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Comparison of interferon-γ-, interleukin (IL)-17- and IL-22-expressing CD4 T cells, IL-22-expressing granulocytes and proinflammatory cytokines during latent and active tuberculosis infection

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Summary

In this study, we investigated the role and expression of T helper type 17 (Th17) cells and Th17 cytokines in human tuberculosis. We show that the basal proportion of interferon (IFN)-γ-, interleukin (IL)-17- and IL-22-expressing CD4$^+$ T cells and IL-22-expressing granulocytes in peripheral blood were significantly lower in latently infected healthy individuals and active tuberculosis patients compared to healthy controls. In contrast, CD4$^+$ T cells expressing IL-17, IL-22 and IFN-γ were increased significantly following mycobacterial antigens stimulation in both latent and actively infected patients. Interestingly, proinflammatory IFN-γ and tumour necrosis factor (TNF)-α were increased following antigen stimulation in latent infection. Similarly, IL-1β, IL-4, IL-8, IL-22 and TNF-α were increased in the serum of latently infected individuals, whereas IL-6 and TNF-α were increased significantly in actively infected patients. Overall, we observed differential induction of IL-17-, IL-22- and IFN-γ-expressing CD4$^+$ T cells, IL-22-expressing granulocytes and proinflammatory cytokines in circulation and following antigenic stimulation in latent and active tuberculosis.

Keywords: active tuberculosis, human, interleukin 17, interleukin 22, latent tuberculosis

Introduction

Human tuberculosis (TB) is primarily a disease of the lungs caused by an obligatory intracellular pathogen, *Mycobacterium tuberculosis*. The majority of infected individuals do not develop clinical disease yet bacteria can persist, resulting in a state of latent infection [1]. Latency requires a balanced interaction between host immunity and bacterial pathogenicity. It is well established in both animals and humans that the T helper (Th) cell type 1 cytokines interleukin (IL)-12 and interferon (IFN)-γ play a crucial role in controlling mycobacterial infection [2,3]. Th17 cells, a newly identified subset of Th cells, have been shown to play an important role in tuberculosis [4,5]. IL-17 is primarily a proinflammatory cytokine secreted by Th17 cells. It acts on a variety of cell types, including epithelial cells and fibroblasts, resulting in the secretion of cytokines [IL-6, IL-8, granulocyte–macrophage colony-stimulating factor (GM-CSF)], chemokines (CXCL1, CXCL10) and metalloproteinases, which in turn attract neutrophils at the site of infection [4,6,7]. Differentiation of Th17 cells is mediated by IL-6, transforming growth factor (TGF)-β and IL-21 [8–10], whereas IL-23 induces further development and amplification of differentiated Th17 cells, thus promoting secretion of IL-17 from this cell type [4,11]. IL-17 has been implicated in many inflammatory diseases, including rheumatoid arthritis, multiple sclerosis, asthma and systemic lupus erythematosus [12–14].

The role of Th17 cytokines in tuberculosis has recently been investigated. IL-17 plays a key role in early neutrophil-mediated pulmonary inflammatory responses, T cell-mediated IFN-γ production and granuloma formation in the lung in response to infection with bacillus Calmette–Guérin (BCG) [15,16]. Studies in IL-23- and IL-12/23-deficient mice have highlighted the importance of the role played by the IL-23/Th17 pathway in immune responses against mycobacterial infection [2,17]. Furthermore, IL-17 accelerates memory Th1 responses in vaccinated mice infected subsequently with *Mycobacterium tuberculosis* [15]. IL-22, a member of the IL-10 family of cytokines, is also produced by Th17 cells [13,18]. It acts primarily on non-immune cells, as IL-22R is not expressed on immune cells [19]. IL-22 plays a protective role during inflammation of various tissues, including liver, intestine and heart [20–22], perhaps by inducing the release of anti-microbial agents such as β-defensin-2 and proinflammatory molecules belonging to

Table 1. Demographic data of subjects in the study.

	Healthy controls	Latent TB	Active TB
No. of participants	11	24	9
Age (mean)	32.09	40.04	52.13
Age range	20–57	19–82	26–77
Gender (% male, % female)	37.5, 62.5	37.5, 62.5	50, 50
Ethnicity	6 Caucasians, 2 Asians, 1 Hispanic, 2 Indo-Canadians	8 Caucasians, 7 Asians, 4 East Europeans, 5 Africans	3 Caucasians, 3 Asians, 1 Afghanistan, 2 Africans

TB, tuberculosis.

the S100 family of calcium-binding proteins [18]. In contrast to IL-17, the role of IL-22 in tuberculosis is not well defined; however, in patients with active tuberculosis (TB), elevated levels of IL-22 in bronchoalveolar lavage specimens have been reported [23].

IL-17 has also been shown to mobilize, recruit and activate neutrophils [24] which appear early during mycobacterial infection. The role of granulocytes in tuberculosis is not clear, but reports suggest that they release chemokines to recruit monocytes and contribute to granuloma formation [25,26]. The lack of neutrophils during the early stages of infection increases bacterial burden in infected tissues because of decreased production of TNF-α, IL-1 and IL-12 [27]. Moreover, neutrophils directly affect mycobacterial killing activity by releasing anti-microbial peptides such as cathelicidin LL-37 and lipocalin-2 [28]. In addition to a protective response, neutrophils may be involved in the destructive immune responses in active tuberculosis [29,30]. Mice infected with the virulent strains of *M. tuberculosis* exhibited formation of granulomas with lymphopenic and granulocytic lesions which resulted ultimately in the death of the host [29]. Furthermore, IL-27-deficient mice infected with mycobacteria succumbed to death due to hyperinflammatory responses when granulomatous lesions have abundant neutrophils [30].

To gain insight into the involvement of Th17 cells, we measured basal levels of IL-17/IL-22 expressing lymphocytes and granulocytes and secretion of proinflammatory cytokines including IL-17 and IL-22 in circulation as well as following peripheral blood mononuclear cell (PBMC) stimulation with mycobacterial antigens in individuals with both latent and active stages of disease. Our results show a differential induction of IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells, IL-22-producing granulocytes and proinflammatory cytokines in circulation and following antigenic stimulation during latent and active tuberculosis.

Subjects and methods

Study subjects and specimen collection

All participants were recruited between May 2008 and March 2009 at the Ottawa Hospital, Ontario, Canada. The participants were classified into three groups, namely healthy controls, latent TB and active TB. The demographic data including age, gender and ethnicity are listed in Table 1. Eleven participants who were tested negative to tuberculin skin test (TST), which was defined as having induration of ≤5 mm, were considered to be healthy controls. Twenty-four participants with latent TB infection were diagnosed by positive TST (induration ≥10 mm) without any clinical and radiological evidence of active disease. Nine active TB patients were diagnosed on the basis of positive acid-fast bacilli staining and culture from sputum, bronchoalveolar lavage or lymph nodes. Two patients had extrapulmonary tuberculosis (TB lymphadenitis and cystitis). None of the latent TB individuals had any active infections at the time of blood acquisitions. All participants with latent and active TB infection were enrolled prior to receiving medication for tuberculosis. All participants were HIV seronegative. Informed consent was given by all participants based on the study protocol, which was approved by the Research Ethics Boards of the Ottawa Hospital Research Institute. The peripheral heparinized blood (20–30 ml) was collected and used for whole blood cytokine assay and for PBMC intracellular cytokine assay.

Antigens, antibodies and cytokines

M. bovis culture filtrate (CF) was a gift from Dr Bryan D. Rennie (Health Canada, Ottawa, Ontario, Canada). This culture filtrate is 99% identical to *M. tuberculosis.* Phorbol 12-myristate-13 acetate (PMA) (Sigma-Aldrich, St Louis, MO, USA) and ionomycin (Invitrogen, Burlington, Ontario, Canada) were used to stimulate cells as a positive control in a whole blood assay. The following antibodies were used for surface and intracellular staining: anti-human-CD3-fluorescein isothiocyanate (FITC), IFN-γ-FITC, CD8-phycoerythrin (PE)-Texas Red (ECD), CD14-ECD (Beckman Coulter, Mississauga, Ontario, Canada); CD4-allophycocyanin and cyanin (APC Cy7), CD8-PE, CD25-PE, IL-22-PE, IL-17-APC Cy7 (R&D Systems, Minneapolis, MN, USA) and CD15-FITC (Sigma-Aldrich). Anti-CD4-APC Cy7 antibody listed above was used in all experiments gating for CD4 T cells. Up to five antibodies were used in each experiment. The antibody combinations used in each panel included:

Panel 1: IFN-γ-FITC, IL-22-PE, CD8-ECD, IL-17-APC, CD4-APC-Cy7
Panel 2: CD3-FITC, CD25-PE, CD8-ECD, IL-17-APC, CD4-APC-Cy7
Panel 3: CD15-FITC, IL-22-PE, CD14-ECD, IL-17-APC, CD3-PC7

The following recombinant human cytokines and TLR ligands were used to stimulate isolated granulocytes: IL-1β (10 ng/ml), IL-6 (10 ng/ml), TGF-β (4 ng/ml), IL-23 (20 ng/ml), IL-17 (25 ng/ml) (R&D Systems), zymosan (10–100 μg/ml) (InvivoGen, San Diego, CA, USA), poly I:C (1–10 μg/ml) (InvivoGen) and cytosine–guanine dinucleotide (CpG) (1 mM) (Cell Sciences, Canton, MA, USA).

Whole blood assay

Heparinized whole blood was usually received from TB clinics in the late afternoon. Blood was then kept overnight at room temperature on a rocker. Whole blood (1 ml) was cultured the next day in the morning at 37°C, 5% CO_2 in 24-well tissue culture plate with or without PMA (50 ng/ml)/ionomycin (1 μg/ml) for 4 h in the presence of BD GolgistopTM (BD Biosciences, Mississauga, Ontario, Canada). The whole blood (40 μl) was incubated with saturating concentration of appropriate fluorochrome-labelled antibodies. Cell fixation, permeabilization and RBC lysis were performed using Intraprep™ permeabilization solution (Beckman Coulter), as described by the manufacturer. Generally, 20 000 leucocytes were acquired. Cells were analysed by Cytomics FC 500 MPL (Beckman Coulter) using CXP Analysis software.

Intracellular cytokine assay

PBMCs (1×10^6 cells/ml) isolated from peripheral blood by centrifugation on Ficoll-Hypaque Plus (Amersham Bioscience, Pittsburgh, PA, USA) were cultured in RPMI-1640 medium (Invitrogen) containing 10% serum at 37°C in 24-well tissue culture plate with or without mycobacterial culture filtrate (5 μg/ml) for 7 days. BD Golgistop™ was added 4 h prior to the cell staining. Cultured PBMCs (100 μl) were incubated with appropriate fluorochrome-labelled antibodies to surface molecules for 15 min at room temperature in the dark. Stained cells were washed with phosphate-buffered saline (PBS) containing 0·1% sodium azide and 0·5% fetal bovine serum (FBS). Cells were then fixed and permeabilized with Hanks's buffered salt solution containing 4% paraformaldehyde and 0·1% saponin for 15 min and subsequently washed twice with PBS containing 0·1% saponin, 0·1% sodium azide and 0·5% FBS. Fluorochrome-labelled anti-cytokine antibodies were then added. Cells were washed again after 15 min incubation and suspended in 300 μl of 1% paraformaldehyde in PBS. IL-17$^+$, IL-22$^+$ and IFN-γ$^+$ CD4$^+$ T cells were quantified by flow cytometry using CXP analysis software.

Cytokine quantitation

For cytokine quantitation, supernatants were collected from 7-day-old *M. bovis*-stimulated and -unstimulated PBMC cultures. Serum was collected from the blood samples obtained from 11 healthy TST non-responders, 21 individuals with latent TB infection and nine patients with active TB infection. Cytokine levels were measured using the FlowCytomix human Th1/Th2 11plex kit, IL-17A and IL-22 simplex kits (Bender Medsystems GmbH, Vienna, Austria), as per the manufacturer's instructions. The detection limit for IFN-γ, IL-17A, IL-22, IL-8, IL-6, TNF-α, IL-1β, IL-4, IL-5, IL-10, IL-2, IL-12p70 and TNF-β were 1·6, 2·5, 43·3, 0·5, 1·2, 3·2, 4·2, 20·8, 1·6, 1·9, 16·4, 1·5 and 2·4 pg/ml, respectively. Data were analysed using FlowCytomix™ Pro 2·3 software.

Granulocyte stimulation assay

Granulocytes were isolated from peripheral blood by gradient centrifugation using Polymorphoprep™ (PN1114683; Axis-Shield, Oslo, Norway), according to the procedure described by the manufacturer. The isolated granulocytes were 95% pure and contained 1–3% CD3$^+$ T cells. Granulocytes (2×10^6/ml) were stimulated with PMA/ionomycin or Toll-like receptor (TLR) ligands [10–100 μg/ml zymosan, 1–10 μg/ml poly I:C, 0·1–1 μg/ml lipopolysaccharide (LPS) or 1 mM CpG] or cytokine cocktails (10 ng/ml IL-1β + 20 ng/ml IL-23, 4 ng/ml TGF-β + 10 ng/ml IL-6 + 20 ng/ml IL-23 or 25 ng/ml IL-17) for 24 h in 24-well plates. Cell pellets were collected for RNA extraction.

RNA isolation and semiquantitative reverse transcription–polymerase chain reaction (RT–PCR) analysis

RNA was extracted from 2×10^6 granulocytes by using RNeasy Kit (Qiagen) as described by the manufacturer. RNA was reverse-transcribed to cDNA using MultiScribe RT (Applied Biosystems, Streetsville, Ontario, Canada). cDNA was then amplified using *Taq*Man Universal PCR Master Mix (Applied Biosystems). Primers for IL-17 (product number: Hs99999082_m1), IL-22 (Hs00220924_m1) and β-actin (Hs99999903_m1) genes were purchased from Applied Biosystems. The fold increase in signal relative to the controls was determined with the change in cycling threshold ($\Delta CT_{sample} - \Delta CT_{control}$) and was calculated as follows: $R = 2 - {}^{(Ct\ sample - Ct\ control)}$, where R is relative expression and Ct is cycle threshold. β-actin was used as an endogenous control.

Statistical analysis

Statistical analysis was performed using Prism software (GraphPad) version 2.7.2. Two-tailed *P*-values were calcu-

lated using Wilcoxon test, Fisher's exact test and non-parametric one-way analysis of variance (ANOVA), as indicated in various figure legends. Because the data are not distributed normally, the non-parametric Kruskal–Wallis test with Dunn's post-test was performed. The receiver operating characteristic (ROC) cut-off values were generated using sensitivity and specificity values with GraphPad prism software.

The area under the curve of a ROC curve is related closely to the Mann–Whitney or Wilcoxon's rank test, which test whether positives are ranked higher than the negatives. As the data are not distributed normally (non-Gaussian), a non-parametric Fisher's exact test was used to generate a ROC curve to create a cut-off in order to identify TB patients based on the presence of IL-17, IL-22 and IFN-γ-positive $CD4^+$ cells compared to the healthy controls.

Results

Circulating levels of IFN-γ-, IL-17- and IL-22-expressing $CD4^+$ T cells are lower in patients with active TB

The circulating levels of IFN-γ-, IL-17- and IL-22-expressing $CD4^+$ T cells in whole blood were determined by intracellular cytokine assay. The frequencies of IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells were found to be lower in active TB patients compared to healthy controls and latent TB subjects (Fig. 1). The gating strategy employed for the identification of IL-17-, IL-22- and IFN-γ-expressing cells is shown (Fig. S1). Due to high variability, the data were analysed using cut-off values. The ROC curve was used to generate the cut-off values maximizing the sensitivity and specificity for predicting the true positives and true negatives within the healthy, latent TB and active TB patient group. In this study, patients with active TB exhibited minimal levels of IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells, the cut-off values for which were calculated as 2·5, 3 and 4, respectively. Individuals with values above these were identified as positive responders. Hence, 50% of healthy controls demonstrated positive IFN-γ responses compared to only 11% of individuals with latent infection and 0% for individuals with active TB infection ($P = 0{\cdot}02$). Similar results were observed for IL-17- and IL-22-producing $CD4^+$ T cells with *P*-values of 0·03 for both groups. One individual with active TB had a very high proportion of IL-17-producing $CD4^+$ T cells (83·2%), which was excluded from analysis due to suspected systematic error. Four out of 10 latent TB individuals co-expressed elevated proportions of $IL\text{-}17^+$ CD4 T cells and $IL\text{-}22^+$ CD4 T cells.

Circulating levels of IL-22-expressing granulocytes are lower in individuals with latent and active infection

Because Th17 cells produce IL-17 and IL-22 and recruit neutrophils to the site of inflammation [18,31], we determined if circulating neutrophils also produce IL-17 and IL-22. As neutrophils comprise approximately 90% of granulocytes, we measured the expression of IL-17 and IL-22 in total granulocytes. The granulocytes were gated according to size and granularity using forward-scatter and side-scatter by flow cytometry (Fig. 2a, left panel). $CD4^-CD8^-$ cells were then gated from the granulocyte-enriched cell population (Fig. 2a, middle panel) and analysed for IL-17 and IL-22 expression (Fig. 2a, right panel). The intracellular IL-22 was detected in a significant proportion of granulocytes from healthy individuals (20–90%). However, intracellular IL-17 was not detected in granulocytes from normal controls and individuals with latent and active TB infection (data not shown). The proportion of IL-22-expressing granulocytes was significantly lower in individuals with latent and active TB infection compared to healthy controls ($P = 0{\cdot}02$; Fig. 2b). IL-22 expression in pure granulocytes isolated from blood was confirmed by counterstaining with another granulocyte marker CD15 (data not shown). To confirm whether IL-22 is transcribed in granulocytes, IL-22 mRNA expression was evaluated at the mRNA level by quantitative real-time PCR (qPCR) in granulocytes isolated from three healthy individuals. Granulocytes were either unstimulated or were stimulated with PMA for 4, 24 and 48 h. Surprisingly, IL-22 mRNA was not detected in unstimulated granulocytes after isolation. However, IL-22 was induced in granulocytes stimulated with PMA and ionomycin (Fig. 2c) with the peak expression at 24 h post-stimulation.

IL-17-, IL-22- and IFN-γ-expressing $CD4^+$ T cells are induced in individuals with latent and active TB infection following stimulation with mycobacterial antigens

To determine whether antigen-specific $CD4^+$ T cells in latent and active TB subjects produce IL-17, IL-22 and IFN-γ in response to mycobacterial antigens, PBMC were stimulated with mycobacterial culture filtrate for 7 days prior to analysis of intracellular cytokines. The induction of cytokine expressing cells was calculated as a percentage increase following stimulation with mycobacterial antigens compared to the unstimulated cells. The increase in IFN-γ ($P = 0{\cdot}008$)-, IL-17 ($P = 0{\cdot}008$)- and IL-22 ($P = 0{\cdot}031$)-expressing $CD4^+$ T cells following mycobacterial antigens stimulation was significantly higher in individuals with active TB infection compared to healthy controls (Fig. 3). In latent TB patients, mycobacterial stimulation increased the number of IFN-γ expressing cells significantly ($P = 0{\cdot}004$); however, a significant increase in IL-17- and IL-22-expressing cells was not observed (Fig. 3). The magnitude of increase in induction of IFN-γ-, IL-17- and IL-22-expressing cells before and after stimulation with mycobacterial antigens is also shown (Fig. S2). The results suggest that although the proportion of IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells in whole blood is significantly low in individuals with active TB

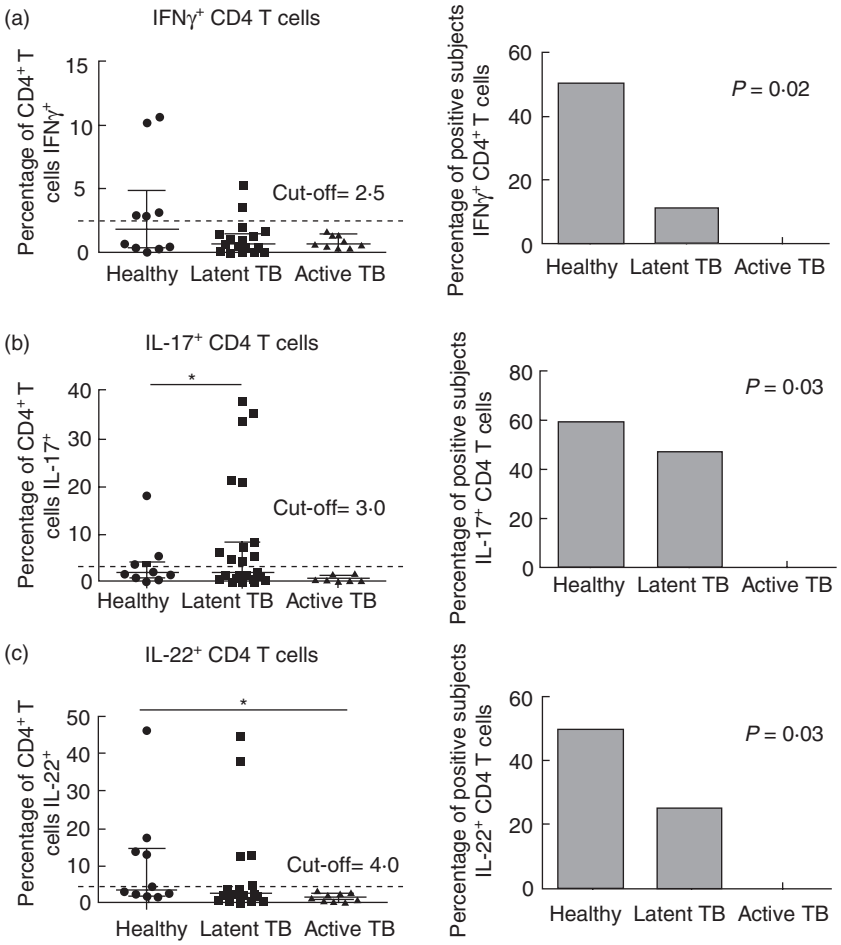

Fig. 1. The frequency of interferon (IFN)-γ-, interleukin (IL)-17- and IL-22-expressing CD4$^+$ T cells are lower in tuberculosis (TB)-infected subjects. Left panel: the percentage of IFN-γ-, IL-17- and IL-22-expressing CD4$^+$ T cells of each participant is shown in (a), (b) and (c), respectively. The broken horizontal lines represent the cut-off values of each test. The cut-off value is generated using receiver operating characteristic (ROC) curves for (a), (b) and (c), which are 2·5, 3 and 4, respectively. The ROC cut-off values are based on optimal sensitivity and specificity values between each group. In (a), the sensitivity values between healthy *versus* latent TB and healthy *versus* active TB subjects was 0·88 and 1, respectively, whereas the specificity value between both the groups was 0·5. In (b), the sensitivity values between healthy *versus* latent TB and healthy *versus* active TB subjects was 0·52 and 1, respectively, whereas the specificity value between both the groups was 0·4. In (c), the sensitivity values between healthy *versus* latent TB and healthy *versus* active TB subjects was 0·75 and 1, respectively, whereas the specificity value between both the groups was 0·5. Left panel group comparisons were performed with Wilcoxon tests. Right panel: based on the cut-off values, the percentage of subjects expressing IFN-γ, IL-17 and IL-22 on CD4$^+$ T cells was compared for the healthy ($n = 10$), latent ($n = 21$) and active TB groups ($n = 9$). The *P*-values are calculated by Fisher's exact tests. *$P < 0·05$. All other combinations of groups are not significant, with *P*-values greater than 0·05.

infection (Fig. 1), mycobacteria-specific IFN-γ-, IL-17- and IL-22-expressing CD4$^+$ T cells can be induced readily in individuals with latent and active TB infection (Fig. 3).

Proinflammatory IL-17, IL-22 and IFN-γ are up-regulated following mycobacterial stimulation in individuals with latent TB infection

To understand further the role of Th17 cytokines in innate and acquired immunity in tuberculosis, we measured the concentration of IL-17, IL-22 and IFN-γ following stimulation of PBMCs with mycobacterial antigens. Upon stimulation, IL-17 and IL-22 were up-regulated, but not significantly, in individuals with latent TB infection. However, these cytokines were not induced following antigenic stimulation in individuals with active TB infection or in healthy controls (Fig. 4). Similarly, IFN-γ levels were increased significantly following mycobacterial stimulation of PBMCs from individuals with latent TB infection compared to the healthy controls ($P = 0·03$; Fig. 4). IFN-γ levels in the supernatants of mycobacterium-stimulated PBMC were 10 times higher in individuals with latent TB infection compared to the corresponding values in healthy individuals. The levels of IFN-γ were higher in supernatants of mycobacterium-stimulated PBMCs compared to the unstimulated cells from individuals with active

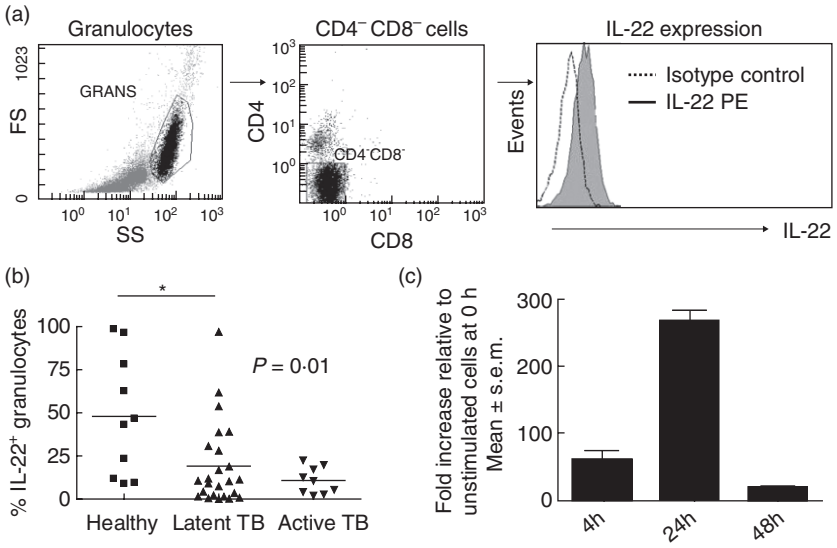

Fig. 2. Circulating levels of interleukin (IL)-22-expressing granulocytes are lower in individuals with latent and active infection. Cells enriched in granulocytes were gated according to size and cytoplasmic structure (forward-scatter and side-scatter; a, left panel) and further gated on $CD4^-CD8^-$ cells (a, middle panel). These cells express IL-22 as detected by flow cytometry (a, right panel). The percentage of IL-22-expressing $CD4^-CD8^-$ granulocytes is shown in (b) ($P = 0{\cdot}01$). The percentage of IL-22-producing granulocytes is greater in healthy controls than in latent and active tuberculosis (TB). IL-22 mRNA expression [quantitative polymerase chain reaction (qPCR)] in unstimulated and phorbol myristate acetate (PMA)-stimulated granulocytes (4, 24, 48 h) isolated from healthy controls ($n = 3$) (c). Data were analysed using the Kruskal–Wallis test and each possible pair was analysed further using Dunn's multiple comparison test. $^*P < 0{\cdot}05$. All other combinations of groups are not significant, with *P*-values greater than 0·05.

TB infection, which needs to be confirmed in a larger study (Fig. 4).

Proinflammatory TNF-α is up-regulated following mycobacterial stimulation of lymphocytes from individuals with latent TB infection alone

Significant IL-8 induction was not observed following antigenic stimulation in latent and actively infected TB individuals (Fig. 5). There was an increase in IL-6 production, although not statistically significant, following mycobacterial stimulation of PBMCs from healthy individuals as well as with latent and active TB infection (Fig. 5). Although IL-1β and TNF-α were also up-regulated in response to mycobacterial stimulation of PBMCs in individuals with both latent and active TB infection, significant TNF-α induction to an extent of 10–20-fold was found in individuals with latent TB infection compared to those from healthy controls ($P = 0{\cdot}01$; Fig. 5). Moreover, levels of IL-12p70, IL-2, IL-4 and TNF-β in the culture supernatants obtained from mycobacterium-stimulated PBMCs did not show any change over unstimulated samples in any of the study groups (data not shown).

Circulating levels of IL-22, IL-6 and TNF-α are higher in serum of individuals with both latent and active TB infection, whereas IL-1β and IL-8 are higher in serum of individuals with latent TB infection

Corresponding to the increased IL-6 and TNF-α induction in antigen-activated PBMCs, serum concentrations of TNF-α ($P = 0{\cdot}001$) and IL-6 ($P < 0{\cdot}0001$) were found to be significantly higher in both groups of TB infection compared to the healthy controls (Fig. 6). Interestingly, high levels of IL-22 were also detected in the serum samples of individuals with latent ($P = 0{\cdot}002$) and active TB infection ($P = 0{\cdot}003$) compared to healthy controls (Fig. 6). IL-1β concentrations in serum of individuals with latent TB infection were increased significantly compared to healthy individuals ($P = 0{\cdot}02$). The levels of IL-1β were also higher in individuals with active TB infection but were not statistically significant. Significantly elevated levels of IL-8 were detected in the serum of individuals with latent TB infection only. Mean IL-8 concentrations were significantly higher in latent TB group compared to healthy controls ($P < 0{\cdot}0001$). However, the levels of IL-8 were higher but not statistically significant in individuals with active TB infection when compared to healthy individuals (Fig. 6); there was no difference in the circulating levels of IL-17, IFN-γ (Fig. 6), IL-12p70, IL-2 and TNF-β (data not shown) in serum samples of healthy, latent and active TB subjects.

Among Th2 cytokines, IL-4 levels are increased in serum samples of individuals with latent and active TB infection

The mean levels of IL-4 in serum of individuals with latent and active TB infection were significantly higher ($P = 0{\cdot}02$) than the levels found in healthy subjects (Fig. 6). Levels of IL-5 and IL-10 cytokines were below the detection limit in

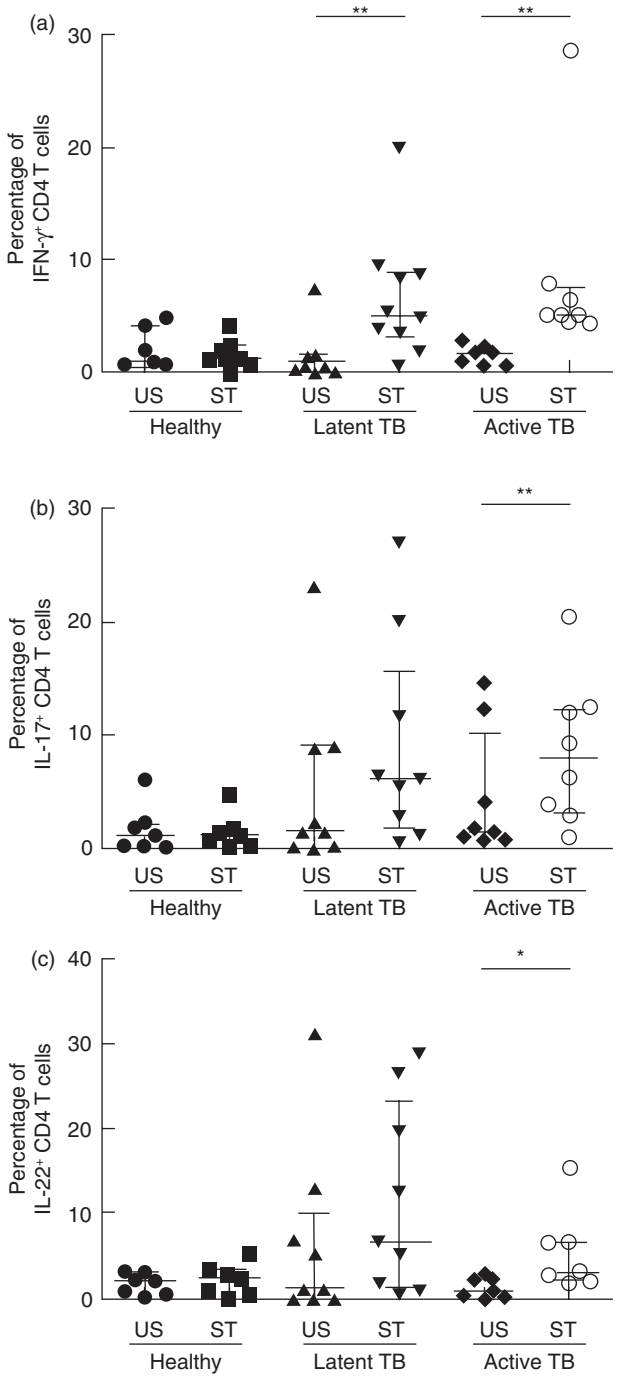

Fig. 3. Interleukin (IL)-17-, IL-22- and interferon (IFN)-γ-expressing $CD4^+$ T cells are induced in individuals with active tuberculosis (TB) infection following stimulation with mycobacterial antigens. Peripheral blood mononuclear cells (PBMCs) (1×10^6/ml) were cultured in the presence or absence of mycobacterial culture filtrate for 7 days. Intracellular IFN-γ (a), IL-17 (b) and IL-22 (c) expression in $CD4^+$ T cells was detected by flow cytometry. (a,b,c) Percentage frequency of IFN-γ^+ ($n = 7$), IL-17^+ ($n = 10$) and IL-22^+ ($n = 8$)-expressing $CD4^+$ T cells, respectively. US, unstimulated group; ST, stimulated group. Data were analysed by the Kruskal–Wallis test. Each possible pair was analysed further by Dunn's multiple comparison tests. All other combinations of groups are not significant, with *P*-values greater than 0·05. $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$.

both antigen-stimulated PBMC culture supernatants as well as in serum samples in all three groups of individuals (data not shown).

Discussion

The present study demonstrates differential induction of IFN-γ-, IL-17- and IL-22-expressing $CD4^+$ T cells in circulation and following specific stimulation with mycobacterial antigens in TST-negative healthy controls, TST-positive latent and active TB subjects. While the expression of IFN-γ and other cytokines has been analysed in human plasma and PBMC supernatants *ex vivo* [32,33], the levels of IL-17- and IL-22-expressing $CD4^+$ T cells and granulocytes in the whole blood of TB patients is not well reported. Herein, we show that the percentage of individuals with active TB expressing IL-17-, IL-22- and IFN-γ-producing $CD4^+$ T cells were decreased significantly compared to the individuals with latent TB infection and healthy controls (Fig. 1). However, such differences were not found in $CD8^+$ T cells (data not shown). The reasons for the decreased IFN-γ-, IL-17- and IL-22-expressing $CD4^+$ T cells in the circulation remain unclear. The differential expression of cytokines in circulation and in affected tissues such as lungs, spleen and lymph nodes have been described in tuberculosis [23,34]. It is possible that antigen-specific IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells are recruited to the affected tissues by chemokines released by infected resident macrophages and dendritic cells. One potential explanation is that IL-17-producing $CD4^+$ T cells are needed to maintain the integrity of granulomas, and thus these cells might migrate out of circulation to the affected tissues [35]. It is also possible that mycobacterial infection itself suppresses Th1, IL-17- and IL-22-producing $CD4^+$ T cells or increases Th2 and regulatory T cells, which may limit the protective immune responses. IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells in individuals with active TB infection can be induced by mycobacterial antigens (Fig. 3). Although not significant, a greater number of mycobacteria-specific IL-17- and IL-22-producing $CD4^+$ T cells compared to the unstimulated cells were found in the latent group than in the active TB group. Although more numbers of patients need to be examined, differential IFN-γ, IL-17 and IL-22 responses could potentially improve our ability to distinguish between latent and active TB infection particularly when a clinical diagnosis is not straightforward [36].

We have shown for the first time that IL-22 is expressed in granulocytes. Interestingly, while intracellular IL-22 protein could be detected, IL-22 mRNA was undetectable in the resting granulocytes. PMA/ionomycin stimulation induced the expression of both IL-22 mRNA as well as intracellular

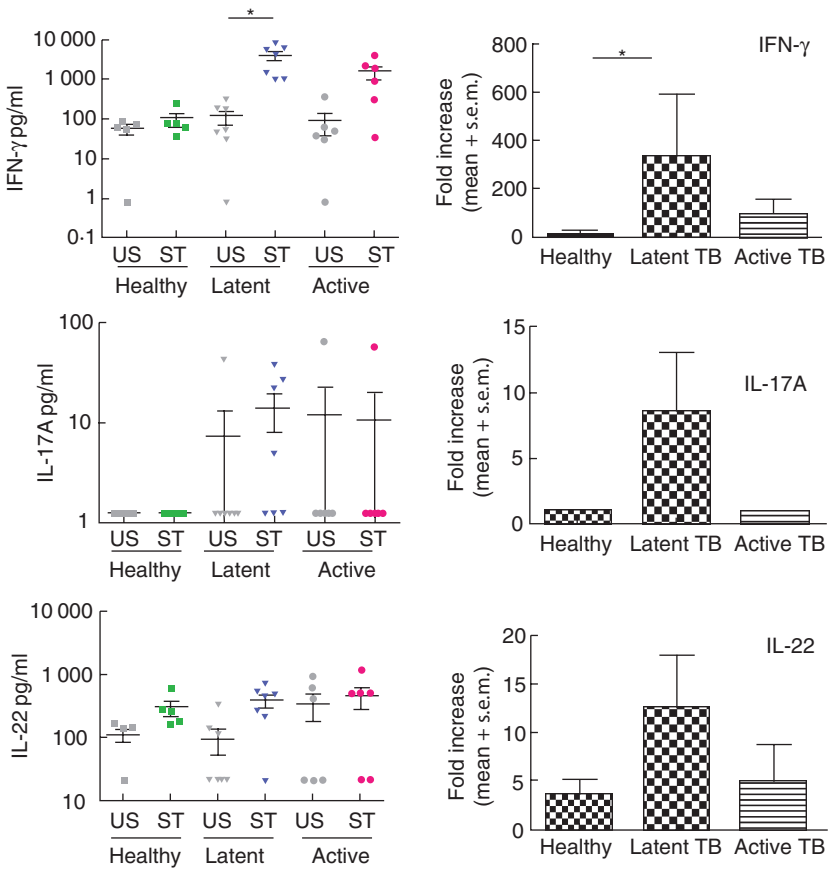

Fig. 4. Proinflammatory interleukin (IL)-17, IL-22 and interferon (IFN)-γ are up-regulated following mycobacterial stimulation in individuals with latent tuberculosis (TB) infection. Purified peripheral blood mononuclear cells (PBMCs) from healthy and *Mycobacterium tuberculosis*-infected subjects were cultured in the presence of *M. bovis* culture filtrate and supernatants were collected after 7 days. Cytokine levels were measured using human T helper type 1 (Th1)/Th2 cytokine assay kit by flow cytometry. A mean ± standard error of the mean value among each group has been shown. Left panel shows the cytokine levels from individual subjects among the groups. Right panel shows the fold increase over unstimulated controls in healthy controls ($n = 11$), latent ($n = 21$) and active TB patients ($n = 9$). Data are analysed using the Kruskal–Wallis test. Each possible pair is analysed further by Dunn's multiple comparison test. All other combinations of groups are not significant, with P-values greater than 0·05. *$P < 0·05$.

IL-22 protein in granulocytes. The presence of IL-22 protein in the absence of detectable mRNA is not a unique phenomenon, as other cytokines such as IL-4 [37], IL-8 [38], macrophage-inflammatory protein 2 (MIP-2) [39], granules and chemokines are also preformed and released rapidly upon stimulation of granulocytes [40,41]. In fact, constitutive expression of MIP-2 mRNA in bone marrow was shown to give rise to peripheral neutrophils with preformed MIP-2 protein [39].

Surprisingly, IL-22-expressing granulocytes in the peripheral blood were found to be higher in healthy controls than in latent TB individuals and even more so in active TB patients. This may be due to localization of IL-22-producing granulocytes in affected tissues. It is also possible that *M. tuberculosis* may affect the expression of IL-22 *in vivo* by inhibiting the synthesis of IL-22. Further studies are needed to investigate IL-22 gene regulation in neutrophils. Although the biological functions of IL-22 have been studied [22,42–45], the regulatory pathway for IL-22 expression is not well characterized. Our preliminary results suggest that neither pathogen-associated molecular patterns including TLR-2, TLR-4 and TLR-9 nor cytokines such as IL-6 and TGF-β, which are known to induce Th17 differentiation [8–10]-induced IL-22 expression in granulocytes (data not shown).

We performed comprehensive analysis of a large number of cytokines (IL-1β, IL-2, IL-5, IL-6, IL-8, IL-4, IL-10, IL-12, IL-17, IL-22, IFN-γ, TNF-α and TNF-β) following mycobacterial stimulation of PBMCs and in a set of serum samples from individuals with latent and active TB infection. Our results show clearly that individuals with latent TB infection express differentially a number of proinflammatory and immunoregulatory cytokines. The proinflammatory cytokines such as IFN-γ and TNF-α were increased significantly following antigen stimulation of PBMC from individuals with latent TB infection alone. Although IL-17 and IL-22 were also induced in antigen-stimulated PBMCs from individuals with latent TB infection, this induction was not statistically significant. In contrast, none of the cytokines, including IL-1β, IL-6, IL-8, IL-4, IL-17, IL-22, IFN-γ or TNF-α, were induced significantly following antigen stimulation of PBMC from active TB patients. The reason for high levels of these cytokines in latent infection is not clear. It is likely that macrophages infected with mycobacteria in individuals with active TB infection may inhibit the production of proinflammatory cytokines to promote their own survival. Age-related immune senescence [46] has been reported, which may possibly explain the low levels of these cytokines in active TB patients, as the average age of individuals in the active TB group is higher than that of the latent TB group in our study. Nevertheless, we did not observe a differential cytokine expression when data were analysed based on age group (data not shown). The significance of

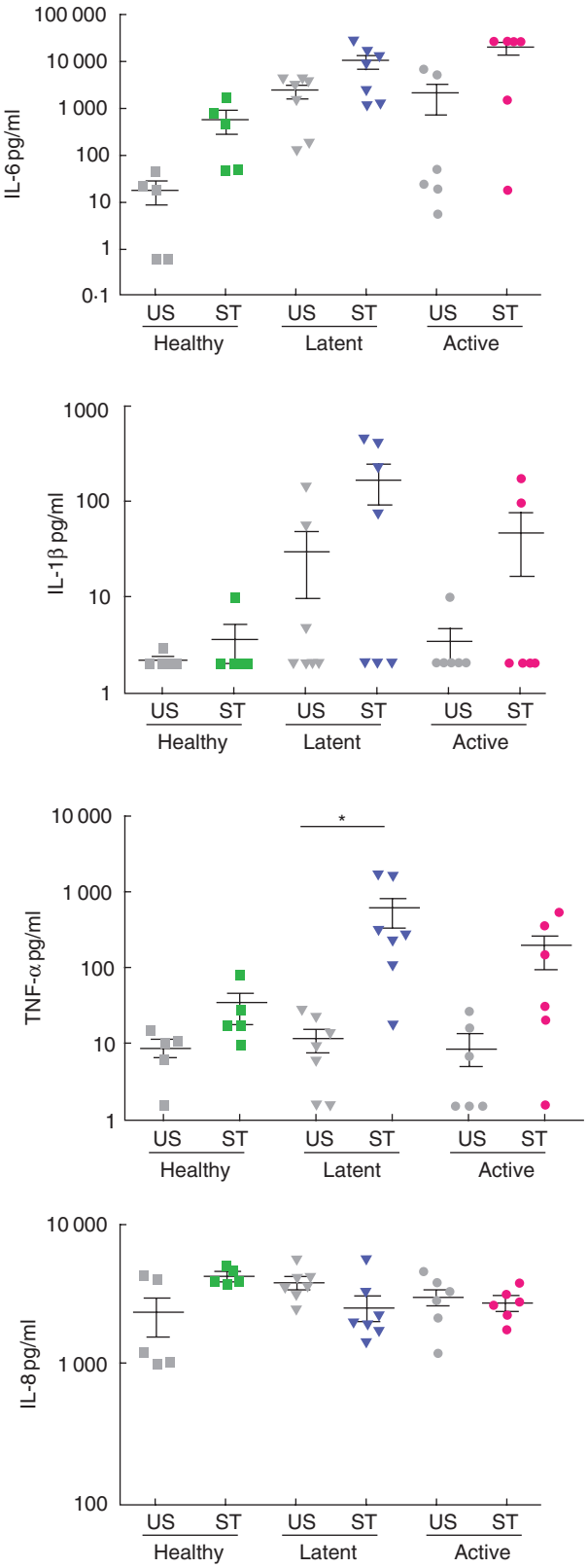

Fig. 5. Secretion of interleukin (IL)-8, IL-6, tumour necrosis factor (TNF)-α and IL-1β in culture supernatants upon stimulation with mycobacterium culture filtrate. Purified peripheral blood mononuclear cells (PBMCs) from healthy latent and active tuberculosis (TB) subjects were cultured in the presence of *M. bovis* culture filtrate and supernatant was collected after 7 days. Cytokine levels were measured using human T helper type 1 (Th1)/Th2 cytokine assay kit by flow cytometry. A mean ± standard error of the mean values among each group has been shown. Data are analysed using the Kruskal–Wallis test. Each possible pair is analysed further by Dunn's multiple comparison tests. US, unstimulated group; ST, stimulated group. All other combinations of groups are not significant, with *P*-values greater than 0·05. $^*P < 0{\cdot}05$.

differential expression of these cytokines in latent and active TB subjects is not clear. Although the expression of these cytokines in latent infection is highly significant, higher numbers of individuals with latent and active TB infection need to be examined to confirm these results.

Our results show clearly that proinflammatory cytokines including IL-6, IL-22 and TNF-α were increased significantly in the serum of individuals with both latent and active TB infection, whereas the levels of IL-1β and IL-8 increased in individuals with latent TB infection. We have also observed that PBMCs from both individuals with latent and active TB infection constitutively express high levels of IL-8. High levels of IL-8 expression in serum may be attributed to several factors. Monocytic cells infected with mycobacteria as well as phenotypically immature monocytes are known to secrete high levels of IL-8 in addition to IL-1β, IL-6 and TNF-α [47]. Mycobacteria-infected monocytic cells also induce IL-8 secretion from pulmonary epithelial cells during the early stages of infection [47,48]. Furthermore IL-1β and IL-6 are known to augment IL-8 expression by epithelial cells [48]. These observations, coupled to the fact that IL-8 is produced by several cell types such as lymphocytes, neutrophils, epithelial cells and endothelial cells [49], may explain our observations of significant IL-8 induction in serum of individuals with latent TB infection and high levels of IL-8 in serum of active TB infection.

The proinflammatory IL-1β, IL-6, IL-8 and TNF-α cytokines are also involved in the regulation and differentiation of the Th17 pathway [8,10,24]. IL-1β and IL-6 regulate Th17 differentiation, whereas IL-8 and TNF-α are secreted from cells stimulated by IL-17 [50]. Although the levels of IL-1β, IL-6, IL-8 and TNF-α are increased in individuals with latent TB infection, serum IL-17 was not found to be different in TB patients and healthy controls in this study. Because Th17 cells are increased significantly following antigen stimulation in TB patients, it is possible that IL-17 expression is increased locally at the lesion site. It is also possible that, like other cytokines [51,52], IL-17 may not be detectable because of its short half-life in serum and body fluids.

It is not clear why latent and active TB-infected individuals respond differently to mycobacterial antigens. It is possible

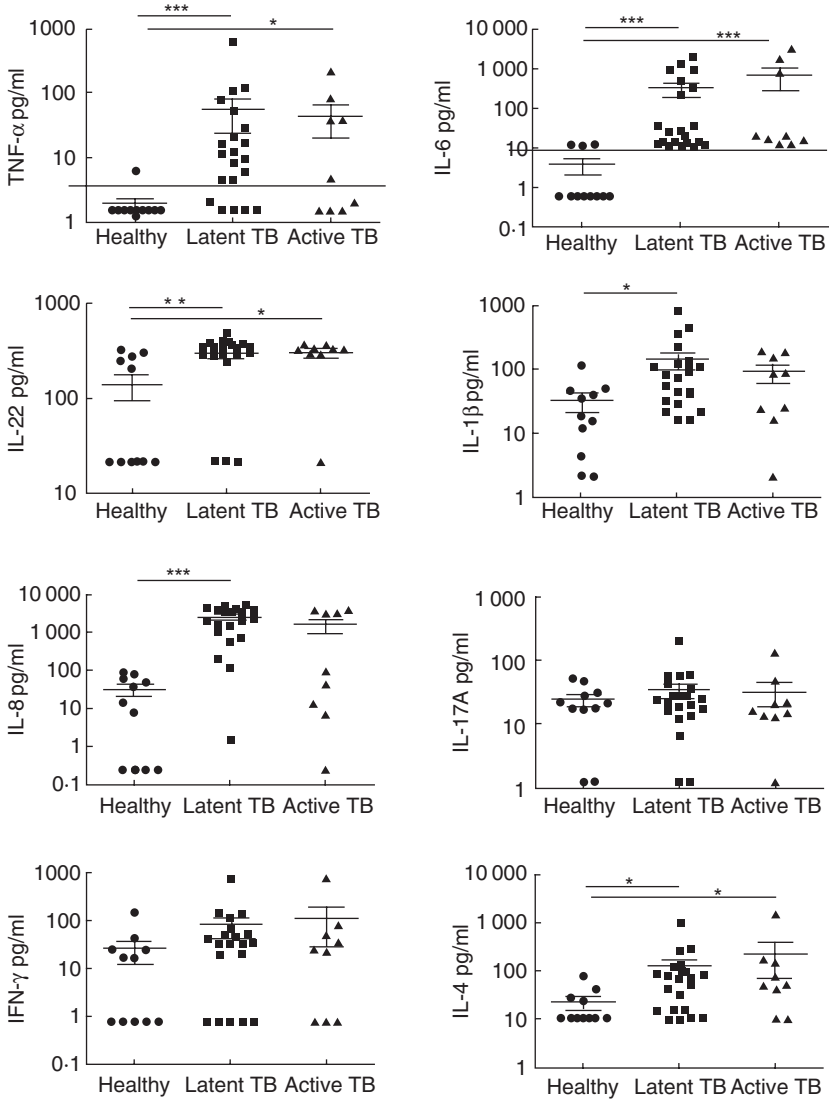

Fig. 6. Serum levels of interferon (IFN)-γ, interleukin (IL)-17A, IL-22, IL-8, IL-6, tumour necrosis factor (TNF)-α, IL-1β and IL-4 in subjects with latent tuberculosis (TB), active TB and healthy controls. Cytokines were measured using human T helper type 1 (Th1)/Th2 cytokine assay kit by flow cytometry. Data are analysed using the Kruskal–Wallis test. Each possible pair is analysed further by Dunn's multiple comparison test or Mann–Whitney *U*-test. Results are shown as mean ± standard deviation. All other combination of groups are not significant, with *P*-values greater than 0·05. $^{*}P < 0{\cdot}05$; $^{**}P < 0{\cdot}01$; $^{***}P < 0{\cdot}001$.

that circulating IFN-γ-, IL-17- and IL-22-producing $CD4^+$ T cells in individuals with latent and active TB infection recognize different antigens in mycobacterial culture filtrate. Mycobacterium expresses different antigens at different stages of the disease [53–56]. For example, during latent TB infection, *M. tuberculosis* is in non-replicating or very slow replicating dormancy stage [57,58], wherein *dosR* regulon gene and 48 *dosR*-regulated genes [53] and 230 genes of enduring hypoxic response [59] are turned on. In contrast, in acute infection, bacteria express early secreted antigens such as Ag85A, Ag85B and early secreted antigenic target-6 (ESAT-6) [60]. Therefore, it is likely that the antigens in mycobacterial culture filtrate used in the present study that induce IL-17 are different from those that induce IL-22 in $CD4^+$ T cells. Greater induction of antigen-specific IL-22-producing $CD4^+$ T cells may be required in active stage to protect tissue damage as shown in the acute liver injury model [22].

Th1/Th2 cytokine balance has been shown to play a major role in the pathogenesis of tuberculosis. Among the Th2 cytokines, only IL-4 serum levels were found to be significantly high in latent and active TB patients. IL-4 production in individuals progressing to active TB has been reported [61,62]. The possible reason for the high levels of IL-4 in the serum of TB patients and its significance is not clear. *M. tuberculosis* itself may induce the expression of IL-4 as its cell wall lipoglycan, ManLAM, has been shown to induce expression of IL-4, TNF-α, IL-1β and IL-6 [63]. IL-4 has the potential to reactivate disease by suppressing the induction of nitric oxide, an important host defence molecule against *M. tuberculosis* [64]. It is likely that high levels of IL-4 expression alone may not be sufficient to induce reactivation and may require other Th2 and immunosuppressive cytokines or factors [65].

In summary, we observed a differential expression of IL-17- and IL-22-producing $CD4^+$ T cells and IL-22-producing granulocytes in human tuberculosis, along with mycobacterium-specific induction of these IL-17- and IL-22-producing $CD4^+$ T cells in culture, thus supporting the

involvement of Th17-specific cytokines during pathogenesis of tuberculosis. Differential induction of these cytokines by mycobacterial antigens during latent and active tuberculosis might be investigated in future for differential diagnosis of the latent and active form of tuberculosis, and for understanding the immunopathogenesis of tuberculosis reactivation from latency.

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Disclosure

The authors declare that there are no conflicts of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Gating strategy for the identification of interleukin (IL)-17$^+$, IL-22$^+$ and interferon (IFN)-γ^+ CD4$^+$ T cells, in the unstimulated peripheral blood mononuclear cells (PBMCs) of healthy controls.

Fig. S2. Interleukin (IL)-17-, IL-22- and interferon (IFN)-γ-expressing CD4$^+$ T cells are induced in individuals with active tuberculosis (TB) infection following stimulation with mycobacterial antigens. Peripheral blood mononuclear cells (PBMCs) (1×10^6/ml) were cultured in the presence or the absence of mycobacterial culture filtrate for 7 days. Intracellular IFN-γ (a), IL-17 (b) and IL-22 (c) expression in CD4$^+$ T cells was detected by flow cytometry. The line graphs of percent frequency of IFN-γ^+ ($n = 7$), IL-17$^+$ ($n = 10$) and IL-22$^+$ ($n = 8$) expressing CD4$^+$ T cells before and after stimulation were generated. US, unstimulated group; ST, stimulated group.

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