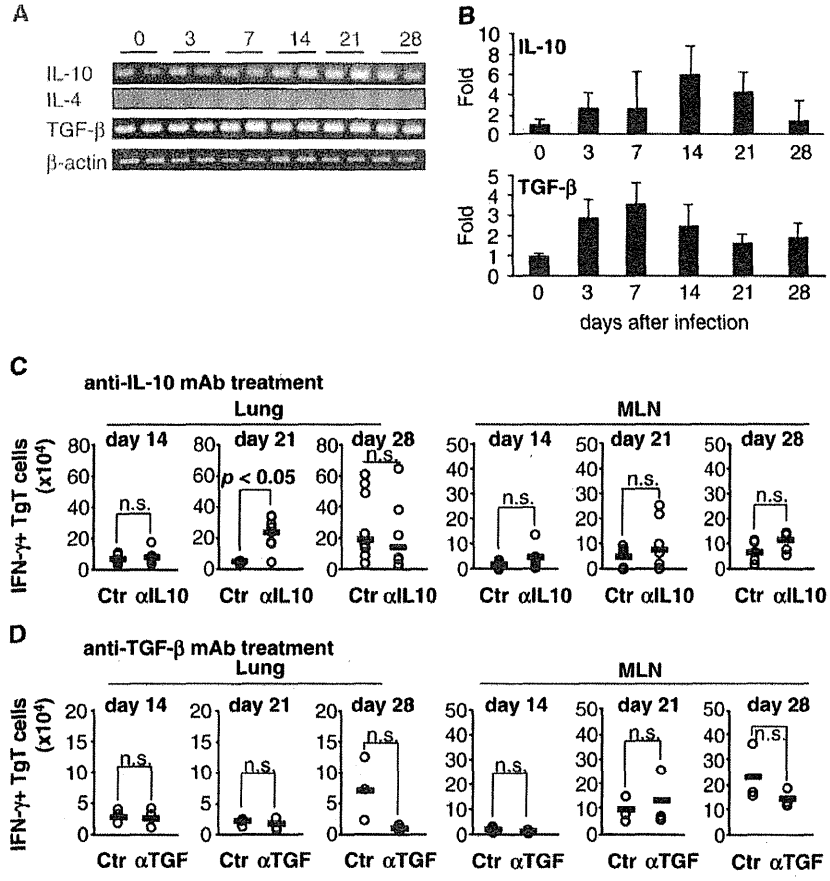

Fig. 7. Effects of regulatory cytokines on the induction of T_h1 response of the P25 TCR-Tg $CD4^+$ T cells after low-dose Mtb lung infection. (A) Transcripts for TGF-β, IL-10, IL-4 and β-actin in the Mtb-infected lung of the wild-type mice were analyzed by electrophoresis of RT-PCR products and ethidium bromide staining. (B) TGF-β and IL-10 mRNA expression levels in the Mtb-infected lung of the wild-type mice were analyzed by real-time RT-PCR with normalization using β-actin mRNA expression level. Expression levels on day 0 were arbitrarily set to 1.0. (C and D) The N-P25 TCR-Tg mice were injected i.v. with anti-IL-10 mAb (αIL10) a day before i.t. inoculation with 1×10^3 Mtb. Control group (Ctr) was left untreated with mAb. The IFN-γ^+ P25 TCR-Tg $CD4^+$ T cells in the lung and MLN were detected by FACS, and absolute number of the IFN-γ^+ T cells was plotted individually. (D) The N-P25 TCR-Tg mice were injected i.v. with anti-TGF-β mAb (αTGF) a day before and once a week after the i.t. inoculation with 1×10^3 CFU of Mtb. The IFN-γ^+ P25 TCR-Tg $CD4^+$ T cells in the lung and MLN were detected by FACS, and absolute number of the IFN-γ^+ T cells was plotted individually. n.s., not significantly different compared with the mAb-untreated group. The data shown are representatives of two independent (A and B) or three independent (C and D) experiments.

induced only transient proliferation of the P25 TCR-Tg $CD4^+$ T cells around day 17 post-infection although underlining mechanism of the transient proliferation is not yet clarified. In our analysis, BrdU incorporation was analyzed on day 7, 14, 21 and 28 after intratracheal Mtb infection, and the point of transient proliferation would not be included in the analysis.

Our data showed that anti-mycobacterial T_h1 response is not observed in the lung when mycobacterial antigen-specific T_h1 cells were detected in the MLN on day 21 of the low-dose Mtb infection. From this observation, we estimated that activated T_h1 cells are suppressed in the lung at this time point. A possible candidate mechanism of this suppression is suppressive cytokines such as IL-4, IL-10 and TGF-β which have been reported to suppress T_h1 response (26). RT-PCR analysis of the infected lung identified induction of IL-10 and TGF-β expression after the pulmonary Mtb infection. When the cytokines were neutralized with mAb, anti-IL-10 mAb resulted in increase of the IFN-γ-producing P25 TCR-Tg $CD4^+$ T cells in the lung on day 21 when T_h1 response was induced in the MLN but anti-TGF-β mAb had no effect on the induction. Therefore, we estimate that T_h1-committed T cells induced in the MLN around day 21 after Mtb infection may migrate into the infected lung but failed to be activated and expand because of IL-10-mediated produced in the Mtb-infected lung.

Regulatory T cell (Treg)-mediated suppression of lung T-cell response is another possible mechanism of suppression of T_h1 T cells in the mycobacteria-infected lung. Scott-Browne *et al.* (30) reported that $FoxP3^+$ Tregs increased in the lung after Mtb infection. Furthermore, depletion of the Treg resulted in enhanced protection in the lung, suggesting suppression of protective immunity against Mtb by Treg in the lung. Interestingly, T_h1 response to Mtb-derived ESAT-6 antigen paradoxically decreased in the Treg-depleted Mtb-infected mice (30), suggesting that Treg may not always suppress T_h1 induction in the mycobacteria-infected lung. In our Tg system,

naive P25 TCR-Tg mice without T-cell transfer contained very low level of FoxP3$^+$ T cells (0.3% of the lung T cells) and transfer of normal T-cell repertoire to prepare the N-P25 TCR-Tg mice resulted in >2-fold increase in the Treg in the lung. However, the P25 TCR-Tg mice and N-P25 TCR-Tg mice contained nearly the same number of IFN-γ-producing P25 TCR-Tg CD4$^+$ T cells on day 21 after Mtb lung infection (data not shown). The experiment did not support Treg-mediated suppression as a major suppressive factor of T_h1 development in the lung after mycobacterial infection.

Alternatively, it is possible that innately programmed pulmonary microenvironment suppress induction of T_h1 response, especially at an early stage of mycobacterial lung infection. It was reported that alveolar macrophages secreted nitric oxide, TGF-β and prostaglandin E_2 that control the function of DC (14, 31, 32). Furthermore, it has been reported that alveolar macrophages suppress maturation of lung DC to express MHC class II (31, 33), which may result in suppression of T_h1 response in the lung. Lung plasmacytoid DC were reported as immunomodulatory cells which shift immune response to T_h2 type (34). Pulmonary DC subpopulation was reported to suppress T_h1 response via IL-10 production (35). Interestingly, adoptive transfer of bone marrow-derived DC into the lung rapidly induced T-cell proliferative response and cytokine production including IFN-γ (36). The innately programmed suppressive mechanisms may suppress early stage of T_h1 immune response after mycobacterial lung infection. To address the hypothesis that pulmonary microenvironment constructed by alveolar macrophages suppress function of lung DC to induce T_h1 response, a preliminary experiment was carried out using mice depleted of pulmonary macrophages and DC and transferred systemically or intratracheally with bone marrow-derived DC before pulmonary BCG infection. Unexpectedly, the mice showed no accelerated induction of mycobacterial antigen-specific T-cell response in the lung. Therefore, alveolar macrophage-mediated suppression would not be an important mechanism of the delay in induction of T_h1 response in the lung. Further analysis on immunoregulatory function of pulmonary DC is on going.

Funding

Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan; Grant-in-Aids for Scientific Research, Japan Society for Promotion of Science.

Acknowledgements

We thank Dr Y. Yoshikai for providing B6 Ly5.1 mice.

Disclosures

The authors have no financial conflict of interest.

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International Immunology, Vol. 22, No. 12, pp. 915–925
doi:10.1093/intimm/dxq446
Advance Access publication 3 November 2010

PD-1–PD-L1 pathway impairs T_h1 immune response in the late stage of infection with *Mycobacterium bovis* bacillus Calmette–Guérin

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Received 16 April 2010, *accepted* 1 October 2010

Abstract

A major concern still prevails as to the reason why various mycobacteria are able to persist within infected host in which protective immunity is generated. To address this question, we monitored the generation of protective T cells during infection with *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). $CD4^+$ T cells obtained 3 weeks after infection conferred protection against *Mycobacterium tuberculosis* challenge and produced IFN-γ and tumor necrosis factor (TNF)-α upon antigen stimulation. However, these abilities were decreased after 6 weeks of infection even though BCG was not thoroughly eliminated from the host. We analyzed the expression of ligands for the CD28/CTLA-4 family receptors on antigen-presenting cells and found that the expression of PD-L1, a ligand for programmed cell death-1 (PD-1), was up-regulated later than 3 weeks of infection. We also found that bacterial numbers in the spleen of PD-1-deficient mice were significantly reduced compared with wild-type mice at 6 and 12 weeks after BCG infection. Furthermore, $CD4^+$ T cells of PD-1-deficient mice showed a higher ability to confer protection and produce IFN-γ and TNF-α even at 12 weeks after infection. These results indicate that the PD-1–PD-L1 pathway impairs T_h1 immunity in the late stage of BCG infection, thereby facilitating the bacterial persistence in the host.

Keywords: Mycobacterium, PD-1, persistent infection, T_h1 immunity

Introduction

Tuberculosis (TB) caused by *M. tuberculosis* (Mtb) is one of the leading threats for humans (1). One-third of the world's population is exposed to Mtb and ~10% of individuals exposed develop TB. Although most of the remaining people do not suffer from disease during their lifetime, TB eventually emerges in those whose immune system is compromised by aging, HIV infection or malnutrition (2, 3). *Mycobacterium bovis* bacillus Calmette-Guérin (BCG), an attenuated vaccine strain, could also persist in the host body for a long time and occasionally causes disease in acquired immunodeficiency syndrome patients even after several decades of vaccination (4–6). These indicate that Mtb and BCG are capable of surviving in the host in which protective immunity is generated. Therefore, an understanding of the mechanisms by which these mycobacteria evade the host defense system is important for the development of effective therapies and the rational design of novel vaccines.

Protective immunity to mycobacteria is mediated by T_h1-type $CD4^+$ T cells with the aid of other types of T cells (7–11). T_h1 cells produce IFN-γ and tumor necrosis factor (TNF)-α in response to mycobacterial antigens, which are critical for macrophage activation and control of bacterial replication (12). Cell-to-cell contact between T cells and antigen-presenting cells (APC) is an important event for induction of the T-cell-mediated immune response. Indeed, the interaction of co-stimulatory and co-inhibitory receptors (e.g. CD28 and CTLA-4) expressed on T cells with the ligands (e.g. B7-1 and B7-2) on APC influences the magnitude and duration of antigen-specific T-cell response (13). Programmed cell death 1 (PD-1, also known as CD279) is a new member of the CD28/CTLA-4 receptor family, which was originally identified in a T-cell hybridoma undergoing apoptotic cell death (14). PD-1 expression is induced on activated T cells and B cells (15), and its constitutive

expression on $CD4^+$ T cells is observed during immunosenescence (16). PD-1 has two ligands, PD-L1 (B7-H1 or CD274) and PD-L2 (B7-DC or CD273). PD-L2 expression is restricted to activated dendritic cells (DC) and macrophages, whereas PD-L1 is constitutively expressed on a wide variety of cells (17). Furthermore, PD-L1 expression on cells including APC is up-regulated after stimulation with IFN-γ and toll-like receptor ligands (18–20). Interaction of PD-1 with the ligands provides an inhibitory signal that regulates T-cell activation to induce and maintain peripheral tolerance (21).

Recent studies have shown that pathogenic microbes exploit the PD-1–PD-L pathway as a strategy for immune evasion and persistent infection (22). For example, PD-1 is highly expressed on functionally impaired (exhausted) T cells in a chronic infection with lymphocytic choriomeningitis virus (LCMV). Blockade of the PD-1–PD-L1 pathway restores T-cell function and promotes clearance of the persisting virus (23). During infection with HIV, hepatitis B virus and hepatitis C virus, T-cell exhaustion and disease progression are associated with up-regulation of PD-1 on antigen-specific T cells (24–26). In addition to chronic viral infection, *Helicobacter pylori* suppresses the T-cell response by up-regulating PD-L1 expression on gastric epithelial cells that are thought to act as APC. *In vitro* blockade of the PD-1 signaling enhances cytokine production and proliferation by T cells on antigen stimulation (27), suggesting that the PD-1–PD-L pathway may also play a role in persistent infection with bacteria.

In mycobacterial infection, $CD4^+$ T cells isolated in the initial stage of infection exert strong cytokine production and proliferation, but these responses are diminished in the late stage of infection (28–30). These findings raised the possibility that the antigen-specific T-cell response is impaired or modulated by some inhibitory mechanism, allowing mycobacteria to achieve persistent infection. In this study, we investigated whether the PD-1–PD-L pathway contributes to the inhibition of T_h1 immune response to BCG. Our results clearly show that although protective T cells were generated by 3 weeks after BCG infection, T-cell responses were impaired in the later period by the PD-1–PD-L1 co-inhibitory pathway.

Methods

Antibodies

The monoclonal antibodies specific for mouse B7-1 (16-10A1), B7-2 (GL1), CD4 (GK1.5), CD11c (N418), CD44 (IM7), CD62L (MEL-14), F4/80 (BM8), herpes virus entry mediator (HVEM, LH1), ICOS ligand (ICOS-L, IHK5.3), PD-1 (RMP1-30), PD-L1 (MIH5) or PD-L2 (TY25) and isotype control antibodies were purchased from eBioscience (San Diego, CA, USA). The antibody to CD16/CD32 (2.4G2), $I\text{-}A^b$ (M5/114.15.2), IFN-γ (XMG1.2) and TNF-α (MP6-XT22) were purchased from BD Biosciences (San Jose, CA, USA).

Mice

C57BL/6 mice were purchased from Japan SLC (Shizuoka, Japan). PD-1-deficient ($PD\text{-}1^{-/-}$) mice on a C57BL/6 background were kindly provided by Dr Tasuku Honjo (Kyoto University) and maintained under specific pathogen-free conditions. All mice used in the experiments were 8–10 weeks old. All the animal experimental procedures were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine, Japan.

Bacteria

Mycobacterium bovis BCG strain Pasteur and Mtb strain H37Rv were grown at 37°C to mid-log phase in Middlebrook 7H9 broth (BD Bioscience) supplemented with 0.2% glycerol and albumin dextrose catalase (ADC) enrichment consisting of 0.5% albumin, 0.2% dextrose and 3 μg ml^{-1} catalase. Bacteria were harvested, stirred vigorously with glass beads (3 mm in diameter) to disperse the bacterial clumps and left to stand for 30 min. The upper part of the suspension, without visible clumps, was collected and stored at −80°C in aliquots. After thawing, the bacterial suspension was centrifuged at 150 × *g* for 2 min to remove any clumps, and only the upper part of the suspension was used for the experiments. Bacterial numbers in the preparation were determined by counting the colonies after plating the diluted suspension on Middlebrook 7H10 agar (BD Bioscience) plates supplemented with ADC enrichment, 0.2% glycerol and 50 μg ml^{-1} oleic acid. The plates were incubated at 37°C and the colony-forming units (CFU) were counted after 3 weeks. The absence of bacterial clumps was confirmed using the LIVE/DEAD BacLight Bacterial Viability kit (Invitrogen, Carlsbad, CA, USA).

Infection and adoptive cell transfer

Mice were infected intravenously (i.v.) with 10^6 CFU of BCG. The spleen and lung were recovered and homogenized in PBS at the indicated time points after infection. The serially diluted homogenates were plated onto 7H10 agar plates and bacterial number was enumerated. In some experiments, spleens were removed at the indicated times after infection and single-cell suspensions were prepared through a 70-μm nylon cell strainer (BD Biosciences) using a 2.5-ml syringe plunger. After RBC lysis with NH_4Cl/Tris solution, $CD4^+$ and $CD8^+$ T cells were isolated at >95% purity using the BD IMag Mouse CD4 T Lymphocyte enrichment set and the CD8 T Lymphocyte enrichment set (both from BD Bioscience), respectively. Purified T cells (5 × 10^6 cells) were injected i.v. into naive WT mice. Mice were infected i.v. with 10^5 CFU of Mtb 1 h after cell transfer. Ten days after Mtb infection, the spleen and lung were homogenized and the number of CFU in the organs was determined.

Analysis of antigen-specific T-cell response

Mice were infected i.v. with BCG and $CD4^+$ T cells were purified from the spleen at 1, 3, 6 and 12 weeks after infection. Bone marrow-derived DC (BMDC) were prepared as described previously (31). In brief, bone marrow cells were removed from the femurs and tibias of WT mice and cultured in RPMI 1640 supplemented with 2 mM glutamine, 10% FCS, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 20 ng ml^{-1} granulocyte macrophage colony-stimulating factor (R&D Systems, Minneapolis, MN, USA). On days 3 and 6, the culture medium was replenished and cells were used on days 7–8 of culture. To prepare BCG-pulsed APC, BMDC were infected with BCG (multiplicity of infection = 5) for 16 h and treated with mitomycin C (50 μg ml^{-1}; Nacalai Tesque,

Kyoto, Japan) for 30 min at 37°C. $CD4^+$ T cells (1×10^6 cells ml^{-1}), obtained from naive and BCG-infected mice, were stimulated with BCG-pulsed BMDC (5×10^5 cells ml^{-1}) for 60 h. Cell proliferation was determined by labeling of cultures for the last 12 h with 5-bromo-2-deoxyuridine using the Cell Proliferation ELISA kit (Roche Diagnostics, Mannheim, Germany). In addition, the culture supernatants were harvested after 48 h of culture and the concentration of cytokines was measured using the BD CBA Mouse Inflammation kit for IFN-γ, IL-10 or TNF-α (BD Biosciences) and the ELISA kit for transforming growth factor (TGF)-β1 (eBioscience). In some experiments, spleen cells (10^6 cells per well) of naive and BCG-infected mice were plated in round-bottom 96-well plates and stimulated with purified-protein derivatives (PPD) (5 μg ml^{-1}; Japan BCG, Kiyose, Japan) for 6 h. Brefeldin A (50 μg ml^{-1}; BD Biosciences) was added 2 h after PPD stimulation. Cells were treated with PE-Cy5-conjugated anti-CD4 antibody for 20 min on ice, followed by fixation for 30 min in 4% PFA solution. Intracellular IFN-γ and TNF-α were stained using the Cytofix/Cytoperm kit (BD Biosciences) according to the manufacturer's instructions and analyzed by flow cytometry.

Flow cytometry

Spleens were recovered from BCG-infected WT mice at the indicated time points and single-cell suspensions were prepared as described above. Cells were incubated with anti-CD16/CD32 antibody for 10 min to block Fc binding and treated with FITC-conjugated anti-I-A^b antibody and PE-conjugated antibody to B7-1, B7-2, HVEM, ICOS-L, PD-L1, PD-L2 or the isotype control antibody for 20 min on ice. In some experiments, cells were stained with PE-Cy5-conjugated anti-CD4 antibody, FITC-conjugated anti-CD44 antibody and PE-conjugated antibody to CD62L or PD-1 for 20 min on ice. To determine apoptosis of memory $CD4^+$ T cells, splenocytes were collected from BCG-infected WT and PD-$1^{-/-}$ mice and treated with PE-Cy5-conjugated anti-CD4 antibody and FITC-conjugated anti-CD44 antibody, followed by staining with Annexin V-PE (Calbiochem, San Diego, CA, USA). Intracellular Foxp3 staining for $CD4^+$ T cells was performed using the FITC anti-Foxp3 staining kit (eBioscience) according to the manufacturer's recommendations. All stained cells were analyzed on a FACSCalibur using CELLQuest software (BD Biosciences) or FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Statistical analysis

For comparisons between two groups, the Student's *t*-test was used when the variances of the groups were judged to be equal by the *F*-test. Multigroup comparisons of mean values were made according to the analysis of variance and the Fisher's protected least significant difference *post hoc* test after the confirmation of homogeneity of the variances among the groups had been confirmed using the Bartlett's test. Statistical significance was determined as $P < 0.05$.

Results

T-cell-mediated protective immunity is attenuated in the late stage of BCG infection

To determine the course of BCG infection, we infected wild-type (WT) C57BL/6 mice i.v. with BCG and monitored the bacterial numbers in the spleen and lung. As shown in Fig. 1A, BCG replicated in these organs within a week after infection. Thereafter, the bacterial number gradually decreased in a time-dependent manner. However, a significant number of bacteria still remained in both organs at 12 weeks after infection.

Because $CD4^+$ T cells play an essential role in protection against mycobacterial infection (7–11), we investigated the generation of protective T cells by an adoptive cell transfer

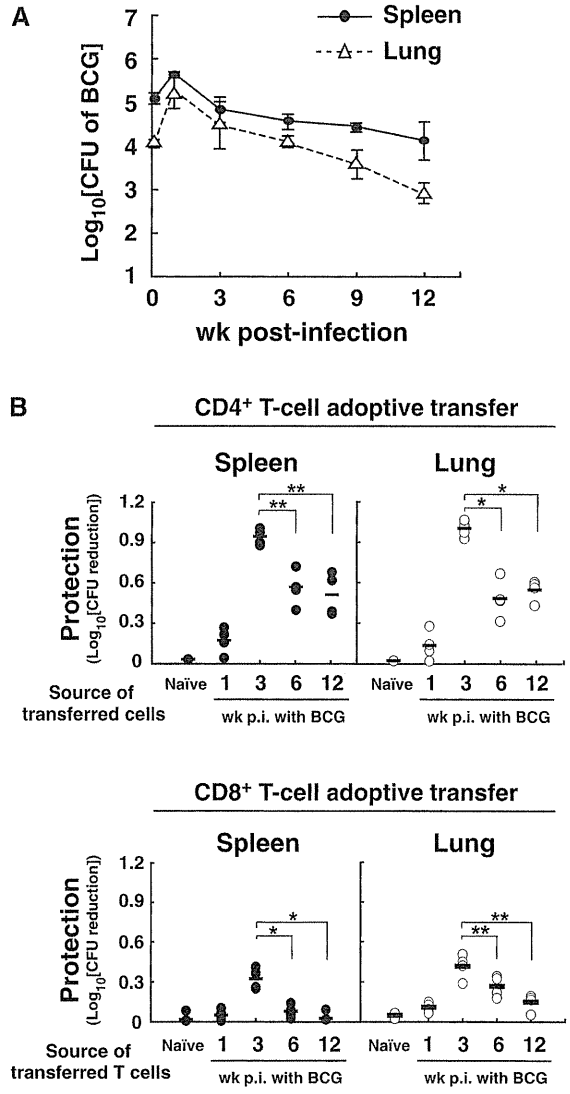

Fig. 1. Kinetics of the bacterial numbers in organs after BCG infection and the protective efficacy of T cells obtained from BCG-infected mice against Mtb infection. (A) WT mice were infected i.v. with 10^6 CFU of BCG. Bacterial numbers in the spleens and lungs were determined. Data represent the means ± SD of CFU in five mice at each time point. (B) $CD4^+$ and $CD8^+$ T cells were purified from spleens at the indicated weeks post-infection (wk p.i.) with BCG and transferred into naïve WT mice. Mice were infected with 10^5 CFU of Mtb 1 h after T-cell transfer. Ten days after Mtb infection, the numbers of bacteria in the spleens and lungs were determined. Each symbol represents a reduction of CFU in the experimental groups compared with that in non-transferred group. Horizontal bars indicate the mean values for each group. $*P < 0.01$, $**P < 0.05$. Results are representative of three independent experiments.

experiment. Naive WT mice were transferred with $CD4^+$ T cells from the spleens of BCG-infected mice and subsequently infected i.v. with Mtb. Ten days later, the bacterial numbers in the spleen and lung were determined. Mice transferred with $CD4^+$ T cells obtained at 1 week after infection with BCG hardly exhibited protection against a challenge infection with Mtb. On the other hand, $CD4^+$ T cells obtained at 3, 6 and 12 weeks after BCG infection conferred protection on recipient mice (Fig. 1B). However, the ability of T cells obtained at 6 and 12 weeks after infection was significantly reduced compared with T cells obtained at 3 weeks after infection, even when BCG could still be detected in the donors (Fig. 1A). These results suggested that although $CD4^+$ T-cells-mediating protective immunity were generated as early as 3 weeks after infection, the protective efficacy was decreased in the later stages. This result may explain why BCG is not easily eradicated from the host.

In addition to $CD4^+$ T cells, it has been shown that $CD8^+$ T cells also play a role in protection against Mtb (7, 8, 32, 33). Thus, we investigated the efficacy of $CD8^+$ T cells from BCG-infected mice on induction of protective immunity by the adoptive cell transfer experiment. Similar to $CD4^+$ T cells, protective $CD8^+$ T cells were generated at 3 weeks after infection (Fig. 1B). However, the protective effect was markedly weaker than that of $CD4^+$ T cells and was mostly decreased to the marginal levels by 12 weeks after infection. In the following experiments, therefore, we examined the mechanism by which $CD4^+$ T-cell functions were decreased during BCG infection.

Antigen-specific $CD4^+$ T-cell response is impaired in the late stage of BCG infection

To clarify whether the reduction of protective efficacy in the late stage of infection is due to the functional impairment of $CD4^+$ T cells, we investigated the ability of $CD4^+$ T cells to produce IFN-γ and TNF-α and assessed their proliferation upon stimulation with BCG-pulsed BMDC. $CD4^+$ T cells obtained at 1 week after infection did not produce IFN-γ or TNF-α and did not exhibit a significant proliferative response (Fig. 2A). On the other hand, T cells obtained at 3 weeks after infection produced a large amount of cytokines and exhibited a significant proliferative response. However, the responses of $CD4^+$ T cells obtained at 6 and 12 weeks after infection were significantly weaker than those of T cells obtained at 3 weeks after infection.

Recently, it has been reported that T cells producing both IFN-γ and TNF-α mediate protection against parasitic and viral infections (34–36). Therefore, the possibility exists that T cells producing both these cytokines may contribute to protection against Mtb infection. We analyzed the profile of cytokine production in $CD4^+$ T cells and determined the kinetics of the T-cell population during the course of BCG infection. As shown in Fig. 2B, $CD4^+$ T cells producing both IFN-γ and TNF-α were virtually undetectable in spleen cells at 1 week after infection but were generated at 3 weeks. However, the number of T-cell populations was markedly reduced at 6 weeks and remained at a low level until 12 weeks after infection. These results showed that protection against Mtb correlates with the generation of the $CD4^+$ T-cell population, suggesting that T cells capable of producing both IFN-γ and TNF-α mediate protective immunity. It is likely that a decrease in protective immunity in the late stage of BCG infection is, at least in part, caused by a reduction of the $CD4^+$ T-cell populations.

It has been shown that $CD4^+$ $CD25^+$ $Foxp3^+$ regulatory T (Treg) cells and IL-10-producing $CD4^+$ T (Tr1) cells are increased during Mtb infection and contribute to suppression of host immune responses (37–40). We investigated whether the number of Treg cells or Tr1 cells is increased in the late stage of BCG infection. First, spleen cells were collected from BCG-infected mice and the population of Treg cells was analyzed by flow cytometry. We found 9.58 ± 1.4% of $CD25^+$ $Foxp3^+$ cells in the naive $CD4^+$ T-cell population and the T-cell population did not increase during BCG infection (Fig. 3A). We next evaluated the generation of IL-10-producing Tr1 cells after BCG infection. However, no significant increase in IL-10 production was observed in $CD4^+$ T cells at 6 or 12 weeks after infection (Fig. 3B). These results suggested that Treg cells and Tr1 cells do not contribute to the impairment of T_h1 response, which was observed later than 6 weeks of BCG infection.

PD-L1 expression on APC is up-regulated in the late stage of BCG infection

It has been shown that the antigen-specific T-cell response is regulated by a sum of co-stimulatory and co-inhibitory signals that are transduced to T cells through the interaction between the CD28/CTLA-4 family receptors expressed on T cells and the ligands expressed on APC (13). We investigated whether these signals are involved in the functional impairment of $CD4^+$ T cells during BCG infection by measuring the expression of ligands for the CD28/CTLA-4 family receptors on MHC class II^{high} ($I\text{-}A^{high}$) APC including DC and macrophages (Fig. 4A). The expression levels of B7-1 (CD80) and B7-2 (CD86) were elevated at 3 weeks after infection and gradually decreased to a level similar to those of uninfected APC at 12 weeks. Because B7 molecules contribute to the control of chronic Mtb infection (41), a decrease in their expression may contribute to the impairment of T-cell response in the late stage of BCG infection. The expression of ICOS-L did not change in the course of BCG infection. On the other hand, a cell population positive for HVEM [a ligand for B and T lymphocyte attenuator (BTLA)] (42) temporarily appeared at 3 weeks and disappeared at 6 weeks after infection. Importantly, the expression of PD-L1 was up-regulated on APC at 3 weeks and the expression level remained higher than that of uninfected APC until 12 weeks after infection. However, the expression of PD-L2 was not induced by BCG infection. We also observed similar patterns of their expressions in DCs ($CD11c^{high}$ cells) and macrophages ($F4/80^+$ cells), respectively (data not shown). These results indicated that PD-L1, as compared with other ligands for the CD28/CTLA-4 family receptors, is predominantly expressed and maintained on APC even at the late stage of infection. To know if the PD-1, the counterpart receptor for PD-L1, is also expressed or not, we looked at the PD-1 expression on $CD4^+$ T cells. As expected, the increase in the PD-1 expression on $CD4^+$ T cells was observed 3 weeks after infection and maintained until 12 weeks (Fig. 4B). The expression of PD-1 was detected preferentially in $CD44^{high}$

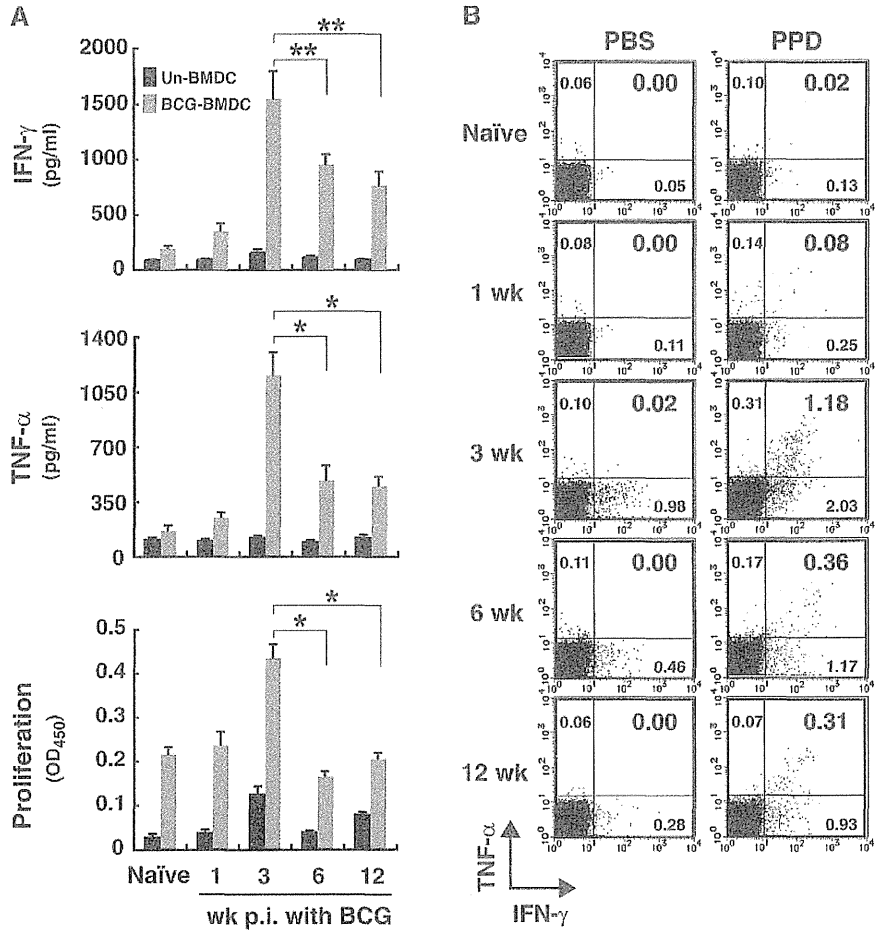

Fig. 2. Antigen-specific responses of $CD4^+$ T cells obtained from BCG-infected mice. (A) $CD4^+$ T cells were isolated from spleens at the indicated weeks post-infection (wk p.i.) with BCG and cultured with unpulsed BMDC (black bars) or BCG-pulsed BMDC (gray bars) for 48 h. The culture supernatants were harvested and the concentration of cytokines was measured. T-cell proliferation was measured by 5-bromo-2-deoxyuridine incorporation. Data are the means ± SD of triplicate cultures. $*P < 0.01$, $**P < 0.05$. (B) Splenocytes were isolated from BCG-infected mice at the indicated times after BCG infection. Cells were stimulated with PPD for 6 h and intracellular IFN-γ and TNF-α were analyzed by flow cytometry. Each value indicates the percentage of cells producing both IFN-γ and TNF-α in $CD4^+$ lymphocytes. Results are representative of four independent experiments.

$CD4^+$ T cells but not in $CD44^{low}$ $CD4^+$ T cells, indicating that PD-1 was exclusively expressed in memory T-cell population (Fig. 4B). These results suggested a possible involvement of PD-1–PD-L1 pathway in the reduction of protective ability of $CD4^+$ T cells after BCG infection.

$CD4^+$ T-cell-mediated protection in PD-$1^{-/-}$ mice is maintained in the late stage of BCG infection

To confirm whether the PD-1–PD-L1 pathway is involved in the reduction of host protective immunity, WT and PD-$1^{-/-}$ mice were infected with BCG and the kinetics of bacterial number was determined. No difference was detected in the number of CFU in the spleens of WT and PD-$1^{-/-}$ mice until 3 weeks after infection (Fig. 5A). However, at 6 and 12 weeks after infection, the number of CFU in PD-$1^{-/-}$ mice was significantly reduced compared with WT mice. We then performed an adoptive cell transfer experiment to compare the ability of $CD4^+$ T cells from WT and PD-$1^{-/-}$ mice to confer protection against challenge infection with Mtb. $CD4^+$ T cells obtained from the spleen of both WT and PD-$1^{-/-}$ mice at 3 weeks after infection conferred a similar level of protection against Mtb in the spleen and lung of recipient mice. However, consistent with the data presented above, the protective ability of WT T cells obtained at 6 and 12 weeks after infection was reduced (Fig. 5B). The ability of PD-$1^{-/-}$ T cells was also reduced at 6 and 12 weeks but was significantly higher than that of WT T cells.

PD-1–PD-L1 pathway is involved in the impairment of $CD4^+$ T-cell response in the late stage of BCG infection

To verify that the PD-1–PD-L1 pathway modulates the immune response of antigen-specific $CD4^+$ T cells generated after BCG infection, we compared the ability of WT and PD-$1^{-/-}$ $CD4^+$ T cells to produce IFN-γ and TNF-α, and also analyzed the proliferative responses upon stimulation with BCG-pulsed BMDC. No difference was found in the production of IFN-γ and TNF-α between WT and PD-$1^{-/-}$ $CD4^+$ T cells obtained at 3 weeks after infection (Fig. 6A). However, $CD4^+$ T cells obtained from PD-$1^{-/-}$ mice at 6 and 12 weeks after infection produced a significantly higher level

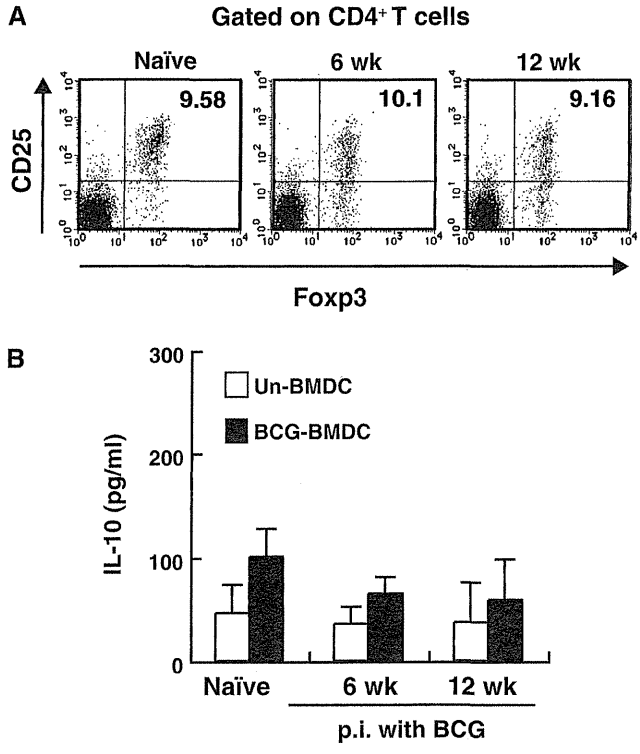

Fig. 3. Analysis of Treg cells and IL-10-producing $CD4^+$ T cells after BCG infection. (A) WT mice were infected with BCG and the frequency of $CD4^+$ $CD25^+$ $Foxp3^+$ T cells in spleens was determined at the indicated times after BCG infection. Numbers indicate the percentage of Treg cells. (B) $CD4^+$ T cells were isolated at the indicated weeks post-infection (wk p.i.) and stimulated with unpulsed BMDC (open bars) or BCG-pulsed BMDC (filled bars) for 48 h. IL-10 concentration in the culture supernatants was determined by using a cytometric bead array kit. Data represent the means ± SD of triplicate cultures. Results are representative of three independent experiments.

of cytokines compared with WT T cells. Similarly, PD-$1^{-/-}$ T cells obtained in the late stage of infection exhibited a higher proliferative response.

We next compared the generation of $CD4^+$ T cells capable of producing both IFN-γ and TNF-α between WT and PD-$1^{-/-}$ mice after BCG infection. Both groups of mice generated a similar level of the $CD4^+$ T-cell population at 3 weeks after infection. The frequency of T cells in PD-$1^{-/-}$ mice was maintained at a higher level at 6 and 12 weeks after infection, while the T-cell population of WT mice was reduced after 6 weeks of infection (Fig. 6B). Consistent with the kinetics of cytokine-producing T cells, the frequency of memory $CD4^+$ T cells ($CD44^{high}$ $CD62L^{low}$ $CD4^+$ T cells) was also maintained at the high level in PD-$1^{-/-}$ mice compared with WT mice (45.4% versus 29.1% and 35.7% versus 23.8% at 6 and 12 weeks, respectively) in the late stage of infection (Fig. 6C). In order to determine whether the reduction of memory $CD4^+$ T-cell population in WT mice was due to programmed cell death or not, we compared the levels of cell death of memory $CD4^+$ T cells ($CD44^{high}$ $CD4^+$ T cells) between WT and PD-$1^{-/-}$ mice by Annexin V staining assay. As shown in Fig. 6D, a similar level of apoptosis was detected in the memory T-cell populations of WT and PD-$1^{-/-}$ mice at 6 and 12 weeks after BCG infection. This finding suggested that the PD-1-dependent difference is ascribed to the functional reduction of memory T cells, not to the apoptotic cell death of memory T cells in the late stage of BCG infection. Taken together, these results may account in part for the fact that $CD4^+$ T cells of PD-$1^{-/-}$ mice confer more effective protection compared with WT T cells (Fig. 5B).

Discussion

On infection with Mtb or BCG, protective T cells are generated in the infected host. However, T-cell-mediated immunity does not easily eradicate these mycobacteria because they have evolved effective strategies to overcome host defense mechanisms (43). Recent studies have identified various virulence-associated genes and intracellular survival mechanisms of mycobacteria (44–46). However, the entire survival strategy remains uncertain. The recent emergence of multidrug-resistant Mtb strains highlights the need for research to unravel the mechanisms that enable this bacterium to be successfully parasitic in humans.

Several studies have demonstrated that the PD-1-signaling pathway is activated during persistent infection with various microorganisms and contributes to impairment of protective immunity. A recent study showed that *in vitro* blockade of PD-1 signaling with the specific antibody enhanced IFN-γ production by T cells of TB patients on stimulation with Mtb antigen (47), indicating that this inhibitory pathway also affects the T-cell function during mycobacterial infection. In the present study, we demonstrated that the PD-1 signaling is actually involved in the impairment of T_h1 response during BCG infection *in vivo*. We found that the ability of WT $CD4^+$ T cells to mediate protection and produce T_h1 cytokines was reduced after 6 weeks of BCG infection. However, the ability was maintained in PD-$1^{-/-}$ $CD4^+$ T cells compared with WT T cells in the late stage of infection. Consistent with the functional impairment of $CD4^+$ T cells observed later than 6 weeks after BCG infection, PD-1 expression was induced in the memory $CD4^+$ T cells and maintained even after 6 weeks. Moreover, PD-L1, as compared with other ligands for the CD28/CTLA-4 family receptors, was dominantly expressed on APC at the late stage of infection. Based on these findings, we concluded that the interaction transduces an inhibitory signal to effector T-cells-mediating protection, resulting in the impairment of T-cell responses required for protective immunity.

We investigated here the effect of PD-1 signal pathway on the effector functions of $CD4^+$ T cells generated in mice infected with BCG because $CD4^+$ T cells played a crucial role in protection compared with $CD8^+$ T cells (8–11). A reason for the lower contribution of $CD8^+$ T cells may be an inferior ability of BCG to induce antigen-specific $CD8^+$ T cells (48–50). On the other hand, several studies have shown that the enhanced expression of PD-1 on antigen-specific $CD8^+$ T cells is associated with their functional exhaustion during chronic viral infection (23–26). As Mtb infection induces a strong $CD8^+$ T-cell response in the infected host, therefore, it will be important to analyze the effect of PD-1 signaling on the function of both $CD4^+$ and $CD8^+$ T-cell populations in Mtb infection. We are currently investigating the role of PD-1

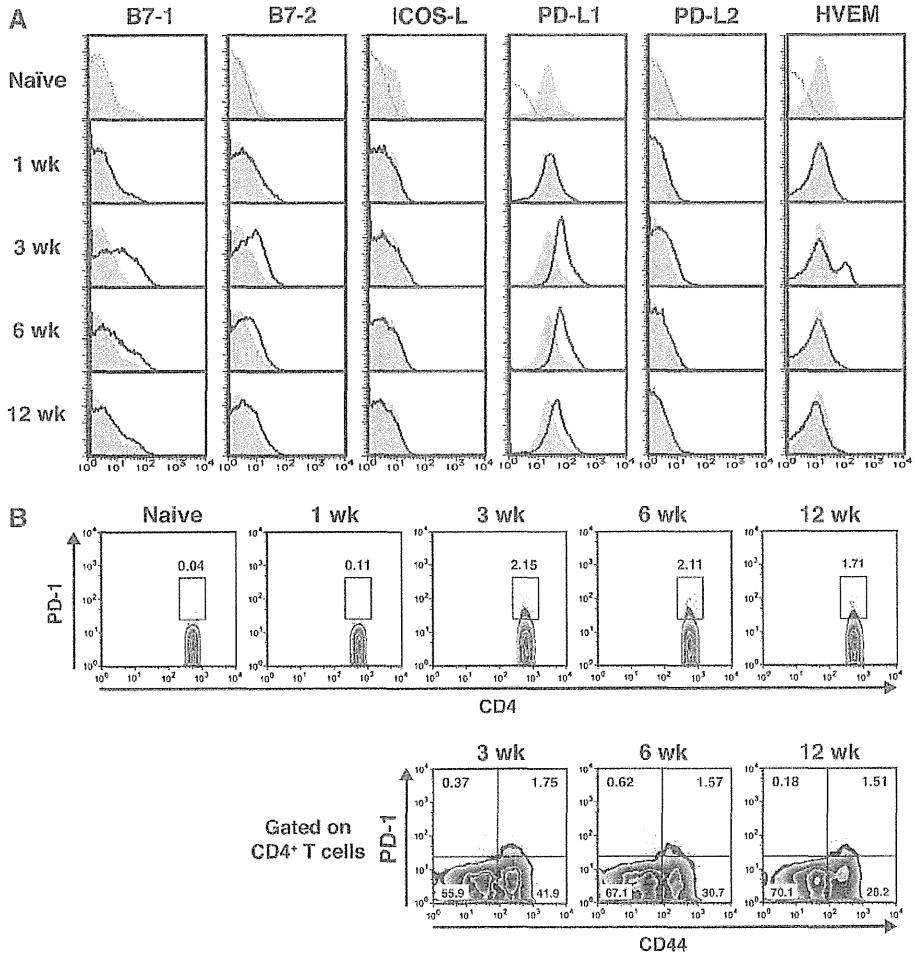

Fig. 4. Kinetics of the expression of co-stimulatory and co-inhibitory molecules on APC and PD-1 on $CD4^+$ T cells after BCG infection. Splenocytes were prepared from BCG-infected mice at the indicated times after infection. (A) Expression level of each molecule on APC ($I\text{-}A^{high}$ cells) was analyzed by flow cytometry. Shaded histograms, naïve cells; solid lines, BCG-infected cells; dotted lines, isotype controls. (B) Splenocytes were collected from WT mice after BCG infection and the kinetics of PD-1 expression on $CD4^+$ T cells was measured by flow cytometry (upper panels). Alternatively, the expression of PD-1 on memory $CD4^+$ T cells was determined on $CD44^{high}$ $CD4^+$ T cells (lower panels). Numbers indicate the percentage of $CD4^+$ T cells in each area. Results are the representative of four independent experiments.

signal pathway in the function of antigen-specific T cells generated by Mtb infection.

The expression of PD-1 was induced in memory $CD4^+$ T cells after 3 weeks of BCG infection, at which stage the protective T cells were generated. Since T-cell receptor-mediated signaling induces PD-1 expression of T cells (15, 17), it appears that the expression was observed concurrently with generation of IFN-γ and TNF-α-producing $CD4^+$ T cells after BCG infection. Similar to the PD-1 expression, PD-L1 expression on APC was enhanced later than 3 weeks after infection. PD-L1 is constitutively expressed on a variety of tissues and cells, and the expression is enhanced by IFN-γ (18, 20). Furthermore, the up-regulation of PD-1 expression on macropahges is shown to be mediated by T_h1 cells (19). From these findings, it seems likely that the up-regulation of PD-L1 is induced by IFN-γ produced from $CD4^+$ T cells that was generated after BCG infection. It is noteworthy that APC at 3 weeks after infection highly expressed not only PD-L1 but also B7 molecules that contribute to control of Mtb growth at the chronic stage of infection (41). It has been shown that PD-1-mediated inhibitory signals can be overcome by co-stimulatory signals through CD28 (51). Therefore, it is possible that the inhibitory signals might be canceled by an interaction of B7 molecules with CD28 at 3 weeks after BCG infection. In the later stage of infection, however, the PD-1-mediated signal might become dominant because of a decrease in the expression of B7 molecules and eventually interfere with protective immunity, allowing the bacteria to infect persistently.

As shown in Fig. 5B, the numbers of CFU in the spleen of $PD\text{-}1^{-/-}$ mice were significantly decreased at 6 and 12 weeks after infection compared with WT mice. However, no difference was found in the bacterial numbers in the lung between WT and $PD\text{-}1^{-/-}$ mice (data not shown). Our preliminary experiments revealed that BCG did not enhance PD-L1 expression on APC in the lung even when the expression on APC in the spleen was up-regulated by infection. This result suggested that activation of the PD-1–PD-L1 pathway is induced by systemic infection with BCG in the spleen but not in the lung. Therefore, the difference in PD-L1 expression

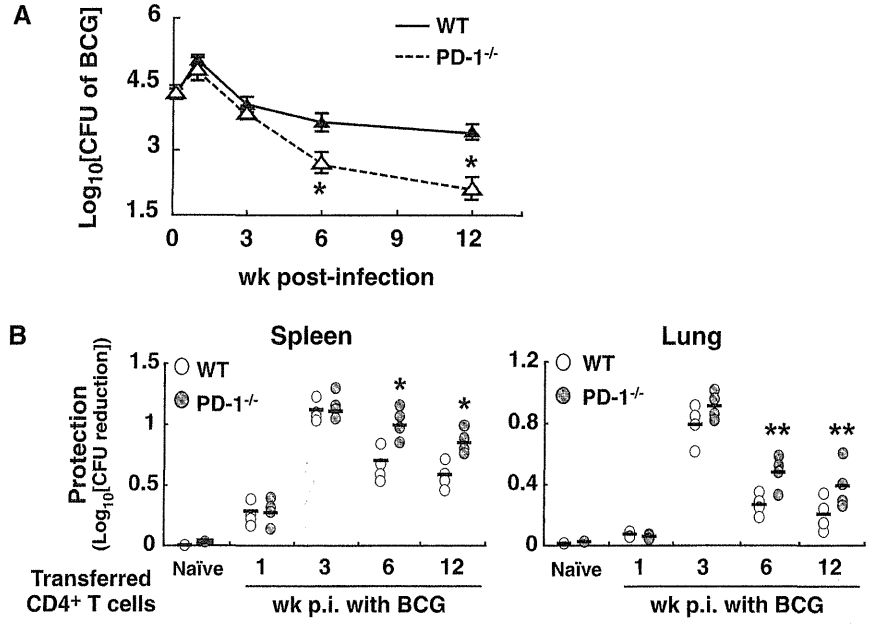

Fig. 5. Comparison of protective immunity to BCG between WT and PD-1$^{-/-}$ mice. (A) WT and PD-1$^{-/-}$ mice were infected with 10^6 CFU of BCG. Bacterial numbers in spleens were evaluated at the indicated time points. Data are the mean ± SD of CFU from four mice per each time point. (B) After BCG infection, CD4$^+$ T cells were purified from the spleens of WT or PD-1$^{-/-}$ mice and transferred into WT recipient mice. Mice were infected with 10^5 CFU of Mtb, and bacterial numbers in the spleen and lung were determined after 10 days. Data are expressed as described in Fig. 1B. *$P < 0.01$, **$P < 0.05$ as compared with WT mice. Results are representative of two independent experiments.

appears to account for the observed difference in bacterial clearance between the spleen and the lung of infected mice. In addition, we found that the expression of PD-Ls was up-regulated on APC in both the lung and the spleen as early as 10 days after Mtb infection (data not shown), suggesting that the inability of BCG to induce PD-L1 expression in the lung might be because of the attenuated virulence or immunogenicity of BCG (52, 53).

Because Treg cells and Tr1 cells are implicated in the suppression of immune response to Mtb (37–40), we investigated whether that these T cells contribute to the reduction of T_h1 response during BCG infection. However, we did not observe a significant increase in the population of Treg cells or IL-10-producing Tr1 cells in the late stage of infection. Previous studies have shown that *in vivo* depletion of Treg cells does not affect protective immunity to BCG (37). Furthermore, bacterial clearance has been observed similarly in C57BL/6 WT and IL-10$^{-/-}$ mice infected with BCG (54, 55). Therefore, it appears that Treg cells and Tr1 cells do not play a major role in the impairment of T-cell-mediated immunity in the late stage of BCG infection. In addition, TGF-β1-producing CD4$^+$ T (T_h3) cells have been postulated to suppress the immune response to Mtb infection (56). However, we were unable to detect a significant increase in TGF-β1 production by CD4$^+$ T cells in the late stage of BCG infection (data not shown).

CD4$^+$ T cells capable of producing both IFN-γ and TNF-α were generated by 3 weeks after infection with BCG. These cytokines are essential for the control of Mtb infection (12). The appearance of cytokine-producing CD4$^+$ T cells strongly correlated with the magnitude of protection because protective efficacy and the frequency of CD4$^+$ T cells producing IFN-γ and TNF-α peaked at 3 weeks after infection and were simultaneously decreased thereafter in WT mice. Consistent with our results, recent studies have shown that the appearance of T cells producing both IFN-γ and TNF-α correlates with protection against infection with various pathogens including Mtb (34–36, 57). Unlike WT mice, however, the T-cell population of PD-1$^{-/-}$ mice was maintained even in the late stage of infection. CD4$^+$ T cells capable of producing both IFN-γ and TNF-α may therefore play an important role in protective immunity to BCG, and the PD-1-dependent signal may be associated with the decrease in the T-cell population.

It has been shown that the number of antigen-specific CD4$^+$ T cells is reduced as a result of a contraction of memory CD4$^+$ T cells after the antigen or pathogen was eliminated from the host (58, 59). In this study, however, we observed the functional reduction of protective CD4$^+$ T cells even when BCG was still detectable in the host. As there was no difference in the level of apoptosis of memory CD4$^+$ T cells between WT and PD-1$^{-/-}$ mice, it is clear that the reduction is not a reflection of cell death. It is therefore assumed that the PD-1-dependent reduction of effector T cells is due to the result of a mechanism distinct from that of the conventional contraction of antigen-specific T cells.

Our study showed that the PD-1–PD-L1 pathway contributes to the impairment of protective immunity in the late stage of BCG infection. In other words, blockade of the co-inhibitory pathway appears to be a useful strategy for therapy of latent TB and enhancement of vaccination efficacy. In fact, during chronic infection with LCMV and simian immunodeficiency virus, treatment with anti-PD-1 antibody resulted in the rapid expansion of virus-specific T cells and

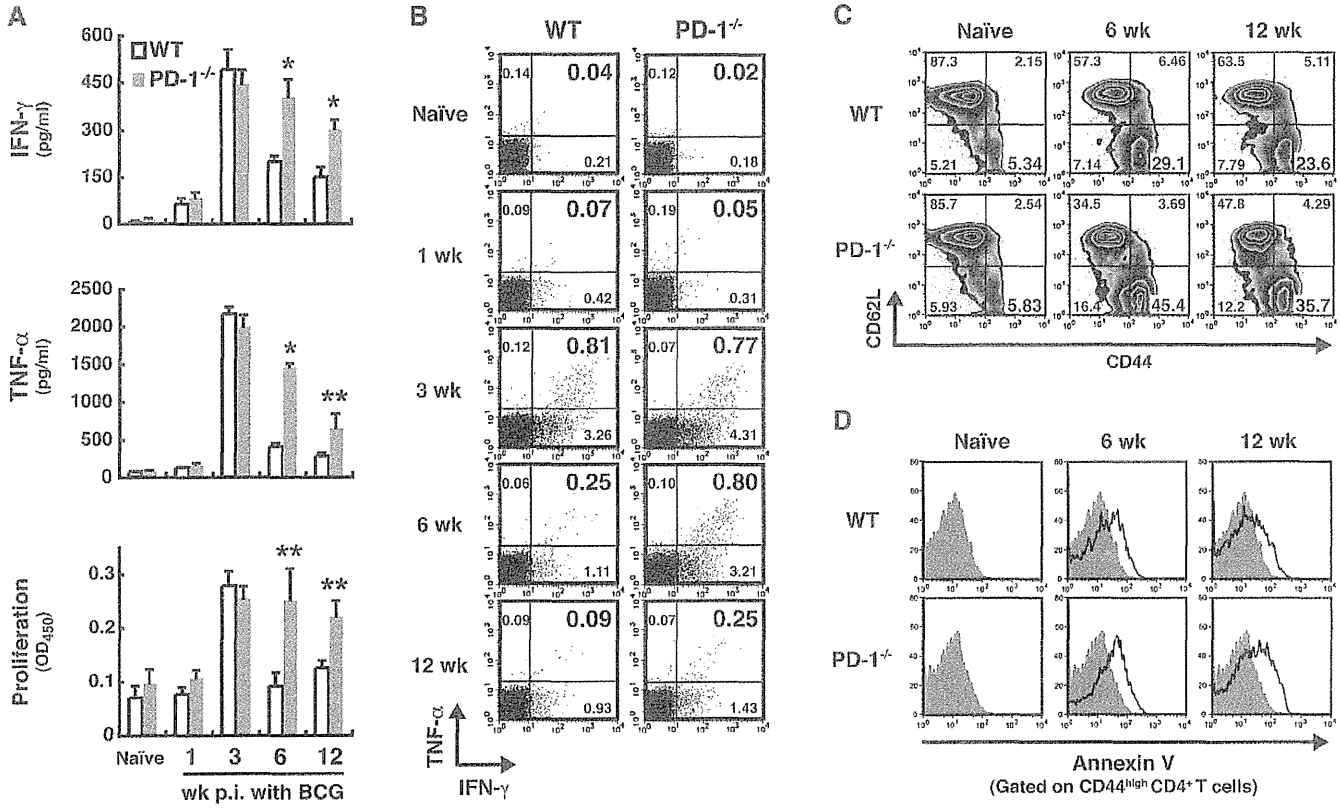

Fig. 6. Functional and phenotypic differences of CD4$^+$ T cells obtained from WT and PD-1$^{-/-}$ mice after BCG infection. (A) CD4$^+$ T cells were purified from WT and PD-1$^{-/-}$ mice after BCG infection and cultured with BCG-pulsed BMDC. IFN-γ and TNF-α production and proliferation of CD4$^+$ T cells were measured. Data are the means ± SD of triplicate cultures. *$P < 0.01$, **$P < 0.05$ as compared with WT mice. (B) Splenocytes were isolated from WT and PD-1$^{-/-}$ mice at the indicated times after BCG infection. Cells were stimulated with PPD for 6 h and intracellular IFN-γ and TNF-α were analyzed by flow cytometry. Each value indicates the percentage of IFN-γ and TNF-α-producing CD4$^+$ T cells. (C) Splenocytes were collected from WT and PD-1$^{-/-}$ mice at 6 and 12 weeks after infection and stained with antibodies to CD4, CD44 and CD62L. The percentage of memory CD4$^+$ T-cell population was analyzed by flow cytometry. Numbers indicate the percentage of CD4$^+$ T cells in each quadrant. (D) Splenocytes were prepared from WT and PD-1$^{-/-}$ mice at the indicated times after infection and stained with anti-CD4 antibody, anti-CD44 antibody and Annexin V. Apoptosis of memory CD4$^+$ T cells (CD44^{high} CD4$^+$ T cells) was measured by Annexin V binding. Shaded histograms, naïve cells; solid lines, BCG-infected cells. Results are the representative of three independent experiments.

reduced viral loads even in hosts suffering from severe lymphopenia (23, 60). Importantly, blockade of the CTLA-4 co-inhibitory pathway does not enhance protection against infection with these viruses and BCG (23, 61, 62). Therefore, the PD-1–PD-L1 pathway appears to be critically involved in the impairment of protective immunity and blockade of the co-inhibitory signal pathway may be a key to augmentation of protection against persistent infection with various pathogens including mycobacteria. Further studies are needed to understand the complete inhibitory mechanism and the potential application of PD-1 in the therapeutic treatment and vaccination for TB.

Funding

Grant-in-Aid for Scientific Research on Priority Areas from the Ministry of Education, Culture, Sports, Science and Technology, Japan; Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science; a Grant-in-Aid for Research on Emerging and Re-emerging Infectious Diseases form the Ministry of Health, Labour and Welfare, Japan; and a research grant from the Waksman Foundation of Japan.

Acknowledgement

We are grateful to Dr Tasuku Honjo (Kyoto University, Kyoto, Japan) for generously providing the PD-1$^{-/-}$ mice used in this study.

Disclosure

The authors have no conflicting financial interests.

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PLoS NEGLECTED TROPICAL DISEASES

A Lipopeptide Facilitate Induction of *Mycobacterium leprae* Killing in Host Cells

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Abstract

Little is known of the direct microbicidal activity of T cells in leprosy, so a lipopeptide consisting of the N-terminal 13 amino acids lipopeptide (LipoK) of a 33-kD lipoprotein of *Mycobacterium leprae*, was synthesized. LipoK activated *M. leprae* infected human dendritic cells (DCs) to induce the production of IL-12. These activated DCs stimulated autologous $CD4^+$ or $CD8^+$ T cells towards type 1 immune response by inducing interferon-gamma secretion. T cell proliferation was also evident from the CFSE labeling of target $CD4^+$ or $CD8^+$ T cells. The direct microbicidal activity of T cells in the control of *M. leprae* multiplication is not well understood. The present study showed significant production of granulysin, granzyme B and perforin from these activated $CD4^+$ and $CD8^+$ T cells when stimulated with LipoK activated, *M. leprae* infected DCs. Assessment of the viability of *M. leprae* in DCs indicated LipoK mediated T cell-dependent killing of *M. leprae*. Remarkably, granulysin as well as granzyme B could directly kill *M. leprae in vitro*. Our results provide evidence that LipoK could facilitate *M. leprae* killing through the production of effector molecules granulysin and granzyme B in T cells.

Citation: Maeda Y, Tamura T, Fukutomi Y, Mukai T, Kai M, et al. (2011) A Lipopeptide Facilitate Induction of *Mycobacterium leprae* Killing in Host Cells. PLoS Negl Trop Dis 5(11): e1401. doi:10.1371/journal.pntd.0001401

Editor: Kenji Hirayama, Institute of Tropical Medicine (NEKKEN), Japan

Received May 16, 2011; **Accepted** October 6, 2011; **Published** November 22, 2011

Funding: This work was supported by a Grant-in-Aid from the Ministry of Health, Labour and Welfare of Japan for "Research on Emerging and Re-emerging Infectious Diseases" (Grant no. H21-Shinko-Ippan-007 to T.M.), and a grant from Ohyama Health Foundation (to Y.M.), Tokyo. The funders had no role in study design, data collection and analysis, decision to publish or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

The introduction of multidrug therapy in 1982 and the WHO campaign for the 'elimination of leprosy as a public health problem', have contributed greatly to the decrease in the prevalence rate over the past three decades. But leprosy still remains to be a public health problem in some countries, and the number of new cases detected during the last three years, remain steady [1]. The disease presents as a clinical spectrum that correlates with the level of the immune response to the pathogen [2]. Patients with lepromatous form of the disease have poor cellular immunity, resulting in extensive intracellular proliferation of *Mycobacterium leprae* bacilli in the skin and nerves. On the other hand, patients with the tuberculoid form of the disease are relatively resistant to the bacilli, so that few, if any, demonstrable bacilli are seen in the lesions [2,3]. For patients with abundant bacilli, whose lesions are characterized by type-2 cytokines, there is a need to up-regulate the T-cell mediated type 1 immune responses, by immunotherapeutic means to kill the bacilli.

We have previously identified a lipoprotein of *M. leprae*, a 33-kD lipoprotein (ML0603) [4]. Truncated protein, having the N-terminal 60 amino acids of 33-kD lipoprotein, had cytokine inducing ability in human monocytes, in contrast to the C-terminal 192 amino acids having no such ability [5]. In this study, we synthesized the lipopeptide (LipoK) having the N-terminal 13 amino acids of the 33-kD *M. leprae* lipoprotein linked to tri-palmitoylated portion of a lipid. Since GC mass spectrometry of mycobacterial lipoproteins provided evidence for the presence of three fatty acids (either palmitic, stearic or tuberculostearic acid), we assumed that tri-palmitoylated peptide would represent the natural lipoprotein of *M. leprae* [6,7]. Further, N-acyl transferase (Lnt) activity was identified in mycobacteria, which transfers the amide-linked acyl group to the N-terminal cysteine residue [6]. This presence of Lnt activity would indicate the presence of triacylated lipoproteins in mycobacteria, although the exact lipid structure of *M. leprae* lipoprotein is still to be determined. Previously, it was observed that hexameric peptides with tri-palmitoyl modification, corresponding to 19-kD and 33-kD lipoproteins of *M. leprae*, partially activates cells through TLR2-TLR1 heterodimers [8,9]. Since dendritic cells (DCs) are the most potent antigen presenting cells capable of bacilli uptake, antigen presentation and initiating acquired immune responses, DCs were used as target antigen presenting cells, in the present study [10,11]. As expected, it was found that LipoK, delivered signals through TLR2, and activated *M. leprae* infected DCs to produce abundant IL-12, although, LipoK does not produce IL-12, in non-infected DCs. Several mechanisms are known to be involved in the clearance of intracellular bacteria, including interferon gamma (IFN-γ) release, apoptosis induction of the host cells and anti-microbial activity of $CD8^+$ cytotoxic T lymphocytes (CTL) [12–15]. CTL mediated killing of mycobacteria, was demonstrated in tuberculosis by Thoma-Uszynski *et al.* They showed that $CD8^+$ CTL-mediated killing of *M. tuberculosis* was dependent on granule exocytosis [16].

In the present study, we analyzed whether *M. leprae* infected DCs, activated through LipoK could undergo functional changes and subsequently induce type 1 T cell activation to kill the bacilli. We observed that LipoK is a potent inducer of T cells equipped

Author Summary

We observed that LipoK (*Mycobacterium leprae* lipopeptide with 13 amino acids) is capable of inducing a good immune response in *M. leprae* infected human dendritic cells (DCs). These activated DCs had up-regulated expression of costimulatory molecule CD86 as well as CD83 (well known maturation marker) on their surface, and secreted IL-12, which is an important cytokine involved in the host defense against pathogens. Importantly, these mature DCs were capable of further driving type 1 responses by stimulating $CD4^+$ T cells and $CD8^+$ T cells for proliferation and interferon-gamma production. Further, both subsets of T cells were capable of producing cytotoxic granules: granulysin and granzyme B. *In vitro* experiments proved that these molecules are capable of killing *M. leprae* directly. It is the first report of the type, which proves that granulysin as well as granzyme B could partially kill *M. leprae*. LipoK would facilitate in inducing the immune responses in patients' harboring *M. leprae*.

with cytolytic function, which can largely contribute to the killing of *M. leprae* in host cells.

Materials and Methods

Ethics statement, cell culture and preparation of the bacteria

Peripheral blood was obtained from healthy Japanese individuals under informed consent. But no information of the donor (exposure to bacilli) was provided. In Japan, BCG vaccination is compulsory for children (0~4 years old). Monocyte-derived DCs were differentiated from monocytes using GM-CSF and IL-4 as described earlier [17,18]. Animal studies were carried out in strict accordance with the recommendations from Japan's Animal Protection Law. The protocol was approved by the Experimental Animal Committee, of the National Institute of Infectious Diseases, Tokyo (Permit Number: 210001). *M. leprae* (Thai-53 strain) is passaged in athymic *nu/nu* mice (Clea Co, Tokyo) [19]. At 8 to 9 months post-infection, the footpads were processed to recover *M. leprae* [20]. For all experiments, *M. leprae* was freshly prepared. The multiplicity of infection (MOI) was determined based on the assumption that DCs were equally susceptible to infection with *M. leprae* [21], and immature DCs were infected with *M. leprae* at MOI 50 in all experiments. Human cells without the bacilli was cultured at 37°C, but when infected with the bacilli, the cells were cultured at 35°C, which is the minimal temperature at which the cells can survive in *in-vitro* experiments. LipoK having the structure Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy) -propyl)-Leu-Pro-Asp-Trp-Leu-Ser-Gly-Phe-Leu-Thr-Gly-Gly-OH, was synthesized by Bachem (Bubendorf, Switzerland). Using LAL assay (QCL-1000, Lonza), endotoxin was undetectable in original LipoK preparation (50 μg/ml). Therefore, any contaminating LPS in the synthesized product could be ruled out. Monoclonal Ab to TLR2 was kindly provided by Genentech, and mAb to mannose receptor and DC-SIGN were obtained from BD Biosciences. Parthenolide obtained from Santa-Cruz was used at a concentration of 2 and 5 μM. CD40L (Pepro Tech) was used at the concentration of 1 μg/ml, whenever needed.

Analysis of cell surface Ags on DCs and measurement of IL-12 production

Immature DCs were stimulated with *M. leprae* and/or LipoK for 48 hours. The expression of cell surface antigens on DCs, were analyzed using FACSCalibur flow cytometer (BD Biosciences). Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D stain. For analysis of cell surface Ag, the following mAb were used: FITC-conjugated mAb against HLA-ABC (G46-2.6), HLA-DR (L243) and CD86 (FUN-1), purchased from PharMingen, and CD83 (HB15a, Immunotech). The ability of DCs to produce IL-12 on stimulation with either LipoK and/or *M. leprae*, was assessed. DCs were stimulated with the Ags on day 4 after the start of culture from monocytes. After 24 hours, OptEIA Human IL-12 (p70) ELISA Set (BD Biosciences) was used to determine the concentration of IL-12 p70 in the culture supernatant.

DC-T cell co-cultures

The ability of *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-T cell co-culture. $CD4^+$ T cells and $CD8^+$ T cells were purified using respective T cell enrichment Set (BD IMag) from freshly thawed PBMCs. The purity of $CD4^+$/$CD8^+$T cells was determined to be more than 95%. The purified responder cells (1×10^5 per well) were plated in 96-well round-bottom tissue culture plates, and mitomycin C-treated DCs which were pulsed with Ag, were added to give the indicated DC: $CD4^+$ or $CD8^+$ T cell ratio. Supernatants of DC-T cell co-cultures were collected on day 4, and IFN-γ production was measured by ELISA, using Opt EIA Human IFN-γ ELISA Set (BD Biosciences). In other experiments, Ag-pulsed DCs were treated with mAb to HLA-ABC (W6/32), HLA-DR (L243), CD86 (IT2.2) or normal mouse IgG. For obtaining naïve T cells, anti-CD45RO mAb (Dako) and anti-mouse IgG Ab Dynabeads M-450 (Invitrogen) were used to negatively select the cells. Since BCG is compulsory for children in Japan, it is likely that naïve T cells respond to *M. leprae* antigens, some of which are cross reactive to *M. bovis* BCG.

Measurement of T cell proliferation by CFSE labeling

DCs stimulated with Ags were co-cultured with the CFSE labeled total T cells. CFSE (Molecular Probes) was added at the concentration of 1 μM and incubated at 37°C for 10 min and stabilized according to the manufacturers' protocol. A total of 1×10^6 cells/well were seeded in a 24-well plate at a DC:T cell ratio of 1:6. After 8 days co-culture, cells were co-stained with PE conjugated anti-CD4 mAb and APC conjugated anti-CD8 mAb (BD Biosciences). CFSE signal of gated T cells were analysed.

Confocal microscopy

Imaging of cells was performed using laser scanning microscope LSM5-Exciter (Carl Zeiss). DCs grown on a 13-mm coverglass in a 24-well plate, were infected with *M. leprae* and/or stimulated with LipoK for 48 hours. T cell from the same donor was purified using the Dynal T cell isolation kit, and co-cultured with DCs for additional 3 days, after washing out extracellular bacilli. Cells were fixed in 2% paraformaldehyde, and the bacilli stained with 0.01% auramine O as described [22]. Anti-*M. leprae* membrane (minus LAM) polyclonal antibody was kindly provided by Dr. John S. Spencer through the NIH/NIAID Leprosy Research Support (N01 A1-25469). Fixed cells were blocked with normal human IgG (10 μg/ml), and stained with the above polyclonal antibody (1 μg/ml) for 30 min in PBS containing 0.1% saponin and 0.5% BSA, and the secondary antibody used was Alexa Fluor 633-conjugated goat anti-rabbit IgG (Molecular Probes), and images were recorded on fluorescent confocal microscope using a 63× oil objective, 488-nm and 633-nm lasers. Data was processed using the LSM software ZEN 2007. All bacilli observed were not surface attached as observed by section scanning (Z-stack Navigation).

Determination of intracellular levels of perforin, granzyme B and granulysin

After 7 days co-culture of purified T cells with DC pulsed with *M. leprae* and/or LipoK, intracellular detection of cytolytic effector molecules was performed. Briefly, GolgiStop (BD Biosciences) was added to the media for the last 12 hours of culture. Cells were first surface stained, fixed, permeabilized, and finally stained with FITC conjugated anti-perforin mAb or anti-granzyme B mAb or isotype control IgG2a (BD Biosciences). For the determination of intracellular levels of granulysin, the procedure was followed as for the intracellular stain of perforin, except that the surface stain used was FITC conjugated-CD4 and APC conjugated anti-CD8 mAb (BD Biosciences), and subsequently PE conjugated granulysin (eBioscience, GmbH, Germany) was used to determine the percentage of granulysin producing cells.

Determination of *M. leprae* viability in DCs

Since *M. leprae* cannot be cultured in vitro, we measured the viability of the bacilli, by the measurement of radioactive CO_2 production from oxidation of palmitic acid as described previously [23]. DCs were infected with *M. leprae* with or without LipoK, and co-cultured with T cells in some cases. Six days later, cells were harvested and washed 3 times in PBS, and centrifuged, so that *M. leprae* that might have escaped from the DCs into the media could be eliminated from our assay. Cell lysates were prepared as follows: 0.1 N NaOH solution was added to the cells for few minutes and then neutralized with the equal volumes of 0.1 N HCl solution. Subsequently, equal volume of 2 times concentrated Middlebrook 7H9 broth supplemented with ADC was added. ^{14}C labeled palmitic acid was added to the lysates of DCs and cultured at 33°C. Seven days later, the amount of $^{14}CO_2$ evolved and trapped on the filter paper was measured using a Packard 1500 TRI-CARB liquid scintillation analyzer. In a likewise manner, direct effect of *M. leprae* killing was observed by incubation of the bacilli with 3 μg/ml of granulysin (R&D systems) or granzyme B (Calbiochem) for a period of 3 days at 33°C, and then ^{14}C labeled palmitic acid was added to determine the viability as described above.

Statistical analysis

The unpaired student's t test was used to find the significance of the two sets of data. Differences were considered as statistically significant if $p<0.05$. All experiments were performed at least 3 times with different blood donors, unless otherwise stated, and the reproducibility of the experiment was evaluated. In some cases, ANOVA was used for probability calculation.

Results

LipoK activated human dendritic cells

We investigated the effect of LipoK stimulation on human monocyte derived DCs. All DCs were CD1a positive and CD14 negative [21]. When LipoK was used as a stimulant for immature DCs, maturation of DCs was observed as shown in Fig. 1. Up-regulation in the expression of CD83 (maturation marker of DCs) and CD86 (co-stimulatory molecule) was observed in LipoK stimulated DCs, the level of which, was similar to that of *M. leprae* infected DCs. *M. leprae* was used at the multiplicity of infection (MOI): 50 in all the experiments. The expression of the CD83 and CD86 molecules was more pronounced when LipoK was used to stimulate *M. leprae* infected DCs. The expression of HLA-ABC and HLA-DR molecules was not significantly different in LipoK stimulated *M. leprae* infected DCs from non-infected DCs, after 48 hours. Although, at earlier time points (18 hours after stimulation with antigen), a higher expression of HLA-ABC and

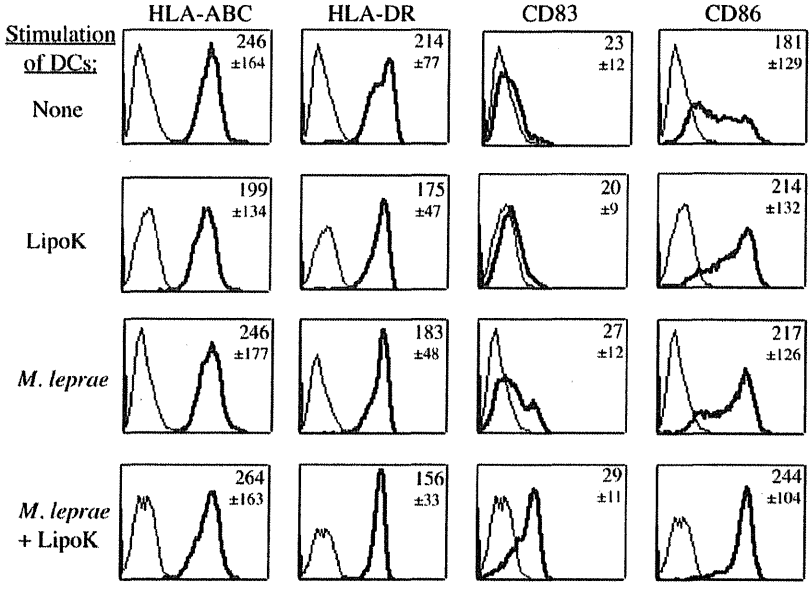

Figure 1. Expression of the surface markers on DCs after stimulation of *M. leprae* infected DCs with LipoK. The expression of cell surface markers on DCs, was analyzed using FACSCalibur. Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D (7-AAD) stain. LipoK was used at a concentration of 0.3 μg/ml. The following mAb were used: FITC-conjugated mAb against HLA-ABC, HLA-DR, CD83 and CD86. Black light lines, isotype-matched control IgG. Black solid lines show the fluorescence intensity of the respective surface markers of DCs. Numbers indicate the mean fluorescence intensity with SD of the respective surface markers. Representative data of three separate experiments with different donors is shown.
doi:10.1371/journal.pntd.0001401.g001

HLA-DR is observed in LipoK stimulated *M. leprae* infected DCs compared to non-stimulated.

Alternatively, when the IL-12 p70 secreted by DCs was measured, increasing dose of LipoK on *M. leprae* infected DCs produced the cytokine, with maximal cytokine production at LipoK concentration of 0.3 μg/ml (Fig. 2A). LipoK alone did not produce statistically significant amounts of IL-12 at the concentration of 0.3 μg/ml compared to the non-stimulated DCs. Another TLR-2 agonist, peptidoglycan could produce IL-12 (data not shown), probably due to the heterogeneous nature of the peptidoglycan which contains long peptide linkages. LipoK probably need other protein/peptide molecules to activate IL-12 production in DCs. Also, *M. leprae* infection alone did not produce IL-12 in DCs. When CD40 ligand (CD40L) was used to stimulate *M. leprae* infected DCs, IL-12 production was negligible.

As could be expected, TLR-2 antagonistic Ab completely blocked IL-12 production, whereas mannose receptor Ab did not, suggesting that IL-12 production from LipoK stimulated *M. leprae* infected DCs was TLR-2 dependent (Fig. 2B). When DCs were pre-treated with parthenolide, which is known to inhibit NF-kB activity [24], it was found that both 2 μM and 5 μM could significantly inhibit the production of IL-12 in a dose-dependent manner (Fig. 2C), indicating that NF-kB is involved in the IL-12 production from these LipoK stimulated DCs.

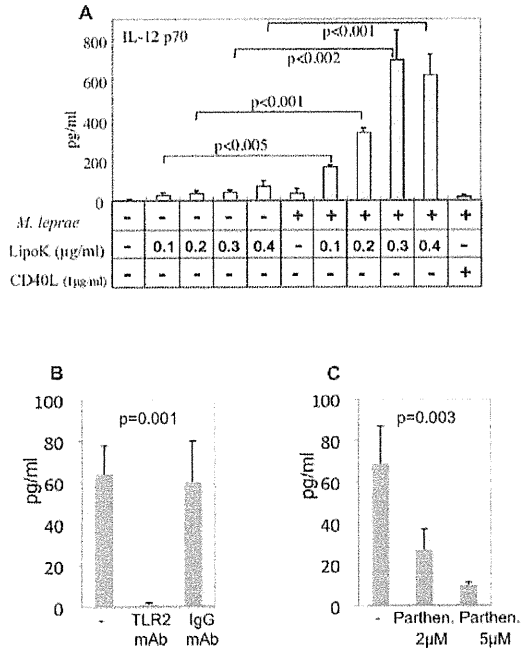

Figure 2. Production of IL-12 p70 from DCs. **(A)** Enhanced induction of IL-12 p70 from DCs by stimulation with LipoK and *M. leprae*. DCs were stimulated with the antigens on day 4 after the start of culture from monocytes. After 24 hours, IL-12p70 concentration in the culture supernatant was measured by the enzyme assay kit Opt EIA Human ELISA Set. The antigens used for the stimulation were: *M. leprae* and LipoK at different concentrations 0.1 μg/ml, 0.2 μg/ml, 0.3 μg/ml, and 0.4 μg/ml, CD40L was used at 1 μg/ml. **(B)** IL-12 p70 production from LipoK stimulated *M. leprae* infected DCs, is inhibited by antagonistic antibodies to TLR-2, and not by control normal IgG. **(C)** Effect of 2 μM and 5 μM of parthenolide (parthen.) on the IL-12 production was observed. Representative data of three separate experiments with different donors is shown. The probability by ANOVA was calculated to be 0.001 for (B) and 0.003 for (C).
doi:10.1371/journal.pntd.0001401.g002

LipoK pulsed human DCs activated human T cells *ex vivo*

To investigate the effect of LipoK on T cell responses, purified $CD4^+$ and $CD8^+$ T cells from autologous donors were cultured with activated DCs. IFN-γ release was measured as correlates of T cell activation. When the IFN-γ levels were compared, DCs activated with *M. leprae* and LipoK produced significantly higher dose of IFN-γ from $CD4^+$ T cells, when compared to that produced by DCs stimulated with *M. leprae* or LipoK alone, or *M. leprae* in presence of CD40L (Fig. 3A), at both high (T:DC = 20:1) and low (T:DC = 40:1) dose of DCs. Note that *M. leprae*-infection or LipoK-stimulation alone was not efficient in stimulating T cells. Similarly, secretion of IFN-γ was also observed from $CD8^+$ T cells but at lower level compared to that from $CD4^+$ T cells. Again there was significantly high production of IFN-γ from $CD8^+$ T cells co-cultured with LipoK stimulated *M. leprae*-infected DCs compared to that from CD40L stimulated *M. leprae*-infected DCs (Fig. 3A). Although the IL-12 p70 production differed in LipoK stimulated *M. leprae*-infected DCs and CD40L stimulated DCs, no IL-12 production was observed from these mitomycin treated DCs which were co-cultured with T cells. In addition, as shown in Fig. 3B, although normal murine IgG did not affect the T cell stimulating activity of both $CD4^+$ and $CD8^+$ T cells, mAbs to HLA-ABC and HLA-DR, inhibited $CD8^+$ T cells and $CD4^+$ T cell activation of LipoK-stimulated *M. leprae*-infected DCs' respectively. The results indicated that the activation of these T cells were MHC Class II- and Class I-dependent in $CD4^+$ T cell and $CD8^+$ T cells respectively. The inhibition was comparable to that of inhibition of IFN-γ production by mAb to co-stimulatory molecule CD86.

Proliferation of these LipoK activated $CD4^+$ and $CD8^+$ T cells, was confirmed by the CFSE labeling of T cells. The labeling experiment was preferable because it could measure proliferation of individual T cell subsets even in the presence of the other subsets. *M. leprae* stimulation of DCs resulted in proliferation of 39.7% of total $CD4^+$ T cells, but stimulation with both LipoK and *M. leprae* resulted in proliferation of 67.5% of total $CD4^+$ T cells. LipoK stimulation alone did not induce any significant proliferation of $CD4^+$ T cells (Fig. 3C). The profiles of flow cytometric analyses showed that 25.3% of $CD8^+$ T cells proliferated by stimulation with *M. leprae* alone, but higher number of cells proliferated (38.9%) in presence of LipoK stimulus.

Subsequently, we examined the response of naïve T cells to LipoK activated DCs. When naïve $CD4^+$ T cells were cultured with DCs activated with *M. leprae* and LipoK, significantly higher dose of IFN-γ was produced in comparison to those cultured with DCs stimulated with *M. leprae* alone or LipoK alone. Production of IFN-γ was low from those activated with *M. leprae* and CD40L (Fig. 3D). It was observed that the IFN-γ production from naïve $CD8^+$ T cells, co-cultured with DCs stimulated with *M. leprae* and LipoK was meager.

When *M. bovis BCG* was used for infecting DCs, the MOI of the bacilli had to be lowered to almost 1~10, because higher MOI (50) would kill the DCs in *in-vitro* culture. BCG when infected at MOI:1 produced 156 pg/ml of IFN-γ from CD8 T cells, but when LipoK was used to stimulate BCG infected DCs, the amount of IFN-γ increased to 380 pg/ml, indicating that LipoK could lead to further T cell activation of BCG infected DCs. It is also likely that LipoK stimulation could increase the production of perforin and granulysin in *M. tuberculosis* infected host cells.

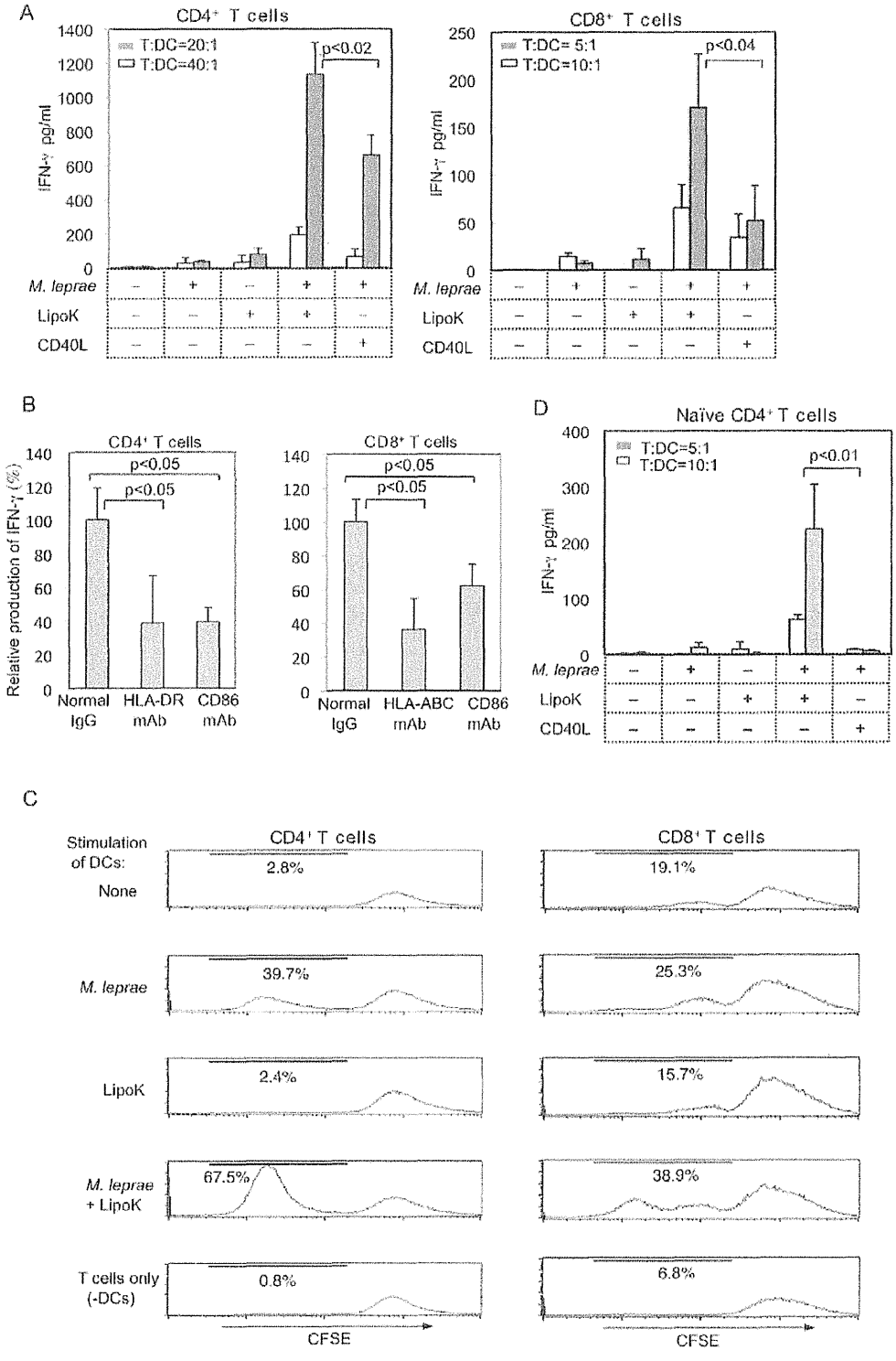

Figure 3. T cell activity as determined IFN-γ production and T cell proliferation. (**A**) The effect of LipoK on *M. leprae*-infected DCs to stimulate T cells was assessed using an autologous DC-CD4$^+$ or DC-CD8$^+$ T cell co-culture. IFN-γ production in the supernatant was measured by ELISA, after 4 days co-culture. (**B**) Effect of normal murine IgG or mAb to HLA-ABC/HLA-DR or CD86 on IFN-γ production from T cells co-cultured with *M. leprae* infected DCs simulated with LipoK. The production of IFN-γ from Ab non-treated T cells, cultured with LipoK and *M. leprae* stimulated DCs, is considered 100% and the actual value of IFN-γ produced from CD4$^+$ T cells is 250 pg/ml and that from CD8$^+$ T cells is 47 pg/ml at T cell:DC ratio of 10:1. (**C**) Proliferation of CD4$^+$ and CD8$^+$ T cells as assessed by CFSE labeling of T cells. DCs were mixed with autologous CFSE labeled T cells at a T cell:DC ratio of 10:1. Proliferating T cells were analysed by FACSCalibur on day 7 after co-culture. The percentage of proliferated cells is indicated. The

lowest histogram shows unstimulated T cells. (**D**) IFN-γ production from DC-naïve $CD4^+$ T cell co-culture. IFN-γ production was measured after 4 days co-culture with stimulated DCs. Representative data of four separate experiments with different donors is shown. Assays were performed in triplicate and the results are expressed as the mean +SD.
doi:10.1371/journal.pntd.0001401.g003

Up-regulation of perforin, granzyme B and granulysin production in $CD4^+$ and $CD8^+$ T cells

To determine whether cytotoxic effect could be induced in highly activated T cells, we analysed the intracellular production of perforin and granzyme B in DC co-culture system with unseparated T cells. As seen in Fig. 4A, 15.8% of activated $CD8^{high}$ T cells produced perforin and 24.9% produced granzyme B when stimulated with DCs activated with *M. leprae* and LipoK, in comparison to those co-cultured with DCs activated with *M. leprae*, showing 1.4% of perforin and 1.8% of granzyme B-producing T cells. Thus, prominent enhancement of both perforin and granzyme B producing $CD8^+$ T cells was observed. Recently, since $CD4^+$ T cells are also known to possess direct cytotoxic potential [25], we measured the percentage of $CD4^+$ T cells producing perforin and granzyme B. When LipoK and *M. leprae* stimulated DCs were co-cultured with T cells, 12.7% of $CD4^{high}$ T cells produced perforin and 14.6% of those cells produced granzyme B, whereas in presence of *M. leprae* stimulated DCs, 6.6% produced perforin and 8.3% produced granzyme B (Fig. 4B). These data indicated that in addition to $CD8^+$ T cells, $CD4^+$ T cells also had the capacity to produce significant amounts of perforin and granzyme B. Nevertheless, the percentage of $CD8^+$ T cells producing these cytolytic proteins was 1.2~1.7 fold higher than $CD4^+$ T cell. Then, we examined, whether $CD8^+$ T cells alone without the direct contact with $CD4^+$ could have the same capacity. When $CD4^+$ T cells were allowed to culture in inserts, so that there was no direct contact between $CD8^+$ and $CD4^+$ T cells, there was decreased production of both perforin (7.3% v/s 15.8%) and granzyme B (9.5% v/s 24.9%) producing $CD8^+$ T cells (Fig. 4A). So, a direct contact of $CD4^+$ and $CD8^+$ T cells was necessary for sufficient production of cytolytic proteins. When we examined whether exogenous IL-2 could substitute the action of $CD4^+$ T cells, we found that addition of 50 U/ml of IL-2 (excess amount) to $CD8^+$ T cells, could produce both perforin and granzyme B equivalent to that of $CD8^+$ T cells co-cultured with LipoK stimulated, *M. leprae* infected DCs in the presence of $CD4^+$ T cells. However such high levels of IL-2 cannot be produced from host cells, in our experimental setting.

The intracellular level of another cytolytic protein, granulysin, was then examined. Enhancement of granulysin producing $CD8^+$ T cells was observed when co-cultured with DCs activated with *M. leprae* and LipoK. As seen in Fig. 4C, 18.9% of activated $CD8^{high}$ T cells and 28.4% of activated $CD4^{high}$ T cells produced granulysin when co-cultured with DCs activated with *M. leprae* and LipoK, in comparison to those co-cultured with DCs activated with *M. leprae*, (1.7% of $CD8^{high}$ T cells and 0.6% of $CD4^{high}$ T cells).

Mycobacterium leprae components were observed at the periphery of the infected DCs stimulated with LipoK, and co-cultured with T cells

To examine the fate of *M. leprae* in activated DCs, the cells were stained with anti-*M. leprae* membrane polyclonal antibody. Confocal microscopy revealed rod shaped *M. leprae* as observed by auramine-O stain, and membrane components seem to be rather localized in the region where *M. leprae* are present (Fig. 5). Strickingly, those DCs stimulated with LipoK for 48 hours and co-cultured with T cells for additional 3 days showed membrane staining at the periphery of the DCs (Fig. 5 arrowheads shown), probably due to processing of the bacilli in activated DCs.

Killing of *M. leprae* in DCs, by the LipoK stimulation

We determined the viability of *M. leprae* in DCs after stimulation with LipoK in the presence of autologous $CD4^+$ and $CD8^+$ T cells. Since *M. leprae* is uncultivable *in vitro*, the viability of *M. leprae* in DCs, after co-culture with the T cells for a week, was determined by the radiorespirometric assay. The amount of radioactive CO_2 evolved which reflects the rate of ^{14}C-palmitic acid oxidized by *M. leprae*, was measured by the scintillation counter. No significant reduction in $^{14}CO_2$ production was observed, from DCs, not co-cultured with T cells, even in the presence of LipoK stimulation (Fig. 6A). But, when the bacilli were recovered from DCs stimulated with LipoK and co-cultured with T cells, $^{14}CO_2$ production were significantly lower ($p<0.001$) than those recovered from DCs not stimulated with LipoK or T cells. The result indicates that approximately 50% reduction in the viability of *M. leprae* was observed in LipoK activated DCs and co-cultured with T cells compared to those obtained from DCs not stimulated with LipoK (Fig. 6B), indicating that T cells were essential and LipoK stimulation to DCs, was necessary to kill *M. leprae* in DCs. To further determine whether the cytolytic granules namely, granulysin and granzyme B could directly kill *M. leprae*, the bacilli was incubated with human granulysin or granzyme B for a period of 3 days at 33°C. Statistically significant reduction of $^{14}CO_2$ was observed when the bacilli were incubated with granulysin as well as granzyme B (Fig. 6C).

Discussion

In the present study we investigated the role of *M. leprae*-derived synthetic lipopeptide (LipoK), which consists of N-terminal 13 amino acids of the 33-kD *M. leprae* lipoprotein (Accession no. ML0603) linked to Palmitoyl-Cys((RS)-2,3-di(palmitoyloxy)-propyl group in the induction of intracellular killing of *M. leprae* through immuno-activation. Previously, we observed that the 33-kD lipoprotein and the truncated form of the protein induced the production of IL-12 in human peripheral blood monocytes [4,5]. Although human DCs are potent inducers of acquired immune responses, when DCs were exposed to *M. leprae*, they are inefficient in activating T cells [21,26]. It is generally recognized that, stimulation of T cells by intracellular pathogens, such as mycobacteria, is achieved by the coordinated processing of the antigens in the phago-lysosome of APCs and the expression of the antigenic determinants on APCs. Furthermore, CD40-CD40L interaction on immature DCs, are known to contribute to cell mediated responses in leprosy [27,28]. In fact, when *M. leprae* infected DCs were stimulated with CD40L, up-regulation of CD83 and CD86 molecules was observed (not shown). However, we found that CD40L failed to induce the production of IL-12 p70 in *M. leprae* infected DCs. In contrast to CD40L stimulation, LipoK stimulation on *M. leprae* infected DCs induced significant production of IL-12. Further, the expression of CD40 on DCs was not enhanced by stimulating *M. leprae* infected DCs with LipoK. It was evident that IL-12 inducing ability of these matured DCs was mediated by TLR2, and not by other receptors such as mannose receptor or DC-SIGN, as observed in DCs exposed to *M. tuberculosis* or *M. bovis* BCG [29,30,31]. The TLR2 antagonistic