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Circulating Biomarkers of Pulmonary and Extrapulmonary Tuberculosis in Children

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Tuberculosis (TB) in children is not only more likely to cause more severe disease than that seen in adults, it is also more likely to be extrapulmonary. Moreover, pediatric TB is very difficult to diagnose and suffers from a lack of understanding of host biomarkers for monitoring the progression of disease. Hence, we sought to identify the expression patterns of a variety of biomarkers in the plasma of children with pulmonary TB (PTB) and extrapulmonary TB (ETB), as well as in healthy control (HC) children. Thus, we examined a variety of circulating markers reflecting tissue inflammation, oxidative stress, innate immune activation, fibrosis, and the cytokine response. Children with active TB, compared to HC children, showed markedly elevated plasma levels of matrix metalloproteinases and their endogenous inhibitors. In addition, children with active TB had significantly elevated levels of C-reactive protein, α-2 macroglobulin, and haptoglobin, as well as hemoxygenase 1. Markers of innate immune activation (lipopolysaccharide [LPS] and lipopolysaccharide-binding protein [LBP]) were significantly lower in ETB than in PTB children. Although there were no significant differences between the two groups in their levels of cytokines (type 1 [gamma interferon (IFN-γ), tumor necrosis factor α (TNF-α), interleukin 2 (IL-2), and IL-12], type 2 [IL-4, IL-5, IL-13, and IL-33], and most type 17 [IL-17A, IL-22, IL-1β, and IL-6] and type 1 interferons [IFN-α and IFN-β]) or most of the cytokines associated with immune modulation (IL-10 and IL-20), pediatric TB was associated with elevated plasma transforming growth factor β (TGF-β), IL-21, and IL-23 levels. Thus, pediatric TB is characterized by elevated levels of a variety of biomarkers at homeostasis, suggesting that these responses may play a crucial role in disease pathogenesis.

Mycobacterium tuberculosis is one of the leading killers among infectious pathogens, with an estimated 8.9 million new infections and 1.7 million deaths per year. Although *M. tuberculosis* infects ~2 billion people worldwide, 90% of *M. tuberculosis*-infected individuals are able to resist overt disease development and manifest only latent infection; however, children belong to the category of relatively susceptible individuals, and young children are highly likely to develop active disease (active tuberculosis [TB]) after infection (1). Indeed, the risk of developing active TB following exposure ranges from 20 to 40% in children below 5 years of age (2). The mechanisms by which children become more susceptible to developing active pulmonary disease as well as extrapulmonary disease following exposure are an active area of research. Young age (2, 3) and HIV coinfection (4, 5) are the most important risk factors for severe or disseminated disease. In those children with extrapulmonary manifestations of TB, lymphadenopathy (TB lymphadenitis) and central nervous system (CNS) involvement (neuro-TB) are the most common (6).

The global burden of childhood tuberculosis is unknown because of the lack of recording and reporting of such cases in children by national programs. It is generally reported that at least 10 to 15% of cases in the world and up to 25% of the cases arising in high-burden countries occur in children (1). Thus, childhood TB represents a significant but still neglected clinical and public health problem (7). Microbiological culture, which is the gold standard for diagnosing TB in adults, often fails in children due to the paucibacillary nature of the infection and the difficulty of obtaining sputum from children (8). Recent studies have shown that nucleic acid amplification-based tests, such as the Xpert MTB/RIF assay, are also hampered by the same limitation (9, 10). Because of the difficulty in confirming the diagnosis of childhood TB microbiologically, immune-based assays have always been considered important in diagnostics. However, very few validated assays exist for diagnosing childhood TB. The commercially available blood-based gamma interferon (IFN-γ) release assays are primarily intended to detect latent TB and do not have a role in active TB detection (11). Finally, since forms of extrapulmonary TB in children present with a variety of clinical features depending on the site of involvement, diagnosis might require examination of non-respiratory samples or other investigations (12). The current challenge therefore is to understand the molecular nature of the complex network of interactions between the immune system and the bacteria.

The commonly used biomarkers in adult tuberculosis are markers of pulmonary damage, acute-phase proteins, markers of innate immune activation, markers of fibrosis, and cytokines. To study the associations of these markers with either susceptibility or resistance to TB infection and to extrapulmonary dissemination, we examined the plasma levels of these biomarkers at homeostasis in children with pulmonary TB (PTB), children with extrapulmonary TB (ETB), and healthy control (HC) children.

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TABLE 1 Demographics of the study population

Demographic characteristic	Study participants with: Pulmonary TB ($n = 14$)	Extrapulmonary TB ($n = 22$)	Controls ($n = 19$)
Median (range) age (yrs)	9 (1–15)	7 (2–15)	9 (2–15)
Gender (no. M/no. F)	6/8	13/9	13/6
BCG status (no. +/no. −)	11/3	11/11	5/14
Results of QuantiFERON assay (no. +/no. −)	6/8	7/15	0/0

We showed that matrix metalloproteinases (MMPs)/tissue inhibitors of MMPs (TIMPs), hemoxygenase 1, acute-phase proteins, and certain cytokines (transforming growth factor β [TGF-β], interleukin 21 [IL-21], and IL-23) are the main biomarkers distinguishing children with active pulmonary TB from HC children and that children with extrapulmonary TB only were characterized by decreased levels of the innate immune activation markers (lipopolysaccharide [LPS] and lipopolysaccharide-binding protein [LBP]). Our findings have implications for the understanding of susceptibility to TB disease as well as the development of immune-mediated pathology in different forms of TB and provide the preliminary framework for an in-depth analysis of these biomarkers.

MATERIALS AND METHODS

Study population. We studied a group of 36 children with TB—14 with PTB and 22 with ETB—as well as 19 HC children (Table 1). All of the children were recruited from the Childs Trust Hospital, Chennai. The diagnosis of active tuberculosis was made on the basis of sputum microscopy and culture, and TB-infected children were studied before the commencement of treatment. All PTB children had positive results for sputum smears and cultures. The diagnosis of ETB was made on the basis of clinical symptoms, physical examination, and radiological and other criteria, e.g., cerebrospinal fluid analysis for TB meningitis, fine-needle aspiration for TB lymphadenitis, etc. The healthy control children were children attending the hospital for routine vaccinations. Blood was collected and routine diagnostic and hematological procedures were performed. QuantiFERON TB Gold-in-Tube enzyme-linked immunosorbent assay (ELISA) (Cellestis) was performed (according to the manufacturer's instructions) to exclude the presence of latent infection in the HC children (13, 14). The QuantiFERON assay was positive in 6 out of 14 PTB and 7 out of 22 ETB children, confirming previous findings that the QuantiFERON assay is not an interpretable assay in active TB (15). All the children were HIV negative. The demographics of the children in the study are shown in Table 1. Children with ETB ($n = 22$) comprised those with TB meningitis and spinal TB ($n = 14$), abdominal TB (including peritonitis or tuberculomas [$n = 2$]), or TB lymphadenitis ($n = 6$). All the HC children were negative by QuantiFERON ELISA. A clinical protocol approved by the Institutional Review Board of the National Institute for Research in Tuberculosis was used to examine all children, and informed written consent was obtained from the parents of all participants.

Immunoassays. All assays were performed on EDTA plasma. Plasma levels of MMPs (1, 7, 8, and 9) and TIMPs (1, 2, 3, and 4) (R&D Systems) were measured on a multiplex ELISA platform using Luminex technology, according to the manufacturer's instructions. Plasma levels of C-reactive protein (CRP), haptoglobin, serum amyloid A (SAA), and α-2 macroglobulin (α-2m) (Bio-Rad) were measured using a Bio-Plex multiplex ELISA system according to the manufacturer's instructions. Hemoxygenase 1 (HO-1) (Assay Designs) in plasma was measured by ELISA. Lipopolysaccharide (LPS) levels were measured using a *Limulus* amebocyte lysate assay (Cell Sciences Hycult Biotech) according to the manufacturer's protocol. Commercially available ELISA kits were used to measure plasma levels of lipid-binding protein (LBP), IgG endotoxin core antibodies (EndoCAb) (Cell Sciences Hycult Biotech), and soluble CD14 (sCD14) (R&D Systems). Plasma levels of vascular endothelial growth factor (VEGF), fibroblast growth factor 2 (FGF-2), granulocyte colony-stimulating factor (G-CSF), and platelet-derived growth factor AA (PDGF-AA) were measured using the Milliplex map kit system (Merck Millipore). Plasma levels of cytokines were measured using a Bio-Plex multiplex cytokine assay system. The cytokines analyzed were IL-2, IFN-γ, tumor necrosis factor α (TNF-α), IL-12p70, granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-4, IL-5, IL-6, IL-10, IL-13, IL-17A, and IL-1β. TGF-β, IL-20, IL-21, IL-22, and IL-23 were measured by ELISA using a kit from R&D Systems. IFN-α (multiple subtypes) and IFN-β were measured using the VeriKine serum ELISA kit from PBL Interferon Source.

Statistical analysis. Geometric means (GMs) were used as measures of central tendency. Comparisons between groups were done using the Kruskal-Wallis test with Dunn's multiple comparisons. All statistical analyses were performed using GraphPad Prism version 5 for Windows (GraphPad Software, Inc., San Diego, CA).

RESULTS

Children with TB have significantly elevated plasma levels of MMP-1, -7, and -8 and TIMP-1 and -3. Since MMPs/TIMPS are associated with tissue damage/inflammation as well as matrix remodeling in TB (16), we examined the plasma levels of MMP-1, -7, -8, and -9 as well as TIMP-1, -2, -3, and -4 in PTB, ETB, and HC children (Fig. 1). As shown in Fig. 1a, we found that levels of MMP-1 in PTB (geometric mean [GM], 0.505 ng/ml) and ETB (GM, 0.495 ng/ml) children were significantly higher than those in HC children (GM, 0.283 ng/ml) and levels of MMP-7 (GM, 2.12 versus 1.18) and MMP-8 (GM, 9.93 versus 6.05) in PTB children were significantly higher than those in HC children. As shown in Fig. 1b, we also found that levels of TIMP-3 in PTB (GM, 0.606 ng/ml) and ETB (GM, 0.386 ng/ml) children were significantly higher than those in HC (GM, 0.230 ng/ml) children and levels of TIMP-1 in PTB (GM, 3.63 ng/ml) children were significantly higher than those in HC (GM, 2.15 ng/ml) children. No significant differences were found in the plasma levels of MMP-9 or TIMP-2 and -4 among PTB, ETB, and HC children. Also, there were no significant differences between MMP/TIMP levels in PTB children and those in ETB children, with the exception of MMP-7 levels (GMs of 2.12 ng/ml in PTB versus 1.25 ng/ml in ETB children). Thus, certain markers of pulmonary damage/inflammation are significantly elevated in children with active TB.

Children with TB have significantly elevated plasma levels of CRP and HO-1. Since acute-phase proteins are important markers of inflammation and CRP has been reported to be a biomarker of adult pulmonary TB (17), we examined the plasma levels of CRP, α-2m, haptoglobin, and SAA in TB-infected and HC children. As shown in Fig. 2a, we found that levels of CRP in PTB (GM, 30.11 ng/ml) and ETB (GM, 26.71 ng/ml) children were significantly higher than those in HC (GM, 18.03 ng/ml) children. In addition, we also found that levels of α-2m (GM, 2.04 versus 1.51) and haptoglobin (GM, 0.331 versus 0.223) were significantly higher in PTB than in HC children. Also, the levels of acute-phase proteins in PTB and ETB children did not differ significantly. Since HO-1 is an important marker of oxidative stress in a variety of diseases (18, 19), we also examined the plasma levels of HO-1 in TB-infected and HC children. As shown in Fig. 2b, PTB (GM, 0.758 ng/ml)

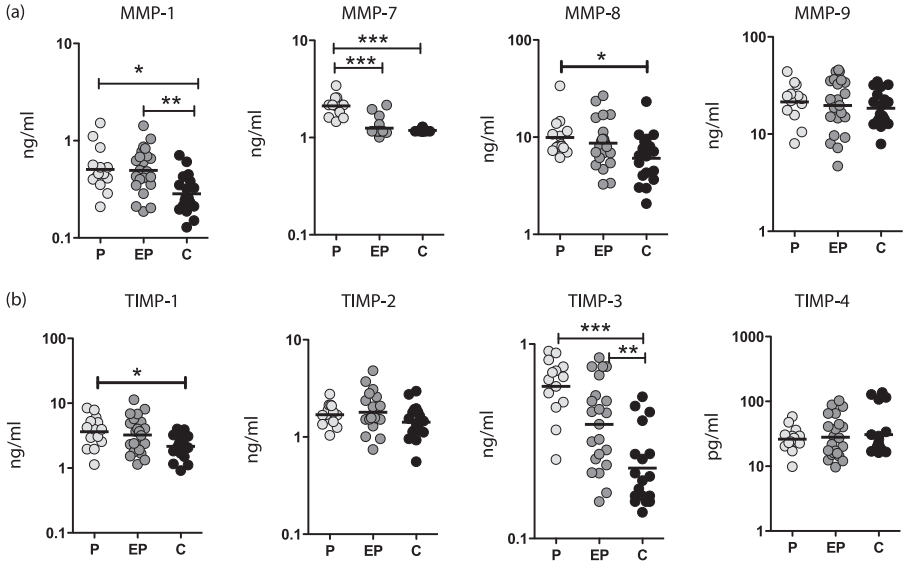

FIG 1 Children with TB have significantly elevated plasma levels of MMP-1, -7, and -8 and TIMP-1 and -3. (a) Plasma levels of MMP-1, -7, -8, and -9 in children with pulmonary TB (P) ($n = 14$), in children with extrapulmonary TB (EP) ($n = 22$), and in HC (C) ($n = 19$) children were measured by ELISA. (b) Plasma levels of TIMP-1, -2, -3, and -4 in children with pulmonary TB ($n = 14$), children with extrapulmonary TB ($n = 22$), and HC ($n = 19$) children were measured by ELISA. The data are shown as scatter plots, with each circle representing a single child. *P* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

and ETB (GM, 0.843 ng/ml) children exhibited levels of HO-1 that were significantly higher than levels in HC (GM, 0.513 ng/ml) children. No significant differences were found between the two TB-infected groups. Thus, certain markers of acute inflammation are also significantly elevated in children with active TB.

Markers of innate immune activation distinguish pulmonary from extrapulmonary TB in children. Since markers of innate immune activation are associated with the pathogenesis of various chronic infectious diseases (20) and the association of innate immune activation with TB is unknown, we examined the plasma levels of LPS, LBP, EndoCAb, and sCD14 in TB-infected and HC children. As shown in Fig. 3, we found significantly higher levels of LPS (GM, 18.44 EU/ml in PTB versus 8.63 EU/ml in ETB children) and LBP (GM, 24.45 μg/ml in PTB versus 21.36 μg/ml in ETB children) in PTB than in ETB children. In contrast, we

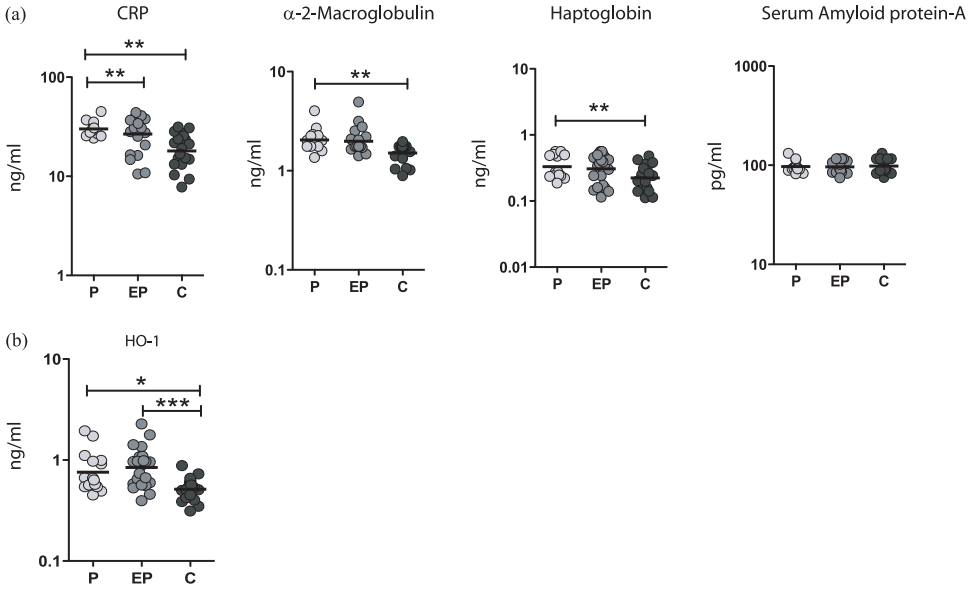

FIG 2 Children with TB have significantly elevated plasma levels of CRP, α-2 macroglobulin, haptoglobin, and HO-1. (a) Plasma levels of C-reactive protein (CRP), α-2 macroglobulin, haptoglobin, and serum amyloid protein A in children with pulmonary TB (P) ($n = 14$), children with extrapulmonary TB (EP) ($n = 22$), and HC (C) ($n = 19$) children were measured by ELISA. (b) Plasma levels of HO-1 in children with pulmonary TB ($n = 14$), children with extrapulmonary TB ($n = 22$), and HC ($n = 19$) children were measured by ELISA. The data are shown as scatter plots with each circle representing a single child. *P* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

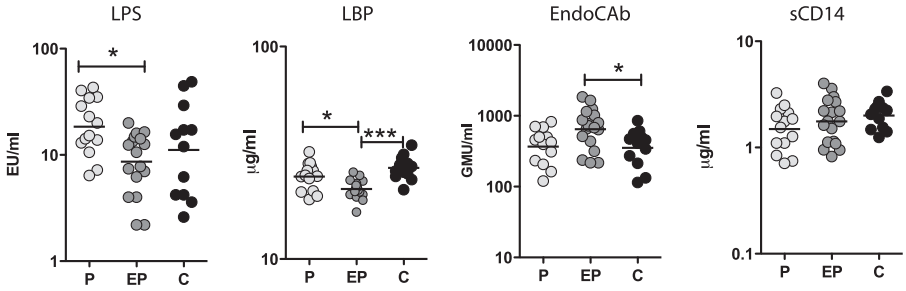

FIG 3 Children with ETB have significantly lower plasma levels of LPS and LBP and higher levels of EndoCAb. Plasma levels of LPS, LBP, EndoCAb, and sCD14 in children with pulmonary TB (P) ($n = 14$), children with extrapulmonary TB (EP) ($n = 22$), and HC (C) ($n = 19$) children were measured by immunoassays. The data are shown as scatterplots, with each circle representing a single child. *P* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons (*, $P < 0.05$; ***, $P < 0.001$).

found no significant differences between PTB and HC children for any of these markers. We also found significantly lower levels of LBP in ETB than in HC children (GM, 21.36 μg/ml in ETB versus 26.85 μg/ml in HC children) and higher levels of EndoCAb in ETB than in HC children (GM, 642.5 GMU/ml in ETB versus 353.4 GMU/ml in HC children). Thus, levels of certain markers of innate immune activation are significantly different in PTB and ETB children.

Children with TB exhibit no significant elevations in profibrotic factors. Since certain profibrotic factors, especially VEGF and PDGF-AA, have been reported to be useful biomarkers in adults with TB (21, 22), we performed *ex vivo* assessments of plasma levels of VEGF, FGF-2, G-CSF, and PDGF-AA from TB-infected and HC children. As shown in Fig. 4, we found no significant differences in the levels of these profibrotic factors among PTB, ETB, and HC children.

Children with TB have significantly elevated levels of TGFβ, IL-21, and IL-23 but not most other cytokines. Since type 1 and type 17 cytokines are associated with protection against infection/disease, and type 2 and immunomodulatory cytokines and type 1, type 2, type 17, and immune-regulatory cytokines as well as type 1 IFNs are associated with pathogenesis (23), we performed *ex vivo* assessments of plasma levels of type 1, 2, and 17 and regulatory cytokines as well as type 1 IFNs from TB-infected and HC children. As shown in Fig. 5a, no significant differences in levels of type 1 cytokines (IFN-γ, TNF-α, IL-2, and IL-12) were observed among PTB, ETB, and HC children. Similarly, as shown in Fig. 5b, no significant differences in levels of type 2 cytokines (IL-4, IL-15, IL-13, and IL-33) were observed. In addition, as shown in Fig. 5c, most type 17 cytokines (IL-17, IL-22, IL-1β, and IL-6) were not significantly different among the three groups. In contrast, two of the type 17 cytokines, IL-21 and IL-23, exhibited significant differences in plasma levels, with IL-21 (GM, 13.88 pg/ml in PTB and 10.42 pg/ml in ETB versus 6.15 pg/ml in HC children) being significantly higher in PTB and ETB children compared to HC children and IL-23 (GM, 219.3 pg/ml versus 103.8 pg/ml) being significantly higher in PTB children compared to HC children. Also, compared to HC children, children with PTB exhibited significantly higher levels of TGF-β (GM, 529.8 pg/ml in PTB versus 257.4 pg/ml in ETB and 237.4 pg/ml in HC children) but did not exhibit significantly higher levels of other regulatory (IL-10 and IL-20) cytokines (Fig. 5d). Finally, no significant differences were observed in the levels of type 1 IFNs (IFN-α and -β) (Fig. 5e). Thus, very few cytokines appear to be differentially expressed in children with active TB compared to HC children.

DISCUSSION

In order to identify biomarkers of pathogenesis in TB, we examined the plasma levels of MMPs/TIMPs, acute-phase proteins, profibrotic factors, oxidative stress proteins, markers, or innate immune activation and type 1 IFNs as markers of innate immunity and type 1-, type 2-, and type 17-associated cytokines as markers of adaptive immunity in a cohort of TB-infected and uninfected children. Our data suggest that MMPs/TIMPs, CRP, α-2m, haptoglobin, HO-1, and TGF-β could potentially serve as biomarkers of infection/disease, while LPS, LBP, and TGF-β could also potentially help distinguish pulmonary from extrapulmonary forms of TB.

MMPs and TIMPs are important factors in the pathogenesis of TB due to their ability to drive immunopathology (16, 24). MMPs

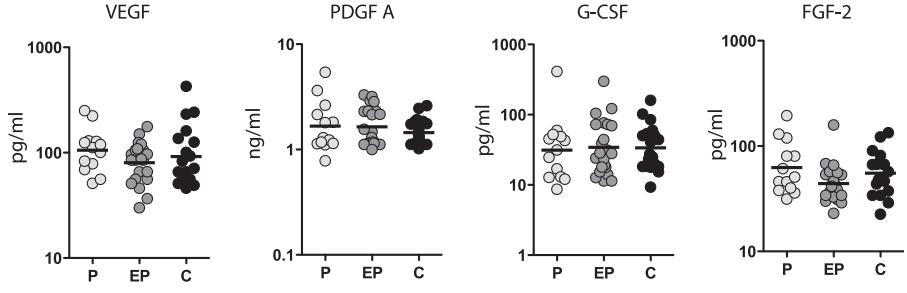

FIG 4 Children with TB do not have significantly altered plasma levels of profibrotic factors. Plasma levels of VEGF, PDGF-AA, G-CSF, and FGF-2 in children with pulmonary TB (P) ($n = 14$), children with extrapulmonary TB (EP) ($n = 22$), and HC (C) ($n = 19$) children were measured by ELISA. The data are shown as scatter plots, with each circle representing a single child.

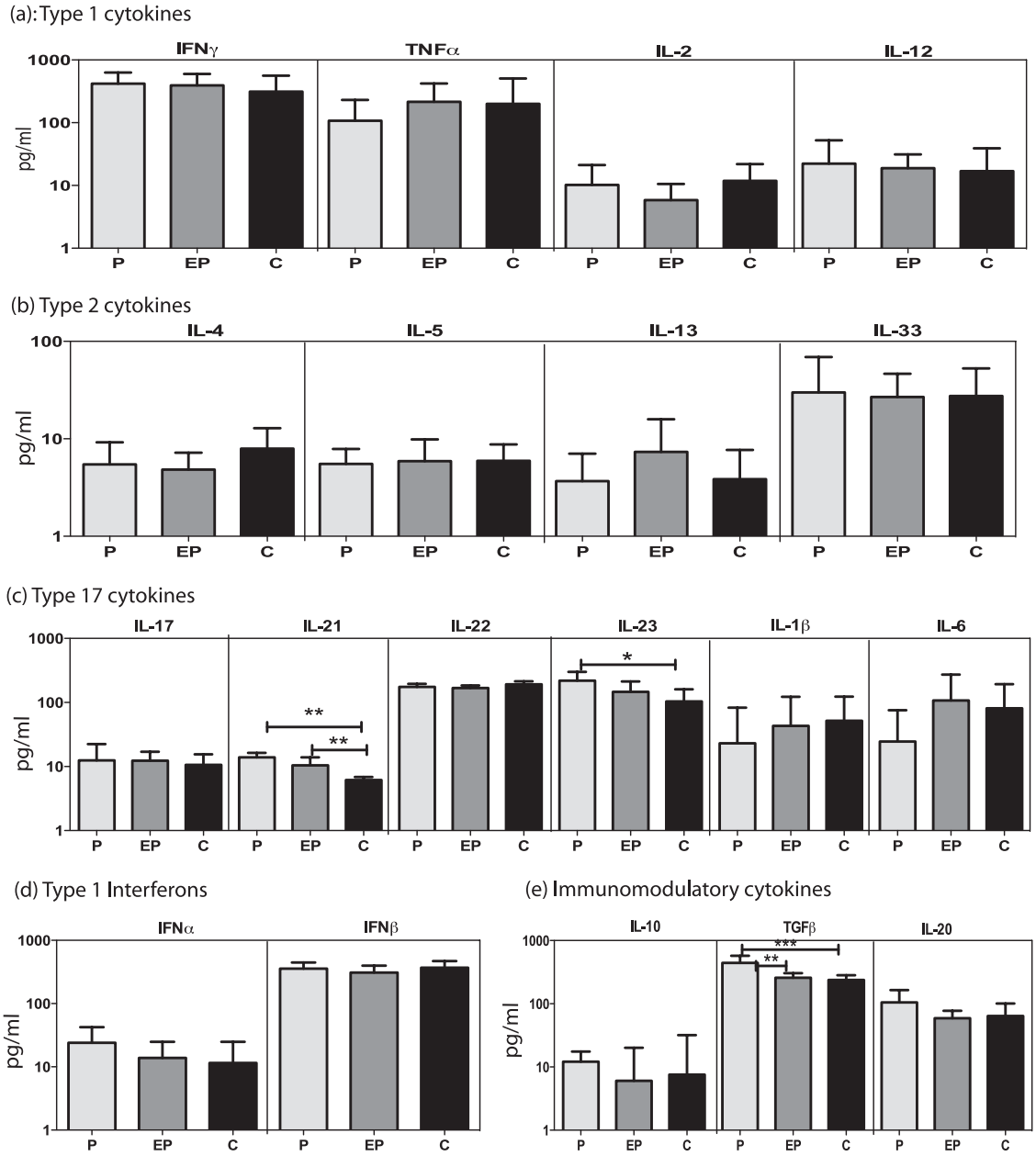

FIG 5 Children with TB have elevated levels of TGF-β, IL-21, and IL-23 but not of other cytokines at homeostasis. Plasma levels of type 1 cytokines (IFN-γ, TNF-α, IL-2, and IL-12p70) (a), type 2 cytokines (IL-4, IL-5, IL-13, and IL-33) (b), type 17 cytokines (IL-17, IL-21, IL-22, IL-23, IL-1β, and IL-6) (c), type 1 IFNs (IFN-α and IFN-β) (d), and regulatory cytokines (IL-10, TGF-β, and IL-20) (e) in children with pulmonary TB (P) ($n = 14$), children with extrapulmonary TB (EP) ($n = 22$), and HC (C) ($n = 19$) children were measured by ELISA. Data are shown as bar graphs, with bars representing the geometric means and 95% confidence intervals. *P* values were calculated using the Kruskal-Wallis test with Dunn's multiple comparisons (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

are zinc-dependent proteases associated with the breakdown of the extracellular matrix and with tissue remodeling (25). TIMPs are specific inhibitors of MMPs and help control tissue pathology (25). While various MMPs have been shown to be upregulated in peripheral blood and at the site of disease in TB infection (16), their role in the extrapulmonary dissemination of TB is less well understood. Similarly, very few studies have examined the expression of TIMP in TB infections (26). Our results suggest that MMP-1, -7, and -8 and TIMP-1 and -3 are expressed at higher levels in TB-infected children and could potentially serve as biomarkers to distinguish individuals with TB disease from healthy controls. However, our results also suggest that plasma levels of MMPs/TIMPs, with the exception of MMP-7, are probably not particularly useful in discriminating between pulmonary and extrapulmonary TB in children. Acute-phase proteins are nonspecific serum proteins that are elevated in patients with TB (23). Recently, CRP has been proposed as a candidate biomarker for active infection with *M. tuberculosis* (27). Point-of-care CRP testing has been shown to be of use in the clinical evaluation of respiratory tract infections in adults and of fever in children (17). Our

study reveals that since CRP levels are significantly elevated in TB-infected children, CRP might serve as an important biomarker in pediatric TB, as has been described in adults for whom CRP is being utilized as a point-of-care test to aid in TB diagnosis (28). In addition, two other acute-phase proteins (α-2m and haptoglobin) are also differentially expressed and could be used in conjunction with CRP for TB evaluation. Although the des-arginine subtype of SAA has been previously described as a candidate marker for TB (29), total SAA (detected in our study) appears not to be associated with infection/disease.

Infection with *M. tuberculosis* has been shown to result in the upregulation of HO-1, an oxidative stress response protein, and to lead to the induction of the bacterial dormancy regulon, perhaps driving infection toward latency (30, 31). HO-1 has also been shown to be a useful biomarker in other infections (18, 19). Interestingly, our data also suggest that HO-1 is probably a useful marker to distinguish individuals with active infection from healthy controls and could potentially serve as an important surrogate marker of disease. However, since we have not examined the expression of HO-1 in children with latent TB, further studies are needed to verify this finding. Markers of innate immune activation include circulating microbial products, such as LPS and innate host proteins responding to LPS, such as LBP, sCD14, and antibodies to the LPS core (EndoCAb) (20). These markers have been shown to be persistently elevated in various infections, including HIV (32), hepatitis (33), and parasitic infections (34, 35). Our study examined the expression patterns of these markers and our findings indicate that LPS, LBP, and perhaps EndoCAb levels could serve as markers for discrimination between pulmonary and extrapulmonary disease in children. Levels of LPS and LBP and increased levels of EndoCAb in ETB children are suppressed compared to levels in PTB children, but interestingly the levels in PTB and HC children do not differ significantly, suggesting that persistent immune activation is not necessarily a hallmark of pulmonary TB in children. Profibrotic markers, such as FGF and PDGF, as well as endothelial activation markers, such as VEGF, are thought to play important roles in the pathogenesis of TB disease (21, 22), and therefore we examined the expression pattern of these factors. However, our findings did not reveal any significant differences in the expression levels of these factors in TB-infected children compared to those in control children. Since VEGF in particular has been shown to be a useful biomarker in adult TB (36, 37), our findings suggest that more studies are needed on VEGF expression patterns before the utility of VEGF can be universally applied.

We wanted to examine the roles of type 1-, type 2-, and type 17-associated cytokines both in disease susceptibility, which would manifest as differences in responses to these biomarkers in TB-infected and noninfected children, and in disease dissemination or extrapulmonary manifestations, which would manifest as differences in responses in PTB- and ETB-infected children. Interestingly, we did not find any significant differences between the groups in their responses to the homeostatic levels of the type 1-, type 2-, and type 17-associated cytokines or their responses to most immunoregulatory cytokines. The only cytokines that exhibited a significantly increased expression pattern were TGF-β, IL-21, and IL-23, for which levels in TB-infected children were found to be elevated compared to those in HC children. Interestingly, TGF-β expression also enabled us to discriminate pulmonary from extrapulmonary TB in children. Among the various factors found to play important roles in dampening immune responses in TB, the most important is TGF-β, which has been shown to mediate the suppression of immune responses in anergic TB patients (38–40). However, to our knowledge, this is the first report of differential expression levels of TGF-β in different forms of TB in children. Similarly, the differential expression levels of IL-21 and IL-23 have not been reported before and need to be evaluated further. Although protective immunity to TB is clearly dependent on type 1 responses, specifically IFN-γ- and TNF-α-mediated responses (41–43), and to a lesser degree on type 17 responses, specifically those to IL-1b, IL-17, IL-22, and IL-23 (44–46), our study revealed no significant differences between TB-infected and uninfected children in regard to the expression levels of these cytokines at homeostasis. Thus, plasma levels of these cytokines measured *ex vivo* might not serve as true indicators of their function *in vivo*, and the utility of these cytokines as biomarkers for TB pathogenesis (as has been suggested previously [23]) needs to be questioned. Type 1 IFNs have been shown to promote TB infection in animal models (47, 48). In addition, IFN-inducible genes have been shown to be highly expressed in the leukocytes of patients with active TB but not in those of patients with latent TB, and this gene signature pattern is thought to predict the development of active TB in infected individuals (49). However, our data fail to show any significant differences in the levels of type 1 IFNs examined—IFN-α and IFN-β. Although the lack of differential expression of type 1 IFNs in plasma does not exclude a potential role in resistance or susceptibility to TB, it certainly suggests limited utility for the measurement of type 1 IFNs as biomarkers of TB infection/disease. Also, another potential explanation for the differences in the results of this study compared to the results of previous studies of cytokines in TB disease could be the presence of an immature immune system in children.

Finally, because *Mycobacterium bovis* BCG vaccination of children has been demonstrated to induce complex cytokine profiles in both $CD4^+$ and $CD8^+$ T cells (50), we wanted to exclude a role for BCG vaccination in the observed differential biomarker expression in TB-infected or healthy children. When we analyzed the production of the different biomarkers on the basis of BCG vaccination status in all children or in TB-infected children, we found no significant difference in any of the markers, indicating that BCG had very little, if any, role in inducing a differential immune response in pediatric TB (data not shown).

The need for TB biomarkers arises, in part, from the difficulty of accurately diagnosing TB infection. This need is increased in children, in whom diagnosis is more difficult due to the various problems associated with even the routine diagnostic tests (smear and culture), including the paucibacillary nature of infection and the difficulty in producing sputum (51). Host biomarkers, therefore, can serve as surrogate or complementary modalities to confirm TB infection in children as well as to provide correlates of risk of TB and correlates of protection against active disease (23, 51). Our study is one of the first to comprehensively evaluate a variety of host factors in TB-infected and uninfected children. We report the utility of certain biomarkers—MMPs/TIMP-1, HO-1, CRP, and TGF-β—in helping to increase our understanding of the host immune factors that might contribute to the pathogenesis of TB and facilitate or impede its extrapulmonary spread in children. A limitation of our study is that it is only a preliminary examination of host molecules that are present at different levels in different clinical phenotypes and therefore would not necessarily constitute

qualified biomarkers. Another limitation is the small sample size and the absence of children with latent TB and with other pulmonary infections. Finally, since most of these markers could potentially also be elevated nonspecifically in plasma, they should be used in conjunction with clinical and other investigations to aid diagnosis. Nevertheless, this study provides valuable insights on factors that might need further exploration as diagnostic and prognostic biomarkers in pediatric tuberculosis.

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