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Effects of *M. tuberculosis* antigens on CD4+ T cells

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ABSTRACT

One-third of the world's population is infected with *M. tuberculosis* (TB), of which the vast majority is latently infected (1). A 5% lifetime risk exists of latent infection becoming active disease (2). A positive purified protein derivative (PPD) skin test suggests previous or latent TB infection. We hypothesize that PPD positive individuals have a higher frequency of TB specific lymphocytes than PPD negative individuals, who are presumed to be uninfected. CD4+ T cells are the most important lymphocyte type in controlling TB infection. For these studies, CD4+ T cells were stimulated by monocytes, which are antigen presenting cells (APCs). APCs digest, process, and present antigens to CD4+ T cells in a form that CD4+ T cell receptors can recognize. CD4+ T-cells and monocytes were isolated by immunomagnetic bead methods. These purified cell populations were stimulated with different TB antigens including live TB, PPD, and whole cell TB lysate. Pro-inflammatory cytokine production (TNF-alpha, IL-2, and IFN-gamma) and CD154 expression were measured in each individual's cells by flow cytometry to determine the frequency of responding cells. TB activated cells express CD154, a protein that co-stimulates CD4+ T cells and APCs. As hypothesized, PPD positive individuals had a higher frequency of responding cells than PPD negative individuals. This more frequent response from PPD positive individuals suggests that these cytokine producing CD154-expressing CD4+ T cells were indeed TB antigen specific. PPD negative individuals have less TB specific CD4+ T cells since these individuals were presumably never infected with TB and thus their immune systems never developed a memory response to the bacilli.

INTRODUCTION

M. tuberculosis (TB) is a bacillus that causes the infectious disease tuberculosis. Tuberculosis infects over one-third of the world population. The majority of these people have a latent infection, meaning it is not active. About a 5% chance exists for the latent form to turn into active disease. TB is an aerobic bacterium that mostly affects the pulmonary tract. It divides every 16 to 20 hours, much slower than most other bacteria. TB is known as an opportunistic infection (OI) (3). OI are infections that generally do not cause the disease in people with healthy immune systems but in those with a poorly functioning or suppressed immune systems. HIV increases the risk of developing OI because the virus attacks the immune system and compromises its ability to respond to infections. TB is an OI that infects HIV+ individuals earlier in the course of HIV disease than any other OI, even before the HIV is detected in the individual. TB also infects individuals with a healthy immune system making it a more virulent OI than most of the others in this group. TB activated cells express CD154, a protein that co-stimulates CD4+ T cells and antigen presenting cells (APC). In studying the expression of CD154 in PPD positive versus PPD negative individuals, we hypothesize that PPD positive individuals have a higher frequency of TB specific lymphocytes than PPD negative individuals, who are presumed to be uninfected.

METHODS AND MATERIALS

PBMC Isolation:

The human subjects protocol was approved by the Institutional Review Committee at University Hospitals and Case Western Reserve University and informed consent was obtained from all donors. Blood was drawn from PPD positive and PPD negative individuals. Peripheral Blood Mononuclear Cells (PBMC) were purified by Ficoll (Pharmacia) per the manufacturer's instructions.

APC and T cell Purification:

CD14+ monocyte isolation was performed using immunomagnetic bead methods (Miltenyi) according to the manufacturer's instructions. CD4+ T cells were isolated using immunomagnetic Dynal bead methods as per the manufacturer's instructions (Invitrogen).

T cell Assays:

Purified CD4+ T cells (5x105/well) were incubated with CD14+ monocytes (5x105/well) in 96 well plates. All assays were performed in standard medium which consisted of RPMI 1640, supplemented with 2% pooled human serum (Gemini Bio-Products), 100 U/ml penicillin, 100 Ag/ml streptomycin, 1% non-essential amino acids, 10 mM HEPES, 2-ME (5 _10_ 5 M), and 2 mM glutamine (Cambrex). Six wells were used per donor with the following antigens diluted in each well: Media, TB 5 x 106(ATCC), TB 1 x 106, PPD (Wyeth Pharmacuticals), TB Lysate (CO State TB Contract), and SEB (staphylococcal enterotoxin B, Sigma). Anti- CD 28/49 at 0.5 ug/ml (BDBiosciences) was added to each well for costimulation. Brefeldin A 10 ug/ml (Sigma) was added after overnight culture and then cells were incubated for four hours before harvest.

Intracellular Staining:

Cells were incubated with the surface antibodies, anti-CD4-FITC (BDBioscicnes) and anti-CD154 -APC (Ebiosciences), for 15 minutes at room temperature. After fixation in 4% paraformaldehyde, cells were incubated in 0.1% saponin (Sigma) with anti TNF-alpha

-PE, IL-2-PE, and IFN-gamma-PE (all 3 EBiosciences) for 20 minutes at room temperature for intracellular staining. Cells were finally re-suspended in 2% paraformaldehyde and analyzed using flow cytometry within 24 hours. Pro-inflammatory cytokine production (TNF-alpha, IL-2, and IFN-gamma) and CD154 expression were measured by flow cytometry to determine the frequency of responding cells.

CD154+ cell Purification:

In selected experiments, CD154+ cell isolation using the immunomagentic anti-CD154 bead kit (Miltenyi was performed as per the manufacturer's instructions. Cell purity was determined by anti-biotin-APC (Miltenyi) staining of CD154 selected cells

RESULTS

To confirm our basic hypothesis that that PPD positive individuals have a higher frequency of TB specific lymphocytes than PPD negative individuals we obtained blood from 4 PPD- and 6 PPD+ donors. Monocytes and CD4+ T cells were purified from their blood using immunomagentic bead methods. The cells were then incubated with TB antigens overnight and the levels of pro-inflammatory cytokines and surface CD154 expression were determined by flow cytometry. The cells could either express CD154 alone, cytokines alone or be dual expressing. Using flow cytometry we were able to determine this on a per cell basis. Figure 1 below shows flow cytometry data for a PPD+ and a PPD - individual. As can be seen there is a clear difference in CD154 and cytokine expression.

Results for all individuals are shown as percentage positive expression in the CD4+ cells for each of the groups. Table 1 summarizes these results for all individuals tested. Also included is the percentage of total CD4+ T cells that expressed CD154 whether there was cytokine production or not. PPD positive individuals had a significantly higher frequency of cells that responded to the mycobacterial antigens than the PPD negative individuals for many of the TB antigens.

This table shows that on average, PPD positive individuals had a higher frequency of CD154+ cells, cytokine-producing cells, and cells that were both CD154+ and cytokine producing. The two different con-

No Antigen		PPD- Individuals Mean (SD)	PPD+ Individuals Mean (SD)	p value
	CD154	0.20 (0.02)	0.15 (0.12)	0.30
	cytokine	0.63 (0.67)	0.81 (0.72)	0.70
	Total CD154	0.23 (0.03)	0.47 (0.82)	0.50
	Dual	0.03 (0.03	0.01 (0.01)	0.34
TB High Dose				
	CD154	3.40 (1.31)	7.48 (4.87)	0.10
	cytokine	0.47 (0.25)	1.65 (0.81)	0.01
	Total CD154	3.57 (1.35)	8.18 (5.10)	0.08
	Dual	0.16 (0.08)	0.71 (0.29)	0.00
TB Low Dose				
	CD154	3.42 (2.26)	8.49 (5.22)	0.07
	cytokine	0.88 (1.11)	3.45 (2.25)	0.04
	Total CD154	3.53 (2.22)	10.08 (5.99)	0.05
	Dual	0.10 (0.07)	1.59 (1.22)	0.03
PPD				
	CD154	0.23 (0.07)	1.79 (1.04)	0.01
	cytokine	1.73 (1.99)	2.56 (1.36)	0.50
	Total CD154	0.27 (0.03)	2.46 (1.41)	0.01
	Dual	0.04 (0.04)	0.68 (0.42)	0.01
TB Lysate				
	CD154	0.62 (0.93)	1.42 (0.76)	0.21
	cytokine	1.06 (1.21)	1.75 (0.50)	0.34
	Total CD154	0.65 (0.93)	1.69 (0.94)	0.13
	Dual	0.02 (0.02)	0.27 (0.21)	0.03
SEB				
	CD154	2.34 (3.23)	8.58 (3.11)	0.02
	cytokine	2.88 (1.80)	11.97 (4.13)	0.00
	Total CD154	2.77 (3.91)	17.35 (10.97)	0.02
	Dual	0.43 (0.68)	6.35 (4.54)	0.02

Table 1. Mean cell expression of PPD positive versus PPD negative individuals. Mean values for PPD positive and PPD negative individuals are displayed on the left side of the respective columns and standard deviation is given in parenthesis. The last column gives P-values.

centrations of live TB had the highest and most significant differences between the two groups. The P-values for the majority of the different cell types are under 0.05. This means that there is less than a 5% chance that the results were accidental, meaning that the results were not a matter of chance, they were obtained through the proper methods.

Staphylococcal enterotoxin B (SEB) was supposed to function as the control, since it was an antigen not related to TB. SEB should have yielded similar values for both PPD positive and PPD negative individuals. However, overall, the number of cells activated by SEB were higher for PPD positive individuals than for PPD negative individuals.

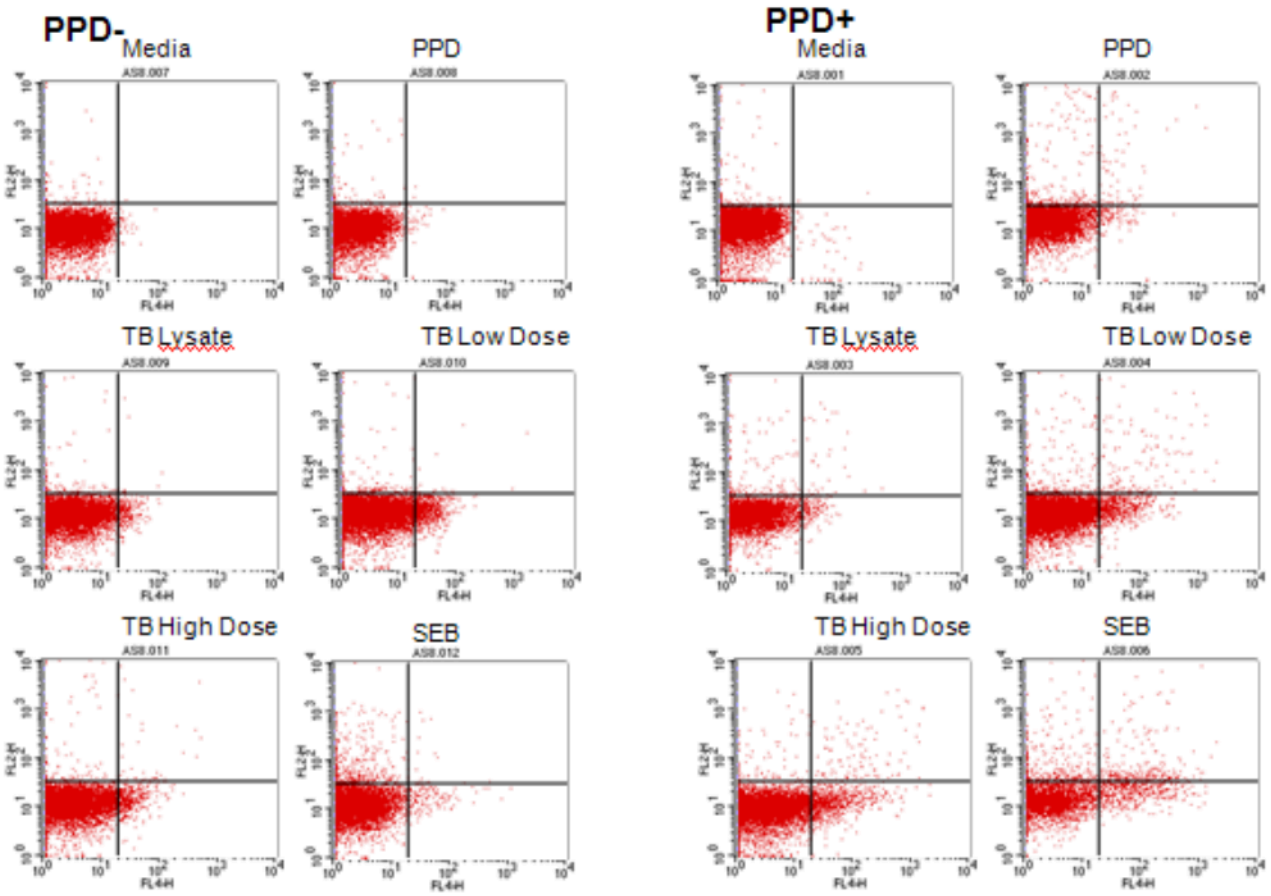

Figure 1: Sample flow cytometry results of a PPD- and a PPD+ donor. The six different environments cells were incubated in overnight were Media (no antigen), TB High dose, TB low dose, PPD, TB Lysat, and SEB. The PPD negative individuals produced a lower number of pure CD154+ cells (lower right quadrant), cytokine-producing cells (upper left and upper right quadrants), all CD154+ cells that did and did not produce cytokines (upper right and lower right quadrants), and CD154+ cells that produced cytokines (upper right quadrant). The PPD positive individual produced a larger number of cells for the aforementioned four cell types as can be determined from abundance of cells in the upper right, upper left, and lower right quadrants of the PPD negative and PPD positive individual results above. The x-axis represents CD154+ cell production. Cells in the lower left quadrant are not CD154+. The y-axis reprsents the production of the three cytokines, TNF-alpha-PE, IL-2-PE, and IFN-gamma-PE.

Figure 2 shows the actual data for every donor that was studied. It is apparent that the PPD- donors (Fig 2A) had a very low frequency of cells that were positive for CD154 or cytokines. PPD negative individuals also responded modestly to the antigens. Even when stimulated with no antigen, the frequency of production of the measured cell types was very low. This means that the PPD negative individuals produced very little CD154 overall.

The PPD positive donors' results (Fig 2B) show these individuals had an overall stronger response to the different antigens. SEB and the two doses of TB yielded in the highest cell responses. PPD and RVL were less effective at stimulating signal but as shown in Table 1 still had significant differences in most cases. These individuals had a higher percentage of CD154+, cytokine producing CD4+ T cells.

CD154+ cells were purified in selected PPD+ donors after stimulation with live TB by a two-step anti-CD154 immunomagentic bead kit. The yield could then be calculated by knowing the number of cells present before CD154+ purification after and the percent CD154+ cells as determined by the data in Fig. 1B. Yield was over 90% on the two donors shown. The purity of the CD154+ cells was determined by using anti-biotin-APC antibody. The purity was not that great as determined by this method. It is likely an underestimation of the purity of the cells as the biotin epitopes may have been obscured in the purified T cells by the anti-biotin beads used in the purification process.

DISCUSSION

As Table 1 and Fig. 2 show, PPD positive individuals expressed more CD154+, CD4+ T cells than PPD negative individuals did. They also produced more pro-inflammatory cytokines than PPD negative individuals did. The more frequent response from PPD positive individuals suggests that these cytokine producing CD154-expressing CD4+ T cells were indeed TB antigen specific. PPD negative individuals have less TB specific CD4+ T cells since these individuals were presumably never infected with TB and thus their immune systems never developed a memory response to the bacilli. This was the anticipated result. For this project, we wanted to develop the method to stimulate the TB specific cells and then try to purify the responding CD154 cells when they were live. This was the rationale in using the CD154 purification method described in Table 2. It has been demonstrated that CD154 up-regulation is a reasonable way to obtain live antigen specific cells (4-6).

With this knowledge, we hope to stimulate HIV+ cells with TB and observe the CD154+, CD4+ T cell frequency responses. We will then go on and purify the TB specific cells using this CD154 method that we have developed and confirmed. Since TB infects HIV+ individuals at a high rate, carrying out such experiments may yield an answer as to why this occurs. HIV+ individuals should be expected to have a lower frequency of TB specific CD 154+, CD4+ T cells since these individuals are unable to battle the infection effectively.

Further developing and characterizing these techniques will allow us to study responses in HIV infected individuals. We hypothesize that MTB specific CD4+ T cells are preferentially infected with HIV. This would

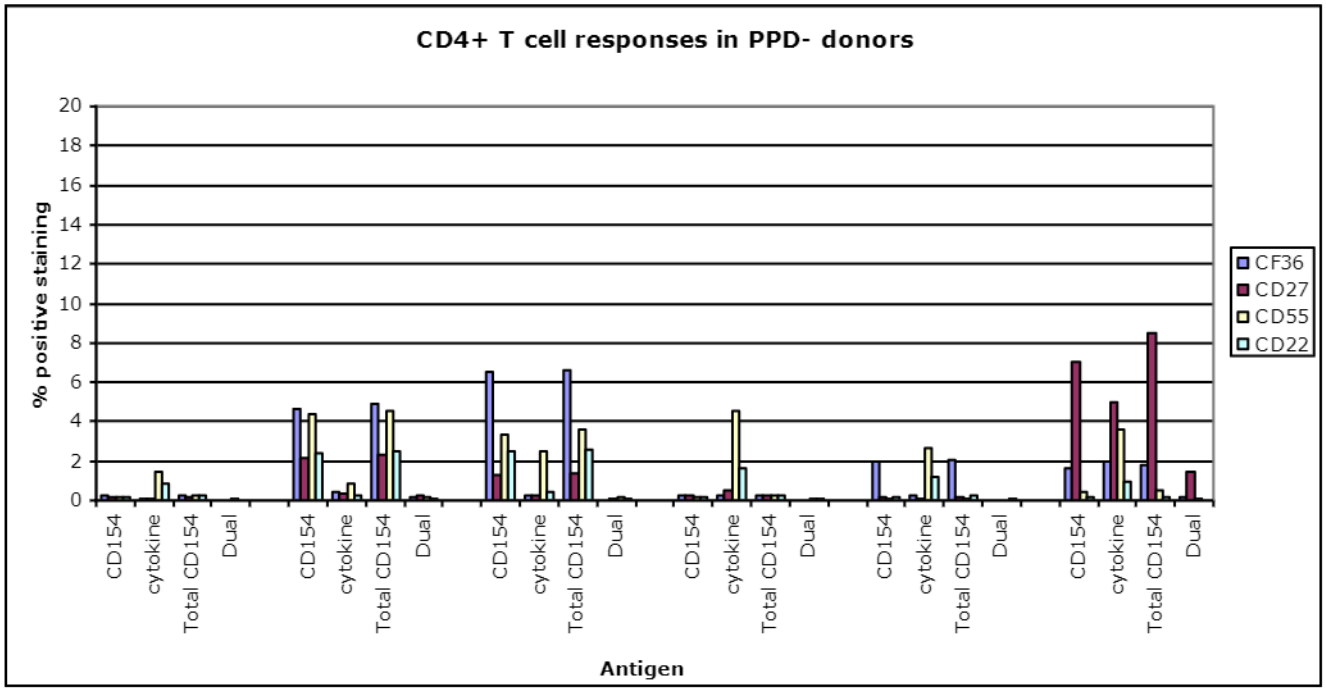

Figure 2A. CD4+ T cell responses to TB antigens in PPD negative donors. Each donor's cells were placed in six different antigens, with one having no antigens as a negative control, shown on the x-axis. The antigens used were TB at two different concentrations, PPD, TB Lysate, and SEB. The x-axis also shows the four different types of cell expression that were stained for and measured; pure CD154+ cells, cytokine-producing cells, all CD154+ cells that did and did not produce cytokines, and cells that were CD154+ and produced cytokines. The four cell types have been shown for the six different environments the cells were incubated in. The percentage of cells and types of cells that were activated by each of the individual antigens is shown.

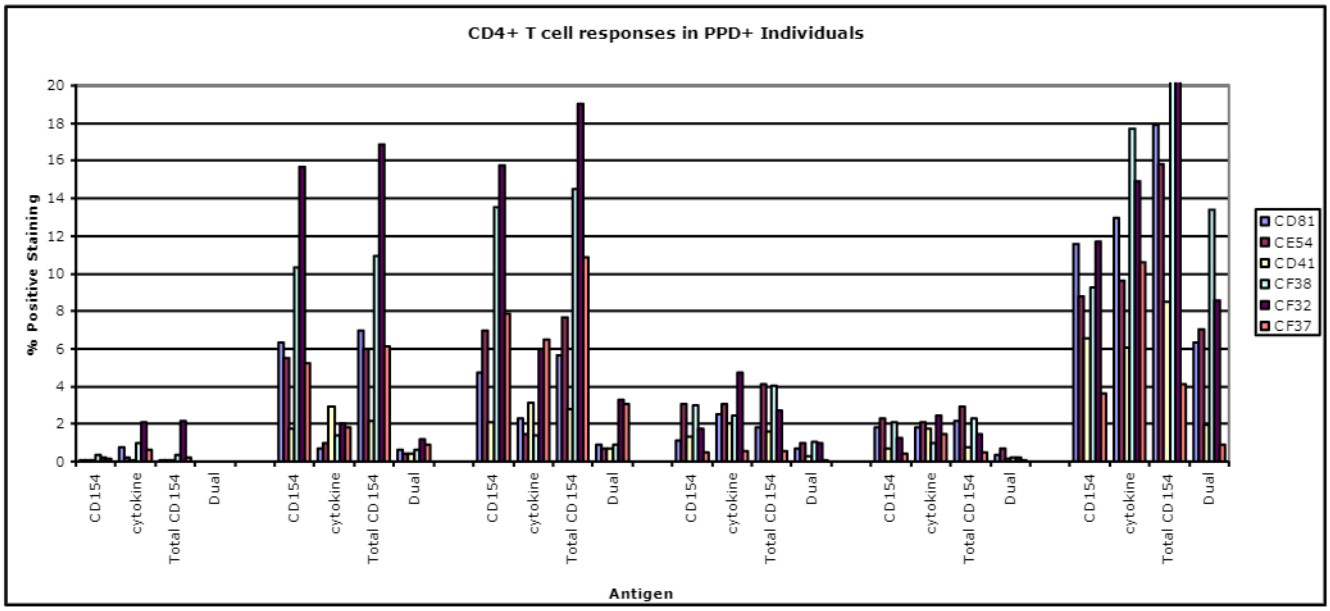

Figure 2B. CD4+ T cell responses to TB antigens in positive donors. Each donor's cells were placed in six different antigens, with one having no antigens as a negative control, shown on the x-axis. The antigens used were TB at two different concentrations, PPD, TB Lysate, and SEB. The x-axis also shows the four different types of cell expression that were measured; pure CD154+ cells, cytokine-producing cells, all CD154+ cells that did and did not produce cytokines, and cells that were CD154+ and produced cytokines. The four cell types have been shown for the six different environments in which the cells were incubated. The percentage of cells and types of cells that were activated by each of the individual antigens is shown.

Donor #	# cells before CD154+ purification	# cells after CD154+ purification	Purity of CD154+ cells (%)	CD154+ cells from flow cytometry (%)
CF38	8.82×10^6	4×10^5	4.35	61
CF32	5.68×10^6	3.2×10^5	5.63	56

Table 2. Yield and purity of CD154+ CD4+ T cells.

increase their elimination and could be a partial explanation for the increased active TB disease in HIV infected individuals. A better understanding of how to induce CD154 expression on live MTB specific cells will allow physical isolation of CD154 expressing MTB specific cells in HIV+ individuals to test if there actually is increased HIV infection in these cells over the total memory CD4+ T Cell population. This information could have ramifications concerning the recommendation of treating HIV+ individuals in anti-retroviral therapy before or during anti-TB therapy. The results of these studies can be utilized to help understand mechanisms of evasion of host immunity by microbes such as *M. tuberculosis*. A better understanding of the mechanisms of immune evasion would help in developing optimal vaccines.

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