FoxP3 (11, 12). Some subsets of $CD4^+CD25^+$ Treg cells also produce effector cytokines, such as IL-10 and transforming growth factor (TGF)-β (13, 14). The defining feature of $CD4^+CD25^+$ Treg cells is their ability to inhibit the proliferation of T cells and IFN-gamma production through cell–cell contact (15, 16), possibly mediated by CTLA-4, and/or through the production of immunosuppressive cytokines, such as TGF-β and IL-10 (13, 14).

Recently, it has been reported that $CD4^+CD25^+$ Treg cells are also involved in suppressive immune responses during several infectious diseases. Depletion of $CD4^+CD25^+$ Treg cells enhances anti-microbial activity against diverse pathogens including the protozoan *Leishmania major* and *Plasmodium yoelii*, viruses such as HIV and Herpes simplex virus and bacteria such as *Helicobacter pylori* (17–21).

In spite of suggested importance of $CD4^+CD25^+$ Treg cells in parasitic pathogens, the knowledge in mycobacterial infection remains controversial (22–25). Kursar *et al.* and Scott-Browne *et al.* showed that Treg cells prevented protective immunity against *M. tuberculosis* infection by utilizing reconstituted and chimeric mice, respectively (22, 25). In contrast, Quinn *et al.* suggested minor role of $CD4^+CD25^+$ in both *Mycobacterium bovis* bacillus Calmette Guérin (BCG)-induced protection and natural mycobacterial infection (23, 24). In order to elucidate the roles of $CD4^+CD25^+$ Treg cells in mycobacterial infection more precisely, we carried out the experiments using Treg-deleted mice by antibody to CD25 molecule and SCID mice reconstituted with T cell subsets. We found that mycobacterial antigen-specific $CD4^+CD25^+$ Treg cells were hardly developed after mycobacterial infection in mice and therefore the function of $CD4^+CD25^+$ Treg cells was limited after the infection was established. Thus, $CD4^+CD25^+$ Treg cells have little impact on the overall course of mycobacterial infection.

Methods

Mice

Specific pathogen-free, female DBA/2 mice aged 6 weeks were purchased from Japan SLC (Shizuoka, Japan). BALB/c and SCID/BALB/c mice were purchased from Japan CLEA (Tokyo, Japan). All mice were maintained under specific pathogen-free conditions in the animal facilities of Osaka City University Graduate School of Medicine and in a biosafety-level-3 facility at The Research Institute of Tuberculosis according to the standard guidelines for animal experiments at each institute with approval of their ethical committees.

Depletion of $CD4^+CD25^+$ cells

A hybridoma cell line expressing anti-mouse CD25 monoclonal IgM [a monoclonal antibody against mouse CD25 (7D4), American Type Culture Collection, Manassas, VA, USA] was expanded as ascites in pristine-primed SCID mice (Wako, Osaka, Japan). The Ig-rich fraction was obtained by 30% ammonium sulfate precipitation of ascitic fluid followed by dialysis in PBS. An isotype-matched control IgM was purchased from eBioscience (San Diego, CA, USA). The protein concentration was determined by Bradford's method using BSA (Sigma–Aldrich, St Louis, MO, USA) as a standard. For depletion of $CD25^+$ cells in early stage of infection, mice were injected with 1 mg of 7D4 or control IgM intraperitoneally (i.p.) 1 day before, and then 3 and 10 days after *M. tuberculosis* infection. For depletion of $CD25^+$ cells in late stage of infection, mice were injected (i.p.) with 1 mg of 7D4 or control IgM at 60, 65 and 70 days after *M. tuberculosis* infection. Depletion of $CD4^+CD25^+$ cells was assessed by flow cytometry using a FACScan (Becton Dickinson, Franklin Lakes, NJ, USA). Peripheral blood leukocytes (PBLs) were obtained by incubation with 0.83% ammonium chloride solution at 37°C for 5 min to induce erythrocyte lysis. PBLs or splenocytes were stained with PE-conjugated anti-CD4 mAb (GK1.5, eBioscience) and FITC-conjugated anti-CD25 mAb (PC61, eBioscience). The data were analyzed by flow cytometry with using Cellquest™ software (Becton Dickinson).

Bacteria and infection

Mycobacterium bovis BCG Tokyo, *M. tuberculosis* H37Rv, *M. tuberculosis* Kurono (ATCC 35812) and *M. tuberculosis* Erdman were grown in 7H9 medium (Difco, Detroit, MI, USA) supplemented with 10% BSA, dextrose and catalase enrichment (Difco) and 0.05% Tween 80 at 37°C to mid-logarithmic phase, then stored in frozen aliquots as previously described (26). For infection with *M. tuberculosis* Kurono and *M. tuberculosis* Erdman, the nebulizer of a Middlebrook airborne infection apparatus (Glas-col, Terre Haute, IN, USA) was filled with 5 ml PBS containing 5×10^6 colony-forming units (CFU) of bacteria and the mice were airborne infected for 90 min by Glas-Col aerosol generator. This procedure deposits ~10 CFU of bacteria into the lungs. At 0, 3 and 5 weeks (and also 2 weeks in some experiments) post-infection, three to five mice per group were euthanized and the lungs, livers and spleens were harvested. The organs were homogenized in 1 ml sterile distilled water using a mortar and pestle and serial dilutions were plated onto Middlebrook 7H11 agar containing oleic acid, dextrose, albumin and catalase enrichment (Difco) (7H11-OADC agar). Bacterial numbers were counted using CFU after culturing at 37°C for 20–30 days. To investigate the role of $CD4^+CD25^+$ Treg cells in the late stage of infection, mice were airborne-infected with *M. tuberculosis* H37Rv as the same method described above and $CD25^+$ cells were depleted by 7D4 treatment on days 60, 65, and 70. At 75 days post-infection, eight mice per group were euthanized and bacterial numbers in lungs and spleens were determined by CFU count and histological evaluation were performed as the same procedures described above.

Isolation of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells

BALB/c mice were infected i.p. with 5×10^4 CFU of *M. bovis* BCG Tokyo. $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were purified from spleens of normal mice or chronically BCG-infected (>6 months post-infection) BALB/c mice using $CD4^+CD25^+$ regulatory T cell isolation kit (Miltenyl Biotec, Bergisch Gladgach, Germany) after depleting erythrocytes with 0.83% ammonium chloride solution. Obtained cells were labeled with PE-conjugated anti-CD25 mAb, stained with FITC-conjugated anti-CD4 mAb (eBioscience) and analyzed

by flow cytometer. The purity of selected populations was confirmed as >96%. Expression of foxp3 in the $CD4^+CD25^+$ T cell population was confirmed using flow cytometer after intracellular staining with anti-FITC-conjugated anti-mouse foxp3 mAb (eBioscience). $CD4^+CD25^+$ T cells stained by this procedure were >90% foxp3-positive. Non-$CD4^+$ cells of normal mice retained in the MACS separation column were flushed out and incubated for >2 h. Attached cells were used as antigen-presenting cells (APCs) after treatment with 20-Gy radiation. T cell populations and APCs were also isolated from DBA/2 mice chronically infected with *M. tuberculosis* as the similar procedure with BCG-infected mice described above. However, in this case, to obtain APCs, spleen cells of normal DBA/2 mice were incubated for >2 h and attached cells were treated with mitomycin C (50 μg zml^{-1}) for 30 min at 37°C instead of radiation.

In vitro T cell proliferation assay and measurement of cytokines

$CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were prepared to be 1×10^6 cells ml^{-1}. Various ratios of $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were cultured for 5 or 7 days with 10 μg ml^{-1} of purified protein derivative of tuberculin (PPD) or anti-CD3 mAb (CEDARLANE, Canada) in the presence of 1×10^5 cells ml^{-1} of APC in 96-well plates in RPMI 1640 supplemented with 10% FCS, 2 mM L-glutamine, penicillin (100 U ml^{-1}), streptomycin (100 mg ml^{-1}) and 50 mM 2-mercaptoethanol. Proliferation was evaluated by pulsing cells with 1 μCi (37 kBq) per well [^{3}H]thymidine ([^{3}H]TdR) for 6 h and [^{3}H]TdR incorporation measured using a scintillation counter. In the experiments to analyze the function of T cells derived from *M. tuberculosis*-infected mice, proliferation was evaluated by incorporation of 5-bromo-2′-deoxyuridine (BrdU) using a commercially available kit (Cell proliferation ELISA, BrdU colorimetoric, Roche, Germany). Production of IFN-gamma, IL-10, IL-2 and IL-6 in the culture supernatant was measured using a commercially available ELISA kit (R&D System, Minneapolis, MN, USA).

Transfer of T cell population into SCID mice

$CD4^+CD25^+$ T cells and $CD4^+CD25^-$ T cells were purified from the spleens of BALB/c mice chronically infected with BCG using $CD4^+CD25^+$ Treg cell isolation kit. Totally, 7.5×10^5 $CD4^+CD25^-$ T cells or 7.5×10^5 $CD4^+CD25^+$ T cells either alone or in combination (7.5×10^5 $CD4^+CD25^-$ T cells and 7.5×10^4 $CD4^+CD25^+$ T cells) were transferred intravenously to cognate SCID mice (17). One day after transfer, recipient mice were infected aerogenically with *M. tuberculosis* Erdman as described above. Three weeks post-infection, five to eight mice were euthanized and bacterial burden was counted. The survival time course of seven mice per group was observed for up to 165 days post-infection.

Neutralization of IL-6 in culture supernatant

$CD4^+CD25^-$ T cells, $CD4^+CD25^+$ T cells and APCs were isolated from chronically BCG-infected mice according to the procedures described above. Anti-mouse IL-6-neutralizing mAb (Biolegend, San Diego, CA, USA) or isotype-matched control antibody (Southern Biotech, Birmingham, AL, USA) was added to the culture of $CD4^+CD25^-$ effector T cells either alone or in combination with $CD4^+CD25^+$ T cells at concentration of 0.02 μg ml^{-1} and cultured for 4 days in the presence of PPD or anti-CD3 mAb. IFN-gamma production and [^{3}H]TdR incorporation were measured after 4 days incubation.

Transfer of culture medium

T cell subsets were obtained from chronically BCG-infected mice as described above. $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ T cells/APCs (1:0:0.1) or $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ T cells/APCs (1:1:0.1) were cultured with PPD or anti-CD3 mAb for 7 days and each culture supernatant stored at −80°C until later use. Freshly isolated $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ cells/APCs (1:0:0.1) or $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ T cells/APCs (1:1:0.1) were cultured with stored supernatant:new medium (1:1) in the presence or absence of anti-CD3 mAb. On day 4, [^{3}H]TdR incorporation was measured as described above.

In vitro activation of $CD4^+CD25^+$ T cells

$CD4^+CD25^+$ T cells isolated from chronically infected BALB/c mice with BCG or *M. tuberculosis* H37Rv were incubated with Dynabeads Mouse CD3/CD28 T cell Expander (Invitrogen, Norway) at a bead:cell ratio of 2:1 adding 2000 U ml^{-1} of recombinant mouse IL-2 according to the manufacturer's protocol. Two days after incubation, the beads were

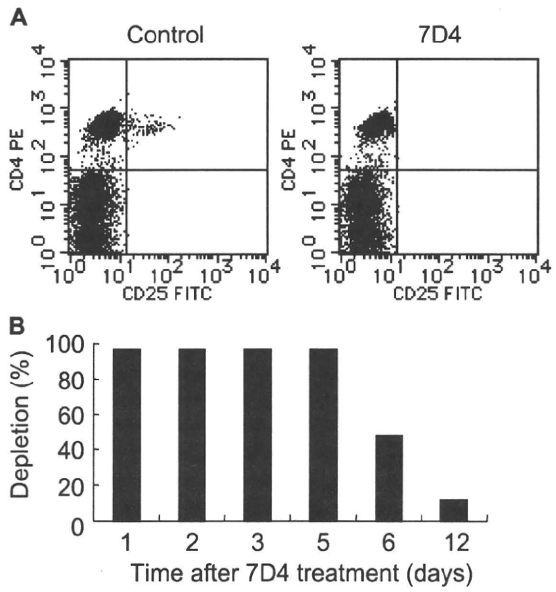

Fig. 1. Selective loss of $CD4^+CD25^+$ T cells by treatment with anti-CD25 mAb, 7D4. (A) Flow cytometric analysis of PBLs obtained from i.p.-injected mice with 1 mg of anti-CD25 mAb (7D4) or control IgM (control) 1 day after injection. Cells were stained with FITC-conjugated anti-CD25 mAb (PC61) and PE-conjugated anti-CD4 mAb (GK1.5). (B) Time course of the level of depletion of $CD4^+CD25^+$ T cell in PBL after a single dose of 7D4. Data are expressed as percent depleted relative to the $CD4^+CD25^+$ cell population in control IgM-treated mice. Data are mean of three mice per time point.

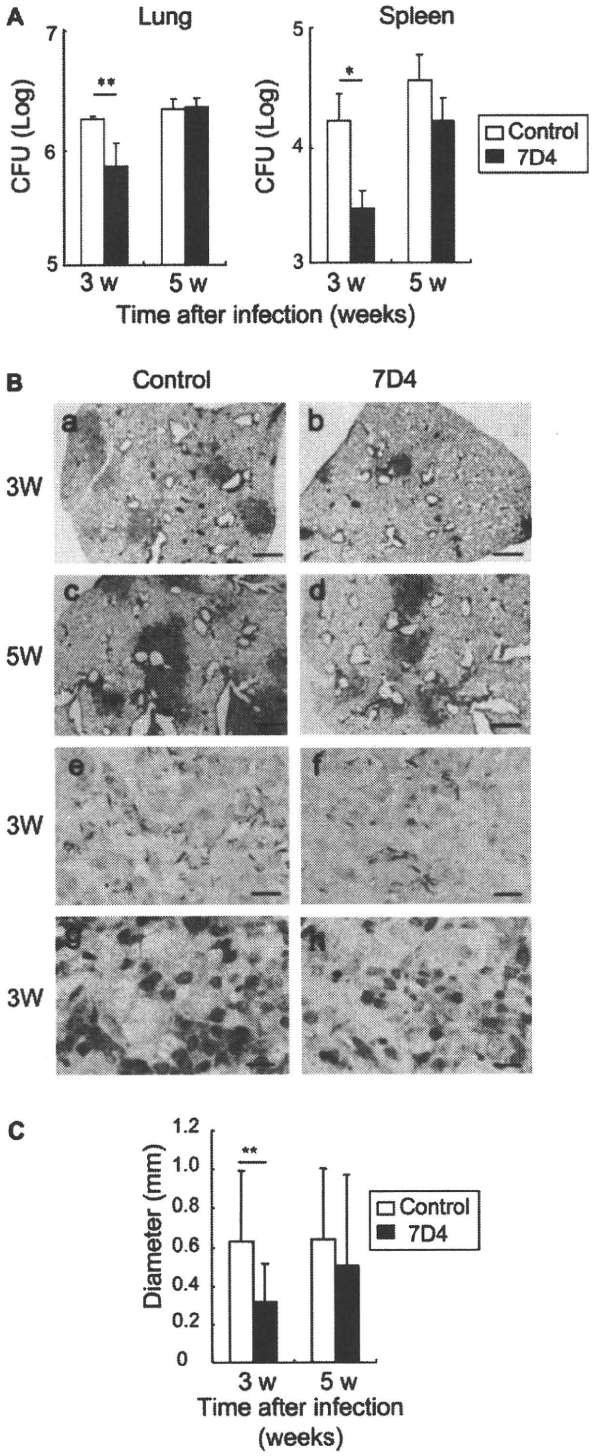

Fig. 2. The effect of $CD25^+$ cell depletion in *Mycobacterium tuberculosis* Kurono infection in mice. DBA/2 mice treated with 1 mg of 7D4, anti-CD25 mAb or control IgM were aerogenically infected with 5×10^6 CFU of *M. tuberculosis* Kurono. (A) Bacterial numbers were counted in lungs (left panel) and spleens (right panel) of mice treated with control IgM (open bars) or with 7D4, anti-CD25 mAb (closed bars). (B) Histopathological features of lungs from *M. tuberculosis* Kurono-infected mice. Lung sections were stained with H&E (a–d), Ziehl–Neelsen (e and f) and Giemsa (g and h). Granulomas mainly consisted of epithelioid macrophages (g and h). Numerous acid-fast bacteria were observed in granulomas of both control IgM-treated and anti-CD25 mAb-treated (7D4) mice. (a, c, e and g) Lungs sections from mice treated with control IgM. (b, d, f and h) Lungs sections from mice treated with 7D4. (a, b and e–h) Three weeks after infection. (c and d) Five weeks after infection. Bars, 500 μm (a–d), 10 μm (e-h). (C) The diameter of granulomatous lesions was measured in the lung sections from mice treated with control IgM (open bars) or with 7D4, anti-CD25 mAb (closed bars). Bars represent mean ±standard deviation of three to five mice. $*P < 0.05$ versus control mice; $**P < 0.01$ versus control mice.

removed from $CD4^+CD25^+$ T cells by magnet. After washing with medium, cells were used as activated $CD4^+CD25^+$ T cells. Freshly isolated $CD4^+CD25^-$ effector T cells were incubated with activated $CD4^+CD25^+$ T cells in the presence of PPD.

Histological analysis

Tissues were removed from mice at various intervals, fixed in 10% formalin and embedded in paraffin blocks. Sections (5 μm) were stained with hematoxylin and eosin (H&E), Ziehl–Neelsen or Giemsa methods. To evaluate the intensity of inflammatory response of the lung, the mean diameters of pulmonary granulomas were measured in three sections per mouse using Microanalyzer (Poladigital, Tokyo, Japan).

Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) by SAS system R.8.1. Data were expressed as mean values ± standard deviation and considered significant if $P < 0.05$.

Results

Depletion of $CD25^+$ cells in early stage of infection causes transient effect on in vivo growth of M. tuberculosis Kurono and M. tuberculosis Erdman

7D4 is a mAb against mouse CD25. Administration of 1 mg 7D4 into a mouse resulted in the loss of >96% of $CD4^+CD25^+$ T cells in the peripheral blood and spleens (Fig. 1A). Loss of $CD25^+$ cells maintained at least for 5 days after 7D4 treatment (Fig. 1B). Depletion of $CD25^+$ cells by 7D4 protects mice from death caused by infection of *Plasmodium yoelii*, suggesting a role for $CD4^+CD25^+$ Treg cells in exacerbating malaria (21).

Using 7D4, we first depleted $CD25^+$ cells of DBA/2 mice and then infected animals with 5×10^6 CFU/mouse of *M. tuberculosis* Kurono, which was clinically isolated strain in Japan, by airborne infection. Bacterial load in lung and spleen, and histopathology of the lung were monitored at 3 and 5 weeks post-infection. *Mycobacterium tuberculosis* Kurono multiplied to approximately 2×10^6 CFU per lung 3 weeks post-challenge and maintained these bacterial numbers 5 weeks post-challenge. In spleens, we detected 2×10^4 and 3×10^4 CFU per organ 3 and 5 weeks

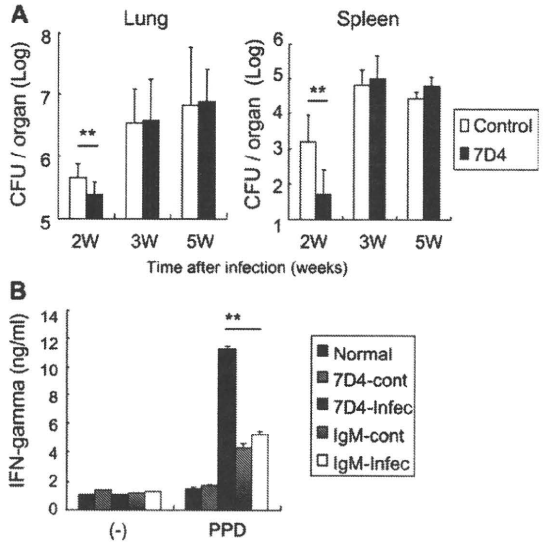

Fig. 3. The effect of $CD25^+$ cell depletion in *Mycobacterium tuberculosis* Erdman infection. (A) Control IgM-treated mice (open bars) and 7D4 anti-CD25 antibody-treated-mice (closed bars) were aerogenically infected with 5×10^6 CFU of *M. tuberculosis* Erdman. Bacterial numbers of lungs and spleens were measured at 2, 3 and 5 weeks post-infection. $^{**}P < 0.01$ versus control mice. (B) $CD4^+$ T cells from non-infected mice with 7D4 treatment (7D4-cont), *M. tuberculosis*-infected mice with 7D4 treatment (7D4-Infec), non-infected mice with control IgM treatment (IgM-cont) or *M. tuberculosis*-infected mice with control IgM treatment (IgM-Infec) were cultured with APC in the presence (PPD) or absence (−) of 10 μg ml^{-1} PPD for 7 days. Production of IFN-gamma in the culture supernatants was analyzed. $^{**}P < 0.01$: *Mycobacterium tuberculosis*-infected mice with control IgM treatment (IgM-Infec) versus *M. tuberculosis*-infected mice with 7D4 treatment (7D4-Infec).

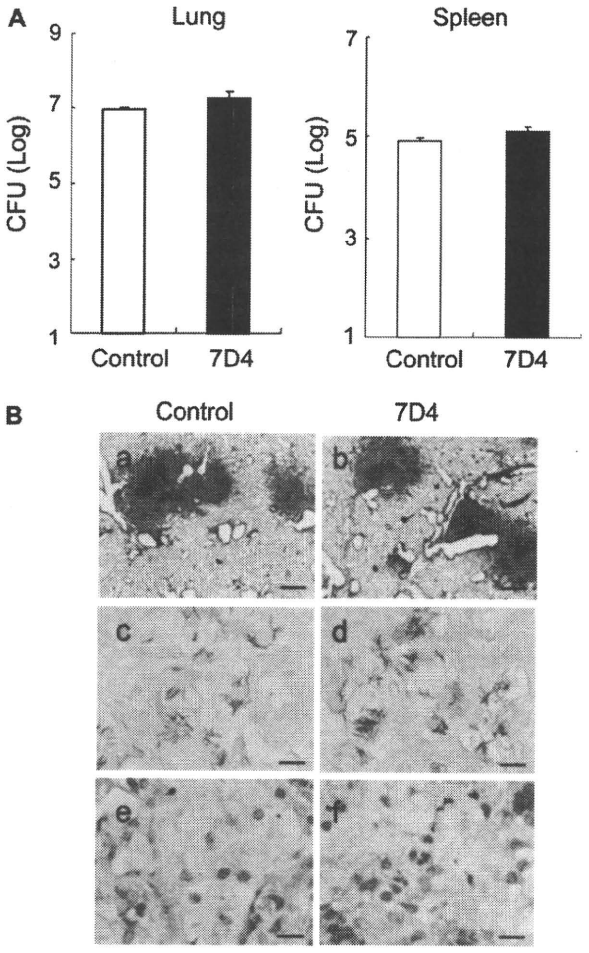

Fig. 4. The effect of depletion of $CD25^+$ cell in chronically infected mice with *M. tuberculosis*. DBA/2 mice were aerogenically infected with 5×10^6 CFU of *M. tuberculosis* H37Rv. Two months after infection, mice were treated with 1 mg of 7D4 or control IgM three times with 4 days interval. Five days later from final treatment, mice were sacrificed and analyzed. (A) Bacterial numbers in lungs (left panel) and spleens (right panel) of mice treated with control IgM (open bars) or 7D4 (closed bars). (B) Lung sections were stained with H&E (a and b), Ziehl–Neelsen (c and d) and Giemsa (e and f). Granulomas mainly consisted epithelioid macrophages (e and f). Numerous acid-fast bacteria were observed in granulomas of both control IgM- and 7D4-treated mice. (a, c and e) Lungs sections from mice treated with control IgM. (b, d and f) Lungs sections from mice treated with 7D4. Bars, 500 μm (a and b), 10 μm (c–f). Bars represent mean ±standard deviation of eight mice.

post-challenge, respectively (Fig. 2A). Depletion of $CD25^+$ cells resulted in significantly lower bacterial number in both lung and spleen 3 weeks after challenge; however, this effect became marginal 5 weeks post-challenge (Fig. 2A). Numerous bacteria were observed in granulomas of 7D4-treated and control mice after infection (Fig. 2B, e and f), consistent with higher bacterial burdens revealed by plating of organ homogenates (Fig. 2A). Histological examination of the lung correlated with the CFU results; 3 weeks post-infection, depletion of $CD25^+$ cells resulted in decreased granuloma formation compared with mice treated with control IgM, but normalized 5 weeks after challenge [Fig. 2B(a–d) and C]. Histopathology showed that granuloma cellular composition did not differ between 7D4-treated mice and control mice, which consisted predominately of epithelioid macrophages (Fig. 2B, g and h). Thus in *M. tuberculosis* Kurono infection, the effect of $CD25^+$ cell depletion was limited to the early phase of infection only.

To determine whether the transient effect of $CD25^+$ cells is specific for *M. tuberculosis* Kurono, we performed similar experiments employing another commonly used mycobacterial strain, *M. tuberculosis* Erdman. Similar results were observed in bacterial burdens: 7D4-treated mice revealed significantly lower bacterial numbers than those of IgM-treated mice at early stage (2 weeks post-infection), but not 3 weeks or 5 weeks post-challenge (Fig. 3A). Splenic $CD4^+$ T cells derived from 7D4-treated mice at this time point produced significantly higher levels of IFN-gamma than those of IgM-treated mice when stimulated with PPD (Fig. 3B).

We also examined the effects of depletion of $CD25^+$ cells on the survival of another mycobacterial strain, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG). DBA/2 or BALB/c mice depleted of $CD25^+$ cells by 7D4 were challenged with BCG intravenously and the survival of BCG in the lungs

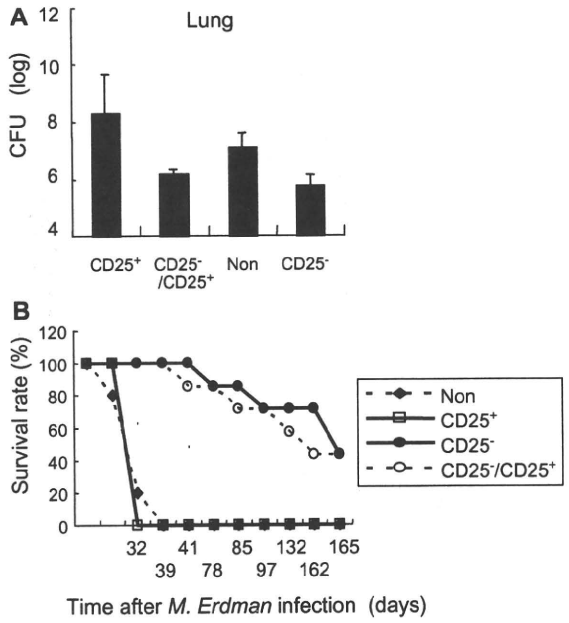

Fig. 5. Bacterial burden and survival kinetics of reconstituted SCID mice with T cell subsets after infection of *Mycobacterium tuberculosis* Erdman. (A) T cell subsets were isolated from spleens of chronically BCG-infected mice. SCID mice were reconstituted with 7.5×10^5 of $CD4^+CD25^+$ T cells only ($CD25^+$), 7.5×10^5 of $CD4^+CD25^-$ T cells and 7.5×10^4 of $CD4^+CD25^+$ T cells ($CD25^-/CD25^+$), untransferred (Non), and 7.5×10^5 of $CD4^+CD25^-$ T cells only ($CD25^-$). One day after reconstitution, naive or T cell subset-reconstituted SCID mice were aerogenically infected with 5×10^6 CFU *M. tuberculosis* Erdman. (B) Survival rates of naive or reconstituted SCID mice after infection. Time course of survival was examined up to 165 days post-infection. Five to eight mice per group were analyzed.

post-challenge was monitored. Unlike *M. tuberculosis*, there was no marked increase in BCG levels in the mouse lungs. Depletion of $CD25^+$ cells did not alter the survival ratio of BCG in the lungs of DBA/2 and BALB/c mice 3 and 5 weeks post-infection, although *in vitro* stimulation with PPD, lymphocytes derived from 7D4-treated mice at 3 weeks after challenge produced higher amount of IFN-gamma than those from control IgM-treated mice (data not shown).

Depletion of $CD25^+$ cells in the chronic stage of infection does not affect the bacterial burdens and pathology

We next examined the effects of depletion of $CD25^+$ cells in the late stage of mycobacterial infection. DBA/2 mice were airborne-infected with *M. tuberculosis* H37Rv and $CD25^+$ cells were depleted by 7D4 treatment after 60, 65 and 70 days later. Five days later from the final treatment of 7D4, we analyzed the bacterial burden and histology in the organs. Bacterial numbers of lungs and spleens in 7D4-treated mice were rather slightly higher than those in control IgM-treated mice; however, significant differences were not observed (Fig. 4A). Pulmonary granuloma formation was conspicuous in both 7D4-treated mice and control IgM-treated mice (Fig. 4B, a and b). Cellular composition of granuloma did not differ between 7D4-treated mice and control mice and numerous bacteria were observed in both groups of mice (Fig. 4B, c–f).

$CD4^+CD25^+$ T cells do not suppress protection induced by $CD4^+CD25^-$ T cells against M. tuberculosis infection in reconstituted mice

To further evaluate the role of $CD4^+CD25^+$ Treg cells in mycobacterial infection at late stage (after developing acquired immunity), the following experiment was conducted. $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells were purified from chronically BCG-infected mice: >90% of the $CD4^+CD25^+$ T cells obtained expressed FoxP3 as estimated by FACScan (data not shown). Each T cell subset, either alone or in combination, was then transferred into SCID mice and mice were infected with *M. tuberculosis* Erdman by airborne exposure. Three weeks post-infection, the bacterial number in lungs (Fig. 5A) and survival kinetics of mice (Fig. 5B) were analyzed.

Observed increases in *M. tuberculosis* were similar in both naive SCID mice and SCID mice reconstituted with $CD4^+CD25^+$ T cells alone, suggesting that $CD4^+CD25^+$ T cells offer no protection against *M. tuberculosis*. In contrast, SCID mice reconstituted with $CD4^+CD25^-$ T cells controlled *M. tuberculosis* infection, at a similar level to that of mice reconstituted with the combination of $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells (Fig. 5A). The survival kinetics showed similar outcomes between mice reconstituted with $CD4^+CD25^-$ T cells plus $CD4^+CD25^+$ T cells and $CD4^+CD25^-$ effector T cells alone (Fig. 5B). These data suggest that the role of $CD4^+CD25^+$ Treg cells in host protection is marginal against *M. tuberculosis* in the overall course of infection (Fig. 5B).

Stimulation with mycobacterial antigens fails to express the function of $CD4^+CD25^+$ Treg cells in vitro

To ascertain why $CD4^+CD25^+$ Treg cells have only a minor role in the late stage of mycobacterial infection, we compared the action of $CD4^+CD25^+$ T cells to $CD4^+CD25^-$ T cells *in vitro*. $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells were isolated from normal mice or mice chronically infected with BCG or *M. tuberculosis* and stimulated with PPD or anti-CD3 mAb in the presence of APCs. $CD4^+CD25^+$ T cells alone showed characteristics of Treg cells, which neither proliferate nor produce cytokines in response to neither PPD nor anti-CD3 mAb (Fig. 6, A–J and a–e). Culture experiments using a combination of $CD4^+CD25^+$ and $CD4^+CD25^-$ T cells showed that $CD4^+CD25^+$ T cells derived from both normal and infected mice suppressed proliferation of $CD4^+CD25^-$ T cells and production of cytokines, such as IFN-gamma and IL-10, in a dose-dependent manner following stimulation with anti-CD3 mAb, showing the characteristics in Treg cells. However, following stimulation with PPD, $CD4^+CD25^+$ T cells failed to suppress both proliferation and production of cytokines. In contrast, IL-2 production was suppressed in a dose-dependent manner in the presence of PPD. Definitive IL-6 production was observed when $CD4^+CD25^-$ T cells were incubated alone or combination with $CD4^+CD25^+$ T cells in the presence of PPD (Fig. 6, I, J and e).

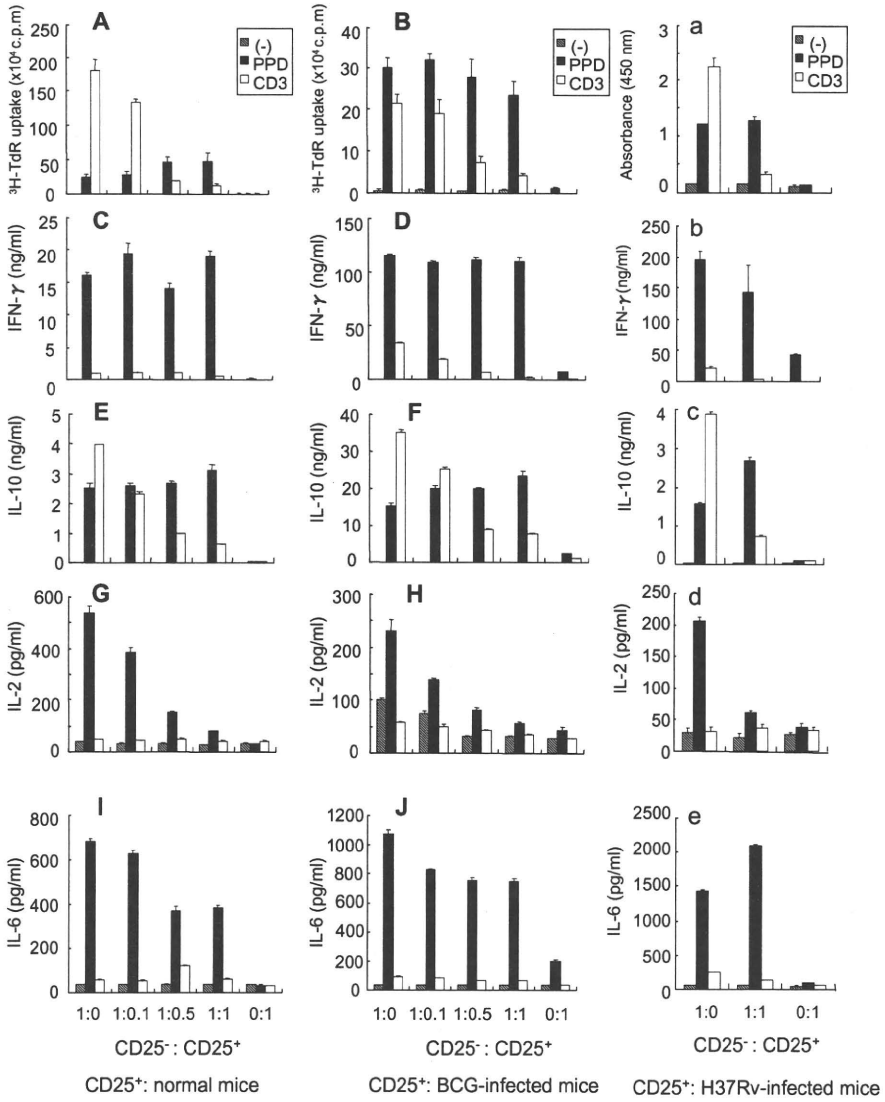

Fig. 6. Stimulation of CD4$^+$CD25$^+$ T cells by PPD fails to suppress the function of PPD-activated CD4$^+$CD25$^-$ T cells. CD4$^+$CD25$^+$ T cells (CD25$^+$) purified from normal (A, C, E, G and I) mice or mice chronically infected with BCG (B, D, F, H and J) or *Mycobacterium tuberculosis* (a–e) were co-cultured with CD4$^+$CD25$^-$ effector T cells (CD25$^-$) purified from mice chronically infected with BCG or *M. tuberculosis* at various ratios with T cell-depleted irradiated spleen cells (APCs) in the presence of PPD (PPD), or anti-CD3 mAb (CD3), or alone (–). Proliferative responses were analyzed at day 5 (A, B and a). Cytokine production in culture supernatants was measured at day 7 (C, E, I, J and b–e) or day 5 (D, F, G and H).

Soluble mediators are not suppressive factors of CD4$^+$CD25$^+$ Treg cell function when stimulated with PPD

Because IL-6 allows effector T cells to overcome suppression by CD4$^+$CD25$^+$ Treg cells (27), we considered the possibility that IL-6 inhibits the function of CD4$^+$CD25$^+$ Treg cells when stimulated with *M. tuberculosis*-derived mycobacterial antigen, PPD. Therefore, we neutralized IL-6 by neutralizing mAb; however, neutralization of IL-6 did not recover suppressive activity of CD4$^+$CD25$^+$ Treg cells (Fig. 7A and B).

To determine whether soluble factors beside IL-6 abrogate the suppressive function of CD4$^+$CD25$^+$ Treg cells upon PPD stimulation, we examined the effects of soluble factors released from T cells and APCs. The culture supernatants from CD4$^+$CD25$^+$ T cells cultured with both CD4$^+$CD25$^-$ T cells and APCs in the presence of PPD or anti-CD3 mAb were collected and then transferred to fresh culture of CD4$^+$CD25$^-$ T cells, CD4$^+$CD25$^+$ T cells and APCs in the presence or absence of anti-CD3 mAb. The proliferative response of CD4$^+$CD25$^-$ effector T cells was analyzed by incorporation of [^{3}H]TdR. The results showed that the supernatants of combined CD4$^+$CD25$^-$ and CD4$^+$CD25$^+$ T cell culture failed to diminish suppressive activity of proliferative response of CD4$^+$CD25$^-$ T cells by CD4$^+$CD25$^+$ T cell stimulated with anti-CD3 mAb (Fig. 8). These results show

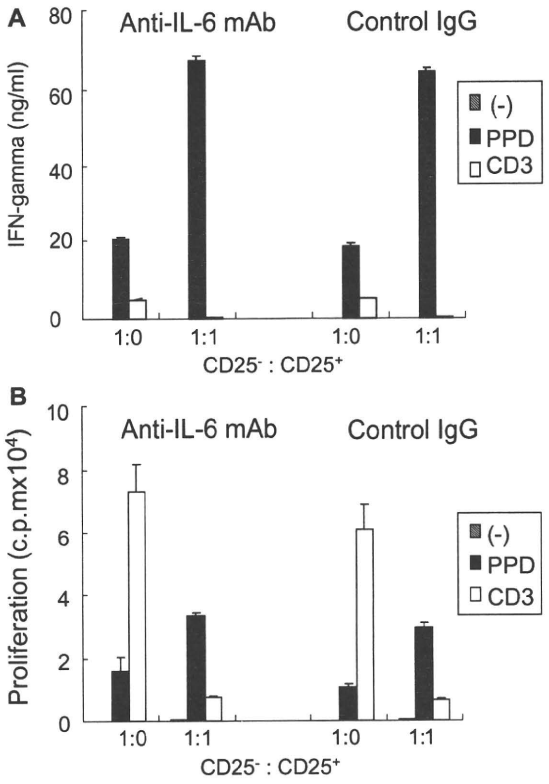

Fig. 7. Neutralization of IL-6 does not affect the $CD4^+CD25^+$ T cell-mediated suppression of the function of effector T cells. $CD4^+CD25^-$ T cells and $CD4^+CD25^+$ T cells were isolated from mice chronically infected with BCG. $CD4^+CD25^-$ effector T cells alone (1:0) or combination of $CD4^+CD25^-$ effector T cells and $CD4^+CD25^+$ T cells (1:1) were cultured with APCs in the presence of PPD (PPD), anti-CD3 mAb (CD3) or absence of these (–). In each well, 0.02 μg ml^{-1} of anti-IL-6 mAb or control IgG were added. IFN-gamma production in culture supernatant (A) and proliferative responses of T cells (B) were analyzed at day 4.

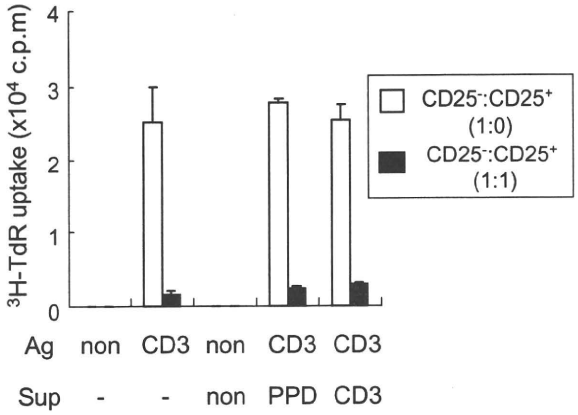

Fig. 8. Soluble mediators upon PPD stimulation do not abrogate $CD4^+CD25^+$ T cell-mediated suppression. $CD4^+CD25^-$ effector T cells ($CD25^-$) and $CD4^+CD25^+$ T cells ($CD25^+$) were isolated from spleens of chronically BCG-infected mice. $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ T cells/APC (1:0:0.1) or $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ cells/APC (1:1:0.1) were cultured with PPD (Sup, PPD), anti-CD3 mAb (Sup, CD3) or alone (Sup, non), for 7 days. Each culture supernatant was stored. Freshly isolated $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ cells/APC (1:0:0.1) or $CD4^+CD25^-$ T cells/$CD4^+CD25^+$ cells/APC (1:1:0.1) were cultured 1:1stored supernatant: fresh culture medium in the presence (Ag, CD3) or absence (Ag, non) of anti-CD3 mAb. Proliferative responses were analyzed at day 4.

that the defective function of $CD4^+CD25^+$ Treg cells following PPD stimulation was not dependent on soluble factors released from T cells and APCs.

Activated $CD4^+CD25^+$ Treg cells suppress the function of PPD-stimulated $CD4^+CD25^-$ effector T cells

Two possibilities could explain the lack of effect of PPD-stimulated $CD4^+CD25^+$ Treg cells on the function of $CD4^+CD25^-$ T cells. First, that activated $CD4^+CD25^+$ Treg cells fail to suppress the function of $CD4^+CD25^-$ T cells by mycobacterial antigens, and second that $CD4^+CD25^+$ Treg cells are not activated by mycobacterial antigens at the late stage of infection. To investigate these possibilities, we purified $CD4^+CD25^+$ T cells from chronically infected mice with BCG or *M. tuberculosis*, then activated *in vitro* with anti-CD3/CD28 mAb-coated beads in the presence of recombinant IL-2. The cells were then cultured with $CD4^+CD25^-$ T cells derived from BCG- or *M. tuberculosis*-infected mice in the presence of PPD. We found that activated $CD4^+CD25^+$ T cells unequivocally suppressed both proliferation and production of IFN-gamma of $CD4^+CD25^-$ T cells stimulated with PPD (Figs 9 and 10). Thus, our data show that both BCG and *M. tuberculosis* infection activate antigen-specific $CD4^+CD25^-$ effector T cells, but not $CD4^+CD25^+$ Treg cells, at the late stage of infection.

Discussion

$CD4^+CD25^+$ Treg cells play a pivotal role in self-tolerance and autoimmune diseases and also in the progression of infectious diseases. It has been shown that $CD4^+CD25^+$ Treg cells are preventive against eradication of persistent pathogens, such as *Leishmania* protozoa, herpes simplex virus and HIV (17–20). Mycobacteria are major parasitic bacteria for eukaryotes (28). In this study, we investigated the role of $CD4^+CD25^+$ Treg cells in mycobacteria infection in mice.

We first studied the effects of Treg cell depletion against infection of mycobacteria. At the early stage of infection, depletion of $CD25^+$ cells significantly suppressed the growth of virulent *M. tuberculosis* strains, such as Kurono and Erdman, suggesting a role for $CD4^+CD25^+$ Treg cells in exacerbation of tuberculosis at the early stage of infection. This is consistent with the previous study performed by Kursar *et al.* (22). This effect of $CD4^+CD25^+$ Treg cells is presumably mediated through naturally occurring $CD4^+CD25^+$ Treg cells, which can be activated through Toll-like receptor (TLR)-mediated signaling (29–32). Mycobacterial DNA [TLR9 ligand (33)] and lipoproteins [TLR2 ligand (34)] may participate in activation of naturally occurring $CD4^+CD25^+$ Treg cells at this stage.

Two to three weeks post-infection, acquired immunity is evident (35). IFN-gamma producing T-helper 1 cells (T_h1) are major effectors in suppressing intracellular survival of

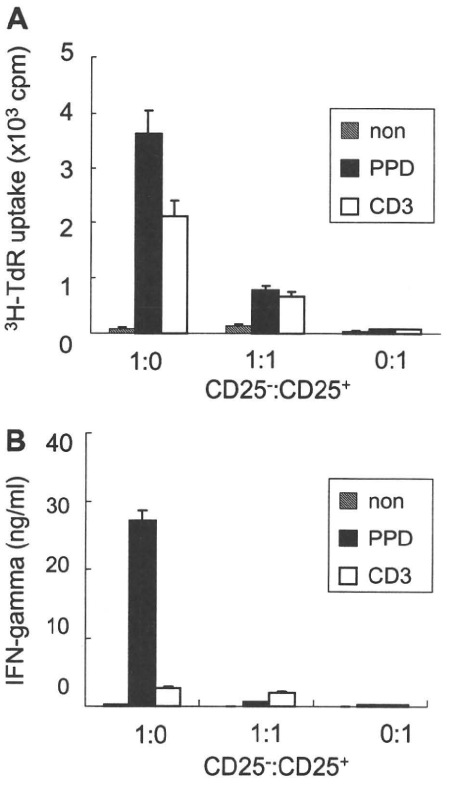

Fig. 9. *In vitro* activation of CD4$^+$CD25$^+$ T cells derived from BCG-infected mice inhibits the function of PPD-stimulated CD4$^+$CD25$^-$ effector T cells. CD4$^+$CD25$^+$ T cells obtained from chronically BCG-infected mice were activated by incubation with anti-CD3/CD28 mAb-coated beads at a bead:cell ratio 2:1 in the presence of 2000 U ml^{-1} of recombinant mouse IL-2 for 48 h. Activated CD4$^+$CD25$^+$ T cells were co-cultured with freshly isolated CD4$^+$CD25$^-$ effector T cells in the presence of PPD (filled column), anti-CD3 mAb (open column) or alone (gray column). (A) Proliferation of CD4$^+$CD25$^-$ effector T cells wase analyzed at day 4. (B) IFN-gamma production in the culture supernatant as measured at day 7.

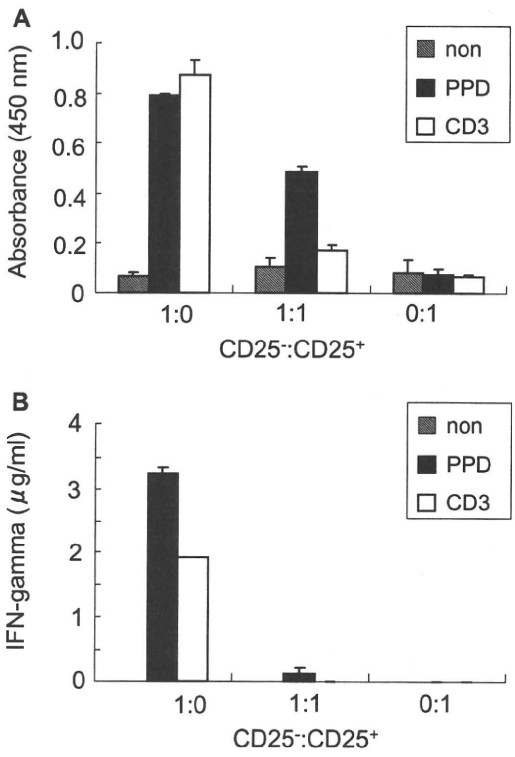

Fig. 10. *In vitro* activation of CD4$^+$CD25$^+$ T cells derived from *Mycobacterium tuberculosis*-infected mice inhibit the function of PPD-stimulated CD4$^+$CD25$^-$ effector T cells. CD4$^+$CD25$^+$ T cells obtained from chronically *M. tuberculosis* H37Rv-infected mice were activated by incubation with anti-CD3/CD28 mAb-coated beads at a bead:cell ratio of 2:1 in the presence of 2000 U ml^{-1} of recombinant mouse IL-2 for 48 h. Activated CD4$^+$CD25$^+$ T cells were co-cultured with freshly isolated CD4$^+$CD25$^-$ effector T cells in the presence of PPD (filled column), anti-CD3 mAb (open column) or alone (gray column). (A) Proliferation of CD4$^+$CD25$^-$ effector T cells was analyzed at day 4. (B) IFN-gamma production in the culture supernatant as measured at day 7.

mycobacteria (36–38). In the CD25$^+$ cell-depletion experiments, the advantages of CD25$^+$ cell depletion were diminished 3 and 5 weeks after the challenge of *M. tuberculosis* Erdman and Kurono, respectively (Figs 2 and 3). We also found that persistence of BCG in mice is not altered by depletion of CD25$^+$ cells by 7D4 (data not shown). These data can be explained by the short-action profile of antibodies. However, we could not find any effect of depletion of CD25$^+$ cells at the chronic stage of infection (Fig. 4), when bacterial numbers were sustained at the same level (39). Similar results were obtained using another anti-CD25 mAb PC6C1, which causes significant reduction of the number of persistent *Leishmania major* in mice by suppressing CD4$^+$CD25$^+$ Treg cells (personal communication with Dr Alan Sher). Furthermore, our *in vivo* experiments in reconstituted SCID mice further suggest that the role of CD4$^+$CD25$^+$ Treg is minimal after infection is established (Fig. 5). The survival kinetics of mice reconstituted with CD4$^+$CD25$^-$ T cells alone are comparable to those in mice reconstituted with both CD4$^+$CD25$^-$ effector T cells and CD4$^+$CD25$^+$ Treg cells (10:1). These data indicate that CD4$^+$CD25$^+$ Treg cells have no impact on the overall outcome of *M. tuberculosis* infection. Kursar *et al.* suggested that CD4$^+$CD25$^+$ Treg cells prevent the bactericidal immune response based on data analyzed in RAG-KO mice reconstituted with each T cell subset (22). However, they reconstituted mice with T cells from naive animals at an unphysiological ratio of CD4$^+$CD25$^-$ T cells to CD4$^+$CD25$^+$ T cells (2:1). These differences may explain the discrepancy between studies.

In order to elucidate the cellular mechanisms of the minimal effect of CD4$^+$CD25$^+$ Treg cells in *M. tuberculosis* infection after the infection was established, we evaluated the function of CD4$^+$CD25$^+$ Treg cells *in vitro*. We activated each population of CD4$^+$ T cells derived from naive and BCG- or *M. tuberculosis*-chronically infected mice with anti-CD3 mAb or *M. tuberculosis*-derived antigens, PPD. BCG has >99.5% identical genome with that of *M. tuberculosis* (40) and therefore BCG and *M. tuberculosis* share almost identical antigens. CD4$^+$CD25$^+$ T cells suppressed anti-CD3-induced

activation (proliferation, production of IFN-gamma and IL-10) of $CD4^+CD25^-$ effector T cells whereas, reflecting our *in vivo* data, PPD stimulation failed to suppress the function of $CD4^+CD25^-$ effector T cells. Both $CD4^+CD25^-$ and $CD4^+CD25^+$ T cells consume IL-2 to proliferate or maintain the state but only $CD25^+CD25^-$ effector T cells produce IL-2. Thus, the level of IL-2 inversely correlated with the number of $CD4^+CD25^+$ T cells (Fig. 6G, H and d) is considered the results of consumption of IL-2 by $CD4^+CD25^+$ T cells but not functional suppression.

One of mechanisms of diminished Treg cell function is mediated by IL-6, which is produced by activated APC through TCR signaling (27). We observed obvious production of IL-6 with PPD stimulation, although which cells produced IL-6 was unknown (Fig. 6, I, J and e). However, neither IL-6 nor other soluble factors released from cells were involved in the non-functional property of $CD4^+CD25^+$ Treg cells following PPD stimulation (Fig. 8). An explanation for this phenomenon is that PPD-specific $CD4^+CD25^+$ Treg cells are not activated at the late stage of mycobacterial infection because $CD4^+CD25^+$ Treg cells activated by anti-CD3/CD28 suppress the function of $CD4^+CD25^-$ effector T cells following stimulation with PPD (Figs 9 and 10).

With the exception of one recent study on herpes simplex virus infection (41), $CD4^+CD25^+$ Treg cells are thought to support parasite persistence in the host by inhibiting the function of effector T cells by a variety of mechanisms. According to this theory, several reports regarding mycobacterial infection have suggested a role for $CD4^+CD25^+$ Treg cells in disease progression and establishment of latent infection (22, 25, 42). However, our findings refute this theory, because $CD4^+CD25^+$ cells did not affect the total infectious load of *M. tuberculosis* in mice (Fig. 5) and mycobacterial infection did not activate mycobacteria-specific $CD4^+CD25^+$ Treg cells (Figs 6, 9 and 10). Several reports showed that FoxP3-positive Treg cells are found in the site of infection with BCG or *M. tuberculosis* (23, 25, 42). However, we consider that these Treg cells are unresponsive to mycobacterial antigens, rather than responding to self-antigens in the disrupted tissues of the infectious legion (43).

In contrast, $CD4^+CD25^+$ Treg cells responding to parasite antigens are activated during infection of *Leishmania* (17, 44) and *Plasmodium* (21). These parasites more closely resemble mammals in the history of evolution; therefore, it can be speculated that they express antigens similar to mammalian self-antigens, which leads to activation of self-antigen-reactive $CD4^+CD25^+$ Treg cells (43). This may be a possible reason for the discrepancy of the host response to mycobacteria versus protozoa. The host must recognize pathogens to survive and mycobacteria represent major bacterial pathogens for vertebrates. In our study, the fact that effector T cells are activated in response to mycobacterial antigens, while suppressive $CD4^+CD25^+$ Treg cells are comparatively silent, is rational based on the host's need to protect itself from mycobacterial infection.

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