Expansion of parasite-specific CD4+ cells expressing IL-10 superfamily cytokine members and their regulation in human lymphatic filariasis

Study - 7
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Introduction

Lymphatic filariasis (LF) is associated with a variety of clinical outcomes [146]. From an immunological perspective, the most intriguing and common clinical manifestation of LF is the subclinical condition associated with circulating microfilariae or circulating adult worm antigen [90]. Clinically asymptomatic LF has been shown to be associated with impaired parasite – specific proliferative responses as well as a down regulation of CD4\(^+\) T cell responses [44]. While parasite antigens specifically down modulate CD4\(^+\) Th1 responses, live parasites appear to induce a global down regulation of both Th1 and Th2 responses in vitro [116]. The clinically asymptomatic infection, filarial disease has been associated with increased frequencies of CD4\(^+\) T cells expressing IFN\(\gamma\) in response to parasite antigen [155] and elevated production of pro-inflammatory Th1 and Th17 cytokines [79] possibly driven by microbial products translocated through lymphatic endothelium [182]. Interestingly, at steady state, patent LF has been shown to be associated with an expanded number of regulatory T cells expressing IL-10 in comparison to uninfected individuals from the same endemic area [183].

Chronic filarial infection has been shown to be associated with a regulatory environment dominated by parasite antigen-driven IL-10 that primarily modulates antigen specific CD4\(^+\) T cells responses but also provides some spillover suppression of bystander responses as well [126,184]. While IL-10 has remained the main focus of studies examining immune-regulation in LF and other infectious diseases, it is also known that IL-10 belongs to
a superfamily of cytokines that consist of 9 members – IL-10, IL-19, IL-20, IL-22, IL-24, IL-26 and the more distantly related IL-28A, IL-28B and IL-29 [77,185]. This IL-10 superfamily is felt to be essential for maintaining the integrity and homeostasis of tissues, modulating innate immune responses from tissues to limit the damage caused by viral and bacterial infections and facilitating wound healing processes in infection and inflammation [77,185]. Unlike IL-10, the role of IL-19, IL-24 and IL-26 in regulating functions of immune cells populations has not been explored. IL-19 has been demonstrated to increase Th2 cytokine expression in activated T cells and activated monocytes and its expression has been shown to be increased in certain Th2-mediated diseases such as atopic dermatitis and asthma [186,187,188]. IL-24, in contrast, has been implicated to play an important role in host defense against tumors [189]. More recently, IL-19 and IL-24 have been shown to have an unequivocal immunosuppressive role in the skin by inhibiting the production of IL-1β and IL-17 and promoting infection with *Staphylococcus aureus* [131] whereas IL-26 has been shown to be more pro-inflammatory, as it can induce cytokine production and Th17 cell generation in rheumatoid arthritis [190].

To examine the association of IL-19, IL-24 and IL-26 with immune responses in human filarial infections, we examined the expression pattern of these cytokines in CD4\(^+\) T cells at homeostasis (ex vivo) and following antigen – stimulation in clinically asymptomatic filarial-infected and -uninfected individuals as well as those with disease associated with LF (lymphedema and/or elephantiasis). We demonstrate that the expression pattern of IL-19 and IL-24 in T cells closely mirrors that of IL-10 and that, similar to IL-10, active filarial infection is characterized by elevated frequencies of CD4\(^+\) T cells expressing IL-19 and IL-24. Increased IL-26 expression, in contrast, appears to be associated with the pathological
Table-7 Characteristic of study Population

<table>
<thead>
<tr>
<th></th>
<th>CP a (n=33)</th>
<th>INF (n=39)</th>
<th>UN (n=15)</th>
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</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>43 (29–65)</td>
<td>39 (23–65)</td>
<td>36 (24-65)</td>
</tr>
<tr>
<td>Gender male / female</td>
<td>21 / 12</td>
<td>29 / 10</td>
<td>9 / 6</td>
</tr>
<tr>
<td>Lymphedema/Elephantiasis</td>
<td>Yes</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>ICT card test</td>
<td>Negative</td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td><em>W. bancrofti</em> circulating antigen levels (U/ml)</td>
<td>&lt; 32 b</td>
<td>1409</td>
<td>&lt; 32</td>
</tr>
<tr>
<td></td>
<td>(138–22377)</td>
<td></td>
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a CP refers to individuals with filarial pathology, INF refers to individuals with asymptomatic, filarial infection and NL refers to endemic normal individuals.

b Below the limits of detection.
consequences of infection (e.g. lymphedema and/or elephantiasis). Finally, we also uncover a novel role for the pro-inflammatory cytokines - IL-1β and IL-23, as drivers of the expansion of IL-19 and IL-24 expressing T cells.

**Study population**

We studied a group of 58 clinically asymptomatic infected (hereafter INF) individuals, 23 individuals with filarial lymphedema (hereafter CP) individuals and 15 uninfected, endemic normal (hereafter UN) individuals in a *W. bancrofti*-area endemic Tamil Nadu, South India (Table 7). All CP and UN 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 in those with CP was made by history and clinical examination as well as positive *Brugia malayi* antigen (BmA) -specific IgG4. BmA-specific IgG4 and IgG ELISA were performed. All INF individuals tested positive for by both the ICT filarial antigen test and the TropBio Og4C3 ELISA and had not received any anti-filarial treatment prior to this study. The UN individuals were from the same area / community as the INF individuals and were recruited during the same time period. Another set of 17 INF individuals were used for the in vitro cytokine blocking and addition studies. There were no differences between the groups in terms of demographics or socio-economic status.
Figure 7.1. Filarial infection is associated with increased frequencies of IL-10, IL-19 and IL-24 expressing and decreased frequencies of IL-26 expressing CD4⁺ T cells.

Figure 7.1.A

CD4⁺ T Cells

Figure 7.1.B
Figure 7.1. Filarial infection is associated with increased frequencies of IL-10, IL-19 and IL-24 expressing and decreased frequencies of IL-26 expressing CD4⁺ T cells. (A) A representative dot plot from a filarial - infected individual showing CD4⁺ T cell expression of IL-10, IL-19, IL-24 and IL-26 in response to BmA and PMA/Ionomycin and representative dot plots from CP, INF and UN individuals for CD4⁺ T cell expression of IL-19 and IL-24. The frequencies of CD4⁺ T cells expressing IL-10 (B), IL-19 (C), IL-24 (D) and IL-26 (E) at baseline and following stimulation with BmA, MF, PPD and PMA/ Ionomycin in CP (n=23), INF(n=25) and UN (n=15) individuals. Antigen – stimulated frequencies are shown as net frequencies with the baseline levels subtracted. The data are shown as scatter plots with each circle representing a single individual. P values were calculated using the Kruskal-Wallis test with Dunn’s multiple comparisons (* p < 0.05, ** p < 0.01, *** p < 0.001).
Results

Filarial infection is associated with increased frequencies of spontaneous and antigen-specific CD4+ T cells expressing IL-19 and IL-24

To determine the association of IL-10 and the related family members IL-19, IL-24 and IL-26 in protection from pathology and in susceptibility to or resistance to clinically apparent disease in LF, we measured the frequency of CD4+ T cells expressing these cytokines in INF individuals and in those with LF-associated disease (CP) or UN individuals (Figure 7.1.A). As shown in Figure 7.1.B, INF individuals had significantly increased frequencies of CD4+ T cells expressing IL-10 at homeostasis/steady state in comparison to CP individuals and increased frequencies of CD4+ T cells expressing IL-10 upon filarial antigen stimulation compared to CP and UN individuals. Similarly, INF individuals also exhibited significantly increased frequencies of CD4+ T cells expressing IL-19 at baseline in comparison to CP individuals and following filarial antigen stimulation compared to CP and INF individuals (Figure 7.1.C). In addition, INF had significantly increased frequencies of CD4+ T cells expressing IL-24 at baseline and following filarial antigen stimulation in comparison to CP individuals (Figure 7.1.D). In contrast, INF had significantly lower frequencies of CD4+ T cells expressing IL-26 following filarial antigen stimulation compared to CP individuals (Figure 7.1.E). Interestingly, no significant difference was observed in the frequency of CD4+ T cells expressing IL-10, IL-19, IL-24 or IL-26 upon PPD stimulation or following PMA/ ionomycin stimulation. Thus, filarial infection is associated with elevated frequencies of spontaneous and antigen-specific IL-10, IL-19 and IL-24 expressing CD4+ T cells in comparison to CP individuals, suggesting that these cells are associated with protection against pathology. In addition, filarial infection is also associated with decreased
Figure 7.2. Filarial infection is associated with increased levels of antigen-induced IL-19 and IL-24 and decreased levels of IL-26.

Figure 7.2. Filarial infection is associated with increased levels of antigen-induced IL-19 and IL-24 and decreased levels of IL-26. The total levels of IL-19 (A), IL-24 (B) and IL-26 (C) in PBMC culture supernatants at baseline or following stimulation with BmA, Mf, PPD or PMA/ ionomycin in CP and INF individuals. Antigen–stimulated cytokine levels are shown as net cytokine levels with baseline subtracted. The data are shown as scatter plots with each circle representing a single individual or as bar graphs with geometric means and 95% confidence intervals. P values were calculated using the Mann-Whitney test.
frequencies of antigen – specific IL-26 expressing CD4+ T cells in comparison to CP individuals, suggesting that IL-26 expressing T cells are pre-dominantly associated with pathology.

**Filarial infection is associated with increased production of antigen – specific IL-19 and IL-24 and decreased production of IL-26**

To determine whether the T cell expression pattern of IL-19, IL-24 and IL-26 is reflected in secreted cytokine production following antigen stimulation, we cultured PBMC from INF and CP individuals with filarial and non-filarial antigen (PPD) as well as with PMA/ionomycin as a positive stimulus for 24h and measured the levels of IL-19, IL-24 and IL-26 by ELISA. As shown in Figure 7.2.A, both BmA [Geometric Mean (GM) of 31.3 pg/ml vs 3.1 pg/ml] and Mf (GM of 99.4 vs. 15.6) induced significantly higher production of IL-19 in PBMC from INF compared to CP individuals. As also shown in Figure 7.2.B, both BmA (GM of 29.4 vs. 5.5) and Mf (GM of 44.2 vs. 7.1) induced significantly higher production of IL-24 in PBMC from INF compared to CP individuals. In contrast, as shown in Figure 7.2.C, BmA induced significantly lower production of IL-26 from INF compared to CP individuals (GM of 368 vs. 198). This response was filarial – antigen-specific since neither PPD nor PMA/ionomycin induced any significant differences in the net cytokine production of IL-19, IL-24 or IL-26. Similarly, spontaneous production of IL-19 and IL-24 (but not IL-26) was also not significantly different between INF and CP individuals. Thus, filarial infection is characterized by increased production of parasite antigen – specific IL-19 and IL-24 and decreased production of IL-26.
Figure 7.3. IL-10 regulates the frequencies of CD4$^+$ and CD8$^+$ T cells expressing IL-19 and IL-24 and modulates IL-19 and IL-24 gene expression in filarial infections.

Figure 7.3. IL-10 regulates the frequencies of CD4$^+$ and CD8$^+$ T cells expressing IL-19 and IL-24 and modulates IL-19 and IL-24 gene expression in filarial infections. (A) The frequencies of CD4$^+$ and CD8$^+$ T cells expressing IL-19, IL-24 and IL-26 following IL-10 neutralization and stimulation with BmA in a subset of INF individuals (n=7). (B) The frequencies of CD4$^+$ and CD8$^+$ T cells expressing IL-19, IL-24 and IL-26 following addition of rIL-10 and stimulation with BmA in a subset of INF individuals (n=7). Antigen – stimulated frequencies are shown as net frequencies with the baseline levels subtracted.
Figure 7.3. IL-10 regulates the frequencies of CD4$^+$ and CD8$^+$ T cells expressing IL-19 and IL-24 and modulates IL-19 and IL-24 gene expression in filarial infections. (C) PBMCs from infected (INF) individuals ($n=7$) were cultured with BmA alone or BmA with rIL-10 for 24 h and mRNA expression of IL-19, IL-24 and 18s RNA were measured by RT-PCR. Data are shown as fold change BmA plus IL-10 over BmA alone controls. Each line represents a single individual. P values were calculated using the Wilcoxon signed rank test.
Since, IL-10 altered the frequency of cytokine expressing T cells, we sought to determine if IL-10 altered the transcriptional regulation of IL-19 and IL-24. To this end, we examined the gene expression of IL-19 and IL-24 in RNA from PBMC of INF individuals (n=7) following addition of rhIL-10 and BmA. As shown in Figure 7.2.C, rhIL-10 induced significant upregulation of the transcripts for IL-19 (GM fold change of 3.8) and IL-24 (GM fold change of 4.2) compared to the media control following BmA stimulation. Thus, both IL-19 and IL-24 expression appears to be regulated at the transcriptional level by IL-10 in filarial infections.

We also tested the role of other pro- and anti-inflammatory cytokines in inducing or abrogating the expansion of CD4⁺ T cells expressing IL-10 family cytokines. Among the cytokines tested, (IL-1 β, IL-2, IL-4, IL-6, IL-23 and TGF-β), only IL-1β and IL-23 exhibited any significant effect on the induction of IL-19 and IL-24 expressing T cells. As shown in Figure 7.3.A, blockade of IL-1 β resulted in significantly lower frequencies of CD4⁺ T cells expressing IL-19 and IL-24 in INF individuals (n=10). Similarly, blockade of IL-23R also resulted in significantly lower frequencies of CD4⁺ and CD8⁺ T cells expressing IL-19 and IL-24 (Figure 7.3.B). On the other hand, blockade of IL-6R had no significant effect on the frequencies of CD4⁺ and CD8⁺ T cells expressing IL-19 and IL-24 (Figure 7.3.C). None of these cytokines had any significant effect on the frequencies of IL-26 expressing T cells in filarial infection (data not shown). Thus, IL-1β and IL-23 appear to also regulate IL-19 and IL-24 expression in T cells.
Discussion

Filarial parasites exert profound immuno regulatory effects on the host immune system with both parasite-antigen specific and more generalized levels of immune suppression [184]. Among the host factors influencing immune-regulation, one of the key players is IL-10. IL-10 is a powerful immune-regulatory cytokine known to be induced in a variety of helminth infections [76]. The IL-10 dominated regulatory environment induced in chronic helminth infections is known to modulate the entire repertoire of CD4$^+$ T cell effector functions [76]. Increased levels of spontaneous as well as parasite – specific IL-10 are associated with filarial infections [191] and thought to play a crucial role in down-regulation of T cell-mediated immune responses [76]. Therefore, this IL-10 dominated response has the potential to regulate not only the balance of T cell subsets, but also to modulate the response to both bystander antigens and allergens as well [184,192,193]. Less well studied, however, is the role of other cytokines belonging to the IL-10 superfamily that includes IL-19, IL-20, IL-22, IL-24 and IL-26 [77]. These cytokines elicit diverse host defense responses during infections and are known to facilitate the tissue-healing process in infection and inflammation [77]. With the exception of IL-22 (whose role as a Th17 cytokine has been well characterized) [194], the role of these IL-10 family of cytokines in modulating immune responses in chronic infection is not known. Although IL-19 has been shown to be increased in both Th1- and Th2-dominant diseases such as psoriasis and asthma, respectively [187,188], data from IL-19 deficient mice and human data autoimmune diseases and other inflammatory conditions suggest that IL-19 has a potent anti-inflammatory activity [195,196,197]. IL-24, alternatively, has been predominantly studied in tumor immunology and has exhibited great promise as an anti-tumor therapeutic cytokine [189]. Recent data from a mouse model of *S. aureus*, however, clearly reveals an important
immuno-modulatory role for IL-19 and IL-24 in suppressing IL-1β and IL-17 dependent effector pathways and promoting susceptibility to infection [131].

Since CD4⁺ T cells are known producers of IL-19 and IL-24 [77], we studied the regulation of these cytokines as well as IL-26 in filarial infection. Our study of CD4⁺ T cells expressing IL-10 and its extended family reveals four important features. First, CD4⁺ T cells expressing IL-19 and IL-24 mirror the pattern observed in CD4⁺ T cells expressing IL-10. Thus, both spontaneously and following filarial – antigen stimulation, we consistently detected a significantly higher proportion of CD4⁺ T cells expressing IL-10, IL-19 and IL-24 in the asymptomatic, infected group. Second, CD4⁺ T cells expressing IL-26 show a very different pattern of expression with significantly decreased frequencies being observed following antigen –stimulation suggesting that IL-26⁺ T cells are associated with inflammatory pathology rather than the asymptomatic state. Third, the regulation of IL-10, IL-19, IL-24 and IL-26 in T cells appears to be highly antigen – specific since the elevated frequencies of IL-10, IL-19, IL-24 and IL-26 expressing T cells is observed only upon filarial antigen stimulation but not following stimulation with an unrelated antigen (PPD) or with a mitogen. Finally, the filarial antigen induced increase in IL-10⁺ or IL-19⁺ T cells in INF individuals or IL-26⁺ T cells in CP individuals is significant even when compared to UN individuals, indicating that the alterations in expression pattern of IL-10 associated cytokines is directly associated with filarial infection or disease. Our report, while not providing direct mechanistic evidence, nevertheless suggests a putative role for the IL-10 family of cytokines especially IL-19 and IL-24 in mediating protection against pathology, as does IL-10 itself. This is further corroborated by the fact that filarial- antigen stimulation of PBMC cultures also resulted in significantly increased levels of total IL-19 and IL-24 in filarial infected
individuals when compared to those with pathology and inactive infection. Our data also reveal an important association of IL-26 expressing T cells with filarial lymphedema. This association is similar to the role that IL-26 plays in other inflammatory diseases such as rheumatoid arthritis [190] wherein IL-26 has been shown to be over-expressed and to generate pro-inflammatory cytokine production and Th17 cell induction- and Crohn's disease [198] wherein IL-26 modulates intestinal epithelial cell proliferation and pro-inflammatory gene expression. Although we have not explored the mechanism by which IL-26 regulates or exacerbates pathology in LF, the presence of elevated frequencies of IL-26 expressing T cells in filarial lymphedema clearly suggests an association with pathology. Finally, a possible explanation for differential relationship between the effector functions of IL-19/IL-24 and those of IL-26 may be related to receptor usage among these cytokines. While IL-19 and IL-24 signal predominantly through the Type 1 IL-20 receptor (composed of IL-20R1 and IL-20R2), IL-26 signaling occurs through a heterodimeric receptor composed of IL-10R2 and IL-20R1 [77].

In addition to characterizing the expression pattern, we also examined some of the mechanisms regulating the expression of these cytokines. Since IL-10 and the persistence of antigen in chronic infections are known to play a role in modulating T cell expression of cytokines [126], we examined the frequencies of CD4$^+$ T cells expressing IL-19, IL-24 and IL-26 following IL-10 blockade during in vitro stimulation and following addition of rIL-10. Our findings reveal an important role for IL-10 in the induction of IL-19 and IL-24 as IL-10 blockade resulted in partial abrogation of CD4$^+$ T cells expression of these cytokines. Our data also reveal that the addition of exogenous rIL-10 significantly modulated IL-19 and IL-24 expression by T cells and suggest that IL-10 may be a transcriptional regulator of IL-19 and
IL-24 in filarial infections. While it would be tempting to speculate that IL-10 induction in filarial infection is mainly responsible for the subsequent induction of IL-19 and IL-24, our data did not examine the kinetics of induction of these cytokines in filarial infection. In terms of other regulators of IL-19 and IL-24, our data suggest that IL-1β and IL-23 induce the expression of IL-19 and IL-24. To our knowledge, this is the first piece of evidence for a role of IL-1β in regulating IL-10 superfamily members, whereas there has been one previous report demonstrating that IL-23 can induce IL-19 and IL-24 in mouse epidermal cells [199]. Interestingly, neither IL-10 itself nor IL-1β and IL-23 had any effect in regulating the expression of IL-26 in CD4⁺ and CD8⁺ T cells. Whether this differential regulation reflects differences in proximity (at the chromosomal level) or genomic structures between IL-10 and the other family members [77] awaits clarification.

One of the hallmarks of chronic infection is the modulation of immune responses in the presence of active infection. However, very little is known about the regulation of these immunomodulatory pathways after the elimination of infection. Indeed, although IL-10 has been thoroughly investigated as the dominant immune modulator in lymphatic filariasis, no data exist on the T cell expression of IL-10 following successful chemotherapy. Therefore, we examined the frequencies of IL-10⁺, IL-19⁺, IL-24⁺ and IL-26⁺ T cells in patients with active LF before and following anti-filarial chemotherapy (in which there was subsequent elimination of filarial antigens). As a control, we also examined individuals who underwent treatment but failed to eliminate infection. Our findings reveal that curative therapy is associated with changes in the cytokine-secreting repertoire of CD4⁺ T cells. Thus, the frequency of T cells expressing IL-10, IL-19, IL-24 and IL-26 are all decreased upon curative treatment, in contrast to that seen in those who continued to harbor infection. This reduction in
frequencies might represent a reduction in the total frequency of filarial-specific T cells. Nevertheless, our study therefore demonstrates that persistence of antigen is a critical factor in maintaining the elevated frequencies of IL-10, IL-19 and IL-24 expressing T cells in filarial infections.

In summary, we have investigated the role of the IL-10 family of cytokines in filarial infection and disease. While we have not performed decades long longitudinal studies to define the development of pathology in filarial infection, our strategy of contrasting immune responses in individuals with subclinical disease and those with chronic clinical manifestations has yielded important information on the role of IL-10, IL-19, IL-24 and IL-26 in pathogenesis. Future studies to evaluate the mechanism by which IL-19 and IL-24 regulate immune response in LF and the mechanism by which IL-26 is associated with pathogenesis should shed light on the role of the IL-10 family of cytokines in other chronic infections as well.