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Pulmonary responses to pathogen-specific antigens in latent *Mycobacterium tuberculosis* infection

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SUMMARY

In this study, we used ELISPOT to quantify frequencies of bronchoalveolar lavage (BAL) and peripheral blood T cells capable of producing IFNγ in response to PPD, antigen 85B, and *Mtb*-specific antigens CFP-10 and ESAT-6 in individuals with latent tuberculosis infection (LTBI) and *Mtb*-naïve controls. Compared to peripheral blood, BAL cells of LTBI subjects displayed significant enrichment for T cells responding to PPD, antigen 85B, and CFP-10, but not to ESAT-6. Baseline BAL cells of LTBI subjects displayed significant production of Mig (CXCL9) in response to PPD, antigen 85B, and CFP-10 as well. These findings suggest that enrichment for *Mtb*-specific T cells within BAL is not unique to active pulmonary tuberculosis and may, to the contrary, contribute to protection from re-infection in *Mtb* immune individuals.

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1. Introduction

Late 20th century advances in molecular biology of the mycobacteria led to the discovery that highly antigenic *Mycobacterium tuberculosis* (*Mtb*) proteins early secreted antigen target 6 kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10) were part of a gene segment present in all *Mtb* strains, but absent in *Mycobacterium bovis* BCG and most of the nontuberculous mycobacteria [1–3]. This important finding was exploited in the development of IFNγ release assays (IGRAs) utilizing these antigens (such as QuantiFERON-TB Gold In-Tube [QFN-GIT] and T-Spot.*TB*). Unlike traditional skin testing with purified protein derivative of *Mtb* (PPD), IGRAs provide the ability to the distinguish immune responses to *Mtb* infection from those induced by BCG vaccination and most environmental mycobacteria [4]. Further studies have attempted to apply this methodology to another clinical dilemma, that of distinguishing active disease in smear-negative pulmonary tuberculosis from latent tuberculosis infection (LTBI). Specifically, prior studies proposed that enrichment within bronchoalveolar lavage (BAL) for T-cell ELISPOT responses to *Mtb*-specific antigens could provide a means for rapid immunodiagnosis of active pulmonary TB [5–7]. However, our prior studies suggested that BAL is enriched for CD4+ T cells capable of responding to *Mtb* protein antigens even in LTBI [8]. Further, early induction of IFNγ-inducible chemokines, presumably facilitated by the presence of these local effector memory T cells (T_{EM}), was associated with recruitment of additional antigen-specific T cells to the lung in response to bronchoscopic challenge with PPD [8,9]. We therefore evaluated BAL cells of healthy individuals with LTBI and of non-vaccinated *Mtb* naïve subjects for ELISPOT responses to *Mtb*-specific antigens CFP-10 and ESAT-6, as well as to cross-reactive mycobacterial antigens *Mtb* Ag 85B and PPD. We also measured induction of CXCR3 chemokine ligands Mig (CXCL9) and IP-10 (CXCL10) by this same panel of antigens.

2. Results

2.1. Subject characteristics

Healthy non-smokers were recruited to LTBI and *Mtb*-naïve subject groups. Mean age was 28 (range 20–47) for the individuals with LTBI and 34 (22–47) for control subjects. The LTBI group consisted of 5 men and 5 women; 8 were Caucasian, 1 African American, and 1 was of Indian descent. The LTBI status of 8 subjects was confirmed by QuantiFERON TB Gold In-Tube (QFN-GIT) testing, whereas 2 had positive PPD skin tests only. Lack of clinical evidence for active disease (as detailed in Methods) was confirmed by BAL

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fluid culture, which showed no growth in all cases. The control group was composed of 6 men and 4 women; 9 were Caucasian and 1 African American. *Mtb*-naïve status was determined by QFN-GIT testing for 9 subjects and by PPD skin testing for 1 subject. BAL cell differentials for LTBI subjects had 93.7 (+/−2.5)% alveolar macrophages, 3.88 (+/−1.73)% lymphocytes, and 2.27 (+/−1.46)%; BAL of *Mtb*-naïve subjects displayed similar findings, with 92.38 (+/3.09%) alveolar macrophages, 4.59 (+/−2.43)% lymphocytes, and 2.36 (+/−2.71)% neutrophils. Eosinphils represented fewer than 1% of BAL cells for both subject groups.

2.2. Frequency of IFNγ-producing cells in PBMC and BAL

ELISPOT results for paired studies of PBMC and BAL are displayed in Figure 1. As illustrated, the frequency of IFNγ-producing cells with responsiveness to PPD, antigen 85B, and CFP-10 within PBMC did not differ between individuals with LTBI and control subjects ($p = 0.396$, $p = 0.320$, $p = 0.176$, respectively, by Mann–Whitney test). However, significantly more ESAT-6 responsive IFNγ-producing PBMC were observed in LTBI subjects as compared to controls ($p = 0.008$), although the absolute numbers of such cells were relatively infrequent for both subject groups. In contrast, in comparison to those of control subjects, BAL cells of LTBI subjects displayed markedly higher frequencies of IFNγ-producing cells with responsiveness to PPD, antigen 85B, and CFP-10 ($p < 0.001$, $p = 0.007$, and $p = 0.003$, respectively), but not to ESAT-6 ($p = 0.361$).

Calculation of enrichment of antigen-specific lymphocytes with BAL as compared to PBMC was based on the conservative estimation that PBMC are generally composed of approximately 85% lymphocytes, and on the observed percentage of lymphocytes in BAL differential cell counts of each subject (Figure 2). As illustrated, compared to *Mtb*-naïve control subjects, individuals with LTBI displayed significant enrichment for BAL lymphocytes producing IFNγ in response to PPD, antigen 85B, and CFP-10 ($p = 0.013$, 0.041, and 0.003, respectively), but not for ESAT-6 responsive lymphocytes ($p = 0.117$). These findings thus indicate that enrichment within BAL for *Mtb*-specific IFNγ-producing lymphocytes for these

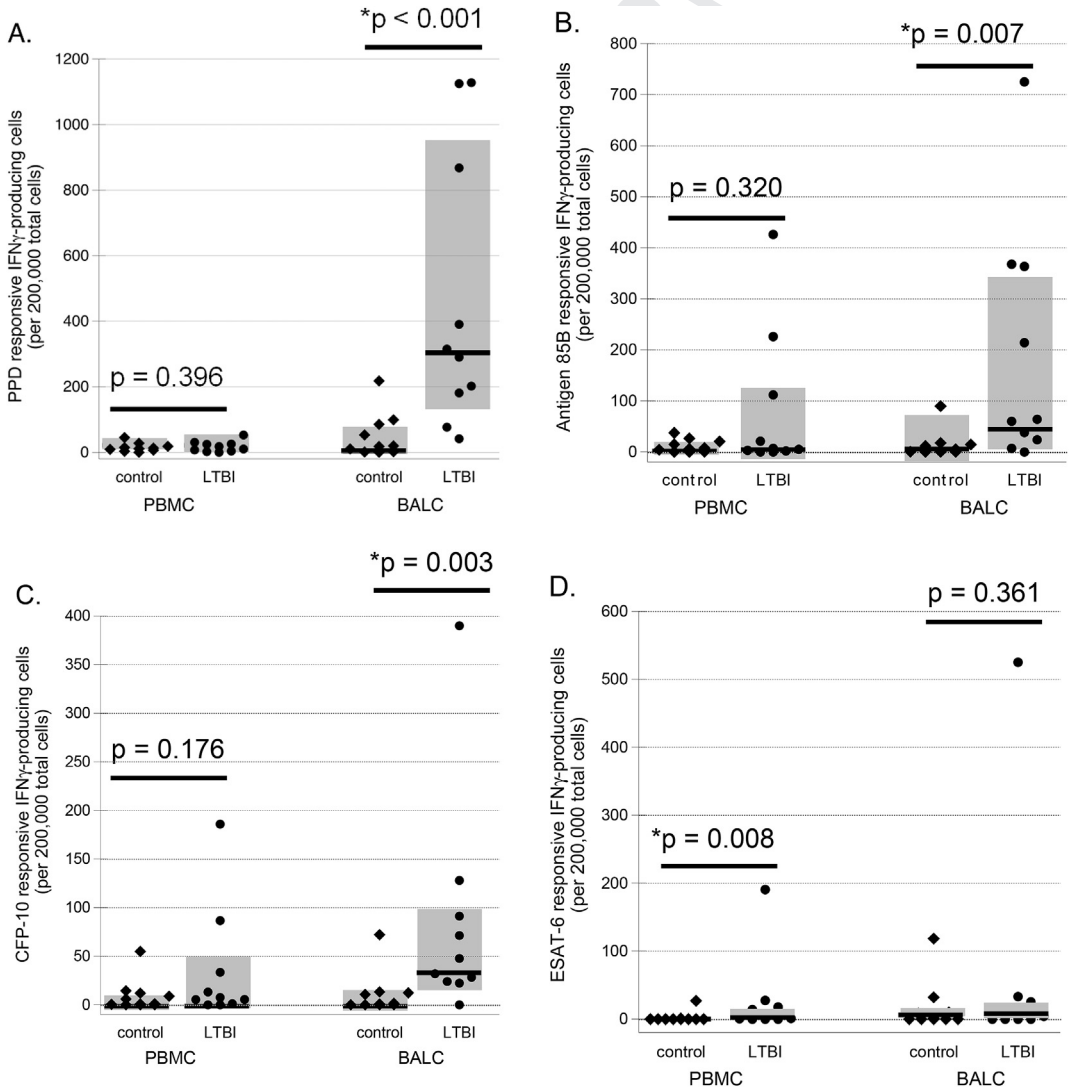

Figure 1. Frequency of antigen-responsive IFNγ-producing cells in peripheral blood and baseline BAL of LTBI subjects and naïve controls. The figures illustrate frequency of cells in PBMC and baseline BAL that produce IFNγ in response to *in vitro* stimulation with PPD (1A), CFP-10 (1B), ESAT-6 (1C) and antigen 85B (1D), as determined by ELISPOT. Diamonds represent findings for individual control subjects, whereas results for subjects with LTBI are represented by circles. In all cases, dark horizontal lines indicate median values and shaded boxes indicate the range of 25th percentile to 75th percentile results. As detailed in the text, individuals with LTBI display higher frequencies of IFNγ-producing PBMC responsive to ESAT-6, but not to PPD, CFP-10, or antigen 85B. In contrast, BAL cells of subjects with LTBI display significantly higher frequencies of IFNγ-producing cells responsive to PPD, CFP-10, and antigen 85B than do *Mtb*-naïve control subjects; however, BAL cell IFNγ responses to *in vitro* stimulation with ESAT-6 do not differ between the two subject groups.

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