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Evaluation of the Contribution of Major T Cell Subsets to IFN-γ Production in TB Infection by ELISPOT

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Interferon gamma remains a key effector molecule that is still widely used as the most informative biomarker for screening human immune responses against tuberculosis, particularly in ELISPOT assays. We investigated the participation of $CD4^+$ and $CD8^+$ T lymphocytes in the PBMC responses to *Mycobacterium tuberculosis* (Mtb) specific antigens in 33 TB cases and 49 contacts. Responses to ESAT-6 were higher than CFP-10. There was no significant difference in responses to both Mtb antigens between cases and contacts. PBMCs response to ESAT-6 but not CFP-10 in cases was significantly reduced by depletion of $CD4^+$ cells whereas $CD8^+$ cell depletion had no impact. In conclusion, ESAT-6 is a more recognized antigen in this population, and $CD4^+$ lymphocytes are the main participants in IFN-γ response by ELISPOT. Thus, a decline of $CD4^+$ T lymphocytes below a critical level might affect the sensitivity of IFN-γ release assays for detecting Mtb infection.

Keywords T lymphocyte subsets, Tuberculosis, IFN-γ, ELISPOT.

INTRODUCTION

It is estimated that one-third of the world population is latently infected with *Mycobacterium tuberculosis* (Mtb), and 5 to 10% of the infected population will develop the disease during their lifetime (Corbett, 2003; Corbett et al., 2003). The outcome of Mtb infection depends on the state of the host immune system:

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the infection may be eliminated, progress directly to active disease, or become latent in the host with a risk of later reactivation (Lin and Ottenhoff, 2008). A major roadblock in TB control is the lack of understanding of what constitutes a protective immunological response against progression of latent infection to active TB disease (Lalvani and Millington, 2008).

Immunological responses to Mtb infection involve both the innate as well as the adaptive immune system (Ottenhoff et al., 2005). A key effector molecule in protective immunity against mycobacteria is IFN-γ, produced by several cells (Flynn et al., 1993; Newport et al., 2003). Studies of T lymphocyte responses show that both $CD4^+$ and $CD8^+$ T cells are important in controlling TB (Flynn, 2004; Smith and Dockrell, 2000; Smith et al., 2000). An analysis of the major T lymphocyte subset responding to the Mtb antigens may inform diagnostic and vaccine development purposes. Therefore, we evaluated the cellular source of the IFN-γ release in an *ex-vivo* ELISPOT assay in TB cases and their contacts.

MATERIALS AND METHODS

Recruitment of Study Participants

Appropriate informed consent was obtained from participants in this study, and protocols used were approved by the Gambia Government / Medical Research Council joint ethics committee.

Newly diagnosed tuberculosis cases from major government health centres and the Medical Research Council Laboratories' (MRC Labs) outpatients' clinic were prospectively and consecutively recruited into the study. They were included if they were aged ≥15 years, had two sputum samples positive for acid-fast bacilli by Ziehl-Neelsen stain and Mtb on culture and had not been previously treated for tuberculosis. Their household contacts were included in this study if they were aged ≥12 years of age and lived the majority of the time with the case.

Tuberculin skin test (TST) was done for all consenting subjects by intradermal injection of PPD (2 TU, RT23, Statens Serum Institute, Copenhagen, Denmark) using the Mantoux technique. Response was determined by the average of the transverse and horizontal diameters of the induration measured by the ball point method after 48–72 hours. Subjects with a positive skin test (mean induration diameter ≥10 mm) were offered a chest X-ray and those with symptoms underwent a clinical assessment. Confirmed TB cases were referred for free treatment through the National TB Control Programme.

HIV Testing

HIV status was determined by a testing algorithm consisting of enzyme linked immunosorbent assays (Murex 1.2.0, Abbott-Murex Biotec, Dartford,

Kent, UK), Hexagon HIV (Human Diagnostics GmbH, Wiesbaden, Germany) and type specific immunoblotting kit (Pepti-LAV I/II, BIORAD, Marnes-la-Coquette, France) for confirmation. HIV positive individuals were referred to a specialist clinic that now offers free anti-retroviral treatment according to set criteria.

Cell Depletion and PBMC Isolation

Heparinized blood samples were collected from study participants before commencement of anti-tuberculosis treatment. The blood samples were depleted of $CD4^+$ and $CD8^+$ T-lymphocytes using RosetteSep® antibody cocktail mix according to the manufacturer's protocol (Stem Cell Technologies, Vancouver). Briefly, 2 mL aliquots of the heparinized blood were placed into three separate tubes and 50 µl/ml of antibody cocktail mix for depleting $CD4^+$ and $CD8^+$ cells was added to tubes 1 and 2, respectively, while the blood in the third tube was used to obtain undepleted PBMCs. The tubes containing the antibody cocktail were mixed briefly and incubated for 20 minutes at room temperature.

The content of each tube was then diluted with an equal volume of RPMI - 1640, layered on lymphoprep medium 1.077 (Axis – Shield PoC AS Oslo, Norway), and the cells harvested, washed twice, resuspended in RPMI-1640 supplemented with 5% heat inactivated human AB serum. Phenotypic analysis was conducted on the purified cell populations by staining with anti-CD3-PerCP, anti-CD4-PE, and anti-CD8-APC (BD BioSciences, USA). The purity of the selected cell populations were >95%.

ELISPOT Assay

Cells were assayed for the frequency of IFN-γ producing cells by ELISPOT as previously described (Hill et al., 2005). Briefly, 96-well nitrocellulose-backed plates (Millipore, Bedford,UK) were coated with anti-human IFN-γ monoclonal antibodies (1-DIK Mabtech, Sweden; 12 µg/ml in sterile PBS). Coated plates were incubated overnight at 4–8ºC. The plates were then washed 6 times in sterile PBS and blocked with 5% fetal calf serum (FCS) in RPMI for 2 hours at room temperature. Two more washes were performed before addition of the cells and antigens. The 3 cell populations (CD4 depleted, CD8 depleted, and bulk PBMCs) were then plated at a concentration of 2×10^5 cells/well with the various antigens and controls and incubated for 18–20 hours at 37°C, 5% CO_2. Antigens used for this study included two synthetic peptide pools each containing sequential 15-mer peptides overlapping by 10 amino acids spanning the length of ESAT-6, and CFP-10 (ABC, Imperial College, London, UK, 2.5 µg/ml each), respectively.

Phytohaemaglutinin (PHA: Sigma-Aldrich, UK, 5 µg/ml) and medium alone, were the positive and negative controls, respectively. All antigens and controls were tested in duplicate wells. After incubation, the contents of the plate were decanted and plate washed 6 times with PBS 0.05% Tween-20.

50 μl/well of biotinylated secondary antibody (7-B6-1-Biotin, Mabtech; 1.0 μg/ml) was added and incubated for 2 hours at room temperature. Thereafter plates were washed 6 times and incubated for an hour with 1 μg/ml of streptavidin alkaline phosphate conjugate (Mabtech). Next the plates were washed as described above and colour developed with a chromogenic alkaline phosphate substrate kit (Bio Rad Laboratories, Hercules, USA) for 3–10 minutes in the dark before stopping the reaction by washing with running tap water. The plates were dried at room temperature in the dark overnight, and counted using an automated ELISpot plate reader (AID-GmbH, Strassberg, Germany). The number of IFN-γ spot forming units (SFU) counted in each well were automatically entered into a database minus background (medium wells), and the data expressed as number of spot-forming cells (SFC) / million.

Data Management and Statistical Analysis

All data were entered using double data entry into an ACCESS database and checked for errors. The Kruskal–Wallis test was used to compare responses from the three different cell populations. Comparison of responses between cases and contacts was by the Student's *t*-test. Statistical significance was assumed at a p-value <0.05. All statistical analyses were conducted using Stata software (version 9; Stata Corp, College Station, TX, USA).

RESULTS

Subjects and Characteristics

A total of eighty four Gambians were recruited. Two (one case and one contact) were excluded from further analyses for being HIV positive. Thirty three of the remaining 82 participants were TB cases (42.2%) and the rest contacts. The mean age of the cases was 33 (range 15–60), and the contacts was 29 (range 12–77) years. Twenty-three cases (69.7%) were male compared to 21 contacts (42.9%). Seven cases (19%) and 25 contacts (42.9%) had a BCG scar. Twenty-four cases (72%) and 47 contacts (57%) were TST positive (≥10 mm). Subsequent analyses were adjusted for presence of a BCG scar and gender.

Responses of T Lymphocyte Subsets to ESAT-6 and CFP-10

To determine the contributions of $CD4^+$ and $CD8^+$ T cells to the immune response, we compared the numbers of IFN-γ producing cells in bulk PBMCs, PBMCs depleted of $CD8^+$ or $CD4^+$ cells obtained from TB cases and contacts after overnight stimulation with either CFP-10 and ESAT-6 (Fig. 1). The purified cell populations were negatively selected. The medians (interquartile range) for the background (non-stimulated) production of the IFN-γ in bulk PBMCs, PBMCs depleted of $CD8^+$ or $CD4^+$ cells for TB cases were 2 (1–5), 2

(0–5) and 2 (0–3), and for household contacts 3 (2–5), 3 (1–5), 2 (1–5), respectively. Thus, the background responses were similar between the different cell populations as well as between cases and contacts. However, there was a significant reduction in the frequency of IFN-γ-producing cells in response to ESAT-6 after depletion of $CD4^+$ T cells compared to bulk PBMCs, but this reached statistical significance only in cases ($p = 0.03$) (Fig. 1A).

The response to ESAT-6 in the $CD4^+$-depleted cells was also significantly lower than that from $CD8^+$-depleted population in cases ($p = 0.015$). The response of $CD8^+$-depleted cells was similar to the bulk PBMC. In contrast, the responses to CFP-10 in bulk PBMCs as well as the $CD8^+$ or $CD4^+$ depleted

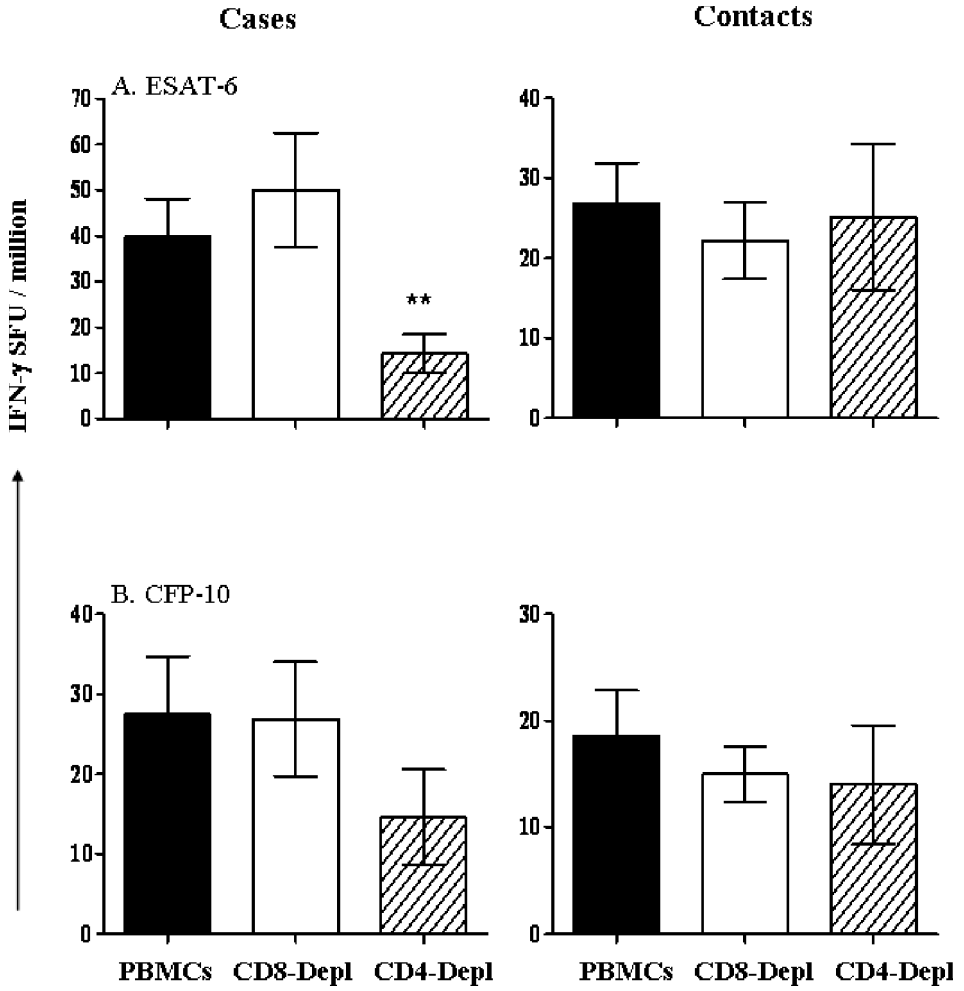

Figure 1: The effect of $CD4^+$- or $CD8^+$- depletion on the frequency of IFN-γ-secreting cells following stimulation with ESAT-6 (A), and CFP-10 (B) in TB cases and contacts. Averages and SEM of responses from 33 TB cases and 49 household contacts are represented. ** p-values = 0.015 and 0.03 as compared to CD8-depleted and undepleted PBMCs, respectively.

populations in cases and contacts were similar (Fig. 1B), implying that both $CD8^+$ and $CD4^+$ lymphocytes contribute to responses to CFP-10. The responses to ESAT-6 by all the cell populations were generally higher than those of CFP-10, but only those of the $CD8^+$-depleted population were significant for both cases and contacts, $p = 0.013$ and 0.04, respectively. Furthermore, to ascertain if differences exist in the response to Mtb antigens of the various cell populations between TB disease and infection, we compared responses from the different cell populations between TB cases and contacts.

The response from the $CD8^+$-depleted population to ESAT-6 in contacts was significantly lower than that of the cases ($p = 0.04$). In contrast, responses obtained from all cell populations to CFP-10 stimulation were similar between cases and contacts. The antigen specificity of these differences is validated by the similarity in the responses to PHA (data not shown). There was no correlation between the frequency of IFN-γ-producing cells with either the presence of BCG scar or the size of tuberculin skin test.

DISCUSSION

Interferon gamma release assays (IGRAs) that measure IFN-γ production by sensitized T-cells in response to stimulation by relatively Mtb-specific antigens are new methods for detecting Mtb infections (Lalvani, 2007; Lalvani et al., 2001; Menzies et al., 2007). Identification of the major T lymphocyte subset responding the Mtb antigens during infection or disease may influence the interpretation of interferon-gamma release assays under certain conditions. Our current study assessed the contribution of $CD8^+$ and $CD4^+$ lymphocytes to the ESAT-6 and CFP-10 antigen-specific IFN-γ response using ELISPOT assay. The ESAT-6 responses tended to be higher than those of CFP-10, and $CD4^+$-depletion significantly reduced the response to ESAT-6 in TB cases. Moreover, the response of the $CD8^+$-depleted cells to ESAT-6 in cases was higher than that of the contacts. These suggest that ESAT-6 specific effector cells are predominant in both TB cases and their contacts, and these are mainly due to the participation of $CD4^+$ T lymphocytes.

ESAT-6 and CFP-10 are secreted Mtb protein antigens whose genes are located in the same operon, and share 40% sequence homology (Berthet et al., 1998). The differences in the responses to both proteins could be related to either their expression and/or antigen processing in the host. Although both $CD4^+$ and $CD8^+$ T cells are important to control TB (Flynn, 2004; Smith and Dockrell, 2000; Smith et al., 2000), our data suggest that the responses are mainly due to $CD4^+$ population, particularly in response to ESAT-6. This could be in keeping with the principal role of $CD4^+$ in protection against Mtb in mice (Flynn et al., 1993). Moreover, these responses were obtained at recruitment, which can be considered an early phase of the disease, thus,

confirming the suggestion that $CD4^+$ T cells are important during early stages of infection whereas $CD8^+$ T cells become more significant for control of chronic infection (van Pinxteren et al., 2000).

Responses from the different cell populations to the Mtb-specific antigens were comparable in both cases and contacts, apart from the of $CD8^+$-depleted population response to ESAT-6 that discriminated between cases and contacts, which is likely due to an enriched ESAT-6-specific $CD4^+$ population in the cases. Thus, it is plausible that ESAT-6-specific $CD4^+$ lymphocytes expand extensively during TB disease resulting in the abrogation and enhancement of responses following their depletion and enrichment, respectively**.** Data have described variable responses to ESAT-6 of TB cases as compared to contacts, and these could be due to variability and difficulty in categorizing the stage of the TB disease in humans. (Cardoso et al., 2002; Roberts et al., 2007; Vekemans et al., 2001).

The similarity of the IFN-γ secretion from PBMCs, PBMCs depleted $CD4^+$, or $CD8^+$ T cells in response to CFP-10 is interesting. We used the same number of cells, 2×10^5, per well for each cell populations studied, which will certainly contain varying proportions of the different lymphocyte subsets. For instance $CD4^+$ cells will predominate in CD8-depleted population, and vice versa. Thus, one obvious implication of the similarity of responses to CFP-10 is that both $CD4^+$ and $CD8^+$ T cells contribute to the response, hence the depletion of one population leads to enhancement of the other, thereby compensating for the loss of response from the depleted cells. Taken together, our data indicate that IFN-γ immune response to CFP-10 is by both $CD4^+$ and $CD8^+$ lymphocytes, while that to ESAT-6 is mainly contributed by $CD4^+$ cells, and these are not compromised by TB disease. This discrepancy in response to ESAT-6 might have consequences on the reliability of IGRAs in conditions associated with low CD4 counts as in HIV/AIDS.

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