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MAJOR ARTICLE

Specific Biomarkers Associated With Neurological Complications and Congenital Central Nervous System Abnormalities From Zika Virus–Infected Patients in Brazil

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Background. Zika virus (ZIKV) infections have been linked to different levels of clinical outcomes, ranging from mild rash and fever to severe neurological complications and congenital malformations.

Methods. We investigated the clinical and immunological response, focusing on the immune mediators profile in 95 acute ZIKV-infected adult patients from Campinas, Brazil. These patients included 6 pregnant women who later delivered during the course of this study. Clinical observations were recorded during hospitalization. Levels of 45 immune mediators were quantified using multiplex microbead-based immunoassays.

Results. Whereas 11.6% of patients had neurological complications, 88.4% displayed mild disease of rash and fever. Several immune mediators were specifically higher in ZIKV-infected patients, and levels of interleukin 10, interferon gamma-induced protein 10 (IP-10), and hepatocyte growth factor differentiated between patients with or without neurological complications. Interestingly, higher levels of interleukin 22, monocyte chemoattractant protein 1, TNF-α, and IP-10 were observed in ZIKV-infected pregnant women carrying fetuses with fetal growth–associated malformations. Notably, infants with congenital central nervous system deformities had significantly higher levels of interleukin 18 and IP-10 but lower levels of hepatocyte growth factor than those without such abnormalities born to ZIKV-infected mothers.

Conclusions. This study identified several key markers for the control of ZIKV pathogenesis. This will allow a better understanding of the molecular mechanisms of ZIKV infection in patients.

Keywords. Zika virus; congenital CNS deformities; cytokines and biomarkers.

Zika virus (ZIKV) has reemerged as an important flavivirus that has caused several Zika fever epidemics in various parts of the world. ZIKV was first isolated in 1947 [1] and is typically transmitted by *Aedes* mosquitoes. Since 2007, of 76 countries and territories that have reported evidence of mosquito-borne ZIKV local transmission, 29 countries have reported congenital anomalies, such as microcephaly, possibly associated with ZIKV infection [2–6]. Most patients remain asymptomatic or suffer from mild symptoms that normally last 2–7 days. Symptoms include fever, arthritis/arthralgia, rash, conjunctivitis, joint pain, and headache [7]. However, there is now evidence of ZIKV with Guillain-Barré syndrome and congenital central nervous system (CNS) abnormalities [8–13]. Therefore, ZIKV infection represents a major public health concern with severe social and economic burden owing to a lack of commercial vaccines or effective antiviral treatments. Treatment is usually symptomatic and pain-relief medicine is the only available option during disease onset. Brazil, hit by the ZIKV epidemics since 2014, accounted >200 000 probable cases of ZIKV infection, with almost 2000 cases of microcephaly in 2015–2016.

Because the mode of transmission of ZIKV and the clinical symptoms caused by ZIKV infection are highly similar to those in multiple arboviruses, including dengue virus (DENV), specific biomarker profiles will be useful for disease prognosis. Based on the current knowledge, DENV and ZIKV are largely similar, but dissimilarities between the 2 viruses (eg, the distinct pattern of E protein glycosylation sites) may have potential effects on viral

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tropism, thermostability of the virus, and immunomodulation during viral replication [14]. Altogether, this may cause different host response and induce distinct clinical observations, particularly in severe cases. In addition, predictive biomarkers that could differentiate between ZIKV-induced neurological complications would be highly desirable. Until now, the association between clinical profile and immune response to ZIKV infection in patients has remained largely unknown.

In the current study, we aimed to analyze the clinical and immunological response by analyzing the cytokine/chemokine profiles of ZIKV-infected patients from the Campinas metropolitan area in Brazil, s home to about 4 million inhabitants and a hub for travelers transiting between different destinations in Brazil and South America. Patients were classified according to their in-house diagnostic test result, type of clinical observation during hospitalization, and fetal development. Comprehensive multiplex microbead-based arrays were performed to associate the differential patterns of immune mediators between adult patients with/without neurological complications; pregnant women who later gave birth to infants with or without congenital CNS deformities; and infants with or without congenital CNS deformities born to ZIKV-infected mothers. These findings will provide critical predictors of severe ZIKV infection and improve current practices of clinical management.

MATERIALS AND METHODS

Ethical Approval

Written informed consent was obtained from all participants and participants' parents or legal guardians (parental consent for age under 17) and study was conducted according to Declaration of Helsinki principles. This study was approved by the Research Ethics Committee of the University of Campinas (Certificate of Presentation for Ethical Consideration [CAAE] No. 56793516.0.0000.5404).

Patients and Serum Collection

Acute-phase serum specimens were collected from 95 patients a median of 3 days after illness onset. Of these patients, 6 were pregnant women who also provided serum samples 1–3 months (convalescent phase) after the first sampling. Serum samples were collected at birth from the 6 newborns born to these women. Additional serum samples from 4 other newborns (born to the 4 ZIKV-infected mothers who were not included in this study) obtained during the same Zika epidemic in Brazil were also included as study samples. Serum samples were obtained from 10 mL of peripheral blood collected in dry tube after peripheral venipuncture. All samples were transported on ice within <6 hours to the Laboratory for Study of Emerging Viruses at the Biology Institute of the University of Campinas. All samples were processed and tested for ZIKV using real-time reverse-transcription polymerase chain reaction (RT-PCR). Frozen serum samples were sent to the Vaccine Development Laboratory at Biomedical Science Institute of University of São Paulo and tested for presence of ZIKV-specific antibodies by enzyme-linked immunosorbent assay (ELISA). Samples from 13 healthy donors were included and prescreened for presence of ZIKV viral RNA and ZIKV-specific antibodies. Clinical data and maternal and perinatal outcomes were retrospectively retrieved from medical records.

ZIKV Detection With Real-Time RT-PCR

RNA samples were extracted from 140 μL of serum using the QIAamp Viral RNA Mini Kit (Qiagen), according to the manufacturer's protocols. ZIKV detection was performed using real-time RT-PCR (TaqMan RNA to-Ct 1-Step Kit; Applied Biosystems) with primers and probes as described elsewhere, with modifications [15] (ZIKV-F, 5'-CCGCTGCCCAACACAAG-3'; ZIKV-R, 5'-CCACTAACGTTCTTTTGCAGACAT-3'; ZIKV-P, 5'FAM-AGCCTACCTTGACAAG CAGTCAGACACTCAA-BHQ1-3'). Briefly, all reactions were performed in a final volume of 12.5 μL with 50 ng of RNA, 400 and 200 nmol/L of primers and probe, respectively, and 6.25 μL of TaqMan PCR master mix (Applied Biosystems). The following cycling algorithm was used: 48°C for 30 minutes, followed by 95°C for 10 minutes, 45 cycles of 95°C for 15 seconds, and 60°C for 1 minute.

ZIKV Detection With In-House Specific ELISA

ZIKV immunoglobulin (Ig) G antibodies were evaluated by means of ELISA serology of patients' serum samples, according to a modified method [16]. Briefly, polystyrene COSTAR microplates (Corning) were coated with 100 ng of a recombinant ZIKV nonstructural protein 1 protein expressed in bacterial cells and suspended in a carbonate buffer solution (pH 9.6). Serum samples were diluted 1:100 and preincubated in sample solution at 37°C for 1 hour. Plate wells were blocked with 5% skimmed milk and 1% bovine serum albumin solution for 2 hours at room temperature, washed 4 times in a phosphate-buffered saline–Tween 0.05% solution and then exposed to the sample solutions at 37°C for 1 hour.

After a new washing cycle, secondary anti-human IgG conjugated to peroxidase (Sigma) was added to wells and incubated again for 1 hour. After a final washing, plate wells were developed with 3,3,5,5-tetramethylbenzidine solution (Sigma). The reaction was stopped after 15 minutes by adding 00 μL of sulfuric acid at 0.2 mol/L. The optical density reading was measured at 450 nm with a plate reader (Thermo Scientific). A signal-to-cutoff was calculated based on known negative samples and applied on the tested samples. After the cutoff determination, the samples were categorized as negative, borderline (undetermined), or positive.

Multiplex Microbead-Based Immunoassay

Serum levels of immune mediators were measured using human cytokine 45-plex immunoassay kits (Procarta), according to

the manufacturer's instruction; cytokines included granulocyte-macrophage colony-stimulating factor, epidermal growth factor, brain-derived neurotrophic factor, beta-nerve growth factor (bNGF), basic fibroblast growth factor (FGF-2), hepatocyte growth factor (HGF), CCL2 (monocyte chemoattractant protein [MCP] 1), CCL3 (macrophage inflammatory protein [MIP] 1α), MIP-1β, CCL5 (RANTES [regulated on activation, normal T-expressed, and presumably secreted]), chemokine (C-X-C motif) ligand 1 (GRO-α), CXCL12α (stromal cell-derived factor 1 [SDF-1α]), CXCL10 (interferon gamma-induced protein 10 [IP-10]), eotaxin, interferon (IFN) α, IFN-γ, interleukin 1α, 1β, 1RA, 10, 13, 15, 17A, 18, 2, 21, 22, 23, 27, 31, 4, 5, 6, 7, 8 (CXCL8), 9, and 12p70, leukemia inhibitory factor, stem cell factor, tumor necrosis factor (TNF) α, TNF-β, vascular endothelial growth factors A and D, platelet-derived growth factor [PDGF-BB], and placental growth factor [PIGF-1]). Briefly, magnetic beads were aliquoted in 96-well plates, followed by the addition of standards and serum samples from patients and control subjects. After an incubation period, plates were washed using a magnetic wash station according to manufacturer's instructions, followed by addition of a detection antibody.

Plates were incubated for another 30 minutes and then washed, followed by a final 10-minute incubation in the presence of streptavidin-phycoerythrin. Results were acquired using the Bio-Plex 200 system (Bio-Rad) with Luminex xPONENT software (version 3.1), based on standard curves plotted through a 5-parameter logistic curve setting. TNF-β was found to be below detection limit and hence excluded from subsequent analysis.

ELISA for Cytokine Quantification

Serum levels of TNF-α (catalog No. DY210-05) were measured using ELISA kits from R&D Systems (DuoSet ELISA; R&D Systems), according to the manufacturer's instructions.

Data Analysis

Sample randomization for the Luminex assays could not be performed for the various sample groups because the sample processing was dependent on the collection at the hospital. Once sufficient samples were collected, the Luminex assays were performed. To remove any potential plate effects, an additional plate was assayed that contained a selected number of samples from all assayed plates. These samples were then used to normalize the assayed plates. A correction factor was obtained from the difference observed between the original assayed plate data and the replicates on the addition plate. This correction factor was then applied to the rest of the samples of the original assayed plate. The concentrations were logarithmically transformed to ensure normality.

One-way analysis of variance with post hoc *t* test corrected using the Bonferroni method was used to detect differences between the various sample groups. These results were corrected for multiple testing using the method of Benjamini and Hochberg. Functional group and canonical pathway analyses were generated using Ingenuity Pathways Analysis software (Qiagen; IPA Spring Release [March 2017]). All statistical analyses were performed with R software (version 3.1.2). Differences were considered statistically significant at $P < .05$. Plots were generated using GraphPad Prism software (version 7).

Table 1. Demographics and Characteristics of 95 ZIKV-Infected Patients Admitted to University of Campinas Hospital and Women's Comprehensive Healthcare Center between February and August 2016

Characteristic	Results[a]
Age, median (range), y	35 (6–82)
Sex ratio (No. male/No. female)	0.6 (29/ 66)
Length of admission, median (range), d	3 (1–10)
Viral load in urine, mean (CT)	37.08 (0.44)
Viral load in blood, mean (CT)	38.04 (0.68)
Fever, No. (%)	62 (65.3)
Body temperature, range, °C	38–40
Rash, No. (%)	67 (70.5)
Conjunctivitis, No. (%)	35 (36.8)
Neurological syndrome, No. (%)	11 (11.6)

Abbreviations: CT, threshold cycle; ZIKV, Zika virus.

[a]ZIKV positivity was confirmed by means of quantitative reverse-transcription polymerase chain reaction and/or by in-house serology assays, as described in Materials and Methods.

RESULTS

Clinical Manifestations in Patients With Zika Fever in Campinas, Brazil

Between February and August 2016, a total of 95 patients were recruited for this study, based on their clinical symptoms during hospital admission (Table 1). All febrile patients' serum samples (median interval between illness onset and sampling, 3 days) were screened with quantitative RT-PCR and/or in-house anti-ZIKV ELISA. According to the guidelines from the World Health Organization, ZIKV infection cannot be ruled out using only the PCR result [17]. Studies have shown that ZIKV-infected patients can display negative PCR results as early as 2 days after symptom onset [18–20]. Another study has also demonstrated the sensitivity and specificity of the ZIKV NS1-specific ELISA for accurate diagnostic of ZIKV infection [21].

Therefore, in addition to PCR, we used an in-house ZIKV NS1-specific ELISA to validate the presence of ZIKV-specific antibodies from the patients. The majority of patients (83 of 95) were PCR positive for ZIKV during hospitalization, and the 12 who were PCR negative patients were serologically confirmed as ZIKV positive by ELISA. In line with the observation that ZIKV is generally self-limiting with mild symptoms, 84 of 95 of ZIKV-infected patients were observed to display mild symptoms, with no neurological complications during the acute phase of infection (Table 1).

In this cohort, 6 of 83 patients who were positive for ZIKV at PCR were pregnant women. Of these 6 women, 1 (17%) was later found to be carrying a fetus with fetal growth–associated

Table 2. Maternal and Perinatal Outcomes in Women Infected With ZIKV During Pregnancy and Their Newborns

Women	No. (%)[a]
Total no. of women	6
Age, mean (SD), y	28.1 ± 5.1
Gestational age at onset of symptoms, mean (SD), wk	24.2 ± 11.7
Onset of symptoms by trimester of pregnancy	
1st trimester	1 (16.7)
2nd trimester	2 (33.3)
3rd trimester	3 (50.0)
Interval between onset of symptoms and sample collection, mean (SD), d	2.6 ± 1.9
Delivery at the reference maternity	6 (100)
Type of delivery	
Normal vaginal delivery	3 (50.0)
Cesarean section	3 (50.0)
Overall birth weight at delivery, mean (SD), g	3066.0 ± 912.2
Birthweight adequacy to gestational age	
Normal	5 (83.3)
Underweight	1 (16.7)
5-min Apgar index	
≤7	1 (16.7)
>7	5 (83.3)
Symptoms during pregnancy	
Exanthema	6 (100)
Pruritus	4 (66.7)
Headache	4 (66.7)
Fever	2 (33.3)
Fetal growth restriction	2 (33.3)
Arthralgia	2 (33.3)
Myalgia	1 (16.7)
Newborns	
Total no. of newborns	10
Intrauterine growth restriction	2 (20)
Neonatal malformations	1 (10)
Microcephaly (25.5 cm), craniosynostosis, hyperexcitability syndrome, hypertonia syndrome, and evidence of congenital cataract	1 (10)
Neonatal deaths	1 (10)

Abbreviations: ZIKV, Zika virus; y, year; wk, week; d, day; g, gram; SD, Standard deviations.
[a]Data represent No. (%) of women or newborns, unless otherwise specified.

malformations. The maternal and perinatal outcomes in these women and their newborns are listed in Table 2. Rash remains one of the most frequent symptoms [7] and was displayed by all pregnant women in this study (Table 2). As expected, of the 6 pregnant women, 5 delivered healthy newborns who tested negative for ZIKV by RT-PCR or ELISA. One woman delivered a newborn with congenital CNS deformities. In addition, 4 other newborn infants born to ZIKV-infected mothers (not part of the original 95 patients) were also included in this study. Of the total 10 infants born to ZIKV-infected mothers, 2 (20%) had intrauterine growth restrictions, 1 (10%) had paraplegia, 1 (10%) had microcephaly, and 1 (10%) had meningoencephalitis.

Profiles of Immune Mediators in ZIKV-Infected Patients With or Without Neurological Complications

Using a multiplex microbead-based immunoassay, the immune profile comprising of up to 45 immune mediators were determined and quantified from serum samples obtained from the 95 ZIKV-infected patients. Samples from 13 healthy adults were also included in the analysis as controls. All ZIKV-infected febrile patients had high levels of a set of common immune mediators compared with the healthy controls (Figure 1). There was no difference in the pattern of immune mediators' profiles whether the patients were classified either by ZIKV PCR (Figure 1A and 1B) or by clinical features (Figure 1C and 1D).

Nineteen factors were significantly higher in febrile patients than in healthy adults: proinflammatory cytokines (interleukin 18, 8, 6, and 7 [IL-18, IL-8, IL-6, and IL-7], TNF-α, IFN-γ, and GRO-α), anti-inflammatory cytokines (interleukin 10, 1RA, and 4 [IL-10, IL-1RA, and IL-4]), chemokines (IP-10, MCP-1, MIP-1β, eotaxin, and SDF-1α), growth factors and others immune mediators (HGF, brain-derived neurotrophic factor, leukemia inhibitory factor, and stem cell factor) (Figure 1 and Supplementary Figures S1 and S2). However, 2 more mediators, FGF-2 and PDGF-BB, also had significantly higher levels in febrile patients than in healthy controls when patients were classified based on ZIKV PCR status (Supplementary Figure S1). This observation suggests that an active production of a network of immune mediators, along with detectable levels of ZIKV RNA, could provide a strong antiviral environment during the acute phase of disease, resulting in a milder clinical outcome. However, no significant correlations were found between the immune mediator profiles and viral load from patients' blood and urine samples (data not shown).

Differential Immune Mediators Profiles in Pregnant Women and Fetuses During Gestation

Profiles between the ZIKV-infected pregnant women were assessed using serum samples collected during the acute and convalescent phases of disease (Figure 2A and 2B). Profiles showed that the pregnant woman carrying a fetus with fetal development anomalies had very high levels of 12 immune mediators: IL-8, IL-18, IL-4, interleukin 22 (IL-22), 23 and 27, MCP-1, TNF-α, IP-10, epidermal growth factor, eotaxin, FGF-2 compared with the other pregnant women carrying normal fetuses. Levels of IL-22, MCP-1, IP-10, and TNF-α were observed to be significantly higher during the acute phase of disease (Figure 2A and Supplementary Figure S3).

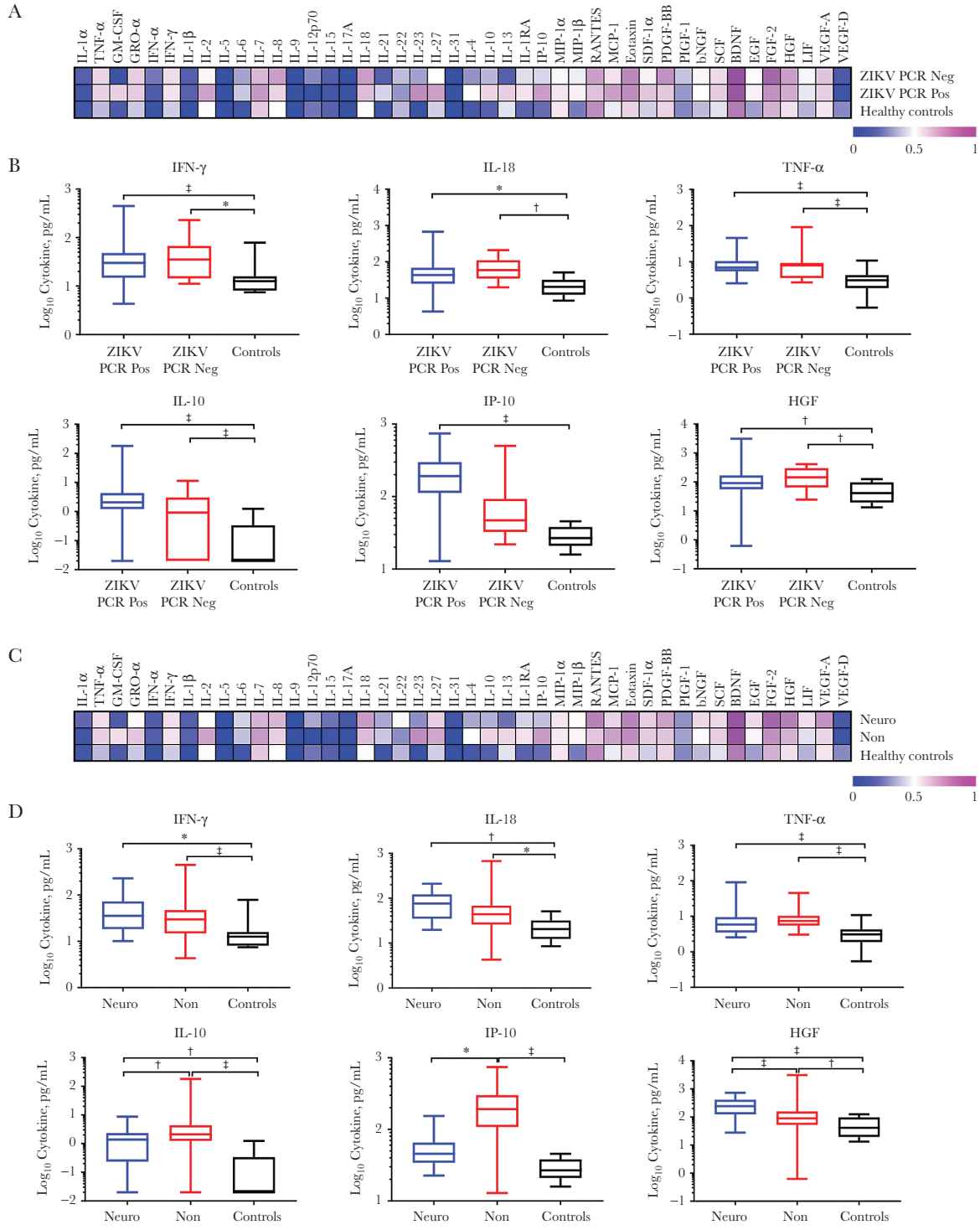

Figure 1. Pattern of immune mediators from a Zika virus (ZIKV) patient cohort in Campinas, Brazil, during the acute phase of disease. *A, B,* Acute-phase patient samples were grouped according to ZIKV polymerase chain reaction (PCR) status (12 patients ZIKV PCR negative [Neg] and 83 ZIKV PCR positive (Pos). *C, D,* clinical observation during hospitalization (11 patients with neurological complications [Neuro] and 84 without [Non]). Levels of immune mediators (proinflammatory cytokines, anti-inflammatory cytokines, chemokines, and growth and other factors) during acute ZIKV infection were analyzed and presented in heat maps of normalized scores. In the heat \-map presentation, the immune mediator concentrations were scaled between 0 and 1 for each measured immune mediator, and the average scaled value was then computed for each group. Selected immune mediators are depicted as Tukey box plots. One-way analyses of variance were conducted on the logarithmically transformed concentrations with Bonferroni-corrected post hoc *t* tests; results were corrected for multiple testing using the method of Benjamini and Hochberg. **P* < .05; †*P* < .01; ‡*P* < .001. Abbreviations: BDNF, brain-derived neurotrophic factor; bNGF, beta-nerve growth factor; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, chemokine (C-X-C motif) ligand 1; HGF, hepatocyte growth factor; IFN, interferon; IL-1α (etc), interleukin 1α (etc); IP-10, interferon gamma-induced protein 10; LIF, leukemia inhibitory factor, MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF-BB, platelet-derived growth factor; PIGF, placental growth factor; RANTES, regulated on activation, normal T-expressed, and presumably secreted; SCF, stem cell factor; SDF, stromal cell-derived factor 1α; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

Higher IL-18 and IP-10 and Lower HGF Levels in Infants with Congenital CNS Deformities Born to ZIKV-Infected Mothers

Profiles in infants with congenital CNS abnormalities and healthy infants born to ZIKV-infected mothers were analyzed next (Figure 3A). Surprisingly, only 3 immune mediators—IL-18, IP-10, and HGF—showed a significant difference between the 2 groups (Figure 3B). Infants with congenital CNS deformities have higher levels of IL-18 and IP-10 but much lower levels of HGF than healthy infants without any specific clinical outcomes (Figure 3B). This is the first observation showing a differential up- and down-regulation of immune mediators in infants born to ZIKV-infected mothers.

DISCUSSION

Immune mediators can be detrimental to or protective of diseases [22]. Brazil has recently declared an end to ZIKV epidemics because the number of reported cases has decreased significantly since the later part of 2016; this may be linked to the development of herd immunity in populations [23, 24], but the effect of herd immunity (generation of protective antibodies against a particular pathogen after an epidemic) on the reemergence of ZIKV and/or DENV epidemics in the future is still unknown. In the current study, to better define ZIKV infection in patients, 95 patients were stratified according to their clinical features, and multiplex microbead-based immunoassays were carried out to determine the immune profiles of ZIKV-infected patients with different clinical features, ranging from mild symptoms to neurological complications. Most of the patients in this study displayed fever and rash only, confirming that Zika fever is generally self-limiting. However, in a small fraction of patients (11.6%), neurological complications developed 0–3 days after the appearance of acute symptoms, consistent with other observations [7, 9].

Currently, there is only 1 publication describing the cytokine profiles of 6 ZIKV-infected patients from acute to recovery phases [25]. Owing to the small number of patients, larger cohorts of patients should be accessed to strengthen the observations. In the current cohort, levels of 21 immune mediators were significantly higher in ZIKV-infected patients than in the healthy controls. This pattern is similar to an earlier study in another cohort that showed high levels of multiple immune mediators, including IL-1RA, IP-10, and HGF in DENV-infected patients [26]. IPA analysis further revealed that these 21 mediators are highly interconnected and are involved in the NF-κB signaling pathway (Figure 4A). The NF-κB pathway has been reported to be activated in DENV infection and responsible for proinflammatory response [27]. Thus, the exact mechanistic pathway involving NF-κB activation should be further explored.

Given the observation that fetal development malformations could occur in a significant proportion of

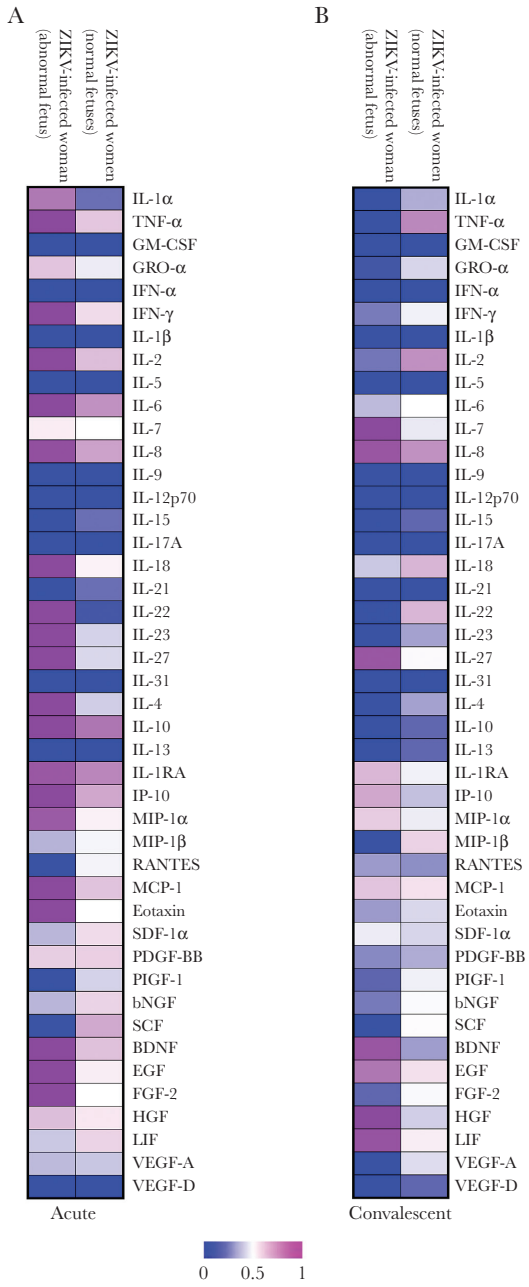

Figure 2. Predictive immune mediators determined from 6 pregnant women with Zika virus (ZIKV) infection. Level of immune mediators from 5 ZIKV-infected pregnant women with normal fetuses and 1 ZIKV-infected pregnant woman whose fetus had congenital anomalies, from serum samples collected during the acute (*A*) and convalescent (*B*) phases of disease were analyzed and presented in heat maps of normalized scores. In the heat-map presentation, the immune mediator concentrations were scaled between 0 and 1 for each measured immune mediator, and the average scaled value was then computed for each group. Blue shading represents the lowest average scaled value; pink shading, the highest average scaled value. Abbreviations: BDNF, brain-derived neurotrophic factor; bNGF, beta-nerve growth factor; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; GRO-α, chemokine (C-X-C motif) ligand 1; HGF, hepatocyte growth factor; IFN, interferon; IL-1α (etc), interleukin 1α (etc); IP-10, interferon gamma-induced protein 10; LIF, leukemia inhibitory factor, MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF-BB, platelet-derived growth factor; PIGF, placental growth factor; RANTES, regulated on activation, normal T-expressed, and presumably secreted; SCF, stem cell factor; SDF, stromal cell-derived factor 1α; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

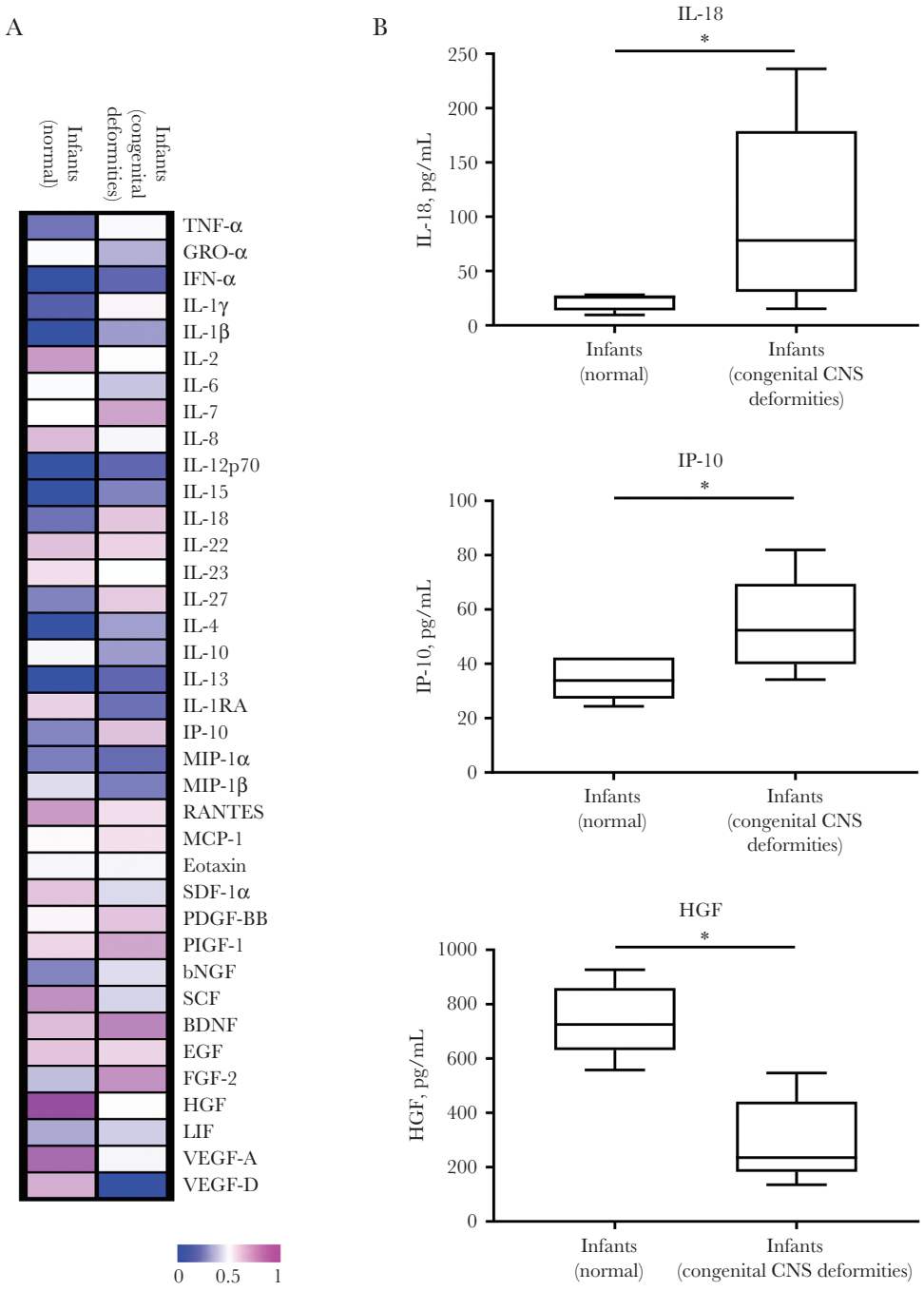

Figure 3. Predictive immune mediators from 10 infants born to women with Zika virus (ZIKV) infection. *A,* Level of immune mediators in serum samples collected from 5 normal infants and 5 infants with congenital central nervous system (CNS) deformities born to ZIKV-infected women were analyzed and presented in heat maps of normalized scores. *B,* Cytokines from individual infants were compared between healthy normal infants and infants with congenital CNS deformities. Selected immune mediators are depicted as Tukey box plots. One-way analyses of variance were conducted on the logarithmically transformed concentrations with Bonferroni-corrected post hoc *t* tests. Results were corrected for multiple testing using the method of Benjamini and Hochberg; $*P < .05$. Abbreviations: BDNF, brain-derived neurotrophic factor; bNGF, beta-nerve growth factor; EGF, epidermal growth factor; FGF-2, basic fibroblast growth factor; GRO-α, chemokine (C-X-C motif) ligand 1; HGF, hepatocyte growth factor; IFN, interferon; IL-1β (etc), interleukin 1β (etc); IP-10, interferon gamma-induced protein 10; LIF, leukemia inhibitory factor, MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF-BB, platelet-derived growth factor; PIGF, placental growth factor; RANTES, regulated on activation, normal T-expressed, and presumably secreted; SCF, stem cell factor; SDF, stromal cell-derived factor 1α; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.

ZIKV-infected pregnant women, it is necessary to unravel the link between ZIKV and congenital CNS abnormalities, given the severe socioeconomic impacts. The differential levels of IL-22, MCP-1, IP-10, and TNF-α in pregnant women could serve as useful prognostic biomarkers to predict the possible outcome in infants born to ZIKV-infected

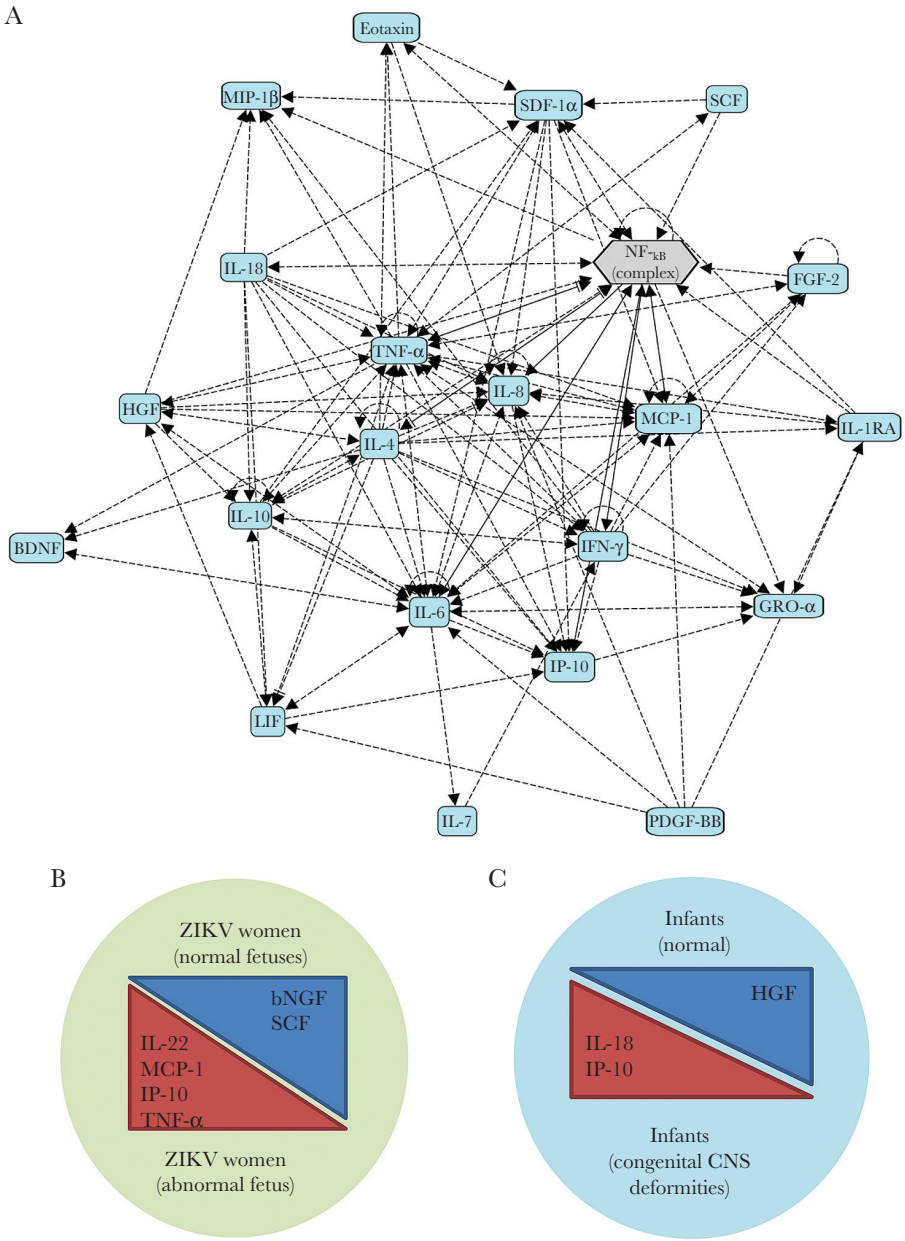

Figure 4. Signature of immune mediators in patients with acute Zika virus (ZIKV) infection. *A,* All immune mediators up-regulated relative to healthy controls were analyzed, and the predicted network was depicted with Ingenuity Pathway Analysis software. *B,* Relative levels of specific immune mediators between ZIKV-infected pregnant women carrying fetuses with or without congenital anomalies. *C,* Relative levels of specific immune mediators infants born to these ZIKV-infected women. Abbreviations: BDNF, brain-derived neurotrophic factor; bNGF, beta-nerve growth factor; FGF-2, basic fibroblast growth factor; GRO-α, chemokine (C-X-C motif) ligand 1; HGF, hepatocyte growth factor; IFN, interferon; IL-4 (etc), interleukin 4 (etc); IP-10, interferon gamma-induced protein 10; LIF, leukemia inhibitory factor, MCP, monocyte chemoattractant protein; MIP, macrophage inflammatory protein; PDGF-BB, platelet-derived growth factor; SCF, stem cell factor; SDF, stromal cell-derived factor 1α; TNF, tumor necrosis factor.

mothers (Figure 4B). Furthermore, a recent study has demonstrated the presence of high levels of TNF-α in ZIKV-infected human fetal brain cells, further implying its role in neuroinflammation [28]. The independent detection of high levels of TNF-α (Supplementary Table S1) further strengthened the possibility of using this set of identified mediators as ZIKV prognostic biomarkers to improve clinical management and potential treatments in future ZIKV outbreaks.

Hypercytokinemia, more commonly known as "cytokine storm," has been well reported in viral diseases [29]. In the current study, ZIKV-infected patients had higher levels of multiple proinflammatory cytokines, including IL-6, IL-7, IL-8, IL-18, GRO-α, TNF-α, and IFN-γ. Notably, IL-18 and IFN-γ have been shown to be important proinflammatory cytokines in host defense against infection and in natural killer (NK) cell activation [30]. Classically, activation of NK cells could initiate

neuroprotection or neurotoxicity through different immune cascades in the presence of specific immune mediators [31]. In the presence of IL-18, NK cells could be activated and induce IFN-γ. Although little is known about the role of NK cells in patients infected by flaviviruses, the involvement of NK cells during the acute phase of ZIKV infection could be inferred from the high levels of IL-18 and IFN-γ. Moreover, studies in other flaviviruses have demonstrated the pathogenic role of IFN-γ in Japanese encephalitis virus infections through the down-regulation of a tight-junction protein within the blood-brain barrier [32]. Establishing the role of IFN-γ in the neuropathogenesis of ZIKV will be of interest in the future.

Interestingly, higher levels of anti-inflammatory cytokines, including IL-4, IL-1RA, and IL-10, were observed in patients with mild disease outcome. Specifically, the level of IL-10 was higher in patients without any neurological complications. A similar pattern of the immune mediators' network that contributes to the neurological status after pathogen infection has also been reported in various studies [31, 33, 34]. Therefore, the current observation suggests a plausible link between NK cells through the cytokine network, involving IL-18, IFN-γ, and IL-10 to protect against ZIKV-induced neuropathogenesis.

The level of HGF was significantly lower in infants with congenital CNS deformities, suggesting an important role in the development of fetal CNS development in during gestation (Figure 4C). The presence of HGF typically activates the HGF/MET signaling pathway that further induces mechanistic target of rapamycin (mTOR) activation [35]. Animal studies have demonstrated the crucial role of mTOR in overall brain growth and postnatal survival [36], while whereas role of HGF in neuroprotection has been indicated in both autoimmune and infectious diseases [37, 38]. Therefore, it is now of prime importance to understand the mechanisms behind the pathogenesis of ZIKV infection that could lead to a down-regulation of HGF during gestation in developing fetuses.

In conclusion, ours is the first systematic large-scale analysis of immune mediators reported in ZIKV-infected patients including pregnant women and infants with congenital CNS abnormalities. Data sets from other cohorts combined with meta-analysis will further verify and validate the list of biomarkers identified in this study, to improve prognostic efficiency and clinical management in the face of emerging new outbreaks.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Author contributions. Y. W. K. and J. A. L. performed immunological microbead-based assays. M. A. V. performed and analyzed the single-plex cytokine ELISA results. L. R., J. L. P. M., L. F. P. N., and F. T. M. C. conceptualized the study. C. C. J., D .A. D. T. T., R. A. S., R. A., M. R. R., A. R. R. F., E. A., R. P. J., M. L. C., J. P. G., C. W. A., L. C. F., and the Zika-Unicamp Network contributed materials. Y. W. K., B. L., J. J. L. T., F. M. L., L. R., L. F. P. N., and F. T. M. C. analyzed the data. Y. W. K., J. A. L., J. J. L. T., F. M. L., J. L. P. M., L. F. P. N., and F. T. M. C. wrote the manuscript. All authors read and approved the manuscript.

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