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Short communication

Impaired toll like receptor 9 response in pulmonary tuberculosis

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ABSTRACT

Background & aim: Innate immune responses are important in susceptibility to pulmonary tuberculosis (TB). In order to test the hypothesis that Toll-like receptor (TLR) 2 function would be abnormal in patients with active pulmonary TB we compared the cytokine responses of peripheral blood mononuclear cells (PBMC) to innate immune ligands in a case-control study.
Methods: PBMC from 19 untreated pulmonary TB patients, 17 healthy controls, and 11 treated pulmonary TB patients, were cultured for 24 h with TLR 2 ligand (PAM-CSK) and other TLR ligands (muramyl dipeptide, flagellin, lipopolysaccharide (LPS), CpG oligodeoxynucleotide (CpG-ODN)). Interleukin-8 (IL-8) was estimated in the supernatant by ELISA. Messenger RNA expression for inflammatory cytokines was quantitated using real time PCR.
Results: The important findings were (1) reduced PBMC secretion of IL-8 in response to all ligands in active TB; (2) normal to increased PBMC secretion of IL-8 in response to all ligands except CpG ODN (TLR 9 ligand) in TB patients who had recovered; (3) absence of difference in mRNA expression for a consortium of inflammatory pathway genes between healthy controls, active pulmonary tuberculosis and treated pulmonary tuberculosis patients.
Conclusion: There was a generalized post-translational suppression of the IL-8 response to innate immune ligands in active TB. There appears to be a defect of TLR 9 signaling in patients with tuberculosis, the nature of which needs to be further explored.

1. Introduction

About one third of the world's population has latent tuberculosis, i.e. they are infected with *Mycobacterium tuberculosis* (MTB) but are not ill with the disease [1]. Only ten percent of these individuals will ever develop active disease. Therefore we infer that host factors are important in determining development or severity of active TB. The innate immune system plays an important role in TB pathogenesis [2–5]. Cells of the innate immune system respond to conserved microbial motifs or pathogen associated molecular patterns (PAMPs) with the production of a number of effector molecules particularly cytokines and chemokines.

Peptidoglycan and lipoteichoic acid, from cell walls of mycobacteria bind to Toll-like receptor (TLR) protein on innate immune cells, resulting in the secretion of a variety of cytokines and chemokines. Activation of TLR 2 by cell wall components of mycobacteria has been shown to be associated with heightened interferon-gamma production that is necessary for protection against TB [6–10]. Another pattern recognition receptor, nucleotide-binding oligomerization domain 2 (NOD 2), recognizes muramyl dipeptide (MDP) present in cell walls of MTB [11]. NOD 2 interacts with TLR 2 for the recognition of *Mycobacterium tuberculosis* to induce a cascade of cytokines that contribute to the containment of TB [11,12]. Evidence from TLR 2-deficient mice suggests that this receptor is important in clearing high dose MTB infection [6,10,13].

Peripheral blood mononuclear cells (PBMC) secrete interleukin-8 (IL-8) on exposure to innate immune receptor ligands; absence of this response has been used to infer defective function of signaling via the corresponding receptor [14]. IL-8 responsiveness to innate immune ligands may thus be used to identify functional defects in innate immunity. We hypothesized that patients with pulmonary tuberculosis may have an underlying defect in TLR 2 signaling. This study was designed to compare IL-8 secretion by PBMC from patients with pulmonary tuberculosis and healthy controls after exposure to TLR 2 and other TLR ligands.

2. Methods

2.1. Participants

Patients with active pulmonary TB (PTB), of either sex, aged between 18 and 60 years, were recruited from the Directly Observed Treatment short course (DOTs) Clinic of the Christian

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Medical College, Vellore, India. Inclusion criteria for PTB were (1) sputum positivity for acid fast bacilli by smear with appropriate chest X-ray evidence of TB (2) no prior treatment, and (3) consent to participate. A second group of treated pulmonary TB (TPT) patients was also recruited from scrutinizing the DOTs Clinic records. This group had (1) completed six months of directly observed treatment three months or more prior to enrolment (2) demonstrated sputum negativity for AFB by smear and (3) had a chest X-ray showing clearance of TB. This group was chosen on the premise that if there was an underlying innate immune defect it would be present in both this group and in the patients with active PTB. Healthy control (HC) subjects were chosen from consenting adults (18–60 years) drawn from the same background and locality as the patients with pulmonary tuberculosis. The study was approved by the Research and Ethics Committees of the Christian Medical College, Vellore. Informed written consent was obtained from all participants.

2.2. Peripheral blood mononuclear cell (PBMC) isolation and incubation with ligands

Four mL of venous blood was collected, mixed with 4 ml RPMI 1640 medium, layered into a Ficoll-Hypaque gradient (Catalog no. H8889, Sigma-Aldrich, St. Louis, MO, USA) and centrifuged for 20 min. PBMC were removed, washed twice with medium, and centrifuged at 200*g* for 10 min to remove platelets. Isolated PBMCs were resuspended in 2 ml of complete RPMI-1640 medium with 10% FBS (Gibco, Invitrogen, USA), cell numbers were adjusted to 1.5 million/mL, and used immediately for culture. The PBMC suspensions were cultured with the following ligands (specifications and concentrations used are mentioned in parentheses): synthetic diacylated lipoprotein Pam3CSK4 (PAM) (Catalog no. tlrlpms, InvivoGen, San Diego, CA, USA, 100 ng/mL); lipopolysaccharide (LPS) from *Escherichia coli* 055:B5 (Catalog no. L2637, Sigma, Poole, UK, 1 ng/mL); flagellin (FLA) from *Salmonella typhimurium* (Catalog no. tlrlstfla, InvivoGen, San Diego, CA, USA, 1 μg/mL); Class B stimulatory human CpG oligodeoxynucleotide (CpG ODN) (Catalog no. tlrl2006, InvivoGen, San Diego, CA, USA, 10 μg/mL); and muramyl dipeptide (MDP) (Daiichi Pharmaceutical, Japan, 1 μg/mL). These ligands were chosen for specificity to the pattern recognition receptors TLR 2 (PAM), TLR 4 (LPS), TLR 5 (FLA), TLR 9 (CpG ODN, stimulatory ligand) and NOD2 (MDP). Phytohemagglutinin (PHA) (*Phaseolus vulgaris* lectin, Catalog no. L8902, Sigma-Aldrich, St. Louis, MO, USA, 10 μg/mL) was used for nonspecific immune stimulation as a control. One million cells per well were seeded into 24 well culture plates, with added ligand and medium in 1 ml volume per well and incubated in a CO_2 incubator at 37 °C for 24 h, at which time period mRNA expression of inflammatory pathway cytokine genes has been shown to be maximal [15]. Cells were then harvested and centrifuged at 5000 rpm. The cell supernatant was separated and stored at −20 °C for ELISA and the pellets were stored in Tri-Reagent (Catalog no. T9424, Sigma-Aldrich, St. Louis, MO, USA) at −80 °C for gene expression studies.

2.3. Interleukin-8 (IL-8) assay

IL-8 concentrations in the supernatant were measured by ELISA (OptEIA Set, Catalog no. 555244, BD Biosciences, San Jose, CA, USA) as per manufacturer's instructions. Samples were diluted to 1 in 10,000 prior to use, to provide optical density readings between 0.1 and 1. One hundred μL samples per well were used with incubation for 2 h at room temperature before adding detection antibody and avidin-horseradish peroxidase. After further one hour incubation and washing, tetramethyl benzidine/hydrogen peroxide reagent (Catalog no. 555214, BD Biosciences, San Jose, CA, USA) was added, incubated in the dark, and the reaction stopped at 30 min. The absorbance was read at 450 nm in a Victor 3 (PerkinElmer, Waltham, MA, USA) multilabel plate reader and IL-8 concentration calculated from a standard curve. All assays were done in duplicate using blanks and standards.

2.4. RNA extraction and reverse transcription

RNA was isolated from the PBMC pellets of 4 PTB and 4 HC participants using RNeasy Plus Mini kit (Catalog no. 74134, Qiagen GmBH, Dusseldorf, Germany), evaluated for concentration and purity using NanoDrop 2000 (Thermo Fisher Scientific, Waltham, MA, USA) at 260/280 nm, and converted to cDNA using RT^2 Easy First Strand kit (Catalog no. 330421, Qiagen GmBH, Dusseldorf, Germany).

2.5. Real time PCR quantitation of cytokine mRNA

A custom designed pathway-targeted PCR array was used to quantitate gene expression of selected cytokines and pathways related to innate immune signaling. The conditions studied included unstimulated controls, LPS-stimulated cells, MDP-stimulated cells and CpG ODN-stimulated cells. The pathway targeted array included 40 genes of interest (Table 1), 5 housekeeping genes, and appropriate controls. The array contained optimized and validated primers for the genes of interest in a 96 well plate format (Catalog no. CAPH11261, SA Biosciences, Qiagen India, Delhi, India). The cDNA derived from the PBMCs was diluted and quantitative PCR was performed using 2× Master Mix (RT^2 qPCR Master Mix, SA Biosciences, Qiagen India, Delhi, India) on a Chromo4 (Bio-Rad Laboratories Inc, Hercules, CA, USA) real time PCR system. The PCR cycling protocol included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s and final extension. Melting curve analysis was performed for each well to check the quality of the products. Amplifications showing more than one peak in each reaction at temperatures greater than 80 °C were not considered for analysis in order to avoid non-specific amplification.

2.6. Analysis

The data was analyzed using the PCR array software (RT^2 PCR Array Data Analysis Version 3.5, SA Biosciences, Qiagen India, Delhi, India) provided by the manufacturer. Expression of mRNA was normalized to expression of the housekeeping genes and expressed as fold-change using the online software provided by the manufacturer. The fold-change for each gene was calculated as $2^{(-\Delta\Delta Ct)}$, where $\Delta\Delta Ct = [\Delta Ct_{(stimulated)}] - [\Delta CT_{(unstimulated)}]$, and $\Delta CT = [CT_{(gene\ of\ interest)}] - [CT_{(average\ of\ housekeeping\ genes\ in\ that\ sample)}]$. $P < 0.05$ was taken as statistically significant.

2.7. Statistics

Sample sizes were calculated on the basis of a study in which IL-8 secretion from peripheral blood mononuclear cells was measured in healthy controls and patients with defective NOD2 signaling [14]. Based on observing similar differences between groups, we calculated a sample size of 17 per group with a study power of 80% and alpha of 0.05. Values were expressed as median (interquartile range, IQR). Comparisons for IL-8 secretion were done using the Kruskal-Wallis test with Dunn post-hoc comparisons between groups. For the PCR array studies of mRNA expression, paired comparisons were done between ligand stimulated and unstimulated control values separately for each ligand in each group of patients.