though all components that are involved in ESX-1 have not yet been fully characterized, a number of proteins with known functional domains are identified. They include a putative chaperone with an AAA+ ATPase (Rv3868), a subtilisin-like serine protease (Rv3883c), and an FtsK/SpoIIIE-like ATPase (Rv3870, Rv3871). Rv3877 is predicted to be a membrane-spanning protein that could be part of the translocation pore in the cytoplasmic membrane. It has also been shown that RD1 is a critical region for bacterial virulence (6, 25, 31, 40). Furthermore, intensive studies have demonstrated the role of RD1 in the generation of protective immunity (5, 41) as well as necrosis induction (25, 27) and granuloma formation (48). To determine how RD1 contributes to the development of host immune response, we compared the cytokine-inducing ability between wild-type *M. tuberculosis* H37Rv and the mutant strain deficient for RD1. We found that RD1 was essential for activation of caspase-1 and subsequent secretion of IL-18 and IL-1β from macrophages infected with *M. tuberculosis*. The activation of caspase-1 was induced via a potassium ion efflux that is highly dependent on RD1 but independent of the P2X7 receptor. Moreover, we found that the type I interferon is not required for the activation of caspase-1 and cytokine production.

MATERIALS AND METHODS

Mice. Female C57BL/6 and BALB/c mice were purchased from Japan SLC (Shizuoka, Japan). INF-α, INF-β, and INF-ω receptor 1 knockout mice (IFNAR1$^{-/-}$ mice, on a C57BL/6 background) were kindly provided by Shigekazu Nagata (Kyoto University Graduate School of Medicine, Kyoto, Japan). P2X7 receptor knockout mice (P2X7R$^{-/-}$ mice, on a C57BL/6 background) were obtained from Tatsuro Ishibashi (Kyushu University, Fukuoka, Japan). Mice were maintained under specific pathogen-free conditions and used at 7 to 9 weeks of age. All the experimental procedures were approved by the Animal Ethics and Research Committee of Kyoto University Graduate School of Medicine, Kyoto, Japan.

Bacterial strains. *M. tuberculosis* H37Rv, an H37Rv mutant deficient for RD1 (ΔRD1) and an RD1-complemented strain (ΔRD1::RD1) (pYUB412::Rv3860-Rv3885c) were kindly provided by William R. Jacobs (Albert Einstein Institute, Bronx, NY) (25). These *M. tuberculosis* strains were grown at 37°C to the mid-log phase in Middlebrook 7H9 broth supplemented with 0.5% albumin, 0.2% dextrose, 3 μg ml^{-1} catalase, and 0.2% glycerol. Bacteria were harvested, stirred vigorously with glass beads (3 mm in diameter), and centrifuged at 300 × *g* for 3 min to remove the bacterial clumps. The suspension was stored at −80°C in aliquots. After thawing, the viable bacteria were enumerated by counting the colonies after plating the diluted suspension on Middlebrook 7H10 agar plates containing 50 μg ml^{-1} oleic acid, 0.5% albumin, 0.2% dextrose, 4 μg ml^{-1} catalase, and 0.85 mg ml^{-1} sodium chloride. In each experiment, bacteria were added to the macrophage culture based on the concentration after the absence of bacterial clumps was confirmed by Kinyoun staining.

Cells. Peritoneal exudate cells of C57BL/6 and BALB/c mice were obtained by a peritoneal lavage 4 days after an intraperitoneal injection with 3 ml of thioglycolate medium (EIKEN Chemical, Osaka, Japan). Peritoneal exudate cells were washed and plated at 5.0 × 10^5 cells well^{-1} in 48-well plates and incubated for 3 h at 37°C in RPMI 1640 medium supplemented with 10% fetal calf serum. Nonadherent cells were removed by washes with warmed RPMI 1640 medium, and adherent cells were used as macrophages in the following experiments. Bone marrow cells were collected from tibiae of C57BL/6 mice and cultured with 100 ng ml^{-1} mouse M-CSF (R&D Systems, Minneapolis, MN) for 5 days. After washes, adherent bone marrow-derived macrophages (BMDM) were collected and seeded at 5.0 × 10^5 cells well^{-1} in 48-well plates (21).

Quantitative real-time RT-PCR. Total cellular RNA was extracted from peritoneal macrophages 9 h after infection with *M. tuberculosis* strains by using Nucleospin RNA II (Macherey-Nagel, Düren, Germany). RNA (0.2 μg) was treated with RNase-free DNase (Promega, Tokyo, Japan) to eliminate contaminating DNA and then subjected to reverse transcription (RT) using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen, Tokyo, Japan). Quantitative real-time RT-PCR was performed on ABI PRISM 7000 (Applied Biosystems, Tokyo, Japan) using Platinum Sybr green quantitative PCR SuperMix-uracil DNA glycosylase (Invitrogen) according to the manufacturer's instructions. The level of each cytokine mRNA expression was normalized on the basis of β-actin mRNA expression, and results were analyzed with ABI PRISM 7000 sodium dodecyl sulfate (SDS) software. The following DNA sequences were designed and used as PCR primers: Il-1β (5′-AAGCTCTCCACCTCAATGGACAG-3′, forward; and 5′-CTCAAACTCCACTTTGCTCTTGA-3′, reverse), Il-18 (5′-ACTGTACAACCGCAGTAATACGG-3′, forward; and 5′-AGTGAACATTACAGATTTATCCC-3′, reverse), and β-actin (5′-TGGAATCCTGTGGCATCCATGAAAC-3′, forward; and 5′-TAAAACGCAGCTCAGTAACAGTCCG-3′, reverse).

Cytokine production and detection of caspase-1. Peritoneal macrophages were infected with *M. tuberculosis* strains at a multiplicity of infection (MOI) of 5 for 3 h. Cells were washed to remove extracellular bacteria and then incubated for 1, 9, and 21 h in the presence or absence of 500 to 2,000 NU/ml anti-IFN-β immunoglobulin G (IgG) (PBL Biomedical Laboratory, Piscataway, NJ), or 10 to 40 mM potassium chloride. Alternatively, macrophages were infected with H37RvΔRD1 at an MOI of 5 for 3 h. Cells were washed to remove extracellular bacteria and incubated for 21 h, and then nigericin (5 μM) and/or KCl (30 mM) was added and incubated for another 3 h. The culture supernatant was collected, and concentrations of cytokines were determined by enzyme-linked immunosorbent assay (ELISA) as reported previously (17, 19, 20). TNF-α, IL-6, and IL-1β were detected by using ELISA kits (eBioscience, San Diego, CA). IL-18 was detected using a pair of biotin-labeled and unlabeled monoclonal antibodies specific to IL-18 (MBL, Aichi, Japan).

In order to detect the activated form of caspase-1, 6 ml of the culture supernatants was incubated with 7 μg of rabbit anti-caspase-1 p10 IgG (Santa Cruz Biotechnology, Santa Cruz, CA) and protein G Sepharose (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to pull down caspase-1. Concurrently, infected macrophages were washed and lysed in phosphate-buffered saline containing 1% Nonidet P-40, 1 μg ml^{-1} leupeptin, 1 μg ml^{-1} pepstatin A, 1.5 μg ml^{-1} aprotinin, and 2 mM dithiothreitol. The lysate was used for detection of procaspase-1. The samples were subjected to SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes by electroblotting. The membrane was sequentially treated with rabbit anti-caspase-1 p10 IgG, anti-rabbit IgG conjugated with peroxidase, and ECL Plus (GE Healthcare). The bands representative of procaspase-1 and caspase-1 were detected by LAS-4000 Mini (Fujifilm, Tokyo, Japan). In addition, mature and proform types of IL-1β were detected by Western blotting using anti-IL-1β antibodies (R&D Systems) in the culture supernatant and the cell lysate, respectively. β-Actin was detected using anti-mouse β-actin monoclonal antibody (Sigma-Aldrich, Tokyo, Japan).

Statistical analysis. Student's *t* test was used to determine the statistical significance of the values obtained, and a *P* value of <0.05 was considered to be statistically significant.

RESULTS

RD1 participates in the production of IL-18 and IL-1β, but not IL-6 or TNF-α, in macrophages infected with *M. tuberculosis*. To investigate whether RD1 in the *M. tuberculosis* genome contributes to cytokine production, we analyzed the production of IL-1β, IL-18, IL-6, and TNF-α after infection with *M. tuberculosis* strains H37Rv, ΔRD1, and ΔRD1::RD. High levels of cytokine production were detected in peritoneal exudate macrophages of C57BL/6 mice in response to H37Rv infection at an MOI of 5 (Fig.1A to D). The production of these cytokines was increased in a time-dependent manner, and the significant production was detected later than 12 h after infection. On the other hand, ΔRD1 did not induce such high levels of IL-1β and IL-18 production, whereas the production of TNF-α and IL-6 was comparable with that induced by H37Rv. In contrast with ΔRD1, the strain ΔRD1::RD1, an RD1-complemented strain, was capable of inducing the production of IL-1β and IL-18 as well as IL-6 and TNF-α, and the levels were almost similar to those induced by H37Rv. The results described above clearly indicate that RD1 is dispens-

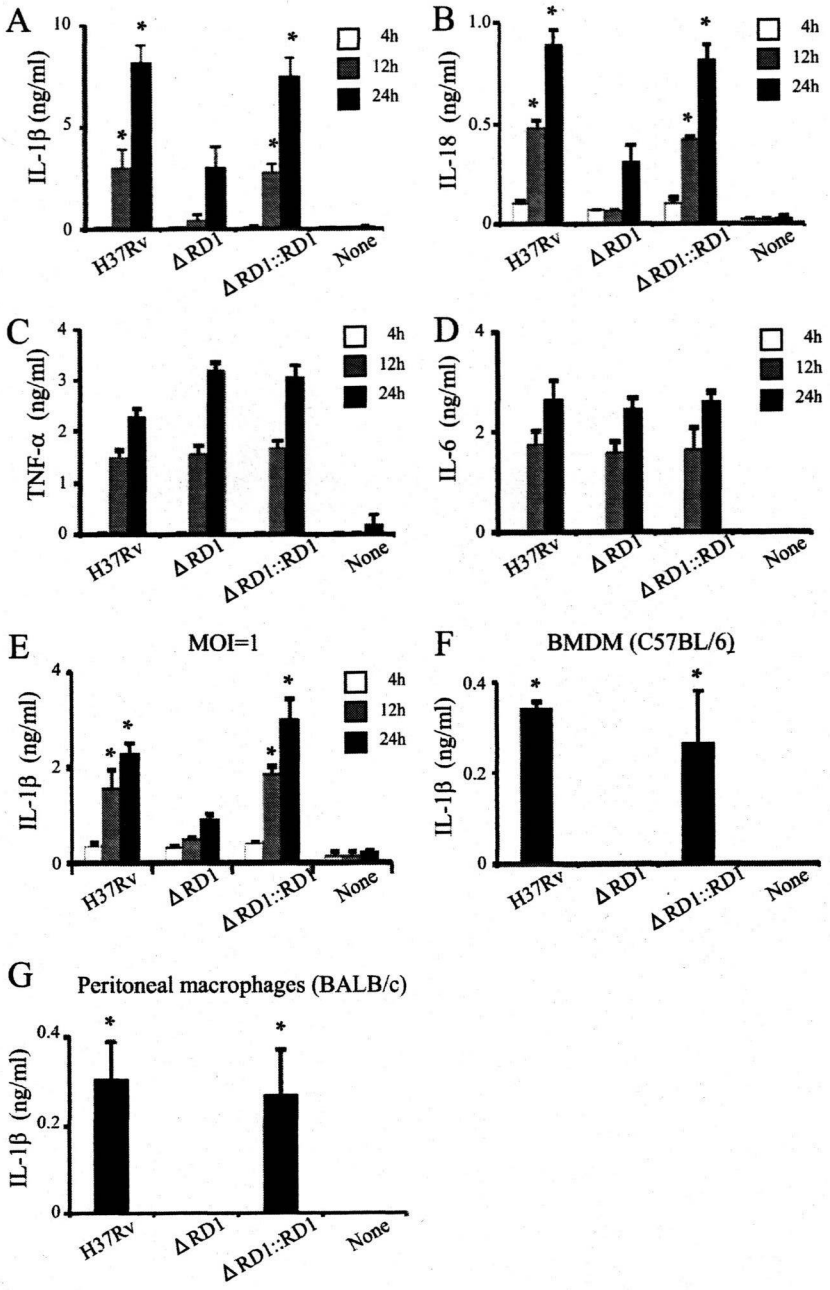

FIG. 1. RD1 participates in the production of IL-1β and IL-18, but not IL-6 or TNF-α, in *M. tuberculosis*-infected macrophages. Thioglycolate-induced peritoneal exudate macrophages of C57BL/6 mice were infected with H37Rv, ΔRD1, and ΔRD1::RD1 at an MOI of 5 for the indicated periods of time. The culture supernatant was collected, and the amounts of IL-1β (A), IL-18 (B), TNF-α (C), and IL-6 (D) were measured by ELISA. Peritoneal exudate macrophages were infected with *M. tuberculosis* strains at an MOI of 1 for the indicated periods of time, and IL-1β production was measured (E). BMDM of C57BL/6 mice (F) and peritoneal exudate macrophages of BALB/c mice (G) were infected with *M. tuberculosis* strains at an MOI of 5 for 24 h. The culture supernatant was collected, and the amount of IL-1β was measured by ELISA. Data represent the mean ± standard deviations of triplicate assays and are representative of three independent experiments. *, a P value of <0.05 for ΔRD1-infected cells compared to either H37Rv-infected cells or ΔRD1::RD1-infected cells.

able for TNF-α and IL-6 production but that it contributes to the production of IL-1β and IL-18 from infected macrophages.

It has been shown that virulent *M. tuberculosis* induces a distinct response in macrophages if cells were infected with either a high or low dose (29). To rule out the possibility that the difference in cytokine production between H37Rv- and ΔRD1-infected macrophages is due to a high load of bacteria, we infected macrophages with *M. tuberculosis* strains at an MOI of 1 and measured the IL-1β production. Similar to the response at the high-dose infection, the response from H37Rv and ΔRD1::RD1 infection showed that cytokine production was induced as early as 12 h after infection (Fig. 1E), while

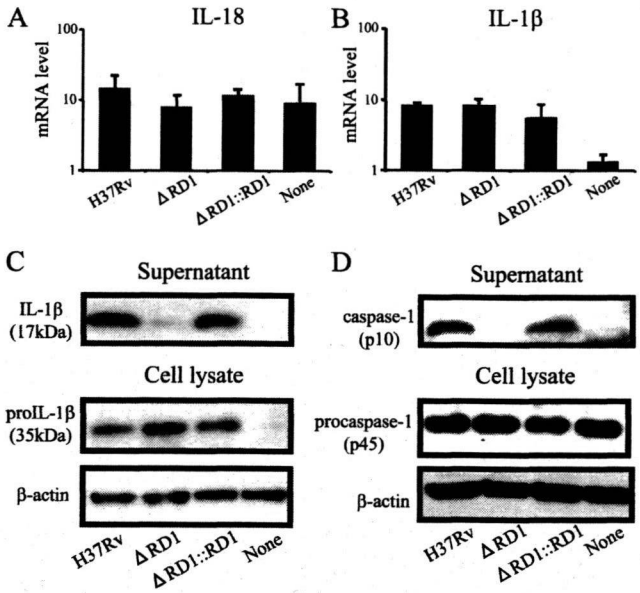

FIG. 2. RD1 contributes to secretion of IL-1β and IL-18 through the activation of caspase-1. Peritoneal macrophages were infected with *M. tuberculosis* strains at an MOI of 5 for 9 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to compare the expression levels of IL-18 (A) and IL-1β (B). Peritoneal macrophages were infected with *M. tuberculosis* strains at an MOI of 5 for 24 h. The cell lysate was prepared, and the amounts of proIL-1β (C) and procaspase-1 (D) were determined by Western blotting. (C) To detect mature IL-1β, the culture supernatant was collected and Western blotting was done. As the direct detection of the activated form of caspase-1 in the supernatant was difficult, the culture supernatant was treated with anti-caspase-1 p10 antibodies plus protein G Sepharose beads to enrich caspase-1. (D) The sample was then subjected to SDS-polyacrylamide gel electrophoresis, and the relative amount of mature caspase-1 (p10) was determined by Western blotting. β-Actin was used as a loading control of the cell lysate.

ΔRD1 did not induce production, suggesting that the observed pattern of cytokine production had not resulted from an unreasonable load of bacteria. In addition, thioglycolate-induced macrophages are known to be in an activated state. We thus employed BMDM to determine whether RD1-dependent IL-1β production is observed in resting macrophages. As expected, infection with H37Rv and ΔRD1::RD1 induced a significant level of IL-1β production, but ΔRD1 infection did not (Fig. 1F). Furthermore, the requirement of RD1 in IL-1β production was observed for peritoneal macrophages of BALB/c mice, though the magnitude of the cytokine response was weaker than that of C57BL/6 macrophages (Fig. 1G).

RD1 contributes to the activation of caspase-1 in macrophages infected with *M. tuberculosis*. We next measured the expression of IL-18 and IL-1β mRNAs by real-time RT-PCR after infection with *M. tuberculosis* strains. IL-18 mRNA was detected in unstimulated macrophages (Fig. 2A). The level was almost similar to that observed after infection with H37Rv and ΔRD1::RD1. Despite the absence of IL-18 secretion in macrophages infected with ΔRD1 (Fig. 1A), there was no difference in the level of IL-18 mRNA expression between H37Rv and this mutant. Compared to the control response (no infection), IL-1β mRNA expression was dramatically increased after infection with all three strains. This profile showed a significant contrast to the profile of secreted IL-1β (Fig. 1). These results clearly showed that though the proforms of IL-18 and IL-1β were generated after ΔRD1 infection, the mutant failed to induce the secretion of the mature forms of these cytokines.

Both IL-1β and IL-18 are members of the IL-1 family of cytokines and are produced as immature proteins. It has been shown that IL-1β and IL-18 are secreted after conversion into mature forms by activated caspase-1 (4, 32). Based on the level of these cytokine transcripts, it appeared that pro-IL-1β and pro-IL-18 were similarly induced after infection with *M. tuberculosis* strains. To determine the level of transcripts, we carried out Western blotting for IL-1β. As shown in Fig. 2C, the 35-kDa band corresponding to pro-IL-1β was similarly detected in the lysates of cells infected with three *M. tuberculosis* strains (Fig. 2C). We next determined whether the activation of caspase-1 was induced after infection with *M. tuberculosis* strains by analyzing the amount of procaspase-1 (p45) and a fragment of the activated form of caspase-1 (p10) (Fig. 2D). It has been shown that activated caspase-1 is secreted from cells along with mature IL-1β and IL-18 (36, 42). Concordantly, we detected caspase-1 in the culture supernatant but not in the cell lysate, suggesting that caspase-1 is mostly secreted after conversion from procaspase-1 to activated caspase-1 in this experimental system. Therefore, we measured the amount of procaspase-1 in the cell lysate and evaluated the activation of caspase-1 by measuring the amount of p10 in the culture supernatant. A large amount of procaspase-1 (p45) was detected in the lysate of uninfected macrophages. A similar amount of p45 was detected in macrophages infected with three *M. tuberculosis* strains. On the other hand, p10 was detected only in the culture supernatant of macrophages infected with H37Rv or ΔRD1::RD1 and was hardly detected in the culture supernatant of ΔRD1-infected macrophages. In proportion to the caspase-1 activation, the mature IL-1β was detected in the culture supernatant of cells infected with H37Rv and ΔRD1::RD1 (Fig. 2C). The results clearly showed that RD1 contributes to the activation of caspase-1, leading to the secretion of IL-1β and IL-18 from H37Rv-infected macrophages.

Endogenous IFN-β does not participate in the activation of caspase-1 in macrophages infected with *M. tuberculosis*. Henry et al. have shown that IFN-β is necessary for the activation of caspase-1 in macrophages infected with *Francisella tularensis* and *Listeria monocytogenes*, whereas *Salmonella enterica* serovar Typhimurium, another intracellular bacterium, induces activation of caspase-1 independently of IFN-β (22). We examined whether IFN-β contributes to the activation of caspase-1 in macrophages infected with *M. tuberculosis*. We first infected macrophages with H37Rv in the presence of anti-IFN-β IgG and measured the production of IL-1β and IL-18. As shown in Fig. 3A and B, neutralization of IFN-β did not affect the production of these cytokines. The antibody employed in this study could block the secretion of IL-1β and IL-18 from macrophages infected with *Listeria monocytogenes* at the concentrations used in this experiment (data not shown). We also determined the effect of anti-IFN-β IgG on the activation of caspase-1. The Western blot clearly showed that the antibody did not affect the amount of p10 fragment of caspase-1 released after infection with H37Rv (Fig. 3C). The absence of an IFN-β contribution, as suggested by the findings described above, could be further confirmed by using type I IFN receptor

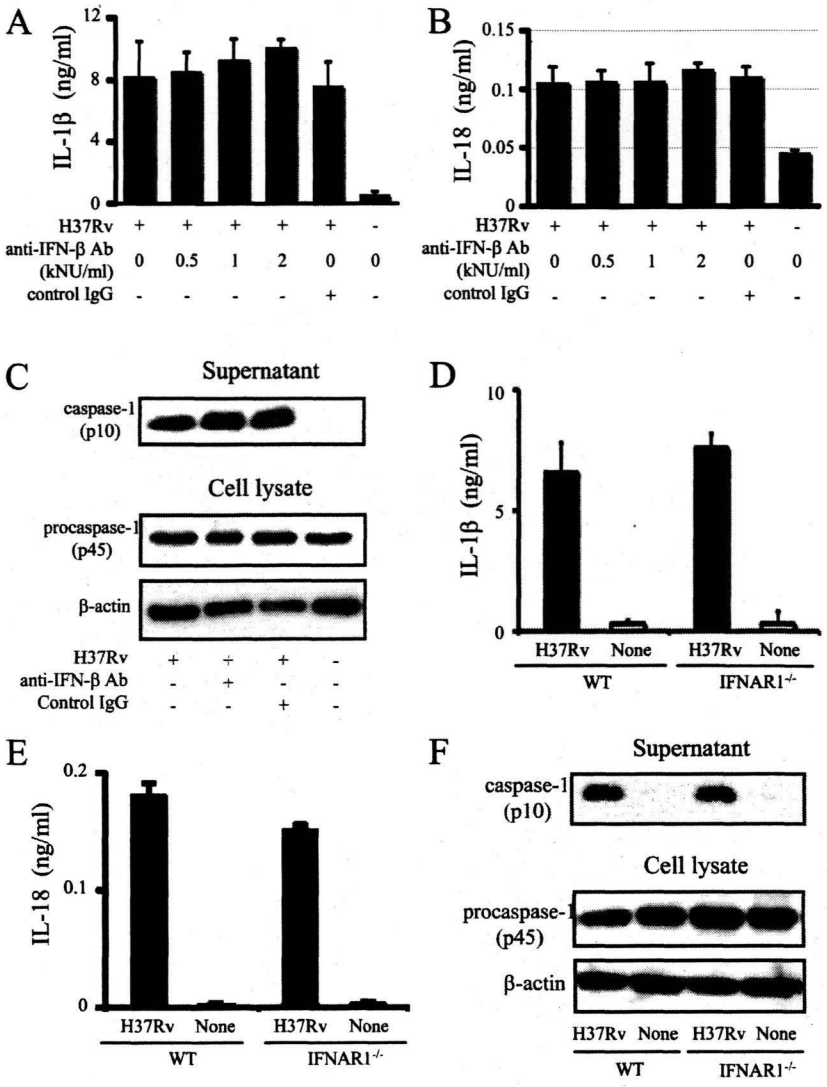

FIG. 3. IFN-β does not contribute to the activation of caspase-1 in *M. tuberculosis*-infected macrophages. Peritoneal macrophages were infected with H37Rv at an MOI of 5 for 24 h in the presence or absence of anti-IFN-β IgG or control IgG. The amount of IL-1β (A) and IL-18 (B) in the culture supernatant was measured by ELISA. Data represent the mean ± standard deviations of triplicate assays and are representative of three independent experiments. Peritoneal macrophages were infected with H37Rv at an MOI of 5 for 24 h in the presence or absence of anti-IFN-β IgG (2×10^3 NU/ml) or control IgG. Procaspase-1 (p45) in the culture supernatant and the activated form of caspase-1 (p10) in the cell lysate were detected by Western blotting (C). Peritoneal exudate macrophages from C57BL/6 and IFNAR1$^{-/-}$ mice were infected with H37Rv at an MOI of 5 for 24 h. The production of IL-1β (D) and IL-18 (E) in the culture supernatant was measured by ELISA. In addition, the amounts of procaspase-1 (p45) in the cell lysate and the fragment (p10) of mature caspase-1, which was immunoprecipitated from the culture supernatant, were detected by Western blotting (F). β-Actin was used as a loading control of the cell lysate. WT, wild type; Ab, antibody.

knockout (IFNAR1$^{-/-}$) macrophages. As shown in Fig. 3D and E, the level of these cytokines produced from IFNAR1$^{-/-}$ macrophages was comparable to that of wild-type macrophages. We also analyzed the activation of caspase-1 after infection with H37Rv. There was no difference in the amount of the fragment of activated caspase-1 (p10) between wild-type and IFNAR1$^{-/-}$ macrophages (Fig. 3F). Therefore, we concluded that IFN-β was not necessary for caspase-1 activation in *M. tuberculosis* infection.

Activation of caspase-1 in *M. tuberculosis* infection is dependent on potassium ion efflux. It has been shown that nigericin, anthrax lethal toxin, monosodium urate, and ATP efficiently induce the activation of caspase-1. These reagents cause a temporal decrease in intracellular potassium concentration and then promote the activation of caspase-1 (38, 39). To know whether potassium efflux is involved in the activation of caspase-1, we examined the effect of increasing concentrations of extracellular KCl on the production of cytokines. The significant levels of IL-1β and IL-18 secretion induced after infection with H37Rv were clearly decreased with increasing concentrations of KCl (Fig. 4A and B). On the other hand, the production of TNF-α and IL-6 was not affected by even the highest concentrations of KCl (Fig. 4C and D). Using Western blot analysis, we determined the effect of extracellular KCl on the activation of caspase-1 in H37Rv-infected macrophages. As expected, the amount of p10 fragment was decreased with

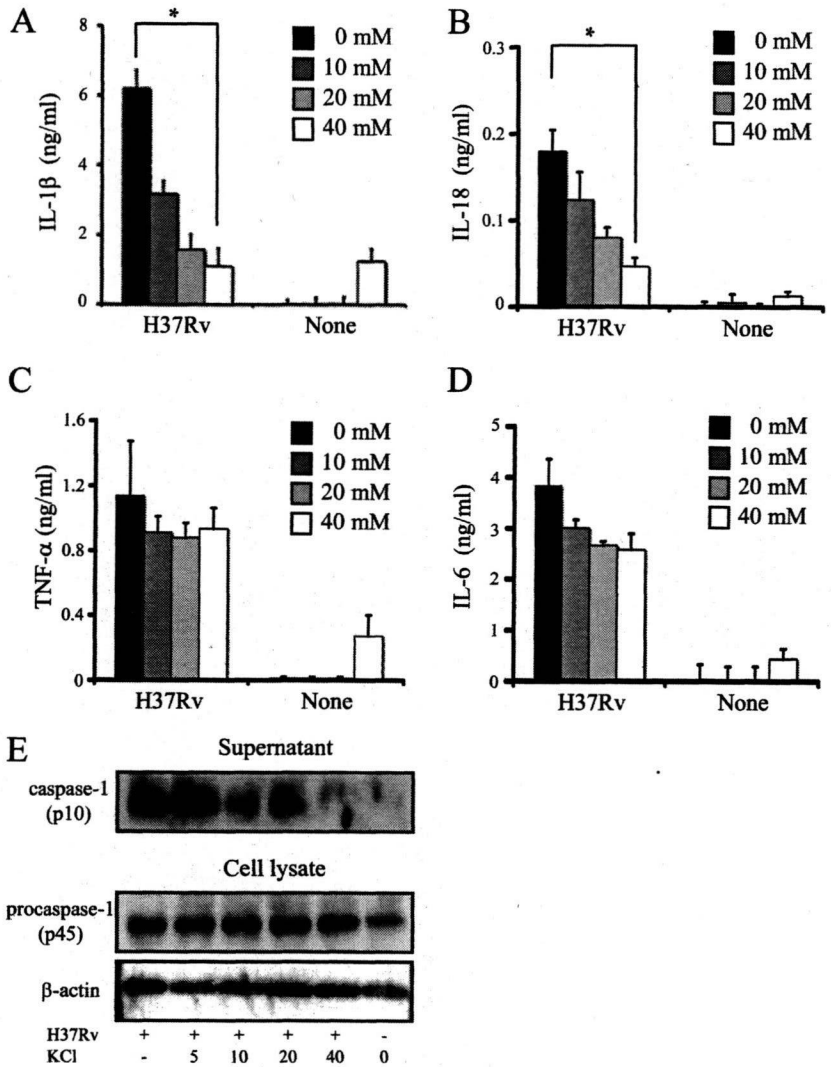

FIG. 4. Potassium ion efflux is essential for the secretion of IL-1β and IL-18 and activation of caspase-1 in *M. tuberculosis*-infected macrophages. Peritoneal macrophages were infected with H37Rv at an MOI of 5 for 24 h in the presence or absence of KCl at 0 mM (black columns), 10 mM (dark gray columns), 20 mM (light gray columns), and 40 mM (white columns). The amounts of IL-1β (A), IL-18 (B), TNF-α (C), and IL-6 (D) were measured by ELISA. Data represent the mean ± standard deviations of triplicate assays. *, a P value of <0.05 for H37Rv-infected cells in the presence of 40 mM KCl compared to H37Rv-infected cells in the absence of KCl. Peritoneal macrophages were infected with H37Rv at an MOI of 5 for 24 h in the presence of increasing concentrations of KCl. The amounts of procaspase-1 (p45) in the cell lysate and the fragment (p10) of mature caspase-1, which was immunoprecipitated from the culture supernatant, were detected by Western blotting (E). β-Actin was used as a loading control of the cell lysate.

increasing concentrations of KCl and was diminished to the level of the noninfected control when cells were cultured with 40 mM KCl (Fig. 4E). These results suggested that the potassium ion efflux is essential for the secretion of IL-1β and IL-18 and that the inability of ΔRD1 to induce the production of these cytokines may be due to the inability of the potassium ion efflux to be induced.

P2X7 receptor does not participate in the activation of caspase-1 in macrophages infected with *M. tuberculosis*. The P2X7 receptor was identified as an important component for caspase-1 activation through promotion of potassium efflux (10, 26). Recently, it has been shown that in response to TLR agonists or infection with *Staphylococcus aureus* or *Escherichia coli*, caspase-1 activation is triggered by the addition of ATP, a signal that promotes caspase-1 activation through depletion of intracellular potassium caused by stimulation of the P2X7 receptor. On the other hand, caspase-1 activation induced by *Salmonella* or *Listeria* was not affected in macrophages deficient in the P2X7 receptor (15). In view of this reported finding, we analyzed whether the P2X7 receptor contributes to *M. tuberculosis*-induced caspase-1 activation by measuring the cytokine response in P2X7 receptor knockout ($P2X7R^{-/-}$) macrophages after H37Rv infection. As shown in Fig. 5, there was no difference in the secretion of IL-1β and IL-18 (Fig. 5A and B), and the activation of caspase-1 (Fig. 5C), between wild-type and $P2X7R^{-/-}$ macrophages. The result indicated that the P2X7 receptor does not play any role in caspase-1 activation induced by *M. tuberculosis*.

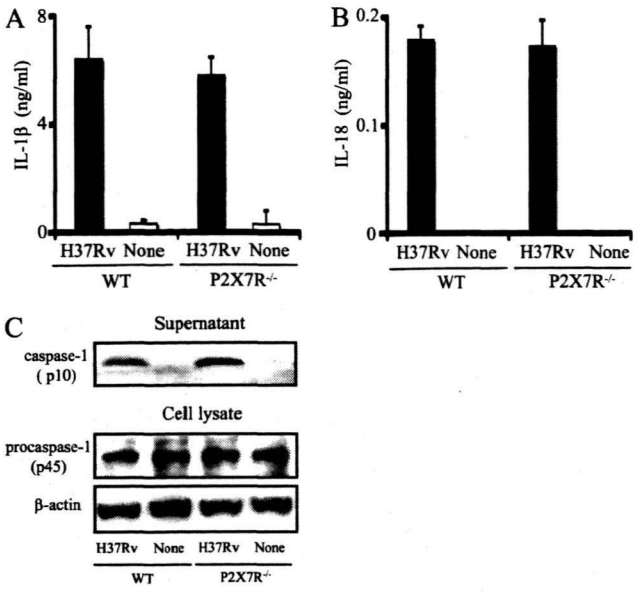

FIG. 5. P2X7 receptor does not contribute to the activation of caspase-1 in *M. tuberculosis*-infected macrophages. Peritoneal exudate macrophages from C57BL/6 and P2X7R$^{-/-}$ mice were infected with H37Rv at an MOI of 5 for 24. The levels of IL-1β (A) and IL-18 (B) in culture supernatants were determined by ELISA. (C) The amounts of procaspase-1 (p45) in the cell lysate and the fragment (p10) of mature caspase-1, which was immunoprecipitated from the culture supernatant, were detected by Western blotting. β-Actin was used as a loading control of the cell lysate. WT, wild type.

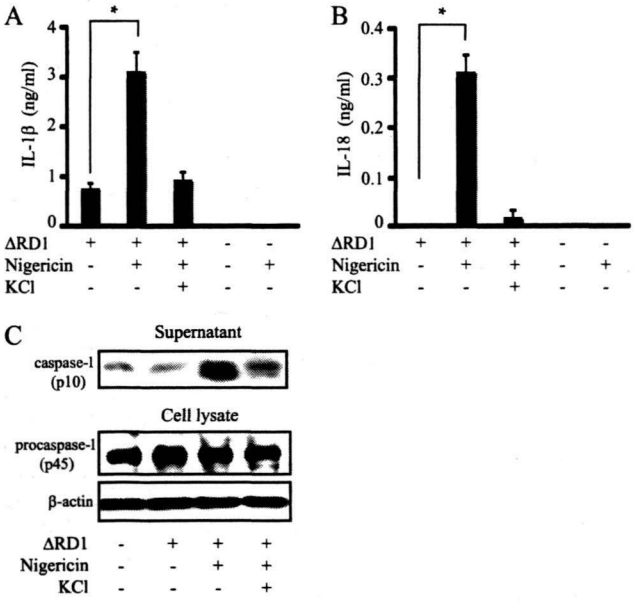

FIG. 6. The inability of H37RvΔRD1 to induce production of IL-18 and IL-1β is compensated by the addition of potassium ionophore (nigericin). Peritoneal macrophages were infected with ΔRD1 at an MOI of 5 for 21 h. Nigericin (5 μM) and/or KCl (30 mM) was added, and the culture was continued for another 3 h. The culture supernatant was collected, and the production of IL-1β (A) and IL-18 (B) was measured by ELISA. Data represent the mean ± standard deviations of triplicate assays. *, a P value of <0.05 for ΔRD1-infected cells in the presence of nigericin compared to either ΔRD1-infected cells in the absence of nigericin or ΔRD1-infected cells in the presence of both nigericin and KCl. (C) The amounts of procaspase-1 (p45) in the cell lysate and the fragment (p10) of mature caspase-1, which was immunoprecipitated from the culture supernatant, were detected by Western blotting. β-Actin was used as a loading control of the cell lysate.

Inability of ΔRD1 to induce production of IL-18 and IL-1β is compensated for by nigericin. The findings described above implied that *M. tuberculosis*-induced caspase-1 activation is through the induction of the potassium ion efflux that is dependent on RD1 but not on the P2X7 receptor. If this is the case, the inability of ΔRD1 to induce cytokine maturation may be compensated for by the induction of the potassium ion efflux by some means. To test this possibility, macrophages were infected with ΔRD1 and then stimulated with nigericin, a potassium ionophore. The culture supernatant was collected, and the production of cytokines and the activation of caspase-1 were measured. The production of IL-1β and IL-18 was not induced by infection with only ΔRD1 or treatment with nigericin alone (Fig. 6A and B). However, a strong cytokine response was observed when ΔRD1-infected cells were stimulated with nigericin. Furthermore, the enhanced cytokine response was diminished mostly by the addition of 30 mM KCl. In proportion to the cytokine production, the amount of p10 was also increased by treatment with nigericin and was reduced by the addition of 30 mM KCl (Fig. 6C). Based on these data, we concluded that RD1 participates in the caspase-1-dependent cytokine production via induction of the potassium ion efflux in infected macrophages.

DISCUSSION

In the present study, the RD1 locus in the *M. tuberculosis* genome is implicated in the activation of caspase-1 via induction of the potassium ion efflux in infected macrophages. Koo et al. have shown recently that *M. tuberculosis* stimulates the secretion of IL-1β and IL-18, and the activity is closely related to the RD1 locus (28). Our results are consistent with their findings. In addition, we newly demonstrated in this study that *M. tuberculosis*-induced caspase-1 activation is not dependent on IFN-β. Furthermore, *M. tuberculosis* caused a potassium ion efflux independently of the P2X7 receptor. We also found that H37Rv has a higher ability to induce cell death of infected macrophages than ΔRD1 (data not shown). ESX-1-dependent cytolysis may be involved in the cytokine and lysosome secretion, as reported previously (28). However, the exact relationship between the intracellular molecular events and the cytokine secretion still remains to be elucidated.

It has been shown that caspase-1 is activated after infection with several bacteria, including *Salmonella enterica* serovar Typhimurium, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Shigella flexneri*, and *Francisella tularensis*, and that various components or cascades are involved in the activation of caspase-1 (32, 50) Recently, Henry et al. have shown that IFN-β participates in the activation of caspase-1 in macrophages infected with *Francisella tularensis* and *Listeria monocytogenes*, whereas it is dispensable for the activation of caspase-1 in infections with *Salmonella enterica* serovar Typhimurium (22). In the case of *M. tuberculosis* infection, it has been shown that IFN-β is produced from macrophages in-

fected with *M. tuberculosis* and that the production is dependent on ESX-1 (46). Our preliminary study also showed that H37Rv induced higher IFN-β production than ΔRD1 (data not shown). However, the present study clearly showed that IFN-β was not required for the activation of caspase-1 in *M. tuberculosis* infection. It has been shown that *M. tuberculosis* may induce the formation of the NALP3/ASC inflammasome (28). As reported previously, *L. monocytogenes* induces caspase-1 activation via the formation of the NALP3/ASC inflammasome. However, there was a difference in the requirement of IFN-β in the formation of inflammasome after infection with *M. tuberculosis* and *L. monocytogenes*. Although the nature of IFN-β-dependent signaling events is not known, it is probable that the inflammasome is completed via the formation of a multiprotein complex. There may be differences in the composition of inflammasome induced by infection with different bacterial species.

Franchi et al. have shown that the requirement for the P2X7 receptor and intracellular potassium in caspase-1 activation is different between infection with intracellular and extracellular parasitic bacterial species (15). According to their report, regarding infection with *Staphylococcus aureus* and *Escherichia coli*, caspase-1 activation was triggered by P2X7 receptor-mediated intracellular potassium depletion, which is induced by the addition of ATP. In contrast, infection with *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* induced both caspase-1 activation and the cytokine secretion independently of the P2X7 receptor and potassium ion efflux. In *M. tuberculosis* infection, as shown here, the P2X7 receptor was not required for caspase-1 activation. However, a potassium ion efflux was necessary for caspase-1 activation, and RD1 was implicated in triggering the intracellular event. Several studies revealed that bacteria or bacterial components secreted in the cytosol induced caspase-1 activation (3, 19, 35) In this context, it has been shown that flagellin secreted by a type III secretion system of *S. enterica* serovar Typhimurium and listeriolysin O produced by *L. monocytogenes* are identified as effector molecules for inducing caspase-1 activation (14, 21). Recent evidence suggested that the ESX-1 secretory system of *M. tuberculosis* is capable of delivering several effector proteins to the host cytosol (1). Therefore, it is likely that some bacterial components induce caspase-1 activation during *M. tuberculosis* infection by intracellular potassium ion efflux.

It has been found that NALP3 and ASC are involved in the activation of caspase-1 in *M. tuberculosis*-infected macrophages (28). Pétrilli et al. have shown that the formation of the NALP inflammasome (NALP1 or NALP3) is induced under a low intracellular potassium concentration (39). Hentze et al. further demonstrated that cathepsin B contributes to the formation of the NALP3 inflammasome that is induced by potassium ion efflux (23). Taken together, it is probable that an ESX-1-dependent potassium ion efflux might cause the formation of the NALP3/ASC inflammasome through a release of cathepsin B from the lysosomal compartment. On the other hand, Fernandes-Alnemri et al. have shown that potassium depletion induces the formation of a pyroptosome, distinct from an inflammasome, which is largely composed of oligomerized ASC and can activate caspase-1 and release IL-1β (9). Although the formation of an inflammasome or pyroptosome may be involved in the activation of caspase-1 in infected macrophages, there was no information about the *M. tuberculosis* factors responsible for the potassium ion efflux followed by the activation of caspase-1. In this study, we demonstrated the close relationship between RD1 and the potassium ion efflux. *M. tuberculosis* and *Mycobacterium marinum* have been shown to induce permeation of the cell membrane (13, 18). Smith et al. have shown that the permeation is caused by pore formation (45). It has been suggested that ESAT-6 of *M. tuberculosis* has a membrane-lysing activity and that ESAT-6 of *M. marinum* could play a direct role in causing pore formation (7, 45). In addition, there are other *M. tuberculosis* components that are secreted through the ESX-1 secretion system, although their functions have not yet been identified (13, 34). Therefore, it is probable that one or more of the effector proteins secreted by the ESX-1 secretory system cause changes in the membrane integrity, leading to a decrease in the intracellular potassium level. We are attempting to identify the *M. tuberculosis* factors which may lead to such intracellular potassium perturbations in our future study.

The roles of IL-18 and IL-1β in the pathogenesis of tuberculosis still remain controversial. There is one recent report demonstrating that *M. tuberculosis* and *Mycobacterium bovis* BCG actively prevent inflammasome activation by use of a putative Zn^{2+} metalloprotease (33), while another report (28) and ours demonstrate that RD1-sufficient strains of mycobacteria can induce inflammasome activation. From our point of view, however, it can be suggested that IL-1β and IL-18 induced by *M. tuberculosis* are important for the protection (16, 44) and formation (37) of tuberculous granuloma. These cytokines, in concert with other cytokines or chemokines, may exert both beneficial and detrimental effects to the host, resulting in a complex pathology. Considering the fact that a RD1-deficient strain of *M. tuberculosis* cannot induce a strong activation of caspase-1 and secretion of IL-18 and IL-1β, it is tempting to assume that the limited efficacy of the BCG vaccine against adult pulmonary tuberculosis (11) is due, at least in part, to the absence of RD-1-dependent induction of mature IL-1β and IL-18. In fact, it has been shown that BCG or *Mycobacterium microti* strains that were transformed with the RD1 region show enhanced efficacy of vaccination in animal models (5, 41), although it is still unknown to what extent IL-1β and IL-18 play roles in this vaccination. Further studies are needed to obtain a comprehensive idea about the roles played by IL-1β and IL-18 in the pathogenesis of tuberculosis and to develop effective vaccines against tuberculosis.

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IL-15 is critical for the maintenance and innate functions of self-specific CD8$^+$ T cells

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IL-15 is a pleiotropic cytokine involved in host defense as well as autoimmunity. IL-15-deficient mice show a decrease of memory phenotype (MP) CD8$^+$ T cells, which develop naturally in naïve mice and whose origin is unclear. It has been shown that self-specific CD8$^+$ T cells developed in male H-Y antigen-specific TCR transgenic mice share many similarities with naturally occurring MP CD8$^+$ T cells in normal mice. In this study, we found that H-Y antigen-specific CD8$^+$ T cells in male but not female mice decreased when they were crossed with IL-15-deficient mice, mainly due to impaired peripheral maintenance. The self-specific TCR transgenic CD8$^+$ T cells developed in IL-15-deficient mice showed altered surface phenotypes and reduced effector functions *ex vivo*. Bystander activation of the self-specific CD8$^+$ T cells was induced *in vivo* during infection with *Listeria monocytogenes*, in which proliferation but not IFN-γ production was IL-15-dependent. These results indicated important roles for IL-15 in the maintenance and functions of self-specific CD8$^+$ T cells, which may be included in the naturally occurring MP CD8$^+$ T-cell population in naïve normal mice and participate in innate host defense responses.

Key words: Bacterial infections · CD8$^+$ T cells · Cytokines · KO mice · Self/non-self discrimination

Introduction

IL-15 is a pleiotropic cytokine involved in both innate and adaptive immune responses and plays important roles in host defense, graft transplantation as well as autoimmunity [1]. IL-15 utilizes the β-chain of IL-2R (CD122) and common cytokine receptor γ-chain (CD132) for signal transduction in lymphocytes and therefore shares many biological properties with IL-2. IL-15-mediated signaling includes Jak-STAT pathways, namely Jak1-STAT3 and Jak3-STAT5, as well as PI3K-Akt and Ras-MAPK pathways, leading to upregulation of Bcl-2 and activation of c-Myc, which promotes cell survival and division, respectively [2–4]. By using IL-15 KO mice, it has been demonstrated that IL-15 is an important factor for the development and/or maintenance of several subsets of lymphocytes, including NK cells, NKT cells, and intraepithelial lymphocyte (IEL) T cells [5, 6]. It is also known that a subset of CD8$^+$ T cells that express memory T cell markers are reduced in IL-15 KO mice.

Although the origin of such IL-15-dependent "memory-phenotype (MP)" CD8$^+$ T cells that develop naturally in naïve mice is unclear, they have been utilized as surrogates for *bona fide* memory CD8$^+$ T cells, which by definition arise from foreign antigen-specific naïve CD8$^+$ T cells and are responsible for the secondary immune response. However, MP T cells were also detected in naïve germ-free mice [7]. CD8$^+$ T cells developed in athymic mice, which lack memory T-cell responses, also express memory markers [8]. In addition, while IL-15-dependency of *bona fide* memory CD8$^+$ T cells was clearly shown [9, 10], IL-15-independent maintenance of memory CD8$^+$ T cells that emerged from chronic viral infection was also reported [11, 12]. Recently, a different origin/lineage of naturally occurring MP CD8$^+$ T cells was demonstrated by the studies on mice deficient for Tec kinases, which lack only conventional naïve CD8$^+$ T cells but not MP CD8$^+$ T cells [13, 14]. Importantly, the MP CD8$^+$ T cells developed in Tec kinase-deficient mice were decreased in the absence of IL-15 [15]. Naïve CD8$^+$ T cells that have undergone

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lymphopenia-induced proliferation also exhibited memory-like phenotypes [16] and used IL-15 for sustained proliferation, while their initial proliferation was IL-7-dependent [17]. Thus, the IL-15-dependent naturally occurring MP $CD8^+$ T cells in normal mice likely consist of heterogeneous $CD8^+$ T-cell populations whose relative contribution is still unclear.

We and others have reported unique features of self-specific $CD8^+$ T cells developed in male H-Y antigen-specific TCR Tg mice (H-Y TCR Tg) [18]. In the thymus of male mice, H-Y antigen-specific T cells ($T3.70^+CD8^+$) are deleted, whereas a large number of $T3.70^+CD8^+$ cells exist in the periphery. We found male but not female $T3.70^+CD8^+$ cells able to develop in the absence of thymus [19]. Interestingly, $T3.70^+CD8^+$ cells in male mice express CD44 and CD122 similar to naturally occurring MP $CD8^+$ T cells in normal mice [20]. They also share similarities in their functions. Thus, $T3.70^+CD8^+$ cells in male H-Y TCR Tg mice and MP $CD8^+$ T cells in naïve normal mice produced IFN-γ and showed CTL activities upon TCR-triggering *ex vivo* [21]. Both subsets of $CD8^+$ T cells express several NK receptors such as CD94 and NKG2D especially after *in vitro* activation with IL-2 [22, 23]. These similarities raise a possibility that the naturally occurring MP $CD8^+$ T-cell population in normal mice includes such self-specific $CD8^+$ T cells. However, *in vivo* IL-15-dependency of $T3.70^+CD8^+$ cells in male H-Y TCR Tg mice has not been clarified. It is also unknown whether IL-15-signaling affects the functions of the self-specific $CD8^+$ T cells in H-Y TCR Tg mice as well as naturally occurring MP $CD8^+$ T cells in normal mice, although an impaired effector function was demonstrated for NK cells and TCR γδ intestinal IEL in IL-15 KO mice [24].

MP $CD8^+$ T cells have been shown to contribute to the host defense against bacterial infection by producing IFN-γ [25–27]. *In vitro* experiments demonstrated the ability of MP $CD8^+$ T cells to produce IFN-γ in response to IL-12 and IL-18 or IL-12 and IL-15 without antigenic stimulation [10, 25, 26, 28, 29]. *In vitro* or *in vivo* stimulation with IL-15 induced their expansion [8, 30]. Thus, naturally occurring MP $CD8^+$ T cells can be activated in an antigen-nonspecific manner and are also called innate $CD8^+$ T cells [31]. Interestingly, $T3.70^+CD8^+$ cells in male H-Y TCR Tg mice and MP $CD8^+$ T cells in normal mice share similarities in their innate functions as well. IL-15-induced expansion of $T3.70^+$ $CD8^+$ cells in male mice *in vitro*, and the IL-15-stimulated $T3.70^+$ $CD8^+$ cells produced IFN-γ in the presence of IL-12 [20]. Furthermore, Dhanji demonstrated that male $T3.70^+CD8^+$ cells were activated and conferred protection against bacterial infection comparable to naturally occurring MP $CD8^+$ T cells in normal mice [32]. These observations further support the possible involvement of self-specific $CD8^+$ T cells in the MP $CD8^+$ T-cell population in naïve normal mice. A role for IL-15 in innate protection by the self-specific $CD8^+$ T cells against bacterial infection is also suggested.

In this study, we assessed the importance of IL-15 in the development, maintenance, and functional maturation of self-specific $CD8^+$ T cells in male H-Y TCR Tg mice by using IL-15 KO mice. We further examined *in vivo* roles of IL-15 in bacterial infection-induced activation of the self-specific $CD8^+$ T cells.

Results

Self-specific $CD8^+$ T cells are decreased in IL-15 KO mice

To examine the roles of IL-15 in the development of H-Y TCR Tg T cells, we crossed H-Y TCR Tg mice with IL-15 KO mice and analyzed the number of $T3.70^+CD8^+$ cells in the periphery. As has been known, there were a large number of $T3.70^+CD8^+$ cells in the spleen of male mice despite negative selection in the thymus (Fig. 1) [33]. The percentage of $T3.70^+CD8^+$ cells in male mice was even higher than female mice due to a large number of Tg TCR^- cells developed in female mice. We found the number of $T3.70^+CD8^+$ cells in male mice decreased in the absence of IL-15, while there was no difference in the number of $T3.70^+CD8^-$ cells in male mice or $T3.70^+CD8^+$ cells in female mice, both of which are $CD44^{low}$ and do not respond to IL-15 *in vitro* [20, 34], between WT and IL-15 KO. A similar reduction in male $T3.70^+CD8^+$ cells was observed in the liver and lymph nodes in IL-15 KO mice (data not shown). Thus, it was revealed that self-specific $CD8^+$ T cells in H-Y TCR Tg mice were IL-15-dependent, similar to naturally occurring MP $CD8^+$ T cells in normal mice.

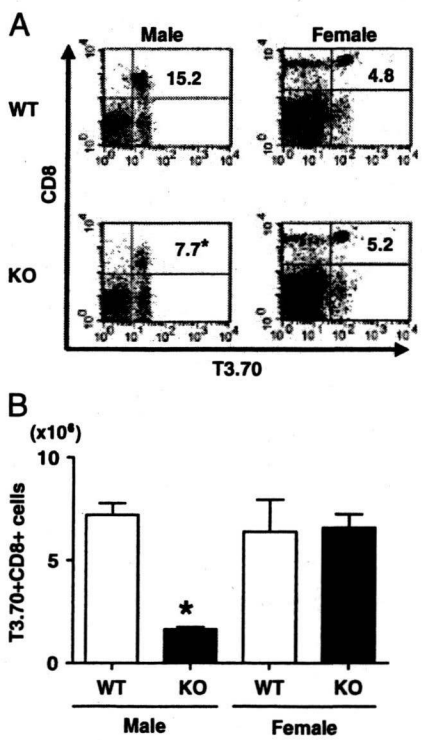

Figure 1. Number of self-specific $CD8^+$ T cells in the spleen of IL-15 KO mice. (A) CD8 and T3.70 profile of splenocytes in the lymphocyte gate in H-Y TCR Tg mice. Numbers in the upper right quadrant indicate the mean percentage of $T3.70^+CD8^+$ cells. (B) Absolute number of $T3.70^+$ $CD8^+$ cells in the spleen (mean±SEM). $^*p<0.05$ between male WT ($n = 3$) and IL-15 KO ($n = 3$), Student's *t*-test.

Although T3.70$^+$CD8$^+$ cells in male mice developed even in the absence of the thymus, their cell number was reduced compared with euthymic conditions [32], suggesting partial dependence on the thymus for their development. Therefore, we examined the number of T3.70$^+$CD8$^+$ cells in the thymus. The thymus of male mice contained a few mature CD8 single-positive T cells, and the number of T3.70$^+$CD8$^+$ cells was 100 times less than the female thymus (Fig. 2A, B). There was no difference in the number between WT and IL-15 KO mice (Fig. 2C). These results suggested intrathymic development of self-specific CD8$^+$ T cells independent of IL-15.

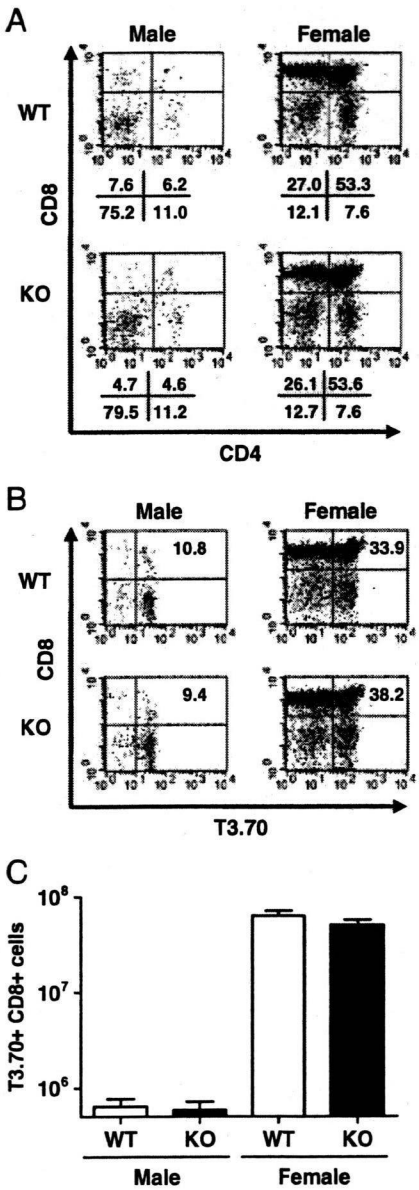

Figure 2. Development of self-specific CD8$^+$ T cells in the thymus of IL-15 KO mice. (A) CD8 and CD4 profile of thymocytes in H-Y TCR Tg mice. The numbers below each panel indicate the mean percentage of cells in the corresponding quadrants. (B) CD8 and T3.70 profile of thymocytes. Number in the upper right quadrant indicates mean percentage of T3.70$^+$CD8$^+$ cells ($n = 3$). (C) Absolute number of T3.70$^+$ CD8$^+$ cells in the thymus (mean ± SEM). No statistical difference was detected between male WT ($n = 3$) and IL-15 KO ($n = 3$), Student's *t*-test.

IL-15 is critical to the maintenance of self-specific CD8$^+$ T cells

As IL-15 is known to be essential for the maintenance of MP CD8$^+$ T cells in normal mice [10, 35], the decreased number of male T3.70$^+$CD8$^+$ cells in IL-15 KO mice might have resulted from impaired maintenance in the periphery. To investigate the roles of IL-15 in the maintenance of mature T3.70$^+$CD8$^+$ cells, we transferred the same number of CFSE-labeled T cells from the spleens of male or female WT H-Y TCR Tg mice into sex-matched WT and IL-15 KO recipient mice. Two weeks after transfer, the male T3.70$^+$CD8$^+$ cells that had undergone cell division were clearly detected in WT recipients but not in IL-15 KO recipients (Fig. 3A). Although the numbers of T3.70$^+$CD8$^+$ cells undergoing cell division were reduced in IL-15 KO recipients, those not undergoing cell division did not differ between WT and IL-15 KO recipients (Fig. 3B). Consistent with these results, the expression levels of Bcl-2 and active Caspase 3 did not differ in T3.70$^+$CD8$^+$ cells recovered from WT and IL-15 KO mice (Fig. 3C). In contrast to the case of male T3.70$^+$CD8$^+$ cells, divided cells were hardly found in female T3.70$^+$CD8$^+$ cells irrespective of the presence of IL-15 (Fig. 3A). In addition, a comparable number of female T cells were recovered from WT and IL-15 KO recipients (data not shown). Taken together, IL-15 is selectively involved in the maintenance of self-specific CD8$^+$ T cells, which might be the main mechanism for the reduced cell number in IL-15 KO mice.

Expression of surface molecules on self-specific CD8$^+$ T cells is regulated by IL-15

We next compared qualitative differences of the self-specific CD8$^+$ T cells developed in WT and IL-15 KO mice. As reported previously [20], the majority of T3.70$^+$CD8$^+$ cells in male mice were CD44^{high}CD122^{high}, while those in female mice were CD44^{low}CD122^{low} (Fig. 4A). Here we found the percentage of CD44^{high}CD122^{high} cells in male T3.70$^+$CD8$^+$ cells to be significantly lower in IL-15 KO mice. The percentage of cells expressing Ly6C, CD62L, as well as NK receptors such as CD94 and NKG2D in male T3.70$^+$CD8$^+$ cells, was also lower in IL-15 KO mice (Fig. 4B). In contrast, there was no difference in the expression of surface markers on female T3.70$^+$CD8$^+$ cells between WT and IL-15 KO mice. Expression of NK receptors was not detected on female CD8$^+$ T cells. In order to test whether the above changes in the expression of surface molecules in the absence of IL-15 are unique to H-Y TCR Tg CD8$^+$ T cells, we also examined CD44^{high} MP CD8$^+$ T cells in naïve normal mice. As reported previously [35], there were fewer CD44^{high} CD8$^+$ T cells, the majority of which were CD122^{high}, in IL-15 KO mice (Fig. 4C). In addition, expression of Ly6C, CD62L, CD94, and NKG2D was reduced in CD44^{high} CD8$^+$ T cells in IL-15 KO mice (Fig. 4D).

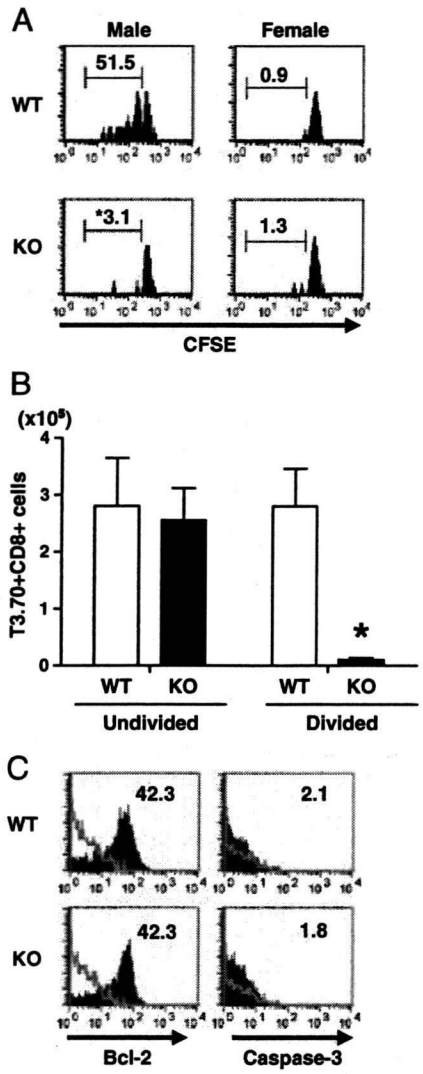

Figure 3. Maintenance of self-specific $CD8^+$ T cells in the periphery of IL-15 KO mice. (A) Spleen T cells from male and female H-Y TCR Tg mice were labeled with CFSE and were transferred into sex-matched WT and IL-15 KO mice. The histograms represent CFSE staining of $T3.70^+CD8^+$ cells in the spleen 14 days after transfer. The number in each panel indicates the mean percentage of divided cells ($n = 3$) $^*p < 0.05$ between male WT and IL-15 KO, Student's t-test. (B) Absolute number of male $T3.70^+CD8^+$ cells with or without cell division recovered from spleens of the recipient mice (mean ± SEM, $n = 3$). $^*p < 0.05$ between divided WT and IL-15 KO, Student's t-test. (C) Bcl-2 (left panel) and active caspase-3 (right panel) expression in $T3.70^+CD8^+$ cells recovered from the spleen of WT and IL-15 KO male was analyzed by intracellular staining. Open histograms indicate isotype controls. The numbers in the histograms indicate the mean fluorescent intensity ($n = 3$). Data are the representative of three independent experiments.

IL-15 is involved in acquisition of effector functions by self-specific $CD8^+$ T cells

Since we have found that the self-specific $CD8^+$ T cells in H-Y TCR Tg mice are equipped with effector functions *in situ*, including IFN-γ production and CTL activity [21], these $CD8^+$ T-cell functions were compared between WT and IL-15 KO mice. IFN-γ production was first examined by intracellular staining after stimulation with PMA and ionomycin. As shown in Fig. 5A, many $T3.70^+CD8^+$ cells in naïve male WT mice were already differentiated to produce IFN-γ, while none in female mice produced IFN-γ. IFN-γ^+ cells were also detected in $T3.70^+CD8^+$ cells in male IL-15 KO mice, but the frequency was significantly lower than in WT mice. The reduction of IFN-γ^+ cells in IL-15 KO mice was not simply attributable to the reduced percentage of $CD44^{high}$ cells, because the percentage of IFN-γ^+ cells among $CD44^{high}$ cells was also reduced in IL-15 KO mice. We further examined IFN-γ production of male $CD8^+$ T cells in response to various doses of H-Y antigen peptides. We previously found that even male $T3.70^+CD8^+$ T cells respond to a high dose of the self-antigen peptide, although they did not respond to male APC [21]. There was a difference in the amount but not in the activation threshold of peptide-induced IFN-γ production by the self-specific $CD8^+$ T cells in WT and IL-15 KO mice (Fig. 5B). *Ex vivo* IFN-γ production by $CD8^+$ T cells in normal mice was also examined (Fig. 5A). Similar to the case of male $T3.70^+CD8^+$ cells, there was a significant reduction in IFN-γ production by $CD44^{high}CD8^+$ T cells in IL-15 KO mice. CTL activity of H-Y TCR Tg T cells was measured by using H-Y peptide-pulsed EL-4 cells as targets. CTL activity was not detected in $CD8^+$ T cells in female mice *ex vivo* (Fig. 5C), although the female $CD8^+$ T cells could differentiate into effector CTL after *in vitro* stimulation with male antigens for 5 days [21]. In contrast, $CD8^+$ T cells in male mice exhibited significant CTL activity *ex vivo*, which was reduced in IL-15 KO mice. In support of these data, expression levels of granzyme B in male $T3.70^+CD8^+$ cells in IL-15 KO mice were significantly lower than those in WT mice (Fig. 5D). Thus, IL-15 regulates not only the expression of surface molecules but also *in situ* acquisition of effector functions of the self-specific $CD8^+$ T cells.

IL-15 is required for proliferation of self-specific $CD8^+$ T cells in response to bacterial infection

$CD8^+$ T cells in male H-Y TCR Tg mice proliferate and produce IFN-γ during *Listeria monocytegenes* (LM) infection and contribute to host defense similar to MP $CD8^+$ T cells in normal mice [22, 32]. We here examined the involvement of IL-15 in the *in vivo* responses of male $T3.70^+CD8^+$ cells during LM infection. $CD8^+$ T cells in male WT H-Y TCR Tg mice were labeled with CFSE and injected intraperitoneally into sex-matched WT or IL-15 KO mice, which were subsequently infected with LM in the peritoneal cavity. Three days after infection, division of male $T3.70^+CD8^+$ cells was clearly detected in WT recipients, whereas cell division was strikingly reduced in IL-15 KO recipients (Fig. 6A). Nearly no cell division was detected in female $T3.70^+$ $CD8^+$ cells even after LM infection, suggesting LM-induced bystander activation specific to the male $CD8^+$ T cells. It also indicated the lack of crossreactivity of Tg TCR to any LM antigens. We examined IFN-γ production by incubating the recovered cells *ex vivo* in the presence of brefeldin A (Fig. 6B). Male $T3.70^+CD8^+$ cells produced IFN-γ after infection with LM.

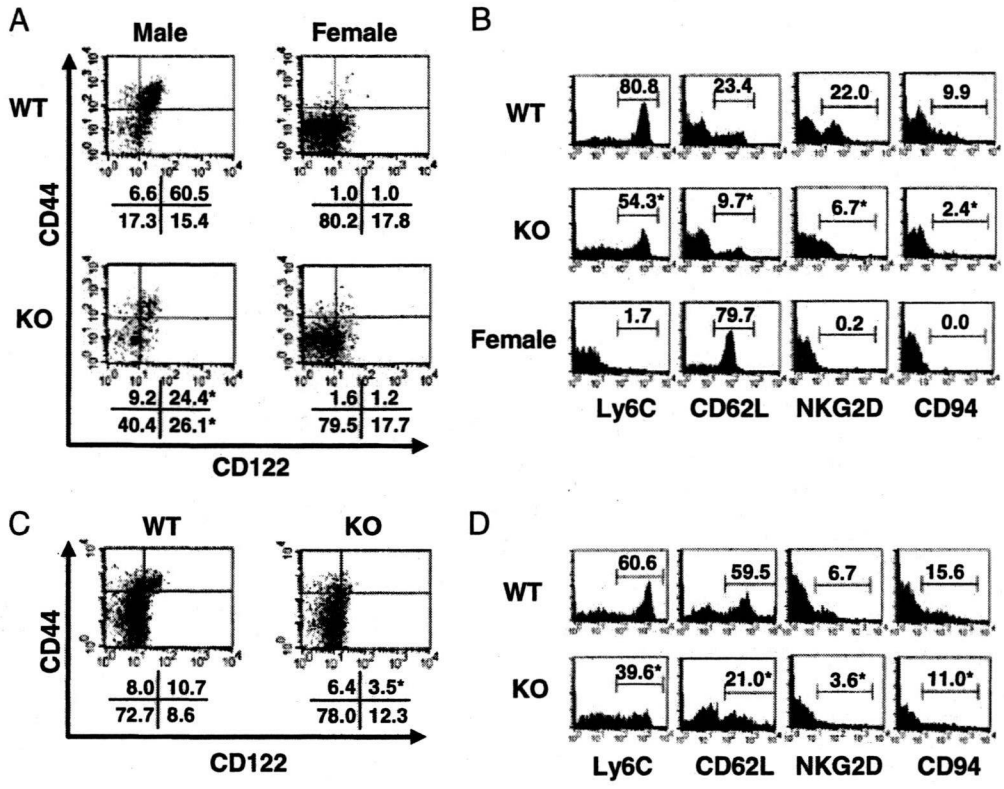

Figure 4. Expression of surface molecules on self-specific $CD8^+$ T cells in IL-15 KO mice. (A) CD44 and CD122 profile of $T3.70^+CD8^+$ cells in the spleen of WT and IL-15 KO male and female H-Y TCR Tg mice. The numbers below each panel indicate the mean percentage of cells in the corresponding quadrants ($n = 3$). $^*p < 0.05$ between male WT and IL-15 KO, Student's t-test. (B) Expression of Ly6C, CD62L, NKG2D, and CD94 on $T3.70^+CD8^+$ cells. The number in each histogram indicates the mean percentage of positive cells ($n = 3$). $^*p < 0.05$ between male WT and IL-15 KO, Student's t-test. (C) Dot plots depict representatives of CD44 and CD122 profile of $CD8^+$ T cells in the spleen of normal WT and IL-15 KO mice. The numbers below each panel indicate the mean percentage of cells in the corresponding quadrants. $^*p < 0.05$ between WT and IL-15 KO, Student's t-test. (D) Expression of Ly6C, CD62L, NKG2D, and CD94 on $CD44^{high}$ $CD8^+$ T cells in WT mice. The number in each histogram indicates the mean percentage of positive cells ($n = 3$). $^*p < 0.05$ between WT and IL-15 KO, Student's t-test.

Most IFN-γ^+ cells had undergone cell division. Interestingly, IFN-γ^+ cells were also detected in $T3.70^+CD8^+$ cells in IL-15 KO recipients despite a large reduction of divided cells. Finally, we examined involvement of IL-12 in the bystander IFN-γ production of the self-specific $CD8^+$ T cells by neutralizing IL-12 *in vivo*. As shown in Fig. 6C, IFN-γ production, but not cell division, was severely impaired in male $T3.70^+CD8^+$ cells in mice administered with anti-IL-12 mAb. These results indicate the independent regulation of bystander IFN-γ production and proliferation of the self-specific $CD8^+$ T cells by IL-12 and IL-15, respectively.

Discussion

Accumulating evidence suggests the importance of naturally occurring MP $CD8^+$ T cells in naïve mice in host defense mechanisms as innate lymphocytes, although their origin and antigen-specificity are still unclear. We and others have reported unique features of self-specific $CD8^+$ T cells developed in male H-Y TCR Tg mice and their similarities with the MP $CD8^+$ T cells in normal mice [8, 22]. In the present study, we demonstrated that the self-specific TCR Tg $CD8^+$ T cells also closely resemble MP $CD8^+$ T cells in normal mice in their IL-15-dependency, which further supports the hypothesis that a naturally occurring MP $CD8^+$ T-cell population in normal mice might involve self-specific $CD8^+$ T cells.

Maintenance of male $T3.70^+CD8^+$ cells was impaired in IL-15 KO mice, which might have resulted from reduced basal proliferation or impaired maintenance of divided cells. Similar to our results, Becker found after transferring virus-specific memory $CD8^+$ T cells as well as naturally occurring MP $CD8^+$ T cells into WT and IL-15 KO mice that the number of divided cells but not undivided cells was decreased in IL-15 KO mice [10]. Consistent with these data, the number of MP $CD8^+$ T cells in c-$myc^{+/-}$ mice, which exhibited an impaired basal proliferation but not survival, was greatly reduced [4]. Although it is still possible that IL-15-deficiency facilitated apoptosis of divided cells, we did not detect a reduction in the expression of Bcl-2 and active caspase 3 in $T3.70^+CD8^+$ cells transferred into IL-15 KO mice. This result is somewhat surprising, as IL-15 is known to upregulate Bcl-2, which is thought to be important for the survival of MP $CD8^+$ T cells [2]. However, a recent study demonstrated that Bcl-2 was actually indispensable for *in vivo* maintenance of MP $CD8^+$ T cells [36]. Since it was shown that intestinal IEL expressing Vγ7 was

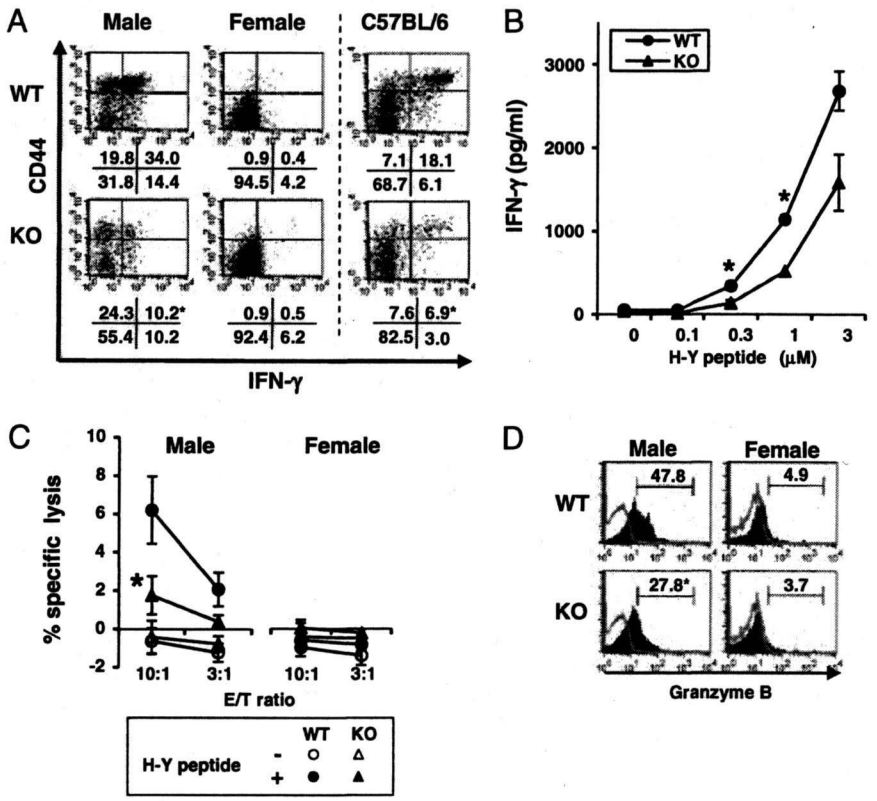

Figure 5. *Ex vivo* effector functions of self-specific $CD8^+$ T cells in IL-15 KO mice. (A) Splenocytes from WT and IL-15 KO male and female H-Y TCR Tg mice and C57BL/6 mice were stimulated with PMA and ionomycin in the presence of brefeldin A for 5 h. Expression of IFN-γ was detected by intracellular staining. Representative dot plots are shown after gating on $T3.70^+CD8^+$ cells (male and female) or TCR-β^+CD8^+ cells (C57BL/6). The numbers below each panel indicate the mean percentage of cells in the corresponding quadrant. (B) IFN-γ production of male $CD8^+$ T cells in response to indicated dose of the H-Y antigen peptides. IFN-γ was measured by ELISA. Data are the representatives of two experiments using pooled T cells and are expressed as mean ± SEM of duplicate samples. (C) WT (circles) and IL-15 KO (triangles) CD8 T cells in male and female H-Y TCR Tg mice were incubated with EL-4 cells ($H\text{-}2^b$) with (filled symbols) or without (open symbols) H-Y peptide (1 μM) in triplicate at indicated effector:target ratios. % specific lysis was calculated as described in the *Materials and methods*. Data are the representative of three independent experiments. (D) Expression of granzyme B in $T3.70^+CD8^+$ cells in male and female WT and IL-15 KO mice was analyzed by intracellular staining. Open histograms indicate negative controls. The number in each panel indicates mean percentage of positive cells. $^*p < 0.05$ between male WT ($n = 3$) and IL-15KO ($n = 3$), Student's *t*-test.

reduced in IL-15 KO mice due to suppressed recombination of the TCR repertoire [37], we also examined the thymus of male H-Y TCR Tg mice, where a small number of $T3.70^+CD8^+$ cells exist. However, we found no difference in the number of $T3.70^+CD8^+$ cells between WT and IL-15 KO mice. Because male but not female $T3.70^+CD8^+$ cells can also develop independently of the thymus [19], it is still possible that IL-15 affects their extrathymic development, which is however practically indistinguishable from peripheral maintenance.

Differential expression of surface markers on the self-specific CD8 T cells between WT and IL-15 KO mice suggests IL-15-signaling-mediated expression of these molecules. In fact, it was shown that expression of NK receptors on self-specific $CD8^+$ T cells in H-Y TCR Tg mice and MP $CD8^+$ T cells in normal mice was upregulated after *in vitro* activation with IL-2 [22, 23]. However, it is possible that the $CD44^{high}$ population of H-Y TCR Tg $CD8^+$ T cells has higher sensitivity to IL-15 than the $CD44^{low}$ population, which simply explains the reduced percentage of $CD44^{high}$ cells among $T3.70^+CD8^+$ cells in IL-15 KO mice. It is unclear why such heterogeneous populations of T cells expressing the same Tg TCR arose. This unlikely resulted from expression of the endogenous TCR α-chain, because similar bimodal distribution in CD44 expression was seen on $CD8^+$ T cells in RAG-deficient H-Y TCR Tg mice (data not shown). Expression levels of CD44 on male $T3.70^+CD8^+$ T cells are reduced in $H\text{-}2^{b/d}$ mice or in female mice [22, 32]. We found male $CD8^+$ T cells developed in athymic mice were highly enriched with $CD44^{high}$ cells [19]. Thus, various factors could be involved in the generation of heterogeneous populations of H-Y TCR Tg $CD8^+$ T cells.

In addition to the expression of surface molecules, we found differences in the effector functions of the self-specific $CD8^+$ T cells, even within $CD44^{high}$ populations, between WT and IL-15 KO mice. Interestingly, the impaired effector functions were also observed in NK cells and TCR γδ intestinal IEL that developed in IL-15 KO mice [24]. There have been studies suggesting a role for IL-2R-signaling in $CD8^+$ T-cell effector functions. Stat5, a transcription factor involved in IL-2R-mediated signaling, binds in the

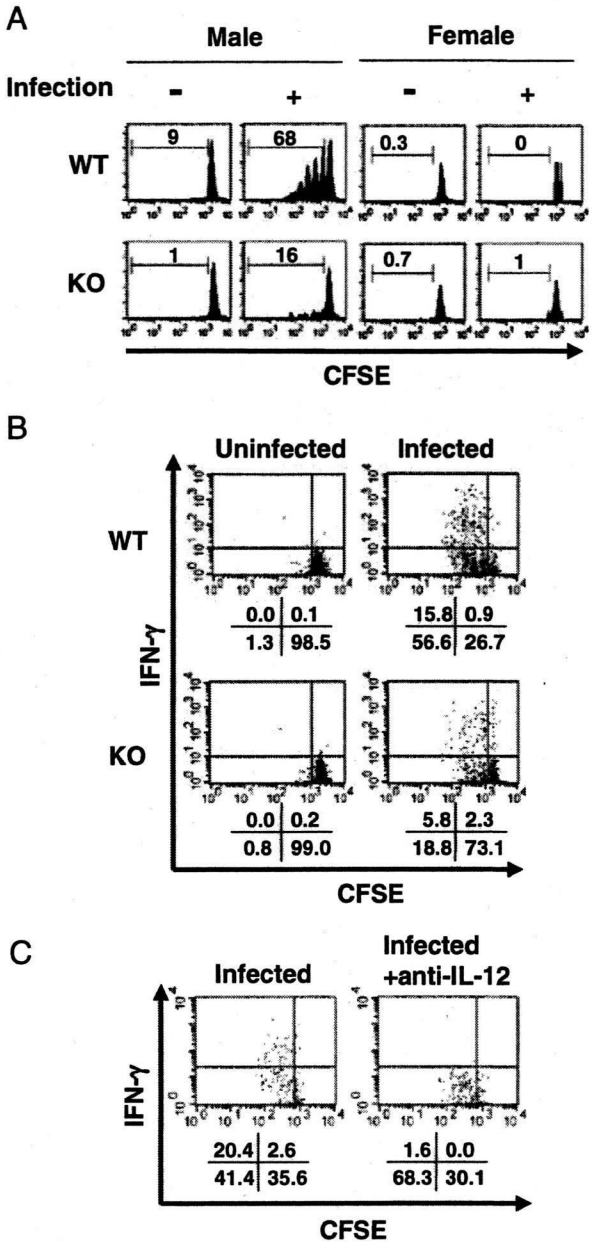

Figure 6. Bystander activation of self-specific $CD8^+$ T cells during bacterial infection in IL-15 KO mice. (A) Splenic T cells in male and female H-Y TCR Tg mice were labeled with CFSE and were injected intraperitoneally into sex-matched WT and IL-15 KO mice, which were subsequently infected with 5×10^5 cfu of LM intraperitoneally. After 3 days the cells were harvested from the peritoneum and analyzed. The numbers in the histograms indicate the percentages of divided cells and are representative of four independent experiments. (B) The harvested cells were immediately incubated with brefeldin A for 6 h and were subjected to intracellular IFN-γ staining. CFSE and IFN-γ profiles of male $T3.70^+CD8^+$ cells in WT or IL-15 KO recipients. The numbers below each panel indicate the mean percentage of cells in corresponding quadrants ($n = 3$). Data are the representative of three independent experiments. (C) In a similar experiment using male H-Y TCR mice as donors and male WT mice as recipients, a group of recipient mice were injected with anti-IL-12 mAb just before LM administration. CFSE and IFN-γ staining of harvested male $T3.70^+CD8^+$ cells. The numbers below each panel indicate the mean percentage of the cells in corresponding quadrants ($n = 2$). Data are representative of two independent experiments.

distal element of the *ifng* gene [38]. Involvement of the Jak3-Stat5 pathway in chromatin remodeling at the *ifng* locus was recently reported [39]. Stat5 activation induced by IL-2R-signaling is also involved in the cytotoxic activity by regulating expression of the *perforin* gene [40]. Furthermore, IL-2R-mediated signaling augments CTL activity in the self-specific $CD8^+$ T cells in H-Y TCR Tg mice and naturally occurring MP $CD8^+$ T cells in normal mice [22, 23]. We here demonstrated using IL-15 KO mice that even basal levels of IL-15-signaling affected functional maturation of these $CD8^+$ T cells.

Male $T3.70^+CD8^+$ cells were activated and conferred significant protection against LM infection with the same efficacy as naturally occurring MP $CD8^+$ T cells in normal mice [22, 32]. Furthermore, male $CD8^+$ T-cell-mediated protection was reduced in female recipients, suggesting a requirement of self-antigen recognition for full activation. We also observed that LM infection-induced proliferation of male $T3.70^+CD8^+$ T cells was reduced, although not abolished, in female recipients (data not shown). In this study we demonstrated that IL-15 is critical to the proliferation of the self-specific T cells during LM infection. Consistently, MP $CD8^+$ T cells in normal mice proliferated after poly I:C or LPS injection, which was mediated by IL-15 [35]. Therefore, the self-specific $CD8^+$ T cells require both TCR- and IL-15-mediated signaling to be fully activated during infection. We found that IFN-γ production by the self-specific $CD8^+$ T cells was induced in the absence of IL-15, while IFN-γ production, but not proliferation, was impaired by neutralizing IL-12, indicating a selective role for IL-15 in bacterial infection-induced proliferation of the self-specific $CD8^+$ T cells. It is noteworthy that foreign antigen-specific *bona fide* memory $CD8^+$ T cells can also be activated during antigenically unrelated bacterial infection [41]. Thus, OVA-specific memory $CD8^+$ T cells were protective against LM infection. However, it remains unclear whether such bystander activation of the OVA-specific memory $CD8^+$ T cells still requires interaction with self-MHC molecules. Interestingly, OT-I T cells, which were used in that study, have high sensitivity to self-MHC molecules and exhibit strong lymphopenia-induced expansion [42].

Antigen specificity of naturally occurring MP $CD8^+$ T cells in normal mice seems to be diverse. Even naïve foreign antigen-specific $CD8^+$ T cells can give rise to MP $CD8^+$ T cells after lymphopenia-induced proliferation, although weak interaction with self-MHC molecules is required [42]. MHC class Ib-restricted $CD8^+$ T cells, if not all, are also $CD44^{high}$ [43]. Notably, H2-M3, a member of class Ib molecules, presents peptides with *N*-formyl methyonine, which are expressed in mammalian mitochondrial proteins as well as bacteria [44, 45]. Some MHC class Ia-restricted MP $CD8^+$ T cells in naïve normal mice are also suggested to show self-specificity [23]. Taken together with our results, it is suggested that any naturally occurring MP $CD8^+$ T cells in naïve normal mice are more or less self-specific. Notably, self-specificity has also been suggested for other subsets of IL-15-dependent T lymphocytes, including NKT cells, CD8αα IEL T cells, and γδ T cells [46–50]. Therefore, self-specificity and IL-15-dependency may be common

features of innate T lymphocytes. However, there might be criticisms on the relevance of H-Y TCR Tg T cells. Premature expression of recombinated TCR-α and -β chains allows expression of TCR at the double negative stage. In fact, delayed expression of the same Tg TCR resulted in a different pattern of T-cell development in H-Y TCR Tg mice [51]. It was rather suggested that the Tg T cells resemble γδ T cells, which develop from early in ontogeny. However, the presence of immature double negative thymocytes expressing αβ TCR was also demonstrated in normal mice [52]. Furthermore, female T3.70$^+$ CD8$^+$ cells, which also express TCR from early in ontogeny, are indistinguishable from conventional naïve CD8$^+$ T cells in normal mice. Therefore, it is unreasonable to attribute all the unique properties of the self-specific CD8$^+$ T cells to the unusual timing of TCR expression. Thus, antigen specificity of the TCR, namely self or non-self, is the only factor for generating differences between male and female T cells in H-Y TCR Tg mice.

We demonstrated in this study the presence of self-specific CD8$^+$ T cells, which are maintained and also activated by IL-15, suggesting their involvement in autoimmunity, because upregulation of IL-15 are observed in many autoimmune diseases [1]. It is of particular interest that IL-15-induced NKG2D expression on CD8$^+$ T cells is involved in the pathogenesis of celiac disease, though the CD8$^+$ T cells kill epithelial cells independently of TCR specificity [53]. Although the self-specific CD8$^+$ T cells in H-Y TCR Tg mice do not respond to male APC due to an increased activation threshold for TCR stimulation [8], they could be involved in inflammatory diseases as well as host defense in a similar IL-15-dependent manner.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Japan (Yokohama, Japan). C57BL/6-background IL-15 KO mice and H-Y-TCR Tg mice were purchased from Taconic (Germantown, NY, USA). The mice were maintained in specific pathogen-free conditions and were used for the experiments at 7–12 wk of age. The study design was approved by the Committee of Ethics on Animal Experiment at the Faculty of Medicine, Kyushu University. Experiments were carried out under the control of the Guideline for Animal Experiment.

Microorganisms

LM, strain EGD, was used in this study. Bacterial virulence was maintained by serial passages in C57BL/6 mice. Fresh isolates were obtained from infected spleens grown in Trypto-Soya broth (Nissui Pharmaceutical, Tokyo, Japan), washed repeatedly and resuspended in 50% glycerol-containing PBS, and small aliquots were stored at −80°C until use.

Antibodies and flow cytometric analysis

mAb and reagents used for flow cytometric analysis were as follows: FITC-conjugated anti-CD4 (RM4-5), anti-CD44 (IM7), anti-CD122 (TMβ1), anti-CD62L (MEL-14), anti-H-Y TCR (T3.70) mAb, PE-conjugated anti-H-Y TCR (T3.70), CD94 (18D3), NKG2D (CX5), anti-IFN-γ (XMG1.2), and anti-granzyme B (16G6) mAb were purchased from eBioscience (San Diego, CA, USA); Allophycocyanin-conjugated anti-CD8α (CT-CD8α) mAb was purchased from Caltag Laboratories (Burlingame, CA, USA); FITC-conjugated anti-Ly6c (AL-21), PE-conjugated anti-Bcl-2 (3F11), anti-active caspase-3 (C92-605), and Armenian hamster isotype control mAb were purchased from BD Biosciences (San Jose, CA, USA). For cell surface staining, single-cell suspension was incubated with an optimal concentration of fluorescent mAb in Hanks balanced salt solution with 0.5% FCS for 20 min at 4°C. Intracellular staining was performed using the BD Cytofix/Cytoperm Kit (BD Biosciences) according to the manufacturer's instruction. Stained cells were run on a FACSCalibur flow cytometer (BD Biosciences). In some experiments, we added propidium iodide (1 μg/mL) to the cell suspension just before run on a flow cytometer to detect and exclude dead cells for the analysis. The data were analyzed using BD CELLQuest software (BD Biosciences).

Adoptive transfer experiments

Nylon wool non-adherent splenocytes were labeled with 1 μM CFSE (Invitrogen Life Technologies, Grand Island, NY, USA) in PBS for 15 min at 37°C. After stopping the reaction by the addition of an equal volume of FCS, the cells were washed with PBS. To examine *in vivo* maintenance of the cells, approximately 2×10^6 labeled cells were injected intravenously into sex-matched WT or IL-15 KO mice. Splenocytes were harvested 14 days after transfer and used for the analysis. To examine *in vivo* responses to LM infection, WT or IL-15 KO mice were injected intraperitoneally with 2×10^6 CFSE-labeled cells, which were subsequently injected intraperitoneally with 5×10^5 LM. After 3 days, peritoneal cells were harvested and used for the analysis. For *in vivo* neutralization of IL-12, mice were also injected with 100 μg of anti-IL-12 mAb (C17.8; Biolegend, San Diego, CA, USA) in PBS at the time of infection.

In vitro stimulation of T cells

To examine *ex vivo* effector functions, freshly isolated splenocytes from H-Y TCR Tg mice and normal mice were stimulated with 10 ng/mL PMA (Sigma-Aldrich) and 1 μg/mL of ionomycin (Sigma-Aldrich) in the presence of 10 μg/mL of brefeldin A (Sigma-Aldrich) for 5 h. Then the cells were subjected to intracellular staining for IFN-γ or granzyme B. To qualitatively measure IFN-γ secretion by H-Y TCR Tg T cells, splenic CD8$^+$ T cells containing 1×10^5 T3.70$^+$CD8$^+$ cells/mL were stimulated with various concentrations of H-Y antigen peptide

(KCSRNRQYL) for 4 days. IFN-γ in the culture supernatants was measured by using an ELISA kit (R&D systems, Minneapolis, MN, USA) according to the manufacturer's protocols. Cytotoxic activities of H-Y TCR Tg T cells were measured by a conventional ^{51}Cr-release assay. Freshly isolated CD8$^+$ T cells from the spleens were incubated with 1×10^4 ^{51}Cr-labeled EL-4 lymphoma cells (H-2^b) with or without 1 μM of H-Y antigen peptide in 96-well plates at various effector:target ratios according to the number of T3.70$^+$CD8$^+$ cells. After 5 h, radioactivity of supernatants was measured, and specific lysis was calculated as follows: specific lysis (%) = [(experimental release−spontaneous release)/(maximum release−spontaneous release)] × 100. Maximal and spontaneous ^{51}Cr release was obtained by incubation of target cells with 1% Triton X and with medium only, respectively.

Statistical analysis

Statistical significance was calculated by using Prism software (GraphPad, San Diego, CA, USA). Differences with p-values of <0.05 were considered statistically significant.

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Abbreviations: **IEL:** intraepithelial lymphocyte · **LM:** *Listeria monocytegenes* · **MP:** memory phenotype

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Article

IL-15 protects antigen-specific CD8$^+$ T cell contraction after *Mycobacterium bovis* bacillus Calmette-Guérin infection

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ABSTRACT

We reported previously that IL-15 plays a critical role in protecting effector CD8$^+$ T cells from apoptosis during the contraction phase following acute infection with *Listeria monocytogenes* by inducing antiapoptotic molecules. In the present study, we examined the effects of in vivo administration of rIL-15 on contraction of CD8$^+$ T cells after chronic infection with *Mycobacterium bovis* BCG and on the efficacy of BCG vaccination against *Mycobacterium tuberculosis* infection. Antigen-specific CD8$^+$ T cells reached an expansion peak at approximately Day 21, followed by a contraction after inoculation with rBCG expressing OVA. In vivo administration of rIL-15 from Days 22 to 42 after BCG inoculation inhibited apoptosis of effector CD8$^+$ T cells by up-regulating their Bcl-2 expression, resulting in a significant increase of antigen-specific memory CD8$^+$ T cells producing IFN-γ. However, the IL-15 treatment did not elicit improved efficacy of BCG vaccination against *M. tuberculosis*. These results suggest that IL-15 plays a critical role in protecting activated CD8$^+$ T cells from apoptosis during the contraction phase following BCG inoculation, although IL-15 administration alone at the contraction phase might not be sufficient to protect the efficient memory T cell responses against subsequent infection with *M. tuberculosis*. *J. Leukoc. Biol.* 86: 187–194; 2009.

Introduction

TB has been a major worldwide cause of death for centuries. One-third of the world's population is infected with *Mycobacterium tuberculosis*, which causes 2 million deaths per year [1]. *Mycobacterium bovis* BCG vaccine confers incomplete protection against TB in adults, although it can protect children efficiently against the early manifestations of TB [2, 3]. This is partly because the BCG vaccine is not effective for inducing long-term cellular immunity mediated by antigen-specific memory CD4$^+$ and CD8$^+$ T cells. The size of the memory T cell pool is at least partly dependent on the amount of surviving T cells from T cell contraction by apoptosis after primary TCR-mediated activation. Several studies have shown that death of the majority of activated CD8$^+$ T cells responding to a foreign antigen in vivo can be prevented by the Bcl-2 family [4, 5], which is known to be induced via signaling from the cγ-chain [5, 6]. Thus, cγ cytokine family members may play a critical role in the survival of effector CD8$^+$ T cells, resulting in increased size of memory CD8$^+$ T cells, and may be useful in increasing the efficacy of BCG vaccine for TB.

IL-15 is a member of the cγ-chain cytokine family, which uses β- and γ-chains of IL-2R for signal transduction and acts as a growth factor for memory CD8$^+$ T cells [7]. We found previously that IL-15 Tg mice, expressing IL-15 cDNA, encoding a secretable isoform, had an increased number of memory CD8$^+$ T cells in a naïve state and showed enhanced protection against infection with *M. bovis* BCG accompanied by an increase of antigen-specific CD8$^+$ T cells [8–10]. On the other hand, we and others [11, 12] have reported that IL-15$^{-/-}$ mice showed an impaired protective immunity to BCG or *M. tuberculosis* infection associated with a dramatic reduction in the numbers and function of CD8$^+$ T cells, although there was a conflicting report about protection against *M. tuberculosis* in IL-15$^{-/-}$ mice [13]. Thus, IL-15 may play important roles in the generation and maintenance of immunological memory mediated by memory CD8$^+$ T cells against mycobacterial infection.

We have reported recently that treatment of IL-15 only at the contraction phase was able not only to protect effector CD8$^+$ T cells from apoptosis during the contraction phase but also to increase the memory CD8$^+$ T cell pool following *Listeria monocytogenes* infection [14]. In the present study, we examined the effects of in vivo administration of rIL-15 on contrac-

Abbreviations: BCG=bacillus Calmette-Guérin, CD62L=CD62 ligand, cγ=common cytokine receptor γ, i.t.=intratracheally, MNC=mononuclear cell, PEC=peritoneal exudates cell, PPD=purified protein derivative, rBCG-Ag85B-IL15=*M. bovis* rBCG-secreting fusion protein Ag85B/IL-15, TB=tuberculosis, Tg=transgenic

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