Comparative Evaluation of Cytokines, T-Cell Apoptosis, and Costimulatory Molecule Expression in Tuberculous and Nontuberculous Pleurisy

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Abstract

In this study, we compared several immune parameters in tuberculosis (TB) and nontuberculosis (NTB) pleurisy to gain an understanding of the mechanism behind enhanced Th1 apoptosis that occurs at sites of active *Myobacterium tuberculosis* (*M. tuberculosis*) infection. An initial evaluation of the accumulated cytokines in pleural fluid (PF) demonstrated that both TB and NTB pleurisy were associated with proinflammatory cytokines, while only TB pleurisy had augmented expression of interferon (IFN)-γ and soluble Fas ligand (sFASL). Despite enhanced expression of the apoptosis-inducing molecule in TB pleurisy, T cells derived from both types of pleurisy exhibited significant apoptosis. In both groups, T-cell apoptosis correlated with low expression of CD80 on PF-derived macrophages and elevated accumulation of TGF-β in the PF. A causative correlation between TGF-β and low CD80 expression in the two groups was established by *in vitro* studies demonstrating TGF-β inhibition of CD80 upregulation in a macrophage cell line. Together, the findings allude to the possibility that activation in the absence of appropriate CD80 costimulation is the mechanism that leads to T-cell apoptosis at sites of active *M. tuberculosis* infection. Furthermore, the findings also indicate that T-cell apoptosis is perhaps a host regulatory mechanism to limit inflammation, rather than a pathogen-induced immune deviation.

Keywords: cytokines, infectious disease, inflammation

Introduction

With one-third of the world's population infected with *Myobacterium tuberculosis* (*M. tuberculosis*), tuberculosis (TB) remains a major threat to public health.[1] Although much is known about the immunology of TB, the precise nature of the protective immune mechanisms against the tubercle bacillus has not been completely defined. Nevertheless, existing evidence suggest that cellular Th1-mediated immunity plays a critical role in host defense against *M. tuberculosis*. The protective role of Th1 cell-mediated immune response in TB is perhaps best illustrated by the enhanced susceptibility to *M. tuberculosis* in individuals with human immunodeficiency virus (HIV) infection, a disease characterized by profound loss of CD4 T cells, including the Th1 subtype.[2]

Pathogens induce apoptosis of macrophages[3] and T cells[4,5] for enhancing their survival in the host and also as a means of evading host immune response. Studies designed to examine the significance of apoptosis in tuberculosis have focused on macrophages, the primary host cell of the tubercle bacillus, and also on T cells, the primary effector cell mediating protection. Although it remains to be proven definitively, these research efforts have collectively yielded the hypothesis that apoptosis of *M. tuberculosis*-infected macrophages may constitute a host defense mechanism against the tubercle bacillus.[6,7] *M. tuberculosis* has evolved strategies to inhibit macrophage apoptosis for its survival,[8] and virulent strains evade apoptosis of infected alveolar macrophages.[9] The recognition of the presence of antiapoptotic genes in the genome of *M. tuberculosis* is a strong evidence that induction of macrophage death is an immune evasion strategy for this pathogen.[10] Enhanced apoptosis of macrophages, however, is also important for initiating adaptive immunity to *M. tuberculosis* in the host.[11] Indeed inactivation in *M. tuberculosis* of a gene that encodes a component of a virulence-associated protein secretion system (secA2) resulted in enhanced macrophage apoptosis and increased priming of adaptive immunity to *M. tuberculosis*.[12]

In contrast to the role of macrophage apoptosis as a host defense mechanism, apoptosis of T cells seen in murine[13,14] and human[15] TB may instead attenuate the host immune response to *M. tuberculosis*. Given the significance of Th1 immunity in host defense against *M. tuberculosis*, it is not unreasonable to postulate that the tubercle bacillus, notorious for persistence in the host, may have evolved mechanisms to attenuate the protective Th1 immune response during tuberculous infection. Indeed, T cell hyporesponsiveness has long been recognized in individuals with acute TB. In humans, it has long been known that apoptosis occurs in tuberculous tissues.[16,17] In *in vitro* studies using peripheral blood mononuclear cells from tuberculous patients,[18] the well-described phenomenon of T cell hyporesponsiveness has been linked to spontaneous or *M. tuberculosis*-induced apoptosis of T cells. The observed apoptosis is associated with diminished *M. tuberculosis*-stimulated IFN-γ and IL-2 production. A relationship of Th1 apoptosis to disease progression is also demonstrated in mice infected with virulent *M. tuberculosis*.[19] These results therefore suggest that apoptotic pathways operative during tuberculous infection could lead to the depletion of *M. tuberculosis*-reactive T cells, thereby contributing to the immunopathogenesis of TB. A detailed analysis of paraformaldehyde-fixed human tuberculous tissues revealed that apoptotic $CD3^+$ and $CD45RO^+$ (a marker of T-cell activation) cells are present in productive tuberculous granulomas, particularly those harboring a necrotic center.[20]

The objective of the present study was to examine the mechanism behind the enhanced Th1 apoptosis that occurs at sites of active *M. tuberculosis* infection.

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Materials, Study Populations, and Methods

Patient population

During the study period, 45 patients with pleural effusions were recruited from the Government General Hospital (GGH), Chennai. Clinical signs and symptoms, demographic data, and radiologic results were recorded. The patients recruited were newly diagnosed for pleural involvement with normal immune function and were not relapsed cases. The age of these patients varied from 20 to 75 years. These patients were found to be seronegative for HIV and were grouped into tuberculous (TB) and nontuberculous (NTB) pleuritis based on various diagnostic criteria.

The TB group consisted of 27 patients (19 men and 18 women; average age 36 years; range 19–65 years) with lymphocyte predominance exudative effusions. The diagnosis for tuberculosis was on the basis of positivity to any two of the following criteria: (i) smear positivity for sputum/pleural fluid (PF) for *M. tuberculosis* (Ziehl–Neelson method), (ii) culture positivity for the growth of *M. tuberculosis* on Lowenstein–Jensen medium, (iii) clinical picture that include chest X-ray and clinical symptoms for tuberculosis, (iv) PCR positivity for the presence of insertion sequence IS6110 specific for *M. tuberculosis*, and (v) favorable response to antituberculosis treatment (ATT) during the first3 months of follow-up period. The samples were collected before the start of the treatment.

The NTB group consisted of 18 patients (15 men and 2 women; average age 52 years; range 25–75 years) with pleural effusions due to malignancy, congestive cardiac failure, and liver disorders. The exudative effusion with lymphocytic predominance was found in malignancy cases, whereas transudative effusion was observed in cardiac, hepatic, and kidney failure cases and in secondary infection in systemic lupus erythematosis (SLE). The PFs or bronchoscopy specimens were subjected to cytological/histology examination for malignant cells and/or other etiology. Other cases were diagnosed on the basis of their compatible clinical presentation and radiological findings. When no diagnosis could be made with all available information, the effusion was deemed idiopathic. The diagnosis was retrospectively proven as the disease was cured following the appropriate therapy.

The study was approved by the institutional ethical committee of Tuberculosis Research Centre (study number TRC/2003/7/Imm/4), Chennai, and followed the ethical guidelines of GGH, Chennai. A written and informed consent was obtained from each patient. An approval was also obtained from the institutional review board at the University of Medicine and Dentistry of New Jersey.

Sample collection and processing

Diagnostic thoracocentesis for pleural aspiration was performed. The volume of PF collected from each patient ranged from 100 to 700 mL. The collected PF was immediately processed to separate the cell-free PF, which was subsequently stored in aliquots at –70°C and thawed at the time of cytokine assays. Cells/CMM ranged from 15 to 230 in the TB group and from 150 to 700 in the NTB group.

Measurement of cytokines and soluble FasL

We assayed the various secretory cytokines in PF of both the groups using the cytometric bead array (CBA) (BD Biosciences Pharmingen, San Diego, CA, USA). The human cytokine kit-II was used to quantify the levels of IFN-γ, TNF-α, IL-4, and IL-10 according to the manufacturer's instructions. Briefly, 50 µL of standard or sample was mixed with 50 µL of premixed capture beads and 50 µL of PE-labeled detection reagent. After 3 hours of incubation at room temperature, excess of detection reagent was removed by washing and the samples were analyzed on FACS using the CBA software from BD Biosciences Pharmingen. The concentrations of samples were calculated by extrapolating the mean fluorescence intensity (MFI) on the respective standard curves. The lower and upper detection limits were 20 and 5,000 pg/mL for these cytokines. The IL-6, TGF-β, IL-12p40, IL-1β, and soluble FasL levels were measured by ELISA (R & D Systems, Minneapolis, MN, USA). The samples were diluted (1 in 5) with assay diluent for all cytokines except IL-6 (1 in 50) and TGF-β (1 in 10). The latent TGF-β present in the samples was acid activated (2.5 N acetic acid/10 M urea), mixed well, incubated and neutralized with base (2.7 N NaOH/1 M HEPES), and subsequently used for the assay of TGF-β. The cytokine concentrations in specimens were determined by referring to a standard curve generated with standards. The average of the duplicate readings was taken as the final concentration and expressed as pg/mL or ng/mL, as appropriate. The assay ranges (in pg/mL) for cytokine ELISA were as follows: IL-6 (4.7–300), TGFβ (62.5–4,000), IL-12p40 (31.2–2,000), IL-1β (3.6–250), and sFasL (31.25–2,000).

Mononuclear cell preparation

Pleural fluid mononuclear cells (PFMC) were isolated from the PF by Ficoll–Hypaque (Amersham Biosciences, Uppsala, Sweden) density gradient centrifugation at 1800 rpm for 30 minutes. The cells were then washed with HBSS (BioWhittaker, Petit Rechain Belgium) and RPMI 1640 (Sigma Chemical Co, St. Louis, MO, USA) at 1,500 rpm for 10 minutes. The PFMC were reconstituted in RPMI 1640 with 10% FCS to get 1×10^6 cells/mL. The viability of the cells was assessed by the trypan blue exclusion method.

Purification of $CD2^+$ and $CD14^+$ cells

The $CD2^+$ T cells and $CD14^+$ monocytes were isolated from fresh 20×10^6 PFMC by positive selection using anti-CD2- and anti-CD14-conjugated magnetic microbeads and purified using MACS column according to the manufacturer's instruction (Miltenyi Biotech, Auburn, CA, USA). The resulting cell population was >95% pure, as assessed by flow cytometric analysis using anti-CD3, -CD4, and -CD14 antibodies.

Spontaneous and induced apoptosis

The 48-well culture plates (Sigma-Aldrich, St. Louis, MO, USA) were prepared for the initial stimulation of CD2-purified T cells by coating wells with 100 µL of PBS containing anti-CD3 (1 µg/mL) for 2 hours at room temperature and washed with Ca^{2+}/Mg^{2+}-free PBS. The wells were seeded with 1×10^6 cells/mL and incubated for 4 hours at 37°C. The spontaneous and induced apoptosis were measured using APO LOGIX kit (Cell Technologies, Mountain View, CA, USA). The cells were treated with 10 µL of 30× working dilution of FAM-peptide-FMK 1 hour before termination. The cells were washed and acquired on flow cytometer. The uninduced control gave the spontaneous apoptosis.

Expression of costimulatory molecule

The CD14-purified monocytes from the PF or THP-1 cells (human macrophage cell line) were directly labeled with anti-human CD14-FITC, CD80-PE, and CD86-PE-Cy5 (BD Biosciences) and incubated at 4°C for 30 minutes. The stained cells were washed twice with 1X PBS and fixed with 1% paraformaldehyde. The

specificity of the staining technique was assessed using nonspecific isotype-matched control. The THP-1 cells were reacted with CD80-PE and CD86-APC (BD Biosciences). The pleural cells were analyzed on FACSCallibur (BD Biosciences) using the Cell quest pro software, and the THP-1 cells were analyzed on LSR2 (BD Biosciences) using the DiVa software.

Statistical analysis

Data are represented as bar graphs in the figures. Comparisons between groups were done by an unpaired *t*-test using GraphPad Prism (version 4; La Jolla, CA, USA). $p < 0.05$ was considered to be statistically significant.

Results

Augmented Th1 cytokines in TB pleurisy

We first determined the levels of the Th1 cytokines IFN-γ and IL-12p40 in pleural effusions and found that the levels of both these cytokines were significantly upregulated in the TB group compared to the NTB group (*Figure 1*). These data support and extend previous observations of Th1 activation in the pleural cavity in TB patients.[15,18,21–23] The absence of Th1 cells in the NTB group provided us an opportunity to examine whether apoptosis is confined to the Th1 phenotype *in vivo*, akin to what was previously observed by us *in vitro*.[24]

To confirm that inflammation was present in the pleura of NTB patients, we also examined the pleural effusion for the levels of the proinflammatory cytokines TNF-α, IL-1β, and IL-6. In contrast to what was observed with the Th1 cytokines, both groups exhibited similar levels of all three proinflammatory cytokines tested (*Figure 2*). This suggests that inflammation is present in both TB and NTB pleural cavity, but the former is Th1 dominant.

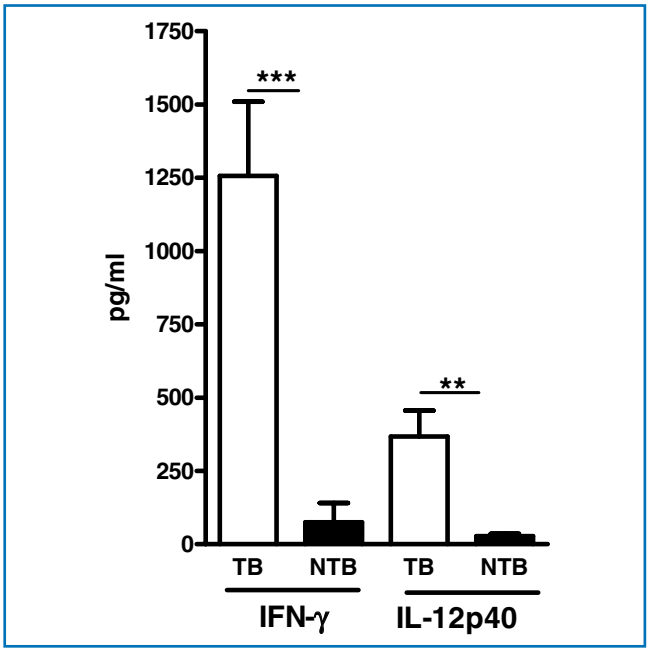

Figure 1. Unequal expression of IFN-γ and IL-12p40 in tuberculous and nontuberculous pleuritis. Pleural fluid levels of IFN-γ and IL-12p40 was assessed by cytometric bead array and ELISA, respectively. The levels were quantified in 27 tuberculous (TB) and 18 nontuberculous (NTB) patients and the concentration of the cytokines is expressed in pg/mL. Data are represented as mean ± standard error of mean (SEM). *Represents $p < 0.05$.

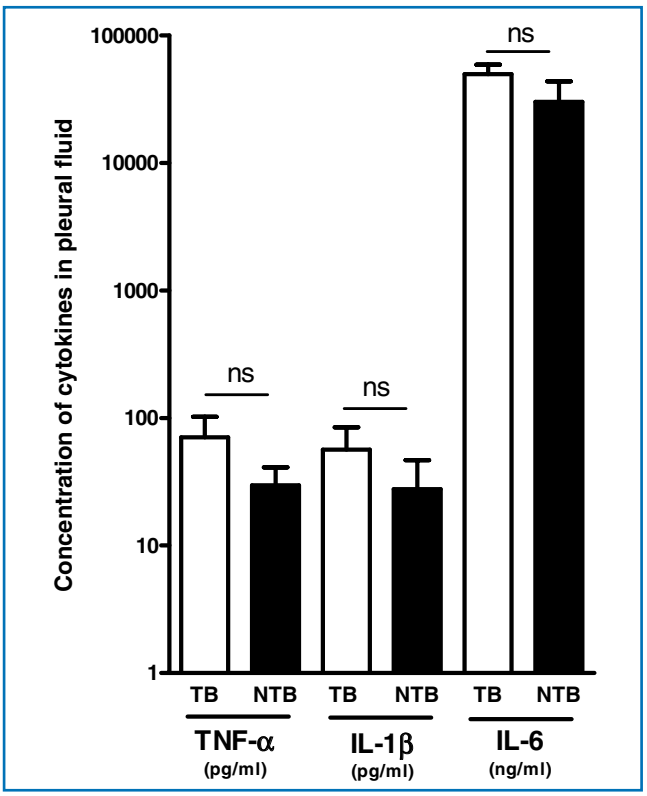

Figure 2. Equivalent level of proinflammatory cytokines in TB and NTB pleural fluid. The mean levels of the cytokines IL-1β and IL-6 were assayed by ELISA and TNF-α by cytometric bead array in pleural fluid of 27 tuberculous (TB) and 18 nontuberculous (NTB) subjects. Only the IL-6 estimation was carried after 1:50 dilution of the samples in reagent diluent and its concentration is expressed in ng/mL. The bars represent mean ± standard error of mean (SEM).

Similar pattern of spontaneous and activation-induced apoptosis of T cells isolated from TB and NTB PF

T cells from both groups exhibited spontaneous apoptosis, and furthermore, CD3 stimulation enhanced activation-induced apoptosis in both groups (*Figure 3*). These data, in contrast to the *in vitro* observations,[24] indicate that apoptosis is not Th1 specific under *in vivo* conditions of activation.

To further understand the cause of the enhanced T cell apoptosis, we determined the level of sFasL, a known mediator of apoptosis, in the pleural effusion from the two groups. Despite similar apoptosis profiles, sFasL level was significantly higher in TB compared to NTB PF (*Figure 4*). These data indicate that other factors may be responsible for the enhanced apoptosis seen in NTB PF.

Low CD80 expression on $CD14^+$ cells isolated from TB and NTB PF

Although the antigen specificity of the T cells in the NTB PF is not known, we argued that antigen presentation to T cells in the absence of second signals from CD80 and CD86 may be the cause of enhanced T-cell apoptosis in the inflamed pleura. The expression of CD80 and CD86 was therefore analyzed on $CD14^+$ cells isolated from the PF of TB and NTB patients. As shown in *Figure 5*, the majority of $CD14^+$ cells isolated from the PFs of both TB and NTB patients expressed CD86, while only 10% of the cells from both groups expressed CD80. The percentage of cells expressing CD80 is significantly upregulated in monocyte cultures when exposed to inflammatory stimuli.[25] Thus, despite elevated levels of inflammatory cytokines, TNF-α and IFN-γ in the pleural effusions, CD80 expression was not upregulated in the

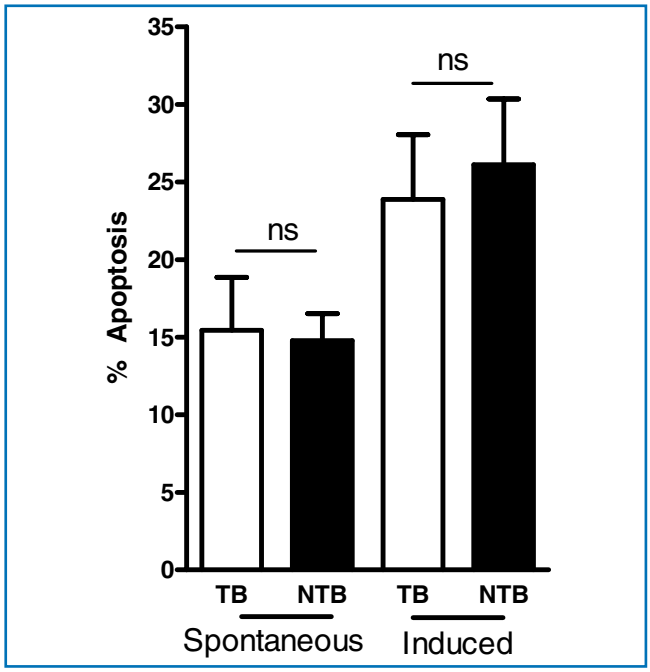

Figure 3. Spontaneous and activation-induced apoptosis of T cells isolated from TB and NTB pleural fluid. Induced apoptosis of CD2 purified T cells from pleural fluid was assessed by pulsing with 0.5 μg of anti-CD28 (0.5 μg/mL) and cultured with anti-CD3 (1 μg/mL). The spontaneous and anti CD3/CD28-induced apoptosis was evaluated by measuring the cleaved caspase proteins after incubation with FAM-peptide-FMK by flow cytometry. The assay was carried out in 18 tuberculous (TB) and 9 nontuberculous (NTB) subjects and the results are expressed as mean ± standard error of mean (SEM).

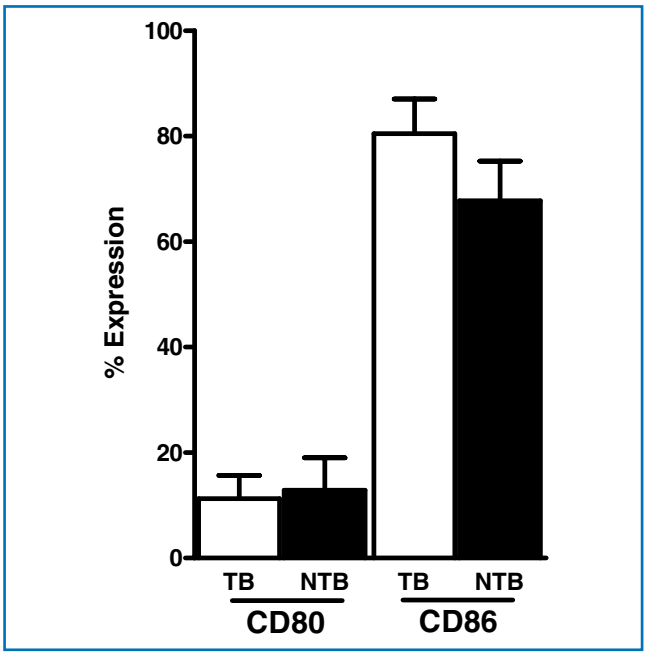

Figure 5. Expression of costimulatory molecules on CD14⁺ cells isolated from pleural fluid of tuberculous and nontuberculous pleuritis. The CD14 purified monocytes from the pleural fluid were directly labeled with anti-human CD14-FITC, CD80-PE, and CD86-PE-Cy5 and their expression were assessed by flow cytometry in 22 tuberculous (TB) and 16 nontuberculous (NTB) subjects. The bars represent the mean percentage expression of CD80 and CD86 molecules on monocytes ± standard error of mean (SEM).

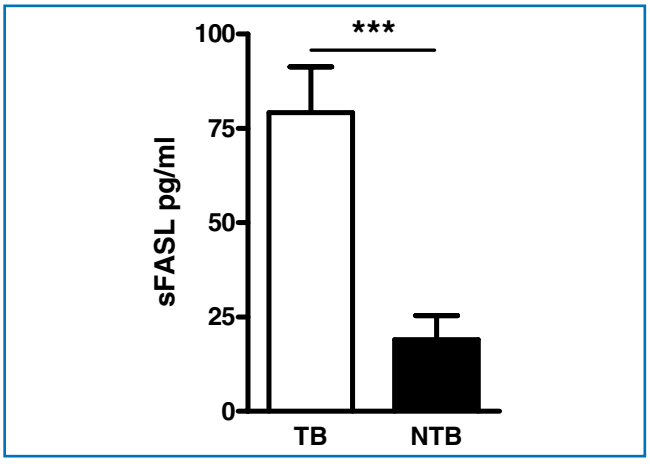

Figure 4. Soluble FasL levels in tuberculous and nontuberculous pleural fluid. The soluble FasL in pleural fluids was estimated by ELISA in 27 tuberculous (TB) and 18 nontuberculous (NTB) subjects. The concentration is expressed in pg/mL and the data are represented as mean ± standard error of mean (SEM). *Represents $p < 0.05$.

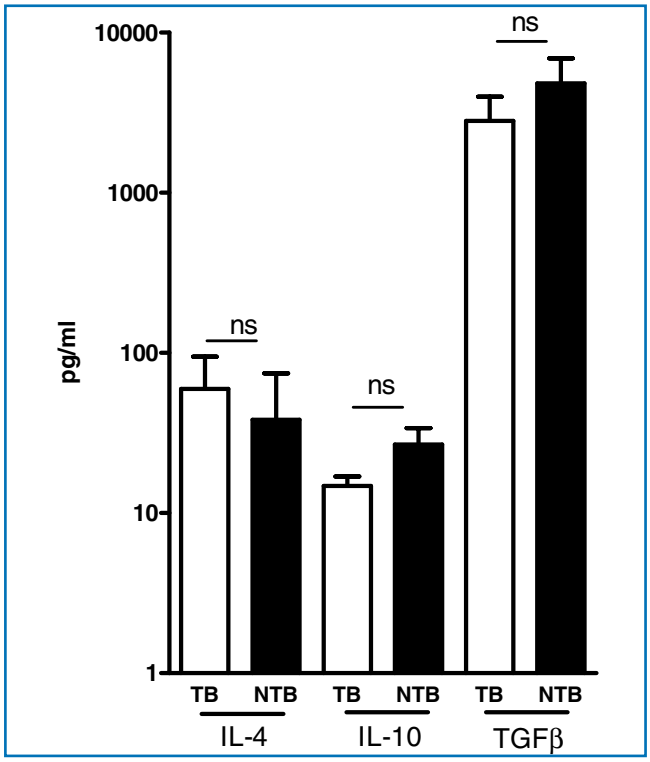

Figure 6. The expression of immunosuppressive cytokines in tuberculous and nontuberculous pleural fluid. The mean levels of the cytokines IL-4 and IL-10 were assayed by CBA and TGF-β by ELISA in pleural fluid of 27 tuberculous (TB) and 18 non tuberculous (NTB) subjects. The estimation of TGF-β was carried after 1:10 dilution of the samples in reagent diluent. The bars represent mean ± standard error of mean (SEM).

CD14⁺ cells derived from both groups, suggesting an association of low CD80 expression with enhanced T-cell apoptosis.

TGF-β regulates CD80 expression

To gain insight into the mechanism behind the lack of CD80 upregulation on CD14⁺ cells, we examined the levels of TGF-β, IL-10, and IL-4, which are the known downregulators of CD80 and CD86 expression. In both groups, equivalent amount of IL-4, IL-10, and TGF-β accumulated in the PF but the level of TGF-β was significantly elevated (*Figure 6*). To determine if there was

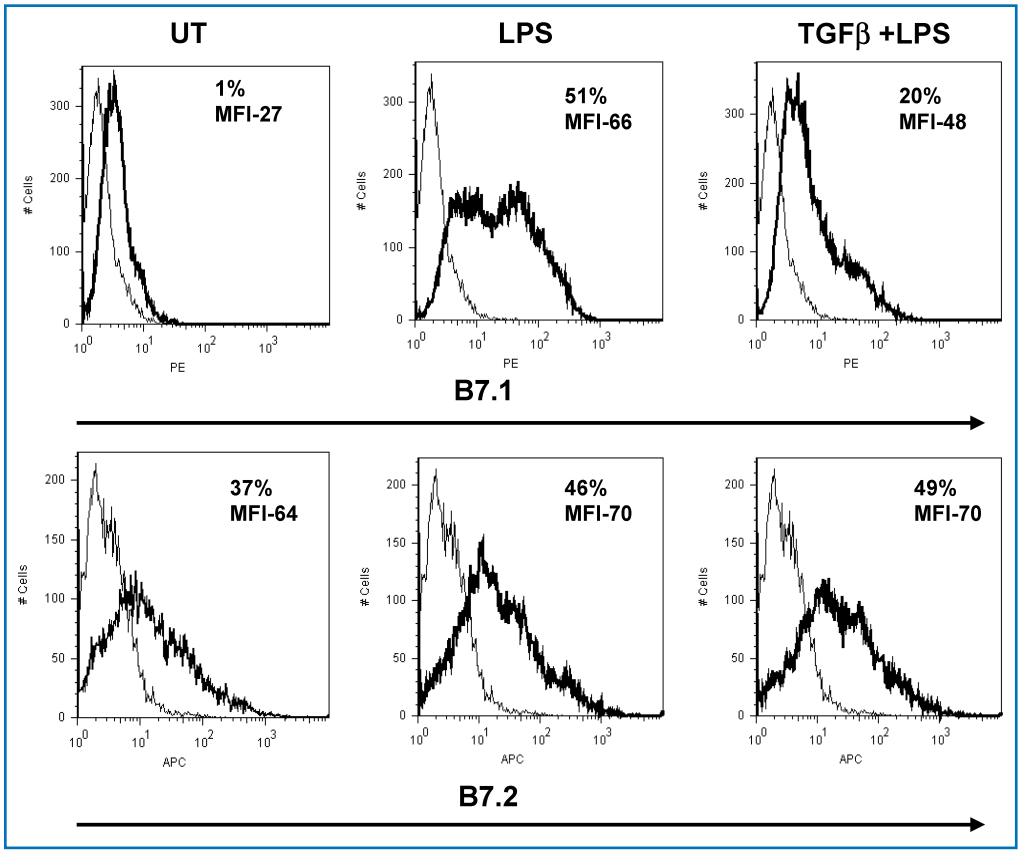

Figure 7. Effect of TGFβ on CD80 and CD86 expression by LPS-stimulated macrophages. A total of 1 × 10^6 THP-1 cells were pretreated with 1 ng/mL of TGF-β (BD Biosciences) for 2 hours, followed by 1 μg/mL LPS (Sigma) for 24 hours. Following which the cells were harvested, reacted with PE-conjugated anti-CD80 and CD86 antibodies (black-colored histogram) and isotype control (gray-colored histogram), and analyzed by flow cytometry for CD80 and CD86 expression. MFI = mean fluorescence intensity. The data are representative of one of three individual experiments.

a causal relationship between the reduced CD80 expression and elevated TGF-β level in the PF, we examined *in vitro* whether TGF-β downmodulated the expression of CD80. This was tested in the human macrophage cell line, THP-1. Examination by flow cytometry showed that only 1% of unstimulated THP-1 cells expressed CD80, and that the expression increased to 51% following stimulation with LPS, but to only 20% when stimulated in the presence of TGF-β (*Figure 7*, upper three panels). In contrast, the THP-1 cells exhibited constitutive CD86 expression, and this was not further modulated by LPS stimulation, with or without TGF-β (*Figure 7*, lower three panels). The MFI also paralleled the percentage of positive cells for the two costimulatory molecules under the different stimulating conditions (*Figure 7*). These data suggest that TGF-β abrogates upregulation of CD80 expression on macrophages, while not interfering with the constitutively expressed CD86 molecules.

Discussion

In this study, we show that while both TB and NTB effusions are characterized by a strong inflammatory response, it is only TB effusions that exhibit a strongly Th1-polarized response, with significantly high levels of IL-12 and IFN-γ, consistent with previous data.[26–28] The cellular source of the IFN-γ in TB effusions could be both Th1 and NK cells, and most likely driven by the presence of IL-12 in the effusions. In addition, *M. tuberculosis* can directly ligate TLRs on NK cells and induce IFN-γ secretion,[29] providing an additional means to enhance IFN-γ secretion from NK cells. Despite the expression of a strong proinflammatory response, the NTB effusions showed neither a Th1- nor a Th2-dominant cytokine milieu. A similar observation was made in a study comparing malignant and tuberculous pleurisies, where PF from malignant patients lacked IFN-γ expression.[30] However, addition of IL-12 and IL-18 to T cells explanted *in vitro* from malignant PF enhanced their IFN-γ production.[30] In the present study, we did not examine whether external factors would enhance IFN-γ secretion from T cells derived from NTB effusions. Nevertheless, it raises an interesting possibility that T cells in malignant effusions are Th1-like and therefore susceptible to spontaneous and induced apoptosis, akin to fully differentiated Th1 cells present in TB effusions.

Another striking difference in the pleural effusions derived from the two groups of patients was the increased level of sFASL only in TB effusions, providing a mechanism for several previous studies that have reported enhanced T-cell apoptosis in TB PF cells. A recent study similarly reports that sFasL is detectable only in tuberculosis pleurisy and not in other conditions of clinical pleurisy.[31] However, we observed that despite low sFASL in NTB effusions, cells derived from these sites exhibited both spontaneous and induced apoptosis. This suggests that apoptotic death of T cells is an important regulator of inflammation that is caused not only from bacterial infection but also from other nonbacterial factors, and furthermore, mediators other than sFasL may contribute to the observed apoptosis.

Previously, we had reported that Th1 cells are highly susceptible to activation-induced apoptosis when costimulation from antigen-presenting cells is lacking,[24] and provision of costimulation by ligation of CD28 molecules rescued them from activation-induced apoptosis.[32] Furthermore, murine macrophages infected with *M. tuberculosis* express reduced levels of costimulatory molecules.[33] Based on these studies, we argued that the enhanced apoptosis occurring in TB pleurisy is not due to increased expression of mediators of apoptosis but due to attenuation of costimulation from macrophages. CD80 and CD86 are two important costimulatory molecules that T cells engage on antigen-presenting cells, and the former is upregulated following macrophage activation by TLR agonists and TNF-α, while the latter is constitutively expressed.[34] Examination of costimulatory molecules on purified CD14+ macrophages revealed that CD86 was expressed by a majority of the cells, while only a small percentage expressed CD80. So

it is very likely that *in vivo*, in the pleural compartment, the CD80-attenuated macrophages do not provide appropriate second signal to T cells, which results in their enhanced apoptosis.

Findings from the present study and that of others[35] demonstrate that TGF-β interferes with CD80 upregulation on antigen-presenting cells, suggesting that *in vivo* also this cytokine may be responsible for the attenuated CD80 expression on macrophages in both TB and NTB pleural effusions. Although, lower levels of IL-4 and IL-10 are detected in the pleural effusions, it is nevertheless possible that these two cytokines have significant biological activity *in vivo* and work together with TGF-β to alter CD80 expression on macrophages. Consistent with TGF-β's role in T-cell apoptosis, Hirsch and colleagues also report that excessive expression of this cytokine along with enhanced levels of TNF and FasL predisposes CD4 T cells derived from TB patients to apoptosis.[36]

Together, the data presented here suggest that a deviation in the expression of CD80 molecules most likely underlies the enhanced T cell apoptosis seen in pleural effusions. Interestingly, the requirement for second signal to resist apoptosis is not confined to the Th1 subtype, since T cells from NTB that are not Th1 polarized also undergo apoptosis under conditions of diminished CD80 expression on macrophages. These findings raise the possibility that apoptosis of T cells *in vivo* during an inflammatory response in the lung may be a way to prevent excessive inflammation and protect the host from tissue damage, rather than an immune deviation strategy of *M. tuberculosis*.

Acknowledgments

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