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Interleukin 1 (IL-1)- and IL-23-Mediated Expansion of Filarial Antigen-Specific Th17 and Th22 Cells in Filarial Lymphedema

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Lymphatic filarial disease is known to be associated with elevated Th1 responses and normal or diminished Th2 responses to parasite-specific antigens. The roles of Th17 cells and the recently described Th22 cells have not been examined in detail in either filarial infection itself or in filarial disease (e.g., lymphedema and elephantiasis). To explore the roles of Th17 and Th22 cells and their subsets, we examined the frequencies of these cells in individuals with filarial lymphedema (chronic pathology [CP]), in clinically asymptomatic infected (INF) individuals, and in uninfected (UN) individuals *ex vivo* and in response to parasite and nonparasite antigens. Those with disease (CP) had significantly expanded frequencies of Th17 and Th22 cells, compared with either INF or UN individuals, at baseline (*ex vivo*) and in response to parasite antigens. This antigen-driven expansion of Th17 and Th22 cells was dependent on interleukin 1 (IL-1), IL-23, and, to lesser extent, transforming growth factor β (TGF-β), as blockade of any of these cytokines resulted in significantly diminished frequencies of Th17 and Th22 cells. Our findings, therefore, suggest that filarial parasite-driven expansion of Th17 and Th22 cells is associated with the pathogenesis of filarial infections and disease.

Th17 cells and the more recently described Th22 cells are thought to play major roles in defenses against extracellular pathogens, as well as being involved in a variety of inflammatory and autoimmune diseases (1–4). Transforming growth factor β (TGF-β), interleukin 1β (IL-1β), IL-6, and IL-23 have each been implicated in driving both Th17 and Th22 responses in mice and in humans (5).

Infections with lymphatic tissue-dwelling filarial parasites (e.g., *Wuchereria bancrofti*, *Brugia malayi*, or *Brugia timori*) are associated with a wide variety of clinical outcomes. While the majority of the 120 million infected individuals are clinically asymptomatic, a significant minority of individuals (~40 million) develop pathology associated with lymphatic filariasis (LF), typically lymphedema, hydroceles, and/or adenolymphangitis (6). Studies using animal models of lymphatic disease have clearly delineated an important role for T cells (particularly $CD4^+$ T cells) in the development of disease associated with filarial infections (7, 8). T cells have also been shown to play a major role in human lymphatic disease (9–15), in which there appears to be a failure in the regulation of the normally modulated filaria-specific $CD4^+$ responses (16).

Since Th17 and Th22 cells are clearly important in the pathological manifestations of autoimmune diseases and other diseases, we sought to determine whether $CD4^+$ Th17 and Th22 cells and their subsets were associated with the unregulated parasite-specific inflammatory responses that are the immunological hallmark of filarial lymphedema (16). Our data reveal an important association between the expansion of Th17 and Th22 cells and the presence of lymphedema in filarial infections, and they demonstrate that these responses are driven by IL-1, IL-23, and TGF-β.

MATERIALS AND METHODS

Study population. We studied a group of 38 individuals with filarial lymphedema (i.e., chronic pathology [CP]), 23 of whose samples were used for whole-blood culture, 25 clinically asymptomatic filaria-infected (INF) individuals, and 15 uninfected (UN) healthy individuals from an area in which LF is endemic, in Tamil Nadu, South India. All CP individuals tested negative for circulating filarial antigen with both the ICT filarial antigen test (Binax, Portland, ME) and the TropBio Og4C3 enzyme-linked immunosorbent assay (ELISA) (TropBio Pty. Ltd., Townsville, Queensland, Australia), indicating a lack of current active infection. The diagnosis of prior filarial infection was made on the basis of history and clinical examination findings, as well as positive *Brugia malayi* antigen (BmA)-specific IgG4 results. BmA-specific IgG4 and IgG ELISAs were performed exactly as described previously (17). All CP individuals had grade I or grade II lymphedema and no secondary infections. All INF individuals demonstrated positive results for the ICT filarial antigen test and the TropBio Og4C3 ELISA and had not received any antifilarial treatment prior to enrollment in this study. All UN individuals tested negative for circulating filarial antigen, without signs or symptoms of infection or disease. There were no differences between the groups in terms of demographic characteristics or socioeconomic status. All individuals were examined as part of clinical protocols approved by the institutional review boards of the National Institute of Allergy and Infectious Diseases and the National Institute for Research in Tuberculosis (protocols NCT00375583 and NCT00001230), and written informed consent was obtained from all participants.

Parasite and control antigens. Saline extracts of *B. malayi* adult worms and microfilariae (Mf) were used for parasite antigens, and mycobacterial purified protein derivative (PPD) (Serum Statens Institute, Copenhagen, Denmark) was used as the control antigen. Final concentrations were 10 μg/ml for BmA, Mf, and PPD. Endotoxin levels in the BmA preparation were <0.1 endotoxin units/ml, as determined with the QCL-1000 Chromogenic LAL test kit (BioWhittaker). Phorbol myristoyl ace-

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tate (PMA) and ionomycin at concentrations of 12.5 ng/ml and 125 ng/ml, respectively, were used together as the positive-control stimuli.

***In vitro* culture.** Whole-blood cell cultures were performed to determine the frequencies of cytokine-producing $CD4^+$ T cells (CP, $n = 23$; INF, $n = 25$; UN, $n = 15$). Briefly, whole blood was diluted 1:1 with RPMI 1640 medium supplemented with penicillin-streptomycin (100 U and 100 mg/ml, respectively), L-glutamine (2 mM), and HEPES (10 mM) (all from Invitrogen, San Diego, CA) and was placed in 12-well tissue culture plates (Costar, Corning Inc., NY, USA). The cultures were then stimulated with BmA, Mf, PPD, PMA/ionomycin, or medium alone, in the presence of the costimulatory reagent CD49d/CD28 (BD Biosciences, San Diego, CA, USA), at 37°C for 6 h. FastImmune brefeldin A solution (10 μg/ml; BD Biosciences) was added after 2 h. After 6 h, whole blood was centrifuged and washed with cold phosphate-buffered saline (PBS), and then 1× fluorescence-activated cell sorting (FACS) lysing solution (BD Biosciences, San Diego, CA, USA) was added. The cells were fixed using Cytofix/Cytoperm buffer (BD Biosciences), cryopreserved, and stored at −80°C until use. For cytokine neutralization experiments, whole-blood samples from a separate set of prospectively enrolled CP individuals ($n = 7$ to 15) were cultured for 18 h in the presence of anti-interleukin 1 receptor (IL-1R), anti-IL-23R, anti-TGF-β, anti-IL-10, anti-tumor necrosis factor receptor 1 (TNFR1), or isotype control antibody (all 5 μg/ml; R& D Systems), after which BmA was added and samples were cultured for another 6 h.

Intracellular cytokine staining. The cells were thawed, washed first with PBS and then with PBS with 1% bovine serum albumin (BSA), and then stained with surface antibodies for 30 to 60 min. The surface antibodies used were CD3-AmCyan, CD4-allophycocyanin-H7, and CD8-phycoerythrin-Cy7 (all from BD Biosciences). The cells were washed and permeabilized with BD Perm/Wash buffer (BD Biosciences) and were stained for intracellular cytokines for an additional 30 min before washing and data acquisition. The cytokine-specific antibodies used were anti-IL-17, anti-IL-22, and anti-gamma interferon (IFN-γ) (BD Biosciences). Flow cytometry was performed with a FACSCanto II flow cytometer with FACSDiva software (version 6; Becton, Dickinson). The lymphocyte gating was set by forward and side scatter, and 100,000 gated lymphocyte events were recorded. Data were collected and analyzed using FlowJo software (version 9.6.4; TreeStar). All data are depicted as the frequency of $CD4^+$ T cells expressing the cytokines. Values recorded following medium stimulation are depicted as baseline frequency, while frequencies recorded following stimulation with antigens or PMA and ionomycin are depicted as net frequencies (with baseline values subtracted).

Statistical analysis. Data analyses were performed using GraphPad Prism (GraphPad Software, Inc., San Diego, CA, USA). Geometric means were used for measurements of central tendency. Differences among the three groups were analyzed using the Kruskal-Wallis test, with Dunn's correction for multiple comparisons.

RESULTS

Expansion of filaria-specific Th17 and Th22 cells and their subsets in filarial lymphedema. To determine the role of $CD4^+$ T cells expressing IL-17 and IL-22 in filarial lymphedema, we measured the frequencies of Th17 and Th22 cells in subjects with lymphedema/elephantiasis (CP) and compared these with frequencies in filaria-infected subjects with no disease (INF) and in filaria-uninfected (UN) individuals, in terms of both their baseline (*ex vivo*) levels and the levels following stimulation with parasite antigens (BmA and Mf), control antigen (PPD), or PMA and ionomycin. The BmA- and PMA/ionomycin-induced expression of Th17 and Th22 cells in a representative individual with disease (CP) is shown in a contour plot in Fig. 1A, which demonstrates the expression of Th17- and Th22-associated cytokines following BmA and PMA/ionomycin stimulation of $CD4^+$ T cells. As shown in Fig. 1B and C, CP individuals exhibited significantly higher frequencies of Th17 cells ($P < 0.001$) and Th22 cells ($P < 0.001$) in the absence of antigen stimulation than did INF or UN individuals. The frequencies of Th17 and Th22 cells in subjects with CP were increased not only at baseline but even more following stimulation with filarial antigens (BmA and Mf). The responses to PPD and PMA/ionomycin were similar in all 3 groups studied. Interestingly, INF individuals exhibited significantly increased frequencies of filarial antigen-driven Th17 and Th22 cells, in comparison with UN individuals. Thus, filarial lymphatic disease, and to a lesser extent filarial infection itself, is associated with expansion of antigen-stimulated Th17 and Th22 cells.

Since Th17 and Th22 subsets secreting different combinations of cytokines have been shown to perform discrete functions in other infections (2) and since their roles in filarial infection/disease are not well characterized, we sought to determine the roles of these subsets in filarial lymphedema. To this end, we measured the frequencies of $CD4^+$ T cells expressing IL-17/IFN-γ, IL-17/IL-22, or IL-22/IFN-γ in response to parasite or nonparasite antigens or PMA and ionomycin, and we compared them with values for INF and UN individuals. As shown in Fig. 2A, CP individuals exhibited significantly increased *ex vivo* and BmA- and Mf antigen-stimulated frequencies of Th17 cells coexpressing IL-17 and IFN-γ, in comparison with INF or UN individuals. Similarly, as shown in Fig. 2B, CP individuals exhibited significantly increased *ex vivo* and filarial antigen-specific frequencies of Th17 cells coexpressing IL-17 and IL-22, in comparison with INF or UN individuals. Finally, as shown in Fig. 2C, CP individuals exhibited significantly increased baseline and filarial antigen-specific frequencies of Th22 cells coexpressing IL-22 and IFN-γ, in comparison with INF or UN individuals. Of interest, the frequencies of these Th17 and Th22 subsets in response to PPD or PMA and ionomycin did not differ significantly among the groups. Thus, filarial lymphatic disease is characterized by filarial antigen-driven expansion of Th17 and Th22 subsets.

IL-1 and IL-23 receptor blockade results in significantly decreased frequencies of Th17 and Th22 cells in CP individuals. Having demonstrated that filarial lymphedema is associated with both *ex vivo* and parasite antigen-driven expansion of Th17 and Th22 subsets, we sought to understand how this expansion might occur; therefore, we examined the roles of cytokines known to induce Th17 and Th22 differentiation and/or expansion (1). To accomplish this goal, we next measured the frequencies of Th17 and Th22 cells in samples from a prospectively enrolled group of 7 to 15 CP individuals, following *in vitro* neutralization of IL-1, IL-23, TGF-β, IL-10, and tumor necrosis factor (TNF) signaling. As shown in Fig. 3A, CP individuals exhibited significantly decreased net frequencies of Th17 cells following IL-1R, IL-23R, and TNFR blockade and following TGF-β neutralization. CP individuals exhibited significantly decreased net frequencies of Th22 cells following IL-1R and IL-23R blockade and significantly increased net frequencies of Th22 cells following TGF-β and IL-10 neutralization (Fig. 3B). Thus, filarial lymphatic disease is characterized by expansion of Th17 cells, regulated positively by IL-1, IL-23, TNF, and TGF-β, and expansion of Th22 cells, regulated positively by IL-1 and IL-23 and negatively by IL-10 and TGF-β.

DISCUSSION

Filarial infections are characterized by different clinical manifestations, including an asymptomatic (or subclinical) form seen among the majority of infected people (18). Although adaptive immune responses, especially T cell responses, are clearly impor-

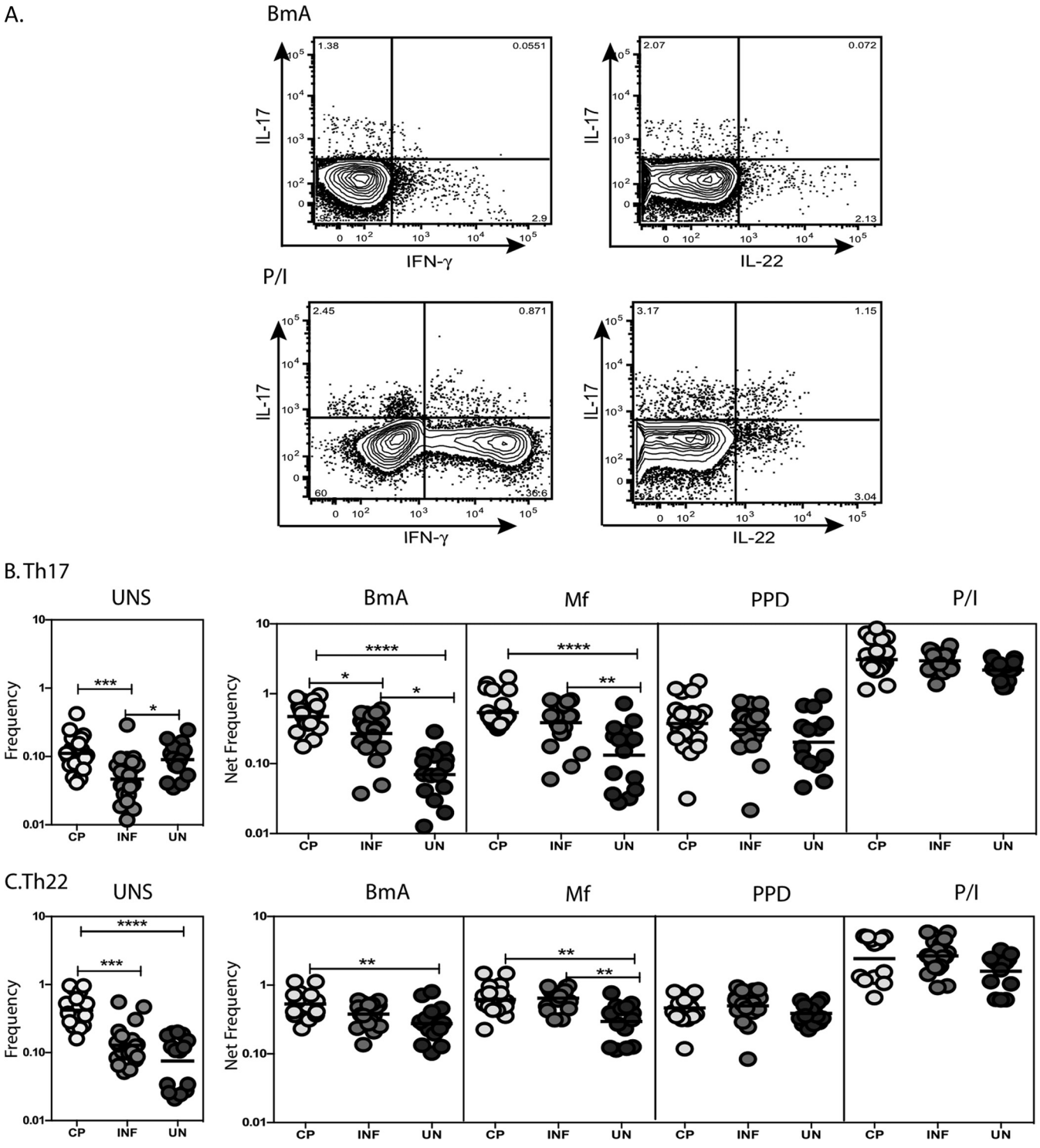

FIG 1 Filarial lymphedema is associated with higher baseline and antigen-stimulated frequencies of Th17 and Th22 cells. (A) Representative flow plot depicting the BmA- and PMA/ionomycin-stimulated frequencies of $CD4^+$ T cells expressing IL-17, IL-22, and IFN-γ in a CP individual. (B) Baseline, antigen-stimulated, and PMA/ionomycin-stimulated frequencies of Th17 cells in CP ($n = 23$), INF ($n = 25$), and UN ($n = 15$) individuals. UNS, unstimulated; P/I, PMA/ionomycin. (C) Baseline, antigen-stimulated, and PMA/ionomycin-stimulated frequencies of Th22 cells in CP ($n = 23$), INF ($n = 25$), and UN ($n = 15$) individuals. The data are depicted as scatter plots, with each circle representing one individual and the lines representing the geometric means. The data for antigen or PMA/ionomycin stimulation are shown as net frequencies, with the baseline frequencies subtracted. *P* values were calculated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

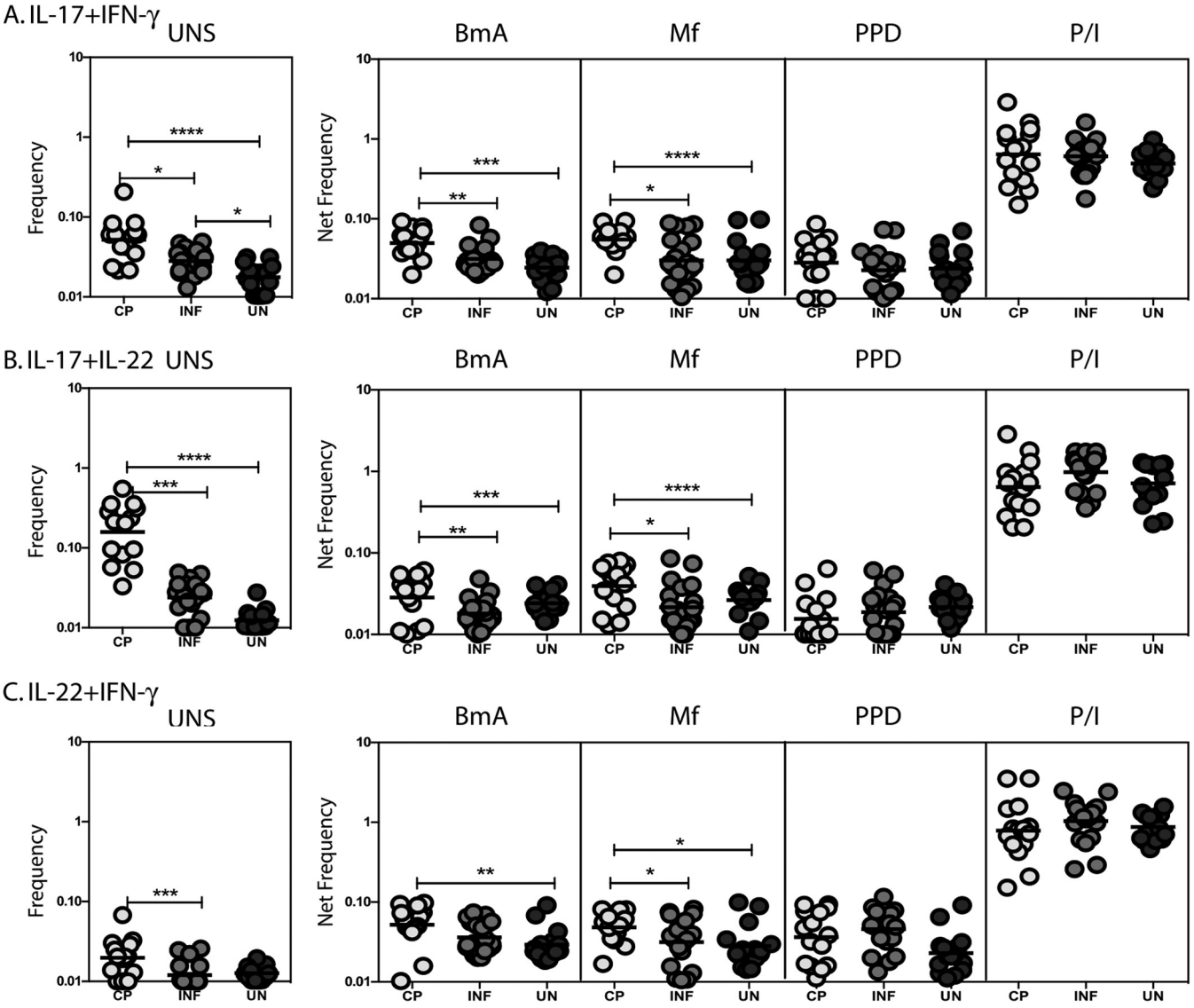

FIG 2 Filarial lymphedema is associated with higher baseline and antigen-stimulated frequencies of Th17 and Th22 subsets. (A) Baseline, antigen-stimulated, and PMA/ionomycin-stimulated frequencies of Th17 cells coexpressing IL-17 and IFN-γ in CP ($n = 23$), INF ($n = 25$), and UN ($n = 15$) individuals. UNS, unstimulated; P/I, PMA/ionomycin. (B) Baseline, antigen-stimulated, and PMA/ionomycin-stimulated frequencies of Th17 cells coexpressing IL-17 and IL-22 in CP, INF, and UN individuals. (C) Baseline, antigen-stimulated, and PMA/ionomycin-stimulated frequencies of Th22 cells coexpressing IL-22 and IFN-γ in CP, INF, and UN individuals. The data are depicted as scatter plots, with each circle representing one individual and the lines representing the geometric means. The data for antigen or PMA/ionomycin stimulation are shown as net frequencies, with the baseline frequencies subtracted. *P* values were calculated using the Kruskal-Wallis test with Dunn's correction for multiple comparisons. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.

tant in mediating the diseases associated with *Brugia* and *Wuchereria* infections, it has been found that dysregulation of $CD4^+$ T-cell-mediated immune activation can lead to the development of tissue inflammation and pathology (16). Antigen-driven expansion of Th1-type $CD4^+$ T cells has long been considered to be the hallmark of chronic pathology in filariasis (16). More recently, the involvement of Th17 responses has also been implicated, based largely on T cell cytokine mRNA expression (15).

$CD4^+$ T cells that express IL-17 are important mediators of inflammation and fibrosis (19). IL-17 expression has been implicated in the pathogenesis of pulmonary fibrosis (20), chronic allograft rejection (21), myocardial fibrosis (22), and hepatic fibrosis (23). Moreover, Th17 cells are clearly associated with the development of pathology in other parasitic infections, including *Schistosoma mansoni* (24, 25), *Leishmania major* (26, 27), and *Toxoplasma gondii* (28) infections. Similarly, although Th22 cells (which primarily express the prototypical cytokine IL-22) are thought to play an anti-inflammatory role in certain settings (29), they have been implicated in the pathogenesis of a variety of inflammatory diseases, including psoriasis (30), systemic lupus erythematosus (31), and rheumatoid arthritis (32).

Our study identifies a clear association between Th17 and Th22 cell expansion and the presence of lymphatic pathology in *W. bancrofti* infections. Our study provides the first detailed examination of Th17 and Th22 cells in filarial lymphedema. Since Th17

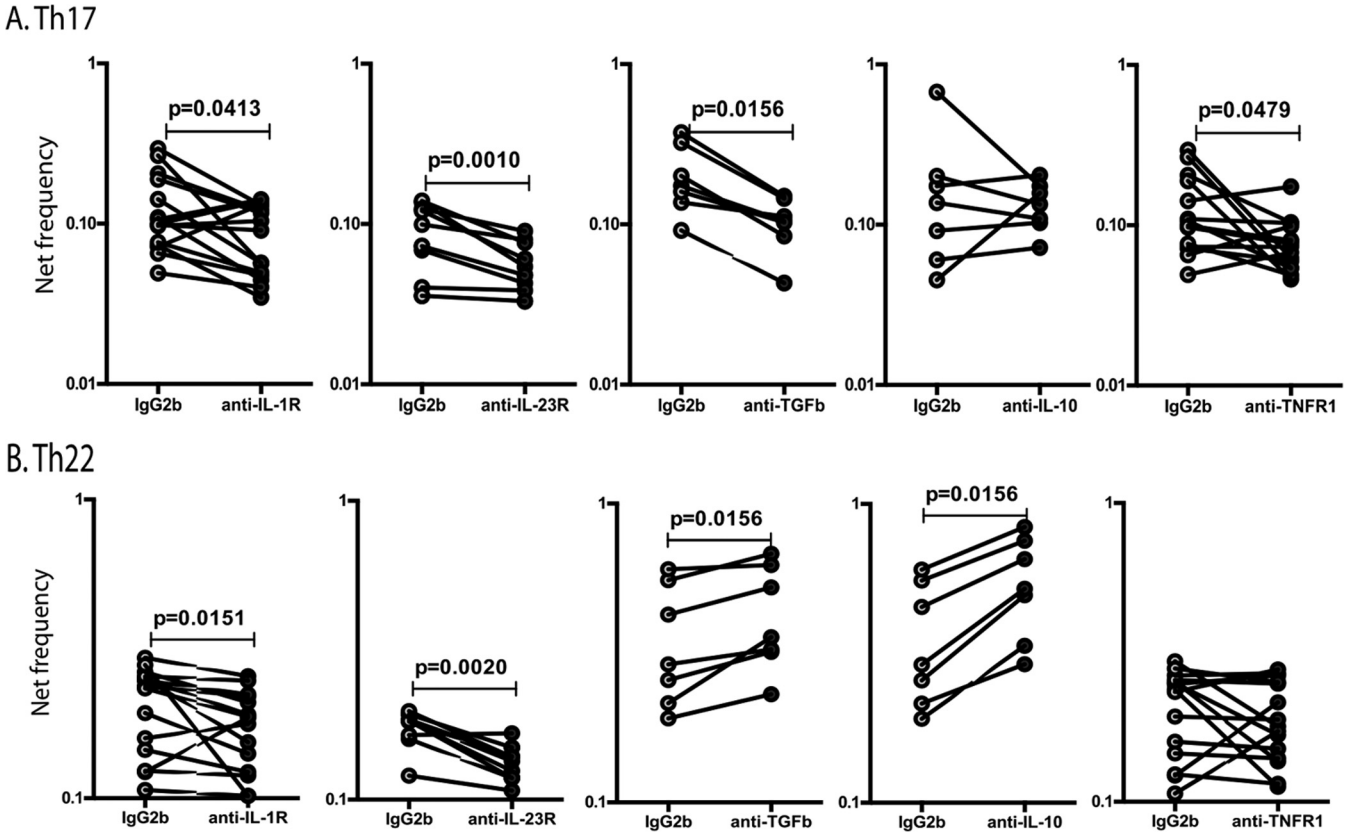

FIG 3 Cytokines regulate the frequencies of Th17 and Th22 cells in filarial lymphedema. (A) Net frequencies of Th17 cells following stimulation with BmA in the presence of blocking antibodies to IL-1R, IL-23R, or TNFR or neutralizing antibodies to TGF-β or IL-10 in CP individuals ($n = 7$ to 15). (B) Net frequencies of Th22 cells following stimulation with BmA in the presence of blocking antibodies to IL-1R, IL-23R, or TNFR or neutralizing antibodies to TGF-β or IL-10 in CP individuals ($n = 7$ to 15). Antigen-stimulated frequencies are shown as net frequencies, with the baseline levels subtracted. Each line represents a single individual. *P* values were calculated using the Wilcoxon signed-rank test.

cells are expressed at significantly higher frequencies in infected and CP individuals than in uninfected individuals, we speculate that this enhanced induction of Th17 subsets could reflect the subsets' roles in promoting the development of pathology. Our data also suggest an important association of Th22 cells with the pathogenesis of lymphatic filarial disease. The exact mechanisms by which IL-17 and IL-22 potentially mediate pathogenic responses in filarial infections need to be elucidated, including postulated mechanisms such as neutrophilic inflammation and induction of matrix metalloproteinases (19). Moreover, our data on the PPD and PMA/ionomycin responses suggest a role for antigen-specific induction of Th17 and Th22 responses in filarial lymphedema.

Our study also provides evidence for important roles for upstream cytokines in the regulation of Th17 and Th22 cells. We showed previously that filarial infection or disease is associated with high systemic levels of IL-1β, TNF-α, IL-23, TGF-β, and IL-10 (33). While it is well known that TGF-β as well as IL-6, IL-1β, and IL-23 can regulate the outgrowth of Th17 cells (34), the identity of the cytokines involved in the regulation of Th22 cells is not well defined. It has been proposed that IL-6 in the absence of TGF-β is an important inducer of Th22 cells (3). Our data clearly reveal that IL-1, IL-23, TGF-β, and TNF-α play important roles in the expansion of Th17 cells in filarial pathology, whereas only IL-1 and IL-23 promote Th22 cell expansion. TGF-β and IL-10 act as down-modulators of this response. Thus, TGF-β has diametrically opposite effects on Th17 and Th22 cell induction, by promoting Th17 cell expansion but limiting Th22 cell expansion. Surprisingly, IL-10 in the context of filarial lymphedema appears to regulate Th22 cells, although the mechanism by which this is accomplished needs further exploration.

Our study highlights important roles of Th17 and Th22 cells in filarial pathology. Since fibrotic pathology is the final common outcome of many chronic inflammatory diseases, our study of Th17 and Th22 cells in filarial disease has important implications for other inflammatory diseases as well. Our data suggest that targeting the IL-17/IL-22 pathway or its upstream inducers could hold promise in ameliorating pathological disease manifestations in filarial infections and other pathologies of similar etiology.

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