CHAPTER 1

Influence of Helminth Infections on the Immune Responses to TB

Antigens in Latent Tuberculosis

Soil transmitted helminths (STHs) are complex eukaryotic organisms, characterized by their ability to maintain long-standing infections in humans, sometimes lasting decades. Hence, parasitic helminths are a major health care problem worldwide, infecting more than two billion people, mostly in resource-limited countries. In addition, helminth parasites are often clinically asymptomatic due, in large part, to the parasites’ ability to manipulate the host immune system to enhance their survival and to restrict local inflammatory pathology (Maizels and Yazdanbakhsh, 2003).

Intestinal helminth infections are common helminth infections (affecting 2 billion people worldwide) known to cause intestinal injury and blood loss (Hotez et al., 2004). These infections occur throughout the tropics and subtropics and in many regions of the world have an overlapping geographic distribution with Mycobacterium tuberculosis (Mtb). The control of Mtb infection requires a clearly delineated Th1 response (IL-12, IFN-γ and TNF-α) and, to a lesser extent, Th17 response (IL-17 and IL-23). Both Th1 and Th17 responses have been shown to be important in the induction and maintenance of protective immune responses in mouse models of Mtb infection or for control of human Mtb infection (as seen in latent TB) (Cooper, 2009; Walzl et al., 2011; Ernst, 2012). During latency, Mtb is contained within granulomas, where the mycobacteria reside in macrophages and in which growth and replication appears to be constrained. Maintenance of the granulomatous lesion is mediated by CD4+ and CD8+ T cells (Ulrichs and Kaufmann, 2006). Modulation of the host immune response involves a variety of strategies including induction of regulatory
networks and dysregulation of innate and adaptive immune responses (Maizels and Yazdanbakhsh, 2003). The immune down modulation associated with helminth infections is mostly parasite-antigen specific, but some bystander effects on routine vaccinations, allergic processes, and autoimmune diseases have been noted (van Riet et al., 2007; Cooper et al., 2009).

Thus, in co-infected individuals, helminth infections often precede the acquisition of latent TB. We hypothesized that immune responses in latent TB would be modulated by the regulatory immune networks often seen in chronic helminth infection. To this end, we examined the induction of Th1, Th2, and Th17 responses in latent TB individuals with or without active helminth infections.

**Materials and methods**

**Study population**

In the first and second part of the study we studied a group of 42 individuals with latent TB, 21 of who were positive by stool microscopic examination for hookworm infection in Tamil Nadu, South India. Latent TB was diagnosed by a positive IGRA, using the QuantiFERON-TB Gold In-Tube (Cellestis, Valencia, CA). All subjects had normal chest radiographs. None of the subjects had pulmonary symptoms (cough, fever, chest pain, hemoptysis) nor positive sputum for Mtb by smear microscopy and culture.

In the third part of the study, we studied a group of 42 individuals with LTB, 14 of whom were positive for Strongyloides infection by recombinant NIE ELISA (Ss/LTB), 14 of whom were positive for *W. bancrofti* infection (FIL/LTB) and the remaining 14 were LTB only. LTB was diagnosed by a positive IFN-γ release assay, using QuantiFERON-TB Gold In-Tube (Cellestis, Valencia, CA).
In the last part of the study, we studied a group of 88 individuals with LTB, 44 of whom were positive for Strongyloides infection by recombinant NIE ELISA (LTB+Ss) and the remaining 44 were LTB only and diagnosed by a positive IFN-γ release assay, using QuantiFERON-TB Gold In-Tube (Cellestis, Valencia, CA). All individuals were examined as part of a clinical protocol approved by Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the National Institute for Research in Tuberculosis (NCT00375583), and informed written consent was obtained from all participants.

**Latent TB diagnostic procedure**

*QuantiFERON-TB Gold In-Tube IFN-γ release assay*

The interferon gamma release assay is one of those assays that could be used as a screening tool to determine latent TB infection in people from a highly endemic area for TB and the procedure is as follows:

- 1 mL of blood was collected in each of the QuantiFERON-TB Gold In-Tubes labelled as NIL (unstimulated), TB ANTIGEN (ESAT-6/CFP-10) and MITOGEN (positive control), the tubes were inverted well and stored at 37°C for not more than 24 hrs

- The tubes were then centrifuged at 2500 g for 15 minutes and the supernatant were collected in individually labelled 1.8 mL screw cap tubes respectively and stored in freezer until running the IFN-γ release assay

- All samples and reagents, except for conjugate 100x concentrate, must be brought to room temperature (22°C ± 5°C) and allowed to sit for approximately 60 minutes to equilibrate
➢ Extra strips were removed from the ELISA Plate that are not required from the frame, it was sealed in the foil pouch, and returned to the refrigerator for storage until required
➢ The Human Interferon-γ kit standard was reconstituted with desired volume of de-ionized or distilled water as printed on the label of the standard vial and ensured complete resolubilization
➢ Reconstitution of the standard to the correct volume will produce a solution with a concentration of 8.0 IU/mL and then it was serially diluted into 8 aliquots as desired for the assay
➢ 6 mL of the green diluent was transferred into a 15 mL falcon and 60 μl of the stock conjugate was added to the falcon and mixed well to produce a working strength conjugate for the assay
➢ 50 μl of freshly prepared working Strength conjugate was added to each well
➢ 50 μl of test samples were added to appropriate wells finally and 50 μl each of the standards 1 to 8 were added and assayed in at least duplicate appropriately
➢ The conjugate and samples/standards were mixed thoroughly using a micro plate shaker for 1 minute at 500 to 1,000 rpm
➢ The plate was covered and incubated at room temperature for 120 ± 5 minutes
➢ Plates should not be exposed to direct sunlight during incubation
➢ Deviation from specified temperature range can lead to erroneous results
➢ Wells were washed at least 6 times on an automated plate washer
➢ If there were residual wash buffer in the wells following the final wash, plates were tapped face down on absorbent towel to remove
100 µl of Enzyme substrate solution was added to each well and mixed for 1 minute at 500 to 1,000 rpm using a micro plate shaker.

The plate was covered and incubated at room temperature for 30 minutes.

Plates should not be exposed to direct sunlight during incubation.

Following the 30 minute incubation, 50 µl of Enzyme stopping solution was added to each well and mixed thoroughly.

Enzyme stopping solution was added to wells in the same order and at approximately the same speed as the substrate was added in the previous step.

Optical Density (OD) of each well was measured within 5 minutes of stopping the reaction using a micro plate reader fitted with a 450 nm filter and with a 620 nm to 650 nm reference filter.

OD values were used to calculate results and a positive result indicates TB exposure.

Parasitologic examination procedures

Stool samples were collected, transported to the laboratory at ambient temperatures, and examined by direct microscopy or by floatation and bile stained techniques to identify hookworms. Stool microscopy was used to exclude the presence of other intestinal helminths including Ascaris, Trichuris, Enterobius, Taenia and Hymenolepsis.

Quantifying circulating W. bancrofti antigen for lymphatic filariasis

Asymptomatic filarial infection was diagnosed by the presence of circulating filarial antigen by the TropBio Og4C3 enzyme-linked immunosorbent assay (ELISA) (Trop Bio Pty. Ltd, Townsville, Queensland, Australia).

A monoclonal antibody (Og4C3) specific to W. bancrofti antigen was used for the estimation of circulating filarial antigen. The monoclonal capture antibody used in this
experimental procedure, is highly specific only to *bancrofti* antigen in human sera and will not cross react with human sera infected with *Brugia* sp, *Loa loa*, and other parasites.

- Microcentrifuge tubes were taken and labeled first
- 300 µl of sample diluent was added to the tubes which were followed by addition of 100 µl of corresponding serum sample
- The diluted serum samples were boiled (100°C for five minutes) in water bath to favor dissociation of antigen antibody complex
- The lids of the tubes were pierced with a fine needle to allow air to escape during boiling
- Since the target antigen (*bancrofti* antigen) is heat stable it was retained in the supernatant, while all other contaminating proteins (antigens and antibodies) precipitate out
- Following heat treatment, the sample was centrifuged (10000 rpm for 5 minutes) for clear separation of supernatant and precipitate
- Seven standards were used in this procedure and 50 µl of each standard was added in duplicates to respective wells
- 50 µl of the sample diluent was added in duplicates and was used as control
- 50 µl of the supernatant was added in duplicates in respective wells
- After adding the standards and samples, the plate was sealed with acetate sealer and incubated at 37°C for 45 minutes
- Wells were washed at least 6 times on an automated plate washer
- Detection antibody obtained by vaccinating rabbit with purified *Onchocerca gibsoni* antigen was used after dilution in antibody diluent (50 µl/6 ml of diluent)
50 µl of diluted rabbit antibody was added to all the wells and the plate was incubated at 37°C for 45 minutes

The HRP tagged conjugate was diluted in antibody diluent (50 µl/6 ml of diluent)

50 µl of diluted conjugate was added to all wells and the plate was incubated at 37°C for 45 minutes

Plate was then washed and 100 µl of substrate which contains a chromogen TMB was added to each well

Plate was incubated for 15 minutes at room temperature before measurement of OD, so as to favor color development

Elisa reader (molecular devices) was switched on and following self calibration of the instrument, the plate was placed in platform and OD was measured at 405 nm or 414 nm

It is to be noted that, OD values greater than 128 IU/mL were considered to be positive for the presence of circulating filarial antigen and OD values below 128 IU/mL were considered negative.

**Quantifying circulating recombinant NIE antigen for Strongyloides stercoralis**

Strongyloides infection was diagnosed by the presence of IgG antibodies to the 31-kDa recombinant NIE antigen by ELISA.

- Stock NIE antigen (1 µg/mL) of Strongyloides stercoralis was added in coating buffer to obtain working buffer

- 100 µL/well of the prepared working buffer was coated in a 96 well ELISA plate and incubated overnight at 4°C
➢ Wash the plate with the ELISA washer and block the plate by adding 190 μL/well of blocking buffer and incubated for 2 hours for 37°C.

➢ Serum/plasma and positive controls of 1 in 500 dilution and blank were prepared and 100 μL/well of the samples and controls were added and incubated overnight at 4°C.

➢ IgG-ALP conjugate was prepared and then Wash the plate with the ELISA washer.

➢ Add 100 μL/well of the prepared conjugate and incubated for 2 hours for 37°C.

➢ Substrate buffer was prepared and then Wash the plate with the ELISA washer.

➢ Add 50 μL/well of the substrate buffer and incubated for 10-15 minutes in the dark at room temperature.

➢ After 15 minutes OD was measured at 405/650 nm dual wavelength in a ELISA reader.

It is to be noted that, OD values greater than 244 LU/mL were considered to be positive for the presence of Strongyloides antigen.

**Total T cells and naïve, memory, and regulatory T cell subsets**

Absolute CD4⁺T cell counts were enumerated in whole blood using BD Multiset 6-Color TBNK cocktail (BD Biosciences). Naïve and memory T cell phenotyping was performed using FITC-CD45RA (BD Pharmingen, BD Biosciences) and APC-CCR7 (eBioscience, San Diego, CA, USA) staining in CD4⁺ and CD8⁺ T cells. Naïve cells were classified as CD45RA⁺CCR7⁺, effector memory cells as CD45RA⁻CCR7⁻, and central memory cells as CD45RA⁻CCR7⁺. Natural Tregs (nTregs) were classified as CD4⁺CD25⁺Foxp3⁺CD127dim (BD Pharmingen and eBioscience).
Antigens used for cell culture assay

Mycobacterial Ags—recombinant early secreted Ag (ESAT)-6 and culture filtrate protein (CFP)-10 (Fitzgerald Industries International, Acton, MA)— as well as total CFP from *M. tuberculosis* H37Rv (*M. tuberculosis* CFP) were used as the antigenic stimuli. Final concentrations were 10 mg/ml for ESAT-6/CFP-10 and 10 mg/ml for *M. tuberculosis* CFP. Phorbol ester (PMA) and ionomycin at concentrations of 12.5 and 125 ng/ml, respectively, were used as the positive control stimuli.

Whole Blood cell culture assay

- Heparanized whole blood was collected and centrifuged at 2600 rpm for 10 minutes and the plasma stored in 1.8 mL screw cap tubes
- Whole blood was diluted 1:1 with RPMI-1640 medium supplemented with penicillin/ Streptomycin (100 U/100 mg/ml), L-glutamine (2 mM), and HEPES (10 mM) and distributed in 12-well tissue culture plates
- The cultures were then stimulated with ESAT-6, CFP-10, W-CFP or PMA/Ionomycin or media alone in 12-well culture plates
- 20 μl of CD28/CD49d co-stimulatory reagent was added to all the labeled wells
- The plates were incubated in CO₂ incubator at 37 °C for 2 hrs
- After 2 hrs Brefeldin (4μl/well) was added to the each well in the culture plates and incubated in CO₂ incubator at 37 °C for 4 hrs
- After incubation the cells were transferred to 50 mL falcons and made up to 15 mL with PBS
- The falcons were vortexed and centrifuged at 1200 rpm for 10 minutes at 4°C and the supernatant was discarded using a serological pipette
The cell pellets were re-suspended in 1:10 dilution of BD lyses solution, make the final volume 20 times of the pellet.

The falcons were vortexed and left at room temperature for 10 minutes, spin at 1200 rpm for 10 minutes at 4ºC.

The supernatant was discarded and the cell pellets were re-suspend in 10 ml of PBS and centrifuged at 1200 rpm for 10 minutes at 4ºC.

The supernatant was discarded and the cell pellets were re-suspend in 200 μl of cytofix/cytoperm buffer and incubated at 4ºC for 20 minutes and then 12 ml of cold 1% PBS/BSA was added to the falcons.

The falcons were vortexed and centrifuged at 2600 rpm for 10 minutes at 4ºC.

The supernatant was discarded and the cell pellets were re-suspend in 1 ml of cold PBS/10% DMSO and stored into two separate 1.8 mL screw cap tubes at -80 ºC.

**Flow cytometry Intracellular Cytokine Staining procedure**

- The cryopreserved fixed cells were thawed slowly at 37 ºC in water bath.
- 10 mL of cold PBS was added to the cells, vortexed and centrifuged at 2600 rpm for 10 minutes at 4ºC.
- The supernatant was discarded and the cell pellets were re-suspend in 10 mL of cold 1% PBS/BSA and centrifuged at 1200 rpm for 10 minutes at 4ºC.
- The supernatant was discarded and the cell pellets were re-suspend in desired volume of 1% PBS/BSA.
- 500 μL of cells were added as aliquots to each appropriately labeled facs tubes.
- The cells were stained with surface antibodies, covered with aluminum foil and incubated for 30 minutes at 4ºC.
Surface antibodies used were CD3 (AmCyan), CD4 (allophycocyanin-H7), and CD8 (PE-Cy7)

After incubation 1 mL of PBS was added to each tube

The tubes were vortexed and centrifuged at 1200 rpm for 10 minutes at 4°C and discard the supernatant

1 mL permeabilization buffer was added to each tube and incubated for 20 minutes at room temperature

The tubes were vortexed and centrifuged at 1200 rpm for 10 minutes at 4°C and discard the supernatant

The cell pellets were re-suspend in 200 μL of cold PBS to the tubes were surface antibodies were

To the remaining tubes the cells were stained with intracellular monoclonal antibodies

The tubes were incubated for 30 minutes at 4°C

Cytokine antibodies used were IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-10, IL-13, IL-17A, IL-17F, and IL-22

1 mL permeabilization buffer was added to each tube

Vortex and Spin at 1200 rpm for 10 minutes at 4°C, discard the supernatant

Add 1 mL permeabilization buffer to each tube

The tubes were vortexed and centrifuged at 1200 rpm for 10 minutes at 4°C and discard the supernatant

The cell pellets were re-suspend in 200 μl of PBS to analyze on the flow cytometer
Flow cytometry analysis

Flow cytometry acquisition was done on BD FACS Canto II (BD Biosciences, San José, CA, USA). Analysis was done using FlowJo software v9.4.10 (TreeStar Inc., Ashland, OR, USA).

Immunoassays

Plasma cytokines on all individuals were measured using Bioplex multiplex cytokine assay system (Biorad). The cytokines analyzed were IL-2, IFN-γ, TNF-α, IL-4, IL-5, IL-13, 10 and IL-17A. IL-17F and IL-22 were measured using classical ELISA by R&D biosystems

Statistical analysis

Data analyses were performed using GraphPad PRISM (GraphPad Software, Inc., San Diego, CA, USA). Geometric means (GM) were used for measurements of central tendency. Statistically significant differences between two groups were analyzed using the nonparametric Mann-Whitney U test and multiple comparisons were corrected using the Holm’s correction.

Results

1.1 Modulation of CD4⁺ producing mono- and dual and CD8⁺ cytokine expressing Th1, Th2 and Th17 cell subsets in latent tuberculosis with concomitant hookworm infection

We hypothesized that immune responses in latent TB would be modulated by the regulatory immune networks often seen in chronic helminth infection. To this end, we examined the induction of Th1, Th2, and Th17 responses in latent TB individuals with or without active hookworm infections. We studied a group of 42 individuals with latent TB, 21 of who were positive by stool microscopic examination for hookworm infection in
Tamil Nadu, South India (Table 1.1). All subjects had normal chest radiographs. None of the subjects had pulmonary symptoms (cough, fever, chest pain, hemoptysis) nor a positive sputum for Mtb by smear microscopy and culture.

Pseudocolor FACS plots were shown (Figure 1A and B) on how to determine the frequencies of T cell subsets – naive (CD45RA⁺CCR7⁺), central memory (CD45RA⁻CCR7⁺), effector memory (CD45RA⁻CCR7⁻) and regulatory T CD4⁺CD127dimCD25⁺FoxP3⁺ cells in a co-infected individual.

i. **Hookworm infection is associated with constitutively decreased frequencies of mono- and dual-functional CD4⁺ Th1 and Th17 cells and increased frequencies of Th2 cells**

To determine the impact of hookworm infection on the steady state (or constitutive) Th1, Th2 and Th17 profile of latent TB individuals, we cultured whole blood from co-infected (HW/LTB) and latent TB only (LTB) individuals with media alone for 6 hrs and measured the frequency of CD4⁺ T cells expressing each of the Th1⁻, Th2⁻ and Th17⁻ associated cytokines Figure 1.1A. As shown in Figure 1.1B and C the frequencies of CD4⁺ T cells expressing TNF-α alone or co – expressing TNF-α/IFN-γ, TNF-α/IL-2, IL-17A/IFN-γ or IL-17A/IL-10 were all significantly reduced in co-infected individuals compared to latent TB infected individuals.

On the other hand, the frequencies of CD4⁺ T cells expressing IL-5 or IL-13 alone or co-expressing IL-5/IL-13 were significantly increased in co-infected individuals. Also, hookworm infection was associated with constitutively higher frequencies of CD4⁺ T cells expressing IL-10 (adaptive Tregs) but not natural regulatory T (nTregs) cells. (Figure
Therefore, hookworm infection is associated with profound alterations in the repertoire of Th1, Th2 and Th17 subsets at steady state.

**ii. Hookworm infection is associated with decreased antigen – specific frequencies of mono – and dual – functional Th1 and Th17 cells and increased Th2 cells**

To determine the impact of coexisting hookworm infection on mycobacterial antigen-specific Th1, Th2 and Th17 responses in latent TB, we stimulated whole blood from co-infected or singly infected individuals with mycobacterial antigens - ESAT-6/CFP-10 or total CFP for 6 hrs and measured the frequencies of mono – and dual – functional Th1, Th2 and Th17 cells (Figure 1.1D). As shown in Figure 1.1E, the frequency of CD4$^+$ T cells expressing IL-2, TNF-α or IL-17A alone was significantly reduced while the frequency of CD4$^+$ T cells expressing IL-5 or IL-13 alone was significantly increased following antigen - stimulation in HW/LTB individuals compared to those with LTB. Similarly, as shown in Figure 1.1F, the frequencies of CD4$^+$ T cells co-expressing IL-2/TNF-α, IFN-γ/TNF-α, IL-17A/IFN-γ, IL-17A/IL-22 and IL-17A/IL-10 were all significantly reduced following ESAT-6/CFP-10– stimulation. In contrast, the frequency of CD4$^+$ T cells co-expressing IL-5/IL-13 (but not IL-4/IL-13) was significantly increased following Mtb antigen – stimulation. In addition, as shown in Figure 1.1G and Figure 1.1H, the frequencies of CD4$^+$ T cells expressing IL-2 or TNF-α alone or co-expressing IL-2/TNF-α, IFN-γ/TNF-α, IL-17A/IFN-γ or IL-17A/IL-10 were significantly reduced following total CFP stimulation. Therefore, hookworm infection profoundly alters the antigen – stimulated Th1 and Th17 responses in latent TB.
iii. *Hookworm infection is not associated with alterations in phorbol ester/ionomycin induced Th1, Th2 and Th17 responses*

To determine whether the altered baseline repertoire of Th1, Th2 and Th17 cells influences the ability of CD4$^+$ T cells to respond to a mitogenic stimulus, we stimulated whole blood from HW/LTB or LTB individuals with PMA/ionomycin for 6 hrs and measured the frequencies of mono – and multi – functional CD4$^+$ T cells expressing Th1, Th2 and Th17 cytokine (Figure 1.1I). As shown in Figure 1.1J, the net frequency of mono – functional CD4$^+$ Th1, Th2 and Th17 cells was not significantly increased in co-infected (HW/LTB) individuals compared to LTB infected individuals. Also as shown in Figure 1.1K, the frequencies of dual – functional CD4$^+$ T cells expressing different combinations of dual cytokines of the Th1, Th2 and Th17 family were not significantly altered in co-infected individuals. Therefore, hookworm infection modulated the repertoire of CD4$^+$ T cells in latent TB but did not impair the ability of these cells to respond appropriately to mitogenic stimulation.

iv. *Coincident hookworm infection is associated with decreased frequencies of mycobacterial-antigen specific Th1 and Th17 and increased frequencies of Th2 cytokine expressing CD8$^+$ T cells*

Since CD8$^+$ T cells play an important role in protection against TB (Rafi et al., 2012), we sought to determine the impact of helminth infection on the CD8$^+$ Th1 Th2 and Th17 cytokine responses in LTB individuals. To this end, we cultured whole blood from co-infected (HW/LTB) and LTB only individuals with media alone, ESAT-6/CFP-10, Mtb CFP and PMA/Ionomycin and measured the frequency of CD8$^+$ T cells expressing each of the Th1, Th2 and Th17-associated cytokines. As shown in Figure 1.1L, HW/LTB
individuals exhibited significantly lower frequencies of CD8+ T cells expressing IFN-γ or TNF-α or IL-17A in comparison to LTB alone individuals \textit{ex vivo}. Also, HW/LTB individuals exhibited significantly higher frequencies of CD8+ T cells expressing IL-5 or IL-4 or IL-13 in comparison to LTB alone individuals \textit{ex vivo}. In addition, HW/LTB individuals exhibited significantly lower frequencies of CD8+ T cells expressing IL-2 or INF-γ or TNF-α or IL-17A or IL-17F or IL-22 in comparison to LTB only individuals upon mycobacterial-antigen specific antigens such as ESAT-6/CFP-10 and Mtb CFP stimulations (Figures 1.1M and N). In contrast, the frequencies of CD8+ T cells expressing Th1 and not Th17 cytokines were not significantly different between LTB and HW/LTB individuals upon PMA/Ionomycin stimulation (Figure 1.1O). Thus, hookworm infections are also associated with a down modulation of CD8+ T cell responses in latent TB.

1.2 Systemic levels of Th1 and Th2 cytokines in latent tuberculosis with concomitant hookworm infection

To examine whether alterations in the frequency of CD4+ Th1 and Th2 cells results in alterations in the circulating levels of the prototypical Th1 and Th2 cytokines, we measured the levels of IFN-γ, TNF-α, IL-2, IL-4, IL-5 and IL-13 in the plasma of HW/LTB co-infected and LTB-infected individuals.

As shown in Figure 1.2, we observed significantly lower plasma levels of IFN-γ (GM of 873.6 pg/ml in LTB vs. 394.8 pg/ml in HW/LTB-infected, \( p = 0.0009 \)) and TNF-α (GM of 1561 pg/ml in LTB vs. 842.1 pg/ml in HW/LTB, \( p = 0.0053 \)) but not IL-2 in co-infected individuals compared to latent TB infected individuals. We also observed significantly higher levels of IL-5 (GM of 33.0 pg/ml in LTB vs. 417.6 pg/ml in HW/LTB, \( p < 0.0001 \)) and IL-13 (GM of 54.5 pg/ml in LTB vs. 477.3 pg/ml in HW/LTB, \( p < 0.0001 \)) in co-
infected individuals compared to latent TB infected individuals. Thus, alterations in CD4+ T cell cytokine expression is reflected in the corresponding changes in systemic levels of Th1 and Th2 cytokines in co-infected individuals.

Discussion

Modulation of CD4+ producing mono- and dual and CD8+ T cell producing cytokine expressing Th1, Th2 and Th17 cell subsets and their respective systemic levels in latent tuberculosis with concomitant hookworm infection

A wide variety of studies have been performed to examine the possible effect of helminth infection on the induction of a protective immune response against mycobacteria (Metenou et al., 2012; Rafi et al., 2012). Both intestinal and systemic helminths have been shown to modulate proliferation and IFN-γ production in response to PPD in helminth – TB co-infected individuals (Metenou et al., 2012; Rafi et al., 2012). Some of these defects have been shown to be reversible following anti-helmintic chemotherapy (Metenou et al., 2012). We have previously demonstrated that concurrent filarial infection could inhibit the generation of potentially protective Th1 immune responses in latent TB infected individuals (Babu et al., 2009). In addition to a significant reduction of Th1 responses by PPD-specific T cells in helminth infected patients, it was shown that IL-23 and IL-17 production in response to both PPD and Mtb culture filtrate was also significantly lower in filarial-infected latent TB individuals when compared with latent TB controls without lymphatic filariasis (Babu et al., 2009). Furthermore, patients co-infected with filariasis and Mtb exhibited a significant reduction of both Toll-like receptor (TLR)-2 and TLR-9 expression and in pro-inflammatory cytokine production and that treatment with anti-helmintic drugs
restored these responses suggesting that helminth-induced immunomodulatory effects are transient (Babu et al., 2009).

Intestinal helminth co-infection has also been shown to adversely impact antimycobacterial immune responses (Elias et al., 2007; Resende et al., 2007). Other studies have also demonstrated that intestinal helminth infections may be one of the risk factors for the development of active pulmonary TB (Elias et al., 2006). Similarly, a recent study found that there was an association between *Schistosoma mansoni* infection and progression to TB disease in HIV-infected Ugandan individuals (Brown et al., 2006). Finally, the immunogenicity of BCG vaccination has been shown to be impaired in helminth-infected individuals, and this is associated with enhanced TGF-β production, but not enhanced Th2 responses (Elias et al., 2007). Apart from human studies, a variety of animal model of co-infection have confirmed the influence of helminth infection on the immune response to TB (Potian et al., 2011; Rafi et al., 2012). Thus, although a variety of epidemiological studies have demonstrated potential association between the presence of helminth infections and the susceptibility to TB disease, very few studies have examined the mechanism behind this potential connection.

The interplay among different CD4+ T cell effector subsets plays a key role in defining immune responses to pathogens as well as to a variety of inflammatory stimuli and the prevention/development of autoimmunity. Not only do Th1 and Th17 cells play an important role in the establishment/maintenance of a variety of chronic inflammatory and autoimmune disorders, they appear to be critical in mediating resistance to a variety of intracellular infections (Ouyang et al., 2008; Sallusto et al., 2012). In Mtb, for example, Th1 responses are absolutely necessary for inducing resistance, with Th17 responses
important in inducing and maintaining memory and recall responses (Cooper, 2009). Because immune-mediated protection against Mtb is characterized by strong mycobacterium-specific Th1 responses, it has been postulated that coincident infections with helminth parasites could modulate these immune responses by driving Th2 and/or Tregs that induce anti-inflammatory responses (van Riet et al., 2007). However, the effect of co-infection on antigen – specific induction of protective or pathogenic Th1, Th2 and Th17 subsets have not been carefully examined.

The examination of constitutive or mitogen – induced immune responses revealed certain interesting differences between hookworm co-infected or LTB alone infected individuals. First, the constitutive frequencies of CD4+ cells producing Th1 and Th17 cytokines were significantly down-regulated in co-infected individuals. Since TNF-α and IL-17 have been reported to exhibit anti-mycobacterial activity either in primary or memory responses to infection (Keane et al, 2001; Khader et al, 2007; Scriba et al, 2008), the fact that hookworm co-infection influences the induction of these T cells suggests a potential compromise in anti-bacterial immunity in the presence of this helminth infection. Second, hookworm infections were also associated with constitutively increased frequency of CD4+ and CD8+ T cells expressing antigen-driven Th2 cells, cells that have been implicated in susceptibility to TB infection because of their potential ability to down-modulate protective Th1 responses (Rook, 2007), to induce alternative activation of macrophages leading to diminished bactericidal responses (Kahnert et al, 2006) and to inhibit autophagy, also involved in bacterial killing (Harris et al, 2007). Thus, the increased frequency of Th2 cells observed in HW/LTB co-infections could potentially serve another possible mechanism by which there is an increased risk of promoting development of active TB. Third, while
hookworm infection was associated with a profound modulation of Th1, Th2 and Th17 baseline repertoires, it did not impair the ability of these CD4+ T cells to respond appropriately to a positive stimulus. While hookworm infection mediates alterations in the baseline subsets of CD4+ and CD8+ T cells, we were interested in examining the effect of these alterations on mycobacterial – antigen specific responses in these latent TB infected individuals. We primarily focused on the expression of mono – and dual – functional expressing CD4+ Th1, Th2 and Th17 cells and also look at mono –functional expressing CD8+ Th1, Th2 and Th17 cells. While the role of CD4+ and CD8+ T cells expressing IFN-γ and TNF-α in resistance to TB is well established, the role multi – functional CD4+ T cells is still not clear (Wilkinson and Wilkinson, 2010). CD4+ T cells expressing IL-2 alone or those co-expressing IL-2 and IFN-γ or TNF-α and IFN-γ has been show to be potential correlates of protective immunity to Mtb (Millington et al., 2007; Day et al., 2008). Similarly, multi – functional CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 has also been shown to correlate with immunity to Mtb in a study comparing smear-positive TB to those with smear-negative TB or latent TB (Day et al, 2011). Thus, mono – and dual – functional Th1 cells clearly play an important role in susceptibility or resistance to infection and/or disease. Our data in HW/LTB co-infected individuals suggests that CD4+ mono – and dual – functional antigen – specific Th1 and Th17 cells and the CD8+ mono - functional antigen – specific Th1 and Th17 cells are functionally impaired following antigen – stimulation. Therefore, one of the potential mechanisms by which helminth infections impair the ability to respond to Mtb is by altering the antigen – specific immune responses of Th1, Th2 and Th17 cells, alterations that could have a profoundly detrimental effect by leading to reactivation of TB.
Helminth infections establish persistent infections, chronicity that is facilitated by mechanisms that dampen immune responses. The two major mechanisms by which helminth infections can modulate immune responses are by the induction of nTregs and CD4+ T cells expressing IL-10 (aTregs) (Allen and Maizels, 2011). Foxp3-expressing T cells are nTregs that play an important role in peripheral tolerance and attenuation of immune responses (Josefowicz et al., 2012). Foxp3 expression is known to be upregulated in human hookworm infections (Ricci et al., 2011), and CD4+CD25+ T cells are known to mediate suppression as evidenced by decreased allergic inflammation in an animal model of helminth infection (Babu et al., 2006; Wilson and Maizels, 2006). Similarly, IL-10 is known to mediate immune suppression in a variety of helminth infections (Jankovic et al., 2010). Hence, we wanted to examine the nature of the cells that might be responsible for this regulation. Interestingly, while we observed no significant differences in the frequency of nTregs, hookworm co-infection was associated with a significantly enhanced frequency of IL-10 expressing CD4+ T cells, suggesting that adaptive Tregs might play the most important role in modulation of the T cell subsets observed in the present study. In addition, hookworm infections did not modulate the absolute numbers or the frequencies of effector memory T cells in latent TB – infecting individuals, suggesting that the altered CD4+ T cell repertoire is not due to alterations in T cell numbers or memory subsets.

There is a great deal of geographic overlap in the regions of high hookworm endemicity and susceptibility to TB (Lipner et al, 2006). We therefore examined whether established immune responses against a given infection could affect the immune response to a subsequent infection. Our study clearly demonstrates the modulation of immune responses to Mtb by coincident hookworm infection. Hookworm-induced regulatory networks appear
to be associated with a significant bystander/spillover effect on the immune responses against mycobacterial antigens in latent TB patients. Our findings may have significant implications for vaccine efficacy in helminth-endemic countries and potentially for understanding how latency in Mtb is broken. Understanding the pathways that helminth infections utilize to mediate bystander suppression/modulation to exogenous antigens and infections should enable new strategies to antagonize suppression for controlling deleterious infections and optimal boosting of vaccine efficacy.

1.3 Modulation of CD4⁺ producing mono- and dual and CD8⁺ cytokine expressing Th1, Th2 and Th17 cell subsets in latent tuberculosis with concomitant helminth infections

Helminth infections commonly occur throughout the tropics and subtropics and in many regions of the world have an overlapping geographic distribution with *Mycobacterium tuberculosis* (Mtb) (Cooper et al, 1998). Of late there is growing evidence that the immune responses associated with both intestinal and tissue-invasive systemic helminths have shown to influence PPD responses to *M. tuberculosis*. Additionally, it was also understood that the presence of helminth infections mediate a Th2 type of response that could thwart the IFN-\(\gamma\) responses necessary to control Mtb infection as a consequence to bystander suppression.

Hence, both filarial parasites (present in the circulation) and Strongyloides (which is an intestinal helminth but has a lung migratory larval stage) could directly influence the outcome of TB infection. We therefore hypothesized that immune responses in latent TB might be modulated by the regulatory immune networks often seen in chronic helminth infections that could have a negatively impact on the course of TB.
To determine the impact of helminth infection on the hematological parameters of latent TB individuals at baseline (or steady state), we performed hematological analysis on LTB (n=14) alone, FIL/LTB (n=14) and Ss/LTB (n=14) individuals. As shown in Table 1.3, infection with *W. bancrofti* or *S. stercoralis* in the context of latent TB infection was not associated with significant alterations in either blood cell counts and or differential counts.

In this study we examined CD4⁺ and CD8⁺ Th1 and Th17 responses in patients with latent TB with or without concomitant filarial or Strongyloides infection. Our data suggest that coincidental helminth infection has a profound inhibitory effect on multi-functional Th1 and Th17 responses as well as on systemic cytokine responses in latent TB.

### i. Coincident helminth infection is associated with decreased frequencies of mycobacterial-antigen specific mono- and multi-functional CD4⁺ Th1 cells

We sought to determine the impact of helminth infection on both the mono-functional and multifunctional CD4⁺ Th1 responses in latent TB individuals. To this end, we cultured whole blood from FIL/LTB, Ss/LTB and LTB only individuals with media alone, CFP-10/ESAT-6, Mtb CFP and PMA/Ionomycin and measured the frequency of CD4⁺ T cells expressing each of the Th1-associated cytokines (Figure 1.3 A). As shown in Figure 1.3B, co-incidental filarial infection was associated with significantly lower frequencies of CD4⁺ T cells expressing IFN-γ, TNF-α and IL-2 alone or co-expressing IL-2/IFN-γ at baseline. Similarly, Strongyloides co-infection was associated with decreased frequencies of CD4⁺ T cells expressing IFN-γ, TNF-α and IL-2 alone at baseline in comparison to individuals with latent TB only. In addition, as shown in Figures 1.3C and D, co-incidental filarial infection was associated with significantly lower frequencies of CFP-10/ESAT-6 induced net frequencies of CD4⁺ T cells expressing IL-2 or IFN-γ (in the
case of Mtb CFP stimulation) or TNF-α alone or co-expressing IL-2/IFN-γ or TNF-α/IFN-γ in comparison to individuals with latent TB only. Similarly, Strongyloides co-infection was also associated with significantly decreased frequencies of the same set of mono and multifunctional CD4⁺ Th1 subsets in response to CFP-10/ESAT-6 and Mtb CFP stimulations. Finally, no significant differences in the net frequency of CD4⁺ Th1 cells were observed between the helminth-infected and -uninfected groups following PMA/Ionomycin stimulation (Figure 1.3E). Thus, helminth infections are associated with a down modulation of spontaneous and/or antigen - specific mono - and multifunctional Th1 responses in latent TB.

ii. **Coincident helminth infection is associated with decreased frequencies of mycobacterial-antigen specific mono- and dual functional CD4⁺ Th17 cells**

We sought to determine the impact of helminth infection on both the mono-functional and multifunctional CD4⁺ Th17 responses in latent TB individuals. To this end, we cultured whole blood from FIL/LTB, Ss/LTB and LTB only individuals with media alone, CFP-10/ESAT-6, Mtb CFP and PMA/Ionomycin and measured the frequency of CD4⁺ T cells expressing each of the Th17-associated cytokines (Figure 1.3F). As shown in Figure 1.3G, co-incidental filarial infection was associated with significantly lower frequencies of CD4⁺ T cells expressing IL-17A, IL-17F and IL-22 alone or co – expressing IL-17A/IFN-γ at baseline in comparison to individuals with latent TB only. Similarly, Strongyloides co-infection was associated with decreased frequencies of CD4⁺ T cells expressing IL-17F and IL-22 alone or co – expressing IL-17A/IFN-γ and IL-17A/IL17F at baseline in comparison to individuals with latent TB only. In addition, as shown in Figure 1.3H, co-incidental filarial infection was associated with significantly lower frequencies of
CFP-10/ESAT-6 induced net frequencies of CD4\(^+\) T cells expressing IL-17A, IL-17F and IL-22 alone or co-expressing IL-17A/IFN-γ in comparison to individuals with latent TB only. Similarly, strongyloides co-infection was associated with decreased frequencies of CD4\(^+\) T cells expressing IL-17F and IL-22 alone or co-expressing IL-17A/IFN-γ in comparison to individuals with latent TB only. Also, as shown in Figure 1.3I, co- incidental filarial and strongyloides infection was associated with significantly lower frequencies of Mtb CFP induced net frequencies of CD4\(^+\) T cells expressing IL-17A and IL-22 alone in comparison to individuals with latent TB only, but no differences were seen in multifunctional CD4\(^+\) Th17 cells. Finally, no significant differences in the net frequency of CD4\(^+\) Th1 cells were observed between the helminth-infected and -uninfected groups following PMA/Ionomycin stimulation (Figure 1.3J), except IL-17F cytokine alone which was downregulated in both the helminth co-infected individuals in comparison with latent TB. Thus, helminth infections are associated with a down modulation of spontaneous and/or antigen - specific mono- and dual functional Th17 responses in latent TB.

iii. **Coincident helminth infection is associated with increased frequencies of mycobacterial-antigen specific mono- and multi-functional CD4\(^+\) Th2 cells**

We sought to determine the impact of helminth infection on both the monofunctional and multifunctional CD4\(^+\) Th2 responses in latent TB individuals. To this end, we cultured whole blood from FIL/LTB, Ss/LTB and LTB only individuals with media alone, CFP-10/ESAT-6, Mtb CFP and PMA/Ionomycin and measured the frequency of CD4\(^+\) T cells expressing each of the Th2-associated cytokines (Figure 1.3 K). As shown in Figure 1.3L, co- incidental filarial and strongyloides infection was associated with significantly increased frequencies of CD4\(^+\) T cells expressing IL-4 and IL13 (apart from
IL-5 which was increased only in strongyloides infection) alone or co – expressing either IL-4/IL-5 or IL-4/IL-13 or IL-5/IL-13 at baseline in comparison to individuals with latent TB only. In addition, as shown in Figure 1.3M, co-incidental filarial infection was associated with significantly increased frequencies of CFP-10/ESAT-6 induced net frequencies of CD4\(^+\) T cells expressing IL-13 alone or, co – expressing IL-4/IL-5 or IL-4/IL-13 or IL-5/IL-13 in comparison to individuals with latent TB only. Similarly, Strongyloides co-infection was associated with increased frequencies of both mono and multifunctional CD4\(^+\) Th2 cells in comparison to individuals with latent TB only. Also, as shown in Figure 1.3N, co-incidental filarial and strongyloides infection was associated with significantly higher frequencies of Mtb CFP induced net frequencies as seen similarly in the CFP-10/ESAT-6 induced net frequencies of CD4\(^+\) Th2 cells in comparison to individuals with latent TB only. Finally, as shown in Figure 1.3O it was interesting to see that almost all the mono and multifunctional CD4\(^+\)Th2 cells were significantly increased in PMA/Ionomycin stimulation. Thus, helminth infections are associated with increased frequencies of spontaneous and/or antigen - specific mono - and multifunctional Th2 responses in latent TB.

iv. **Coincident helminth infection are associated with decreased frequencies of mycobacterial-antigen specific Th1 and Th17 and increased frequencies of Th2 cytokine expressing CD8\(^+\) T cells**

Since CD8\(^+\) T cells play an important role in protection against TB [Rafi et al., 2012], we sought to determine the impact of helminth infection on the CD8\(^+\) Th1 Th2 and Th17 cytokine responses in LTB individuals. To this end, we cultured whole blood from co-infected (HW/LTB) and LTB only individuals with media alone, ESAT-6/CFP-10, Mtb
CFP and PMA/Ionomycin and measured the frequency of CD8\(^+\) T cells expressing each of the Th1, Th2 and Th17-associated cytokines. As shown in Figure 1.3P, both FIL/LTB and Ss/LTB individuals exhibited significantly lower frequencies of CD8\(^+\) T cells expressing IFN-\(\gamma\) or TNF-\(\alpha\) or IL-2 (Type-1 cytokines) and IL-17A or IL17-F or IL-22 (Type 17 cytokines) in comparison to LTB alone individuals ex vivo. Also, both FIL/LTB and Ss/LTB individuals exhibited significantly increased frequencies of CD8\(^+\) T cells expressing IL-5 or IL-4 or IL-13 in comparison to LTB alone individuals ex vivo. In addition, both FIL/LTB and Ss/LTB individuals exhibited significantly lower frequencies of CD8\(^+\) T cells expressing IL-2 or INF-\(\gamma\) or TNF-\(\alpha\) or IL-17A or IL-17F or IL-22 in comparison to LTB only individuals upon mycobacterial-antigen specific antigens such as ESAT-6/CFP-10 and Mtb CFP stimulations (Figures 1.3Q and R). In contrast, the frequencies of CD8\(^+\) T cells expressing Th1, Th2 and Th17 cytokines were not significantly different between LTB and both groups of co-infected individuals upon PMA/Ionomycin stimulation (Figure 1.3S). Thus, helminth infections are also associated with a down modulation of CD8\(^+\) T cell responses in latent TB.

1.4 **Modulation of the systemic levels of Th1, Th2 and Th17 cytokines in latent tuberculosis with concomitant strongyloides infection**

To examine whether alterations in the frequency of CD4\(^+\) Th1, Th2 and Th17 cells results in alterations in the circulating levels of the prototypical Th1, Th2 and Th17 cytokines, we had recruited a total of 88 individuals positive for latent TB diagnosed by a positive IFN-\(\gamma\) release assay, using QuantiFERON-TB Gold In-Tube (Cellestis, Valencia, CA). Out of the 88 individuals, 44 of them were diagnosed for Strongyloides infection (LTB+Ss) by measuring antibody responses to the circulating recombinant NIE
antigen for strongyloides and the remaining 44 individuals remained LTB alone. To
determine the impact of helminth infection on the hematological parameters of latent TB
individuals at baseline (or steady state), we performed hematological analysis on LTB
(n=44) alone, LTB+Ss (n=44) individuals. As shown in Table 1.4, infection with S.
stercoralis in the context of latent TB infection was not associated with significant
alterations in either blood cell counts and or differential counts. We measured the levels of
IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-13, IL-10, TGF-β, IL17A, IL17F and IL-22 in the
plasma of LTB+Ss co-infected and LTB-infected individuals. Further, we also measured
Type-1 IFNs and IL-1 family of cytokines and other pro-inflammatory cytokines such as
IL-6, IL-12 and GM-CSF which are thought to play a role in TB immunity. We have shown
in Figure 1.4A that Type-1 cytokine levels that constitute the IFN-γ, TNF-α and IL-2 as
well the Type-17 cytokine levels that constitute the IL-17A and IL-17F were all
significantly decreased in LTB+Ss individuals in comparison with LTB individuals alone.
Also, the presence of strongyloides infection mounted Type-2 cytokines such as the IL-4,
IL-5 and IL-13 in LTB+Ss individuals (Figure 1.4B). Since one of the mechanisms of
parasites mediating immune responses is by regulatory networks, we looked at the plasma
levels of the regulatory cytokines such as IL-10 and TGF-β and have shown to be
significantly increased in association with strongyloides infection in LTB individuals
(Figure 1.4C). Finally, we have shown that IFN-α, IL-6, IL-12 and GM-CSF levels where
all significantly decreased in the LTB+Ss individuals in comparison with LTB individuals
alone. These set of data shows that, the systemic levels of the above cytokines mirrored the
frequencies of CD4+ and CD8+ T cell responses in helminth co-infected individuals.
Discussion

Modulation of CD4+ producing mono- and dual and CD8+ T cell cytokine expressing Th1, Th2 and Th17 cell subsets and their respective systemic levels in latent tuberculosis with concomitant helminth infections

Infections caused by intestinal helminths are very common in South India. One study by (Kang et al., 1998) had demonstrated that the prevalence of hookworm infections was 62%, whereas the prevalence of Strongyloides and Ascaris was about 15.4% and 6.4%, respectively in the city of Chennai, South India. In the same city the prevalence of exposure to mycobacterial antigens, as measured by reactivity to purified protein derivative (PPD), in the general population is estimated to be virtually 60-100% to PPD (Radhakrishna et al., 2003; Lipner et al., 2006). This clearly shows that both tuberculosis and helminth infections have an overlapping geographical distribution although it has not been studied extensively, and the information regarding coinfection in coendemic regions are sparse (Bentwich et al., 1999; Bentwich, 2000). Of late there is growing evidence that the immune responses associated with both intestinal and tissue-invasive systemic helminths have shown to influence PPD responses to M. tuberculosis. Additionally, it was also understood that the presence of helminth infections could modulate the IFN-γ responses necessary to control Mtb infection as a consequence to bystander suppression (Newport et al., 1996).

Many studies have shown that helminth infections are associated with Th2 type of immune responses (IL-4, IL-5 and IL-13) (Maizels and Yazdanbakhsh, 2003). Whereas, mycobacterial infections and BCG vaccination have shown to primarily induce Th1 (IFN-γ–dominated) CD4+ responses. Hence this interaction between the two opposite arms of the CD4+ response and presumably along with regulatory T cells capable of modulating type 1
and type 2 responses suggest that the equity among the antigen specific responses and the systemic cytokine levels critically influences the ability to handle pathogens. However, the effect of co-infection on antigen – specific induction of protective or pathogenic Th1, Th2 and Th17 subsets have not been carefully examined.

In this FIL/LTB and Ss/LTB study we have shown that constitutive or mitogen – induced immune responses revealed certain interesting differences between helminth co-infected or LTB alone infected individuals. First, the constitutive frequencies of CD4+ and CD8+ cells producing Th1 and Th17 cytokines were significantly down-regulated in co-infected individuals. Although it is well established that TNF-α and IL-17 have been reported to exhibit anti-mycobacterial activity either in primary or memory responses to infection (Keane et al., 2001; Khader et al., 2007; Scriba et al., 2008), the fact co-incident helminth infections (either filariasis or strongyloidiasis) influences the induction of these T cells suggests a potential compromise in anti-bacterial immunity in the presence of helminth infections. Second, helminth infections were also associated with constitutively increased frequency of CD4+ and CD8+ T cells expressing antigen-driven Th2 cells, representing IL-5, IL-4 and IL-13 cytokines that have been shown to play a major role TB susceptibility due to their potential ability to down-modulate protective Th1 responses (Rook, 2007), to induce alternative activation of macrophages leading to diminished bactericidal responses (Khanert et al., 2006) and to inhibit autophagy, also involved in bacterial killing (Harris et al., 2007). Interestingly, the systemic levels of Th2 cytokines (IL-5, IL-4 and IL-13) reflected the very aspect that even plasma levels of these cytokines were skewed in SS/LTB individuals. Thus, the increased frequency of Th2 cells observed in FIL/LTB and SS/LB co-infections could potentially serve as another possible mechanism by which there is an
increased risk of promoting development of active TB. Helminth infections are known to establish persistent infections that are mediated by mechanisms that dampen immune responses. One such mechanism by which helminth infections can modulate immune responses is by the induction of regulatory cytokines IL-10 (Jankovic et al., 2010). This has been supported from our data which clearly shows that the systemic levels IL-10 and TGF-β were significantly up-regulated by the influence of Ss infection on LTB individuals.

We primarily focused on the expression of mono– and dual–functional expressing CD4+ Th1, Th2 and Th17 cells in addition to mono–functional expressing CD8+ Th1, Th2 and Th17 cells. While the role of CD4+ and CD8+ T cells expressing IFN-γ and TNF-α in resistance to TB is well established, the role multi–functional CD4+ T cells is sparse (Wilkinson and Wilkinson, 2010). CD4+ T cells expressing IL-2 alone or those co-expressing IL-2 and IFN-γ or TNF-α and IFN-γ has been show to be potential correlates of protective immunity to Mtb (Millington et al., 2007; Day et al., 2008). Additionally, multi–functional CD4+ T cells co-expressing IFN-γ, TNF-α and IL-2 has also been shown to correlate with immunity to Mtb in a study comparing smear-positive TB to those with smear-negative TB or latent TB (Day et al., 2011). Thus, mono– and dual–functional Th1 cells clearly play an important role in susceptibility or resistance to infection and/or disease. Our data in FIL/LTB and SS/LTB co-infected individuals suggests that CD4+ mono– and dual–functional antigen–specific Th1 and Th17 cells and the CD8+ mono–functional antigen–specific Th1 and Th17 cells are functionally impaired following antigen–stimulation. Interestingly, the systemic levels of Th1 cytokines (IFN-γ, TNF-α and IL-2) and Th17 cytokines (IL-17A and IL-17F) reflected the very aspect that even plasma levels of these cytokines were impaired in SS/LTB individuals, suggesting the
fact that these cytokines indeed play an important role in susceptibility or resistance to infection and/or disease. Therefore, one of the potential mechanisms by which helminth infections impair the ability to respond to Mtb is by altering the antigen – specific immune responses of Th1, Th2 and Th17 cells, alterations that could have a profoundly detrimental effect by leading to reactivation of TB.

Since IL-12 is important in promoting the emergence of Th1, IFN-γ producing T cells, the role of this cytokine in resistance to M. tuberculosis infection is important. Early studies showed that administration of exogenous IL-12 enhanced resistance of mice upon Mtb infection, which was associated with greater splenocyte production of IFN-γ upon stimulation with mycobacterial antigens than that of splenocytes from control mice while treatment with neutralizing antiIL-12 antibody led to heightened bacillary burden in the organs and poorly formed granulomas (Cooper et al., 1995; Noton and Petros, 2014). Also Th17 cells producing IL-17 enhances T cell priming and stimulates the production of pro-inflammatory molecules such as IL-1, IL-6, resulting in inflammation during TB infection (McGeachy et al., 2009; van de Wetering et al., 2009).

To understand better about how helminth infections could impair immune responses pertaining to TB, we also measured plasma levels of these cytokines and showed that influence of Ss infection could impair the type 1 IFNs, IL-1 family of cytokines and other pro-inflammatory cytokines like IL-6, IL-12 and GM-CSF, suggesting that helminth infections could potentially impair the responses leading to TB immunity and heighten the risk of promoting development of active TB.

Since both helminth incidence and TB endemicity share a major portion of overlapping geographical distribution, we therefore examined whether established immune responses
against one infection could affect the immune response to an already exiting infection. Our study clearly demonstrates the modulation of immune responses to Mtb by coincident helminth infection. Understanding the pathways that helminth infections utilize to mediate bystander suppression/modulation to exogenous antigens and infections should enable new strategies to antagonize suppression for controlling deleterious infections and optimal boosting of vaccine efficacy.