Adaptive Immune Response to Lymphatic Filariasis

Chapter - 2
Parasite-antigen driven expansion of $IL-5^-$ and $IL-5^+$ Th2 human subpopulations in lymphatic filariasis and their differential dependence on $IL-10$ and $TGF-\beta$

Study -3
Parasite-antigen driven expansion of IL-5 and IL-5+ Th2 human subpopulations in lymphatic filariasis and their differential dependence on IL-10 and TGF-β

Introduction

Th2 cells were initially characterized as expressing the cytokines – IL-4, IL-5 and IL-13 [104]. Although Th2 cells can express a variety of other cytokines, these three cytokines remain the hallmark Th2 cytokines. Each Th2 cytokine has a well-defined and relatively specific function. While IL-4 is the major driving force behind Th2 differentiation, IgE class switching and alternative macrophage activation, IL-13 is an important mediator of goblet cell hyperplasia, mucus secretion and airway hyper reactivity [41]. IL-5, in contrast, acts primarily on eosinophils and their precursors in the bone marrow to induce enhanced production, survival and activation of these cells [105]. While Th2 cells have generally been considered a homogenous population, recent reports provide evidence for subpopulations within the Th2 lineage [106]. Two of the main subsets identified recently are the IL-5 expressing Th2 subset (co-expressing IL-4, IL-5 and IL-13) and the non IL-5 expressing Th2 subset (co-expressing only IL-4 and IL-13) [106]. Since the three established Th2 cytokines each play a non-redundant role in allergic disease pathology, it was postulated that these Th2 subsets might play an important role in allergic diseases. Indeed, IL-5+Th2 cells have been found in greater frequencies (F_o) in patients with eosinophilic gastrointestinal disease, while peanut allergy was found to be associated with higher F_o of IL-5 Th2 cells [107].

The canonical host immune response seen in human filarial infections is of the Th2 type and involves the production of cytokines – IL-4, IL-5, IL-9, IL-10 and IL-13, the
antibody isotypes – IgG1, IgG4 and IgE, and expanded populations of eosinophils and immunoregulatory monocyte [108]. Human filarial infection is known to be associated with down regulation of parasite-specific Th1 responses and T cell proliferation and but with augmented Th2 responses [44]. Thus, in human lymphatic filariasis (LF) patent filarial infection is associated with an antigen – specific expansion of Th2 cells (mostly defined by IL-4 expression) and enhanced production of IL-4 and IL-13 [44]. However, antigen – driven IL-5 production has been shown to be diminished in patently infected individuals [109,110] in some studies. Similarly, although protective immunity to filarial infections in mice is dependent primarily on IL-4, IL-5 does not appear to play a role in resistance to infection [111,112]. Hence, filarial infections provide a natural setting in which to explore the differential role (if any) of Th2 subsets. We wanted to explore the hypothesis that Th2 subsets would be differentially regulated in asymptomatic infection compared to uninfected or individuals with chronic pathology.

We, therefore, examined the Th2 cytokine expression patterns of CD4+ T cells in clinically asymptomatic patently infected (INF) individuals, filarial-uninfected endemic normal (UN) individuals, and in previously infected patients with filarial lymphedema (CP) both at homeostasis and following stimulation with parasite and control antigens. We show that active human LF is characterized by a significant enhancement in the Fo of both spontaneously expressed and parasite antigen – driven IL-5+ and IL-5+Th2 cells. We show that the Fo of IL-5+Th2 subpopulation is positively correlated with peripheral eosinophil and neutrophil numbers in filarial infections, while the IL-5+Th2 cells are strongly positively related to the levels of parasite specific IgE and IgG4. We also show that these Th2
Table 5 Characteristic of study Population

<table>
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<th></th>
<th>CP&lt;sup&gt;a&lt;/sup&gt; (n=23)</th>
<th>INF (n=39)</th>
<th>UN (n=15)</th>
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</table>

<sup>a</sup> CP refers to individuals with filarial pathology, INF refers to individuals with asymptomatic, filarial infection and NL refers to endemic normal individuals.

<sup>b</sup> Below the limits of detection.
subpopulations appear to have differing programs of regulation by both IL-10 and TGF-β in filarial-infected individuals.

**Study population**

We studied a total of 70 individuals comprising of 32 clinically asymptomatic, filarial infected (hereafter INF) individuals, 23 individuals with filarial lymphedema or elephantiasis (hereafter CP), and 15 uninfected, endemic normal (hereafter EN) individuals in an area endemic for LF in Tamil Nadu, South India (Table 5). All CP individuals were circulating filarial antigen negative by both the ICT filarial antigen test (Binax, Portland, ME) and the TropBio Og4C3 enzyme-linked immunosorbent assay (ELISA) (Trop Bio Pty. Ltd, Townsville, Queensland, Australia), indicating a lack of current active infection. The diagnosis of prior filarial infection was made by history and clinical examination as well as by positive *Brugia malayi* antigen (BmA) -specific IgG4. BmA-specific IgE, IgG4 and IgG ELISA were performed . All INF individuals tested positive for active infection by both the ICT filarial antigen test and the TropBio Og4C3 ELISA and had not received any anti-filarial treatment prior to this study. All INF individuals were treated with a standard dose of diethylcarbamazine (DEC) and albendazole and follow – up blood draws were obtained one year later. We also used 7 of the 32 INF individuals exclusively for performing cytokine blocking studies. All EN individuals were circulating filarial antigen negative and without any signs or symptoms of infection or disease. There were no differences between the groups in terms of demographics or socio-economic status.
Parasite-antigen driven expansion of IL-5 and IL-5+ Th2 human subpopulations in lymphatic filariasis and their differential dependence on IL-10 and TGF-β

Figure 3.1. A Representative dot plot showing the BmA – stimulate expression of CD4+ T cells expressing various Th2 cytokines. CD4+ T cells expressing IL-4, IL-5 and IL-13 at baseline and following filarial antigen stimulation are shown in a representative flow cytometry plot from an INF individual.
Figure 3.2. Expanded F₀ of Th2 subsets in filarial infected individuals in comparison to uninfected individuals.

(A) IL-5- Th2 Cells

(B) IL-5+ Th2 Cells

Figure 3.2. Expanded F₀ of Th2 subsets in filarial infected individuals in comparison to uninfected individuals. (A) Baseline as well as filarial antigen (BmA and Mf), PPD and PMA/ionomycin stimulated F₀ of CD4⁺ T cells co-expressing IL-4 and IL-13 but not IL-5 (IL-5-Th2 cells) in INF (n=25) and UN (n=15) individuals. (B) Baseline as well as filarial antigen (BmA and Mf), PPD and PMA/ionomycin stimulated F₀ of CD4⁺ T cells co-expressing IL-4, IL-5 and IL-13 (IL-5⁺Th2 cells) in INF and UN individuals. The data are depicted as bar graphs with the bars representing the geometric means and 95% confidence intervals. P values were calculated using the Mann-Whitney test.
Results

Increased frequency of IL-5⁻ and IL-5⁺ Th2 subsets following filarial – antigen stimulation in INF compared to UN and CP individuals

We first measured the spontaneously expressed and antigen-stimulated $F_o$ of CD4⁺ T cells expressing IL-4 and IL-13 but not IL-5 (CD4⁺IL4⁺IL13⁻IL5⁻) as well as those expressing IL-4, IL-13, and IL-5 (CD4⁺IL4⁺IL13⁺IL5⁻) in INF (for representative flow plot, see Figure 3.1) and contrasted these with the $F_o$ of these subpopulations in UN and CP. As shown in Figure 3.2A, INF individuals exhibit significantly higher $F_o$ of IL-5⁻ Th2 cells in response to the parasite antigens BmA (2.2 fold) and Mf (2.1 fold) but not at baseline nor following PPD or PMA/ionomycin stimulation in comparison to UN individuals, INF individuals also had significantly higher $F_o$ of IL-5⁺ Th2 cells at baseline (1.2 fold) and following BmA (2.6 fold) and Mf (1.9 fold) (but not PPD or PMA/ionomycin) stimulation in comparison to UN (Figure 3.2.B). Similarly, INF individuals exhibit significantly higher $F_o$ of IL-5⁻ Th2 cells at baseline (1.8 fold) and in response to BmA (3 fold) and Mf (2.2 fold) but not following PPD or PMA/ionomycin stimulation in comparison to CP individuals (Figure 1.A). Finally, INF individuals also had significantly higher $F_o$ of IL-5⁺ Th2 cells both at baseline (1.2 fold) and following stimulation with BmA (2.1 fold) and Mf (1.7 fold) (but not with PPD or PMA/ionomycin) in comparison to CP (Figure 3.2.B). Thus, filarial infection in this population was associated with expanded $F_o$ of antigen – stimulated Th2 subpopulations, perhaps indicating a role for these cells in filarial infection and in the prevention of overt pathology.
Figure 3. Positive relationship between IL-5+Th2 cells and absolute eosinophil and neutrophil counts in filarial infections. Baseline Fo of CD4+ T cells co-expressing IL-4, IL-5 and IL-13 (IL-5+Th2 cells) or co-expressing IL-4 and IL-13 but not IL-5 (IL-5-Th2 cells) were correlated to absolute eosinophil (A), neutrophil (B) and basophil (C) counts in INF (n = 32) individuals. The data are shown as scatterplots with each circle representing a single INF individual. P and r values were calculated using the Spearman rank test.
Figure 3.4. Positive relationship between IL-5-Th2 cells and BmA specific IgE and IgG4 levels in filarial infections.

(A). IgE

(B). IgE

Figure 3.4. Positive relationship between IL-5-Th2 cells and BmA specific IgE and IgG4 levels in filarial infections. Baseline Fo of CD4+ T cells co-expressing IL-4, IL-5 and IL-13 (IL-5+Th2 cells) or co-expressing IL-4 and IL-13 but not IL-5 (IL-5-Th2 cells) were correlated to BmA specific IgE (A) or IgG4 (B) antibody titres in INF (n = 32) individuals. The data are shown as scatterplots with each circle representing a single INF individual. P and r values were calculated using the Spearman rank test.
IL-5^+Th2 cells exhibit a positive correlation with absolute eosinophil and neutrophil counts in INF individuals

To determine whether the IL-5^- and IL-5^+Th2 subsets were associated with differential functions in filarial infections, we examined the relationship between the baseline F_o of IL-5^- and IL-5^+Th2 cells and peripheral eosinophil, neutrophil and basophil numbers in INF individuals (n=32). As shown in Figure 3.3.A, IL-5^+ (but not IL-5^-) Th2 cells had a significantly positive correlation between their F_o ex vivo and the absolute eosinophil count (r=0.4667, p=0.0071) as determined by Spearman rank correlation. Similarly, as shown in Figure 3.3.B, IL-5^+ (but not IL-5^-) Th2 cells exhibited a significant positive association with the absolute neutrophil count (r=0.4115, p=0.0193). In contrast, both IL-5^- and IL-5^+Th2 cells showed no correlation with the absolute basophil numbers in INF individuals (Figure 3.3.C).

IL-5^-Th2 cells exhibit a positive correlation with IgE and IgG4 isotypes in INF individuals

We next examined the relationship between the F_o of IL-5^- and IL-5^-Th2 cells ex vivo and IgE and IgG4 levels in INF individuals (n=32). As shown in Figure 3.4.A, IL-5^- (but not IL-5^+) Th2 cells exhibited a significantly positive correlation between baseline F_o and the BmA – specific IgE levels (r=0.7717, p<0.0001) as determined by Spearman rank correlation. Similarly, as shown in Figure 3.4.B, IL-5^- (but not IL-5^-) Th2 cells exhibited a significant positive association with the BmA – specific IgG4 levels (r=0.4115, p=0.0193).
Figure 3.5. IL-10 and TGF-β regulate the Fo of Th2 subsets in filarial infections.

(A) IL-5- Th2 Cells

(B) IL-5+ Th2 Cells

Figure 3.5. IL-10 and TGFβ regulate the Fo of Th2 subsets in filarial infections. (A) IL-10 and TGF-β neutralization (with anti-IL-10 and anti-TGF-β antibodies, respectively) significantly decreases the Fo of CD4+ T cells expressing IL-4 and IL-13 but not IL-5 (IL-5-Th2 cells) following stimulation with BmA in a subset of INF individuals (n = 7). (B) IL-10 and TGF-β neutralization significantly increases the Fo of CD4+ T cells expressing IL-4, IL-5 and IL-13 (IL-5+Th2 cells) following stimulation with BmA in a subset of INF individuals. Antigen – stimulated Fo are shown as net Fo with the baseline levels subtracted. Each line represents a single individual. P values were calculated using the Wilcoxon signed rank test.
**Th2 subsets are differentially regulated by IL-10 and TGF-β in INF individuals**

To determine the role of IL-10 and TGF-β in the modulation of Th2 subpopulations in INF, we measured the frequency of IL-5−Th2 cells and IL-5+Th2 cells following short-term stimulation with the parasite antigen BmA in the presence or absence of anti-IL-10 or anti-TGF-β neutralizing antibody in INF individuals (n=7). As shown in Figure 3.5.A, both IL-10 and TGF-β neutralization resulted in significantly decreased F₀ of IL-5−Th2 cells in INF individuals (2.3 and 1.9 fold respectively). In marked contrast, as shown in Figure 3.5.B, both IL-10 and TGF-β blockade resulted in significantly increased F₀ of IL-5+Th2 cells following BmA stimulation (1.5 and 1.3 fold respectively). Thus, both IL-10 and TGF-β play an important role in the modulation of Th2 subset F₀ in filarial infections.

**Discussion**

Th2 responses are considered to be the hallmark of helminth infection and are indeed required for host resistance to a variety of helminths in animals [108]. The three prototypical Th2 cytokines – IL-4, IL-5 and IL-13 have all been shown to play important but non-redundant roles in helminth immunity [113]. In addition, the recent explosion of interest in CD4+ T cell subpopulations and the availability of polychromatic cytokine staining has helped define heterogeneity within the Th2 compartment [106]. Thus, two major subsets of Th2 cells were recently described – an IL-5 expressing Th2 population, which is thought to play an important role in eosinophilic inflammation and an IL-5 non expressing Th2 population, which is thought to play an important role in other forms of allergic inflammation [106]. Moreover, it has been demonstrated that IL-5+ and IL-5−Th2 cells represent more and less
highly differentiated Th2 subpopulation, respectively [114]. However, the role of these subsets in helminth infections is not known.

The induction of prototypical Th2 response with high IL-4, IL-5 and IL-13 secretion has long been considered to be the hallmark of active infection in human LF [44]. However, not all studies have consistently shown a predominant prototypical Th2 response in filarial infections. A recent study in Mali suggested that patent, long-standing filarial infection is associated with expanded adaptive regulatory T cell cells rather than an expansion of classical Th2 cells environment [115]. Previous studies have reported a down regulation of IL-5 upon parasite stimulation [109,110,116]. In addition, the role of Th2 responses in protection from or in the pathogenesis underlying the disease associated with LF has not been well characterized either. We therefore utilized two sets of comparisons to help elucidate the role of Th2 subsets in human filarial infections – (1) comparisons of Th2 subsets in INF and UN individuals, (2) comparisons of these subsets in INF and CP individuals. We were able to demonstrate that both Th2 subsets are expanded preferentially in active, subclinical infection but not in filarial disease (without active infection). Our data on Ag–induced expression of CD4+ Th2 cell subpopulations also reveal interesting facets of T cell driven immune regulation in filarial infection and disease. First, the alterations in the CD4+ Th2 cell cytokine repertoire is filarial – antigen specific since the since these alterations in Th2 subpopulation Fo were primarily observed only in response to the filarial-derived BmA and Mf antigens but not to PPD nor in response to polyclonal stimulation. Second, the importance of antigen – persistence is clearly illustrated by our data on a small subset of individuals who cleared infection following treatment and were therefore proven to be filarial antigen negative. The follow up data on these individuals indicate a clear reversion to the normal/homeostatic levels of Th2 subset
populations. On the other hand, the different Th2 subpopulations continue to expand in a control group of individuals, who also received treatment but failed to clear infection. Therefore, the expansion of antigen-specific Th2 subsets is closely associated with the presence of parasite antigen in vivo.

Th2 cells are thought to play a counter-regulatory role in a variety of infectious and inflammatory conditions to offset pathology and promote tissue repair and wound healing mechanisms [117]. Th2 responses are considered to be fundamentally important in protection against the development of pathology both because of their ability to ameliorate Th1 induced inflammatory responses and because of their propensity to promote wound healing and tissue repair [118]. For example, IL-5 and IL-13 have pro-fibrotic activity and, in addition, IL-4 and IL-13 are critical mediators of alternative activation of macrophages. Our study of the Ag–stimulated expansion of Th2 subpopulations reveals a significant association of these cells with asymptomatic infection, confirming a previously reported association [119]. By contrasting these Th2 subpopulations in clinically asymptomatic patients to those with filarial lymphedema (CP) we may be able to infer a role for these expanded Th2 subsets in protection from the development of clinical pathology. Moreover, our data on the lower levels of Th2 subpopulations in CP supports the suggestion that unchecked parasite-specific Th1/Th17 cells may contribute to the pathological process in LF.

Our data reveal clear distinctions in the relationship between IL-5+ and IL-5+Th2 cells and expansion of innate leukocyte populations in filarial infections. Eosinophils are considered to be important innate effectors in immunity to helminth infections and have been shown to play a role in protection against S. mansoni and other helminths [120,121]. Similarly, basophils are known to act as effectors to promote parasite killing during challenge
infections of immunized animals, perhaps through antibody dependent mechanisms [122,123], while neutrophils have been demonstrated to attack helminth larvae in response to IL-4 and IL-5 [124,125]. Our study implicates the IL-5+ Th2 subpopulation in this innate defense mechanism by promoting the recruitment and/or activation of eosinophils and neutrophils. Our study also demonstrates an important association of IL-5− Th2 cells in promoting Th2 associated (IgE and IgG4) antibody responses in filarial infection. All helminth infections are characterized by the induction of antibody isotypes of the class – IgE and IgG4 (IgG1 in mice), that are largely dependent on the IL-4 [126].

Not only did we assess the expansion of these Th2 subpopulations, we also examined the mechanisms regulating the expression of these cytokines in these two subpopulations. Since IL-10 and the TGFβ in chronic infections are known to play a role in modulating T cell expression of cytokines in filarial infections [119], we examined the Fo of IL-5+ and IL-5− Th2 cells following either IL-10 or TGF-β blockade during in vitro stimulation with filarial antigen. Our data, through preliminary due to the small number of samples able to obtained, show clear differences in the modulation of the Th2 subsets. We demonstrate that the expansion of IL-5− Th2 cells is dependent on both IL-10 and TGF-β since blockade of these cytokines significantly reduces the frequency of IL-5− Th2 cells. Conversely, both IL-10 and TGF-β appear to impair the induction of the IL-5+ Th2 subset. While it has been previously shown that IL-5 expression in Th2 cells is limited to the effector memory subset whereas IL-4 is expressed in both central and effector memory subsets [106], this finding that IL-10 and TGF-β signaling may be critical to Th2 subpopulation expansion provide new insight into the interrelationship between the IL-5+ and IL-5− Th2 subpopulations and provides new avenues
for the study of filarial-specific immune regulation and protection from immune-mediated pathology in LF.

In summary, our study examines in depth the CD4+ Th2 cell subset repertoire in a chronic parasitic infection and sheds light on the role of these subsets in both the regulation of immune responses in active infection and in the pathogenesis of filarial lymphedema. While we have not performed longitudinal studies to define the development of pathology in filarial infection and this was a study using a combination of previously (pre-treatment) and prospectively (post-treatment) collected samples with inherent potential limitations with respect to bias, our strategy of contrasting immune responses in individuals with early or subclinical disease and those with late or clinical disease yields important information on the association of Th2 subsets in pathogenesis. However, the potential drawbacks in the study include potential bias in using both prospectively and retrospectively collected samples and lack of rigorous controls in eliminating potential confounders including socio-economic status of individuals in the study. The lack of proper information in the study area regarding the prevalence of the different clinical groups, adds to the problem of potential bias, which means that our conclusions cannot be generalized. In addition, while we have demonstrated the presence of Th2 subsets in filarial infections, disease association is not formal proof of function and the elucidation of function needs to be explored in the future. Nevertheless, our study clearly defines and important association of filarial infection with heightened expansion of Th2 cells suggesting that these subsets play an important role in infection.