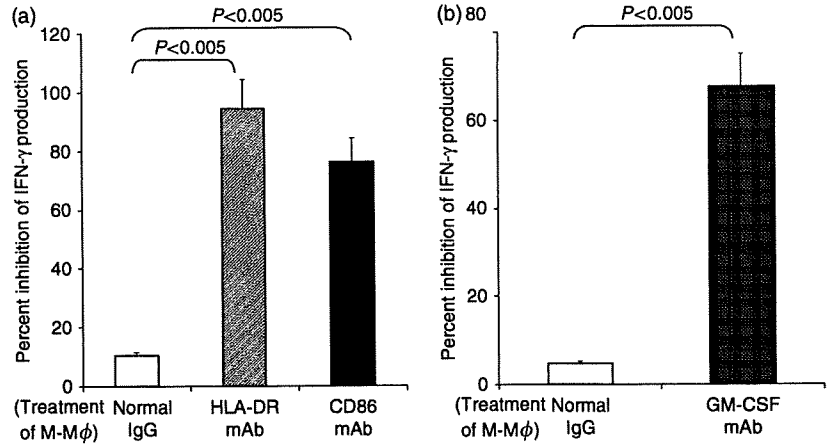

Fig. 4. (a) Inhibition of IFN-γ production by $CD4^+$ T cells by pretreatment of BCG-SM-infected M-Mϕ with mAb to HLA-DR or CD86. M-Mϕ differentiated from monocytes using rM-CSF were infected with BCG-SM at an MOI of 0.25 on day 5 of culture and cultured for another 2 days. The BCG-SM-infected M-Mϕ were treated with mAb to HLA-DR or CD86, or isotype-matched control IgG (10 μg mL^{-1}), and used as a stimulator of $CD4^+$ T cells, at a T cell : M-Mϕ ratio of 20 : 1 and cultured for another 4 days. The optimal concentration of mAb was determined in advance. Non-pretreated BCG-SM-infected M-Mϕ induced the production of 220.8 pg mL^{-1} of IFN-γ by $CD4^+$ T cells. This titre was taken as 0% inhibition. (b) Inhibition of IFN-γ production by $CD4^+$ T cells by neutralizing GM-CSF produced from BCG-SM-infected M-Mϕ. M-Mϕ, differentiated from monocytes by culturing for 5 days with rM-CSF, were infected with BCG-SM (MOI 0.25) in the presence of neutralizing mAb to GM-CSF or isotype-matched control IgG (10 μg mL^{-1}). These M-Mϕ were used as a stimulator of $CD4^+$ T cells as in (a). The optimal concentration of mAb was determined in advance. M-Mϕ infected with BCG-SM in the absence of any Ab induced the production of 168.3 pg mL^{-1} of IFN-γ by $CD4^+$ T cells. This titre was taken as 0% inhibition. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is shown. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

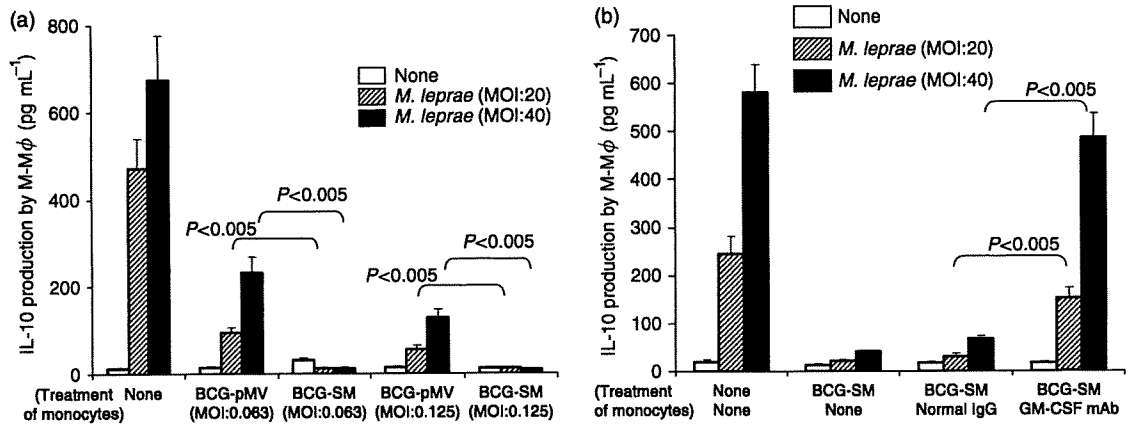

Fig. 5. (a) Production of IL-10 by M-Mϕ. Monocytes were infected with the indicated dose of BCG-pMV or BCG-SM, and subsequently differentiated into M-Mϕ by culturing for 5 days in the presence of M-CSF and rBCGs. These rBCG-preinfected M-Mϕ were stimulated with *Mycobacterium leprae* at the indicated MOI for 24 h. (b) Recovery of IL-10 production by M-Mϕ. Monocytes were infected with BCG-SM (MOI 0.063) in the presence of neutralizing GM-CSF mAb or isotype-matched control IgG and were subsequently differentiated into M-Mϕ by culturing for 5 days. These BCG-SM-preinfected M-Mϕ were stimulated with *M. leprae* at the indicated MOI for 24 h. The optimal concentration of mAb was determined in advance. A representative experiment based on three separate experiments conducted using three separate PPD-positive individuals is show. Assays were performed in triplicate and the results are expressed as the mean ± SD. Titres were statistically compared using Student's *t*-test.

monocytes also produced GM-CSF on stimulation with BCG in a BCG-SM-predominant fashion (not shown), we examined the effect of infection with rBCGs in monocytes on IL-10 production by M-Mϕ challenged with *M. leprae* (Fig. 5). M-Mϕ differentiated from monocytes untreated with any bacteria produced > 400 pg mL^{-1} of IL-10 on stimulation with *M. leprae*; however, the production of cytokine by M-Mϕ pretreated with rBCGs was significantly inhibited (Fig. 5a). The inhibition was more significant when BCG-SM was used as a stimulator of monocytes, and IL-10 production by M-Mϕ was almost completely inhibited. The inhibition was dependent on the dose of BCGs

used for pretreatment. In addition, pretreatment of monocytes with BCG-SM inhibited the IL-10 production induced even by lipopolysaccharide (not shown).

Furthermore, M-Mϕ differentiated from monocytes infected with BCG-SM in the presence of normal IgG did not produce IL-10 on stimulation with *M. leprae* (Fig. 5b). However, a significant level of IL-10 was produced when monocytes were infected with BCG-SM in the presence of the neutralizing mAb to GM-CSF. These results indicate that endogenously produced GM-CSF can inhibit IL-10 production.

Discussion

The host defence against intracellular parasitic pathogens such as *M. leprae* is closely associated with the activation of IFN-γ-producing type 1 T cells (Hashimoto *et al.*, 2002). In fact, in patients with paucibacillary leprosy, the activation of $CD4^+$ T cells results in inhibition of the intracellular multiplication and intercellular spread of *M. leprae* (Sieling *et al.*, 1999). The T-cell activation largely depends on the extent of the activation of APC, in which DC play an extremely important role, as DC are the most powerful T-cell stimulators among the APC (Hashimoto *et al.*, 2002). However, if T cells are not efficiently activated due to poor participation of DC, *M. leprae* may be predominantly retained in macrophages. In fact, multibacillary leprosy patients retain numerous *M. leprae* in their macrophages which, in some cases, allow the multiplication and intercellular spread of the bacteria (Ridley & Jopling, 1966).

The tissue resident macrophages, represented by GM-Mϕ and M-Mϕ, are heterogeneous in terms of function (Nakata *et al.*, 1991; Randolph *et al.*, 1999; Akagawa, 2002), despite being similarly susceptible to mycobacterial infection (Makino *et al.*, 2007). GM-Mϕ infected with *M. tuberculosis* or *M. leprae* significantly stimulated $CD4^+$ T cells, whereas M-Mϕ failed to stimulate $CD4^+$ T cells (Verreck *et al.*, 2004; Makino *et al.*, 2007). In this study, we found that, similar to those pathogenic mycobacteria, vector control BCG (BCG-pMV)-infected GM-Mϕ significantly stimulated $CD4^+$ T cells, whereas the BCG-pMV-infected M-Mϕ were less efficient in stimulating these cells. These results indicate the possibility that parental BCG may long reside in M-Mϕ and stimulate T cells inadequately, like the *M. leprae*-infected resident macrophages in multibacillary leprosy. In contrast to BCG-pMV, rBCG that secretes MMP-II (BCG-SM) has the ability to enlist not only GM-Mϕ, but also M-Mϕ, for T-cell activation. Further, the production of IFN-γ by $CD4^+$ T cells stimulated with BCG-SM-infected M-Mϕ was significantly inhibited by pretreatment of the M-Mϕ with the mAb to HLA-DR or CD86 antigens. In addition, the pretreatment of M-Mϕ infected with both BCG-SM and BCG-pMV effectively inhibited $CD4^+$ T-cell activation (not shown). Therefore, the BCG-SM-infected M-Mϕ seemed to stimulate $CD4^+$ T cells in an antigen-specific manner. Furthermore, there was a striking difference between BCG-pMV and BCG-SM in the induction of GM-CSF production. Not only from M-Mϕ, but also from GM-Mϕ, BCG-SM more efficiently induced GM-CSF production than BCG-pMV, and, further, rMMP-II protein, though less efficient, induced significant GM-CSF production. Previously, we reported that rMMP-II is highly immunogenic and induces production of various cytokines, including IL-12 and TNF-α, from APCs such as macrophages and DC (Maeda *et al.*, 2005). These findings indicate that the enhanced production of GM-CSF on stimulation by BCG-SM was at least partially associated with the secretion of MMP-II from BCG-SM.

As the activation of T cells by BCG-SM-infected M-Mϕ was largely inhibited when endogenously produced GM-CSF was neutralized by the mAb to GM-CSF, the endogenously produced GM-CSF may be closely associated with the enhanced T-cell activation by BCG-SM. Although we could not identify the most relevant antigen for T-cell activation, GM-CSF may change the activation status of macrophages or may at least partially transform the BCG-SM-infected M-Mϕ to GM-Mϕ (Makino *et al.*, 2007). Therefore, BCG-SM seems to be a unique rBCG capable of producing GM-CSF and utilizing M-Mϕ for T-cell stimulation.

Another important characteristic of mycobacteria which contributes to the inhibition of T-cell activation is the abundant production of IL-10 by M-Mϕ (Jonuleit *et al.*, 2001; Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004). The major purpose of a vaccination is the production of memory T cells which can rapidly respond to subsequently invading pathogenic mycobacteria. However, IL-10 inhibits the re-activation of memory T cells *in vitro*. We found that the ability of BCG-SM to induce production of GM-CSF is useful to inhibit IL-10 production. Monocytes were quite sensitive in the production of GM-CSF, and both BCG-pMV and BCG-SM induced cytokine production by monocytes, although BCG-SM predominated at lower doses (not shown). Thus, even BCG-pMV inhibited IL-10 production at higher doses. However, macrophages differentiated from monocytes which were infected with a small dose of BCG-SM completely inhibited IL-10 production upon subsequent challenge with *M. leprae*, and the inhibitory activity was at least partially cancelled out by the neutralization of endogenously produced GM-CSF. Further, heat-killed BCG-SM, which does not secrete MMP-II (Makino *et al.*, 2006), did not inhibit IL-10 production. These observations indicate that macrophages treated with GM-CSF endogenously diminished the ability to produce IL-10 upon stimulation with *M. leprae*. Previously, we observed that addition of GM-CSF exogenously blocked the ability to produce IL-10 (Makino *et al.*, 2007), which agrees with the

present data. The benefit of inhibition of IL-10 production for host defence has been previously demonstrated *in vivo*. IL-10-deficient mice displayed increased anti-mycobacterial immune responses and decreased bacterial burden (Murray & Young, 1999). In the absence of IL-10, antigen-specific memory T cells, which are efficiently produced by vaccination with BCG-SM for instance, may be fully activated for elimination of *M. leprae*. Although these are still preliminary findings, in one experiment BCG-SM more efficiently inhibited the multiplication of *M. leprae* in footpads of mice than in parent BCG. Therefore, BCG-SM may wipe out favourable conditions for the survival of *M. leprae*. The molecules that are present in the parental BCG and are associated with GM-CSF production remain undefined in the present study, but identification of these molecules may be useful to further enhance the T-cell-stimulating activity of BCG-SM. Also, the identification of such molecules may contribute greatly to the control of the pathogenic mycobacterial diseases using modified BCG.

In this study, we demonstrated that BCG-SM which can induce abundant GM-CSF production, may be more potent than parent BCG in immunostimulation and in the inhibition of IL-10 production, for preventing the survival of *M. leprae*.

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The RD1 Locus in the *Mycobacterium tuberculosis* Genome Contributes to Activation of Caspase-1 via Induction of Potassium Ion Efflux in Infected Macrophages[▽]

Takeshi Kurenuma,[1] Ikuo Kawamura,[1]* Hideki Hara,[1] Ryosuke Uchiyama,[2] Sylvia Daim,[1] Sita Ramyamali Dewamitta,[1] Shunsuke Sakai,[1] Kohsuke Tsuchiya,[1] Takamasa Nomura,[1] and Masao Mitsuyama[1]

Department of Microbiology, Kyoto University Graduate School of Medicine, Kyoto 606-8501, Japan,[1] and Department of Microbiology, Hyogo College of Medicine, Nishinomiya 663-8501, Japan[2]

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A genomic locus called "region of difference 1" (RD1) in *Mycobacterium tuberculosis* has been shown to contribute to the generation of host protective immunity as well as to the virulence of the bacterium. To gain insight into the molecular mechanism, we investigated the difference in the cytokine-inducing ability between H37Rv and a mutant strain deficient for RD1 (ΔRD1). We found that RD1 is implicated in the production of caspase-1-dependent cytokines, interleukin-18 (IL-18) and IL-1β, from infected macrophages. The expression of these cytokines was similarly induced after infection with H37Rv and ΔRD1. However, the activation of caspase-1 was observed only in H37Rv-infected macrophages. The cytokine production and caspase-1 activation were induced independently of type I interferon receptor signaling events. We also found that the activation of caspase-1 was markedly inhibited with increasing concentrations of extracellular KCl. Furthermore, the production of IL-18 and IL-1β and caspase-1 activation were induced independently of a P2X7 purinergic receptor, and the inability of ΔRD1 in caspase-1 activation was compensated for by nigericin, an agent inducing the potassium ion efflux. Based on these results, we concluded that RD1 participates in caspase-1-dependent cytokine production via induction of the potassium ion efflux in infected macrophages.

Mycobacterium tuberculosis, an etiologic agent of human tuberculosis, is one of the leading threats to humans. It has been reported that *M. tuberculosis* still causes 9.2 million new cases of tuberculosis worldwide and 1.7 million deaths annually (49). The recent emergence of multidrug-resistant and extensively drug-resistant *M. tuberculosis* strains highlights the urgent need for extensive research unraveling the complex mechanism enabling the bacterium to be successfully parasitic in humans.

The protective immunity against *M. tuberculosis* is mediated mainly by Th1-type $CD4^+$ T cells and $CD8^+$ T cells. These T cells produce a large amount of cytokines, including gamma interferon (IFN-γ) and tumor necrosis factor alpha (TNF-α), resulting in the enhancement of macrophage bactericidal activity and the development of granulomas in which *M. tuberculosis* is killed and prevented from disseminating to the bloodstream and other tissues (12, 47). It has been demonstrated that T cells differentiate into Th1 cells in cooperation with several proinflammatory cytokines, such as interleukin-12 (IL-12), IL-18, and IFN-γ, that are produced by infiltrating dendritic cells, macrophages, and NK cells. A number of in vitro studies have shown that these cytokines are produced via recognition of pathogen-associated molecular patterns of *M. tuberculosis* by Toll-like receptors (TLRs) (43). However, the role of TLR-mediated signaling pathways in the protective immunity against *M. tuberculosis* is controversial. Abel et al. have shown that TLR4-deficient mice display reduced bacterial clearance during a long-term infection and develop chronic pneumonia (2). Drennan et al. have also reported that TLR2-deficient mice initially control an aerosol infection with *M. tuberculosis*, but develop increased bacterial burden and succumb to chronic pneumonia (8). On the other hand, recent studies have shown that TLRs are dispensable in the development of T-cell-mediated adaptive immunity, while myeloid differentiation protein 88 (MyD88) is required for restriction of the intracellular growth of *M. tuberculosis* (44). In addition, Fremond et al. (16) and Hölscher et al. (24) have shown that mice deficient for IL-1 receptor (IL-1R) succumbed to acute *M. tuberculosis* infection in a manner similar to mice deficient for MyD88, whereas mice deficient for TLR2, TLR4, TLR9, or Toll-IL-1R domain-containing adaptor protein could control acute *M. tuberculosis* infection to the same extent as wild-type mice. These findings suggest that MyD88 plays a much more prominent role in adaptive immunity than functioning as an adaptor molecule of TLRs, and the role of the MyD88-dependent IL-1R signaling pathway is necessary for induction of efficient protection against *M. tuberculosis*.

A genomic locus of *M. tuberculosis* called "region of difference 1" (RD1) was first discovered as a locus that is absent in a genome of *Mycobacterium bovis* BCG (30). RD1 is 9.5 kb in length and comprises nine genes, including the genes that encode the secretory proteins ESAT-6 (6-kDa early secreted antigen target) and CFP-10 (10-kDa culture filtrate protein). The other genes encode components of a secretion system that is called ESX-1 (ESAT-6 system 1). It is supposed that more than 14 proteins contribute to this secretion system (1). Al-

* Corresponding author. Mailing address: Department of Microbiology, Kyoto University Graduate School of Medicine, Yoshidakonoecho, Sakyo-ku, Kyoto 606-8501, Japan. Phone: 81-75-753-4447. Fax: 81-75-753-4446. E-mail: ikuo_kawamura@mb.med.kyoto-u.ac.jp.

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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-

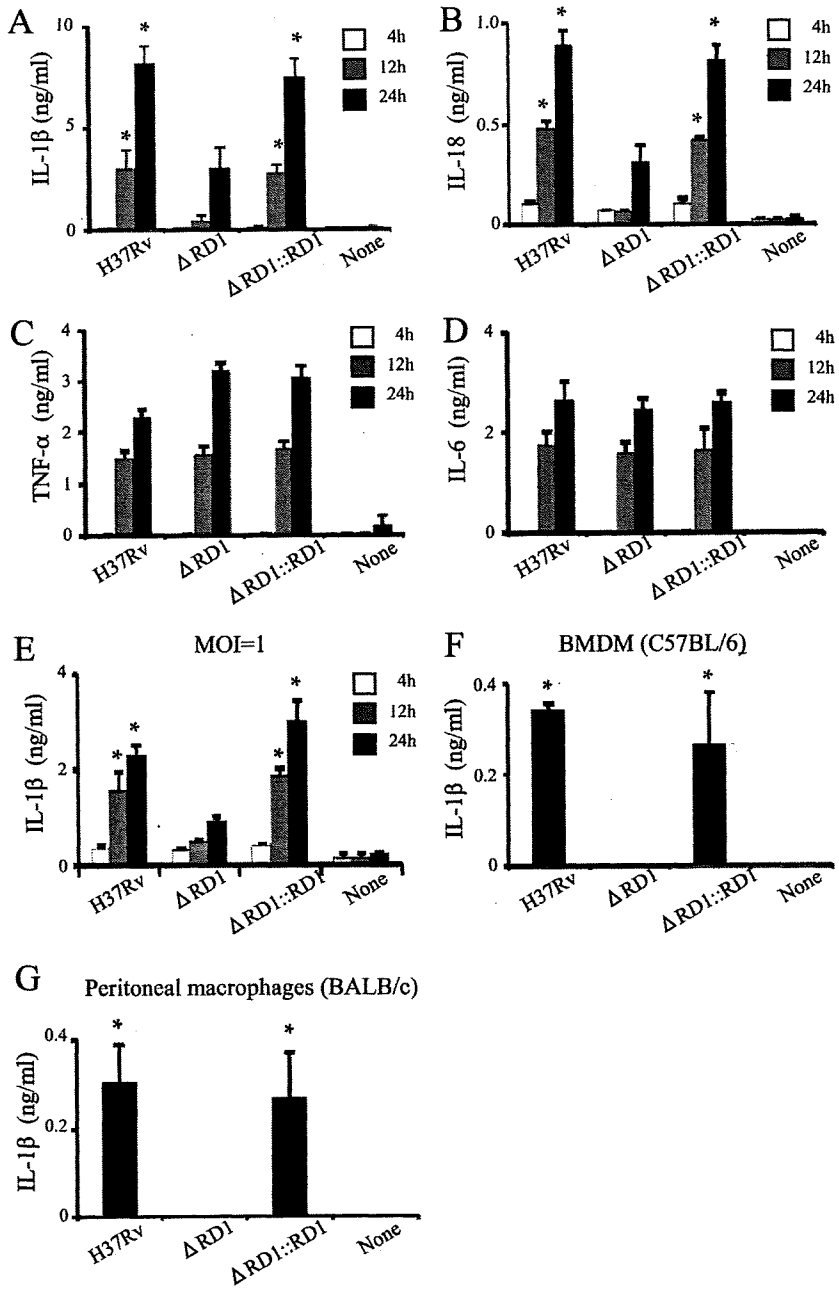

FIG. 1. RD1 participates in the production of IL-1β and IL-18, but not IL-6 or TNF-α, in *M. tuberculosis*-infected macrophages. Thioglycolate-induced peritoneal exudate macrophages of C57BL/6 mice were infected with H37Rv, ΔRD1, and ΔRD1::RD1 at an MOI of 5 for the indicated periods of time. The culture 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

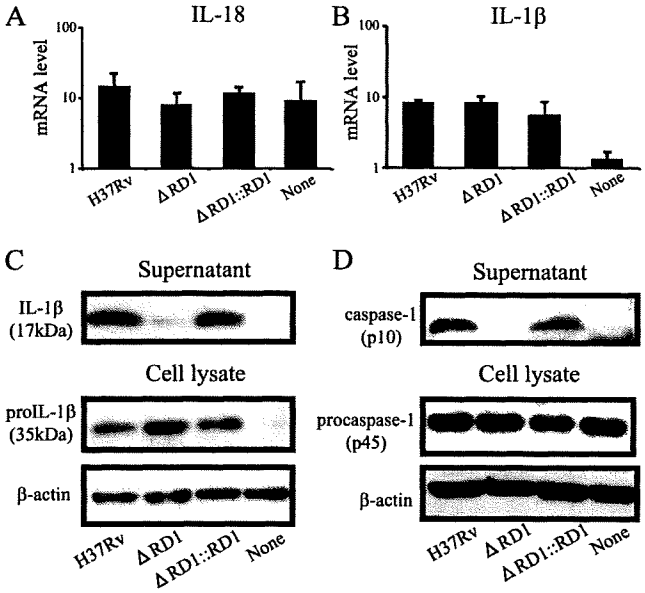

FIG. 2. RD1 contributes to secretion of IL-1β and IL-18 through the activation of caspase-1. Peritoneal macrophages were infected with *M. tuberculosis* strains at an MOI of 5 for 9 h. Total RNA was extracted and subjected to quantitative real-time RT-PCR to compare the expression levels of IL-18 (A) 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

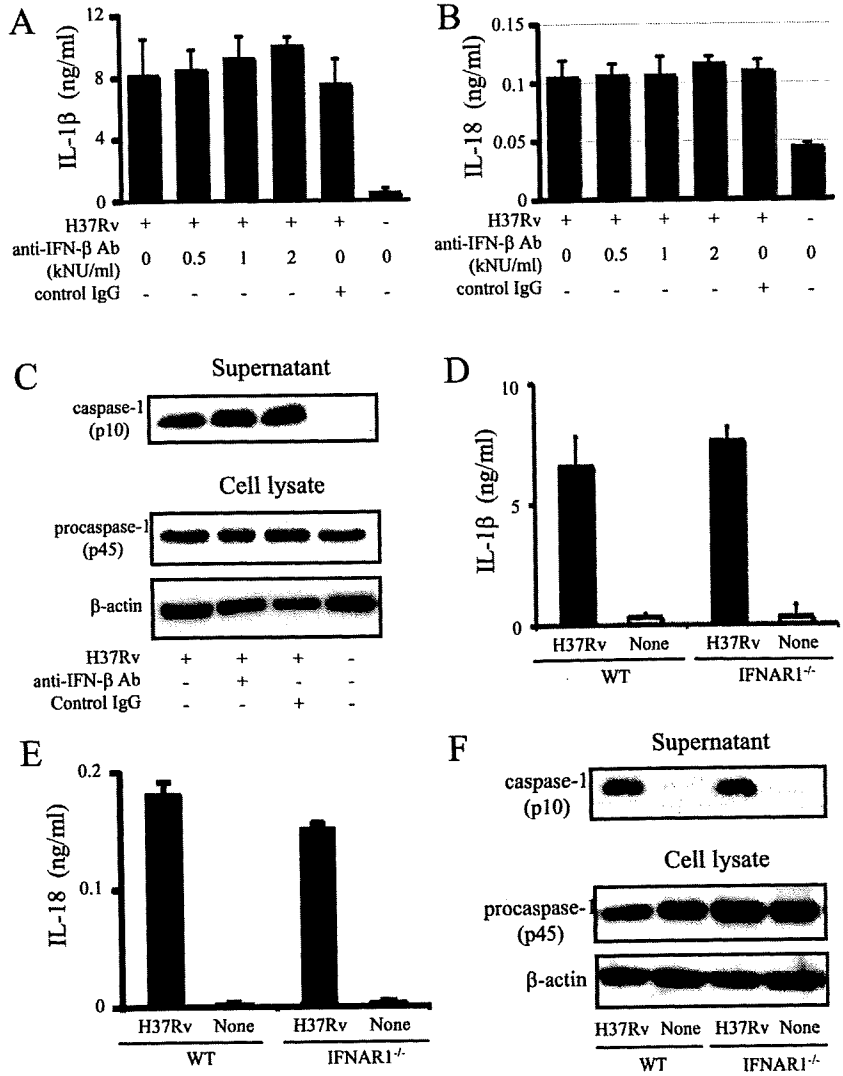

FIG. 3. IFN-β does not contribute to the activation of caspase-1 in *M. tuberculosis*-infected macrophages. Peritoneal macrophages were infected with H37Rv at an MOI of 5 for 24 h in the presence or absence of anti-IFN-β IgG or control IgG. The amount of IL-1β (A) and IL-18 (B) in the culture supernatant 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

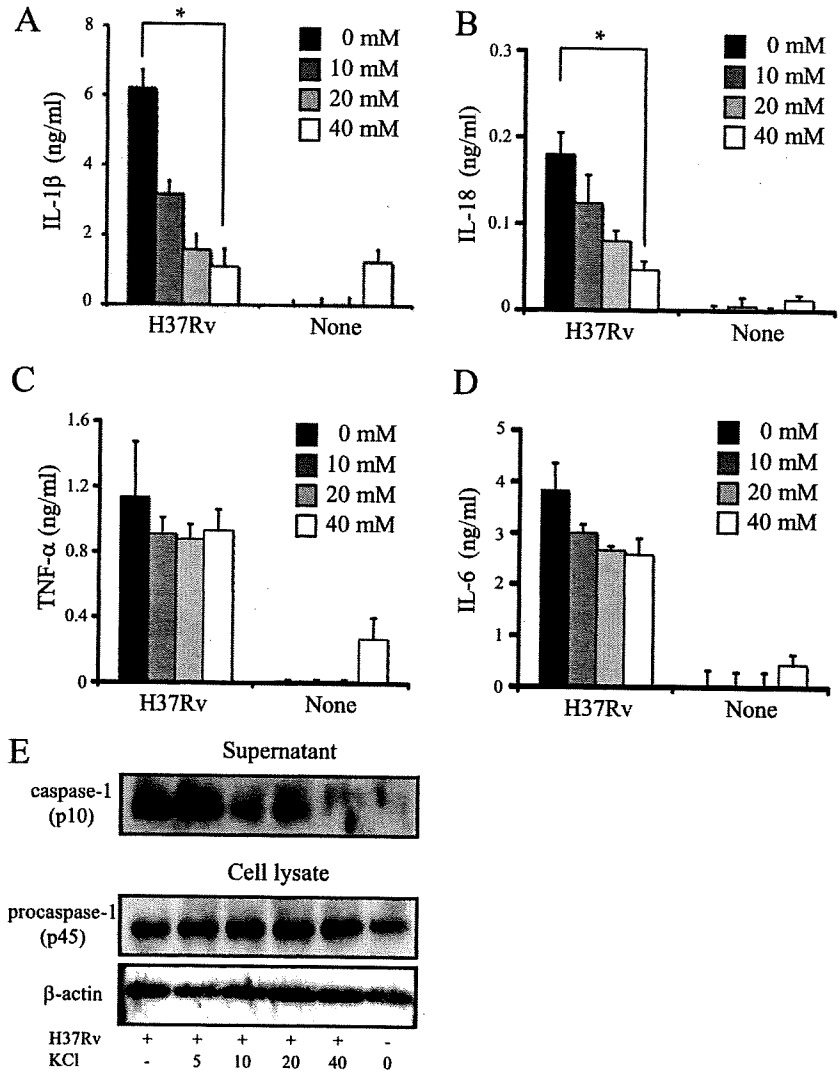

FIG. 4. Potassium ion efflux is essential for the secretion of IL-1β and IL-18 and activation of caspase-1 in *M. tuberculosis*-infected macrophages. Peritoneal macrophages were infected with H37Rv at an MOI of 5 for 24 h in the presence or absence of KCl at 0 mM (black columns), 10 mM (dark gray columns), 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*.

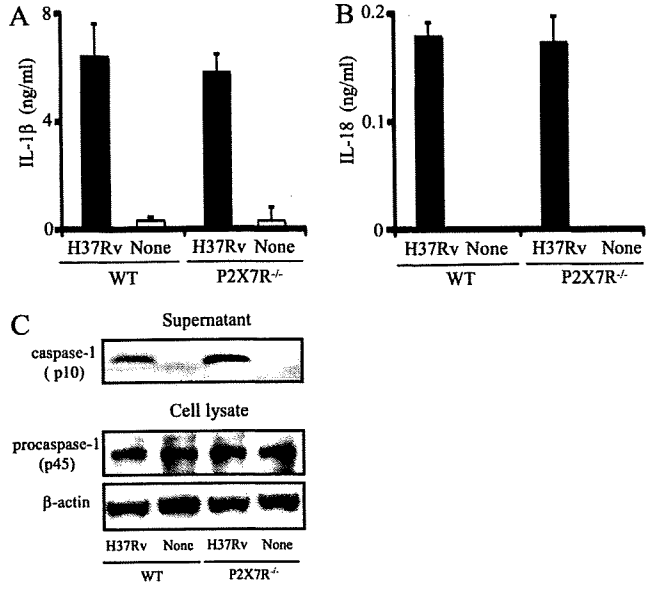

FIG. 5. P2X7 receptor does not contribute to the activation of caspase-1 in *M. tuberculosis*-infected macrophages. Peritoneal exudate macrophages from C57BL/6 and P2X7R$^{-/-}$ mice were infected with H37Rv at an MOI of 5 for 24. The levels of IL-1β (A) and IL-18 (B) in culture supernatants were determined 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.

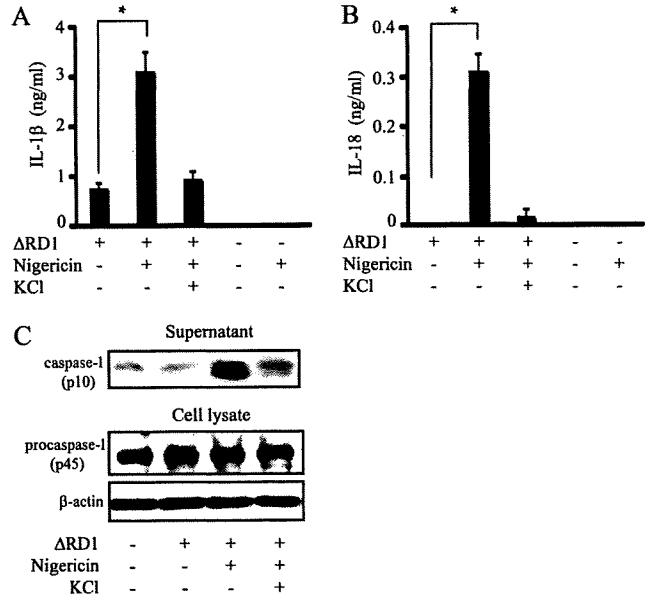

FIG. 6. The inability of H37RvΔRD1 to induce production of IL-18 and IL-1β is compensated by the addition of potassium ionophore (nigericin). Peritoneal macrophages were infected with ΔRD1 at an MOI of 5 for 21 h. Nigericin (5 μM) and/or KCl (30 mM) was added, and the culture was continued for another 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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Virulence of *Mycobacterium avium* complex strains isolated from immunocompetent patients

Yoshitaka Tateishi [a,b,*], Yukio Hirayama [a], Yuriko Ozeki [a,c], Yukiko Nishiuchi [a,d], Mamiko Yoshimura [a], Jing Kang [a], Atsushi Shibata [a], Kazuto Hirata [e], Seigo Kitada [b], Ryoji Maekura [b], Hisashi Ogura [f], Kazuo Kobayashi [g], Sohkichi Matsumoto [a,*]

[a] Department of Bacteriology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
[b] Department of Internal Medicine, National Hospital Organization Toneyama National Hospital, 5-1-1 Toneyama, Toyonaka, Osaka 560-8552, Japan
[c] Sonoda Women's University, 7-29-1 Minamitsukaguchi-cho, Amagasaki, Hyogo 661-8520, Japan
[d] Toneyama Institute for Tuberculosis Research, Osaka City University Medical School, Toyonaka 5-1-1, Toneyama, Toyonaka, Osaka 560-8552, Japan
[e] Department of Respiratory Medicine, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
[f] Department of Virology, Osaka City University Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan
[g] Department of Immunology, National Institute of Infectious Diseases, 1-23-1, Toyama, Shinjuku-ku, Tokyo 162-8640, Japan

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ABSTRACT

Mycobacterium avium complex (MAC) disease has been increasing worldwide not only in immunocompromised but also in immunocompetent humans. However, the relationship between mycobacterial strain virulence and disease progression in immunocompetent humans is unclear. In this study, we isolated 6 strains from patients with pulmonary MAC disease. To explore the virulence, we examined the growth in human THP-1 macrophages and pathogenicity in C57BL/6 mice. We found that one strain, designated 198, which was isolated from a patient showing the most progressive disease, persisted in THP-1 cells. In addition, strain 198 grew to a high bacterial load with strong inflammation in mouse lungs and spleens 16 weeks after infection. To our knowledge, strain 198 is the first isolated MAC strain that exhibits hypervirulence consistently for the human patient, human macrophages *in vitro*, and even for immunocompetent mice. Other strains showed limited survival and weak virulence both in macrophages and in mice, uncorrelated to disease progression in human patients. We demonstrated that there is a hypervirulent clinical MAC strain whose experimental virulence corresponds to the serious disease progression in the patients. The existence of such strain suggests the involvement of bacterial virulence in the pathogenesis of pulmonary MAC disease in immunocompetent status.

1. Introduction

Mycobacterium avium complex (MAC) is the most common cause of human infection due to nontuberculous mycobacteria. Initially MAC was regarded as only an opportunistic pathogen, primarily in acquired immunodeficiency syndrome (AIDS) patients [1]; however, it has now been shown to cause progressive pulmonary disease even in immunocompetent humans [2]. The American Thoracic Society indicates a wide range of clinical manifestation in patients with non-AIDS MAC disease; some patients keep a stable condition for years, whereas others progress their illness rapidly [3]. Furthermore, MAC infection can be more difficult to treat than *M. tuberculosis* due to even fewer available anti-microbial agents [3].

The pathogenesis of MAC infection has been recently investigated with respect to the host immune response. Interferon-gamma (IFN-γ) activates macrophages to produce proteolytic enzymes and other metabolites, which exhibit mycobactericidal effects. Tumor necrosis factor-alpha (TNF-α), of which production is also stimulated by IFN-γ, augments the bactericidal capacity of macrophages and plays a key role in the induction of the acquired immune response against mycobacteria [4]. A defective IFN-γ response has been shown recently to cause disseminated MAC disease in IFN-γ knock out mice and in humans with genetic mutations of IFN-γ receptor [5,6] or autoantibodies to IFN-γ in some young non-AIDS patients [7,8]. In addition to that, the activity of interleukin-10 (IL-10), which is known to inhibit cytokine synthesis by IFN-γ-producing type1 helper T cells (Th1 cells), has been shown to increase susceptibility to MAC infection in immunocompetent mice [9].

* Corresponding authors. Department of Bacteriology, Osaka City University, Graduate School of Medicine, 1-4-3 Asahi-machi, Abeno-ku, Osaka 545-8585, Japan. Tel.: +81 6 6645 3746; fax: +81 6 6645 3747.
E-mail address: y-tateishi@med.osaka-cu.ac.jp (Y. Tateishi).

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Besides genetic factors of the host, bacterial virulence should play an important role for the development of MAC disease. While isolates of *M. tuberculosis* are genetically homogeneous at the nucleotide level [10], MAC has high genetic diversity, including the presence of multiple plasmids [11], and thus likely to have a large corresponding diversity in virulence. In the most complete study examining virulence, forty-one MAC isolates from the environment as well as infected humans and animals were compared for virulence in C57BL/6 mice by intravenous injection [12]. Monitoring of the virulence by CFU counts in lungs, livers, and spleens over 4 months revealed three virulence phenotypes; high (logarithmically increasing load), intermediate (chronic infection at a constant load), and low (initial load increase followed by a decrease until clearance). In addition, clinical studies have suggested severe disease outcome in patients infected with some specific strain type of MAC. For example, MAC serovars 1, 4, and 8 *Mycobacterium avium* are associated with disease severity in AIDS patients [13], and a serovar 4 *M. avium* isolate from an AIDS patient was more invasive and proliferative in blood mononuclear cell-derived human macrophages than a serovar 2 strain from chickens [14]. In non-AIDS MAC disease, *Mycobacterium intracellulare* is associated with greater disease progression [15], and moreover, our previous prospective study on 68 non-AIDS patients suggests that serovar 4 *M. avium* is linked to greater disease progression with a pulmonary MAC infection [16]. Taking these previous data into consideration, we hypothesize that relatively hypervirulent MAC strains exist and may be associated with serious disease progression in immunocompetent patients. In order to elucidate the involvement of mycobacterial virulence in the pathogenesis of human pulmonary MAC disease, in this study we examined the difference of mycobacterial virulence of clinical isolates from patients with different disease types using human macrophages and immunocompetent mice.

2. Results

2.1. Characteristics of mycobacterial strains

Six clinical isolates of MAC were isolated from sputum of non-AIDS patients with pulmonary MAC disease, and designated 27, 33, 36, 198, 288, and 347 (Table 1). Strains 33, 198 and 288 were derived from patients with progressive disease against combination chemotherapy recommended by the American Thoracic Society guideline (progressive type) [3]. The patients with progressive disease exhibited higher levels of erythrocyte sedimentation rate (ESR), diffuse and severe pulmonary lesions in chest X-ray findings, and numerous bacteria in the sputum. The patient infected with strain 198 exhibited the most serious disease outcome among study patients in that a right pneumonectomy was needed to prevent disease progression. Strains 27, 36, and 347 were derived from patients with little progression of disease without chemotherapy (silent type). They exhibited lower levels of ESR, segmental pulmonary lesions in chest X-ray findings, and fewer bacteria in the sputum. The isolates belonging to the progressive type consisted of *M. intracellulare* unclassified serovar similar to serovar 12 (strain 198) and *M. avium* apolar type (strains 33 and 288). The isolates belonging to the silent type consisted of *M. intracellulare* serovar 1 (strain 27) and *M. avium* apolar type (strains 36 and 347). For comparison, we employed 2 veterinary strains of *M. avium* ATCC 25291 (serovar 2) as a highly virulent strain in mice [12] and ATCC 35767 (serovar 4) as a low virulent strain. Four clinical isolates other than strains 33 and 347, and ATCC 25291 formed the transparent colony morphology. Strain 33 produced both transparent and rough colony morphologies. Strain 347 and ATCC 35767 displayed smooth opaque colony morphology.

2.2. Growth of clinical isolates in 7H9 broth

All strains showed logarithmic growth from 3 days after culture in 7H9 broth (Table 2). At day 5, two isolates from progressive type (strains 198 and 288) and one isolate from silent type (strain 36) grew significantly slower than ATCC 25291 ($P < 0.005$), and all clinical strains grew significantly slower than ATCC 35767 ($P < 0.0001$). The growth of strain 198 at day 5 was significantly slower than that of strain 27 ($P = 0.001$), and was not significantly different from that of other clinical isolates.

2.3. Virulence of clinical isolates in THP-1 monocyte-derived macrophages

We next studied intracellular survival of the isolates. THP-1 cells, a human monocytic cell line, were differentiated into macrophages by treatment with phorbol 12-myristate 13-acetate (PMA) and infected with MAC strains. Strain 198 grew in THP-1 cells significantly higher than any other strains during 7 days of infection ($P < 0.0001$) (Table 3). Strain 198 grew to approximately 20-fold during 2 days of infection ($P = 0.005$), and even at day 7, it kept the same level of bacterial load as day 0. Strain 36 also grew to approximately 2-fold during 2 days of infection ($P = 0.008$); however, it was rapidly eliminated at day 7, similar to the other strains except for strain 198. There was no significant difference in

Table 1
Characteristics of isolated strains and clinical findings.

Isolates	Species and serovar	Age	Sex	Duration of illness (years)	Erythrocyte sedimentation rate (mm/h)	Chest X-ray findings[a]	Sputum[b]	
							Smear	Culture
Progressive type								
33	*M. avium* apolar type	58	M	17	62	Advanced	2+	3+
198	*M. intracellulare* unclassified serovar[c]	62	F	3	108	Advanced	2+	2+
288	*M. avium* apolar type	56	F	12	78	Advanced	2+	2+
Silent type								
27	*M. intracellulare* serovar 1	67	F	17	50	Moderate	–	1+
36	*M. avium* apolar type	54	F	9	29	Moderate	–	1+
347	*M. avium* apolar type	79	F	14	50	Moderate	1+	1+

Data and sputum samples were collected at the enrollment of the study in 2003.

[a] Advanced chest X-ray findings were defined as bilateral cavities, giant cavities, or bilateral bronchiectasis, and moderate findings were defined as focal inflammation, small or fewer cavities, or mild bronchiectasis.

[b] Smear findings of sputum were defined as follows in high performance fields of microscopy; -: no bacteria in all fields, 1+: less than one bacteria in several fields, 2+: approximately 1–12 bacteria in one field. Culture findings were defined as follows using Ogawa egg agar; 1+: colonies less than 200, 2+: colonies more than 200 and less than 500, 3+: colonies more than 500 and less than 2000.

[c] The serovar of strain 198 was identified as a new type similar to serovar 12 determined by the liquid chromatography/mass spectrometry.

Table 2
Growth rate of MAC in 7H9 broth.

Strain	Ratio of CFUs at[a]		
	Day 1	Day 3	Day 5
33	0.91 ± 0.28	38 ± 0.86	63 ± 10
198	0.93 ± 0.17	12 ± 1.9	18 ± 4.0
288	0.85 ± 0.20	4.3 ± 1.9	16 ± 0.95
27	1.1 ± 0.25	7.3 ± 2.2	120 ± 21
36	0.83 ± 0.093	5.4 ± 0.21	11.0 ± 1.7
347	0.96 ± 0.16	2.7 ± 1.1	44 ± 19
25291	1.6 ± 0.25	4.8 ± 0.24	110 ± 16*
35767	0.97 ± 0.12	11 ± 3.0	370 ± 43**

* Significantly different ($P < 0.005$) from values for strain 198, 288, and 36 as calculated by Scheffé's test.
** Significantly different ($P < 0.0001$) from values for all clinical strains as calculated by Scheffé's test.
[a] Means ± standard deviations of the ratio of CFUs to those at day 0.

the growth rate among these strains except strain 198 during infection.

On light microscopic observation, THP-1 cell morphologies were not different between infected and uninfected cells (data not shown). We then assessed cytotoxicity by the levels of lactate dehydrogenase (LDH) released into the culture supernatants at day 7. The LDH release was detectable in strain 33 and the laboratory strains (strain 33; 5.8 ± 1.5%, ATCC 25291; 11 ± 1.0%, ATCC 35767; 12 ± 1.9%, without significant difference among these strains); however, it was not detectable in other clinical isolates.

2.4. Pathogenesis of clinical isolates in mice

Female C57BL/6 mice were infected by intratracheal instillation with each strain. Bacterial load in lungs, livers, and spleens were evaluated, and histological inflammation was visually analyzed in 5-mice per strain at defined time points during 16 weeks of infection. There was no significant difference in lung CFUs among strains tested 1 day after the inoculation.

Strain 198 showed high bacterial load, and tended to increase gradually both in lungs and spleens during 16 weeks of infection ($P = 0.08$ between day 1 and 16 weeks) (Fig. 1). Strain 198 was loaded in lungs significantly higher than strain 27 ($P = 0.04$) and 33 ($P = 0.0006$) at 8 weeks of infection, and than strain 33 ($P = 0.0009$), 288 ($P = 0.001$), 36 ($P = 0.0003$), and 347 ($P = 0.004$) at 16 weeks. Histologically, strain 198 induced strong inflammation in lungs, which was paralleled with bacterial loads (Fig. 2). ATCC 25291, known as highly a virulent strain in mice [12], showed initial reduction of bacterial load in lungs at 4 weeks of infection ($P = 0.01$ between day 1 and 4 weeks) and rapid increase in bacterial load in lungs after 4 weeks of infection. ATCC 25291 was comparatively virulent to strain 198 with respect to the high bacterial load in lungs and spleens, and severe pulmonary inflammation at 16 weeks of infection. By contrast, other clinical isolates did not increase profoundly in lung CFUs; however, these strains were never eliminated from lungs. ATCC 35767 was rapidly decreased and undetectable in lungs, spleens and livers within 16 weeks of infection. Overall, the clinical isolates other than strain 198 exhibited limited histological lesions with transient inflammatory changes in lungs 4 weeks after the inoculation, and thereafter the inflammation subsided at 16 weeks.

Table 3
Growth rate of MAC in THP-1 cells.

strain	Ratio of CFUs at[a]	
	Day 2	Day 7
33	0.29 ± 0.12	0.25 ± 0.16
198	18 ± 12*	1.30 ± 0.68*
288	0.47 ± 0.26	0.097 ± 0.055
27	0.54 ± 0.37	0.28 ± 0.11
36	2.4 ± 1.2	0.31 ± 0.11
347	1.1 ± 0.49	0.36 ± 0.23
25291	0.16 ± 0.048	0.11 ± 0.038
35767	0.059 ± 0.029	0.0070 ± 0.0048

* Significantly different ($P < 0.0001$) from values for any other strains studied as calculated by Scheffé's test.
[a] Means ± standard deviations of the ratio of CFUs to those at day 0.

3. Discussion

Virulence is defined as the quantitative ability of an agent to cause disease. The virulence of mycobacteria can be evaluated by the infection to macrophages and animals [17]. This is the first study that examined the virulence of MAC isolates from immunocompetent patients with different types of disease outcome. We found that strain 198, which derived from a patient with most serious disease, revealed high bacterial load both in THP-1 cells and in C57BL/6 mice among isolates studied. Strain-specific virulence of MAC has been implicated by some previous studies of the serovar 4 *M. avium* isolated from patients. In AIDS-related MAC disease, a serovar 4 *M. avium* isolate has shown to be one of the frequently isolated type [13], and a previous analysis of a serovar 4 isolate and ATCC strain has shown the superior virulence of serovar 4 *M. avium* in human macrophages [14]. In non-AIDS pulmonary MAC disease, our recent prospective study indicates that patients infected with serovar 4 *M. avium* has poorer prognosis than those infected with MAC of other serovars [16]; however, to our best knowledge, no study has shown the direct data of mycobacterial virulence of clinical isolates and clinical disease outcome. Strain 198 is the first MAC isolate whose experimental virulence corresponds to the serious disease outcome in humans. Thus, strain 198 has strain-specific strong virulence for immunocompetent humans and mice. We consider that strain 198 is worth further genetic investigation of virulence factors.

MAC strains hypervirulent for mice has been isolated previously by Pedrosa et al. including ATCC 25291 and MAC 101, which proliferate profoundly in mouse macrophages and in mice *in vivo* [12]. In this study, strain 198 proliferated in human macrophages, in correspondence with rapid clinical disease progression and additionally in mouse lungs. The consistency between experimental virulence in human cells and clinical disease outcome suggests that the capability of inducing such strong pathogenesis may be attributed mostly to the characteristics of the pathogen, i.e. virulence factor(s) for mammalian cells unique to strain 198. Previously Birkness et al. has shown the strong cytotoxic effect and growth of serovar 4 *M. avium* isolated from an AIDS patient in blood mononuclear cell-derived human macrophages compared with a serovar 2 strain from chickens (ATCC 35713). Therefore, we evaluated cytotoxicity of MAC strains by the microscopic morphology and by the LDH release from infected THP-1 cells; however, contrary to the expectation, strain 198 was not cytotoxic to THP-1. In addition, the release of LDH was lower in strain 33, ATCC 25291, and ATCC 35767 than in the previous experiment of *M. tuberculosis* infection to THP-1 cells (cytotoxicity in cases of *M. tuberculosis* H37Rv and H37Ra; approximately 30%) [18]. We assume that cytotoxic effect may not play a major role in displaying the virulence of MAC during infection, suggested by the similar result by Huttunen et al. showing the lack of cytotoxic effect of MAC in human 28SC macrophage and A549 lung epithelial cell lines evaluated by 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay [19]. We speculate the virulence of strain 198 depends on the ability to survive or proliferate in macrophages rather than cytotoxic effect, which, may be causative for severe pulmonary MAC disease with rapid disease progression within a few years.

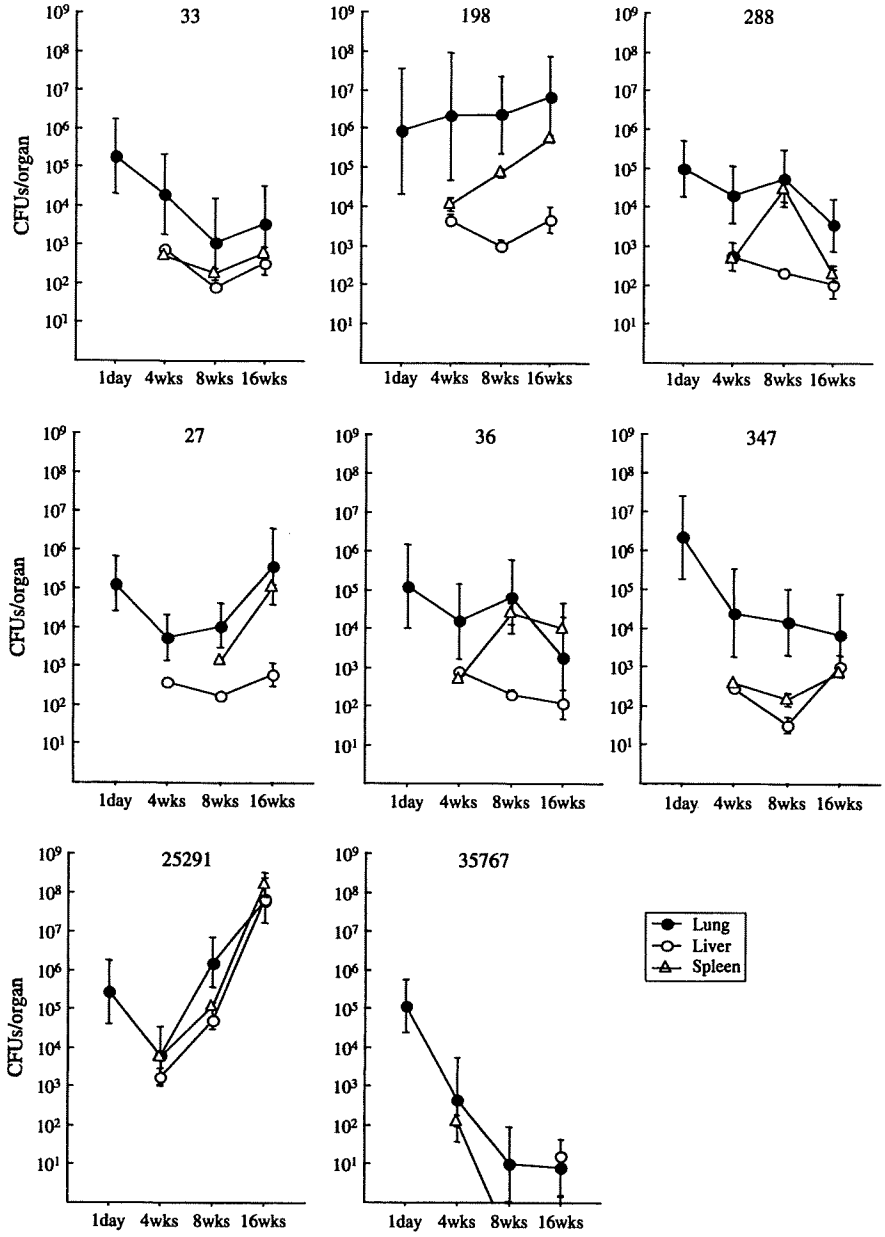

Fig. 1. Time course of mycobacterial growth in lungs, spleens and livers of C57BL/6 mice. Bacterial suspensions containing 1×10^5 CFUs were inoculated intratracheally to female C57BL/6 mice at the age of 7 weeks ($n = 20$ per strain). The lungs, livers and spleens of 5 mice per strain were sectioned at day 1 (only lungs), 4, 8, and 16 weeks later from challenge Data were presented as means ± standard deviations of CFUs/organ.

In this study, strain 198 showed strong virulence for mice at 16 weeks of infection similar to ATCC 25291; however, virulence for THP-1 cells was quite different, and pathogenic effects for mice within 4 weeks of infection was dissimilar between these two strains. These differences can be explained by the difference of immune response between host species and by the difference of immune phase. First, strain 198 could proliferate, but ATCC 25291 was rapidly eliminated in THP-1 cells (Table 3). In mouse macrophages mycobactericidal activity is attributed to nitric oxide produced by inducible nitric oxide synthase [20], whereas in human macrophages, it is attributed to Toll-like receptor signaling-dependent production of anti-microbial peptides [21,22]. Strain 198 is capable of proliferating under these two patterns of mycobactericidal activities, which suggests that strain 198 may have some virulence factors advantageous to survive both in human and mouse macrophages against mycobactericidal activity of the hosts, in contrast to ATCC 25291 which may lack virulence factors to survive in human macrophages. Second, strain 198 showed high bacterial load in lungs continuingly during 16 weeks of infection in mice, while ATCC 25291 proliferated after initial reduction in lungs at 4 weeks of infection (Fig. 1). The *in vitro* infection model using cell lines and the *in vivo* infection model using mice within 4 weeks reflects early stages of infection; on the other hand, the *in vivo* model after 8 weeks reflects chronic phase of infection [17,23]. The difference of pathogenic effects for mice within 4 weeks of infection suggests that strain 198 may resist both innate and acquired immunity, while ATCC 25291 may resist acquired immunity only. We assume that strain 198 and ATCC 25291 may possess different virulence mechanisms to persist in *in vivo* after development of acquired immunity.

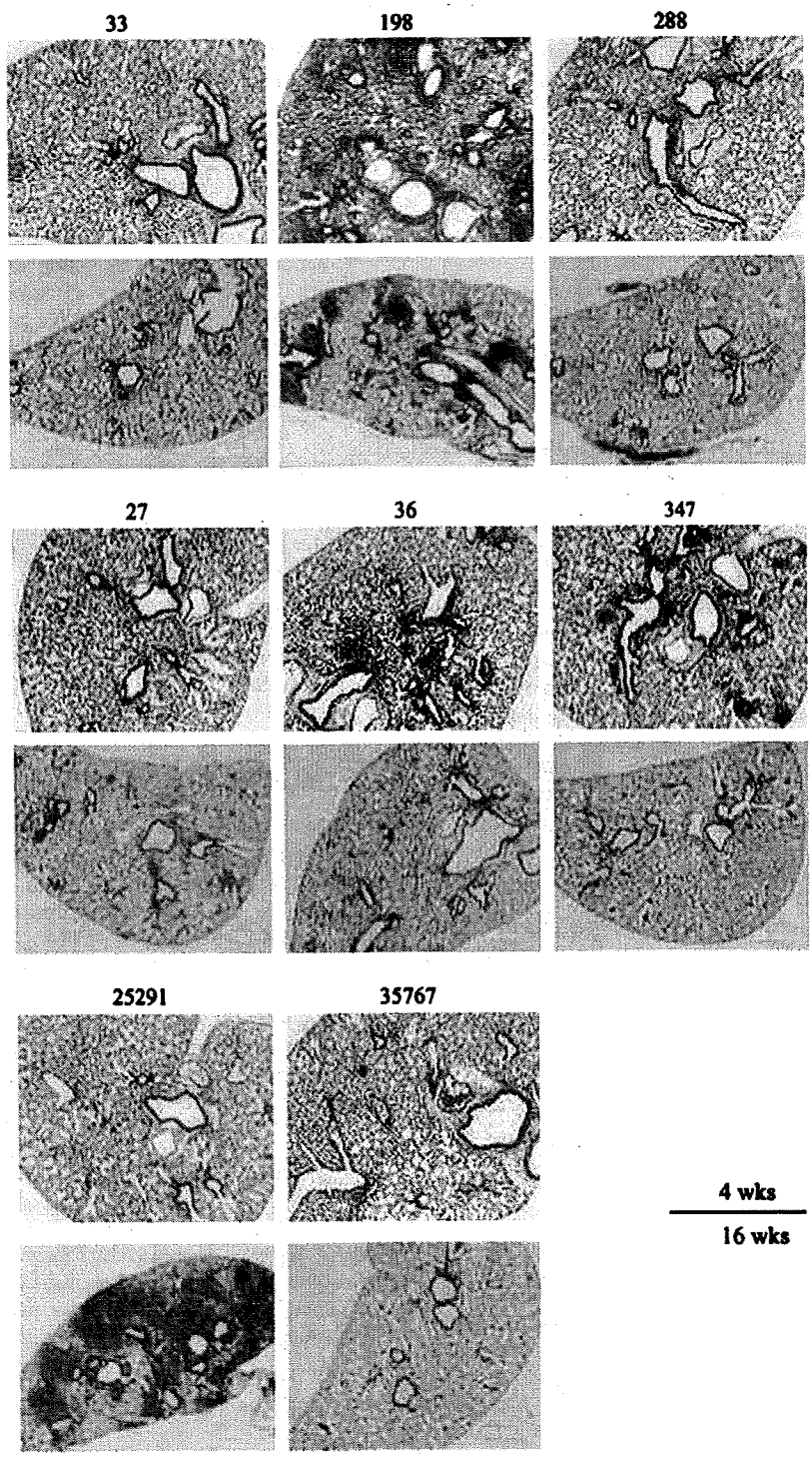

Fig. 2. Histological pictures of the lungs during 4 weeks or 16 weeks of infection in C57BL/6 mice by hematoxylin–eosin staining. Magnification, ×40.

In this study, clinical strains except for strain 198 did not show consistent virulence-associated phenotype among THP-1 cells, C57BL/6 mice, and clinical disease outcome. Similarly, Pedrosa et al. has also revealed that the growth of MAC in bone-marrow derived macrophages does not necessarily predict the virulence in mice by comparing the growth of 41 MAC isolates from various derives including humans, animals, and environment [12]. Although some clinical cases of pulmonary MAC disease may be caused by hypervirulent strains such as strain 198, these findings of clinical and natural isolates suggest that virulence may not the only determinant of the pathogenesis of pulmonary MAC disease in the majority of clinical cases. The development of pulmonary MAC disease depends on the balance between bacterial virulence and host defense. It is widely accepted that patients with pulmonary MAC disease have some characteristics of clinical background, such as males in their 40s and early 50s who have a history of cigarette smoking and excessive alcohol use, and such as postmenopausal, nonsmoking females [3], and these patient characteristics might

possibly indicate unknown predisposing conditions which enhance susceptibility for pulmonary MAC infection. The diverse phenotype of clinical MAC strains may be attributed to the disease susceptibility of the hosts. We propose that pathogenic mechanism of human pulmonary MAC disease include two patterns; one is that the strong virulence of MAC strains such as strain 198 induces rapid mycobacterial growth and serious disease outcome, and the other is that relatively weak to moderate virulence interacts with predisposing conditions of the host, leading the wide range of clinical outcome.

This study was preliminary in that we did not identify the mechanism of hypervirulence of strain 198. We observed the consistency between hypervirulence in human macrophages bedsides in immunocompetent mice and severe clinical outcome only in strain 198, not in any other isolates studied. From this finding, we speculate the existence of strain-specific virulence factors of strain 198. Recent exponential advances have enabled whole genome sequence of two *M. avium* strains, *M. avium* 104 and *M. avium* subsp. *paratuberculosis* K-10. Based on these exhaustive information, comparative genomics of MAC organisms has revealed the different genomic components regarding virulence factors, such as *ser2* encoding glycosylation enzyme of the lipopeptide core to generate the glycopeptidolipids, mammalian cell entry (*mce*) gene homologs, and PE/PPE genes (i.e., with Pro Glu and Pro Pro Glu motifs) [24]. In addition, there are large sequence polymorphisms among MAC organisms, suggesting a large corresponding diversity in virulence [11,24]. We speculate that the virulence of MAC strains including strain 198 may be determined by insertion or deletion of virulence genes encoding known [24] or unknown virulence factors.

In summary, we demonstrated that certain clinical strain derived from patients of the progressive pulmonary MAC disease exhibits strong virulence in human macrophages and in immunocompetent mice. Among clinical isolates, strain 198 is the first isolate hypervirulent to both human macrophages and mice. Our data suggest that strain-to-strain differences in virulence may play a significant role in disease progression in humans. Although Sarmento et al. showed that capability of TNF-α production from macrophages inversely correlates with the virulence of MAC strains [25], we could not find such relationship among the isolates (data not shown). In future studies, we will identify the virulence/pathogenicity-associated factor(s) of strain 198 and survey the frequency of strain variation in immunocompetent patients with pulmonary MAC disease.

4. Materials and methods

4.1. Bacterial strains

We used six clinical isolates from non-AIDS patients with pulmonary MAC disease and two laboratory strains, *M. avium* ATCC 25291 (serovar 2) and *M. avium* ATCC 35767 (serovar 4), in this study. Clinical isolates were obtained between September and November in 2003 at Toneyama National Hospital. Informed consent was obtained from all patients according to the guideline of Institutional Review Board of Toneyama National Hospital. Diagnosis of pulmonary MAC disease was made according to the American Thoracic Society guideline [3]. The samples were derived from two groups of patients; one group exhibited progressive disease in spite of the combination chemotherapy including clarithromycin, ethambutol and rifampin recommended by the American Thoracic Society guideline (progressive type) [3], the other displayed no exacerbation without anti-microbial chemotherapy for approximately ten years or more (silent type). These types were determined by the laboratory findings at the period of sputum sampling (including sputum smear and culture, chest X-ray findings, and erythrocyte sedimentation rate) and the rapidness of disease progression (Table 1). Sputum specimens were mixed with 2% sodium hydroxide, and *N*-acetyl-L-cysteine and then centrifuged for 15 min at 3000 *g*. The supernatants were discarded, and the sediment was mixed at 1:10 (vol/vol) with sterile water. The bacteria were cultivated in Middlebrook 7H9 broth supplemented with albumin–dextrose–catalase, 0.02% glycerin and 0.05% Tween 80, and then kept at −80 °C until following experiments. Identification of MAC was made by polymerase chain reaction using a commercially available kit (AMPLICOR Mycobacterium Tuberculosis Test, Roche, Basel, Switzerland). The serovars of clinical isolates were identified by the liquid chromatography/mass spectrometry as described previously [26]. Strains not containing serovar-specific oligosaccharides were defined as apolar type.

4.2. Growth in 7H9 broth

Bacterial suspension was adjusted to be 0.2 by optical density (OD) at 630 nm. The samples were cultured in 5 ml of 7H9 media in plastic tubes without agitation After vortexing to dissolve aggregates, cultivated bacterial suspensions were inoculated at days 1, 3, and 5 by serial 10-fold dilutions on Middlebrook 7H11 agar plates supplemented with oleic acid–albumin–dextrose–catalase, and 0.05% glycerol (7H11-OADC) agar plates in triplicate. The number of CFUs was counted after cultivating at 37 °C for 3 weeks.

4.3. Infection of THP-1 cells with MAC in vitro

THP-1 cells were purchased from Health Science Research Resources Bank (Tokyo, Japan). The cells were cultured in RPMI1640 containing 10% heat-inactivated fetal bovine serum (FBS; Equitech-bio, TX), and subcultured every 3–4 days. THP-1 cells were differentiated by 100 nM PMA (Sigma–Aldrich, St Louis, MO) for 48 h before infection. Before 48 h of infection, 1 ml of 2×10^5/ml cells was cultured in RPMI1640 containing 5% human serum (AB-blood group) in 24-well plates. Then, 1 ml of 2×10^4 CFUs/ml bacteria was exposed to the cultured cells for 24 h without opsonization (multiplicity of infection; 0.1 bacteria/cell). After that, the cells were treated with 20 μg/ml of gentamicin for 3 h to kill extracellular bacteria, followed by washing 4 times by RPMI1640. The infected cells were cultured in 2 ml of RPMI1640 containing 5% human serum. At days 0 and 7, uninfected bacteria were removed by washing with RPMI1640 4 times, and 500 μl of filter-sterilized phosphate buffered saline containing 0.5% Triton X-100 (Wako, Osaka, Japan) was treated per well to lyse cell membrane. The intracellular survival of bacteria was determined by counting CFUs by inoculating the cell lysate on 7H11-OADC agar plates. The experiment was performed in triplicate.

4.4. Assays for cytotoxicity

Cytotoxic effects were evaluated by the release of LDH from the cells. LDH activity of culture supernatants was determined by a commercially available kit (Roche, Basel, Switzerland). Supernatants were diluted to be 10^{-1} by distilled water for optimal reaction. The diluents were reacted with reaction mixture for 30 min, and then the OD was measured at 492 nm. Supernatants of completely lysed uninfected cells with filter-sterilized phosphate buffered saline containing 20% Triton X-100 and those of uninfected cells untreated with Triton X-100 were served as high and low controls, respectively. Cytotoxicity (%) was calculated as follows; $(OD_{sample} - OD_{low\ control}) \times 100/(OD_{high\ control} - OD_{low\ control})$. The measurement was performed in triplicate.