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Immunological Aspects

Diminished type 1 and type 17 cytokine expressing - Natural killer cell frequencies in tuberculous lymphadenitis

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ABSTRACT

Tuberculous lymphadenitis (TBL) is associated with the expansion of $CD4^+$ and $CD8^+$ T cells expressing Type 1 and Type 17 cytokines in the peripheral blood. However, the expression pattern of cytokine producing natural killer (NK) cells in both the peripheral blood and affected lymph nodes i.e. site of infection in TBL have not been examined. Hence, we have analyzed the baseline and mycobacterial antigen specific NK cell cytokine frequencies in whole blood of TBL and pulmonary tuberculosis (PTB) individuals. We have also examined the NK cell frequencies before and after treatment completion and in peripheral blood versus affected lymph nodes (LN) of TBL individuals. TBL is characterized by diminished frequencies of NK cells expressing Type 1 (IFNγ, TNFα), Type 17 (IL-17F) cytokines compared to PTB individuals upon antigen-specific stimulation. In contrast, TBL individuals did not exhibit any significant differences in the frequencies of NK cells expressing Type 1 and Type 17 cytokines upon completion of anti-tuberculosis treatment. LN of TBL is associated with altered frequencies of NK cells expressing Type 17 (increased IL-17F and decreased IL-22) cytokines when compared to peripheral blood. Thus, we conclude that TBL individuals are characterized by diminished frequencies of NK cells expressing Type 1/Type 17 cytokines.

1. Introduction

Tuberculosis (TB) remains a major global threat with 1.4 million deaths and 10 million active TB cases reported in the year 2017 [1]. Lymph node tuberculosis (LNTB) or tuberculous lymphadenitis (TBL) is the common form of extra pulmonary TB (EPTB) with 15–20% prevalence rate in TB endemic nations. TBL causes significant disease burden with large tuberculin reactions and strong female preponderance [2–4]. Different animal and human studies have shown both innate and adaptive immune mechanisms are crucial in host defense against TB disease. In previous studies it has been shown that immunity to *Mycobacterium tuberculosis* (Mtb) requires both innate and adaptive immune responses [5,6].

Natural killer (NK) cells are important in the early stages of protection against intracellular pathogens. These NK cells are a distinct subset of lymphocytes (10–15%), which circulate in the peripheral blood and form part of the innate immune repertoire. NK cells can kill Mtb infected monocytes and alveolar macrophages directly and also have the potential cytotoxic ability to kill tumour cells, viruses and intracellular parasites [7–10]. These cells are activated by Type I interferons, IL-12, IL-2, IL-15 and IL-18 and generate specialized function depending upon different tissue locations [11–14]. Further, human NK cells play a vital role in bridging innate and adaptive immunity and express Type 1/17 cytokines or lytic production against microbial pathogens [15,16] Apart from this, NK cells also mediate the anti-viral and anti-tumour immunity as well and produce anti-bacterial mediators and immunoregulatory cytokines [17]. NK cells also express the pathogen recognition receptors through which they activate the effector molecules by recognising the pathogen-associated molecular patterns [18,19]. Apart from producing cytokines, NK cells also have the ability to produce chemokines (MIP-1α and MIP-1β), which further creates a proinflammatory milieu [20].

In pulmonary TB (PTB), NK cells are a major source of TNFα, IFNγ and IL-22 cytokine production [21,22]. It was observed that in the absence of T cells, the presence of NK cells can limit Mtb infection in animal models and their reduction enhances the growth of

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Mycobacterium avium [23,24]. Even in healthy individuals, NK cells can lyse the infected monocytes and decrease Mtb growth [7,25]. BCG re-vaccination in latent TB infected individuals has been shown to promote NK cell mediated immune responses [26]. Elevated NK cells are associated with TB pleurisy and play a crucial role against Mtb disease [27]. However, the role of NK cells expressing Type 1/Type 17 cytokines has not been fully delineated in TBL. Hence, we have performed a detailed analysis of NK cells expressing Type 1 and Type 17 cytokines in TBL compared to PTB, in TBL before and after ATT, and in lymph nodes versus peripheral blood of TBL individuals. TBL individuals exhibited reduced and altered frequencies of NK cells expressing Type 1 and Type 17 cytokines in whole blood and at the site of infection.

2. Materials and methods

2.1. Ethics

This study was sanctioned and approved by the Internal Ethics Committee of National Institute for Research in Tuberculosis (NIRT IEC 2010 007). The written informed consent form was obtained from all study participants.

2.2. Study population

We recruited a group of TBL (n = 10) and pulmonary TB (n = 10) individuals in the present study (Table 1). In addition, lymph node (n = 10) samples were collected from TBL individuals. Similarly, for TBL individuals, blood samples (n = 10) were also collected following completion of anti-tuberculosis treatment (ATT). For TBL individuals, we have taken the matched blood and lymph node samples and that is the reason for us in limiting our sample size to n = 10 in each group. Likewise, we did not have the ethical approval to collect the peripheral blood of PTB patients after the completion of ATT for this study. TBL diagnosis was done on the basis of clinical presentation for the swelling of lymph node and either fine needle aspiration cytology (FNAC) or culture positive for *Mycobacterium tuberculosis* from the excised lymph node biopsy samples. PTB individuals were diagnosed on the basis of sputum smear positivity for acid fast bacillus (AFB) by Ziehl-Neelsen staining. All the study participants were HIV negative and devoid of any steroid treatment.

2.3. Antigens

TB antigens used in the study were purified protein derivative (PPD; Statens Serum Institute) with the final concentration of 10 μg/mL, recombinant early-secreted antigen 6 (ESAT-6) and culture filtrate protein 10 (CFP-10) peptide pools (BEI Resources, National Institute of Allergy and Infectious Diseases [NIAID], National Institutes of Health [NIH]) with the final concentration of 10 μg/mL. The human immunodeficiency virus Gag peptide pool (HIVPP; AIDS Reagent Program, Division of AIDS, NIAID, NIH) of 10 μg/mL was used as non-TB specific antigen stimuli. Finally, the combination of Phorbol 12-myristate 13-acetate (PMA)–ionomycin (PMA-Iono; Calbiochem) was used as a positive antigen control with the final concentration of 12.5 ng/mL and 125 ng/mL.

2.4. In-vitro whole blood and lymph node culture

The *in-vitro* cell cultures were performed to measure the intracellular cytokine levels. Briefly, whole blood was diluted to 1:1 with the Roswell Park Memorial Institute (RPMI) 1640 medium provided with penicillin-streptomycin (100 U/100 mg/mL), L-glutamine (2 mM), and HEPES (10 mM) (Invitrogen, San Diego, CA) buffer. The excised lymph nodes were harvested in RPMI 1640 medium and processed immediately. The LN samples were washed twice, cut down into smaller pieces in RPMI 1640 medium, treated with Liberase (0.1 mg/mL) and DNase (0.1 mg/mL, Roche Diagnostics) enzymes and then incubated for 20–30 min at 37 °C. Again, the cells were washed with RPMI 1640 medium and centrifuged at 2,600 rpm for 10 min and the supernatant was discarded. The extracted whole blood and lymph node cells were uniformly dispersed (2 million cells in 2 mL/well) in 12-well tissue culture plates (Costar; Corning Inc., Corning, NY). Whole blood and lymph node cells were stimulated with following antigens such as PPD, ESAT-6, CFP-10, HIVPP, PMA/I or were left unstimulated (UNS) and incubated at 37 °C in 5% CO_2 for 18 h. On the course (after 2 h of incubation), Fast Immune™ brefeldin A solution (10 μg/mL) was added to the cultures. After 18 h of incubation, the stimulated cells were meticulously transferred to 50 mL sterile falcon tubes. The samples were centrifuged at 2,600 rpm for 10 min; the supernatant was collected in 2 mL screw-cap tubes and stored at −80 °C. Further the samples were washed with 1x Phosphate-buffered saline (PBS-LONZA) and red blood cells were lysed using BD FACS™ lysing solution. Finally, the cells were fixed using BD cyto fix/cyto perm™ and cryopreserved at −80 °C in PBS/Dimethyl sulfoxide (DMSO [HiMedia]).

2.5. Lymph node and whole blood intracellular cytokine staining

The cryopreserved cells were thawed, washed with 1x PBS and permeabilized using permeabilization buffer™ (ebiosciences) and incubated for 60 min before washing. After washing, permeabilization buffer™ was added to the cells and stained with both surface as well as with intracellular cytokines and incubated for overnight at 4 °C. The surface markers like CD3-AmCyan (BD Biosciences), CD56-PerCP (Ebioscience) and cytokines like IFNγ-PE (BD Biosciences), TNFα-FITC (BD Biosciences), IL-17A-FITC (Miltenyi Biotech), IL-17F-APC and IL-22-PE (R&D systems) were used. Finally, the cells were washed with permeabilization buffer and PBS was added to the cells following which

Table 1
Demographics of the study population.

Study Demographics	TBL baseline	Lymph node	Pulmonary TB
No of subjects recruited (n)	10	10	10
Gender (M/F)	4/6	4/6	7/3
Median age in years (range)	27.8 (18–47)	27.8 (18–47)	32.1 (19–62)
Lymph node culture grade (0/1+/2+/3+)	2/8/0/0	2/8/0/0	Not done
Smear Grade (0/1+/2+/3+)	Not done	Not done	0/2/5/3

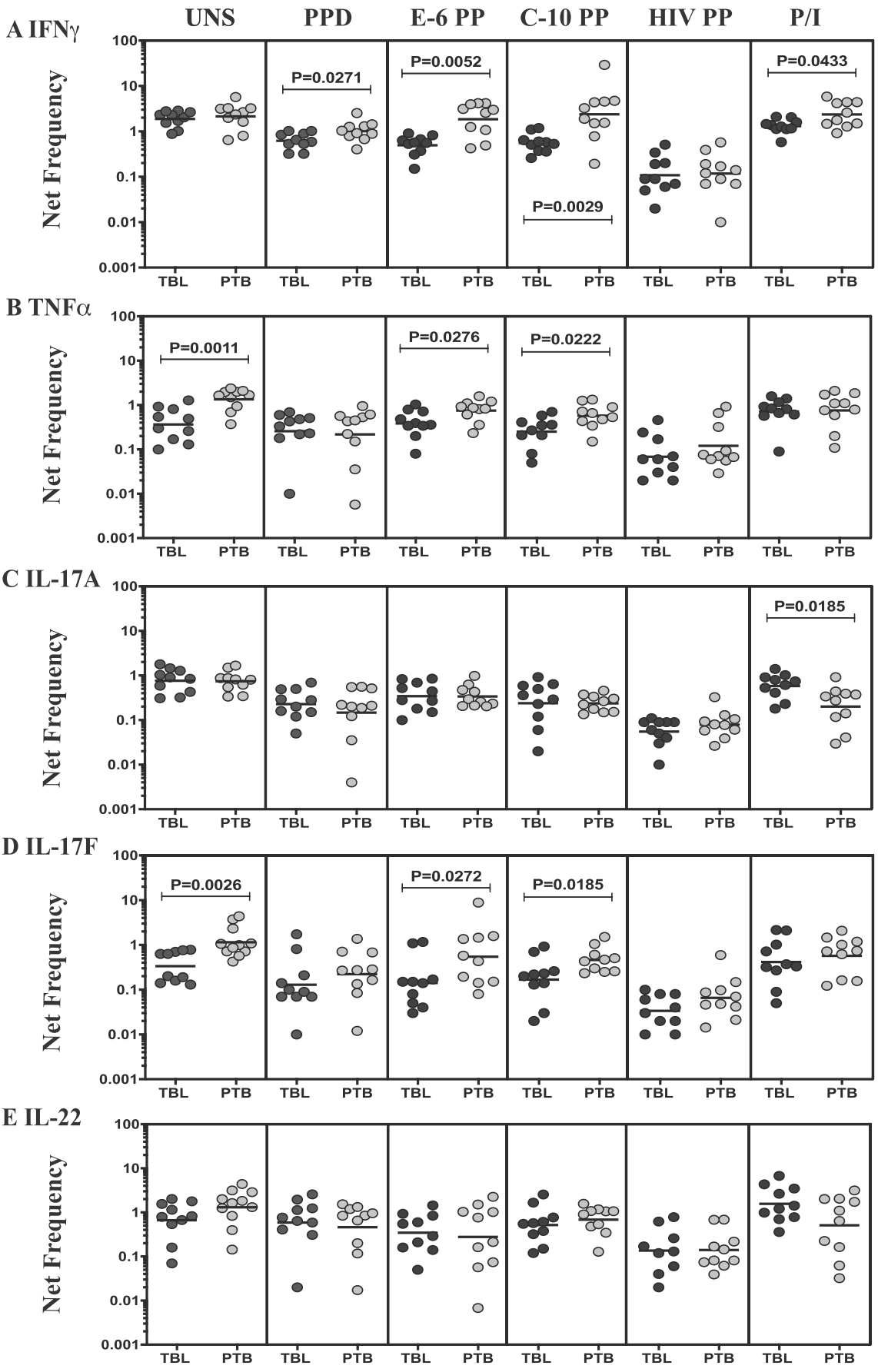

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Fig. 1. Diminished antigen-specific frequencies of NK cells expressing Type 1 (IFNγ, TNFα) and Type 17 (IL-17F) cytokines in TBL. Whole blood of TBL (n = 10) and PTB (n = 10) individuals were cultured with medium alone or mycobacterial or control antigens for 18 h. By using the harvested cells both baseline and antigen-stimulated cytokine frequencies were analyzed using multi-color flow cytometry. **(A–E)** Baseline (left panels), **(A–E)** PPD, ESAT-6 PP and CFP-10 PP (middle three panels), **(A–E)** HIV Gag PP (second last panels) and **(A–E)** PMA/I (last right panels) antigen stimulated frequencies of NK cells expressing respective Type 1 (IFNγ, TNFα and IL-2), and Type 17 (IL-17 A, IL-17F, IL-22) cytokines (each black colored round circle represents single TBL individuals, each grey colored round circle represents single PTB individuals). The bar represents the geometric mean values and P values were calculated using the Mann-Whitney *U* test. The net frequencies were calculated by subtracting the baseline from antigen-stimulated values for each individual. P value < 0.05 is considered as statistically significant.

acquisition was done. We performed the experiments using eight-color flow cytometry on a FACSCanto II flow cytometer with FACSDiva software v.6 (Becton Dickinson and Company, Cockeysville, MD). The cytokines and cytotoxic molecules were stained in diverse sets of panels. We used the forward vs side scatter to set the lymphocyte gating and 100 000 gated lymphocyte events were acquired. The gating strategy for $CD56^+$ NK cells expressing cytokines was determined by FMO. All the data were collected and analyzed with the help of Flow Jo software (TreeStar Inc., Ashland, OR). All the data were represented as frequencies of $CD56^+$, $CD3^-$ cells expressing cytokine(s). The baseline values following media stimulation alone used to determine the baseline frequency, whilst the net frequencies were represented with the stimulated antigens or PMA/I (with the baseline values subtracted). Live/dead marker to analyse the viability of the cell population was not used in the study which is a limitation. The human peripheral blood and lymph node NK cell gating strategy is given in the Supplementary Fig. 1.

2.6. Data analysis

Statistical analyses were performed using GraphPad PRISM (version 8) software (Graph- Pad Software, Inc., San Diego, CA). The geometric means (GM) were used for measuring the central tendency. Intergroup comparisons were analyzed using the nonparametric Mann-Whitney *U* test and pre and post-treatment cytokine frequencies were compared using the Wilcoxon signed rank test.

3. Results

3.1. Diminished frequencies of type 1/type 17 cytokine expressing NK cells in TBL

The representative plots of peripheral blood NK cells expressing Type 1 and Type 17 cytokines are shown in Supplementary Fig. 2. To delineate the role of NK cells expressing cytokines in the peripheral blood of TBL and PTB individuals, we examined the frequencies of Type 1 (IFNγ, TNFα) and Type 17 (IL-17A, IL-17F, IL-22) expressing NK cells (Fig. 1). We observed no significant differences in the unstimulated or baseline frequencies of NK cells expressing Type 1 (except TNFα) and Type 17 (except IL-17F) cytokines between TBL and PTB individuals (Fig. 1A–E, left panels). In contrast, in response to TB antigenic stimuli, we observed that TBL was associated with diminished frequencies of NK cell expressing Type 1 (IFNγ in PPD), (IFNγ and TNFα in ESAT-6 PP and CFP-10 PP) and Type 17 (IL-17F in ESAT-6 PP, CFP-10 PP) cytokines in comparison to PTB individuals (Fig. 1A–E, middle three panels). As shown in Fig. 1A–E (second last panels), no significant difference was observed in the frequencies of NK cells expressing cytokines when stimulated with HIV Gag PP. Finally, upon stimulation with P/I, no significant difference in the frequencies of NK cells expressing Type 1 and Type 17 cytokines was observed between TBL and PTB individuals (Fig. 1A–E last right panels). Thus, TBL is associated with reduced frequencies of mycobacterial antigen stimulated NK cells expressing Type 1 and Type 17 cytokines.

3.2. Frequencies of type 1/Type 17 cytokine expressing NK cells are not altered upon anti-tuberculosis treatment

To characterize the treatment induced modulation of NK cells expressing Type 1 (IFNγ, TNFα) and Type 17 (IL-17A, IL-17F, IL-22) cytokines in TBL, we examined those cytokine frequencies before and after completion of ATT (Fig. 2). As we have shown in Fig. 2A–E (left panels), we did not observe any significant difference in the baseline or unstimulated Type 1 or Type 17 cytokine expressing NK cell frequencies. In addition, we did not observe any significant alterations in the frequencies of NK cells expressing Type 1 (except IFNγ in ESAT-6 PP) cytokines upon different antigenic (PPD, ESAT 6-PP and CFP-10 PP) stimuli (Figure A–B middle three panels) following ATT. Similarly, we did not observe any significant alterations in the frequencies of NK cells expressing Type 17 (IL-17A, IL-17F and IL-22) cytokines (except IL-22 in ESAT-6 PP and CFP-10 PP) following ATT (Figure C–D middle three panels). Finally, we also did not observe any significant alterations in the frequency of cytokine expressing NK cells in response to HIV Gag PP (Fig. 2A–E, second last panels) or P/I stimulation (Fig. 2A–E, last right panels). Thus, in TBL, no major changes were seen in the frequencies of NK cells expressing Type 1 and Type 17 cytokines after the completion of ATT.

3.3. Diminished frequencies of NK cells expressing type 17 cytokines and TNFα at the site of infection

Next, we examined the frequency of NK cells expressing Type 1 (IFNγ, TNFα) and Type 17 (IL-17A, IL-17F, IL-22) cytokines in lymph node and peripheral blood of TBL individuals (Fig. 3). The representative plots of NK cell expressing Type 1 and Type 17 cytokines in TBL LN are shown in Supplementary Fig. 3. At baseline, TBL individuals did not exhibit significant differences in the frequencies of Type 1 and Type 17 (except IL-17F) cytokine expressing NK cells in LN when compared to whole blood (Fig. 3A–E, left panels). As shown in Fig. 3A and B (middle three panels), upon different antigenic (PPD, ESAT 6-PP and CFP-10 PP) stimuli (except TNFα in ESAT-6 PP), there was no significant difference observed between the frequencies of Type 1 cytokine expressing NK cells between LN and blood. In contrast, upon different antigen stimulation (PPD, ESAT-6 PP and CFP-10 PP), Type 17 (IL-17F, IL-22 (PPD alone)) cytokine expressing NK cell frequencies were significantly diminished in LN when compared to blood. Upon HIV Gag PP stimulation, the frequency of NK cells expressing Type 1 or Type 17 cytokines did not show any significant differences between LN and blood (Fig. 2A–E, second last panels). Upon P/I stimulation, we observed significant difference in the frequency of NK cells expressing Type 1 (IFNγ and IL-22 alone) cytokines in LN of TBL compared to blood (Fig. 3A–E (last right panels)). Hence, TBL LN associated with decreased frequencies of NK cell expressing Type 17 cytokines.

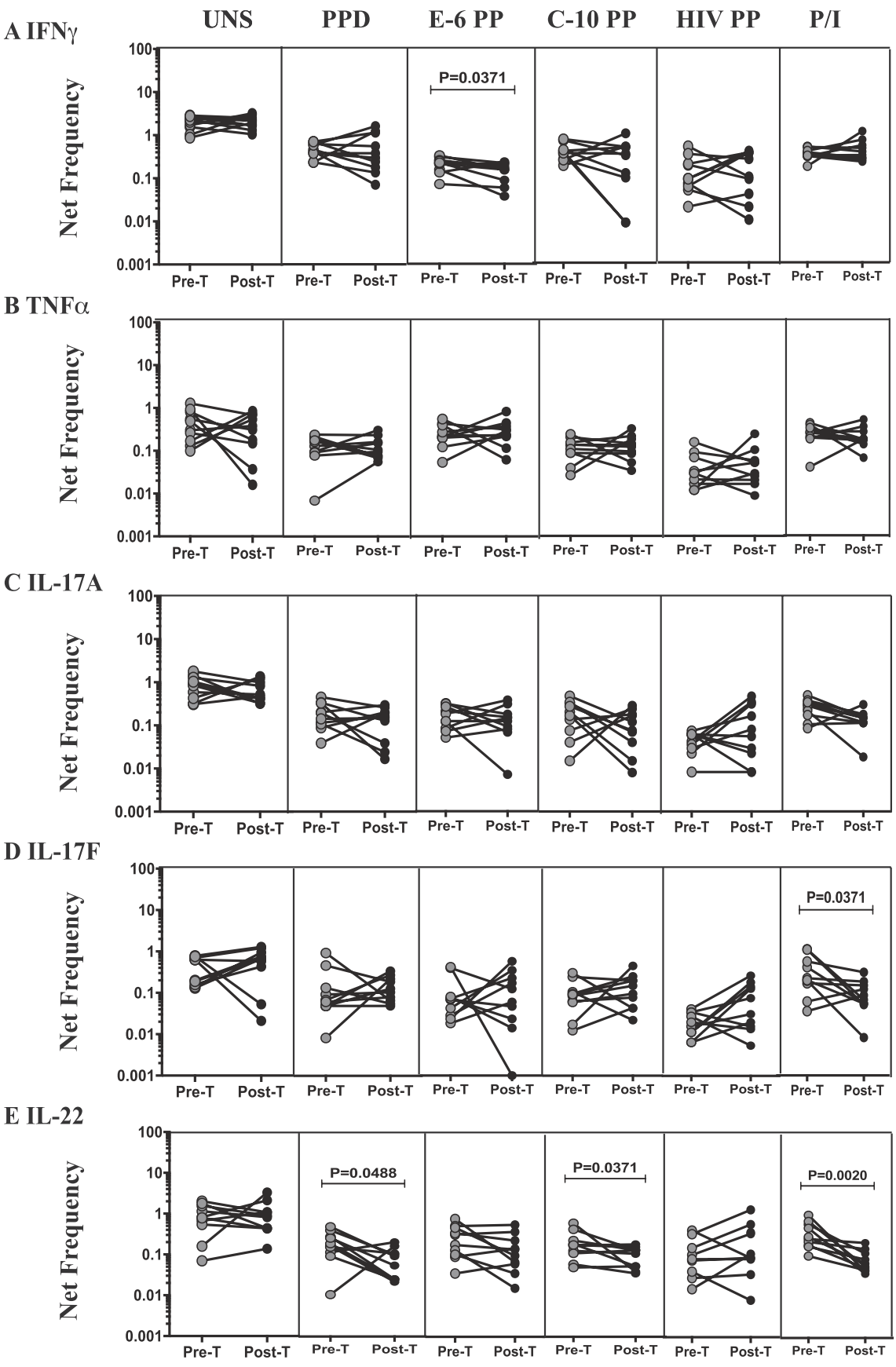

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Fig. 2. No alterations in the NK cell frequencies of Type 1 and Type 17 expressing cytokines upon completion of ATT in TBL. Whole blood from TBL [pre-treatment (pre-T, n = 10) and post treatment (post-T, n = 10)] individuals were cultured with medium alone or mycobacterial or control antigens for 18 h. By using the harvested cells both baseline and antigen-stimulated cytokine frequencies were analyzed using multi-color flow cytometry. **(A–E)** Baseline (left panels), **(A–E)** PPD, ESAT-6 PP and CFP-10 PP (middle three panels), **(A–E)** HIV Gag PP (second last panels) and **(A–E)** PMA/I (last right panels) antigen stimulated frequencies of NK cell expressing respective Type 1 (IFNγ, TNFα and IL-2), and Type 17 (IL-17 A, IL-17F, IL-22) cytokines. The bar represents the geometric mean values and P values were calculated using the Wilcoxon signed rank test. The net frequencies were calculated by subtracting the baseline from antigen-stimulated values for each individual.

4. Discussion

TB is primarily a disease of the lung with extra-pulmonary dissemination (especially the involvement of lymph node) occurring mainly due to immune-suppression [2,4]. Both, $CD4^+$ and $CD8^+$ T cells are important in fighting against TB infection but the outcome of TB infected patients not only depends on these cells [5,28]. NK cells also exert a parallel effect in killing microbes by using effector molecules such as perforin and granzymes [29–31]. NK cell mediated immune response is crucial against several viruses and intracellular pathogens [32–34]. Even in the secondary lymphoid tissues (especially in lymph nodes), NK cells acts as a critical role in mediating immune responses against infections [35]. NK cells are a potent source of early production of IFNγ and IL-22. NK cells help Th1 clonal expansion and also aid cytotoxic $CD8^+$ T cells to lyse the infected monocytes [32,36–38].

However, the knowledge on NK cell cytokine profile in peripheral blood and lymph node is scant and no studies have previously been done in TBL disease. Previous studies from our lab shown the data on $CD4^+$ and $CD8^+$ T cell frequencies in TBL and PTB individuals. We revealed that TBL individuals exhibited increased frequencies of $CD4^+$ and $CD8^+$ Th1 and Th17 cells compared to PTB individuals at baseline and following ESAT-6 PP and CFP-10 PP antigen stimulation. In contrast, reduced frequencies of $CD8^+$ T cells expressing perforin, granzyme B and CD107a were associated with TBL individuals [39,40]. Hence, we have concentrated specifically on NK cell expressing cytokines in this study population. A previous study has described that $CD4^+CD69^+$ T cells produce significantly enhanced levels of IFNγ, IL-2 and TNFα when compared to $CD4^+CD69^-$ T cells upon stimulation with Mtb specific antigens in tuberculous pleurisy (TBP) individuals [41]. Similarly, $CD4^+$ T cells in TBP individuals express Th1 (IFNγ, TNFα) and Th17 (IL-22) cytokines after stimulation with ESAT-6/CFP-10 PP [42]. It was also shown that NK cells in pleural fluid have heightened levels of ICAM-1 [43]. The pleural fluid cells tends to express significantly elevated frequencies of NK cell producing IL-22 and IFNγ upon stimulation with IL-15 or IL-15 and IL-12 co-stimulation [44].

T helper cells expressing IFNγ and TNFα cytokines are crucial in the formation and maintenance of granuloma, macrophage activation and effector molecule production [45]. In addition, NK cells are the major source of IFNγ and TNFα and thereby mediate the immune response against Mtb by activating the macrophages, dendritic cells and increasing the bactericidal activity [21]. We reveal in our study that the homeostatic immune milieu is profoundly diverse in TBL compared to PTB and associated with diminished baseline Type 1 (TNFα) cytokine expressing NK cell frequencies. But upon antigen (PPD, ESAT-6 PP, CFP-10 PP) stimulation, both IFNγ and TNFα cytokine expressing NK cell frequencies were significantly reduced in TBL compared to PTB individuals. This is perhaps due to the fact that TBL is more paucibacillary form of disease and therefore optimal NK cells stimulation fails to occur in this setting. Moreover, the alterations in the frequencies were pathogen specific, since the cytokine expression profile were not changed upon stimulation with HIV Gag PP and P/I (except IFNγ) between the two study groups. Interestingly, when compared to blood, the frequencies of TNFα (only with ESAT-6 PP) expressing NK cells was also significantly diminished in lymph nodes of TBL individuals. Similarly, the frequencies of NK cells expressing cytokines were not significantly altered upon post treatment. Our analysis clearly delineates an important association of NK cell producing Type 1 cytokines with TBL and suggests that reduction in the frequencies possibly reflects the pathogenesis of TBL disease. Thus, NK cell production of Type 1 cytokines needs further studies to determine if it is a potential correlate of protective immunity in TBL.

Like Type 1 cytokines, NK cells expressing Type 17 (IL-17A, IL-17F and IL-22) cytokines are also integral part of providing immune protection and inflammatory pathology in TB disease. These cells play a dominant role in arbitrating immune response to both extra and intracellular bacteria, including Mtb [46–49]. IL-17F shares numerous characteristics with IL-17A [43]. With respect to pulmonary immunity, both IL-17 and IL-22 chiefly act on the lung epithelium, stimulate antimicrobial proteins and neutrophil chemo attractants [50,51]. $CD4^+$ T cells producing IL-17 and IL-22 cytokines provide anti-mycobacterial responses both in human and bovine models [49,52]. Our study shows that frequencies of NK cells producing IL-17F were significantly diminished either at baseline or following mycobacterial antigen stimulation in TBL individuals in comparison with PTB individuals. Indeed, IL-17 is an important player in the immune response to TB and is involved in both protection and pathogenesis of Mtb disease [53,54]. Therefore, the presence of decreased frequencies of IL-17F expressing NK cells suggests that these cells might be another important correlate of protective immunity to TBL. In contrast, both baseline and antigen stimulated NK cells expressing IL-17F were significant elevated in the LN when compared to whole blood and this represents the role of cellular immune responses engendered by IL-17F. Surprisingly, our analysis on NK cells expressing IL-17A and IL-22 cytokine demonstrated no significant alteration either at baseline or after Mtb antigen stimulation in TBL individuals compared with PTB. However, the frequency of NK cells expressing IL-22 showed significant changes following treatment and also demonstrated decreased frequencies in LN compared to whole blood, indicating that NK cells expressing IL-22 might play a role in pathogenesis of TBL.

On the whole, the frequencies of NK cell expressing cytokines were compromised in TBL individuals after stimulated with Mtb antigens. The diminished levels of NK cells might result in impaired activation of protective Th1 (IFNγ) or Th17 (IL-22) cytokines to control or to eliminate the pathogen in TB infected individuals. In addition, the diminished cytokine production of NK cells at the site of infection could be a predisposing factor to increased disease severity in TBL. Our study suffers from certain limitations, including the small sample size, absence of live/dead marker to analyse viability of cells and absence of follow up of PTB patients. Nevertheless, our data clearly emphasize the importance of NK cells expressing Type 1 and Type 17 cytokines in TBL disease. The expansion or down regulation of different cytokines might not necessarily correlate with host resistance but might serve as a signature for disease severity and dissemination of this poorly documented form of TB infection.

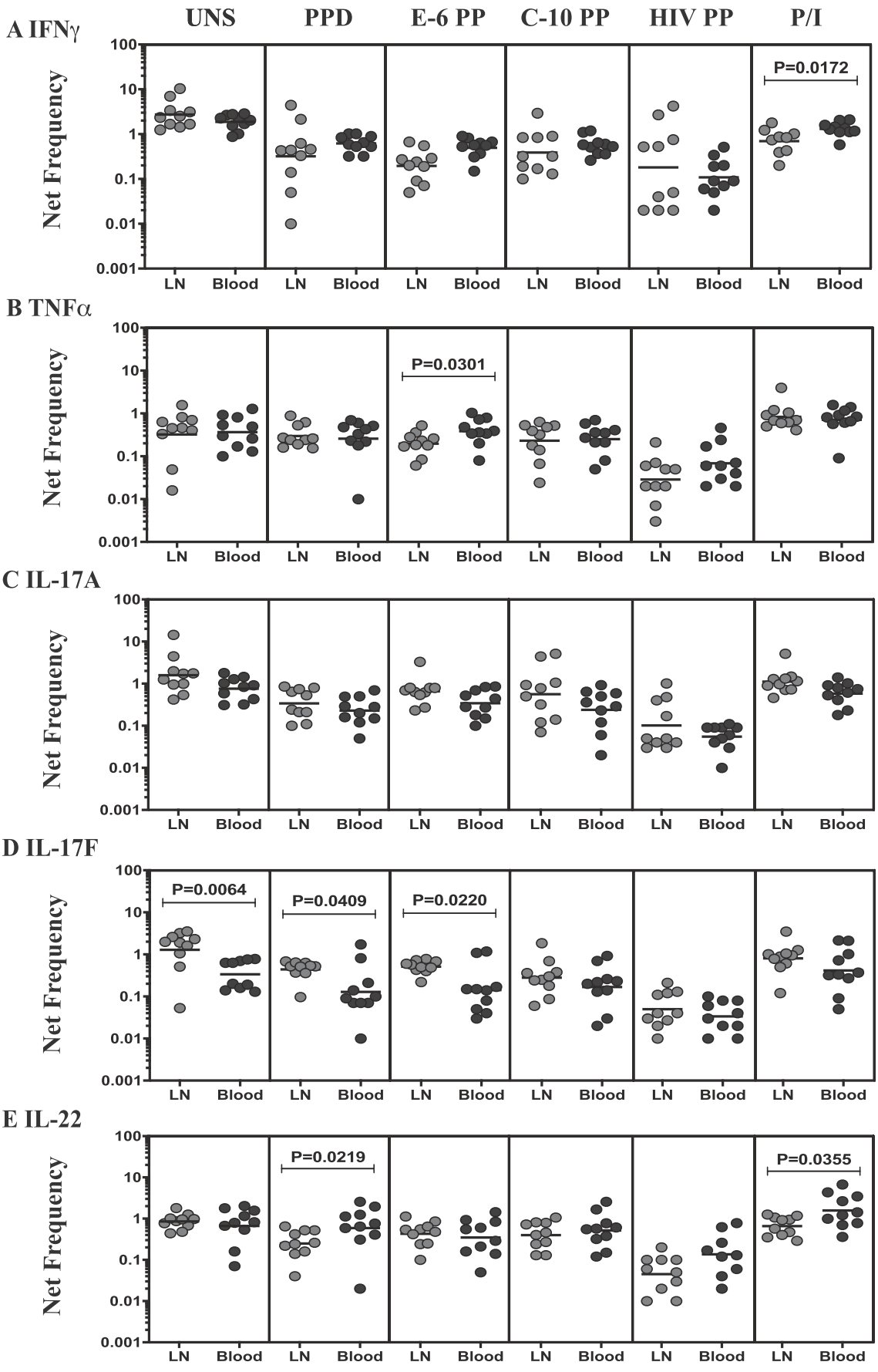

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Fig. 3. TBL is characterized by altered antigen-specific frequencies of NK cells expressing Type 17 cytokines at the site of infection. Lymph node (n = 10) and whole blood (n = 10) from TBL individuals were cultured with medium alone or mycobacterial or control antigens for 18 h. By using the harvested cells both baseline and antigen-stimulated cytokine frequencies were analyzed using multi-color flow cytometry. **(A–E)** Baseline (left panels), **(A–E)** PPD, ESAT-6 PP and CFP-10 PP (middle three panels), **(A–E)** HIV Gag PP (second last panels) and **(A–E)** PMA/I (last right panels) antigen stimulated frequencies of NK cell expressing respective Type 1 (IFNγ, TNFα and IL-2), and Type 17 (IL-17 A, IL-17F, IL-22) cytokines (each grey colored round circle represents lymph node and each black colored round circle represents whole blood of single TBL individuals). The bar represents the geometric mean values and P values were calculated using the Mann-Whitney *U* test. The net frequencies were calculated by subtracting the baseline from antigen-stimulated values for each individual.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tube.2019.101856.

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