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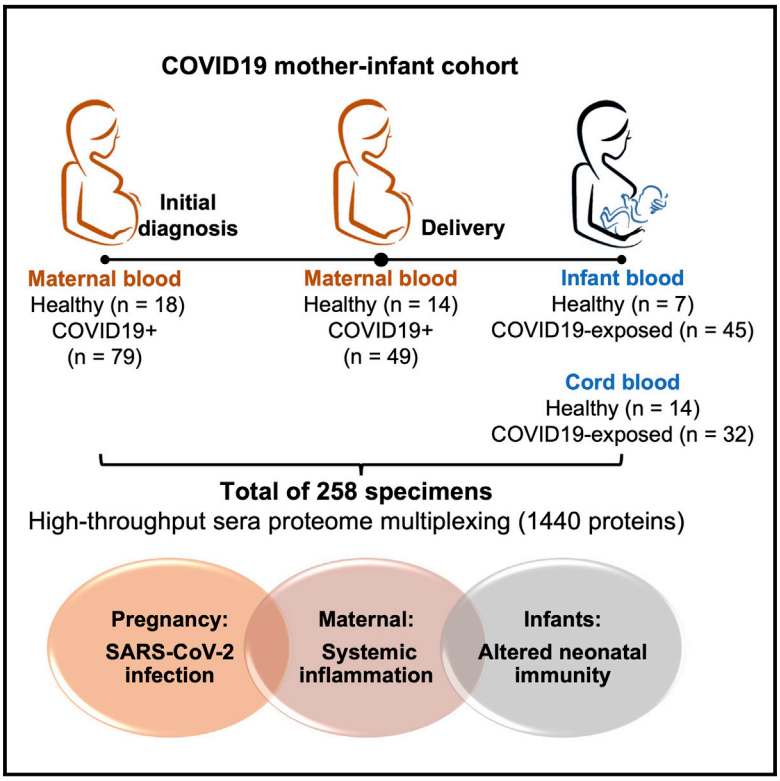
The systemic inflammatory landscape of COVID-19 in pregnancy: Extensive serum proteomic profiling of mother-infant dyads with *in utero* SARS-CoV-2

Graphical abstract



Highlights

- Prenatal SARS-CoV-2 infection triggers NF-κB-dependent immune activation
- Pregnant women with severe COVID-19 show antiviral IFN-λ signaling
- SARS-CoV-2 infection re-shapes maternal immunity at delivery
- COVID-19-exposed infants exhibit altered neonatal immunity at birth

Authors

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In brief

The study performed by Foo et al. unveils distinct immune alterations of mothers and infants induced by SARS-CoV-2 infection during pregnancy. These findings highlight the importance of long-term postpregnancy clinical follow-up of mother-infant dyads to prevent potential unforeseen health conditions following prenatal SARS-CoV-2 infection.

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The systemic inflammatory landscape of COVID-19 in pregnancy: Extensive serum proteomic profiling of mother-infant dyads with *in utero* SARS-CoV-2

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SUMMARY

While pregnancy increases the risk for severe COVID-19, the clinical and immunological implications of COVID-19 on maternal-fetal health remain unknown. Here, we present the clinical and immunological landscapes of 93 COVID-19 mothers and 45 of their SARS-CoV-2-exposed infants through comprehensive serum proteomics profiling for >1,400 cytokines of their peripheral and cord blood specimens. Prenatal SARS-CoV-2 infection triggers NF-κB-dependent proinflammatory immune activation. Pregnant women with severe COVID-19 show increased inflammation and unique IFN-λ antiviral signaling, with elevated levels of IFNL1 and IFNLR1. Furthermore, SARS-CoV-2 infection re-shapes maternal immunity at delivery, altering the expression of pregnancy complication-associated cytokines, inducing MMP7, MDK, and ESM1 and reducing BGN and CD209. Finally, COVID-19-exposed infants exhibit induction of T cell-associated cytokines (IL33, NFATC3, and CCL21), while some undergo IL-1β/IL-18/CASP1 axis-driven neonatal respiratory distress despite birth at term. Our findings demonstrate COVID-19-induced immune rewiring in both mothers and neonates, warranting long-term clinical follow-up to mitigate potential health risks.

INTRODUCTION

The immunological consequences of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in pregnancy remain poorly understood. It is well documented that pregnant women are more vulnerable to infectious illnesses, including certain respiratory pathogens, likely related to the paradox of maternal-fetal immune tolerance.[1,2] During the H1N1 pandemic of 2009, the

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incidence of mortality and acute respiratory distress syndrome (ARDS) in pregnant women highly exceeded those observed in the general population.[3,4] Similar to influenza studies, rates of ARDS and extracorporeal membrane oxygenation (ECMO) are higher among pregnant women with SARS-CoV-2.[5,6] SARS-CoV-2 joins the list of highly pathogenic coronaviruses, which include SARS-CoV-1 and the Middle East respiratory syndrome coronavirus (MERS-CoV). Both SARS-CoV-1 and MERS-CoV were found to cause adverse pregnancy outcomes, including miscarriage, premature delivery, and maternal death.[7,8] The clinical spectrum of COVID-19 in pregnancy ranges from asymptomatic to critical disease, requiring mechanical ventilation or even ECMO, and in some cases, death.[9–11] Recent surveillance studies suggest that pregnant women are at an increased risk of severe COVID-19, marked by a dysregulated inflammatory response.[12]

While episodes of SARS-CoV-2 mother-to-child transmission are estimated to be low,[5] the long-term consequences following *in utero* exposure are unknown. Although the clinical presentation in most neonates with SARS-CoV-2 appears relatively benign,[13,14] some infants develop RD, requiring supplemental oxygen or intubation.[15,16] Infants whose mothers have severe/critical SARS-CoV-2 infection but do not themselves become infected are at risk because of maternal hypoxia and multi-organ failure.[17] It is unclear to what extent immune dysregulation in severe COVID-19 in pregnancy, a state marked by tightly regulated T cell balance, augments the risk of systemic inflammation.[18] Systemic inflammation in pregnancy is associated with severe fetal outcomes such as fetal demise,[19] raising concern about the immediate and long-term infant consequences of severe SARS-CoV-2 infection in pregnancy following exposure to a maternal cytokine storm. Maternal immune activation in pregnancy has been linked to potential long-term neurodevelopmental pathology such as autism spectrum disorder in early childhood and schizophrenia in young adulthood.[20–23]

Emerging pathogens should always be considered potentially hazardous to maternal-fetal health. Therefore, it is crucial to define the obstetrical, clinical, and infant outcomes associated with SARS-CoV-2 infection in pregnancy to further elucidate the mechanisms of pathogenesis in this vulnerable population. The COVID-19 Outcomes in Mother-Infant Pairs (COMP) study is a prospective observational cohort of mother-infant dyads diagnosed with SARS-CoV-2 infection in pregnancy in the United States and in Brazil, two countries disproportionately affected by COVID-19. Here, we describe the clinical outcomes and extensive serum cytokine profiles of the initial mother-infant dyads from our longitudinal study enrolled in Los Angeles, California.

RESULTS

Clinical outcomes

A total of 93 women diagnosed with SARS-CoV-2 infection by nasopharyngeal (NP) RT-PCR (N = 90 maternal cases) or serology (N = 3 maternal cases) at any point during gestation from April 15, 2020 to March 1, 2021 were enrolled in our study. Women with a history of COVID-19 vaccination were excluded from the analysis. Table 1 describes the demographics and clinical characteristics of the women infected with SARS-CoV-2 at any point during gestation stratified by the National Institutes of Health (NIH) COVID-19 severity of illness categories,[24,25] and includes infants born before March 1, 2021. The median maternal age was 33 years (ranges 16–44). Nearly half (47.3%) of the cohort was Latina, and 60.0% of those with severe/critical disease were also Latina ($p < 0.001$). Over half of the participants with severe/critical disease (55.0%) had public insurance, compared to 39.8% in the overall cohort ($p = 0.039$). The majority of participants with severe/critical disease were diagnosed during the second trimester (60.0%), compared to only one-third (33.3%) in the overall cohort ($p < 0.001$). Participants with severe/critical disease were more likely to have any comorbidity compared to the overall cohort (75.0% versus 45.2%; $p < 0.001$). Of the 20 participants with severe/critical disease, 13 (65.0%) had a prepregnancy body mass index (BMI) > 30, compared to 31.2% of the overall cohort. One-fifth of the overall cohort required enhanced oxygen requirements, and 7.5% were intubated. Five of our participants developed ARDS, and 3 required ECMO. A total of 16 participants (17.2%) in the total cohort had evidence of cytokine storm syndrome/cytokine release syndrome, compared to 75.0% of those with severe/critical disease. Participants with asymptomatic and mild/moderate disease did not receive any treatment, although 75.0% of women with severe/critical disease received remdesivir and 70.0% received dexamethasone. Seventy pregnancies were completed by March 1, 2021 (Table 1, B), and 32.9% were diagnosed with a hypertensive disorder. Nearly one-fifth of the cohort (15.7%) developed preeclampsia or HELLP (hemolysis, elevated liver enzymes, and low platelets). There was no difference in mode of delivery across the COVID-19 severity categories. Overall, there were 2 miscarriages (<20 weeks), 2 fetal losses (≥20 weeks), 2 pregnancy terminations, and 1 maternal-fetal demise. There were 5 multiple gestations. While 69 infants within the cohort were born as of March 1, 2021 to 60 women, infant outcomes with associated O-link data were reported for the first 45 infants (Table 1, C). Infants born to mothers with severe/critical disease were more likely to be preterm (100% versus 31.1%; $p < 0.001$), have low birth weight (<2,500 g; 100% versus 41.7%; $p < 0.001$), and show RD (100% versus 33.3%; $p < 0.001$). The median head circumference was 25.5 cm (range 24.4–28.5) for infants born to mothers with severe/critical disease, compared to 33.0 cm (range 24.4–38.5) for all infants ($p < 0.001$). However, the median head circumference based on the Fenton growth chart was 41.2% for both groups.

Prenatal COVID-19$^+$ status dysregulates maternal immune response

We sought to characterize the immunological impact of *in utero* SARS-CoV-2 infection using maternal and neonatal sera (Figure 1A). Maternal blood specimens (N = 93 maternal participants total) were collected at the time closest to initial laboratory-confirmed diagnosis of infection (N = 79), and at the time of labor and delivery (N = 49). Gestational age-matched healthy pregnant women were included as controls (N = 18). Cord blood (N = 32) was collected at delivery when possible, and infant blood (N = 45) specimens were collected at day 1 of life. Sera isolated from blood specimens were subjected to high-throughput

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Table 1. Demographics and clinical characteristics of mother-infant dyads infected with SARS-CoV-2 during pregnancy[a]

A. Maternal demographics and medical history (N = 93)	All women, N = 93	Asymptomatic, N = 12 (12.9%)	Mild/moderate, N = 61 (65.6%)	Severe/critical, N = 20 (21.5%)	*P*
Median age, y, median (range)	33 (16–44)	33 (19–40)	34 (16–44)	33 (18–44)	0.74
Race/ethnicity, N (%)					<0.001
Latina	44 (47.3)	4 (33.3)	28 (45.9)	12 (60.0)	
White	23 (24.7)	3 (25.0)	17 (27.9)	3 (15.0)	
Black/African American	8 (8.6)	2 (16.7)	2 (3.3)	4 (20.0)	
Asian/other	18 (19.4)	3 (25.0)	14 (23.0)	1 (5.0)	
Insurance, N (%)					0.039
Public	37 (39.8)	5 (41.7)	21 (34.4)	11 (55.0)	
Private	56 (60.2)	7 (58.3)	40 (65.6)	9 (45.0)	
Occupation, N (%)					<0.001
Healthcare worker	16 (17.2)	1 (8.3)	13 (21.3)	2 (10.0)	
Other	77 (82.8)	11 (91.7)	48 (78.7)	18 (90.0)	
Parity, median (range)	2 (1–10)	3 (1–6)	2 (1–10)	3 (1–7)	0.32
Gestational age at diagnosis, N (%)					<0.001
1st trimester	18 (19.4)	1 (8.3)	15 (24.6)	2 (10.0)	
2nd trimester	31 (33.3)	2 (16.7)	17 (27.9)	12 (60.0)	
3rd trimester	44 (47.3)	9 (75.0)	29 (47.5)	6 (30.0)	
Days of symptoms in relation to diagnosis, median (range)	2 (0–21)	–	2 (0–21)	3 (0–9)	0.62
Days of symptoms in relation to enrollment, median (range)	14 (0–111)	–	16 (0–111)	9 (0–85)	0.091
Medical history prepregnancy, N (%)					<0.001
Any comorbidities	42 (45.2)	5 (41.7)	22 (36.1)	15 (75.0)	
Obesity (prepregnancy BMI >30)	29 (31.2)	3 (25.0)	13 (21.3)	13 (65.0)	
Diabetes mellitus (not gestational)	4 (4.3)	1 (8.3)	2 (3.3)	1 (5.0)	
Pulmonary arterial hypertension	1 (1.1)	0 (0.0)	0 (0.0)	1 (5.0)	
Congenital heart disease	4 (4.3)	1 (8.3)	2 (3.3)	1 (5.0)	
Asthma	11 (11.8)	1 (8.3)	5 (8.2)	5 (25.0)	
Autoimmune disorders[b]	4 (4.3)	1 (8.3)	3 (4.9)	0 (0.0)	
HIV	2 (2.2)	0 (0.0)	1 (1.6)	1 (5.0)	
COVID symptoms at time of diagnosis, N (%)					<0.001
None	21 (22.6)	12 (0.0)	7 (11.5)	2 (10.0)	
Fever	26 (28.0)	0 (0.0)	13 (21.3)	13 (65.0)	
Cough/sore throat/rhinorrhea	59 (63.4)	0 (0.0)	43 (70.5)	16 (80.0)	
Dyspnea	21 (22.6)	0 (0.0)	4 (6.6)	17 (85.0)	
Abdominal pain/nausea/emesis/diarrhea	23 (24.7)	0 (0.0)	20 (32.8)	3 (15.0)	
Anosmia/dysgeusia	21 (22.6)	0 (0.0)	16 (26.2)	5 (25.0)	
Fatigue/myalgias/arthralgias	45 (48.4)	0 (0.0)	32 (52.5)	13 (65.0)	
COVID-19 related complications[c]					N/A
Pneumonia/pneumonitis	11 (11.8)	0 (0.0)	2 (3.3)	9 (45.0)	
Admission to intensive care unit	13 (14.0)	0 (0.0)	0 (0.0)	13 (65.0)	
Enhanced oxygen requirements	18 (19.4)	0 (0.0)	0 (0.0)	18 (90.0)	
Intubated	7 (7.5)	0 (0.0)	0 (0.0)	7 (35.0)	
Need for extracorporeal membrane oxygenation (ECMO)	3 (3.2)	0 (0.0)	0 (0.0)	3 (15.0)	
Acute respiratory distress syndrome (ARDS)	5 (5.4)	0 (0.0)	0 (0.0)	5 (25.0)	

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Table 1. *Continued*

A. Maternal demographics and medical history (N = 93)	All women, N = 93	Asymptomatic, N = 12 (12.9%)	Mild/moderate, N = 61 (65.6%)	Severe/critical, N = 20 (21.5%)	*P*
Need for vasopressors	5 (5.4)	0 (0.0)	0 (0.0)	5 (25.0)	
Myocarditis	1 (1.1)	0 (0.0)	0 (0.0)	1 (5.0)	
Thromboembolic events	3 (3.2)	0 (0.0)	0 (0.0)	3 (15.0)	
Maternal death	1 (1.1)	0 (0.0)	0 (0.0)	1 (5.0)	
Cytokine storm syndrome/cytokine release syndrome	16 (17.2)	0 (0.0)	1 (1.6)	15 (75.0)	
Need for blood transfusion	4 (4.3)	0 (0.0)	1 (1.6)	3 (15.0)	
COVID-19 treatment					N/A
Any treatment	16 (17.2)	0 (0.0)	0 (0.0)	16 (80.0)	
Use of remdesivir	15 (16.1)	0 (0.0)	0 (0.0)	15 (75.0)	
Use of dexamethasone	14 (15.1)	0 (0.0)	0 (0.0)	14 (70.0)	
Use of convalescent plasma	8 (8.6)	0 (0.0)	0 (0.0)	8 (40.0)	
Use of other immunomodulators	2 (2.2)	0 (0.0)	0 (0.0)	2 (10.0)	
B. Pregnancy-related clinical findings (N = 70[d])	All women, N = 70	Asymptomatic, N = 12 (17.14%)	Mild/moderate, N = 44 (62.86%)	Severe/critical, N = 14 (20%)	*P*
Complications during the course of pregnancy pre-delivery, N (%)					
Gestational diabetes	9 (12.8)	2 (16.7)	3 (6.8)	4 (28.6)	0.10
Hypertensive disorder	23 (32.9)	3 (25.0)	14 (31.8)	6 (42.9)	0.61
Late pregnancy and postpartum complications, N (%)					
Fetal growth restriction	11 (15.7)	2 (16.7)	8 (18.2)	1 (7.1)	0.07
Chorioamnionitis	6 (8.6)	1 (8.3)	3 (6.8)	2 (14.3)	0.05
Postpartum hemorrhage	11 (15.7)	0 (0.0)	5 (11.4)	6 (42.9)	<0.001
Preeclampsia/HELLP	11 (15.7)	3 (25.0)	6 (13.6)	2 (14.3)	0.72
Preterm rupture of membranes	4 (5.7)	0 (0.0)	2 (4.5)	2 (14.3)	0.02
Unknown	4 (5.7)	0 (0.0)	1 (2.3)	3 (21.4)	0.05
Mode of delivery/pregnancy endpoint, N (%)					0.10
NSVD	33 (47.1)	6 (50.0)	25 (56.8)	2 (14.3)	
C-section	25 (35.7)	5 (41.7)	14 (31.8)	6 (42.9)	
Vacuum-assisted vaginal delivery	1 (1.4)	0 (0.0)	1 (2.3)	0 (0.0)	
Unknown	4 (5.7)	0 (0.0)	1 (2.3)	3 (21.4)	
Miscarriage/termination/fetal loss	7 (10.0)	1 (8.3)	3 (6.8)	3 (21.4)	
Miscarriage (<20 weeks)	2 (2.9)	0 (0.0)	1 (2.3)	1 (7.1)	
Fetal loss (≥20 weeks)	2 (2.9)	0 (0.0)	1 (2.3)	1 (7.1)	
Pregnancy termination	2 (2.9)	1 (8.3)	1 (2.3)	0 (0.0)	
Maternal-fetal demise	1 (1.4)	0 (0.0)	0 (0.0)	1 (7.1)	
Pregnancies resulting in live births (N = 70)	63 (90.0)	11 (91.7)	41 (93.2)	11 (78.6)	0.28
No. multiple gestations[e]	5 (7.1)	1 (8.3)	2 (4.5)	2 (14.3)	0.46
No. infants born as of March 1, 2021	69 (98.6)	13 (18.57)	43 (61.43)	13 (18.57)	
C. Infant outcomes with associated O-link data[f] (N = 45), N (%)	All women	Asymptomatic, N = 11 (24.44%)	Mild/moderate, N = 28 (62.22%)	Severe/critical, N = 6 (13.34%)	*P*
Preterm delivery	14 (31.1)	0 (0.0)	8 (28.6)	6 (100)	<0.001
Small-for-gestational-age	5 (12.5)	1 (9.1)	4 (14.3)	0 (0.0)	0.58
Low birth weight (<2,500 g)	13 (41.7)	1 (9.1)	6 (21.4)	6 (100)	<0.001

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Table 1. *Continued*

A. Maternal demographics and medical history (N = 93)	All women, N = 93	Asymptomatic, N = 12 (12.9%)	Mild/moderate, N = 61 (65.6%)	Severe/critical, N = 20 (21.5%)	*P*
Respiratory distress	15 (33.3)	3 (27.3)	6 (21.4)	6 (100)	<0.001
Head circumference, median (range)	33.0 (24.4–38.5)	33.5 (31.3–38.5)	33.3 (29.5–36.8)	25.5 (24.4–28.5)	<0.001

[a]Women were grouped into the following NIH COVID-19 severity of illness categories. (1) Asymptomatic: individuals who test positive for SARS-CoV-2, but who have no symptoms that are consistent with COVID-19. (2) Mild/moderate: includes the "mild" category, individuals who have any of the various signs and symptoms of COVID-19, but who do not have shortness of breath, dyspnea, or abnormal chest imaging, and the "moderate" category, individuals who show evidence of lower respiratory disease during clinical assessment or imaging and who have saturation of oxygen (SpO_2) $\geq$94% on room air at sea level. (3) Severe/critical: includes the "severe" category, individuals who have SpO_2 <94% on room air at sea level, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO_2/FiO_2) <300 mm Hg, respiratory frequency >30 breaths/min, or lung infiltrates >50%, and the "critical" category, individuals who have respiratory failure, septic shock, and/or multiple organ dysfunction.
[b]Includes systemic lupus erythematosus, cutaneous lupus, multiple sclerosis, and mixed connective tissue disorder.
[c]Represents comparison of mild/moderate versus severe/critical.
[d]70 pregnancies completed to date, including 66 deliveries at UCLA and 4 at an outside hospital; 23 were expecting as of March 1, 2021.
[e]Two viable twin deliveries, 2 triplet deliveries, 1 twin demise.
[f]There have been 69 infants born to date, although outcomes are presented only for those with available O-link data.

next-generation sequencing (NGS)-based proteomics multiplexing to detect for >1,400 cytokines and serum proteins (Figures 1A and S1).

To characterize the prenatal immune responses elicited by acute SARS-CoV-2 infection, the sera proteome profiles from 79 infected pregnant women (whose illness ranged from asymptomatic to critical) at initial diagnosis were compared to those from gestational age-matched healthy current and prepandemic controls (n = 18). Across >1,400 sera cytokines screened, 171 cytokines were significantly altered ($p < 0.05$ and $-2 <$ mean fold change [FC] > 2); specifically, the levels of 169 cytokines and 2 cytokines were induced and repressed, respectively (Figure 1B). In comparison to healthy pregnant controls, paired immunoglobin-like type 2 receptor alpha (PILRA) (mean FC = 271.27), cathepsin B (CTSB) (mean FC = 152.06), kallikrein-related peptidase 4 (KLK4) (mean FC = 140.18), adenosine deaminase 2 (ADA2) (mean 114.79), and interferon-γ (IFN-γ) (mean 107.93) were the highest induced serum proteins in pregnant women with COVID-19 (Figure 1C). However, only 2 cytokines, KLK13 (mean FC = -2.37) and interleukin-13 (IL-13) (mean FC = -5.77), were repressed during acute infection (Figure 1D). This is strikingly different from the immunoprofiles of non-pregnant infected adult patients who showed high serum levels of IL-6, IL-1β, tumor necrosis factor (TNF), and C-X-C motif chemokine ligand 10 (CXCL10) during SARS-CoV-2 infection.[24,26] Although TNF (mean FC = 2.11) was also detectably elevated in pregnant women with COVID-19, only modest increases of IL-6 (mean FC = 1.64), IL-1β (mean FC = 0.51), and CXCL10 (mean FC = 1.07) were observed (Figure 1E).

Ingenuity Pathway Analysis (IPA) suggested nuclear factor κB (NF-κB) complex as the top upstream regulator to generate serum cytokine profiles of pregnant women with COVID-19, promoting a robust proinflammatory cytokine-mediated immune activation such as IFN-γ, C-C motif chemokine ligand 2 (CCL2), IL-32, and TNF (Figure 1F). Using peripheral blood mononuclear cells (PBMCs) derived from healthy pregnant controls (n = 6) and SARS-CoV-2-infected pregnant women (n = 11) at the time of initial diagnosis, we validated the robust transcriptional induction of NF-κB p50 as well as IL-1β, CXCL10, and TNF during COVID-19-affected (COVID-19$^+$) pregnancies (Figure S2A). These results collectively suggest that SARS-CoV-2 infection in pregnant women induces changes in serum cytokine profiles. However, these are clearly distinct from what is seen in non-pregnant populations.[24,27–29]

Immune signatures of asymptomatic COVID-19 during pregnancy

Sera collected from our participants at the time point closest to their initial diagnosis (n = 79) consisted of 9 asymptomatic, 46 mild, 4 moderate, 6 severe, and 14 critical cases. Unbiased clustering revealed that the immune profiles of pregnant infected patients shifted away from those of healthy pregnant controls as the disease progressed toward severe/critical outcomes, creating immune profiling clusters of pregnant women with varying disease severity of COVID-19 (Figure 2A). Asymptomatic patients with COVID-19 displayed overlapping immune signatures to those of pregnant women with mild/moderate COVID-19 (Figure 2A). While a small set of 43 serum cytokine levels was exclusively altered in the asymptomatic COVID-19$^+$ group (n = 9) when compared to healthy pregnant controls, the levels of 188 and 185 cytokines were significantly altered in the mild/moderate COVID-19$^+$ pregnant group (n = 50) and the severe/critical COVID-19$^+$ pregnant group (n = 20), respectively (Figure 2B). Consistent with the higher number of cytokines altered in the symptomatic group compared to the asymptomatic COVID-19 group, lower cycle threshold (Ct) values (inversely proportional to viral load) were detected in pregnant women with symptomatic COVID-19 (n = 46 for all available Ct values; Figure 2C). Angiotensin-converting enzyme 2 (ACE2), the receptor for SARS-CoV-2, was also highly induced in pregnant women with symptomatic COVID-19 compared to the asymptomatic group (Figure 2D).

Among the 43 immune signatures that were significantly altered ($p < 0.05$) in pregnant women with asymptomatic COVID-19, 41 cytokines were significantly upregulated, while only 2 cytokines, CXCL3 (mean FC = -0.17) and TP53INP1 (mean FC = -0.35), were downregulated (Figures 2E and S2B). The highest induced serum proteins during asymptomatic COVID-19$^+$ pregnancy included X-prolyl aminopeptidase 2

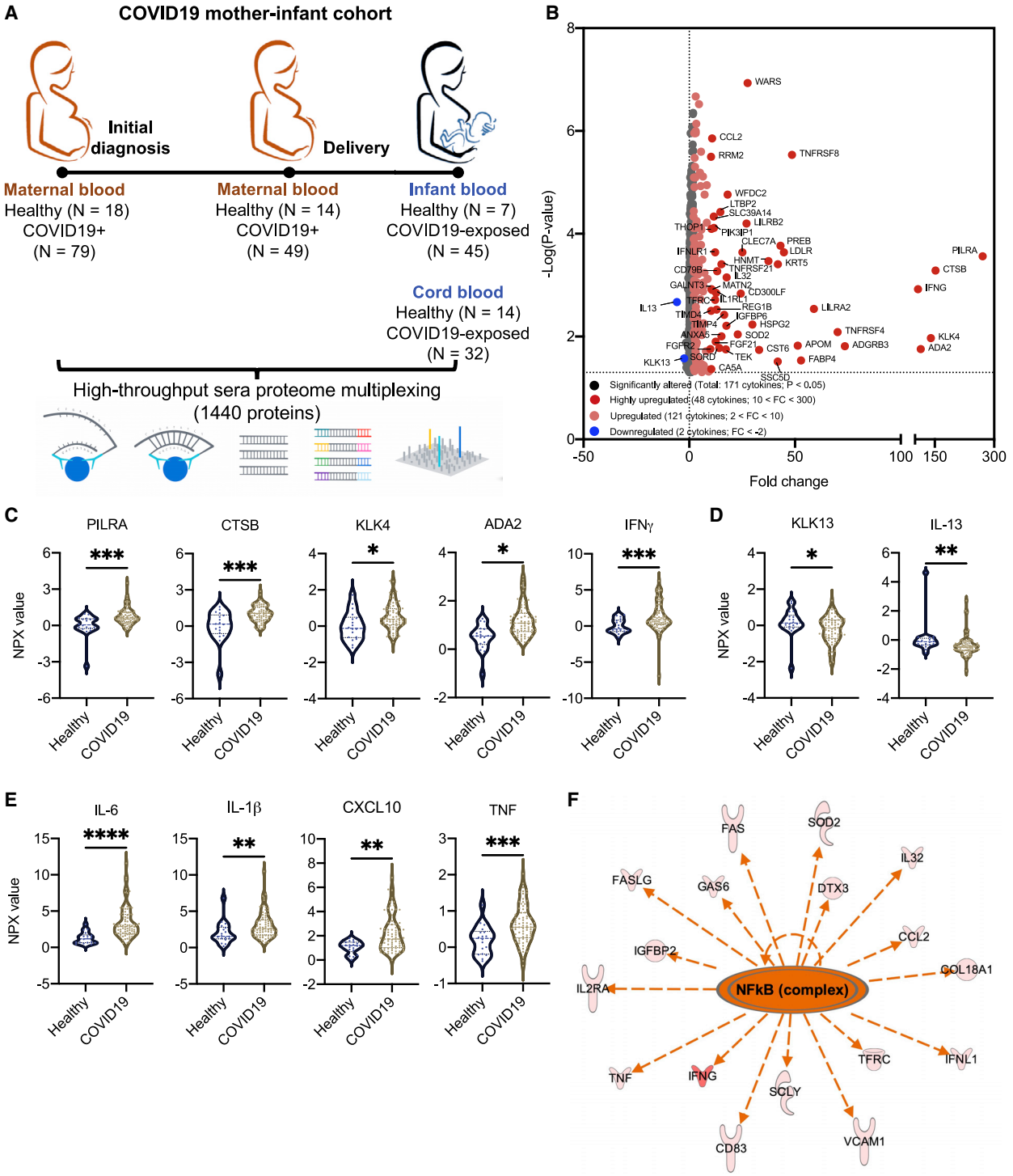

Figure 1. Acute SARS-CoV-2 infection reshapes maternal immune responses

(A) Schematic representation of serum proteome multiplexing of COVID-19-affected (COVID-19^{+}) mother-infant pairs. Maternal blood specimens (n = 93 maternal participants total) were collected at the time of diagnosed infection (n = 79) and delivery (n = 49). Neonatal (n = 45) and cord (n = 32) blood specimens were collected within 24 h of birth.

(B) Volcano plot illustrating the cytokines that are significantly altered ($p < 0.05$ and $-2 < FC > 2$) during COVID-19^{+} pregnancy.

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(XPNPEP2) (mean FC = 29.63), N-terminal prohormone of brain natriuretic peptide (NTproBNP) (mean FC = 26.7), serine peptidase inhibitor Kazal type 6 (SPINK6) (mean FC = 10.28), and ectodysplasin A2 receptor (EDA2R) (mean FC = 8.16; Figures 2F and S2C). In addition, allograft inflammatory factor 1 (AIF-1) (mean FC = 1.01), a marker for activated macrophage, and matrix metalloproteinase 7 (MMP7) (mean FC = 1.06), IL-19 (mean FC = 0.65), Forkhead box protein O1 (FOXO1) (mean FC = 0.28), and MMP9 (mean FC = 0.1) were also modestly induced in the pregnant women with asymptomatic COVID-19, suggestive of an anti-inflammatory M2 macrophage activation. Using STRING cluster analysis, MMP9 was identified as a central protein in modulating extracellular matrix (ECM) disassembly and collagen metabolism (Figures 2H and 2I). The dysregulated MMP9:TIMP1 (TIMP metallopeptidase inhibitor 1) ratio has been associated with respiratory disorders; increased ratio has been implicated with chronic obstructive pulmonary disease,[30] whereas decreased ratio has been linked to airway narrowing.[31] Despite significant increases in MMP9 and TIMP1 expressions in asymptomatic cases, their MMP9:TIMP1 ratio was not disrupted, but pregnant women with symptomatic COVID-19 exhibited a significantly lower ratio when compared to healthy controls (Figures 2I, S2D, and S2E). Our findings suggest that a skewed M2 anti-inflammatory response could contribute to the lack of sequelae in pregnant women with asymptomatic COVID-19.

Severe/critical COVID-19 promotes prenatal immune activation and overt inflammation

Next, we sought to identify the differential immune responses elicited during SARS-CoV-2 infection of pregnant women with symptomatic COVID-19 onset. We identified 188 and 185 cytokines that were specifically affected in mild/moderate and severe/critical COVID-19 groups, respectively (Figure 3A). In both mild/moderate and severe/critical COVID-19 groups, the majority (96.8% of mild/moderate; 67% severe/critical) of the affected cytokines were induced in their expressions. A common predicted activation of inflammatory and antiviral signaling pathways included NF-κB signaling, IFN induction, nitric oxide (NO) and reactive oxygen species (ROS) production in macrophages, coronavirus pathogenesis pathway, and STAT3 signaling. Interestingly, IL-15 canonical production pathway was detectably higher in the mild/moderate COVID-19 groups than in the severe/critical COVID-19 groups (Figure 3B; Table S2).

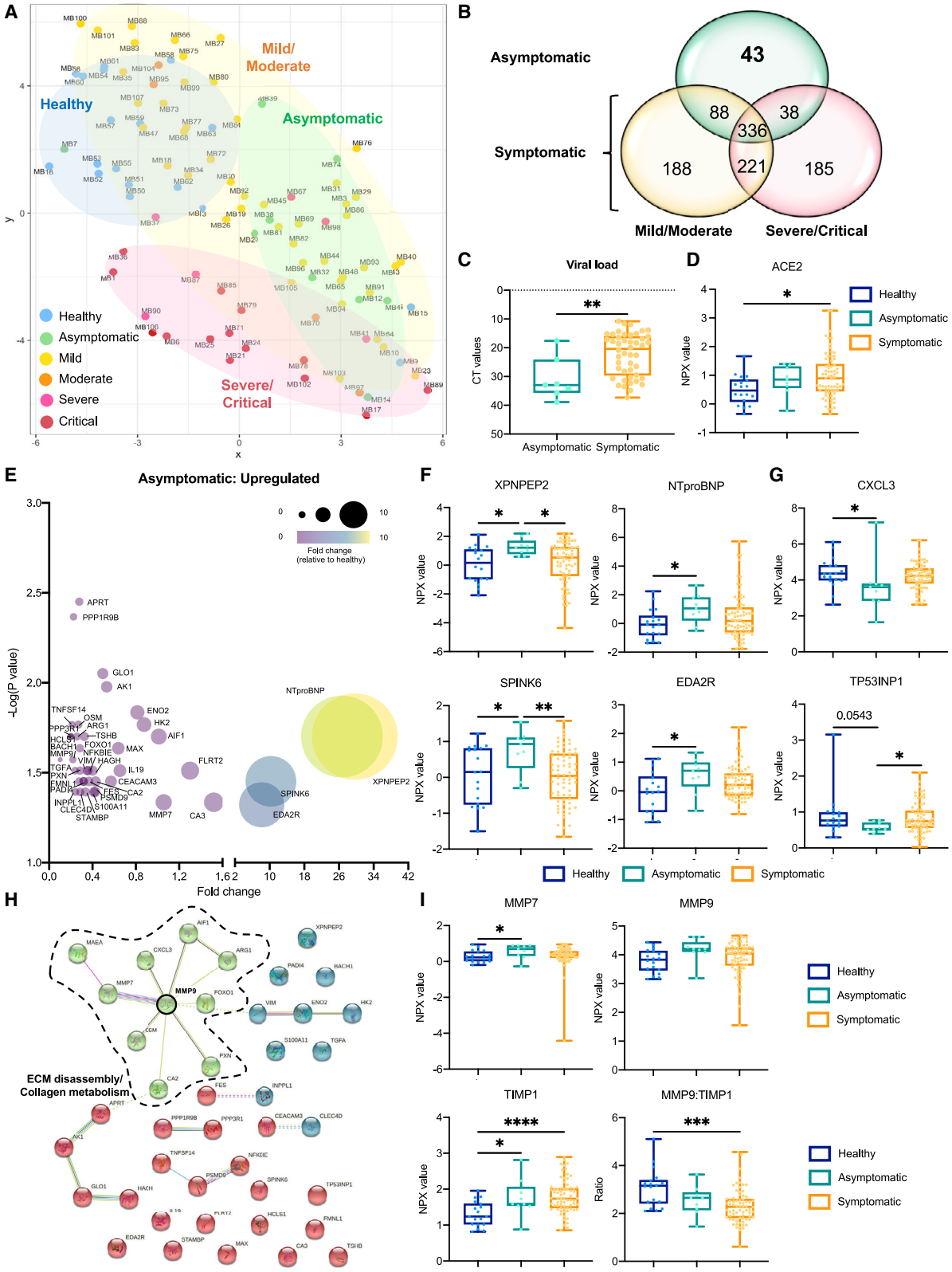
Of the 188 significantly affected cytokines, the top immune signatures induced in mild/moderate COVID-19$^+$ pregnancies were KLK4 (mean FC = 142.07), adhesion G protein-coupled receptor B3 (ADGRB3) (mean FC = 97.4), TNF receptor superfamily member 4 (TNFRSF4) (mean FC = 77.21), scavenger receptor cysteine-rich family member with 5 domains (SSC5D) (mean FC = 50.63), cystatin E/M (CST6) (mean FC = 40), TIMP4 (mean FC = 23.52), TEK receptor tyrosine kinase (TEK) (mean FC = 18.93), galanin and GMAP prepropeptide (GAL) (mean FC = 10.86), peptidase domain containing associated with muscle regeneration 1 (PAMR1) (mean FC = 10.72), and KLK11 (mean FC = 10.67) when compared to healthy controls (Figure 3A). However, severe/critical COVID-19$^+$ pregnancies triggered overt expressions of fatty acid-binding protein 4 (FABP4) (mean FC = 71.68), superoxide dismutase 2 (SOD2) (mean FC = 55.64), sorbitol dehydrogenase (SORD) (mean FC = 29.42), carbonic anhydrase 5A (CA5A) (mean FC = 23.13), IFNL1 (mean FC = 17.67), Fc receptor-like B (FCRLB) (mean FC = 16) and ACE2 (mean FC = 2.29), along with repressions of selectin P ligand (SELPLG) (mean FC = −77.37), transmembrane serine protease 5 (TMPRSS5) (mean FC = −28.51), and cerebellin 4 precursor (CBLN4) (mean FC = −12.29) (Figures 3A and 3C). As IFN-λ has been reported to play a crucial role in preventing vertical transmission of Zika virus (ZIKV) during prenatal infection,[32] high expressions of IFNL1 and IFNLR1 during severe/critical COVID-19$^+$ pregnancies may contribute to blocking the vertical transmission of SARS-CoV-2 (Figure 3D).

Further comparison analysis of the predicted canonical pathways between mild/moderate COVID-19 groups and severe/critical COVID-19 groups showed the pronounced activation of (1) inflammatory responses—high mobility group box protein 1 (HMGB1) signaling, triggering receptor expressed on myeloid cells' triggering receptor expressed on myeloid cells 1 (TREM1) signaling, IL-6 signaling, and IL-17 signaling, and (2) cell death signaling—death receptor signaling, apoptosis, and ferroptosis in severe/critical COVID-19$^+$ pregnancies, indicating the association of a robust inflammatory immune response with the cell death signaling pathway present during severe COVID-19 disease progression (Figure 3E; Table S3). These biological processes were evidenced by pronounced increases of proinflammatory cytokines IFN-γ (mean FC = 251.08), TNF (mean FC = 4.49), IL-6 (mean FC = 3.25), IL-18 (mean FC = 2.4), and IL-1β (mean FC = 0.54) in pregnant women with severe/critical COVID-19 (Figures 3F and 3G). These data point toward a highly inflammatory systemic immune signature in severe/critical COVID-19$^+$ pregnancies.

Prenatal SARS-CoV-2 infection re-shapes maternal immunity at delivery

To determine whether prenatal SARS-CoV-2 infections affected maternal immunity at delivery, we compared immune profiles at delivery between serum specimens of control healthy women (n = 14) and pregnant women with COVID-19 (n = 49) (Figure 4A). When cases at delivery were compared to gestational age-matched healthy controls at delivery (Figures 1 and 2), only 17 cytokines with threshold for FC <−2 (2 cytokines) or >2 (15 cytokines) were altered at delivery in women who had a history of COVID-19. The significantly altered cytokines were further classified into (1) pregnancy complication associated or (2) immune activation associated (Figures 4B and 4C). Pregnancy complication-associated cytokines included the upregulation of MMP7

(C–E) Highly upregulated and downregulated cytokines (C and D) and (E) proinflammatory cytokines that are significantly altered in COVID-19$^+$ pregnancy (n = 79) compared to healthy pregnancy (n = 18). Data are presented as means ± SEMs, using Mann-Whitney *U* test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. (F) Predicted upstream regulator of COVID-19-induced immune activation.



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(mean FC = 21.07), midkine (MDK) (mean FC = 2.04) and endothelial cell-specific molecule 1 (ESM1) (mean FC = 2.88), and the downregulation of biglycan (BGN) (mean FC = −4.24) and dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin/cluster of differentiation 209 (DC-SIGN/CD209) (mean FC = −2.36) (Figure 4B). Induction of other cytokines such as protein kinase C theta (PRKCQ) (mean FC = 12.97),[33] surfactant protein A2 (SFTPA2) (mean FC = 18.56),[34] TNFRSF6B (mean FC = 8.62),[35] TNFRSF8 (mean FC = 3.26),[36] and FCRLB (mean FC = 3.18)[37] in COVID-19$^+$ pregnancies indicated possible immune activation at the time of delivery (Figure 4C).

When we compared the serum immune profiles of healthy pregnant women between delivery and 2nd or 3rd trimester of their pregnancies, we observed a large number of significantly altered cytokines ($p < 0.05$; Figure 4D). Among the 666 cytokines that were affected ($p < 0.05$) at delivery compared to 2nd trimester pregnancies, 169 cytokines were significantly induced (FC > 2) and 6 cytokines were repressed (FC <−2; Figure 4E). However, 623 cytokines were altered at delivery when compared to 3rd trimester pregnancies, with 188 cytokines significantly induced (FC > 2) and 2 cytokines repressed (FC <−2; Figure 4E). This suggests that the progression of pregnancy may be associated with the reshaping of serum immune profiles.

To characterize how COVID-19 diagnosis at different pregnancy stages affected immune response at delivery, we analyzed serial serum specimens from patients diagnosed with COVID-19 during the 2nd trimester (n = 9) or the 3rd trimester of pregnancy (n = 18) and at the time of their respective deliveries (Figure 4D). To identify the immune alterations at the time of delivery specifically associated with prenatal SARS-CoV-2 infection, we made comparisons across the various pregnancy groups: (1) healthy controls—delivery (n = 14) versus 2nd trimester (n = 5); (2) healthy controls—delivery (n = 14) versus 3rd trimester (n = 6); (3) COVID-19 follow-up—delivery versus 2nd trimester (n = 9); and (4) COVID-19 follow-up—delivery versus 3rd trimester (n = 18; Figure 4E). This analysis identified 7 cytokines and 21 cytokines that were significantly altered ($p < 0.05$; $-2 < FC > 2$) at delivery when SARS-CoV-2 infection occurred in the 2nd and 3rd trimesters of pregnancy, respectively (Figure 4E). Specifically, CA9 (mean FC = 162.64), plasminogen activator, tissue type (PLAT) (mean FC = 15.63), osteomodulin (OMD) (mean FC = 3.56), and fibroblast growth factor 21 (FGF21) (mean FC = 2.32) were induced upon delivery of pregnant women infected during the 2nd trimester, while CA11 (mean FC = −3.11), RuvB-like AAA ATPase 1 (RUVBL1) (mean FC = −17.52), and fructose-bisphosphatase 1 (FBP1) (mean FC = −384.73) were repressed (Figure 4F). In contrast, SARS-CoV-2 infection during the 3rd trimester of pregnancy led to the downregulation of 20 cytokines and the upregulation of SPINK6 only (mean FC = 2.8) (Figure 4G). IPA analysis predicted an opposing trend of organismal death in SARS-CoV-2 infection in the 2nd versus 3rd trimesters (Figures 4H and 4I). Pregnant women infected during the 3rd trimester exhibited a pronounced repression of cytokines such as BGN, vasorin (VASN), scavenger receptor class B member 1 (SCARB1), and alcohol dehydrogenase 4 (ADH4), which is linked to the promotion of organismal death (Figure 4I, S3A and S3B). This finding correlated with a high incidence of late pregnancy- and postpartum-related complications observed in our cohort, particularly, preeclampsia and fetal growth restriction (Figure 4J; Table 1B). These data demonstrated that SARS-CoV-2 infection at different stages of pregnancy may differentially dysregulate maternal peripheral immunity, with potential implications for pregnancy-related complications.

Gestational exposure to SARS-CoV-2 alters infant immunity at birth

Inflammatory responses in pregnant women may modulate infant immunity.[38] To examine the effects of *in utero* exposure of SARS-CoV-2 on neonatal immune responses, peripheral blood (healthy, n = 7; COVID-19 exposed, n = 45) and cord blood specimens (healthy, n = 14; COVID-19 exposed, n = 32) were collected at birth and subjected to high-throughput NGS-based proteomics multiplexing (Figure 5A). COVID-19-exposed infants showed minor changes in serum cytokines in peripheral blood (9 cytokines) and cord blood (7 cytokines) in comparison to infants from healthy mothers (Figure 5B). This was considerably different from the maternal blood of women with COVID-19, which showed drastic changes (171 cytokines) in serum cytokines when compared with healthy maternal blood. Induction of the IL-6R was commonly observed in both COVID-19$^+$ maternal blood (mean FC = 3.24) and COVID-19-exposed infant blood (mean FC = 12.6; Figure 5C), but not in cord blood (Figure 5C). Sera cord blood from 32 COVID-19-exposed infants was distinct from sera obtained in the first day of life from these infants with no overlap (Figure 5B). There was also minimal overlap between the cytokine profile of mothers at the time of acute infection during pregnancy or delivery with that of infants at the time of birth (Figures 5B and S3C).

Figure 2. Immune biomarkers of asymptomatic COVID-19 in pregnancy

(A) t-SNE plot of serum proteomic profiles of healthy (n = 18) and SARS-CoV-2-infected (n = 79) pregnant women at the time of diagnosis.

(B) Comparison of significantly altered cytokines ($-2 < FC > 2$, $p < 0.05$) of asymptomatic or symptomatic COVID-19 pregnant women relative to healthy controls.

(C) Ct values of nasal swabs derived from symptomatic (n = 46) versus asymptomatic (n = 7) COVID-19 pregnant women. Data are presented as means ± SEMs, using Mann-Whitney *U* test. *$p < 0.05$, **$p < 0.01$.

(D) ACE2 expression in healthy controls (n = 18), asymptomatic (n = 9), and symptomatic (n = 70) COVID-19 groups. Data are presented as means ± SEMs, using 1-way ANOVA Kruskal-Wallis with uncorrected Dunn's test. *$p < 0.05$.

(E) Bubble plot of significantly upregulated cytokines of asymptomatic COVID-19 pregnant women.

(F and G) Cytokines highly upregulated and downregulated in asymptomatic COVID-19 pregnant women. Data are presented as means ± SEMs, using 1-way ANOVA Kruskal-Wallis with uncorrected Dunn's test. *$p < 0.05$, **$p < 0.01$.

(H) STRING cluster analysis of 43 significantly altered cytokines in asymptomatic COVID-19 pregnant women.

(I) MMP7, MMP9, and TIMP1 expression, and MMP9:TIMP1 ratio in all groups. Data are presented as means ± SEMs, using 1-way ANOVA Kruskal-Wallis with uncorrected Dunn's test. *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

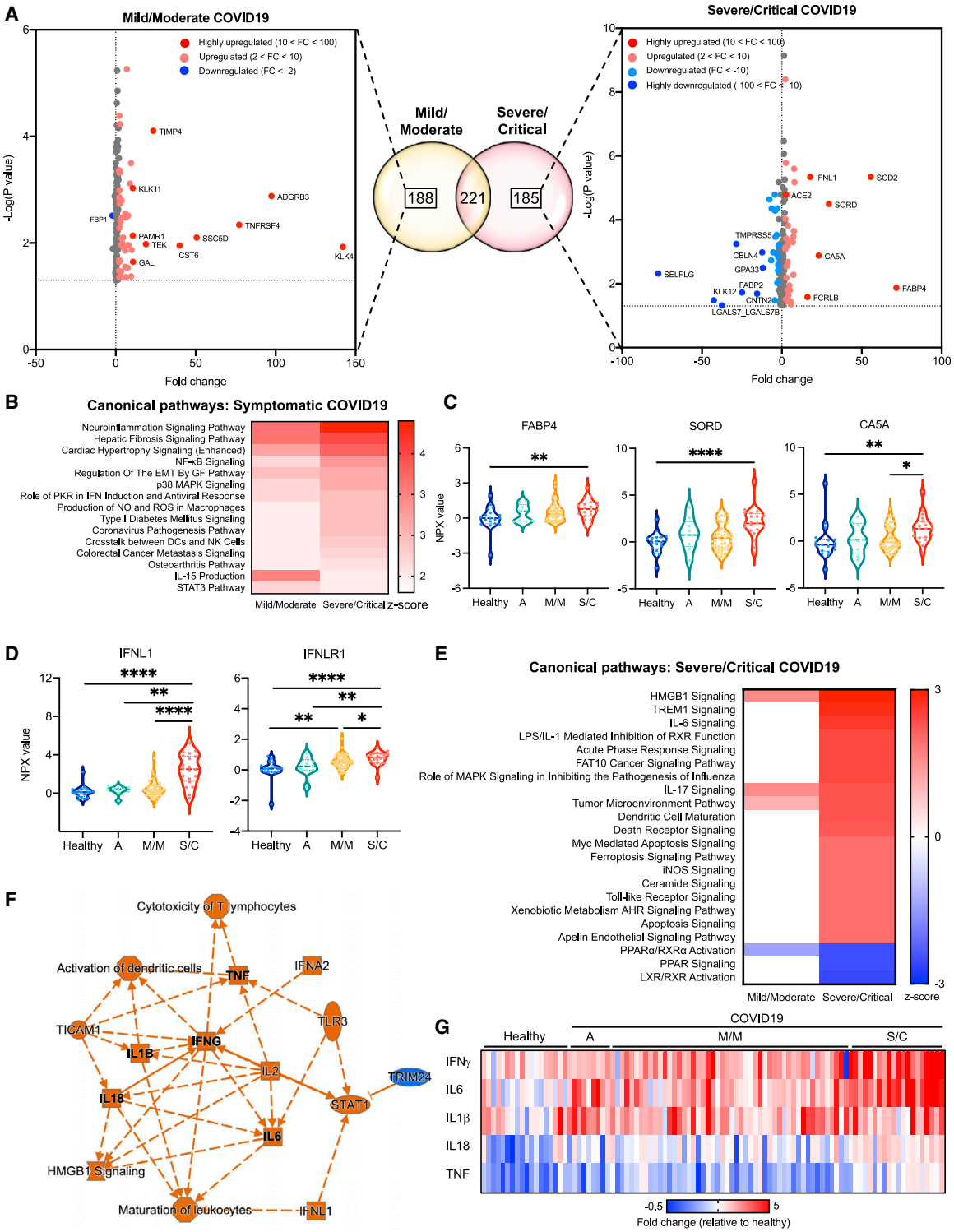

Figure 3. Overt prenatal immune activation in severe COVID-19 pregnant women

(A) Volcano plots illustrating the serum cytokines of mild/moderate (n = 50) and severe/critical (n = 20) COVID-19^{+} pregnant women that are significantly altered ($p < 0.05$ and $-2 < FC > 2$) when compared to healthy controls (n = 18).

(B) Heatmap illustrating the common canonical pathways ($p < 0.05$, Z score > 2) induced in symptomatic COVID-19 pregnancy.

(C and D) Expressions of highly upregulated serum cytokines in severe/critical COVID-19 pregnancy. Data are presented as means ± SEMs, using 1-way ANOVA Kruskal-Wallis with uncorrected Dunn's test. *$p < 0.05$, **$p < 0.01$, ****$p < 0.0001$. A, asymptomatic; M/M, mild/moderate; S/C, severe/critical.

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While 90 cytokines were detectably altered ($p < 0.05$) in COVID-19-exposed infants as compared to healthy infants, 8 cytokines were significantly induced (FC > 2) and 2 cytokines, CD33 (mean FC = −2.06) and PLAT (mean FC = −10.79), were repressed (FC <−2; Figures 5D and 5F). Those 8 cytokines were IL-6R, Dickkopf-like acrosomal protein 1 (DKKL1; mean FC = 10), LY6/PLAUR domain containing 1 (LYPD1; mean FC = 9.16), glycoprotein A33 (GPA33; mean FC = 6.36), SELPLG (mean FC = 4.82), sphingomyelin phosphodiesterase acid-like 3A (SMPDL3A; mean FC = 3.39), proteasome 20S subunit alpha 1 (PSMA1; mean FC = 3.03), and CCL21 (mean FC = 2.36). Furthermore, the cord blood of COVID-19-exposed infants exhibited the induction of 5 serum proteins (Figures 5E and 5G).

Maternal COVID-19 disease severity reshapes the infant immune landscape

To further correlate maternal COVID-19 disease severity with the neonatal immune landscape, COVID-19-exposed infants (n = 45) were grouped according to maternal COVID-19 disease severity: (1) asymptomatic (n = 11), (2) mild/moderate (n = 28), and (3) severe/critical (n = 6). Seven infants from healthy mothers were included as controls. The immune landscape of infants born to mothers with severe/critical COVID-19 was distinct from that of infants from mothers with asymptomatic or mild/moderate illness and infants from healthy mothers (Figure 6A). Comparison analysis between the asymptomatic and symptomatic groups of COVID-19-exposed infants revealed a stronger immune activation in infants born to mothers who had severe illness (Figure 6B). Upon comparison to infants from healthy mothers, 60 cytokines in infants born to asymptomatic COVID-19 mothers, 272 cytokines in infants born to mild/moderate COVID-19 mothers, and 455 cytokines in infants born to severe/critical COVID-19 mothers were significantly altered ($p < 0.05$) (Figures 6C, S4A, and S4B). Among 60 cytokines that were significantly altered ($p < 0.05$) in infants born to asymptomatic COVID-19 mothers, only SIRPA was markedly altered and specific to the exposure of asymptomatic COVID-19 mothers (mean FC = 9.86; Figure 6D). Seven cytokines—IL-6R (mean FC = 13.01), nicalin (NCLN) (mean FC = 3.94), PSMA1 (mean FC = 3.71), eukaryotic translation initiation factor 5A (EIF5A) (mean FC = 2.79), granzyme B (GZMB) (mean FC = 2.06), CD33 (mean FC = −2.35), and SLAM family member 8 (SLAMF8) (mean FC = −2.85)—were markedly altered in infants from mild/moderate COVID-19 mothers, when compared to healthy controls (Figures 6E and S4C). Upon comparison with infants from healthy mothers, infants from severe/critical COVID-19 mothers showed markedly increased 44 soluble factors, including bone morphogenetic protein 4 (BMP4) (mean FC = 68.9), proprotein convertase subtilisin/kexin type 9 serine protease (PCSK9) (mean FC = 67.8), and DKKL1 (mean FC = 25), and markedly decreased 13 soluble factors, including N-acylethanolamine acid amidase (NAAA) (mean FC = −534.41), leukocyte immunoglobulin-like receptor B2 (LILRB2) (mean FC = −411.35), and Fc fragment of IgG receptor IIIb (FCGR3B) (mean FC = −235.09) (Figures 6E–6G).

In our infant cohort, 33.3% of the infants had RD (Table 1C). Since premature infants frequently have RD requiring oxygen supplementation, we sought to delineate the specific immune alterations resulting from possible COVID-19 exposure-associated RD in term infants. We categorized infants as: (1) no (−) RD, preterm birth (n = 3), (2) (−) RD, term birth (n = 27), (3) (+) RD, preterm birth (n = 11), and (4) (+) RD, term birth (n = 4) (Figure 6H). Compared with healthy control infants (n = 7), preterm COVID-19-exposed infants with RD exhibited the most significant immune alterations of 36 cytokines (Figure S4C). We identified 5 specific cytokines that were affected in COVID-19-exposed term birth infants with RD, including iduronidase (IDUA) (mean FC = 2.03), TREM2 (mean FC = −11.77), ghrelin and obestatin prepropeptide (GHRL) (mean FC = −2.06), anterior gradient 3, protein disulfide isomerase family member (AGR3) (mean FC = −2.61), and procollagen C-endopeptidase enhancer (PCOLCE) (mean FC = −2.69) (Figures 6H, 6I, and S4D). Overall, infants born to COVID-19 mothers with severe/critical disease exhibited more pronounced immune alterations as compared to infants born to mothers with asymptomatic illness. In addition, several serum factors were identified as potential biomarkers for prenatal SARS-CoV-2 exposure and neonatal RD.

DISCUSSION

Infection during pregnancy can have dire consequences. Several respiratory viruses, including influenza and other highly pathogenic coronaviruses, are associated with increased maternal risk of ARDS and adverse pregnancy outcomes, including spontaneous abortion and preterm delivery.[3,4,7,8] However, little is known regarding the immunological implications of prenatal SARS-CoV-2 infections in pregnant women and their fetuses. Our comprehensive characterization of the systemic immunity of COVID-19^{+} pregnancies revealed distinct alterations of SARS-CoV-2-driven immune landscapes in mother-infant dyads, which may explain some of the adverse clinical outcomes associated with severe/critical COVID-19 infection in pregnancy.

COVID-19 mother-infant cohort

While the morbidity and mortality associated with COVID-19 are high in some subgroups, most SARS-CoV-2-infected individuals remain asymptomatic or develop mild symptoms. Our COVID-19 pregnant cohort was relatively ill, reflective of the status of our institution as a quaternary referral center for complex cases. Despite this referral bias, 12.9% of women included in our series were asymptomatic and 65.6% had mild or moderate disease. Although our study is not a pregnancy registry and the analysis is not powered to focus on clinical outcomes, our findings illustrate the degree to which disease severity of pregnant women

(E) Heatmap illustrating the specific canonical pathways ($p < 0.05$, $-2 < Z$ score > 2) affected in severe/critical COVID-19 in pregnancy.
(F) Graphical summary of upstream regulators and biological pathways, based on altered serum cytokine profiles in pregnant women with severe/critical COVID-19.
(G) Heatmap illustrating the expression profiles of proinflammatory cytokines in healthy and COVID-19 affected-pregnancies.

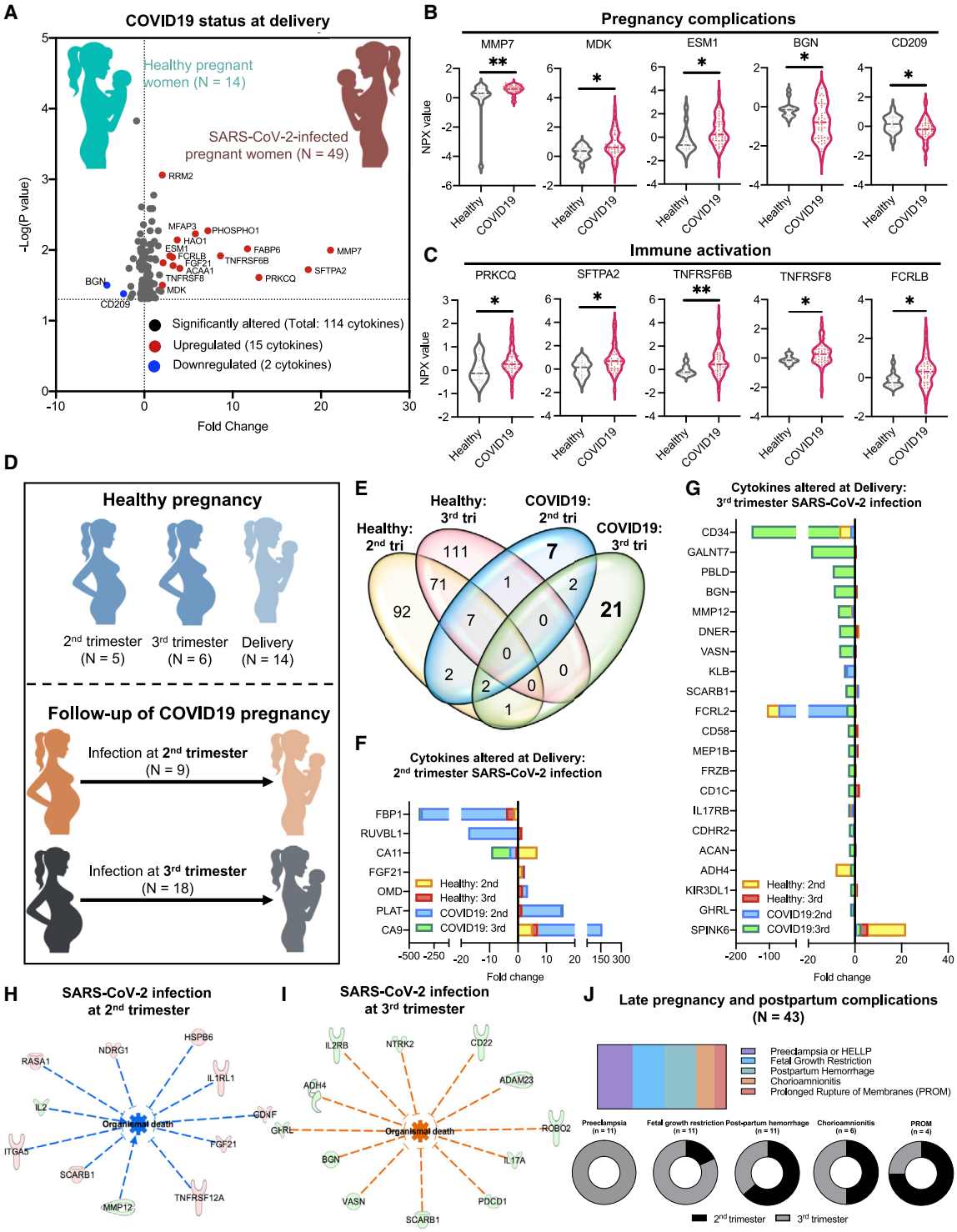

Figure 4. Prenatal SARS-CoV-2 infection alters maternal immune landscape at delivery

(A) Volcano plots illustrating significantly altered serum cytokines ($p < 0.05$) of SARS-CoV-2-infected pregnant women (n = 49) at the time of delivery ($p < 0.05$ and $-2 < FC > 2$) when compared to those of healthy pregnant women (n = 14) also at the delivery time point.

(B and C) Expression of pregnancy complication-associated (B) or immune activation-associated (C) cytokines in SARS-CoV-2-infected pregnant women at delivery. Data are presented as means ± SEMs, using 1-way ANOVA Kruskal-Wallis with uncorrected Dunn's test. *$p < 0.05$, **$p < 0.01$.

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with COVID-19 can manifest, including ARDS requiring ECMO and maternal death. Of note, nearly half of our cohort is Latina, and the majority of participants with severe/critical disease had public insurance, reflective of systemic inequities that manifested in the national data as well.[5,10] It was notable that 60.0% of our participants with severe/critical disease were diagnosed during the 2nd trimester, which warrants further investigation. The vast majority of participants with severe/critical disease had specimens collected before or within hours of treatment initiation, and thus, COVID-19 treatments unlikely influenced the immune profiles. The lower virus load observed in asymptomatic pregnant women could be a real phenomenon or it may reflect the fact that they are further along in the viral clearance process based on the timing of infection. Nevertheless, there are recent data to suggest asymptomatic patients may contribute less to transmission, and a lower virus load in this population would support this premise.[39]

It is well documented that several comorbidities, including obesity, hypertension, and diabetes mellitus, increase the risk of severe/critical COVID-19 among pregnant people[6] and in non-pregnant populations.[40,41] While cytokine profiles must be interpreted with caution in this exploratory study, the purpose of our study was to describe the immunologic landscapes of mother-infant dyads diagnosed with *in utero* COVID-19. These data provide important insights into the pathophysiology of COVID-19 in pregnancy that has not been adequately described until now.

Prenatal immune adaptations in women with asymptomatic COVID-19

Asymptomatic COVID-19 remains a perplexing aspect of SARS-CoV-2 infection. Despite detectable levels of SARS-CoV-2 in the nasal discharge and retaining maintained transmissibility, as high as 50% of infected individuals report asymptomatic disease.[42] We demonstrated that lower levels of serum ACE2 appeared to be correlated with reduced viral loads in the nasal swabs of asymptomatic COVID-19 women compared to those with symptomatic COVID-19. Specifically, the abundant expressions of XPNPEP2, NTproBNP, SPINK6, and EDA2R were detected in the sera of asymptomatic COVID-19 pregnant women. Interestingly, ACE2 and XPNPEP2 are involved in the metabolism of kinins, and SPINK6 is a potent inhibitor for several KLKs, including KLK4. Coincidentally, all three proteins are involved in the metabolic cascade of the kinin-kallikrein system, which leads to a short burst of vasoactive kinin production upon activation to induce vasodilation and inflammation.[43,44] Here, we detected reduced levels of KLK4 and robust expression of SPINK6 in asymptomatic COVID-19 pregnant women. While these findings suggest the contribution of the kinin-kallikrein cascade activation in COVID-19$^+$ pregnancies, XPNPEP2 and SPINK6 may prevent the development of COVID-19 symptoms in pregnant women.

Overt inflammation in pregnant women with symptomatic COVID-19

Pregnant women are at increased risk of developing severe manifestations of COVID-19 compared to the general population.[6] Consistent with national surveillance data, there was a high prevalence of obesity and diabetes in this cohort.[6] Our study showed that prenatal SARS-CoV-2 infection led to overt inflammation, correlating with the severity of COVID-19 during pregnancy, particularly in those with severe/critical illness. We demonstrated inflammatory signaling of HMGB1, IL-17, and inducible NO synthase (iNOS), which are associated with the severity of influenza pneumonia[45,46] and play a part in ARDS due to MERS-CoV and influenza A.[47,48]

Antiviral action of type III IFN in COVID-19-affected pregnancies

The rate of vertical transmission in COVID-19 is estimated to be ~2%[49] and heavily influenced by active infection at the time of delivery.[5] We showed that symptomatic pregnant women with COVID-19 had a higher viral burden compared to asymptomatic pregnant women. A recent study suggested a major paradox in SARS-CoV-2-induced antiviral defense, in which proinflammatory responses were activated before a weak induction of IFNs, the first line of innate immune defense against pathogens.[50] Patients with COVID-19 were found to have delayed and suboptimal induction of type I (IFN-α and IFN-β) and III (IFN-λ) IFN responses, noted in a subset of patients who became critically ill.[50] In our present cohort, both IFN-λ and its receptor IFNLR1 emerged as prominent biomarkers of pregnancies affected by COVID-19. High levels were detected in the sera of pregnant women with severe/critical COVID-19, possibly explaining the relatively rapid virus clearance. Notably, IFN-λ has been linked to a protective role in preventing the vertical transmission of ZIKV.[32] It is possible that the high level of IFN-λ during pregnancies of COVID-19 patients may contribute to the relatively low incidence of vertical transmission, although this warrants further study.

SARS-CoV-2-triggered prenatal immune activation may promote late pregnancy and postpartum complications

We detected increased MMP7 and ESM1 and reduced CD209, which has been associated with gestational hypertension and

(D) Schematic representation of patient specimens used for (E) and (J). Comparison analyses were made between delivery blood specimens and predelivery 2nd/3rd trimester blood specimens of healthy pregnant women or SARS-CoV-2-infected pregnant women.

(E) Comparison analysis of cytokines significantly altered ($p < 0.05$; $-2 < FC > 2$) at delivery in healthy or COVID-19 pregnant women in contrast to cytokines observed in the 2nd/3rd trimester of pregnancy in both groups.

(F and G) Cytokines of SARS-CoV-2-infected pregnant women significantly altered at delivery compared to (F) 2nd trimester or (G) 3rd trimester infection cytokine profiles.

(H and I) Predicted functional processes in SARS-CoV-2-infected pregnant women at delivery in contrast to cytokines present during the (H) 2nd trimester or (I) 3rd trimester infection before delivery.

(J) Diagram illustrating the distribution of late pregnancy and postpartum complications observed in COVID-19$^+$ pregnancies resulting from infection in the 2nd or 3rd trimester ($n = 43$).

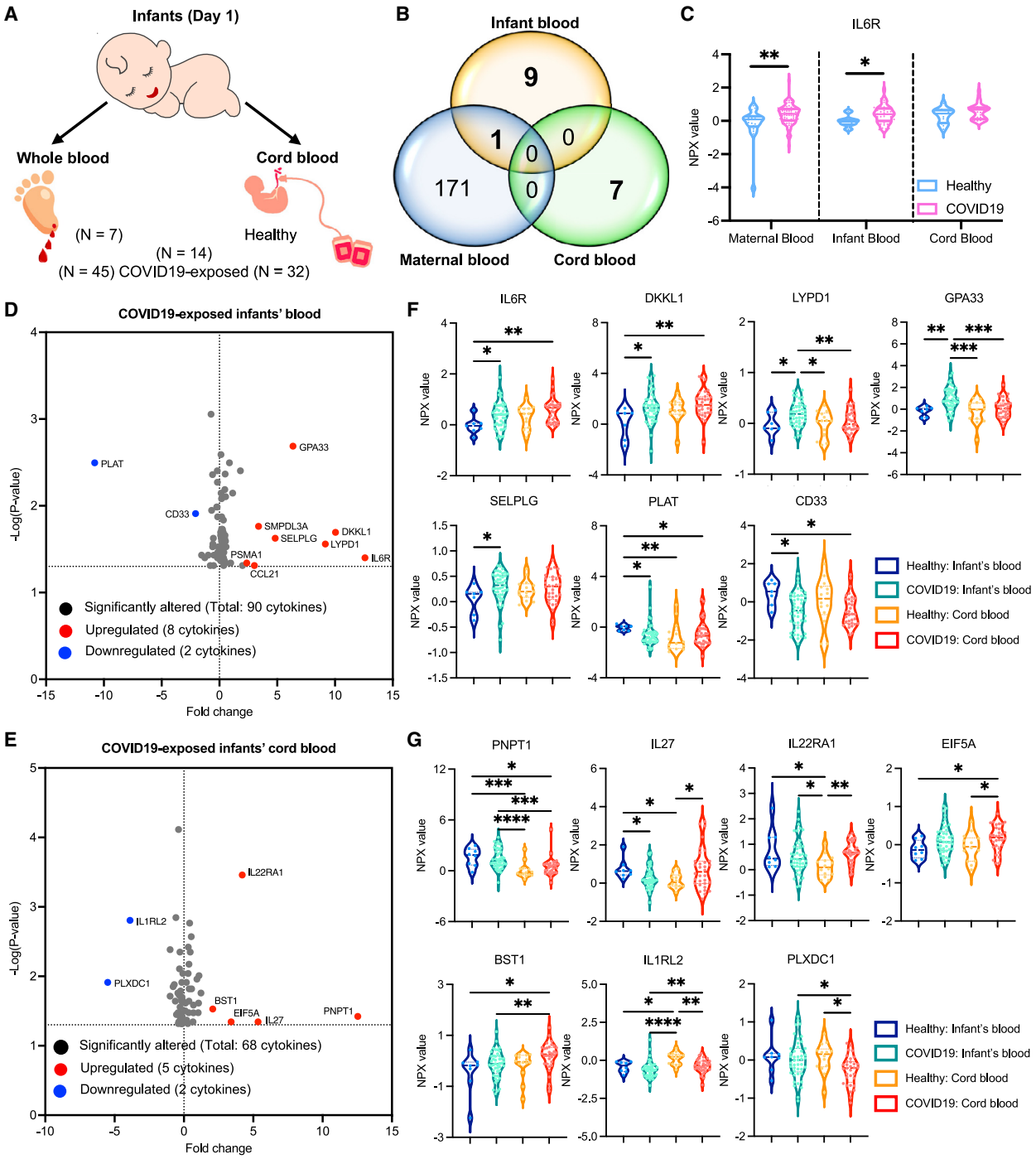

Figure 5. Serum cytokine profiles of infants from SARS-CoV-2-infected mothers

(A) Schematic representation of COVID-19-exposed infants in this study. Blood specimens were collected from infants at day 1 of life born to healthy mothers (n = 7) or to SARS-CoV-2-infected mothers (n = 45). Cord blood specimens were collected at delivery from healthy (n = 14) or COVID-19-exposed (n = 32) pregnancies.

(B) Comparison analysis of COVID-19-induced serum cytokines of mothers (time of initial diagnosis; n = 79), infant (n = 45) and/or cord blood (n = 32) specimens.

(C) IL-6R expression in mother-infant pairs. Data are presented as means ± SEMs, using Mann-Whitney *U* test. *p < 0.05, **p < 0.01.

(D and E) Volcano plot illustrating the cytokines that are significantly altered (p < 0.05, −2 < FC > 2) in whole blood (D) or cord blood (E) of COVID-19-exposed infants compared to infants born to healthy mothers.

(F and G) Cytokines exclusively affected in whole blood (F) or cord blood (G) of COVID-19-exposed infants compared to that of infants born to healthy mothers. Data are presented as means ± SEMs, using Mann-Whitney *U* test. *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

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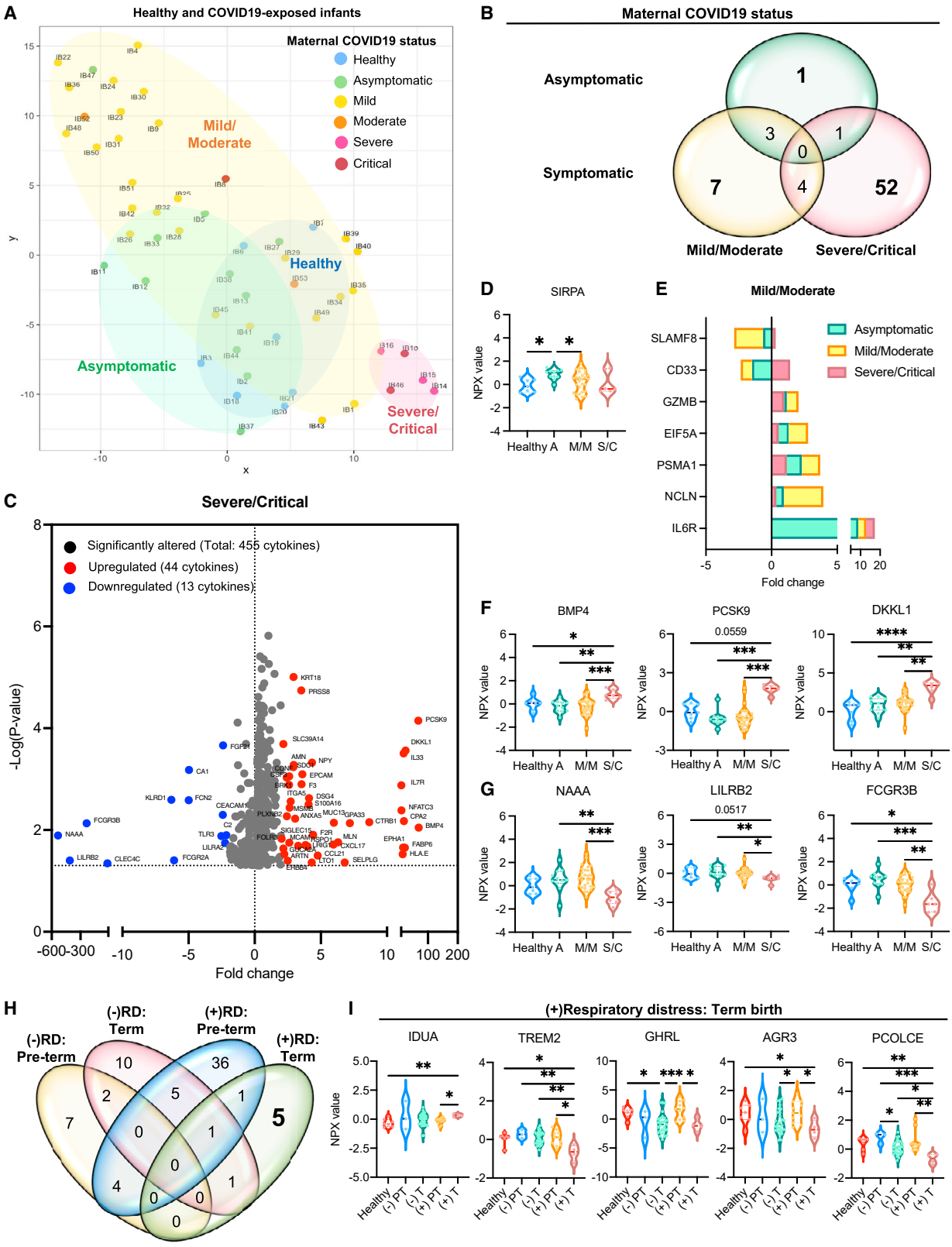

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preeclampsia,[51–53] along with reduced BGN expression, which is involved in fetal growth restriction.[54] While most of our cohort had healthy live births, pathway analysis indicated an increase in the predicted organismal death pathway observed at delivery, specifically in pregnant women infected during the 3rd trimester. This may partially explain the high incidence of preeclampsia and fetal growth restriction in our cohort. Intrauterine inflammation may lead to adverse pregnancy outcomes, including preterm birth and fetal loss.[55] Elevated proinflammatory cytokines such as IL-6R were observed in both maternal blood and infant blood of COVID-19 pregnant mothers, suggesting perinatal systemic inflammation. The cytokine storm associated with severe/critical COVID-19$^+$ pregnancies may contribute to late pregnancy and postpartum complications, potentially contributing to poor maternal and neonatal outcomes.

Prenatal COVID-19 exposure may alter neonatal T cell immunity and neurodevelopment

The neonatal immunity is heavily skewed toward the innate immune arm rather than the adaptive T cell immunity, which is considered immature and immunodeviant.[56,57] In our COVID-19-exposed infant cohort, we observed overt expressions of T cell-related cytokines such as IL-33, NFATC3, and CCL21 in infant blood and IL-27 in cord blood, compared to infants born to healthy women. CCL21 is involved in the chemotaxis of naive T cells,[58] IL-33 promotes the expansion of regulatory T cells (Tregs),[59] NFATC3 is crucial in regulating naive T cell activation and differentiation into T helper 2 cells (Th2),[60] and IL-27 may facilitate the maturation of Tregs during viral infections.[61] The induction of these lymphokines is suggestive of the occurrence of Th2-skewed T cell activation in the COVID-19-exposed infants as early as the first day of birth. Despite the immunodeviant state of adaptive immunity, the neonatal immunity is dominated by an imbalanced Th2-skewed response, which becomes a predisposing factor for the development of allergic inflammation toward pro-Th2 vaccines.[62] The overwhelming Th2 responses in the COVID-19-exposed infants is something that requires further study; it is important to further dissect the immunological consequences of *in utero* SARS-CoV-2 infection on immune maturation.

Sustained inflammation during pregnancy at the maternal-fetal interface has been linked to neurological disorders.[55,63] The neurodevelopment of a child begins as early as gastrulation, continuing into early and mid-childhood, governed by a sophisticated network of cell signaling pathways, including Wnt/β-catenin signaling.[64,65] Dysregulation of Wnt/β-catenin signaling in either direction is implicated in altered neurodevelopment, potentially contributing to neurological disorders such as autism.[66] While the characterization of serum immune profiles of infants born to mothers with asymptomatic COVID-19 revealed minimal changes, infants of mothers with mild/moderate and severe/critical illness exhibited pronounced immune alterations. Abundant expressions of human R-spondin1 (RSPO1) and RSPO3, positive regulators of the Wnt signaling, were observed in infants born to severe/critical COVID-19 mothers. Interestingly, DKKL1, a regulator of Wnt/β-catenin signaling pathway,[67] was also induced in these infants. These opposing effects derived from the high expressions of RSPO1, RSPO3, and DKK1 suggest a dysregulated Wnt/β-catenin signaling in the infants exposed to severe/critical COVID-19 during gestation. Moreover, PCSK9, which is involved in neuronal apoptosis through the induction of NF-κB-mediated inflammation,[68,69] was also highly expressed in infants born to mothers with severe/critical COVID-19. The negative impact of gestational inflammation and the deregulated Wnt/β-catenin signaling pathway on neurodevelopment suggests that infants exposed to severe/critical COVID-19 may be at risk of neurodevelopmental complications, warranting long-term clinical monitoring.

RD of term COVID-19-exposed infants is associated with NLRP3 inflammasome activation

In our study, 33.3% of the COVID-19-exposed infants developed RD. While some of these RD cases were likely due to preterm births and cesarean delivery, 4 infants were born at term to mothers with either asymptomatic or symptomatic COVID-19. Immunological evaluations of term COVID-19-exposed infants who developed RD identified high levels of IL-18, IL-1β, and CASP1, indicative of an activated NLRP3 inflammasome pathway. High levels of TREM2, known to promote macrophage survival and viral-induced lung pathogenesis,[70] were detected in the COVID-19-exposed infants. Excessive degradation of ECM makeup of glycosaminoglycans[71] and dysregulated ciliary beating of the airway epithelium[72] may contribute to RD. In the COVID-19-exposed infants, we observed increased IDUA levels that degrade glycosaminoglycans, and decreased AGR3, which is essential in regulating ciliary beat frequency in the airway. Therefore, these proteins are potential pathogenic factors

Figure 6. Neonatal immune signatures of infants exposed to maternal severe COVID-19 or infants who had respiratory distress (RD) at birth

(A) Serum proteomic profiles of healthy control infants (n = 7) and COVID-19-exposed infants according to maternal disease presentation (asymptomatic, n = 11; mild/moderate, n = 28; and severe/critical, n = 6) are displayed as a t-distributed stochastic neighbor embedding (t-SNE) plot.

(B) Comparison analysis of significantly altered cytokines ($p < 0.05$; $-2 < FC > 2$) present in infants born to asymptomatic, mild/moderate, and severe/critical COVID-19$^+$ mothers and infants born to control healthy women.

(C) Volcano plot illustrating the cytokines that are significantly altered ($p < 0.05$ and $-2 < FC > 2$) in serum specimens of COVID-19-exposed infants born to women who had severe/critical illness in comparison to cytokines present in infants born to healthy mothers.

(D and E) Cytokines specifically altered in infants born to asymptomatic (D) or mild/moderate (E) COVID-19$^+$ mothers are presented as a mean FC relative to cytokines present in infants born to healthy mothers.

(F and G) Expression of the top 3 serum cytokines induced (F) or repressed (G) in infants born to mothers with severe/critical COVID-19.

(H) Comparison of serum cytokines altered in infants exposed to COVID-19 in pregnancy stratified by (−) RD: term (n = 27), (−) RD: preterm (n = 3); or (+) RD: term (n = 4) and (+) RD: preterm (n = 11), relative to healthy infants (n = 7).

(I) Serum cytokines specifically altered in (+) RD: term (n = 4) COVID-19-exposed infants. Data presented as means ± SEMs, using 1-way ANOVA Kruskal-Wallis with uncorrected Dunn's test. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

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implicated in RD associated with prenatal COVID-19-exposure in term infants.

Conclusions

In this study, we performed comprehensive serum immunoprofiling to characterize the immunological repercussions of COVID-19 on maternal and infant systemic immunity of 93 COVID-19$^+$ pregnancies enrolled in our COMP study cohort. Pregnant women with COVID-19 mounted pronounced inflammatory responses in conjunction with a robust activation of the IFNL1/IFNLR1 axis. In addition, we identified biomarkers for severe/critical COVID-19 during pregnancy, many of which are implicated in cardiac and hepatic damage. Despite the lack of robust evidence for vertical transmission, SARS-CoV-2 infection in pregnancy appears to trigger prenatal immune activation that may lead to adverse maternal and neonatal outcomes. Notably, prenatal COVID-19 exposure led to sustained inflammation during gestation and dysregulation of key signaling pathways that could affect long-term infant immune maturation and neurodevelopment. In summary, our findings highlight the importance of long-term clinical monitoring of mother-infant dyads following COVID-19$^+$ pregnancies. Follow-up of our cohort is ongoing with continued monitoring of these mother-infant pairs.

Limitations of the study

This study is an exploratory study and correlation should always be interpreted with caution in an observational study, as confounding and reverse causality cannot be excluded. Next, the University of California, Los Angeles (UCLA) is a quaternary referral hospital; therefore, our cohort was biased toward severe/critical cases. However, the heterogeneity of our cohort was also a major strength, as we were able to explore differences in proteomic profiles across disease severity classes as a result. Finally, our study does not propose a conclusive, underlying biological mechanism to explain these findings. Rather, this work generated provocative questions that will be explored in the future with animal models and warrants long-term clinical monitoring of mother-infant dyads.

STAR★METHODS

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SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xcrm.2021.100453.

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AUTHOR CONTRIBUTIONS

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DECLARATION OF INTERESTS

J.U.J. is a scientific adviser to Vaccine Stabilization, a California corporation.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Serum specimens from peripheral blood of SARS-CoV-2-diagnosed pregnant women	UCLA	N/A
Serum specimens from peripheral blood of healthy pregnant women	UCLA, USC+LAC	N/A
Serum specimens from peripheral blood of COVID-19-exposed infants	UCLA	N/A
Serum specimens from peripheral blood of healthy infants	UCLA	N/A
Serum specimens from cord blood of COVID-19-exposed infants	UCLA	N/A
Serum specimens from cord blood of healthy infants	UCLA	N/A
Chemicals, peptides, and recombinant proteins		
Triton X-100	Sigma-Aldrich	X100
Nuclease-free water	Sigma-Aldrich	W4502
SsoAdvanced Universal SYBR® Green Supermix	BIO-RAD	1725272
iScript cDNA Synthesis Kit	BIO-RAD	1708891
Critical commercial assays		
Olink Explore 1536	Olink	N/A
Deposited data		
Olink proteomics data	This paper	https://dx.doi.org/10.17632/mdnb359tp9.1
Oligonucleotides		
Real time-PCR primers (refer to Table S1)	Integrated DNA Technologies	N/A
Software and algorithms		
RStudio	Database: https://www.rstudio.com/	v4.0.5
R	Database: https://cran.r-project.org/	v1.3.1093
Graphpad Prism	Graphpad	v9.0.0 (86)
QIAGEN Ingenuity Pathway Analysis (IPA)	QIAGEN	v01-19-00

RESOURCE AVAILABILITY

Lead contact

Futher information and drequests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Jae U. Jung (jungj@ccf.org).

Materials availability

This study did not generate new unique reagents.

Data and code availability

- The Olink protein data generated in this study is available for download at Mendeley Data: https://dx.doi.org/10.17632/mdnb359tp9.1.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this work paper is available from the Lead Contact upon request.

EXPERIMENTAL MODELS AND SUBJECT DETAILS

Pregnant women and sample collection

Pregnant women aged 16 and above with confirmed SARS-CoV-2 infection by NP RT-PCR or serology at any point during gestation were eligible for enrollment at the University of California, Los Angeles. Pregnant women with pre-existing conditions, including obesity (pre-pregnancy BMI > 30), chronic hypertension, and diabetes mellitus, were eligible for participation. Participants, including healthy controls were primarily recruited through the maternal-fetal medicine service at UCLA. Beginning in April 2020, all women admitted to the labor and delivery (L&D) floor were screened for active SARS-CoV-2 infection through NP RT-PCR. Healthy pregnant controls without COVID-19 or upper respiratory infection symptoms and negative NP RT-PCR were concurrently recruited. Exclusion criteria for healthy pregnant controls at both institutions included: (i) vaccination within last 14 days, (ii) suspected/recent illness within last 14 days, (iii) steroid administration during pregnancy, (iv) immunodeficiency/immunocompromised conditions, (v) autoimmune disorders, and (vi) suspected fetal anomalies on transfontanelle ultrasonography. Peripheral blood specimens were obtained from pregnant women at the time of enrollment (acute infection), and admission for delivery. Cord blood was collected at delivery when feasible, and infant blood specimens were collected between 24 and 48 h of life. NP swabs for SARS-CoV-2 PCR were collected between hours 4 and 48 of life for all infants born to mothers with active SARS-CoV-2 infection at the time of delivery. All specimens were collected in accordance with the World Health Organization (WHO) guidelines.[73] Serum aliquots were stored at −80°C.

Pregnancy was categorized into three trimesters: first trimester (0 – 13 weeks), second trimester (14 – 27 weeks) and third trimester (≥28 weeks). Women with SARS-CoV-2 infections were grouped into the following NIH COVID-19 severity of illness categories[25]:

a) Asymptomatic: individuals who test positive for SARS-CoV-2, but have no symptoms consistent with COVID-19;
b) Mild Illness: individuals who have any of the various signs and symptoms of COVID-19, but do not have shortness of breath, dyspnea, or abnormal chest imaging;
c) Moderate Illness: individuals who show evidence of lower respiratory disease during clinical assessment or imaging and have saturation of oxygen (SpO2) ≥ 94% on room air;
d) Severe Illness: individuals who have SpO2 < 94% on room air, a ratio of arterial partial pressure of oxygen to fraction of inspired oxygen (PaO2/FiO2) < 300 mmHg, respiratory frequency > 30 breaths per minute, or lung infiltrates > 50%; and
e) Critical Illness: individual who have respiratory failure, septic shock, and/or multiple organ dysfunction.

The clinical categories were collapsed into asymptomatic, mild/moderate and severe/critical for the analyses. Clinical, lab and hospital data was abstracted from the chart by a multidisciplinary team of infectious disease specialists, maternal-fetal medicine specialists, and neonatologists. Gestational aged-matched healthy pregnant subjects were recruited at UCLA and Los Angeles County + University of Southern California (LAC + USC) Medical Center (pre-pandemic). Serum aliquots from all patients were stored at −80°C until the performance of the present experiments.

Informed consent for study participation was obtained for all participants prior to enrollment. If the participant was incapacitated during an acute hospitalization and consented by a surrogate, the participant was asked whether they wanted to continue with the study once they regained capacity. The study was approved by the UCLA Institutional Review Board which oversaw study conduct.

METHOD DETAILS

Serological testing for COVID-19

All maternal and cord blood samples were tested for quantitative anti-SARS-CoV-2 IgA, IgG and IgM. Sera were analyzed by enzyme-linked immunosorbent assay (ELISA) to detect the spike receptor-binding domain (RBD) IgA, IgG and IgM[25] as previously described.[74] A combination of four current (including two cord bloods) controls with negative SARS-CoV-2 NP PCR testing, and 18 pre-pandemic healthy pregnant controls (including eight cord bloods) were tested for validation of the serologic assays.

Next-generation sequencing (NGS)-based Olink Explore sera proteomics profiling

Sera proteomics profiling was performed using Olink Explore 1536, a high-multiplex, high-throughput protein biomarker platform that utilizes Proximity Extension Assay (PEA) technology coupled to NGS using the Illumina NovaSeq instrument for readout. All sera specimens were inactivated in 10% Triton X-100 to a final concentration of 1%, followed by 2 h incubation at room temperature. Inactivated sera specimens were stored at −80°C until protein assays were performed.

PEA uses matched pairs of antibodies conjugated to unique DNA oligonucleotides. PEA probe pairs are incubated with 2.8 μL of sera and incubated overnight. The antibody pairs bind to the target protein, allowing for the DNA oligonucleotides to hybridize and create a double-stranded DNA template for each bound protein. The DNA templates are then extended and amplified with PCR before pooling and an additional round of PCR. The PCR amplification of the templates provides an assay sensitivity equal to or greater than traditional enzyme-linked immunosorbent assays (ELISA). The resulting samples are pooled again and checked for quality on an Agilent Bioanalyzer 2100 before sequencing on an Illumina NovaSeq 6000 S1 flowcell with paired end 50bp reads. The forward and reverse sequencing reads are then matched with their correct DNA barcodes and converted into normalized protein

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expression (NPX). NPX units are relative protein quantification units where counts are normalized to a known standard, log-2 transformed, and normalized again to a plate control. NPX values are then compared to a limit of detection (LOD) for quality control before use in downstream analysis.

The PEA technology allows the Olink Explore 1536 panel to simultaneously assay 1472 proteins across four 384-plex panels that includes 48 controls and three interpanel quality control protein markers (IL-6, CXCL8 and TNF). The Olink Explore 1536 panel interrogates proteins related to one of five categories: low-abundant inflammation proteins, actively secreted protein, drug target proteins, organ-specific proteins in circulation, and potential biomarkers. The four panels allow for the measurement of the 1472 proteins across 88 samples and eight control samples in less than 36 h.

To mitigate the possibility of batch effect, 10 samples used in the first analysis were included again in the second analysis as bridging samples. The differences in NPX values of the bridging samples in both batches of Olink analyses were used to normalize the data between the two batches of analyses. Additionally, Olink performs stringent quality control and data normalization through the inclusion of multiple internal controls including immuno/incubation controls, extension controls and detection controls on each 96-well plate (88 samples/plate). These measures remove the possibility of batch effect in the interpretation of our data and also ensure the greatest accuracy of the data presented.

Viral load analysis

The UCLA Clinical Microbiology Laboratory performed the in-house SARS-CoV-2 PCR testing. NP swabs were analyzed with one of three assays: 1)The TaqPath COVID-19 Combo Kit (Thermo Fisher Scientific Inc), which uses probes targeting the ORF1ab, N and S genes.[75] The DiaSorin Simplexa COVID-19 Direct RT-PCR (DiaSorin Molecular LLC), which targets the ORF1ab and S genes.[76] The US Centers for Disease Control and Prevention (CDC) 2019-nCoV RT-PCR Diagnostic Panel Protocol which probes the N1 and N2 genes. An in-house analysis found comparable Ct ranges without significant differences across the three assays, suggesting semi-quantitative grouping was acceptable across our in-house PCR assays.[77] Serial maternal NP swabs were analyzed by the Abbott RT-PCR platform for SARS-CoV-2 in accordance with the manufacturing instructions.[78]

RNA extraction and real time PCR analysis

Total RNA extractions were performed using RNeasy mini/micro Kit (QIAGEN) according to manufacturer's instructions. RNA concentration was determined by NanoDrop 1000 spectrophotometer (Thermo Scientific). Extracted total RNA was reverse-transcribed using iScript cDNA synthesis kit (BIO-RAD) according to the manufacturer's instructions. Gene expression qRT-PCR were performed with 10 ng of cDNA/well using SsoAdvanced Universal SYBR Green Supermix (BIO-RAD). All qRT-PCR reactions were performed using BIO-RAD CFX96 Touch Real-Time PCR Detection System on 96-well plates.

QUANTIFICATION AND STATISTICAL ANALYSIS

Sera proteome analysis

NPX values for all proteins and samples are received directly from Olink after sequencing. NPX values have already been normalized to controls and log-2 converted. We compared these NPX values against limits of detection (LOD) for each protein. LOD are calculated from three negative controls (buffer alone) to measure background levels within the assay. NPX values were compared against the LOD for each protein and proteins where greater than 80% of NPX values are lower than the LOD were excluded from the analysis. Samples from the two sets of the Olink Explore assay runs were then normalized using 10 repeated samples.

Heatmaps comparing cytokine expression for this study were generated in R[79] (using the pheatmap package[80] and RColorBrewer.[81]. Volcano plots in this study were generated in R[79] using the ggplot2 package.[82] FC was calculated by first averaging the expression of each group and then using the following equation: $\frac{(Test\ NPX\ -\ Control\ NPX)}{|Control\ NPX|}$. This formula accounts for negative NPX values that were generated during the initial log-2 conversion and nomralization. tSNE plots in this study were generated in R[80] using the Rtsne package.[83,84,85] Pathway analysis was conducted using either Ingenuity Pathway Analysis (QIAGEN). No custom code was generated in this study.

Gene expression analysis

Gene expression Fold Change (FC) was calculated with the ΔΔCt method using Microsoft Excel. Briefly, ΔΔCt = ΔCt(COVID-19-positive)-ΔCt(healthy control) with ΔCt = Ct(gene-of-interest)-Ct(housekeeping gene-GAPDH). The FC for each gene is calculated as 2-ΔΔCt. All primers sequences used in this study is available upon request.

Statistical analysis for sera proteome profiling

Statistical tests for this study were done either in R (R Core Team, 2020) or in Graphpad PRISM 9.0 software. Chi-square tests were used to assess whether COVID-19 severity differed based on maternal demographic variables, medical history, pregnancy-related clinical outcomes, or infant outcomes. Welch t tests or Wilcoxon Rank Sum Tests were done using the R base package, t test. Comparison between (i) healthy and COVID-19+ pregnancies; (ii) healthy and various COVID-19 disease severity pregnancies, were performed using Wilcoxon rank sum test. Comparison between (iii) healthy delivery and COVID-19+ delivery; (iv) healthy and

COVID-19-exposed infants; (v) healthy and various COVID-19 severity-exposed infants or infants presenting respiratory distress, were performed using Welch t test. Comparison between COVID-19-initial diagnosis and COVID-19-delivery was performed using paired t test. Comparison analyses (i) to (v) were performed using R. For all other analyses, Mann-Whitney U test was used for comparison between two groups and one-way ANOVA Kruskal-Wallis with uncorrected Dunn's test was used for comparison among three groups. According to G*Power analysis, the Mann-Whitney test with 79 COVID-19+ maternal cases and 18 healthy controls in our study achieves 80% power assuming an effect size of 0.675.