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Tuberculous pleurisy drives marked effector responses of γδ, CD4+, and CD8+ T cell subpopulations in humans

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ABSTRACT

Although tuberculous pleurisy (TP) presumably involves a hypersensitivity reaction, there is limited evidence indicating overreactive effector responses of γδ T cells and αβ T cells and their interrelation with Foxp3+ T_{regs} in pleural and other compartments. We found that TP induced reciprocal representations of Foxp3+ T_{regs} and Mtb phosphoantigen-specific Vγ2Vδ2 T cells in different anatomic compartments. Patients with TP exhibited appreciable numbers of "proliferating" Ki-67+ Vγ2Vδ2 T cells in the airway where Foxp3+ T_{regs} were not dominant, whereas striking increases in Foxp3+ T_{regs} in the blood and pleural compartments coincided with low frequencies of Vγ2Vδ2 T cells. Interestingly, anti-tuberculosis chemotherapy control of Mtb infection in patients with TP reversed reciprocal representations of Foxp3+ T_{regs} and proliferating Vγ2Vδ2 T cells. Surprisingly, despite high-level Foxp3+ T_{regs}, TP appeared to drive overreactive responses of IFN-γ-producing Vγ2Vδ2, CD4+CD25+, and CD8+CD25+ T effector subpopulations, whereas IL-22-producing Vγ2Vδ2 T cells increased subtly. Th1 effector responses were sustained despite remarkable declines in Foxp3+ T_{regs} at 1 mo after the treatment. Overreactive T effector responses of Mtb-reactive γδ T cells, αβ CD25+CD4+, and CD25+CD8+ T cell subpopulations appear to be immune features for TP. Increased Foxp3+ T_{regs} might be responsive to overreactive TP but unable to influence T effector responses despite having an inverse relation with proliferating Vγ2Vδ2 T cells. ***J. Leukoc. Biol.*** **98: 851–857; 2015.**

Abbreviations: BAL = bronchoalveolar lavage, BALF = bronchoalveolar lavage fluid, HMBPP = (*E*)-4-hydroxy-3-methyl-2-butenyl pyrophosphate, HV = health volunteer, ICS = intracellular cytokine staining, Mtb = *Mycobacterium tuberculosis*, PE = pleural effusion, TB = tuberculosis, T_{eff} = effector T cell, TP = tuberculous pleurisy, T_{reg} = regulatory T cell

The online version of this paper, found at www.jleukbio.org, *includes supplemental information.*

Introduction

TB remains one of the leading causes of global morbidity and mortality among infectious diseases largely because of HIV pandemics and multidrug-resistant TB [1]. TP represents one of the most common forms of extrapulmonary TB and often results from Mtb infection of the pleura, with an intensive accumulation of immune cells in the pleural space [2–4]. Our studies and those of others [5–11] have shown that $CD4^+$ and $CD8^+$ T cells have a role in immunity against Mtb infection. Our recent studies [12–14] also show that Mtb phosphoantigen-specific Vγ2Vδ2 T cells, which exist only in primates, also have a role in immune resistance to TB. IFN-γ and TNF-α produced by αβ $CD4^+/CD8^+$ and γδ T cells have been shown to be critical for controlling Mtb infection [5–11]. However, overproduction of IFN-γ and TNF-α by $CD4^+$ and $CD8^+$ T cells might exacerbate inflammation and tissue damage in Mtb infection. Potential overreactive host responses might be regulated or inhibited by $CD4^+CD25^+Foxp3^+$ T_{regs}. T_{regs} have been shown to suppress T cells and APCs in vitro and to inhibit transplant rejection and autoimmune reaction in vivo [15, 16]. Although T_{regs} undergo expansion during human TB [17–20], the in vivo roles of T_{regs} and their effects on responses of αβ $CD4^+/CD8^+$ and γδ T cells remain incompletely understood in human TB or TP.

It is generally presumed that TP reflects a delayed hypersensitivity response to mycobacterial antigens in the pleural space, with consequent pleural effusion. Although several groups have reported that T_{regs} are increased in patients with TP [21–25], studies have not investigated a detailed correlation between T_{regs} and αβ $CD4^+/CD8^+$ and γδ T cells in respective pleural and airway or lung compartments or the dynamic changes in these T effector

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subpopulations after anti-TB chemotherapy. Importantly, although T cells are most frequently found in TP fluid [3], there is no in vivo evidence indicating that hypothetical, overreactive T cells in the pleural compartment produce large amounts of proinflammatory cytokines [26–28]. One of the reasons for this might be due to a lack of optimal and timely assays catching effector function for producing cytokines by pleural lymphocytes. T cells that have undergone trafficking/accumulating in pleural space may be highly activated effector cells with "terminated" phenotypes, and conventional ICS after in vitro Ag stimulation may not optimally detect effector function for producing cytokines by these pleural T cells. In addition, the inflammatory nature of pleural effusion fluid may predispose immune cells/cytokine proteins to disadvantageous viability/detectability, making it difficult to detect by conventional ICS after Ag stimulation.

In the current study, we used direct ICS without Ag stimulation in vitro to measure potential overreactive effector responses of Vγ2Vδ2 T and αβ $CD25^+CD4^+/CD8^+$ T cell subpopulations. The direct ICS approach has been validated recently for its ability to detect large numbers of T_{effs} constitutively producing cytokines in macaques infected with TB or other pathogens [9, 13, 29–31] and in patients with TB [32] in comparison with control settings. We also examined the interrelation between $Foxp3^+$ T cells and dominant Vγ2Vδ2 T subset or αβ $CD25^+CD4^+/CD8^+$ T subsets in different anatomic compartments as well as dynamic changes in these effector cells after anti-TB chemotherapy.

MATERIALS AND METHODS

Ethics statement

All samples were collected with informed, written consent, according to protocols approved by the internal review and the ethics boards of Guangdong Medical College.

Patients

Twenty-one patients with TP, from 22 to 60 yr old, and 18 healthy individuals, from 20 to 55 yr old, were used in this study (Supplemental Table 1). TP subjects were inpatients of the pulmonary departments of the Affiliated Hospital of Guangdong Medical College, the Affiliated Houjie Hospital of Guangdong Medical College, and the Dongguan Hospital for Prophylaxis and Treatment of Chronic Disease. TP was confirmed based on typical clinical symptoms, chest X-radiography with no evidence of lung TB lesions, positive Ziehl-Neelsen acid fast bacilli staining, positive Lowenstein-Jensen slants bacterial culture for pleural fluid sediments, or pleural membrane biopsies or apparent anti-TB drugs efficacy. Bronchoscopy was performed to confirm a lack of evidence for respiratory diseases, lesions, or tumors and to collect BAL fluid. Subject exclusion criteria included HIV^+ test results, diabetes, cancer, autoimmune diseases, immunosuppressive treatment, or previous pulmonary TB. All blood, pleural effusion fluid, or BAL samples were collected before or within ~1 wk before the patients received anti-TB drugs of individualized isoniazid, rifampicin, pyrazinamide, and ethambutol. All patients responded well to anti-TB chemotherapy, with apparent clinical improvement before discharge. The 18 healthy, uninfected individuals served as controls.

Preparation of PBMC and lymphocytes from PE or BAL fluid

These methods were described in details elsewhere [13, 32]. Briefly, PBMC and lymphocytes were isolated from fresh blood and pleural effusion, respectively, by standard Ficoll (GE Healthcare, Little Chalfont, United Kingdom) density gradient centrifugation. For isolation of lymphocytes from BAL fluid, fresh BAL fluid was filtered through 20 ml of 2% FBS-PBS into 50 ml tubes followed by 5 min × 1500 rpm centrifugation. Cell pellets were then treated with 5 ml RBC blood lysis buffer (eBioscience, San Diego, CA, USA) for 10 min or until the suspension became clear and washed once with 2% FBS-PBS. Isolated lymphocytes were stained with trypan blue to identify the viability and to enumerate cell counts.

Antibodies and reagents

The following Abs were used for flow cytometry: anti-human CD3 (SP34-2; BD Biosciences, San Jose, CA, USA), anti-human CD4 (OKT4; eBioscience), anti-human CD8 (DK25; Dako, Glostrup, Denmark), anti-human CD25 (M-A251; BD Biosciences), CD127 (eBioRDR5, eBioscience), anti-human Vγ9 (7A5; Thermo Fisher Scientific, Rockford, IL, USA), anti-human Vδ2 (15D; Pierce, Rockford, IL, USA), anti-human Foxp3 (206D; BioLegend, San Diego, CA, USA), anti-human Ki-67 (Ki-67; BioLegend), anti-human IFN-γ (4S.B3; BD Biosciences), anti-human IL-22 (clone C8.6; Miltenyi Biotec, Bergisch Gladbach, Germany), and mouse IgG isotype control (eBioscience). Phosphoantigen compound HMBPP (>98% pure) was provided by Dr. Hassan Jomaa (Justus-Liebig-Universität Giessen, Giessen, Germany). Purified protein derivative and PMA were purchased from Mycos Research (Loveland, CO, USA) and GenScript (Piscataway Township, NJ, USA), respectively. Anti-CD28 (CD28.2; BD Biosciences) and anti-CD49d (9F10; BD Biosciences) were used as costimulatory Abs.

ICS

ICS was performed as previously described (13, 32). Lymphocytes ($3\sim10 \times 10^5$) were incubated for 6 h in the absence or presence of HMBPP (40 ng/ml) medium, purified protein derivative (20 μg/ml), and PMA, plus costimulatory CD28 (1 μg/ml) and CD49d (1 μg/ml) mAbs. After 6 h incubation, cells were transferred into 5 ml polystyrene, round-bottom tubes (BD Biosciences) for staining. Cells were washed once with 2% FBS-PBS and stained at room temperature for 25 min with surface marker Abs. For ICS, PBMCs were further washed twice with 2% FBS-PBS and permeabilized with BD FACS permeabilizing solution (BD Biosciences) for 30 min at room temperature, and then stained for another 45 min with IFN-γ and IL-22 Abs, followed by 2 final washes with 2% FBS-PBS buffer and analysis with BD FACSCanto II (BD Biosciences) flow cytometry. To ensure specific immune staining in ICS, matched isotype IgG served as negative controls for staining cytokines or surface markers. Direct ICS was used for measuring T_{effs} producing cytokines without in vitro antigen stimulation, as we recently described [9, 13, 29–32].

CFSE proliferation assay

PBMCs were stained with fluorescence-labeled antibodies against CD3, CD4, CD25, and CD127 and sorted into $CD25^+CD127^-$ T_{regs} and $CD25^{low/-}CD127^+$ T_{effs} using a BD FACSAria II cell sorter (BD Biosciences). Sorted T_{regs} and T_{effs} were further characterized by intracellular staining with Foxp3. T_{effs} were then labeled with 20 μM of CFSE (Beyotime, Shanghai, China) for 10 min at 37°C and protected from light, which was followed by 3 washes to remove the excess CFSE. After that, CFSE-labeled T_{effs} (1×10^4) were cocultured with or without T_{regs} (1×10^4) in the presence or absence of anti-CD3/CD28 mAbs and cultured for 3 d at 37°C with 5% CO_2. The CFDA intensity of the gated cells was measured through a 518 nm filter (FL1) with a BD FACS Canto II flow cytometry, and the proliferation index (average number of cell divisions) and percentage of proliferation (cells that divided at least once) were analyzed and calculated by FlowJo.7.6.1 software (Tree Star, Ashland, OR, USA).

Statistical analysis

The normality evaluation was first performed to determine whether the data set was well-modeled with a normal distribution. If data passed the normality distribution evaluation, Student's *t* test was used for 2-tailed comparisons; if data did not pass the normality, a Mann-Whitney *U* test was employed, as previously described [13, 32] using GraphPad Prism version 5.0 (GraphPad Software,

La Jolla, CA, USA). Results are expressed as means ± SEM In all cases, $P < 0.05$ was considered as statistically significant.

RESULTS

Patients with TP exhibit appreciable numbers of airway "proliferating" Vγ2Vδ2 T cells when Foxp3$^+$ T cells are not dominant

Comparative studies of Mtb-reactive γδ T and T_{regs} in the blood, PE, and alveoli or airway in patients with TP have not previously been reported. Here, we comparatively measured the frequencies of Foxp3$^+$ T cells and Vγ2Vδ2 T cells in PBMC, pleurisy lymphocytes in PE, and alveoli cells in BALF from patients with TP using flow cytometry. The flow cytometry gating strategy is shown in Supplemental Figure 1. Representative flow cytometry diagrams are shown in **Fig. 1A**. Interestingly, percentages of Vγ2Vδ2 T cells in PE appeared lower than those in BALF and blood (Fig. 1A and B), although there were no apparent differences in the frequencies of blood Vγ2Vδ2 T cells between patients with TP and HV controls (Fig. 1B). Notably, when Ki-67 expression was measured as a surrogate marker for cellular proliferation of Vγ2Vδ2 T cells, patients with TP had fewer blood Ki-67$^+$ Vγ2Vδ2 T cells than did HV controls (Fig. 1C). However, Ki-67$^+$ Vγ2Vδ2 T cells in the airway were significantly higher than those in blood and PE lymphocytes in patients with TP ($P < 0.001$; Fig. 1C) because almost 30% of γδ T cells in BALF were indeed Ki-67$^+$ Vγ2Vδ2 T cells.

Interestingly, high levels of Ki-67$^+$ Vγ2Vδ2 T cells in the airway coincided with low frequencies of Foxp3$^+$CD25$^+$CD4$^+$ T_{regs} (Fig. 1D). Consistently, low levels of Vγ2Vδ2 T cells in the blood and PE lymphocytes of patients with TP were associated with remarkably high frequencies of Foxp3$^+$CD25$^+$CD4$^+$ T cells. These Foxp3$^+$CD25$^+$CD4$^+$ T cells exhibited immune suppressive T_{reg} functions in vitro (Supplemental Fig. 2). In fact, the frequencies of T_{regs} in the blood of patients with TP were significantly higher than those in HV controls, with up to 50% Foxp3$^+$ cells in CD4$^+$CD25$^+$ T cells (Fig. 1D). Control patients

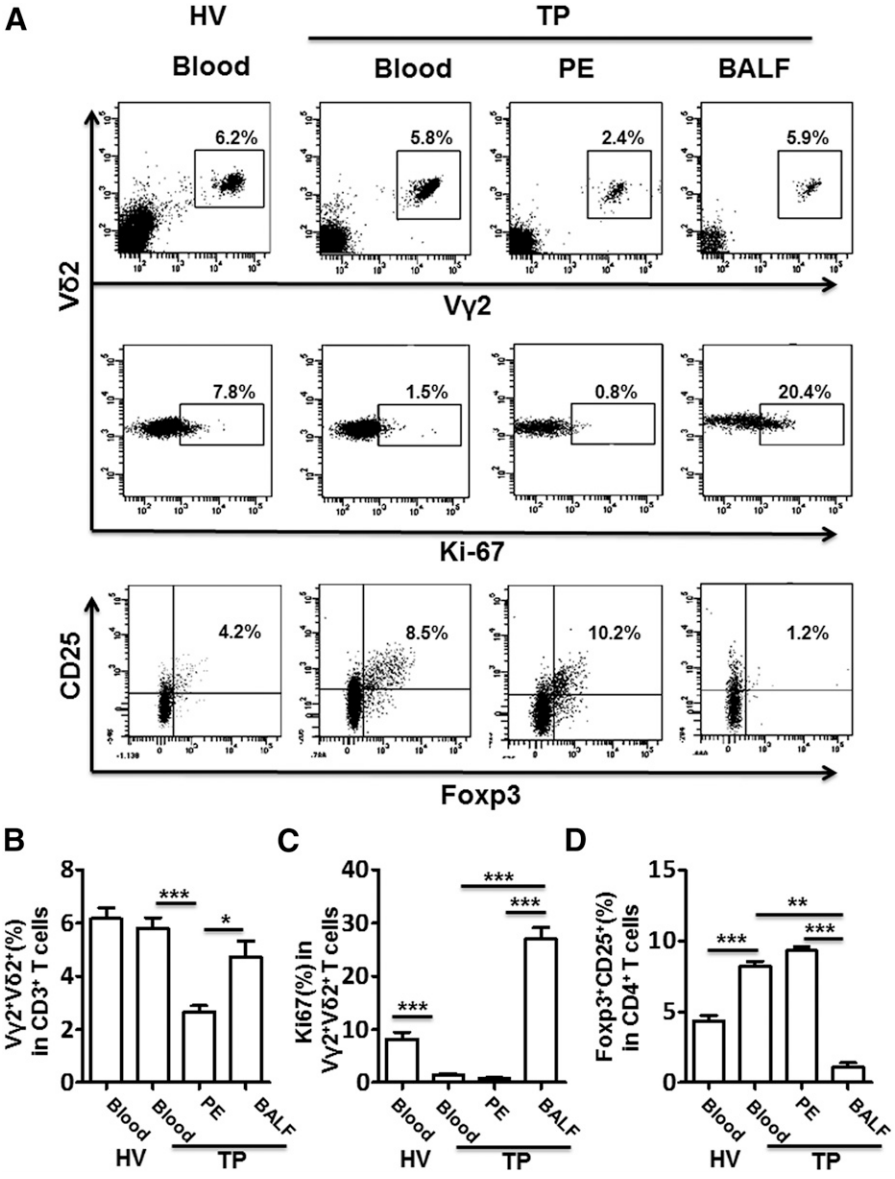

Figure 1. Frequencies of Vγ2Vδ2 T cells, Ki67$^+$Vγ2Vδ2 T cells, and CD4$^+$CD25$^+$Foxp3$^+$ T cells in blood, PE, and BALF from patients with TP. PBMCs were prepared from the patients with TP ($n = 21$) and HV ($n = 18$) controls, and the lymphocytes were isolated from PE and BAL fluid from patients with TP. Cells were assessed for frequencies of Vγ2Vδ2 T cells, Ki-67$^+$Vγ2Vδ2$^+$ T cells and CD4$^+$CD25$^+$Foxp3$^+$ T cells. Ki-67 expression was measured as a surrogate marker for cellular proliferation of Vγ2Vδ2 T cells. (A) Representative histograms for flow cytometry analysis of Vγ2Vδ2 T cells (left, gated on CD3), Ki-67 in Vγ2$^+$Vδ2$^+$ T cells (left, gated on Vγ2$^+$Vδ2$^+$), and Foxp3$^+$ CD25$^+$ expression in CD4$^+$ T cells (left, gated on CD4$^+$). (B) Graph data showing the mean frequencies of Vγ2Vδ2 T cells in CD3$^+$ T cells of PBMCs from patients with TP and HV controls and PE and BALF from patients with TP. (C) Graph data showing the mean frequencies of Ki-67$^+$ cells in Vγ2Vδ2 T cells in PBMCs from patients with TP and HV controls and from PE and BALF of patients with TP. (D) Graph data showing the mean frequencies of Foxp3$^+$ CD25$^+$ cells in CD4$^+$ T cells in PBMCs from patients with TP and HV controls and from PE and BALF of patients with TP. The P value is shown in each column. M0 and M1 indicate pretreatment and 1 mo after treatment, respectively. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

with intraparenchymal TB exhibited means of ~6.4% of $Foxp3^+CD25^+CD4^+$ T_{regs} and ~5% Vγ2Vδ2 T cells in total circulating T cells (data not shown). When we compared T_{regs} in different compartments from patients with TP, we found that frequencies of T_{regs} in the blood and PE were significantly higher than those in the BALF ($P < 0.01$ and $P < 0.001$, respectively; Fig. 1D).

Thus, these results demonstrate that patients with TP exhibited appreciable numbers of proliferating $Ki\text{-}67^+$Vγ2Vδ2 T cells in airways in which $Foxp3^+$ T cells were not dominant, and that striking increases in $Foxp3^+$ T cells in pleurisy lymphocytes in PE were coincident with reduced numbers of Vγ2Vδ2 T cells.

Anti-TB drugs reverse reciprocal representations of $Foxp3^+$ T cells and proliferating Vγ2Vδ2 T cells in patients with TP

We then sought to determine whether treatment with anti-TB drugs could alter the relative representations of Vγ2Vδ2 T cells and $Foxp3^+CD25^+CD4^+$ T cells in patients with TP. Representative flow diagrams are shown in **Fig. 2A**. Interestingly, after anti-TB chemotherapy, the frequencies of blood $Foxp3^+$ T cells from patients with TP decreased significantly to levels comparable to those in HV controls (Fig. 2A and B). Despite no significant alterations in nonreplicating γδ T cells in the blood of these patients with TP after treatment (Fig. 2C), we found significant increases in the frequencies of proliferating $Ki\text{-}67^+$ Vγ2Vδ2 T cells in the blood 1 mo after treatment (Fig. 2D). These results demonstrate, therefore, that anti-TB chemotherapy control of Mtb infection in patients with TP can reciprocally alter the representations of $Foxp3^+$ T cells and proliferating Vγ2Vδ2 T cells.

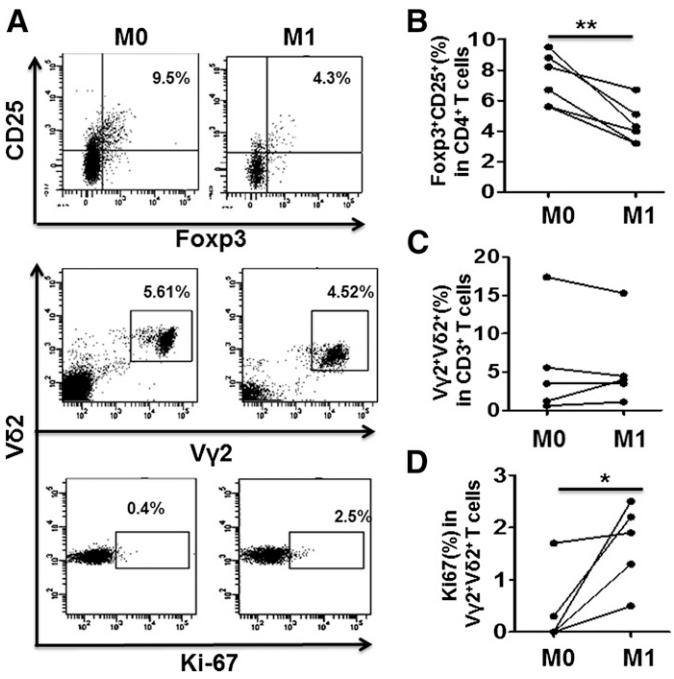

Figure 2. Anti-TB chemotherapy in patients with TP reversed reciprocal representations of $Foxp3^+$ T cells and proliferating Vγ2Vδ2 T cells. Blood samples were collected from 5 patients who were followed for 1 mo after treatment. PBMCs were prepared and assessed for frequencies of $V\gamma2^+V\delta2^+$ T cells, $Foxp3^+$ $CD25^+$ T cells, and $Ki67^+V\gamma2^+V\delta2^+$ T cells using flow cytometry. (A) Representative histograms for flow cytometry analysis of Vγ2Vδ2 T cells (left, gated on CD3), Ki-67 in $V\gamma2^+V\delta2^+$ T cells (left, gated on $V\gamma2^+V\delta2^+$), and $Foxp3^+$ $CD25^+$ expression in $CD4^+$ T cells (left, gated on $CD4^+$). (B) Changes in $Foxp3^+$ $CD25^+$ T cells in PBMCs from patients with TP before and after 1 mo of treatment. (C) Changes in Vγ2Vδ2 T cells in PBMCs from patients with TP at 1 mo after treatment. (D) Changes in Ki-67 expression in Vγ2Vδ2 T cells in the blood of patients with TP. M0 and M1 indicate pretreatment and after 1 mo of treatment, respectively. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Dominant representation of $Foxp3^+$ T cells in the blood and pleurisy compartments of patients with TP is associated with subtle increases in IL-22-producing Vγ2Vδ2 T cells

Because TP might represent an overreactive clinical subtype of tuberculosis, we sought to examine whether Vγ2Vδ2 T cells and αβ $CD4^+/CD8^+$ T cells displayed effector functions for production of selected inflammatory cytokines. We focused on IFN-γ and IL-22 because these 2 cytokines can either function as anti-TB elements [33, 34] or act as proinflammatory cytokines [35]. We examined the effector responses of Vγ2Vδ2 T cells and $CD4^+/CD8^+$ T cells using direct intracellular staining without antigen stimulation. PBMC or cells isolated from the PE of patients with TP were directly stained for cytokines without antigen stimulation in vitro, as we recently described [9, 13, 29–32], to examine the ability of T cells to constitutively produce IFN-γ or IL-22. We have validated the specificity and utility of the direct ICS approach during Mtb infection of macaques and humans [9, 13, 29–32]. To facilitate detection, we examined CD25 coexpression in CD3/CD4 or CD3/CD8 for constitutive cytokine production because $CD25^+CD4^+/CD8^+$ T cells were supposed to be activated cells.

We first investigated whether these cells were constitutive IL-22-producing T effector subpopulations in patients with TP, as we previously found in the setting of severe TB [29, 34]. We found that Vγ2Vδ2 T effector cells constitutively producing IL-22 without in vitro HMBPP stimulation were measurable in the blood of patients with TP and were higher than those in HV controls (**Fig. 3A** and **B**), with similar frequencies in the blood and pleurisy compartments of patients with TP (Fig. 3B). At 1 mo after treatment, we observed a reduction in the percentages of $IL\text{-}22^+$ Vγ2Vδ2 T cells (Fig. 3C). $CD4^+CD25^+$ and $CD8^+CD25^+$ T cell subpopulations in patients with TP contained low frequencies of constitutively $IL\text{-}22^+$ effector cells in either the blood and pleurisy compartments (Fig. 3D) and exhibited no apparent changes at 1 mo after treatment (Fig. 3E).

These results suggest that the dominant distribution of $Foxp3^+$ T cells in the blood and pleurisy compartments of patients with TP is associated with detectable, minor increases in constitutive IL-22-producing Vγ2Vδ2 T cells.

TP drive overreactive responses of IFN-γ-producing Vγ2Vδ2, $CD4^+CD25^+$ and $CD8^+CD25^+$ T effector subpopulations despite high levels of $Foxp3^+$ T cells, and responses are sustained despite dramatic decline in $Foxp3^+$ T cells 1 mo after treatment with anti-TB drugs

We then measured T_{effs} constitutively producing IFN-γ without antigen stimulation in vitro. We found that the mean percentages of constitutive IFN-γ-producing T_{effs} in $V\gamma2V\delta2^+$ T cell, $CD4^+CD25^+$ T cell, and $CD8^+CD25^+$ T cell subpopulations in

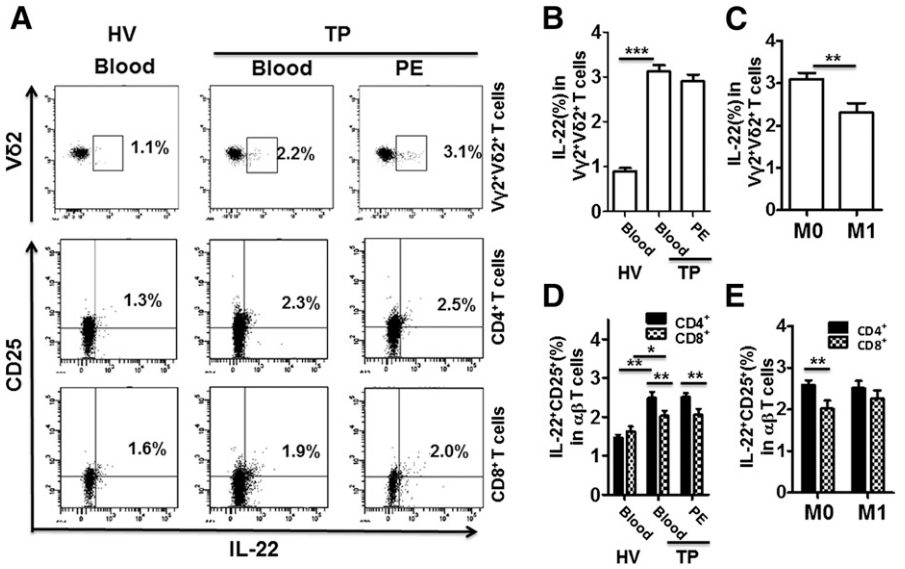

Figure 3. Constitutive IL-22 production by γδ and αβ T cell subpopulations in PBMCs and cells isolated from PE of patients with TP. (A) Representative flow cytometry histograms showing percentages of IL-22-producing cells within Vγ2Vδ2 T cells, αβCD4$^+$CD25$^+$, or CD8$^+$ CD25$^+$ T cells. (B) Graph data showing percentages of IL-22-producing cells within Vγ2Vδ2 T cells ($n = 21$). (C) Percentage of IL-22-producing cells in Vγ2Vδ2 without antigen stimulation of PBMC after 1-mo of ATD treatment ($n = 5$). (D) Graph data showing percentages of IL-22-producing cells within CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cells ($n = 21$). (E) Percentage of IL-22-producing cells in CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cells without antigen stimulation of PBMC after 1 mo of ATD treatments ($n = 5$); M0 and M1 indicate pretreatment and 1 mo after treatment, respectively. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the blood circulation were ~8, 10.5, and 10%, respectively, which were significantly higher than those in HV controls (**Fig. 4A–E**). In control patients with intraparenchymal TB, means of ~6% Vγ2Vδ2 T cells and means of ~5% CD25$^+$CD4$^+$ T cell subpopulation in the blood could constitutively produce IFN-γ (data not shown). Surprisingly, despite high-levels of Foxp3$^+$ T cells, the mean percentages of T_{effs} that constitutively produced IFN-γ within these 3 T cell subpopulations in the pleurisy compartment were significantly higher than those in the blood circulation (Fig. 4A, B, and D). Approximately 18% of Vγ2Vδ2$^+$ T cell subset in PE lymphocytes from patients with TP could constitutively produce IFN-γ without the need for antigen stimulation (Fig. 4B), although frequencies of these γδ T_{effs} were lower than those within CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cell subpopulations in PE lymphocytes from patients with TP (Fig. 4B and D). Interestingly, at 1 mo after treatment, the percentages of constitutively IFN-γ-producing effector cells within Vγ2Vδ2, CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T cell subpopulations were sustained and somewhat increased (Fig. 4C and E). These short-term increases could be explained by anti-TB chemotherapy-driven improvement of both the presumably activated CD25$^+$ T subset and the Vγ2Vδ2$^+$ T cell subpopulation comprising both activated and nonactivated cells. These results suggest that T_{effs} constitutively producing IFN-γ are engaged by Mtb antigens in vivo in association with delayed hypersensitivity syndrome (fever and pleurisy/breathing compromise).

Thus, TP induced large numbers of constitutive IFN-γ-producing Vγ2Vδ2, CD4$^+$CD25$^+$ and CD8$^+$CD25$^+$ T effector subpopulations despite high levels of Foxp3$^+$ T cells, and constitutive T_{effs} were sustained even after T_{regs} declined to within reference range at 1 mo after treatment.

DISCUSSION

The current work extends our previous studies of T_{eff} response in primate TB to the setting of patients with TP and demonstrates

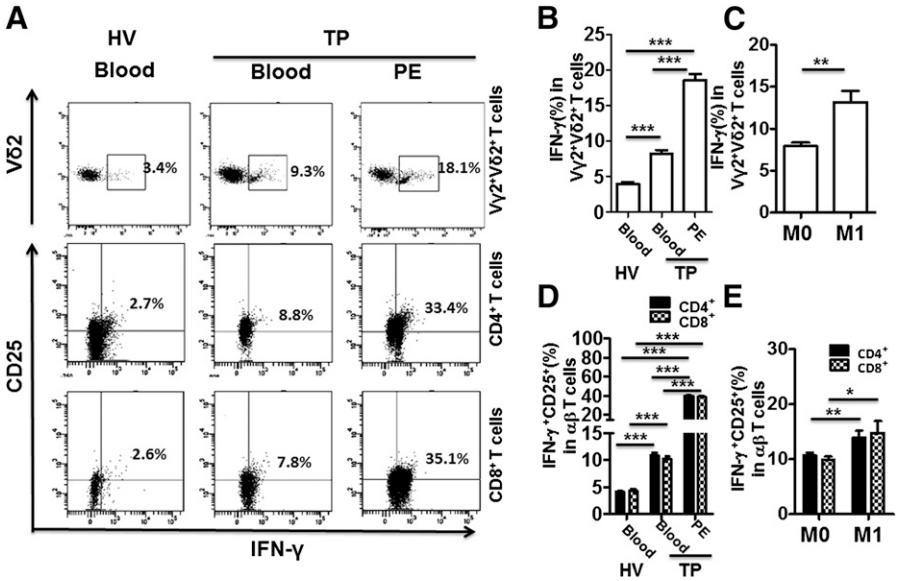

Figure 4. Constitutive IFN-γ production by γδ and αβ T cell subpopulations in PBMCs and cells isolated from the PE of patients with TP. (A) Representative flow cytometry histograms showing percentages of IFN-γ-producing cells in Vγ2Vδ2 T cells, αβ CD4$^+$CD25$^+$, or CD8$^+$CD25$^+$ T cells. (B) Graph data showing percentages of IFN-γ-producing Vγ2Vδ2 T cells in PBMCs or in PE of patients with TP without Ag stimulation ($n = 21$). (C) Percentages of IFN-γ-producing cells within Vγ2Vδ2 T cells in PBMCs after 1 mo of ATD treatment ($n = 5$). (D) Graph data showing percentages of IFN-γ-producing αβ CD4$^+$CD25$^+$ or CD8$^+$CD25$^+$ T cells in PBMCs or in PE of patients with TP without Ag stimulation ($n = 21$). (E) Percentages of IFN-γ-producing cells within αβ CD4$^+$CD25$^+$ or CD8$^+$CD25$^+$ T cells in PBMCs after 1 mo of ATD treatment ($n = 5$). M0 and M1 indicate pretreatment and 1 mo after treatment, respectively. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the following findings, which, to our knowledge, have not been previously described: 1) Mtb-specific Vγ2Vδ2 T cells can appreciably travel to the airway with a proliferating phenotype in inverse relationship to Foxp3$^+$ T_{regs} in TP, and anti-TB chemotherapy control of Mtb infections can reverse the reciprocal representatives of Foxp3$^+$ T cells and proliferating Ki-67$^+$ Vγ2Vδ2 T cells; 2) TP represents overreactive proinflammatory responses of IFN-γ-producing Vγ2Vδ2 T and αβ CD4$^+$/CD8$^+$ CD25$^+$ T effector subpopulations, despite high levels of Foxp3$^+$ T_{regs}, and such remarkable T_{eff} responses are sustained even after T_{regs} decline to within reference range 1 mo after treatment.

The current study provides interesting data illustrating the distribution or homeostasis of proliferating Vγ2Vδ2 T cells in the airway and pleural and circulating compartments, as well as their inverse in vivo relationship with Foxp3$^+$ T cells in patients with TB. "Proliferating" Ki-67$^+$Vγ2Vδ2 T cells in the blood or PE in the setting of high levels of Foxp3$^+$ T cells in patients with TP are significantly lower than those in blood of HV controls. However, much higher frequencies of Ki-67$^+$Vγ2Vδ2 T cells with proliferating Ki-67$^+$ phenotype in the airway correlate with lower levels of Foxp3$^+$ T cells. Thus, although earlier studies did not compare representatives of proliferating Vγ2Vδ2 T cells and Foxp3$^+$ T cells in the airway and PE of patients with TP [21–25], we have established an inverse relationship between proliferating Vγ2Vδ2 T cells and Foxp3$^+$ T cells in the anatomic compartments studied. These results suggest that increased Foxp3$^+$ T cells in patients with TP might predominantly travel to TB-driven, overreactive pleural compartments, but not to TB-free airways, for immune regulation. The inverse relationship between Foxp3$^+$ T cells and Vγ2Vδ2 T cells in TP also suggests that a dominance of Foxp3$^+$ T cells in the tissue interface may affect the trafficking of proliferating γδ T cells.

Surprisingly, TP induces remarkable, constitutive Th1-effector responses in Vγ2Vδ2 T cells and αβ CD25$^+$CD4$^+$/CD8$^+$ T subpopulations despite high frequencies of Foxp3$^+$ T_{regs}. To our knowledge, this is the first report demonstrating highly reactive, proinflammatory T cell responses that involve such large numbers of TB-reactive γδ T_{eff} and αβ T_{eff} subpopulations in patients with TP. Here, we temporarily use the term "highly reactive" T_{eff} response because surprisingly high frequencies of Vγ2Vδ2 T cells and αβ CD25$^+$CD4$^+$/CD8$^+$ T subpopulations in the blood and PE can constitutively produce IFN-γ without the need for TB antigen stimulation in culture. We used direct ICS to detect the constitutive T_{eff} function producing IL-22 or IFN-γ, and we validated it in macaques and humans actively infected with TB or other infections [9, 13, 29–32]. Moreover, the relevant isotype IgG controls or irrelevant Ab could not stain or detect such constitutive T_{effs} in samples from patients with TP (data not shown). In parallel, direct ICS methods detected very low levels of constitutive IL-22$^+$ T cells in the same samples from those patients with TP. Furthermore, our data are consistent with recent T SPOT.*TB* (Oxford Immunotec Ltd., Abingdon, United Kingdom) and ELISA results indicating high frequencies IFN-γ$^+$ lymphocytes [26] and elevated IFN-γ levels [27, 28] in PE fluid. The sustained T_{eff} responses at 1 mo after the TB treatment do not appear to be attributable to a coincidence of TB lesions and the effusion because repeated chest X-ray tests did not detect any lung TB lesions with negative acid-fast staining in the BAL fluid. Thus, high frequencies of constitutive IFN-γ-producing T_{eff} responses in patients with TP may indeed represent highly reactive host responses. These Th1 effector cells are likely vigorously stimulated in vivo by TB antigens, stimulatory cytokines, or both, which makes it possible to uncover the ability of these cells to produce IFN-γ without the need for restimulation by TB antigens.

High frequencies of IFN-γ$^+$ Vγ2Vδ2 T cells and αβ CD4$^+$/CD8$^+$ CD25$^+$ T effector subpopulations may have a role in the immune pathogenesis of TP. Although these highly reactive T_{effs} tend to respond vigorously to pleural TB for infection control, the overreactive proinflammatory responses of these IFN-γ$^+$ cells may contribute to severe pleural inflammation and effusion. Importantly, these overreactive T_{eff} responses correlate with clinical features of PE resulting in respiratory compromise and fever. In fact, we have recently shown that highly productive malarial infection from simian HIV coinfection can induce overreactive, constitutive Th1 effector responses in the acute blood stage and lead to the development of life-threatening malaria [36], and vaccination against the coinfection-induced attenuation of these highly reactive Th1 responses leads to protection against fatal malaria in primates [37].

Dynamic changes in Foxp3$^+$ T cells suggest they are responsive to TP, rather than dysfunctional. Although earlier studies reported TB-driven increases in T_{regs} in TP [21–25] and their ability to inhibit immune cells in vitro [17–20], our study extends previous findings by demonstrating that the frequencies of Foxp3$^+$ T cells in BALF are much lower than those in the blood and PE of patients with TP. Whether this is due to antagonizing effect from Vγ2Vδ2 T cells remains unknown. Moreover, 1 mo anti-TB chemotherapy can control TP and reduce Foxp3$^+$ T cells to within reference range. These results suggest that remarkable increases in Foxp3$^+$ T cells might respond to a TB-induced high inflammatory reaction in pleural compartments, but not in the airways if no TB lesions are present in the lungs. The scenario that increases in Foxp3$^+$ T cells respond to the initial high reaction to pleural TB in the host may help explain why increased Foxp3$^+$ T cells fail to influence the robust, constitutive T_{eff} response or prevent pleural inflammation/effusion in patients with TP.

Thus, our study provides previously undescribed findings, and data support our hypothesis that TP appears to be characterized by a highly reactive host response involving large numbers of T_{eff} subpopulations constitutively producing 1 or more proinflammatory cytokines, and Foxp3$^+$ T cells may be responsive to such vigorous T_{eff} responses.

AUTHORSHIP

Z.W.C. and J.X. designed the research. J.Z and B.K. analyzed data. Z.S., X.C., D.L., G.L., and L.Y. collected samples. Z.W.C. and J.X. wrote the manuscript. J.Z., S.H., W.W., Y.Z., W.X., and Y.H. performed experiments. C.Y.C., D.H, L.S., H.S., and Z.W.C. provided reagents and protocols, and gave scientific advice while evaluating and discussing data.

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DISCLOSURES

The authors declare no competing financial interests.

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human Vγ2Vδ2 T cells · Foxp3$^+$ T$_{reg}$ · *Mycobacterium tuberculosis* · Th1 · Th22

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