IMMUNE ACTIVATION IN AMNIOTIC FLUID FROM ZIKA VIRUS ASSOCIATED MICROCEPHALY

Running Title: Immune activation associated with Zika infection.

Alice M. M. Ornelas (MSc)[1], Paula Pezzuto (PhD)[1], Paola P. Silveira (MSc)[1], Fabiana O. Melo (MD) [2], Thales A. Ferreira (MD)[2], Patricia S. Oliveira-Szejnfeld (MD, PhD)[3], Jeime I. Leal (MD)[2], Melania M. R. Amorim (MD, PhD) [2], Stuart Hamilton (PhD) [4], William D. Rawlinson (PhD)[4], Cynthia C. Cardoso (PhD)[1], Douglas F. Nixon (MD, PhD)[5], Amilcar Tanuri (MD, PhD)[1], Adriana S. Melo (MD, PhD)[2] and Renato S. Aguiar (PhD)[1‡]

[1]- Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Rio de Janeiro, Brazil.

[2]- Instituto de Pesquisa Professor Joaquim Amorim Neto (IPESQ), Campina Grande, Paraíba, Brazil.

[3]-Fundação Instituto de Ensino e Pesquisa em Diagnóstico por Imagem (FIDI), Departamento de Diagnóstico por Imagem, Universidade Federal de São Paulo, São Paulo, Brazil

[4]- Virology Research, POWH & UNSW Research Laboratories, Prince of Wales Hospital, Randwick, 2031 NSW, Australia.

[5]-Department of Microbiology, Immunology & Tropical Medicine, The George Washington University, Washington DC, USA.

‡ Corresponding author.

Address for correspondence: Renato S. Aguiar, Departamento de Genética, Instituto de Biologia, Universidade Federal do Rio de Janeiro, Av. Carlos Chagas Filho 373, 21941-902, Brazil.

email:santanarnt@gmail.com; **phone:** +55-21-25643353; **fax:**+55-21-25643353.

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Abstract

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Recent advances in the understanding of neuropathogenesis associated with Zika virus (ZIKV) infection has led to descriptions of neonatal microcephaly cases. However, none of these reports has evaluated the humoral response during ZIKV infection. We report here polyfunctional immune activation associated with increased IP-10, IL-6, IL-8, VEGF, MCP-1 and G-CSF levels in the amniotic fluid of ZIKV positive pregnant women with neonatal microcephaly. These cytokines have been associated not only with neuronal damage, but also with differentiation and proliferation of neural progenitor cells. Our results suggested that the immune activation caused by ZIKV infection in the uterine environment could also interfere with the fetal development.

Introduction

Zika virus (ZIKV) is a flavivirus that belongs to the *Flaviviridae* family. A recent epidemic of human ZIKV infection started in 2014 in Brazil, and has spread to more than 30 countries worldwide (1). While many infected individuals are asymptomatic or have only mild symptoms, a major concern has been driven by the association of ZIKV and neurological disorders, including Guillain-Barre Syndrome (GBS), acute myelitis, meningoencephalitis and fetal and neonatal microcephaly (1, 2), which led the World Health Organization to declare a Public Health Emergency. In the northeast region of Brazil, the numbers of microcephaly cases in 2015 jumped twenty-fold in comparison to previous years (4). A potential causal relationship between ZIKV and neurological damage has been suggested due to the detection of ZIKV in the blood of microcephalic newborns, and in the amniotic fluid (AF) or brains (BR) of microcephalic fetuses. More recently, ZIKV has been shown to infect and cause death of neural stem cells (NSC) (2-4).

AF is a complex biological fluid that performs multiple functions at different stages of fetal development. AF cells originate from the three germ cell layers of the embryo, ranging from

unspecified progenitors to mature differentiated cells (5). The acellular part of AF contains suspended RNA from the fetus, as well as cytokines and growth factors, which are important for fetal development including central nervous system formation and cell-fate determination (5,6). Here, we investigated the AF cytokine and growth factors profiles of pregnant women with diagnosis of fetus microcephaly and ZIKV infection.

Methods

We analyzed AF cytokine levels from nine pregnant women aged 18-34 years (median 24 years) with microcephalic fetuses or neonates. All the cases were from Campina Grande, Paraiba state, Brazil. Almost all women developed acute Zika virus symptoms during pregnancy. AFs were collected at different times after disease onset in midtrimester, and ZIKV infection was diagnosed by One Step Taqman RT-qPCR (Thermo Fisher Scientific) with primers and probe described elsewhere (7) (Table 1).

The pregnant women had 3-6 follow-up visits with fetal ultrasonography (USG). Other arboviruses such as dengue and chikungunya virus were negative by ELISA and PCR examination in the blood and AF. STORCH (syphilis, toxoplasmosis, HIV, measles, rubella, cytomegalovirus, and herpes simplex) panels of all participants were also negative, as well as specific HIV, syphilis, cytomegalovirus, and parvovirus B19 screens. Fetal and neonatal microcephaly was confirmed by intrauterine ultrasonography or head circumference measure following WHO recommendations (8). This study was approved by the local internal review board (IRB). All patients agreed to participate in this study, and signed informed consent. In order to evaluate the immune response during ZIKV infection we investigated the levels of proinflammatory cytokines, chemokines, adhesion molecules and growth factors in AF of the

pregnant woman enrolled in this study using the Bio-Plex Panel 27-Plex (Bio-Rad, California, USA). Cytokine levels of twenty seven normal amniotic fluids sampled in early midtrimester (16 weeks gestation), shown to be uninfected with a range of common congenital viruses and pathogens, were used in parallel as negative controls (12).

Results

The analysis of cytokines during ZIKV infection in the intrauterine environment amniotic fluid showed a decreasing amount (up to 2 fold) of interleukin (IL) 13, IL-9, fibroblast growth factor (FGF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) between ZIKV positive AF and the negative controls (Table 2). However, a remarkable increase in the quantity of different cytokines was found in the AF. The IL-17, IL-15, interferon γ (IFN γ), Eotaxin, IFN-gamma-inducible protein (IP-10), granulocyte colony stimulating factor (G-CSF) and IL-7 levels were increased 5 – 20 fold. IL-6, IL-5, IL-10, IL-4, IL-1β, tumor necrosis factor α (TNF α), IL-8, macrophage inflammatory protein (MIP) 1α and 1β, regulated on activation, normal T cell expressed and secreted (RANTES), monocyte chemoattractive protein (MCP-1), vascular endothelial growth factor (VEGF) and Platelet-Derived Growth Factor BB (PDGF-BB) levels were increased 1.3 – 4.3 fold in ZIKV positive pregnant woman AF, with neonatal microcephaly, relative to the control group (Table 2).

Discussion

Brazil is facing a health emergence situation since 2015 with the ZIKV outbreak. Furthermore, ZIKV infection has been associated with microcephaly and neurological disorders in adults (1-2). Our study measured cytokine concentrations in the intrauterine environment from nine ZIKV

infected pregnant women with neonatal microcephaly providing valuable information for future comparative assays.

Animal studies have shown that the activation of the maternal immune system by infections alters cytokine levels in the placenta, amniotic fluid and fetal brain (13-15). The inflammatory microenvironment caused by ZIKV infection could in part determine the differentiation, proliferation, migration and survival of neural progenitor cells (9). The cytokine storm that we observed in AF from ZIKV women could be associated with neuro-developmental defects. The downregulation of MCP-1 or IL-6 promotes the differentiation of human amniotic fluid derived-mesenchymal progenitor cells (MePR-2B) to a neuro-glial phenotype (6), and the increase in IL-6 (2.5 fold) and to lesser extent MCP-1 levels (1.3 fold), could be a critical key to the differentiation of derived-mesenchymal progenitor cells to mature neuro-glial cells in the fetus, contributing to microcephaly. Higher levels of IL-6, IL-8, MCP-1, IP-10, VEGF and decreased levels of IL-13 and IL-9 are associated with undifferentiated MePR-2B cells (6). We also found these cytokines elevated in ZIKV positive AF, with IP-10 at high level (19.65 fold). As reported before, human placental macrophages are permissive to ZIKV and secrete pro-inflammatory cytokine such as IL-6, MCP-1 and IP-10 (10).

Previous studies already showed that the excess of pro- or anti-inflammatory cytokines during an infectious response might be involved in disrupting normal fetal brain development (16,17). Our results showed a remarkable polyfunctional immune activation, increasing the levels of Th1 (IFN-γ and TNFα), Th2 (IL- 4, IL-5 and IL-10) and Th17 (IL-17) immune responses in ZIKV positive pregnant women. Furthermore, we found elevated levels of G-CSF in the AF of ZIKV positive pregnant women, and a previous study had suggested that this growth factor acts as an autocrine protective signaling mechanism in response to neural injury (11).

Several groups have demonstrated elevated AF cytokine levels, in particular IL-6 and IL-8 (18), as markers for intrauterine infection in the second trimester. However, other diseases such as pre-eclampsia, as well as preterm labor associated or not with bacterial or viral infections may trigger the production of inflammatory cytokines as IL-6, IL-8, IL-10, IL-15 and G-CSF (12, 18-20)

The inflammatory response we describe here in the ZIKV infected uterine environment should be further investigated, allowing additional studies of Th1- and Th2-biased cell responses, since pro-inflammatory cytokines could also damage neuron cells and interfere with fetal development. We have included in our analyses data from two liquid amniotic collections after the second trimester (Table 1, cases 5 and 8) where there was no change in the quantification of any cytokines. To our knowledge this study is the first to report midtrimester amniotic fluid data for twenty seven cytokines in ZIKV pregnant women.

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Author Contributions:

AMMO, SH, WDR, CCC, DFN, AT, ASM and RSA conceived and designed the study. AMMO, PP, PPS, FOM, TAF, PSOS, JIL, MMRA, SH, WDR, CCC, DFN performed the data acquisition and analysis. AMMO, CCC, DFN, AT, ASM and RSA wrote and edited the initial drafts. All authors reviewed the final draft here submitted.

Conflicts of Interest

Nothing to report

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Table 1: Demographic and clinical characteristics of women enrolled in this study.

Case	Maternal Age (yr)	GT of Zika acute symptoms	GT of amniotic fluid sampling	PCR Diagnosis amniocentesis or at birth in AF and PL or post mortem tissue (BR)	Newborn Outcome
1	23	(18 wk) rash, itching, arthralgia	20	POS (AF)	Deceased
2	34	(8 wk) rash, arthralgia, conjunctivitis	20	POS (AF)	Alive
3	29	(12 wk) rash, arthralgia, itching	24	POS (AF)	Alive
4	25	None	23	POS (AF)	Alive
5	18	(10 wk) rash	37	POS (AF)	Alive
6	19	(6 wk) rash, headache, asthenia	20	POS (AF)	Alive
7	31	(7 wk) rash, itching, feet edema	17	POS (AF)	Deceased
8	19	(10 wk) rash, asthenia, itch	35	POS (PL)	Alive
9	18	(12 wk) rash, itch, headache	17	POS (BR)	Deceased

GT, Gestational Time; WK, weeks; YR, years; AF, Amniotic Fluid; PL, Placenta; BR, brain.

Table 2: Pro-inflammatory cytokines, chemokines and growth factors levels in amniotic fluid from ZIKV positive pregnant woman with a diagnosis of fetal microcephaly

	Control (n= 23)	ZIKV (+)		
	Cytokine Level, pg/mL, average (IC 95%)	Cytokine Level, pg/mL, average (IC 95%)	Fold Change (sample/control)	*p value*
Inflammatory Cytokines				
IL-1ra	961.87 (622.01 – 1301.74)	554.08 (338.78 – 769.38)	0.58	NS
IL-6	191.35 (139.39 – 243.31)	484.42 (189.89 – 778.96)	2.53	$p < 0.05$
IL-2	4.26 (2.84 – 5.68)	-		
IL-5	2.20 (1.65 – 2.75)	3.75 (2.05 – 5.46)	1.71	NS
IL-10	1.17 (0.27 – 2.08)	3.18 (0.90 – 5.46)	2.71	$p < 0.01$
IL-12	1.95 (0.73 – 3.18)	-		
IL-13	1.82 (1.45 – 2.19)	0.65 (0.29 – 1.02)	0.36	$p < 0.01$
IL-17A	0.65 (0.01 -1.30)	10.41 (3.35 – 17,46)	15.94 16.01	$p < 0.0001$
IL-4	2.38 (1.87 – 2.89)	10.30 (6.03 – 14.57)	4.33	$p < 0.0001$
IL-1β	0.58 (0.30 – 0.85)	2.31 (0.82 – 3.79)	4.00	$p < 0.01$
IL-9	34.71 (28.85 – 40.56)	9.47 (0 – 20.37)	0.27	$p < 0.01$
IL-15	1.23 (0 – 2.47)	17.55 (9.22 – 25.88)	14.24	$p < 0.0001$
IFN-γ	19.58 (14.04 – 25.11)	97.46 (55.94 – 138.97)	4.98	$p < 0.0001$
TNF-α	6.21 (0 – 17.79)	21.08 (7.85 – 34.31)	3.39	$p < 0.0001$
Chemokines				
IL-8	147.45 (111.62 – 183.28)	267.80 (167.81 – 367.80)	1.82	$p < 0.05$
MIP-1α	2.17 (1.15 – 3.20)	4.95 (0 – 9.89)	2.27	NS
RANTES	8.57 (4.43 – 12.71)	28.59 (0 – 66,20)	3.34	NS
Eotaxin	7.39 (4.42 – 10.36)	64.80 (43.28 – 86.31)	8.77	$p < 0.0001$
IP-10	891.38 (690.25 – 1092.51)	17512.09 (4621.31 – 30402.87)	19.65	$p < 0.0001$
MCP-1	206.73 (186.88 – 235.30)	271.67 (224.64 – 429.96)	1,31	$p < 0.05$
MIP-1β	14.91 (12.23 – 17.58)	44.60 (19.04 – 70.15)	2.99	$p < 0.0001$
Adhesion Molecules and Growth Factors				
G-CSF	33.81 (24.71 – 42.90)	169.65 (126.06 – 213.25)	5.02	$p < 0.0001$
IL-7	3.40 (2.06 – 4.14)	20.13 (13.02 – 27.23)	5.92	$p < 0.0001$
FGF Basic	27.73 (22.70 – 32.76)	10.86 (10.26 – 11.45)	0,39	NS
VEGF	6.25 (2.78 – 9.71)	21.87 (0 – 46.47)	3.50	NS
PDGF-BB	47.18 (37.83 – 56.53)	78.89 (33.62 – 124.15)	1.67	NS
GM-CSF	2.70 (0 – 6.41)	1.32 (0 – 2.81)	0.49	$p < 0.05$

- Indeterminate

Abbreviations: FGF, fibroblast growth factor; G-CSF, granulocyte colony-stimulating factor; IFN- γ, interferon γ; IL, interleukin; TNF-α, tumor necrosis factor α; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractive protein; IP, IFN-gamma-inducible protein ; VEGF, vascular endothelial growth factor; regulated on activation, normal T cell expressed and secreted (RANTES). Fold Change value is relative to cytokine level average of healthy donors AF. Mann-Whitney U test was used to evaluate significant differences between cytokine concentration from healthy (normal) and Zika positive amniotic fluid (ZIKV +). NS – not significant *p*-value.