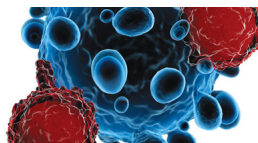


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This information is current as of July 14, 2022.

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*J Immunol* 2015; 195:2241-2250; Prepublished online 22 July 2015;  
doi: 10.4049/jimmunol.1500745  
<http://www.jimmunol.org/content/195/5/2241>

**Supplementary Material** <http://www.jimmunol.org/content/suppl/2015/07/22/jimmunol.1500745.DCSupplemental>

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The American Association of Immunologists, Inc.,  
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.



# Parasite Antigen-Specific Regulation of Th1, Th2, and Th17 Responses in *Strongyloides stercoralis* Infection

Rajamanickam Anuradha,\* Saravanan Munisankar,\* Chandrakumar Dolla,<sup>†</sup> Paul Kumaran,<sup>†</sup> Thomas B. Nutman,<sup>‡</sup> and Subash Babu\*<sup>\*,‡</sup>

Chronic helminth infections are known to be associated with modulation of Ag-specific CD4<sup>+</sup> T responses. However, the role of CD4<sup>+</sup> T cell responses in human infection with *Strongyloides stercoralis* is not well defined. To examine the role of CD4<sup>+</sup> T cells expressing Th1, Th2, and Th17 cytokines in strongyloidiasis, we compared the frequency (F<sub>o</sub>) of these subsets in infected (INF) individuals with F<sub>o</sub> in *S. stercoralis*-uninfected (UN) individuals. INF individuals exhibited a significant decrease in the spontaneous and Ag-specific F<sub>o</sub> of both monofunctional and dual-functional Th1 cells compared with UN. Similarly, INF individuals also exhibited significantly decreased F<sub>o</sub> of monofunctional and dual-functional Th17 cells upon Ag stimulation compared with UN. In contrast, both the spontaneous and the Ag-induced F<sub>o</sub> of monofunctional and dual-functional Th2 cells was significantly increased in INF compared with UN individuals. This differential T cell response was predominantly Ag specific because it was abrogated upon control Ag or mitogen stimulation. The regulation of Th1, Th2, and Th17 cells was predominantly dependent on IL-10, whereas the regulation of Th2, but not Th1 or Th17, cells was also dependent on TGF-β. In addition, treatment of *S. stercoralis* infection significantly increased the Ag-specific F<sub>o</sub> of Th1 and Th17 cells and decreased the F<sub>o</sub> of Th2 cells in INF individuals. Thus, *S. stercoralis* infection is characterized by a parasite Ag-dependent regulation of monofunctional and dual-functional Th1, Th2, and Th17 cells, a regulation also reversible by antihelminthic treatment. *The Journal of Immunology*, 2015, 195: 2241–2250.

CD4<sup>+</sup> T cells can be classified into different subsets based on the pattern of cytokine expression (1). Thus, Th1 cells typically express IFN-γ, IL-2, and/or TNF-α, Th2 cells express IL-4, IL-5, and/or IL-13, whereas Th17 cells express IL-17, IL-22, and/or IFN-γ. Moreover, based on the expression of one or more cytokines within a single cell, CD4<sup>+</sup> T cells can also be further classified into single cytokine-producing (hereafter monofunctional), dual cytokine-producing (dual-functional), and triple cytokine-producing (multifunctional) T cells (2). Multifunctional Th1 cells expressing IFN-γ, IL-2, and TNF-α are thought to play an important role in resistance to a variety of infections including viral, bacterial, and parasitic infections (2). Dual-functional or multifunctional T cells are also thought to produce more cytokines on a per-cell basis and for an extended period than monofunctional T cells (3, 4). Moreover, the presence of these multifunctional T cells is thought to be a better correlate of protective immunity to several infections and to vaccine-mediated immunity (5). Th2 cells expressing a dual-functional or multifunctional phenotype have been described in both allergic and parasitic infection (6). Th17 cells can also exhibit multifunctionality, and certain Th17

cell subsets are thought to play preferential roles in resistance to different infections (7). Although the concept of multifunctionality in cytokine production has been extensively investigated in intracellular infections, little is known about the role of multifunctional T cells in extracellular infections.

Human infections with *Strongyloides stercoralis* appear to be controlled through a Th2 response based on observations in patients with human T lymphotropic virus 1 and *S. stercoralis* coinfection (8–10). Moreover, protective immunity to *S. stercoralis* larvae in mice is dependent on CD4<sup>+</sup> T cells, and these cells typically produce IL-4 and IL-5 (11). Finally, primary infections of rats or mice with the rodent parasites, *S. ratti* and *S. venezuelensis*, respectively, result in a Th2 response, with production of IL-4, IL-5, and IL-13 and concomitant suppression of IFN-γ (12). The role of Th1 cells expressing other type 1 cytokines (IL-2 and TNF-α) has not been examined in animal models or human *S. stercoralis* infection; nor has the role played by Th17 cells been explored in any form of *S. stercoralis* infection. Finally, a detailed examination of the role of Th2 subsets in human *S. stercoralis* infection is lacking.

Therefore, we sought to determine the expression pattern of the major functional subsets of CD4<sup>+</sup> Th1, Th2, and Th17 cells in *S. stercoralis* infections. Comparison of asymptomatic, infected (INF) individuals with uninfected control individuals reveals a distinct expression pattern of CD4<sup>+</sup> T cell subset expression in *S. stercoralis* infection, a pattern that was associated with diminished frequency (F<sub>o</sub>) of spontaneously expressed or Ag-induced monofunctional and dual-functional CD4<sup>+</sup> Th1 and Th17 cells and an increased F<sub>o</sub> of homeostatic and Ag-induced monofunctional and dual-functional Th2 cells. The changes associated with *S. stercoralis* infection were dependent on IL-10 and were reversible after chemotherapy. Thus, *S. stercoralis* infection is characterized by a complex repertoire of CD4<sup>+</sup> T cells subsets whose interaction might determine both susceptibility and resistance to infection.

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Received for publication March 30, 2015. Accepted for publication June 24, 2015.

This work was supported by the Intramural Research Program of the Division of Intramural Research, National Institute of Allergy and Infectious Diseases, National Institutes of Health.

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The online version of this article contains supplemental material.

Abbreviations used in this article: F<sub>o</sub>, frequency; GM, geometric mean; INF, infected; LIPS, Luciferase Immunoprecipitation System Assay; P/I, PMA/ionomycin; PPD, purified protein derivative; SsAg, *S. stercoralis* somatic larval Ag; UN, uninfected.

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## Materials and Methods

### Study population

We studied a total of 66 individuals: 43 clinically asymptomatic, *S. stercoralis* INF individuals and 23 uninfected (UN), endemic normal individuals in Tamil Nadu, South India (Table I). Twenty-eight of the INF individuals were used for in vitro culture and flow cytometry, whereas 15 of the INF individuals were used for cytokine neutralization experiments alone. *S. stercoralis* infection was diagnosed by the presence of IgG Abs to two recombinant Ags: NIE and SsIR by the Luciferase Immunoprecipitation System Assay (LIPS), as described previously (13). Only those individuals who tested positive by LIPS assay to both Ags were classified as INF. All individuals were also negative for filarial infection by filarial Ag tests and for other intestinal helminths by stool microscopy. All INF individuals were treated with a standard dose of ivermectin and albendazole, and follow-up blood draws were obtained 6 months later in 15 individuals. All UN individuals were LIPS assay negative and without any signs or symptoms of infection or disease. There were no differences between the groups in terms of demographics or socioeconomic status. All individuals were examined as part of a natural history study protocol approved by Institutional Review Boards of both the National Institutes of Allergy and Infectious Diseases and the National Institute for Research in Tuberculosis (NCT00375583 and NCT00001230), and informed, written consent was obtained from all participants.

### Hematologic analysis

Hematology was performed on all individuals using the Act-5 Diff hematology analyzer (Beckman Coulter, Brea, CA).

### Parasite and control Ag

Saline extracts of *S. stercoralis* somatic larval Ags (hereafter SsAg) and recombinant NIE Ag (hereafter NIE) were used for parasite Ags, and mycobacterial purified protein derivative (PPD) (Serum Statens Institute, Copenhagen, Denmark) was used as the control Ag. Final concentrations were 10 µg/ml for SsAg, NIE, and PPD. Endotoxin levels in the SsAg was <0.1 endotoxin units/ml using the QCL-1000 Chromogenic LAL test kit (BioWhittaker). PMA and ionomycin at concentrations of 12.5 and 125 ng/ml, respectively, were used as the positive control stimuli.

### In vitro culture

Whole blood cell cultures were performed to determine the F<sub>o</sub> of intracellular cytokine-producing cells. In brief, 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) (all from Invitrogen, San Diego, CA), and placed in 12-well tissue culture plates (Costar, Corning, NY). The cultures were then stimulated with SsAg, NIE, PMA/ionomycin (P/I), or media alone in the presence of the costimulatory reagent, CD49d/CD28 (BD Biosciences, San Diego, CA) at 37°C for 6 h. Fast Immune Brefeldin A Solution (10 µg/ml; BD Biosciences) was added after 2 h. After 6 h, whole blood was centrifuged, washed using cold PBS, and then 1× FACS lysing solution (BD Biosciences) was added. The cells were fixed using Cytofix/Cytoperm buffer (BD Biosciences), cryopreserved, and stored at -80°C until use. For cytokine neutralization experiments (*n* = 15), whole blood from INF individuals was cultured in the presence of anti-IL-10 (5 µg/ml) or anti-TGF-β (5 µg/ml) or isotype control Ab (5 µg/ml; R&D Systems) for 1 h, after which NIE and brefeldin A were added and cultured for a further 23 h.

### Intracellular cytokine staining

The cells were thawed and washed with PBS first and PBS/1% BSA later and then stained with surface Abs for 30–60 min. Surface Abs used were CD3, CD4, and CD8 (all from BD Biosciences). The cells were washed and permeabilized with BD Perm/Wash buffer (BD Biosciences) and stained with intracellular cytokines for an additional 30 min before washing and acquisition. Cytokine Abs used were IFN-γ, TNF-α, IL-2, IL-4, IL-5, IL-13, IL-17, and IL-22 (all from BD Pharmingen). Flow cytometry was performed on a FACS Canto II flow cytometer with FACSDiva software v.6 (Becton Dickinson). The lymphocyte gating was set by forward and side scatter, and 100,000 gated lymphocyte events were acquired. Data were collected and analyzed using FlowJo software. All data are depicted as F<sub>o</sub> of CD4<sup>+</sup> T cells expressing cytokine(s). Thus, F<sub>o</sub> of any particular subset would be the F<sub>o</sub> of CD4<sup>+</sup> T cells expressing that particular cytokine divided by the total F<sub>o</sub> of CD4<sup>+</sup> T cells. Monofunctional Th1 cells were defined as CD4<sup>+</sup> T cells expressing only one of three cytokines: IFN-γ, TNF-α, or IL-2; dual-functional cells were those expressing any two of the above; and multifunctional cells were those

expressing all three cytokines. Multifunctional Th1 cells were below the threshold of detection in our study. Monofunctional Th2 cells were defined as CD4<sup>+</sup> T cells expressing only one of either IL-4, IL-5, or IL-13, and dual-functional Th2 cells expressed either IL-4/IL-5 or IL-4/IL-13 or IL-5/IL-13. Monofunctional Th17 cells were defined as CD4<sup>+</sup> T cells expressing only one of either IL-17 or IL-22, and dual-functional Th17 cells expressed either IL-17/IFN-γ or IL-17/IL-22 or IL-22/IFN-γ. Frequencies after media stimulation are depicted as baseline F<sub>o</sub>, whereas F<sub>o</sub> after stimulation with Ags or P/I are depicted as net F<sub>o</sub> (with baseline F<sub>o</sub> subtracted).

### Statistical analysis

Data analyses were performed using GraphPad PRISM (GraphPad Software, San Diego, CA). Geometric means (GMs) were used for measurements of central tendency. Statistically significant differences between the two groups were analyzed by Mann-Whitney *U* test and multiple comparisons corrected by Holm's correction. Statistically significant differences between pretreatment and posttreatment, as well as after cytokine blockade, were analyzed by Wilcoxon signed rank test.

## Results

### *S. stercoralis* infection is associated with an increased F<sub>o</sub> of total white cells, eosinophils, and basophils

As shown in Table I, INF individuals differed from UN individuals in having significantly higher levels of total white cell counts, absolute eosinophil, and basophil counts. Also, as shown in Table I, no significant differences in ages or sex were observed between the two groups.

### *S. stercoralis* infection is associated with a spontaneous and an Ag-specific decrease in the F<sub>o</sub> of monofunctional and dual-functional CD4<sup>+</sup> Th1 cells

To examine the baseline (or steady-state) and Ag-stimulated expression pattern of Th1 cells in *S. stercoralis* infections, we cultured whole blood from INF and UN individuals with media alone or with SsAg, NIE, PPD, or P/I and measured the F<sub>o</sub> of CD4<sup>+</sup> T cells expressing each of the Th1 cytokines. A representative flow cytometry plot is shown in Supplemental Fig. 1A. As shown in Fig. 1A, the baseline F<sub>o</sub> of CD4<sup>+</sup> T cells expressing IFN-γ or IL-2 alone (monofunctional Th1 cells) or the F<sub>o</sub> of CD4<sup>+</sup> T cells coexpressing TNF-α/IFN-γ or IFN-γ/IL-2 or TNF-α/IL-2 (dual-functional Th1 cells) was significantly reduced in INF individuals. Similarly, the F<sub>o</sub> of monofunctional and dual-functional Th1 cells was also reduced significantly in response to SsAg and NIE stimulation (Fig. 1B, 1C). In contrast, neither PPD (Fig. 1D) nor P/I (Fig. 1E) induced any significant difference in the F<sub>o</sub> of CD4<sup>+</sup> Th1 cells. Thus, *S. stercoralis* infection is associated with profound alterations in the repertoire of monofunctional and dual-functional Th1 cells at steady-state and after parasite Ag stimulation.

### *S. stercoralis* infection is associated with a spontaneous and an Ag-specific increase in the F<sub>o</sub> of monofunctional and dual-functional CD4<sup>+</sup> Th2 cells

To examine the baseline (or steady-state) and Ag-stimulated expression pattern of Th2 cells in *S. stercoralis* infections, we cultured whole blood from INF and UN individuals with media alone or with SsAg, NIE, PPD, or P/I and measured the F<sub>o</sub> of CD4<sup>+</sup> T cells expressing each of the Th2 cytokines. A representative plot is shown in Supplemental Fig. 1B. As shown in Fig. 2A, the baseline F<sub>o</sub> of CD4<sup>+</sup> T cells expressing IL-4, IL-5, or IL-13 alone (monofunctional Th2 cells) or the F<sub>o</sub> of CD4<sup>+</sup> T cells coexpressing IL-4/IL-13 or IL-5/IL-13 (dual-functional Th2 cells) was significantly increased in INF individuals. Similarly, the F<sub>o</sub> of monofunctional and dual-functional Th2 cells was also increased significantly in response to SsAg and NIE stimulation (Fig. 2B, 2C). In contrast, neither PPD (Fig. 2D) nor P/I (Fig. 2E) induced any significant difference in the F<sub>o</sub> of CD4<sup>+</sup> Th2 cells. Thus, *S. stercoralis* infection is associated with profound alterations in the

Table I. Demographics of the study population and hematology data

	INF (n = 43)	UN (n = 23)	p Value
M/F, n	21/22	10/13	
Age, y	40.18 (18–61)	38 (20–63)	
NIE and SsIR LIPS	Positive	Negative	
Hemoglobin, g/dl, GM (range)	15.58 (6.9–17.2)	10.75 (4.9–16.3)	NS
RBC, 10 <sup>6</sup> /ml, GM (range)	5.5 (2.94–5.59)	5.78 (3.71–5.47)	NS
WBC, 10 <sup>3</sup> /ml, GM (range)	12.42 (8.7–14)	10.64 (6.1–12.9)	0.0228
Hematocrit, %, GM (range)	36.44 (23–45.6)	40.10 (30.7–46.7)	NS
Platelets, 10 <sup>3</sup> /ml, GM (range)	286.39 (171–742)	264.06 (124–378)	NS
Neutrophils, 10 <sup>3</sup> /ml, GM (range)	3.93 (2.15–8.17)	3.42 (1.92–6.14)	NS
Lymphocytes, 10 <sup>3</sup> /ml, GM (range)	2.79 (1.72–3.71)	2.76 (1.55–4.41)	NS
Monocytes, 10 <sup>3</sup> /ml, GM (range)	0.47 (0.11–0.87)	0.77 (0.27–2.32)	NS
Eosinophils, 10 <sup>3</sup> /ml, GM (range)	1.68 (0.27–3.52)	0.54 (0.19–1.2)	<0.0001
Basophils, 10 <sup>3</sup> /ml, GM (range)	0.44 (0.057–0.17)	0.17 (0.051–0.16)	0.0028

repertoire of monofunctional and dual-functional Th2 cells at steady-state and after parasite Ag stimulation.

*S. stercoralis* infection is associated with an Ag-specific decrease in the F<sub>o</sub> of monofunctional and dual-functional CD4<sup>+</sup> Th17 cells

To examine the baseline (or steady-state) and Ag-stimulated expression pattern of Th17 cells in *S. stercoralis* infections, we cultured whole blood from INF and UN individuals with media alone or with SsAg, NIE, PPD, or P/I and measured the F<sub>o</sub> of CD4<sup>+</sup> T cells expressing each of the Th17 cytokines. A representative plot is shown in Supplemental Fig. 1C. As shown in Fig. 3A, the baseline F<sub>o</sub> of CD4<sup>+</sup> T cells expressing IL-17 or IL-22 alone (monofunctional Th17 cells) or the F<sub>o</sub> of CD4<sup>+</sup> T cells coexpressing IFN- $\gamma$ /IL-17 or IL-17/IL-22 (dual-functional Th17 cells) was significantly increased in INF individuals. However, upon Ag stimulation, the profile was reversed, with the F<sub>o</sub> of monofunctional and dual-functional Th17 cells being reduced significantly in response to SsAg and NIE stimulation (Fig. 3B, 3C). In contrast, neither PPD (Fig. 3D) nor P/I (Fig. 3E) induced any significant difference in the F<sub>o</sub> of CD4<sup>+</sup> Th17 cells. Thus, *S. stercoralis* infection is associated with profound alterations in the repertoire of monofunctional and dual-functional Th17 cells at steady-state and after parasite Ag stimulation.

*IL-10 modulates the F<sub>o</sub> of monofunctional and dual-functional Th1, Th2, and Th17 cells in S. stercoralis infections*

To determine the role of IL-10 in the modulation of Th1, Th2, and Th17 subpopulations in INF, we measured the F<sub>o</sub> of these major CD4<sup>+</sup> T cell subsets after stimulation with the parasite Ag NIE in the presence or absence of anti-IL-10 neutralizing Ab in INF individuals (n = 15). As shown in Fig. 4A, IL-10 neutralization resulted in significantly increased F<sub>o</sub> of monofunctional (IL-2- or INF- $\gamma$ -expressing) and dual-functional (IL-2/IFN- $\gamma$ - or IL-2/TNF- $\alpha$ -coexpressing) Th1 cells in INF individuals. In contrast, as shown in Fig. 4B, IL-10 neutralization significantly decreases the F<sub>o</sub> of monofunctional (IL-4- or IL-13-expressing) and dual-functional (IL-4/IL-5 or IL-4/IL-13) Th2 cells in INF individuals. Finally, as shown in Fig. 4C, IL-10 blockade resulted in significantly increased F<sub>o</sub> of monofunctional (IL-17- or IL-22-expressing) and dual-functional (IFN- $\gamma$ /IL-22- or IL-17/IL-22-coexpressing) Th17 cells in INF individuals. Thus, IL-10 plays an important role in the modulation of Th1, Th2, and Th17 cells F<sub>o</sub> in *S. stercoralis* infections.

*TGF- $\beta$  also modulates the F<sub>o</sub> of monofunctional and dual-functional Th2 cells in S. stercoralis infections*

To determine the role of TGF- $\beta$  in the modulation of Th1, Th2, and Th17 subpopulations in INF, we measured the F<sub>o</sub> of these cells after stimulation with the parasite Ag NIE in the presence or

absence of anti-TGF- $\beta$  neutralizing Ab in INF individuals (n = 15). As shown in Fig. 5A, TGF- $\beta$  neutralization had no significant effect on the F<sub>o</sub> of monofunctional and dual-functional Th1 cells in INF individuals. In contrast, as shown in Fig. 5B, TGF- $\beta$  blockade resulted in significantly diminished F<sub>o</sub> of monofunctional (IL-4 or IL-5 expressing) and dual-functional (IL-4/IL-5 or IL-4/IL-13 or IL-5/IL-13) Th2 cells in INF individuals. Finally, as shown in Fig. 5C, TGF- $\beta$  blockade had minimal effect on the F<sub>o</sub> of monofunctional and dual-functional Th17 cells in INF individuals, with the exception of IL-17 monofunctional cells, which were significantly decreased. Thus, TGF- $\beta$  plays a major role in the modulation of Th2 cells F<sub>o</sub> in *S. stercoralis* infections.

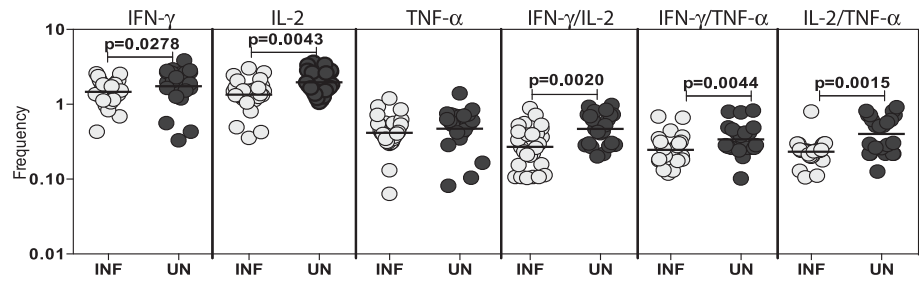
*Anti-S. stercoralis therapy results in significantly increased F<sub>o</sub> of Ag-stimulated Th1 and Th17 cells and significantly decreased F<sub>o</sub> of Ag-stimulated Th2 cells*

To determine the role of active infection in the regulation of monofunctional and dual-functional Th1, Th2, and Th17 cells in *S. stercoralis* infections, we measured the F<sub>o</sub> of Th1, Th2, and Th17 cells in a subset of INF individuals (n = 15) who had been treated with anti-*S. stercoralis* chemotherapy 6 months earlier. As shown in Fig. 6A and Supplemental Fig. 2, treatment of *S. stercoralis* infection resulted in significantly increased F<sub>o</sub> of monofunctional Th1 cells expressing IFN- $\gamma$ , IL-2, or TNF- $\alpha$  alone or dual-functional Th1 cells expressing IFN- $\gamma$ /IL-2 or IFN- $\gamma$ /TNF- $\alpha$  or IL-2/TNF- $\alpha$  upon NIE or SsAg stimulation when compared with their pretreatment levels. Interestingly, this effect was specific to the parasite Ag-stimulated F<sub>o</sub> of CD4<sup>+</sup> Th1 cells as neither PPD (Fig. 6B) nor P/I (Supplemental Fig. 2) significantly altered the F<sub>o</sub> of these subsets after treatment. In contrast, as shown in Fig. 6A and Supplemental Fig. 2, treatment of *S. stercoralis* infection resulted in significantly decreased F<sub>o</sub> of monofunctional Th2 cells expressing IL-4 or IL-5 or IL-13 alone or dual-functional Th2 cells expressing IL-4/IL-5 or IL-4/IL-13 or IL-5/IL-13 upon NIE or SsAg stimulation, but not in response to PPD or P/I stimulation (Fig. 6B, Supplemental Fig. 2). In addition, as shown in Fig. 6A and Supplemental Fig. 2, treatment of *S. stercoralis* infection resulted in significantly increased F<sub>o</sub> of monofunctional Th17 cells expressing IL-17 or IL-22 alone or dual-functional Th17 cells expressing IFN- $\gamma$ /IL-17 or IL-22/IFN- $\gamma$  upon NIE or SsAg stimulation, but not in response to PPD or P/I stimulation (Fig. 6B, Supplemental Fig. 2). Thus, the Ag-driven modulation of CD4<sup>+</sup> T cell subpopulations in *S. stercoralis* infection is reversible for the most part after treatment of infection.

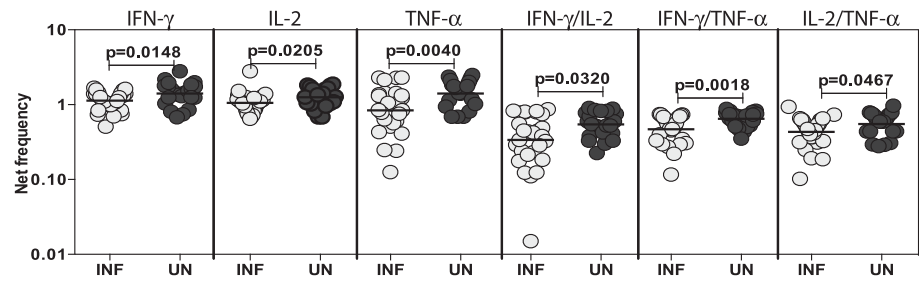
*F<sub>o</sub> distribution of monofunctional and dual-functional Th1, Th2, and Th17 cells in S. stercoralis infections*

To determine the relative contributions of monofunctional or dual-functional cells to the composite Th1, Th2, and Th17 responses in

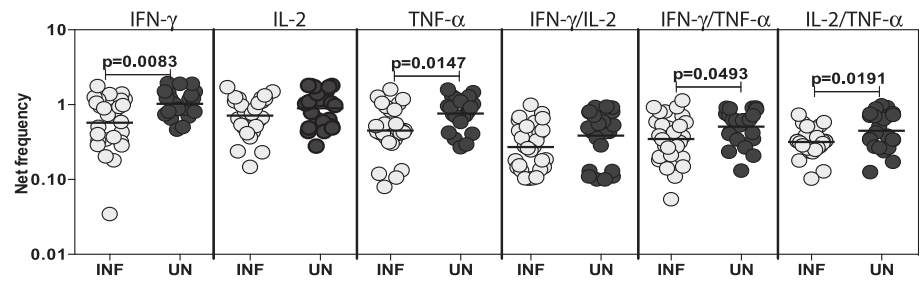
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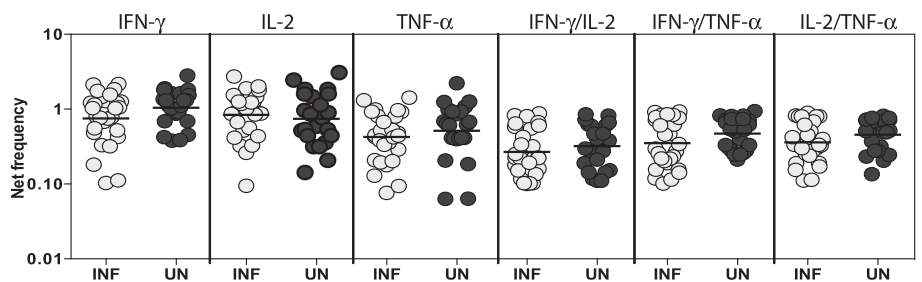
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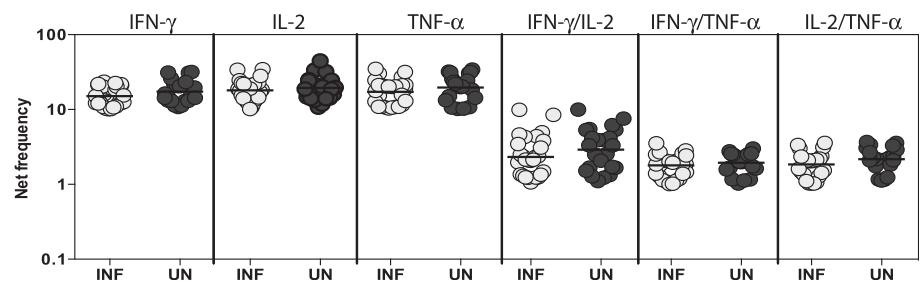
## C NIE



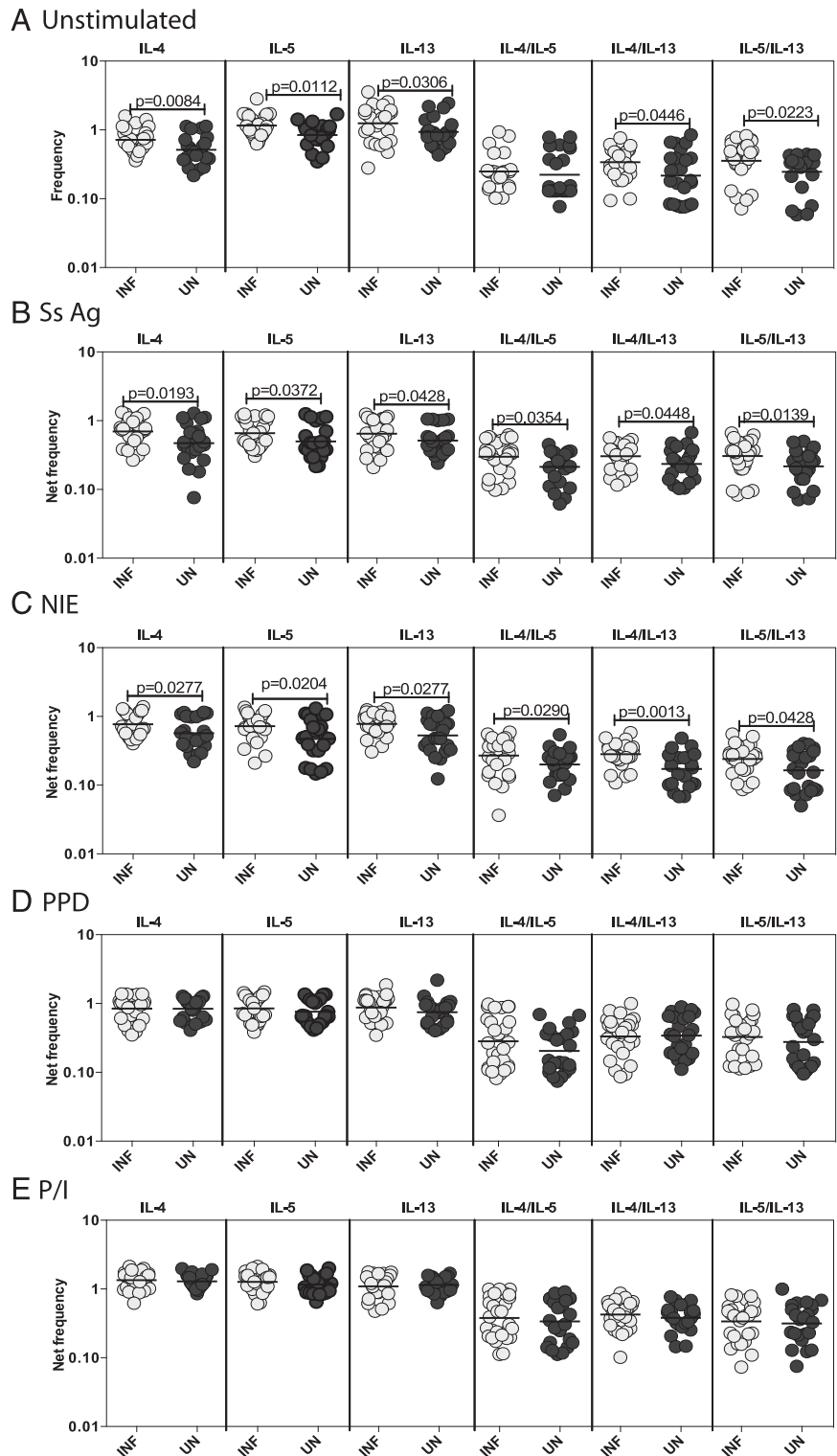
## D PPD



## E P/I



**FIGURE 1.** *S. stercoralis* infection is associated with decreased spontaneously expressed and Ag-induced  $F_o$  of CD4<sup>+</sup> monofunctional and dual-functional Th1 cells. Whole blood was cultured with media alone for 6 h and the baseline and Ag-stimulated  $F_o$  of Th1 cells determined. The baseline (A) as well as SsAg (B), NIE (C), PPD (D), and P/I (E) stimulated  $F_o$  of monofunctional and dual-functional CD4<sup>+</sup> Th1 cells in INF ( $n = 28$ ) and UN ( $n = 23$ ) individuals are shown. Each circle represents a single individual and the bars represent the GM values. Net  $F_o$  were calculated by subtracting baseline  $F_o$  from the Ag-induced  $F_o$  for each individual. The  $p$  values were calculated using the Mann-Whitney  $U$  test.

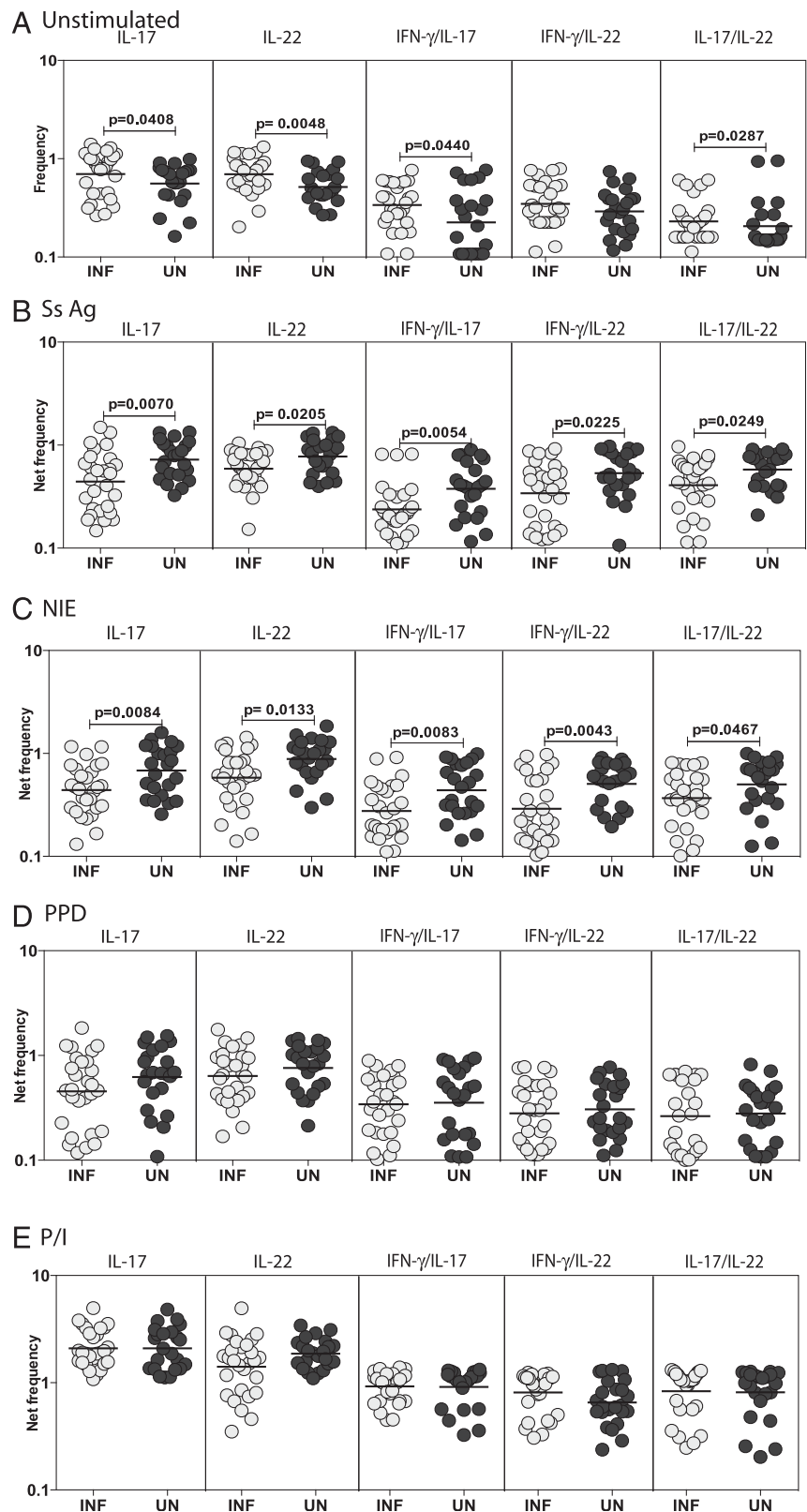


**FIGURE 2.** *S. stercoralis* infection is associated with increased spontaneously expressed and Ag-induced  $F_o$  of  $CD4^+$  monofunctional and dual-functional Th2 cells. Whole blood was cultured with media alone for 6 h and the baseline and Ag-stimulated  $F_o$  of Th2 cells determined. The baseline (A) as well as SsAg (B), NIE (C), PPD (D), and P/I (E) stimulated  $F_o$  of monofunctional and dual-functional  $CD4^+$  Th2 cells in INF ( $n = 28$ ) and UN ( $n = 23$ ) individuals are shown. Each circle represents a single individual and the bars represent the GM values. Net  $F_o$  were calculated by subtracting baseline  $F_o$  from the Ag-induced  $F_o$  for each individual. The  $p$  values were calculated using the Mann–Whitney  $U$  test.

UN and INF (before and after treatment) individuals, we plotted the  $F_o$  of each cell subset in response to different Ags. As shown in Supplemental Fig. 3, the data are plotted as pie charts with each piece of the pie depicting the percentages of each subset (monofunctional or dual-functional) in the total Th1, Th2, or Th17 cell population. Our data clearly reveal that although Th1 and Th2 cell responses to different Ags appear comparable in terms of these subsets, the percentages of Th17 cell subsets undergo dramatic alterations upon P/I stimulation compared with antigenic stimulation.

### Discussion

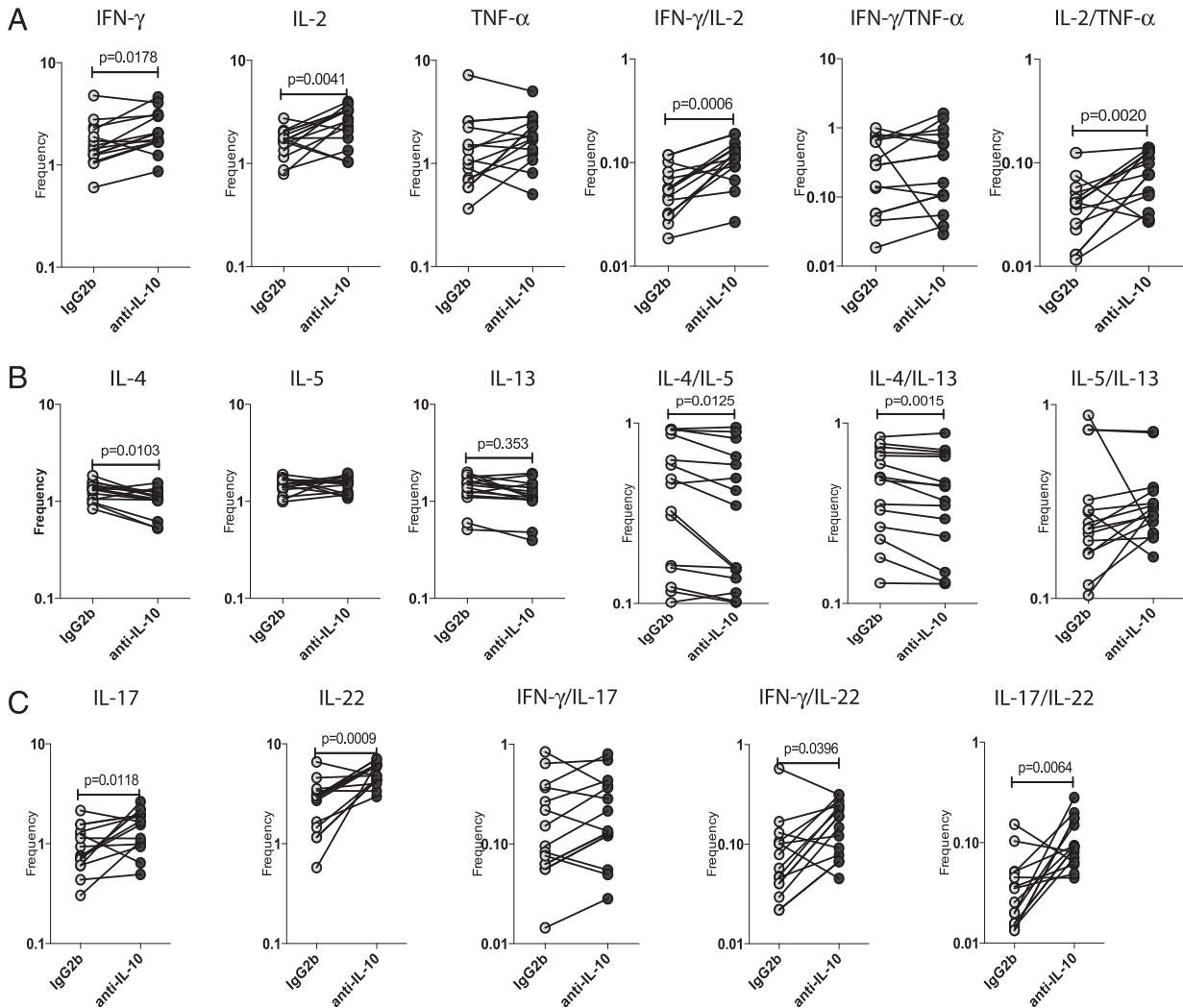
*S. stercoralis*, an intestinal parasitic nematode, infects >100 million people worldwide (14). Infection with *S. stercoralis* can occur without any clinical symptoms whatsoever, with mild abdominal symptoms, or as a potentially fatal hyperinfection syndrome, or as a disseminated infection (14, 15). In addition, chronic infection is also associated with the autoinfection/tissue migration aspect of the life cycle (14, 15). The most common risk factors for these severe manifestations are immunosuppression caused by corticosteroids or by infection with human T lymphotropic virus (16). Despite the



**FIGURE 3.** *S. stercoralis* infection is associated with decreased spontaneously expressed and Ag-induced F<sub>o</sub> of CD4<sup>+</sup> monofunctional and dual-functional Th17 cells. Whole blood was cultured with media alone for 6 h and the baseline and Ag-stimulated F<sub>o</sub> of Th17 cells determined. The baseline (A) as well as SsAg (B), NIE (C), PPD (D), and P/I (E) stimulated F<sub>o</sub> of monofunctional and dual-functional CD4<sup>+</sup> Th17 cells in INF ( $n = 28$ ) and UN ( $n = 23$ ) individuals are shown. Each circle represents a single individual and the bars represent the GM values. Net F<sub>o</sub> were calculated by subtracting baseline F<sub>o</sub> from the Ag-induced F<sub>o</sub> for each individual. The  $p$  values were calculated using the Mann–Whitney  $U$  test.

widespread global prevalence and its propensity to cause fatal disease, the immune responses to *S. stercoralis* infection in either humans or in animal models has been largely neglected (17). One of the major factors for this lacuna is the relative insensitive methods to diagnose asymptomatic infection. However, with recent advances in *S. stercoralis*-specific serology and/or PCR, diagnostic capabilities have been considerably enhanced (14, 15).

The canonical host immune response to helminth parasites in animal models is of the Th2 type and involves the production of cytokines—IL-4, IL-5, IL-9, IL-10, and IL-13, the Ab isotypes, IgG1, IgG4, and IgE—and expanded populations of eosinophils, basophils, mast cells, and alternatively activated macrophages (18). Over time, these responses are thought to be modulated in chronic infection by both adaptive and natural regulatory T cells,



**FIGURE 4.** IL-10 regulates the  $F_0$  of monofunctional and dual-functional Th1, Th2, and Th17 cells in *S. stercoralis* infections. **(A)** IL-10 neutralization (with anti-IL-10 Ab) significantly increases the  $F_0$  of monofunctional or dual-functional  $CD4^+$  Th1 cells after stimulation with NIE in a subset of INF individuals ( $n = 15$ ). **(B)** IL-10 neutralization significantly decreases the  $F_0$  of monofunctional or dual-functional  $CD4^+$  Th2 cells after stimulation with NIE in a subset of INF individuals ( $n = 15$ ). **(C)** IL-10 neutralization significantly increases the  $F_0$  of monofunctional or dual-functional  $CD4^+$  Th17 cells after stimulation with NIE in a subset of INF individuals ( $n = 15$ ). Ag-stimulated  $F_0$  are shown as net  $F_0$  with the baseline levels subtracted. Each line represents a single individual. The  $p$  values were calculated using the Wilcoxon signed rank test.

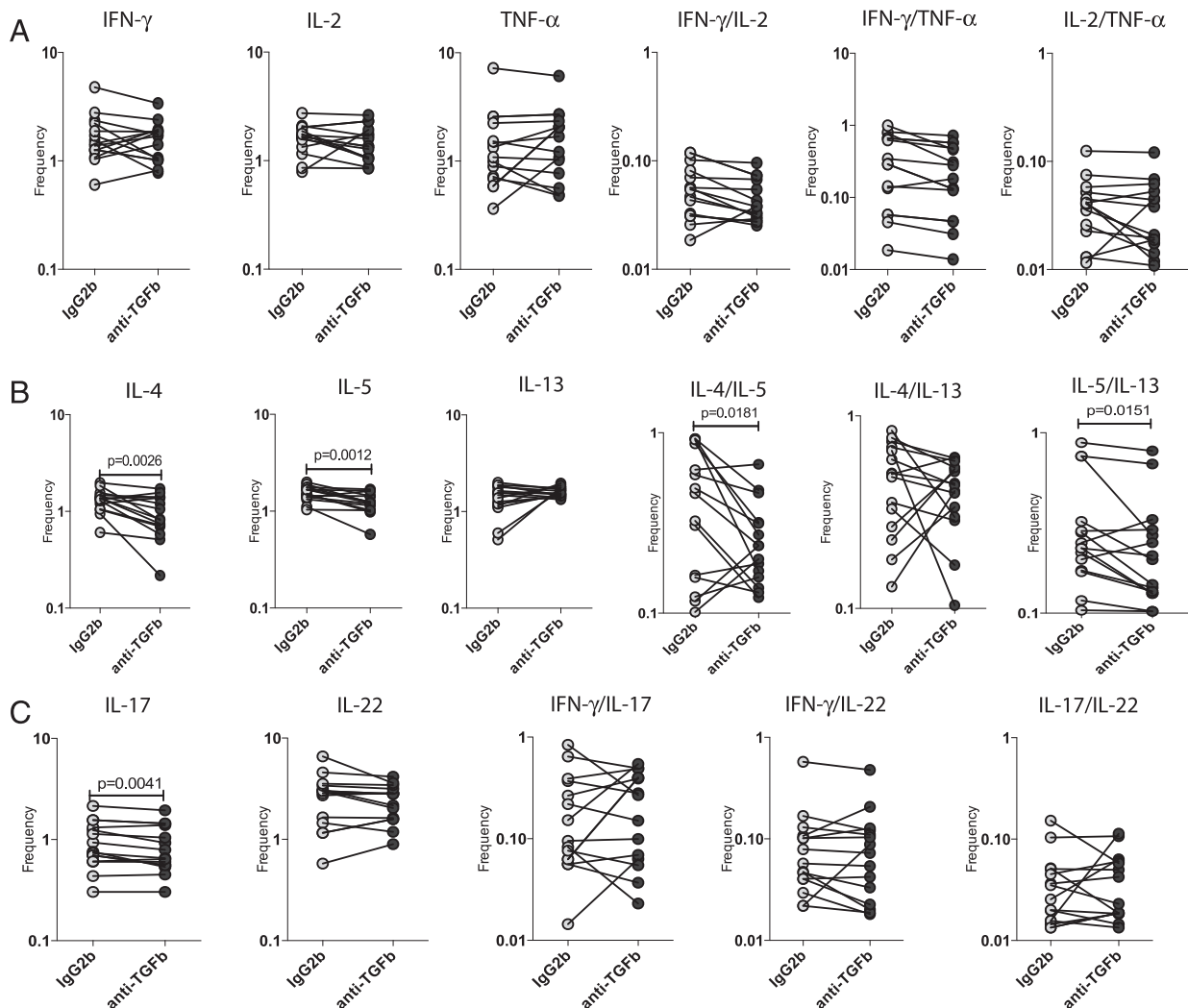
alternatively activated macrophages, and other cell types (19). Although Th1 cells are known to be downmodulated and Th2 responses are known to be required for resistance to infection (20, 21), the role of other subsets of  $CD4^+$  T cells, especially Th17 cells in helminth infections, is not well defined. Moreover, little is known about the regulation of these subsets in helminth infections in general, and in *S. stercoralis* in particular.

Our study expands the limited knowledge to date on the human  $CD4^+$  T cell response to *S. stercoralis* infection and offers certain major insights into the understanding of the regulation of  $CD4^+$  T cell responses in *S. stercoralis* infection. First, we established the downregulation of both monofunctional and dual-functional Th1 cells expressing the panel of cytokines: IFN- $\gamma$ , TNF- $\alpha$ , and IL-2 in chronic *S. stercoralis* infection. More detailed analyses reveal that Th1 cells in *S. stercoralis* infection are downmodulated both at homeostasis (baseline) and after parasite Ag stimulation. Moreover, the basic downregulated response is remarkably similar in response to two very different sets of Ags: a somatic extract of larval Ags (SsAg) (22) and a defined recombinant Ag (NIE), shown serologically to be immunodominant (23). Interestingly, this regulation is highly parasite Ag specific because neither control Ag nor mitogenic

stimulation could induce the downmodulation of Th1 cells in IN individuals. Thus, we show that T cells in *S. stercoralis* infections are not intrinsically defective in their ability to respond to antigenic stimulation but appear to exhibit a very active impairment only in response to *S. stercoralis*-specific parasitic Ags. Finally, we also provide data that this downmodulation of Ag-specific Th1 responses is reversible, because treatment with a standard regimen of anti-helminthic chemotherapy at least partially reverses this downregulation. Our studies using the pretreatment and posttreatment data from INF individuals clearly illustrates a pivotal role for active infection in the maintenance of impaired Th1 responses in chronic *S. stercoralis* infections. Th1 responses are typically associated with the development of pathology in helminth infections (18). Thus, in filarial infections (24), as well as in schistosomiasis (25), Th1 cells are closely involved in the development of pathology. Our data on the downregulation of Th1 responses in *S. stercoralis* infection would therefore appear to reflect an active ongoing regulatory process that might act to prevent tissue damage in this chronic infection.

The second major finding in our study is the induction of monofunctional and dual-functional Th2 cell responses in *S. stercoralis* infection. Different Th2 subsets have been previously



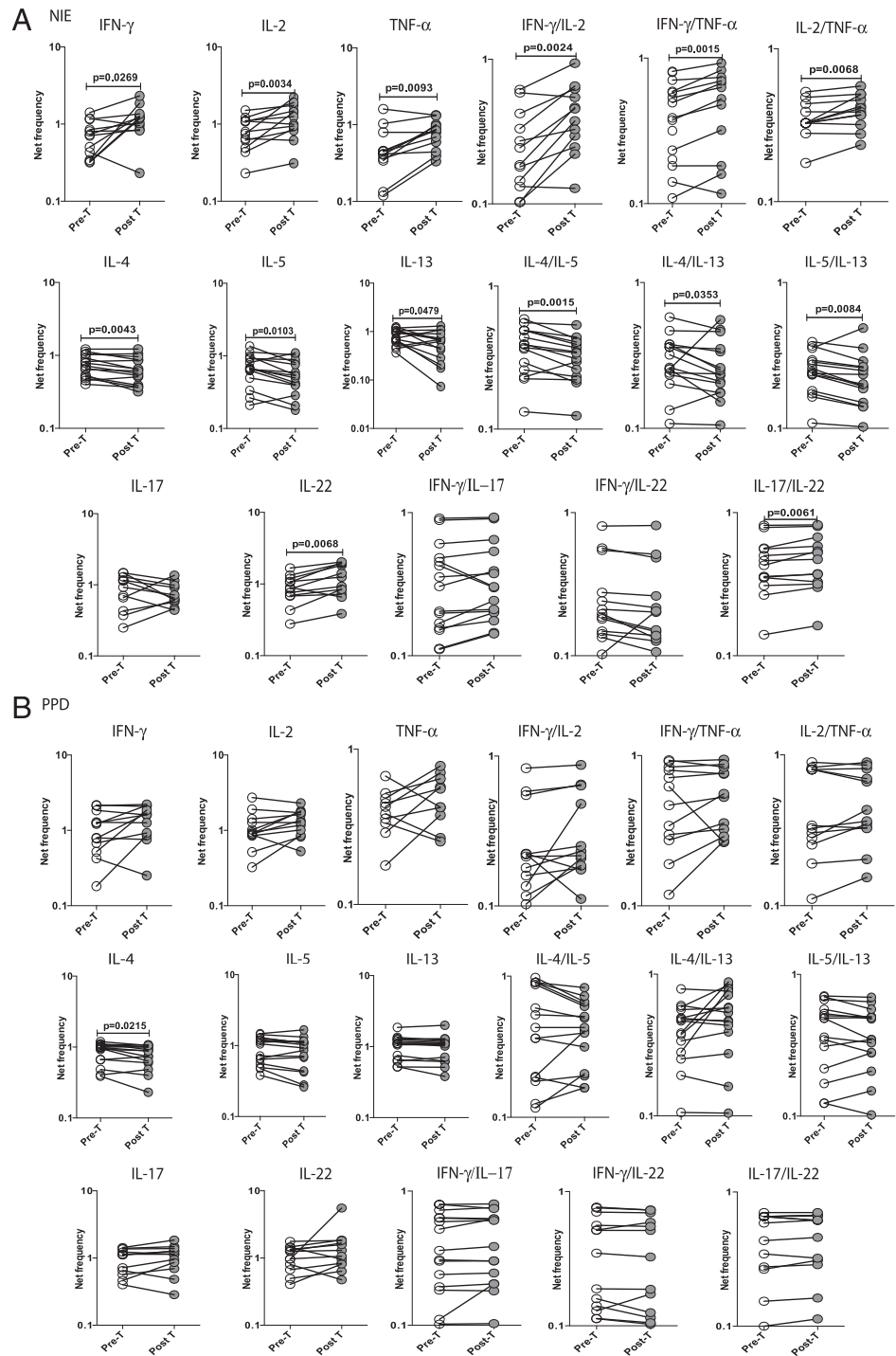


**FIGURE 5.** TGF- $\beta$  significantly regulates the  $F_0$  of monofunctional and dual-functional Th2 cells in *S. stercoralis* infections. **(A)** TGF- $\beta$  neutralization (with anti-TGF- $\beta$  Ab) does not significantly alter the  $F_0$  of monofunctional or dual-functional CD4<sup>+</sup> Th1 cells after stimulation with NIE in a subset of INF individuals ( $n = 15$ ). **(B)** TGF- $\beta$  neutralization (with anti-TGF- $\beta$  Ab) significantly diminishes the  $F_0$  of monofunctional or dual-functional CD4<sup>+</sup> Th2 cells after stimulation with NIE in a subset of INF individuals ( $n = 15$ ). **(C)** TGF- $\beta$  neutralization (with anti-TGF- $\beta$  Ab) does not significantly alter the  $F_0$  of monofunctional or dual-functional CD4<sup>+</sup> Th17 cells (with the exception of IL-17 monofunctional cells) after stimulation with NIE in a subset of INF individuals ( $n = 15$ ). Ag-stimulated  $F_0$  are shown as net  $F_0$  with the baseline levels subtracted. Each line represents a single individual. The  $p$  values were calculated using the Wilcoxon signed rank test.

shown to play different roles in infections and allergic disorders (6). Thus, IL-5<sup>+</sup> Th2 cells have been found in greater  $F_0$  in patients with eosinophilic gastrointestinal disease, whereas peanut allergy was found to be associated with higher  $F_0$  of IL-5<sup>+</sup> Th2 cells (26). In addition, parasite Ag-driven Th2 subsets have been shown to be expanded in filarial infections, a process dependent on IL-10 and TGF- $\beta$  (27). Moreover, whereas the IL-5<sup>+</sup> Th2 cells are predominantly involved in Ab class switching to IgG4 and IgE in filarial infection, IL-5<sup>+</sup> Th2 cells primarily regulate eosinophilia (27). Our data reveal that both monofunctional and dual-functional Th2 cells were expanded in the context of *S. stercoralis* infection at steady-state and that this expansion was further upregulated upon parasite Ag stimulation. Similar to the data from filarial infection (27), this regulation of Th2 subsets also appears to be dependent on IL-10 and TGF- $\beta$ . Finally, the regulation of Th2 responses in *S. stercoralis* infection, similar to Th1 cells, also appears to be modulated by active infection because the  $F_0$  of these cells is considerably reduced after antihelminth chemotherapy. Thus, chronic *S. stercoralis* infection is clearly characterized by modulation of Th2 cell subsets that probably play an effector role in the immune response to in-

fection by either limiting the density of infection or by modulating pathology. Also, our data suggest that the mere expansion of Th2 cells in INF individuals is not sufficient to mediate protection and, therefore, the timing, intensity, and/or duration of expansion could potentially modulate immune resistance mechanisms.

The third major insight provided by our study is the identification of a modulated Th17 response in *S. stercoralis* infection. Our study provides a detailed examination of Th17 cell subsets in *S. stercoralis* infections and reveals that CD4<sup>+</sup> Th17 cells are decreased in  $F_0$  in an Ag-specific manner, similar to Th1 cells. Th17 cells are known to be predominantly involved in the immune responses in inflammatory and autoimmune diseases, but their role in infectious diseases is not very clear (28). Th17 cells are central to host protection against bacterial infections at barrier sites and play an important role in defense against extracellular infections (29). However, Th17 cells are also associated with pathogenesis of disease in a number of infectious diseases, including schistosomiasis and filariasis (24, 30). Our data suggest that Th17 cells are present at decreased  $F_0$  in *S. stercoralis* infections and, therefore, could reflect either a failed resistance mechanism against the



**FIGURE 6.** Treatment of *S. stercoralis* infection is associated with increased  $F_0$  of Ag-specific Th1 and Th17 cells and decreased  $F_0$  of Ag-specific Th2 cells. **(A)** The  $F_0$  of Th1, Th2, and Th17 cells after stimulation with NIE before and after treatment with a standard dose of ivermectin and albendazole in a subset of INF individuals ( $n = 15$ ). **(B)** The  $F_0$  of Th1, Th2, and Th17 cells after stimulation with PPD before and after treatment with a standard dose of ivermectin and albendazole in a subset of INF individuals ( $n = 15$ ). Ag-stimulated  $F_0$  are shown as net  $F_0$  with the baseline levels subtracted. Each line represents a single individual. The  $p$  values were calculated using the Wilcoxon signed rank test.

parasite or an active process of inhibiting excessive inflammation. This is further reinforced by the finding that treatment of active infection results in a significant increase in Th17 cells in an Ag-dependent manner.

The central players in regulating the immune response in parasitic infections are IL-10 and TGF- $\beta$  (19). The role of IL-10 and TGF- $\beta$  in the regulation of helminth infections, including schistosomiasis and filariasis, has been documented previously (31, 32). Our data reveal that IL-10 is the most important regulator of monofunctional and dual-functional Th1, Th2, and Th17 cells. TGF- $\beta$  appears to play a minimal role in the regulation of Th1 and Th17 cells in *S. stercoralis* infection but does appear to regulate the expansion of Th2 cells. This dichotomy in regulation of im-

mune responses by the central regulatory cytokines might reflect different sources of production or different modes of action of these cytokines, which deserves further study. Nevertheless, our data implicate *S. stercoralis* as yet another infectious model, where IL-10 appears to exert important immunomodulatory controls on the immune response.

Our study therefore highlights an important role for CD4<sup>+</sup> T cell subsets in the regulation of immune responses in *S. stercoralis* infections. Our study also highlights the complexity of the T cell network underlying the susceptibility or resistance to infection and also suggests that the role of Th1, Th2, and Th17 cells in *S. stercoralis* infections warrants further investigation, perhaps using animal models of infection. Finally, our work emphasizes

the growing importance of multifunctional CD4<sup>+</sup> T cells in immunity to parasitic infections.

## Acknowledgments

We thank Dr. M. Satiswaran, Dr. J. Jeevan, Prabhu Balakrishnan, Jai Ganesh, and the staff of the National Institute for Research in Tuberculosis Epidemiology Unit for assistance with patient recruitment.

## Disclosures

The authors have no financial conflicts of interest.

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