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CD4$^+$ CD25^{high} Foxp3$^+$ regulatory T cells downregulate human Vδ2$^+$ T-lymphocyte function triggered by anti-CD3 or phosphoantigen

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Summary

Vδ2$^+$ T cells, the major circulating T-cell receptor-γδ-positive (TCR-γδ$^+$) T-cell subset in healthy adults, are involved in immunity against many microbial pathogens including *Mycobacterium tuberculosis*. Vδ2$^+$ T cells recognize small phosphorylated metabolites (phosphoantigens), expand in response to whole *M. tuberculosis* bacilli, and complement the protective functions of CD4$^+$ T cells. CD4$^+$ CD25^{high} Foxp3$^+$ T cells (Tregs) comprise 5–10% of circulating T cells and are increased in patients with active tuberculosis (TB). We investigated whether, in addition to their known role in suppressing TCR-αβ$^+$ lymphocytes, Tregs suppress Vδ2$^+$ T-cell function. We found that depletion of Tregs from peripheral blood mononuclear cells increased Vδ2$^+$ T-cell expansion in response to *M. tuberculosis* (H37Ra) in tuberculin-skin-test-positive donors. We developed a suppression assay with fluorescence-activated cell sorting-purified Tregs and Vδ2$^+$ T cells by coincubating the two cell types at a 1 : 1 ratio. The Tregs partially suppressed interferon-γ secretion by Vδ2$^+$ T cells in response to anti-CD3 monoclonal antibody plus interleukin-2 (IL-2). In addition, Tregs downregulated the Vδ2$^+$ T-cell interferon-γ responses induced by phosphoantigen (BrHPP) and IL-2. Under the latter conditions there was no TCR stimulus for Tregs and therefore IL-2 probably triggered suppressor activity. Addition of purified protein derivative (PPD) increased the suppression of Vδ2$^+$ T cells, suggesting that PPD activated antigen-specific Tregs. Our study provides evidence that Tregs suppress both anti-CD3 and antigen-driven Vδ2$^+$ T-cell activation. Antigen-specific Tregs may therefore contribute to the Vδ2$^+$ T-cell functional deficiencies observed in TB.

Keywords: regulatory T cells; tuberculosis; Vδ2$^+$ T cells

Introduction

Development of protective immunity against *Mycobacterium tuberculosis* depends on the interplay between various T-lymphocyte subsets and professional antigen-presenting cells (APCs). Conventional CD4$^+$ and CD8$^+$ T-cell receptor-αβ-positive (TCR-αβ$^+$) T cells play a major role in adaptive immunity against *M. tuberculosis*.[1–6] Among unconventional T cells, human γδ T cells have also been implicated in protective immunity against *M. tuberculosis*.[7,8] In adults, γδ T cells normally constitute between 2 and 5% of peripheral blood T cells. The majority of circulating human γδ T cells expresses the Vγ9Vδ2 TCR (also termed Vγ2Vδ2 TCR). Vγ9Vδ2$^+$ T cells (Vδ2$^+$ T cells) are readily activated by mycobacterial extracts *in vitro* and expand greatly in response to systemic infections.[9–11] In non-human primates, Vδ2$^+$ T-cell responses increase during primary mycobacterial infection and after challenge with bacillus Calmette–Guérin (BCG).[7] The BCG vaccination of human adults enhances Vδ2$^+$ T-cell responses *in vitro* suggesting the development of a memory-like phenotype.[12] Although the role of Vδ2$^+$ T cells in immune defence against mycobacterial infections remains poorly characterized, Vδ2$^+$ T cells may contribute to early immune responses to *M. tuberculosis* and serve as a bridge between innate and adaptive immune responses.[13,14] Unlike TCR-αβ$^+$ T cells, human Vδ2$^+$ T cells do not recognize peptides presented by classical or non-classical

major histocompatibility complex molecules. Instead Vδ2$^+$ T cells recognize a group of non-peptidic prenyl pyrophosphate antigens known as phosphoantigens.[15–17] While Vδ2$^+$ T cells and αβ TCR$^+$ T cells display similar functional properties, their differential contribution to the immune defence against *M. tuberculosis* may be linked to the type of antigen they recognize and the mode of recognition.[11,18]

Regulation of Vδ2$^+$ T-cell function during *M. tuberculosis* infection is poorly understood. In contrast to acute systemic infections such as malaria, in tuberculosis (TB) the number and function of Vδ2$^+$ T cells are downregulated.[19–22] A variety of mechanisms have been proposed for the decrease in Vδ2$^+$ T-cell function in TB, including redistribution to the lung parenchyma, lack of CD4$^+$ T helper activity, activation-induced cell death and immune suppression.[14,23] Regulatory T cells (Tregs) have a major role in suppressing other immune cells such as CD4$^+$ TCR-αβ$^+$ T cells, CD8$^+$ TCR-αβ$^+$ T cells, natural killer T (NKT) cells, monocytes and dendritic cells.[24] The role of Tregs in suppression of Vδ2$^+$ T-cell function has not been studied.

Although the primary role of Tregs in the immune system is to prevent autoimmunity, Tregs have also been shown to suppress immune responses against persistent pathogens.[25–29] In the murine model of TB two studies demonstrated that decreased Treg numbers correlated with lower mycobacterial load.[29,30] More importantly, increased Treg numbers have been demonstrated both in blood and at sites of infection in patients with active TB.[31–33] Two main subsets of Tregs have been described: naturally occurring CD4$^+$ CD25$^+$ Tregs (nTregs) and inducible Tregs (iTregs). The nTregs develop in the thymus and constitutively express the α-chain of the interleukin-2 (IL-2) receptor (CD25) and the transcription factor Foxp3. The iTregs develop in the periphery from conventional CD4$^+$ T cells and, upon TCR triggering under tolerogenic conditions, they express CD25 and, in humans, Foxp3.[34–36] There is no single marker that distinguishes these two Treg subsets in humans and it remains unclear if they are two completely distinct cell populations. Our current study focuses on the role of Tregs that constitutively express high levels of CD25 and Foxp3 (CD4$^+$ CD25^{high} - Foxp3$^+$) referred to as Tregs throughout this manuscript.

Here, we demonstrate that CD4$^+$ CD25^{high} Foxp3$^+$ T cells suppress both anti-CD3 and phosphoantigen-triggered Vδ2$^+$ T-cell responses. Taking advantage of the different antigen specificities of αβ and Vδ2$^+$ T cells we developed an assay for simultaneous stimulation of Tregs and Vδ2$^+$ T cells with purified protein derivative (PPD) and phosphoantigen. We establish that PPD triggers Treg suppression of Vδ2$^+$ T-cell function. Our data suggest that antigen-specific Tregs contribute to the Vδ2$^+$ T-cell functional deficiencies observed in TB. This constitutes the first report on the suppression of phosphoantigen-reactive Vδ2$^+$ T cells by antigen-specific Tregs.

Materials and methods

Monoclonal antibodies and antigens

The following monoclonal antibodies (mAbs) were used for T-cell purification and fluorescence-activated cell sorting (FACS): fluorescein isothiocyanate (FITC)-conjugated anti-Vδ2 TCR (clone B6.1); phycoerythrin (PE)-conjugated CD25, peridinin chlorophyll protein (PerCP)-labelled anti-CD4 and allophycocyanin-labelled anti-CD3. The anti-CD3 mAb clone Hit3a was used for T-cell stimulation. All mAbs and corresponding isotypic controls were purchased from BD Pharmingen, San Diego, CA.

Mycobacterium tuberculosis H37Ra (American Type Culture Collection, Manassas, VA) was cultured in Middlebrook 7H9 with albumin dextrose catalase enrichment and frozen stocks were prepared as described previously.[9] Bacterial counts and viability were performed by light microscopy and by counting colony-forming units on 7H10 medium. Before use in T-cell assays, mycobacteria were washed three times in RPMI-1640, sonicated for 40 seconds, passed multiple times through a 25-gauge needle to disrupt clumps, and diluted in non-heat-inactivated serum-containing media.

BrHPP (Phosphostim) was kindly provided by Christian Belmant, Innate Pharma, Marseille, France.[37] The PPD from *M. tuberculosis* (PPD) was obtained from Wyeth Lederle Vaccines, Pearl River, NY.

Cell culture reagents

Cells were cultured in RPMI-1640 supplemented with 10% pooled human serum (SeraCare Life Sciences, Milford, MA), 20 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, non-essential amino acids, 100 U/ml penicillin and 100 μg/ml streptomycin (all from BioWhittaker, Walkersville, MD) in 96-well U-bottomed plates (Becton Dickinson, Franklin Lakes, NJ).

Isolation of peripheral blood mononuclear cells and monocytes

Peripheral blood mononuclear cells (PBMC) were isolated from 240 cm^3 of blood from six healthy tuberculin-skin-test-positive (TST$^+$) donors (18–45 years old) recruited from among laboratory staff. All protocols were approved by Case Western Reserve University's institutional review board. Informed written consent was obtained from all participants. PBMC were isolated by density gradient centrifugation over sodium diatrizoate/

Hypaque (GE HealthCare, Uppsala, Sweden). For monocyte isolation, PBMC were incubated on plastic tissue culture dishes (Falcon, Becton Dickinson) precoated with pooled human serum for 1 hr at 37°; non-adherent cells were removed, dishes were extensively washed and adherent cells were collected by scraping with a plastic policeman. Monocytes were resuspended in culture media and irradiated with 3000 rads before adding to 96-well plates in the suppression assays.

CD25$^+$ T-cell depletion assay

CD25$^+$ T cells were depleted from PBMC using the MACS CD25 Microbeads magnetic cell sorting kit and LS columns according to the manufacturer's instructions (Miltenyi Biotec, Bergisch Gladbach, Germany). Depletion was confirmed by staining with anti-CD25 mAbs and FACS analysis. Depleted and not depleted PBMC (2×10^6 cells/well) were cultured at 37° in 12-well tissue culture plates with or without H37Ra (2×10^5 CFU/ml). Cells were harvested on day 7 and viable cells were determined using the Trypan blue exclusion method. Baseline (day 1) and day 7 samples were stained with FITC-anti-Vδ2TCR and allophycocyanin-anti-CD3 mAbs. Cells were acquired using a FACSCalibur flow cytometer (BD, San Jose, CA) and analysed with the FLOWJO SOFTWARE (Tree Stop, Stanford University, CA). Ten thousand events were recorded for each cell surface marker. The cut-off lines for positive and negative fluorescence were set manually based on the distribution of cells stained with FITC- and PE-conjugated isotypic controls, and were kept constant within each experiment.

Purification of CD4$^+$ CD25^{high} (Tregs), CD4$^+$ CD25$^-$, Vδ2$^+$ T cells

Non-adherent cells were labelled with the following cocktail of mAbs: FITC-conjugated anti-Vδ2 TCR, PerCP-conjugated anti-CD4 and PE-conjugated-anti-CD25 plus or minus allophycocyanin-conjugated anti-CD3. CD4$^+$ CD25$^-$, CD4$^+$ CD25^{high} and Vδ2$^+$ T cells (purity > 95% for all three groups) were isolated by FACS using a FACSAria flow cytometer (BD Biosciences, San Jose, CA). The analysis and sort gates were restricted to the population of lymphocytes by means of their forward and side scatter properties. Foxp3 staining was performed with the human regulatory T-cell staining kit (eBioscience, San Diego, CA) following the manufacturer's instructions.

In vitro *suppression assay*

To analyse Treg-mediated suppression by polyclonal stimulation, 96-well U-bottom plates (Becton Dickinson) were coated overnight at 4° with anti-CD3 mAbs (10 μg/ml) in 0·1 M Tris–HCl pH 9·5, and washed three times with PBS. The FACS-purified CD4$^+$ CD25$^-$ or Vδ2$^+$ T cells ($2{\cdot}5 \times 10^4$ cells/well) were cultured in the absence or presence of CD4$^+$ CD25^{high} cells at a 1 : 1 ratio. IL-2 (25 U/ml) was added as costimulatory stimulus.

To measure suppression by antigen-specific Tregs, FACS-purified CD4$^+$ CD25$^-$ or Vδ2$^+$ T cells ($2{\cdot}5 \times 10^4$ cells/well) were cultured in 96-well U-bottom plates with or without CD4$^+$ CD25^{high} T cells at a 1 : 1 ratio. Irradiated monocytes ($2{\cdot}5 \times 10^4$ cells/well) were used as APCs. For stimulation of the Vδ2$^+$ T cells, IL-2 (25 U/ml) plus BrHPP (10 μM) was added. For antigenic stimulation of the CD4$^+$ T-cell subsets PPD was added at 10 μg/ml.

Interferon-γ enzyme-linked immunosorbent assay

Plates were incubated at 37° for 5 days and 100 μl of cell culture supernatant was harvested and stored at −20°. Interferon-γ (IFN-γ) was determined in culture supernatants by sandwich enzyme-linked immunosorbent assay with the anti-IFN-γ antibody pairs M-700A and biotinylated M-701B (Endogen, Cambridge, MA).

Statistical analysis

Statistical analyses were performed using the paired Student's *t*-test. P values < 0·05 were considered significant. Results are expressed as means ± SEM.

Results

Depletion of peripheral blood CD25$^+$ T cells increases *M. tuberculosis*-induced Vδ2$^+$ T-cell expansion

To explore the role of Tregs on suppression of γδ T-cell function, we depleted CD25$^+$ T cells from PBMC of TST$^+$ donors (Fig. 1a,b). We then compared the expansion of Vδ2$^+$ T cells in response to *M. tuberculosis* in CD25$^+$ T-cell-depleted and non-depleted PBMC. As shown in Fig. 1(c), Vδ2$^+$ T-cell expansion in response to *M. tuberculosis* was higher in CD25$^+$-depleted compared to non-depleted PBMC ($P < 0{\cdot}01$). After stimulation with *M. tuberculosis*, Vδ2$^+$ T cells expanded from 6705 cells/ml (range 864–12 935 cells/ml) to 66 050 cells/ml (range 15 318–134 160 cells/ml) in PBMC cultures containing CD25$^+$ T cells and from 9795 cells/ml (range 1026–21 158 cells/ml) to 111 700 cells/ml (range 67 200–197 763 cells/ml) in CD25$^+$-depleted cultures ($n = 4$). On average there was a 42% greater increase in Vδ2$^+$ T-cell expansion in cultures depleted of CD25$^+$ T cells compared to non-depleted cultures ($n = 4$, $P < 0{\cdot}01$). Our results suggest that CD25$^+$ T cells are activated by *M. tuberculosis* and suppress the expansion of mycobacteria reactive-Vδ2$^+$ T cells.

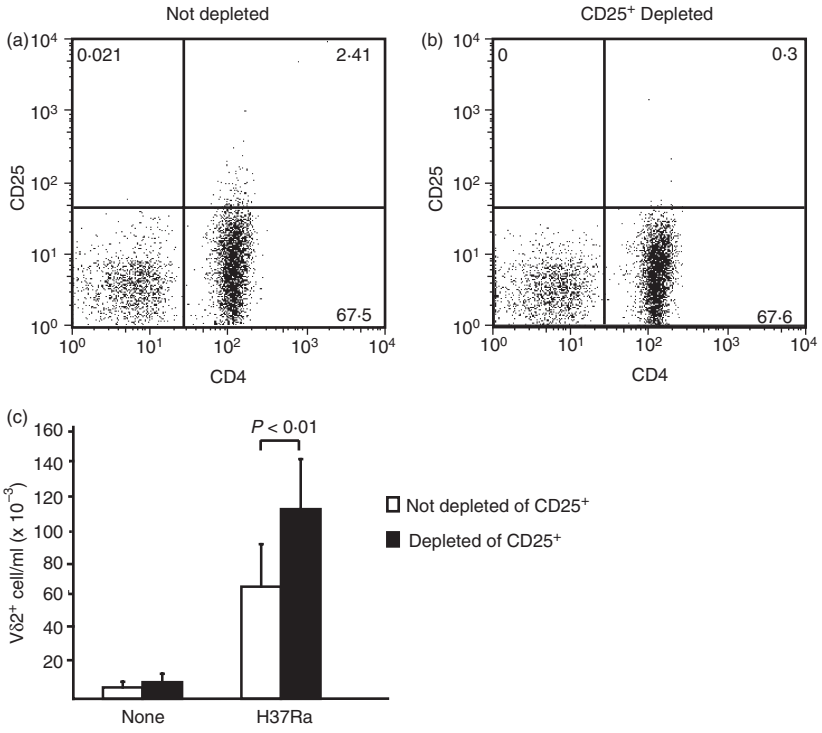

Figure 1. Depletion of $CD25^+$ T cells increases $V\delta2^+$ T-cell expansion in response to *Mycobacterium tuberculosis*. Peripheral blood mononuclear cells (PBMC) isolated from tuberculin-skin-test-positive (TST^+) donors were not depleted (a) or $CD25^+$ depleted with anti-CD25-labelled magnetic beads (b). Not depleted or $CD25^+$-depleted PBMC (2×10^6 cells/well) were stimulated for 7 days with or without whole *M. tuberculosis* (H37Ra, 0.2×10^5 CFU/ml) (c). Total cell numbers were determined by the trypan blue exclusion method and the percentage of $V\delta2^+$ T cells was determined by flow cytometry. Means ± SEM are shown for four experiments.

$CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells suppress anti-CD3-triggered $V\delta2^+$ T-cell responses

The results described above indicate that $CD25^+$ T cells suppress $V\delta2^+$ T-cell expansion in response to *M. tuberculosis*. However, inhibition may be indirect, resulting from suppression of $CD4^+$ T cells and reduced T-cell helper activity in the form of IL-2. In addition, because only $CD4^+$ T cells expressing high levels of CD25 are suppressor cells, depletion of all $CD25^+$ cells decreases non-Treg subsets.[38] As shown in Fig. 2(a), only $CD4^+$ $CD25^{high}$ T cells coexpressed Foxp3 (87%), a specific marker for Tregs. To better characterize the effect of Tregs on $V\delta2^+$ T cells we developed a suppression assay using FACS-purified $CD4^+$ $CD25^{high}$, $CD4^+$ $CD25^-$ and $V\delta2^+$ T cells. The average purity was 96 ± 2·94% for $CD4^+$ $CD25^{high}$, 95 ± 2·28% for $CD4^+$ $CD25^-$ and 96 ± 2·95% for $V\delta2^+$ T cells ($n = 6$).

Upon stimulation with plate-bound anti-CD3 plus IL-2, Tregs suppressed the IFN-γ responses of both $CD4^+$ $CD25^-$ and $V\delta2^+$ T cells (Fig. 3a,b). The magnitude of Treg-mediated inhibition of $V\delta2^+$ T-cell responses (75 ± 12%; range 52–100%) was not significantly different from that of $CD4^+$ $CD25^-$ T-cell responses (57 ± 7%; range 48–71%; $P > 0·05$) (Fig. 3c). As expected, Tregs were anergic when stimulated with anti-CD3 plus IL-2.

Therefore, $CD4^+$ $CD25^{high}$ T cells purified by flow cytometry had the Treg phenotype, i.e. they expressed Foxp3 and were anergic and suppressive upon polyclonal stimulation. Thus $CD4^+$ $CD25^-$ and $V\delta2^+$ T cells were equally susceptible to suppression by polyclonally stimulated Tregs.

Antigen-specific Tregs partially suppress phosphoantigen-triggered IFN-γ secretion by $V\delta2^+$ T cells

In addition to polyclonal stimulation, antigen-specific stimulation can trigger the suppression of $CD4^+$ T cells by Tregs.[39,40] We hypothesized that Tregs can be rendered suppressive by antigen stimulation and inhibit $V\delta2^+$ T-cell function. Taking advantage of the different antigen repertoire of TCR-$\alpha\beta^+$ and TCR-$\gamma\delta^+$ T cells, we developed a suppression assay using PPD as CD4 T-cell antigen and BrHPP plus IL-2 as $V\delta2^+$ T-cell stimulus. As shown before,[39] $CD4^+$ $CD25^-$ T-cell responses to PPD were almost completely abolished in most donors in the presence of Tregs (mean suppression 95 ± 3%, $n = 4$) (Fig. 4a). $V\delta2^+$ T-cell responses to BrHPP plus IL-2 were also significantly inhibited by coincubation with Tregs (mean suppression 55 ± 10%, range 42–73%, $n = 4$) (Fig. 4b). Suppression by Tregs of $V\delta2^+$ T responses to phosphoantigen was significantly less than Treg suppression of $CD4^+$ $CD25^-$ responses to PPD ($P < 0·05$) (Fig. 4c). In summary, our data suggest that PPD triggers antigen-specific Tregs that completely suppress $CD4^+$ $CD25^-$ T-cell responses and partially inhibit phosphoantigen-reactive $V\delta2^+$ T cells.

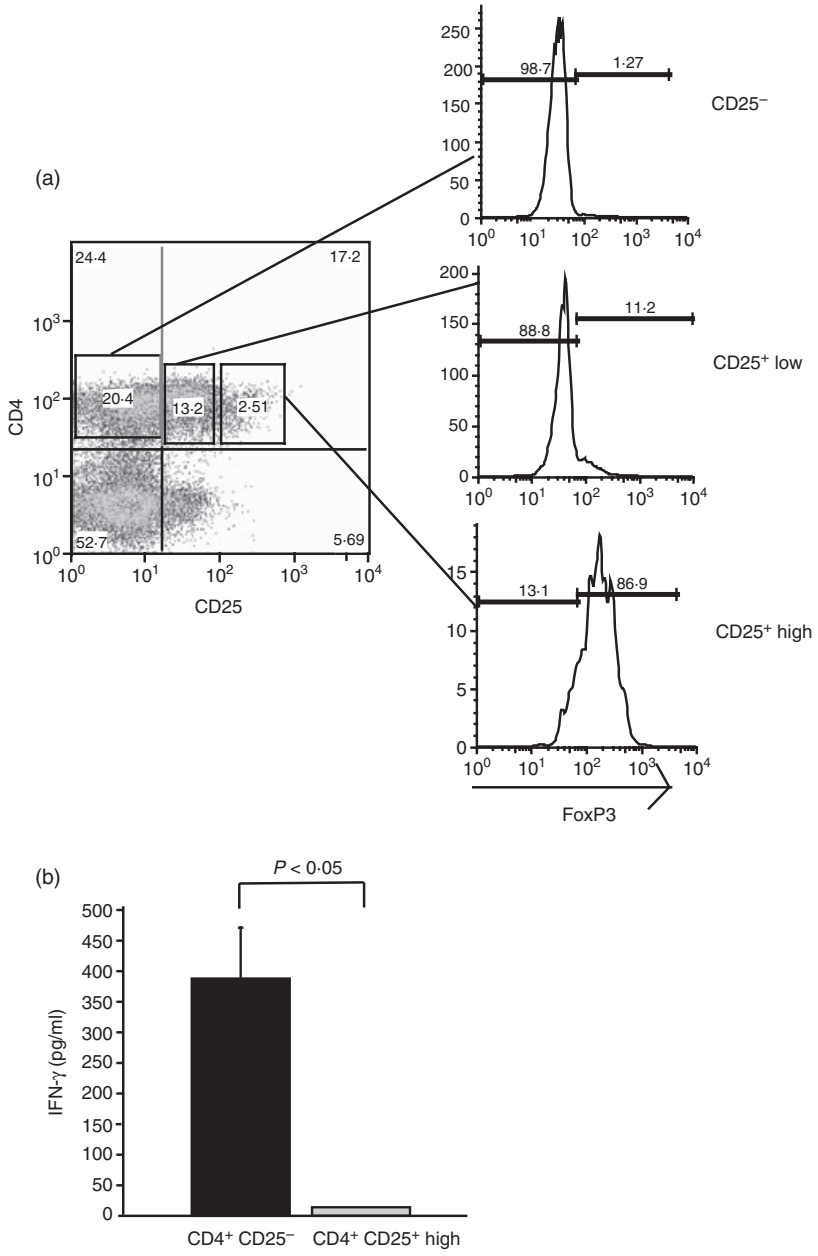

Figure 2. Purification and functional characterization of $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells. (a) Peripheral blood mononuclear cells (PBMC) were labelled with anti-CD4 and anti-CD25 monoclonal antibodies and purified by fluorescence-activated cell sorting based on the level of CD25 expression. $CD25^{high}$ gate was drawn around the events with $> 10^2$ mean fluorescence on the CD25 axis. Foxp3 expression was determined by intracellular staining in the sorted populations. (b) Flow-sorted $CD4^+$ $CD25^-$ and $CD4^+$ $CD25^{high}$ T cells were stimulated with plate-bound anti-CD3 monoclonal antibody plus interleukin-2. Interferon-γ (IFN-γ) was measured by enzyme-linked immunosorbent assay in 5-day culture supernatants. One representative experiment of three is shown.

Treg suppression can be triggered in the absence of TCR stimulation

Tregs have not previously been shown to suppress human effector cells in the absence of TCR stimulation. To determine if TCR triggering of Tregs is an absolute requirement for suppression of Vδ2 T-cell function, we compared suppression in the presence or absence of TCR-αβ stimulus, i.e. PPD. IFN-γ production by $V\delta2^+$ T cells stimulated with BrHPP and IL-2 was partially inhibited when cocultured with Tregs in the absence of PPD ($P < 0{\cdot}05$) (Fig. 5a). Addition of PPD to these cocultures significantly increased Treg-mediated suppression presumably as the result of TCR triggering of Tregs (Fig. 5b) ($P < 0{\cdot}01$). These results suggest that exogenous IL-2 alone can trigger Tregs to suppress $V\delta2^+$ T cells significantly; and that providing Tregs with a TCR stimulus such as PPD increases the suppression induced by IL-2 alone.

Discussion

$V\delta2^+$ T cells complement the protective role of αβ T cells against *M. tuberculosis*. We have previously demonstrated that patients with TB have depressed $V\delta2^+$ T-cell responses to phosphoantigen.[22] The mechanisms of $V\delta2^+$ T-cell-depressed responses during TB are poorly understood. Here we investigated the role of Tregs as

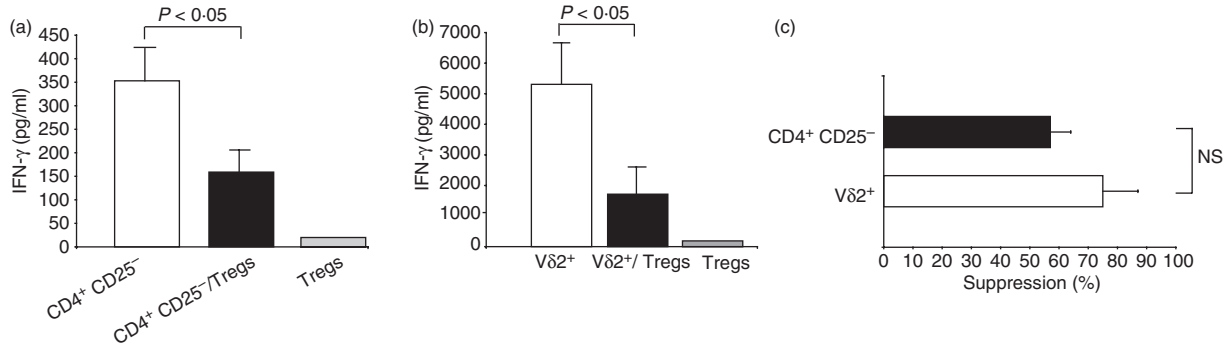

Figure 3. $CD4^+$ $CD25^{high}$ T cells suppress polyclonally stimulated $CD4^+$ $CD25^-$ and $V\delta2^+$ T cells. Fluorescence-activated cell sorted $CD4^+$ $CD25^-$ (a) and $V\delta2^+$ T cells (b) (2.5×10^4 cells/well) were stimulated with plate-bound anti-CD3 monoclonal antibody (10 μg/ml) and interleukin-2 (25 U/ml) in the presence or absence of $CD4^+$ $CD25^{high}$ regulatory T cells (Tregs) in a 1 : 1 effector to suppressor ratio. Tregs were also stimulated without effector cells. Cell culture supernatants were harvested on day 5 and interferon-γ (IFN-γ) was determined by enzyme-linked immunosorbent assay. Shown are mean values ± SEM of three independent experiments. (c) Percentage suppression of IFN-γ production was determined as follows: (IFN-γ in cultures with $CD4^+$ $CD25^{high}$)/(IFN-γ in cultures without $CD4^+$ $CD25^{high}$) × 100. Means ± SEM of three independent experiments are shown.

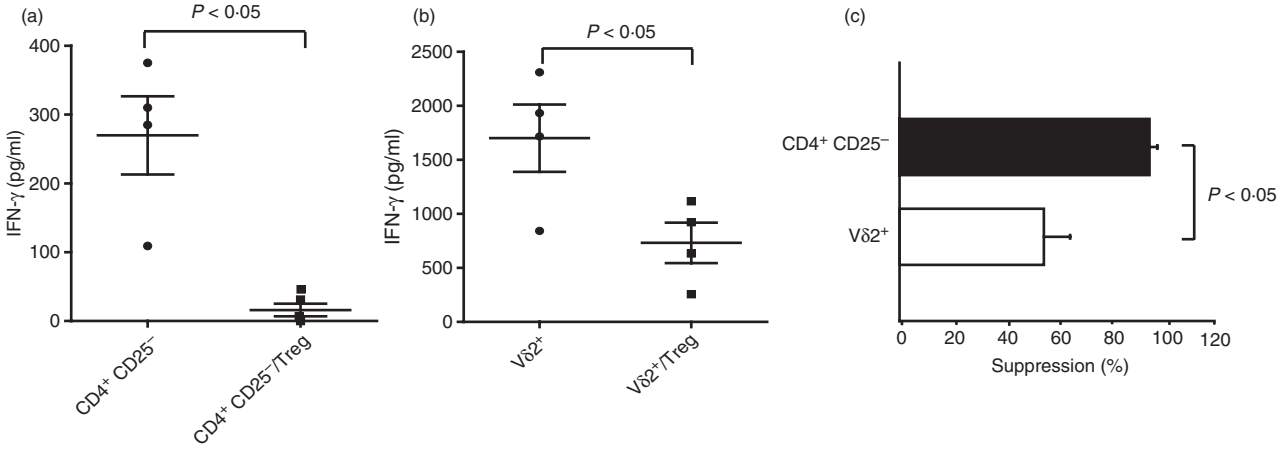

Figure 4. $CD4^+$ $CD25^{high}$ T cells suppress antigen-specific $CD4^+$ $CD25^-$ and $V\delta2^+$ T-cell responses. (a) Fluorescence-activated cell sorting (FACS) sorted $CD4^+$ $CD25^-$ (2.5×10^4 cells) isolated from tuberculin-skin-test-positive (TST^+) donors were stimulated with purified protein derivatve (PPD; 10 μg/ml) in the presence or absence of $CD4^+$ $CD25^{high}$ T cells (Tregs) at a 1 : 1 effector : suppressor ratio. Interferon-γ (IFN-γ) in 5-day culture supernatants was determined by enzyme-linked immunosorbent assay (ELISA). Mean values ± SEM of four independent experiments are shown. (b) FACS-sorted $V\delta2^+$ T cells (2.5×10^4 cells) isolated from TST^+ donors were stimulated with BrHPP (10 μM) plus interleukin-2 (25 U/ml) and PPD (10 μg/ml) in the presence or absence of $CD4^+$ $CD25^{high}$ T cells (Tregs) at 1 : 1 effector : suppressor ratio. IFN-γ in 5-day culture supernatants was determined by ELISA. Mean values ± SEM of four independent experiments are shown. (c) Percentage suppression was determined as follows: (IFN-γ in cultures with $CD4^+$ $CD25^{high}$)/(IFN-γ in cultures without $CD4^+$ $CD25^{high}$) × 100. Means ± SEM are shown for four independent experiments.

suppressors of $V\delta2^+$ T cells. We made use of the different antigenic repertoire of $V\delta2^+$and $CD4^+$ αβ T cells to elucidate if mycobacterial antigens can trigger Treg suppression.

We demonstrated that $CD4^+$ $CD25^{high}$ $Foxp3^+$ T cells isolated from human blood suppressed IFN-γ secretion by both $CD4^+$ and $V\delta2^+$ T cells. While previous studies have shown Treg suppression of a variety of cell targets including $CD4^+$ and $CD8^+$ T cells, monocytes and dendritic cells, this is the first report of Tregs suppressing $V\delta2^+$ T cells.[38–44]

Mycobacterium tuberculosis induces both $CD4^+$ and $V\delta2^+$ T-cell responses in PBMC isolated from TST^+ individuals. Our observation that depletion of $CD4^+$ $CD25^+$ T cells increased $V\delta2^+$ T-cell expansion in response to *M. tuberculosis* supports the idea that Tregs are triggered in an antigen-specific manner. Elegant studies by Suffia *et al.* have demonstrated that nTregs not only recognize

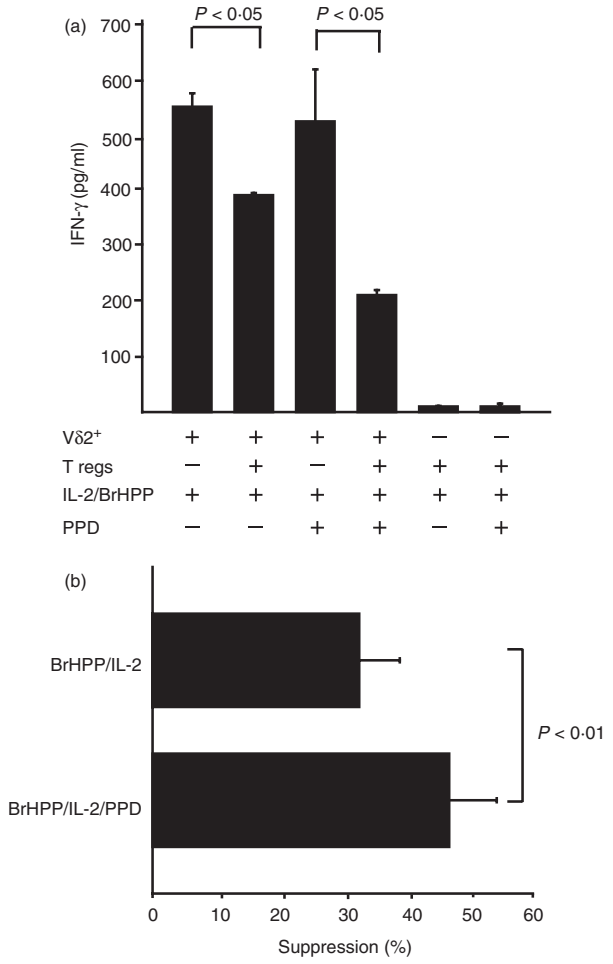

Figure 5. $CD4^+$ $CD25^{high}$ T cells can suppress $V\delta2^+$ T cells in the absence of TCR-αβ triggering. (a) Fluorescence-activated cell-sorted $V\delta2^+$ T cells (2.5×10^4 cells) isolated from tuberculin-skin-test-positive donors were stimulated with BrHPP (10 μM) plus interleukin-2 (IL-2; 25 U/ml) with or without purified protein derivative (PPD; 10 μg/ml) in the presence or absence of $CD4^+$ $CD25^{high}$ T cells (Tregs) at 1 : 1 effector : suppressor ratio. Interferon-γ (IFN-γ) in 5-day culture supernatants was determined by enzyme-linked immunosorbent assay. Mean values ± SEM are shown of triplicates of one representative experiment. (b) Percentage suppression was determined as follows: (IFN-γ in cultures with $CD4^+$ $CD25^{high}$)/(IFN-γ in cultures without $CD4^+$ $CD25^{high}$) × 100. Means ± SEM of seven independent experiments are shown.

self-antigens but also respond to foreign antigens.[45] In this context, our results suggest *M. tuberculosis* antigens are recognized by and activate Tregs, which in turn suppress $V\delta2^+$ expansion.

Because $V\delta2^+$ T-cell activation is known to depend on IL-2 secreted by $CD4^+$ T cells,[46] decreased $V\delta2^+$ T-cell expansion in the presence of $CD25^+$ T cells could be secondary to the suppression of $CD4^+$ T-cell helper activity. Therefore we developed a system to directly study the effect of Tregs on $V\delta2^+$ T cells independent of fluctuations in $CD4^+$ T-cell help or IL-2. In addition, we focused on $CD4^+$ $CD25^{high}$ $Foxp3^+$ Tregs as this population has been shown to exhibit the true suppressor phenotype. In our experimental model Tregs suppressed polyclonal responses to anti-CD3 mAb plus IL-2 in both CD4 and $V\delta2^+$ T cells. As described before for suppression of $CD4^+$ $CD25^-$ T cells, suppression of $V\delta2^+$ T cells by Tregs was dependent on cell–cell contact (data not shown).[38,42,47] Even though $V\delta2^+$ T cells secreted 10 times more IFN-γ than $CD4^+$ T cells, both T-cell subsets were equally susceptible to inhibition by Tregs. Therefore Tregs can downregulate potent IFN-γ responses by $V\delta2^+$ T cells.

It has been recently proposed that Tregs function in the immune system beyond control of self-reactive T cells. Tregs may be critical in regulating the responses to microbial infections by limiting the collateral damage caused by excessive inflammation. On the other hand, Tregs could represent a mechanism for pathogens to evade the immune response. Two pieces of evidence point to a role of Tregs in human TB: first, TB patients have higher numbers of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells both in peripheral blood and at sites of infection;[31–33] and second, Tregs expand *in vitro* in response to whole *M. tuberculosis* bacilli or mycobacterial ManLAM (Hirsch *et al.*, unpublished data).[48] Our results demonstrate that mycobacterial antigen-reactive Tregs suppress antigen-specific $V\delta2^+$ T-cell responses and this may explain $V\delta2^+$ T-cell deficits in TB. Therefore activation of Tregs by *M. tuberculosis* may represent a mechanism to suppress a broad range of protective T-cell responses, including $V\delta2^+$ T cells, in *M. tuberculosis* infection.

While both $V\delta2^+$ and $CD4^+$ $CD25^-$ polyclonal responses were equally downregulated by Tregs, antigen-specific $V\delta2^+$ T-cell responses were less susceptible to inhibition than antigen-specific $CD4^+$ T-cell responses. There are at last three possible explanations for this difference. First, phosphoantigen-stimulated $V\delta2^+$ T cells may be intrinsically more resistant to suppression by Tregs than mycobacterial peptide-reactive $CD4^+$ $CD25^-$ T cells. Second, in the case of antigen-specific stimulation, only a few clones among the $CD4^+$ $CD25^-$ T cells recognize PPD antigens. On the other hand, because of the restricted antigenic repertoire of $V\delta2^+$ T cells, a large proportion will respond to phosphoantigens. Therefore the effective ratio of Tregs to antigen-specific $CD4^+$ $CD25^-$ T cells is higher than the ratio of Tregs to antigen-specific $V\delta2^+$ T cells. This allows better suppressor cell to effector cell contact and suppression of the $CD4^+$ $CD25^-$ T cells compared to the $V\delta2^+$ T cells. Third, as reported by Misra *et al.*[49] and Taams *et al.*,[50] Tregs downregulate APC function and, as a consequence, they may decrease the processing and presentation of peptide antigen to $CD4^+$ T cells. Although $V\delta2^+$ T-cell responses are higher in the presence of APC,[51] phosphoantigen recognition by the $V\delta2^+$ TCR is

independent of processing and presentation by APC. Therefore inhibited APC function would have a greater impact on CD4$^+$ T-cell responses than on Vδ2$^+$ T-cell responses.

Activation through the TCR is thought to be an absolute requirement for Treg function. Most studies of Tregs have been performed using polyclonal stimuli such as anti-CD3 or allogeneic APC, with antigen-specific activation with tetanus toxoid, HSP65, PPD or leishmania antigens.[25,39] In these experiments Tregs and the cells they affect are stimulated at the same time. Therefore, it is difficult to separate TCR stimulation of Tregs from that of the cells being suppressed. Dieckmann *et al.* overcame these limitations by using Tregs and APC from syngeneic and allogeneic donors and provided evidence that stimulation of human Tregs through the TCR induces suppression of CD4$^+$ T cells.[42] However, these studies do not rule out the possibility that suppression could be triggered without activation through the TCR, by cytokines for example. Our experimental system allowed concomitant stimulation of effector and suppressor cells with different antigens, and suggested that some Tregs can be suppressed in the absence of αβ TCR stimulation. We hypothesize that IL-2 may activate TCR-independent suppression.

While we have not directly investigated the mechanism of suppression of Vδ2$^+$ T cells by Tregs, previous reports have demonstrated that CD4$^+$ CD25$^+$ Foxp3$^+$ Treg suppression of human CD4$^+$ T cells is contact dependent and independent of IL-10 or surface molecules such as cytotoxic T-lymphocyte antigen-4, programmed death-1 ligand and glucocorticoid-induced tumour necrosis factor receptor.[52] Therefore it is likely that suppression of Vδ2$^+$ T cells responses by Tregs will use a similar mechanism. In addition, mouse nTregs isolated from lymph node or spleen suppress mouse, δ T cells in a contact-dependent manner and independently of IL-10 or transforming growth factor-β (Silva-Santos B. M., personal communication).

In summary, our study represents the first report on the suppression of Vδ2$^+$ T-cell function by circulating CD4$^+$ CD25^{high} Foxp3$^+$ T cells. In addition, we confirmed that mycobacterial antigen-reactive suppressor cells are part of the CD4$^+$ CD25^{high} Foxp3$^+$ T-cell repertoire in human peripheral blood.

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