Activated TLR2/4-positive T cells boost cell exhaustion during lepromatous leprosy infection via PD-1 upregulation

Soumi Sadhu, Sanjay Kumar, Dipendra Kumar Mitra, Beenu Joshi

Department of Transplant Immunology & Immunogenetics, All India Institute of Medical Sciences, New Delhi 110029, India
Department of Life Sciences, Sharda School of Basic Sciences and Research, Sharda University, Greater Noida, UP 201310, India
National JALMA Institute for Leprosy and OMD, Taj Ganj, Agra, UP 282004, India

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ABSTRACT

The most important stage in activating an appropriate immune response during an infection is pathogen detection. Pattern recognition receptors (PRRs) are innate sensors used for pathogen detection that mould and link the innate and adaptive immune responses by the host. Toll Like receptors (TLRs), specifically TLR2 and TLR4, are PRRs, which have gained prominence due to their exceptional capacity to recognize unique molecular patterns from invading pathogens. They also play a critical role in maintaining the balance between Th1 and Th2 responses, which are necessary for the host’s survival. Leprosy is a spectral disease with a wide range of immunological manifestations in the host. Cells of both the innate and adaptive branches play crucial roles in this polarized immune state. Here, we have analysed the proportional expression patterns of TLR2 and TLR4 on the surface of CD3⁺, CD4⁺, CD8⁺, CD19⁺ and CD161⁺ lymphocytes and CD14⁺ monocytes in different groups of leprosy patients. Further, these TLRs positive cells were correlated with the surface markers of cell exhaustion such as Programmed Death-1 (PD-1) and its ligand (PD-L1), which indicated their role in immunosuppression. Additionally, blocking the interaction of PD-1 with PD-L1 in lymphocytes demonstrated visible improvement in their immune activation status through release of pro-inflammatory cytokines (IFN-γ and TNF-α).

1. Introduction

Leprosy is a neglected infectious disease that spreads from person to person through infected respiratory droplets, and affecting approximately 200,000 individuals/year over the world. It is reported in intertropical countries mainly India, Brazil, Indonesia, Bangladesh and Ethiopia, which accounts 80% of cases [1, 2]. The clinical phenotype that emerges is determined by the immune response of host. Leprosy patients exhibit a wide range of clinical symptoms that include discolored patches of skin, with loss of sensation, muscle weakness or paralysis, enlarged nerves or even blindness [3, 4]. The polar manifestations of the disease are thought to follow an immunological spectrum. Tuberculoid illness is caused by a predominantly Th1 immune response with a high level of cell mediated immunity. Low cell-mediated immunity and a humoral Th2 response characteristically define lepromatous leprosy [5, 6]. Mycobacterium leprae (M. leprae), the causal organism of leprosy, is an intracellular pathogen, therefore innate immune components play a critical role in mounting the initial immune response.

Toll like receptors (TLRs) are proteins that play crucial role in the innate immune system. They are non-catalytic, single membrane-spanning receptors that detect structurally conserved microbe-derived compounds [7]. TLRs sense bacteria that have been broken through physical barriers such as the skin or mucosa of the digestive tract, have been reported in triggering immune cell responses [8]. The highly conserved TLRs on the surface of monocytes and macrophages identify mycobacterial lipoproteins. This appears to be mediated mostly by the TLR1/2 heterodimer, which results in monocyte differentiation into macrophages and dendritic cells (DCs) [9, 10]. The later displays antigen and triggers IL-12 production, which activates naïve T cells. TLR activation also activates NF-κB, a nuclear transcription factor that regulate the expression of several immune response genes [10]. It has been demonstrated that interferon IFN-γ, IL-2 and lymphotoxin-α are released in tuberculoid lesions, resulting with increased phagocytic

* Corresponding author.
E-mail address: beenuj2002@yahoo.co.in (B. Joshi).

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activity [11]. The cytokine TNF-α have been shown to stimulate macrophages and lymphocytes leading to the formation of granulomas [12, 13]. Additionally, CD4+ Lymphocytes have been shown mostly within the granuloma, while CD8+ cells have been mostly observed in the mantle region of granuloma [14]. Furthermore, T-cells have been reported to produce the antibacterial protein granulysin in tuberculous granulomas during immune reaction [15]. However, poor granuloma development was shown as the hallmark of lepromatous leprosy (LL). The cytokines IL-4, IL-5, and IL-10 were the most often produced [13]. TLR2 expressed monocytes has been found to be downregulated by IL-4, while IL-10 has been proven to reduce IL-12 production [16]. Research study implied that TLR4 polymorphisms could be linked to leprosy susceptibility, and that could mediate the regulation of M. leprae pathogenesis at the cellular level via TLR4 signalling [17]. Additionally, the higher level of LTR2 along with IL-10 has been linked with formation of TLR2/2 dimers required for bacillary survival and evasion mechanisms for lepromatous leprosy [18]. The immunologically borderline part of the spectrum is very dynamic and keeps shifting between the two polar forms of leprosy. The reactions that are a characteristic of borderline states are caused by alterations in the immune response mediated mostly by T cells [19]. The delicate balance and interplay of cytokines, chemokines, adhesion molecules, receptors expressed and released by the innate and adaptive immune cells all play roles in modulating the individual’s immunological response to the pathogen [20].

Programmed Death 1 (PD-1) is a Type I membrane protein of 268 amino acids that belongs to the CD28/CTLA 4 family of T cell regulators [21]. The expression of PD-1 has been found on the surfaces of activated amino acids that belongs to the CD28/CTLA 4 family of T cell regulators are members of the B7 family [21, 22]. The expression of PD-L1 protein is immunosuppressive effect. PD-1 has two ligands, PD-L1 and PD-L2, both T cells, B cells, and macrophages, implying that PD-1 leads to broader reactions that are a characteristic of borderline states are caused by alterations in the immune response mediated mostly by T cells [19]. The delicate balance and interplay of cytokines, chemokines, adhesion molecules, receptors expressed and released by the innate and adaptive immune cells all play roles in modulating the individual’s immunological response to the pathogen [20].

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Studies have demonstrated that PD-1 and its ligands inhibit immune responses. In anti-CD3 driven T cells treated with PD-L1-Ig has shown suppression of T cell proliferation and IFN-γ secretion under in vitro condition [24]. Additionally, diminished T cell proliferation has been linked to reduced IL-2 secretion, which could be improved by using anti-CD28 cross-linking antibodies or addition of exogenous IL-2, suggesting that PD-1 acts as a negative regulator of T cell responses [25, 26]. Furthermore, PD-L1 transfected DCs and PD-1 transgenic CD4+ and CD8+ T cells revealed CD8+ T cells are more vulnerable to PD-L1 suppression, albeit this could be dependent on TCR signalling intensity [27]. The interaction of PD-1 and PD-L1 has been shown to suppress virus-specific CD8+ T cell activation, expansion, and acquisition of effector functions, which may be reversed by blocking PD-1 and PD-L1 binding, thereby indicating that PD-1-PD-L1 binding is important in negatively regulating CD8+ T cell responses [28].

However, the association between the surface expression of TLRs (TLR2 and TLR4) and the apoptotic markers, PD-1 and PD-L1 has not been investigated in mycobacterial infections. The current study was undertaken to investigate the role of the important TLR2 and TLR4 and the negative co-stimulatory molecule PD-1 and ligand PD-L1 in the immune response in polarized forms of leprosy and their relationship.

2. Materials and methods

2.1. Patients and controls

The approval for conducting this study was taken from the Institutional Ethics Committee of the National JALMA Institute for Leprosy & Other Mycobacterial Diseases (NJILOMD), Indian Council of Medical Research, Agra, India. The study comprised 30 treatment naive leprosy patients (17 males, 13 females, ages 19–60 years), who visited the Out Patient Department of the NJILOMD, Agra, India. The patients were classified according to the Ridley-Jopling classification of leprosy [29, 30], and grouped them into two categories (a) Borderline tuberculoid/Tuberculoid type leprosy (BT/TT, n = 15) and (b) Borderline lepromatous/Lepromatous leprosy (BL/LL, n = 15). The patients under the age of 15, pregnant women, clinical indications of anaemia, and other illnesses such as tuberculosis, HIV, and helminthic infestation were excluded. This study also included 10 healthy family subjects as controls (hereafter referred to as Healthy Subjects (HS)) who were relatives of patients diagnosed with leprosy but without clinical symptoms or other co-morbidities. The details of patients and health subjects (HS) are given in Table 1.

2.2. Flow cytometry

Monoclonal antibodies for different immunological markers (anti-human CD3, CD4, CD8, CD161, CD14, CD19, CD11c, CD83, TLR2, TLR4, PD-1, PD-L1 and IFN-γ and TNF-α) were purchased from BD Biosciences, CA, USA. The whole cell lysate (WCL) of M. leprae was collected from the Immunology laboratory of NJILOMD, Agra, India.

**Ficol hypaque** (Sigma Aldrich, St. Louis, USA) density gradient centrifugation was used to isolate Peripheral blood mononuclear cells (PBMCs) from heparinized blood, which were then suspended in RPMI-1640 (Caisson Laboratories, USA) supplemented with L-Glutamine (Sigma, USA), HEPES (Sigma, USA), antibiotics (Biological Industries, Israel), and 10% heat inactivated foetal calf serum (FCS) (Biological Industries, Israel). PBMCs (2 × 10^6 cells/ml) were cultured for 24 h under different conditions such as (i) without any stimulation (as negative control) (ii) with addition of inducer PMA + Ionomycin (as a positive control) for last 6 h of incubation; (iii) with M. leprae antigen (WCL, 20 g/l) at 5% CO2, 37 °C. 1M Monensin (Sigma, USA) was added as Golgi function blocker to all culture conditions for the last 6 h of total incubation. After culture termination, cells were stained for intracellular cytokines IFN-γ and TNF-α, as well as surface markers CD3, CD4, CD8, CD161, CD14, CD19, CD11c, CD83, TLR2, TLR4, PD-1, and PD-L1. Finally, the cells were suspended in staining buffer and the flow cytometry experiment was carried using BD FACS Calibur; USA, with analysis through Flowjo software (Tree Star, Oregon, USA).

PBMCs from LL patients (n = 4) were also used for in vitro blocking cell culture experiments, which were designed with different conditions such as, (i) Unstimulated (without M. leprae WCL), (ii) Stimulated with M. leprae WCL, (iii) Stimulated with M. leprae WCL and antibodies against PD-1 (5 μg/ml, J116; ebioscience), (iv) Stimulated with M. leprae WCL and antibodies against PD-L1 (2 μg/ml, MIH1; ebioscience) and, (v) Stimulated with M. leprae WCL and antibodies against PD-1 and PD-L1. Brefeldin (GolgiPLUG 10 μg/ml; Sigma, USA) was also used as the cytokine blocker to all culture conditions. As an isotype control, purified mouse IgG1 (final concentration of 10 mg/ml; ebioscience) was employed. At 37 °C and 5% CO2, cells under different conditions were cultured in duplicate in a 96-well culture plate. Flow cytometry was used

| Clinical types | Number of Patients | Sex | Age (years) | BI Duration of leprosy (months) |
|----------------|-------------------|-----|-------------|--------------------------------|
| Borderline Tuberculoid (BT) | 15 | 10 / 5 | 20–57 | 0–0.5 | 1–30 |
| Lepromatous Leprosy (LL) | 15 | 7 / 8 | 19–60 | 4.5–6 | 6–14 |
| Healthy controls (HC) | 10 | 5 / 5 | 22–40 | - | - |

Patients were classified on the basis of Ridley Jopling classification, BI: Bacillary Index (mean of six lesional sites). M: male, F: female. BT: Borderline Tuberculoid, LL: Lepromatous Leprosy, HC: Healthy controls.
to determine the percentage of IFN-\(\gamma\) and TNF-\(\alpha\) secreting T cells after a 72-hour incubation period. As previously stated, the surface and intracellular staining protocols were followed. After that, stained cells were captured on a BD FACS Calibur; USA, and Flowjo software (Tree Star, Oregon, USA) was used to analyse them.

2.3. Statistical analysis

Mann-Whitney test was used for analysis of non-paired samples while the Student’s t test was for paired samples. \(p < 0.05\) values were considered significant. GraphPad Prism5 software (La Jolla, CA, USA) was utilized for all statistical analysis.

3. Results

3.1. Increased proportion of TLR2 and TLR4 in T cell subsets of lepromatous leprosy

Immunophenotyping was carried out to investigate the levels of TLR2 and TLR4 in different T lymphocyte subsets using markers such as CD3, CD4, CD8 and CD161 in PBMCs isolated from (BT/TT) vs. BL/LL (\(n = 15\) in each group) and Healthy Subjects (HS, \(n = 10\)) [Suppl. Figure 1]. The significantly higher proportion of both surface markers TLR2 and TLR4 were observed in T-helper (CD3+CD4+) as well as cytotoxic T cells (CD3+CD8+) cells in BL/LL as compared to BT/TT leprosy cases and HS (Figure 1A, B, C, and D). This data suggests that these circulatory T-lymphocytes may be involved in overcoming the \(M. leprae\) bacillus load by upregulating TLR2 and TLR4 in the host.

3.2. Upregulated surface proportion of TLR2 and TLR4 in natural killer (NK) cells and natural killer T (NK-T) cells of lepromatous leprosy

The natural killer cells are one of the first players of the immune response mounted by the host, whilst the NK-T cell subset has been also shown as an interphase between the adaptive and the innate immune components of the host [31]. Therefore, we wanted to look at levels of TLRs (TLR2 and TLR4) in NK cells and NK-T cells [Supp. Figure 2]. The increased proportions of both TLR2 and TLR4 were observed on the surface of NK (CD3- CD161+) cells (Figure 2A, and C) and as well as on NK-T (CD3- CD161+) cells (Figure 2 B, and D) in patients of BL/LL as against BT/TT and HS. This further highlights that these cells may be actively involved in clearing the infection burden of \(M. leprae\).

Figure 1. Proportion of cells showing increase of TLR2 and TLR4 in T cell subsets of LL vs. BT cases of Leprosy. Bar graph showing differential proportion of (A) TLR2 in CD3+CD8+ Cytotoxic T cells; (B) TLR2 in CD3+CD4+ Helper T cells; (C) TLR4 in CD3+CD8+ Cytotoxic T cells; (D) TLR4 in CD3+CD4+ Helper T cells from PBMCs isolated from peripheral blood of BT/TT vs. BL/LL patients (\(n = 15\)) vs. Healthy Subjects (HS, \(n = 10\)). P value \(< 0.05\) was considered to be significant. Data analysis was performed with Flowjo software. Statistical analysis was done using Mann-Whitney test.
3.3. Higher proportion of TLR4 on antigen presenting cells (APCs) as compared to TLR2 in lepromatous leprosy

Mammalian TLRs are crucial for the recognition of microbial pathogens by APCs such as macrophages, dendritic cells, and B cells during immune reaction [7]. Significant upregulation of TLR2 was found on monocytes (Figure 3A) whereas higher proportion of TLR4 was observed in monocytes (CD14$^{+}$) (Figure 3C) as well as in B (CD19$^{+}$) cells (Figure 3D) of patients BL/LL against BT/TT cases or HS. Enumeration was done as shown in Supplementary figures 3 and 4.

3.4. Elevated levels of PD-1 on T, NK-T and NK cell subsets in lepromatous leprosy

It has been reported that interaction of PD-1 with its ligands PD-L1 results into inhibition of activated T cells that promotes apoptosis and anergy leading to cell exhaustion [28, 32]. Here, we found significantly elevated levels of the negative regulatory molecule PD-1 on CD3$^{+}$CD8$^{+}$ cytotoxic T cells (Figure 4A), CD3$^{+}$CD4$^{+}$ T helper cells (Figure 4B), and CD3-CD161$^{+}$ NK cells alone (Figure 4D) in BL/LL as compared to BT/TT and HS. Also, the proportion of PD-1 in CD3-CD161$^{+}$ NK-T cells (Figure 4C) was not statistically higher but trending upward. This clearly indicates that T lymphocyte subsets are expressing higher levels of apoptotic marker, PD-1 on their surface which may be one of the factors leading to death of activated T cells in the infected host with M. leprae.

3.5. Increased proportion of PD-L1 in T cells and APC in lepromatous leprosy

The constitutive expression of PD-L1 has been reported at low levels on hematopoietic cells, including resting T, B, myeloid, and dendritic cells, as well as some non-hematopoietic cells in the lung, heart, and other organs [28]. The upregulated proportion of PD-L1 has been demonstrated during T-cell activation and interacts with PD-1 and CD80, which delivers an inhibitory signal to reduce the activation of T cells [32]. Our study indicates that upon activation of T cells in infected hosts, PD-L1 is upregulated (Figure 4E) in BL/LL against BT/TT cases and HS. Significantly elevated levels of PD-L1 were also observed in different APCs such as monocytes (CD14$^{+}$) (Figure 4F), and dendritic (CD11c$^{+}$CD83$^{+}$) cells (Figure 4H) in BL/LL compared to BT/TT and HS. The same trend was also observed in CD19$^{+}$ B cells (Figure 4G) but not found significant. This upregulation of both PD-1 and its ligand PD-L1 in lepromatous leprosy might be a possible cause wherein chronic stimulation of PD-1 is resulting in T-cell "exhaustion" and the attenuation of immune responses.

Figure 2. Proportion of cells showing upregulated TLR2 and TLR4 in Natural Killer (NK) cells and Natural Killer T (NK-T) cells of Lepromatous Leprosy. Bar graph showing differential proportion of (A) TLR2 in CD3-CD161$^{+}$ Natural Killer cells; (B) TLR2 in CD3+CD161$^{+}$ Natural Killer T cells; (C) TLR4 in CD3-CD161$^{+}$ Natural Killer cells; (D) TLR4 in CD3+CD161$^{+}$ Natural Killer T cells from PBMCs isolated from peripheral blood of BT/TT vs. BL/LL patients (n = 15) vs. Healthy Subjects (HS, n = 10). P value < 0.05 was considered to be significant. Data analysis was performed with Flowjo software. Statistical analysis was done using Mann-Whitney test.
3.6. TLR4 correlates positively with PD-1 in cytotoxic T cells and NK-T cells in lepromatous leprosy

Cytotoxic T cells as well as NK-T cells are known to have important implications in determining the host immunity and subsequent disease manifestation [33]. The results in previous sections have depicted significant increase in TLR2 and TLR4 proportion in T cell subsets and APCs along with increased surface proportion of PD-1 and PDL-1 in different immune cells in BL/LL vs. BT/TT. Hence, we attempted to correlate the surface proportion of TLR2 and TLR4 with PD-1/L-1 in BL/LL patients wherein they might play an important role in immune response generation. We found a positive correlation between levels of TLR4 and PD-1 in CD3⁺CD8⁺ cytotoxic T cells (r = 0.48, P = 0.07; Figure 5A) as well as in CD3⁺CD161⁺ NK-T cells (r = 0.7, p = 0.007; Figure 5B) in case of BL/LL patients. Simultaneously, the proportion of TLR4 were also correlated with the increased proportion of PD-L1 (Supplementary Figure 5), however, there was no significance. Our results indicate that high TLR4 proportion in cytotoxic T cells and NK-T cells in LL patients may be incapacitated by the concomitant upregulation of PD-1/L-1 consequently leading to cell exhaustion and apoptosis.

3.7. Polarized immunity displayed by cytotoxic T cells and NKT cells highlighting the immunosuppression in lepromatous leprosy

The intracellular production of IFN-γ, TNF-α and IL-4 was determined in CD3⁺CD8⁺ cytotoxic T cells and CD3⁺CD161⁺ NKT cells after stimulation with M. leprae WCL for 24 h (Figure 6). Interestingly, cytotoxic T cells obtained from BT/TT patients preferentially produced higher amounts of IFN-γ and TNF-α cytokines (p < 0.05) (Figure 6A, B). On the other hand, CD3⁺CD8⁺ cytotoxic T cells from BL/LL patients showed significantly increased level of IL-4 as compared to BT/TT patients (p < 0.05, Figure 6C). Similar trend of increased IFN-γ and TNF-α vs. IL-4 production was observed in CD3⁺CD161⁺ NKT cells as well after stimulation with M. leprae WCL for 24 h (Figure 6D, E, F). This gave a strong indication of cytokine polarization among cytotoxic T cells and NKT cells alongside Th1/Th2 like differentiation in BT/TT and BL/LL patients.

3.8. Blocking of the PD-1/PDL-1 pathway leads to reversal in the immune response in lepromatous leprosy

PD-1 has recently been shown to inhibit T cell responses during chronic viral infections such as HIV [34]. Here, we found that there is a significant difference in the proportion of both PD1 and PD-L1 in T, B, NKT cells as well monocytes in BL/LL vs. BT/TT cases of human leprosy. Hence, we wanted to examine whether the blocking of this pathway could lead to the reversal in the immune response of the immunocompromised host (BL/LL patients). Using specific antibodies against the PD-1/PDL-1 molecules, we successfully blocked their interaction and measured the production of Th1 cytokines like IFN-γ and TNF-α by CD3⁺ T cells (Figure 7A and B). Our results revealed that the immune response of the host can be reversed to quite some extent through increase in the production of IFN-γ and TNF-α by CD3⁺ T cells after blocking of the PD-1/PDL-1 interaction. The increase was more pronounced in the case of IFN-γ rather than TNF-α. Hence, blocking of the PD-1–PDL-1 interaction restores T cell function in the case of immunocompromised lepromatous leprosy cases.

4. Discussion

Leprosy manifests itself in a variety of clinical and histological ways. The ability of the host to develop a cellular immune response to M. leprae...
was thought to be the source of this vast diversity [9, 10]. The host defence systems that occur early in infection during the indeterminate phase are perhaps the least understood aspects of leprosy immunology [35, 36]. The combination of an effective innate immune response and the poor virulence of the leprosy bacillus may explain disease resistance. However, disease dissemination occurs when host immunity is suppressed due to multiple factors, including genetics and bacillus load [37].

During innate immunity, mammalian TLRs are essential for macrophages and DCs to identify microbial invaders. TLRs are transmembrane proteins with phylogenetically conserved leucine-rich motifs in their extracellular domains [38]. The cytoplasmic signalling domain of TLR has been associated to the IL-1 receptor-associated kinase, which increases cytokine production by activating transcription factors such as NF-κB [39]. TLR1-TLR2 heterodimers, and TLR4 have all been implicated...
in mycobacteria pathogen detection [40, 41]. Additionally, TLRs have been discovered to be necessary for the optimal production of IL-12, a pro-inflammatory cytokine involved in the induction of Th-1-type immunity, as well as TNF-α, a pro-inflammatory cytokine involved in cellular activation and granuloma formation but also implicated in the tissue destruction seen in leprosy reactions [42, 43]. APCs such as DCs are stimulated through TLR ligands and restrict CD4 T-cell proliferation leading to significant reduction of the adaptive immune responses required for disease control [42]. Studies have demonstrated that *M. leprae* activates and signals through the TLR4 receptor in macrophages for the pathogenesis of the disease [42]. However, genetic association studies in human populations suggested that people with TLR2/4 polymorphisms may be protected against leprosy [17, 44]. In our study, it was found that even though both TLRs (TLR2 and TLR4) were enhanced in LL patients, there was a substantial difference in the proportion of TLR4 notably in T cytotoxic (CD3+CD8+) cells. TLR2 on the other hand showed increased proportion on T helper cells (CD3+CD4+) and NKT (CD3+CD161+) cells in LL patients. TLR2 was also significantly upregulated in NK (CD3-CD161+) cells and monocytes (CD14+) whilst TLR4 was increased in B lymphocytes (CD19+) cells, in LL patients. This suggests that TLRs (TLR2 and 4) upregulation on these cells are very important in overcoming the infection load of *M. leprae* in the host. Even though our findings may not be conclusive as to the exact involvement of TLRs in leprosy, more research may offer insight on whether TLRs though our findings may not be conclusive as to the exact involvement of TLRs in leprosy, more research may offer insight on whether TLRs through our *M. lepra* WCL in PBMCs isolated from peripheral blood of leprosy patients and compared against unstimulated negative control and PMA + ionomycin positive control well. Each dot represents a single individual. Median values are shown in each set while P value < 0.05 was considered to be significant. Data analysis was performed with flowjo software. Statistical analysis was done using Mann-Whitney test in the Graphpad Prism5 software.

On the other hand, the B7 family of co-signalling molecules, PD-1 and its ligands PD-L1 and PD-L2 have been demonstrated to play a significant role in down-regulating the T-cell immune response. [28, 36] T cell responses to a persistent viral infections like HIV have recently been demonstrated to be inhibited by PD-1 [45]. However, little is known about the involvement of PD-1 and its ligand PD-L1 in the immunological response of the host in leprosy with polarised clinical manifestations. In comparison to tuberculoid cases of the disease, we found higher levels of the negative regulatory molecule PD-1 on CD3+ T cells, and CD161+ NK cells in lepromatous leprosy, which could be one of the key factors causing host immunological compromise and active disease. Our findings suggest that when T cells are activated, PD-1 along with PD-L1 are also elevated. Consequently, prolonged PD-1 stimulation might lead to T-cell "exhaustion" and attenuation of host immune responses. In lepromatous leprosy patients, APCs such as DCs, monocytes and B cells simultaneously demonstrated elevated levels of PD-L1 surface proportion. Cytokine polarization at the level of effector T cells is well demonstrated in leprosy. Our findings further suggest that cytotoxic T cells as well NKT cells are also playing crucial roles in determining the polarized immunological status of the host, in terms of production of the pro-inflammatory IFN-γ and TNF-α vs. anti-inflammatory IL-4. This is especially relevant for the lepromatous leprosy patients, wherein we observe profound immunosuppression. These factors may be responsible for immunological compromise in the host leading to disseminated disease and massive cellular and nerve damage as observed in reversal reactions [28]. The PD-1-PD-L1 binding has been demonstrated to suppress activation, expansion, and acquisition of effector activities of CD3+ T cells. Studies in lymphomas have shown that the expressions of TLR4 and PD-1 pathway may act as predictors for survival time in patients [46, 47]. Here, the increased proportion of TLR4 in relation to the level of PD-1 was found to be positive, especially in the lymphocyte subsets of double positive CD3+ and CD8 cytotoxic T cells and CD3+ and CD161+ NKT cells. This indicates that these cells are very crucial in preventing dissemination of disease by clearing the intracellular *M. leprae* pathogens residing within the macrophages, but were unable to perform efficiently due to over representation of apoptotic markers PD-1/PD-L1 leading to cell death. Blocking of the interaction between PD-1 and PD-L1 may help to reverse the suppressive effect mounted by this pathway, which was
achieved successfully using specific antibodies against the PD-1/PDL-1 molecules. The increased production of Th1 cytokines like IFN-γ and TNF-α by CD3+ T cells indicates that inhibition of the PD-1 and PD-L1 interaction restores T cell function in immune-compromised lepromatous leprosy patients.

To our knowledge, current research is the first to look at TLR2 and TLR4 levels in the context of polarized forms of leprosy, revealing that higher TLR4 expression could be associated with the dissemination of disease in individuals with lepromatous leprosy. We also found the association between high TLR4 level and PD-1 proportion in the same patients, which could be associated with poor prognosis. Hence, the potential therapeutic implications of these immunological checkpoint molecules should be explored further with prospective studies and clinical trials in order to receive a clear picture regarding their roles.

5. Limitations of the study

The current study has been performed in low sample size of different groups of leprosy patients due to the reduced prevalence of leprosy (especially lepromatous cases) in current times. Another limitation of our study was the use of a 3-color panel for flow cytometry which prevented the co-staining for the markers of suppression, i.e., PD-1 and PD-L1 and TLRs.

Declarations

Author contribution statement

Soumi Sadhu: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.
Sanjay Kumar: Analyzed and interpreted the data; Wrote the paper.
Dipendra Kumar Mitra: Contributed reagents, materials, analysis tools or data.
Beenu Joshi: Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

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Data availability statement
The data that has been used is confidential.

Declaration of interest’s statement
The authors declare no conflict of interest.

Additional information
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