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Coexistent Malnutrition Is Associated with Perturbations in Systemic and Antigen-Specific Cytokine Responses in Latent Tuberculosis Infection

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Malnutrition, as defined by low body mass index (BMI), is a major risk factor for the development of active tuberculosis (TB), although the biological basis underlying this susceptibility remains poorly characterized. To verify whether malnutrition affects the systemic and antigen-specific cytokine levels in individuals with latent TB (LTB), we examined circulating and TB antigen-stimulated levels of cytokines in individuals with LTB and low BMI (LBMI) and compared them with those in individuals with LTB and normal BMI (NBMI). Coexistent LBMI with LTB was characterized by diminished circulating levels of type 1 (gamma interferon [IFN-γ] and tumor necrosis factor alpha [TNF-α]), type 2 (interleukin-4 [IL-4]), type 17 (IL-22), and other proinflammatory (IL-1α, IL-1β, and IL-6) cytokines but elevated levels of other type 2 (IL-5 and IL-13) and regulatory (IL-10 and transforming growth factor beta [TGF-β]) cytokines. In addition, LBMI with LTB was associated with diminished TB antigen-induced IFN-γ, TNF-α, IL-6, IL-1α, and IL-1β levels. Finally, there was a significant positive correlation between BMI values and TNF-α and IL-1β levels and a significant negative correlation between BMI values and IL-2, IL-10, and TGF-β levels in individuals with LTB. Therefore, our data reveal that latent TB with a coexistent low BMI is characterized by diminished protective cytokine responses and heightened regulatory cytokine responses, providing a potential biological mechanism for the increased risk of developing active TB.

Tuberculosis (TB) is one of the most common infectious diseases, with nearly 10 million new cases occurring worldwide each year (1). Tuberculosis manifests as a disease spectrum ranging from latent infection to overt pulmonary or extrapulmonary disease. Healthy individuals are typically successful in containing the infection following exposure to *Mycobacterium tuberculosis*, resulting in a latent, asymptomatic infection (LTB) (1, 2). Only about 5 to 10% of individuals with LTB are thought to progress (or reactivate) to active TB during their lifetime, and this progression is thought to reflect a breakdown of protective immune mechanisms (3). Active TB reflects the progression from asymptomatic, latent infection to active symptomatic disease (1, 2). Cytokines of the innate and adaptive immune systems orchestrate the immune response to *M. tuberculosis*, with type 1, type 17, and interleukin-1 (IL-1) family cytokines having been implicated in protection against TB disease in murine models (4, 5), whereas type 2 and anti-inflammatory cytokines have been associated with increased susceptibility to disease and/or enhanced pathology (6, 7).

Among the many risk factors for the development of active TB, malnutrition is one that has been recognized for a long time, mainly through observational and ecological studies (8). There are several classical observational clinical studies that have provided compelling evidence for the contribution of malnutrition to enhanced susceptibility to TB disease (8). These data were further confirmed by studies using animal models of TB infection, mainly in guinea pigs and mice (8). Data from studies of humans and animals show that susceptibility to progression from infection to disease is related to nutrient deficiencies that induce impairment of cellular immunity and that this impairment is partially reversible following nutritional replenishment (9–11). In addition, animal studies clearly reveal an important role for protective cytokines, especially gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), and the regulatory cytokine transforming growth factor beta (TGF-β) in increased susceptibility to TB in nutritionally deprived animal models (12). However, the roles of these cytokines in human immune responses to TB in malnourished individuals have never been examined. Despite the clinical and public health significance posed by the dual burden of TB and malnutrition, very little is known about the immunological and biochemical mechanisms of susceptibility.

We hypothesized that malnutrition would predominantly attenuate protective cytokine responses in LTB and thereby predispose individuals to an increased risk of developing active TB. To study the influence of malnutrition on LTB, we examined plasma levels of a large panel of type 1, type 2, type 17, regulatory, and other proinflammatory cytokines in individuals with LTB and a coexistent low body mass index (LBMI) and compared them to those in individuals with LTB but a normal BMI (NBMI).

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TABLE 1 Demographics and biochemical parameters of the study groups

Parameter	Value[a]	
	LBMI group ($n = 28$)	NBMI group ($n = 28$)
No. of males/no. of females	15/13	16/12
Age (yr)	28 (18–60)	29 (19–61)
Body mass index	16.98 (12.28–18.44)	22 (18.90–24.97)
Albumin (g/dl)	2.8 (2.5–3.2)	4.2 (3.7–4.8)
Random blood glucose (mg/dl)	87 (84–113)	92.3 (60–129)
HbA1c (%)	5.6 (4.8–6.3)	5.7 (4.9–6.1)
Urea (mg/dl)	19.5 (12–34)	21.9 (11–42)
Creatinine (mg/dl)	0.78 (0.3–1)	0.80 (0.4–1.3)
ALT (U/liter)	17.7 (7–60)	22.4 (7–92)
AST (U/liter)	27.8 (16–110)	24.7 (11–68)

[a] The values represent geometric means and ranges (except for age, for which medians and ranges are shown, and numbers of males and females).

TABLE 2 Hematological parameters of the study groups

Parameter	Value (GM [range])		
	LBMI group ($n = 28$)	NBMI group ($n = 28$)	P value[a]
Hemoglobin (g/dl)	12.4 (8.4–16.3)	13.51 (9.1–18.6)	0.5176
Red blood cell count (10^6/liter)	4.6 (3.8–6.2)	4.85 (3.8–6.4)	0.1551
White blood cell count (10^3/liter)	70 (45–112)	77 (49–117)	0.3766
Hematocrit (%)	38.75 (27.6–54)	40.49 (27.9–58.6)	0.3025
Platelet count (10^3/liter)	286.5 (145–174)	264.11 (172–397)	0.3425
Cell concn (cells/ml)			
Neutrophils	3,555 (2,246–6,328)	4,128 (2,229–6,537)	0.0473
Lymphocytes	2,496 (1,582–3,498)	2,454 (1,612–4,843)	0.5070
Monocytes	410 (25–873)	474 (234–963)	0.5280
Eosinophils	431 (85–1,575)	370 (78–2,817)	0.3659
Basophils	53 (18–265)	60 (14–379)	0.4713

[a] Calculated using the Mann-Whitney test.

MATERIALS AND METHODS

Study population. We studied a group of 56 individuals with latent TB infection: 28 with LBMI and 28 with NBMI (Table 1). This study was conducted in South India, and the individuals were screened as part of a community screening protocol in a rural village on the outskirts of Chennai. All individuals were adults of between 18 and 65 years of age and were enrolled consecutively. All individuals with LTB who had a low or normal BMI were enrolled, provided that they were negative for diabetes, HIV, and parasitic infection. LTB was diagnosed on the basis of being tuberculin skin test (TST) positive and Quantiferon TB Gold in Tube test (QFT) positive, with no symptoms or signs of active TB, no history of previous TB, and normal chest radiographs. TST was performed using 2 tuberculin units of tuberculin purified protein derivative (PPD) RT 23 SSI (Serum Statens Institute). A positive skin test was defined as an induration of at least 12 mm in diameter, based on the previously determined cutoff norms for South India (13). QFT was performed according to the manufacturer's instructions (Qiagen). All the individuals were nondiabetic and HIV negative according to their medical records. Anthropometric measurements, including height, weight, and waist circumference, and biochemical parameters, including plasma glucose, serum albumin, urea, creatinine, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and HbA1c levels, were obtained using standardized techniques. Low and normal BMIs were defined on the basis of the 2013 American Heart Association/American College of Cardiology guidelines (LBMI, <18.5 kg/m^2; and NBMI, between 18.5 and 24.9 kg/m^2). In addition, malnutrition was confirmed by the presence of low serum albumin (<3.4 g/dl) in all the low-BMI individuals. Hematology was performed on all individuals by using an Act-5 Diff hematology analyzer (Beckman Coulter). Stool microscopy was performed to rule out the presence of intestinal parasites. Also, filarial infections were excluded by TropBio enzyme-linked immunosorbent assay (ELISA). All individuals were examined as part of a clinical protocol approved by the Institutional Review Board of the National Institute of Research in Tuberculosis (approval no. NCT00375583 and NCT00001230), and informed written consent was obtained from all individuals.

ELISA. Plasma cytokines were measured using a Bioplex multiplex cytokine assay system (Bio-Rad). The cytokines analyzed were IFN-γ, TNF-α, IL-2, IL-17A, IL-4, IL-5, IL-13, IL-10, IL-6, IL-12, and granulocyte-macrophage colony-stimulating factor (GM-CSF). Plasma levels of TGF-β, IL-1α, IL-1β, IL-18, and IL-22 (all from R&D Systems) and of IL-17F (Biolegend) were measured by ELISA.

QFT ELISA. Whole-blood samples obtained from individuals with an LBMI or NMBI were incubated *in vitro* with either no antigen, a cocktail of TB antigens (ESAT-6, CFP-10, and TB 7.7), or mitogen for 18 h, using a QFT kit (Qiagen) according to the manufacturer's instructions. The TB antigen- or mitogen-stimulated whole-blood supernatants were then used to analyze the levels of IFN-γ, TNF-α, IL-2, IL-22, IL-10, and TGF-β by using Duo-Set ELISA kits from R&D Systems.

Statistical analysis. Geometric means (GM) were used for measurements of central tendency. Sample size was based on convenience, and nonparametric tests were conducted because a normal distribution of data was not assumed. Statistically significant differences between two groups were analyzed using the nonparametric Mann-Whitney U test with Holm's correction for multiple comparisons. Correlations were calculated using Spearman rank correlation. P values of <0.05 were considered significant. Analyses were performed using GraphPad Prism, version 5.01.

RESULTS

Study population characteristics. Individuals with LTB were classified into two groups based on BMI: LBMI was defined as a BMI of <18.5 kg/m^2, while NBMI was defined as a BMI between 18.5 and 24.9 kg/m^2. All the individuals with LBMI had serum albumin levels of <3.4 g/dl, confirming the presence of malnutrition, while the individuals with NBMI had serum albumin levels (3.7 to 4.8 g/dl) in the normal range. The two groups did not differ significantly in the levels of random blood glucose, HbA1c, urea, creatinine, ALT, and AST levels. The hematological parameters are reported in Table 2. As shown, the two groups did not differ significantly in their baseline hematological parameters, with the exception of absolute number of neutrophils, which was marginally significantly lower in the LBMI group. Therefore, individuals with LBMI and LTB did not differ significantly from their NBMI counterparts in age, gender, biochemical, and hematological parameters, excluding a role for these confounding variables in the interpretation of the study findings. Also, the plasma cytokine levels were not different between males and females and did not show any relationship with age (data not shown).

LBMI is associated with decreased circulating levels of type 1 cytokines and IL-22. To determine the influence of BMI on type 1 and type 17 cytokines in LTB, we measured the circulating levels of IFN-γ, TNF-α, and IL-2, as well as IL-17A, IL-17F, and IL-22, in LBMI and NBMI individuals with concomitant LTB (Fig. 1). As shown in Fig. 1, the systemic levels of two of the type 1 cytokines, i.e., IFN-γ (GM of 119.2 pg/ml for LBMI versus 198.9 pg/ml for

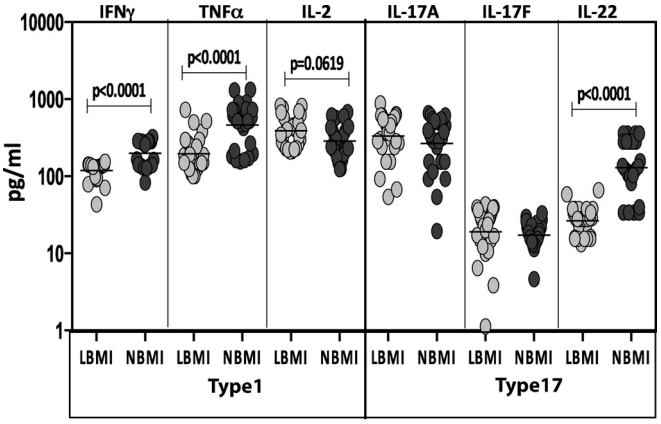

FIG 1 Diminished systemic levels of type 1 cytokines and IL-22 in individuals with LBMI. The plasma levels of type 1 (IFN-γ, TNF-α, and IL-2) and type 17 (IL-17A, IL-17F, and IL-22) cytokines in individuals with LBMI ($n = 28$) or NBMI ($n = 28$) and LTB were measured by ELISA. The data are presented as scatterplots, with each circle representing a single individual (light gray dots, LBMI; and dark gray dots, NBMI). *P* values were calculated using the Mann-Whitney test.

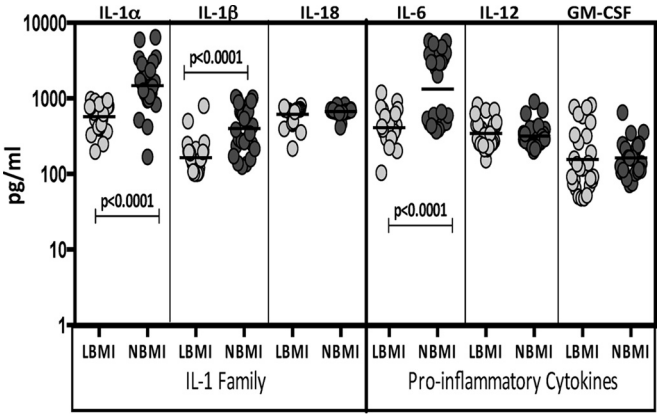

FIG 3 Diminished systemic levels of proinflammatory cytokines in individuals with LBMI. The plasma levels of IL-1 family (IL-1α, IL-1β, and IL-18) and other proinflammatory (IL-6, IL-12, and GM-CSF) cytokines in individuals with LBMI ($n = 28$) or NBMI ($n = 28$) and LTB were measured by ELISA. The data are presented as scatterplots, with each circle representing a single individual (light gray dots, LBMI; and dark gray dots, NBMI). *P* values were calculated using the Mann-Whitney test.

NBMI; $P < 0.0001$) and TNF-α (GM of 196.3 pg/ml versus 462.9 pg/ml; $P < 0.0001$), as well as the type 17 cytokine IL-22 (GM of 26.3 pg/ml versus 129.2 pg/ml; $P < 0.0001$), were significantly lower in individuals with LBMI than in those with NBMI. In contrast, the systemic levels of the prototypical type 17 cytokines—IL-17A and IL-17F—were not significantly different between the two groups. Finally, the systemic levels of IL-2 (GM of 386.5 pg/ml versus 284.1 pg/ml; $P = 0.0619$) were not significantly higher in individuals with LBMI than in those with NBMI. Thus, LBMI is associated with diminished levels of common type 1 cytokines and IL-22 in individuals with LTB.

LBMI is associated with increased circulating levels of type 2 and regulatory cytokines. To determine the influence of BMI on type 2 and regulatory cytokines in LTB, we measured the circulating levels of IL-4, IL-5, and IL-13, as well as IL-10 and TGF-β, in LBMI and NBMI individuals with concomitant LTB (Fig. 2). As shown in Fig. 2, the systemic levels of IL-4 (GM of 50.9 pg/ml versus 78.8 pg/ml; $P = 0.0009$) were significantly lower in individuals with LBMI than in those with NBMI. In contrast, the systemic levels of IL-5 (GM of 255.7 pg/ml versus 172 pg/ml; $P = 0.0005$) and IL-13 (GM of 315.4 pg/ml versus 184.5 pg/ml; $P = 0.0004$) were significantly higher in individuals with LBMI than in those with NBMI. Similarly, the systemic levels of the regulatory cytokines IL-10 (GM of 441 pg/ml versus 209.6 pg/ml; $P < 0.0001$) and TGF-β (GM of 490.4 pg/ml versus 298.7 pg/ml; $P = 0.0015$) were also significantly higher in individuals with LBMI than in those with NBMI. Thus, LBMI is associated with enhanced levels of common type 2 and regulatory cytokines in individuals with LTB.

LBMI is associated with decreased circulating levels of proinflammatory cytokines. To determine the influence of BMI on other proinflammatory cytokines in LTB, we measured circulating levels of these cytokines in LBMI and NBMI individuals with concomitant LTB (Fig. 3). As shown in Fig. 3, the systemic levels of the IL-1 family cytokines IL-1α (GM of 574.3 pg/ml versus 1,473 pg/ml; $P < 0.0001$) and IL-1β (GM of 164.5 pg/ml versus 398.4

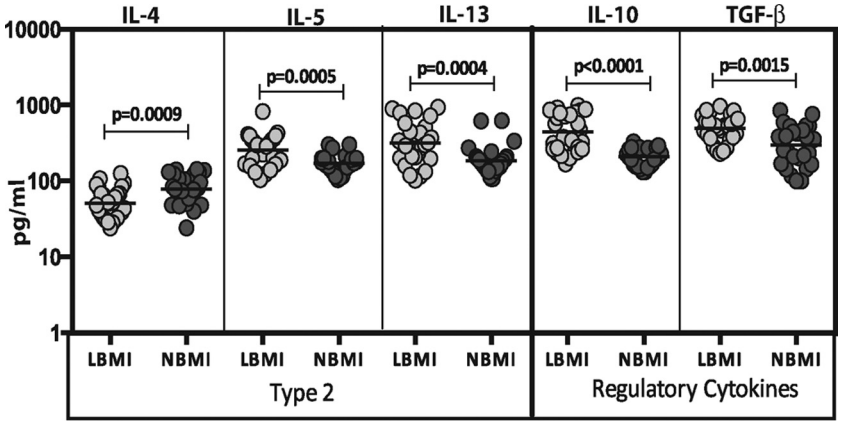

FIG 2 Elevated systemic levels of type 2 and regulatory cytokines in individuals with LBMI. The plasma levels of type 2 (IL-4, IL-5, and IL-13) and regulatory (IL-10 and TGF-β) cytokines in individuals with LBMI ($n = 28$) or NBMI ($n = 28$) and LTB were measured by ELISA. The data are presented as scatterplots, with each circle representing a single individual (light gray dots, LBMI; and dark gray dots, NBMI). *P* values were calculated using the Mann-Whitney test.

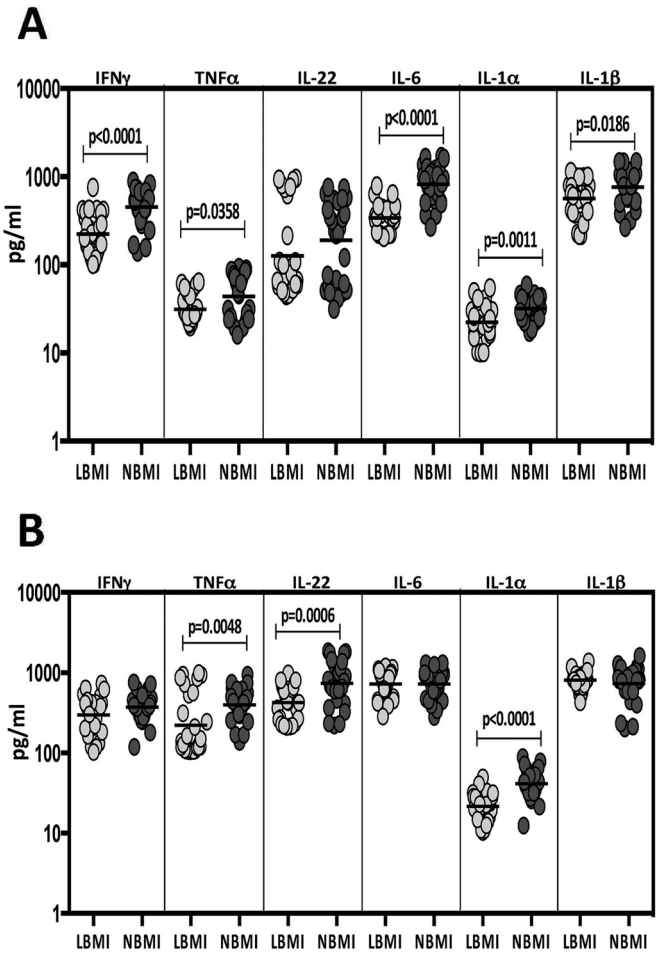

FIG 4 Diminished TB antigen-stimulated levels of proinflammatory cytokines in individuals with LBMI. The TB antigen-stimulated (A) or mitogen-stimulated (B) levels of IFN-γ, TNF-α, IL-22, IL-6, IL-1α, and IL-1β in whole blood from individuals with LBMI ($n = 28$) or NBMI ($n = 28$) and LTB were measured by ELISA. The data are presented as scatterplots, with each circle representing a single individual (light gray dots, LBMI; and dark gray dots, NBMI). *P* values were calculated using the Mann-Whitney test.

pg/ml; $P < 0.0001$), but not IL-18, were significantly lower in individuals with LBMI than in those with NBMI. Similarly, the systemic levels of IL-6 (GM of 462.5 pg/ml versus 1,327 pg/ml; $P < 0.0001$), but not IL-12 or GM-CSF, were also significantly lower in individuals with LBMI than in those with NBMI. Thus, LBMI is associated with diminished levels of certain proinflammatory cytokines in individuals with LTB.

LBMI is associated with decreased TB antigen-stimulated levels of proinflammatory cytokines. To determine the influence of BMI on TB antigen-stimulated cytokine production in LTB, we measured the levels of these cytokines following stimulation of whole blood obtained from LBMI and NBMI individuals with concomitant LTB with a cocktail of TB antigens (ESAT-6, CFP-10, and TB 7.7) or mitogen (Fig. 4). As shown in Fig. 4A, the TB antigen-stimulated levels of IFN-γ (GM of 224.7 pg/ml versus 450.5 pg/ml; $P < 0.0001$), TNF-α (GM of 31.2 pg/ml versus 43.8 pg/ml; $P = 0.0358$), IL-6 (GM of 339.2 pg/ml versus 821.5 pg/ml; $P < 0.0001$), IL-1α (GM of 22.4 pg/ml versus 32.1 pg/ml; $P = 0.0011$), and IL-1β (GM of 563.9 pg/ml versus 8,764.4 pg/ml; $P = 0.0186$) were significantly lower in individuals with LBMI than in those with NBMI. Next, upon mitogen stimulation, as shown in Fig. 4B, the levels of TNF-α (GM of 196.3 pg/ml versus 462.9 pg/ml; $P = 0.0048$), IL-22 (GM of 574.3 pg/ml versus 1,473 pg/ml; $P = 0.0006$), and IL-1α (GM of 26.3 pg/ml versus 129.2 pg/ml; $P < 0.0001$) were also significantly lower in individuals with LBMI than in those with NBMI individuals. Thus, LBMI is associated with diminished levels of TB antigen-stimulated proinflammatory cytokines.

Relationship between systemic cytokines and BMI. Low BMI is an accurate indicator of the level of malnutrition or undernutrition in the individual (14). Thus, to examine the relationships between the systemic levels of type 1, type 2, proinflammatory, and regulatory cytokines and BMI, we assessed the association of all of the above-mentioned cytokines with BMI in all the individuals in the study. As shown in Fig. 5, the systemic levels of TNF-α and IL-1β each exhibited a significant positive association with BMI, while the systemic levels of IL-2, IL-10, and TGF-β each exhibited a significant negative association in individuals with LTB. No correlation was observed with the other cytokines tested (data not shown).

DISCUSSION

Malnutrition is a common cause of secondary immune deficiency and susceptibility to infections in general (15). During severe malnutrition, both acquired immunity and innate host defense mechanisms are affected, with the consequent reduction in immunity rendering individuals susceptible to a variety of infections (15–17). Malnutrition is known to cause direct effects on T cells, with decreased CD4/CD8 ratios and increased numbers of CD4 and CD8 double-negative T cells (18). Malnutrition also affects antibody responses, with diminished antibody production observed for a variety of infections (15, 17, 19). In addition, innate immune responses are diminished, including phagocytosis and production of reactive oxygen and nitrogen intermediates by macrophages and antigen presentation to T cells by dendritic cells (20). Thus, a variety of dysregulated immune parameters may lead to enhanced susceptibility to infections in malnourished individuals. The immunological basis for susceptibility to tuberculosis among those with low BMI is not well understood. One possible mechanism is that an impaired immune response in malnourished patients facilitates either primary infection with tuberculosis or reactivation of latent tuberculosis (8). There is little direct evidence that malnutrition affects the risk of acquiring latent infection, whereas the evidence with regard to malnutrition increasing the risk of reactivation disease is stronger (8, 21). As mentioned above, this is due to the negative effect of macronutrient and micronutrient deficiencies on cell-mediated immune responses (8, 22, 23).

Since cell-mediated immunity is vital for resistance to TB disease (2), we explored cytokine responses, which reflect cell-mediated immunity in latent TB infection. Cytokines are known to play a major role in determining the outcome of infection in the host defense against mycobacterial infections (4, 5). Of major importance are IFN-γ and TNF-α, whose functions have been well documented for both mouse models and human infections (4, 24, 25). In addition, type 17 cytokines, especially IL-17A, are known to play a role in the memory response to infection (26, 27). However, the roles of IL-17F and IL-22 are not well defined. Cytokines of the IL-1 family, especially IL-1α, IL-1β, and IL-18, are all important

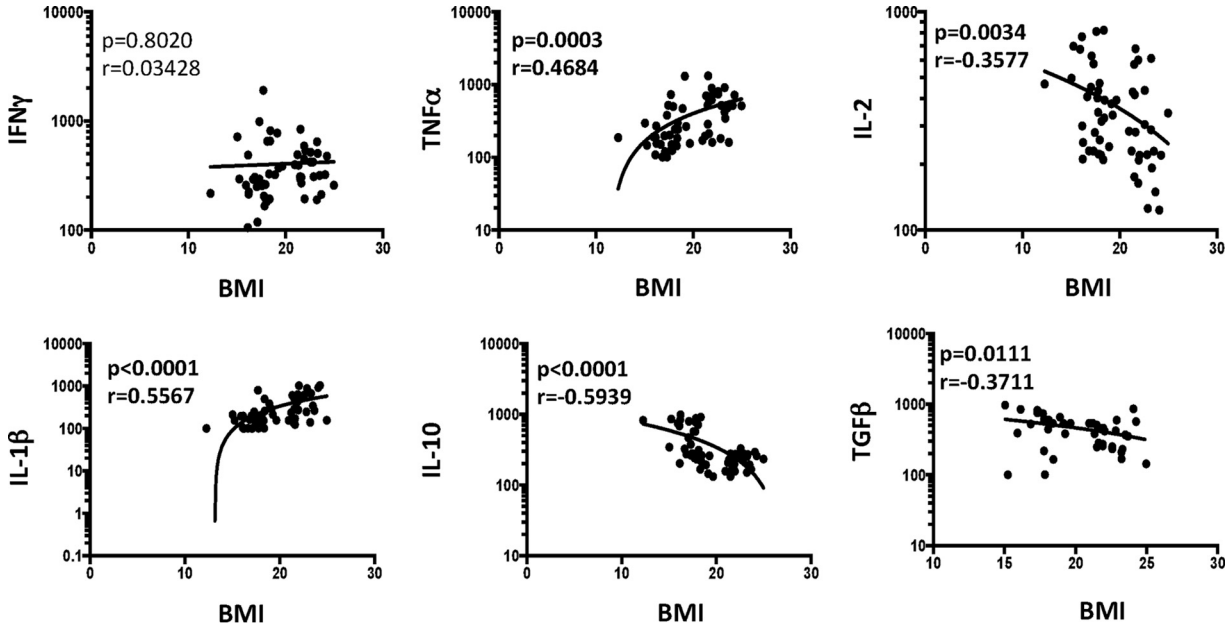

FIG 5 Positive and negative relationships between systemic levels of cytokines and BMI values for individuals with LTB. The relationships between the plasma levels of IFN-γ, TNF-α, IL-2, IL-1β, IL-10, and TGF-β and BMI values were examined for all individuals with LTB ($n = 56$). The data are presented as scatterplots, with each circle representing a single individual. *P* values were calculated using Spearman rank correlation.

for resistance to infection in animal models (28–30). Finally, IL-6, IL-12, and GM-CSF are also known to play important roles in immunity, with IL-12 being known to be crucial for immunity to TB in both mouse models and human infections (31–33). Our examination of the above-mentioned cytokines clearly reveals a systemic deficiency in the circulating levels of most of these cytokines, with the exception of IL-2. In addition to the suppressed systemic cytokine levels, individuals with LTB and LBMI also appear to be compromised in the ability to mount protective cytokine responses to TB antigens. Therefore, the inability to produce optimal levels of IFN-γ, TNF-α, IL-1α, and IL-1β both at baseline and following TB antigen stimulation suggests that individuals with LBMI are potentially at a higher risk for developing active TB due to a compromise in their cell-mediated immune responses.

One potential mechanism for the decrease in baseline levels of proinflammatory cytokines in individuals with LBMI could be a concomitant increase in the levels of systemic type 2 or regulatory cytokines. Indeed, with the exception of IL-4, individuals with LBMI did exhibit enhanced systemic levels of type 2 (IL-5 and IL-13) and regulatory (IL-10 and TGF-β) cytokines. Since both type 2 cytokines and regulatory cytokines are known to play a role in enhancing susceptibility to infection (34, 35), our data indicate that alteration of the balance between pro- and anti-inflammatory cytokines plays a pivotal role in the establishment of cytokine responses in LTB. In addition, since both IL-10 and TGF-β are also known to downmodulate protective type 1 and other proinflammatory responses in TB (1, 34), our data also offer a plausible mechanism for the downmodulation of these cytokines systemically in individuals with LBMI. Finally, our data also offer direct evidence of the association of BMI with the perturbations in plasma cytokine levels in individuals with LTB. BMI appears to directly correlate positively with two of the known protective cytokines (TNF-α and IL-1β) and negatively with the regulatory cytokines (IL-10 and TGF-β). This suggests that BMI *per se* (independent of other covariables in the system) regulates systemic cytokine levels in TB infection. Our data also indicate that TNF-α and IL-1β are very strong positive predictors and IL-10 and TGF-β are very strong negative predictors of the effect of nutrition on TB infection. Thus, our data suggest that low BMI is associated with diminished protective cytokine levels and heightened regulatory cytokine levels in LTB, possibly contributing to the increased risk of developing active TB.

Our study suffers from the limitations of being a cross-sectional study examining only associations, not cause and effect, being limited in terms of sample size, and relying on systemic biomarkers, which could be affected by a variety of other parameters. Also, we used only defined TB antigens, not PPD, for whole-blood stimulation, which could be a limitation. Finally, while we observed an association between malnutrition and cytokine diminution, we cannot infer any causality. While we have excluded most of the known comorbidities that are known to influence cytokine responses in TB, including HIV, diabetes, and intestinal parasites (36), it is theoretically possible that other factors not examined in this study could have contributed to the differential responses. We also observed a bimodal distribution of the levels of certain cytokines that did not relate to age or gender and could be the effect of other confounders. Nevertheless, to our knowledge, this study is the first human study to examine the relationship between cytokine responses and BMI in latent TB. In addition, a recent study showed that the population attributable fraction of malnutrition for the incidence of TB in a setting of high endemicity, such as India, is about 55% (37). This is an alarming figure and underscores the importance of understanding the biological interplay between nutrition and immune responses. Our data clearly confirm the results of animal model studies that also showed that malnutrition is characterized by defective IFN-γ and TNF-α responses and enhanced TGF-β responses and add additional im-

plications for the role of cytokines in malnutrition-TB comorbidity. Our study provides important insights into the influence of nutrition on the pathogenesis of TB. Our study also provides an impetus to perform longitudinal studies examining the role of immunological biomarkers in the development of TB in malnourished patients, especially the roles of IL-10 and TGF-β as predictors of nutritional influence on TB. Finally, by dissecting the innate and adaptive cytokine responses at homeostasis and after *in vitro* antigen stimulation in malnourished individuals with LTB, our study provides the first direct association of an impaired cytokine response with low BMI in this study population.

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