Triggering Through Toll-like Receptor 2 Limits Chronically Stimulated T-helper Type 1 Cells From Undergoing Exhaustion

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Chronic infections result in T-cell exhaustion, a state of functional unresponsiveness. To control the infection, it is important to salvage the exhausted T cells. In this study, we delivered signals through Toll-like receptor 2 (TLR-2) to reinvigorate functionality in chronically activated T-helper type 1 (Th1) cells. This process significantly augmented the expression of T-bet, interferon γ, interleukin 2, and the antiapoptotic molecule Bcl-2, whereas it dampened the display of the exhaustion markers programmed death receptor 1 (PD-1) and lymphocyte activation gene 3 (Lag-3). Additionally, TLR-2 signaling bolstered the ability of chronically stimulated Th1 cells to activate B cells. Finally, the results were substantiated by observing reduced lung pathology upon administration of TLR-2 agonist in the chronic infection model of tuberculosis. These data demonstrated the importance of TLR-2 in rescuing chronically activated Th1 cells from undergoing exhaustion. This study will pave a way for targeting TLR-2 in developing therapeutic strategies to treat chronic diseases involving loss of Th1 cell function.

Keywords. Th1 cells; TLR-2; chronic infection; exhaustion markers; tuberculosis.

Persistent antigenic load in chronic viral infection often results in functional exhaustion of antigen specific CD8 T cells and impaired cell-mediated immunity [1–3]. The T cell exhaustion phenotype is associated with diminished expression of effector molecules, such as interferon γ (IFN-γ), interleukin 2 (IL-2), tumor necrosis factor α (TNF-α), perforins, and granymes, and higher expression of coinhibitory molecules, such as cytolytic T-lymphocyte antigen 4 (CTLA-4), programmed death receptor 1 (PD-1), lymphocyte activation gene 3 (Lag-3), CD160, and T-cell immunoglobulin mucin 3 (Tim-3) [4]. Further, the exhaustion phenotype of T cells is maintained even after antigen withdrawal [5]. The gene expression signature of exhausted CD8 T cells is distinct from effector and memory T cells [6]. Engagement of the above-mentioned coinhibitory molecules through cell-cell contacts results in sustenance of the exhausted phenotype of CD8 T cells [7]. Importantly, blockade of signaling delivered through coinhibitory receptors, such as Lag-3, Tim-3, and PD-1/PDL-1, and anti-inflammatory cytokines, namely interleukin 10 and transforming growth factor β, restored the function of exhausted T cells in Lymphocytic choriomeningitis, Toxoplasma gondii, and Plasmodium vivax infections [8, 9]. The phenomenon of exhaustion is not limited to CD8 T cells but has also been observed in CD4 T cells, especially during persistent infections and malignancies [10, 11]. CD4 T cells are known as helper cells because of their manifold function in assisting the immune system. They activate antigen-presenting cells (APCs) and provide help to B cells and CD8 T cells [12]. Thus, any abnormality in the function of CD4 T cells affects both cell-mediated and humoral immunity. Exhaustion of CD4 T cells adversely influences effector immunity against many diseases, including those due to viruses, bacteria, and protozoa [2, 13, 14]. For example, chronic Mycobacterium tuberculosis infection is associated with...
slow proliferation and impairment in the function of CD4 T cells [15]. Recently, the combined blockade of PD-1, Lag-3, and Tim-3 has been reported in rescuing exhausted T cells [9, 16].

In addition to being the classical activators of APCs, Toll-like receptors (TLRs) have been shown to have an effect on T-cell activation through direct signaling [17]. Further, intrinsic TLR receptors (TLRs) have been shown to have an effect on T-cell function [15]. Recently, the combined blockade of PD-1, Lag-3, and Tim-3 has been reported in rescuing exhausted T cells [9, 16].

Astounding, direct triggering through TLRs also reduces the expression of PD-1 on CD8 T cells [19, 20]. Surprisingly, the role of TLR-2 signaling in rescuing Th1 cells from exhaustion has not been explored, although they play a cardinal role in protecting against many intracellular infections, such as tuberculosis [21].

Taking into consideration the aforesaid facts, we examined the impact of TLR-2 signaling on chronically stimulated Th1 cells in recovering them from exhaustion. Intriguingly, we observed that triggering through TLR-2 using Pam2Cys could significantly protect chronically stimulated Th1 cells from exhaustion. This was demonstrated by the enhancement in the effector function of chronically stimulated Th1 cells and decline in the expression of exhaustion markers. Further, chronically stimulated Th1 cells derived from MyD88−/− mice failed to display the rescued phenotype upon Pam2Cys treatment. Furthermore, administration of Pam2Cys to chronically infected mice with M. tuberculosis rescued Th1 cells from exhaustion and significantly ameliorated the disease symptoms. Thus, this phenomenon may have important implications in developing immunotherapeutic strategies to reinvigorate T cells during chronic infections.

MATERIALS AND METHODS

Experimental Mice

Female, C3He/J and BALB/c mice (age, 4–5 weeks) were procured from the Experimental Animal Facility of the Institute of Microbial Technology, Council of Scientific and Industrial Research (CSIR-IMTECH; Chandigarh, India). C57BL/6 (wild-type and MyD88−/−) mice were a kind gift from Dr Stephanie C. Eisenbarth, Department of Laboratory Medicine, Yale University (New Haven, CT). All experiments were approved by the Institutional Animal Ethics Committee of CSIR-IMTECH and performed according to the National Regulatory Guideline issued by the Committee for the Purpose of Supervision of Experiments on Animals (no. 55/1999/CPCSEA), Ministry of Environment and Forest, Government of India, and in accordance with guidelines of the Institutional Animal Care and Use Committee, Yale University.

Stimulations of Naïve CD4 T Cells

Naïve T cells of C3He and C57BL/6 (WT or MyD88−/−) were purified by magnetic-activated cell sorting, according to the manufacturer’s instructions. The isolated naïve T cells were highly purified and devoid of the non–CD4 T-cell population, as ascertained by flow cytometry (CD3+CD4+CD621hiCD44lo/− CD8+CD19+CD11c−F4/80−; data not shown). Naïve T cells (3 × 10⁵ cells/well) were cultured in 24-well plates (BD Biosciences, San Diego, CA) coated with plate-bound anti-CD3 antibody (Ab: 5 µg/mL) and soluble anti-CD28 Ab (2 µg/mL) under Th1 (interleukin 12: 10 ng/mL; anti-interleukin 4 [IL-4] Ab: 5 µg/mL; and IL-2: 100 U/mL) polarization conditions. Plate bound anti-CD3 Ab was used for the stimulation of T cells.

Acute Stimulation

The cells were stimulated as described above for 60–62 hours. Cells were then harvested and rested for another 60 hours along with cytokines (without washing) and used for the acute stimulation experiments.

Chronic Stimulation

The cells were cultured as described above for 120 hours with or without Pam2Cys (300 ng/mL). Later, without resting, the cells were used for chronic stimulation phenotype of T cell. Naïve T cells that were acutely stimulated, chronically stimulated, or stimulated with Pam2Cys during culture under Th1 polarization conditions are hereafter termed “ASTh1 cells,” “CSTh1 cells,” and “pCSTh1 cells,” respectively.

Flow Cytometry

Cells were surface stained with fluorochrome-labeled antimouse Abs and their respective isotype-matched controls, as described previously [22, 23]. For intracellular staining, ASTh1, CSTh1, and pCSTh1 cells were stained with phorbol 12-myristate 13-acetate (50 ng/mL) and ionomycin (1 µM) for 2 hours, followed by brefeldin for additional 2 hours, to check intracellular cytokine expression. The cells were stained with fluorochrome-labeled Abs and isotype-matched controls. Flow cytometry data were acquired using the FACS Aria II and analyzed by FACS Diva software. For histogram overlay, FlowJo_V10 (TreeStar) software was used. Data are represented as percentage population or as mean fluorescence intensity (MFI). Fold-change was determined by dividing the MFI values of CSTh1 cells or pCSTh1 cells by the MFI of ASTh1 cells.

Determination of T-Cell Proliferation by the Carboxyfluorescein Succinimidyl Ester (CFSE) Dye-Dilution Assay

ASTh1, CSTh1, and pCSTh1 cells were stained with CFSE (1 µM) by incubating in phosphate-buffered saline (PBS) for 8 minutes at 37°C. The cells were washed 3 times and restimulated with anti-CD3 (5 µg/mL) Ab for 72 hours, and proliferation was monitored by flow cytometry.

T-Cell and B-Cell Coculture

ASTh1, CSTh1, and pCSTh1 cells (1 × 10⁵ cells/well) were co-cultured with B cells (1 × 10⁵ cells/well), labeled with CFSE, and
stimulated with plate-bound anti-BCR Ab. B cells were checked for activation markers and proliferation after 48 hours and 72 hours, respectively, by flow cytometry. After 48 hours, intracellular expression of CD40L was monitored on ASTh1, CSTh1, and pCSTh1 cells.

**Experimental Model of Chronic M. tuberculosis Infection**

BALB/c mice underwent aerosol challenge with low (100 colony-forming units [CFU]) and high (1000 CFU) doses of M. tuberculosis (H37Rv) to create models of acute and chronic infections, respectively. After 45 days, the chronically infected mice were anaesthetized with isoflurane and administered Pam2Cys (10 nmol/50 µL/animal) intranasally, and a booster dose was given 7 days after primary immunization. Control mice were injected with PBS (placebo). Mice were euthanized 15 days after receipt of the booster immunization. T cells were examined for the surface markers (Lag-3, PD-1, CD44, CD62L, KLRG-1, CD27, and CD43) ex vivo. Intracellular expression of IFN-γ and cell proliferation was checked by in vitro restimulation of CD4 T cells isolated from lungs with purified protein derivative (10 µg/mL). Lung sections underwent histopathologic examination by hematoxylin-eosin staining, and the bacterial burden was assessed by plating tissue homogenates on 7H11 agar plates.

**IFN-γ Enzyme-Linked Immunosorbent Assay (ELISA)**

ASTh1, CSTh1, and pCSTh1 cells were restimulated with plate-bound anti-CD3 Ab (5 µg/mL) for 12 hours, and the IFN-γ level was estimated in the culture supernatants by sandwich ELISA, as per the manufacturer’s instructions [24].

**Statistic Analysis**

The statistical analysis of the data was done by the Student t test, the nonparametric Mann–Whitney 2-tailed test, and repeated-measures analysis of variance with the post hoc Student-Newman–Keuls multiple-comparisons test, using Graph Pad InStat 3 software. Additional methods are specified in the Supplementary Materials.

**RESULTS**

**Triggering Through TLR-2 Rescues CSTh1 Cells From Exhaustion**

The significant role of TLRs as pathogen sensors in imparting prompt immune response is now a thoroughly established paradigm [25]. Although the direct effect of TLR-2 has been investigated in the biology of CD4 T cells, its influence on exhaustion is still unknown. Therefore, we designed an in vitro experimental system involving chronic stimulation of purified naive CD4 T cells to mimic antigen persistence during exhaustion [26]. We could compare CSTh1 cells with ASTh1 cells, for which TCR stimulation was removed once the cells were activated. Chronic stimulation manifested the hallmarks of exhaustion phenotype upon restimulation, as revealed by a loss of effector functions, notably a decline in the expression of IFN-γ and IL-2, and up-regulation of exhaustion markers PD-1, Lag-3, Fas ligand (FasL), and Fas, compared with acute stimulation (Figure 1A–C, 1F, and 1G and Supplementary Figure 1A and 1B). Thus, continuous signaling leads to functional exhaustion of Th1 cells. Naïve CD4 T cells do not express high levels of TLR-2 but is upregulated upon activation and further enhanced in the presence of the TLR-2 ligand Pam2Cys (Supplementary Figure 1C). We enquired whether TLR-2 stimulation could prevent CSTh1 cells from exhaustion. pCSTh1 cells exhibited significant (P < .01) augmentation in the percentage of IFN-γ–expressing cells (Figure 1A). The increment in the expression of IFN-γ was observed in a dose-dependent manner. The optimum secretion was observed with 300 ng/mL of Pam2Cys, and therefore this concentration was selected for all the subsequent experiments (Supplementary Figure 1D). The IFN-γ induction was further confirmed at the messenger RNA level and in the culture supernatants (Supplementary Figure 1E and 1F). Further, pCSTh1 showed substantial (P < .01) increase in the percentage of PD-1hi/IFN-γlo cells and a reduction in the percentage of PD-1hi/IFN-γlo cells (Figure 1A). Furthermore, an increase in the expression of IL-2 (P < .05) and a reduction in Lag-3 (P < .001), was noticed (Figure 1B and 1C). Consequently, indicating a rescued phenotype of Th1 cells [8]. Additionally, a considerable decrease (P < .05) in the PI/annexin-V+ apoptotic cells and Fas/FasL expression and an increased cell number were noted in pCSTh1 cells, compared with CSTh1 cells (Supplementary Figure 1A, 1B, 1G, and 1H). The electron microscopic analysis of pCSTh1 cells revealed a refractile cell surface and enhanced viability, compared with CSTh1 cells (Figure 1D). Although, CSTh1 cells expressed effector memory markers (CD44hi/CD62Llo), Pam2Cys treatment skewed them toward a central memory phenotype (CD44hi/CD62Lhi; Figure 1E). Compared with ASTh1 cells, we observed higher expression of CD44 on CSTh1 cells (Figure 1E), an indicator of exhaustion [27]. Pam2Cys induced moderate increases in IFN-γ, IL-2, and T-bet expression in ASTh1 as well, but no changes in PD-1 and Lag-3 expression were observed (Supplementary Figure 2A–E). Compared with ASTh1 cells, our data also indicated noticeable difference in the activation/exhaustion markers on CSTh1 cells (Figure 1A–G).

Next, we evaluated the specificity of Pam2Cys in rescuing CSTh1 cells. MyD88−/− mice completely lack TLR-2 signaling. Importantly, the expression of IFN-γ and PD-1 on CSTh1 cells derived from MyD88−/− animals failed to display any change with the treatment of Pam2Cys. However, a significant change was noticed for both markers in the cells generated from wild-type mice (Figure 1F and 1G), confirming the specific action of Pam2Cys on CSTh1 cells. Controlling the expression of molecules that promote T-cell exhaustion may have great potential in restoring effector function and can safeguard CSTh1 cells from exhaustion.
Figure 1. Stimulation through Toll-like receptor 2 (TLR-2) rescues chronically stimulated T-helper type 1 (CSTh1) cells from exhaustion. Acutely stimulated Th1 (ASTh1), CSTh1, and Pam2Cys-exposed chronically stimulated Th1 (pCSTh1) cells were monitored for the functional and exhaustion phenotypes. A, Contours and their bar diagrams designate coexpression of programmed death receptor 1 (PD-1)lo/interferon γ (IFN-γ)hi. The numbers in the inset of contours (left panel) and bar diagram (right panel) indicate percentages. B, Histograms signify intracellular expression of interleukin 2 (IL-2), and inset data represent mean fluorescence intensities (MFIs; left panel); the bar diagram denotes the percentage of IL-2hi cells (right panel). C, Histograms depict lymphocyte activation gene 3 (Lag-3) expression (left panels), and bar diagrams reveal fold-changes in MFIs (right panels) from pooled data of 5 and 2 experiments, respectively. D, Images show cells revealed by bright-field microscopy (40× original magnification; upper panel) and scanning electron microscopy (5000× original magnification; lower panel). E, Contour plots show percentages of cells coexpressing CD44/CD62L. F and G, Bar diagrams depict MFIs of IFN-γ- and PD-1-expressing cells generated from MyD88−/− and wild-type (WT) mice. Fold-changes in MFI were calculated with respect to MFIs for ASTh1 cells. Data are means ± standard errors of the mean in histograms, contours, dot plots, and bar diagrams for 3 independent flow cytometry experiments (A–B and E–G). *P < .05, **P < .01, and ***P < .001.
Figure 2. Triggering of chronically stimulated T-helper type 1 (CSTh1) cells through Toll-like receptor 2 (TLR-2) augments the expression of T-bet and restores the effector phenotype. Acutely stimulated Th1 (ASTh1), CSTh1, and Pam2Cys-exposed chronically stimulated Th1 (pCSTh1) cells were examined for the phenotype that facilitates rescuing from exhaustion. A, Flow cytometry histogram plots (left panel) represent the expression of T-bet, and the numbers in the inset pertain to mean fluorescence intensities. The bar diagram (right panel) depicts the fold-change in the expression of T-bet with respect to the value for ASTh1 cells. B and C, Bar diagrams of Western blot (B) and reverse transcription quantitative polymerase chain reaction (RT-qPCR; C) analysis of T-bet expression. D, Contour plots depict coexpression of programmed death receptor 1 (PD-1)/T-bet (left panel), and numbers in the inset indicate percentages of cells with the indicated phenotype. The bar diagram shows the percentages of PD-1hi/T-betlo cells (right panel). E, The histogram shows expression of Bcl-2. Flow cytometry histograms, contours, and bar diagrams (A, D, and E) and the bar diagram of RT-qPCR findings (C) are means ± standard errors of the mean of 3 independent experiments. *P < .05 and ***P < .001.
Figure 3. Toll-like receptor 2 (TLR-2) signaling enables chronically stimulated T-helper type 1 (CSTh1) cells to provide better help to B cells. Acutely stimulated Th1 (ASTh1), CSTh1, and Pam2Cys-exposed chronically stimulated Th1 (pCSTh1) cells were cocultured with B cells. A, Flow cytometry histograms (left panel) and the bar diagram (right panel) represent B-cell proliferation revealed by the carboxyfluorescein succinimidyl ester (CFSE) dye-dilution assay. The y-axis in bar graph represents the percentage of proliferating cells (CFSE<sup>lo</sup>). B–D, Histogram plots (left panels) and bar diagrams (right panels) designate the surface expression of CD80, CD86, and CD40 on CD19-gated B cells. E, Histograms (left panel) and the bar diagram (right panel) denote intracellular expression of CD40L on CD4-gated T cells. In panels B–E, the y-axes represents the mean fluorescence intensity of respective markers. Data in the bar diagrams are means ± standard errors of the mean of 3 independent experiments. *P< .05 and ***P< .001. Abbreviation: MFI, mean fluorescence intensity.
Figure 4. Intranasal immunization with Pam2Cys restricts T-cell exhaustion during chronic Mycobacterium tuberculosis infection. Models of acute and chronic M. tuberculosis infection were established as described in "Materials and Methods" section. Sixty-seven days after infection, acutely infected mice (AI), chronically infected mice (CI), and Pam2Cys-exposed chronically infected mice (pCI) were euthanized. CD4 T cells were isolated from the perfused lungs and examined for various activation and exhaustion markers by flow cytometry. A–G, Bar diagrams represent the mean fluorescence intensities (MFIs) of ex vivo expression of lymphocyte activation gene 3 (Lag-3), programmed death receptor 1 (PD-1), KLRG-1, CD43, CD44, CD62L, and CD27. H, Histograms (left panel) and the bar diagram (right panel) refer to the percentages of proliferating CD4 T cells revealed by the carboxyfluorescein succimidyl ester dye-dilution assay on in vitro stimulation with purified protein derivative (PPD). I, Dot plots (left panel) and the bar diagram (right panel) represent the percentages and integrated MFIs, respectively, of CD4/interferon γ (IFN-γ)+ cells stimulated with PPD. J, Hematoxylin–eosin staining of lung sections (100× original magnification); arrows denote granulomas. K, The bar diagram depicts bacterial loads assessed by plating lung homogenates and represented as log_{10} colony-forming units (CFU) per gram of lung. Data are representative of 2–3 independent experiments with 3–4 mice/group. *P<.05, **P<.01, and ***P<.001.
**pCSTh1 Cells Maintained an Effector Phenotype by Persistent Expression of T-bet**

Next, we investigated the mechanisms for restoration of the effector function in pCSTh1. It has been reported that transcription factor T-bet restricts the exhaustion of CD8 T cells during viral infections [28]. Hence, we checked whether prevention of CSTh1 cell exhaustion via TLR-2 signaling was a result of T-bet induction. We observed a significant ($P < .05$) increase in T-bet expression in pCSTh1 cells by flow cytometry (Figure 2A). This observation was also supported by Western blot and reverse transcription quantitative polymerase chain reaction data (Figure 2B and 2C). The pool of pCSTh1 cells expressing PD-1hi/T-betlo was substantially ($P < .001$) reduced, compared with the pool of CSTh1 cells (Figure 2D). It has been reported that PD-1hi T cells exhibit low levels of the antiapoptotic molecule Bcl-2 and are prone to apoptosis [29]. Intriguingly, we noticed considerable upregulation of Bcl-2 in pCSTh1 cells (Figure 2E). It was important for us to monitor whether the activity of Pam2Cys in rescuing T-cell exhaustion was restricted to CSTh1 cells or could be extended to CSTh2 and CSTh17 cells. Even though there was a slight increase in IL-4 and IL-17 expression by Th2 and Th17 cells, respectively, upon Pam2Cys treatment, no apparent change was noticed in PD-1 and Lag-3 expression (data not shown). Thus, rescuing of exhaustion by Pam2Cys may occur solely by T-bet induction and thus will not be reflected in Th2 or Th17 cells. These results suggest that prevention of exhaustion by TLR-2 signaling is T-bet dependent and is therefore restricted to CSTh1 cells.

**pCSTh1 Cells Acquire an Enhanced Ability to Activate B Cells**

CD4 T cells play a crucial role in helping B cells [30, 31]. Cognate interaction between CD40 and CD40L, expressed on B cells and T cells, respectively, enhances the B-cell survival and antibody formation. Further, cytokines released by CD4 T cells increase B-cell activation [32, 33]. Apparently, any defect in the function of CD4 T cells would lead to compromised activation of B cells. Recent reports suggest that blockade of PDL-1 and Lag-3 restored the generation of protective antibody responses and amplified the number of follicular B cells in murine malarial infection [9]. We next asked whether stimulation through TLR-2 bolsters the ability of pCSTh1 cells to activate B cells. Interestingly, the capacity of pCSTh1 cells to help B cells was improved, as evidenced by a significant increase in B-cell proliferation ($P < .001$) and expression of the costimulatory molecules CD80 ($P < .001$), CD86 ($P < .001$), and CD40 ($P < .05$; Figure 3A–D). Further, an enhanced level of CD40L ($P < .05$) was observed on CSTh1 cells (Figure 3E). The interaction of CD40L with its receptor CD40 on B cells is reported to be involved in B-cell activation. Boosting the activity of CSTh1 cells by Pam2Cys and their enhanced ability to stimulate B cells may be an interesting avenue for therapeutic intervention in diseases related to B-cell deficiencies.

**Intranasal Administration of Pam2Cys Limits T-Cell Exhaustion During Chronic M. tuberculosis Infection**

We investigated whether Pam2Cys administration would be effective in vivo in chronic diseases to rescue T cells undergoing exhaustion. This proposition was evaluated in an experimental model of chronic *M. tuberculosis* infection. Chronically infected and acutely infected mice exhibited differential expression of CD4 T-cell surface markers, lung pathology, and bacterial burden (Figure 4A–G and Supplementary Figure 3A–C). CD4 T cells isolated from acutely infected, chronically infected, and Pam2Cys-exposed chronically infected mice were ex vivo examined for exhaustion markers. Compared with AI mice, a higher expression of Lag-3, PD-1, KLRG-1, and CD43 was noted in chronically infected mice (Figure 4A–D). Fascinatingly, there was significant suppression of Lag-3 ($P < .01$), PD-1 ($P < .05$), KLRG-1 ($P < .001$), and CD43 ($P < .05$) expression in Pam2Cys-exposed chronically infected animals (Figure 4A–D). Further, significant increases in levels of the activation/memory T-cell markers CD44 ($P < .01$), CD62L ($P < .001$), and CD27 ($P < .05$) were also noticed (Figure 4E–G). Considerable increases in the proliferation ($P < .01$) and IFN-γ expression ($P < .05$) by CD4 T cells isolated from the lungs of Pam2Cys-exposed chronically infected mice were observed upon in vitro culturing of cells with purified protein derivative of *M. tuberculosis* (Figure 4H and 4I). Furthermore, amelioration of the lung pathology and a significant reduction in the bacterial burden ($P < .05$) were observed (Figure 4J and 4K). There was a significant difference in the status of exhaustion/activation markers on the cells of acutely infected and chronically infected mice. Excitingly, findings for cells from Pam2Cys-exposed chronically infected mice were fairly comparable with those of acutely infected mice (Figure 4A–4K). Furthermore, purified CD4 T cells isolated from Pam2Cys-exposed chronically infected animals showed enhanced proliferation and IFN-γ expression upon TCR stimulation (Supplementary Figure 3D and 3E). It may be concluded from these experiments that Pam2Cys is quite efficacious in bolstering immunity and protecting mice against chronic *M. tuberculosis* infection.

**DISCUSSION**

Th1 cells are crucial in maintaining cell-mediated immunity and defending against intracellular pathogens [34]. The Th1 cytokines IFN-γ and IL-2 are involved in the activation of macrophages and B cells and in augmenting their microbialcidal and antibody production, respectively [32, 33]. Additionally, Th1 cells can directly lyse infected cells [35]. Upon infection, antigen-specific CD4 and CD8 T cells undergo activation and perform effector functions. But in the case of chronic infections, pathogens persist in the host for a prolonged period and alter the function of pathogen-specific T cells, ultimately resulting in their exhaustion. CD4 T cells are known to become...
functionally unresponsive following persistent infections and malignancies [3, 10]. CD4 T cells have multiple effects on the overall immune response. Many studies have reported reversion of exhausted CD8/CD4 T cells. Nonetheless, no definitive study is available signifying a direct role of innate receptors in restoring the activity of exhausted CD4 T cells [8, 36–39]. Several studies have suggested the role of TLRs in T-cell activation [17]. Further, TLRs operate synergistically to induce optimal immune responses against intracellular pathogens [40]. Administration of TLR-9 agonist in mice with tuberculosis resulted in the generation of PD-1hi-expressing T cells [20]. TLR-2 signaling exerts antitumor activity [41, 42]. Based on these findings, we created in vitro and in vivo models of exhaustion to monitor the impact of TLR-2 signaling on the rescue of CSTh1 cells. The following major findings emerged from this study. First, continuous TCR stimulation of naive CD4 T cells under Th1-polarizing conditions resulted in an exhausted phenotype. Second, signaling through TLR-2 limited the exhaustion of CSTh1 cells by downregulating the expression of PD-1 and Lag-3 and increasing the expression of IFN-γ, Bcl-2, and IL-2. Third, the rescue mechanism observed through TLR-2 signaling was T-bet dependent and, hence, restricted to Th1 cells. Fourth, the reinvigorated CSTh1 cells restored their activity to help B cells. Fifth, the specificity of Pam2Cys in rescuing CSTh1 cells was authenticated by using MyD88−/− mice. Sixth, TLR-2 agonist administration protected mice from chronic M. tuberculosis infection.

pCSTh1 cells exhibited decreased expression of the co-inhibitory molecules PD-1 and Lag-3 and showed improved effector function. This has immense clinical relevance as PD-1− and Lag-3− blocking therapy is a promising strategy against chronic infections and tumors [9, 11]. Interestingly, pCSTh1 cells display enhanced expression of T-bet. T-bet repressed the transcription of genes encoding PD-1, Lag-3, and other inhibitory receptors [28]. Therefore, it can be inferred that TLR-2 signaling induced the enhancement in T-bet, which resulted in improved IFN-γ release and suppression of PD-1 and Lag-3. CSTh1 cells exhibited a higher frequency of cell death, which corroborated with the increased levels of Fas/Fasl. In contrast, pCSTh1 cells showed increased cell viability and a decline in levels of Fas/Fasl expression. Fas-mediated cell death is partially regulated by Bcl-2. Overexpression of Bcl-2 prevents T-cell apoptosis on hyperactivation [43]. Interestingly, pCSTh1 cells displayed increased levels of the antiapoptotic molecule Bcl-2. Thus, pCSTh1 cells may be resistant to death, which ultimately would enhance their effector function. Similar results were achieved in restoring the activity of human CD4 T cells upon stimulation with TLR-2 agonist (data not shown). It was also noticed that pCSTh1 cells retained their optimum function, as verified by their ability to activate B cells. Importantly, we also proved the specificity of our concept by generating CSTh1 cells from MyD88−/− mice. Intriguingly, these cells failed to respond to Pam2Cys in attaining the rescued phenotype, thus validating the authenticity of the phenomenon.

Besides defending the host from many intracellular pathogens, Th1 cells also play a cardinal role in protecting against M. tuberculosis infection [21]. However, chronic M. tuberculosis infection is associated with impaired proliferation and effector functions of T cells [15]. Thus, it is important to investigate the factors involved in reverting them from exhaustion. Emerging evidence suggests the critical role of TLRs in promoting the protective Th1 response against M. tuberculosis [40]. TLR-2 stimulation mainly induces a Th1 response [44]. Conjugation of Pam2Cys, a TLR-2 agonist, with M. tuberculosis peptidoglycan generated enduring Th1 memory cells and reduced the bacterial burden [22].

Based on our results, we were curious whether signaling via TLR-2 can be effective in vivo in protecting animals with chronic M. tuberculosis infection. We established models of acute and chronic M. tuberculosis infection in mice by infecting with low and high doses of M. tuberculosis. The severity of acute versus chronic infection was established on the basis of (1) widespread confluent tuberculosis pneumonia, based on the presence of aggregates of lymphohistiocytes and lipogranulomatous cells and (2) the number and size of granulomas. Further, chronic infection was substantiated on the basis of the exhaustion phenotype (Lag-3hi/KLRG-1hi/PD-1hi/CD43hi) among the CD4 T cells isolated from the lungs of the mice. Importantly, administration of TLR-2 agonist improved the production of IFN-γ, upregulated the expression of memory markers (CD44hi/CD62Lhi) on CD4 T cells, and decreased the M. tuberculosis-inflicted pathology and mycobacterial burden. These observations strongly suggest that triggering through TLR-2 restores the in vivo function of T cells, as well. It has been reported that TLR-2 stimulation can reinstate the functionality of exhausted CD8 T cells obtained from patients with diffuse cutaneous leishmaniasis [19]. Very recently, it was reported that stimulation of CD4 T cells through TLR-2 can enhance their protective efficacy against M. tuberculosis [45]. In summary, our study is the first to demonstrate a novel role of TLR-2 in rescuing Th1 cells from exhaustion and therefore shows that TLR-2 can be an important target in the treatment of patients with chronic infections.

Supplementary Data

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyrighted. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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