CHAPTER 2

Influence of Helminth Infections on the Immune Responses to TB Antigens in Active Tuberculosis

While it has long been recognized that helminth infections alter the pathophysiology of allergic and autoimmune disease, data suggested that helminth infections also exert an important immunological effect on concomitant infections and vaccine responses. In particular, helminth coinfection can modulate the severity, pathogenesis and transmission of other infectious diseases. In this chapter, we examined the mechanism by which helminth infections modulate the immunological responses to tuberculosis antigens in individuals with active pulmonary tuberculosis. Our data suggests that two different helminth infections, with different life cycles, tissue localization and modes of transmission essentially exerted very similar effects on the adaptive immune response to tuberculosis antigens in pulmonary tuberculosis. This included a compromised induction of protective cytokine-expressing T cells as well as inhibitory effects on systemic cytokines that are potentially protective in tuberculosis. The strength of this study lies in the fact that this is the first study to demonstrate that two different helminth infections essentially impair cytokine responses in a similar manner in pulmonary tuberculosis.

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) (Salgame et al., 2013). Moreover, age-specific prevalence studies have indicated that helminth infections usually precede the acquisition of pulmonary tuberculosis (Lipner et al., 2006). Finally, 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 active 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 active TB.

Materials and Methods

Study population

The first and second part of this study deals with a group of 50 individuals with active pulmonary TB, 17 of whom were infected with *W. bancrofti* (hereafter FIL/TB) and 13 of whom had *S. stercoralis* (hereafter STR/TB) infection in Tamil Nadu, South India. Another set of 10 individuals with active pulmonary TB and coincident filarial infection and 10 individuals with active TB alone were used for cytokine neutralization experiments. In the third part of the study that deals with the various biomarkers associated with active tuberculosis with concomitant strongyloides infection, we studied a group of 69 individuals with active pulmonary TB, 33 of whom were infected with *S. stercoralis* (hereafter ATB+Ss) infection and 36 of whom had active TB alone (ATB) in Tamil Nadu, South India. Another set of 46 individuals were used as TB - uninfected controls (hereafter NTB), of whom 23 were infected with *S. stercoralis* (hereafter NTB+Ss) infection. Individuals were recruited from patients and their relatives attending the outpatient clinic at the Stanley Medical Hospital, Chennai. All individuals were examined as part of a clinical research protocol approved by Institutional Review Board of the National Institute for Research in Tuberculosis, and informed written consent was obtained from all participants.
Pulmonary TB diagnostic procedures

Active pulmonary TB was diagnosed microbiologically on the basis of being at least culture positive for Mtb by solid cultures in LJ medium and some were also sputum smear positive.

Löwenstein–Jensen medium culture for M.tb

- 4 to 5mL of sputum was collected in the McCartney bottles.
- Double the volume of sterile 4% NaOH solution was added into the Mc Cartney bottles.
- The caps of the bottles were tightened and inverted repeatedly so that the NaOH solution was mixed thoroughly inside the bottles.
- The bottles were placed in a shaker and incubated at 37°C for 15 minutes.
- After 15 minutes, the bottles were removed from the shaker and 15 mL of sterile distilled water was added to the bottles and mixed thoroughly.
- The bottles were then centrifuged at 3000 g for 15 minutes.
- The McCartney bottles were carefully removed from the centrifuge without shaking and the supernatant were discarded slowly into a container with 5% phenol solution.
- The pellet was washed again with 15 mL of sterile distilled water was added to the bottles and mixed thoroughly.
- The bottles were again centrifuged at 3000 g for 15 minutes and the supernatant decanted.
- From the sediment two slopes of LJ medium were inoculated using a sterile 5 mm inoculating loop made up of Nichrome wire (22SWG) and one loop full of sediment was used for each inoculation.
The LJ medium slopes were incubated at 37°C and the growths were checked regularly every week for a period of eight weeks.

**Acid-Fast Bacteria staining procedure for M.tb**

- A small amount of growth was removed from the culture using a loop and gently rubbed into a drop of saline on a slide to form a smear.
- The smear was allowed to dry and the slide was heat fixed initially.
- The heat fixed slide was then flooded with Carbol Fuchsin stain and ensured that the stain is covered throughout the slide.
- With the help of a Bunsen burner the slides were heated slowly until they steam.
- The steam was maintained for 5 minutes by gently passing the burner along the bottom of the slides.
- The slides were then rinsed with water and flooded with 3% acid-alcohol in order to decolourize for 5 minutes till the slides are clear of the stains visible to the naked eye.
- The slides were then rinsed with water thoroughly and then drained any excess from the slides.
- The slides were then flooded with a counter stain namely Methylene Blue and kept for 1 minute.
- The slides were then rinsed with water thoroughly and mounted on a microscope to visualize the presence of *Mtb* colonies which would turn out to be bright-red in colour against a blue background in a field.
Parasitological examination procedures

**Quantifying circulating *Wucheria 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) (TropBio Pty. Ltd, Townsville, Queensland, Australia). A monoclonal antibody (Og4C3) specific to *Wucheria 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 labelled 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 *Onchocera 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 favour colour 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 at 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 at 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.
**Microscopic stool examination**

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

**Hematological parameters**

Leukocyte counts and differentials were performed on all individuals using the Act-5 Diff hematology analyzer (Beckman Coulter).

**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 antigens— recombinant early secreted antigen-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) (Fitzgerald Industries Intl. Inc, Acton, MA) — were used as the antigenic stimuli. These antigens contain epitopes reactive to both CD4+ and CD8+ T cells (Lindestam et al., 2014). Final concentrations were 10 µg/ml for ESAT-6 and CFP-10. Anti-CD3 at a concentration of 10 µg/ml was 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, anti-CD3 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 (4 µl/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 4°C.
- 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 4°C.
The supernatant was discarded and the cell pellets were re-suspend in 200 μl of cytotox/cytoperm buffer and incubated at 4°C 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 4°C

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°C.

For cytokine neutralization experiments, whole blood from individuals with filariasis and active TB or active TB alone (n = 10) was cultured in the presence of anti-IL-10 (5 μg/ml) or anti-TGFβ (5 μg/ml) or isotype control antibody (5 μg/ml) (R& D Sytems) for 6 h following which CFP-10 and brefeldin A was added and cultured for a further 12 h.

**Flow cytometry Intracellular Cytokine Staining procedure**

- The cryopreserved fixed cells were thawed slowly at 37 ºC in water bath
- 10mL of cold PBS was added to the cells, vortexed and centrifuged at 2600 rpm for 10 minutes at 4°C
- 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 4°C
- 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 labelled facs tubes
- The cells were stained with surface antibodies, covered with aluminium foil and incubated for 30 minutes at 4°C.
- 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 @ 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 mins 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 mins 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-10, IL-17A, IL-17F and IL-22. TGFβ levels were measured using a standard ELISA kit from R&D Systems. Plasma samples were assayed for MMP-1, MMP-7, MMP-8 and MMP-9 using the R&D (Minneapolis, MN) multiplex ELISA system according to the manufacturer's instructions. TIMP-1, TIMP-2, TIMP-3, and TIMP-4 levels were measured using the R&D multiplex ELISA system according to the manufacturer's instructions. Plasma levels of immune activation markers such as sCD14 (Bio-Rad), sCD163 (R&D), sPD-1 (R&D) and Platelet derived growth factor (PDGF; Biorad) were measured by ELISA.

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. Comparisons were made using either the Kruskal-Wallis test with Dunn's multiple comparisons (unpaired comparisons) or the Wilcoxon signed rank test (paired comparisons) or the Mann-Whitney U test with Holm's correction for multiple comparisons.
Results

To determine the impact of helminth infection on the hematological and immunological parameters of active TB individuals at baseline (or steady state), we performed hematological and flow cytometry analysis on these individuals. As shown in Table 2.1, infection with *W. bancrofti* or *S. stercoralis* in the context of active pulmonary TB was not associated with significant alterations in the absolute numbers of CD4$^+$ and CD8$^+$ T cells nor in the frequency distribution of the various T cell subsets - naive, central memory, effector memory and regulatory T cells - when compared to helminth-uninfected individuals with active TB. Similarly, all other hematological and immunological parameters examined including total leukocyte and differential cell counts were similar between those helminth-infected and –uninfected individuals with active TB.

Pseudocolor FACS plots were shown (Figure 2A-C) 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$^+$CD127$^{dim}$CD25$^+$FoxP3$^+$ cells and monocyte subsets- classical monocytes (CD14$^{++}$CD16$^-$), intermediate monocytes CD14$^{++}$CD16$^+$) and non-classical monocytes (CD14$^{++}$CD16$^{++}$) in a co-infected individual.

2.1. Modulation of CD4$^+$ producing mono- and dual cytokine expressing Th1, and Th17 cell subsets and CD8$^+$ producing cytokine expressing Th1, and Th17 cell subsets in active tuberculosis with concomitant filarial and strongyloides infection

In this study we examined CD4$^+$ and CD8$^+$ Th1 and Th17 responses in patients with active 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 active pulmonary TB.
Our data also suggest that IL-10 is an important mediator of these inhibitory effects for filarial co-infections.

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

Since a decrease in multifunctional CD4+ Th1 cells is known to be associated with increased bacterial burdens in active TB (Day et al., 2011) and since both mono- and multifunctional CD4+ Th1 cells are potential correlates of protective immunity in TB (O’Garra et al., 2013), we sought to determine the impact of helminth infection on both the mono-functional and multifunctional CD4+ Th1 responses in TB infected individuals. To this end, we cultured whole blood from FIL/TB, STR/TB and TB only individuals with media alone, CFP-10, ESAT-6 and anti-CD3 and measured the frequency of CD4+ T cells expressing each of the Th1-associated cytokines (Figure 2.1A). As shown in Figure 2.1B, co-incidental filarial infection was associated with significantly lower frequencies of CD4+ T cells expressing IL-2 alone or co-expressing TNF-α/IFN-γ or IL-2/IFN-γ at baseline. Similarly, Strongyloides co-infection was associated with decreased frequencies of CD4+ T cells co-expressing TNF-α/IFN-γ or IL-2/IFN-γ/TNF-α at baseline in comparison to individuals with active TB only. In addition, as shown in Figures 2.1C and D, co-incidental filarial infection was associated with significantly lower frequencies of CFP-10 and ESAT-6 induced net frequencies of CD4+ T cells expressing IL-2 or IFN-γ or TNF-α alone or co-expressing TNF-α/IFN-γ or IL-2/IFN-γ or IL-2/TNF-α or IL-2/IFN-γ/TNF-α in comparison to individuals with active TB only. Similarly, Strongyloides co-infection was also associated with significantly decreased frequencies of almost all of the above-mentioned mono- and multifunctional CD4+ Th1 cell subsets in response to CFP-10
and ESAT-6 (Figures 2.1C and D). Finally, no significant differences in the net frequency of CD4+ Th1 cells was observed between the helminth-infected and -uninfected groups following anti-CD3 stimulation, with the exception of CD4+ T cells expressing IL-2 alone in FIL/TB individuals (Figure 2.1E). Thus, helminth infections are associated with a down modulation of spontaneous and/or antigen - specific mono - and multifunctional Th1 responses in active TB.

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

Since both mono - and multifunctional CD4+ Th17 cells have also been implicated as being important in the immune response in active TB (O’Garra et al., 2013), we sought to determine the impact of helminth infection on the CD4+Th17 responses in TB infected individuals. To this end, we cultured whole blood from co-infected (FIL/TB or STR/TB) and TB only (TB) individuals with media alone, CFP-10, ESAT-6 and anti-CD3 and measured the frequency of CD4+ T cells expressing each of the Th17-associated cytokines (Figure 2.1F). As shown in Figure 2.1G at baseline, the frequencies of CD4+ T cells expressing IL-22 or co – expressing IL-17A/IFN-γ or IL-17A/IL-17F or IL-17A/IL-22 was significantly reduced in FIL/TB compared to TB alone individuals. In addition, as shown in Figures 2.1H and I, upon Mtb-specific antigen stimulation, the frequencies of CD4+ T cells expressing IL-17A or IL-22 or co – expressing IL-17A/IFN-γ or IL-17A/IL-17F was significantly reduced in FIL/TB compared to TB alone individuals. Moreover, similar to the pattern observed in Th1 cells, the differential frequencies of Th17 cells was also mycobacterial - antigen specific since anti-CD3 stimulated frequencies of these cells did not exhibit any major significant differences (Figure 2.1J). In contrast, STR/TB individuals
did not exhibit any significant differences in the frequencies of mono- or multifunctional CD4⁺Th17 cells in comparison to TB alone individuals ex vivo or following stimulation with TB antigens or anti-CD3. Thus, helminth infections, and more specifically filarial infections, are associated with a modulation of spontaneous or antigen-specific Th17 responses in active TB.

**iii. Coincident helminth infection is associated with decreased frequencies of mycobacterial-antigen specific Th1 and Th17 cytokine expressing CD8⁺ T cells**

Since CD8⁺ T cells play an important role in protection against TB (North and Jung, 2004), we sought to determine the impact of helminth infection on the CD8⁺ Th1 and Th17 cytokine responses in TB infected individuals. To this end, we cultured whole blood from co-infected (FIL/TB or STR/TB) and TB only individuals with media alone, CFP-10, ESAT-6 and anti-CD3 and measured the frequency of CD8⁺ T cells expressing each of the Th1 and Th17-associated cytokines. As shown in Figure 2.1K, FIL/TB individuals exhibited significantly lower frequencies of CD8⁺ T cells expressing IL-2 or TNF-α or IL-17A in comparison to TB alone individuals ex vivo. Similarly, STR/TB individuals also exhibited significantly decreased frequencies of CD8⁺ T cells expressing IL-17A or IL-17 or IL-22 ex vivo (Figure 2.1K). In addition, FIL/TB individuals exhibited significantly lower frequencies of CD8⁺ T cells expressing IL-2 or INF-γ or TNF-α or IL-17A or IL-17F in comparison to TB only individuals upon CFP-10 and ESAT-6 stimulation (Figures 2.1L and M). Similarly, as shown in Figures 2.1L and M, STR/TB individuals also exhibited significantly lower frequencies of CD8⁺ T cells expressing INF-γ or TNF-α or IL-17A or IL-17F in comparison to TB only individuals upon CFP-10 and ESAT-6 stimulation. In contrast, the frequencies of CD8⁺ T cells expressing Th1 and Th17 cytokines were not
significantly different between the 3 groups upon anti-CD3 stimulation (Figure 2.1N). Thus, helminth infections are also associated with a down modulation of CD8+ T cell responses in active TB.

**iv. IL-10 modulates the frequencies of mono- and multifunctional CD4+ Th1 cells in filarial-TB co-infection**

To determine the role of IL-10 and other known immunomodulatory cytokines (e.g. TGFβ) in the modulation of CD4+ Th1 cells in active TB with concomitant helminth infection, we measured the frequency of cells following stimulation with the TB antigen - CFP-10 in the presence or absence of anti-IL-10 or anti-TGFβ neutralizing antibody in FIL/TB and TB alone individuals (n = 10). As shown in Figure 2.1O, IL-10 neutralization resulted in significantly increased frequencies of monofunctional (IL-2 or INF-γ or TNF-α expressing) and multifunctional (IL-2/IFN-γ or IFN-γ/TNF-α or IL-2/TNF-α co-expressing) Th1 cells in FIL/TB individuals. In marked contrast, as shown in Figure 2.1P, TGFβ neutralization had no significant effect on the frequencies of mono- or multi-functional Th1 cells. On the other hand, IL-10 neutralization resulted in significantly increased frequencies of monofunctional (IL-2 or INF-γ or TNF-α expressing) but not multifunctional (IL-2/IFN-γ or IFN-γ/TNF-α or IL-2/TNF-α co-expressing) Th1 cells in TB alone infected individuals (Figure 2.1Q). Thus, IL-10 plays an important role in the modulation of CD4+ Th1 cells in FIL/TB co-infection.

**2.2. Modulation in the systemic levels of Th1, and Th17 cytokines in active tuberculosis with concomitant filarial and strongyloides infection**

Since Th1 and Th17 cytokines are cytokines important components of the immune response in active TB (O’Garra et al., 2013), we wanted to explore the effect of coincident
helminth infection on systemic levels of these cytokines. To determine the impact of helminth infections on the circulating levels of the prototypical Th1 and Th17 cytokines as well as regulatory cytokines, we measured the levels of IFN-γ, TNF-α, IL-2, IL-17A, IL-17F, IL-22, IL-10 and TGFβ in the plasma of three groups of individuals with active TB - FIL/TB, STR/TB or TB alone.

As shown in Figure 2.2, we observed significantly lower plasma levels of Th1 associated cytokines - IFN-γ (Geometric Mean of 936.6 pg/ml in TB alone vs. 59.2 pg/ml in FIL/TB and 60.5 pg/ml in STR/TB), IL-2 (GM of 27.6 pg/ml in TB alone vs. 11.7 pg/ml in FIL/TB and 13.7 pg/ml in STR/TB) and TNF-α (GM of 1017 pg/ml in TB alone vs. 493.1 pg/ml in FIL/TB and 202.7 pg/ml in STR/TB) as well as Th17 - associated cytokines - IL-17A (GM of 219.2 pg/ml in TB alone vs. 84.1 pg/ml in FIL/TB and 90.6 pg/ml in STR/TB) and IL-17F (GM of 110.2 pg/ml in TB alone vs. 59.9 pg/ml in FIL/TB and 73.6 pg/ml in STR/TB) but not IL-22 in co-infected individuals compared to TB infected individuals. In contrast, we observed significantly higher plasma levels of IL-10 (GM of 116.9 pg/ml in TB alone vs. 209.7 pg/ml in FIL/TB and 177.7 pg/ml in STR/TB) but not TGFβ (data not shown) in helminth co-infected individuals compared to TB-infected individuals. Thus, both helminth infections are associated with profound alterations systemic levels of Th1 and Th17 cytokines in co-infected individuals.

2.3. **Various biomarkers associated with active tuberculosis with concomitant strongyloides infection**

We studied a group of 69 individuals with active pulmonary TB, 33 of whom were infected with *S. stercoralis* (hereafter ATB+Ss) infection and 36 of whom had active TB alone (ATB) in Tamil Nadu, South India (Table 2.3A). Another set of 46 individuals were
used as TB - uninfected controls (hereafter NTB), of whom 23 were infected with *S. stercoralis* (hereafter NTB+Ss) infection.

**i. Coincident helminth infection is associated with decreased systemic levels of acute phase proteins in active pulmonary tuberculosis**

To determine the impact of *Ss* infection on the acute phase protein elevations seen in ATB, circulating levels of α-2M, CRP, SAA and haptoglobin in ATB and ATB+Ss individuals. As shown in Figure 2.3A, infection with *S. stercoralis* in the context of active pulmonary TB was associated with significantly lower levels of α-2M (Geometric Mean of 4.0 ng/ml in ATB vs. 2.7 ng/ml in ATB+Ss), CRP (GM of 6.2 ng/ml in ATB vs. 4.3 ng/ml in ATB+Ss) and SAA (GM of 4.3 ng/ml in ATB vs. 3.1 ng/ml in ATB+Ss) - when compared to Ss-uninfected individuals with active TB. On the other hand, *Ss* infection was not associated with any significant alterations in the systemic levels of acute phase proteins (with the exception of SAA) in NTB individuals (Figure 2.3A), indicating that helminth modulation of inflammatory markers is specific to active TB. Thus, *Ss* infection is associated with the dampening of systemic inflammation in active pulmonary TB.

**ii. Coincident helminth infection is associated with decreased systemic levels of MMPs and TIMPs in active pulmonary tuberculosis**

To determine the impact of *Ss* infection on markers associated with tissue inflammation and remodeling at baseline (or steady state), we measured the circulating levels of MMP-1, 7, 8, 9 and TIMP-1, 2, 3, 4 in ATB and ATB+Ss individuals. As shown in Figure 2.3B, infection with *S. stercoralis* in the context of active pulmonary TB was associated with significantly lower levels of MMP-1 (GM of 5.3 ng/ml in ATB vs. 3.9 ng/ml in ATB+Ss) and MMP-9 (GM of 646.4 ng/ml in ATB vs. 331.2 ng/ml in ATB+Ss) - when compared to
Ss-uninfected individuals with active TB. Similarly, the plasma levels of TIMP-1 (GM of 230.4 ng/ml in ATB vs. 189.4 ng/ml in ATB+Ss), TIMP-2 (GM of 291 ng/ml in ATB vs. 228.6 ng/ml in ATB+Ss) and TIMP-4 (GM of 11.0 ng/ml in ATB vs. 9.3 ng/ml in ATB+Ss) were all significantly lower in ATB+Ss compared to ATB individuals (Figure 2.3B).

On the other hand, strongyloides infection was not associated with any significant alterations in the systemic levels of MMPs or TIMPs (with the exception of TIMP-1 and TIMP-3) in NTB individuals with or without Ss infection (Figure 2.3B). Interestingly, TIMP-3 was the exception to both patterns with higher levels in ATB+Ss (compared to ATB) and lower levels in NTB+Ss (compared to NTB) individuals. Thus, Ss infection is associated with a TB specific modulation of circulating MMP and TIMP levels, indicating amelioration of disease severity in the context of helminth co-infection.

iii. Coincident helminth infection is associated with decreased systemic levels of immune activation markers in active pulmonary tuberculosis

To determine the impact of Ss infection on systemic immune activation markers at baseline (or steady state), we measured the circulating levels of sCD14, sCD163, sPD-1 and PDGF in ATB and ATB+Ss individuals. As shown in Figure 2.3C, infection with S. stercoralis in the context of active pulmonary TB was associated with significantly lower levels of sCD14 (GM of 7.3 ng/ml in ATB vs. 4.1 ng/ml in ATB+Ss) and sCD163 (GM of 2.7 ng/ml in ATB vs. 1.9 ng/ml in ATB+Ss) - when compared to Ss-uninfected individuals with active TB. On the other hand, as shown in Figure 2.3C, Ss infection was associated with significant elevations in the systemic levels of sCD163 and PDGF in NTB individuals.
Thus, Ss infection is associated with the dampening of systemic inflammation in active pulmonary TB.

**iv. Active TB associated elevation in the systemic levels of acute phase proteins, MMPs and TIMPs is independent of co-existent helminth infection**

Since strongyloides infection was found to impart a profound effect on the systemic immune profile in active TB, we wanted to examine the impact of tuberculosis disease in helminth-infected individuals. To this end, we measured the circulating levels of all the above mentioned parameters, including acute phase proteins, MMPs, TIMPs and immune activation markers in ATB+Ss individuals and compared them to NTB+Ss individual to determine the contribution of Ss infection to changes in systemic markers in active TB versus controls. As shown in Table 2.3B, active TB in the presence of Ss infection exhibited significantly higher levels of α-2m, CRP, SAA and haptoglobin in comparison to NTB individuals with Ss infection. Similarly, as shown in Table 2.3B, active TB in the presence of Ss infection exhibited significantly higher levels of MMP -1, 8 and 9, TIMP -1, 2 and 4 in comparison to NTB individuals with Ss infection. Finally, as shown in Table 2.3B, active TB in the presence of Ss infection exhibited significantly higher levels of sPD-1 and PDGF in comparison to NTB individuals with Ss infection. Thus, active TB, independent of Ss co-infection, profoundly alters the circulating levels of inflammatory and immune activation markers.
Discussion

Modulation of CD4\(^+\) producing mono- and dual cytokine expressing Th1, and Th17 cell subsets, CD8\(^+\) mono cytokine expressing Th1, and Th17 cell subsets and their systemic levels in active tuberculosis with concomitant filarial and strongyloides infection

Helminth infections afflict over 1.5 billion people worldwide, while Mtb infects one third of the world's population resulting in a million deaths per year (Salgame et al., 2013). The overlapping geographic distributions of the helminth infections and tuberculosis demonstrate very clearly that, on a population level, the potential for interaction among these various pathogens can occur. 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 mycobacterial (Metenou et al., 2012), (Rafi et al., 2012). Both intestinal and systemic helminths have been shown to modulate proliferation and IFN-\(\gamma\) production in response to PPD in helminth – latent TB coinfected individuals (Metenou et al., 2012), (Rafi et al., 2012). Some of these effects have been shown to be reversible following antihelmintic chemotherapy (Metenou et al., 2012). Indeed, we have previously demonstrated that concurrent filarial infection could inhibit the generation of potentially protective Th1 and Th17 immune responses in latent TB infected individuals (Babu et al., 2009). In addition, we have also shown that concomitant hookworm infection modulates the frequency of Th1 and Th17 cytokine-producing cells in latent TB (George et al., 2013). The immunogenicity of BCG vaccination has been shown to be impaired in helminth-infected individuals, and this is associated with enhanced TGF-\(\beta\) production but not enhanced Th2 responses (Elias et al., 2007), while there exist an inverse association
between BCG immunization and intestinal nematode infection (Elliot et al., 1999). Despite these studies on the interaction of helminth infection and latent TB or TB vaccination, the relationship of helminth infection on the development of active tuberculosis or outcome following treatment is not completely clear.

The two major subsets of CD4$^+$ T cells that form an important component of adaptive immune responses to TB are Th1 and Th17 cells (Cooper, 2009), (O’Garra et al., 2013). Th1 responses are known to be important in resistance to TB, while Th17 responses are known to be important in inducing and maintaining memory and recall responses to TB (Cooper, 2009). Finally, multifunctional Th1 cells are also thought to play an important role in protection against TB disease (Wilkinson et al., 2010). Because immune-mediated protection against Mtb is characterized by strong mycobacterium-specific Th1 and Th17 responses (Cooper, 2009), 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 (Salgame et al., 2013). Therefore, we have examined the effect of helminth infection on TB - antigen specific immune responses in individuals with active microbiologically confirmed pulmonary TB.

Our data reveal significant alterations in the baseline frequencies of mono - and multifunctional CD4$^+$ and CD8$^+$ Th1 and Th17 cells in TB-infected individuals with active helminth infection. This is associated with perturbations in the homeostatic or steady - state levels of Th1 and Th17 cytokines in pulmonary TB individuals in comparison to co-infected individuals as well. Our examination of plasma levels of these cytokines clearly reveals that a profound depression of both Th1 and Th17 cytokines is found in those with helminth infection and active TB. Amongst all the cytokines, IFN-$\gamma$ and TNF-$\alpha$ are known
to be critically responsible for protection against TB (O’Garra et al., 2013). Therefore, the diminished circulating levels of these cytokines in helminth co-infected individuals, suggest impairment in Th1 responses in pulmonary TB with coincident filarial infection. In addition, the diminished systemic production of IL-2, IL-17A and IL-17F also indicate a more extensive impairment in Th1 and Th17 responses in co-infection settings. Thus, helminth infection appears to be associated with homeostatic alterations in the Th1 and Th17 cellular responses in pulmonary TB.

Our study highlights the association of filarial co-infection with a profound impairment in TB - antigen specific CD4$^+$ Th1 and Th17 responses. Our data on STR/TB co-infection also reveals remarkably similar yet more pronounced effects of helminth infection on CD4$^+$ T cell responses in active TB. Thus, co-infected individuals exhibit a spontaneous deficiency in the frequencies of Th1 and Th17 cells and a much more potent deficiency in the expansion of mono- and multifunctional Th1 and Th17 cells in response to Mtb-specific antigens. In contrast, our data suggest that the intrinsic potential of CD4$^+$ T cells to respond to polyclonal stimulation and induce Th1 and Th17 cytokine expression is unaltered in the presence of coincident helminth infection. 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, multifunctional CD4$^+$ T cells co-expressing IFN-γ, TNF-α and IL-2 have 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 multifunctional Th1 cells clearly play an important role in susceptibility or resistance to infection and/or disease. In addition, Th17 cells, secreting one or more cytokines, are also known to play an
important role in protective memory responses in TB infection (Khader and Cooper, 2008). Since multifunctional T cell responses are known to be better correlates of protective immunity and also to be more persistent (Darrah et al., 2007), the impairment of multifunctional CD4\(^+\) Th1 and Th17 cells could potentially have an impact on the clinical course of TB disease in co-infected individuals.

Although a role for CD4\(^+\) T cells in protection against Mtb is well established, there is also a large body of evidence derived from both humans and animal models that suggest an essential role for CD8\(^+\) T cells (North and Jung, 2004) and (Cooper, 2009) as well. CD8\(^+\) T cells are known producers of Th1 and Th17 associated cytokines and also possess direct antimicrobial activity through granule-exocytosis dependent mechanisms (Cooper, 2009). Since helminth infections can also modulate CD8\(^+\) T cell responses, we examined the effect of co-incidental helminth infection on CD8\(^+\) T cell cytokine responses in active TB. Similar to the effect on CD4\(^+\) T cells, helminth infections appear to exhibit a profound inhibitory effect on the expression of Th1 and Th17 associated cytokines in the context of pulmonary TB. Alterations in cytokine producing CD4\(^+\) and CD8\(^+\) T cell subsets could be the result of altered T cell numbers at baseline. Our data suggest that helminth infections are not associated with any such alterations (see Table 2.1). Moreover, our data also clearly reveal that helminth infections have very little effect on the naive and memory compartmentalization of CD4\(^+\) and CD8\(^+\) T cells in active TB. In addition, while the induction of natural Tregs by filarial infections is a major mechanism by which these infections could dampen host immune responses (Metenou et al., 2010), our data also clearly indicate no significant difference in the frequency of nTregs between helminth -
infected and uninfected individuals, suggesting that nTreg expansion might not play an important role in modulation of the T cell subsets observed in the present study.

The other major mechanism by which helminth infections are known to alter immune responses to bystander antigens is by the production of immuno-modulatory cytokines - IL-10 and TGFβ (Allen and Maizels, 2011). Indeed, filarial infections are known to be associated with an IL-10 dominant cytokine milieu (Metenou et al., 2010). Moreover, helminth infections were associated with elevated circulating levels of IL-10 in the co-infected individuals, implicating a potential regulatory role for IL-10 in co-infections. Our data on the role of IL-10 and TGFβ in the helminth infection associated modulation of CD4+ Th1 responses implicate IL-10 as the major player in the down modulation of Th1 responses in active TB, at least in the context of filarial infections. Moreover, our data also reveal an important role for IL-10 in the down regulation of both mono- and multifunctional Th1 cells in this setting. Interestingly, TGFβ appeared to have a negligible effect on the modulation of the Th1 response to TB antigen, although an effect on Th17 responses or CD8+ T cell responses cannot be excluded. In addition, while IL-10 also appears to play an important role in down modulation of Th1 responses in active TB individuals without helminth infection, this effect appears to be selective to mono-functional Th1 cells only. In contrast, filarial infection modulated effector CD4+ T cell responses encompass both mono- and multi-functional Th1 cells. Our data, therefore, suggest a major role for IL-10 in the regulation of immune responses of active TB.
Our findings suggest that in the presence of coincident helminth infection, the ability to restore homeostatic CD4+ and CD8+ T cell responses in active disease could be worsened. Our study did not have the sample size required to assess the impact of helminth infection on severity of disease or bacterial burdens but the immunological correlates nevertheless highlight a potentially deleterious effect of filarial infection on active TB. In addition, the major strength of our study is the finding that two different helminth infections, with different modes of transmission as well as localization, are both associated with down modulation systemic and antigen-specific immune responses in active TB. Our findings, therefore, have significant implications for treatment and vaccine discovery in TB and suggest that treatment of concomitant helminth infections could have an impact on both the clinical course of TB as well as on vaccine studies in TB-endemic areas.

**Concomitant helminth infection has a secondary effect on systemic markers of disease severity/activity in pulmonary tuberculosis**

Epidemiological and clinical studies in humans as well as experimental studies in animal models strongly indicate that helminth infections can confer protection from a variety of inflammatory diseases such as allergy, autoimmunity and inflammatory bowel disease (van Riet et al., 2007; Harnett and Harnett, 2010). The propensity of helminths to produce modulatory molecules to suppress anti-parasite and immuno-pathological responses at multiple levels renders them also with the ability to modulate host pathology during other chronic infections (Harnett and Harnett, 2010; Finlay et al., 2014). Furthermore, although helminths are typically inducers of strong Th2 responses, they also induce regulatory T cells, alternatively activated macrophages and anti-inflammatory
cytokines and antibodies to suppress host - protective (and possibly pathological) pro-inflammatory responses (Allen and Maizels, 2011). Thus, helminth products have been shown to modulate both Th1/Th17-mediated inflammation and Th2 dependent pathology (Finlay et al., 2014). Recent data suggest that parasitic worms can potentially provide benefits to humans in a clinical setting, and infection with helminths or their products have shown promise as potential therapeutics for inflammatory bowel disease and other inflammatory disorders (Elliot and Weinstock, 2009; Elliot and Weinstock, 2012). We and others have previously shown that helminth infections can modulate both the innate and adaptive arms of the immune system in active and latent TB (Metenou et al., 2012). In this study, we sought to elucidate the modulatory function (if any) of a chronic helminth infection on the systemic pathological responses that characterize disease activity and severity in pulmonary TB. S. Stercoralis infection is known to overlap geographically with M. tuberculosis (Lipner et al., 2006) and, more significantly, Ss infection in the mouse has been shown to impair immune responses to TB infection (Salgame et al., 2013). Therefore, we elected to examine the interaction of Ss and M. tuberculosis at the systemic level.

Acute phase proteins are non-specific serum proteins that are elevated in patients with TB. Recently, CRP has been proposed as a candidate biomarker for active infection with Mtb (Wilson et al., 2014). Point-of-care CRP testing has been shown to be of use in the clinical evaluation of respiratory tract infections in adults and of fever in children (Pfafflin and Schleicher, 2009). In addition, previous studies also report that SAA is another important candidate biomarker for TB (Agranoff et al., 2006). Our study reveals clearly reveals that in addition to CRP and SAA, α-2M is also a remarkably good biomarker in
distinguishing TB from non-TB individuals. More importantly, however, our data also show that all three markers of systemic inflammation in TB are significantly modulated by the presence of coincidental Ss infection and that this modulation is relatively TB disease specific. While acute phase proteins are typically (but not always) markers of acute inflammation, systemic immune activation markers more accurately reflect inflammatory pathology in chronic infections. sCD14 and sCD163 are markers of monocyte/macrophage activation and their levels in the blood usually reflect chronic immune activation involving myeloid cells (Wilson et al., 2014). In addition, sCD163 and sCD14 have been shown previously to server as plasma markers of active TB (Knudsen et al., 2005; Feruglio et al., 2013). PDGF is a pro-fibrotic growth factor that has been directly linked to increased fibrosis in TB patients (Ameglio et al., 2005). Finally, we also examined the circulating levels of sPD-1, which is a soluble form of the T cell co-receptor PD-1 and has been implicated in disease activity in various inflammatory conditions (Greisen et al., 2014), (Wan et al., 2006). Our data first confirms the utility of these molecules (with the exception of sCD163) as putative biomarkers of active TB. More interestingly, our data reveal a profound impact on Ss co-infection on the systemic levels of some of these biomarkers, especially the ones associated with monocyte/macrophage activation. Helminth infections are known to have a major impact on the function of antigen-presenting cells, including dendritic cells, monocytes and macrophages (Allen and Maizels, 2011). Hence, it is not surprising to find an important effect of Ss infection on monocyte activation markers in active TB.

MMPs and TIMPs are important additional factors in the pathogenesis of TB due to their ability drive immune-mediated pathology (Elkington et al., 2007; Ong et al., 2014). MMPs
are zinc dependent proteases, associated with breakdown of the extracellular matrix and tissue remodelling (Amalinei et al., 2010; Khokha et al., 2013). TIMPs are specific inhibitors of MMPs and help control tissue pathology (Amalinei et al., 2010; Khokha et al., 2013). Thus, various MMPs have been shown to be upregulated in peripheral blood and at the site of disease in TB infection and their circulating levels have been shown to accurately reflect lung pathology and TB disease severity (Elkington et al., 2007). This is because MMPs play a major role in the underlying mechanism of lung extracellular matrix destruction in TB owing to their unique ability to degrade fibrillar collagens and other matrix components (Ong et al., 2014). This matrix destruction is central to the development of lung cavitations and necrosis, which in turn is the mainstay of TB transmission (Elkington et al., 2013). Very few studies have examined the expression of TIMP in TB infections. While TIMPs are clearly known to bind and inhibit the function of MMPs, it is also becoming evident that TIMP binding to MMP can enhance the activity of certain MMPs (Khokha et al., 2013). Moreover, TIMPs appear to also exert MMP independent activities in tissue remodelling (Moore and Crocker, 2012). Our data clearly reveal two very interesting features concerning the family of tissue remodeling enzymes: (i) systemic MMPs and TIMPs appear to be very good biomarkers in distinguishing TB from non-TB individuals and (ii) the concentrations of MMPs and TIMPs are downregulated by the presence of Ss co-infection. Helminth infections are known to modulate the expression pattern of MMPs and TIMPs (Wynn, 2007) and in the case of certain helminth infections can act as inducers of these factors themselves (Anuradha et al., 2012). This data, however, suggest that Ss infection in the context of TB disease plays a modulatory role with respect to the production of pro-fibrotic factors and hence can potentially impact the degree of lung
pathology. Unfortunately, we were unable to systematically collect radiological information on the extent of pulmonary disease in this study, and therefore we are unable to corroborate the systemic findings at the pulmonary level.

It has been speculated that there are two evolutionarily conserved defence strategies against infection that limit host disease severity (Schneider and Ayres, 2008; Medzhitov et al., 2012). The first is dependent on the capacity of the host's immune system to reduce pathogen burden. In our study, since we did not observe any significant differences in the sputum smear grades between the two groups, we conclude that the bacterial load was not affected by the presence of coincidental Ss infection. The second defense strategy is commonly known as “disease tolerance” and is thought to affect the fitness cost of infection i.e. metabolic or other pathways that limit disease severity (Medzhitov et al., 2012). While we have not directly identified the mechanism by which Ss infection affects disease severity in TB, it is tempting to speculate that both alterations in the host immune and non-immune defence pathways are being modulated by co-infection. It is likely that the regulatory pathways induced during chronic helminth infections play a role in restoring homeostasis and normal tissue function and in promoting wound healing/repair and anti-inflammatory responses (Gause et al., 2013). One of the major problems in terms of current TB research and clinical demands is the increasing number of cases of extensively drug resistant and treatment refractory TB (Zumla et al., 2014). To combat this problem, a great deal of emphasis is now laid on host-directed therapies targeting inflammatory processes that can be deleterious and lead to immune exhaustion in TB (Kaufmann, 2006). Candidates for such interventions may be biological agents or already approved drugs repurposed to interfere with inflammatory processes (Hawn et al., 2013). Helminth
immuno-modulators, if approved, could feasibly serve as another approach to tackle this issue.

Our findings have important implications for the design of studies investigating immunologically-based biomarkers to distinguish active from latent TB and to monitor response to therapy. Patients from helminth-endemic regions have previously been reported to have less extensive disease compared to those from helminth-free regions (Fox et al., 1956), but it is not clear whether this is due to ethnic differences, to underlying host-pathogen differences, or to access to therapy. Our data adds another layer of complexity to this conundrum and suggest that the presence of a different chronic infection could also have an effect on the disease manifestations in TB. While our study is clearly preliminary and needs to be confirmed in a much larger setting – and while longitudinal studies examining the effect of anti-helminth treatment on TB pathology needs to be examined – our data clearly illustrate the powerful regulatory effects that helminth infections can exert on the immune response to third party infections. A more detailed examination of the regulatory pathways influencing this effect of helminth infection on TB could provide clues to unravel potentially beneficial avenues to combat this pervasive infection and disease.