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Cytotoxic T-Lymphocyte-Associated Antigen 4 (CTLA-4)- and Programmed Death 1 (PD-1)-Mediated Regulation of Monofunctional and Dual Functional CD4$^+$ and CD8$^+$ T-Cell Responses in a Chronic Helminth Infection

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ABSTRACT Chronic helminth infections are known to be associated with the modulation of antigen-specific T-cell responses. *Strongyloides stercoralis* infection is characterized by the downmodulation of antigen-specific Th1 and Th17 responses and the upregulation of Th2 and Th9 responses. Immune homeostasis is partially maintained by negative regulators of T-cell activation, cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4) and programmed death 1 (PD-1), which dampen effector responses during chronic infections. However, their roles in *S. stercoralis* infection are yet to be defined. Therefore, we sought to determine the role of CTLA-4 and PD-1 in regulating CD4$^+$ and CD8$^+$ T-cell responses and examined the frequencies of monofunctional and dual functional Th1/T cytotoxic type 1 (Tc1), Th17/Tc17, Th2/Tc2, and Th9/Tc9 cells in *S. stercoralis* infection in 15 infected individuals stimulated with parasite antigen following CTLA-4 or PD-1 blockade. Our data reveal that CTLA-4 or PD-1 blockade results in significantly enhanced frequencies of monofunctional and dual functional Th1/Tc1 and Th17/Tc17 cells and, in contrast, diminishes the frequencies of monofunctional and dual functional Th2/Tc2 and Th9/Tc9 cells with parasite antigen stimulation in whole-blood cultures. Thus, we demonstrate that CTLA-4 and PD-1 limit the induction of particular T-cell subsets in *S. stercoralis* infection, which suggests the importance of CTLA-4 and PD-1 in immune modulation in a chronic helminth infection.

KEYWORDS CTLA-4 and PD-1 blocking, chronic helminth infection, immune regulation, *Strongyloides stercoralis*, T-cell subsets, monofunctional and dual functional T-cell subsets

Strongyloides stercoralis, a soil-transmitted nematode which dwells in the small intestine of human hosts, infects more than 50 million to 100 million people worldwide (1). The clinical manifestations of *S. stercoralis* infection can range from the clinically asymptomatic to, at its most severe, a potentially fatal disseminated infection. *S. stercoralis* infection is characterized by the downmodulation of antigen-specific T helper 1 (Th1) and Th17 responses and the upregulation of Th2 and Th9 responses (2, 3). Chronicity is the hallmark of most helminth infections (4) and is a state that requires the dampening of effector responses, which is largely seen with parasite-specific T-cell responses.

T-cell activation is dependent upon signals delivered both through the T-cell receptor (TCR) and through particular costimulatory receptors. Signaling through these costimulatory receptors can be inhibited through the members of the CD28:B7 superfamily of molecules, namely, cytotoxic T lymphocyte antigen 4 (CTLA-4; CD152) and

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programmed death 1 (PD-1; CD279). These receptors play a critical role in the down-regulation of T-cell responses, the regulation of T-cell tolerance, and autoimmunity (5–12). Both CTLA-4 (13) and PD-1 (14) bind to their respective ligands, found most commonly on antigen-presenting cells (APCs) (10, 13, 15).

There are relatively few data on the role of these inhibitory signaling pathways in human helminth infection. Previous studies have reported that the increased expression of CTLA-4 and PD-1 on T cells is detected in helminth infections (16, 17) and that blocking of CTLA-4 can alter the Th1/Th2 balance in human filarial infections (17). Since the regulatory pathways induced by helminth parasites are highly conserved, we wanted to examine the functional responses in *S. stercoralis* infection (18), although the increased expression of CTLA-4 and PD-1 had not been demonstrated in this infection. Herein, we sought to determine the impact of both CTLA-4 and PD-1 on the function of $CD4^+$ and $CD8^+$ Th1/T cytotoxic type 1 (Tc1) cells (defined by the expression of gamma interferon [IFN-γ], interleukin-2 [IL-2], and/or tumor necrosis factor alpha [TNF-α]), Th2/Tc2 cells (defined by the expression of IL-4, IL-5, and/or IL-13), Th9/Tc9 cells (defined by the expression of IL-9 and/or IL-10), and Th17/Tc17 cells (defined by the expression of IL-17 and/or IL-22) in chronic *S. stercoralis* infection. Our data show that these checkpoint inhibitors play a crucial role in modulating the nature of antigen-specific $CD4^+$ and $CD8^+$ T-cell subsets.

RESULTS

CTLA-4 and PD-1 regulate the antigen-stimulated frequencies of monofunctional $CD4^+$ T-cell subsets in *S. stercoralis* infection. To examine the effect of CTLA-4 and PD-1 on monofunctional $CD4^+$ T cells in *S. stercoralis* infection, we measured the frequencies of Th1 (IFN-γ, TNF-α, or IL-2), Th17 (IL-17, IL-22), Th2 (IL-4, IL-5, IL-13), and Th9 (IL-9, IL-10) cells following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4 or anti-PD-1 in *S. stercoralis*-infected individuals ($n = 15$). As shown in Fig. 1A, CTLA-4 blockade resulted in significantly increased frequencies of monofunctional $CD4^+$ Th1 (except for TNF-α) and Th17 (as well as IL-13 single expressers) cells and significantly decreased the frequencies of $CD4^+$ Th2 (IL-4 and IL-5) and Th9 cells. As shown in Fig. 1B, PD-1 blockade resulted in similarly significantly increased frequencies of monofunctional $CD4^+$ Th1 (except for IFN-γ) and Th17 (as well as IL-13 single expressers) cells and significantly decreased frequencies of $CD4^+$ Th2 (IL-4 and IL-5) cells. Also, CTLA-4 or PD-1 blockade in unstimulated samples had no effect on $CD4^+$ T-cell frequencies.

CTLA-4 and PD-1 regulate the antigen-stimulated frequencies of dual functional $CD4^+$ T-cell subsets in *S. stercoralis* infection. To examine the effect of CTLA-4 and PD-1 on dual functional $CD4^+$ T cells in *S. stercoralis* infection, we measured the frequencies of Th1, Th17, Th2, and Th9 cells following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4 or anti-PD-1 in *S. stercoralis*-infected individuals. As shown in Fig. 2A, CTLA-4 blockade resulted in significantly increased frequencies of dual functional $CD4^+$ Th1 (IFN-γ/IL-2, IFN-γ/TNF-α, or IL-2/TNF-α) and Th17 (IFN-γ/IL-17 or IL-17/IL-22) cells and significantly decreased frequencies of dual functional $CD4^+$ Th2 (IL-4/IL-5) and Th9 (IL-9/IL-10) cells. As shown in Fig. 2B, PD-1 blockade resulted in significantly increased frequencies of dual functional $CD4^+$ Th1 (IFN-γ/IL-2 or IFN-γ/TNF-α) and Th17 (IFN-γ/IL-17 or IL-17/IL-22) cells and significantly decreased frequencies of dual functional $CD4^+$ Th2 (IL-4/IL-5, IL-4/IL-13, IL-5/IL-13) and Th9 (IL-9/IL-10) cells. Also, the blockade of CTLA-4 or PD-1 in unstimulated samples had no effect on dual functional $CD4^+$ T-cell frequencies. Multifunctional T cells were present at levels below the threshold of detection in our study.

No alterations in the frequencies of monofunctional and dual functional $CD4^+$ T-cell subsets following CTLA-4 and PD-1 blockade in healthy controls. To examine the effect of CTLA-4 and PD-1 on monofunctional and dual functional $CD4^+$ T cells in healthy control individuals, we measured the frequencies of Th1, Th17, Th2, and Th9 cells following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4

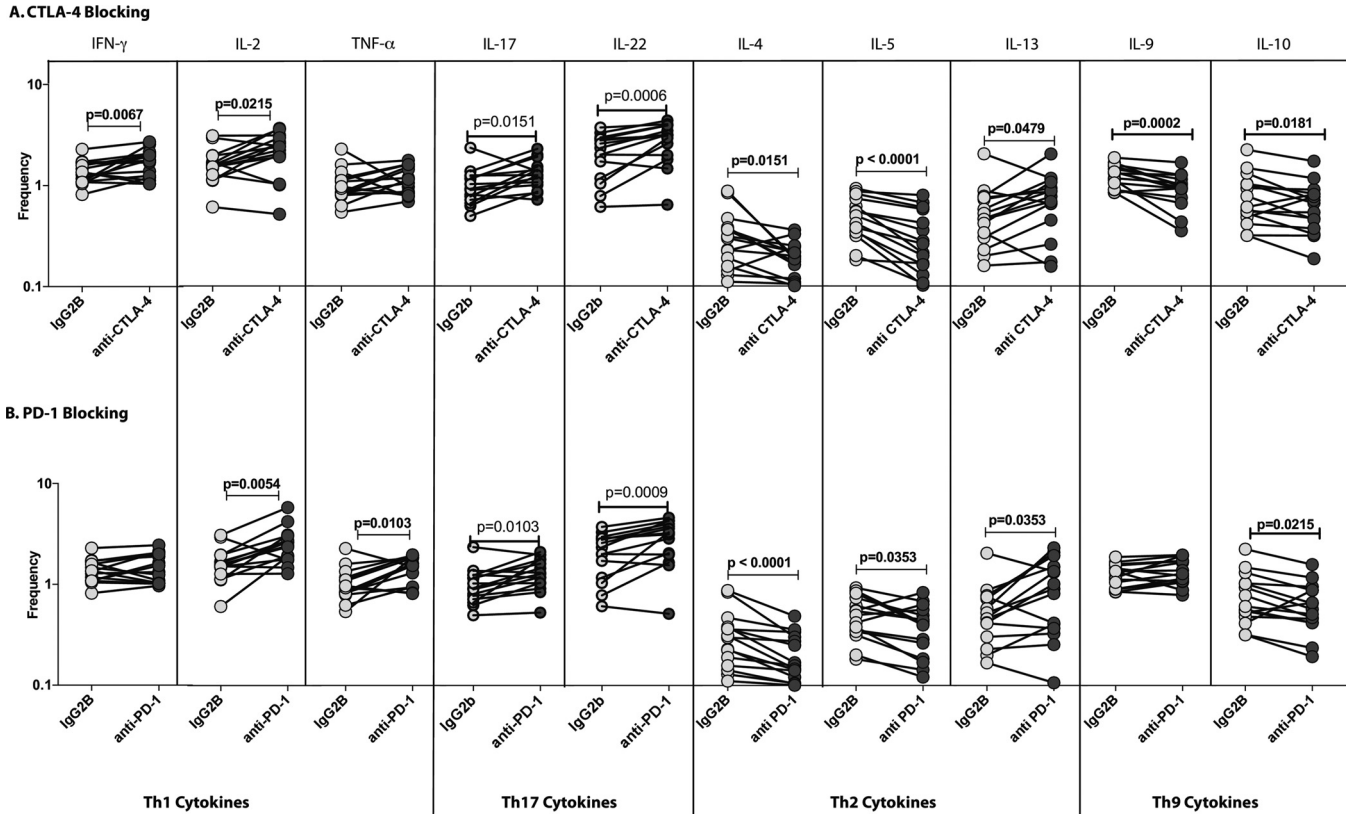

FIG 1 CTLA-4 and PD-1 regulate the antigen-stimulated frequencies of monofunctional CD4$^+$ T-cell subsets in *S. stercoralis* infection. The frequencies of monofunctional CD4$^+$ Th1, Th2, Th9, and Th17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 15 *S. stercoralis*-infected individuals. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction. Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

or anti-PD-1 in healthy controls. As shown in Fig. 3A to D, the blockade of CTLA-4 or PD-1 had no effect on monofunctional and dual functional CD4$^+$ T-cell frequencies.

Regulation of monofunctional CD8$^+$ T-cell subsets by CTLA-4 and PD-1 in *S. stercoralis* infection. To determine the role of CTLA-4 and PD-1 in the induction of Tc1, Tc2, Tc9, and Tc17 cytokine responses in *S. stercoralis* infection, we measured the CD8$^+$ T-cell frequencies following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4 or anti-PD-1 in *S. stercoralis*-infected individuals. As shown in Fig. 4A, CTLA-4 blockade resulted in significantly increased frequencies of monofunctional CD8$^+$ Tc1 and Tc17 (except for IL-17) cells (as well as IL-13 single expressers) and significantly decreased frequencies of CD8$^+$ Tc2 and Tc9 cells. As shown in Fig. 4B, PD-1 blockade resulted in significantly increased frequencies of monofunctional CD8$^+$ Tc1 (except for IL-2) cells and significantly decreased frequencies of CD8$^+$ Tc2 (IL-13 alone) cells. Also, the blockade of CTLA-4 or PD-1 in unstimulated samples had no effect on CD8$^+$ T-cell frequencies.

Regulation of dual functional CD8$^+$ T-cell subsets by CTLA-4 and PD-1 in *S. stercoralis* infection. To examine the effect of CTLA-4 and PD-1 on dual functional CD8$^+$ T cells in *S. stercoralis* infection, we measured the frequencies of dual functional Tc1, Tc17, Tc2, and Tc9 cells following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4 or anti-PD-1 in *S. stercoralis*-infected individuals ($n = 15$). As shown in Fig. 5A, CTLA-4 blockade resulted in significantly increased frequencies of dual functional CD8$^+$ Tc1 (IFN-γ/IL-2, IFN-γ/TNF-α, or IL-2/TNF-α) and Tc17 (IL-17/IL-22) cells and significantly decreased frequencies of dual functional CD8$^+$ Tc2 (IL-4/IL-5, IL-4/IL-13, IL-5/IL-13) and Tc9 (IL-9/IL-10) cells. As shown in Fig. 5B, PD-1 blockade

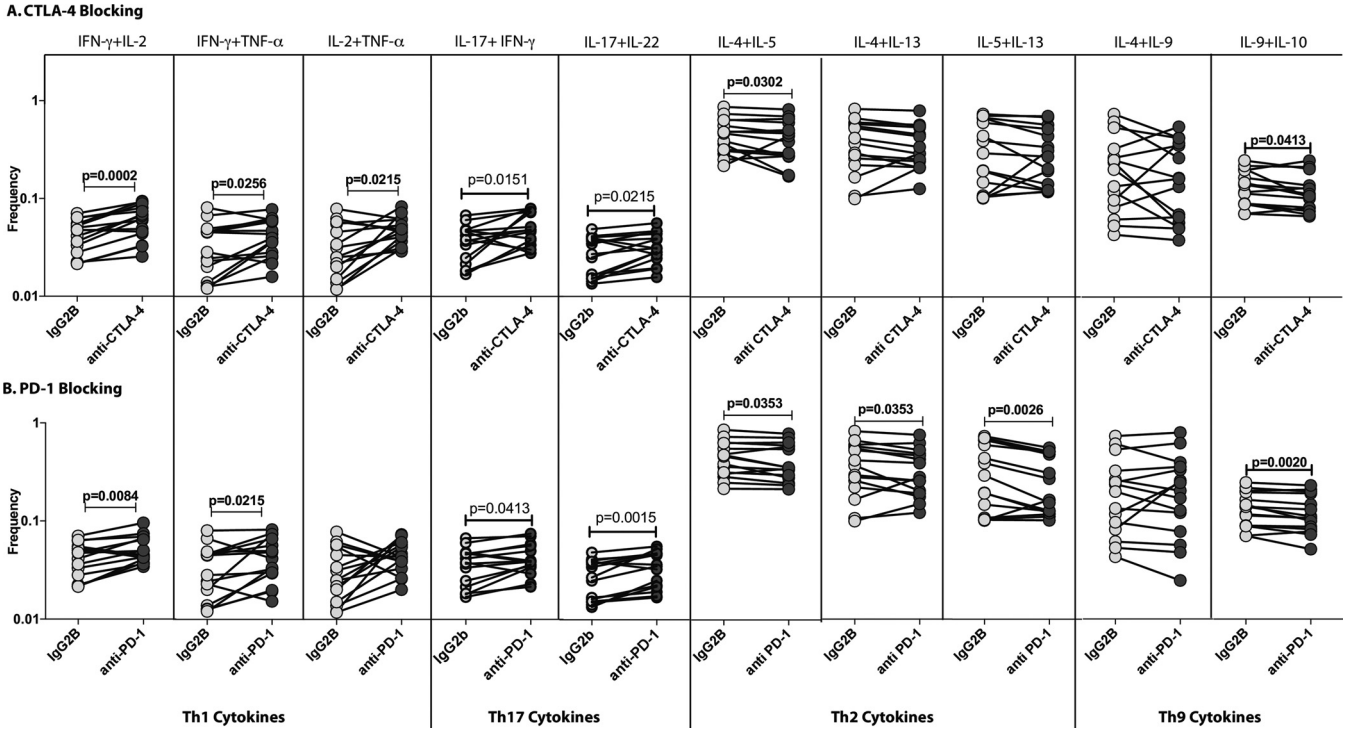

FIG 2 CTLA-4 and PD-1 regulate the antigen-stimulated frequencies of dual functional CD4$^+$ T-cell subsets in *S. stercoralis* infection. The frequencies of dual functional CD4$^+$ Th1, Th2, Th9, and Th17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 15 *S. stercoralis*-infected individuals. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction. Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

resulted in significantly increased frequencies of dual functional CD8$^+$ Tc1 (IFN-γ/IL-2) cells and significantly decreased frequencies of dual functional CD8$^+$ Tc9 (IL-4/IL-9, IL-9/IL-10) cells. Also, the blockade of CTLA-4 or PD-1 in unstimulated samples had no effect on dual functional CD4$^+$ T-cell frequencies. Multifunctional T cells were present at levels below the threshold of detection in our study.

No alterations in the frequencies of monofunctional and dual functional CD8$^+$ T-cell subsets following CTLA-4 and PD-1 blockade in healthy controls. To examine the effect of CTLA-4 and PD-1 on monofunctional and dual functional CD8$^+$ T cells in healthy control individuals, we measured the frequencies of Tc1, Tc17, Tc2, and Tc9 cells following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4 or anti-PD-1 in healthy controls. As shown in Fig. 6A to D, the blockade of CTLA-4 or PD-1 had no effect on monofunctional and dual functional CD8$^+$ T-cell frequencies.

CTLA-4 and PD-1 regulate the MFI of cytokine expression on CD4$^+$ and CD8$^+$ T cells. To examine the effect of CTLA-4 and PD-1 on the mean fluorescence intensity (MFI) of cytokine expression on T cells, we measured the MFI of Th1 (IFN-γ, TNF-α, or IL-2), Th17 (IL-17, IL-22), Th2 (IL-4, IL-5, IL-13), and Th9 (IL-9, IL-10) cytokines following stimulation with the parasite antigen NIE in the presence of anti-CTLA-4 or anti-PD-1 in *S. stercoralis*-infected individuals ($n = 15$). As shown in Fig. 7A, CTLA-4 blockade resulted in a significantly increased MFI of CD4$^+$ Th1 and Th17 cytokines and also IL-13 single expressers and a significantly decreased MFI of CD4$^+$ Th2 (IL-4 and IL-5) and Th9 cytokines. As shown in Fig. 7B, PD-1 blockade resulted in a significantly increased MFI of CD4$^+$ Th1 and Th17 cytokines and also IL-13 single expressers and a significantly decreased MFI of CD4$^+$ Th2 (IL-4 and IL-5) and Th9 cytokines.

As shown in Fig. 7C, CTLA-4 blockade resulted in a significantly increased MFI of CD8$^+$ Tc1 and Tc17 cytokines and a significantly decreased MFI of CD8$^+$ Tc2 and Tc9 cytokines. As shown in Fig. 7D, PD-1 blockade resulted in a significantly increased MFI

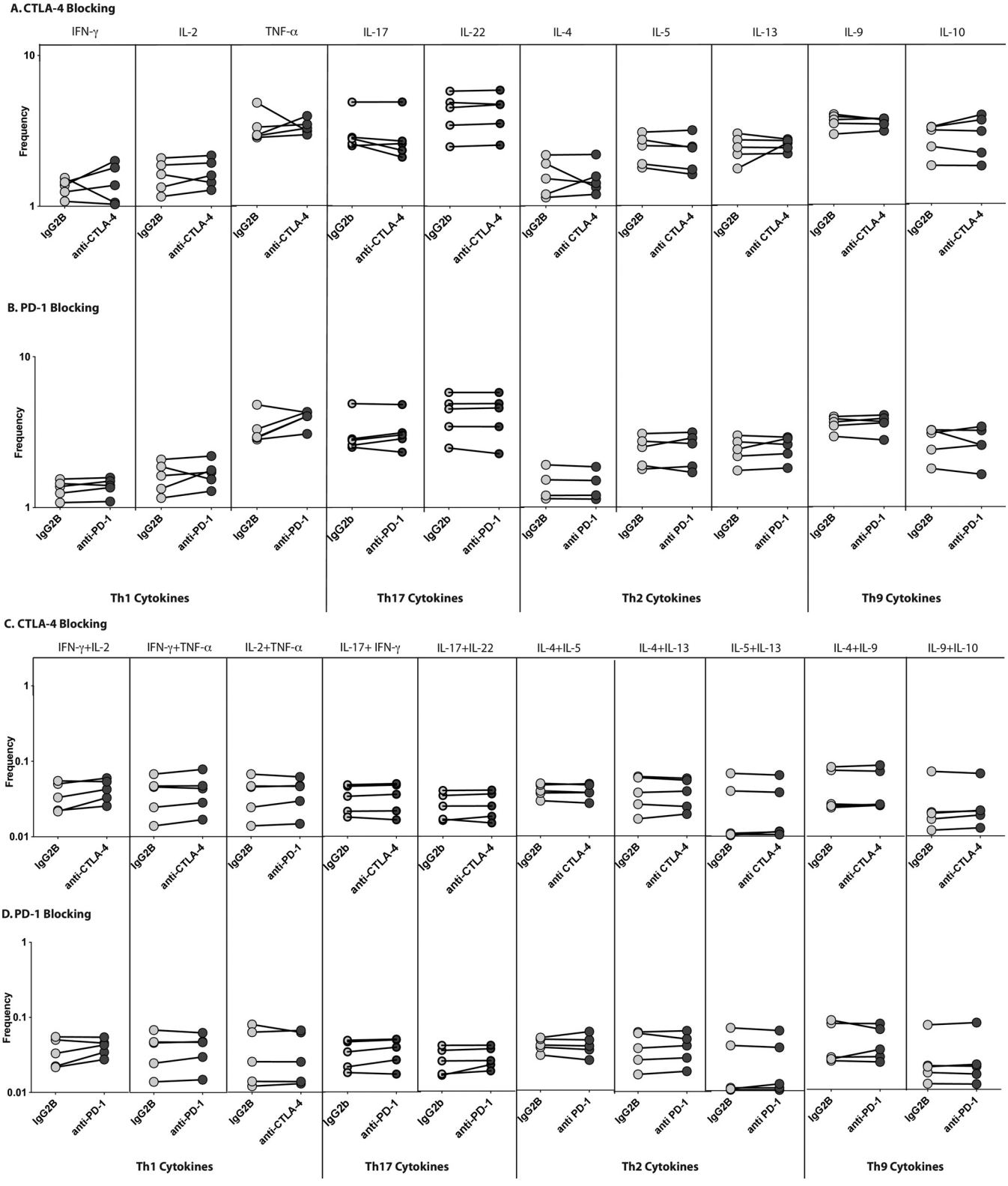

FIG 3 No alterations in the frequencies of monofunctional and dual functional CD4$^+$ T-cell subsets following CTLA-4 and PD-1 blockade in healthy controls. (A and B) The frequencies of monofunctional CD4$^+$ Th1, Th2, Th9, and Th17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 5 healthy individuals. (C and D) The frequencies of dual functional CD4$^+$ Th1, Th2, Th9, and Th17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (C), anti-PD-1 (D), or isotype control (C and D) antibody blockade in 5 uninfected individuals. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction. Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

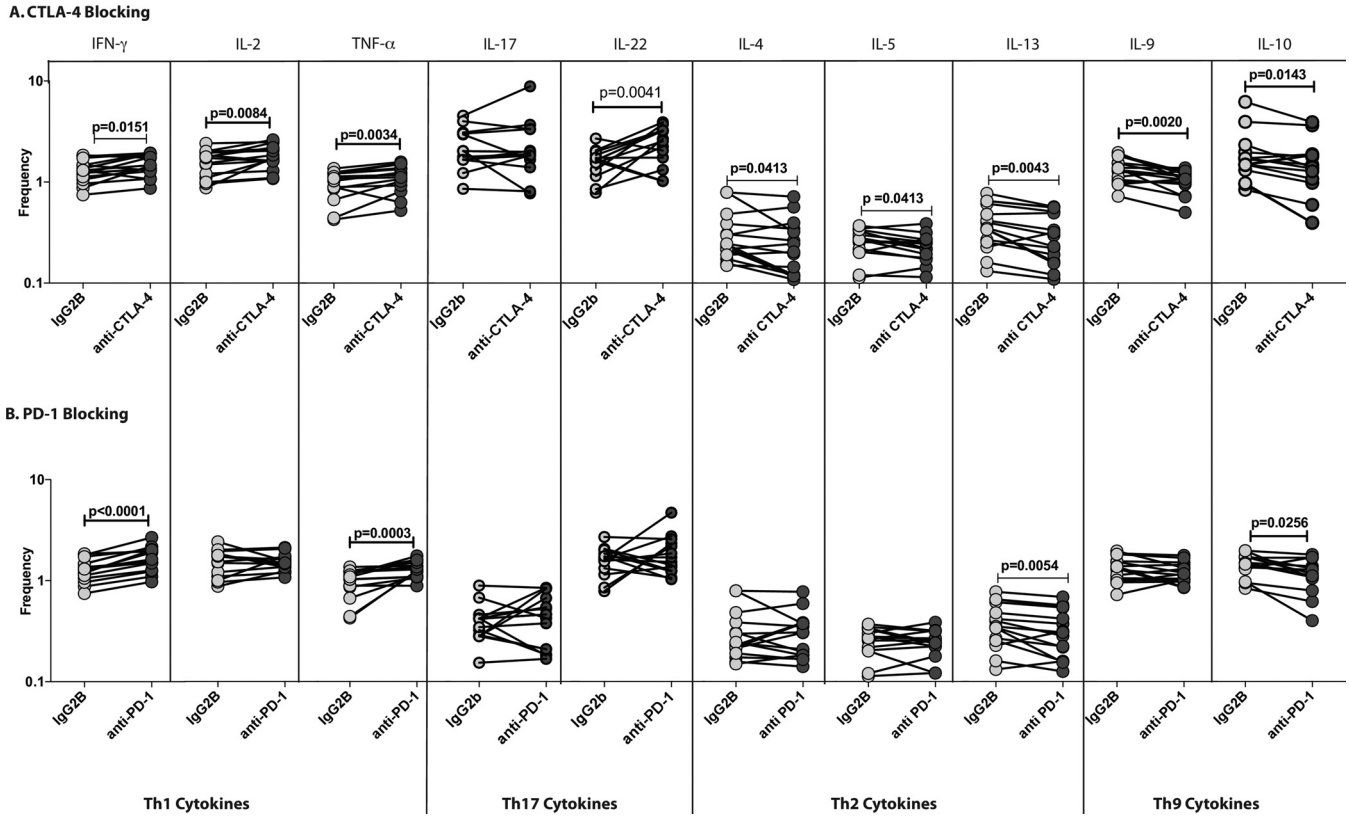

FIG 4 Regulation of monofunctional CD8$^+$ T-cell subsets by CTLA-4 and PD-1 in *S. stercoralis* infection. The frequencies of monofunctional CD8$^+$ Tc1, Tc2, Tc9, and Tc17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 15 *S. stercoralis*-infected individuals. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction. Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

of CD8$^+$ Tc1 and Tc17 cytokines and a significantly decreased MFI of CD8$^+$ Tc2 and Tc9 cytokines. Thus, CTLA-4 and PD-1 blockade impacts the intensity of expression of different cytokines on a per cell basis.

DISCUSSION

Upon antigenic stimulation, CD4$^+$ T cells undergo a differentiation process that ultimately can result in the expansion of various Th-cell subsets, based on the pattern of transcription factors induced and cytokines produced (19). Based on the expression of one or more cytokines within a single cell, CD4$^+$ and CD8$^+$ T cells can also be further classified into single cytokine-producing (monofunctional), dual cytokine-producing (dual functional), and triple cytokine-producing (multifunctional) T cells (20). Dual functional T cells are also thought to secrete more cytokines on a per cell basis and to secrete cytokines for a more prolonged period than monofunctional T cells (21, 22). The presence of these multifunctional T cells is believed to be a better associate of protective immunity with viral, bacterial, and parasitic infections (23) and with vaccines (24, 25).

The hallmark of helminth infection is a T helper 2 (Th2)-cell response, which is required for host resistance to a variety of helminths (26). Th2 cell subsets play an effector role in the immune response to infection either by reducing the density of infection or by regulating pathology and promoting tissue repair (27). Th2 cells expressing a dual functional or multifunctional phenotype have been described in both allergic disease and parasitic infection (28). Blocking of PD-1 signaling has been shown to enhance the CD4$^+$ Th2-cell responses, which resulted in more severe liver immu-

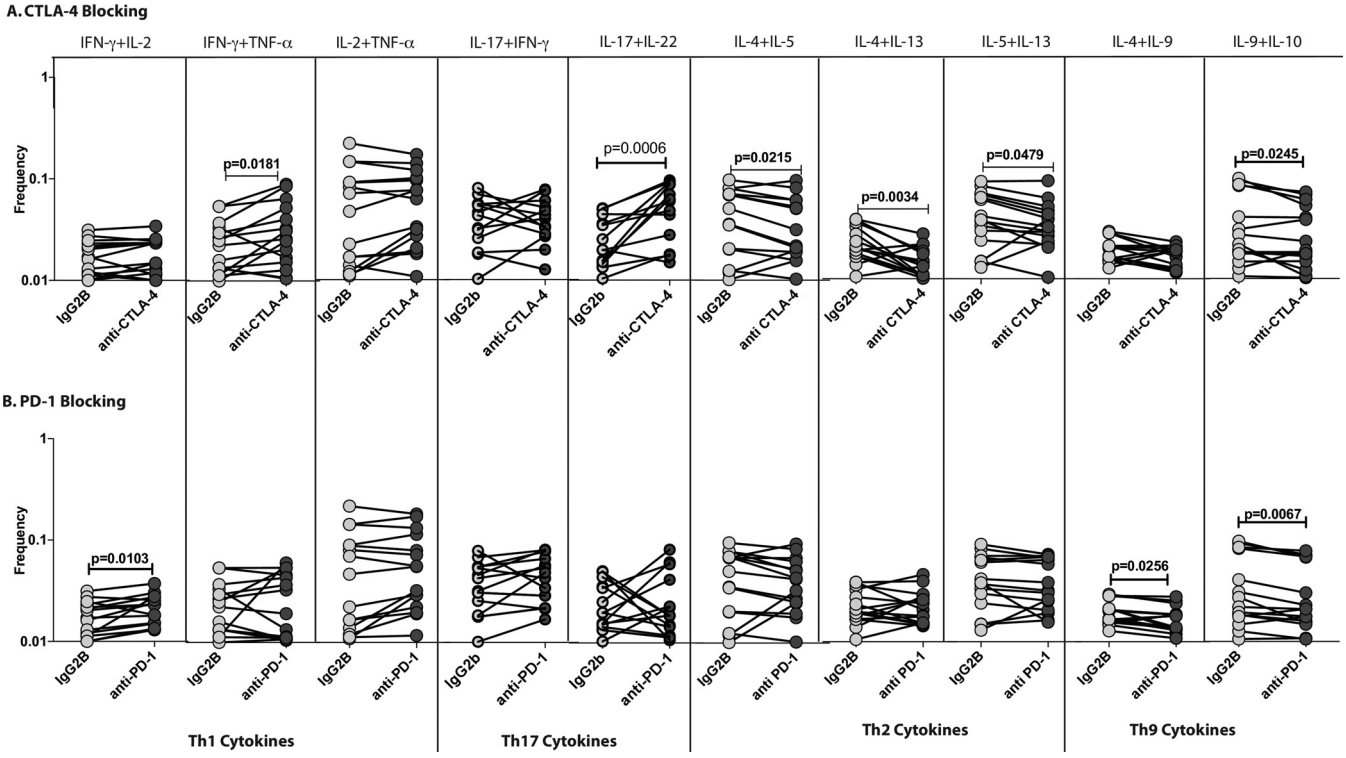

FIG 5 Regulation of dual functional CD8$^+$ T-cell subsets by CTLA-4 and PD-1 in *S. stercoralis* infection. The frequencies of dual functional CD8$^+$ Tc1, Tc2, Tc9, and Tc17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 15 *S. stercoralis*-infected individuals. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction. Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

nopathology in *S. japonicum*-infected mice (29). *In vitro* PD-1 blockade enhanced the antigen-specific ability of hyporesponsive Th2 cells to produce Th2 cytokines (30). CTLA-4 has been shown to promote the expression of T-cell anergy factors and inhibit protective Th2-cell immunity during filarial infections (16, 31). Th9 cells have been associated with resistance to intestinal helminth infection in animal models (32–34). In humans, Th9 cells are known to play a protective immune response and also control the pathology (35). In humans, Th9 cells play role in a variety of different disease conditions, such as atopy (36), melanoma (37), asthma (38), and autoimmunity (39).

The regulation of effector T-cell function is crucial for immune homeostasis during infection. Immune homeostasis is maintained, in part, by the negative regulators of T-cell activation, CTLA-4 and PD-1 (40). Earlier studies with mouse models have shown that CTLA-4 blockade augments T-cell responses in chronic infections, such as those caused by *Helicobacter pylori* (41), *Leishmania* spp. (42, 43), and *Trypanosoma cruzi* (44). Enhanced T-cell responses and more effective infection control in *Nippostrongylus brasiliensis* (45) and *Listeria monocytogenes* (46) infections following the blockade of CTLA-4 have also been reported. PD-1 is an important immune checkpoint molecule that functions to keep immune balance and to exert critical inhibitory functions in the setting of persistent antigenic stimulation, such as during encounters with self-antigens, in chronic infections, and in tumors (10, 47). In recent years, many studies have indicated that helminths may exploit the PD-1 pathway to modulate the host immune system to minimize excessive inflammation and promote the chronicity of helminth infection (30).

PD-1 is upregulated on both CD4$^+$ and CD8$^+$ T cells during human immunodeficiency virus (HIV) infection and on CD8$^+$ T cells during hepatitis C virus (HCV) infections and is linked with T-cell functional deficiency and the development of disease (48). The

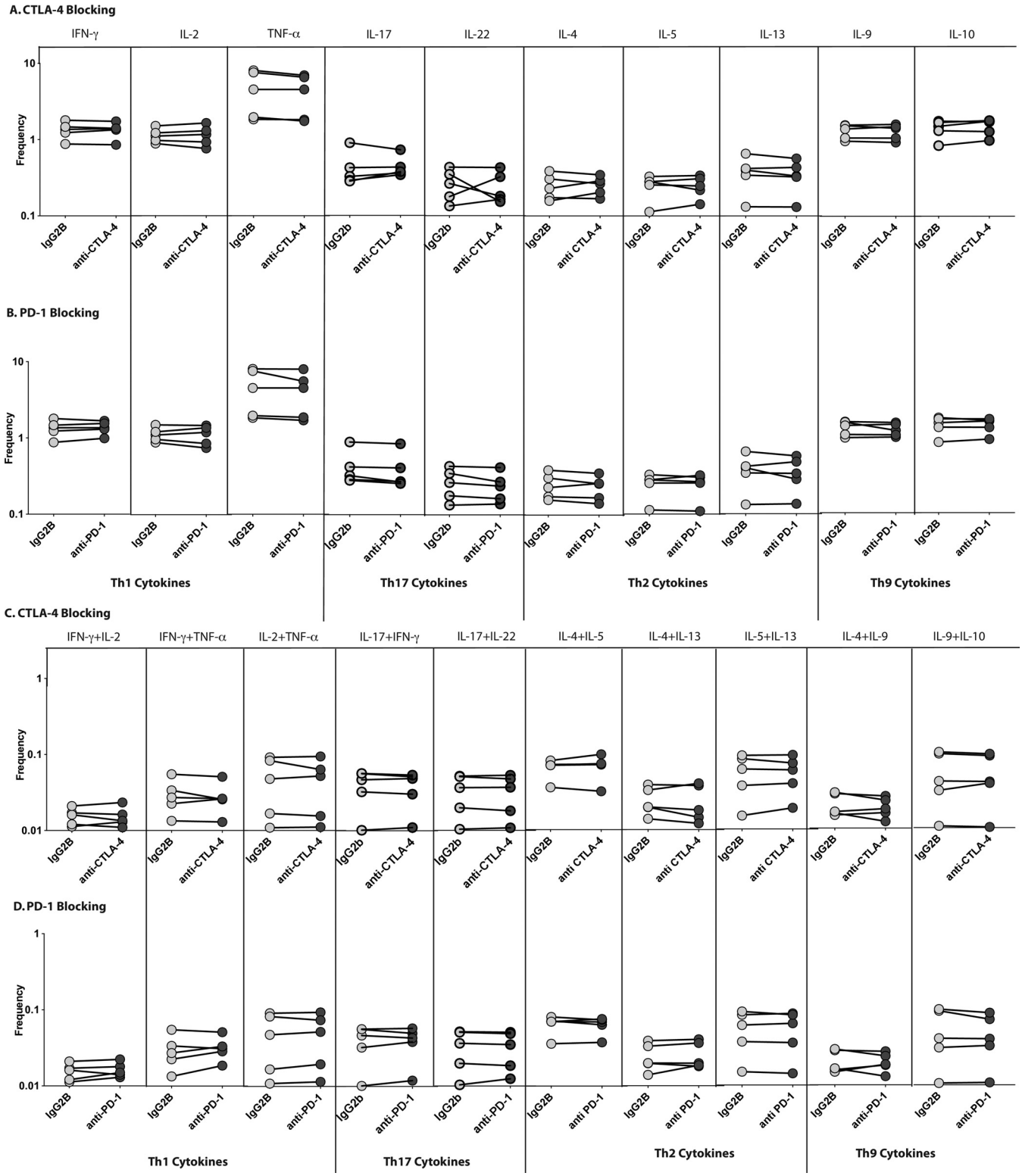

FIG 6 No alterations in the frequencies of monofunctional and dual functional CD8$^+$ T-cell subsets following CTLA-4 and PD-1 blockade in healthy controls. (A and B) The frequencies of monofunctional CD8$^+$ Tc1, Tc2, Tc9, and Tc17 cells stimulated by the parasite antigen NIE were measured by ow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 5 healthy individuals. (C and D) The frequencies of dual functional CD8$^+$ Tc1, Tc2, Tc9, and Tc17 cells stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (C and D) antibody blockade in 5 healthy individuals. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction. Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

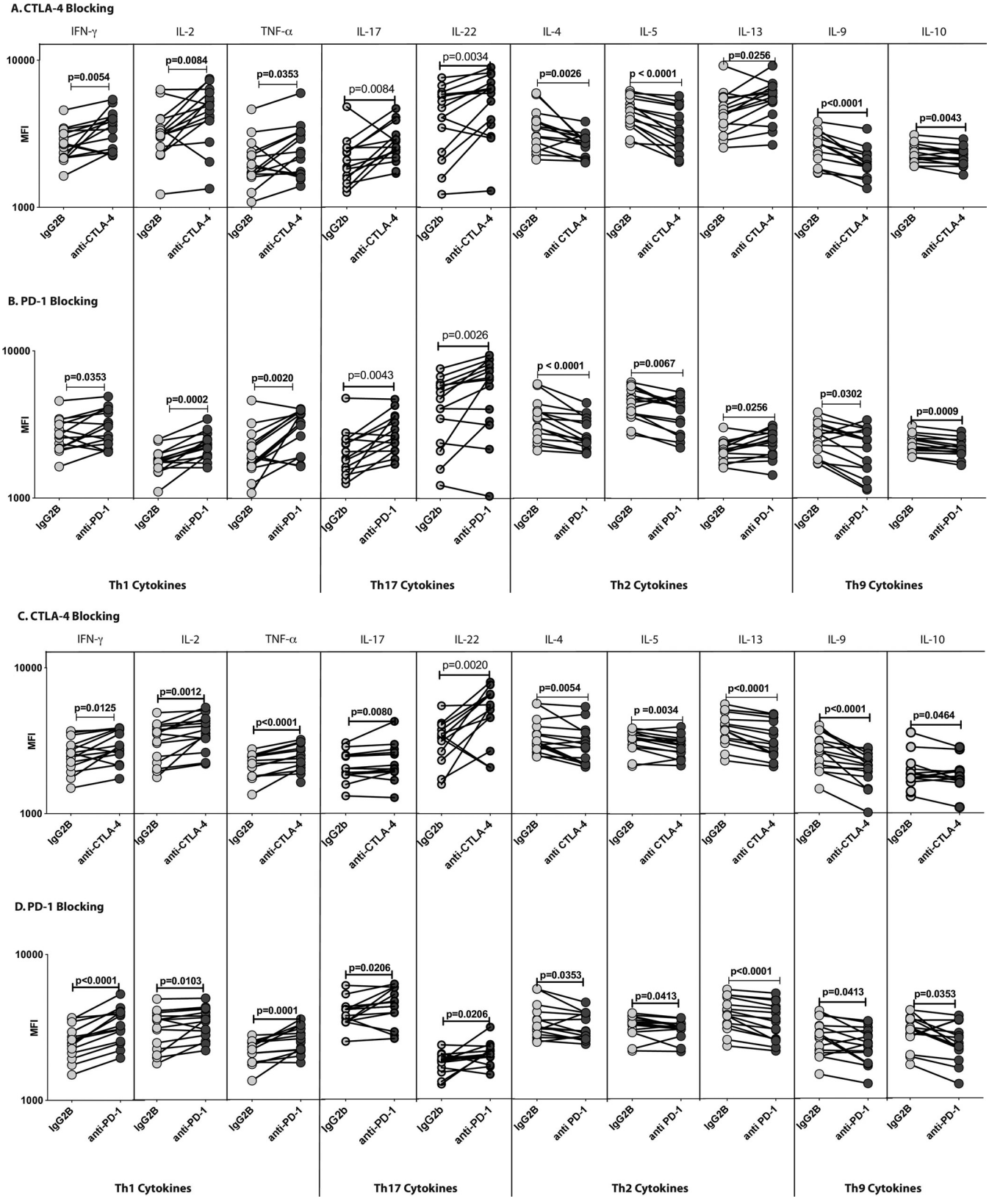

FIG 7 CTLA-4 and PD-1 regulate the mean fluorescence intensity (MFI) of cytokine expression on $CD4^+$ and $CD8^+$ T cells. (A and B) The MFI of $CD4^+$ Th1, Th2, Th9, and Th17 cytokines stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (A), anti-PD-1 (B), or isotype control (A and B) antibody blockade in 15 *S. stercoralis*-infected individuals. (C and D) The MFI of $CD8^+$ Tc1, Tc2, Tc9, and Tc17 cytokines stimulated by the parasite antigen NIE were measured by flow cytometry following anti-CTLA-4 (C), anti-PD-1 (D), or isotype control (C and D) antibody blockade. The data are represented as line diagrams, with each line representing a single individual. *P* values were calculated by the Wilcoxon signed-rank test, followed by the Holms correction.

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in vitro blockade of PD-1/PD-L1 led to the reversal of immune dysfunction in HCV infection (49). The *in vitro* blockade of CTLA-4 enhances the HIV-specific $CD4^+$ T-cell function (48).

Our study examined the modulation of monofunctional and dual functional $CD4^+$ and $CD8^+$ T cells following the *in vitro* blockade of CTLA-4 and PD-1 in *S. stercoralis* infection. Previously, we have shown the presence of markedly modulated Th1 and Th17 responses in *S. stercoralis* infection (3). In the present study, the blockade of CTLA-4 and PD-1 led to increased frequencies of $CD4^+$ and $CD8^+$ monofunctional and dual functional Th1 and Th17 cells. In addition to an increased frequency of Th1 and Th17 cells, the density of expression of cytokines on a per cell basis was also increased. The expansion of Th1 and Th17 cytokines following the blockade of inhibitory receptors suggests that CTLA-4 and PD-1 could either promote susceptibility to helminth infection or limit tissue damage by inhibiting Th1/Th17 responses. Previous studies have shown that the *in vitro* blockade of CTLA-4 augments Th2 production (17, 50, 51). The blockade of CTLA-4 in peripheral blood mononuclear cell cultures increases Th2 cytokine responses in patients with filarial infections (16) and promotes resistance to filarial and gastrointestinal nematodes in murine models (45, 52).

Our study suggests that in *S. stercoralis* infection, both CTLA-4 and PD-1 represent pathways essential for the regulation of Th2/Th9-cell responses and T-cell homeostasis during chronic infection. Our study reveals a dichotomous effect of CTLA-4 and PD-1 blockade on Th1/Th17-cell versus Th2/Th9-cell expansion. While CTLA-4 and PD-1 appear to downregulate Th1/Th17 responses, they might either upregulate or indirectly influence the expansion of Th2/Th9 cells. In addition to the decreased frequency of Th2 and Th9 cells, the density of expression of cytokines on a per cell basis is also decreased. Little is known about the mechanism by which CTLA-4 exerts its inhibitory function. It has been speculated that CTLA-4 may scavenge B7 ligands, rendering them unable to bind CD28 and thus reducing T-cell responses. The mechanism by which both CTLA-4 and PD-1 mediate this dual regulation of T-cell expansion needs to be examined in the future. Our study also reveals the specificity of checkpoint blockade inhibition, since the use of PD-1 and CTLA-4 in healthy control individuals had no significant effect on monofunctional or dual functional $CD4^+$ and $CD8^+$ T-cell cytokine production.

Our study examined $CD4^+$ and $CD8^+$ T-cell responses in *S. stercoralis* infection following CTLA-4 and PD-1 blockade, and taken together, the findings shed light on the role of these T-cell subsets in the regulation of immune responses. Our study suffers from the limitations of having a small sample size, of not using nonspecific control comparators, and of not determining PD-1 and CTLA-4 expression on T cells from our samples. Nevertheless, our study clearly demonstrates the importance of CTLA-4 and PD-1 in the regulation of immune responses in *S. stercoralis* infection, with expansion of the Th1 and Th17 cytokine responses and suppression of the Th2 and Th9 cytokine responses. Our data reveal certain interesting differences in the effects of checkpoint blockade inhibitors in helminth infections, with CTLA-4 exerting a profound effect on both $CD4^+$ and $CD8^+$ T cells and PD-1 exerting a major effect on $CD4^+$ T cells but only a moderate effect on $CD8^+$ T cells. Thus, in summary, both CTLA-4 and PD-1 have an essential role in regulating the immune responses to chronic *S. stercoralis* infection.

MATERIALS AND METHODS

Study population. We studied a group of 15 clinically asymptomatic, *S. stercoralis*-infected individuals and a group of 5 healthy control individuals in Tamil Nadu in southern India. Demographics and hematological parameters are provided in Tables S1 and S2, respectively, in the supplemental material. All infected individuals were NIE enzyme-linked immunosorbent assay (ELISA; *S. stercoralis* diagnostic antigen) positive. Samples with values above the cutoff of >299 absorbance units were defined to be positive by the NIE IgG ELISA. This finding was further confirmed by specialized examination of stool samples with nutrient agar plate cultures. All individuals were adults between 18 and 65 years of age and

FIG 7 Legend (Continued)
Abbreviations: IFN-γ, interferon gamma; IgG2B, immunoglobulin G2B; IL-2, interleukin-2; TNF-α, tumor necrosis factor alpha; IL-4, interleukin-4; IL-5, interleukin-5; IL-9, interleukin-9; IL-10, interleukin-10; IL-13, interleukin-13; IL-17, interleukin-17; IL-22, interleukin-22.

were enrolled consecutively. HIV infection, diabetes, and other helminth infections were exclusion criteria. The presence of other intestinal parasitic infections was ruled out by performing stool microscopy using the formyl ether concentration method. The TropBio ELISA method was used to exclude the possibility of filarial infection. None of the subjects had received anthelmintic treatment prior to enrollment in this study. All healthy control individuals were negative for all of the above-described infections. All individuals were examined as part of a natural history study, with the protocol being approved by the Institutional Review Boards of both the National Institute of Allergy and Infectious Diseases and the National Institute for Research in Tuberculosis (approval number 12-I-073), and informed written consent was obtained from all participants.

Parasite antigen. Recombinant NIE antigen, a previously characterized immunodominant, 31-kDa *S. stercoralis* larval antigen, was used as the parasite antigen (53). Previous studies have shown that the *S. stercoralis* NIE antigen derived from *S. stercoralis* L3 parasites has a performance comparable to that of the crude antigen (53, 54).

***In vitro* culture.** Whole blood from *S. stercoralis*-infected individuals was cultured in the presence of anti-CTLA4 (5 μg/ml; Ancell), anti-PD-1 (5 μg/ml; eBioscience, San Diego, CA), or an isotype control antibody (5 μg/ml; R&D Systems) with NIE antigen (10 μg/ml) for 18 h. 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 placed in 12-well tissue culture plates (Costar, Corning, Inc., NY). FastImmune brefeldin A solution (10 μg/ml; BD Biosciences) was added 2 h before the end of the culture. Whole blood was then centrifuged and washed with cold phosphate-buffered saline (PBS), and then fluorescence-activated cell sorter (FACS) lysing solution (BD Biosciences, San Diego, CA) was added. The cells were fixed using Cytofix/Cytoperm buffer (BD Biosciences, San Diego, CA), cryopreserved, and stored at −80°C until use.

Intracellular cytokine staining. The cells were thawed and washed with PBS first and PBS–1% bovine serum albumin (BSA) next and then stained with antibodies to surface markers expressed on the cell surface for 30 to 60 min. The following antibodies were used for surface marker staining: CD3-Amcyan, CD4-allophycocyanin (APC)-conjugated H7, and CD8-phycoerythrin (PE)-conjugated Cy7 (all from BD Biosciences). The cells were washed, permeabilized with BD Perm/Wash buffer (BD Biosciences), and stained with intracellular cytokines for an additional 30 min before washing and acquisition. The following cytokine antibodies were used: IFN-γ–PE, (BD Pharmingen), TNF-α–fluorescein isothiocyanate (FITC) (BD Biosciences), IL-2–APC (eBioscience), IL-4–FITC (BD Biosciences), IL-5–APC (BD Biosciences), IL-3–PE (BD Biosciences), IL-9–PE (BD Pharmingen), IL-10–APC (BD Pharmingen), IL-17–FITC (Miltenyi Biotech), and IL-22–PE (R&D Systems). Flow cytometry was performed on a FACSCanto 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 (TreeStar). All data are depicted as frequencies, denoted by the percentage of $CD4^+$ and $CD8^+$ T cells expressing the respective cytokine(s). Data are also depicted as the mean fluorescence intensities of cytokine expression on $CD4^+$ and $CD8^+$ T cells. Monofunctional Th1/Tc1 cells were defined as $CD4^+$/$CD8^+$ 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-mentioned cytokines, and multifunctional cells were those expressing all three cytokines. Monofunctional Th2/Tc2 cells were defined as $CD4^+$/$CD8^+$ T cells expressing only one of either IL-4, IL-5, or IL-13, and dual functional Th2/Tc2 cells expressed either IL-4/IL-5, IL-4/IL-13, or IL-5/IL-13. Monofunctional Th17/Tc17 cells were defined as $CD4^+$/$CD8^+$ T cells expressing only IL-17, and dual functional Th17/Tc17 cells expressed either IL-17/IFN-γ or IL-17/IL-22. Monofunctional Th9/Tc9 cells were defined as $CD4^+$/$CD8^+$ T cells expressing only IL-9, and dual functional Th9/Tc9 cells expressed either IL-4/IL-9 or IL-9/IL-10. A representative flow cytometry plot is shown in Fig. S1 in the supplemental material, depicting $CD4^+$ and $CD8^+$ T cell cytokine expression in an *S. stercoralis*-infected individual, as well as blockade of anti-CTLA-4 and antigen-stimulated frequencies of $CD4^+$ and $CD8^+$ Th1, Th2, Th17, and Th9 cells.

Statistical analysis. Data analyses were performed using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA). Geometric means (GM) were used for measurements of central tendency. Statistically significant differences were analyzed using the Wilcoxon signed-rank test followed by Holm's correction for multiple comparisons.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/IAI.00469-19.

SUPPLEMENTAL FILE 1, PDF file, 1.4 MB.

SUPPLEMENTAL FILE 2, PDF file, 0.1 MB.

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S.B. conceived of and designed the experiments. A.R. and S.M. performed the experiments. A.R. and S.B. analyzed the data. C.D. and T.B.N. contributed reagents/materials/analysis tools. A.R., S.B., and T.B.N. wrote the paper. All authors contributed to manuscript revision and read and approved the submitted version.

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