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Single-cell RNA sequencing reveals immunological rewiring at the maternal-fetal interface following asymptomatic/mild SARS-CoV-2 infection

Graphical abstract

Highlights

- Asymptomatic and mild COVID-19 significantly remolds the maternal-fetal interface
- Altered frequency of decidual macrophages, T regs, and activated T cells
- Antigen presentation and type I IFN signaling are attenuated in decidual macrophages
- T cell repertoire diversity in circulation and decidua is reduced

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In brief

Sureshchandra et al. describe extensive changes in blood and decidua obtained from pregnant women experiencing asymptomatic and mild SARS-CoV-2 infection. These data indicate that, regardless of severity, COVID-19 re-models the maternal-fetal interface with potentially significant ramifications for pregnancy and neonatal outcomes.
Single-cell RNA sequencing reveals immunological rewiring at the maternal-fetal interface following asymptomatic/mild SARS-CoV-2 infection

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SUMMARY

While severe coronavirus 2019 (COVID-19) is associated with immune activation at the maternal-fetal interface, responses to asymptomatic/mild severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infection during pregnancy remain unknown. Here, we assess immunological adaptations in blood and term decidua in response to asymptomatic/mild disease in pregnant women. We report attenuated antigen presentation and type I interferon (IFN) signaling pathways, loss of tissue-resident decidual macrophages, and upregulated cytokine/chemokine signaling in monocyte-derived decidual macrophages. Furthermore, we describe increased frequencies of activated tissue-resident T cells and decreased abundance of regulatory T cells with infection while frequencies of cytotoxic CD4/CD8 T cells are increased in the blood. In contrast to decidual macrophages, type I IFN signaling is higher in decidual T cells. Finally, infection leads to a narrowing of T cell receptor diversity in both blood and decidua. Collectively, these observations indicate that asymptomatic/mild COVID-19 during pregnancy results in remodeling of the immunological landscape of the maternal-fetal interface, with a potential for long-term adverse outcomes for the offspring.

INTRODUCTION

The current pandemic of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) continues to be a significant global threat (Huang et al., 2020). Overwhelming evidence suggests that pregnant women are a high-risk group for COVID-19 (Subbaraman, 2021). Indeed, a study on severe COVID-19 infections in pregnant women from 18 countries has reported higher rates of adverse outcomes such as mortality, preeclampsia, and preterm birth (Villar et al., 2021). While pregnant women with a severe COVID-19 diagnosis are at 62% higher odds of getting admitted to the intensive care unit (ICU) compared with non-pregnant women of reproductive age (Subbaraman, 2021; Yap et al., 2020), a majority of those who get exposed to the virus experience asymptomatic or mild COVID-19 (Edlow et al., 2020b; Li et al., 2020; Lokken et al., 2020; Panagiotakopoulos et al., 2020). Both vulnerability and immune responses to viral infections during pregnancy can be distinct compared with non-gravid individuals, as observed in influenza, hepatitis E, varicella, and measles (Jamieson et al., 2006; Kourtis et al., 2014; Sappenfield et al., 2013). These differences are primarily driven by peripheral immune adaptations during pregnancy (Aghaeepour et al., 2017, 2018) that balance fetal tolerance and growth with host defense. A recent analysis of the peripheral immune system of mothers with asymptomatic disease revealed an increase in low-density neutrophils (LDNs) but no gross changes in leukocyte frequencies, activation, or function (De Biasi et al., 2021). Furthermore, the cytokine storm that is characteristic of severe COVID-19 in the general population is hardly reported in cases of severe COVID-19 among pregnant patients (Hojyo et al., 2020; Wang et al., 2020). These findings support the hypothesis that pregnancy limits the induction of exuberant peripheral inflammatory responses to SARS-CoV-2 infection that are widely reported in non-pregnant individuals.

The immunological landscape of the maternal-fetal interface (placenta) undergoes significant changes during pregnancy. The decidual compartments of the placenta harbor maternal immune cells (macrophages, natural killer [NK] cells, and T cells [Vento-Tormo et al., 2018]), which exhibit mixed signatures of activation and regulatory phenotype that correlate with gestation (van der Zwan et al., 2018) and can respond to foreign particles at the maternal-fetal interface (Ander et al., 2019). However, details of decidual adaptations to a respiratory infection such as
COVID-19 are slowly beginning to emerge. Although available data strongly suggest a lack of vertical transmission (Edlow et al., 2020b; Garcia-Flores et al., 2021; Schwartz and Morotti, 2020; Vivanti et al., 2020) with rare detection of viral RNA in the placenta (Edlow et al., 2020a), severe COVID-19 has been shown to trigger maternal inflammatory responses at the maternal-fetal interface (Garcia-Flores et al., 2021; Lu-Culligan et al., 2021). Specifically, an increase in markers associated with preeclampsia and activation of placental NK cells and T cells and increased expression of interferon-related genes and stress-associated heat-shock proteins have been reported (Lu-Culligan et al., 2021). However, placental immune rewiring with asymptomatic/mild infections and how that relates to peripheral immune adaptations remain poorly understood.

Therefore, in this study, we examined the effect of maternal asymptomatic/mild SARS-CoV-2 infection occurring during late pregnancy on peripheral and decidual immune cells of the maternal-fetal interface at term. We used single-cell RNA sequencing and multicolor flow cytometry to profile changes in the immune landscape of maternal decidua. Our analysis revealed an expansion of CD69+ T cells and selective loss of HLA-DRhigh regulatory macrophages and regulatory T cells (Tregs) with asymptomatic/mild SARS-CoV-2 infection in the decidua. Single-cell analysis revealed broad activation of myeloid cells in the decidua, with enrichment of cytokine-secreting subsets, attenuation of interferon signaling, and a concomitant drop in expression of major histocompatibility complex (MHC) class II molecules in blood monocyte-derived macrophage subsets. Activated T cells and terminally differentiated CD8 T cells (TEMRAs) were enriched with infection, but an expansion of cytotoxic CD4 and antiviral CD8 T cells was only observed in the blood. While cytokine-signaling modules were attenuated in the blood, elevated interferon-signaling signatures were detected in the decidua. These findings highlight that even asymptomatic/mild infection can trigger placental immune activation with potential long-term consequences for the developing fetus.

RESULTS

Peripheral and decidual immunological changes in response to asymptomatic/mild SARS-CoV-2 infection

To comprehensively assess the impact of asymptomatic/mild SARS-CoV-2 infection on the immune landscape of the maternal-fetal interface, we collected decidua basalis (maternal membrane) and blood at delivery from mothers who experienced mild symptoms consistent with an upper respiratory tract infection not requiring medical care during pregnancy and had a positive SARS-CoV-2 PCR+ (mild) or those who tested positive during mandatory screening at the time of delivery but experienced no symptoms (asymptomatic) (Figure 1A; Table S1). We observed no differences in antibody titers (p = 0.599 for NP immunoglobulin G [IgG] and p = 0.87 for receptor-binding domain [RBD] IgG) between subjects in the mild and asymptomatic groups. Therefore, all subsequent analyses were performed on all SARS-CoV-2-exposed subjects (referred to as the asymptomatic/mild group) and compared with SARS-naive healthy pregnant subjects (healthy donors [HDs]). No difference in age or body mass index was observed between SARS-CoV-2-exposed subjects and HDs. Blood samples were used to obtain complete blood counts and plasma antibody titers and to assess changes in peripheral immune blood cell (peripheral blood mononuclear cells [PBMCs]) composition by flow cytometry (Figure 1A). Decidual immune cells were phenotyped using multispectral flow cytometry and single-cell RNA sequencing to assess immune perturbations at the maternal/fetal interface.

Complete blood cell counts indicated that absolute numbers of circulating granulocytes, monocytes, and platelets increased with infection, but no changes in lymphocyte numbers were detected (Figure S1A). Multicolor flow analysis of PBMCs (Figure S1B) revealed a redistribution of naive and memory T cell subsets in the absence of changes in total CD4 and CD8 T cell frequencies (Figures S1C and S1D). Specifically, infection status was associated with a reduced abundance of naive CD4 T cells but a modest expansion of central memory T cells (TCMs) (Figure S1D). The frequency of total B cells and relative abundance of naive/memory subsets did not vary significantly with infection (Figure S1E). Although proportions of total NK cells did not vary with infection, the relative proportions of CD16+CD56dim expanded with infection while those of the CD16+CD56+ subset decreased (Figure S1F). Similarly, while frequencies of total monocytes and dendritic cells (DCs) were comparable between the two groups (Figure S1G), a modest expansion of CD16++ non-classical monocytes and a significant drop in plasmacytoid DCs (pDCs) was noted (Figure S1H). Finally, no differences in surface expression of MHC class II molecule HLA-DR (Figure S1I) or activation marker CD86 (Figure S1J) were observed with infection.

Flow analysis of decidual leukocytes revealed no changes in total T or B cell frequencies (Figures 1B and S2A). However, we observed a modest expansion of CD4 EMs and a modest reduction in the CD8 naive T cells (Figure 1C) as well as frequencies of CD69+CD103+CD4 and CD8 tissue-resident T cells with asymptomatic/mild disease (Figure 1D). No differences in total decidual NK cell frequencies or subsets were observed (Figures S2B and S2C). However, proportions of both HLA-DRhigh macrophages and DCs decreased significantly with asymptomatic/mild infection (Figure 1E).

Unsupervised single-cell analysis of decidual leukocytes reveals heightened myeloid cell activation with asymptomatic/mild SARS-CoV-2 infection

We next assessed the rewiring of immune (CD45+) cell states with asymptomatic/mild infection in an unbiased way at the single-cell resolution. Decidual leukocytes from 4 individuals per group were multiplexed using lipid-tagged oligos (compatible with 3’ single-cell gene expression), sorted to remove dead cells, and analyzed using single-cell RNA sequencing (scRNA-seq) (Figure 1A). Following doublets and dead cell removal, dimension reduction, and clustering, our analysis identified 11 unique immune cell clusters (Figure 2A). Cluster annotations were derived from differential marker analysis (Figure 2B; Table S2) and confirmed using markers previously described for the
first-trimester decidual immune landscape (Vento-Tormo et al., 2018). Within the lymphoid clusters, B cells were identified based on high expression of CD79A; CD4 T based on IL7R levels; CD8 T on CD8A and GZMH expression; and NK cell subsets on differential expression of CSF1, GZMH, CXCR4, and ITGB2 (Vento-Tormo et al., 2018) (Figures 2A and 2B; Table S2). Myeloid clusters consisted primarily of macrophages, which were broadly divided by the magnitude of HLA-DRA expression (as observed in flow cytometry; Figure S2A; Table S2) into dMac1 (HLA-DR<sup>high</sup>) and dMac2 (HLA-DR<sup>low</sup>) (Vento-Tormo et al., 2018) and DCs, expressing high FCER1A (Figures 2A and 2B; Table S2).

We next stratified clusters by SARS-CoV-2-exposure status (Figures 2C and S3A) and patient of origin (since the cells from each subject were barcoded using oligo-tagged lipids) and compared cluster frequencies between the two groups (Figure S3B). No changes were observed in the relative frequency of decidual T cells (Figures S3C and S3D), B cells (Figure S3E), or NK cells (Figure S3F). Additional analysis shows a modest increase in dNK2 (Vento-Tormo et al., 2018) (Figure S3G); therefore, we identified genes differentially expressed with SARS-CoV-2 infection within decidual NK cells. Modest gene-expression changes (73 upregulated and 33 downregulated with infection) were identified with pathways associated with protein folding, MAPK signaling, and type I interferon signaling upregulated with infection, whereas those associated with immune activation and adhesion were downregulated (Figure S3H).

Interestingly, and in contrast to the flow-cytometry data presented above, no differences in DC frequencies were detected from the scRNA-seq data (Figure S3I). However, as observed with flow cytometry, we observed a selective loss of HLA-DR<sup>high</sup> macrophages (dMac1) with asymptomatic/mild SARS-CoV-2 infection (Figure 2D). Additional investigation on this subset...
Figure 2. Unsupervised analysis of CD45+ compartment within term maternal decidua and adaptations with asymptomatic/mild SARS-CoV-2 infection

(A) Uniform manifold approximation and projection (UMAP) representation of 22,566 immune cells within the term decidual CD45+ compartment showing 11 clusters.

(B) Violin plots of key gene markers used for cluster annotations. The y axis represents the normalized transcript counts.

(C) Stacked bar graph comparing the distribution of decidual immune cell clusters in healthy donors (n = 5, 10,153 cells) and mothers with asymptomatic/mild SARS-CoV-2 infection (n = 4, 12,413 cells).

(D) Box and whiskers comparing single-cell frequencies of two macrophage subsets dMac1 and dMac2 with asymptomatic/mild SARS-CoV-2 infection.

(E) UMAP of term decidual myeloid cells highlighting the diverse states within the dMac1 (HLA-DRA high) macrophage subset. Each color indicates one of 6 dMac1 clusters identified with a prominent marker highlighted.

(legend continued on next page)
revealed a high degree of heterogeneity, with the presence of 6 distinct macrophage states (Figure 2E). These included regulatory macrophages expressing TREM2 and activated subsets expressing high levels of complement gene C1Q, tetraspanin CD9, pro-inflammatory cytokine IL1B, chemokine CXCL10 (IP-10), or growth factor VEGFA (Figures 2E and 2F).

Although frequencies of dMac1 were reduced with infection (Figure 2G), the IL1B+ dMac1 subset expressed cytokines IL1B, CCL3, and CCL20 expanded with infection (Figure 2G). Moreover, robust gene-expression changes were observed within both macrophage subsets in response to infection (Figure 3A). In dMac1 macrophages, infection was associated with the induction of genes involved in the antiviral response, viral sensing, nuclear factor κB (NF-κB) signaling, and cytokine production, while a small number of genes that play a role in myeloid cell activation and antigen processing and presentation were suppressed (Figure 3B). More robust gene-expression differences were observed in the dMac2 subset (Figure 3A), altering signatures associated with immune activation, upregulating genes involved in chemotaxis, cell death, and interleukin-17 (IL-17) signaling, and suppressing genes involved in antigen processing and presentation (Figure 3C).

We next compared scores for specific signaling pathways across both groups. SARS-CoV-2 infection was associated with an increase in the scores of modules associated with cytokines and chemokine signaling (Figure 3D), notably NF-κB, tumor necrosis factor (TNF), and IL-17 signaling (Figure S4A) as well as pathogen-sensing pathways such as Toll-like receptor (TLR) and RIG-I signaling (Figure S4B). The higher chemokine module score is illustrated by increased expression of several chemokine genes (such as CCL3, CCL3L1, CCL4, CXCL3, and CCL5), notably in the dMac1 subset (Figures 3D and 3E). Interestingly, only the dMac2 subset exhibited heightened IL-6 STAT signaling (Figure 3D), including an upregulation of inflammatory genes such as alarmins (S100A8, S100A9, S10012), TREM1, and cytokines (IL1A, IL1B, IL6) with infection (Figure 3E). Additionally, only the dMac2 macrophage subset exhibited dampened type I interferon signaling (Figure 3D) with key interferon-stimulated genes (IFIT2, GBP1, IRF1) downregulated with infection (Figure 3F).

Finally, we observed a differential impact of mild infection of MHC signaling in decidual macrophage subsets. Module scores for MHC class II signaling were upregulated in dMac1 but downregulated in dMac2 macrophages (HLA-DRA, HLA-DRB1, HLA-DRB1) (Figures 3D and 3F), while MHC class I signaling was downregulated in both subsets (Figures 3F and 4C). Interestingly, surface expression of activation markers CD40 and CD86 did not change with infection (Figure S4D). On the other hand, a higher expression level of M2 marker CD206 was detected on dMac1 macrophages, and modest increases in interferon receptor IFNAR1 expression were seen on both subsets (Figure S4E).

**Expansion of activated CD4 T cells and upregulation of type I interferon signaling in CD4 and CD8 T cells following asymptomatic/mild SARS-CoV-2 infection**

To investigate the impact of asymptomatic/mild SARS-CoV-2 infection in pregnant mothers on diverse T cell states within term placentas, we profiled sorted CD3+ T cells from decidual leukocytes and performed single-cell analysis (gene expression and T cell repertoire) following multiplexing and feature barcoding (5’ technology for parallel gene expression and T cell repertoire analyses) while simultaneously staining for CD4, CD8, CD45RA, tissue markers CD69 and CD103, and regulatory markers PD1 and CD25. Orthogonal readouts were measured in T cells from matched blood samples (Figure 4A). After doublets and ambient RNA removal, we identified 10 memory T cell clusters in addition to a naive cluster (Figure 4B; Tables S3 and S4) within the decidua. Both surface expression of CD4, CD8, CD69, CD103, CD25, and PD-1 from feature barcoding (Figure S5A) and highly expressed RNA markers (Figure 4C; Tables S3 and S4), were used to annotate these memory T cell clusters. All memory CD4s expressed CD69, with three additional clusters expressing either high levels of PD-1 or RNA levels of HLA-DRA or KLRB1 (Tables S3 and S4). Memory CD8 T cells, on the other hand, expressed either CD69 (including a subset of gamma delta T cells) or CD103 and PD-1 (including a subset of proliferating CD8s) (Figures 4B, 4C, and S5A; Tables S3 and S4).

We next compared the proportions of decidual T cell clusters with infection (Figures S5B and S5C). A modest reduction in naive T cells and a statistically significant reduction in Treg frequencies were observed with infection (Figures 4D and S5D). This reduction was accompanied by an overall expansion of CD69+ memory subsets (Figures S5C and S5D), as observed with flow cytometry (Figure 1D). Interestingly, a subset of activated CD4+CD69+HLA-DRA+ was significantly expanded with infection (Figure 4D). This subset of T cells, which was nearly absent in HDs, expressed high levels of genes involved in the regulation of viral processes, stress response, cell adhesion, and apoptosis (Figure 4E). Differential gene-expression analysis of memory CD4 T cells showed upregulation of IFNG, antiviral genes (OASL, ISG15, XAF1), and heat-shock proteins (HSPA6, HSPA8) but downregulation of Th2 genes (GATA3, PDE4D) (Figure 4F). Although frequencies of CD8 T cell subsets did not vary significantly with infection (Figure S5E), differential gene expression analysis revealed elevated signatures of T cell apoptosis, viral processes, myeloid leukocyte activation, and antigen processing and presentation (Figures 4G and 4H). On the other hand, genes associated with NF-κB signaling were downregulated with infection (NFKBIA, CCL4) (Figures 4G and 4H). Finally, increased module scores for IL-2, type I interferon, and T cell signaling, as well as T cell activation and exhaustion, were observed with SARS-CoV-2 infection (Figure S5F).

(F) Heatmap of normalized transcript counts of top 20 genes within each of the 6 dMac1 clusters. A handful of highly expressed markers within each cluster are highlighted. Color in the heatmap represents scaled average expression ranging from low (blue) to high (red).

(G) Box and whiskers comparing changes in macrophage subset frequencies with asymptomatic/mild SARS-CoV-2 infection. Two group differences were tested using an unpaired t test with Welch’s correction (p < 0.05).

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Distinct impact of asymptomatic/mild SARS-CoV-2 infection during pregnancy on peripheral and decidual T cells

We next investigated peripheral T cell adaptations to asymptomatic/mild SARS-CoV-2 infection and compared it with changes observed in placental T cells. Dimension reduction and clustering identified naive, central memory, and several clusters of effector memory T cells, particularly within CD8 EMs (Figure 5A) that were annotated using a combination of gene expression (Figure S6A) and protein markers (Figure S6B). Several clusters of naive CD4 T cells varying in their T cell receptor (TCR) gene usage (TRA or TRB) were all pooled as a naive CD4 cluster (Figure 5A). We compared frequencies of T cell clusters with infection and analyzed differential gene expression changes within effecter memory T cells, particularly within CD8 EMs.
Figure 4. Decidual T cell adaptations to maternal asymptomatic/mild SARS-CoV-2 infection

(A) Experimental design for deep profiling of T cells from blood and maternal decidua (n = 4/group). Isolated immune cells from decidua basalis were surface stained with a fluorescent marker for T cells (CD3), DNA oligo-tagged antibodies for CD4, CD8, naive, and memory, and tissue-resident markers, along with hashing antibodies, enriched for T cells using fluorescence-activated cell sorting (FACS), and subjected to 50 gene expression and TCR analysis (immune repertoire) on the 10X platform.

(B) Dimension reduction and clustering of decidual T cells identifies 11 distinct T cell clusters at term, annotated by unique surface- and gene-expression markers.

(C) Stacked violin plots highlighting key gene-expression markers of T cell subsets within term maternal decidua. The y axis represents the normalized transcript counts.

(D) Box and whiskers plot of decidual T cell subsets changing with asymptomatic/mild SARS-CoV-2 infection.

(E) Functional enrichment of top genes (log2 fold change $\geq 0.4$, q value $\leq 0.05$) within the CD4+CD69+HLA-DRA$^{high}$ T cell cluster. The y axis represents q values (negative log10 transformed).

(F) Violin plots of select statistically significant (log2 fold change $\geq 0.4$, up- or downregulated, q value $\leq 0.05$) differentially expressed genes with asymptomatic/mild SARS-CoV-2 infection in memory CD8 T cell clusters.

(G) Bubble plots representing two-way functional enrichment of genes up- and downregulated with asymptomatic/mild SARS-CoV-2 infection in memory CD8 T cell clusters. The size of the bubble mapping the number of genes, whereas the color represents the level of statistical significance.

(H) Violin plots of select statistically significant (log2 fold change $\geq 0.4$, up- or downregulated, q value $\leq 0.05$) differentially expressed genes with asymptomatic SARS-CoV-2 infection in memory CD4 T cell clusters. Two group differences were tested using an unpaired t test with Welch’s correction (**p < 0.01; ***p < 0.001; ****p < 0.0001).
Within CD4 T cells, this analysis revealed an expansion of EMs and a cluster of CXCR4+ cells expressing cytotoxic molecules with infection (Figure 5B). Differentially expressed genes with infection in the blood were quite distinct from ones observed in decidua (Figure 5C). For example, while decidual T cells exhibited upregulation of antiviral (IFI44L, ISG15, OASL, IFNG, MX1, GBP1) and MHC class II signaling (HLA-DRA, HLA-DRB1), matched blood T cells exhibited elevated cytotoxic signatures (CCL5, GZMA, GZMH). More importantly, genes associated with apoptotic (FAS, PIM3,
DAD1, MAL, TRADD), TNF (CD27, TNFRSF4, STAT1), and TCR signaling (TRAT1, TRAC, PSMB9) were exclusively downregulated in blood CD4 T cells (Figure 5C).

Within CD8+ T cells, infection was associated with an expansion of TEMRA and PD1+ CD8 T cells expressing antiviral transcripts (Figures 5B and S6C). Additionally, distinct antiviral signatures were upregulated in decidua (ISG15, MX1, IFI6, IFITM2) compared with blood (OASL, CXCR4, IFI2, TNF, TNFAIP3, FOS) (Figure S6D). Infection was associated with the upregulation of MHC class II genes in both decidual and blood CD8 T cells (Figure S6D). However, blood CD8 T cells exhibited profound dampening of cytotoxicity genes such as GNLY, NKG7, PRF1, KLRD1, and KLRK1. Furthermore, several genes involved in TCR signaling (CD247, LCK, TRAC, TRAB1, CSK, UBB) were significantly downregulated in blood. Finally, clonal analysis of T cells revealed expansion of large clones (>100 cells) with infection in blood but not in decidua (Figures 5D and S6E), where only small clones were detected. Furthermore, T cell repertoire diversity in blood was lower than that of decidual T cells after infection (Figure 5E).

**DISCUSSION**

Placental immune cells facilitate implantation, fetal growth, and tolerance and promote labor (Vento-Tormo et al., 2018). Immune responses at the maternal-fetal interface are highly fine-tuned, balancing protecting the fetus from pathogens while also limiting excessive inflammatory exposure associated with stress or infection (Ander et al., 2019). In vitro studies have demonstrated the ability of the virus to enter ACE2-expressing maternal and fetal cells in the placenta; however, this observation was not the ability of the virus to enter ACE2-expressing maternal and fetal cells in the placenta; however, this observation was not associated with an expansion of TEMRA and PD1+ CD8 T cells expressing antiviral transcripts (Figures 5B and S6C). Additionally, distinct antiviral signatures were upregulated in decidua (ISG15, MX1, IFI6, IFITM2) compared with blood (OASL, CXCR4, IFI2, TNF, TNFAIP3, FOS) (Figure S6D). Infection was associated with the upregulation of MHC class II genes in both decidual and blood CD8 T cells (Figure S6D). However, blood CD8 T cells exhibited profound dampening of cytotoxicity genes such as GNLY, NKG7, PRF1, KLRD1, and KLRK1. Furthermore, several genes involved in TCR signaling (CD247, LCK, TRAC, TRAB1, CSK, UBB) were significantly downregulated in blood. Finally, clonal analysis of T cells revealed expansion of large clones (>100 cells) with infection in blood but not in decidua (Figures 5D and S6E), where only small clones were detected. Furthermore, T cell repertoire diversity in blood was lower than that of decidual T cells after infection (Figure 5E).

**DISCUSSION**

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Mild COVID-19 in the non-gravid population is characterized by lymphopenia, albeit with a lower magnitude than that observed with severe disease (Chen et al., 2020). Patients with mild COVID-19 also experience an early increase in cytokines but a progressive reduction in type I and III interferon responses as well as increased levels of reparative growth factors (Lucas et al., 2020). Furthermore, the increase in innate immune cell frequencies (monocytes, neutrophils, eosinophils) was less dramatic with moderate disease, suggesting a less inflammatory immune response with mild/moderate disease (Lucas et al., 2020). In line with these previous observations, we report elevated frequencies of granulocytes, monocytes, and platelets but with no overt signs of lymphopenia. Moreover, as previously described (Arunachalam et al., 2020; Laing et al., 2020), we show a reduction in pDCs. Finally, we did not observe a redistribution of monocyte subsets or changes in the surface expression of HLA-DR and CD86, similar to a recent report (De Biasi et al., 2021). The minimal immune activation may be driven by the Th2 bias observed with pregnancy (Aghaeepour et al., 2017). Indeed, elevated plasma levels of TCR signaling (CD247, LCK, TRAC, TRAB1, CSK, UBB) were significantly downregulated in blood. Finally, clonal analysis of T cells revealed expansion of large clones (>100 cells) with infection in blood but not in decidua (Figures 5D and S6E), where only small clones were detected. Furthermore, T cell repertoire diversity in blood was lower than that of decidual T cells after infection (Figure 5E).
MHC class II module scores was observed in dMac2, consistent with studies on circulating monocytes with COVID-19 (Schulte-Schrepping et al., 2020; Wilk et al., 2020). Macrophages in the decidua play diverse roles ranging from clearance of apoptotic bodies and wound healing to host defense, pathogen clearance, and initiation of labor. Aberrantly activated macrophages could potentially disrupt these processes and provide a potential mechanism for increased adverse outcomes for pregnant women with COVID-19 (Schwartz et al., 2022).

We report a modest loss of naïve T cells and an expansion of memory subsets in both blood and decidua, including PD1+ CD8 T cells. As recently described for patients hospitalized with severe COVID-19 (Lu-Culligan et al., 2021), we detected an expansion of CD8+CD69+ and CD4+CD69+ tissue-resident T cells with asymptomatic/mild disease. We also report a loss of Tregs in decidua but an expansion of HLA-DRA-expressing activated CD4 T cells. Additionally, upregulation of MHC class II molecules on CD8 T cells was reported exclusively in the decidua, whereas the appearance of cytotoxic CD4 T cells expressing CXCR4 and high levels of GZMA, GZMH, and CCL5 was seen only in the blood. In circulation, circulating CD4 T cells showed downregulated signatures of apoptotic signaling, TNF, and IFNγ signaling not observed in the decidua. Finally, T cell repertoire analysis suggests that, unlike in blood, T cells in decidua undergo minimal clonal expansion following asymptomatic infection. Taken together, these findings suggest that while antiviral cytotoxic responses are likely restricted to the blood, activated tissue-resident decidual T cells are expanded with infection and exhibit signs of heightened IFN signaling. These changes in the T cell compartment coupled with the loss of regulatory tissue-resident macrophages (dMac1) may skew the balance of decidual immune cells toward a pro-inflammatory state, thereby contributing to pregnancy complications.

Limitations of the study

The main limitation of this study is the small sample size, which, combined with the wide window of infectivity during pregnancy and the presence of samples from subjects with both asymptomatic and mild disease, precluded the assessment of gestational age and disease severity on immunological outcomes. Given the limited amount of maternal blood obtained, we were unable to conduct a comprehensive measurement of antigen-specific T and B cell responses. Finally, it remains unclear if the placenta harbors virus-specific T cells that migrate from blood. Additionally, certain aspects of COVID-19-associated immune responses at the maternal-fetal interface such as IL-10 expression, antibody transfer, and interferon-stimulated gene (ISG) expression have been shown to be more prominent in mothers carrying male fetuses (Bordt et al., 2021). Given the relatively small sample size of our study, we were unable to dissect fetal sex-specific adaptations in the placenta. Finally, maternal infections have been shown to mildly affect the umbilical cord blood cytokine environment, affecting fetal T cell responses with no dramatic changes in their repertoire diversity (Garcia-Flores et al., 2022; Gee et al., 2021). However, given how sensitive the fetal myeloid compartment is to maternal inflammation, it remains to be seen if asymptomatic/mild maternal infection can have long-term consequences on immunity in the offspring.

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.celrep.2022.110938.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.S., N.E.M., and I.M.; methodology, S.S., N.E.M., and I.M.; investigation, S.S., M.Z.Z., B.M.D., and A.J.; writing, S.S. and I.M.; funding acquisition, N.E.M. and I.M.; resources, M.R., D.T., and R.E. All authors have read and approved the final draft of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| CD3-FITC            | BD Pharmigen | Cat#556611 Clone-SP34 |
| CD20-FITC           | Biolegend | Cat#302304 Clone-2H7 |
| CD56-BV711          | Biolegend | Cat#318336 Clone-HCD56 |
| CD57-PE-Cy7         | Biolegend | Cat#359624 Clone-HNK-1 |
| KLRG1-APC           | Biolegend | Cat# 367716 Clone-SA231a2 |
| CD16-PB             | Biolegend | Cat#302032 Clone-3G8 |
| CD14-AF700          | Biolegend | Cat#301822 Clone-MSE2 |
| HLA-DR-APC-Cy7      | Biolegend | Cat#307618 Clone-L243 |
| CD11c-PE-eF610      | ThermoFisher Scientific | Cat#61-0116-42 Clone-3.9 |
| CD123-PCP-Cy5.5     | Biolegend | Cat#306016 Clone-6H6 |
| CD86-BV605          | Biolegend | Cat#305430 Clone-IT2.2 |
| CD4-APC-Cy7         | Biolegend | Cat#317418 Clone-OKT4 |
| CD8b-ECD            | Beckman Coulter | Cat#6607123 Clone-2ST8.5H7 |
| CD45RA-PerCP-Cy5.5  | TONBO Biosciences | Cat#65-040458-t100 Clone-HI100 |
| CCR7-PE-Cy7         | Biolegend | Cat#353226 Clone-GO43H7 |
| CD19-PE             | Biolegend | Cat#302208 Clone-HIB19 |
| IgD-BV605           | Biolegend | Cat#348232 Clone-IA6-2 |
| CD27-BV711          | Biolegend | Cat#356430 Clone-M-T271 |
| PD-1-BV510          | Biolegend | Cat#329932 Clone-Eh12.2h7 |
| CD45- FITC          | Biolegend | Cat#368501 Clone-HI30 |
| CD66b-FITC          | Biolegend | Cat#305104 Clone-G10F5 |
| CD3-PE              | BD Pharmigen | Cat#556612 Clone-SP34 |
| Total-seq-C0072-CD4 | Biolegend | Cat#300567 Clone-RPA-T4 |
| Total-seq-C0046-CD8 | Biolegend | Cat#344753 Clone-SK1 |

(Continued on next page)
### REAGENT or RESOURCE SOURCE IDENTIFIER

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Total-seq-C0148-CCR7 | Biolegend | Cat#353251, Clone-G043H7 |
| Total-seq-C0063-CD45RA | Biolegend | Cat#304163, Clone-HI100 |
| Total-seq-C0146-CD69 | Biolegend | Cat#310951, Clone-FN50 |
| Total-seq-C0145-CD103 | Biolegend | Cat#350233, Clone-Ber-ACT8 |
| Total-seq-C0088-PD-1 | Biolegend | Cat#329983, Clone-EH12.2H7 |
| Total-seq-C0085-CD25 | Biolegend | Cat#302649, Clone-BC96 |
| Total-seq-C0251 | Biolegend | Cat#394661, Clone-LNH-94, 2M2 |
| Total-seq-C0254 | Biolegend | Cat#394667, Clone-LNH-94, 2M2 |
| Total-seq-C0256 | Biolegend | Cat#394671, Clone-LNH-94, 2M2 |
| Total-seq-C0260 | Biolegend | Cat#394679, Clone-LNH-94, 2M2 |
| Human IgG | BD Pharmigen | Cat#55788, Clone-G17-4 |

#### Biological samples

| Biological samples | SOURCE | IDENTIFIER |
|--------------------|--------|------------|
| Fetal Bovine Serum, USDA Certified, Heat Inactivated | Omega Scientific | Cat#FB-02 |
| FetalPlex™ Animal Serum Complex | GeminiBio | Cat#100-602 |

#### Chemicals, peptides, and recombinant proteins

| Chemicals, peptides, and recombinant proteins | SOURCE | IDENTIFIER |
|-----------------------------------------------|--------|------------|
| SARS-CoV-2 Spike Protein | Genscript | Cat#Z03483-1 |
| SARS-CoV-2 Nucleocapsid protein | Genscript | Cat# Z03480-1 |
| o-phenylenediamine dihydrochloride | ThermoFisher Scientific | Cat# 34005 |
| Collagenase | Sigma | Cat# C9722, Source: Clostridium histolyticum |
| Percoll Density Gradient | Neta Scientific | Cat# 17-0891-01 |
| Ghost Dye 540 | TONBO Biosciences | Cat#13-0879 |
| SYTOX Red Dead Cell Stain | ThermoFisher Scientific | Cat#S34859 |
| SYTOX Blue Dead Cell Stain | ThermoFisher Scientific | Cat#S34857 |
| Human TruStain FcX | Biolegend | Cat# 422302 |
| Bovine Serum Albumin (BSA), Fraction V—Molecular Biology Grade | GeminiBio | Cat#700-106P |

#### Critical commercial assays

| Critical commercial assays | SOURCE | IDENTIFIER |
|----------------------------|--------|------------|
| 3' CellPlex Kit Set A | 10X Genomics | PN-1000261 |
| Chromium Single Cell 3' Reagent Kits v3 | 10X Genomics | PN-1000075 |
| 3' Feature Barcode Kit | 10X Genomics | PN-1000262 |
| Chromium Single Cell 5' Reagent Kits V2 | 10X Genomics | PN-1000263 |
| 5' Feature Barcode Kit | 10X Genomics | PN-1000256 |

#### Deposited data

| Deposited data | SOURCE | IDENTIFIER |
|----------------|--------|------------|
| 3' Single Cell GEX data | This paper | NCBI Sequence Read Archive: PRJNA817521 |
| 5' Single Cell TCR data | This paper | NCBI Sequence Read Archive: PRJNA817521 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Ilhem Messaoudi (ilhem.messaoudi@uky.edu).

Materials availability
This study did not generate new unique reagents.

Data and code availability
- Single-cell RNA-seq data have been deposited at GEO and are publicly available as of the date of publication. Accession numbers are listed in the key resources table.
- This paper does not report original code.
- Additional Supplemental Items are available from Mendeley Data at https://doi.org/10.17632/pxgb3cnxvd.1.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study was approved by the University of California Irvine Institutional Review Boards (Protocol ID HS# 2012-8716) and Oregon and Health Sciences University (eIRB# 22713 and 16328). Informed consent was obtained from all enrolled subjects. All participants in this study were healthy and did not report any significant comorbidities or complications with pregnancy. Detailed characteristics of participants and experimental breakdown by samples are provided in Table S1. Subjects with the asymptomatic disease are defined as those who tested positive during the mandatory screening at the time of admission to labor and delivery. Subjects with mild disease are defined as those who experienced (self-reported) symptoms consistent with mild upper respiratory tract infection but did not necessitate a hospital visit or stay.

METHOD DETAILS

Blood processing
Whole blood samples were collected in EDTA vacutainer tubes. PBMC and plasma samples were isolated after whole blood centrifugation 1200 g for 10 min at room temperature in SepMate tubes (STEMCELL Technologies). Plasma was stored at -80°C until analysis. PBMC were cryo-preserved using 10% DMSO/FBS and Mr. Frosty Freezing containers (Thermo Fisher Scientific) at -80°C then transferred to a cryogenic unit 24 h later until analysis.

PBMC phenotyping
Frozen PBMCs were thawed, washed in FACS buffer (2% FBS, 1 mM EDTA in PBS), and counted on TC20 (Biorad) before surface staining using two independent flow panels. For the innate panel, the following antibodies were used: CD3 (SP34, BD Pharmingen) and CD20 (2H7, BioLegend) for the exclusion of T & B lymphocytes, respectively. We further stained for CD56 (HNK-1, BioLegend), KLRG1 (SA231A2, BioLegend) CD16 (3G8, BioLegend), CD14 (M5E2, BioLegend), HLA-DR (L243, BioLegend), CD11c (3.9, ThermoFisher Scientific), CD123 (6H6, BioLegend) and CD86 (IT2.2, BioLegend). For the adaptive panel, the following antibodies were used: CD4 (OKT4, BioLegend), CD8b (2STB.5H7, Beckman Coulter), CD45RA (HI100, TONBO Biosciences), CCR7 (G043H7, Biolegend), CD19 (HIB19, BioLegend), IgD (Ia6-2, BioLegend), CD27 (M-T271, BioLegend), KLRG1 (SA231A2, BioLegend) and PD-1 (Eh12.2h7, BioLegend). Cells were stained with Ghost Dye viability dye (TONBO biosciences) for
30 min at 4C per manufacturer’s instructions, washed, surface stained with either innate or adaptive panels for 30 min at 4C. Samples were then washed and analyzed on Attune NxT Flow Cytometer (ThermoFisher Scientific, Waltham MA).

Serology
RBD and NP end-point titers were determined using standard ELISA and plates coated with 500 ng/mL SARS-CoV-2 Spike–protein Receptor-Binding Domain (RBD) (GenScript, Piscataway NJ) or 1ug/mL SARS-CoV-2 nucleocapsid protein (NP). Heat inactivated plasma was added in 3-fold dilutions starting at 1:50. Responses were visualized by adding HRP-anti-human IgG (BD Pharmingen) followed by the addition of o-phenylenediamine dihydrochloride (ThermoFisher Scientific). ODs were read at 490 nm on a Victor3™ Multilabel plate reader (Perkin Elmer). Batch differences were minimized by normalizing to positive control samples run on each plate.

Placenta processing
Decidua basalis and villous chorion membranes were separated and immediately immersed in RPMI supplemented with 10% FBS and antibiotics. Samples were processed within 24 h of collection. Decidua basalis membranes were washed thoroughly in HBSS to remove contaminating blood. Tissues were minced into approximately 0.2–0.3 mm3 cubes and enzymatically digested in 0.5 mg/mL collagenase V (Sigma, C-9722) solution in 50 mL R3 media (RPMI 1640 with 3% FBS, 1% Penicillin-Streptomycin, 1% L-glutamine, and 1M HEPES) at 37C for 1 h. The disaggregated cell suspension was passed through tissue strainers to eliminate large tissue chunks. Cells were pelleted from the filtrate, passed through 70-um cell sieve, centrifuged, and resuspended in R3 media. Red blood cells were lysed using RBC lysis buffer (155 mM NH4Cl, 12 mM NaHCO3, 0.1 mM EDTA in double-distilled water) and resuspended in 5 mL R3 media. The cell suspension was then layered on a discontinuous 60% and 40% percoll gradient and centrifuged for 30 min with brakes off. Immune cells at the interface of 40% and 60% gradients were collected, washed in HBSS, counted, and cryopreserved for future analysis.

Decidua immunophenotyping
1-2 X 106 fresh decidual leukocytes were washed with PBS and stained using the following cocktail of antibodies: CD45 (HI30, Biolegend), CD66b (G10F5, BioLegend), CD20 (2H7, BioLegend), CD4 (OKT4, BioLegend), CD8b (2ST8.5H7, Beckman Coulter), CD14 (M5E2, BioLegend), HLA-DR (L243, BioLegend), CD56 (HCD56, Biolegend), CD16 (3G8, BioLegend), CD11c (3.9, ThermoFisher Scientific), CD123 (6H6, BioLegend), for 20 min in dark at 4C. Samples were washed twice in FACs buffer and resuspended in 400 uL. All samples were acquired with the Attune Nxt Flow Cytometer (ThermoFisher Scientific, Waltham MA), immediately after addition of SYTOX Red Dead Cell Stain (1:1000). Data were analyzed using FlowJO (Ashland OR).

3' multiplexed single-cell RNA sequencing
Freshly thawed decidual immune cells (1-2 10e6 cells) were incubated with Fc blocker (Human TruStain FcX, Biolegend) in PBS with 1% BSA for 10 min at 4C and then surface stained with CD45-FITC (HI30, Biolegend) for 30 min in the dark. Samples were then washed twice in PBS with 0.04% BSA and incubated with individual 3' CellPlex oligos (10X Genomics) per manufacturer’s instructions for 5 min at room temperature. Pellets were washed three times in PBS with 1% BSA, resuspended in 300 uL FACs buffer, and sorted on BD FACs Aria Fusion into RPMI (supplemented with 30% FBS) following the addition of SYTOX Blue stain for live versus dead discrimination. Sorted live cells were counted in triplicates on a TC20 Automated Cell Counter (BioRad), washed, and resuspended in PBS with 0.04% BSA in a final concentration of 1500 cells/µL. Single cell suspensions were then immediately loaded on the 10X Genomics Chromium Controller with a loading target of 20,000 cells. Libraries were generated using the V3 chemistry (for gene expression) and Single Cell 3’ Feature Barcode Library Kit per the manufacturer’s instructions (10X Genomics, Pleasanton CA). Libraries were sequenced on Illumina NovaSeq with a sequencing target of 30,000 gene expression reads and 5,000 multiplexed CellPlex reads per cell.

5' multiplexed single cell RNA sequencing with feature barcoding
Matched PBMC and decidual leukocytes were thawed, washed, filtered, and stained with Ghost Violet 540 live-dead stain (Tonbo Biosciences) for 30 min in the dark at 4C. Given the parallel assessment of both TCR and gene expression, we used an antibody-based multiplexing technology as it is the only compatible with 5’ gene expression analysis. Samples were washed thoroughly with a cell staining buffer (1X PBS with 0.5% BSA), and Fc blocked for 10 min (Human TruStain FcX, Biolegend) and incubated with a cocktail containing CD3-PE (SP34, BD Pharmingen), 0.5 ug each of oligo tagged CD4 (TotalSeq™-C0072, Biolegend), CD8 (TotalSeq™-C0046, Biolegend), CCR7 (TotalSeq™-C0148, Biolegend), CD45RA (TotalSeq™-C0063, Biolegend), CD69 (TotalSeq™-C0146, Biolegend), CD103 (TotalSeq™-C0145, Biolegend), PD-1 (TotalSeq™-C0088, Biolegend), CD25 (TotalSeq™-C0085, Biolegend), and unique 5’ hashing antibody (TotalSeq™-C0251, C0254, C0256, and C0260, Biolegend) for an additional 30 min at 4C. Samples were washed four times with 1X PBS (serum and azide free), filtered using Flowmi 1000 uL pipette strainers (SP Bel-Art) and resuspended in 300 uL FACs buffer. CD3+ T cells were sorted on the BD FACs Aria Fusion into RPMI (supplemented with 30% FBS). Sorted pellets were counted in triplicates on a TC20 Automated Cell Counter (BioRad), washed and resuspended in PBS with 0.04% BSA in a final concentration of 1500 cells/uL. Single cell suspensions were then immediately loaded on the 10X Genomics Chromium Controller with a loading target of 20,000 cells. Libraries were generated using the 5’ V2 chemistry (for gene expression) and Single Cell 5’ Feature Barcode Library Kit per manufacturer’s instructions (10X Genomics, Pleasanton CA). Libraries were sequenced on Illumina NovaSeq with a sequencing target of 30,000 gene expression reads and 5,000 multiplexed CellPlex reads per cell.
were sequenced on Illumina NovaSeq with a sequencing target of 30,000 gene expression reads and 10,000 multiplexed CellPlex reads per cell.

**Single-cell RNA-Seq data analysis**

For 3\(^\text{rd}\) gene expression with CellPlex, raw reads were aligned and quantified using Cell Ranger (version 6.0.2, 10X Genomics) against the human reference genome (GRCh38) using the multi option and CMO information. Only singlets identified from each sample were included in downstream analyses. For 5\(^\text{th}\) gene expression, alignments were performed using the feature and vdj option in Cell Ranger. Following alignment, hashing (HTO) and cell surface features (Antibody Capture) from feature barcoding alignments were manually updated in cellranger generated features file. Doublets were then removed in Seurat using the HTODemux function, which assigned sample identity to every cell in the matrix. Droplets with ambient RNA (cells with fewer than 400 detected genes), and dying cells (cells with more than 20% total mitochondrial gene expression) were excluded during initial QC. Data normalization and variance stabilization were performed on the integrated object using the NormalizeData and ScaleData functions in Seurat where a regularized negative binomial regression corrected for differential effects of mitochondrial and ribosomal gene expression levels. Dimension reduction was performed using RunPCA function to obtain the first 30 principal components and clusters visualized using Seurat's RunUMAP function. Cell types were assigned to individual clusters using FindMarkers function with a log\(_2\) fold change cutoff of at least 0.4 (FDR<0.05) and using a known catalog of well-characterized scRNA markers for human PBMC and decidual leukocytes. For T cells, 5\(^\text{th}\) feature barcoding reads were normalized using centered logratio (CLR) transformation. Differential markers between clusters were then detected using FindMarkers function. A combination of gene expression and protein markers was used to define T cell subsets. CCR7 staining did not exhibit a significant positive peak, and hence was excluded from all downstream analyses. A list of cluster specific markers is provided in Tables S2, S3, and S4. Differential expression analysis was performed using the wilcoxon rank-sum test using default settings in Seurat. Only statistically significant genes (log\(_{10}\)(fold change) cutoff \( \geq 0.4\); adjusted \( p \)-value \( \leq 0.05 \)) were included in downstream analyses. Module scores for specific gene sets were incorporated cluster-wise using AddModuleScores function.

**scTCR analysis**

TCR reads were aligned to VDJ-GRCh38 ensembl reference using Cell Ranger 6.0 (10X Genomics) generating sequences and annotations such as gene usage, clonotype frequency, and cell-specific barcode information. Only cells with one productive alpha and/or one productive beta chain were retained for downstream analyses. CDR3 sequences were required to have a length between 5 and 27 amino acids, start with a C, and not contain a stop codon. Clonal assignments from cellranger were used to perform all downstream analyses using the R package immunarch. Data were first parsed through repLoad function in immunarch, and clonality was examined using repExplore function. Family and allele level distributions of TRA and TRB genes were computed using geneUsage function. Diversity estimates (Hill numbers) were calculated using repDiversity function.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

Data sets were first tested for normality using the Shapiro-Wilk test. Two-way comparisons for normally distributed data were tested for significance using an unpaired t-test with Welch's correction. For comparisons involving multiple groups, differences were tested using one-way ANOVA followed by Holm Sidak’s multiple comparisons tests. \( p \) values less than or equal to 0.05 were considered statistically significant. Values between 0.05 and 0.1 are reported as trending patterns.