ERJ Express. Published on November 1, 2006 as doi: 10.1183/09031936.00111205

Lung and blood mononuclear cell responses of TB patients to mycobacterial proteins

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Running title **– Lung and blood immunity in tuberculosis**

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Informed consent was obtained from all tuberculosis and non-tuberculosis patients participating in this study. Ethical approval was obtained from the Ethics Committee of Postgraduate Institute of Medical Education and Research, Chandigarh, India

Authors do not have a commercial or other association that might pose a conflict of interest.

Financial Support –This study was funded by a grant from Department of Biotechnology and Indian Council of Medical Research, Govt. of India (New Delhi, India). S B. Sable is the recipient of a Senior Research Fellowship of the Council of Scientific and Industrial Research (New Delhi, India).

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Abstract

The differences in specificity of human lung and peripheral lymphocytes for the mycobacterial antigens need to be evaluated to identify vaccine candidates against pulmonary tuberculosis (TB).

Therefore, responses to low molecular weight secretory proteins of *Mycobacterium tuberculosis* were examined in bronchoalveolar lavage (BAL) and peripheral blood mononuclear cells (PBMCs) from minimal pulmonary TB and non-TB patients.

Ag85A, Ag85B, CFP-31, CFP-22.5, CFP-21, MPT-64 and as yet uncharacterized 19 kDa protein were found to be predominantly recognized by BAL cells of TB patients on the basis of lymphocyte proliferation and significant IFN- γ release. However, recognition of CFP-8, ESAT-6, CFP-10, CFP-14.5, MTSP17 and five other as yet uncharacterized low molecular weight polypeptides was found to be high on the basis of lymphocyte proliferation at the level of PBMCs. Further, BAL macrophages and not blood monocytes were found to produce nitric oxide in response to mycobacterial antigens. Among polypeptides predominantly recognized by BAL lymphocytes, only Ag85A and B were found to induce both nitric oxide and IL-12 (p40) by alveolar macrophages.

These results indicate heterogeneity in antigen recognition by BAL cells and PBMCs of minimal TB patients and also suggest the utility of Ag85 complex polypeptides for the development of future mucosal antituberculous vaccine.

Key words – Immune response, Low molecular weight polypeptides, Lung, Peripheral blood, Tuberculosis, Vaccine.

Introduction

For the rational design of new efficacious and safe vaccines against tuberculosis, it is essential to define and characterize the specificities of T-lymphocytes for mycobacterial antigens, which can be related to protective or deleterious responses. Most studies of the human immune responses to mycobacterial antigens have been focused on blood cells [1-8]. Tuberculosis, however, is predominantly a lung disease and local mucosal immune responses play an important role against invasion and subsequent spread of causative tubercle bacilli. Therefore, studies involving characterization of immune responses to mycobacterial antigens from the actual site of infection are needed.

Limited information is available regarding the differences between mycobacterial antigens recognized by T-lymphocytes from peripheral blood and from the site of infection [9, 10]. The capacity of different mycobacterial antigens to either activate or down regulate alveolar macrophages, the cells that primarily act as habitat for tubercle bacilli is also largely unknown. We, however, have recently observed that the identical repertoire of mycobacterial antigens from the low molecular mass secretory proteome are recognized by peripheral blood mononuclear cells (PBMCs) of healthy TB contacts or treated TB (memory immune) subjects and pleural fluid mononuclear cells (PFMCs) of tuberculous pleurisy patients [11]. These findings lead us to conclude that considerable homogeneity exists between the specificities of PBMCs and PFMCs for

mycobacterial antigens in human subjects representing a state of protective immunity. However, the lung and alveolar cavity represents the true site of infection as compared to pleura or peripheral blood and to date; no report is available regarding the fine specificities of bronchoalveolar lavage (BAL) cells to mycobacterial antigens in TB subjects. Moreover, comparative specificities of autologous blood and BAL cells to mycobacterial antigens are not studied in detail. Therefore, in the present study, local (BAL cell) immune responses to mycobacterial antigens were compared with systemic (peripheral blood) immune responses in minimal TB patients.

Materials and Methods

Study subjects

Minimal TB patients [n=21] and non-TB patients [n=10] having an indication for fiberoptic bronchoscopy, examined and diagnosed at Department of Pulmonary Medicine, PGIMER, and Chandigarh, India were used as study subjects. A chest roentgenogram was obtained at the start of the study and the patients were graded as minimal TB patients according to National Tuberculosis Association criteria [12]. In brief, patients classified as having minimal TB had only one or few lesions on chest X-ray that did not involve more than one segment of lung, and usually had milder symptoms for a shorter period of time than patients with severe disease (4). Active pulmonary TB was confirmed by positive sputum smear or sputum/BAL culture for acid-fast bacilli. Exclusion criteria for TB patients included disseminated or advanced TB, pulmonary

diseases other than TB or associated immunocompromized conditions including HIV infection and the start of anti-tuberculous drug therapy. Non-TB group included patients suffering from oesophagitis or haemaptysis of unknown etiology. These subjects did not show any confirmed clinical, radiological or microbiological evidence of TB. Exclusion criteria for non-TB patients included pulmonary diseases including bronchial asthma or upper respiratory tract infections in preceding 8 weeks, immunosuppressive diseases or medications. All the study subjects received BCG as childhood vaccination and were from the north Indian states with high incidence of disease. The Mantoux tests were not performed due to the poor specificity of the test in this population and the unwillingness of most donors to remain at the clinic for several days for test to be read. All the non-TB patients had no known contacts with TB patients. However, 33.3% (3/9) and 66.6% (6/9) of non-TB patients responded to PPD in *invitro* lymphocyte proliferation assay employing BAL cells and PBMCs respectively and their exposure to environmental mycobacteria is not known. However, widespread exposure to environmental mycobacterial species in India and in this region is well hypothesized for failure of BCG vaccination and interference with responses to *M. tuberculosis* [13]. Bronchoscopy [14] and venipuncture was carried out after written consent from all the study subjects and the Institutional Ethics Committee approved the study.

Isolation of BAL cells and PBMCs

Bronchoscopy was performed by an Olympus fiberoptic bronchoscope as described previously [14] as a part of confirmatory procedure for TB. Sterile isotonic saline (100ml) was instilled into the lungs after local anesthesia of upper airways with 2% lignocaine and the percentage of retrieved saline was 70±10% in both the groups. The bronchoalveolar cells were obtained from BAL as described by others [9, 15]. The cellular profiles of BAL cells in TB and non-TB patients are shown in table 1. Venous blood was obtained from all the study subjects and PBMCs were isolated by centrifugation of heparinized blood over Ficoll-Hypaque. BAL cells and PBMCs were adjusted to 10^6 cells/ml after determining the viability of cells by trypan-blue exclusion method.

Antigens

Total culture filtrate proteins of *M. tuberculosis* H_{37}Rv were obtained by stationary growth of tubercle bacilli in liquid modified Youman's medium [16] for 4 weeks and were termed as RvCFP. In a previous study, 104 purified mycobacterial polypeptides (<40kDa) were evaluated for the immune responses induced in healthy TB contacts [11]. Based on the type of immune responses, immunodominant polypeptides were divided in two groups. Group- I consisted of 18 low molecular weight polypeptides prominently recognized by peripheral blood T-lymphocytes while group-II consisted of 10 low molecular weight polypeptides recognized by both T and B lymphocytes of healthy TB contacts

[11]. All the individual polypeptides used in the study were purified using combination of the anion exchange column chromatography and the high-resolution preparative SDS-PAGE followed by electroelution [11] and is described in table 2.

Lymphocyte proliferation assay

BAL cells and PBMCs were added to flat-bottom 96 well tissue culture plates (Greiner Bio-one, Germany) at 1 x 10^5 cells/well in RPMI-1640 medium (Sigma, St. Louis, MO, USA) supplemented with 50 µg/ml gentamicin sulphate, 200 mM L-glutamine, 5 x 10^{-5} M β-mercaptoethanol, 25 mM HEPES (Fluka, Switzerland), 1 mM sodium pyruvate and 10% heat inactivated autologous serum. Selected purified polypeptides (2 µg/ml), RvCFP and PPD (2 µg/ml) and the mitogen PHA (1 µg/ml) were used for *in vitro* stimulation. Cultures were incubated for 5 days at 37^0C in an atmosphere containing 5% CO_2 and 100% humidity. At the end of the incubation period, 0.25 µCi [^{3}H]-thymidine was added followed by incubation for 18-22 hr. The cells were harvested on to glass fiber filters using the Nunc cell harvester (Intermed, Denmark) and the incorporated radioactivity was measured in LKB Rack Beta liquid scintillation counter (Model 1214; LKB-Wallac, Palo Alto Calif).The proliferation responses were expressed as stimulation indices (SI). SI was calculated by dividing mean counts per minute in antigen-stimulated wells by mean counts per minute in unstimulated wells. SI of 3.0 was used as a positive cut off value to determine the

percentage recognition of different mycobacterial antigens by PBMCs and BAL cells.

IFN-γ and IL-12 (p40) assay

Levels of IFN-γ released by PBMCs and BAL cells in response to the individual antigen were estimated in culture supernatants collected on day 5 using human IFN-γ reagent set (Opt EIA™ set, BD Pharmingen, USA). The assay was performed as per the manufacturer's instructions. Quantitative IL-12 (p40) measurements were performed similarly with 24hr supernatants from PBMC and BAL cell cultures using human IL-12 (p40) reagents (Duoset R& D system). The lower limit of detection for IFN-γ was 2.35 pg /ml and for IL-12 (p40) was 16.0 pg/ml.

Nitric oxide (NO) assay

BAL cells ($1x10^5$ cells/well) were cultured for different time intervals ranging from day 0 to day 10 in DMEM (phenol red free; Sigma, St.Lous, MO, USA) supplemented with 50 µg/ml gentamicin sulphate, 200 mM L-glutamine, 2.5 mM L-arginine and 10% heat inactivated autologous serum. *M. tuberculosis* RvCFP, PPD and selected purified polypeptides were used at a concentration of 2 µg/ml while *E. coli* lipopolysaccharide (LPS) was used at the concentration of 10 µg/ml for the in *vitro* stimulation. NO in terms of nitrite released in culture supernatants was estimated using Griess reagent as described by Green *et al* [17].

Statistical analysis

All the results were presented as mean values of triplicate wells. The data obtained was analyzed by the Mann-Whitney two-tailed test and the analysis of variance. $P<0.05$ was considered statistically significant.

Results

Lymphocyte responses of BAL cells and PBMCs to mycobacterial antigens

Lymphocyte proliferation in response to group-I (fig. 1) and group-II (fig. 2) antigens was assessed in BAL cells (A and C) and PBMCs (B and D) from minimal TB (A and B) and non-TB patients (C and D).

When proliferative responses of BAL cells of minimal TB patients in response to *in vitro* stimulation with group-I antigens were evaluated, the polypeptides 2, 14, 3, 18 and 15 induced prominent lymphocyte proliferation (median SI ≥ 3.0; fig.1A) and the maximum SI was observed for polypeptide 2, a putative ESAT-6 family member (table2) [median SI, 4.47; 58.82% recognition]. Although increased response was observed for all the polypeptides in TB patients, statistically no significant difference was observed when BAL cell responses of group-I polypeptides in TB patients (fig1A) were compared with BAL cell proliferative responses of non-TB patients (fig1C). Amongst group-II polypeptides, all the polypeptides except polypeptide 26 and 27 induced prominent BAL cell proliferation (median SI ≥ 3.0; fig.2A) and the maximum SI was observed in response to polypeptide 24 (Ag85 B; table 2) [median SI, 7.21;

70.58% recognition]. Significantly high proliferative response in BAL cells of TB patients (fig2A) as compared to non-TB patients (fig2C) was observed for polypeptides 22(MPT-64), 24(Ag85B), 23(Ag85A), 25 (CFP-31), 28 (CFP-11) ($p<0.01$) and polypeptide 19 (CFP-22.5) ($p<0.05$).

When proliferative responses of PBMCs of TB patients in response to stimulation with group-I antigens were evaluated, the top five polypeptides which induced prominent lymphocyte proliferation were polypeptide 9, 4, 3, 16 and 6 (median SI > 3.0; fig.1B) with maximum SI for polypeptide 9 (CFP-10, table2) [median SI, 3.84; 70.58% recognition]. On the other hand, when PBMC responses of group-II polypeptides were evaluated, none induced prominent lymphocyte proliferation (median SI≥ 3.0, fig2B) and the maximum SI was observed in response to polypeptide 27 (CFP12.5, table2) [median SI 2.86; 47.05% recognition].

When proliferative responses of PBMCs and BAL cells of TB patients in response to group-I polypeptides were compared, no significant difference was observed. However, the BAL cell responses of TB patients were significantly high as compared to PBMC responses for polypeptides 25 (CFP-31) ($p<0.001$), 22 (MPT-64), 24 (Ag85B), 23 (Ag85A) ($p<0.01$), 19(CFP 22.5), 20(CFP-21) and 21 ($p<0.05$) (fig2A and B) from group II polypeptides. Comparison of proliferative responses of BAL cells and PBMCs of non-TB patients demonstrated no significant difference for majority of mycobacterial antigens evaluated (fig1C and D; fig2C and D).

IFN-γ levels in response to group-I (fig. 3) and group-II (fig. 4) antigens was estimated in BAL cells (A and C) and PBMCs (B and D) culture supernatants from minimal TB (A and B) and non-TB (C and D) patients. BAL cells of TB patients were found to release significantly high levels ($p<0.01$ to $p<0.001$) of IFN-γ in *in vitro* culture supernatants than those of non-TB patients, in response to all mycobacterial antigens tested (fig3A and C; fig4A and C). Among group-I polypeptides evaluated, polypeptides 9 (CFP-10), 4 (ESAT-6), 6 (CFP-8), 7 and 2 (both putative ESAT-6 family members) were found to induce prominent IFN-γ release (median IFN-γ, ≥100.0 pg/ml, fig3 A) in BAL cell culture supernatants of TB patients with maximum IFN-γ release in response to polypeptide 9 (CFP-10) [median IFN-γ, 110.0pg/ml]. When group-II polypeptides were evaluated, polypeptide 24 (Ag85B), 23 (Ag85A), 22 (MPT-64), 19(CFP -22.5), 20 (CFP-21), 27 (CFP 12.5), 28 (CFP-11) were found to induce prominent IFN-γ release (median IFN- γ ≥100.0 pg/ml) in BAL cell culture supernatants with maximum response to polypeptide 24 (Ag85B) [median IFN-γ 240.0 pg/ml] (fig.4A).

In case of PBMCs of TB patients, maximum IFN-γ release in culture supernatant was observed for polypeptide 4 (ESAT-6) [median IFN-γ, 78.0 pg/ml] from group I (fig3B) and for polypeptide number 24 (Ag85B) [median IFN-γ 45.0 pg/ml] from group II (fig4B). The IFN-γ responses of BAL cells of TB patients were significantly high ($p<0.05$ to $p<0.001$) as compared to that induced by PBMCs in response to all antigens evaluated except

polypeptides 4 (ESAT-6), 5 (putative ESAT-6 family member), 10 (uncharacterized) and 11 (MTSP-17) (fig 3 A and B, Fig 4 A and B). No significant difference was observed for group-I and group-II antigens, when IFN-γ responses of BAL cells and PBMCs of non-TB patients were compared (fig 3 C and D, fig 4 C and D).

Though not statistically significant, the proliferative and IFN-γ responses of BAL cells of minimal TB patients in response to stimulation with group-II antigens were high as compared to group-I antigens. In group-I, 5/18 polypeptides demonstrated median SI in the range of 3.0-5.0 and median IFN-γ in the range of 100.0-150.0 pg/ml. However, in group-II, 8/10 polypeptides induced median SI in the range of 3.0-7.5 and 6/10 polypeptides induced median IFN-γ in the range of 100.0-250.0 pg/ml. On the contrary, although not statistically significant there was a trend toward increased recognition of majority of group-I antigens as compared to group-II antigens by PBMCs of TB patients. In group-I, 7/18 polypeptides demonstrated median SI > 3.0 and 4/18 polypeptides demonstrated median IFN-γ responses in the range of 50.0-100 pg/ml. However, in group II, all the 10 polypeptides induced median SI < 3.0 with median IFN-γ responses < 50.0 pg/ml.

Macrophage responses of BAL cells and PBMCs to mycobacterial antigens

Alveolar macrophages and blood monocytes activating ability of group-I and group-II CFPs was evaluated by estimating levels of nitric oxide (nitrite) and IL-12 (p 40) in the culture supernatants of BAL cells and PBMCs respectively. To determine the kinetics of nitric oxide release in culture supernatants of BAL cells in response to *in vitro* stimulus, BAL cells from active minimal TB patients were cultured with different stimuli i.e. *M. tuberculosis* H_{37}Ra (5:1 *M. tuberculosis* : BAL cells), RvCFP (10 µg/ml) and *E. coli* LPS (10 µg/ml) for varying period of time. Nitric oxide levels measured in terms of nitrite release were negligible at initial periods of 24 and 48 hr. Levels of nitrite release in response to RvCFP were found to be low when compared to whole bacilli (Data not shown). Since, significant levels of nitrite were observed by day seven; supernatants collected at this time point were used for the nitrite estimation in subsequent studies. On the other hand, studies carried out using blood monocytes derived macrophages produced negligible NO (nitrite) in culture supernatants (<4µM) when *M. tuberculosis* H_{37}Rv CFPs or purified polypeptides were used as *in vitro* stimulus (Data not shown). Fig. 5 depicts nitric oxide (nitrite) levels released by BAL cells of TB (A and C) and non-TB patients (B and D), in response to group-I (A and B) and group-II polypeptides (C and D).

When NO responses of BAL cells of TB patients in response to stimulation with group-I antigens were evaluated, the polypeptides 14, 5, 8, 4, 9 and 17 induced prominent NO release (median NO > 5.0 µM; fig.5A). The maximum NO

production was observed in response to polypeptide 14, yet uncharacterized antigen [median NO, 6.29]. Statistically no significant difference was observed for majority of the group-I polypeptides to induce nitrite in BAL cell cultures of TB and non-TB patients. However, for polypeptides 2, 10, 11(CFP 11) and 13, high nitrite response was observed in BAL cell cultures of non-TB subjects ($p<0.01$) as compared to that of TB patients, suggesting increased alveolar macrophage response for above polypeptide in non-TB patients (fig 5A and B). When NO responses of group-II antigens were evaluated in TB patients, the prominent nitrite release was observed for all the polypeptides except polypeptide 21, 22 and 26 (median NO > 5.0 µM; fig.5 C). The maximum NO release was induced by polypeptide 27, a mixed sample [median NO, 10.80] followed by polypeptide 24 (Ag 85 B) [median NO, 9.79]. However, Levels of nitrite release were found to be significantly higher in BAL cell cultures of TB patients for polypeptides 24 (Ag85B), 23(Ag85A), 27(CFP12.5), 28 (CFP11) ($p<0.01$) as compared to that induced in BAL cell culture of non-TB patients (fig 5 C and D).

Group-II polypeptides were found to release high levels of nitrite in BAL cell cultures of minimal TB patients as compared to group I polypeptides. Among group-II polypeptides, 7/10 polypeptides demonstrated high nitrite responses > 5.0 µM (median range 3.49-10.80 µM) while only 6/18 group-I polypeptides induced nitrite levels > 5.0 µM (median range 2.00-6.29 µM).

Simultaneously, BAL cells and PBMCs of minimal TB and non-TB patients were stimulated with mycobacterial antigens for 24 hr and IL-12 levels in culture supernatants were measured by sandwich ELISA. IL-12 (p40) levels above the detection limit were observed only for RvCFP, PPD, Ag85B and Ag85A in BAL cell cultures of minimal TB patients. None of the mycobacterial antigens tested induced significant IL-12 (p40) release in BAL cells or PBMCs culture supernatants of non-TB patients (Data not shown).Thus, this study demonstrate the increased recognition of antigen 85 complex (85A and B) polypeptides by BAL cells of minimal TB patients and is evident by high lymphoproliferative, IFN-γ, NO and IL-12 responses.

Discussion

Differences in mycobacterial antigen specificity of lung cells derived from bronchoalveolar spaces and mononuclear cells isolated from peripheral venous blood of human subjects have not been evaluated. Therefore, in this study eighteen predominant T-cell response (group-I) and ten T and B-cell response (group-II) inducing polypeptides, recognized by PBMCs of healthy TB contacts and PFMCs of tuberculous pleurisy patients [11] were investigated. Due to ethical constraints of bronchoscopy of healthy TB contacts, a human model of protective immunity and non-responsiveness of BAL cells to *M. tuberculosis* antigens in healthy human subjects [9,10], we evaluated mycobacterial antigens for their recognition by BAL cells of minimal TB patients demonstrating least pathology. The advanced TB patients were not investigated considering

extensive lung pathology and peripheral anergy in these subjects indicative of diminished operative beneficial responses. ^{3}[H] thymidine uptake assay (DNA synthesis) using BAL cells from radiologically affected lung of minimal TB patients demonstrated increased responsiveness to majority of mycobacterial antigens evaluated. It was associated with increased IFN-γ secretion in *in vitro* cultures. In contrast, PBMCs from same TB patients were generally hypo-responsive to these stimuli in terms of IFN-γ production with the exception of ESAT-6, MTSP-17, polypeptides 5 (putative ESAT-6 family member) and 10 (uncharacterized) where no statistically significant difference between the BAL and PBMC responses was observed. Increased proliferative and IFN-γ responses of BAL cells of TB patients over that of non-TB patients in response to *in vitro* stimulation of mycobacterial antigens indicate mycobacterial antigen specific hyper responsiveness of BAL cells of TB patients. The disparity between specific cell count in BAL cell preparations of TB and non-TB patients (table 1) might be suspected to account for these differences. Though data is not corrected for these differences in the present study, the cell number adjusted ELISPOT assay in a study by Schwander *et al* [9] indeed suggested that there is an absolute increase in *M. tuberculosis* antigen specific IFN-γ secreting cells in BAL of TB patients than control subjects. Since IFN-γ is generally produced by lymphocytes and not other cells, these results support the likelihood that the mycobacterial antigens induced increased stimulation indices could not be accounted for only by an increase in numbers of lymphocytes. Our observations of such

compartmentalization of antimycobacterial immune responses at the site of infection during active pulmonary tuberculosis are in accordance with that reported by others [9, 10]. This may be due to localization of circulating specific cells or local proliferation and expansion of few specific precursors or a combination of the two mechanisms.

In our study, although responses of group-I and group-II polypeptides were not statistically significant, majority of group-II polypeptides were found to be prominently recognized by BAL cells of minimal TB patients in terms of both lymphocyte proliferation and IFN-γ induction with predominant responsiveness to Ag85 B, Ag 85A, CFP-31, CFP-21, CFP-22.5, MPT-64 and yet uncharacterized 19 kDa protein. Reasons for such an increased recognition of group-II antigens by BAL cells is not clear, but can be attributed to strong inherent immunogenicity of these polypeptides [18, 19]. The polypeptide recognition pattern of BAL cells of minimal TB patients observed in the present study was found to be different from that of PBMCs of healthy TB contacts and PFMCs of tuberculous pleurisy patients, where an increased recognition of group- I polypeptitdes was observed [11]. Further, more pronounced diversity was observed in the antigen recognition by autologous PBMCs and BAL cells of minimal TB patients. Nonetheless, disparity between spleen and lung immune responses in mice [20,21] as well as PBMCs and BAL cell responses in human using few characterized antigens like ESAT-6 and Ag85 complex proteins have been

reported earlier [9,10] and is consistent with the observation made in the present study.

Apart from alveolar lymphocytes, alveolar macrophages have been shown to play an important role in defense against *M. tuberculosis* [15, 22]. The capacity of alveolar macrophages obtained from affected lung of minimal TB patients to produce NO and IL-12 in response to *in vitro* stimulation with *M. tuberculosis* and its secretory antigens was investigated and compared with that obtained in blood monocytes. Some studies failed to show production of NO by human macrophages [23, 24], while numerous other studies successfully demonstrated the capacity of human macrophages to produce NO [22, 25]. In this study, alveolar macrophages and not blood monocytes were capable of producing NO after *in vitro* stimulation with *M. tuberculosis* and its secretory antigens. Our study demonstrate that alveolar macrophages activated with various stimuli failed to produce NO at early time points of 24-48 hr and NO production was seen only after several days of culture (4-7 days). It can be speculated that IFN-γ produced by the alveolar lymphocytes after 72-96 hr of stimulation might be responsible for it's activation and subsequent release of detectable NO levels. It has earlier been well documented that antigen specific lymphocytes enhance nitric oxide production by macrophages through increased IFN-γ release [26, 27]. This might be responsible for differences in the NO levels observed in response to different antigens that were screened. BAL cell responses to Ag85A and Ag85B proteins observed in this study can be cited to explain the link between alveolar

macrophages and lymphocyte responses. BAL cell responses to these antigens were characterized by high lymphocyte proliferation, prominent IL-12 (p40), IFN-γ and NO responses in minimal TB patients. In addition, detectable *in vitro* IL-12 (p40) responses in BAL cells of minimal TB patients were also observed to Ag 85 complex proteins only. Previously, Ag 85A and B proteins have been demonstrated as suitable candidates for the development of future antituberculous vaccine [20, 28-30]. Predominant recognition of these proteins by BAL cells; characterized by prominent release of IL-12, IFN-γ and NO *in vitro* suggests potential of these antigens as constituents of a future mucosal anti-TB vaccine. The recent observations of improved protection by these vaccine candidates following intranasal vaccination of mice against *M. tuberculosis* challenge [31, 32] and their entry into clinical trials [29, 33] bodes well for the use of these candidates as mucosal vaccines for humans and need further evaluation.

Thus the overall data presented in this study suggests that the lymphocytes and macrophages responses at the actual site of disease are different from responses by the same cells in the periphery. Therefore, it seems imperative to evaluate vaccine candidates, both at the level of lung and blood cells for their recognition.

Acknowledgements

The help of Dr. J.T. Belisle and Dr. K.Dobos, Mycobacterial Research Laboratories, Dept of Microbiology, Immunology and Pathology, C.S.U. Fort Collins, Colorado, USA in LC-MS-MS analysis of mycobacterial antigens as well

as in providing MAbs from WHO collection and Dr.I. Rosenkrands, Statens Serum Institute, Copenhagen, Denmark in providing MAbs against RD1 and RD2 proteins is gratefully acknowledged. Authors sincerely thank Mr Virendra Singh for his help with the graphics and gratefully acknowledge the participation of all the volunteers in this study.

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Table 1 – Cellular profile of BAL preparations in minimal tuberculosis and non-tuberculosis patients

Group	Alveolar macrophages	Alveolar lymphocytes	Alveolar neutrophils
Tuberculosis patients	58±3%	30±5%	12±5%
Nontuberculosis patients	88±4%	10±2%	1±0%

Proportion of cells within BAL preparations was characterized morphologically by Wright's stain. Data are % (mean ± SEM).

Table 2 – Characterization of Group-I and Group-II purified polypeptides

Group I			Group II		
SNo.	Apparent Mol. Wt. (kDa)	Identity#	**SNo.**	Apparent Mol. Wt (kDa)	Identity#
1	4.5	Unknown	19	22.5	CFP22.5* Mixed sample (Enoyl-CoA-hydratase,Two component response regulator) [c] **Rv0632C, Rv1626**
2	4.0	ESAT-6 family member? [a]	20	21-22.0	CFP-21 (cutinase precursor) [a] **Rv1984C**
3	4.5	TB10.4 [a] **Rv0288**	21	19.0	Unknown
4	6.0	ESAT-6 [a, c] **Rv3875**	22	24.0	MPT-64[a, b, c] **Rv1980C**
5	7.0	ESAT-6 family member? [a,c]	23	30.5	Ag85A (MPT-44, mycolyl transferase) [a, b, c] **Rv3804C**
6	8.0	CFP-8* (conserved hypothetical protein) [c] **Rv0496**	24	29.5	Ag85B (MPT-59, mycolyl transferase) [a, b, c] **Rv1886C**
7	9.0	ESAT-6 family member? [a]	25	31	CFP-31* (hypothetical protein) [c] **Rv0831C**
8	9.5	ESAT-6 family member? [a]	26	5.5	ESAT-6 family member? [a]
9	10.0	CFP-10 [a] **Rv3874**	27	12.5	CFP 12.5* Mixed sample (10kDa, 11kDa conserved hypothetical protein, 12.5kDa thioredoxin/MPT46) [c] **Rv3874, Rv3592, Rv3914**
10	11.0	Unknown	28	13.5	CFP-11 * (conserved hypothetical protein) [b] **Rv2433C**
11	15.0	MTSP17 [c] **Rv0164**			
12	5.5	Unknown			
13	6.0	Unknown			
14	9.0	Unknown			
15	10.0	GroES (hsp-10/MPT-57) [a,c] **Rv3418C**			
16	14.5	CFP 14.5* (ndkA) [c] **Rv2445C**			
17	16.0	MTSP14/CFP-17[c] **Rv1827**			
18	18.0	CFP-18* (mixed sample) [c] **Rv2185C, Rv1827**			

#Identified using http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi; Identity was determined by **a**-monoclonal/polyclonal antibody reactivity, **b**-N-terminal sequencing and **c**-LC-MS-MS analysis; *Identified previously as novel T- Cell antigens (11,19).

Legends to Figures:

Fig 1: Proliferative responses of BAL cells (A and C) and PBMCs (B and D) of minimal TB (A and B) and non-TB patients (C and D) in response to *in vitro* stimulation with group-I purified polypeptides. Standard box plots with median (25^{th} and 75^{th} percentiles) and whiskers at minimum and maximum values are shown. The median counts per minutes of BAL cell and PBMC cultures without antigen in TB patients was 780 and 650 while that in non-TB patients was 632 and 710 respectively. The median SI in response to PHA of BAL cells and PBMCs of TB patients was 20.81 and 13.04 while that in non-TB patients was 14.22 and 16.0 respectively.

Fig 2: Proliferative responses of BAL cells (A and C) and PBMCs (B and D) of minimal TB (A and B) and non-TB patients (C and D) in response to *in vitro* stimulation with group-II purified polypeptides. Standard box plots with median (25^{th} and 75^{th} percentiles) and whiskers at minimum and maximum values are shown. The median SI in response to RvCFP of BAL cells and PBMCs of TB patients was 12.44 and 6.13 while that in non-TB patients was 3.81 and 6.0 respectively. The median SI in response to PPD of BAL cells and PBMCs of TB patients was 8.22 and 4.40 while that in non-TB patients was 2.44 and 5.8 respectively. *: $p<0.05$; **: $P<0.01$ *versus BAL*, Non-TB and #: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$ *versus* PBMC, TB.

Fig 3: IFN-γ responses of BAL cells (A and C) and PBMCs (B and D) of minimal TB (A and B) and non-TB patients (C and D) in response to group-I purified polypeptides. Standard box plots with median (25^{th} and 75^{th} percentiles) and whiskers at minimum and maximum values are shown. The median IFN-γ released in BAL cell and PBMC cultures without antigen was 9.0 pg/ml and below detection limit (BDL) in TB patients while that in non-TB patient was 4.0 pg/ml and BDL respectively. The median IFN-γ released in response to PHA in BAL cell and PBMC cultures of TB patients was 440.0 pg/ml and 272.0 pg/ml while that in non-TB patients was 264.0 pg/ml and 360.0 pg/ml respectively. **: $p<0.01$; ***: $P<0.001$ *versus BAL*, Non-TB and #: $p<0.05$; ##: $p<0.01$; ###: $p<0.001$ *versus* PBMC, TB.

Fig 4: IFN-γ responses of BAL cells (A and C) and PBMCs (B and D) of minimal TB (A and B) and non-TB patients (C and D) in response to group-II purified polypeptides. Standard box plots with median (25^{th} and 75^{th} percentiles) and whiskers at minimum and maximum values are shown. The median IFN-γ released in response to RvCFP of BAL cell and PBMC cultures of TB patients was 190.0 pg/ml and 69.0 pg/ml, while that in non-TB patients was 44.0 pg/ml and 64.0 pg/ml respectively. The median IFN-γ released in response to PPD in BAL cell and PBMC culture supernatants of TB patients was 190.0 pg/ml and 58.0 pg/ml while that in

non-TB patients was 28.0 pg/ml and 54.0 pg/ml respectively. **: p<0.01; ***: P<0.001 *versus BAL*, Non-TB and #: p<0.05; ##: p<0.01; ###: p<0.001 *versus* PBMC, TB.

Fig 5: Nitric oxide responses of BAL cells of minimal TB patients (A and C) and non-TB patients (B and D) induced by group-I (A and B) and group-II purified polypeptides (C and D). Standard box plots with median (25^{th} and 75^{th} percentiles) and whiskers at minimum and maximum values are shown. The median NO released in BAL cell culture supernatants of TB patients and non-TB patients in response to RvCFP was 10.60 μM and 6.28 μM while that released in response to PPD was 11.71μM and 5.95 μM respectively. The median NO levels released in BAL cell culture supernatants of TB patients and non-TB patients in response to medium alone were 2.90 μM and 2.79 μM respectively. **: p<0.01 *versus BAL*, Non-TB

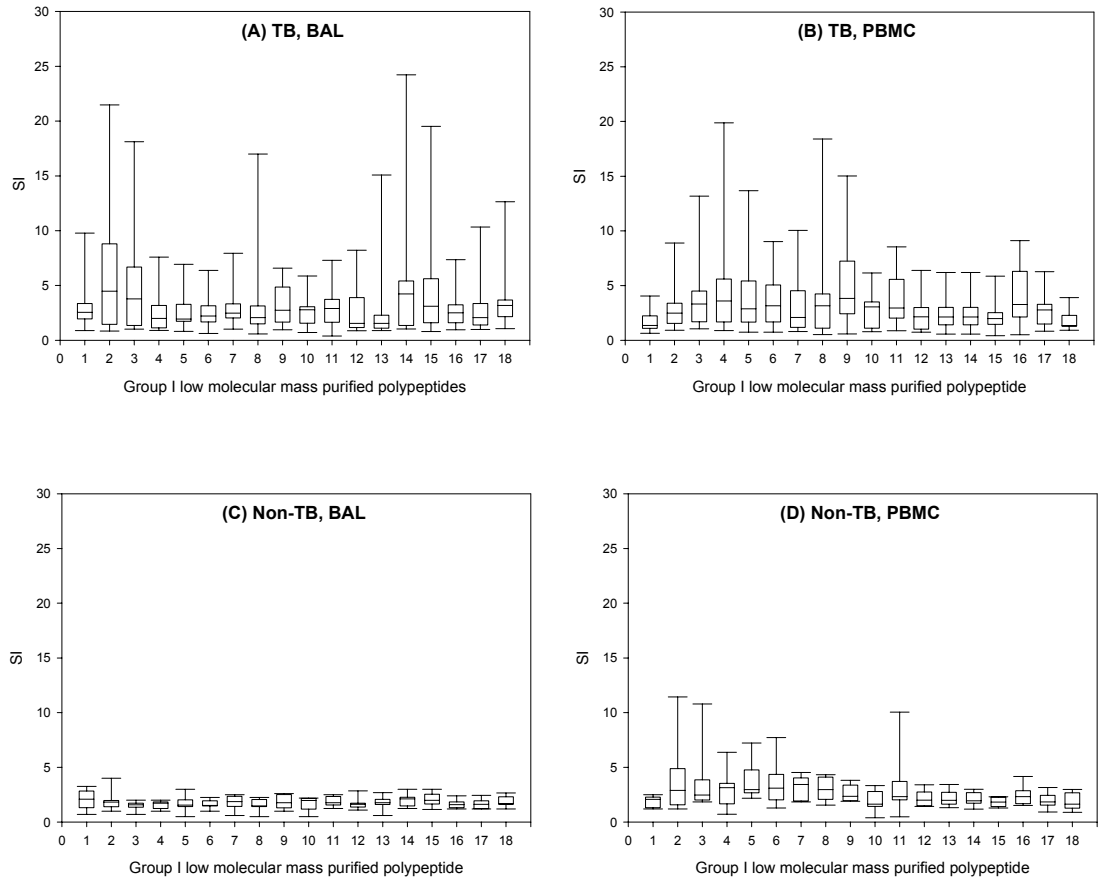

(A) TB, BAL
(B) TB, PBMC
(C) Non-TB, BAL
(D) Non-TB, PBMC
SI
Group I low molecular mass purified polypeptides
Group I low molecular mass purified polypeptide

Figure 1

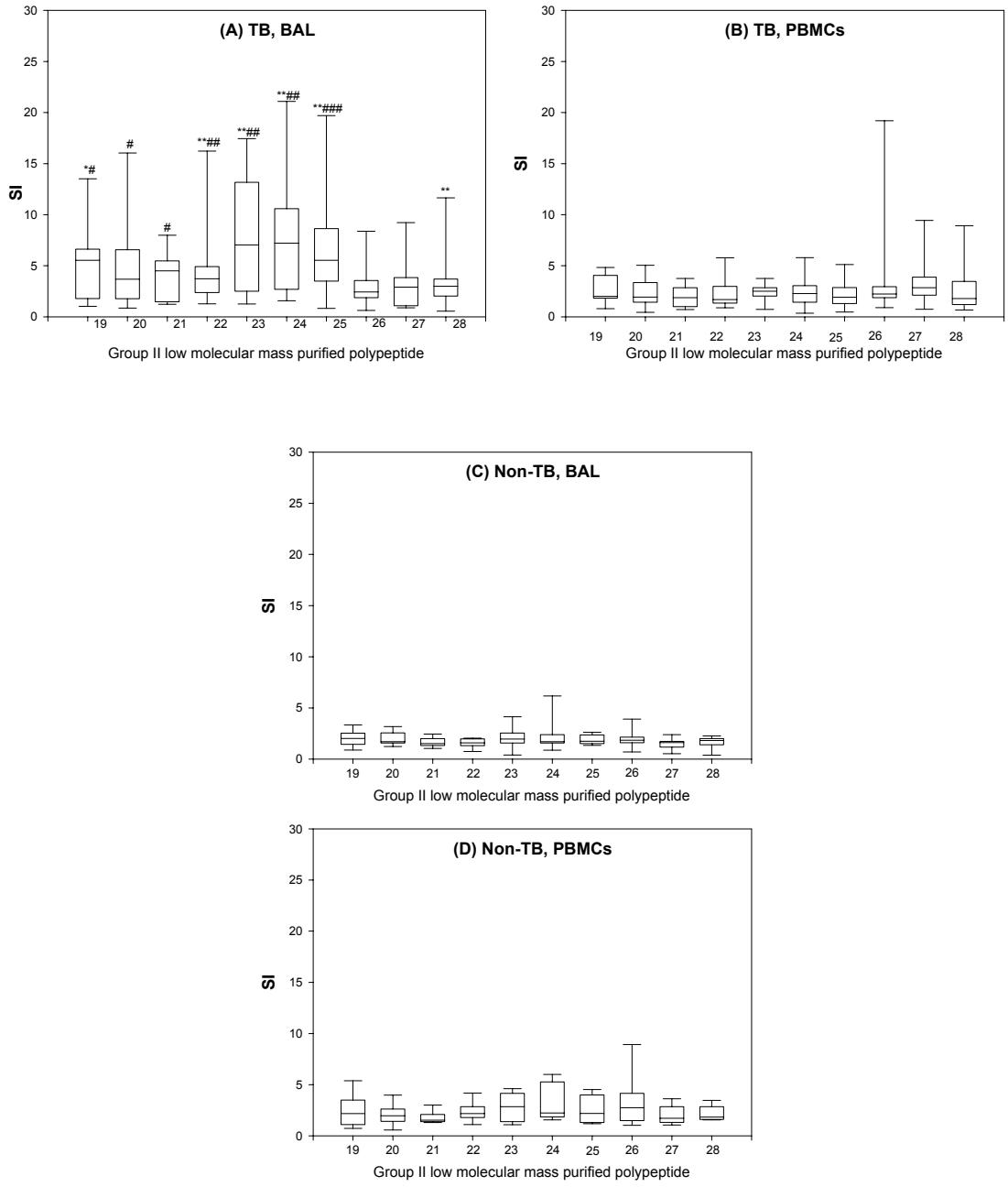
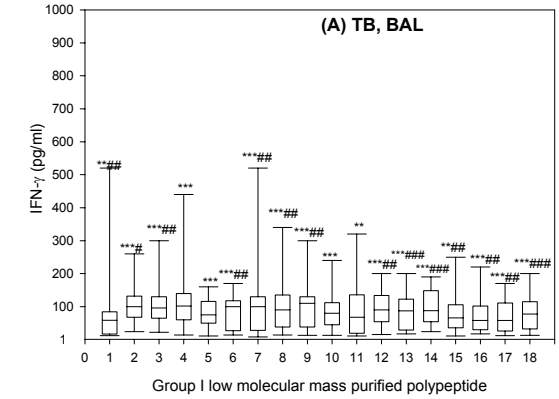
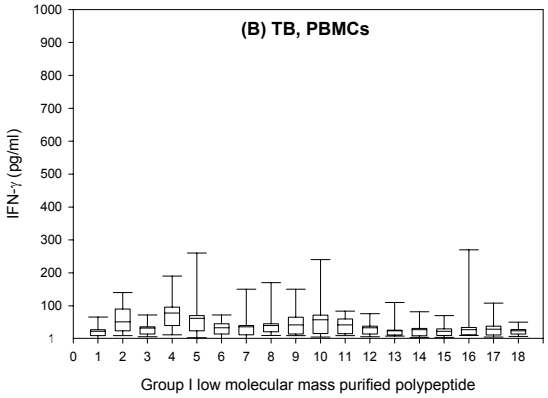

(A) TB, BAL
(B) TB, PBMCs
(C) Non-TB, BAL
(D) Non-TB, PBMCs
SI
Group II low molecular mass purified polypeptide
*#
#
#
**##
**##
**##
**###
**
19
20
21
22
23
24
25
26
27
28

Figure 2


(A) TB, BAL
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Group I low molecular mass purified polypeptide


(B) TB, PBMCs
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Group I low molecular mass purified polypeptide

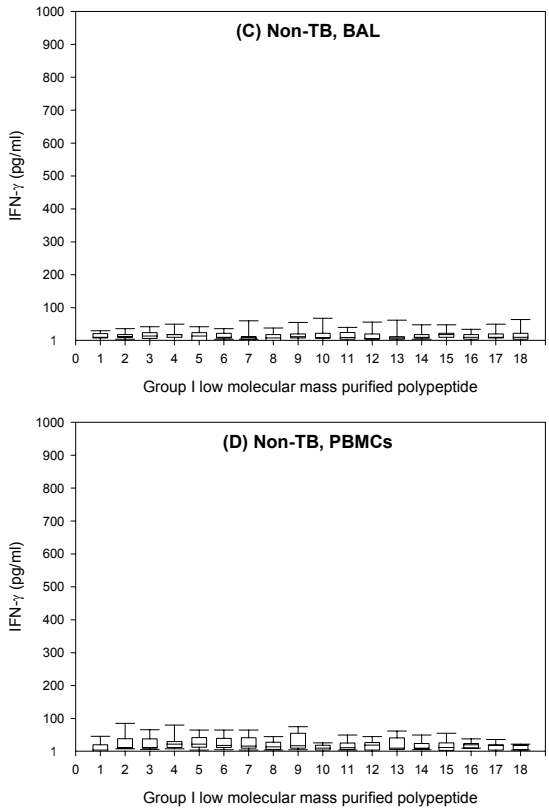
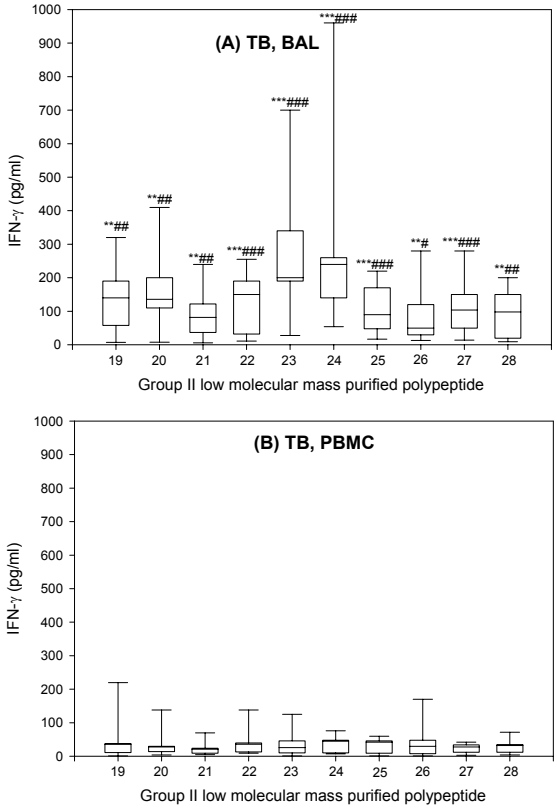

(C) Non-TB, BAL
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Group I low molecular mass purified polypeptide
(D) Non-TB, PBMCs
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
1
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
Group I low molecular mass purified polypeptide

Figure 3


(A) TB, BAL
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
**##
**##
**##
***###
***###
***###
***###
**#
***###
**##
19
20
21
22
23
24
25
26
27
28
Group II low molecular mass purified polypeptide
(B) TB, PBMC
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
19
20
21
22
23
24
25
26
27
28
Group II low molecular mass purified polypeptide

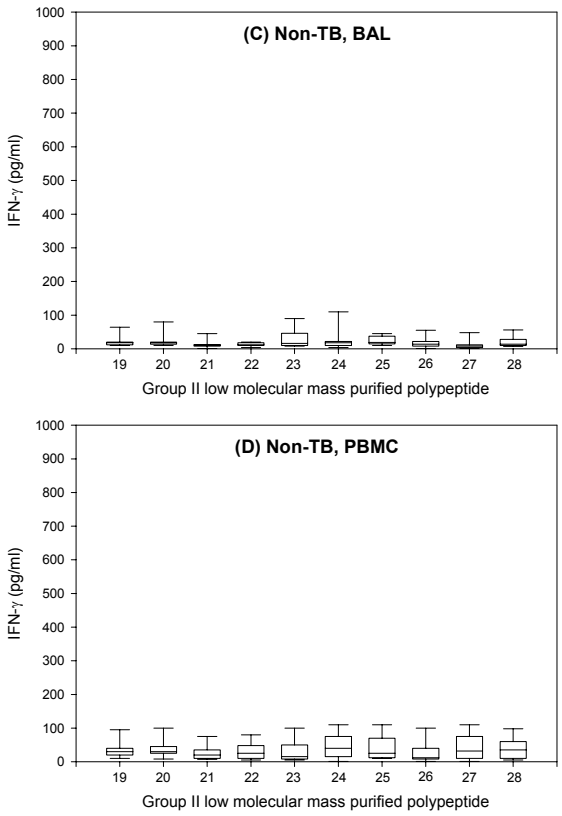

(C) Non-TB, BAL
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
19
20
21
22
23
24
25
26
27
28
Group II low molecular mass purified polypeptide
(D) Non-TB, PBMC
IFN-γ (pg/ml)
1000
900
800
700
600
500
400
300
200
100
0
19
20
21
22
23
24
25
26
27
28
Group II low molecular mass purified polypeptide

Figure 4

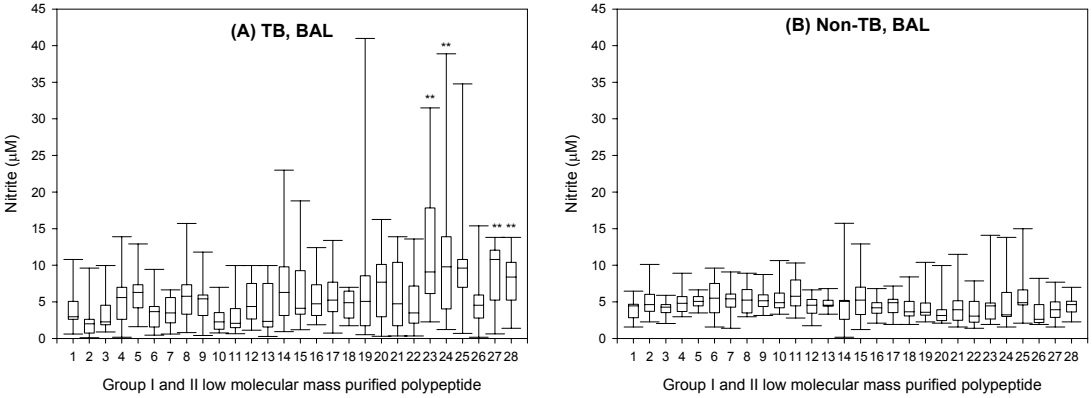

(A) TB, BAL
(B) Non-TB, BAL
Nitrite (μM)
Group I and II low molecular mass purified polypeptide

Figure 5