**Fig. 2.** Activation and $T_h1$ response of the P25 TCR-Tg CD4$^+$ T cells after BCG s.c. infection. The N-P25 TCR-Tg mice were s.c. infected with $5 \times 10^6$ CFU of BCG, and bacterial burden and the P25 TCR-Tg CD4$^+$ T cells in the DLN were analyzed. (A) Bacterial number in the DLN was analyzed on day 7, 14 and 28 after BCG s.c. infection. (B) The absolute number of the P25 TCR-Tg CD4$^+$ T cells detected as KN7$^+$ CD4$^+$ cells was measured on day 0, 3 and 7 after BCG s.c. infection at the indicated doses. (C) FACS profile on the CD69 expression, BrdU incorporation or IFN-$\gamma$ production of the P25 TCR-Tg CD4$^+$ T cells was analyzed in the DLN. Representative data on day 0, and 3 after s.c. infection of BCG are shown. Analysis gate was set on the P25 TCR-Tg CD4$^+$ T cells. (D–F) The absolute number of the CD69$^+$ (D), BrdU$^+$ (E) or IFN-$\gamma^+$ (F) P25 TCR-Tg CD4$^+$ T cells was measured on day 0, 3, 7, 14 and 28 after s.c. infection BCG. For data given in the panel (D–F), results are shown as mean ± SD, and the asterisks indicate significance of difference ($P < 0.05$) compared with the cell numbers on day 0. The data shown are as the representatives of two to five independent experiments with 3–5 mice used and individually analyzed for each experiment.

of the P25 TCR-Tg CD4$^+$ T cells at early stage of the infection (data not shown). These results suggest that the Ag85B-specific P25 TCR-Tg CD4$^+$ T cells in the DLN are activated and differentiated into $T_h1$ cells at early stage after s.c. BCG infection such as on day 3–7 when high dose ($1–5 \times 10^6$ CFU) of BCG was infected.

*The Ag85B-specific P25 TCR-Tg CD4$^+$ T cells were activated rapidly but differentiated to $T_h1$ cells at later stages in the lung and MLN after pulmonary BCG infection*

We next analyzed kinetics of mycobacterial Ag85B-specific CD4$^+$ T-cell response after pulmonary BCG infection in the N-P25 TCR-Tg mouse system. We used high dose ($5 \times 10^6$ CFU) of BCG in the pulmonary infection because the dose of BCG induced strong T-cell response while mice remained healthy after both s.c. and i.t. infection and maintained high bacterial burden in the lung and MLN until day 28 after the i.t. infection (Fig. 3A). After i.t. BCG infection, the P25 TCR-Tg CD4$^+$ T-cell number peaked on day 3 in the lung (Fig. 3B).

In the MLN, the TCR-Tg T cell slightly increased on day 3 although the increase was not statistically significant (Fig. 3B). Representative FACS profiles of CD69 and IFN-$\gamma$ expression of the P25 TCR-Tg CD4$^+$ T cells in the lung and MLN before and 28 days after BCG i.t. infection are shown in Fig. 3(C), and the kinetics of the CD69$^+$, BrdU$^+$ and IFN-$\gamma^+$ P25 TCR-Tg CD4$^+$ T cells are demonstrated in the Fig. 3(D–F). The data indicated that the CD69$^+$ and BrdU$^+$ P25 TCR-Tg CD4$^+$ T cells were vigorously increased (up to 270-folds) in the lung and MLN on day 3–7 of i.t. BCG infection followed by decrease of the number (Fig. 3D and E). In contrast, the $T_h1$-type IFN-$\gamma^+$ P25 TCR-Tg CD4$^+$ T cells were hardly detected on day 3 (Fig. 3F). Although low level of increase in the number of IFN-$\gamma^+$ P25 TCR-Tg CD4$^+$ T cells (<10-folds) was observed on day 7–21, there was no statistical significant increase when compared with that before i.t. BCG infection. Robust increase (20- to 30-folds) of the IFN-$\gamma^+$ P25 TCR-Tg CD4$^+$ T cells was seen on day 28 in the lung and MLN (Fig. 3F). These data indicate that
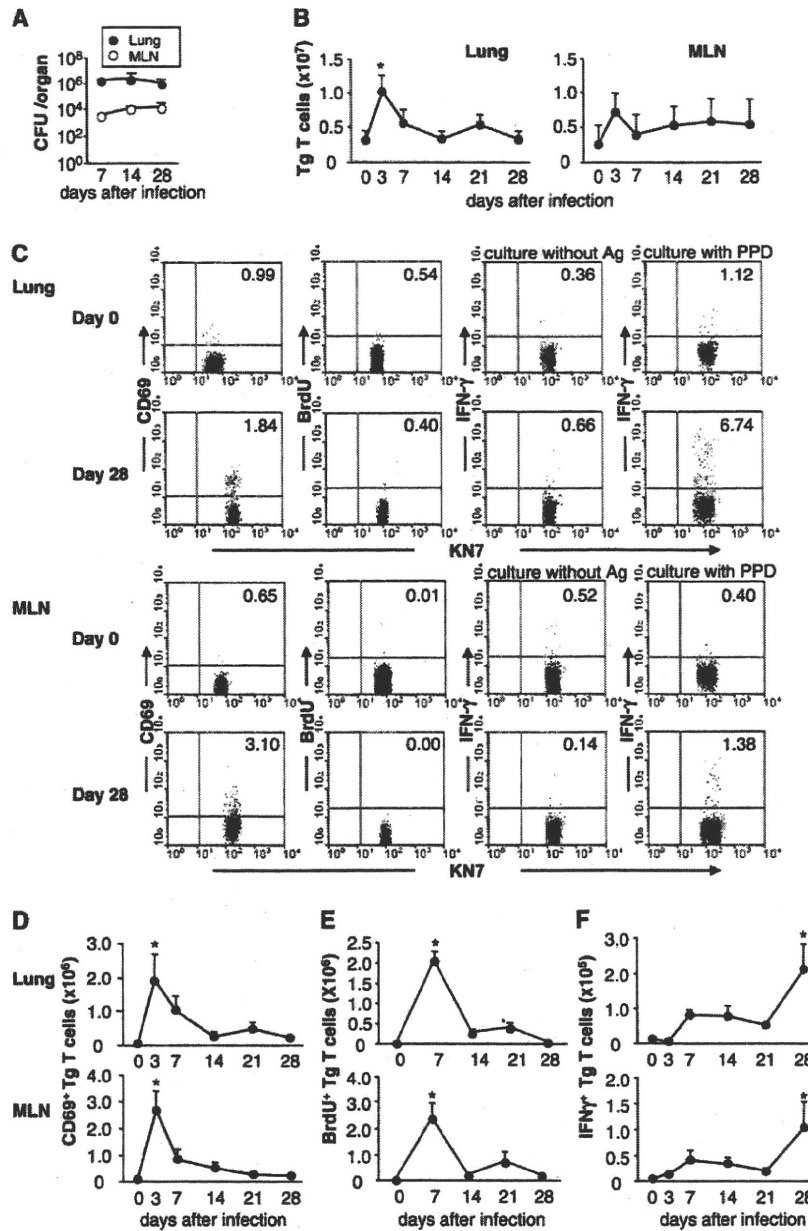
**Fig. 3.** Activation and $T_h1$ differentiation of the P25 TCR-Tg CD4^{+} T cells after BCG lung infection. The N-P25 TCR-Tg mice were i.t. infected with $5 \times 10^6$ CFU of BCG, and the lung and MLN were analyzed. (A) Bacterial number in the lung and MLN was analyzed on day 7, 14 and 28 after BCG i.t. infection. (B) Kinetics of the absolute number of P25 TCR-Tg CD4^{+} T cells in the lung and MLN after the infection. (C) Expression of the CD69 and IFN-γ in the lung and MLN determined on day 0 and 28 after i.t. infection. Analysis gate was set on P25 TCR-Tg CD4^{+} T cells determined as KN7^{+} CD4^{+} cells. (D–F) The absolute number of CD69^{+} (D), BrdU^{+} (E) and IFN-γ^{+} (F) P25 TCR-Tg CD4^{+} T cells in the lung or MLN. Data in Fig. 3(A, B, D–F) are shown as mean ± SD, and the asterisks indicate significance of difference ($P < 0.05$) compared with the cell number on day 0. The data shown are as the representatives of two to five independent experiments with 3–5 mice used and individually analyzed for each experiment.

mycobacterial Ag85B-specific $T_h1$ response is fully established 3–4 weeks after activation and proliferation of the T cells in the lung and MLN of mice i.t. infected with high dose of BCG, whereas Ag85B-specific $T_h1$ response was induced in parallel to activation of the T cells after s.c. BCG infection. This suggests the presence of suppressive mechanisms against induction of $T_h1$ T-cell response in the lung.

*Kinetics of activation and T_h1 differentiation of the P25 TCR-Tg CD4+ T cells after low-dose Mtb s.c. or i.t. infection*

To analyze kinetics of activation and T_h1 induction of Ag85B-specific CD4+ T cells after Mtb infection and compare the responses with those seen in the BCG-infected mice, the N-P25 TCR-Tg mice were s.c. or i.t. infected with low dose $(1 \times 10^3$ CFU) of Mtb. After the s.c. infection with low dose of Mtb, bacterial number was low on day 7, increased on day 14 and maintained the level until day 28 in the DLN (Fig. 4A). The P25 TCR-Tg CD4+ T-cell number slightly increased on day 3 and remarkably increased on day 28 in the DLN (Fig. 4B). The infection also induced significant increase of the CD69+ (Fig. 4C) and IFN-γ+ (Fig. 4E) P25 TCR-Tg CD4+ T cells on day 28 but not on day 3. BrdU+ proliferating P25 TCR-Tg CD4+ T cells showed no significant increase at the analyzed time points (Fig. 4D). Therefore, low-dose Mtb s.c. infection induced activation and T_h1 differentiation of the P25 TCR-Tg CD4+ T cells at a later stage of infection but not at an early stage of the infection.

Kinetics of mycobacterial Ag85B-specific CD4+ T-cell activation and differentiation in the lung and MLN was also analyzed after i.t. low-dose Mtb infection of the N-P25 TCR-Tg mice. Bacterial burden in the lung and MLN was low on day 7, increased on day 14 and maintained the level until day 28, as observed in the DLN of low-dose s.c. Mtb-infected mice (Fig. 5A). The cell number of total, CD69+ and IFN-γ+ P25 TCR-Tg CD4+ T cells showed significant increase in the lung on day 28 of the Mtb i.t. infection (Fig. 5B, C and F), whereas MLN showed significant increase of the CD69+ and IFN-γ+ P25 TCR-Tg CD4+ T cells on day 21 (Fig. 5C and E). Similar kinetics of induction of IFN-γ+ CD4+ T cells was observed in the wild-type C57BL/6 mice infected i.t. with low dose of Mtb (data not shown) and the mice infected with low-dose Mtb containing aerosol (19). Increase of the CD69+ T cells in the lung was not statistically significant on

day 3. Induction of the BrdU+ P25 TCR-Tg CD4+ T cells was not observed even on day 28 after the infection (Fig. 5D). These data demonstrate that both activation and T_h1 differentiation of the mycobacterial Ag85B-specific CD4+ T cells became detectable 4 weeks and 3 weeks after low-dose Mtb i.t. infection in the lung and MLN, respectively, and the response may requires increase of bacterial burden in the infected organs. This is in consistent to the observation that proliferative response of the adoptively transferred P25 TCR-Tg CD4+ T cells was not detected until bacterial burden increased to $>1.5 \times 10^3$ CFU in MLN (19).

*Early activation and T_h1 differentiation of the P25 TCR-Tg CD4+ T cells after high-dose Mtb s.c. or i.t. infection*

Since low-dose BCG infection failed to induce early activation and T_h1 response of the P25 TCR-Tg CD4+ T cells, we estimated that low bacterial burden at an early stage of low-dose Mtb infection is a cause of the discrepancy between high-dose BCG infection and low-dose Mtb infection in induction of the response. To address the issue, we inoculated high dose $(5 \times 10^6$ CFU) of Mtb and compared number of the CD69+ and IFN-γ+ P25 TCR-Tg CD4+ T cells between high dose-infected, low dose-infected and uninfected mice (Fig. 6). The analysis was carried out on day 3 after the infection because intratracheal infection of the high-dose Mtb is lethal but the infected mice survived for 4 to 5 days. The high-dose Mtb s.c.-infected mice showed significantly higher number of the P25 TCR-Tg CD4+ T cells expressing CD69 and IFN-γ in the DLN compared with the low-dose Mtb s.c.-infected or uninfected mice. In contrast, we detected no significant increase of the IFN-γ+ P25 TCR-Tg CD4+ T cells in the lung and MLN of the high-dose Mtb i.t.-infected mice compared with those in the low-dose Mtb i.t.-infected or uninfected mice although the CD69+ P25 TCR-Tg CD4+ T cells significantly increased in the lung by
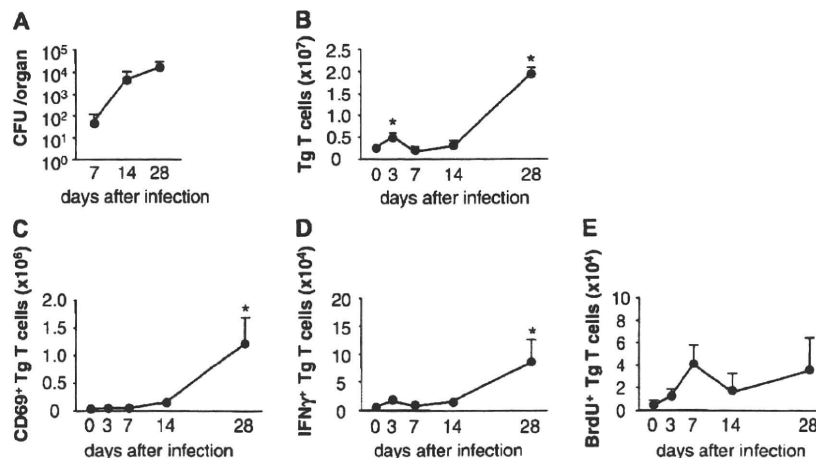


**Fig. 4.** Activation and T_h1 differentiation of the P25 TCR-Tg CD4+ T cells after low-dose Mtb s.c. infection. The N-P25 TCR-Tg mice were s.c. infected with $1 \times 10^3$ CFU of Mtb, and the DLN was analyzed (A). Bacterial number in the DLN was analyzed on day 7, 14 and 28 after Mtb s.c. infection. (B) Kinetics of the absolute number of the P25 TCR-Tg CD4+ T cells. (C–E) The absolute number of CD69+ (C), BrdU+ (D) and IFN-γ+ (E) P25 TCR-Tg CD4+ T cells in the DLN. Results are shown as mean ± SD, and the asterisks indicate significance of difference ($P < 0.05$) compared with the cell number on day 0. The data shown are representatives of two to five independent experiments with 3–5 mice individually analyzed for each experiment.
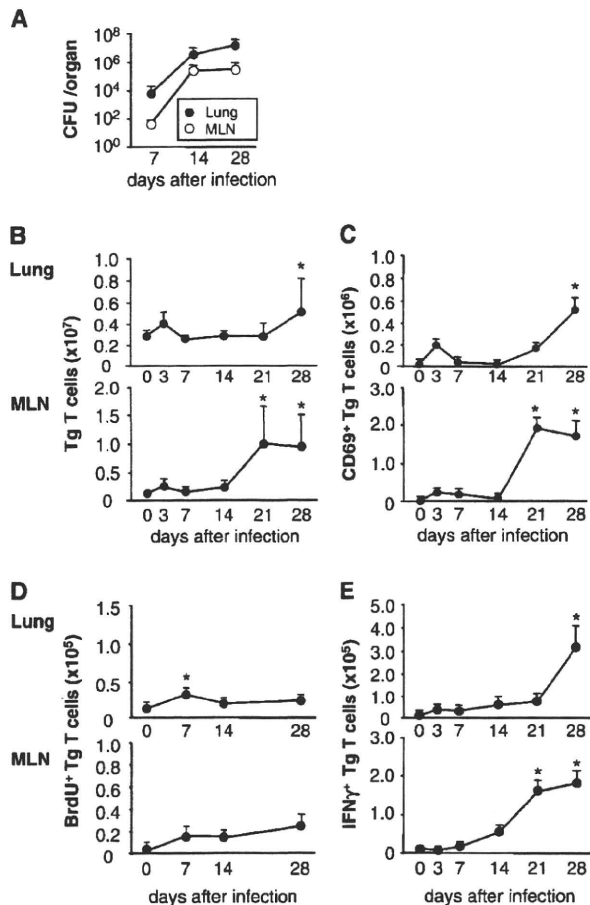
**Fig. 5.** Activation and $T_h1$ differentiation of the P25 TCR-Tg CD4$^+$ T cells after low-dose Mtb lung infection. The N-P25 TCR-Tg mice were i.t. infected with $1 \times 10^3$ CFU of Mtb, and the lung and MLN were analyzed. (A) Bacterial number in the lung and MLN was analyzed on day 7, 14 and 28 after Mtb i.t. infection. (B) Kinetics of the absolute number of the P25 TCR-Tg CD4$^+$ T cells in the lung and MLN. (C–E) Absolute number of the CD69$^+$ (C), BrdU$^+$ (D) and IFN-γ$^+$ (E) P25 TCR-Tg CD4$^+$ T cells in the lung and MLN. Results are shown as mean ± SD, and the asterisks indicate significance of difference ($P < 0.05$) compared with the cell number observed on day 0. The data shown are representatives of two to five independent experiments with 3–5 mice individually analyzed for each experiment.

the high-dose Mtb i.t. infection. The results suggest that early induction of $T_h1$-type immune response to Mtb is suppressed in the lung and MLN even in the presence of high dose of Mtb in the lung.

### IL-10 partially suppressed induction of Mtb-specific $T_h1$ immune response in the lung

In low-dose Mtb i.t.-infected N-P25 TCR-Tg mice, IFN-γ$^+$ P25 TCR-Tg CD4$^+$ T cells were detected on day 21 after the infection but not in lung (Fig. 5). Since $T_h2$-type cytokines (IL-4 and IL-10) and TGF-β have been reported to suppress IFN-γ production (26), we next analyzed the possibility that the cytokines participate in the suppression of mycobacterial

Ag85B-specific $T_h1$ response in the lung after mycobacterial infection. Reverse transcription (RT)–PCR analysis detected expression of *Il10* and *Tgfβ* but not *Il4* in the lung of naive and low-dose Mtb-infected mice (Fig. 7A). Real-time RT–PCR analysis showed enhancement of *Il10* and *Tgbβ* expression by Mtb i.t. infection (Fig. 7B), and therefore, we examined the influence of IL-10 and Tgfβ in the induction of $T_h1$ response in the lung of the Mtb-infected N-P25 TCR-Tg mice.

To address the possible involvement of IL-10 or TGF-β for suppression of $T_h1$ induction in the Mtb-infected lung, we examined the effects of the cytokine neutralization by anti-IL-10 or anti-TGF-β mAb treatment. The mAb were i.v. administered a day before Mtb i.t. infection to the N-P25 TCR-Tg mice. On day 14, 21 and 28 after the infection, the lung and MLN were analyzed for IFN-γ production. As shown in Fig. 7(C), anti-IL-10 mAb-treated mice showed significant increase in the number of the IFN-γ$^+$ P25 TCR-Tg CD4$^+$ T cells in the lung on day 21 but not on day 14 and 28 after the infection. IFN-γ$^+$ P25 TCR-Tg CD4$^+$ T cells in the MLN did not increased by the same mAb treatment. The kinetics of CD69 expression was not affected by the anti-IL-10 mAb treatment (data not shown). In contrast, anti-TGF-β mAb treatment showed no effect on the number of IFN-γ$^+$ P25 TCR-Tg CD4$^+$ T cells after Mtb infection (data not shown). Since it is possible that a single anti-TGF-β mAb treatment was not sufficient to fully neutralize the biological function of TGF-β, the anti-TGF-β mAb was repeatedly inoculated i.v. every week. However, it failed to accelerate induction of the IFN-γ$^+$ P25 TCR-Tg CD4$^+$ T cells (Fig. 6D). CD69 induction of the P25 TCR-Tg CD4$^+$ T cells was not influenced by the anti-TGF-β mAb treatment (data not shown). These data suggested that IL-10, but not TGF-β, is partially involved in the suppression of $T_h1$ induction after Mtb i.t. infection in the lung.

### Discussion

In the present report, we analyzed kinetics of appearance of $T_h1$-type CD4$^+$ T cells in the lung of the mice with high number of naive TCR-Tg CD4$^+$ T cells specific for mycobacterial Ag85B (N-P25 TCR-Tg mice) after mycobacterial pulmonary infection. The results suggest that it requires 4 weeks after pulmonary infection of Mtb or BCG to fully establish mycobacterial antigen-specific $T_h1$ response in the lung and MLN, whereas it takes only a week to establish $T_h1$ response in DLN after s.c. infection of the mycobacteria. Therefore, we concluded that $T_h1$-type immune response is controlled in the mycobacteria-infected lung through lung-specific mechanisms which may include suppression of $T_h1$ induction as discussed below.

Our data showed that $T_h1$ induction of the Ag85B-specific P25 TCR-Tg CD4$^+$ T cells requires >3 weeks in the lung and MLN even when activated (CD69$^+$) or proliferated (BrdU$^+$) P25 TCR-Tg CD4$^+$ T cells increased quickly by day 3 after high-dose BCG or Mtb lung infection. From this observation, we speculated that antigen presentation to the P25 TCR-Tg CD4$^+$ T cells occurred in the MLN and the lung after high-dose lung mycobacterial infection but $T_h1$ induction was not induced at this stage. Wolf *et al.* (19, 27) reported using the P25 TCR-Tg CD4$^+$ T cell-transferred mice that lung DC

captured Mtb and migrated into the MLN on day 17 of low-dose aerosol Mtb infection and induced proliferative response of the P25 TCR-Tg CD4^{+} T cells (19). They detected IFN-γ-producing P25 TCR-Tg CD4^{+} T cells on day 17 in the lung and MLN in proliferating P25 TCR-Tg CD4^{+} T cells (19). Gallegos *et al.* (20) also reported using another Tg mice expressing TCR derived from CD4^{+} T cells specific for a major Mtb antigen ESAT-6 that adoptively transferred CD4^{+} Tg T cells were activated in MLN on day 7–10 and differentiated into $T_h1$ T cells by day 15. Furthermore, Mittrücker *et al.* (28) demonstrated that pulmonary Mtb infection of normal mice induced mycobacterial Ag85A- and Ag85B-specific $T_h1$-type CD4^{+} T cells in the lung on day 28 but not on day 14 of the infection. These data are consistent to our results in that induction of mycobacterial antigen-specific $T_h1$-type CD4^{+} T cells was detected at relatively late stage of immune response after encounter with mycobacteria. Wolf *et al.* (27) attributed the delay of the appearance of mycobacterial antigen-specific $T_h1$ T cells to the delay of antigen presentation by DC. However, our data suggest that antigen presentation to the P25 TCR-Tg CD4^{+} T cells occur at an early stage of high-dose BCG infection and induced T cell activation, but induction of IFN-γ production still requires >3 weeks. Therefore, not only delayed migration of antigen loaded DC into the MLN but also other immune regulatory mechanisms may be involved in the control of $T_h1$ response in the mycobacteria-infected lung.

Although bystander activation of T cells have been reported, we consider that the activation and IFN-γ production of the P25 TCR-Tg CD4^{+} T cells in the N-P25 TCR-Tg mice were induced by TCR-mediated mycobacterial antigen recognition after mycobacterial infection. Bystander activation was reported in various infections *in vivo*, especially on CD8^{+} T cells (29). However, there was only marginal IFN-γ production of the P25 TCR-Tg CD4^{+} T cells when the cells were cultured without mycobacterial antigen, which suggest that the T cells recognize specific antigen to produce IFN-γ. Antigen-specific *in vivo* response of the P25 TCR-Tg CD4^{+}

T cells was also demonstrated by Wolf *et al.* (19). They demonstrated that infection of Ag85B-deficient Mtb failed to induce proliferation of adoptively transferred P25 TCR-Tg CD4^{+} T cells *in vivo* although bacterial burden in the lung and MLN of the Ag85B-deficient Mtb-infected mice was nearly the same as that of mice infected with wild-type Mtb. CD69 expression could also be served as a hallmark of TCR-mediated T-cell activation. CD69 expression was induced in parallel to IFN-γ production in the P25 TCR-Tg T cells after s.c. BCG infection although the number of the IFN-γ^{+} cells were less than that of the CD69^{+} cells. In contrast, i.t. mycobacterial infection induced early CD69 expression without IFN-γ expression in the lung and MLN. All the observation suggest that the P25 TCR-Tg CD4^{+} T cells are activated *in vivo* by antigen recognition after mycobacterial infection and express early T-cell activation marker CD69.

It is also intriguing that the CD69^{+} and IFN-γ^{+} P25 TCR-Tg CD4^{+} T cells increased in the absence of detectable BrdU incorporation at later stage of low-dose Mtb infection. Although the mechanism is not yet clarified, there are several possibilities. First, it is possible that Mtb-infected DC deliver inefficient signals to the T cells which is enough to induce CD69 expression but not sufficient to induce strong clonal expansion. This is in consistent to the observation that total P25 TCR-Tg CD4^{+} T-cell number in the lung and MLN marginally increased after the low-dose Mtb infection. Second, it is possible that the P25 TCR-Tg CD4^{+} T cells proliferate transiently at a restricted time point after pulmonary Mtb infection. Wolf *et al.* (19) demonstrated that absolute number of the adoptively transferred P25 TCR-Tg CD4^{+} T cells increased in the Mtb-infected lung and MLN on day 17 but not on day 14 or day 21. *In vivo* proliferation assay using carboxyfluorescein diacetate succimidyl ester-labeled cells also showed that the P25 TCR-Tg CD4^{+} T cells proliferated on day 17 but not on day 14 after the Mtb infection, and the ratio of proliferated P25 TCR-Tg CD4^{+} T cells seems to be nearly the same on day 21 compared with that on day 17 (19). All the data suggest the possibility that Mtb infection
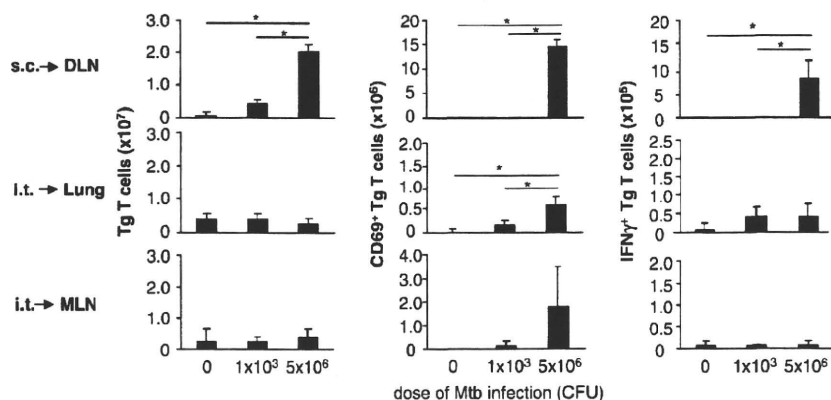


**Fig. 6.** Activation and $T_h1$ differentiation of the P25 TCR-Tg CD4^{+} T cells at an early stage of high-dose Mtb infection. The N-P25 TCR-Tg mice were s.c. or i.t. infected with 1 × 10³ CFU or 5 × 10⁶ CFU of Mtb, and the DLN, lung and MLN were analyzed before or 3 days after the infection. Total number of P25 TCR-Tg CD4^{+} T cells (left panels), and absolute number of CD69^{+} (middle panels) or IFN-γ^{+} (right panels) P25 TCR-Tg CD4^{+} T cells in the DLN after s.c. Mtb infection or in the lung and MLN after i.t. Mtb infection are demonstrated. Results are shown as mean ± SD, and the asterisks indicate significance of difference (*P* < 0.05) compared with the cell number observed on each groups. The data shown are representatives of two independent experiments with 3–5 mice individually analyzed for each experiment.
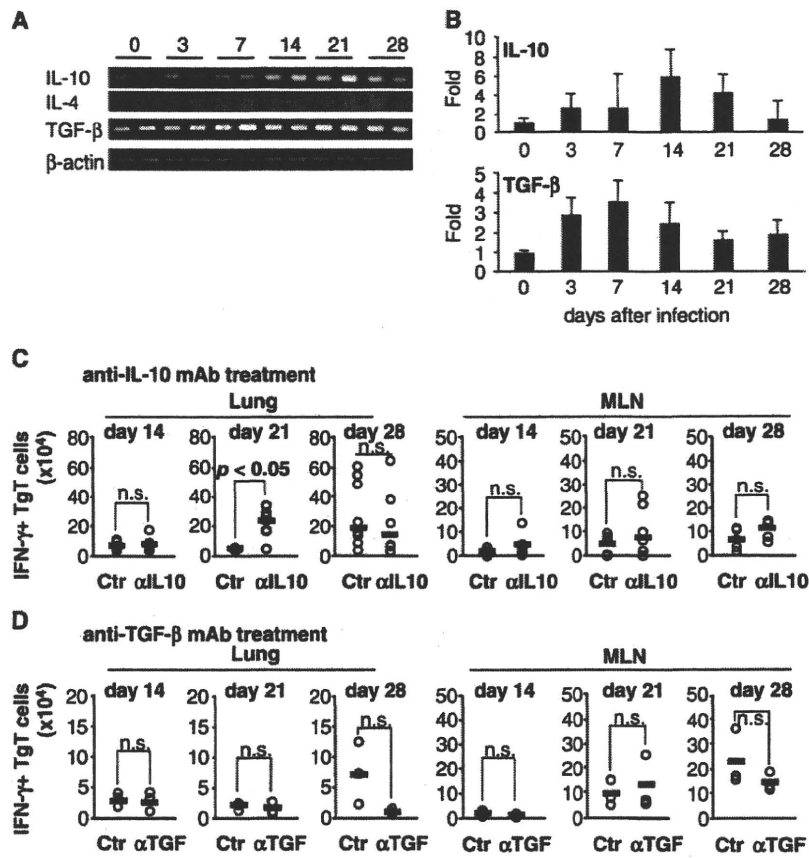
**Fig. 7.** Effects of regulatory cytokines on the induction of $T_h1$ response of the P25 TCR-Tg CD4+ T cells after low-dose Mtb lung infection. (A) Transcripts for TGF-β, IL-10, IL-4 and β-actin in the Mtb-infected lung of the wild-type mice were analyzed by electrophoresis of RT–PCR products and ethidium 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 $1 \times 10^3$ 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 $1 \times 10^3$ 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.

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## Disclosures

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

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## 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-$\gamma$ and tumor necrosis factor (TNF)-$\alpha$ 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-$\gamma$ and TNF-$\alpha$ even at 12 weeks after infection. These results indicate that the PD-1–PD-L1 pathway impairs $T_h$1 immunity in the late stage of BCG infection, thereby facilitating the bacterial persistence in the host.

*Keywords*: Mycobacterium, PD-1, persistent infection, $T_h$1 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_h$1-type CD4$^+$ T cells with the aid of other types of T cells (7–11). $T_h$1 cells produce IFN-$\gamma$ and tumor necrosis factor (TNF)-$\alpha$ 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$_h$1 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-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-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$_4$Cl/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
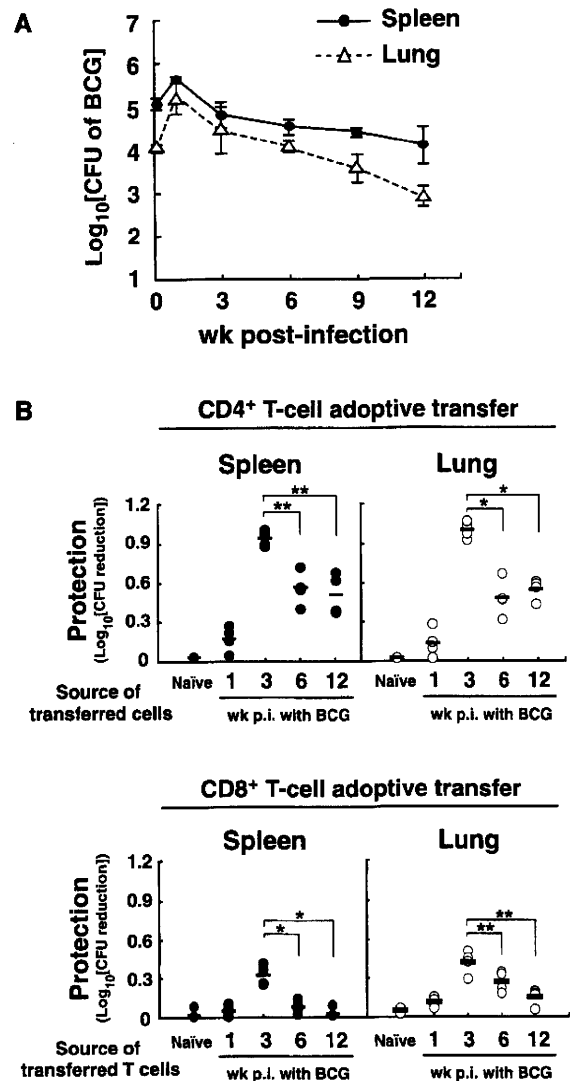


**Fig. 1.** Kinetics of the bacterial numbers in organs after BCG infection and the protective efficacy of T cells obtained from BCG-infected mice against Mtb infection. (A) WT mice were infected i.v. with 10$^6$ CFU of BCG. Bacterial numbers in the spleens and lungs were determined. Data represent the 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-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}
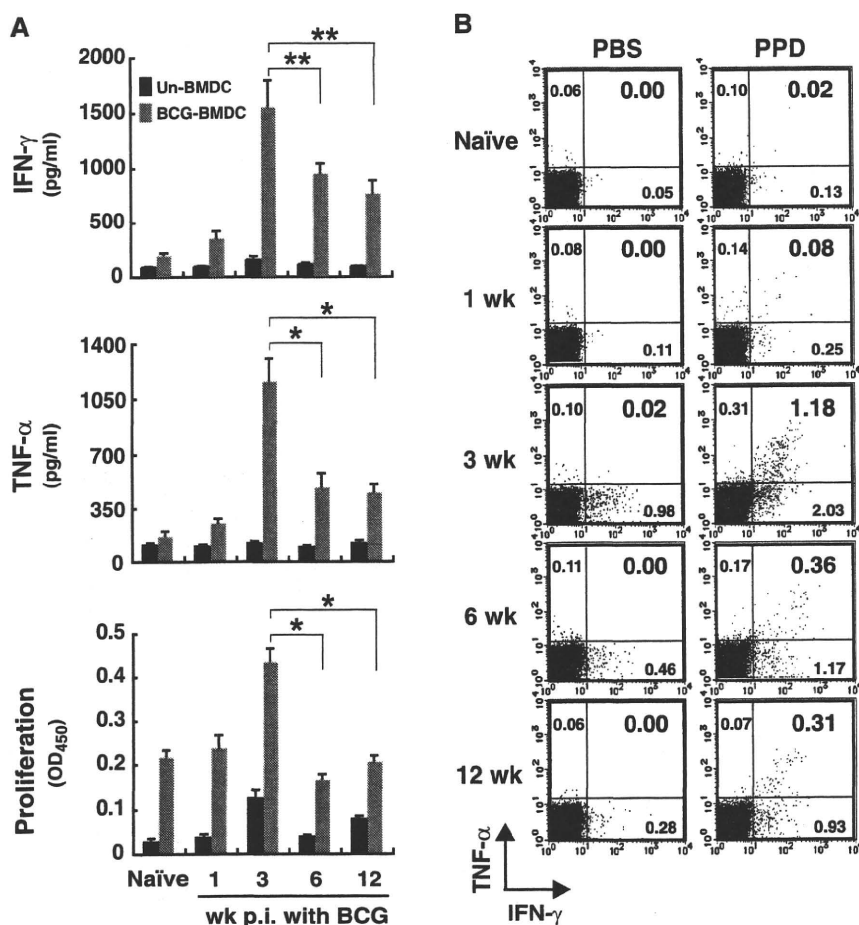
**Fig. 2.** Antigen-specific responses of CD4⁺ T cells obtained from BCG-infected mice. (A) CD4⁺ T cells were isolated from spleens at the indicated weeks post-infection (wk p.i.) with BCG and cultured with unpulsed BMDC (black bars) or BCG-pulsed BMDC (gray bars) for 48 h. The culture supernatants were 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
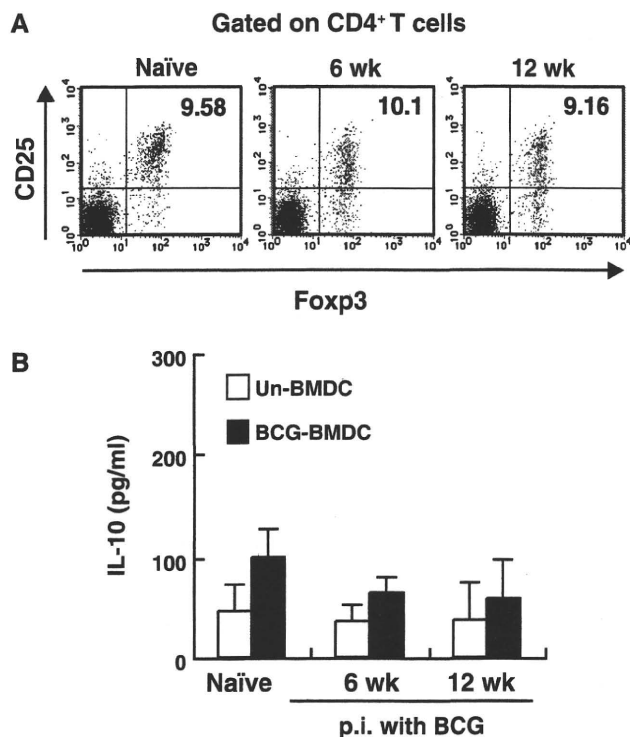
**A**

**Gated on CD4⁺ T cells**



**B**



**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 Th1 response during BCG infection *in vivo*. We found that the ability of WT CD4⁺ T cells to mediate protection and produce Th1 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
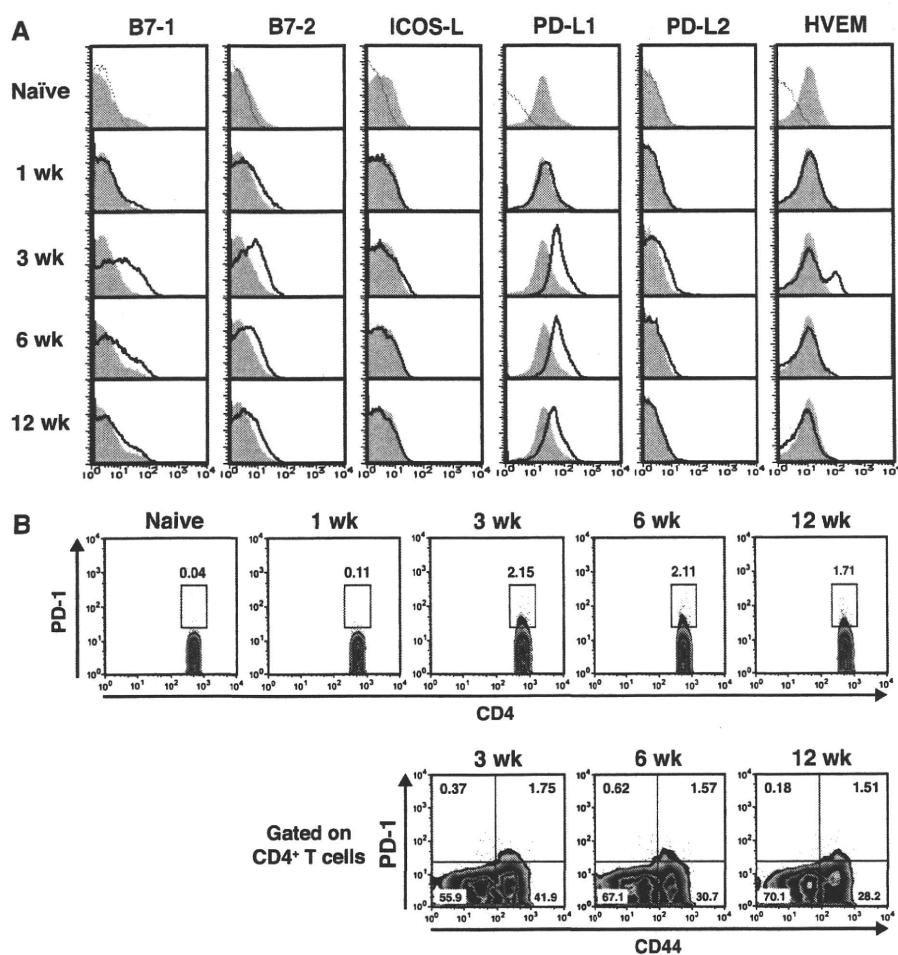
**Fig. 4.** Kinetics of the expression of co-stimulatory and co-inhibitory molecules on APC and PD-1 on CD4⁺ T cells after BCG infection. Splenocytes were prepared from BCG-infected mice at the indicated times after infection. (A) Expression level of each molecule on APC (I-A$^{high}$ cells) was analyzed 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$_h$1 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-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-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
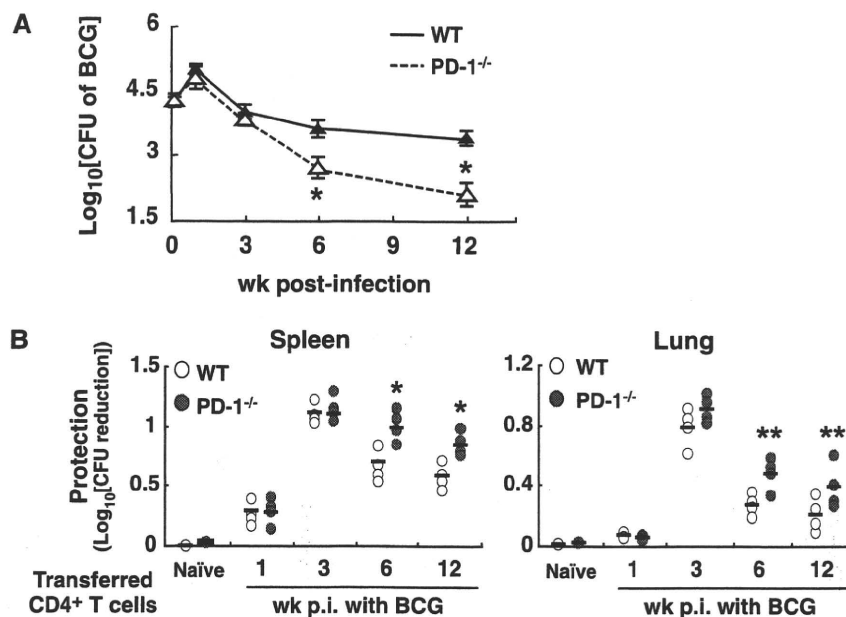
**Fig. 5.** Comparison of protective immunity to BCG between WT and PD-1$^{-/-}$ mice. (A) WT and PD-1$^{-/-}$ mice were infected with $10^6$ CFU of BCG. Bacterial numbers in spleens were evaluated at the indicated time points. Data are the mean ± SD of CFU from four mice per each time point. (B) After 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$_h$1 response during BCG infection. However, we did not observe a significant increase in the population of Treg cells or IL-10-producing Tr1 cells in the late stage of infection. Previous studies have shown that *in vivo* depletion of Treg cells does not affect protective immunity to BCG (37). Furthermore, bacterial clearance has been observed similarly in C57BL/6 WT and IL-10$^{-/-}$ 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$_h$3) cells have been postulated to suppress the immune response to Mtb infection (56). However, we were unable to detect a significant increase in TGF-β1 production by CD4$^+$ 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 coinhibitory 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
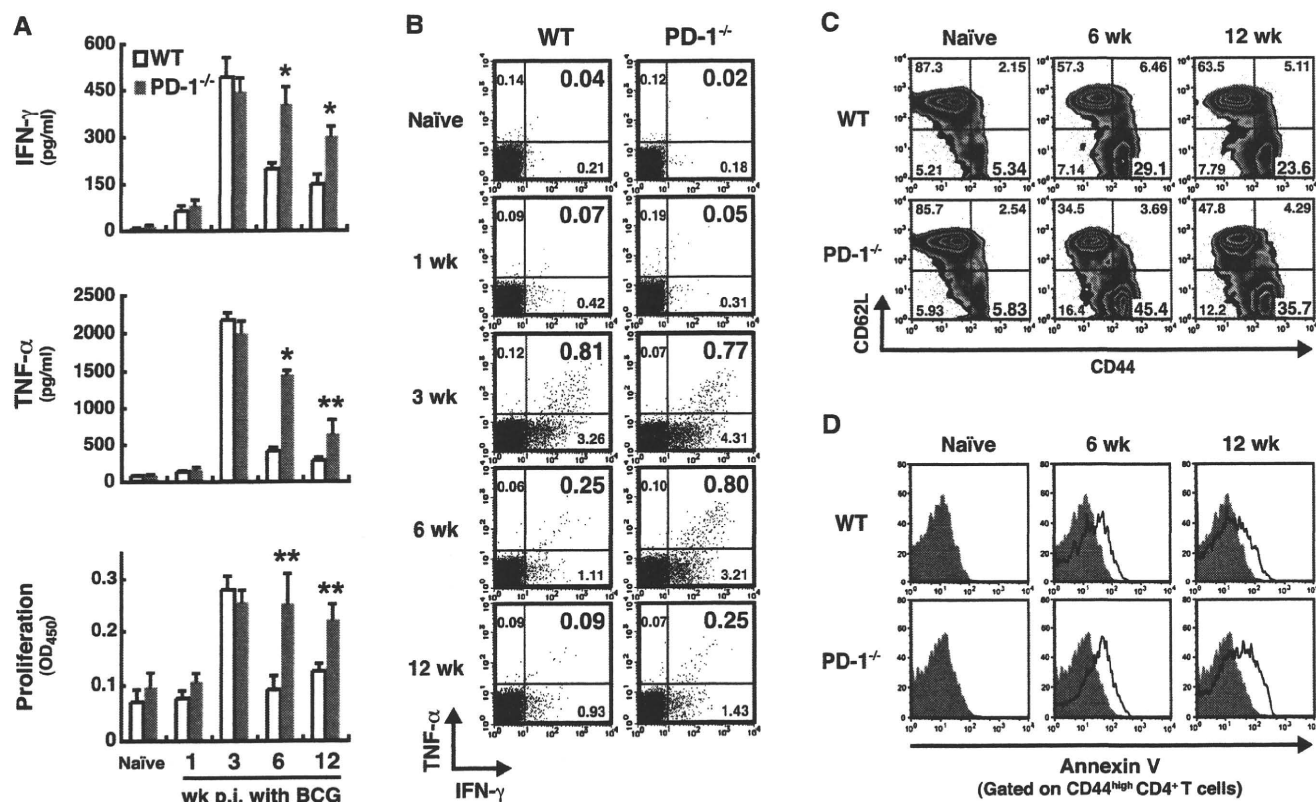
**Fig. 6.** Functional and phenotypic differences of CD4$^+$ T cells obtained from WT and PD-1$^{-/-}$ mice after BCG infection. (A) CD4$^+$ T cells were purified from WT and PD-1$^{-/-}$ mice after BCG infection and cultured with BCG-pulsed BMDC. IFN-$\gamma$ and TNF-$\alpha$ production and proliferation 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-$\gamma$ and TNF-$\alpha$ were analyzed by flow cytometry. Each value indicates the percentage of IFN-$\gamma$ and TNF-$\alpha$-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.

## Disclosure

The authors have no conflicting financial interests.

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