Chapter 4 - Integrating scRNA-seq with single cell-level protein-level data and established gene signatures to characterize maternal resident immune cell populations

4.1 Overview

Following the identification of several novel maternal immune subsets from scRNA-seq, we sought to examine markers distinguishing these populations at the protein level as well as begin defining their potential functions at the maternal-fetal interface. In this chapter, I first describe how protein abundance data from FACS coupled with plate-based scRNA-seq data, along with high-dimensional protein expression data derived from CyTOF, were used to validate identified decidual immune cell populations. Next, I present our further analysis of the maternal resident mononuclear phagocyte populations, in which we utilized established macrophage gene signatures and public tissue-level gene expression data of the placenta and endometrium to functionally annotate these cell types.

4.2 FACS and CyTOF validation of maternal resident immune cell subpopulations

Overlapping gating during FACS index sorting allowed us to enrich for the main immune subsets in the decidua using a 14-antibody panel selected based on previous knowledge of decidual immune cell surface markers (see Methods). The proportions of immune cells that were captured and sorted for sequencing therefore do not represent the true proportions observed *in vivo*, but are rather a consequence of our enrichment strategy. Following full-length scRNA-seq and unbiased transcriptome-based clustering of the sorted immune cells from five maternal decidua and two matched peripheral blood samples (sample information in **Table 1**), novel subsets of dNK cells, myeloid cells, $CD4^+$ and $CD8^+$ T cells, and Treg cells were defined as the main decidual immune populations, with their separation from the subsets containing peripheral blood-derived cells on t-SNE suggesting that these populations are tissue-resident cells rather than intravascular or circulating immune cells (**Figure 1a**). Next, FACS index sorting metadata was overlaid on the t-SNE mapping to link the gated identities of the cells with their transcriptome-defined identities (**Figure 1b**).

Table 1. Plate-based scRNA-seq sample information.

Donor	Post-conceptional weeks	Pregnancy	SmartSeq2 - Decidua	SmartSeq2 - Peripheral Blood	Fetus sex
D5	7 to 9	3rd	879 cells		male
D6	11 to 13	3rd	630 cells		female
D7	8 to 10	6th	1295 cells		female
D8	11 to 13	1st	1021 cells	539 cells	male
D9	8 to 10	1st	493 cells	724 cells	female

Overall, FACS gating was well-correlated with the transcriptome-based cell annotations. CD9 is a unique marker of dNK cells[37], and as expected, the $CD9^+$ NK cells are predominantly located among dNK cell clusters and absent among peripheral blood NK cells. In both decidua and blood, T cell clusters are definable by $CD4^+$ and $CD8^+$ identities. Interestingly, the two decidual mononuclear phagocyte populations (dMP1 and dMP2) are distinguishable by $CD14^+$HLA-DR^+ (Myeloid+) and $CD14^{++}$HLA-DR^{++} (Myeloid++) gating, suggesting that their transcriptome-level differences are paralleled by distinct surface protein expression profiles. A previous study of decidual macrophages identified two subsets defined by high and low expression of the integrin protein CD11C (ITGAX)[70]. Differential expression analysis between the two identified decidual mononuclear monocyte subsets confirmed *CD11C* as one of genes upregulated in dMP1 (Myeloid+ cells) relative to dMP2 (Myeloid++ cells) (**Figure 1c**).

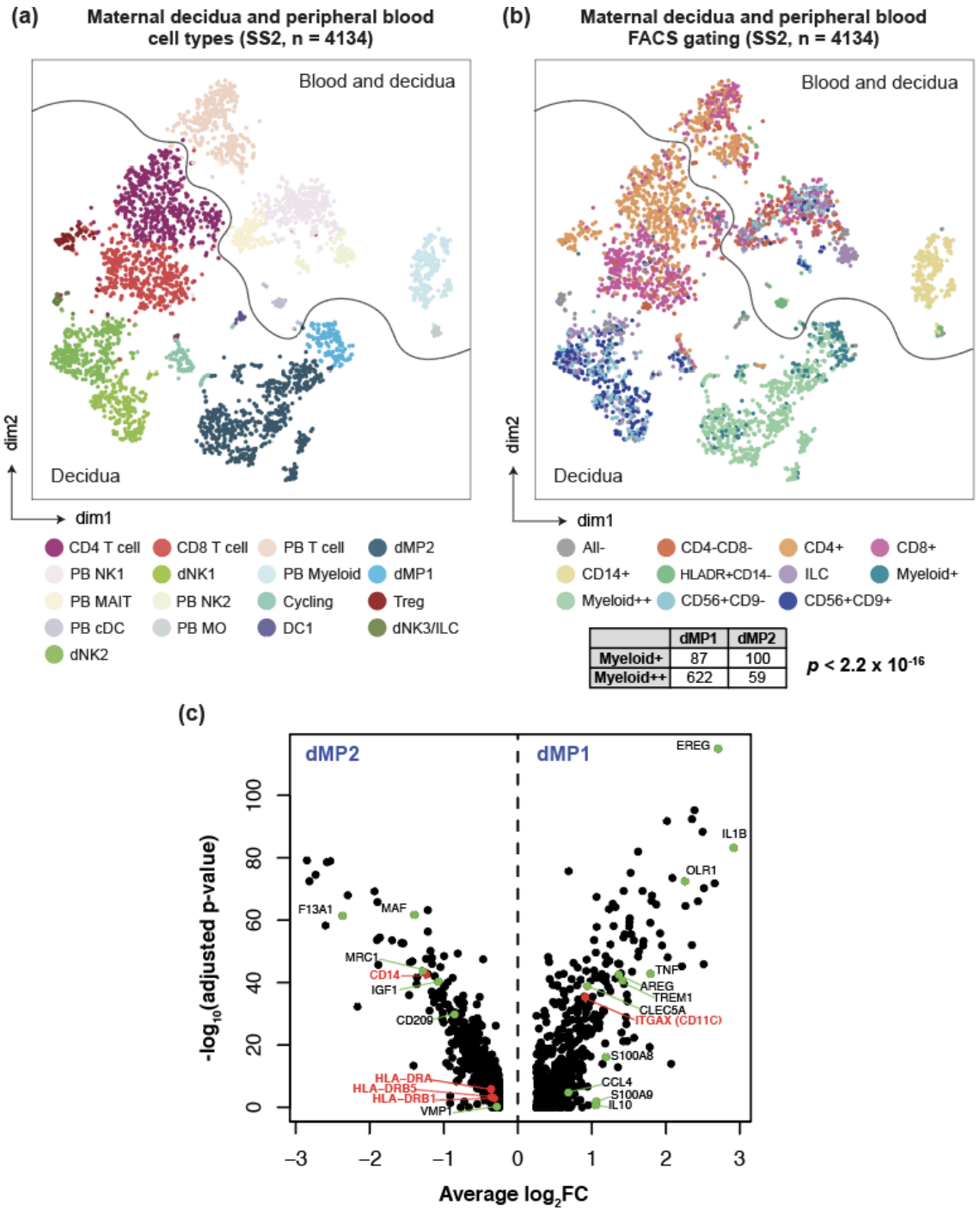

Figure 1. t-SNE visualizations juxtaposing cell type annotations and FACS gating distinguish two decidual mononuclear phagocyte populations. (a) Cells colored by predicted cell type or cell state as inferred by unbiased clustering on decidua SmartSeq2 scRNA-seq data and marker-based annotation (from Vento-Tormo R, Efremova E, et al., unpublished). **(b)** Cells colored by gates from FACS index sorting. Student's t-test shows significant enrichment of Myeloid++ cells in the dMP1 subset and Myeloid+ cells in the dMP2 subset ($p < 2.2 \times 10^{-16}$). **(c)** Volcano plot of differentially expressed genes between dMP1 and dMP2 showing upregulation of *CD14* and *HLA-DR* mRNA in dMP2, as well as upregulation of *CD11C* in dMP1 and differential expression of additional genes (in green) highlighted in a previous study of decidual macrophages[70].

We next performed CyTOF on the decidual samples in order to independently validate identified subpopulations and their transcriptome-defined markers at the protein level. CyTOF facilitated the

simultaneous quantification of 39 protein markers of interest (Appendix 3) at single-cell resolution by leveraging metal isotope-labeled antibodies coupled with mass spectrometry[167]. Analysis of the CyTOF data from $CD14^+$ immune cells in three maternal decidua confirms that there are indeed two distinct decidual mononuclear phagocyte populations, defined by high and low expression of CD11C. Additionally, we detected the presence of two dendritic cell subsets: DC1, which expresses high levels of the dendritic cell marker CLEC9A, and DC2, which expresses high levels of the dendritic cell marker CD1C (**Figure 2**).

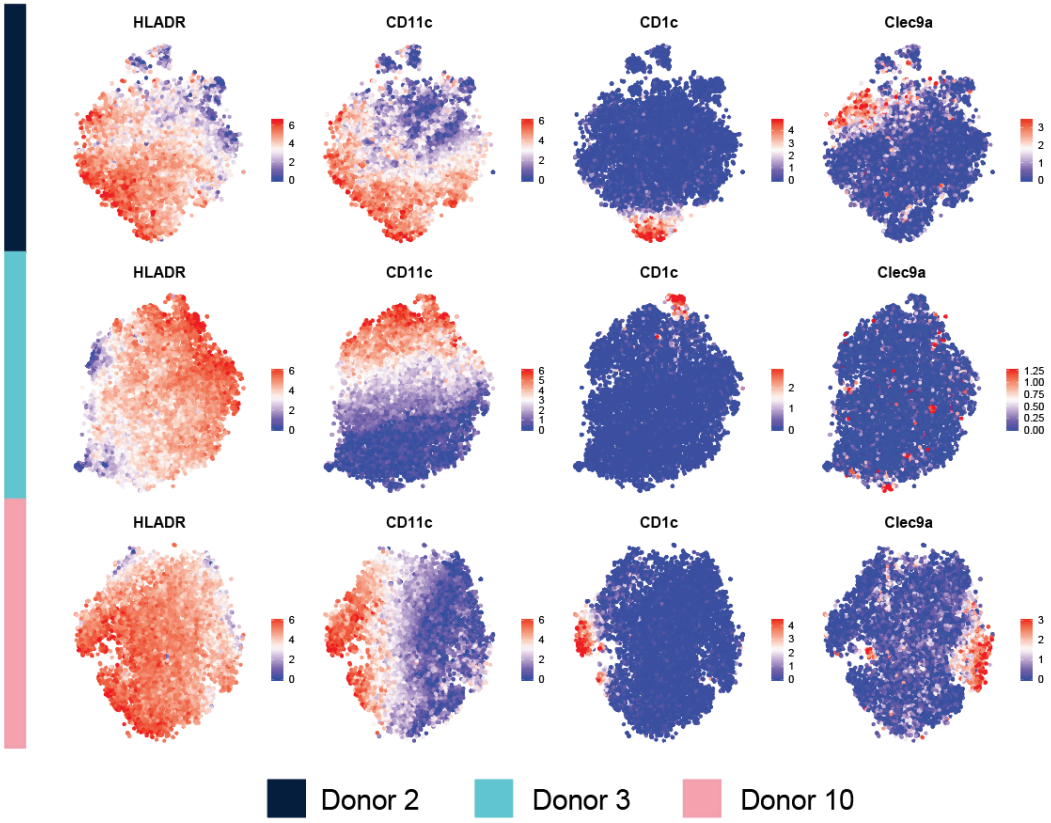

Figure 2. CyTOF validation of decidual myeloid cell subsets. t-SNE plots show protein expression of CD11C, CD1C, and CLEC9A on live cells gated on $CD45^+CD14^+$ events from three maternal decidua (Donors 2, 3, and 10).

Both droplet encapsulation and plate-based scRNA-seq approaches identified three main NK subsets in the decidua (Vento-Tormo R, Efremova M et al., under submission): dNK1, which exhibits high levels of cytotoxic granule (*GNLY, GZMA, GZMB*) and HLA-C-binding KIR genes (*KIR2DL1/DL2/DL3* and *KIR2DS1/DS4*); dNK2, which exhibits lower expression of both classes of genes; and dNK3/ILC1, which does not significantly express classical granzymes or HLA-C-binding KIRs, but rather expresses genes encoding the ILC1 markers *CD160*, *CD161* (*KLRB1*), and *CD103* (*ITGAE*), as well as *CD49d* (*ITGA4*),

a marker expressed by ILCs migrating into tissues[168]. Through CyTOF on $CD56^+$ immune cells from three maternal decidua, we confirmed that these dNK populations are also distinguishable by a subset of these markers at the protein level (**Figure 3a**, Appendix 7). The dNK3/ILC1 population expresses CD103, CD161 and CD49d while exhibiting low expression of GZMB. The dNK1 population highly expresses both KIR2DL1/DL3 and GZMB, while the population expressing GZMB but lower levels of the inhibitory KIRs likely corresponds to dNK2.

Finally, transcriptome-based clustering resolved populations of decidual $CD4^+$ T cells, $CD8^+$ T cells and $FOXP3^+$ Treg cells, which we were also able to identify through CyTOF analyses of $CD3^+$ T cells from three maternal decidua (**Figure 3b**, Appendix 7). While we observed a population of naive ($CD45RA^+$) $CD8^+$ T cells by CyTOF, most decidual T cells appear to be memory ($CD45RO^+$) T cells. A subset of the memory $CD8^+$ T cells additionally expressed the tissue residency marker CD103. Interestingly, the $CD8^+$ T cells exhibit low or absent expression of CD127 (IL-7R), a major mediator of homeostatic regulation and survival in most T cell populations[169]. Finally, we also detected small populations of $CD8^+$ gamma-delta T cells.

Overall, these results demonstrate that pairing of plate-based scRNA-seq data with FACS index sorting metadata enables us to generally validate transcriptome-level cell annotations at the protein level. At the same time, it allows us to reciprocally define certain cell types, such as the two decidual mononuclear phagocyte populations, on the basis of distinct surface protein expression. Meanwhile, CyTOF facilitates independent validation of identified cell subsets and their distinguishing markers. Its high-parameter capacity also allows for the simultaneous profiling of additional protein markers of interest that may prove useful in further describing these populations.

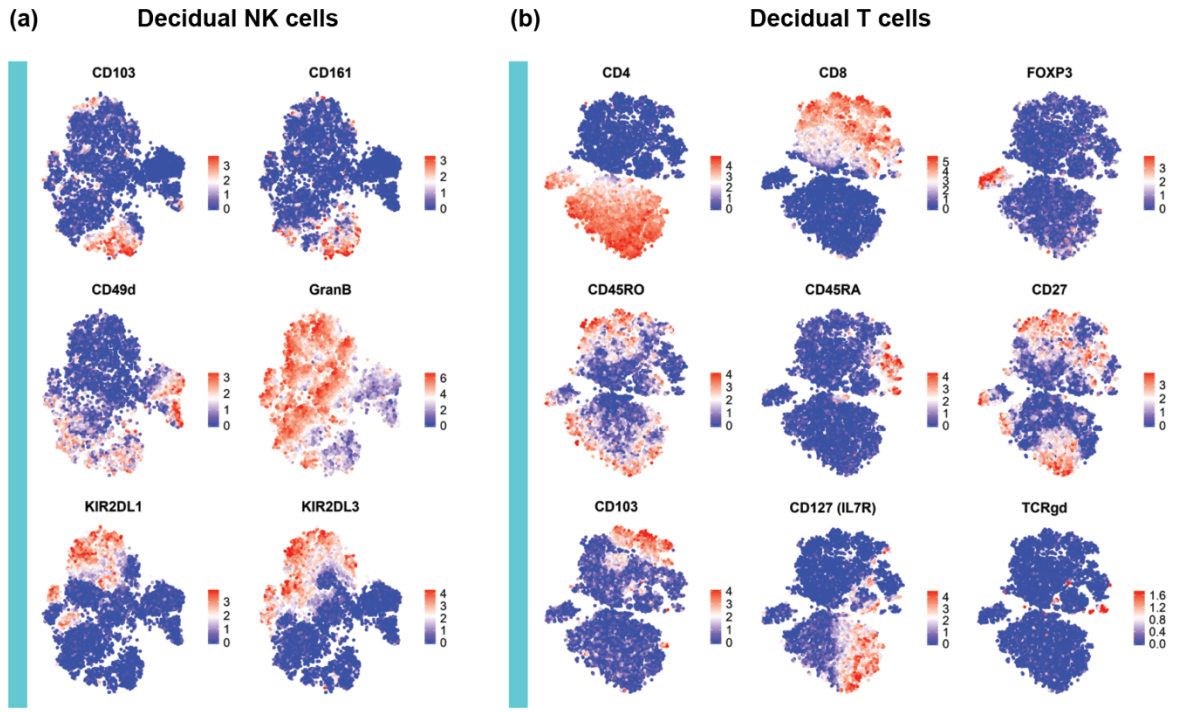

Figure 3. CyTOF validation of dNK and decidual T cell subsets. (a) t-SNE plots showing protein expression of dNK and ILC markers on live cells gated on $CD45^{+}CD56^{+}CD14^{-}HLA\text{-}DR^{-}CD3^{-}$ events from Donor 3 maternal decidua. **(b)** t-SNE plots showing protein expression of T cell markers on live cells gated on $CD45^{+}CD3^{+}CD14^{-}HLA\text{-}DR^{-}CD56^{-}$ events from Donor 3 maternal decidua. (CyTOF data from Donors 2 and 10 shown in Appendix 7.)

4.3 Further characterization of maternal mononuclear phagocyte populations

Given the evidence for three maternal mononuclear phagocyte populations from the droplet-based scRNA-seq data ($CD11c^{hi}$ dMP1, $CD11C^{lo}$ dMP2, and plMP, the placenta-localized maternal mononuclear phagocytes) (**Chapter 3**), we sought to further profile these cell populations based on their respective gene signatures and relate their characteristics to existing knowledge about macrophages and genes enriched in the placenta and endometrium.

Using the droplet-based scRNA-seq data, we first visualized the most highly upregulated genes in each subset relative to all other cell types at the maternal-fetal interface (**Figure 4**, additional upregulated genes in Appendix 8). We also performed differential gene expression analyses specifically between the three mononuclear phagocyte populations (Appendix 9). The $CD11c^{hi}$ dMP1 population exhibits high expression of monocyte genes (e.g. *S100A8* and *S100A9*) along with macrophage markers (e.g. *CD68*), suggesting a monocyte-derived macrophage phenotype. Meanwhile, the $CD11C^{lo}$ dMP2 population exhibits high expression of tissue-resident macrophage markers *CD209* (*DC-SIGN*), *CD206* (*MRC1*), and *MAF*, indicating a more differentiated macrophage phenotype. The expression of monocyte markers is also evident in plMP, suggesting its monocyte-derived origin. In addition, the plMP

population expresses *GCHFR*, a regulator of nitric oxide production, and high levels of *APOC1* and *APOE*, genes involved in lipoprotein synthesis.

Next, in order to functionally annotate the mononuclear phagocyte populations, we performed gene ontology (GO) and Reactome enrichment analysis on all genes significantly upregulated (log_2(fold-change) ≥ 0.5, adjusted $p < 0.05$) in each subset relative to other decidual and placental cell populations (**Figures 5-7**). We then examined the overrepresented Reactome pathways and GO terms from the biological process (BP) and molecular function (MF) categories. The results from our enrichment analyses suggest that the mononuclear phagocytes collectively play important roles in balancing immunity, inflammation, tolerogenicity, and tissue remodeling. Overrepresented terms describe processes related to phagocytosis and to pathogen response and debris clearance (e.g. dMP1: endopeptidase activity, pattern recognition receptor activity; dMP2: endopeptidase activity, antigen processing-cross presentation; plMP: intracellular pH reduction, scavenging by class A receptors), regulation of inflammatory and immunosuppressive responses (e.g. dMP1: arachidonic acid binding, chemokine receptors bind chemokines; dMP2: icosanoid biosynthetic process, myeloid leukocyte cytokine production; plMP: neutrophil aggregation, regulation of IL-10 secretion), and modulation of cell adhesion and tissue remodeling (e.g. dMP1: smooth muscle adaptation; dMP2: VEGFR signaling pathway; plMP: blood vessel morphogenesis, collagen degradation). Additionally, dMP1 and plMP specifically exhibit overrepresentation of terms associated with ROS production and detoxification (e.g. dMP1: respiratory burst, cellular oxidant detoxification; plMP: ROS biosynthetic process, detoxification of ROS), as well as lipid metabolism and transport (e.g. dMP1: fat cell differentiation, LDL receptor activity; plMP: positive regulation of cholesterol storage, lipid binding).

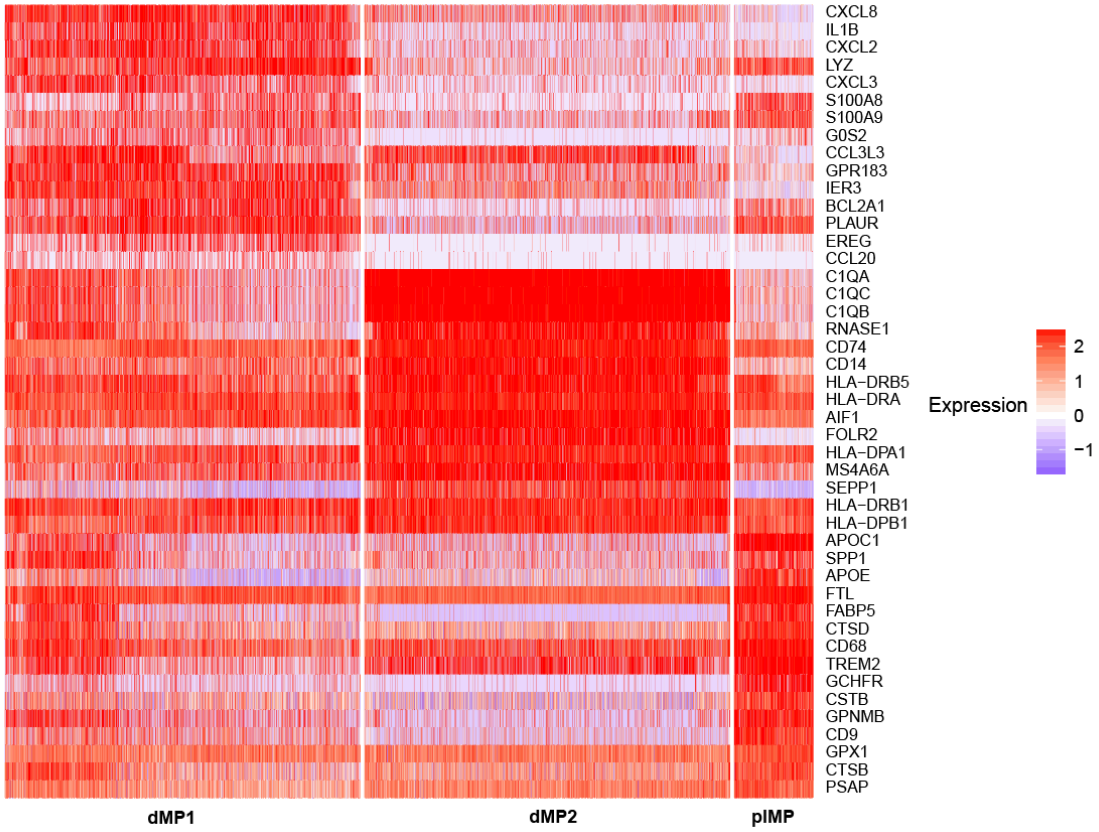

Figure 4. Heatmap of marker genes for the three identified maternal mononuclear phagocyte populations. The 15 most highly upregulated genes distinguishing each mononuclear phagocyte subset from all other cell types at the maternal-fetal interface are plotted in order of decreasing $\log_2$(fold-change). Genes which are significantly upregulated in multiple mononuclear phagocyte subsets are plotted with the subset in which they exhibit the greatest $\log_2$(fold-change). In particular, dMP1 and pIMP express high levels of the monocyte markers *S100A8* and *S100A9*, suggesting a monocyte-derived origin.

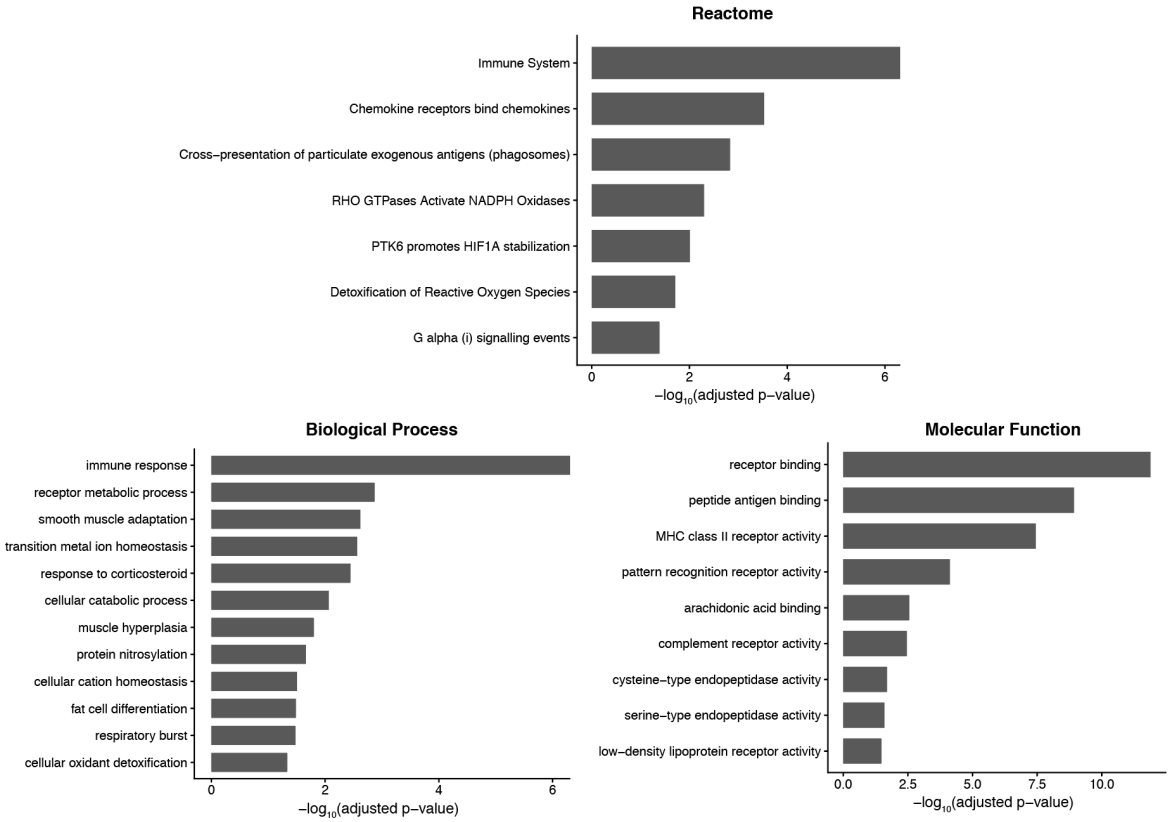

Figure 5. Genes involved in phagocytic activity and immune response, arachidonic acid signaling, ROS detoxification, tissue remodeling, and lipid metabolism are overexpressed in dMP1. GO and Reactome term enrichment analysis was performed on the genes upregulated by $\log_2$(fold-change) ≥ 0.5 in dMP1 relative to all other cell types at the maternal-fetal interface. Enriched terms are ordered by adjusted *p*-value.

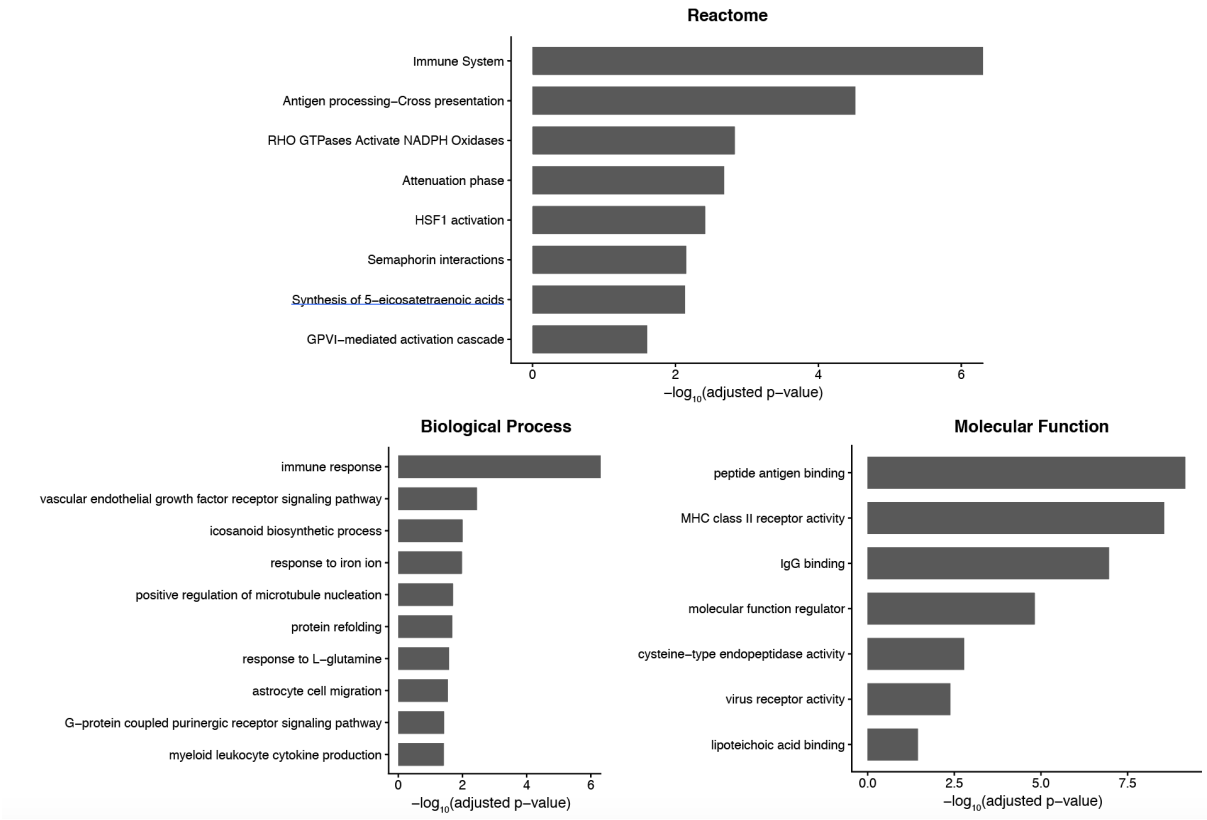

Figure 6. Genes involved in phagocytic activity and immune response, arachidonic acid signaling, and tissue remodeling are overexpressed in dMP2. GO and Reactome term enrichment analysis was performed on the genes upregulated by $\log_2$(fold-change) ≥ 0.5 in dMP2 relative to all other cell types at the maternal-fetal interface. Enriched terms are ordered by adjusted *p*-value.

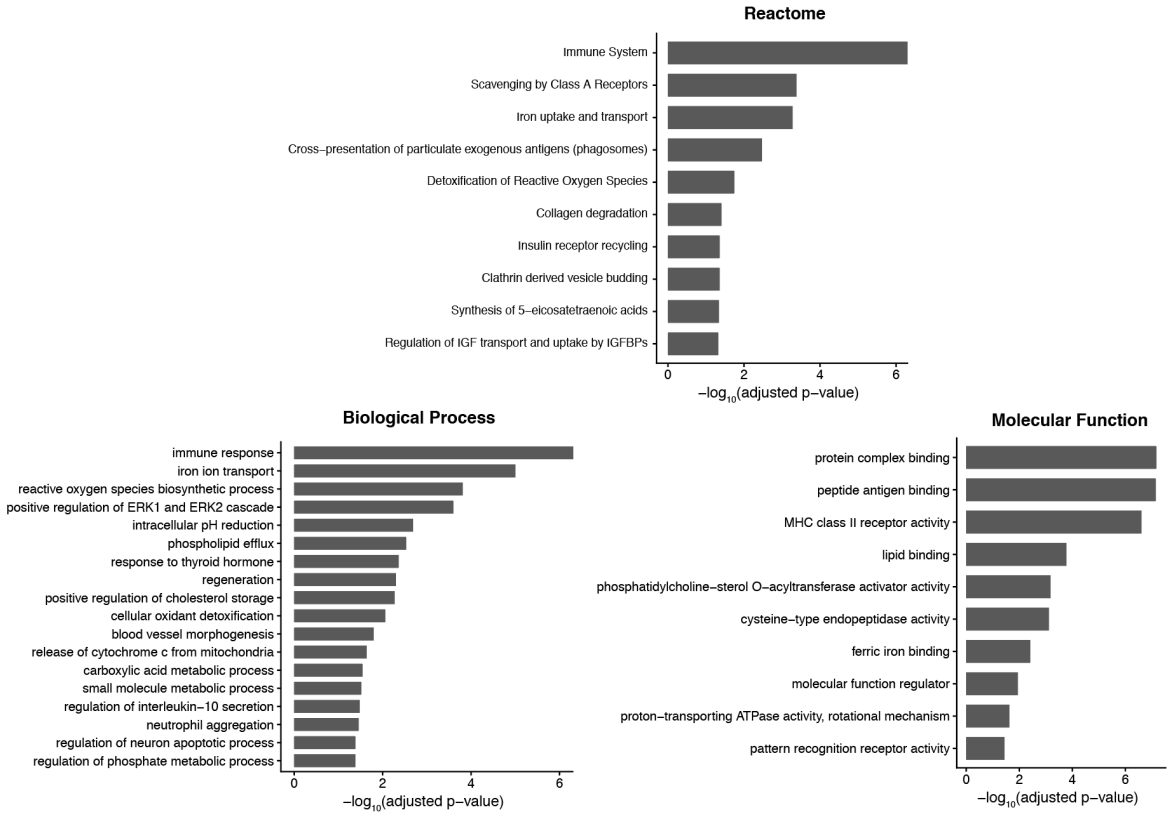

Figure 7. Genes involved in phagocytic activity and immune response, arachidonic acid signaling, ROS detoxification, tissue remodeling, and lipid metabolism are overexpressed in plMP. GO and Reactome term enrichment analysis was performed on the genes upregulated by $\log_2$(fold-change) ≥ 0.5 in plMP relative to all other cell types at the maternal-fetal interface. Enriched terms are ordered by adjusted *p*-value.

To further characterize the three mononuclear phagocyte subsets, we next examined their gene expression patterns in relation to the classical model of macrophage polarization into M1 (inflammatory) and M2 (non-inflammatory) populations[64]. We intersected a list of canonical M1 macrophage and M2 macrophage marker genes[139] with the upregulated gene sets associated with each of our three populations (**Figure 8**, Appendix 10). CD11c^{hi} dMP1 expressed significantly elevated levels of several classical M1 genes, including the inflammatory cytokines *CCL20* and *TNF*, while also expressing M2 genes such as *CXCR4* and *IL-10*, which have been implicated in resolving inflammation and tissue remodeling. CD11C^{lo} dMP2 exhibited enriched expression of several M2 genes, including *TGF-β*, the scavenger receptor *MSR1* and mannose receptor *MRC1*, and insulin-like growth factor *IGF2*, but also highly expressed the M1-associated interleukin receptor *IL2RA*. Finally, plMP showed upregulation of *SLC31A2*, associated with copper ion-mediated antimicrobial responses in M1 macrophages, along with M2 macrophage-linked *LIPA* (lysosomal lipase), scavenger receptor *CD36*, and *FN1* (fibronectin). Collectively, these results suggest that decidual and placental maternal mononuclear phagocytes appear to exhibit a mixture of conventional M1-like and M2-like characteristics, although dMP1 appears

to express more pro-inflammatory cytokines and dMP2 more genes involved in tissue clearance. Overall, however, these subsets cannot be fully distinguished along the classical M1/M2 polarization axis (p = 0.3806, Fisher's exact test).

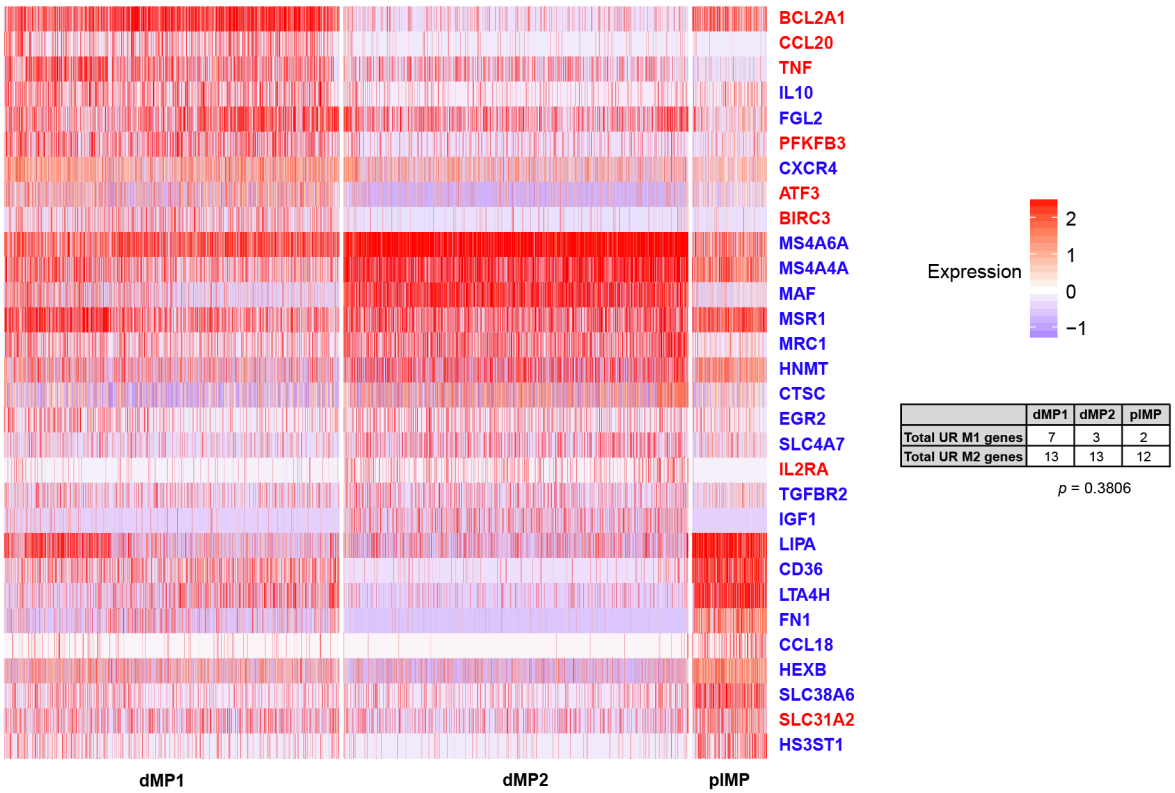

Figure 8. Each of the maternal mononuclear phagocyte populations exhibits upregulation of both canonical M1 and M2 macrophage genes. Heatmap plotting in order of decreasing $\log_2$(fold-change) genes that distinguish M1 macrophages (red) and M2 macrophages (blue)[64] and that are significantly upregulated in each mononuclear phagocyte population (full results in Appendix 10). Genes which are significantly upregulated in multiple mononuclear phagocyte subsets are plotted with the subset in which they exhibit the greatest $\log_2$(fold-change). The subsets express a mixture of pro-inflammatory and anti-inflammatory genes, suggesting they do not significantly align with the conventional M1/M2 transcriptional and functional polarization of macrophages (p = 0.3806, Fisher's exact test).

An emerging paradigm is that macrophages, in addition to their conventional phagocytic capacity and roles in regulating host immunity to infections, assume an array of specialized functions tailored to their tissue of residence, as reflected by distinct transcriptional and epigenetic programs[74]. For example, the specific roles of osteoclasts in regulating bone resorption and synthesis are mediated by their expression and activation of genes in the RANK signaling pathway[77]. Additionally, existing knowledge about the extensive tissue adaptation of populations such as the dNK cells suggests that this degree of specialization exists in other components of the immune cell compartment as well. We therefore sought to further profile the potential functions of our identified resident immune cell subsets from a tissue-

centered perspective, hypothesizing that these populations might upregulate certain genes which are enriched in the placenta and endometrium that would be indicative of their tissue-specific functions.

To compile a list of placenta- and endometrium-enriched genes to study in our resident immune cell subsets, we used tissue-level gene expression data deposited in the Human Protein Atlas[127,128], which was collectively generated from bulk RNA-seq of 37 different human tissues from 122 individuals. For each gene, we calculated a tissue-specificity index, Tau[140], and determined the tissue(s) in which the gene was most highly expressed, ultimately identifying 410 genes with Tau ≥ 0.8 (Appendix 5) to be significantly enriched in the placenta and/or endometrium relative to other tissues (see Methods). We then visualized the overlap between these tissue-enriched genes and the significantly upregulated genes among each of the maternal resident immune populations (**Figure 9**).

Overall, we observed that the mononuclear phagocyte populations express a large proportion of the immune cell marker genes which are enriched in placenta and endometrium, suggesting their high degree of tissue adaptation relative to even other resident immune cell populations. The upregulated placenta- and endometrium-enriched genes which characterize mononuclear phagocytes collectively span a number of potential tissue-specialized functions. Among these are lipid uptake and metabolism, as suggested by upregulation of genes encoding LDL receptor *OLR1* in dMP1, and *PLTP* and *ENPP2*, which modulate phospholipid transfer and lipid signaling, in dMP2. The mononuclear phagocytes additionally express a number of genes involved in cell adhesion and tissue or vascular remodeling, including *PECAM1* in dMP1; *PDGFB* (platelet-derived growth factor) in dMP2; and *SPP1* (osteopontin), *FN1* (fibronectin), and *CTSL* (cathespin L1, a collagen proteinase) in plMP. Furthermore, they appear to regulate inflammation and immunity through multiple mechanisms, including via immunoglobulin gamma Fc receptors *FCGR2A/B* in dMP2/1 and folate (*FOLR2*) or prostaglandin synthesis (*HPGDS*) and signaling in dMP2.

We also examined the tissue-resident profiles of other decidual immune populations. DCs exhibit upregulation of *IDO1*, an immunomodulatory molecule which dampens T cell function, and the peptidase inhibitor *SERPINB9*. dNK cells exhibit upregulation of genes including hypoxia-induced *EPAS1*, the NK receptor *KIR2DL1*, and *AREG*, which regulates gland development. Interestingly, these are all molecules defining the dNK1 subset (Vento-Tormo R, Efremova M et al., under submission), suggesting that this dNK population exhibits the strongest tissue-resident phenotype. Overall, intersection of the placenta- and endometrium-enriched gene set with the maternal resident immune

cell marker genes underscores the tissue specialization of all maternal immune cell populations, and in particular that of the decidual and placental mononuclear phagocytes.

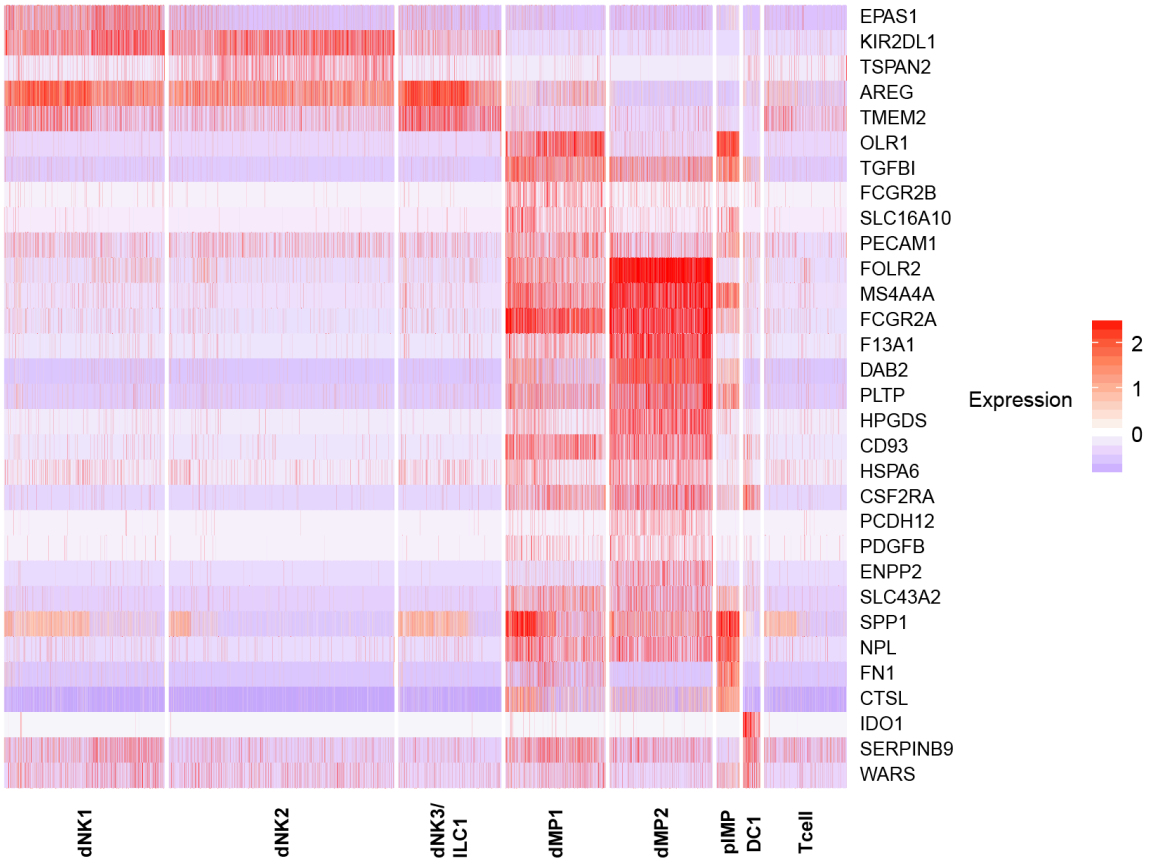

Figure 9. Maternal resident immune cell subsets exhibit upregulation of placenta- and endometrium-enriched genes. Heatmap plotting in order of decreasing $\log_2$(fold-change) the genes that are enriched in the placenta and endometrium and that are significantly upregulated in each maternal resident immune cell population. Genes which are significantly upregulated in multiple subsets are plotted with the subset in which they exhibit the greatest $\log_2$(fold-change). The maternal mononuclear phagocyte populations exhibit upregulation of a large number of the tissue-enriched genes relative to other immune subsets.

4.4 Discussion

In this chapter, I have integrated single cell-level protein and transcriptomic data to study the decidual immune cell populations in depth. I first presented results demonstrating the utility of FACS and CyTOF in validating the distinct immune subsets identified from transcriptome-based clustering and annotation. I then utilized gene ontology (GO) and Reactome enrichment analysis, M1/M2 macrophage signatures, and data from the Human Protein Atlas to functionally characterize the maternal mononuclear phagocyte subsets and dissect the tissue-resident transcriptional profiles of the decidual immune cell populations.

FACS gating revealed two myeloid cell populations distinguishable by $CD14^{+}HLADR^{+}$ and $CD14^{++}HLADR^{++}$ surface phenotypes that corresponded to the $CD11c^{hi}$ and $CD11C^{lo}$ decidual mononuclear phagocyte subsets defined by analysis of single-cell transcriptomes (**Figure 1**). Through CyTOF, we further confirmed the presence of $CD11c^{hi}$ and $CD11C^{lo}$ mononuclear phagocytes at the protein level, while also detecting two DC subsets, $CLEC9A^{+}$ DC1 and $CD1C^{+}$ DC2, in the decidua (**Figure 2**). CyTOF also validated the presence of $CD8^{+}$ and $CD4^{+}$ T cells and $FOXP3^{+}$ Treg cells in the maternal decidua, and identified a small population of gamma-delta T cells (**Figure 3b**). We show that most decidual T cells are antigen-experienced ($CD45RO^{+)}$, consistent with previous studies of T cell surface markers in early pregnancy decidua[170], and that a proportion of $CD8^{+}$ T cells express CD103, a marker of tissue residency. Interestingly, the CyTOF data confirms the absence of CD127 (IL-7R) among $CD8^{+}$ T cells. In most naive and mature T cell populations, IL-7/IL-7R signaling supports T cell survival and homeostasis through regulation of pro- and anti-apoptotic genes[169], induction of the JAK/STAT pathway, which mediates cell survival and proliferation[171], and modulation of glucose metabolism[172]. The lack of IL-7R in decidual $CD8^{+}$ T cells suggests that these populations are maintained and regulated through differential, perhaps decidua-specific, signaling mechanisms. Lastly, our CyTOF analysis also validated the three novel dNK subsets initially identified by scRNA-seq, confirming the presence of a dNK1 population expressing high levels of inhibitory KIR molecules and Granzyme B, dNK2 cells expressing Granzyme B but lower levels of KIR molecules, and a dNK3/ILC1 population with low Granzyme B expression but high expression of ILC1 markers CD161 and CD103 (**Figure 3a**). Overall, CyTOF independently validated the scRNA-seq-driven annotations of immune cell types by demonstrating that the distinctions between these cell subsets exist at the protein level as well as at the transcriptomic level. This has particular implications for defining the functional roles assumed by each identified cell population. For example, as evidenced by studies reporting the influence of maternal KIR molecules on susceptibility to preeclampsia[150] and other adverse pregnancy outcomes[44,47,48], the KIR repertoire expressed by dNK cells strongly shapes regulation of their activation and function. Thus, our demonstration through CyTOF that KIRs are variably expressed between dNK subsets underscores the potential that these populations can engage in differing interactions with other cell types at the maternal-fetal interface, and that the functional outcomes of these cell-cell interactions differ. In contrast to previous studies that consider dNK as a homogeneous population[39,150], we have

identified and validated the presence of dNK subsets that likely assume distinct functions at the maternal-fetal interface which reflect their respective transcriptional and phenotypic profiles.

Although decidual mononuclear phagocyte populations have been characterized previously through sorting based on CD11C and microarray gene expression profiling[70], scRNA-seq enabled unbiased, high-resolution dissection of the transcriptomes and potential functions corresponding to these subsets. Moreover, demuxlet analysis uncovered the presence of an additional maternal mononuclear phagocyte population in the placenta (plMP) that we further analyzed and compared with the two decidual mononuclear phagocyte subsets (CD11c^{hi} dMP1 and CD11C^{lo} dMP2). We first evaluated the most highly upregulated markers in each of the subsets relative to other cell populations at the maternal-fetal interface (**Figure 4**; **Appendix 8**). Consistent with previous literature reporting the upregulation of angiogenic factor VEGF[56], scavenger receptor CD163[55], complement protein C1q[54], and matrix metalloproteinases MMP7 and MMP9 in decidual macrophages[54,58], *VEGF* was specifically overexpressed in dMP1, *CD163* and *C1q* genes in dMP2, *MMP7* and *MMP9* in plMP. However, despite previous observations of elevated expression of *PDCD1* (*PD-1*) and B7-family molecules[51], *IDO*[52], and *VEGFR/FLT-1*[59] in decidual macrophages, we did not find them to be significantly overexpressed in any of the mononuclear phagocyte subsets relative to other decidual cell populations in our study.

Next, we performed differential expression analyses between each of the three mononuclear phagocyte populations using the droplet-based scRNA-seq data (Appendix 9). Consistent with the Houser et al. study describing two decidual CD11c$^{hi/lo}$ macrophage subsets[70], we observed upregulation of the pattern recognition receptor *CLEC5A*; growth factors *AREG*, *EREG*, and *IGF1*; lipoprotein receptor *OLR1*, and inflammatory mediator *TREM1* among CD11c^{hi} dMP1 mononuclear phagocytes. Similar to the CD11C^{lo} population described by Houser et al.[70], CD11C^{lo} dMP2 significantly upregulates *CD209* (*DC-SIGN*), *CD206* (*MRC1*), and *SEPP1*, a secreted glycoprotein associated with antioxidant activity[173]. We also found vacuole membrane protein 1 (*VMP1*) to be significantly overexpressed in CD11C^{lo} dMP2, consistent with observations that the CD11C^{lo} population is more highly vacuolated[70]. Finally, we observed that dMP1 exhibits upregulation of the genes encoding *CCL4* (*MIP-1β*), *IL-10*, *TNF-α*, and *IL1-β*, which were also among the cytokines found to be secreted preferentially by CD11c^{hi} relative to CD11C^{lo} macrophages[70]. Meanwhile, in contrast to Houser et al., *CD1C*, *UNC5B*, *DMD*, *HRH1*, *WNT5B*, and *STON2*, additional genes reported to be differentially expressed between CD11c^{hi} and CD11C^{lo} macrophages[70], were not found to be significantly differentially expressed in any of our three

mononuclear phagocyte subsets. The new plMP population we have identified does not express significantly high or low levels of CD11C, but exhibits elevated expression of *PPAR-γ*, lipoprotein receptor *OLR1*, and lipoprotein lipase (*LPL*), all previously associated with CD11c^{hi} macrophages, and upregulation of the insulin-like growth factor *IGF2* and IGF-binding protein *IGFBP3*. plMP also expresses significantly lower levels of *IL1-β*, *TNF-α*, and *CCL4* than the two decidual mononuclear phagocyte populations, which were among the cytokines reported to be highly secreted by CD11c^{hi} macrophages. Thus, taken together, the transcriptional profiles of dMP1 and dMP2 largely align with a previous study of CD11c^{hi} and CD11C^{lo} macrophages in the decidua, while the expression patterns of these same genes in plMP appear to distinguish this subset as a distinct population.

I subsequently performed gene ontology (GO) and Reactome enrichment analysis on the genes significantly overexpressed by $\log_2$(fold-change) ≥ 0.5 in each of the mononuclear phagocyte subsets relative to other cell types at the maternal-fetal interface (**Figure 5-7**). Overall, GO terms and Reactome pathways overrepresented among each of the subsets reflected many of the general functions known to be associated with decidual mononuclear phagocytes, including pattern recognition, scavenger, and chemokine receptor activity (dMP1/2, plMP); antigen processing and cross-presentation (dMP1/2, plMP); regulation by hormones (dMP1 and plMP); and pH reduction, GTPase activity, and catabolic functions indicative of phagosome maturation (dMP1/2, plMP). Moreover, consistent with the functional capabilities proposed by Houser et al. for CD11c^{hi} macrophages[70], dMP1 are associated with proinflammatory response (pattern recognition receptor and complement receptor activity) mediated by hormones (response to corticosteroids), and lipid metabolism functions including fat cell differentiation and LDL receptor activity. Meanwhile, dMP2 are linked to extracellular communication (semaphorin interactions, receptor binding), tissue and vascular remodeling (VEGFR signaling pathway), and cell growth (microtubule nucleation; enriched expression of multiple IGFBPs), as previously described in CD11C^{lo} macrophages[70]. At the same time, our GO analysis suggested that some of these functions are in fact shared across subsets rather than restricted to one population. For example, apart from dMP2, both dMP1 and plMP also appear to be enriched in genes regulating various aspects of tissue and vascular remodeling, as evidenced by the association of dMP1 with smooth muscle adaptation and of plMP with blood vessel morphogenesis and collagen degradation. Intriguingly, the GO and Reactome annotations also highlighted functions not previously well-described among macrophages at the

maternal-fetal interface, including arachidonic acid signaling, lipid transport and metabolism, and ROS generation and detoxification.

Meanwhile, our comparison of upregulated maternal mononuclear phagocyte genes to established M1 and M2 macrophage gene signatures reveals that none of the three subsets exhibited gene expression profiles aligning with this classical macrophage polarization scheme (**Figure 8**). These findings parallel previous observations that resident macrophages in many other tissues tend to exhibit phenotypic plasticity and characteristics which are uniquely specialized to their tissue of origin rather than fully describable by the M1/M2 binary classification[74]. Moreover, these results further reinforce the notion that in the decidual and placental milieu, where processes such as initiation and resolution of inflammation and tissue growth, disruption, and remodeling must be carefully fine-tuned over the duration of pregnancy, cell populations tend to exhibit mixed phenotypes rather than stark polarization in their functions.

The maternal mononuclear phagocytes, notably dMP2, appear to be among the most highly tissue-specialized immune cell populations, based on the number of placenta- and endometrium-enriched genes which are upregulated in these cells relative to other immune cell types (**Figure 9**). However, our analyses suggest that many of the maternal immune populations exhibit some degree of tissue adaptation. For example, the dNK express highest levels of KIR2DL1, an inhibitory receptor known to specifically engage HLA-C-expressing cells such as trophoblasts and a marker gene defined for our newly identified dNK1 population (**Figure 9**). Other dNK-upregulated genes include hypoxia-inducible factor EPAS1 and epithelial growth factor-related ligand AREG, reflecting the specialized roles of dNKs in regulating placentation and underscoring how the particular tissue microenvironment of early pregnancy (e.g. oxygen tension[1]) in turn shapes dNK phenotype and function. One limitation of our tissue-enriched gene analysis, however, is that the Human Protein Atlas gene expression data for placenta and endometrium includes both term placenta and decidua samples as well as samples from the non-pregnant endometrium[128,174], in contrast to our tissues exclusively derived from early pregnancy. Given the profound cellular and molecular changes known to occur at the maternal-fetal interface between the different reproductive stages, the placenta and endometrium gene list produced by our analysis may not completely reflect the genes that are actually expressed in a tissue-enriched manner during early pregnancy.

Taken collectively, our GO, Reactome, and placenta and endometrium gene enrichment analyses suggest some notably interesting new findings about the maternal mononuclear phagocytes in particular. First, it appears that all three of the identified subsets, and not just the CD11C^{lo} dMP2-like macrophages as previously reported[70], appear to be capable of participating in tissue remodeling activities at the maternal-fetal interface, as we found angiogenic factors such as VEGF, matrix metalloproteinases such as MMP7 and MMP9, and ECM-related proteins such as fibronectin and cathespin to also be highly expressed among the dMP1 and plMP populations (**Figure 4**; **Appendix 8**), and processes such as smooth muscle adaptation, blood vessel morphogenesis, and collagen degradation to be overrepresented in these subsets (**Figure 5-7**). Building on previous studies, we show that all maternal mononuclear phagocyte populations, in addition to modulating inflammation through secreting mixtures of proinflammatory and anti-inflammatory cytokines, regulate synthesis and signaling via arachidonic acid, an omega-6 fatty acid, and its metabolites, including prostaglandins and leukotrienes[175]. Arachidonic acid metabolism has previously been described in activated human alveolar macrophages[176] and resident mouse peritoneal macrophages[177,178], and its apparent regulation by the mononuclear phagocytes potentially serves to further modulate inflammatory processes at the maternal-fetal interface.

In addition to arachidonic acid signaling, GO analyses suggest that dMP1 and plMP also appear to be particularly specialized for general lipid processing and metabolism (**Figure 4-7**). While most mononuclear phagocytes are capable of metabolizing lipids obtained via phagocytosis, there is accumulating evidence that macrophages residing in certain tissues are particularly specialized in this function[179]. For instance, relative to other macrophage populations, liver Kupffer cells and alveolar macrophages are both enriched in core lipid metabolism genes, such as PPAR-γ, which modulates lipid uptake and cholesterol efflux[180,181], or liver X receptors (LXRs), which regulate cholesterol sensing and transport[182]. Alveolar macrophages deficient in PPAR-γ exhibit widespread alterations in gene expression[181] and impaired catabolism of pulmonary surfactant, a lipoprotein complex produced by alveolar epithelial cells to facilitate gas exchange in the lung[183]. This results in excess surfactant buildup in the macrophages and alveoli, inducing a state of low-grade inflammation and driving the development of pulmonary alveolar proteinosis[181]. Meanwhile, in atherosclerosis, the aggregation of pro-inflammatory lipoprotein-containing plaques on arterial walls promotes the recruitment of monocyte-derived macrophages[184]. Excess lipoprotein uptake and metabolism converts the macrophages into

cholesterol-laden foam cells, which exacerbate atherosclerotic plaque progression through the production of proinflammatory cytokines, proteases, and other factors[185]. Given the importance of macrophage-mediated lipid processing in other tissues, it is conceivable that macrophages are also central to maintaining lipid homeostasis at the maternal-fetal interface, particularly as apoptotic cell clearance and placental transfer of cholesterol and lipid metabolites are among the important processes occurring in this environment. Notably, pregnancies complicated by maternal obesity are associated with elevated placental triglyceride storage, altered lipid transport[186,187], and a persistent inflammatory state which has been shown to promote the increased recruitment of macrophages to the placenta[188]. Although the fetal or maternal origin of these macrophages was not conclusively determined, these data parallel the observation of increased maternal leukocyte accumulation and infiltration of the placenta in chronic noninfectious villitis, another complication of pregnancy which is positively correlated with increased maternal BMI[189]. Thus, lipid metabolism by the maternal mononuclear phagocytes may represent yet another means by which they promote or resolve inflammation at the maternal-fetal interface.

Additionally, we show that dMP1 and plMac upregulate genes involved in the production and detoxification of reactive oxygen species (ROS) (**Figure 4-7**). Although ubiquitously generated by cells as a byproduct of mitochondrial respiration, ROS are also specifically released by macrophages to mediate intracellular killing of pathogens[190], and have been shown to contribute to regulating macrophage differentiation and polarization[191]. At the maternal-fetal interface, ROS has also been found to assume important roles in facilitating endometrial changes, blastocyst implantation, and placentation, among other processes[192]. Notably, oxidative burst, or the rapid cellular release of ROS, has been shown to induce trophoblast invasion by triggering their switch from a proliferative to invasive state[193], to regulate the ability of trophoblasts to fuse into the syncytiotrophoblast layer of the placenta[194], and to modulate trophoblast autophagy and apoptosis[195]. Additionally ROS promotes increased production of VEGF, HIF-1[196], and other angiogenesis-promoting transcriptional factors[197], while also oxidizing phospholipids, which can independently promote vascular growth factor production by activating TLR2 signaling in endothelial cells[198]. On the other hand, excessive oxidative stress, either through elevated ROS generation or deficient antioxidant activity, has been linked with the onset of preeclampsia, intrauterine growth restriction, and other placenta-associated conditions[192]. Thus, the potential roles that dMP1 and plMP play in modulating the balance of ROS in decidual and placental tissues may have

direct implications for pregnancy success while representing yet another potential dimension through which they contribute to the fine-tuning of tissue homeostasis at the maternal-fetal interface.

As a whole, our analyses of the maternal mononuclear phagocyte compartment at the maternal-fetal interface have enabled us to characterize the transcriptional profiles and potential functional roles assumed by each of the three subpopulations. These annotations collectively highlight the tissue-specific nature of these cells rather than their adherence to the classical M1/M2 model of macrophage polarization.