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Endogenous control of inflammation characterizes pregnant women with asymptomatic or paucisymptomatic SARS-CoV-2 infection

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Article

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Abstract

In 14 pregnant women who had asymptomatic or paucisymptomatic SARS-CoV-2 infection, we performed a detailed 38-parameter analysis of peripheral blood mononuclear cells by mass cytometry, studied the expression of T-cell master regular genes, investigated cell proliferation and cytokine production, and measured plasma levels of 62 cytokines. No patient showed lymphopenia or gross alterations of white blood cells. Unsupervised analyses revealed that most immune parameters were similar in patients and uninfected controls, apart from an increase in low density neutrophils in SARS-CoV-2 positive women. Also, patients did not show altered plasma levels of interleukin-6 or other main inflammatory molecules, but displayed significant increases of anti-inflammatory cytokines such as IL-1RA, IL-10 and IL-19, and decreased levels of IL-17, PD-L1 and D-dimer. The endogenous control of inflammation, as evidenced by plasma levels of soluble molecules, could be a strategy used during pregnancy to avoid virus-induced damages and maintain a normal immune response.

Introduction

SARS-CoV-2 infection has rapidly become a public health emergency of international concern culminating in the WHO declaration of pandemic. Scientists from all over the world are investigating the pathogenetic mechanisms of SARS-CoV-2 action, but still little is known about particular groups of patients. Clinical manifestation of COVID-19 can be characterized by mild or asymptomatic infection of the upper respiratory tract, infection of lower respiratory tract with or without life-threatening pneumonia and eventually acute respiratory distress syndrome [1].

Previous studies have shown a variety of immunological changes in patients of all ages, but limited data are currently available for pregnant women with COVID-19 pneumonia [2], or even with paucisymptomatic or asymptomatic SARS-CoV-2 infection. Even though COVID-19 manifestation seems to be less severe in pregnant women than in elderly patients, it could be not completely absent, or silent. Therefore, investigational studies need to be done to assess the impact of COVID-19 on pregnant women.

Different questions need to be answered, such as why symptoms and severity of the disease seem different in pregnant women, how the infection can cause complications during pregnancy, or which is the frequency, if any, of vertical transmission. In general, it is well known that pregnant women have a biased immune response, with a skewing towards T-helper 2 (Th2) which protect the fetus from mother's aggression, but can leave the mother more susceptible to viral infections, as a Th1 response would better help to contrast viruses [3].

A recent review of 63 observational studies on a total of 637 women with SARS-CoV-2 infection revealed that more than 3 out of 4 experienced mild disease [4]. Poor outcomes were predicted by well-known risk factors such as older age, obesity, diabetes mellitus, and by laboratory findings such as high levels of serum D-dimer and interleukin-6.

In order to better understand the severity of the infection, clinical outcomes on pregnant women with COVID-19 was compared with those of SARS and Middle East Respiratory Syndrome (MERS) [5]. Interestingly, case fatality was 0%, 18% and 25% respectively. In addition, clinical manifestations reported in pregnant women were mild and similar to those reported in non-pregnant women infected by SARS-CoV-2, with predominant features including fever, cough, dyspnea and lymphopenia [6]. Vertical transmission has been reported in SARS as ACE2 receptor is widely expressed in the placenta. Even if the structure of the receptor-binding domain of SARS and SARS-CoV-2 is similar, no cases of vertical transmission of COVID-19 have been reported so far [3, 4, 7].

Few studies have analyzed in depth the immune system of pregnant women with SARS-CoV-2 infection. Thus, to fill, at least in part, this lack of knowledge, we have deeply studied pregnant women positive or negative to SARS-CoV-2 (as revealed by nasopharyngeal swab), compared to non-pregnant women negative to the virus. Using 38 parameter mass cytometry, we investigated peripheral mononuclear cell (PBMC) populations, paying particular attention to the T and B cell compartments, and focusing on their phenotypes both in a supervised and unsupervised analysis. In addition, T cell production of cytokines was measured as well as T and B cell proliferative capacity and the expression of master regulator genes for T lymphocytes. Plasma level of 62 molecules, including cytokines, chemokines and growth factors were measured, along with main biochemical parameters, such as D-dimer.

In pregnant women with or without infection we found different plasma levels of a few pro-inflammatory and anti-inflammatory cytokines. Those infected showed normal IL-6, and lower levels of D-dimer. All women had similar distributions of different populations of PBMC, but significantly higher amounts of circulating low density neutrophils were detected in infected pregnant women. A deep characterization of T and B lymphocyte subsets, along with monocytes, natural killer and dendritic cells showed a substantial lack of effect of the viral infection on such populations.

Results

Plasma cytokine levels in COVID-19 pregnant women: dysregulation of a few pro- and anti-inflammatory cytokines and decreased levels of D-dimer.

Plasma levels of 62 cytokines were measured in 14 infected pregnant women, 28 uninfected and 15 age-matched non-pregnant controls. Results are reported in Fig. 1A and 1B. Regarding growth factors, the levels of platelets derived growth factor (PDGF-AA) and epidermal growth factor (EGF) were higher in pregnant COVID-19 compared to pregnant without COVID-19. Levels of Granzyme B (GRZB), IL-1RA, IL-27, IL-10, IL-19, CXCL12, CCL4, APRIL and BAFF levels were higher in pregnant COVID-19 compared to those without COVID-19. Plasma levels of PD-L1 and FAS, molecules that are linked to apoptosis, were lower in pregnant COVID-19 when compared to those without COVID-19. Interestingly, pregnant women positive to SARS-CoV-2 had significantly lower levels of D-dimer (median value: 785 mg/L) than those who were negative (1,640 mg/L; $p = 0.009$; see details in the Supplementary Table 1).

Pregnant women with or without COVID-19 displayed a similar peripheral blood cell landscape distribution but different amounts of low density granulocytes.

To assess whether a different peripheral blood landscape distribution was present in pregnant women with or without COVD-19, we used mass cytometry and a panel of 38-markers to define different subpopulations of T, B, NK, DC, γδ cells, monocytes and low-density neutrophils (LDN). The representation of different cell distribution of CD45 expressing leukocytes, performed by the Uniform Manifold Approximation and Projection (UMAP) approach, is reported in Fig. 2A. Unsupervised analysis revealed a total of 27 main clusters on the basis of the different markers used (Fig. 2B). First, $CD4^+$ and $CD8^+$ T cells and B lymphocytes have been identified, and separately analyzed as described below. Then, based on the expression of CD16 and CD14, three clusters revealed different subpopulations of monocytes (classical, intermediate and non-classical). CD16 and CD56 were used to classify NK cells, and in particular three different clusters of NK were identified (early NK, mature NK expressing or not CD57). γδ T cells were identified by the expression of γδ TCR, and then three clusters were recognized: naïve cells expressing CD45RA plus CCR7, and effector memory (EM) expressing CD45RA (EMRA), expressing or not CD57. Two clusters, expressing or not CD11c, represented DC, identified by the expression of CD123. Mucosal associated invariant T cells (MAIT) were identified by the expression of CD161. LDN were those cells expressing CD66b, CXCR1, CD11b and CD16 (Fig. 2C and 2D).

We investigated separately $CD4^+$ and $CD8^+$ T cells, and B lymphocytes (Fig. 3A, 3B and 3C, respectively). We could find 14 main clusters indicating different subpopulations of $CD4^+$ T cells, 13 clusters of $CD8^+$ T cells and 6 clusters of B lymphocytes. In general, a few differences were present between healthy controls and pregnant women, (either positive or negative) but women with or without SARS-CoV-2 infection were almost identical as far as all of the clusters of the T cells are concerned. Concerning B cells, pregnant women with or without infection were similar; both groups however displayed more plasmablasts but less memory switched cells than controls.

Overall, we found that almost all analyzed clusters were not statistically different between pregnant women with and without COVID-19. Indeed, cells belonging to adaptive (B and T cells) or innate immunity (subpopulation of monocytes, NK, DC) were represented in a similar percentage in pregnant women with or without COVID-19 infection. Mature NK and dendritic cells were lower in both groups of pregnant women compared to healthy controls. The percentage of LDN was higher in pregnant women with COVID-19 compared to pregnant women without infection (Fig. 2C, green arrow).

Expression of master regulator genes and chemokine receptors on $CD4^+$ and $CD8^+$ T cell are similar in COVID-19 pregnant women and healthy controls.

To further characterize CD4 and CD8 T cells, we investigated the expression of different chemokine receptors together with that of master regulator genes such as CCR6, CD161, CXCR3, CCR4, GATA3 and TBET to better define the helper capability of T cells in terms of acting as Th1, Th2, or Th17. Th1 were

defined as $CXCR3^+TBET^+$, Th2 were defined as $GATA3^+CCR4^+$ and Th17 were defined as $CD161^+CCR6^+$. The gating strategy for the identification of $CD4^+$ and $CD8^+$ T cells is reported in Supplementary Fig. 1.

Similar percentages of $CD4^+$ T cells expressing CCR4, CCR6, CD161, CXCR3, TBET and GATA3 were found among healthy donors and pregnant women with or without COVID-19. Moreover, no differences were found in terms of percentages of Th1, Th2 and Th17 among healthy donors and pregnant woman with or without COVID-19 (Fig. 4A). Concerning $CD8^+$ T cells, we found comparable percentages of cells expressing different master regulator genes or chemokine receptors. Regarding the percentage of $CCR6^+CD161^+$ cells, we found that pregnant COVID-19 were characterized by a lower, even if not significant, percentage of these cells (Fig. 4B).

T cells from COVID-19 pregnant women and healthy pregnant women are fully functional in terms of proliferation and cytokine production.

We characterized $CD4^+$ and $CD8^+$ T cells also from a functional point of view. Proliferative capability and cytokine production were evaluated and quantified after *in vitro* stimulation with anti-CD3/CD28. The gating strategies for studying the proliferation capacities and cytokine production are reported in Supplementary Figs. 2 and 3 respectively. By using the method of CFSE dilution for evaluating cell proliferation (Fig. 4C), we found that $CD4^+$ T cells from pregnant women had a small but significant increase either in the proliferation index or in the division index, more marked in those who were not infected. No differences were found in CD8 + T cells.

We then evaluated the amount of IL-4, TNF, IL-17A, IFN-γ produced by $CD4^+$ T cells and expression of CD107a (Fig. 4D). In CD4 + T cells, we found no differences in terms of single cytokine production (upper panels) as well as in their polyfunctionality, *i.e.*, the ability to produce different cytokines at a time (not shown). Similar results were obtained regarding $CD8^+$ T cells (lower panels), that gave comparable results in the three groups investigated.

Correlogram reveals different correlation among several laboratory parameters.

We used all the data available in some pregnant women with or without the infection and built a correlation matrix, visualized in Fig. 5. It is to note that in those who had the virus, but not in the others, highly significant positive correlations were present among the percentage of low density neutrophils (LDN, boxed in green in the first column) among mononuclear cells and plasma levels of molecules such as CCL2, CCL3, CCL4, CCL-5, CCL11, CCL19, CD40L, CXCL1, CXCL2, IL-1α, IL-1β, IL-2, IL-4, IL-7, IL-10, IL-13, IL-17E, IL-33, PDGF-AA, PDGF-AB/BB, EGF, IFN-α, and IFN-γ. Several positive correlations among plasma cytokines were then found, that were much more marked in SARS-CoV-2 infected pregnant women (Fig. 5 and Supplementary Tables 2–4).

Discussion

In this study, we describe main immunological features in pregnant women with paucisymptomatic or asymptomatic SARS-CoV-2 infection. To this regard, plasma level of 62 cytokines was measured together with the distribution of innate and adaptive immunity cells within PBMC. The main findings of our study are that, in comparison with control pregnant women, those with asymptomatic or paucisymptomatic COVID-19 are characterized by: i) different plasma levels of a few pro-inflammatory and anti-inflammatory cytokines; ii) lack of high plasma levels of IL-6; iii) lower levels of D-dimer; iv) similar distributions of different populations of PBMC; v) significantly higher amounts of circulating low density neutrophils. Moreover, T and B cell subsets were in depth characterized, and we found that these cells were also able to maintain their functional properties, in terms of cell proliferation and cytokine production.

From a clinic point of view, the majority of pregnant women affected by COVID-19 are asymptomatic or paucisymptomatic. In them, immune responses and changes have never been studied in detail, and scanty data describe immunological changes due to SARS-CoV-2 infection. Most studies reported that pregnant women positive for COVID-19 do not experience severe symptoms and gave birth to healthy babies, so the infection seems to impact neither the pregnancy nor the newborns [3], even in a case where a pregnant woman with critical COVID-19 has been described [8]. Both the mother and son showed lymphopenia, along with a delayed immunoglobulins (Ig) response due to a low number of Ig-switched B cells and a very small compartment of naïve T and B cells. In contrast with recent data showing that COVID-patients display profound derangement in the B cell compartment [9], we found that the number and quality of B cells were similar in infected or uninfected women, who both showed small but significant changes in memory B cells and in plasmablasts.

There is a gradual, but marked increase of neutrophils during the first trimester of pregnancy. Here we found that pregnant women with COVID-19 displayed an increase in the population of low density neutrophils, a phenomenon which has been described by several studies, and has been related to the severity of the disease. Increase of LDN could be associated to the light but not significant increase in plasma levels of GM-CSF, whose increase has been recently described in patients with COVID-19 pneumonia [10]. The lack of serious symptoms in our patients could however suggest that the proinflammatory activity exerted by these cells, whose low density could be due to their eventual degranulation in various tissues, is well compensated by anti-inflammatory cytokines. In any case, the correlations between LDN and several soluble molecules, including inflammatory cytokines, that were present only in infected women deserve further attention.

Monocytes are also modified during pregnancy, and in particular the sub-population of intermediate monocytes, while classical decrease and there are no changes to the non-classical [11]. Here, we saw that all monocyte populations were not different between infected or non-infected women, indicating that likely these cells do not participate to the creation of an inflammatory milieu.

NK cells belong to the complex family of innate lymphoid cells, that participate in tissue immunity. They are found in the placental decidua (dNK), are essential for successful fetal implantation, are involved in

tissue modifications and in forming new vessels [12]. They also interact with myelomonocytic cells to favor the development of regulatory T cells (Treg), that have a key role in immunosuppression and induction of tolerance to the fetus. dNK can derive from the expansion of single mature $CD56^{dim}$ clones, from the recruitment and maturation of $CD56^{bright}$ NK cells or from the development of tissue-resident $CD56^{bright}$ NK cells independently of the circulating compartment [13]. It is known that during pregnancy, peripheral blood NK cells and their activity decrease [14]. Even if we could not distinguish between CD56 dim or bright, in both infected or uninfected pregnant women we found a significant decrease in circulating mature NK cells, indicating that SARS-CoV-2 was not able to alter the delicate equilibrium that regulates immunological tolerance.

SARS-CoV-2 infection leads to a process called “cytokine storm”, where a variety of cytokines, from those proinflammatory, to anti-inflammatory or those indicating a skewing towards Th1, Th2 or Th17 are produced [9, 15]. The same process was already described during sepsis, where this overwhelming phenomenon may lead to multiple organ failure. Few data, if any, exist that describe in detail plasma cytokine levels, nor that report cytokine production in lymphocytes from pregnant women with COVID-19. We found differences in the levels of some growth factors, such as EGF and PDGF-AA, that were higher in pregnant women with COVID-19 compared to those uninfected. The level of these molecules was correlated with the severity of the disease, and in particular higher levels of angiogenesis factors were elevated in hospitalized patients with non-critical COVID-19 infection [16]. However, this aspect was not analyzed during pregnancy. Here we found increased level of different soluble molecules, such as pro- and anti-inflammatory cytokines. During the cytokine storm, pro-inflammatory molecules are first produced to contrast the infection, but anti-inflammatory molecules are needed to contrast and dampen the inflammation. We reported that patients affected by COVID-19 showed an increase of cytokines with different properties, such as CCL4, IL-10 and PD-L1 [17]. The same phenomenon has been shown in sepsis where there is the concomitant production of IL-1RA and IL-10 [18] and high level of both APRIL and BAFF [19].

During pregnancy the immune system goes through some modifications to create the proper environment for fetal growth and for immunological tolerance. Any inflammatory situation that triggers a Th1 immune response can clearly be dangerous for the fetus. This can well explain the low levels of cytokines that regulate T cell activities like IL-2, IL-4 and IL-5, that we have found in both groups of pregnant women. Similarly, the balance between pro- and anti-inflammatory cytokines goes in the direction of the latter ones, as shown, for example, by the opposite directions taken by IL-1, IL-33 and IL-1RA.

PD-L1 plays a remarkable role in pregnancy by inducing maternal immune tolerance to fetal tissue and is more present in serum of pregnant women compared to non-pregnant ones [20]. Recently, we found higher levels of soluble PD-L1 in COVID-19 patients [10]. PD-L1 belongs to a class of molecules that regulate the balance between protective immunity and host immune-mediated damage. However, in our cohort of patients, infected pregnant women showed lower levels of PD-L1. We could hypothesize that PD-L1 had been used to contrast the action of inflammatory cells, and thus to further protect the fetus.

Despite changes in the plasmatic levels of a variety of cytokines produced by PBMC, we found similar distribution of cells among PBMC between pregnant women with and without COVID-19 infection. Therefore, it could be hypothesized that the different production of cytokine in pregnant women with COVID-19 does not cause damages to the immune system, but it is probably capable of balancing the opposite effects of the cytokines creating a tolerance environment.

We are well aware of the limitations of this study. First of all, the relatively low number of patients studied. Second, we could only investigate transversally a cohort of asymptomatic or paucisymptomatic pregnant women. Few cases of pregnant woman with severe COVID-19 are described, but no data exists regarding a deep immune characterization of those patients, nor on the presence of predictive markers. Furthermore, data on hormones production would be of interest to better to investigate their importance in this situation. Despite the limitations pointed out, this is likely the first detailed immunological study conducted on pregnant women with COVID-19, and it shows that asymptomatic or paucisymptomatic SARS-CoV-2 positive pregnant women are characterized by a few changes in plasma cytokine levels, that cannot be compared to the classical, well known cytokine storm that has been described during COVID-19. Interestingly, it is likely that the few immune changes described here not only do not alter the efficiency of the global immune response to the virus, but also do not cause any significant change in PBMC asset.

Finally, we underline that in our cohort SARS-CoV-2 had no impact on newborns. None was infected nor suffered of any disturbance after birth. This is in agreement with a recent study showing no virus in maternal or cord blood (despite detection in the women's respiratory system), no signs of the virus in placentas and no evidence of viral transmission to newborns [7]. Thus, it can be hypothesized that infection of fetus does not occur because of the reduced co-expression and colocalization of placental angiotensin-converting enzyme 2 and transmembrane serine protease 2, and that the immunosuppression present during pregnancy could protect not only most women from the cytokine storm, massive immune activation and hyper inflammation, but also the fetus from a possible maternal immune aggression. In conclusion, our data might be useful for clinicians, as if the conditions of patients are stable, and infection remains paucisymptomatic or asymptomatic, there could be no risk of pregnancy complications neither for the mother nor for the newborn, so that in the large majority of the cases an anticipated delivery should not be required.

Materials And Methods

1. Study design

This is a case-control, cross sectional, single-centre study, approved by the local Ethical Committee (Comitato Etico dell'Area Vasta Emilia Nord, protocol number 177/2020, March 11th, 2020) and by the University Hospital Committee (Direzione Sanitaria dell'Azienda Ospedaliero-Universitaria di Modena, protocol number 7531, March 11th, 2020). Each participant, including healthy controls, provided informed consent according to Helsinki Declaration, and all uses of human material have been approved by the

same Committees. A total of 14 pregnant women with SARS-CoV2 infection was included in the study; they had a median age of 33.8 years (range 19-39). Patients were matched for age and gender with 28 pregnant women negative to nasopharyngeal swab (median 33.9 years, range 18-42) and a total of 15 non pregnant healthy women (CTR), median age 39 years (range 25-50 years). We recorded demographic data, medical history, symptoms, signs, temperature, and main laboratory findings from each patient. For details, see supplementary Table 1.

Pregnant women were eligible for inclusion if they were aged 18 years or older, able to provide informed consent and diagnosed with SARS- CoV-2 infection. Confirmed SARS-CoV-2 infection was defined as nasopharyngeal swab reverse transcription–polymerase chain reaction (RT-PCR) test results positive for SARS-CoV-2. According to routine methods, we could measure anti-SARS-CoV-2 IgM and IgG in 12 women positive to the swab, and 9 had detectable plasma levels of IgM and IgG, while three were negative to both antibodies. All the 15 control women that we could test were negative. In all cases, paucisymptomatic symptoms were fever <37.5°C, cough, loss of smell or taste, and no drugs were administered to treat them.

The total number and type of leukocytes in peripheral blood was analyzed by hemocytometer in the Clinical Laboratory of the University Hospital, that also analyze all the biochemical parameters quoted in the manuscript, according to routine methods.

2. Blood collection

Blood samples (up to 20 mL) were obtained after informed consent. In some donors, blood was obtained after diagnosis of SARS-CoV-2 infection. Peripheral blood mononuclear cells (PBMC) were isolated according to standard procedures and stored in liquid nitrogen until use. Plasma was collected and stored at -80°C until use. Measurements were taken from individual patients; in the case of plasma, each measurement was performed in duplicate and only the mean was considered and shown.

3. Mass cytometry analysis

Thawed PBMC were washed twice with PBS and stained with Maxpar® Direct™ Immune Profiling Assay™ (Fluidigm), a dry 30-marker antibody panel (viability marker Cell-ID™ Intercalator-103Rh included) plus the addition of 6 drop-in catalog antibodies (Fluidigm) and 2 custom-conjugated mAbs, for a total of 38 markers. The markers were the following: CD3, CD19, CD45, CD4, CD20, CD45RA, CD8, CD25, CD45RO, CD11c, CD27, CD56, CD14, CD28, CD57, CD16, CD38, CD66b, CCR7, CXCR3, CXCR5, HLA-DR,IgD, TCRγδ, CD123, CD127, CD161, CD294, CCR4, CCR6, CXCR1, PDL1, CD80, CD40, CD24, PD1-1, CD11b/MAC, CD21, IgM. See Supplementary Table 5 for the complete list of mAbs used. At least 300,000 events were acquired per sample. Data in FCS file format were normalized for intra-file and inter-file signal drift using the FCS Processing tab in the CyTOF Software 6.7. The method is a two-step algorithm that first identifies the EQ Four Element Calibration Beads and then applies the dual count values registered by the beads to calculate the normalization factor to be applied to the data.

4. Representation of high parameter cytometry

Compensated and normalized Flow Cytometry Standard (FCS) 3.0 files were imported into FlowJo software version 10 (Becton Dickinson, San Josè, CA) and pre-processed excluding EQ Four Element Calibration Beads and doublet using Gaussian Discrimination parameters. Then, were selected live undamaged $CD45^+$ and excluded artifact cells ($CD3^+CD19^+$ or $CD3^+CD14^+$). All living $CD45^+$ were exported for further analysis in R using Bioconductor libraries CATALYST (version 1.12.2) [21] and diffcyt (version 1.8.8) [22]. The data were transformed using arcsinh with *cofactor* = 5 to make the distributions more symmetric and to map them to a comparable range of expression. The main cell population identification was performed through unsupervised clustering using the FlowSOM algorithm (K= 30). 2D visual representation was performed applying Uniform Manifold Approximation and Projection (UMAP). Then, the clusters identified as $CD4^+$, $CD8^+$, or $CD19^+$ lymphocytes, were selected and re-clusterized separately to describe more in-depth the cellular distribution of each sub-population. We used K= 15 for $CD4^+$ and $CD8^+$ T cells, while K= 9 for $CD19^+$ cells. Clusters with similar markers distribution were merged. Then we re-applied UMAP for dimensionality reduction and visualization purposes. Statistical analysis was performed using generalized linear mixed models (GLMM) applying as FDR cutoff = 0.05.

5. Polychromatic flow cytometry

5.1. T cell characterization

For the analysis of T cell skewing toward Th1, Th2, or Th17, and of chemokine receptor expression, thawed PBMC were washed twice with PBS and stained with the viability marker AQUA LIVE DEAD (ThermoFisher). Then, up to 1 million cells were washed and stained at 37 °C with the following mAbs: anti-CXCR3- AF488, -CXCR4-PE. Cells were washed again and stained at room temperature with anti-CD161-PC7, -CCR6-BV605, -CCR4-PE-CF594, -CD4-AF700, -CD8- APC-Cy7. Cells were washed, fixed, and permeabilized using Foxp3/Transcription Factor Staining Buffer Set (ThermoFisher). Finally, cells were stained with anti- GATA3-BV421 and anti-TBET-APC and washed. A minimum of 500,000 PBMC were acquired by using Attune NxT acoustic focusing flow cytometer (ThermoFisher). mAbs used are listed in Supplementary Table 6.

5.2. Proliferation assay

Cells were stimulated for 6 days in resting conditions, or after stimulation with anti-CD3 plus anti-CD28 mAbs (1 µg/mL each, Miltenyi Biotech, Bergisch Gladbach, Germany) and with 20 ng/mL IL-2. The fluorescent dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CSFE) was used at a concentration of 1 µg/mL (ThermoFisher) according to standard procedures [23]. Flow cytometric analyses for the identification of cycling cells belonging to different T cell populations were performed by gating CD4+, CD8+ T cells and CD19+ B cells. mAbs used are listed in Supplementary Table 2.

5.3 In vitro stimulation and intracellular cytokine staining

For functional assays on cytokine production by T cells, thawed isolated PBMCs were stimulated for 16 h at 37 °C in a 5% CO2 atmosphere with anti-CD3/CD28 (1 µg/mL) in complete culture medium (RPMI 1640 supplemented with 10% fetal bovine serum and 1% each of l- glutamine, sodium pyruvate, nonessential amino acids, antibiotics, 0.1 M HEPES, 55 µM β-mercaptoethanol). For each sample, at least 2 million cells were left unstimulated as negative control, and 2 million cells were stimulated. All samples were incubated with a protein transport inhibitor containing brefeldin A (Golgi Plug, Becton Dickinson) and previously titrated concentration of CD107a-PE. After stimulation, cells were stained with LIVE-DEAD Aqua (ThermoFisher Scientific) and surface mAbs recognizing CD4 AF700, and CD8 APC- Cy7 (Biolegend, San Diego, CA, USA). Cells were washed with stain buffer and fixed and permeabilized with the cytofix/cytoperm buffer set (Becton Dickinson) for cytokine detection. Cells were next stained with previously titrated mAbs recognizing CD3 PE- Cy5, IL-17 BV421, TNF BV605, IFN-γ FITC, IL-4 APC, or granzyme-B BV421 (all mAbs from Biolegend). Then, a minimum of 100,000 cells per sample were acquired on a Attune NxT acoustic cytometer (ThermoFisher) [24]. mAbs used are listed in Supplementary Table 2.

5. Quantification of cytokine plasma levels

The plasma levels of 62 molecular species were quantified using a Luminex platform (Human Cytokine Discovery, R&D System, Minneapolis, MN) for the simultaneous detection of the following molecules: G-CSF, PDGF-AA, EGF, PDGF-AB/BB, VEGF, GM-CSF, FGF, GRZB, IL-1A, IL-1RA, IL-2, IL-27, IL-4, IL-6, IL-10, IL-13, TNF, IL-17C, IL-11, IL-18, IL-23, IL-6RA, IL-19, IFN-B, IL-3, IL-5, IL-7, IL-12p70, IL-15, IL-33, TGF-B, IFN-G, IL-1B, IL-17, IL-17E, CCL3, CCL11, CCL20, CXCCL1, CXCL2, CCL5, CCL2, CCL4, CCL19, CXCL1, CXCL10, PD-L1, FLT-3, TACI, FAS, LEPTIN R, APRIL, OPN, BAFF, LEPTIN, BMP4, CD40 LIGAND, FAS LIGAND, BMP7, BMP2, TRAIL, according to the manufacturer's instruction. Data in the scatter plots represent the mean of two technical replicates.

6. Analysis of the correlations among all parameters

To identify possible correlations among the parameters we have studied, we have designed a table containing: i) all 27 clusters percentages obtained using the unsupervised analysis on living CD45+ cells; ii) 12 biochemical parameters reported in Supplementary Table 1, and iii) 61 plasma cytokines out of 62 because all values of IL-17p70 were identical. Pairwise correlations between variables were calculated and visualized as a correlogram using R function *corrplot* (Figure 5, and Supplementary Tables 2-4). Spearman's rank correlation coefficient (ρ) was indicated by color scale; significance was indicated by *P < 0.05, **P < 0.01, and ***P < 0.001. All variables were displayed using original order without applying any hierarchical clustering.

7. Statistical analysis

High-dimensional cytometric analysis was performed by using differential discovery in high-dimensional cytometry via high-resolution clustering. Quantitative variables were compared using Mann-Whitney test. Statistical analyses were performed using Prism 6.0 (GraphPad Software Inc., La Jolla, USA).

Declarations

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Conflict of interest

AQ, CP, GA, SD, DL, JN are employers of Fluidigm Corporation; LC is the CEO at Labospace. All other authors declare no conflict of interest.

Author contributions

S.D.B., L.G., D.L.T., A.P., A.Q., C.P., G.A., S.D., D.L., J.N., R.B., L.F. and M.Ma. carried out experiments and drafted the figures; L.G. and D.L.T. drafted the tables; C.L., M.Me., M.G., G.G., C.M. and F.F. followed patients; L.C., M.S. and T.T. contributed to experimental procedures; S.D.B., L.G., D.L.T. and A.C. performed bioinformatic and statistical analyses; S.D.B., L.G., C.M., F.F. and A.C. conceived the study. All authors read and approved the paper.

Data availability

The source data underlying Figs. 1–4 are provided as a Source Data file. Original .fcs files concerning cytofluorimetric analysis are deposited at the flowrepository.org in the following folder: FR-FCM-Z3GH.

References

1. Liu H, Wang LL, Zhao SJ, Kwak-Kim J, Mor G, Liao AH. Why are pregnant women susceptible to COVID-19? An immunological viewpoint. *J Reprod Immunol* **139**, 103122 (2020).
2. Chen H, *et al.* Clinical characteristics and intrauterine vertical transmission potential of COVID-19 infection in nine pregnant women: a retrospective review of medical records. *Lancet* **395**, 809–815 (2020).

3. Dashraath P, *et al.* Coronavirus disease 2019 (COVID-19) pandemic and pregnancy. *Am J Obstet Gynecol* **222**, 521–531 (2020).
4. Turan O, Hakim A, Dashraath P, Jeslyn WJL, Wright A, Abdul-Kadir R. Clinical characteristics, prognostic factors, and maternal and neonatal outcomes of SARS-CoV-2 infection among hospitalized pregnant women: A systematic review. *Int J Gynaecol Obstet* **151**, 7–16 (2020).
5. Schwartz DA, Graham AL. Potential Maternal and Infant Outcomes from (Wuhan) Coronavirus 2019-nCoV Infecting Pregnant Women: Lessons from SARS, MERS, and Other Human Coronavirus Infections. *Viruses* **12**, (2020).
6. Yee J, *et al.* Clinical manifestations and perinatal outcomes of pregnant women with COVID-19: a systematic review and meta-analysis. *Sci Rep* **10**, 18126 (2020).
7. Edlow AG, *et al.* Assessment of Maternal and Neonatal SARS-CoV-2 Viral Load, Transplacental Antibody Transfer, and Placental Pathology in Pregnancies During the COVID-19 Pandemic. *JAMA Netw Open* **3**, e2030455 (2020).
8. du Fosse NA, *et al.* Detailed immune monitoring of a pregnant woman with critical Covid-19. *J Reprod Immunol* **143**, 103243 (2021).
9. De Biasi S, *et al.* Expansion of plasmablasts and loss of memory B cells in peripheral blood from COVID-19 patients with pneumonia. *Eur J Immunol* **50**, 1283–1294 (2020).
10. Gibellini L, *et al.* Altered bioenergetics and mitochondrial dysfunction of monocytes in patients with COVID-19 pneumonia. *EMBO Mol Med* **12**, e13001 (2020).
11. Faas MM, Spaans F, De Vos P. Monocytes and macrophages in pregnancy and pre-eclampsia. *Front Immunol* **5**, 298 (2014).
12. Ander SE, Diamond, M. S., & Coyne, C. B.. Immune responses at the maternal-fetal interface. *Science Immunology* **4**, eaat6114 (2019).
13. Cristiani CM, Palella E, Sottile R, Tallerico R, Garofalo C, Carbone E. Human NK Cell Subsets in Pregnancy and Disease: Toward a New Biological Complexity. *Front Immunol* **7**, 656 (2016).
14. Hidaka Y, *et al.* Changes in natural killer cell activity in normal pregnant and postpartum women: increases in the first trimester and postpartum period and decrease in late pregnancy. *J Reprod Immunol* **20**, 73–83 (1991).
15. Cossarizza A, De Biasi S, Guaraldi G, Girardis M, Mussini C, Modena Covid-19 Working G. SARS-CoV-2, the Virus that Causes COVID-19: Cytometry and the New Challenge for Global Health. *Cytometry A* **97**, 340–343 (2020).
16. Pine AB, *et al.* Circulating markers of angiogenesis and endotheliopathy in COVID-19. *Pulm Circ* **10**, 2045894020966547 (2020).
17. De Biasi S, *et al.* Marked T cell activation, senescence, exhaustion and skewing towards TH17 in patients with COVID-19 pneumonia. *Nat Commun* **11**, 3434 (2020).
18. Benjamin JT, *et al.* Cutting Edge: IL-1alpha and Not IL-1beta Drives IL-1R1-Dependent Neonatal Murine Sepsis Lethality. *J Immunol* **201**, 2873–2878 (2018).

19. Lendak DF, Mihajlovic DM, Novakov-Mikic AS, Mitic IM, Boban JM, Brkic SV. The role of TNF-alpha superfamily members in immunopathogenesis of sepsis. *Cytokine* **111**, 125–130 (2018).
20. Okuyama M, Mezawa H, Kawai T, Urashima M. Elevated Soluble PD-L1 in Pregnant Women's Serum Suppresses the Immune Reaction. *Front Immunol* **10**, 86 (2019).
21. Crowell H, Zanotelli, V., Chevrier, S., Robinson, M.. CATALYST: Cytometry dATa anALYSis Tools. *R package version 1140*, (2020).
22. Weber L. diffcyt: Differential discovery in high-dimensional cytometry via high-resolution clustering. *R package version 188*, (2020).
23. De Biasi S, *et al.* Mitochondrial functionality and metabolism in T cells from progressive multiple sclerosis patients. *Eur J Immunol* **49**, 2204–2221 (2019).
24. Cossarizza A, *et al.* Guidelines for the use of flow cytometry and cell sorting in immunological studies (second edition). *Eur J Immunol* **49**, 1457–1973 (2019).