Circulating mycobacterial-reactive CD4+ T cells with an immunosuppressive phenotype are higher in active tuberculosis than latent tuberculosis infection

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Abstract

Background

Previous studies suggest that control of *Mycobacterium tuberculosis* infection is compromised by the activity of regulatory T cells, including those that express CD39, an ectonucleotidase with immunosuppressive properties. Here, we examine the role of CD39 on CD4⁺ T cells reacting to *M. tuberculosis* antigens.

Methods

Cryopreserved PBMC from patients with active TB (n=31) or individuals with LTBI (n=30) were cultured with PPD, ESAT-6 or CFP-10 and antigen-reactive CD4⁺ T cells assessed by: A) intracellular expression of interferon-gamma (IFN- γ), tumour necrosis factor alpha (TNF- α) and interleukin (IL)-2, B) co-expression of CD25 and CD134 with or without CD39, and C) production of IFN- γ , TNF- α and IL-10 in culture supernatants.

Results

Active TB patients were not differentiated from individuals with LTBI by intracellular expression of IFN- γ , TNF- α or IL-2 (alone or together), nor by co-expression of CD25 and CD134. However, active TB patients exhibited higher proportions of CD25⁺,CD134⁺ CD4⁺ T cells expressing CD39 in response to all antigens ($p \leq 0.022$). Furthermore, in response to PPD, CD39 expression on CD25⁺,CD134⁺ CD4⁺ T cells correlated with IL-10 production ($r=0.41$, $p=0.005$) and inhibition of CD39 decreased IL-10 production.

Conclusions

Antigen-reactive CD4⁺ T cells expressing CD39 are more abundant in active TB than LTBI and are associated with production of the immunosuppressive cytokine IL-10. Modulating the effects of CD39 might enhance cellular immune responses against *M. tuberculosis*.

Keywords: Tuberculosis, antigen-reactive T cells, CD39, IL-10

Introduction

Mycobacterium tuberculosis infection affects approximately 2 billion people (about one third of the world's population), the majority of whom achieve immune control of the infection resulting in latent tuberculosis infection (LTBI) [1]. Both innate and adaptive immune responses mediated by CD4⁺ and CD8⁺ T cells, CD1-restricted T cells, B cells, macrophages, neutrophils, fibroblasts and multinucleated giant cells contribute to the formation of granulomas and an environment that contains the infection [2]. Within that cellular environment, multiple chemokines, cytokines, including tumour necrosis factor alpha (TNF- α) and interferon-gamma (IFN- γ), and other cellular mediators play critical roles in that process. Reactivation of *M. tuberculosis* infection results from increased mycobacterial virulence and/or loss of immune control, which leads to the development of active tuberculosis (TB). Failure of immune control mechanisms within granulomas may result from CD4⁺ T cell depletion caused by HIV infection [3], therapeutic inhibition of TNF- α activity [4], immunosuppressant and corticosteroid therapy or other causes of local or systemic impairment of immunocompetence including smoking, aging, diabetes, renal failure and malnutrition [5]. However, the reason for loss of immune control of *M. tuberculosis* infection is often unclear.

Comparison of cellular immune responses against *M. tuberculosis* antigens in patients with active TB and individuals with LTBI has been undertaken to try and define immune mechanisms associated with control of *M. tuberculosis* infection. Proportions of T cells producing IFN- γ in response to RD1 antigens in ELISpot assays are higher in active TB cases than contacts with LTBI [6], suggesting that production of IFN- γ by T cells is not a correlate of protective immune responses against *M. tuberculosis*. Studies of T cells producing multiple cytokines in response to *M. tuberculosis* antigens using polychromatic flow cytometry have demonstrated higher proportions of CD8⁺ T cells producing IFN- γ and interleukin (IL)-2 in individuals with LTBI [7], but higher proportions of CD4⁺ T cells producing a dominant TNF- α only response in patients with active TB [8, 9].

Active TB is also associated with increased proportions of regulatory T (Treg) cells in blood and at sites of *M. tuberculosis* infection [10, 11], including a subset of cells that express the ectoenzyme CD39 [12]. However, the role of Treg cells in the control of *M. tuberculosis* infection is unclear. In murine models of TB, removal of Treg cells (CD4⁺,CD25⁺,FoxP3⁺) decreased mycobacterial burden [13], suggesting that Treg cells

have a detrimental effect, perhaps by suppressing effector T cell responses. In humans, it has been reported that active TB is associated with increased proportions of circulating Treg cells [10, 11] and that CD39⁺ Treg cells suppress Th17 cell production in the pleural compartment leading to an imbalance of Th17 and Treg cells [14].

Human Treg cells have been defined in multiple ways, including the immunophenotypes CD4⁺,CD127^{lo},FoxP3⁺, CD4⁺,CD25^{hi},CD127^{lo} or CD4⁺,CD25^{hi},FoxP3⁺, production of IL-10 and TGF- β , or expression of co-inhibitory molecules such CTLA-4 [15]. Recently, it has been reported that a subset of Treg cells express the ectoenzyme CD39 [12] and that CD39⁺ T cells exhibit an immunosuppressive Treg phenotype and function [16]. CD39 is a member of the ectonucleotidase triphosphate diphosphohydrolase (ENTPDase) family that, in concert with CD73, hydrolyses ATP and ADP to AMP and adenosine, which exert an anti-inflammatory and immunosuppressive effect [17-19]. This effect is, in part, mediated by inhibiting the migration of T cells and their expression of CD28 and production of IL-2 and IFN- γ particularly in older individuals [20, 21]. However, the effect of ENTPDases on T cell function is complex because, while the products of ATP and ADP hydrolysis impair T cell function, inhibition of ENTPDases may impair T cell activation and production of IFN- γ and IL-2 [22]. Although CD39 is an important Treg cell effector molecule [23], several observations suggest that its immunosuppressive effects are not restricted to Treg cells. Firstly, CD39 is also expressed by some FoxP3⁻ memory T cells at sites of inflammation [24]. Secondly, the suppressive effect of regulatory CD8⁺ T cells on proliferation of mycobacterial-specific CD4⁺ T cells is partly mediated by CD39 [25]. Finally, CD39 has recently been shown to regulate the suppressive effect of AMP on *M. tuberculosis*-infected macrophages [26].

Adenosine is deaminated by adenosine deaminase (ADA), which is produced at sites of active TB. An increased level of ADA is a relatively sensitive and specific marker of tuberculosis in pleural fluid and, to lesser extent, pericardial fluid and cerebrospinal fluid [27, 28].

In this study, we have used a novel assay of antigen-reactive T cells to examine the frequency of CD4⁺ T cells activated by *M. tuberculosis* antigens (CD25⁺,CD134⁺) [29, 30], as well as the proportion of those cells that express CD39 [16], in patients with active TB or individuals with LTBI. In addition, we have examined the production of pro-inflammatory (IFN- γ , TNF- α and IL-2) and regulatory (IL-10) cytokines in T cells or

cultures of peripheral blood mononuclear cells (PBMCs) and the relationship of cytokine production with antigen-reactive T cells, including the CD39⁺ subpopulation. We observed higher proportions of mycobacterial-reactive CD4⁺ T cells expressing CD39 in active TB patients. Additionally, expression of CD39 on mycobacterial-reactive CD4⁺ T cells was associated with higher production of IL-10 in PBMC cultures.

Materials and Methods

Study participants

Adults (over 18 years of age) with active TB were enrolled into the study at the clinic of the Western Australian Tuberculosis Control Program. Individuals with active TB had culture positive drug-susceptible pulmonary or extrapulmonary TB and subsequently completed treatment with rifampicin and isoniazid for 6 months together with pyrazinamide and ethambutol during the first 2 months. Individuals with LTBI had a positive whole blood IFN- γ release assay (IGRA) or a tuberculin skin test (TST) reaction >10mm at screening and a positive whole blood IGRA at study entry with no clinical or radiological evidence of active TB. Exclusion criteria were HIV infection, immunosuppressive medication, relapse of TB and previous history of anti-TB treatment or multi-drug resistant TB. Demographic characteristics of the study participants are provided in Table 1. Extra-pulmonary TB was present in 26% of participants with active TB. All study participants provided informed consent and the study was approved by the Human Research Ethics Committees of Royal Perth Hospital and the University of Western Australia.

Antigens

Stimulations were performed with *M. tuberculosis*-derived recombinant proteins of ESAT-6 (Statens Serum Institute, Denmark) and CFP10 (Genway Biotech, USA) and with PPD produced from culture filtrates of *M. tuberculosis* (Statens Serum Institute, Denmark). CFP10 had a purity of >98% by SDS-PAGE and electrophoresis. ESAT-6 is a recombinant dimer of the antigen ESAT-6 from *M. tuberculosis* and consists of 196 amino acids with a molecular weight of 20.5 daltons.

Flow cytometry

Intracellular flow cytometry was used to measure cytokine producing CD4⁺ T cells. Briefly, cryopreserved PBMC (5×10^5 cells) were cultured with costimulatory antibodies

CD28 and CD49d (0.5µg/mL, BD Biosciences, USA) in the presence of no stimulus (control), mitogen (SEB 1µg/mL, Sigma-Aldrich USA), or *M. tuberculosis* antigens (10µg/mL of PPD, ESAT-6 or CFP-10) for 2 hours at 37°C in 5% CO₂ followed by an additional 4 hours with Brefeldin-A (1µg/mL, BD Biosciences, USA). A concentration of 10µg/mL for each of the *M. tuberculosis* antigens was selected following preliminary titration experiments. At the end of stimulations, PBMC were stained with surface markers: CD3-APC-H7 (clone SK7) and CD4-PerCP-Cy5.5 (SK3), permeabilised using Cytotfix/Cytoperm reagents, (BD Biosciences, USA) and stained with intracellular markers: IFN-γ-PE-Cy7 (B27), TNF-α-PE (MAb11) and IL-2-APC (5344.111), (BD Biosciences, USA) according to the manufacturer's protocol.

The frequency of antigen-reactive CD4⁺ T cells in PBMC samples was determined by enumerating CD25⁺,CD134⁺ CD4⁺ T cells after culture of PBMC (5 x 10⁵ cells) with *M. tuberculosis* antigens or with mitogen (concentrations as described above) for 24 hours [29, 30]. The subset of antigen-reactive (CD25⁺,CD134⁺) CD4⁺ T cells co-expressing CD39 was also determined. At the end of stimulation, PBMC were stained with surface markers: CD3-APC-H7, CD4-PerCP-Cy5.5, CD134-PE (ACT35), CD25-APC (M-A251) and CD39-FITC (TU66) (BD Biosciences,USA). For these 24 hour cultures, staining with 4',6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Invitrogen, Carlsbad, CA, USA) was used to exclude dead cells as described previously [30].

After staining, PBMC were resuspended with 200µL of 1% bovine serum albumin in phosphate buffered saline (BSA/PBS) and acquired using a BD FACSCanto™ II flow cytometer (BD Biosciences, USA). The acquisition stopping gate was set at 100,000 CD4⁺ T cell events. Analysis was done using FlowJo software (v7.6, Treestar, USA). For the analysis of cytokine producing CD4⁺ T cells, Boolean gating was performed using FlowJo in order to identify each subset of single, double and triple cytokine producing CD4⁺ T cells. Representative staining of antigen-reactive (CD25⁺,CD134⁺) CD4⁺ T cells expressing CD39 and cytokine producing CD4⁺ T cells are presented in Supplementary Figures 1 and 2, respectively.

Analysis of the effect of CD39 inhibition in antigen-stimulated PBMC cultures

To determine what effect CD39 inhibition had on PPD-stimulated IL-10 production, PBMC from individuals with active TB or LTBI with the highest IL-10 production in PBMC cultures stimulated with PPD (n=10), were cultured again with PPD or SEB with or without the

addition of the CD39 inhibitor ARL67156 (Sigma-Aldrich, St Louis, MO, USA) and both IL-10 and IFN- γ were assayed in culture supernatants by ELISA. ARL67156 was used at a concentration of 200 μ g/mL, determined through analysis of dose response curves (Supplementary Figure 3).

Cytokine ELISA assay

Culture supernatants were collected from PBMC cultures with antigens to measure the concentrations of cytokines by ELISA. BD OptEIA™ Sets (BD Biosciences, USA) were used for IFN- γ , TNF- α and IL-10. The manufacturer's protocol was modified for assays of IFN- γ , TNF- α and IL-10 to use 1% BSA/PBS as diluent instead of 10% foetal calf serum/PBS, as suggested by the manufacturer.

Whole blood interferon-gamma release assays

Whole blood IGRAs (QuantiFERON-TB Gold in tube assay, Cellestis, Melbourne, Australia) were undertaken according to the manufacturer's instructions in the laboratory of PathWest, Laboratory Medicine WA.

Statistical analysis

All statistical analyses were performed using Prism 5 (GraphPad Software, La Jolla, CA). Non-parametric tests were conducted to determine statistical significances. Mann-Whitney tests were used to compare results between active TB and LTBI participants. The Wilcoxon matched-pair signed rank test was used for analysis of the CD39 blocking experiments. Spearman's non-parametric correlation tests were used to calculate correlation coefficients. P values <0.05 were considered to be statistically significant.

RESULTS

Active TB is associated with higher proportions of mycobacterial-reactive CD4+ T cells expressing CD39

To determine if the characteristics of T cells reactive with mycobacterial antigens differed between patients with active TB and individuals with LTBI, we cultured PBMC with PPD, ESAT-6 or CFP10 and examined CD4+ T cells for intracellular expression of IFN- γ , TNF- α or IL-2 after 6 hours, and for surface expression of CD25, CD134 and CD39 after 24 hours, by flow cytometry. The proportion of antigen-reactive CD4+ T cells (CD25+,CD134+) activated by PPD, ESAT-6 or CFP10 did not differ between patients

with active TB or individuals with LTBI (Figure 1A-C). However, in response to all three antigens, the proportion of antigen-reactive CD4⁺ T cells expressing CD39 was higher in patients with active TB than in individuals with LTBI (Figure 1D-F). In contrast, intracellular expression of IFN- γ , TNF- α or IL-2 alone or in combination (i.e. polyfunctional T cells) did not differ between patients with active TB or individuals with LTBI (Supplementary Table 1) with the exception of IFN- γ ⁻ IL-2⁺ TNF- α ⁺ cells in response to ESAT-6, which were higher in active TB patients ($p=0.04$). Proportions of activated CD4⁺ T cells (CD25⁺,CD134⁺) expressing CD39 after incubation with PPD, ESAT-6 or CFP10 were not significantly different between patients with pulmonary or extra-pulmonary TB (data not shown).

Active TB is associated with higher production of TNF- α and IL-10 than IFN- γ in antigen-stimulated PBMC cultures

We also examined the production of IFN- γ , TNF- α and IL-10 by PBMC cultured with PPD, ESAT-6 or CFP10 in patients with active TB and individuals with LTBI. There was a general trend towards lower production of IFN- γ and higher production of TNF- α in patients with active TB when compared to individuals with LTBI (Figure 2A, B), and this became more apparent when TNF- α :IFN- γ ratios were compared between these groups ($p=0.003$ for PPD, 0.03 for ESAT-6 and <0.001 for CFP-10) (Figure 2C). There was no difference in IL-10 production between active TB patients and individuals with LTBI in response to all antigens (data not shown). However, patients with active TB had higher IL-10/IFN- γ ratios than individuals with LTBI when PBMC were cultured with PPD and ESAT-6 ($p= 0.003$ and 0.017, respectively) but not with CFP-10 (Figure 2D). The IL10:TNF α ratio did not differ between groups when PBMC were cultured with PPD ($p= 0.67$), ESAT-6 ($p= 0.65$) or CFP-10 ($p= 0.79$).

Expression of CD39 on mycobacterial-reactive CD4⁺ T cells was associated with higher production of IL-10 in PBMC cultures

To examine the relationship between the phenotype of mycobacterial-reactive CD4⁺ T cells and production of pro-inflammatory or anti-inflammatory cytokines, we correlated the proportion of antigen-reactive CD4⁺ T cells (CD25⁺,CD134⁺) with the proportion of IFN- γ ⁺, IL-2⁺ and/or TNF- α ⁺ CD4⁺ T cells, or levels of IFN- γ , TNF- α or IL-10 in supernatants of PBMC cultured with mycobacterial antigens. The proportion of PPD-reactive CD4⁺ T cells (CD25⁺,CD134⁺) correlated with the proportion of IFN- γ ⁺ IL-2⁺ TNF- α ⁺ ($r=0.54$ $p<0.0001$, Figure 3A), IFN- γ ⁺ IL-2⁻ TNF- α ⁺ ($r=0.63$ $p<0.0001$, Figure 3B), IFN- γ ⁻ IL-2⁺ TNF-

α^+ ($r=0.43$, $p=0.0007$, Figure 3C) and IFN- γ ^{+ IL-2⁻ TNF- α ^{+ ($r=0.35$ $p=0.007$, Figure 3D) CD4⁺ T cells as well as the levels of PPD-induced IFN- γ ($r=0.49$ $p=0.0002$, Figure 3G) and TNF- α ($r=0.45$ $p=0.0003$, Figure 3H) in PBMC cultures. In contrast, levels of IL-10 in PPD-stimulated PBMC culture supernatants only correlated with the proportion of PPD-reactive CD4⁺ T cells (CD25⁺,CD134⁺) that co-expressed CD39 ($r=0.41$ $p=0.005$, Figure 3I). While all correlations were weak ($r<0.63$), it was notable that IL-10 production only correlated with the proportion of PPD-reactive CD4⁺ T cells that co-expressed CD39. The proportion of CFP-10 reactive CD4⁺ T cells (CD25⁺,CD134⁺) correlated with the proportion of IFN- γ ⁺ IL-2⁺ TNF- α ⁺ ($r=0.36$ $p=0.004$, Figure 3E) and IFN- γ ⁺ IL-2⁻ TNF- α ⁺ ($r=0.38$ $p=0.003$, Figure 3F) CD4⁺ T cells stimulated by CFP-10. All other cytokine responses measured by intracellular flow cytometry or by ELISA in PBMC cultures did not correlate with proportions of antigen- reactive T cells after culture with ESAT-6 or CFP-10 (data not shown).}}

CD39 inhibition decreased IL-10 production in PPD-stimulated PBMC cultures

To further investigate the positive correlation between PPD-induced CD39 expression on CD25⁺,CD134⁺ CD4⁺ T cells and IL-10 production in PBMC cultures, we assessed what effect inhibition of CD39 by ARL67156 had on PPD-induced IL-10 production. ARL67156 was chosen as a CD39 inhibitor because others have found it to inhibit CD39 activity on human cells and exhibit greater inhibitory activity than antibodies to CD39 [25, 31]. Production of IL-10 was inhibited by ARL67156 in cultures of PBMC stimulated with both PPD and SEB (Figure 4A,B). We also observed that ARL67156 inhibited IFN- γ production (Figure 4C,D), as would be expected from previous observations that ENTPDase inhibitors impair T cell activation and production of IFN- γ [22].

Negative whole blood IGRA results in patients with active TB were not associated with higher CD39⁺ mycobacterial-reactive CD4⁺ T cells

Similar to previously reported studies [32-34], we demonstrated that 9 out of 31 patients with active TB had a negative whole blood IGRA result. To determine if a negative IGRA result might reflect an effect of mycobacterial-reactive T cells with an immunosuppressive phenotype, we compared the proportions of CD39⁺,CD25⁺,CD134⁺ CD4⁺ T cells in active TB patients with and without a positive whole blood IGRA result. As shown in Figure 4, the proportion of CD39⁺,CD25⁺,CD134⁺ CD4⁺ T cells reactive with ESAT-6 was higher in patients with a negative IGRA but the difference was not statistically significant, possibly because of small participant numbers. In addition, we did not

demonstrate a difference in antigen-induced IL-10 production between patients with and without a positive whole blood IGRA result ($p > 0.46$ for all comparisons; data not shown).

DISCUSSION

We compared circulating CD4⁺ T cell responses to *M. tuberculosis* antigens in patients with active TB and individuals with LTBI and demonstrated that, while the frequency of antigen-reactive CD4⁺ T cells did not differ between the groups, patients with active TB had a higher proportion of antigen-reactive CD4⁺ T cells that expressed CD39. Furthermore, we demonstrated that the proportion of antigen-reactive CD4⁺ T cells correlated with the production of the pro-inflammatory cytokines IFN- γ and TNF- α in antigen-stimulated CD4⁺ T cells and by antigen-stimulated PBMC cultures. Of note, IL-10 production in PPD-stimulated PBMC cultures only correlated with CD39 expression on antigen-reactive CD4⁺ T cells and inhibition of CD39 decreased IL-10 production. We did not address the issue of whether the antigen-reactive CD25⁺,CD134⁺ CD4⁺ T cells expressing CD39 exhibited characteristics of Treg cells, such as FoxP3 expression, as our intention was to study the role of CD39, which is expressed by several cell-types other than Treg cells [24-26].

CD39, in concert with CD73, hydrolyses the conversion of ATP and ADP to AMP and adenosine in response to inflammation in tissue microenvironments [35]. Adenosine increases production of IL-10 from various cells including macrophages, monocytes, microglial cells, dendritic cells and CD1-restricted T cells [36-40]. The correlation between levels of IL-10 in PPD-stimulated PBMC cultures and the proportion of PPD-activated CD4⁺ T cells (CD25⁺,CD134⁺) that co-expressed CD39 in this study may therefore reflect increased production of adenosine induced by CD39. The inhibitory effect of ARL67156 on PPD-stimulated IL-10 production provides further support for this proposal. While we did not assess adenosine production in the PBMC cultures, increased production of ADA at sites of TB [27, 28] is likely to be marker of increased adenosine activity.

Increased production of IL-10 has been associated with suppression of protective immune responses against *M. tuberculosis* (reviewed in [41]) by various mechanisms, including, suppression of Th1 and Th17 immune responses [42, 43], inhibition of antigen

processing and presentation [44], skewing of monocyte/macrophage responses from apoptosis to necrosis [45] and inhibition of granuloma formation [46]. Our findings suggest that CD39 expression by T cells reacting to *M. tuberculosis* antigens may be a cause of increased IL-10 production.

The CD39/Adenosine pathway was also shown to exert an immunosuppressive effect in HIV infection, as indicated by inhibition of T cell proliferation [47]. This effect was mediated via A2A receptors, which are expressed by monocytes and macrophages and exert an inhibitory effect on phagocytic function and influence the balance between Th1 and Th2 immune responses [48].

To our knowledge, this is the first publication demonstrating that increased CD39 expression on *M. tuberculosis*-specific CD4⁺ T cells differentiates patients with active TB from individuals with LTBI. The cause and consequences of the increased CD39 expression are currently unclear. On the one hand, increased CD39 expression might be part of the increased Treg cell response that has been described in patients with TB [10-13]. We did not examine ex vivo CD25⁺,CD127^{lo} or CD25⁺,CD39⁺ Tregs in PBMC from patients in this study. On the other hand, it might be a response to the immune activation associated with active TB and have a beneficial effect by suppressing inflammation. In either case, increased CD39 expression on antigen-reactive T cells might suppress protective cell-mediated immune responses against *M. tuberculosis* in an analogous manner to the suppression of effector CD8⁺ T cell responses by CD39⁺ Treg cells in patients with HIV infection [47]. If so, investigation of the effect of therapies that block the adenosine receptor [49], as a means of modulating the immunosuppressive effects of mycobacterial-reactive T cells, might be undertaken in patients with active TB.

Unlike Harrari *et al.* [8], we did not demonstrate that patients with active TB had an increased proportion of antigen-reactive CD4⁺ T cells expressing intracellular TNF- α , either alone or with IFN- γ and/or IL-2, compared with individuals with LTBI. We did, however, demonstrate higher production of TNF- α and higher TNF- α :IFN- γ ratios in the supernatants of antigen-stimulated PBMC cultures of patients with active TB. The difference in the findings of the two studies might reflect methodological differences. In contrast to Harari *et al.*, cryopreserved PBMC were not rested overnight in our study. Resting PBMC before use enhances the sensitivity of T-cell assays through depletion of dead cells but PBMC may be lost [50]. Another difference between the study of Harari *et*

al [8] and ours was that we included 31 active TB patients compared to 8 active TB patients in their study.

In summary, our data indicate that active TB patients have a higher proportion of circulating antigen-reactive CD4⁺ T cells expressing CD39 than individuals with LTBI and that CD39 expression on PPD-reactive CD4⁺ T cells was associated with higher production of the immunoregulatory cytokine IL-10 by PBMC. We hypothesise that increased expression of CD39 on CD4⁺ T cells responding to *M. tuberculosis* antigens increases IL-10 production and that this suppresses 'protective' T cell responses against *M. tuberculosis* antigens. This hypothesis should be tested in longitudinal studies of individuals with LTBI.

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Table 1. Demographic characteristics of study participants with active TB or latent TB infection

	Active TB	LTBI	p
Number	31	30	NA
Male (%)	67.7	50	NS
Age (years; median and IQR)	35 (29-42.5)	32 (25-39)	0.09
Non-Australian born (%)	100	93.3	NS
Extra-pulmonary TB (%)	25.8	NA	

NA = not applicable

NS = not significant

Supplementary table 1 - Proportion of cytokine-producing CD4+ T cells in response to *Mycobacterium tuberculosis* antigens

% of CD4+ T-cells	PPD		ESAT-6		CFP-10	
	Active TB	LTBI	Active TB	LTBI	Active TB	LTBI
IFNγ+TNFα-IL2-	0 (0-0.3) ^a	0.005 (0-0.34)	0.02 (0-0.37)	0.01 (0-0.35)	0.04 (0-0.5)	0.04 (0-0.49)
IFNγ-TNFα+IL2-	0.10 (0-0.4)	0.09 (0-2.44)	0.08 (0-0.81)	0.04 (0-0.35)	0.09 (0-0.74)	0.08 (0-1.05)
IFNγ-TNFα-IL2+	0 (0-0.17)	0.01 (0-0.3)	0.01 (0-0.58)	0 (0-0.1)	0.02 (0-0.44)	0 (0-0.17)
IFNγ+TNFα+IL2-	0.08 (0-1.23)	0.06 (0-0.58)	0 (0-0.20)	0 (0-0.15)	0.04 (0-0.44)	0.05 (0-0.19)
IFNγ+TNFα-IL-2+	0 (0-0.02)	0 (0-0.03)	0 (0-0.02)	0 (0-0.005)	0 (0-0.03)	0 (0-0.02)
IFNγ-TNFα+IL-2+	0.03 (0-0.10)	0.03 (0-0.15)	0.03 (0-0.24) ^b	0.02 (0-0.09)	0.002 (0-0.04)	0.02 (0-0.22)
IFNγ+TNFα+IL-2+	0.13 (0-2.09)	0.11 (0-0.62)	0.01 (0-0.19)	0 (0-0.29)	0.04 (0-0.78)	0.04 (0-0.31)

^a Data are presented as median (range)

^b Differed significantly from LTBI, p=0.04, Mann-Whitney test

Figure legends

Figure 1.

Active TB is associated with higher proportions of mycobacterial-reactive CD4+ T cells expressing CD39. The proportions of antigen-reactive CD4+ T cells (CD25+,CD134+) activated by PPD (A), ESAT-6 (B) and CFP10 (C), and the proportions of the antigen-reactive CD4+ T cells expressing CD39 activated by PPD (D), ESAT-6 (E) and CFP10 (F) in PBMC of study participants with active TB [(●), n = 31] and LTBI [(○), n = 30] enumerated by flow cytometry are shown. Horizontal lines denote median values. P values obtained by the Mann-Whitney test, $p < 0.05$ was considered significant.

Figure 2.

Active TB is associated with higher production of TNF- α than IFN- γ in antigen-stimulated PBMC cultures. The production of IFN- γ (A) and TNF- α (B), and the ratios of TNF- α :IFN- γ (C) and IL-10:IFN- γ (D) by PBMC cultured with PPD, ESAT-6 or CFP-10, in study participants with active TB [(●), n = 31] and latent TB [(○), n = 30]. Horizontal lines denote median values. P values obtained by the Mann-Whitney test, $p < 0.05$ was considered significant.

Figure 3.

Expression of CD39 on mycobacterial-reactive CD4+ T cells was associated with higher production of IL-10 in PBMC cultures. The proportion of antigen-reactive CD4+ T cells (CD25+,CD134+) activated by PPD correlated with the proportion of CD4+ T cells expressing IFN- γ +,IL-2+,TNF- α + (A), IFN- γ +,IL-2-,TNF- α + (B), IFN- γ -,IL-2+,TNF- α + (C) and IFN- γ -,IL-2-,TNF- α + (D) after PPD stimulation and the proportion of antigen-reactive CD4+ T cells activated by CFP-10 correlated with the proportion of CD4+ T cells expressing IFN- γ +,IL-2+,TNF- α + (E) and IFN- γ +,IL-2-,TNF- α + (F) after CFP-10 stimulation. The frequency of antigen-reactive CD4+ T cells activated by PPD also correlated with the production of IFN- γ (G) and TNF- α (H) in PBMC culture supernatants. The proportion of antigen-reactive CD4+ T cells expressing CD39 after PPD stimulation correlated with the production of IL-10 (F) in all study subjects [(●), n = 61]. P values obtained by the Spearman correlation test, $p < 0.05$ was considered significant.

Figure 4.

IL-10 production in PPD-stimulated PBMC cultures is decreased by the CD39 inhibitor ARL67156. PBMC samples from study participants with active TB (n=5) or LTBI (n=5) that produced the highest amounts of IL-10 in previous PPD-stimulated cultures were incubated with PPD or SEB with or without ARL67156 at a concentration of 200µg/mL and IL-10 (A,B) or IFN-γ (C,D) assayed in culture supernatants. P values obtained by the Wilcoxon matched-pairs signed rank test, $p < 0.05$ was considered significant.

Figure 5.

Negative whole blood IGRA results in patients with active TB are not associated with higher CD39+ mycobacterial-reactive CD4+ T cells. The proportion of antigen-reactive CD4+ T cells (CD25+,CD134+) activated by PPD, ESAT-6 or CFP10 expressing CD39 in study participants with active TB who had a negative IGRA result (▲, n=9) or positive IGRA result (□, n=31). Horizontal lines denote median values. P values obtained by the Mann-Whitney test, $p < 0.05$ was considered significant.

Supplementary Figure 1.

Antigen-reactive CD4+ T cells (CD25+,CD134+) expressing CD39 detected by flow cytometry. Representative staining of unstimulated (A, B), PPD-stimulated (C, D) or ESAT-6-stimulated (E, F) PBMC showing antigen-reactive CD4+ T cells (left panel) expressing CD39 (right panel) after 24-hour culture. Dot-plots were gated on CD3+,CD4+ T cells.

Supplementary Figure 2.

CD4+ T cells expressing IFN-γ, IL-2 or TNF-α after stimulation with M. tuberculosis antigens. Singlet cells were gated based on forward-scatter (FSC) height and FSC area (A) followed by identification of the lymphocyte population using FSC and side-scatter properties (B). From the lymphocyte gate, CD4+ T cells were identified based on co-expression of CD3 and CD4 (C). The proportion of CD4+ T cells expressing IFN-γ, TNF-α or IL-2 after 6 hours of culture either unstimulated (D) or with PPD (E), ESAT-6 (F) or CFP-10 (G) are shown.

Supplementary figure 3.

Titration of the CD39 inhibitor ARL67156. Effect of different concentrations of the CD39 inhibitor ARL67156 on the production of IFN- γ by PBMC stimulated with SEB **(A)** or PPD **(B)** and on the production of IL-10 by PBMC stimulated with SEB **(C)** or PPD **(D)**. Maximal suppression of cytokine production was obtained at a concentration of 200 μ g/mL.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplementary figure 1

Supplementary Figure 2

Supplementary figure 3