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# The immune response to tuberculosis infection in the setting of *Helicobacter pylori* and helminth infections

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## SUMMARY

We screened 176 healthy, adult (aged 18–55 years) US refugees from tuberculosis (TB)-endemic countries to evaluate whether cytokine responses to latent TB infection (LTBI) are modified in the setting of concurrent *H. pylori* and helminth infection. As measured by the Quantiferon-TB GOLD interferon-$\gamma$ release assay, a total 38 (22%) subjects had LTBI, of which 28 (74%) also were *H. pylori* seropositive and/or helminth infected. Relative to ten subjects with LTBI only, 16 subjects with concurrent *H. pylori* infection had significantly elevated levels of IFN-$\gamma$, and nine subjects with both *H. pylori* and helminth infection had significantly elevated levels of IFN-$\gamma$, IL-2, IL-13, and IL-5. *H. pylori* is associated with enhanced IFN-$\gamma$ responses to TB, even in the setting of concurrent helminth infection. Efficacy of TB vaccines may vary with the co-existence of these three infections in the developing world.

**Key words**: *Helicobacter pylori*, helminths, immunology, principal components analysis, tuberculosis infection

## INTRODUCTION

A healthy immune response is essential for control of tuberculosis (TB) infection. In the absence of adequate control, 10% of the 2 billion annual cases of TB infection progress to active disease [1, 2]. Although the mechanisms are not fully understood, latent TB infection (LTBI) appears to be maintained by a complex interplay of immune responses that contain the bacteria while limiting pathology [3]. Cytokine signalling plays a critical role in orchestrating a coordinated protective response [4]. IFN-$\gamma$ secretion by CD4+ cells is considered the quintessential T helper 1 (Th1 type) or pro-inflammatory cytokine response associated with control of TB infection, while high levels of interleukin (IL)-4, IL-13 and other T helper 2 (Th2 type) cytokines, as well as expansion of regulatory cytokines, such as IL-10 and TGF-$\beta$, may be associated with progression of latent infection to active disease [5, 6]. The recently characterized class of Th17 cells may also be important during reactivation [7].

A growing number of studies are exploring geographical variations in immunity to TB. Compared to counterparts in industrialized countries, individuals in the developing world exhibit important differences in immunological profiles that can affect response to TB antigens. For example, healthy HIV-negative

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Ethiopians have lower proportions and absolute numbers of naive CD4+ and CD8+ T cells, and correspondingly greater numbers of central and effector memory T cells compared to Dutch counterparts [8]. Compared to UK infants, Malawian infants have a lower interferon-$\gamma$ response to purified protein derivative (PPD) after bacillus Calmette-Guérin (BCG) vaccination [9], and higher levels of Th2 and regulatory type cytokines IL-4, IL-13, and IL-10 [10]. This developing world immune profile may reflect continuous exposure to environmental mycobacteria, helminths, and enteric bacteria, with important implications for explaining the diminished efficacy of BCG vaccines in populations where these exposures are endemic [11], as well as for the accuracy of delayed type hypersensitivity and T-cell based diagnostic tests [12], and immunogenicity of candidate TB vaccines in different populations [13].

In the developing world, *Helicobacter pylori* and helminth infections frequently co-exist with LTBI [14]. Typically acquired before the age of 5 years through crowded and unsanitary conditions, *H. pylori* actively infects about 80% of individuals living in the developing world [15]. Colonization, which causes gastric inflammation with secretion of T-cell derived IFN-$\gamma$ and other Th1 type cytokines [16] as well as a strong systemic antibody response, may persist asymptomatically for life. In an African cohort, we have previously reported that, compared to TB patients, latently infected household contacts who do not progress to TB within 2 years are more than three times as likely to be *H. pylori* seropositive [17]. Infecting over 2 billion people [18], virtually all in the developing world, helminths are classically associated with Th2 type and humoral responses, including induction of IL-4, IL-13 and IL-5 with eosinophilia [19]. Recurrent throughout early life, helminth infections are well-known immune modulators, capable of polarizing responses to TB and other antigens [20]. In controlled studies, treatment of helminth infection has been associated with phenotypic changes in the T-cell compartments [21], as well as alterations in T-cell proliferative responses and in response to BCG vaccine [22]. In rodent models, concurrent helminth infection may also downregulate the inflammatory response to *Helicobacter* infection [23]. Both types of gut infection also invoke T-cell regulatory networks, such as via IL-10 secretion, which may be important in controlling local inflammation and maintaining immune homeostasis while permitting tolerance [24–26]. The disappearance of these gastrointestinal infections in the industrialized world has been associated with increased risk of Th-2 and T-reg modifying conditions such as asthma [27, 28] and autoimmune diseases, respectively [29]. Studies of immune response to TB infection in the setting of concurrent *H. pylori* and worm infection may help elucidate why some infected individuals can control TB infection whereas others cannot.

We previously reported that, in healthy Hispanics living in Northern California, *H. pylori* infection was associated with an enhancement of IFN-$\gamma$ and other Th1-type cytokine responses to TB infection [17]. We now expand on this work in a population with high prevalence of both *H. pylori* and helminth infection.

## METHODS

### Population and study design

The study was cross-sectional in design. Between June 2008 and June 2011, 322 adult refugees (aged 18–55 years) undergoing routine health screening as part of the California refugee resettlement and health screening programme were recruited through the Santa Clara Valley TB Clinic and the San Francisco General Hospital Refugee Medical Clinic. To be enrolled in the study, refugees had to have come from a high TB incidence ($>20/100\,000$ population [30]) country (excluding North America, Australia, Western Europe and New Zealand), and have resided in the USA for $<2$ years. In addition to completing their regular clinic screening, enrollees agreed to complete a health interview, and provide blood (30 $\mu$l) and three stool specimens to test for common infections. We excluded individuals who self-reported major chronic illnesses or immunocompromising conditions, antibiotic use within the previous 6 months; a history of treatment for TB or *H. pylori*; or recent or current ($<3$ months) prescription treatment for allergies, malaria, or intestinal parasites. We also excluded individuals who had clinical laboratory results outside of the laboratory's normal ranges for liver function, blood counts, T-cell subsets, C-reactive protein, or erythrocyte sedimentation rate; or who tested positive for current hepatitis B or C infection. Pregnant or lactating women were also excluded. We elicited history of BCG vaccination and interviewers recorded the presence or absence of a BCG scar. Clinical laboratory tests were conducted by Stanford Hospital and participating clinic laboratories.

Informed consent was obtained from all participants. The study was approved by the institutional review boards of Stanford University, the Santa Clara Valley Medical Centre, and the University of California, San Francisco.

## Infection assessment

All individuals meeting initial health and clinical laboratory guidelines were classified for TB, *H. pylori*, and helminth infections. The following criteria were used to define eight possible infection strata, including a group with none of the target infections.

### *LTBI*

TB infection was defined by results of the in-tube QuantiFERON-TB GOLD test ('QFT', Cellestis Ltd, Australia), which was conducted by study personnel following the manufacturer's recommendations, as described previously [31]. To be considered TB infected, an individual had to be positive by QFT ($\geqslant$0·35 IU/ml); individuals with IFN-$\gamma$ production $<$0·35 ml/IU were considered uninfected. Individuals with indeterminate QFT results ($<$0·5 IU/ml mitogen reading) were excluded from the study.

### H. pylori *infection*

Enzyme-linked immunosorbent assay (ELISA) was used to test for antibodies to high-molecular-weight proteins of *H. pylori* and to the CagA protein, as described previously [32, 33]. Individuals considered *H. pylori* infected were either *H. pylori* or CagA positive, and individuals considered uninfected tested negative on both assays. Sera with equivocal results were considered indeterminate, and the subjects were excluded from the study.

### *Helminth infection*

Helminth infection was ascertained by stool ova and parasite examination as well as by serology for *Strongyloides* (*S. stercoralis*), schistosomiasis, and cysticercosis using commercial ELISA kits (Scimedex, USA). To be considered helminth infected, subjects had to have at least one positive stool examination or a positive *S. stercoralis* antibody test. To be considered helminth uninfected, individuals had to have returned at least two stool samples and all samples needed to be negative for helminths. Additionally, to be classified as helminth-uninfected, serology must have been negative for strongyloides, cysticercosis and schistosomiasis. Individuals with isolated eosinophil counts ($\geqslant$500 cells/$\mu$l) or with schistosomiasis or cysticercosis positive IgG only were excluded from the study.

### *Multiple infections*

Individuals meeting criteria for two or more infections simultaneously were additionally classified as *H. pylori* and LTBI, *H. pylori* and helminth, LTBI and helminth, or *H. pylori*, LTBI, and helminth infected.

### *No infections*

Subjects meeting criteria simultaneously for absence of all three infections were classified as having none of the target infections.

## Cytokine profiling studies

Excess plasma from the QFT assay, including plasma stimulated with the overlapping peptide pool ESAT-6, CFP-10, and TB 7.7, as well as plasma stimulated with the positive control, PHA, and unstimulated (saline) sample, were frozen at $-20\,^{\circ}$C. After thawing, stimulated and unstimulated plasma samples were prepared for Luminex XMAP analysis using a customized human 'Milliplex' kit mixed according to manufacturer's instructions (Millipore, USA). Cytokines included in the customized kit were IFN-$\gamma$, IL-10, IP-10, IL-2, IL-5, IL-13, tumour necrosis factor (TNF)-$\alpha$, and IL-17. Output was read on a Bio-Rad Luminex reader, expressed as pg/ml. The limits of detection for this standardized assay were 3·2 pg/ml and 10 000 pg/ml.

## Statistical analysis

Statistical analysis was performed with SAS v. 9.2 (SAS Institute, USA). Cytokine output was log-transformed for analysis, with values below 1 coded as 1. To preserve negative values, the difference between a subject's antigen-stimulated and unstimulated well was computed as a log (antigen)-log(nil) well. This 'signal-to-noise' ratio normalizes each subject to his/her unstimulated well.

A generalized linear model (GLM) was used to characterize mean log-transformed cytokine levels in a full factorial model, accounting for each of the three infections, and their interactions. Interpretation of *P* values was based on four *a priori* comparisons

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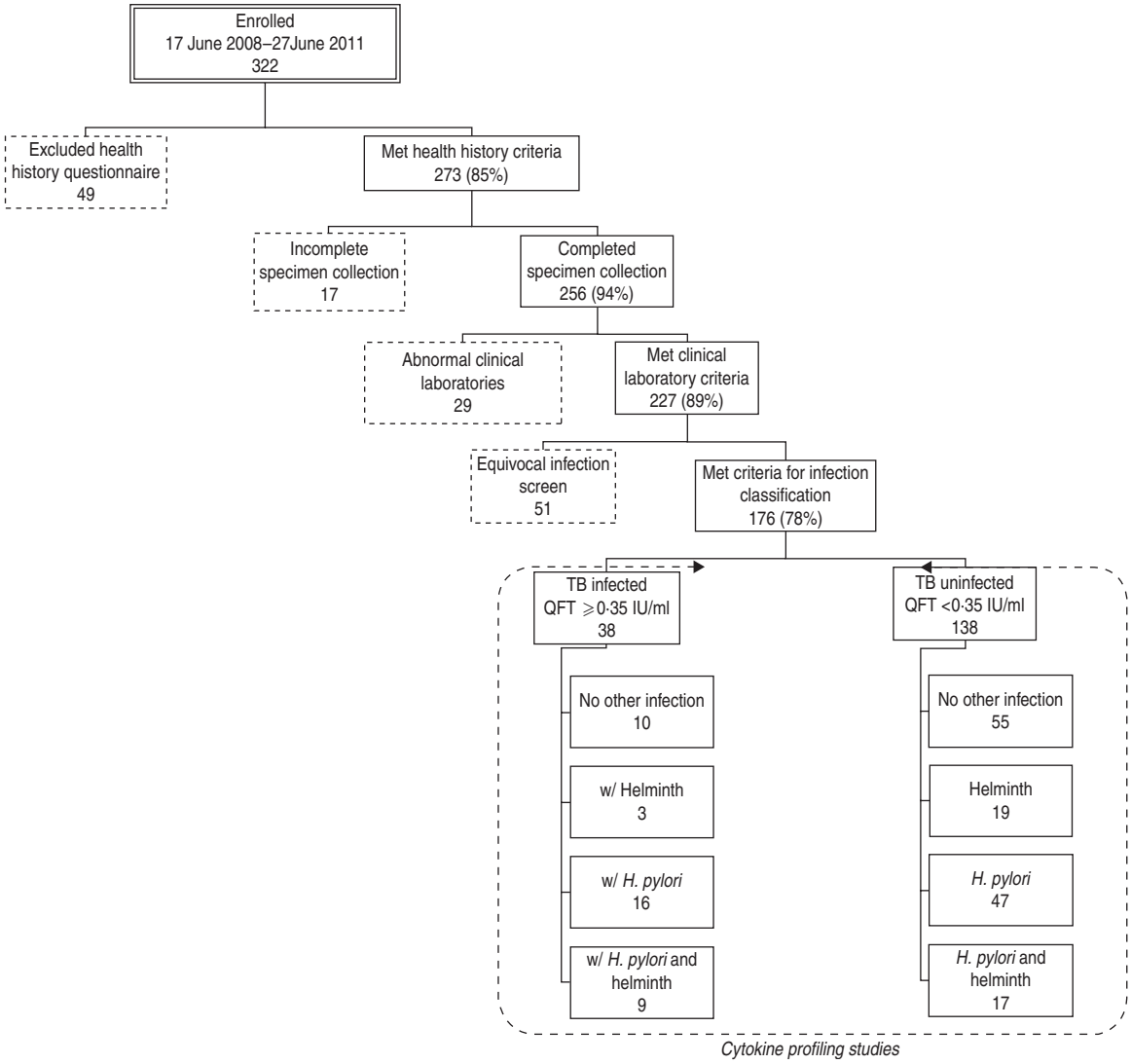


**Fig. 1.** Infection assessment protocol. Flow chart of enrolments, assessments, and classification of infections for cytokine studies. TB, Tuberculosis; QFT, QuantiFERON-TB GOLD in-tube interferon-$\gamma$ release assay.

using the 'LTBI only group' as the reference (Dunnett's test). These comparisons were: LTBI *vs.* no infections; LTBI *vs.* LTBI/*H. pylori* infected, LTBI *vs.* LTBI/helminth infected, and LTBI *vs.* LTBI/*H. pylori*/helminth infected.

Principal components analysis (PCA) [34] was used to characterize the inter-correlation of cytokine responses within the population sample and to elicit clustering profiles. The first two components (eigenvalue $>1$) were selected for graphic representation. A multi-way contingency (log linear) analysis [35] was used to fit the spatial distribution of cases and test (log likelihood ratio test) for inter-independence of the infections. Mean standardized component scores were compared among the eight possible infection strata using a least squares means test.

## RESULTS

### Results of infection assessment

Of 322 enrolled in the study, 227 (71%) met initial health and clinical laboratory screening criteria and were included in the co-infection assessment (Fig. 1). A total 303 (94%) had resided in the USA for $<1$ year. Of the 95 excluded, 49 (52%) were excluded due to self-reported health history, 17 (18%) because of incomplete specimen collection, and 29 (31%) due to abnormal clinical laboratory results. Of the 227 eligible for the infection assessment, 176 (84%) were successfully classified, and 51 (22%) who had equivocal test results for one or more of the chronic infections were excluded. About half (53%) of subjects enrolled during this time period (2008–2011) were

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from Afghanistan or the Middle East, 32% from Asia, 14% from Africa, and 4% from the Americas.

Of the 176 subjects classified for infection profile, 38 (22%) had LTBI as indicated by a positive QFT. LTBI+ and LTBI– subjects did not differ with respect to age, sex, or frequency of markers for hepatitis A, hepatitis B, or helminth infections (Table 1). Subjects with LTBI were somewhat less likely to have an observable BCG scar ($P=0{\cdot}06$), and were more likely to have antibody responses to *H. pylori* ($P=0{\cdot}03$). Helminth infections, 46/48 of which were diagnosed by positive *S. stercoralis* test, were of similar prevalence (32% and 26%) in LTBI+ and LTBI– groups ($P=0{\cdot}50$). Among the 38 subjects with LTBI, 28 (74%) had *H. pylori* and/or helminth infection as well.

Table 1. *Infection profiles in 176 subjects selected for cytokine profiling studies*

| Characteristic | LTBI (QFT ⩾0·35) | No LTBI (QFT <0·35) | *P* value |
|---|---|---|---|
| Total | 38 | 138 | |
| Age (yr), mean (S.D.) | 29·4 (7) | 29·8 (7) | 0·77 |
| Male sex, *n* (%) | 28 (74) | 82 (59) | 0·11 |
| BCG scar observed, *n* (%) | 20 (53) | 95 (69) | 0·06 |
| TST*, *n* (%) | | | |
| ⩾10 mm | 31 (89) | 68 (49) | <0·001 |
| ⩾15mm | 23 (66) | 39 (29) | <0·001 |
| Infection profiles†, *n* (%) | | | |
| *H. pylori* | 25 (66) | 64 (46) | 0·03 |
| HAV | 28 (74) | 88 (64) | 0·25 |
| HepB | 16 (42) | 40 (29) | 0·14 |
| Helminth‡ | 12 (32) | 36 (26) | 0·50 |

LTBI, Latent tuberculosis infection [defined by QuantiFERON-TB GOLD (QFT) interferon-$\gamma$ release assay]; BCG, bacillus Calmette-Guérin; TST, tuberculin skin test.

* 173/176 subjects had concurrent TSTs performed by their refugee clinic.

† See Methods section for infection classifications (HAV, hepatitis A virus antibody; HepB, hepatitis B virus antibody (two individuals missing results).

‡ Only two cases had positive ova and parasite examination (one *A. lumbricoides*, and one mixed *T. trichiuria*, *S. stercoralis* and hookworm), and 46 were ascertained by *S. stercoralis* antibody test, of which five also had elevated eosinophil count.

### Effect of *H. pylori* and helminth infection on IFN-$\gamma$ responses measured by the QFT assay

Concurrent infection with *H. pylori* or *H. pylori* and helminths was associated with significant variation in IFN-$\gamma$ responses to the QFT (Fig. 2). Compared to ten subjects with LTBI only (Fig. 2*b*), mean IFN-$\gamma$ responses were 0·89 log (IU/ml) greater in 16 LTBI subjects with concurrent *H. pylori* infection, and 1·2 log (IU/ml) greater in nine LTBI subjects with both *H. pylori* and helminth infection. Overall, concurrent *H. pylori* infection was associated with a 0·8 log greater IFN-$\gamma$ response to QFT antigens ($P<0{\cdot}001$) accounting for helminth status. As expected, 138 subjects without TB infection had significantly lower IFN-$\gamma$ responses to QFT antigens regardless of co-infection (Fig. 2*a*). The IFN-$\gamma$ difference associated with concurrent LTBI/*H. pylori* infection was unchanged when accounting for hepatitis A virus antibody response ($P=0{\cdot}63$).

*Results of cytokine profiling studies*

Compared to 138 individuals without LTBI, the 38 LTBI+ cases had significantly greater concentrations of the inflammatory cytokines IFN-$\gamma$, IL-2, and chemokine, IP-10, as well as significantly greater concentrations of IL-5, and IL-13 (Fig. 3). Levels of the regulatory cytokine IL-10 ($P=0{\cdot}57$), the inflammatory cytokine, TNF-$\alpha$ ($P=0{\cdot}20$), and IL17 ($P=0{\cdot}10$) were not significantly different between LTBI+ and LTBI– subjects (data not shown). Within the LTBI group, and adjusting for all possible interactions, concurrent infection with *H. pylori* (without helminths) was associated with significantly higher levels of IFN-$\gamma$ ($P=0{\cdot}03$) compared to the ten subjects with isolated LTBI, while concurrent *H. pylori* and helminth infection was associated with higher levels of IFN-$\gamma$ ($P<0{\cdot}001$), IL-2 ($P=0{\cdot}005$), IL-5 ($P<0{\cdot}001$), and IL-13 ($P<0{\cdot}001$), and a trend towards higher levels of TNF-$\alpha$ ($P=0{\cdot}05$). Compared to subjects with *H. pylori*/LTBI infection, subjects with all three infections had significantly greater levels of IL-5 and IL-13 ($P<0{\cdot}01$ in each case), while IFN-$\gamma$ levels did not differ ($P=0{\cdot}27$). Levels of IP-10 and IL-10 did not vary with concurrent *H. pylori* or helminth infection.

### *H. pylori* infection is associated with enhanced Th1-type response to TB infection, even in the setting of concurrent helminth infection

PCA (Table 2) elicited a mixed Th1/Th2-type population profile. The first component ('mixed IFN-$\gamma$ axis') explained 45% variance and consisted of IFN-$\gamma$

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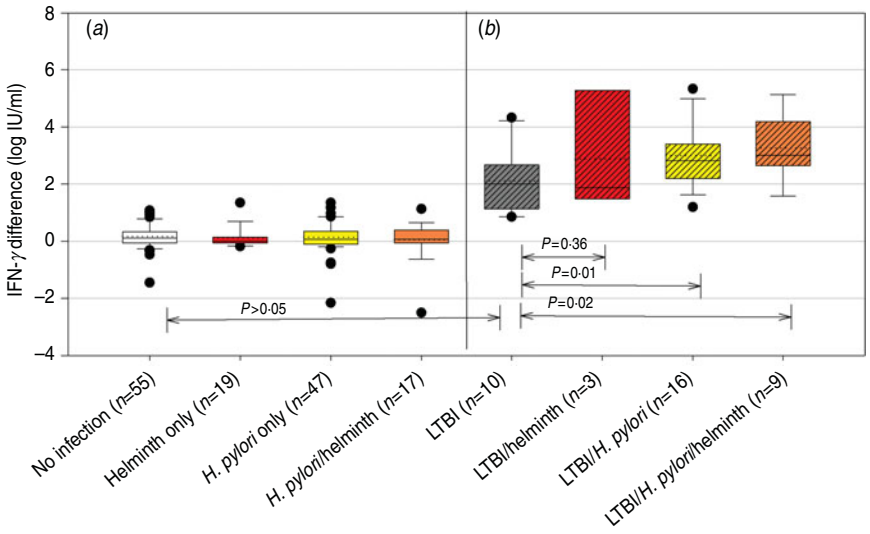


**Fig. 2.** IFN-$\gamma$ responses to QuantiFERON-TB GOLD in-tube (QFT) interferon-$\gamma$ release by infection profile. QFT+, IFN-$\gamma$ response $\geqslant$0·35 IU/ml. ——, Median response; ·····, mean response. Group mean differences evaluated by general linear model accounting for all possible interactions. *P* values interpreted by Dunnett's test using isolated latent tuberculosis infection (LTBI) [box 1 in panel (*b*)] as comparator group to test for differences *vs*. other groups in panel (*a*) (no LTBI) and panel (*b*) (LTBI with *H. pylori* and/or helminth co-infection).

and cytokines correlated with IFN-$\gamma$ in this population, including inflammatory markers IL-2, and IP-10, and non-inflammatory markers, IL-5 and IL-13. The second component explained an additional 18% of total variance and consisted of IL-10, a regulatory marker, and the inflammatory marker, TNF-$\alpha$ ($r=0{\cdot}40$). A third component, accounting for an additional 11% of variance, represented IL-17. This cytokine pattern did not vary appreciably when altering the mix of infections included in the PCA.

Of the 38 LTBI cases, 35 (92%) had an elevated mixed IFN-$\gamma$ axis score, including 21 (60%) who were dominant on the first component (Fig. 4). Of those dominant on the mixed IFN-$\gamma$ axis, 14 (67%) were also *H. pylori* infected ($n=10$) or *H. pylori* infected with helminths ($n=4$). In a log-linear model fitting the distribution of cases with respect to dominance on the IFN-$\gamma$ axis (LR 5·14, $P=0{\cdot}82$), *H. pylori* was significantly associated with LTBI ($P=0{\cdot}037$) after controlling for isolated LTBI ($P<0{\cdot}001$) and an independent effect of helminth infection ($P=0{\cdot}001$). Compared to individuals with isolated LTBI and accounting for helminth infection, mixed IFN-$\gamma$ axis scores were about 2·3-fold greater in subjects with concurrent *H. pylori* infection ($P=0{\cdot}003$), while second component scores were not significantly different (Fig. 5). In addition, subjects with triple infection had higher mixed IFN-$\gamma$ axis scores than subjects with *H. pylori*/TB co-infection ($P<0{\cdot}001$). Thus, the frequency of concurrent *H. pylori* infection in those with LTBI, within a population where concurrent helminth infections were also relatively common, strongly influenced the overall pattern both qualitatively and quantitatively of cytokine responses to TB antigens.

## DISCUSSION

The interaction of *H. pylori*, helminth, and *M. tuberculosis* infections provides an informative model for investigating the properties of immune homeostasis in the developing world, with important implications for vaccine and diagnostic research. In this geographically diverse group of healthy adults from high-incidence TB countries, isolated TB infection was comparatively rare, while mixed infection was common. We found that subjects infected with *H. pylori* infection had significantly higher IFN-$\gamma$ and other Th1-type inflammatory responses to specific TB antigens, even in the presence of helminth infections. This finding complements our previous observations in a US Hispanic population, where cross-sectionally, *H. pylori* was also associated with dominant Th1-type cytokine responses to TB infection [17].

To our knowledge, this is the first epidemiological study attempting to look at a three-way interaction of these three developing world infections. In separate

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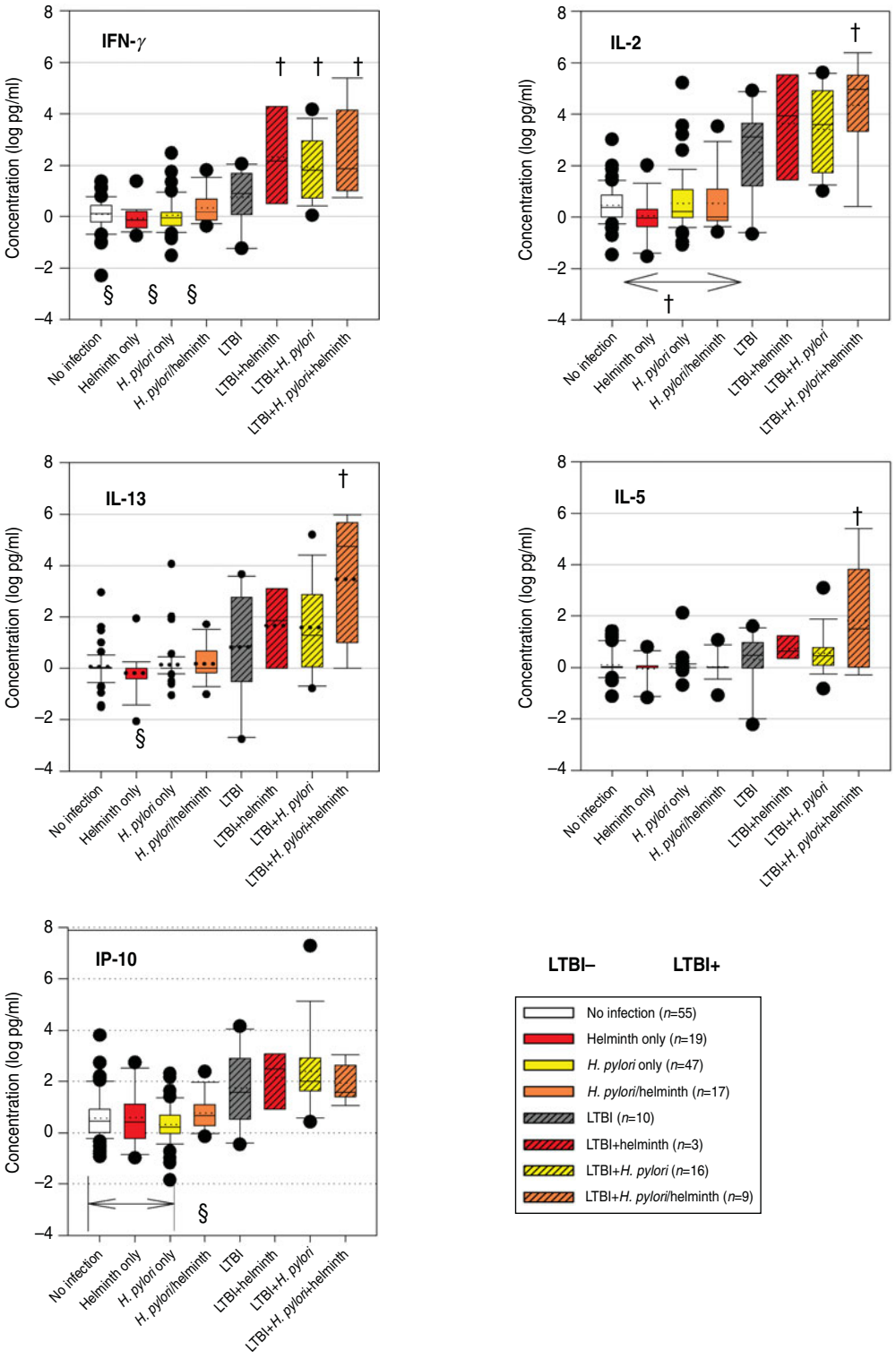


**Fig. 3.** Significant cytokine responses to TB antigens in different infection strata as measured by Luminex (multiplex) assay. Cytokine levels were measured in supernatant stimulated 1 day with overlapping peptides ESAT-6, CFP-10, and TB (7.7) as preserved from the QuantiFERON-TB GOLD assay, with values log-transformed for analysis. ——, Median response. ·····, mean response. Group mean differences evaluated by general linear model accounting for all possible interactions. *P* values interpreted by Dunnett's test using isolated latent tuberculosis infection (LTBI) (box 5 in panels) as comparator group to test for differences *vs.* other groups (†, $0 \leqslant P < 0{\cdot}05$; §, $0{\cdot}05 \leqslant P < 0{\cdot}10$). IFN-$\gamma$, interferon-$\gamma$; IL-2, interleukin 2; IP-10, IFN-$\gamma$ inducible protein 10; IL-5, interleukin 5; IL-13, interleukin 13. Not shown: TNF-$\alpha$, tumour necrosis factor-$\alpha$; IL-10, IL-17 ($P > 0{\cdot}10$).

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Table 2. *Summary of principal components analysis*

| Functional category | Cytokine | Correlation with IFN-$\gamma$* | Contribution to principal component (multiple correlation coefficient) PC1 (eigenvalue 3·6) | PC2 (eigenvalue 1·5) | PC3 (eigenvalue 0·89) |
|---|---|---|---|---|---|
| Cumulative variance explained (%) | | | 45 | 63 | 74 |
| Inflammatory | IFN-$\gamma$ | 1·00 | 0·48 | −0·013 | 0·12 |
| | IL-2 | 0·78 | 0·45 | −0·14 | −0·15 |
| | IP-10 | 0·56 | 0·33 | 0·18 | −0·37 |
| | TNF-$\alpha$ | 0·21 | 0·18 | 0·55 | −0·35 |
| Anti-inflammatory or regulatory | IL-5† | 0·53 | 0·40 | −0·24 | −0·112 |
| | IL-13† | 0·66 | 0·45 | −0·23 | 0·03 |
| | IL-10 | 0·07 | 0·07 | 0·69 | 0·012 |
| Th17 | IL17 | 0·50 | 0·26 | 0·25 | 0·83 |

PC, Principal component; eigenvalue, unitized value of variation explained by each component.
* Pearson's correlation coefficient (for logged values).
† IL-5 and IL-13 were correlated at $r = 0{\cdot}77$.

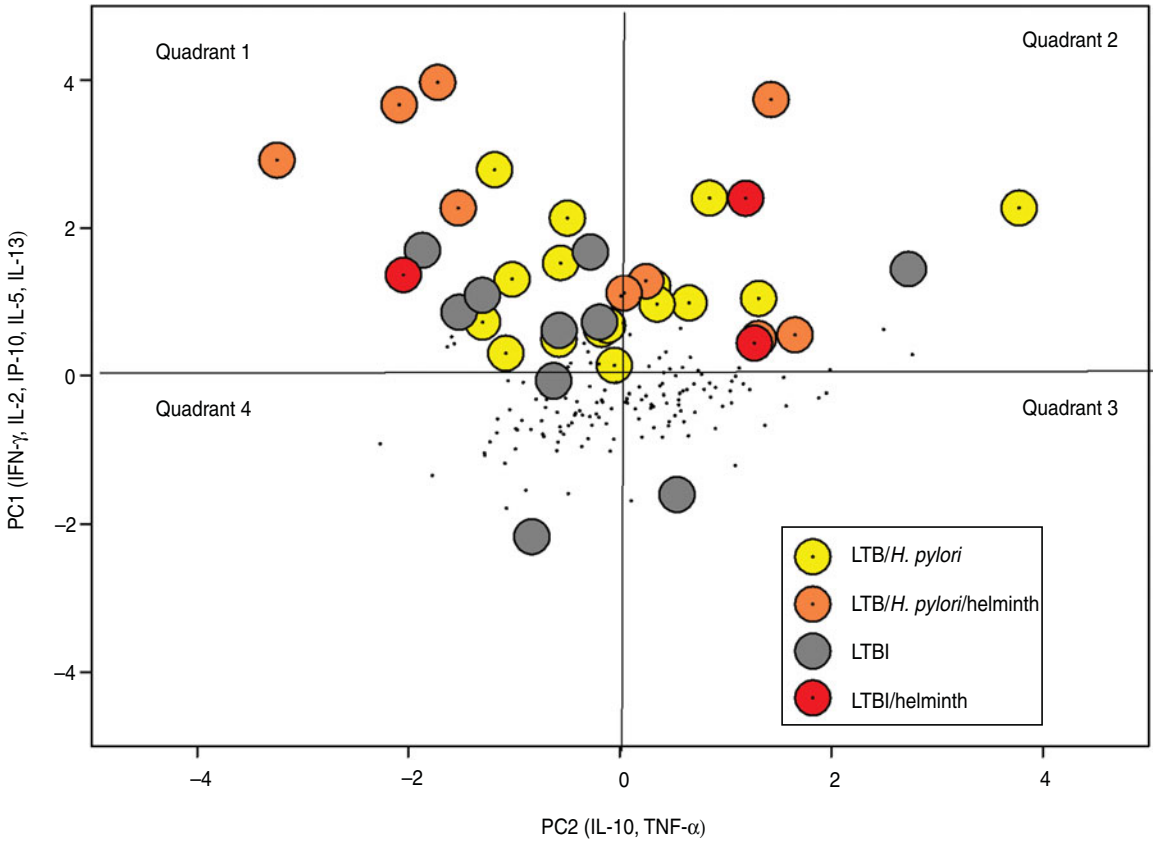


**Fig. 4.** Scatterplot of first two principal components, illustrating distribution of scores by quadrant. All circular symbols, latent tuberculosis infection (LTBI): yellow circle with dot: LTBI/*H. pylori*; orange circle with dot: LTBI/*H. pylori*/helminth; small scattered dots: cases without LTBI.

studies, helminths and *H. pylori* infection have been associated with immunosuppressive [36] and inflammatory responses [17] responses to TB antigens, respectively. However, in this mixed infection population, many individuals with higher IFN-$\gamma$ responses to TB antigens also had higher levels of IL-13 and

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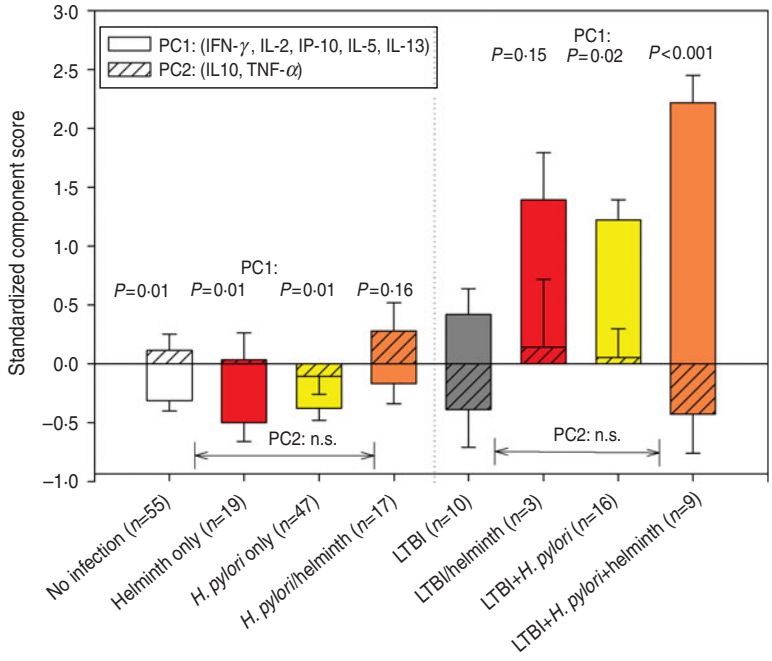


**Fig. 5.** Comparison of mean standardized component scores by infection profile. PC1, First principal component (clear boxes); PC2, second principal component (hatched boxes). Differences evaluated by least squares means test with Dunnett's test for multiple comparisons.

IL-5, as well as of the Th1 markers IL-2 and IP-10, and individuals with all three infections had accentuated Th1-type responses, while Th2-type responses did not differ. This 'mixed Th1 + Th2' signature in response to TB antigens has been previously reported in developing world populations [10]. In these populations, it has been suggested that a helminth-primed Th-2 background or a Th1 response pre-boosted by environmental mycobacteria may help explain the variable geographical efficacy of BCG vaccines, particularly nearer the equator [10–11, 37]. Such a finding may be due to specific host factors or to non-specific environmental factors that participate in regulation of immune responses to specific chronic infections. Although we used a rigorous set of algorithms to exclude concomitant medical conditions and to define the three infections, this does not exclude a background of other immune activators to which the population has been 'primed'.

An IFN-$\gamma$ driven Th1-type response is known to be essential (but not necessarily sufficient) for maintenance of latency [38]. Although our PCA failed to elicit an orthogonal Th1/Th2 matrix, over half of healthy TB-infected individuals were dominant on an axis representing cytokines correlated with IFN-$\gamma$. The correlation of IL-5 and IL-13 with IFN-$\gamma$ was not altered by the mix of infection profiles included in the PCA, and appears to depend on the interaction of LTBI and *H. pylori* infection, as well as independently on helminth infection. In addition, subjects with isolated LTBI had significantly lower IFN-$\gamma$ correlated component scores than latently infected individuals with *H. pylori* or *H. pylori* and helminth infection. Among latently infected individuals with concurrent *H. pylori* infection, first component scores were more than twofold greater after accounting for helminth infection. In contrast with our previous report [17], HAV antibody response was not a significant predictor of IFN-$\gamma$ responses in this more complex model. We hypothesize that *H. pylori* is one, if not an exclusive, marker for enteric infections of early life that heighten protective Th1-type responses to TB infection. In the setting of concurrent *H. pylori*/helminth infection, immunosuppressive Type 2 responses to TB antigens, such as IL-5 and IL-13, may also be heightened but without diminishing the magnitude of Type 1 pro-inflammatory responses. Such a profile may be controlled by more complex regulatory networks involved in tolerance to each infection than the simple Th1/Th2 dichotomy embraces [34].

Our results are consistent with the view that the baseline response to TB antigens, including response to TB vaccines, is 'primed' by the host's microbial environment. Some have suggested that in 'mixed Th1 + Th2' type populations, a BCG (Th1 type) boost

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vaccine may not provide additional protection beyond that of natural environmental stimuli, and that manipulation of regulatory and Th2-type responses may be more critical [13]. If, as has been suggested for environmental mycobacteria [39], *H. pylori* infection, uniquely or as a proxy for chronic enteric infections, represents a pre-existing Th1-type 'boost,' then response to IFN-$\gamma$ based vaccines may be expected to vary with this marker. In this setting, the role of helminth-like infections is complex: in the absence of mixed Th1 + Th2-type infection, responses to TB antigens may appear to be depressed, while in the presence of mixed Th1 + Th2 infection, they may appear to be enhanced. These considerations argue for strong characterizations of prevalent infections in vaccine candidate populations.

Some important caveats about this study include its cross-sectional design and constraints on statistical power exacted by a full factorial analysis. Cross-sectional epidemiological studies cannot establish mechanisms, direction of effect, or reproducibility of results. Small cell sizes for some subgroups can result in spurious statistical results. Although we screened extensively for helminth infections by serology and ova and parasite examination, the overwhelming majority of individuals classified as helminth-infected met this definition by virtue of *S. stercoralis* IgG serology. This may reflect the fact that presumptive anti-helminthic treatment is now widely used in pre-departure US refugee programmes [40], although the standard regimen may be less effective against *Strongyloides* [41]. Although *S. stercoralis* IgG is thought to reflect active disease, serology does not distinguish active from past infection. In support of seropositive subjects harbouring living worms, subjects were not eligible for analysis if they reported any anti-helminthic treatment in the previous 3 months, and all subjects were from regions of the world where helminth infections are endemic. All subjects determined by their clinic to be seropositive for *S. stercoralis* were treated with ivermectin. In addition, responses to the stereotypic helminth cytokines, IL-5 and IL-13, were pronounced in those classified as helminth-infected. Similarly, elevated levels of TB antigen-induced IL-5 and IL-13 were observed in Malawi infants, a helminth-endemic region [10]. For these reasons, it is probable that *S. stercoralis* antibody responses reflected current infection. Nonetheless, we cannot exclude the possibility that individuals classified as helminth-infected had past, as opposed to current, infection, in which case the study's findings reduce to concurrent *H. pylori* infection in a population with probable, recent exposure to helminths.

In addition, we defined LTBI according to responses to the QuantiFERON-TB GOLD, and the multiplex analysis used supernatant from the proprietary QFT kit. Although this added coherence and specificity to our analysis, many of the QFT negatives had positive tuberculin skin test (TST) results. On the other hand, because concordance of positive QFT with a positive TST was high (89%), the results of the PCA were not altered appreciably by substituting the TST for the QFT in the definition of LTBI [42]. An important strength of the study was our algorithm-based approach to health and infection classification. While this approach resulted in the exclusion of many individuals, it also reduced immunological heterogeneity associated with other underlying illnesses or medications, and attempted to focus the analysis on individuals with healthy, or normative, responses to the three infections of interest. Although we cannot rule out misclassification bias, such error would tend to bias results towards no difference.

There is growing evidence that chronic gastrointestinal infections are important regulators of the inadequate immune responses associated with TB progression as well as with many emerging diseases in the developed world. We have observed that in a population with exposure to both Th1- and Th2-type modifying infections, *H. pylori* infection is associated with enhanced Th1-type responses to TB antigens, even in the setting of concurrent helminth infection. This effect may be due to the synergy of responses to Th1-modifying infections as well as to reciprocal regulatory pathways induced in populations with high burden of infectious diseases [43].

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## DECLARATION OF INTEREST

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