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IMMUNOLOGICAL ASPECTS

Programmed death-1 receptor suppresses γ-IFN producing NKT cells in human tuberculosis

Amar Singh [a], Aparajit Ballav Dey [b], Anant Mohan [b], Dipendra Kumar Mitra [a,*]

[a] Department of Transplant Immunology & Immunogenetics, All India Institute of Medical Sciences, New Delhi, India
[b] Department of Medicine, All India Institute of Medical Sciences, New Delhi, India

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SUMMARY

IFN-γ biased Th1 effector immune response is crucial for containment of *Mycobacterium tuberculosis* infection. Various T cell subsets with regulatory function dictate the generation of Th1 like cells. NKT cells are a specialized T cell subset known to be activated early in immune response and control T cell response via release of immunoregulatory cytokines like IFN-γ, IL-4 and IL-10. *M. tuberculosis*, with abundance of its cell wall lipids may potently activate NKT cells resulting in cytokine production and PD-1 expression. In this study, among 49 treatment naive active pulmonary tuberculosis patients, we found a higher percentage of $PD1^+$ NKT cells correlating with sputum bacillary load. Furthermore, blocking PD-1 increased the number of IFN-γ producing NKT cells by inhibiting their apoptosis. Moreover, peripheral frequency of NKT cells declined with therapy suggesting their role in host T cell response. In this study, we concluded that PD-1 preferentially induces apoptosis of IFN-γ producing NKT cells while sparing NKT cells that produce IL-4. Such a polarized NKT cell function may impose a Th2 bias on the ensuing effector T cell response leading to inefficient clearance of *M. tuberculosis*. Inhibiting PD-1 may therefore alter the T cell response in favor of the host by rescuing type 1 NKT cells from apoptosis and boosting Th1 effector T cell functions against *M. tuberculosis*.

1. Introduction

Th1 polarized effector T cell response is believed to be critical for immune containment of intracellular *Mycobacterium tuberculosis* (*Mtb.*). Suppressed state of host T cell response occurs among tuberculosis patients, both in peripheral compartment and pathologic sites [1,2]. Various mechanisms have been documented that underlie such suppression involving various immune-regulatory T cells such as Treg and Natural killer T (NKT) cells [3–5]. Treg cells mediated suppression via IL-10 and PD-1 of antigen specific effector T cells response has been documented [6–8]. NKT cells possess similar regulatory role with potential to undergo cytokine polarization and early activation upon recognition of mycobacterial lipids [9–11]. Our study, in particular is focused on NKT cells with majorly diverse TCRs. As defined [9–11], unlike the mouse NKT cells, human NKT cell population can be broadly categorized into three major categories: i) Type-I NKT cells reactive to CD1d, expressing invariant TCR; ii) type-II NKT cells reactive to CD1d, expressing semi-invariant TCR and iii) non-CD1d reactive cells, expressing diverse TCR. $CD3^+CD161^+$ NKT and CD1d reactive invariant NKT (iNKT) cells are 5–10% and 0.1–1.0% of human peripheral blood T cells, respectively [12–14]. Literature on different types of human NKT cells (especially diverse NKT cells; *d*NKT) is scarce and the regulatory impact of PD-1 on the diverse NKT cell response against *Mtb.* remains relatively less explored. Immunoregulatory cytokines produced by NKT cells early during the infection are believed to decide the effector T cells function in terms of cytokine production and cytotoxicity [12–14]. Activated NKT cells have been associated with early resistance against intracellular pathogens and are known to be potent producers of cytokines IFN-γ and IL-4 that play an important role in immune regulation and enhance the activity of various immune cells. The capacity of

Abbreviations: APCs, antigen presenting cells; ATD, anti-tubercular drugs; Ag, antigen; Cat-I, category-I; Cat-II, category-II; HCs, healthy controls; IFN-γ, interferon gamma; IL-2, interleukine-2; *Mtb.*, *Mycobacterium tuberculosis*; mAB, monoclonal antibody; MDR-TB, multi-drugs resistance tuberculosis; NS, not significant; NKT, natural killer T cells; PBLs, peripheral bloods; PBMCs, peripheral blood mononuclear cells; PD-1, programmed death-1; PD-L1, programmed death ligand-1; PD-L2, programmed death ligand-2; PTB, pulmonary tuberculosis; TB, tuberculosis; Teff, effector T cells; Th-1, T helper-1; Th-2, T helper-2; WCL, whole cell lysate.

* Corresponding author. Division of Cellular Immunology, Department of Transplant Immunology & Immunogenetics, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110029, India. Tel.: +91 11 2659 4638, +91 11 2659 4446; fax: +91 11 2658 8663.
E-mail addresses: amaraiims@gmail.com (A. Singh), salilmitra2@gmail.com (D.K. Mitra).

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activated NKT cells to stimulate innate immunity and modulate the adaptive immunity to promote a potent antimicrobial immune response suggests the importance of these unique lymphocyte subsets [15].

During infection, NKT cells are a major source of IFN-γ through a mechanism that involves NKT cell–APC interaction and *Mtb.*–NKT direct contact [16] and their functions are likely to be regulated by balance between activating and inhibitory receptors expressed on them. Programmed cell death-1 (PD-1), is a well defined negative regulator of T cells and has been recently described to be expressed on NKT, T cells, monocytes, macrophages and mouse dendritic cells [5,17]. Recent evidence from viral infections in humans indicated a critical role of PD-1 for the inhibition of T cell functions [18–23] and NKT cells energy [24] by recruitment of intracellular Src homology region 2 domain-containing phosphatase SHP-1 and SHP-2, deactivating downstream signal pathways.

In several chronic infections, the causative pathogens have been shown to exploit the PD-1 pathway for evading host effector immune response [5,17]. Recently, others and we have demonstrated that PD-1 pathway inhibits T cell effector functions during human tuberculosis [7,8,25]. Expression of PD-1 has been described on iNKT and NK cells and plays crucial role in innate immunity against *Mtb.* infections [24,26,27]. However, little is known about PD-1 expression on NKT cells during tuberculosis and its regulatory impact on effector T cells function. Here, we aimed at studying i) the expression of PD-1 on CD161 + NKT cells representing TCR diverse population and ii) their influence on the polarized cytokine production (IFN-γ vs. IL-4) by *Mtb.* specific effector NKT cells. We studied, 49 treatment-naive pulmonary TB patients from category-I ($n = 27$) and category-II ($n = 22$) tuberculosis, using multiparametric flow cytometry to characterize PD-1 on NKT cells before and after the therapy and functions of PD-1 on NKT cells (Table 1).

Here, we showed PD-1 expression on higher proportion of TCR diverse CD161 + NKT cells among PTB patients, correlating to the sputum bacillary load. Moreover, inhibiting the PD-1 pathway led to increased frequency of *Mtb.* specific IFN-γ producing NKT cells without any significant change in the frequency of IL-4 producers. PD-1 blocking also reduced the apoptosis of IFN-γ producing NKT cells and enhanced their lytic degranulation, as measured by CD107a expression. Longitudinal analysis revealed that effective ATT treatment resulted in decline of PD-1$^+$ NKT cells frequency. All together, our data indicated that PD-1 expression may selectively inhibit IFN-γ producing NKT cells via apoptosis and decreased their lytic potential.

Table 1
Demographic and clinical details of the pulmonary tuberculosis (PTB) patients.

	Pulmonary tuberculosis category I ($n = 27$)	Pulmonary tuberculosis category II ($n = 22$)
Demographic characteristics		
Age (mean SD), range	(29.2 8.8), 20–49	(27.9 9.4), 18–50
Sex (M/F)	19/8	18/4
BMI (Kg/m^2, mean SD)	(20.2 1.9)	(27.7 2.8)
Ethnicity	Indian	Indian
Clinical details		
Radiological classification		
Extent of lesion-chest-radiograph		
Minimal lesions	5	3
Moderately advanced lesions	14	14
Far advanced lesions	8	5
Cavitary (Yes/No)	(19/2)	(19/2)
Bacillary load (sputum positivity)		
3+	8	11
2+	6	9
1+	8	7
Mx (positive/negative)	(22/5)	(18/4)
Diagnostic characteristics		
Smear for *Mycobacterium tuberculosis*		
Sputum (Positive)	27	22
Culture for *Mycobacterium tuberculosis*		
L-J Medium/BACTEC (460)		
Sputum (Positive/Negative)	19/8	(15/7)

Abbreviations: L–J medium = Lowenstein–Jensen medium, TB = tuberculosis.

2. Materials and methods

2.1. Patients

The study was conducted among 49 Pulmonary Tuberculosis (PTB) patients (27 Category I PTB: freshly diagnosed, 22 Category II PTB: treatment failure or relapse). Patients with active tuberculosis were evaluated at the All India Institute of Medical Sciences, New Delhi, India. The diagnosis was established on the basis of clinical and radiological data, identification of acid-fast bacilli in sputum, together with the past history of tuberculosis. Physical examination, complete blood cell count, electrolytes, chest X-ray, and HIV test were performed for each patient. Exclusion criteria included a positive HIV test result or the presence of concurrent infectious diseases. All patients were treated with intermittent (three times a week), Directly Observed Treatment (DOT) in accordance with guidelines of the Revised National Tuberculosis Programme (RNTCP) as Category I treatment (HRZE for initial two months and RH for the next four months; $2H_3R_3Z_3E_3 + 4R_3H_3$) and Category II treatment (SHRZE for two months, HRZE for 1 month and HRE for the next 5 months; $2S_3H_3R_3Z_3E_3 + 1H_3R_3Z_3E_34R_3H_3 + 5H_3R_3E_3$). All patients responded to anti-TB treatment. 23 Healthy Control (H) subjects were also included in the study. These control subjects did not suffer from any disease and their peripheral blood examination and chest radiographs were normal. They were attendants of indoor patients and laboratory volunteers. Peripheral blood samples were collected in heparinised tubes from all individuals after receiving informed consent.

2.2. Cell preparations and culture conditions

After sample collection, peripheral blood mononuclear cells (PBMCs) were freshly isolated by density gradient centrifugation on Ficoll–Paque (Amersham Biosciences, UK) as described elsewhere [8]. The cells were then cultured at 2×10^6 cells per ml in 96 well plates (BD Falcon, NJ, USA) with RPMI 1640 medium (Caisson Laboratories UT, USA), supplemented with L-glutamine (2 mmol/L; Sigma–Aldrich, St Louis, USA), gentamicin, and 10% bovine serum for 48 h at 5% CO_2 and 37 °C. In these experiments, cells were incubated with or without blocking antibodies against PD-1 (5 μg/ml, J116; eBioscience, CA USA), to block the interaction between PD-1 and its ligands, in the presence or absence of *M. tuberculosis* (20 μg/ml, whole cells lysate) and brefeldin (GolgiPLUG 10 μg/ml, Sigma, St Louis, USA) for last 12 h of culture. Purified mouse IgG1 (final concentration of 10 mg/mL; e-bioscience, CA USA) was used as an isotype control. Cultured cells were examined for the percentage of IFN-γ and IL-4 secreting cells using α-IFN-γ and α-IL-4 antibodies (BD Biosciences, CA, USA), were determined by flow cytometry. In separate experiment, these cells were also studied for apoptosis (annexinV) and lytic degranulation (CD107a expression).

2.3. Flow cytometry

Freshly isolated PBMCs were stained for CD3, CD161 and PD-1 expression, In separate experiments, PBMCs were incubated with