-classical MHC class I (HLA-F), and MHC class II (HLA-DRA, HLA-DMB, HLA-DMA, HLA-DPA1) molecules. Moreover, they have several scavenger receptors; surface molecules that typically bind multiple ligands to remove nonself or altered-self targets (PrabhuDas et al., 2017). Finally, we found previously identified Hofbauer cell markers such as CD163, CD68, CD209, FOLR2, ARG2, VSIG4, and MRC1 (Schliefsteiner et al., 2017; Thomas et al., 2021).

Changes in the proteomic profile of placental macrophages in response to bacterial lipopolysaccharide

To determine their response to bacterial infection, we treated HBCs with bacterial LPS. A total of 314 proteins were changed by at least 1.5-fold (adj. $p < 0.05$) in the LPS-treated HBCs compared with NT, with 176 being increased and 138 decreased (Figure 2A) (Supplementary file 2). Among the decreased proteins were MRC1 and CD209, markers of M2 anti-inflammatory macrophages, while CCR7, a marker of M1 pro-inflammatory macrophages, was upregulated (Martinez, Gordon, Locati, & Mantovani, 2006) (Figure 2B), suggesting that HBCs may move away from their M2-like phenotype upon encounter with bacteria. Gene ontology analysis of the LPS-induced differentially abundant proteins revealed their involvement in the immune response, inflammatory response, and response to external stimulus (Figure 2C- D). More specifically, Reactome analysis of the LPS-increased proteins identified two pathways: ‘chemokine receptors bind chemokines’ (proteins involved: CCL20, CXCL8, CXCL1, CCL3, CXCL5, CCL2, CCL4, CCR7, CXCL3) and ‘metallothioneins bind metals’ (proteins involved: MT1M, MT1X, MT2A, MT1E, MT1F) (Figure 2E- F). No pathways were identified by Reactome analysis of the LPS-decreased proteins (adj. p value<0.05). InterPro analysis, which classifies proteins into families according to their domains and important sites, showed that several LPS-upregulated proteins bear basic leucine zipper-domains, chemokine conserved sites, metallothionein domains, and DNA-binding sites (Supplementary file 2). Regarding the proteins decreased by LPS treatment, the top significantly changed families were immunoglobulin E, immunoglobulin-like, and MD-2-related lipid-recognition domains (Supplementary file 2).

Changes in proteomic profile of placental macrophages in response to viral dsRNA

To investigate HBC responses to viral infection, we treated cells with poly(I:C) (PIC) a viral dsRNA mimic. A total of 393 proteins were changed by at least 1.5-fold ($p<0.05$) in the PIC-treated HBCs compared with NT, with 240 being increased and 153 decreased (Figure 3A) (Supplementary file 3). Similarly to LPS, PIC caused a reduction in the expression of the M2 markers MRC1, CD163, and CD209, and an increase of the M1 markers CXCL11 and IL1a (Martinez et al., 2006) (Figure 3B), suggesting that HBCs may become more pro-inflammatory upon virus encounter. Gene ontology analysis of the proteins increased in response to PIC indicated their involvement in biological processes including antigen processing and presentation, viral processes, cell activation, cell death, regulation of cytokine-mediated signalling, and protein transport (Figure 3C). On the other hand, the proteins that were decreased in the PIC-treated HBCs were involved in cytokine production, immune effector processes, extracellular matrix organisation, metabolic processes, response to external stimulus, and endocytosis (Figure 3D). Pathway enrichment analysis revealed induction of several Reactome pathways in HBCs in response to PIC treatment: ‘interferon alpha/beta signalling’, ‘interferon gamma signalling’, ‘ISG15 antiviral mechanism’, ‘antigen presentation: folding, assembly and peptide loading of class I MHC’, ‘endosomal/vacuolar pathway’, ‘DDX58/IFIH1-mediated induction of interferon-alpha/beta’, ‘negative regulators of DDX58/IFIH1 signalling’, and ‘ER-phagosome pathway’ (Figure 3 E- F).

Differential response to bacterial and viral PAMPs by placental macrophages

To explore the differences between bacterial and viral infections, we directly compared the protein abundances in the LPS and PIC treated HBCs (Supplementary file 4). From the 360 differentially abundant proteins, 153 were increased in LPS- and 207 in PIC-treated HBCs (Figure 4A). The differentially abundant proteins consisted, amongst others, of chemokines, cytokines, chemokine/cytokine and scavenger receptors, HLA molecules, and other surface molecules (Figure 4B). LPS increased the levels of the pro-inflammatory chemokines CXCL1, CXCL3, CXCL5, CCL2, CCL3, and CCL20, while PIC induced CCL8, CXCL10, and CXCL11. HBCs treated with PIC were more abundant in IL18, an IFN-γ inducing factor, while LPS-treated HBCs were more abundant in IL6, IL8, IL1A, IL1B, IL36G, and TNF-α. Interestingly, the expression of HLA molecules including HLA-A, HLA-B, HLA-C, HLA-F, and HLA-DRA was augmented in the PIC and reduced in the LPS group. The levels of several scavenger receptors were also different in the two treatment groups, with LPS treated cells being more enriched in SCARF1, SCARB2, LRP1, MRC1, CD163, CD44, CD14, MSR1, CD209, CD36, COLEC12, CD163L1, CD68, and PIC treated cells in MSR1 and CD68. Gene ontology analysis of the proteins increased in LPS, and decreased in PIC, indicated their involvement in biological processes including regulation of metabolic process, regulation of I-kappaB kinase/NF-kappaB signaling, production of molecular mediator of immune response, cell adhesion and motility, and regulation of secretion (Figure 4C, Supplementary file 4). Conversely, the proteins increased in PIC, and decreased in LPS treated HBCs, were involved in antigen processing and presentation of endogenous peptide antigen by MHC class I via ER pathway, TAP-independent viral process, regulation of cell adhesion, cell killing, cytokine production, defence response, and others (Figure 4D, Supplementary file 4). Several interaction networks were observed in both LPS and PIC treated HBCs (Figure 4 E- F). The

most represented Reactome pathways in LPS treated HBCs were ‘Metallothioneins bind metals’, ‘Chemokine receptors bind chemokines’, and ‘Cholesterol biosynthesis’. In PIC treated HBCs, pathway enrichment analysis revealed the induction of several Reactome pathways: ‘interferon alpha/beta signalling’, ‘interferon gamma signalling’, ‘ISG15 antiviral mechanism’, ‘antigen presentation: folding, assembly and peptide loading of class I MHC’, ‘endosomal/vacuolar pathway’, ‘DDX58/IFIH1-mediated induction of interferon-alpha/beta’, ‘negative regulators of DDX58/IFIH1 signalling’, ‘ER-phagosome pathway’, and ‘Negative regulators of DDX58/IFIH1 signaling’ (Supplementary file 4). These data illustrate the distinct responses of HBCs to bacterial and viral ligands, even though some proteins might be commonly up or downregulated compared to the non-treated controls (Figure S2).

Fetal sex differences in placental macrophage phenotype

We next sought to identify differences between HBCs of female and male fetuses by performing differential expression analysis, accounting for treatment, to identify differences due to sex (Figure 5A) (Supplementary file 5). The comparison identified 274 differentially abundant proteins. HBCs from female placentas were more abundant in proteins involved in actin and cytoskeleton organization, cell adhesion, and response to wounding (Figure 5B), while HBCs from male placentas were more abundant in proteins involved in lipid and alcohol metabolic processes and locomotion (Figure 5C). In both sexes, differentially abundant proteins were primarily extracellular region- and vesicle-associated (Figure 5D -E). InterPro analysis showed that the female specific proteins carried Calponin homology domains (TAGLN3, CNN3, TAGLN, FLNB, ACTN1, LMO7, TAGLN2, SPTBN1), Zinc finger motifs LIM-type (LMCD1, FHL2, LMO7, TGFB1, PDLIM7, PDLIM2, PDLIM1, PDLIM7), EF-hand(-like) domains (TPPP3, S100A1, RCN1, SPTAN1, S100P, S100A13, S100A16, ACTN1, CAPS, EHD2, PVALB), and a subset belonged to the Serpin family (SERPINB9, SERPINE2, SERPINA1, SERPINB2, SERPINH1) (Figure 5F). Reactome analysis of the male specific proteins significantly identified just two pathways: ‘Cholesterol biosynthesis’ (proteins involved: MSMO1, SQLE, HMGCS1, CYP51A1, FDFT1, HMGCR) and ‘Activation of gene expression by SREBF (SREBP)’ (proteins involved: SQLE, DHCR7, HMGCS1, SC5D, CYP51A1, FDFT1, HMGCR) (Figure 5G). Sterol regulatory element-binding proteins (SREBP) are activators of transcription for genes involved in lipogenesis (Eberle, Hegarty, Bossard, Ferre, & Foufelle, 2004). These data suggest that sex-dependent differences in HBCs are primarily related to lipid metabolism in males and cytoskeleton organisation in females.

Fetal sex differences in placental macrophage responses to viral and bacterial PAMPs

To explore sex-associated differences in HBC responses to bacterial and viral ligands, we next compared the proteome of female and male HBCs in each treatment group (NT, LPS, PIC) (Figure 6A) (Supplementary file 6). In the resting HBCs (NT), 15 proteins were significantly elevated in females compared with males and ten were higher in males (FC>1.5 adj. p value<0.05). In the LPS treatment group, 73 proteins were higher in females compared with males and 18 in males compared with females, while in the PIC treatment group, 56 proteins were higher in females and 11 in males (FC>1.5 adj. p value<0.05). We then proceeded to identify the male-female differentially abundant proteins that were

common/disparate between the different treatment groups (Figure 6B). The proteins present in both PIC and LPS but not in the NT group, revealed the sex-specific response to infection. Male HBCs expressed fewer proteins (ITGAL, CCR1, PLXND1, COMT) in response to LPS and PIC compared with female HBCs, which expressed proteins involved in actin filament and cytoskeleton organisation, found primarily in cell junctions, and functioning as protein binding molecules (Figure 6C). In conclusion, HBCs exhibit intrinsic sex associated differences that may affect their response to infection.

Discussion

To investigate the response of placental macrophages to infection, HBCs were isolated from term placentas and exposed to bacterial LPS, a cell wall component of Gram-negative bacteria that signals primarily through toll-like receptor 4 (TLR4), or poly(I:C) (PIC), a viral dsRNA analogue that signals through TLR3. Quantitative proteomics was performed to comprehensively interrogate the phenotype of term HBCs, their response to bacterial and viral infection, and the contribution of fetal sex to these profiles.

We used tandem mass tag (TMT) proteomics, which has emerged as a novel high-throughput mass spectrometry method for protein identification and quantification in several experimental settings, including the description of monocyte surface markers (Ravenhill, Soday, Houghton, Antrobus, & Weekes, 2020). Here, a total of 5892 proteins were quantitatively identified in all three treatment groups (NT, LPS, and PIC). We showed that HBCs express a number of macrophage related proteins, whose expression levels change in response to bacterial and viral PAMPs.

According to our data, term HBCs carry both classical and non-classical MHC class I, as well as MHC class II molecules, the expression of which is affected by bacterial and viral cues. A recent study by Thomas et al. (2021) suggested that first trimester HBCs do not express HLA-DR (MHC class II) molecules and their presence may indicate contamination with placenta-associated maternal macrophages during isolation. However, ontogeny of early gestation and term HBCs is thought to be different with early HBCs deriving from mesenchymal cells, while term HBCs may also include recruited yolk sac, fetal liver, and bone marrow monocytes (Castellucci et al., 1987). Importantly, in contrast to Thomas et al. (2021) in which cells were isolated from just two placental enzymatic digests we used an optimised protocol which requires discarding the first three digests, which would contain contaminating cytotrophoblast and maternal blood cells, and only a fourth digest of the villous core was used for subsequent isolation followed by two immunoselection steps (using EGFR and then CD10 antibodies) (Tang et al., 2011). Diversity in term HBCs might be due to their responses to the surrounding microenvironment such as senescent trophoblasts, necrotic tissue, and inflammatory cues (Reyes & Golos, 2018). Though absent in early HBCs (Thomas et al., 2021; Vento-Tormo et al., 2018), HLA-DR may therefore be present in term HBCs, as reported previously (Ben Amara et al., 2013; Blaschitz et al., 2011).

Upon LPS treatment, term HBCs switch towards a more pro-inflammatory phenotype, with increased M1 and decreased M2 phenotype markers. This is in agreement with previous studies reporting elevated

secretion of the pro-inflammatory cytokines IL1B and/or TNF-α after LPS exposure of first trimester (Thomas et al., 2021) and term HBCs (Abrahams et al., 2020). Swieboda et al. (2020) reported that term HBCs were less plastic than early or mid-gestational HBCs and predominantly M2-like, but we showed that term HBCs do respond to bacterial PAMPs by altering their expression profile. Our findings are supported by other studies documenting the capacity of term HBCs for proinflammatory responses (Pavlov, Pavlova, Ailamazyan, & Selkov, 2008; Young et al., 2015). Some variability in the literature could be explained by the different methodological approaches utilized. For example, we analysed HBC responses collectively using proteomics, while Schliefsteiner et al. (2020) used flow cytometry and showed that upon LPS and IFN-γ treatment, M2-like HBCs were reduced in number increasing the ratio of M1/M2 HBCs, suggesting that there is no shift in phenotype but rather a shift in the number of M2-like cells. Moreover, Young et al. (2015) using flow cytometry and immunohistochemistry showed that HBCs, though maintaining M2 marker expression, are exquisitely sensitive to LPS treatment.

Upon PIC treatment, we observed some similar responses, where term HBCs expressed a plethora of pro-inflammatory cytokines and chemokines, including IL1, TNF-α, CCL3, CCL4, and CXCL10 among others. Other studies have shown that treatment of HBCs with PIC increases the mRNA expression and secretion of the pro-inflammatory cytokines IL6 and IL8 (Young et al., 2015). HBCs have been demonstrated to both fight (Hendrix et al., 2020; Johnson & Chakraborty, 2012; Oliveira, Fonseca, & De Bonis, 1992) and permit (Bokun et al., 2019; Johnson et al., 2018; Schliefsteiner et al., 2020) the replication and transmission of viruses. The variability may lie with the viral species, the HBC donor, or the methodology (Tabata et al., 2018). Our data clearly show upregulation of antiviral pathways, such as IFN and ISG15 signalling, as well as overexpression of classical (HLA-A, HLA-B, HLA-C) and non-classical (HLA-F) MHC class I molecules in response to PIC.

After exploring the differences between treated and non-treated cells, we directly compared LPS and PIC treated HBCs which highlighted the same pathways, showing that LPS and PIC have distinct effects on cells, as also seen by principal component analysis. HBCs therefore employ distinct defence mechanisms to fight bacterial and viral infections, as previously observed in human monocyte derived macrophages, with LPS inducing NFκB signalling and PIC elevating the expression of interferon stimulated genes (ISG) (Reimer, Brcic, Schweizer, & Jungi, 2008). However, we did identify some proteins were similarly affected in LPS and PIC treated HBCs, including common upregulation of both IL1 and TNF-α, as expected for these master regulators of inflammation (Fakonti et al., 2022).

We next sought to investigate the role of fetal sex in the phenotype of placental macrophages. We identified >250 proteins differentially expressed between female and male HBCs. These included sex chromosome-associated proteins (DDX3Y upregulated in males and PUDP, PAGE4, PDLIM7, IL3RA, GK, PDLIM7, and EIF1AX in females), but also proteins encoded by genes on autosomes. Proteins more abundant in females were associated with actin and filament organisation, whilst those more abundant in males were associated with metabolic processes including cholesterol (steroid) and alcohol biosynthesis. Others have also attempted to decipher sex differences between HBCs from first trimester

human (Sun et al., 2020) and E17.5 (term) mouse (Ceasrine et al. (2021) PREPRINT) placentas using scRNAseq, however the number of differentially expressed genes identified in these studies was limited (17 and 27 respectively). Functional analysis of the differentially expressed genes between female and male murine HBCs showed distinct immune/ defence responses and signalling via the JAK-STAT pathway (Ceasrine et al. (2021) PREPRINT). Regarding human HBCs, apart from the sex chromosome associated genes, overexpression of the hemoglobin subunit β (HBB) in female HBCs was the only common finding between our study and Sun et al. (2020), which might be due to the difference in gestation, or the type of material analysed (RNA vs protein). The placenta derived C-terminal fragment of HBB was previously reported to have antimicrobial properties, by blocking viral and/or bacterial entry, augmented in acidic (like inflammatory) conditions (Gross et al., 2020). We found that male HBCs had significantly higher levels of HLA-A and HLA-B compared with female HBCs, which might explain why male HBCs are more sensitive to pro-inflammatory cues *in utero* (Na et al., 2021). Indeed, for example SARS-CoV-2 infection seems to affect disproportionally pregnancies with male fetuses, which exhibit increased placental inflammation and expansion of HBCs (Bordt et al., 2021). In fact, pregnancy pathologies like term pre-eclampsia and gestational diabetes tend to be associated with male fetal sex (Broere-Brown et al., 2020). Here, we observed that HBCs from females overexpressed several proteins that were associated with cytoskeleton and organelle organisation, adhesion, and membrane configuration, in response to LPS and PIC. The motility of macrophages is important to facilitate their defence response, which requires reassembly of the actin meshwork for polarization and formation of cell structures, like filopodia, and adhesion with the extracellular matrix (Jones, 2000). A subset of the proteins we found upregulated in female HBCs were calponins, which stabilise actin and are involved in signalling (Yin, Schnoor, & Jun, 2020), and serpins, a superfamily of protease inhibitors with diverse roles including inhibition of inflammatory response and hormone trafficking (Law et al., 2006). We also found increased expression of intermediate filament proteins including vimentin, which can be located on the membrane, in the cytosol or extracellularly, in adjusting cells (Jiu et al., 2015; Mak & Bruggemann, 2016). In macrophages exposed to PAMPs or damage associated molecular patterns (DAMPs) vimentin interacts directly with the intracellular sensor NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3), triggering a proinflammatory response involving caspase-1 (dos Santos et al., 2015). This mechanism was actually found to be adopted by HBCs in response to LPS and adenosine triphosphate (ATP) (Abrahams et al., 2020), and our data now indicate that this might be sex specific.

We found that HBCs from female placentas mounted a much stronger response to bacterial and viral ligands compared to HBCs from male placentas, in terms of more increased proteins. There are many examples where adult females develop more potent immune responses than males, which result in increased vaccine efficiency and successful pathogen elimination, but also in their increased susceptibility to autoimmune diseases (Klein & Flanagan, 2016; Klein, Jedlicka, & Pekosz, 2010). In terms of innate immune responses, IFN-α production due to single-stranded DNA viruses is greater in females, based on exposure of blood samples to TRL7 ligands (Berghofer et al., 2006). Some immune related differences between males and females are attributed to hormones, with transient increase in pro-inflammatory responses in males during puberty (Lamason et al., 2006). Similarly, *in utero* immune

dimorphism may be aided by the production of androgens from male testes from week 10 of gestation (Goldenberg et al., 2006; Klein & Flanagan, 2016). Apart from hormones, environmental (nutrition and microbiota) and genetic mediators differentially influence immune responses in males and females *in utero* and throughout life (reviewed in Klein and Flanagan (2016)). It is reported that spontaneous abortion, pre-eclampsia and other pregnancy complications are more frequent in male fetuses (Byrne & Warburton, 1987; Goldenberg et al., 2006; Vatten & Skjaerven, 2004).

A limitation of this study was that TMT proteomics currently allows only a maximum of 11 samples to be run in one batch. Our number of treatments and the inclusion of fetal sex necessitated the running of several batches, and we observed batch effects. This required us to normalise between batches to enable robust analysis, with the caveat that we were only able to analyse proteins present in all three batches, resulting in some information loss. Another limitation, as a consequence of batch correction, is that only proteins that were present in all three treatment groups could be analysed. However, the benefit of employing TMT-based quantitative proteomics meant that we were able to accurately quantify changes in protein levels between conditions, and perform paired analysis, which resulted in the identification of many more significant changes than could be obtained by non-quantitative proteomics. Additionally, while PAMPs are a widely used tool to simulate bacterial and viral infections in a controlled manner, they might not accurately represent real life conditions (Mezouar et al., 2019). Further functional experiments with whole viral and bacterial pathogens would strengthen the results of this study. Finally, the placenta donors in this study were exclusively White non-Hispanic individuals, which eliminates potential variability, but limits the findings of this study to a specific population. Our results should be validated in a larger cohort including a range of ethnicities to allow generalization.

Overall, we have shown that HBCs respond to bacterial and viral ligands by altering their expression landscape to reflect a more pro-inflammatory phenotype, but separate mechanisms are employed based on the trigger. We also provide the first clear picture of sexual dimorphism in HBC proteomic profiles and responses to pathogens, primarily related to lipid metabolism in males and cytoskeleton organisation in females, which could contribute to the observed relationship between fetal sex and responses to congenital and perinatal infections. To elucidate the sexual dimorphism at the maternal-fetal interface and its implications for the mother and the fetus during the various types of infection and inflammation further studies are needed. Moreover, as also suggested by Schliefsteiner et al. (2020) and Thomas et al. (2021), it may be time to acknowledge the pleiotropic nature of HBCs at the different stages of pregnancy and define specific placental macrophage phenotypes and signatures.

Methods

Patient recruitment

Term placentas (≥37 weeks gestation) were obtained from uncomplicated singleton elective caesarean sections without labor performed at Yale New Haven Hospital. Human placental tissue collection was approved by Yale University's Human Research Protection Program (IRB Protocol ID: 1208010742) and all

samples were collected through the Yale University Reproductive Sciences (YURS) Biobank following patient consent. Exclusion criteria were: known multiple gestations and karyotypically abnormal fetuses, patients with chronic hypertension, preeclampsia, pregestational diabetes, systemic lupus erythematosus, autoimmune disease, congenital heart disease, chronic severe asthma, thrombophilic medical conditions, chronic abruption or vaginal bleeding during pregnancy and maternal infections (HIV, TB, malaria, SARS-CoV-2).

Hofbauer cell isolation and culture

Hofbauer cells (HBCs) were isolated as previously described (Tang et al., 2011). Briefly, chorionic villous tissue from fresh placentas was minced, washed to remove blood, sequentially digested with trypsin (Gibco, 15090-046) and deoxyribonuclease I (DNase I, Roche, 10104159001), overall 3 rounds: 15 min, 30 min and 30 min. The trypsin undigested tissue was washed and digested with collagenase A (Roche, 11088793001) and DNAse I for 1h to obtain HBCs. Digests were passed through 100µm sieves and separated over a discontinuous Percoll (GE Healthcare Biosciences, 17-0891-01) gradient (35%/30%/25%/20%). Cells from the 20%/25% - 30%/35% interfaces were combined and stored in RPMI-1640 (Merk, R6504) supplemented with 5% fetal bovine serum (FBS, GemCell™, 100-500), 1% Pen-Strep, 4 mM L-glutamine, 25 mM HEPES overnight on ice. The following day, HBCs were further purified by negative immunoselection to deplete EGFR+ and CD10+ cells using antibodies conjugated to magnetic beads (anti-EGFR clone #528; anti-CD10 clone #HI10a, Biolegend). Fifteen million cells were plated in a $10cm^2$ culture dish in RPMI-1640 medium supplemented with 5% FBS, 1% Pen-Strep, 4 mM L-glutamine, 25 mM HEPES. After 1 h, any floating and weakly attached cells were removed with two washes of PBS and cells cultured in 10ml DMEM/F12 (Merk, D2906) supplemented with 10% FBS, 1% Pen-Strep, 4mM L-Glutamine. The following day, HBCs were washed twice in PBS and media exchanged for serum-free DMEM/F12 supplemented with 1% Pen-Strep, 4mM L-Glutamine, 50 µg/ml ascorbic acid and ITS+ premix (Corning, 354352), yielding a final concentration of 6.25 µg/ml insulin, 6.25 µg/ml transferrin, 6.25 ng/ml selenous acid, 1.25 mg/ml BSA and 5.35 µg/ml linoleic acid.

Cells were left untreated or were treated with 1 ng/ml Lipopolysaccharides from *Escherichia coli* O55:B5 (LPS, Sigma) or with 1 µg/ml of the viral dsRNA mimic, Poly(I:C) (PIC, high molecular weight, Invivogen) for 24h (Supplemental Figure 1A).

Protein preparation, quantitative Tandem-Mass Tag (TMT) labelling and high pH reversed-phase chromatography

Cells were lysed with 750 µl 1x RIPA buffer (Cayman Chemicals) supplemented with protease and phosphatase inhibitors (cOmplete Protease Inhibitor cocktail and PhosSTOP, Roche). Lysates were incubated on ice for 15 min, centrifuged at 16,100 ×G for 10 min at 4^oC, and the supernatant stored at -80^oC until use. Protein was quantified using the Micro BCA™ Protein Assay Kit (Thermo Fisher Scientific). Samples were run in three batches for tandem mass tag (TMT) labelling: two 9-plex and one 11-plex. Aliquots of 50 µg of each sample were digested with trypsin (2.5 µg trypsin per 100 µg protein; 37°C,

overnight), labelled with TMT ten/eleven plex reagents according to the manufacturer's protocol (Thermo Fisher Scientific) and the labelled samples were pooled. An aliquot of 100 µg of the pooled sample was desalted using a SepPak cartridge according to the manufacturer's instructions (Waters). Eluate from the SepPak cartridge was evaporated to dryness and resuspended in buffer A (20 mM ammonium hydroxide, pH 10) prior to fractionation and nano-LC MSMS using an Orbitrap Fusion Lumos mass spectrometer (Thermo Fisher Scientific; see supplementary methods).

Protein identification and quantification

Raw data files were processed and quantified using Proteome Discoverer software v2.1 (Thermo Scientific) and searched against the UniProt Human database (downloaded August 2020: 167789 entries) using the SEQUEST HT algorithm (see supplementary methods).

Bioinformatic/Statistical analysis

The normalised abundance values were imported into R for statistical analysis (R Core Team (2017)). The abundance values were log2 transformed and only proteins that were measured across all three batches were used for subsequent analysis. Batch effect adjustment was undertaken using SVA (Leek et al., 2021) and Principal Component Analysis using PCATools (Blighe & Lun, 2021) (Figure S1 B). Subsequent differential expression analysis was conducted using limma, fitting linear models in a paired manner, while accounting for treatment, sex and batch (Ritchie et al., 2015). P values were adjusted for multiple testing. Enhanced volcano was used for volcano plot visualisations (Blighe & Lun, 2021).

Gene ontology (GO) enrichment analysis of the generated datasets was performed using DAVID Bioinformatics Resources 6.8 with the default background (Huang da, Sherman, & Lempicki, 2009a, 2009b). Protein functions were classified into three subgroups: biological process (BP), cellular compartment (CC), and molecular function (MF). The subset of the more specific terms represented by the GO_Fat categories was selected for further analysis. The genomic sources Reactome 78.0 (Jassal et al., 2020) and InterPro 87.0 (Blum et al., 2021) were used to identify enriched pathways and protein families in each dataset, respectively. The Benjamini correction was used to assess statistical significance. Enriched GO terms, Reactome pathways, and InterPro entries with adj. p value< 0.05 were considered statistically significant. Interaction networks were produced in STRING v11.5 using experimentally determined interactions and interactions arising from curated databases, and the minimum required interaction score - medium confidence (0.4) (Szklarczyk et al., 2019)

Declarations

Acknowledgements

Research reported in this publication was supported by National Institute for Child Health (NICHD) of the National Institutes of Health (NIH) under award number R01HD093801 (to BH). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National

Institutes of Health. MK is a Sir Henry Wellcome Fellow (206508/Z/17/Z). The authors would like to thank the Yale University Reproductive Sciences Biobank and the staff of Labor and Delivery, Yale-New Haven Hospital for their help with placental sample collection. We thank Dr Kate Heesom and Dr Phil Lewis at Bristol University proteomics facility for performing the proteomics, Georgia Fakonti for assistance with the literature review and Toby Clements for laboratory assistance.

Funding

Open Access funding provided by the National Institutes of Health (NIH).

Data availability

The raw proteomics data are deposited at PRIDE with the experiment code X (to be obtained before publication). Metadata and lists of proteins are available here (link to excel files) and from the authors upon request.

Competing interests

The authors declare no competing interests.

Author contributions

Conceptualization: B.H., S.G., V.M.A., P.P. Experimental work: Z.T., P.P. Data curation: P.P., M.K., A.M. Data analysis: P.P., M.K. Methodology: P.P., M.K., Z.T., A.M., S.G., B.H., Funding acquisition: B.H. Writing—original draft: P.P., Writing—review & editing: P.P., M.K., Z.T., A.M., V.M.A., S.G., B.H.

References

Abrahams, V. M., Tang, Z., Mor, G., & Guller, S. (2020). NLRP3 inflammasome function and pyroptotic cell death in human placental Hofbauer cells. *J Reprod Immunol, 142*, 103214. doi:10.1016/j.jri.2020.103214

Allard, M. J., Giraud, A., Segura, M., & Sebire, G. (2019). Sex-specific maternofetal innate immune responses triggered by group B Streptococci. *Sci Rep, 9*(1), 8587. doi:10.1038/s41598-019-45029-x

Ben Amara, A., Gorvel, L., Baulan, K., Derain-Court, J., Buffat, C., Verollet, C., . . . Mege, J. L. (2013). Placental macrophages are impaired in chorioamnionitis, an infectious pathology of the placenta. *J Immunol, 191*(11), 5501-5514. doi:10.4049/jimmunol.1300988

Berghofer, B., Frommer, T., Haley, G., Fink, L., Bein, G., & Hackstein, H. (2006). TLR7 ligands induce higher IFN-alpha production in females. *J Immunol, 177*(4), 2088-2096. doi:10.4049/jimmunol.177.4.2088

Blaschitz, A., Gauster, M., Fuchs, D., Lang, I., Maschke, P., Ulrich, D., . . . Sedlmayr, P. (2011). Vascular endothelial expression of indoleamine 2,3-dioxygenase 1 forms a positive gradient towards the feto-maternal interface. *PLoS One, 6*(7), e21774. doi:10.1371/journal.pone.0021774

Blighe, K., & Lun, A. (2021). PCAtools: Everything Principal Components Analysis. Retrieved from http://bioconductor.org/packages/release/bioc/html/PCAtools.html

Blum, M., Chang, H. Y., Chuguransky, S., Grego, T., Kandasaamy, S., Mitchell, A., . . . Finn, R. D. (2021). The InterPro protein families and domains database: 20 years on. *Nucleic Acids Res, 49*(D1), D344-D354. doi:10.1093/nar/gkaa977

Bokun, V., Moore, J. J., Moore, R., Smallcombe, C. C., Harford, T. J., Rezaee, F., . . . Piedimonte, G. (2019). Respiratory syncytial virus exhibits differential tropism for distinct human placental cell types with Hofbauer cells acting as a permissive reservoir for infection. *PLoS One, 14*(12), e0225767. doi:10.1371/journal.pone.0225767

Bordt, E. A., Shook, L. L., Atyeo, C., Pullen, K. M., De Guzman, R. M., Meinsohn, M. C., . . . Edlow, A. G. (2021). Maternal SARS-CoV-2 infection elicits sexually dimorphic placental immune responses. *Sci Transl Med, 13*(617), eabi7428. doi:10.1126/scitranslmed.abi7428

Broere-Brown, Z. A., Adank, M. C., Benschop, L., Tielemans, M., Muka, T., Goncalves, R., . . . Schalekamp-Timmermans, S. (2020). Fetal sex and maternal pregnancy outcomes: a systematic review and meta-analysis. *Biol Sex Differ, 11*(1), 26. doi:10.1186/s13293-020-00299-3

Byrne, J., & Warburton, D. (1987). Male excess among anatomically normal fetuses in spontaneous abortions. *Am J Med Genet, 26*(3), 605-611. doi:10.1002/ajmg.1320260315

Castellucci, M., Celona, A., Bartels, H., Steininger, B., Benedetto, V., & Kaufmann, P. (1987). Mitosis of the Hofbauer cell: possible implications for a fetal macrophage. *Placenta, 8*(1), 65-76. doi:10.1016/0143-4004(87)90040-3

Castellucci, M., Zaccheo, D., & Pescetto, G. (1980). A three-dimensional study of the normal human placental villous core. I. The Hofbauer cells. *Cell Tissue Res, 210*(2), 235-247. doi:10.1007/BF00237612

Ceasrine, A. M., Batorsky, R., Shook, L. L., Kislal, S., Bordt, E. A., Devlin, B. A., . . . Edlow, A. G. (2021). Single cell profiling of Hofbauer cells and fetal brain microglia reveals shared programs and functions. *bioRxiv*. doi:https://doi.org/10.1101/2021.12.03.471177

dos Santos, G., Rogel, M. R., Baker, M. A., Troken, J. R., Urich, D., Morales-Nebreda, L., . . . Ridge, K. M. (2015). Vimentin regulates activation of the NLRP3 inflammasome. *Nat Commun, 6*, 6574. doi:10.1038/ncomms7574

Doster, R. S., Sutton, J. A., Rogers, L. M., Aronoff, D. M., & Gaddy, J. A. (2018). Streptococcus agalactiae Induces Placental Macrophages To Release Extracellular Traps Loaded with Tissue Remodeling Enzymes via an Oxidative Burst-Dependent Mechanism. *mBio, 9*(6). doi:10.1128/mBio.02084-18

Eberle, D., Hegarty, B., Bossard, P., Ferre, P., & Foufelle, F. (2004). SREBP transcription factors: master regulators of lipid homeostasis. *Biochimie, 86*(11), 839-848. doi:10.1016/j.biochi.2004.09.018

Fakonti, G., Pantazi, P., Bokun, V., & Holder, B. (2022). Placental Macrophage (Hofbauer Cell) Responses to Infection During Pregnancy: A Systematic Scoping Review. *Frontiers in Immunology, 12*. doi:10.3389/fimmu.2021.756035

Goldenberg, R. L., Andrews, W. W., Faye-Petersen, O. M., Goepfert, A. R., Cliver, S. P., & Hauth, J. C. (2006). The Alabama Preterm Birth Study: intrauterine infection and placental histologic findings in preterm births of males and females less than 32 weeks. *Am J Obstet Gynecol, 195*(6), 1533-1537. doi:10.1016/j.ajog.2006.05.023

Gross, R., Bauer, R., Kruger, F., Rucker-Braun, E., Olari, L. R., Standker, L., . . . Munch, J. (2020). A Placenta Derived C-Terminal Fragment of beta-Hemoglobin With Combined Antibacterial and Antiviral Activity. *Front Microbiol, 11*, 508. doi:10.3389/fmicb.2020.00508

Hendrix, P., Tang, Z., Silasi, M., Racicot, K. E., Mor, G., Abrahams, V. M., & Guller, S. (2020). Herpesvirus-infected Hofbauer cells activate endothelial cells through an IL-1beta-dependent mechanism. *Placenta, 91*, 59-65. doi:10.1016/j.placenta.2020.01.010

Huang da, W., Sherman, B. T., & Lempicki, R. A. (2009a). Bioinformatics enrichment tools: paths toward the comprehensive functional analysis of large gene lists. *Nucleic Acids Res, 37*(1), 1-13. doi:10.1093/nar/gkn923

Huang da, W., Sherman, B. T., & Lempicki, R. A. (2009b). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc, 4*(1), 44-57. doi:10.1038/nprot.2008.211

Jassal, B., Matthews, L., Viteri, G., Gong, C., Lorente, P., Fabregat, A., . . . D'Eustachio, P. (2020). The reactome pathway knowledgebase. *Nucleic Acids Res, 48*(D1), D498-D503. doi:10.1093/nar/gkz1031

Jiu, Y., Lehtimaki, J., Tojkander, S., Cheng, F., Jaalinoja, H., Liu, X., . . . Lappalainen, P. (2015). Bidirectional Interplay between Vimentin Intermediate Filaments and Contractile Actin Stress Fibers. *Cell Rep, 11*(10), 1511-1518. doi:10.1016/j.celrep.2015.05.008

Johnson, E. L., Boggavarapu, S., Johnson, E. S., Lal, A. A., Agrawal, P., Bhaumik, S. K., . . . Chakraborty, R. (2018). Human Cytomegalovirus Enhances Placental Susceptibility and Replication of Human Immunodeficiency Virus Type 1 (HIV-1), Which May Facilitate In Utero HIV-1 Transmission. *J Infect Dis, 218*(9), 1464-1473. doi:10.1093/infdis/jiy327

Johnson, E. L., & Chakraborty, R. (2012). Placental Hofbauer cells limit HIV-1 replication and potentially offset mother to child transmission (MTCT) by induction of immunoregulatory cytokines. *Retrovirology, 9*, 101. doi:10.1186/1742-4690-9-101

Jones, G. E. (2000). Cellular signaling in macrophage migration and chemotaxis. *J Leukoc Biol, 68*(5), 593-602. Retrieved from https://www.ncbi.nlm.nih.gov/pubmed/11073096

Khan, S., Katabuchi, H., Araki, M., Nishimura, R., & Okamura, H. (2000). Human villous macrophage-conditioned media enhance human trophoblast growth and differentiation in vitro. *Biol Reprod, 62*(4), 1075-1083. doi:10.1095/biolreprod62.4.1075

Klein, S. L., & Flanagan, K. L. (2016). Sex differences in immune responses. *Nat Rev Immunol, 16*(10), 626-638. doi:10.1038/nri.2016.90

Klein, S. L., Jedlicka, A., & Pekosz, A. (2010). The Xs and Y of immune responses to viral vaccines. *Lancet Infect Dis, 10*(5), 338-349. doi:10.1016/S1473-3099(10)70049-9

Lamason, R., Zhao, P., Rawat, R., Davis, A., Hall, J. C., Chae, J. J., . . . Nagaraju, K. (2006). Sexual dimorphism in immune response genes as a function of puberty. *BMC Immunol, 7*, 2. doi:10.1186/1471-2172-7-2

Law, R. H., Zhang, Q., McGowan, S., Buckle, A. M., Silverman, G. A., Wong, W., . . . Whisstock, J. C. (2006). An overview of the serpin superfamily. *Genome Biol, 7*(5), 216. doi:10.1186/gb-2006-7-5-216

Leek, J. T., Johnson, W. E., Parker, H. S., Fertig, E. J., Jaffe, A. E., Zhang, Y., . . . Torres, L. C. (2021). sva: Surrogate Variable Analysis. R package version 3.42.0. Retrieved from https://bioconductor.org/packages/release/bioc/html/sva.html

Loegl, J., Hiden, U., Nussbaumer, E., Schliefsteiner, C., Cvitic, S., Lang, I., . . . Desoye, G. (2016). Hofbauer cells of M2a, M2b and M2c polarization may regulate feto-placental angiogenesis. *Reproduction, 152*(5), 447-455. doi:10.1530/REP-16-0159

Mak, T. N., & Bruggemann, H. (2016). Vimentin in Bacterial Infections. *Cells, 5*(2). doi:10.3390/cells5020018

Martinez, F. O., Gordon, S., Locati, M., & Mantovani, A. (2006). Transcriptional profiling of the human monocyte-to-macrophage differentiation and polarization: new molecules and patterns of gene expression. *J Immunol, 177*(10), 7303-7311. doi:10.4049/jimmunol.177.10.7303

Mezouar, S., Benammar, I., Boumaza, A., Diallo, A. B., Chartier, C., Buffat, C., . . . Mege, J. L. (2019). Full-Term Human Placental Macrophages Eliminate Coxiella burnetii Through an IFN-gamma Autocrine Loop. *Front Microbiol, 10*, 2434. doi:10.3389/fmicb.2019.02434

Na, Q., Chudnovets, A., Liu, J., Lee, J. Y., Dong, J., Shin, N., . . . Burd, I. (2021). Placental Macrophages Demonstrate Sex-Specific Response to Intrauterine Inflammation and May Serve as a Marker of Perinatal Neuroinflammation. *J Reprod Immunol, 147*, 103360. doi:10.1016/j.jri.2021.103360

Oliveira, L. H., Fonseca, M. E., & De Bonis, M. (1992). Placental phagocytic cells infected with herpes simplex type 2 and echovirus type 19: virological and ultrastructural aspects. *Placenta, 13*(5), 405-416. doi:10.1016/0143-4004(92)90048-x

Pavlov, O., Pavlova, O., Ailamazyan, E., & Selkov, S. (2008). Characterization of cytokine production by human term placenta macrophages in vitro. *Am J Reprod Immunol, 60*(6), 556-567. doi:10.1111/j.1600-0897.2008.00657.x

PrabhuDas, M. R., Baldwin, C. L., Bollyky, P. L., Bowdish, D. M. E., Drickamer, K., Febbraio, M., . . . El Khoury, J. (2017). A consensus definitive classification of scavenger receptors and their roles in health and disease. *J Immunol, 198*(10), 3775-3789. doi:10.4049/jimmunol.1700373

Przybyl, L., Haase, N., Golic, M., Rugor, J., Solano, M. E., Arck, P. C., . . . Herse, F. (2016). CD74-Downregulation of Placental Macrophage-Trophoblastic Interactions in Preeclampsia. *Circ Res, 119*(1), 55-68. doi:10.1161/CIRCRESAHA.116.308304

R Core Team (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from http://www.r-project.org/index.html

Ravenhill, B. J., Soday, L., Houghton, J., Antrobus, R., & Weekes, M. P. (2020). Comprehensive cell surface proteomics defines markers of classical, intermediate and non-classical monocytes. *Sci Rep, 10*(1), 4560. doi:10.1038/s41598-020-61356-w

Reimer, T., Brcic, M., Schweizer, M., & Jungi, T. W. (2008). poly(I:C) and LPS induce distinct IRF3 and NF-kappaB signaling during type-I IFN and TNF responses in human macrophages. *J Leukoc Biol, 83*(5), 1249-1257. doi:10.1189/jlb.0607412

Reyes, L., & Golos, T. G. (2018). Hofbauer Cells: Their Role in Healthy and Complicated Pregnancy. *Front Immunol, 9*, 2628. doi:10.3389/fimmu.2018.02628

Ritchie, M. E., Phipson, B., Wu, D., Hu, Y., Law, C. W., Shi, W., & Smyth, G. K. (2015). limma powers differential expression analyses for RNA-sequencing and microarray studies. *Nucleic Acids Res, 43*(7), e47. doi:10.1093/nar/gkv007

Rosenfeld, C. S. (2015). Sex-Specific Placental Responses in Fetal Development. *Endocrinology, 156*(10), 3422-3434. doi:10.1210/en.2015-1227

Schliefsteiner, C., Ibesich, S., & Wadsack, C. (2020). Placental Hofbauer cell polarization resists inflammatory cues in vitro. *Int J Mol Sci, 21*(3). doi:10.3390/ijms21030736

Schliefsteiner, C., Peinhaupt, M., Kopp, S., Logl, J., Lang-Olip, I., Hiden, U., . . . Wadsack, C. (2017). Human Placental Hofbauer Cells Maintain an Anti-inflammatory M2 Phenotype despite the Presence of Gestational Diabetes Mellitus. *Front Immunol, 8*, 888. doi:10.3389/fimmu.2017.00888

Seval, Y., Korgun, E. T., & Demir, R. (2007). Hofbauer cells in early human placenta: possible implications in vasculogenesis and angiogenesis. *Placenta, 28*(8-9), 841-845. doi:10.1016/j.placenta.2007.01.010

Sun, T., Gonzalez, T. L., Deng, N., DiPentino, R., Clark, E. L., Lee, B., . . . Pisarska, M. D. (2020). Sexually Dimorphic Crosstalk at the Maternal-Fetal Interface. *J Clin Endocrinol Metab, 105*(12). doi:10.1210/clinem/dgaa503

Sutton, J. A., Rogers, L. M., Dixon, B., Kirk, L., Doster, R., Algood, H. M., . . . Aronoff, D. M. (2019). Protein kinase D mediates inflammatory responses of human placental macrophages to Group B Streptococcus. *Am J Reprod Immunol, 81*(3), e13075. doi:10.1111/aji.13075

Swieboda, D., Johnson, E. L., Beaver, J., Haddad, L., Enninga, E. A. L., Hathcock, M., . . . Chakraborty, R. (2020). Baby's First Macrophage: Temporal Regulation of Hofbauer Cell Phenotype Influences Ligand-Mediated Innate Immune Responses across Gestation. *J Immunol, 204*(9), 2380-2391. doi:10.4049/jimmunol.1901185

Szklarczyk, D., Gable, A. L., Lyon, D., Junge, A., Wyder, S., Huerta-Cepas, J., . . . Mering, C. V. (2019). STRING v11: protein-protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. *Nucleic Acids Res, 47*(D1), D607-D613. doi:10.1093/nar/gky1131

Tabata, T., Petitt, M., Puerta-Guardo, H., Michlmayr, D., Harris, E., & Pereira, L. (2018). Zika Virus Replicates in Proliferating Cells in Explants From First-Trimester Human Placentas, Potential Sites for Dissemination of Infection. *J Infect Dis, 217*(8), 1202-1213. doi:10.1093/infdis/jix552

Tang, Z., Buhimschi, I. A., Buhimschi, C. S., Tadesse, S., Norwitz, E., Niven-Fairchild, T., . . . Guller, S. (2013). Decreased levels of folate receptor-beta and reduced numbers of fetal macrophages (Hofbauer cells) in placentas from pregnancies with severe pre-eclampsia. *Am J Reprod Immunol, 70*(2), 104-115. doi:10.1111/aji.12112

Tang, Z., Tadesse, S., Norwitz, E., Mor, G., Abrahams, V. M., & Guller, S. (2011). Isolation of hofbauer cells from human term placentas with high yield and purity. *Am J Reprod Immunol, 66*(4), 336-348. doi:10.1111/j.1600-0897.2011.01006.x

Thomas, J. R., Appios, A., Zhao, X., Dutkiewicz, R., Donde, M., Lee, C. Y. C., . . . McGovern, N. (2021). Phenotypic and functional characterization of first-trimester human placental macrophages, Hofbauer cells. *J Exp Med, 218*(1). doi:10.1084/jem.20200891

Vatten, L. J., & Skjaerven, R. (2004). Offspring sex and pregnancy outcome by length of gestation. *Early Hum Dev, 76*(1), 47-54. doi:10.1016/j.earlhumdev.2003.10.006

Vento-Tormo, R., Efremova, M., Botting, R. A., Turco, M. Y., Vento-Tormo, M., Meyer, K. B., . . . Teichmann, S. A. (2018). Single-cell reconstruction of the early maternal-fetal interface in humans. *Nature, 563*(7731), 347-353. doi:10.1038/s41586-018-0698-6

Vinnars, M. T., Rindsjo, E., Ghazi, S., Sundberg, A., & Papadogiannakis, N. (2010). The number of CD68(+) (Hofbauer) cells is decreased in placentas with chorioamnionitis and with advancing gestational age. *Pediatr Dev Pathol, 13*(4), 300-304. doi:10.2350/09-03-0632-OA.1

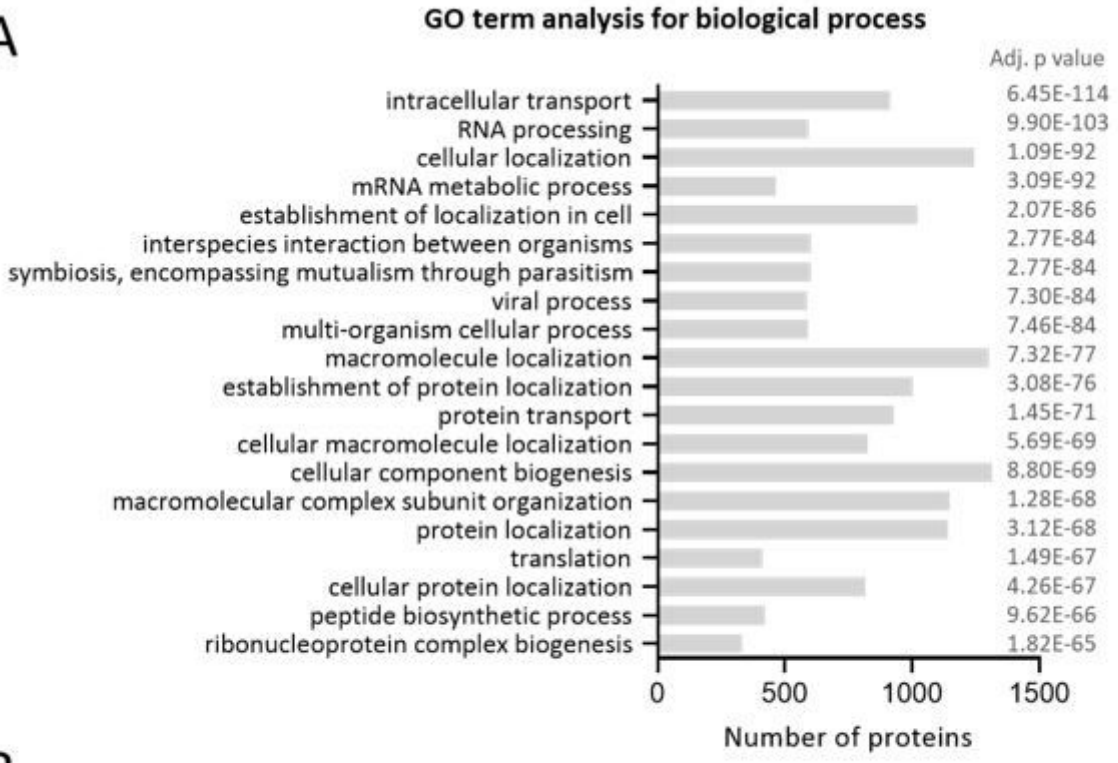
Yang, S. W., Cho, E. H., Choi, S. Y., Lee, Y. K., Park, J. H., Kim, M. K., . . . Kang, Y. S. (2017). DC-SIGN expression in Hofbauer cells may play an important role in immune tolerance in fetal chorionic villi during the development of preeclampsia. *J Reprod Immunol, 124*, 30-37. doi:10.1016/j.jri.2017.09.012

Yin, L. M., Schnoor, M., & Jun, C. D. (2020). Structural Characteristics, Binding Partners and Related Diseases of the Calponin Homology (CH) Domain. *Front Cell Dev Biol, 8*, 342. doi:10.3389/fcell.2020.00342

Young, O. M., Tang, Z., Niven-Fairchild, T., Tadesse, S., Krikun, G., Norwitz, E. R., . . . Guller, S. (2015). Toll-like receptor-mediated responses by placental Hofbauer cells (HBCs): a potential pro-inflammatory role for fetal M2 macrophages. *Am J Reprod Immunol, 73*(1), 22-35. doi:10.1111/aji.12336

Figures

A

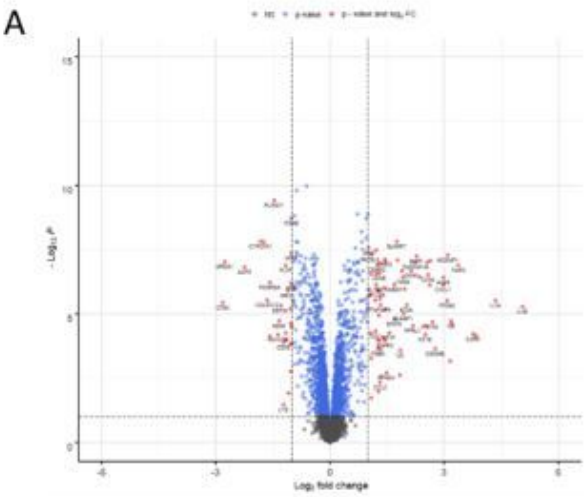


B

Molecules pertinent to macrophage function	
Chemokines	CCL8, CXCL8, CXCL3, CCL20, CXCL1, CXCL5, CCL3, CXCL10, CXCL2, CXCL11, CCL4, CCL2
Cytokines	IL16, IL18, IL6, IL4I1, IL1B, IL1A, IL36γ, TNF, IK
Human leukocyte antigen complexes	HLA-DRA, HLA-DMB, HLA-F, HLA-DMA, HLA-A, HLA-B, HLA-C, HLA-DPA1
Chemokine receptors	CCR1, CCR7
Cytokine receptors	CD40, IL3RA, IL17RA, IL10Rβ
Toll-like receptors	TLR2, TLR8, TLR7, TLR3
C-type lectins	MRC1, CD209, COLEC12, LY75, CLEC4E, CD302, ATRN, CD93
Scavenger receptors	SCARF1, SCARB2, LRP1, MRC1, CD163, CD44, CD14, MSR1, CD209, CD36, COLEC12, CD163L1, CD68

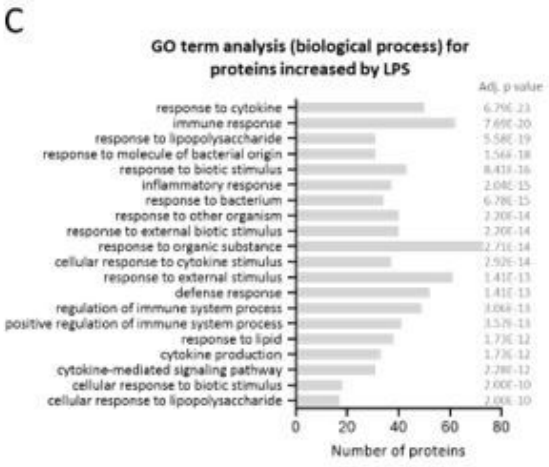
Figure 1

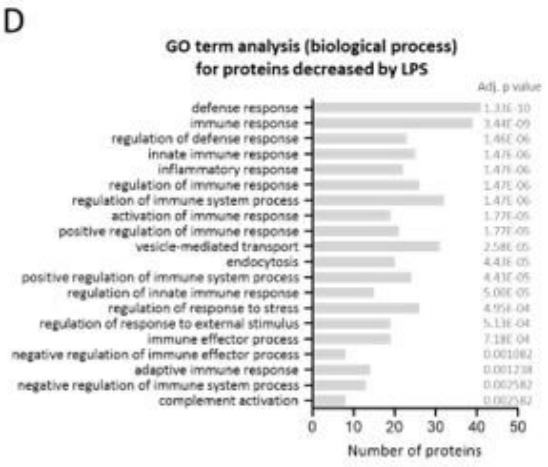
Characterisation of placental macrophages. The proteome of non-treated term HBCs was investigated. A) The proteins were analysed by DAVID functional annotation to produce clusters (≥2 proteins/cluster) and Gene ontology (GO) terms corresponding to biological process (GOTERM_BP_FAT) were extracted. The histogram shows the top 20 GO terms significantly associated (adj. $p \leq 0.05$) with the protein list along with the number of proteins in each cluster. B) List(s) of macrophage-relevant proteins present in resting HBCs.

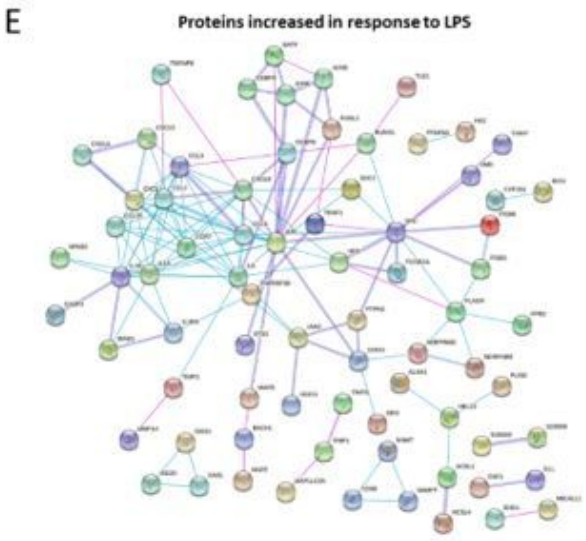


B

Molecules pertinent to macrophage function relative to levels in NT cells	
Chemokines	↑CXCL11, ↑CCL20, ↑CXCL3, ↑CXCL5, ↑CCL3, ↑CCL4, ↑CCL2,
Cytokines	↑IL6, ↑IL1A, ↑IL1B, ↑IL8, ↑IL36G, ↑TNFα
Human leukocyte antigen complexes	↓HLA-DMA, ↓HLA-B
Chemokine receptors	↑CCR7, ↓CCR1
Scavenger receptors	↑ SCARF1, ↓LRP1, ↓MSR1, ↓MRC1, ↓COLEC12, ↓CD36, ↓CD209, ↓CD68
Other surface molecules	↑CD93, ↑CD48, ↑CD55, ↑CD82, ↑CD300E, ↑CD38, ↓LY75, ↓CD302, ↓CD46, ↓CD59, ↓CD109, ↓CD99, ↓CD9







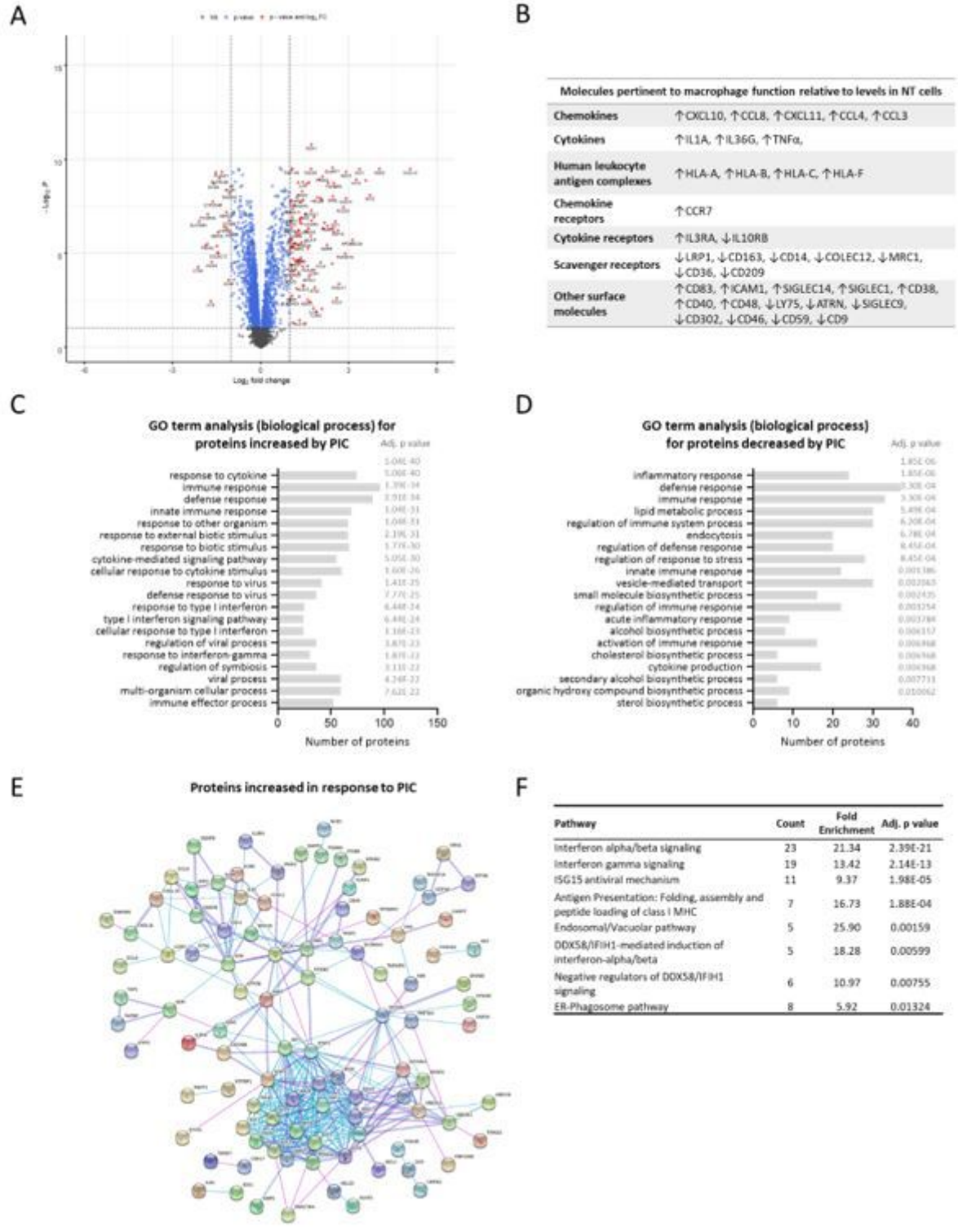
F

Pathway	Count	Fold Enrichment	Adj. p value
Chemokine receptors bind chemokines	9	11.35	2.35E-04
Metallothioneins bind metals	5	33.81	0.001089

Figure 2

Proteomic characterisation of placental macrophages in response to bacterial PAMP. HBCs were exposed to bacterial lipopolysaccharide (LPS) for 24h, followed by TMT-proteomics, to enable quantitative measurement of the LPS response. A) Volcano plot depicting the log2 fold differences in protein abundance between LPS treated and non-treated HBCs. Increased (FC>1.5) and decreased (FC<-1.5) proteins are labelled green and are found on the right and the left side of the graph respectively. A cut-off

of 0.05 was applied for the adjusted p-values (adj. p value; y-axis). B) Proteins pertinent to macrophage function found up (↑) or down (↓) in LPS-treated HBCs compared with non-treated (NT) cells. C,D) Gene ontology (GO) analysis of the (C) increased (FC>1.5 , adj. p value <0.05) and (D) decreased (FC<-1.5 , adj. p value <0.05) proteins in HBC treated with LPS compared with controls (non-treated HBCs). The proteins were analysed by DAVID functional annotation to produce clusters (≥2 proteins/cluster) and GO terms corresponding to biological process (GOTERM_BP_FAT) were extracted. The histogram [NB –not formally a histogram in my understanding, needs a continuous categorical axis, no?) shows the top 20 GO terms significantly associated (adj $p \leq 0.05$) with the protein list along with the number of proteins in each cluster. E) Interaction networks of the up-regulated proteins using STRING v11.5. Colored lines between the proteins indicate the interaction evidence (pink: experimentally determined, blue: from curated databases), minimum required interaction score - medium confidence (0.4), disconnected nodes are hidden from the graph. F) Reactome pathway enrichment analysis. Two pathways were over-represented (adj. p value <0.05) in LPS-treated HBCs. For each pathway, the number of proteins (Count) and the (Fold Enrichment) are given.



Pathway	Count	Fold Enrichment	Adj. p value
Interferon alpha/beta signaling	23	21.34	2.39E-21
Interferon gamma signaling	19	13.42	2.14E-13
ISG15 antiviral mechanism	11	9.37	1.98E-05
Antigen Presentation: Folding, assembly and peptide loading of class I MHC	7	16.73	1.88E-04
Endosomal/Vacuolar pathway	5	25.90	0.00159
DDX58/IFIH1-mediated induction of interferon-alpha/beta	5	18.28	0.00599
Negative regulators of DDX58/IFIH1 signaling	6	10.97	0.00755
ER-Phagosome pathway	8	5.92	0.01324

Figure 3

Proteomic characterisation of placental macrophages in response to viral PAMP. HBCs were exposed to poly(I:C) (PIC), a viral RNA analogue, for 24h, followed by TMT-proteomics, to enable quantitative measurement of the PIC response. A) Volcano plot depicting the fold differences in protein abundance between PIC-treated and non-treated HBCs. Increased (FC>1.5) and decreased (FC<-1.5) proteins are labelled green and are found on the right and the left side of the graph respectively. A cut-off of 0.05

was applied for the adjusted p-values (adj. p value, y-axis). B) Proteins pertinent to macrophage function found up (↑) or down (↓) regulated in PIC-treated HBCs. C,D) Gene ontology (GO) analysis of the (C) up-regulated (FC>1.5 , adj. p value <0.05) and (D) down-regulated (FC<-1.5, adj. p value <0.05) proteins in HBC treated with PIC compared with controls (non-treated HBCs). The proteins were analysed by DAVID functional annotation to produce clusters (≥2 proteins/cluster) and GO terms corresponding to biological process (GOTERM_BP_FAT) were extracted. The histogram shows the top 20 GO terms significantly associated (adj $p \leq 0.05$) with the protein list along with the number of proteins in each cluster. E) Interaction networks of the up-regulated proteins using STRING v11.5. Colored lines between the proteins indicate the interaction evidence (pink: experimentally determined, blue: from curated databases), minimum required interaction score - medium confidence (0.4), disconnected nodes are hidden from the graph. F) Reactome pathway enrichment analysis. A total of 8 pathways were over-represented (adj. p value <0.05) in PIC-treated HBCs. For each pathway, the number of proteins (Count) and the fold enrichment are given.

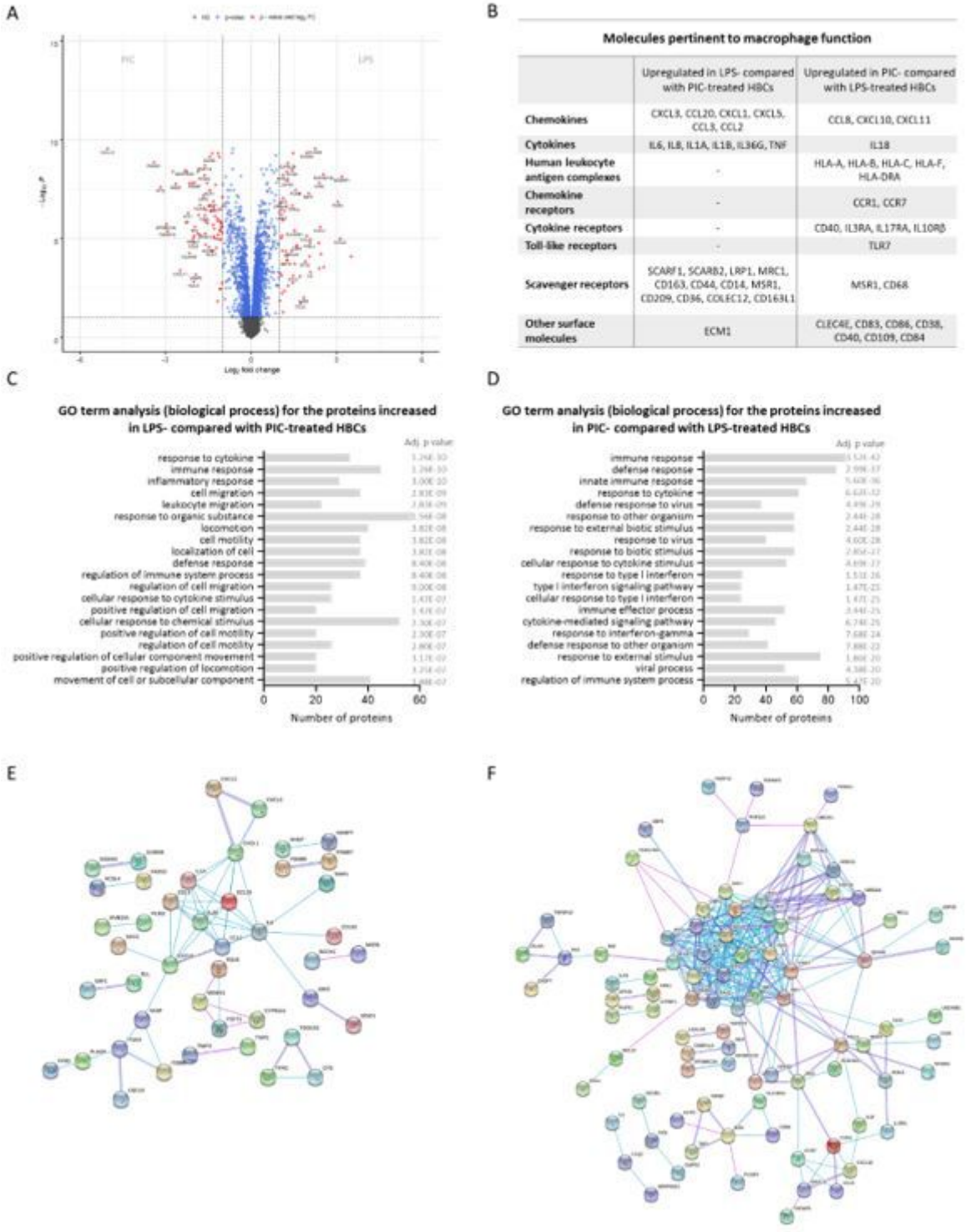
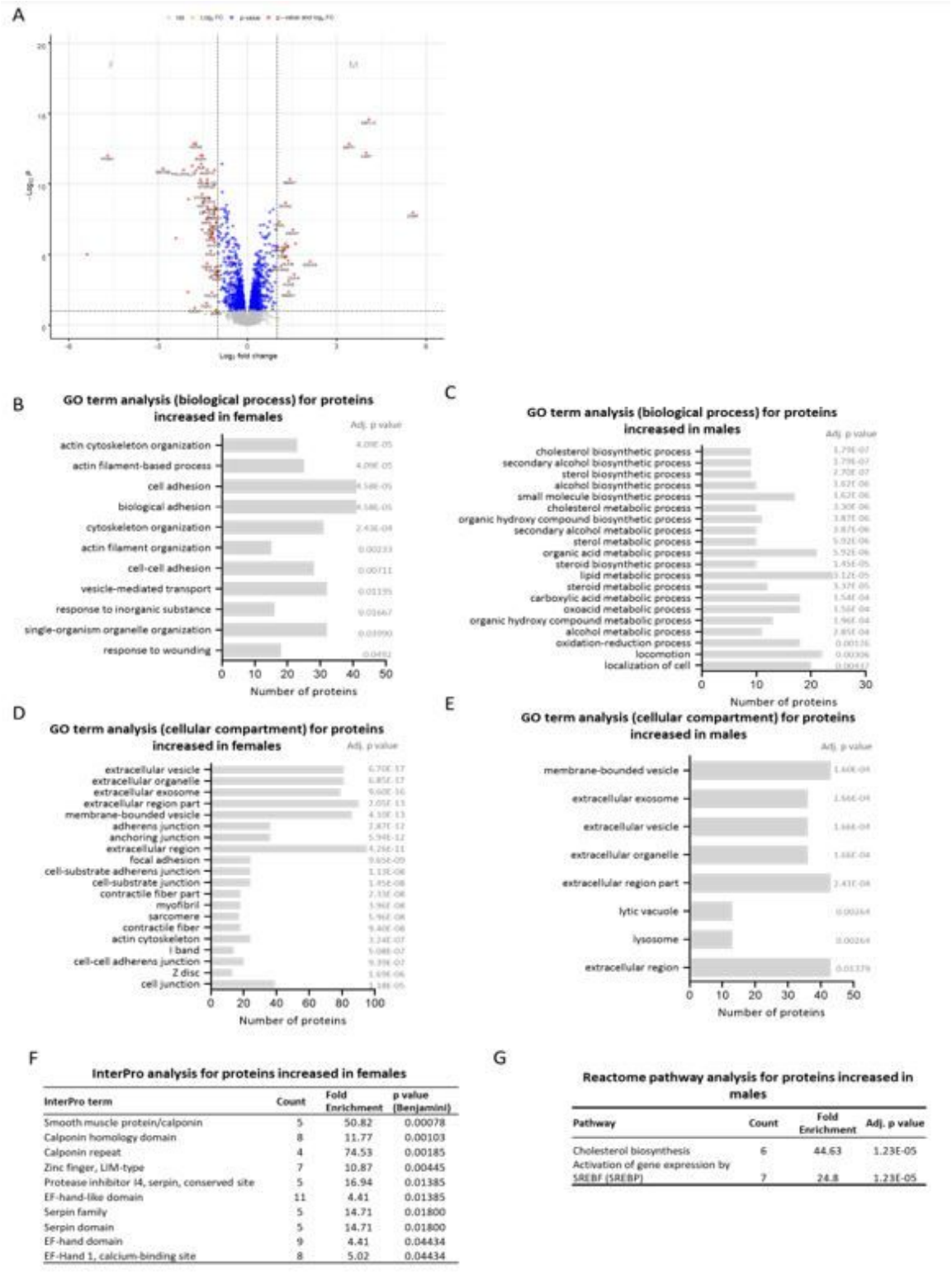


Figure 4

Differential placental macrophage responses to bacterial and viral PAMPs. A) Volcano plot depicting the fold differences in protein abundance between LPS-treated and PIC-treated HBCs. Proteins increased (FC>1.5) in LPS-treated HBCs are found on the right side of the graph, while proteins decreased (FC<-1.5) in LPS-treated and therefore increased in PIC-treated HBCs are found on the left side. A cut-off of 0.05 was applied for the adjusted p-values (adj. p value, y-axis). B) Proteins pertinent to macrophage function found increased in LPS- compared with PIC-treated HBCs and vice versa. C,D) Gene ontology (GO)

analysis of the proteins up-regulated in (C) LPS-treated and (D) PIC-treated HBCs (FC>1.5 , adj. p value <0.05). The proteins were analysed by DAVID functional annotation to produce clusters (≥2 proteins/cluster) and GO terms corresponding to biological process (GOTERM_BP_FAT) were extracted. The histogram shows the top 20 GO terms significantly associated (adj $p \leq 0.05$) with the protein list along with the number of proteins in each cluster. E, F) Interaction networks of the up-regulated proteins in (E) LPS-treated and (F) PIC-treated HBCs using STRING v11.5. Colored lines between the proteins indicate the interaction evidence (pink: experimentally determined, blue: from curated databases), minimum required interaction score - medium confidence (0.4), disconnected nodes are hidden from the graph.



F

InterPro analysis for proteins increased in females

InterPro term	Count	Fold Enrichment	p value (Benjamini)
Smooth muscle protein/calponin	5	50.82	0.00078
Calponin homology domain	8	11.77	0.00103
Calponin repeat	4	74.53	0.00185
Zinc finger, LIM-type	7	10.87	0.00445
Protease inhibitor I4, serpin, conserved site	5	16.94	0.01385
EF-hand-like domain	11	4.41	0.01385
Serpin family	5	14.71	0.01800
Serpin domain	5	14.71	0.01800
EF-hand domain	9	4.41	0.04434
EF-Hand 1, calcium-binding site	8	5.02	0.04434

G

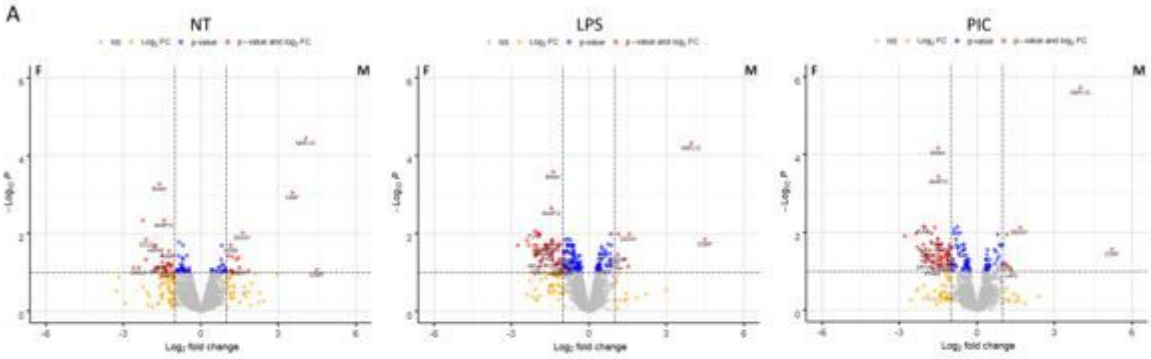
Reactome pathway analysis for proteins increased in males

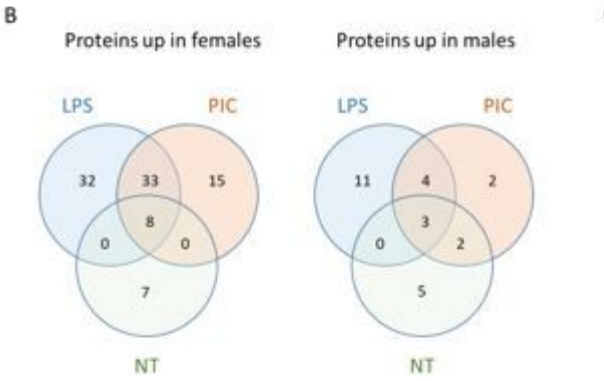
Pathway	Count	Fold Enrichment	Adj. p value
Cholesterol biosynthesis	6	44.63	1.23E-05
Activation of gene expression by SREBF (SREBP)	7	24.8	1.23E-05

Figure 5

Sex-specific proteomic profiles of placental macrophages. Differential expression analysis was performed in HBCs from female and male placentas, accounting for treatment, to identify differences due to sex. A) Volcano plot depicting the fold differences in protein abundance between females (left) and males (right). B, C) Gene ontology (GO) analysis, for the biological process, of the proteins increased (FC>1.5 , adj. p value <0.05) in female HBCs compared with male HBCs (B) and of the proteins increased

(FC>1.5 , adj. p value <0.05) in male HBCs compared with female HBCs (C) . D, E) Gene ontology (GO) analysis, for the cellular compartment, of the proteins increased (FC>1.5 , adj. p value <0.05) in female HBCs compared with male HBCs (D) and of the proteins increased (FC>1.5 , adj. p value <0.05) in male HBCs compared with female HBCs (E). F) Classification of protein families in the female HBC up-regulated proteins using InterPro v86.0. G) Reactome pathway enrichment analysis for the male HBC up-regulated proteins. For each pathway, the number of proteins (Count) and the (Fold Enrichment) are given.





	Females	Males
Only in NT	KIF3B, SLCO2B1, CCL2, FNDC3B, DPP3 , NNMT, VTA1	CAST, PLTP, TST, PDCD4, SFXN2
Only in LPS	SPATS2L, TPM1, RPS26, CKAP4, PDLIM5, CDC42EP2, HNRNPR, PSIP1, MYO1D, CRYAB, H1F0, THBS1, NFIC, FGF2, PLCG1, RBMX, GNAI1, SELENOW, HNRNPA0, MORF4L2, CAV1, HNRNPR, RBM12B, NGDN, PALLD, PDLIM2, ZFR, PRO2051, EHD2, LYDG10, TNRC6B, HEL-176	CLINT1, PLXNB2, SLC16A3, CTSB, CD38, MSMO1, KYNU, SLC37A2, SMPDL3A, SLAMF6, CD209
Only in PIC	PLAU, HDGFRP3, ACTN1, SDC2, MPZL1, PDLIM7, VNN2, IL6, PFKM, FCAR, SERPINB9, BATF, CFL2, TAGLN3, IVNS1ABP	ALCAM, SRXN1
Common in NT-LPS-PIC	FLNB, HSPA2, AKAP12, PLIDP, DGLUCY, ENAH, NDRG1	CD99, DDX3Y, NSFL1C
Common in LPS-PIC	RAB23, GFPT2, MYO1B, B2R6V9 , TGFB1I1, PPL, UGDH, PAGE4, SORBS2, BAG2, SERPINA1, ALB, KRT18, RBP1, VCL, MYH10, ADD1, TAGLN2, SERPINH1, BASP1, TAGLN, FHL2, DPYSL3, CNN3, DES, PTRF, SEPT8, PRKCDBP, FERMT2, MMAB, PDLIM7, LMCD1, SYNPO2	ITGAL, CCR1, PLXND1, COMT

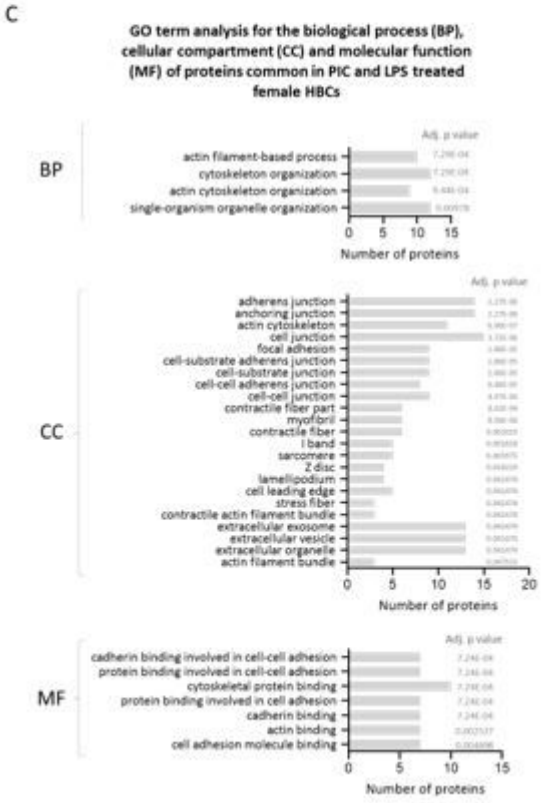


Figure 6

Sex-specific proteomic profiles of placental macrophages separated by treatment. The proteomic profile of female and male HBCs was compared in each treatment group (NT, LPS, PIC). A) Volcano plots depicting the fold differences in protein abundance between females (left) and males (right) in each treatment group separately NT , LPS, PIC (from left to right). B) Venn diagrams of the differentially expressed proteins between female (left) and male (right) HBCs and their subclassification in the different treatment groups. The names of the proteins in each subgroup of the Venn diagrams are given in the table below. C) Gene ontology (GO) analysis, for the biological process (BP), cellular compartment (CC), and molecular function (MF) of the 33 proteins common in PIC and LPS treated female HBCs.

Supplementary Files

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- SupplementaryInformationNatCom.docx