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De Rissio, Susana A. Laucella and Jacqueline Bua

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Serum Cytokines as Biomarkers of Early *Trypanosoma cruzi* infection by Congenital Exposure

Bibiana J. Volta, Patricia L. Bustos, Rita L. Cardoni,¹ Ana M. De Rissio, Susana A. Laucella, and Jacqueline Bua

Trypanosoma cruzi, the causing agent of Chagas disease, leads to an activation of the immune system in congenitally infected infants. In this study, we measured a set of cytokines/chemokines and the levels of parasitemia by quantitative PCR in the circulation of neonates born to *T. cruzi*-infected mothers to evaluate the predictive value of these mediators as biomarkers of congenital transmission. We conducted a retrospective cohort study of 35 infants with congenital *T. cruzi* infection, of which 15 and 10 infants had been diagnosed by detection of parasites by microscopy in the first and sixth month after delivery, respectively, and the remaining 10 had been diagnosed by the presence of *T. cruzi*-specific Abs at 10–12 mo old. Uninfected infants born to either *T. cruzi*-infected or uninfected mothers were also evaluated as controls. The plasma levels of IL-17A, MCP-1, and monokine induced by IFN- γ were increased in infants congenitally infected with *T. cruzi*, even before they developed detectable parasitemia or seroconversion. Infants diagnosed between 6 and 12 mo old also showed increased levels of IL-6 and IL-17F at 1 mo of age. Conversely, infants who did not develop congenital *T. cruzi* infection had higher levels of IFN- γ than infected infants born to uninfected mothers. Monokine induced by IFN- γ , MCP-1, and IFN- γ production induced in *T. cruzi*-infected infants correlated with parasitemia, whereas the plasma levels of IL-17A, IL-17F, and IL-6 were less parasite load dependent. These findings support the existence of a distinct profile of cytokines and chemokines in the circulation of infants born to *T. cruzi*-infected mothers, which might predict congenital infection. *The Journal of Immunology*, 2016, 196: 4596–4602.

Chagas disease, caused by the protozoa *Trypanosoma cruzi*, is a major cause of cardiac failure in Latin America, where an estimated 6–7 million people are currently infected (1). In nonendemic areas, congenital infection has become the most important route of transmission (2, 3). Infected women in reproductive age can transmit the parasite to their offspring during successive pregnancies, making this transmission

route an important health problem that can easily be extended in time and space, through migrations (4, 5).

In Argentina, *T. cruzi* is vertically transmitted in ~7–11% of pregnancies in seropositive women (6). The diagnosis of infected children, according to the Chagas National Program, relies on the visualization of the parasite by microscopy, when passively transferred maternal *T. cruzi* Abs can still be present. If parasites cannot be detected, children have to be followed up and return for a second parasitological control at around 6 mo of age. In the face of new negative findings in parasite detection, a third control visit is pursued around 10 mo after delivery for detection of parasite-specific Abs through serological assays, which will confirm the positive or negative diagnosis for *T. cruzi* infection. Nowadays, this is changing due to the development of new tools that allow the confirmation of *T. cruzi* infection (7–10).

There is increasing evidence that *T. cruzi* infection in pregnant women can induce the activation of T lymphocytes of the fetus in utero (11–13). Although the production of proinflammatory cytokines like IL-1 β , IL-6, and TNF- α in response to *T. cruzi* Ags and the spontaneous release of TNF- α in uninfected infants born to *T. cruzi*-infected mothers (B⁻M⁺) have been described, the levels of these cytokines in congenitally infected newborns are rather low (11, 13–16), suggesting that the immune response might be an important factor in vertical transmission (17).

By using a quantitative PCR (qPCR), we have previously demonstrated that the parasitemia of congenitally infected infants is highly variable at birth (7, 18), and this fact posed the question whether *T. cruzi*-infected children also exhibit variable levels of chemokines and cytokines that might correlate with parasite burden and predict congenital infection. In this context, our study aimed to evaluate the chemokine and cytokine profiles in groups of congenitally infected and uninfected infants born to *T. cruzi*-

Instituto Nacional de Parasitología Dr. M. Fatalla Chaben, Administración Nacional de Laboratorios e Institutos de Salud Dr. C.G. Malbrán, Buenos Aires 1063, Argentina

¹R.L.C. is deceased.

ORCID: 0000-0003-3246-8161 (B.J.V.); 0000-0001-8373-4031 (J.B.).

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R.L.C., A.M.D.R., and J.B. conceived and designed the experiments; B.J.V. performed the experiments; B.J.V., P.L.B., S.A.L., and J.B. analyzed data; A.M.D.R. and R.L.C. contributed with materials; B.J.V., P.L.B., S.A.L., and J.B. wrote the manuscript; and B.J.V., P.L.B., A.M.D.R., S.A.L., and J.B. approved manuscript contents.

Address correspondence and reprint requests to Dr. Jacqueline Bua, Instituto Nacional de Parasitología, Paseo Colón 568, Buenos Aires 1063, Argentina. E-mail address: jacobua@yahoo.com

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Abbreviations used in this article: B⁺C1, newborns diagnosed for *T. cruzi* infection in the first control visit; B⁺C2, newborns diagnosed for *T. cruzi* infection in the second control visit; B⁺C3, newborns diagnosed for *T. cruzi* infection in the third control visit; B⁻M⁺, uninfected infants born to *T. cruzi*-infected mothers; B⁻M⁻, uninfected infants born to *T. cruzi*-uninfected mothers; MCP-1, monocyte chemoattractant protein-1; MIG, monokine induced by IFN- γ ; Pe, parasite equivalent; qPCR, quantitative PCR; RANTES, CCL5 chemokine.

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seropositive mothers and with different parasite loads during a 1-y follow-up study.

Materials and Methods

Study location and participants

This work was conducted at the Instituto Nacional de Parasitología Dr. Mario Fatała Chaben in Buenos Aires, an area that is not endemic for *T. cruzi* infection. Infants born to seropositive mothers were diagnosed according to the Argentine Chagas National Program, as mentioned earlier. Thirty-five infants, who completed three control visits during 1 y after delivery (when live parasites were not detected) or until the time of parasite detection, were recruited from 2008 to 2011 and included in this study. Congenital infection was inferred because these infants were residents in areas nonendemic for *T. cruzi* infection, had not traveled to endemic areas, and had not received any blood transfusion. Treatment-naive seropositive infants in the asymptomatic phase of Chagas disease, with no concomitant infections, were enrolled in this study. *T. cruzi*-infected infants were categorized into three groups based on the time of diagnosis: 15 newborns who had been diagnosed for *T. cruzi* infection by microscopic observation of bloodstream parasites during the first control visit within the first month after delivery (B⁺C1) (i.e., only one blood sample from each infant); 10 babies who had been diagnosed by microscopic observation of bloodstream parasites during the second control visit at 6 months of age (B⁺C2) (i.e., two blood samples from each infant), and 10 babies who had been diagnosed during the third control visit between 10 and 12 mo of age (B⁺C3) by specific serology (i.e., three blood samples from each infant). Two control groups were randomly selected among the pediatric population attending our health center: 10 uninfected infants born to *T. cruzi*-uninfected mothers (B⁻M⁻) and 10 B⁻M⁺, with 3 samples obtained from each child during the first, second, and third control visits, respectively. The infants included in this study did not significantly differ with respect to gestational age and weight at birth (Table I) ($p < 0.05$, ANOVA). Blood from babies (0.5 ml) was drawn by venipuncture into tubes containing guanidine hydrochloride 6M, EDTA 0.2 M, pH 8 (GE), and kept at room temperature for 1 wk and then at 4°C until use for qPCR analysis. Another 1-ml blood sample was allowed to coagulate at 37°C, centrifuged for serum separation, and used for three *T. cruzi* serological assays: ELISA, indirect hemagglutination, and indirect immunofluorescence as previously described (7).

An additional 1 ml blood was centrifuged at 1000 × *g* for 15 min for plasma separation, and samples were used for cytokine/chemokine analysis. By the time of blood sampling, all infants had been vaccinated according to standard guidelines (World Health Organization) for their respective age.

Quantitative *T. cruzi* DNA amplification

An internal standard of DNA extraction (2 ng) was included in each blood-GE sample and DNA was isolated with a commercial kit, as previously described (8). Parasites were quantified amplifying a *T. cruzi* satellite sequence flanked by the Sat Fw and Sat Rv oligonucleotides (9). Several points of the parasite standard curve, two positive samples, two negative samples, and nontemplate DNA were included in every qPCR run. The cutoff value of this method is 0.14 parasite equivalents (Pe)/ml (8).

Cytometric bead array

Levels of IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p70, IL-13, IL-17A, IL-17F, IFN-γ, TNF-α, the CCL5 chemokine (RANTES), monokine induced by IFN-γ (MIG/CXCL9), monocyte chemoattractant protein-1 (MCP-1)/CCL2, and MIP-1β/CCL4 were measured simultaneously in plasma samples using cytometric bead array (BD Biosciences) according to the manufacturer's protocol. Data were acquired

on a FACSAria flow cytometer (BD Biosciences) and analyzed with FCAP Array software (BD Biosciences).

Statistical analysis

Data normality was evaluated by Shapiro–Wilk test. Cytokine levels were presented as the medians with interquartile ranges. Median values between groups were compared by Kruskal–Wallis test, followed by Mann–Whitney posttest to compare pairs. Other data with normal distribution were presented as mean and SD, and differences between groups were examined using the *t* test. Correlations were evaluated using the Spearman's rank test. Statistical analysis was performed using GraphPad Prism 5.0 software (GraphPad Software). A *p* value < 0.05 was considered statistically significant. Three cytokine networks were created after performing the correlation analysis between cytokines of each group, whereas a fourth network was created among cytokine concentrations and parasitemia levels, using the software Cytoscape 2.8.3 (<http://www.cytoscape.org/download.php>).

Ethics statement

The study was approved by the Ethics Committee of Administración Nacional de Laboratorios e Institutos de Salud “Carlos G. Malbrán” and carried out according to the Declaration of Helsinki. Written informed consent was obtained from mothers allowing the enrollment of their infants.

Results

Cytokine and chemokine profile in infants born to

T. cruzi-infected mothers according to the age of diagnosis for *T. cruzi* infection

Circulating levels of Th1, Th2, Th17, regulatory T cells proinflammatory and hematopoietic cytokines, and chemokines were measured in neonates born to *T. cruzi*-infected mothers, who were followed up for 1 y after delivery or until the time of confirmed diagnosis for *T. cruzi* infection, determined either by the presence of bloodstream parasites (between 1 and 6 mo of follow-up) or *T. cruzi*-specific IgG Abs at 10–12 mo of age. B⁻M⁻ were also evaluated to measure the normal concentrations of these molecules. The different parameters were evaluated prospectively, and once positive or negative diagnosis for *T. cruzi* infection was confirmed, subjects were analyzed retrospectively according to the time of diagnosis during follow-up, as indicated in *Materials and Methods* (Table I).

The plasma levels of IL-17A, MCP-1, and MIG were increased in infants congenitally infected with *T. cruzi* (B⁺C1, first column of Fig. 1B–D, respectively, or B⁺C2, second column of Fig. 1B–D, respectively), even before they developed detectable parasitemia (B⁺C2, first column of Fig. 1B–D, respectively) or seroconvert (B⁺C3 in first and second column of Fig. 1B–D, respectively) in comparison with B⁻M⁺ or B⁻M⁻ (Fig. 1B–D). However, the kinetics of the plasma levels of IL-17A and MIG was different between infants diagnosed at 1 mo after delivery (B⁺C1) and those diagnosed between 6 and 12 mo of age (B⁺C2, B⁺C3), because the latter had higher levels of IL-17A but lower levels of MIG than children diagnosed in the first month of life (B⁺C1) (Fig. 1B–D). Conversely, infants who did not develop congenital *T. cruzi* infection (B⁻M⁺) had higher levels of IFN-γ than B⁻M⁻, whereas

Table I. Characteristics of the study population

	<i>N</i>	Males/Females, <i>n</i>	Gestational Age (wk) ^a	Birth Weight (g) ^a
B ⁻ M ⁻	10	6/4	39.6 ± 0.5	3227 ± 105
B ⁻ M ⁺	10	5/5	39.7 ± 0.5	3357 ± 205
B ⁺ 1C	15	8/7	38.9 ± 0.7	3136 ± 168
B ⁺ 2C	10	5/5	39.1 ± 0.5	3215 ± 312
B ⁺ 3C	10	4/6	39.6 ± 0.5	3289 ± 355

^aArithmetic mean ± SEM values are shown.

congenitally infected infants had normal values of IFN- γ levels (Fig. 1A). Infants diagnosed between 6 and 12 mo after delivery showed higher levels of IL-6 at 1 mo of age than uninfected (B^-M^- , B^-M^+) and infected infants diagnosed in the first control visit (B^+C1). These levels were sustained by 6 mo of follow-up and decreased thereafter (Fig. 2A). Likewise, IL-17F was also increased in infants diagnosed between 6 and 12 mo after follow-up, but the levels were sustained until the end of the study (Fig. 2B). No alterations were observed in the levels of the remaining cytokines/chemokines evaluated in the first, second, and third control visits

when compared with uninfected children (Supplementary Tables I–III, respectively).

The correlation analysis performed among cytokines/chemokines with altered plasma levels in *T. cruzi*-infected or uninfected children born to *T. cruzi*-infected mothers showed a moderate positive correlation of IL-6 with the levels of IL-17, and a weak inverse correlation of MIG with IL-6 and IL-17A, only in *T. cruzi* congenitally infected infants (Fig. 3C, Supplemental Table IV). Notably, the moderate correlation between IFN- γ and MCP-1 observed in healthy children born to uninfected mothers

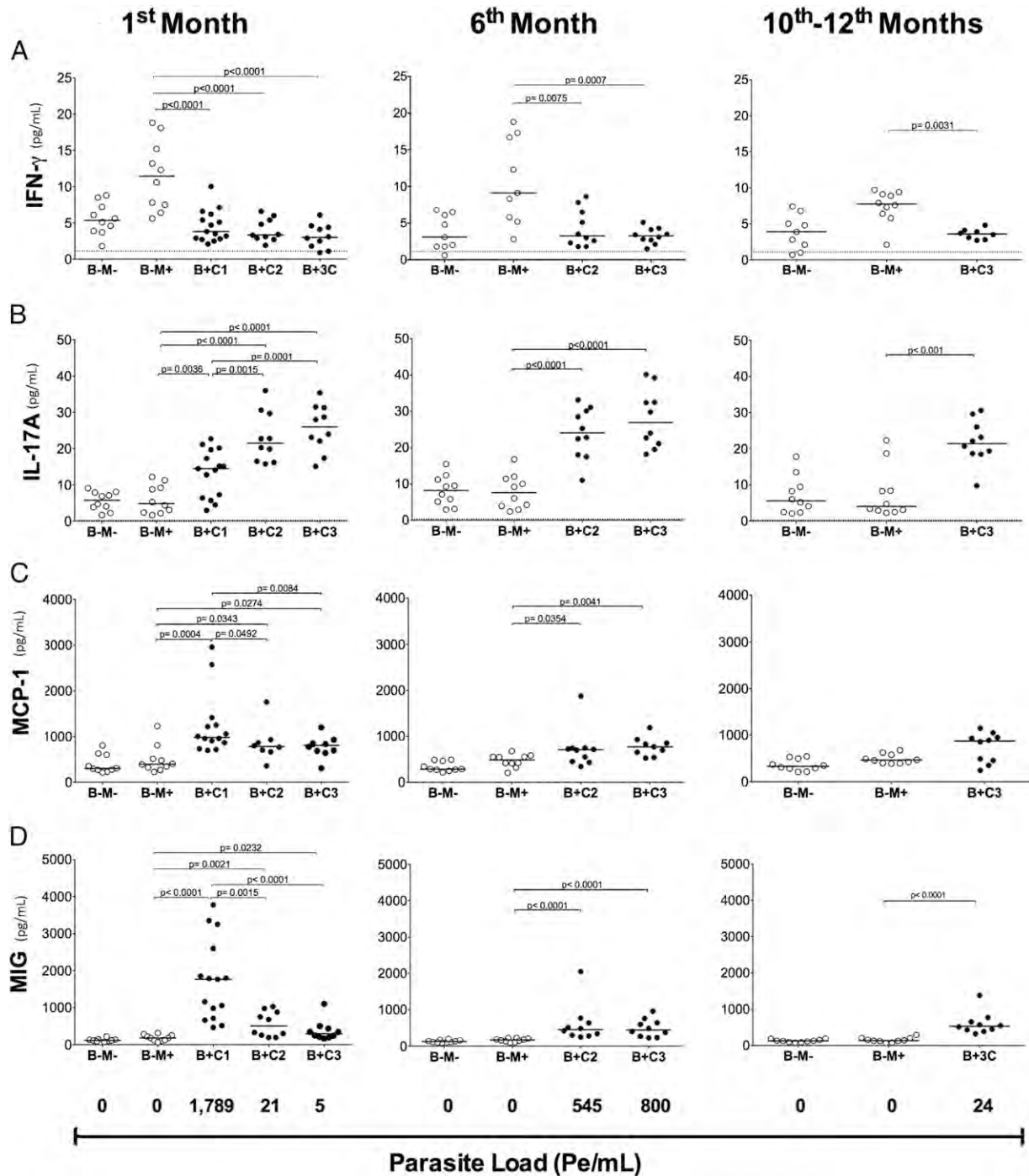


FIGURE 1. Plasma levels of IFN- γ (A), IL-17A (B), MCP-1 (C), and MIG (D) measured by cytometric bead array and expressed as pg/ml in B^-M^- , B^-M^+ , and infected infants diagnosed in their first (B^+C1), second (B^+C2), and third control visit (B^+C3) at 1 mo, 6 mo, and around 1 y of age, respectively. Horizontal lines represent median values for each group. Differences between groups were tested using Kruskal–Wallis, followed by Mann–Whitney *U* test for post hoc comparisons ($p < 0.05$), respectively. Median parasitemia values evaluated for each group in the different control visits are indicated as Pe/ml.

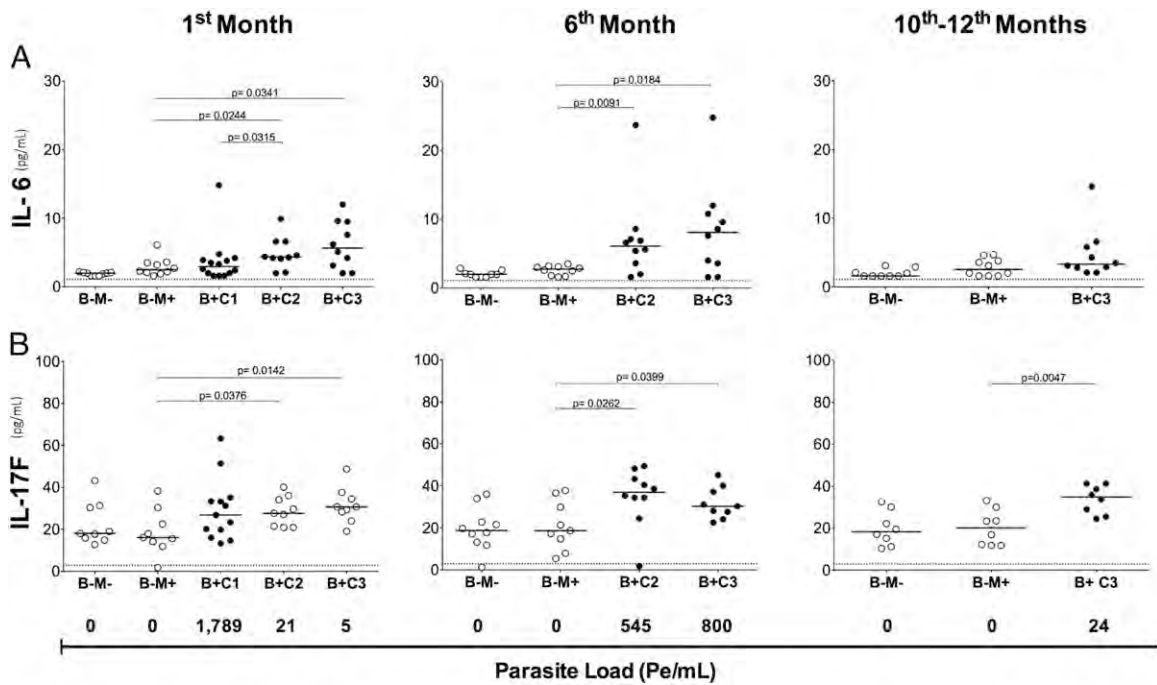


FIGURE 2. Plasma levels of IL-6 (**A**) and IL-17A (**B**) measured by cytometric bead array in $B^{-}M^{-}$, $B^{-}M^{+}$, and infected infants diagnosed in their first ($B^{+}C1$), second ($B^{+}C2$), and third control visits ($B^{+}C3$) at 1 mo, 6 mo, and around 1 y of age, respectively. Horizontal lines represent median values for each group. Differences between groups were tested using Kruskal–Wallis, followed by Mann–Whitney U test for post hoc comparisons ($p < 0.05$). Median parasitemia values evaluated for each group in the different control visits are indicated as Pe/ml.

(Fig. 3A) is lost in children congenitally exposed to *T. cruzi* (Fig. 3B, 3C).

Altogether, these findings support that the measurement of a defined set of circulating immune mediators allows prediction of *T. cruzi* infection early after birth, when maternal Abs are still present and parasitemia is not detectable by microscopy.

Parasite load and plasma cytokine/chemokine secretion in *T. cruzi* congenitally infected children

We have previously reported that *T. cruzi*-infected newborns diagnosed by blood microscopic parasite observation have a very high parasite load by qPCR early after birth and that babies with undetectable parasitemia and diagnosed thereafter, by either the presence of circulating parasites or the development of IgG Abs specific for *T. cruzi* after 10 mo of age, exhibit low but positive parasite load by qPCR (7) (Fig. 1). In this study, we evaluated the correlation between parasite load, as measured by qPCR, and the levels of cytokines/chemokines that were found altered in *T. cruzi*-infected children. This analysis showed that the plasma levels of MIG strongly correlated with parasite burden, whereas MCP-1 and IFN- γ levels exhibited a moderate correlation with parasitemia (Fig. 3D, Supplemental Table IV). In addition, parasitemia levels and serology data of the *T. cruzi*-infected infants included in the study are shown in Supplemental Table V.

T. cruzi infection resulted in high levels of circulating IL-6, IL-17F, and IL-17A. However, at 1 mo of age, the amount of these cytokines was lower in infected neonates with high parasitemia, but higher in infected neonates with low parasitemia diagnosed at 6–10 mo of age (Figs. 1, 2). This feature also can be visualized in Fig. 3D and Fig. 4, in which IL-6 and IL-17A display weak negative correlations with parasite load. In summary, MIG, MCP-1, and IFN- γ production induced in *T. cruzi*-infected infants correlates with parasitemia, contrasting with the less parasite load-dependent production of IL-17A, IL-17F, and IL-6.

Discussion

The bulk of the evidence indicates that the induction of appropriate immune responses in *T. cruzi*-infected pregnant women, fetuses, and newborns might be an important factor to control parasite burden and vertical transmission (10, 12, 17). High parasitemia and reduced innate and parasite-specific Th1 immune responses in *T. cruzi*-infected pregnant women have been associated with a high rate of vertical transmission (10, 17). Likewise, reduced *T. cruzi*-specific CD8 $^{+}$ T cells secreting IFN- γ have been detected in newborns with high parasitemia (12), whereas a strong inflammatory response is developed in uninfected neonates born to *T. cruzi*-infected mothers (11, 14). In this study, we measured a set of cytokines/chemokines in the circulation of a cohort of neonates born to *T. cruzi*-infected mothers to evaluate the predictive value of these immune mediators as biomarkers of congenital transmission.

Increased plasma levels of IL-17A, MCP-1, and MIG, but reduced IFN- γ production were the most predictive molecules of congenital infection because increased values were observed in the first month of life in infants for whom the diagnosis of *T. cruzi* infection was confirmed between 6 and 12 mo later, either through the detection of live parasite or by the induction of *T. cruzi*-specific IgG Abs, respectively.

Consistent with previous studies, congenital *T. cruzi* infection was associated with a decreased production of IFN- γ , which is crucial for the control of the parasite through the production of NO (16). In our study, IFN- γ was weakly correlated with parasite load as measured by qPCR, supporting that only a large production of this cytokine is able to eliminate the parasite, whereas lower concentration might only keep the parasite under control. Morbidity and mortality of congenital disease have also been associated with very high parasitemia (17), reinforcing the role of the immune system in limiting disease severity.

In early stages of the infection, *T. cruzi* can activate macrophages through the interaction of TLRs, thus eliciting innate im-

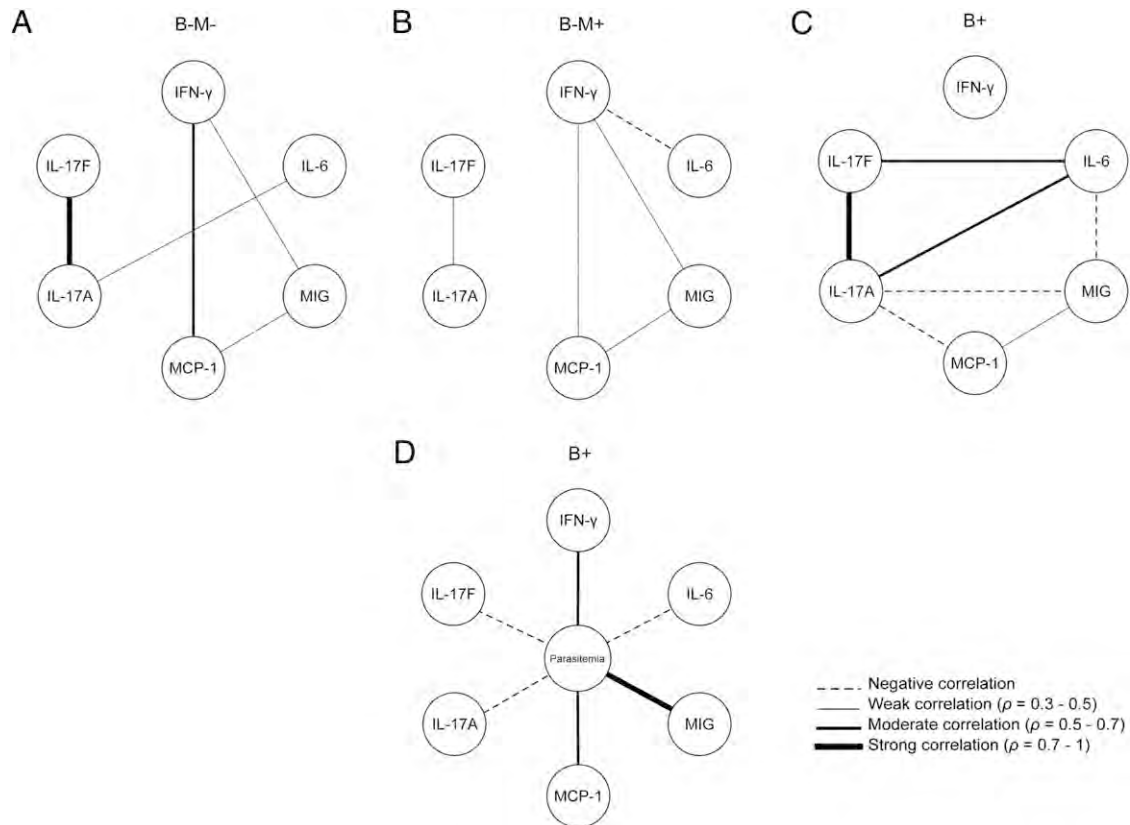


FIGURE 3. Interactions between plasma cytokines/chemokines in $B^{-}M^{-}$ (A), $B^{-}M^{+}$ (B), and infected infants (C). Interaction between parasitemia and plasma cytokines/chemokines (D). Each connecting line represents a significant correlation between a pair of cytokines/chemokines, and its thickness represents the strength. Dashed lines represent negative correlations. Solid lines represent positive correlations. Spearman ρ indexes, with p values <0.05 , were used to classify the connecting edges as negative, moderate, or strong positive correlation, as shown in the figure.

immune responses with the secretion of cytokines and chemokines that promote intracellular death (19). In this study, MIG, a potent chemoattractant for Th1 cells (20) secreted by activated monocytes and macrophages, was increased in congenitally infected infants and positively associated with the risk for vertical transmission. Previous studies have shown that MIG has a parasiticidal effect on *Leishmania mexicana* promastigotes in vitro and that it induces small lesions in the plasma membrane of the parasite that can eventually lead it to death, but also that it is unable to generate immediate lysis (21). Previous studies have also shown increased MIG levels in the circulation of infants infected with malaria (22, 23) and visceral leishmaniasis (24), supporting an important role for this chemokine in vivo. In addition, the levels of MCP-1, a monocyte chemotactic factor produced by macrophages and endothelial cells (25), were also high in the circulation of *T. cruzi*-infected infants. In mice, *T. cruzi* infection induces the production of NO and MCP-1 from peritoneal macrophages (26), whereas in patients infected with *Leishmania infantum*, MCP-1 has been pointed out to be involved in macrophage activation (27). Therefore, MCP-1 might contribute to parasite clearance, either by its monocyte chemoattractant properties or by direct stimulation of monocyte trypanocidal activity.

Infants diagnosed for *T. cruzi* infection later after birth (i.e., 6–12 mo of age) had not only lower IFN- γ levels but also higher IL-17A and IL-6 levels throughout the follow-up period than children diagnosed at 1 mo of age. We speculate that, as reported in experimental models of *T. cruzi* infection, IL-17 might have a protective role recruiting and activating neutrophils and monocytes required for early control of the pathogen (28, 29). Other studies

have shown that IL-17 is also crucial in the control of cardiac inflammation and host survival, playing a negative feedback role in the production of IFN- γ and chemokines during *T. cruzi* infection in humans and mice (30). Thus, the increased production of IL-17A in neonates with low parasitemia could be modulating the inflammatory environment created by their mothers to control the increase in parasite burden. However, the increase in parasite load in children diagnosed between 6 and 12 mo of age indicates that, without the presence of high levels of IFN- γ , parasite replication cannot be maintained under control over time. Whether IL-17 derives from NK, Th17, or both remains to be further investigated.

The expression of IL-6 and IL-17A is high at birth and begins to decline over the first years of life along with an increased expression of the proinflammatory cytokines IL-1 β and TNF- α in whole blood, monocytes, and dendritic cells (31–34). Our findings reveal a similar polarization in our cohort, with greater production of IL-6 and IL-17A than of IL-1 β and TNF- α . Noteworthy, we observed that IL-17F and IL-17A displayed interactions with IL-6 only in *T. cruzi*-infected infants, and that IL-17A displayed negative associations with MIG and MCP-1. These findings demonstrate the induction of a distinct immune profile in congenitally infected infants. The positive correlation of MIG, IFN- γ , and MCP-1 and the negative correlation of IL-6, IL-17A, and IL-17F with parasitemia support that altered levels of these immune mediators are due to an active infection with *T. cruzi*.

The great majority of the infants studied to date with confirmed diagnosis for *T. cruzi* infection showed detectable qPCR, regardless of the time of diagnosis. Moreover, qPCR is

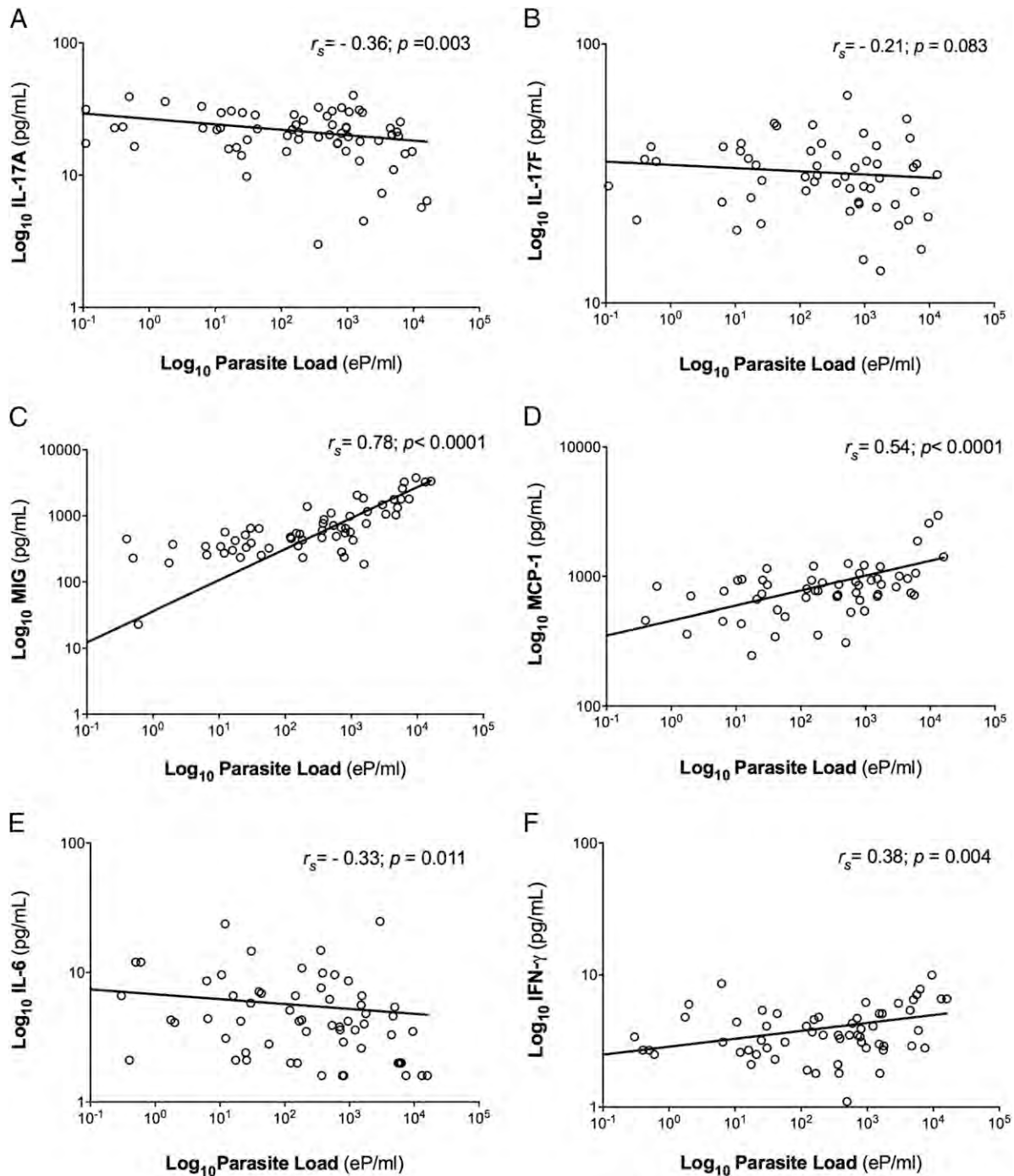


FIGURE 4. Correlations between parasitemia and cytokines/chemokines levels in *T. cruzi*-infected infants. The plasma levels of IL-17A (A), IL-17F (B), MIG (C), MCP-1 (D), IL-6 (E), and IFN- γ (F) were measured by cytometric bead array and expressed as log₁₀ (pg/ml). Parasitemia values are shown as log₁₀ Pe/ml. Spearman ρ indexes and p values are expressed in each panel.

significantly high when live parasites are detected by microscopy, supporting that it might be an early indicator of congenital infection. Our results further sustain the identification of potential markers for an early prediction of congenital infection. In this study, we showed that increased levels of IL-17A, MCP-1, MIG, IL-6, and IL-17F in the presence of detectable qPCR were associated with the development of congenital infection.

In summary, our results show that infants with congenital *T. cruzi* infection mount a vigorous innate immune response with a polarization toward a Th17 profile. Decreased levels of IFN- γ but increased levels of IL-17A, MIG, and MCP-1 are revealed as early predictors of *T. cruzi* infection in the presence of either high or

low parasitemia, whereas *T. cruzi*-infected infants also display increased levels of IL-6 and IL-17F, but only in the presence of low parasitemia.

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Disclosures

The authors have no financial conflicts of interest.

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