observed in HIES patients. Another remarkable feature of HIES is that patients are often afebrile and feel well[1], despite serious pneumonia or dermal pathology[4]. Indeed, acute-phase responses, such as an increase in serum C-reactive protein during severe infections, were diminished in our patients. STAT3 was originally identified as a protein binding to the IL-6-responsive element in the genes encoding hepatic acute-phase proteins[21,22], and the liver-specific inactivation of STAT3 leads to an impaired acute-phase response in mice[23]. Thus, the apparent lack of classical inflammatory responses in HIES patients could be attributed to defective signalling of pro-inflammatory cytokines, including IL-6.

Enhanced IgE production in the patients may reflect dysregulated immune responses owing to the impaired response to IL-10, a critical negative regulator[24], even though the exact mechanism of hyper IgE remains to be determined, as in the case of other disorders such as Wiskott-Aldrich syndrome. HIES patients often suffer from severe staphylococcal infection in the skin and lung. STAT3 plays a critical part in $T_H17$ development[25], and IL-17 produced by $T_H17$ cells is protective in the host defence against extracellular bacteria[26]. IL-22 stimulates cells in the skin and respiratory systems to produce β-defensins through STAT3 activation[27]. Thus, the susceptibility to bacterial infection could be attributed, at least in part, to the defects in $T_H17$ development and IL-22 signalling. Among the 15 sporadic HIES patients investigated in this study, no apparent difference was observed in clinical phenotypes and severity between those with the STAT3 mutations and those without the mutations, indicating that other HIES aetiology might be functionally linked to STAT3.

In summary, the present study identified a human deficiency in STAT3 as a major cause of multisystem HIES. This study highlights the multiple and critical roles of STAT3 in humans. The identification of these STAT3 mutations as causative for HIES, in addition to the previous finding of a causative mutation in TYK2 (ref. 8), underlines the critical involvement of a variety of cytokine signals in the pathogenesis of HIES. The diagnosis of HIES early in life is often hampered by a paucity of specific clinical features. Our discovery of STAT3 as a major causative gene of this disease will facilitate earlier and definitive diagnosis, leading to the prevention of serious complications by prompting the start of prophylactic antibiotic treatment early in life.

## METHODS SUMMARY

**Patients.** This study was approved by the institutional review board at the Tokyo Medical and Dental University; written informed consent was obtained from all the individuals studied. The clinical characteristics of the eight HIES patients investigated in this study are summarized in Supplementary Table 1, and all the patients display definitive phenotypes of multisystem HIES (score ≥ 40).

**Stimulation of cells with cytokines, and measurement of cytokines and IgM production.** Cells were stimulated for the indicated time in culture with cytokines as described previously[8]. The concentration of IFNγ and TNFα in the culture supernatants was determined by ELISA (BD-PharMingen), according to the manufacturer's instructions. The amount of IgM secretion from EBV-infected B cells was determined as previously described[28]

**RT–PCR and direct sequence analysis.** Extraction of total RNA, cDNA synthesis, PCR, semiquantitative RT–PCR, and sequencing were performed as previously described[29].

**Immunoblotting and immunoprecipitation.** Immunoblotting and immunoprecipitation were performed as described previously[8].

**Enzyme-linked DNA–protein interaction assay.** Binding of STAT3 and STAT1 to their target DNA was measured using the Mercury TransFactor kit (Clontech Laboratories) according to the manufacturer's protocol.

**Retroviral infections.** Retroviral infections were done as described previously[8].

**Flow cytometric analysis.** The surface immunophenotype was analysed as described[30].

**STAT3 knock-down.** Transfection of short interfering RNA (siRNA) oligonucleotides (5′-ccugcaagagucgaauguucucuau-3′ and 5′-gcaguuucuucagagcaguaucuu-3′) was performed as described previously[8]. Forty hours after transfection, the cells were treated with IFNα for 5 h. In the experiment shown in Fig. 4a, nucleotide sequences of wild-type and mutant STAT3 cDNAs were modified so that they were insensitive to STAT3 siRNA, but they still encoded the original amino acid sequences of STAT3.

**Luciferase reporter assay.** The reporter construct of STAT3-responsive elements linked to a luciferase reporter gene was transfected with wild-type or mutant STAT3. Forty hours after the transfection, the cells were stimulated with 100 ng ml$^{-1}$ IL-6 for 5 h. Luciferase activity was measured with a dual-luciferase assay system according to the manufacturer's protocol (Promega).

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

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**Author Contributions** Y.M. designed and conducted most of the experiments; M.S. conducted the genetic analysis and the generation of osteoclasts; S.T., I.T., H.T., T.H., N.K., T.A., S.P. and A.M. diagnosed and collected samples; O.S. collected samples; H.K. oversaw the entire project; Y.M. and H.K. wrote the manuscript with comments from all co-authors.

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## METHODS

**Patients.** An immunological work-up revealed high serum IgE in all the patients and eosinophilia in five of them. All other laboratory data examined were within the normal range, including the lymphocyte subpopulations, their proliferative responses to mitogens, the levels and subclasses of serum immunoglobulins, the oxidative burst of granulocytes, and the number and size of platelets.

**Antibodies and cytokines.** Antibodies against STAT3, tyrosine-phosphorylated STAT3, Flag and HA, and HRP-conjugated rabbit anti-mouse and goat anti-rabbit antibodies were purchased from Cell Signaling. The CD117 monoclonal antibody was from BD-PharMingen, and the CD3 monoclonal antibody (OKT3) was from Janssen Pharmaceutical. Recombinant human IL-6, IL-10, IL-12, IFNα, and GM-CSF were purchased from Peprotech, recombinant human IL-2 from Shionogi, and lipopolysaccharide (055:B5) from Sigma-Aldrich.

**Isolation and culture of T cells and macrophages from PBMCs.** Isolation and cell culture of T cells and macrophages were performed as described previously[8]. All the experiments were performed at least three times with three different controls.

**Stimulation of cells with cytokines, and measurement of cytokines and IgM production.** Cells were stimulated for the indicated time in culture with IL-6 (100 ng ml$^{-1}$), IL-10 (100 ng ml$^{-1}$), IL-12 (10 ng ml$^{-1}$), or IFNα (5 ng ml$^{-1}$) as described previously[8].

**RT–PCR and direct sequence analysis.** Sequencing was performed with an ABI Prism dRhodamine Terminator kit and analysed with an ABI Prism 310 DNA Sequencer (Perkin-Elmer Applied Biosystems). At least two independent PCR products were sequenced.

**Enzyme-linked DNA–protein interaction assay.** Thirty micrograms of nuclear extracts were incubated in a 96-well microplate precoated with oligonucleotides encoding the consensus binding sequence for STAT1 or that for STAT3. Bound STAT3 or STAT1 was detected with specific antibodies plus an HRP-conjugated secondary antibody.

**Retroviral infections.** Retroviral infections were done with the retroviral vector pMX-IRES-GFP (a gift from T. Kitamura) carrying the wild-type or one of each mutant STAT3 sequence as described previously[8].

**STAT3 knock-down.** Transfection of siRNA oligonucleotides was performed by using Lipofectamine-RNAiMAX reagent (Invitrogen). Forty hours after transfection, the cells were treated with IFNα (10 ng ml$^{-1}$) for 5 h.

**Luciferase reporter assay.** The reporter construct contained 4 repeated STAT3-responsive elements linked to a luciferase reporter gene. HeLa cells or HepG2 cells were transfected with the pcDNA3 vector bearing wild-type or mutant *STAT3*, the reporter construct, and an expression vector for *Renilla* luciferase driven by the CMV reporter, with FuGENE6 (Roche). The relative luciferase activity was determined by normalizing the values against the *Renilla* luciferase signal.

# H2-M3-Restricted CD8$^+$ T Cells Induced by Peptide-Pulsed Dendritic Cells Confer Protection against *Mycobacterium tuberculosis*[1]

**Takehiko Doi,*† Hisakata Yamada,[2]* Toshiki Yajima,* Worawidh Wajjwalku,‡ Toshiro Hara,† and Yasunobu Yoshikai***

**One of the oligopolymorphic MHC class Ib molecules, H2-M3, presents *N*-formylated peptides derived from bacteria. In this study, we tested the ability of an H2-M3-binding peptide, TB2, to induce protection in C57BL/6 mice against *Mycobacterium tuberculosis*. Immunization with bone marrow-derived dendritic cell (BMDC) pulsed with TB2 or a MHC class Ia-binding peptide, MPT64$_{190-198}$ elicited an expansion of Ag-specific CD8$^+$ T cells in the spleen and the lung. The number of TB2-specific CD8$^+$ T cells reached a peak on day 6, contracted with kinetics similar to MPT64$_{190-198}$-specific CD8$^+$ T cells and was maintained at an appreciable level for at least 60 days. The TB2-specific CD8$^+$ T cells produced less effector cytokines but have stronger cytotoxic activity than MPT64$_{190-198}$-specific CD8$^+$ T cells. Mice immunized with TB2-pulsed BMDC as well as those with MPT64$_{190-198}$-pulsed BMDC showed significant protection against an intratracheal challenge with *M. tuberculosis* H37Rv. However, histopathology of the lung in mice immunized with TB2-pulsed BMDC was different from mice immunized with MPT64$_{190-198}$-pulsed BMDC. Our results suggest that immunization with BMDC pulsed with MHC class Ib-restricted peptides would be a useful vaccination strategy against *M. tuberculosis*. *The Journal of Immunology*, 2007, 178: 3806–3813.**

Tuberculosis is one of the major public health problems. About one-third of the world population has been latently infected with *Mycobacterium tuberculosis* (1). The tuberculosis incidence is increasing in association with increased numbers of HIV/AIDS patients. Furthermore, the emergency of multidrug-resistant strains of *M. tuberculosis* has worsened the problems. To prevent an epidemic of tuberculosis, *Mycobacterium bovis* bacillus Calmette-Guérin (BCG)[3] is the only vaccine currently available against tuberculosis. Although BCG vaccine protect children efficiently against the early manifestations of tuberculosis (2, 3), especially meningeal tuberculosis (4), it confers incomplete protection against tuberculosis in adults presumably because BCG may not be effective for inducing long-term cellular immunity sufficient for protection against pulmonary disease (5). Furthermore, BCG, a live vaccine, may not be safe for immunocompromised hosts such as AIDS and aged patients. Therefore, it is urgently required to develop prophylactic and therapeutic vaccines for tuberculosis in place of BCG (6).

Although protection against infection with intracellular bacteria such as *M. tuberculosis* depends mainly on CD4$^+$ Th1 cells, there are substantial lines of evidence that CD8$^+$ T cells also play a requisite role (7–9). $\beta_2$-microglobulin-deficient mice and TAP-deficient mice, both of which lack functional CD8$^+$ T cells, are susceptible to infection with *M. tuberculosis* (10, 11). Adoptive transfer of immunized CD8$^+$ T cells conferred protection against subsequent challenge with *M. tuberculosis* (12). Thereafter, various vaccination strategies have settled to efficiently induce protective memory CD8$^+$ T cells. Peptide-pulsed mature bone marrow-derived dendritic cells (BMDC) efficiently generate high numbers of effector and memory CD8$^+$ T cells (13–15) and there have been several studies on BMDC-based vaccines against *M. tuberculosis* (16–18) in which a certain level of protection was observed. However, an obstacle for clinical application of these peptide-based vaccination strategies is the polymorphism of MHC molecules (19).

Although most of CD8$^+$ T cells recognize peptides on highly polymorphic class Ia molecules, some CD8$^+$ T cells recognize peptides presented by class Ib molecules which have limited polymorphism (20). H2-M3 is a member of MHC class Ib molecules showing specificity for hydrophobic peptide sequences initiating with *N*-formyl methionine derived from only bacteria or mitochondrial proteins (21, 22). Chun et al. (23) have identified *M. tuberculosis*-derived peptides which bind H2-M3 and showed an involvement of H2-M3-restricted CD8$^+$ T cell response in murine models of *M. tuberculosis* infection. Thus, H2-M3-binding peptides may serve as a good candidate for universal vaccine against *M. tuberculosis* (24). At present, it is unclear whether immunization with H2-M3 peptide induces long-lasting protective immunity to *M. tuberculosis* infection.

In the present study, we examined the effects of vaccination with BMDC pulsed with a H2-M3-binding peptide, TB2 (23) or a MHC

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[3] Abbreviations used in this paper: BCG, *Mycobacterium bovis* bacillus Calmette-Guérin; DC, dendritic cell; BMDC, bone marrow-derived DC; MFI, mean fluorescent intensity.

 0022-1767/07/$2.00

Table I. *Synthetic peptides used in this study*[a]

| Peptide | Sequence and Location | Gene Designation and Putative Identification |
|---|---|---|
| MHC class Ia-restricted (H-2$^b$-restricted) peptide | | |
| MPT64$_{190-198}$ | FAVTNDGVI (190–198) | Rv1980c (immunogenic protein MPB64/MPT64) |
| Mtb32A$_{309-318}$ | GAPINSATAM (309–318) | Rv0125 (probable serine protease PepA) |
| 38 kDa$_{129-137}$ | AQQVNYNLP (129–137) | Rv0934 (periplasmic phosphate-binding lipoprotein PSTS1) |
| OVA$_{257-264}$ | SIINFEKL (257–264) | OVA |
| H2-M3-restricted peptide | | |
| TB2 | f-MLVLLV (1–6) | Rv0476 (possible conserved transmembrane protein) |
| TB4 | f-MFLIDV (1–6) | Rv0277C (conserved hypothetical protein) |
| TB7 | f-MILLV (1–5) | Rv1686C (probable conserved integral membrane protein ABC transporter) |
| LemA | f-MIGWII (1–6) | Listerial peptide |

[a] The sequence and annotation information of *M. tuberculosis* was obtained from The Institute for Genomic Research (http://cmr.tigr.org/tigr-scripts/CMR/CmrHomePage.cgi) and The Institut Pasteur (http://genolist.pasteur.fr/Tubercul.list/).

class Ia (H-2D$^b$)- binding peptide, MPT64$_{190-198}$ (25–27) on *M. tuberculosis* H37Rv infection in mice. We found that immunization with TB2-pulsed BMDC elicited long-lasting Ag-specific CD8$^+$ T cells, leading to protection against intratracheal infection with *M. tuberculosis* at a level comparable to MPT64$_{190-198}$-pulsed BMDC.

## Materials and Methods

### *Animals*

Six- to 8-wk-old female C57BL/6 mice (Charles River Laboratories) and C57BL/6 Ly5.1-congenic mice (The Jackson Laboratory) were used. They were housed in a pathogen-free environment throughout the experiment. This study was approved by the Committee of Ethics on Animal Experiment in Faculty of Medicine (Kyushu University, Kyushu, Japan). Experiments were conducted under the control of the Guidelines for Animal Experiment and were performed mainly under barrier conditions in a level III biosafety animal facility.

### *Microorganisms*

*M. tuberculosis* strain H37Rv was grown in Middlebrook 7H9 medium (Difco) supplemented with albumin-dextrose-catalase enrichment (Difco) and Tween 80 at 37°C. The bacteria in the culture were stored in Middlebrook 7H9 medium supplemented with 20% (v/v) glycerol at −80°C until they were used.

### *Abs and synthetic peptides*

Following Abs were used: FITC-conjugated anti-IFN-γ (R4-6A2), Cy5-conjugated anti-CD8a (53-6.7), PE-conjugated anti-CD44 (IM7), biotin-conjugated anti-CD45.1 (A20), and streptavidin-Cy5 (eBioscience). The H2-D$^b$-restricted peptide, MPT64$_{190-198}$ (FAVTNDGVI) (25–27), Mtb32A$_{309-318}$ (GAPINSATAM) (28, 29), 38 kDa$_{129-137}$ (AQQVNYNLP) (30), the H2-K$^b$-restricted peptide, OVA$_{257-264}$ (SIINFEKL), and the $H_2$-$M_3$-restricted peptide, TB2 (f-MLVLLV), TB4 (f-MFLIDV), TB7 (f-MILLV) (23), and LemA (f-MIGWII) (Table I) were purchased from Greiner Bio-One.

### *Generation of peptide-pulsed BMDCs and immunization*

RBC-depleted bone marrow cells were cultured at 1 × 10$^6$ cells/ml in RPMI 1640 medium (Sigma-Aldrich) supplemented with 20 ng/ml murine IL-4 and 20 ng/ml murine GM-CSF (PeproTech) at 37°C with 5% $CO_2$. Three days after the initial culture, two-thirds of the medium containing small nonadherent cells were removed and fresh RPMI 1640 containing GM-CSF and IL-4 was added back. At day 6, 1 μg/ml LPS (Sigma-Aldrich) was added to induce maturation. After an overnight culture, 5 μM synthetic peptides are added to the cultures 3 h before harvest. The nonadherent cells were harvested and then layered onto 15% metrazimade (Sigma-Aldrich). After a centrifugation at 600 × *g* for 20 min at 20°C, mononuclear cells at the interface were collected and washed twice before immunization. These cells were 80–90% CD11c$^+$ and expressed high levels of CD80, CD86, CD40 molecules. C57BL/6 mice were injected i.v. with 1 × 10$^6$ peptide-pulsed BMDC via the dorsal tail vein. Control mice received either PBS or none peptide-pulsed BMDC.

### *Quantification of Ag-specific CD8$^+$ T cell response*

The number of CD8$^+$ T cells specific for MPT64$_{190-198}$ or TB2 was determined by intracellular staining for IFN-γ. The cells were incubated for 5–6 h with or without 5 μM of synthetic peptides in the presence of 10 μg/ml brefeldin A at 37°C. For the surface staining, cells were first incubated with a mAb directed against the Fcγ II/III receptors (2.4G2) and were incubated with PE-conjugated anti-CD44 mAb and Cy5-conjugated anti-CD8 mAb. The cells were fixed, permeabilized, and further stained with FITC-conjugated anti-IFN-γ mAb. Samples were run on a FACSCalibur flow cytometer (BD Biosciences) and analyzed with CellQuest software (BD Biosciences).

### *Quantification of cytokine by ELISA*

CD8$^+$ T cells in the spleens were purified after depleting nylon wool-adherent cells by positive selection using anti-CD8 magnetic beads (Miltenyi Biotec). CD8$^+$ T cells (2 × 10$^5$) were cocultured with mitomycin C-treated syngeneic splenocytes (2 × 10$^5$) from naive C57BL/6 mice at a volume of 200 μl in the presence or absence of different concentrations of synthetic peptides. The cells were incubated at 37°C and 5% $CO_2$ for 48 h and culture supernatants were collected and stored at −20°C. The amount of IFN-γ and TNF-α was measured by ELISA kit (R&D Systems) according to the manufacturer's protocols.

### *In vivo cytotoxicity assay*

B6-Ly5.1$^+$ splenocytes were divided and labeled with either a high concentration (5 μM) or a low concentration (0.5 μM) of CFSE (Invitrogen Life Technologies). CFSE$^{high}$ cells were pulsed with 5 μM synthetic peptide for 1 h at 37°C, whereas CFSE$^{low}$ cells left uncoated. After washing, the cells were mixed in equal proportions (2 × 10$^7$ total cells/200 μl) and injected i.v. into mice immunized with peptide-pulsed DC 6 days previously. Splenocytes in the recipients were harvested 4 h later for flow cytometric analysis. Percent-specific lysis was calculated according to the formula (1 − (ratio primed/ratio unprimed) × 100), where the ratio unprimed = percent CFSE$^{low}$/percent CFSE$^{high}$ cells remaining in nonimmunized recipients, and ratio primed = percent CFSE$^{low}$/percent CFSE$^{high}$ cells remaining in immunized recipients.

### *Infection of immunized mice with* M. tuberculosis

Mice were anesthetized by i.p. injection of pentobarbital sodium and tracheae were exposed. Infection was effected by intratracheal inoculation with 1 × 10$^5$ viable CFU of *M. tuberculosis* H37Rv diluted in 50 μl of PBS. The numbers of viable bacteria in organs were measured 7 or 28 days after infection by plating serial dilutions of whole organ homogenates on supplemented Middlebrook 7H10 agar (Difco) enriched with 10% oleic acid-albumin-dextrose-catalase (Difco) and 0.5% glycerol, and incubated at 37.5°C for 3 wk. Colonies were counted and total tissue CFU calculated.

### *ELISPOT assay*

The numbers of MPT64$_{190-198}$- or TB2-specific T cells in the lungs and spleen after infection with *M. tuberculosis* were determined by an ELISPOT assay (Mouse IFN-γ ELISPOT Set; BD Biosciences) according to the manufacturer's instructions. Lung mononuclear cells were prepared by collagenase digestion and were pooled from three mice. To supplement

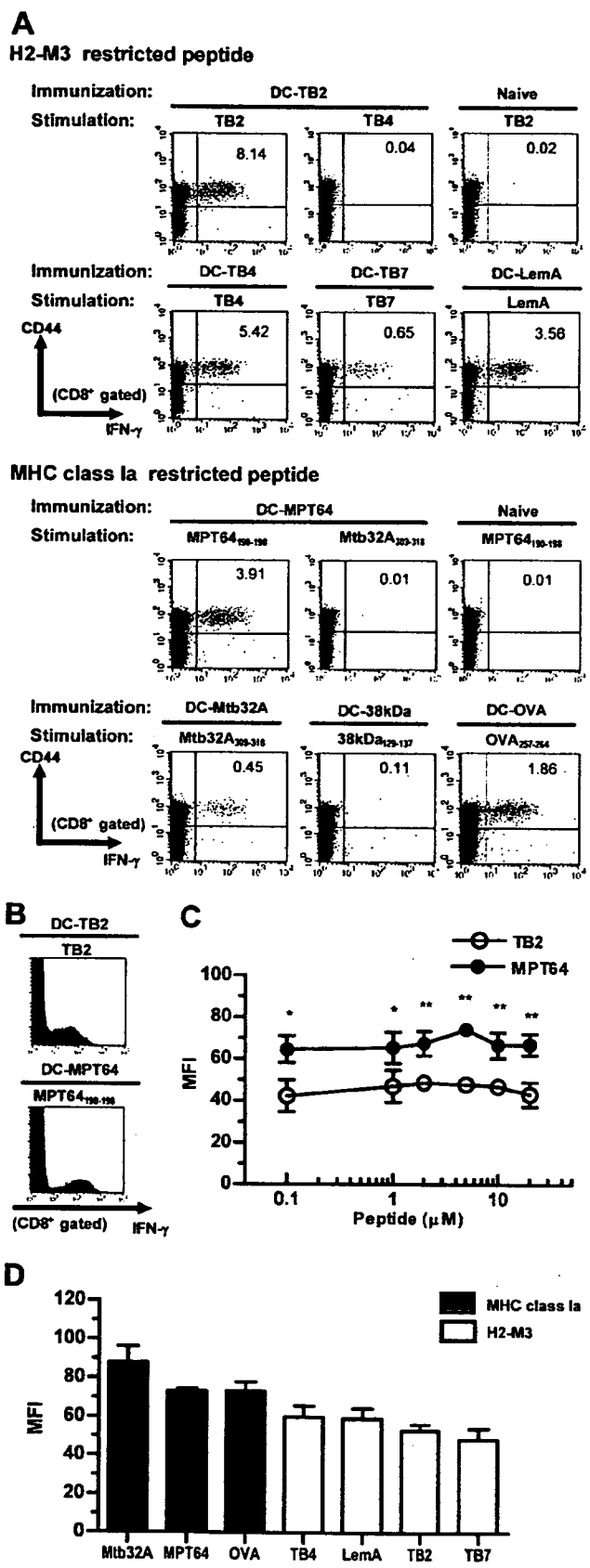


FIGURE 1. Expansion of Ag-specific $CD8^+$ T cells after immunization with *M. tuberculosis*-derived peptide-pulsed BMDCs. *A*, $CD8^+$ T cells in the spleens of naive mice or the mice immunized with H2-M3 (*upper panels*) or MHC class Ia (*lower panels*) restricted peptide-pulsed BMDCs 6 days previously were harvested and restimulated with the indicated peptide in vitro. IFN-γ-producing $CD8^+$ T cells were detected by a flow cytometer. Data are representative of three separate experiments and are expressed as means of three mice in each group. DC-MPT64, $MPT64_{190-198}$-pulsed BMDC; DC-Mtb32, $Mtb32A_{309-318}$-pulsed BMDC; DC-38 kDa, 38-$kDa_{129-137}$-pulsed BMDC; DC-OVA, $OVA_{257-264}$-pulsed BMDC; DC-TB2, TB2-pulsed BMDC; DC-TB4, TB4-pulsed BMDC; DC-TB7, TB7-pulsed BMDC; DC-LemA, LemA-pulsed BMDC. *B*, Expression levels of intracellular IFN-γ of TB2-specific $CD8^+$ T cells (*upper panel*) and $MPT64_{190-198}$-specific $CD8^+$ T cells (*lower panel*) were depicted as histograms. *C*, MFI of IFN-γ staining of $CD8^+$ T cells induced by different concentrations of peptide was shown. Data are representative of three separate experiments and are expressed as means + SD of three mice in each group. *, $p < 0.05$, **, $p < 0.01$, significantly different from the value of TB2-specific $CD8^+$ T cells. *D*, MFI of IFN-γ staining of $CD8^+$ T cells induced by different peptides was shown. Data are representative of three separate experiments and are expressed as means + SD of three mice in each group.

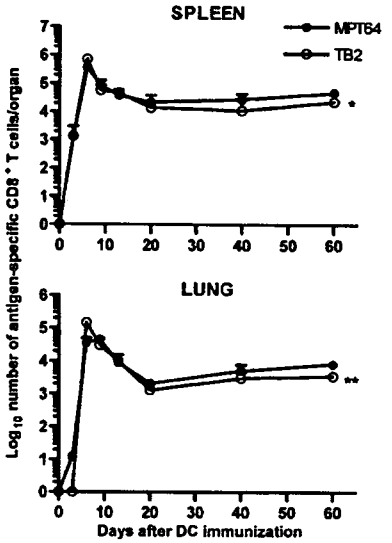


FIGURE 2. Kinetics of the absolute number of $MPT64_{190-198}$-specific or TB2-specific, IFN-γ-producing $CD8^+$ T cells in the spleens and the lungs. To calculate the number of Ag-specific $CD8^+$ T cells, we subtracted the percentage of IFN-$\gamma^+$ $CD8^+$ T cells in unstimulated samples from the peptide-stimulated value. Data are representative of three separate experiments and are expressed as means + SD of three mice in each group. *, $p < 0.05$, significantly different from the value of $MPT64_{190-198}$-specific $CD8^+$ T cells.

APCs in the lung cells, mitomycin C-treated syngeneic splenocytes were added at a ratio of 1:1 lung cells/APCs. Two-fold serial dilutions of the 100-μl admixture were added in triplicate to the wells precoated with anti-mouse IFN-γ mAb starting at $1 \times 10^5$ lung cells/well. The wells were further added with 100 μl of RPMI 1640-FCS containing no Ag, 5 μM $MPT64_{190-198}$ or 5 μM TB2. After 24 h of incubation at 37°C and 5% $CO_2$, unattached cells were aspirated from the wells and the remaining cells were lysed with distilled water. The wells were washed again with PBS containing 0.05% Tween 20 and incubated with a second biotinylated anti-mouse IFN-γ mAb. The wells were then washed with PBS-Tween 20, incubated for 1 h with streptavidin-HRP, washed, and developed with 3-amino-9-ethyl-carbazol as substrate. After washing and drying, the number of spots per well was counted with the aid of a digital microscope at ×40. The number of cells specific for each peptide was calculated by subtracting the number of spots formed in the absence of Ag from that formed in its presence. Experiments were repeated twice.

### *Histopathology*

Tissues were preserved in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. Random sections including hilar of the lung from five mice per group were examined.

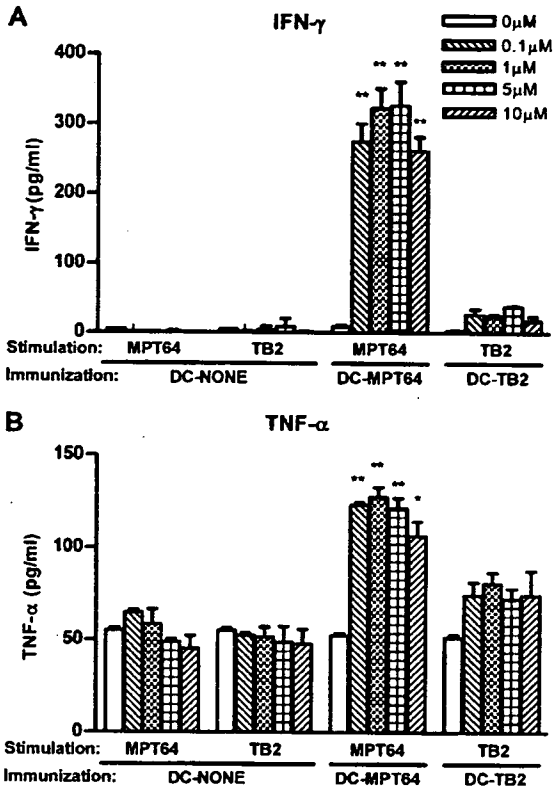


FIGURE 3. Cytokine production by $MPT64_{190-198}$- or TB2-specific $CD8^+$ T cells. Mice were immunized with $MPT64_{190-198}$ (DC-MPT64), TB2 (DC-TB2), or no peptide-pulsed BMDC (DC-NONE). After 6 days, purified $CD8^+$ T cells from the spleens were cultured with PBS or different concentrations of $MPT64_{190-198}$ or TB2 and syngeneic mitomycin C-treated splenocytes for 48 h. IFN-γ (*A*) and TNF-α (*B*) in the supernatants was measured by ELISA. Data are representative of three separate experiments and are expressed as means + SD of triplicate cultures of each group. *, $p < 0.05$, **, $p < 0.01$, significantly different from the value of TB2- specific $CD8^+$ T cells.

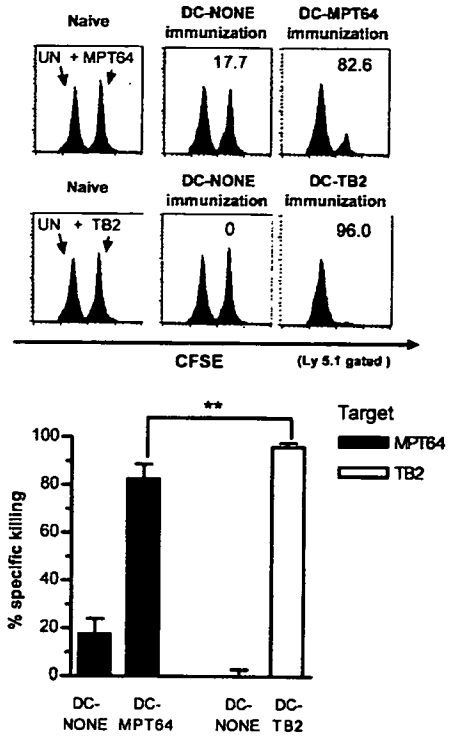


FIGURE 4. In vivo cytotoxic activity of $MPT64_{190-198}$- or TB2-specific $CD8^+$ T cells. Mice were immunized with $MPT64_{190-198}$ (DC-MPT64), TB2 (DC-TB2), or no peptide-pulsed BMDC (DC-NONE). Six days after immunization, CFSE-labeled, $MPT64_{190-198}$-, or TB2-pulsed and untreated (UN) target splenocytes ($Ly5.1^+$) were coinjected. Cytotoxic activity was expressed as the percent of specific killing of the targets. Data are representative of three separate experiments and are expressed as means + SD of four mice in each group. **, $p < 0.01$ significantly different from the value of $MPT64_{190-198}$-specific $CD8^+$ T cells.

*Statistical analysis*

The statistical significance of the bacteria number was determined by one-way ANOVA. Other data were determined by the Student *t* test. Differences with a *p* value of <0.05 were considered significant. Analyses were completed using SPSS software.

## Results

*Induction of MHC class Ia- and H2-M3-restricted $CD8^+$ T cell expansion by peptide-pulsed BMDCs*

Chun et al. (23) have identified several *M. tuberculosis*-derived peptides binding to a MHC class Ib molecule, H2-M3. We first compared immunogenicity of three of these peptides named TB2, TB4, and TB7 (Table I), all of which were shown to be immunogenic in *M. tuberculosis*-infected C57BL/6 mice (23). We examined expansion of Ag-specific T cells after immunization with peptide-pulsed BMDC, which were treated with LPS to induce full maturation. We found this maturation step was necessary for inducing clear expansion of Ag-specific T cells in preliminary experiments (data not shown). As shown in Fig. 1*A*, an expansion of $CD8^+$ T cells producing IFN-γ was observed 6 days after immunization with H2-M3-binding peptides. Among the peptides tested, TB2 induced the strongest expansion of Ag-specific T cells. We also tested three MHC class Ia-restricted peptides derived from *M. tuberculosis* (Table I) for their immunogenicity. Although $MPT64_{190-198}$ gave a strong T cell response similar to the control OVA, the other two *M. tuberculosis*-derived peptides induced only marginal expansion of Ag-specific T cells. Therefore, we used TB2 and $MPT64_{190-198}$ as representative of H2-M3-binding and MHC class Ia-binding peptides, respectively, in the subsequent experiments. It is of note to find that, mean fluorescent intensity (MFI) for IFN-γ staining of $MPT64_{190-198}$-specific $CD8^+$ T cells was higher than that of TB2-specific $CD8^+$ T cells at any concentration of the peptides (Fig. 1, *B* and *C*). Furthermore, $CD8^+$ T cells specific for MHC class Ia-restricted peptides generally showed higher MFI than those specific for H2-M3-binding peptide (average MFI 77.8 vs 55.0, respectively, $p = 0.007$) (Fig. 1*D*).

Kinetic analysis revealed that the number of $MPT64_{190-198}$- or TB2-specific $CD8^+$ T cells in the spleen and the lung peaked on day 6 after immunization and contracted until day 20, and then was maintained an appreciable level at least 60 days (Fig. 2). The absolute number of TB2-specific $CD8^+$ T cells tended to be higher than that of $MPT64_{190-198}$-specific $CD8^+$ T cells in the spleen at the peak, but there were no significant differences. Although the number of TB2-specific $CD8^+$ T cells was lower than that of $MPT64_{190-198}$-specific $CD8^+$ T cells at day 60, it was still above background. These data showed that not only MHC class Ia-restricted $MPT64_{190-198}$- but also H2-M3-restricted TB2-pulsed mature BMDC induced long-lasting Ag-specific $CD8^+$ T cells.

*Effector functions of TB2- and MPT64-specific $CD8^+$ T cells*

As potential of IFN-γ production seemed different between MHC class Ia- and H2-M3-restricted $CD8^+$ T cells by intracellular flow

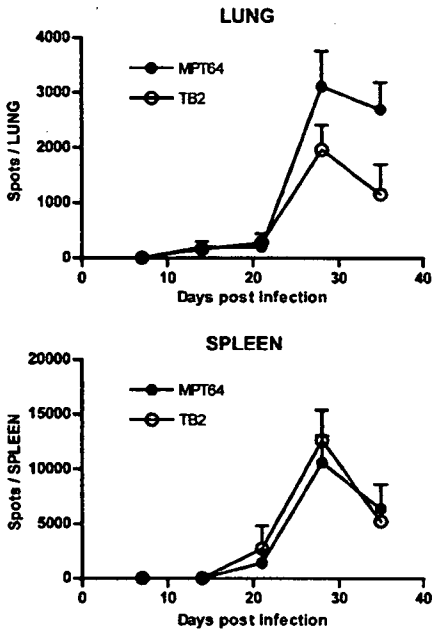


**FIGURE 5.** Kinetics of the number of $MPT64_{190-198}$- or TB2-specific $CD8^+$ T cells in *M. tuberculosis*-infected mice. Mice were infected intratracheally with $2 \times 10^2$ CFU *M. tuberculosis*. Number of $CD8^+$ T cells producing IFN-γ in response to $MPT64_{190-198}$ or TB2 per organ was measured by an ELISPOT assay. Results were obtained with pooled lung and spleen cells from three mice. Shown are the means + SD of the number of spots in triplicate wells. Similar results were obtained in two separate experiments.

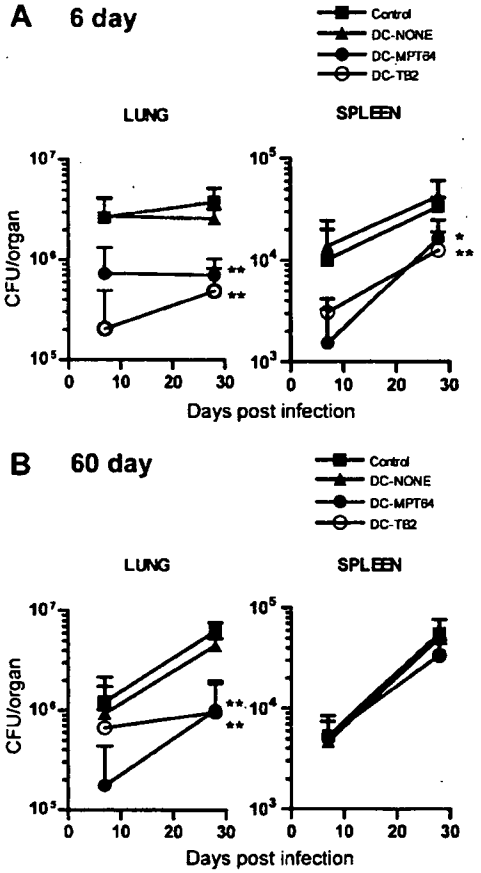


**FIGURE 6.** Protection against virulent *M. tuberculosis* H37Rv by immunization with $MPT64_{190-198}$ or TB2. Mice were immunized with BMDC pulsed with $MPT64_{190-198}$ (DC-MPT64), TB2 (DC-TB2), or no peptide (DC-NONE). Control mice were given PBS alone. At 6 days (*A*) or 60 days (*B*) postimmunization, the mice were challenged intratracheally with $1 \times 10^5$ CFU of live *M. tuberculosis* H37Rv. Data are representative of two separate experiments and are expressed as means + SD of four mice of each group. *, $p < 0.05$, **, $p < 0.01$ significantly different from the values of PBS and DC-NONE-immunized mice.

cytometric analysis (Fig. 1, *B–D*), we further compared the functions of TB2-specific $CD8^+$ T cells and $MPT64_{190-198}$-specific $CD8^+$ T cells 6 days after immunization. IFN-γ and TNF-α production by spleen $CD8^+$ T cells were measured by ELISA. Although there was no significant difference between the number of TB2-specific $CD8^+$ T cells and that of $MPT64_{190-198}$-specific $CD8^+$ T cells in the spleen as shown in Fig. 2, TB2-specific $CD8^+$ T cells produced less IFN-γ than $MPT64_{190-198}$-specific $CD8^+$ T cells at any concentration of the peptide (Fig. 3*A*). The level of TNF-α production was also lower in TB2-specific $CD8^+$ T cells than $MPT64_{190-198}$-specific $CD8^+$ T cells (Fig. 3*B*).

We next evaluated the cytotoxic activity of $MPT64_{190-198}$- or TB2-specific $CD8^+$ T cells by measuring in vivo cytotoxic activity against syngeneic peptide-pulsed splenocytes 6 days after immunization. Both $MPT64_{190-198}$-specific $CD8^+$ T cells and TB2-specific $CD8^+$ T cells lysed peptide-pulsed syngeneic splenocytes (Fig. 4). As opposed to the case of IFN-γ or TNF-α production, the cytotoxic activity of TB2-specific $CD8^+$ T cells was significantly higher than that of $MPT64_{190-198}$-specific $CD8^+$ T cells. Taken together, these data indicated that, although H2-M3-restricted TB2-specific $CD8^+$ T cells and MHC class Ia-restricted $MPT64_{190-198}$-specific $CD8^+$ T cells expand in similar extent with similar time kinetics after immunization with BMDC, they have somewhat different activities of function.

### *Response of $MPT64_{190-198}$- or TB2-specific $CD8^+$ T cells during infection with* M. tuberculosis

To examine whether $CD8^+$ T cell response to $MPT64_{190-198}$ or TB2 was elicited during infection with *M. tuberculosis*, the number of $MPT64_{190-198}$- or TB2-specific T cells in the lung or spleen from $2 \times 10^2$ CFU *M. tuberculosis* H37Rv-infected mice was measured by an ELISPOT assay (Fig. 5). Two weeks after infection, a small number of peptide-specific IFN-γ spots was detected in the lung and the spleen. The frequency of $MPT64_{190-198}$- and TB2-specific $CD8^+$ T cells both rapidly increased from 3 wk after infection then reached a peak at 4 wk after infection. These results clearly indicate that $MPT64_{190-198}$ and TB2 are presented during *M. tuberculosis* infection. There was no clear difference in kinetics of the response between $MPT64_{190-198}$- and TB2-specific $CD8^+$ T cells.

### *H2-M3-restricted TB2-specific $CD8^+$ T cells protect mice from intratracheal* M. tuberculosis *infection*

To examine whether these $CD8^+$ T cells are both protective against *M. tuberculosis*, we challenged the mice intratracheally with *M. tuberculosis* H37Rv 6 days after immunization with $MPT64_{190-198}$-pulsed BMDC (DC-MPT64), TB2-pulsed BMDC (DC-TB2), or BMDC without peptides (DC-NONE) (Fig. 6*A*). One or 4 wk after infection, lungs and spleens were prepared from the mice and the extent of bacterial growth was determined. At 1 wk, the CFU in the lung of DC-TB2-immunized mice tended to be lower than those of DC-NONE-immunized mice or naive mice, but it was not statistically significant. At 4 wk, the CFU in these organs of DC-MPT64 or DC-TB2 immunized mice was significantly lower than that of DC-NONE-immunized mice or naive mice. The

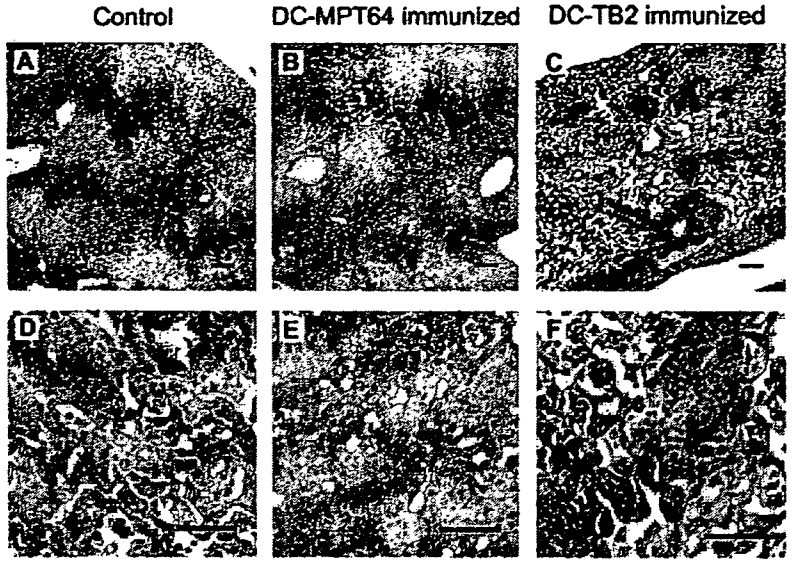


**FIGURE 7.** Lung histopathology of the mice immunized with $MPT64_{190-198}$- or TB2-pulsed BMDC following *M. tuberculosis* infection. Control PBS-injected mice (*A* and *D*) or the mice immunized with BMDC pulsed with $MPT64_{190-198}$ (DC-MPT64) (*B* and *E*) or TB2 (DC-TB2) (*C* and *F*) were challenged intratracheally with *M. tuberculosis*. After 8 wk, histology of the lungs was examined by staining with H&E. Lungs from five mice per group were examined. Representative figures are shown. Original magnification, ×4, scale length, 100 μm (*A–C*); ×20. scale length, 50 μm (*D–F*).

difference of the CFU in the lung was ~1 log10 order. Thus, both $MPT64_{190-198}$-specific $CD8^+$ T cells and TB2 specific-$CD8^+$ T cells were protective against respiratory *M. tuberculosis* infection.

*Vaccination with TB2-pulsed BMDC conferred long-lasting protective immunity against* M. tuberculosis

As shown in Fig. 2, $MPT64_{190-198}$-specific $CD8^+$ T cells and TB2-specific $CD8^+$ T cells were maintained for 60 days after immunization. To evaluate whether BMDC immunization can induce long-lasting protective immunity against *M. tuberculosis*, we challenged intratracheally with *M. tuberculosis* 60 days later after immunization. At 4 wk, the CFU in the lungs of DC-MPT64- or DC-TB2-immunized mice was significantly lower than those of DC-NONE-immunized mice and naive mice (Fig. 6*B*). The difference of the CFU in the lungs was ~1 $\log_{10}$ order. Although the CFU in the spleens was considerably lower than that of DC-NONE-immunized mice or naive mice, there was no statistical difference. These data suggested that both MHC class Ia-restricted $CD8^+$ T cells and H2-M3-restricted $CD8^+$ T cells induced by peptide-pulsed mature BMDC elicited long-lasting protection against respiratory *M. tuberculosis* infection.

*Lung histopathology of the mice immunized with TB2 or $MPT64_{190-198}$ after intratracheal* M. tuberculosis *infection*

As we observed some differences in the activity of function between $MPT64_{190-198}$- and TB2-specific $CD8^+$ T cells in vivo as well as in vitro (Figs. 3 and 4), it is of interest to compare histopathological changes in the lungs of the mice immunized with the different peptides. DC-MPT64-immunized mice had larger pulmonary infiltrates composed of formed macrophages (Fig. 7, *B* and *E*) compared with control (Fig. 7, *A* and *D*) or DC-TB2-immunized mice (Fig. 7, *C* and *F*). Areas of bronchopneumonia were clearly evident and frankly necrotic areas were observed in part (Fig. 7*B*). In contrast, although large infiltrates were also observed in the lungs of DC-TB2-immunized mice, there were few necroses and the structure of the walls of the alveoli comparatively avoided destruction (Fig. 7*C*). There tended to be greater numbers of lymphocytes in the inflammatory infiltrate compared with DC-MPT64-immunized mice. Both perivascular and interstitial lymphoid infiltrates were observed (Fig. 7*F*). These histopathological features suggest that the protection mechanism of TB2-specific $CD8^+$ T cells against *M. tuberculosis* infection is different from that of $MPT64_{190-198}$-specific $CD8^+$ T cells.

## Discussion

Because $CD8^+$ T cells play a requisite role in the resistance to mycobacterial infection, Ag-specific $CD8^+$ T cells are major target for vaccine design for tuberculosis. We here showed the first evidence that immunization with mature BMDC pulsed with either MHC class Ia ($H\text{-}2D^b$) binding $MPT64_{190-198}$ or class Ib (H2-M3) binding TB2 peptide induced long-lasting Ag-specific $CD8^+$ T cells and conferred protection against an intratracheal challenge with *M. tuberculosis*.

There have been several studies on BMDC-based vaccination against *M. tuberculosis* infection models in mice (16–18). McShane et al. (18) reported that mice immunized with immature BMDC pulsed with either MHC class I- or MHC class II-restricted Ag85A peptide was not protective against *M. tuberculosis* challenge. Nakano et al. (16) reported that retroviral *Ag85A* gene-transduced, incompletely matured BMDC immunization was not effective enough in terms of clearance of *M. tuberculosis* from the tissues. In contrast, Malowany et al. (17) reported that adenoviral *Ag85A* gene-transduced mature BMDC induced much longer immune response compare with immature peptide-pulsed BMDC. These findings clearly indicated the importance of maturational stage of BMDC for vaccination. We also observed a clear difference in inducing T cell response between immature and mature BMDC (data not shown). Thereafter, we used LPS-stimulated BMDC for immunization and successfully induced protective $CD8^+$ T cell responses against *M. tuberculosis* infection.

There have been some reports showing an involvement of H2-M3-restricted $CD8^+$ T cells in *M. tuberculosis* infection. Chun et al. (23) identified several H2-M3-binding peptides by scanning the full sequence of the *M. tuberculosis* genome. Although they showed $CD8^+$ T cell responses to some of these peptides including TB2 after infection with *M. tuberculosis*, it has been unknown whether the H2-M3-restricted $CD8^+$ T cells are protective against *M. tuberculosis* infection. Dow et al. (31) also identified several H2-M3-binding peptides derived from *M. tuberculosis* and showed CTL response to these peptides. They also examined protection against *M. tuberculosis* challenged 10 days after immunization with some of these peptides. However, these peptides were longer

than the predicted length of H2-M3-binding peptides revealed by the crystallography (32) and also by bioassays (33), and none of these peptides were identical with the peptides identified by Chun et al. (23). In this study, we found TB2 elicited strongest T cell response after immunization with peptide-pulsed BMDC. Thereafter, we used TB2 and found that immunization with TB2 confers significant protection as vaccine against *M. tuberculosis* challenged even 60 days after immunization.

The importance of H2-M3-restricted $CD8^+$ T cells has been more clearly shown in *Listeria monocytogenes* infection. It was recently reported that H2-M3-deficient mice were impaired in early bacterial clearance during primary *L. monocytogenes* infection (34). H2-M3-restricted $CD8^+$ T cells play a role in early protection against a primary *L. monocytogenes* infection by expanding quicker than class Ia-restricted $CD8^+$ T cells (35, 36). In the present study, however, we did not find difference in the kinetics of expansion between H2-M3-restricted $CD8^+$ T cells and MHC class Ia-restricted $CD8^+$ T cells either after immunization with BMDC or during *M. tuberculosis* infection. In the former case, the discrepancy between the previous observations and our findings may be explained by different efficacy in Ag processing between MHC class Ia-binding peptides and H2-M3-binding peptides. For the presentation by MHC class Ia molecules, antigenic peptides in the cytosol are translocated to the lumen of the endoplasmic reticulum by TAP and loaded onto peptide-receptive MHC class Ia complexes. Stably conformed and peptide-filled class Ia complexes then egress from the endoplasmic reticulum to the cell surface. In contrast, TAP did not appear to be absolutely necessary for the presentation of *N*-formylated peptides by H2-M3 molecules (37). In addition, the supply of endogenous *N*-formylated mitochondrial peptides is limited and a significant pool of H2-M3 exists intracellularly, which can be rapidly mobilized to the surface when provided with appropriate exogenous *N*-formylated peptides (38). Therefore, it is suggested that MHC class Ib-binding Ag peptides are more rapidly presented by APCs. Immunization with peptide-pulsed mature BMDC may circumvent these time-dependent factors. In the case of in vivo infection, one of the major differences between *L. monocytogenes* and *M. tuberculosis* is their growth rate. *M. tuberculosis* divide slowly and their Ags are presented gradually with time, which may conceal the lag of response of MHC class Ia-restricted $CD8^+$ T cells behind that of H2-M3-restricted $CD8^+$ T cells. Additionally, there seems to be a difference in Ag processing between *L. monocytogenes* and *M. tuberculosis*. In contrast to *L. monocytogenes*, which actively escapes phagosomes and enters the cytosol, *M. tuberculosis* resides within phagosomes which has features similar to those of an early endosome (39, 40). Nevertheless, *M. tuberculosis*-derived peptides are cross-presented by MHC class I pathway, which is supposed to be far less efficient than the case of *L. monocytogenes*. These differences in Ag processing and presentation of different microbes may also be involved in the discrepancy.

There were some differences in the activities of effector functions between H2-M3-restricted TB2-specific $CD8^+$ T cells and MHC class Ia-restricted $MPT64_{190-198}$-specific $CD8^+$ T cells, although both were protective against *M. tuberculosis* infection. Cytotoxic activity of TB2-specific $CD8^+$ T cells was higher than that of $MPT64_{190-198}$-specific $CD8^+$ T cells, whereas the ability to produce IFN-$\gamma$ or TNF-$\alpha$ was the opposite. Such differences are usually observed after immunization with different peptides, even restricted by the same MHC molecule and could be related to the stability of the MHC complexes. In this regard, it is notable to find that the expression levels of IFN-$\gamma$ were generally lower in H2-M3-restricted $CD8^+$ T cells than in MHC class Ia-restricted $CD8^+$ T cells by intracellular flow cytometric analysis (Fig. 1*D*), suggesting that the difference in the activities of function between TB2- and $MPT64_{190-198}$-specific $CD8^+$ T cells may be generalized to difference between MHC class Ia- and MHC class Ib-restricted $CD8^+$ T cells. Further detailed analysis is needed to test this possibility. These differences in activities of function of $CD8^+$ T cells might result in the different histopathology of the lung between $MPT64_{190-198}$- and TB2-immunized mice. The lungs of $MPT64_{190-198}$-pulsed BMDC-immunized mice following *M. tuberculosis* infection had large pulmonary infiltrates composed of formed macrophages. In contrast, the lungs of TB2-pulsed BMDC-immunized mice following *M. tuberculosis* infection had less necrosis and reduced pulmonary injury.

In conclusion, our results clearly indicated that vaccination with mature BMDC pulsed with a H2-M3-binding peptide significantly confers protection against *M. tuberculosis*. Because MHC class Ib molecules including H2-M3 have an advantage of limited polymorphism, immunization with MHC class Ib-restricted peptides would be a novel vaccination strategy against *M. tuberculosis* infection for a broad range of recipients.

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## Disclosures

The authors have no financial conflict of interest.

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# Association of *IL12RB1* polymorphisms with susceptibility to and severity of tuberculosis in Japanese: a gene-based association analysis of 21 candidate genes

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## Summary

Tuberculosis (TB) is the second commonest cause of death from infectious disease after HIV/AIDS worldwide. Association studies have revealed that host genetic factors, such as human leukocyte antigen and solute carrier family 11 member A1 (NRAMP1), play roles in susceptibility to TB. To identify host genetic factors involved in the susceptibility to TB in Japanese, we performed a gene-based association analysis of 21 candidate genes on 87 TB patients and 265 controls using marker single nucleotide polymorphisms (SNPs). For the genes with two or more marker SNPs exhibiting significant allele association, we subsequently analysed the association between adjacent coding SNPs (cSNPs) and TB. Among a total of 118 marker SNPs, 3 of *IL1B* and 2 of *IL12RB1* showed association with TB. Non-synomymous cSNPs were not identified in *IL1B*. Association studies on four non-synomymous cSNPs of *IL12RB1* (641A/G, 1094T/C, 1132C/G, 1573G/A) in linkage disequilibrium showed that three of them (641A/G, 1094T/C, 1132C/G) were significantly associated with the development of TB. Haplotype analysis on the four cSNPs demonstrated that frequency of ATGG haplotype was significantly lower in TB patients than in controls. When TB patients were divided into two subgroups according to the severity of lung disease, advanced subgroup showed a prominent association with 641A/G, 1094T/C and 1132C/G SNPs. These data suggested that genetic variants of *IL12RB1*, at least in part, confer genetic susceptibility to TB, and are associated with the progression of the disease, in Japanese.

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**Abbreviations**

TB, tuberculosis; IL, interleukin; NRAMP1, natural resistance associated macrophage protein 1; SLC11A1, solute carrier family 11 member A1; VDR, vitamin D receptor; NTM, non-tuberculous environmental mycobacteria; IFN, interferon; MSMD, Mendelian susceptibility to mycobacterial disease; SNP, single, nucleotide polymorphism; cSNP, coding SNPs; IFN-γR, IFN-γ receptor; IL-12R, IL-12, receptor; STAT, signal transducer and activator of transcription; IL-18R, IL-18 receptor; IL-23R, IL-23 receptor; TNF, tumor necrosis factor; TNFRSF, TNF receptor superfamily; UBE3A, ubiquitin protein ligase E3A; LD, linkage disequilibrium; UTR, untranslated region.

## Introduction

Tuberculosis (TB) is the second commonest cause of death from infectious disease after HIV/AIDS worldwide. The World Health Organization estimated 8–9 million new cases of clinical TB and 2 million deaths resulting from the disease every year (WHO, 2005). Only about 10% of the individuals infected with *Mycobacterium tuberculosis* develop TB, whereas the remaining 90% stay free from the disease throughout their life (Murray *et al.*, 1990). Almost half of the patients show rapid progression and develop clinical disease within 2 years after infection (Frieden *et al.*, 2003). In addition to these clinical observations, epidemiological, twin and adoption studies support the role of host genetic factors in the susceptibility to TB (Comstock, 1978; Sorensen *et al.*, 1988). Previous association studies demonstrated the association of several genes, such as human leucocyte antigen (HLA), natural resistance associated macrophage protein 1 (NRAMP1 or solute carrier family 11 member A1 [SLC11A1]) and vitamin D receptor (VDR) genes and interleukin (IL)-1 locus, with the susceptibility to TB (Singh *et al.*, 1983; Bellamy *et al.*, 1998, 1999; Goldfeld *et al.*, 1998; Wilkinson *et al.*, 1999; Greenwood *et al.*, 2000). A linkage analysis on sib-pairs conducted in Africa (Bellamy *et al.*, 2000) has mapped TB susceptibility loci to chromosomes 15q11–13 and Xq26, although another genome-wide scan for a Brazilian TB patient did not replicate it (Miller *et al.*, 2004).

On the other hand, genetic analysis of severe or recurrent cases with clinical diseases caused by weakly virulent mycobacterial species, such as BCG and non-tuberculous environmental mycobacteria (NTM) revealed the congenital deficiencies of the molecules involved in IL-12/interferon

 

(IFN)-γ axis named 'Mendelian susceptibility to mycobacterial disease (MSMD, MIM 209950)' (Dupuis *et al.*, 2000). Increased susceptibility to TB is also observed in this type of genetic disorders. Therefore, it is possible that mutations causing MSMD are responsible for the development of TB and/or that any functional polymorphisms of the genes encoding molecules of IL-12/IFN-γ axis may affect the genetic control of *M. tuberculosis*.

In the present study, we screened 21 candidate genes for TB susceptibility in Japanese by a gene-based association analysis using marker single nucleotide polymorphisms (SNPs) and subsequently analysed the association between TB and coding SNPs (cSNPs) adjacent to the positive marker SNPs in terms of susceptibility and disease severity.

## Materials and methods

### Subjects

The study population comprised 87 unrelated Japanese patients with TB (mean age: 52.7 ± 21.1 years; 18 women and 69 men) and 265 unrelated healthy Japanese individuals (mean age: 56.5 ± 12.7 years; 112 women and 153 men), who resided in Kyushu Island in the southern part of Japan. All the TB patients had been given a diagnosis of pulmonary TB on the basis of clinical symptoms and chest radiographic findings with bacteriological confirmation (culture, 82 patients; smear and/or polymerase chain reaction [PCR], 5 patients). Eleven patients were having TB relapses. Common clinical symptoms were cough (77%), sputum (53%) and fever (30%). Patients with known immunodeficient states, such as HIV infection and are undergoing immunosuppressive therapy were excluded. Lung disease on standard posterior–anterior chest radiograph of each patient was graded according the International Classification of Tuberculosis (Falk *et al.*, 1969; Van Lettow *et al.*, 2004):

(1) minimal lung disease was defined as infiltrates of slight to moderate density; disease present in a small portion of both lungs; the total volume of infiltrate(s) being the volume of one lung present above the second chondrosternal junction and the spine of the fourth junction or the body of the fifth thoracic vertebra and no cavitations present.
(2) moderately advanced disease was defined as disease present in one or both lungs; the total extending not more than as follows:
    (i) scattered lesions of slight to moderate density do not involve more than the total volume of one lung or the equivalent volume of both lungs
    (ii) dense, confluent lesions do not involve more than one-third of the volume of one lung, and
    (iii) the total diameter of the cavities are less than 4 cm; and
(3) far advanced lung disease was defined as: lesions more extensive than moderately advanced disease. Thirty-four, 38 and 15 patients had minimal, moderately advanced and far advanced lung disease, respectively. Twenty-nine patients had cavitary lesion(s). Subjects with diabetes were not included in the control group. After full explanation of the study by research personnel, written informed consent was obtained from the subjects or guardian(s). This study was approved by the ethical committees of Kyushu University and by the other participating institutions.

### Screening of the candidate genes

Genomic DNAs were extracted from whole blood by using QIAamp DNA Blood Kit (Qiagen, Germantown, MD). Twenty-one candidate genes selected for analysis consisted of three genes whose association with TB has been observed in Japanese and/or other ethnic population (SLC11A1, VDR and IL-1β genes), 14 genes associated with IL-12/IFN-γ axis (IFN-γ, IFN-γR [IFN-γ receptor] P, IFN-γR2, IL-12 p40, IL-12p35, IL-12R [IL-12 receptor] β1, IL-12Rβ2, signal transducer and activator of transcription [STAT]-1, IL-18, IL-18R [IL-18 receptor], IL-23p19, IL-23R [IL-23 receptor], IL-27p28 and IL-27R [IL-27 receptor, WSX-1] genes), three genes associated with tumor necrosis factor (TNF)-α signaling (TNF-α, TNFRSF [TNF receptor superfamily]1 A and TNFRSF1B genes), and ubiquitin protein ligase E3A (UBE3A) gene, a putative TB susceptibility gene in chromosome 15q11–13 based on the sib-pair linkage analysis (Cervino *et al.*, 2002). All of them are located on autosomal chromosomes. HLA genes were not analysed in this study because of their complexity. These candidate genes were screened by association analysis of marker SNPs, which were validated by the TaqMan™ Validated SNP Genotyping Assays (Applied Biosystems, Foster City, CA). A total of 118 marker SNPs with 62–23 572 base pair (bp) interval within each gene (median 5633 bp interval) were genotyped by Assays-On-Demand™ primer and probe sets (Applied Biosystems) using ABI PRISM 7900HT (Applied Biosystems) according to the manufacturer's protocol.

### SNPs detection and genotyping by PCR sequencing

For genes with two or more marker SNPs exhibiting significant allele association with TB (cut-off at $P < 0.05$), we subsequently searched for adjacent cSNPs by PCR and direct sequencing. Genomic DNAs extracted from whole blood of 24 TB patients randomly selected from the total TB population were used. Twenty-four samples are sufficient to detect SNPs with minor allele frequencies over 5%. To analyse exons 1–7 and 3′ UTR of *IL1B* adjacent to three marker SNPs with positive association (rs1143629, rs1143643 and rs3917368), we constructed eight pairs of oligonucleotide primer pairs according to the human *IL1B* gene sequence (GenBank Accession No. AY137079), as follows: 5′-AAACAGCGAGGGAGAAACTG-3′ and 5′-GCATACACACAAAGAGGCAGAG-3′ for exon 1, 5′-ACACATGAACGTAGCCGTCA-3′ and 5′-AGGGGAA-AAATCTGGTCTCC-3′ for exon 2, 5′-GCAGGCT-GTTTGCAGTTTCT-3′ and 5′-TCCTTGGGTTGGGAG-TTAAA-3′ for exon 3, 5′-CTCCCTCCCTCGCTCTCT-3′ and 5′-CTGCCTGCTCTTGGCTAACT-3′ for exon 4,

 

5′-CCTAAACAACATGTGCTCCA-3′ and 5′-AATTAGCAAGCTGCCAGGAG-3′ for exon 5, 5′-CTGCACTGCTGTGTCCCTAA-3′ and 5′-AAGTGGTAGCAGGAGGCTGA-3′ for exon 6, 5′-CCTTGCCCCACAAAAATTC-3′ and 5′-TACCCTAAGGCAGGCAGTTG-3′ for 3′ UTR, and 5′-CTGGCAGAAAGGGAACAGAA-3′ and 5′-ACTTCTTGCCCCCTTTGAAT-3′ for 3′ UTR.

To analyse exons 1–17 of *IL12RB1* adjacent to two marker SNPs with positive association (rs2305739 and rs383483), we constructed 17 pairs of oligonucleotide primer pairs according to the human *IL12RB1* gene sequence (GenBank Accession No. AY771996), as follows: 5′-GCTTCAATGTGTTCCGGAGT-3′ and 5′-CCCACAGCTCTCCACACATA-3′ for exon 1, 5′-GAGGGTGCATAGATGGGAAA-3′ and 5′-ATCCTCAGCCAACAATGAGG-3′ for exon 2, 5′-TGAGGTGACGCTGAAAGATG-3′ and 5′-TGAGGGTTTGGGAATGGTAG-3′ for exon 3, 5′-CACTGACACCCTCCTTCCTG-3′ and 5′-CTGATGGCCTCTCTGGGTAA-3′ for exon 4, 5′-TTCAGGGCCCATTAACTCAC-3′ and 5′-CCTGGACTTGGGAAACAAAC-3′ for exon 5, 5′-TTCAGCACCAAAATGCAAAA-3′ and 5′-CTGAACTATGGGGCAGGGTA-3′ for exon 6, 5′-GGACAATTCTTACGGCCTGA-3′ and 5′-TTGCCCCTGTTCCTGTACTC for exon 7, 5′-AGTTGGTTTGGTTCTGATTGC-3′ and 5′-TCCCTCCATCTACCACTTGC-3′ for exon 8, 5′-TGCCTATGGGATGATGAGTG -3′ and 5′-GAGGCTCAGAGTAGGTGCTCA for exon 9, 5′-CAACTGTCTCGATGCGTCTC-3′ and 5′-AGGGCACAGAGGAGGGGTAG-3′ for exon 10, 5′-CCTGGCCTTTGCTTATCCTT-3′ and 5′-CACTGTGCCCAGCCTCTATT for exon 11, 5′-CCAGCATTCTTGGTGTTGAC-3′ and 5′-CAGGTCTGCACTGCCTCAC-3′ for exon 12, 5′-CCTGGCCTCTGAGGAGTAAA-3′ and 5′-GCAGTGCATGCTGGGTAAAT-3′ for exon 13, 5′-AGGAAGAGGCAGGAGGTAGC-3′ and 5′-CTGCCCAGCATCATTACCAT-3′ for exon 14, 5′-AGCAAGACTCCGTCTCCAAA-3′ and 5′-AATGCGTAACCCTTGTCCAG-3′ for exon 15, 5′-GTGGCCCTACCCCTCCCTCT-3′ and 5′-CTGACCGTCTGGCCCACT for exon 16, and 5′-CTACAACCACCCCCTGAAAG-3′ and 5′-CCATTTCATGGCAGCATCTA-3′ for exon 17.

Approximately 10 ng of genomic DNA and 5 pmol of each primer were used in a standard PCR reaction. Direct sequencing of PCR products was performed using the Big Dye terminator cycle sequencing kit (Applied Biosystems), according to the manufacturer's protocol. Sequencing reactions were run on an ABI 3700 automated sequencer (Applied Biosystems). Data were collected and analysed using the ABI DNA Sequencing Software Version 3.6. cSNPs were identified using the SeqMan II software version 4 (DNASTAR Inc., Madison, WI, USA). Among the cSNPs identified, non-synonymous cSNPs were selected for the second-round association study. Genotyping of 641A/G, 1094T/C, 1132C/G and 1573G/A SNPs of *IL12RB1* was performed by PCR and direct sequencing using primer pairs for exons 7, 10 and 13 listed in previous discussions. Positions given for the four cSNPs are those noted in relation to the transcription start site.

### Statistics

Chi-square tests were employed to evaluate statistical differences in genotype distributions and allele frequencies of each SNP between TB and control groups. Genotype distributions of tested SNPs were compatible with the Hardy–Weinberg equilibrium. *P* values less than 0.05 were considered statistically significant. Linkage disequilibrium (LD) was evaluated by Lewontin's D′ (| D′ |) running all pairs of bi-allelic loci (Hedrick, 1987). All statistical analyses including haplotype estimation and association by $\chi^2$ test were performed by using SNPAlyze version 3.2 software (DYNACOM, Mobara, Japan) (Tanaka *et al.*, 2003).

## Results

A total of 118 marker SNPs listed in Table 1 were genotyped for 87 TB patients and 265 control subjects. Location of these marker SNPs in each gene was as follows: 57 SNPs in intron, 2 synonymous cSNPs, 1 non-synonymous cSNP, 3 SNPs in 5′untranslated region (5′UTR), 5 SNPs in 3′UTR, 23 SNPs in the upstream of the 5′ end of the first exon (5′ upstream) and 27 SNPs in the downstream of the 3′ end of the last exon (3′downstream). These marker SNPs covered the 21 candidate genes, and frequencies of the minor allele observed in control subjects were between 0.01 and 0.50 (average was 0.25). Association analysis revealed that seven SNPs showed a significant difference ($P < 0.05$) in the allele frequencies between the two groups; 3 in *IL1B* (rs1143629 [$P = 0.002$], rs1143643 [$P = 0.002$] and rs3917368 [$P = 0.049$]); 2 in *IL12RB1* (rs383483 [$P = 0.011$], rs2305739 [$P = 0.037$]); and 1 in *STAT1* (rs2280234 [$P = 0.004$]) and *TNFRSF1B* (rs496888 [$P = 0.007$]) (Table 1). With respect to *STAT1*, the distance between rs2280234 and its closest known cSNP, rs1803838 (chromosome position 191670871), is 4.8 kb, whereas rs2280235 with 1.4 kb distance from rs1803838 showed no association ($P = 0.680$). As to *TNFRSF1B*, rs496888 is located 14 kb upstream to exon 1, and marker SNPs closer to exon 1 (rs976881, rs616645 and rs474247) showed no association. Therefore, *STAT1* and *TNFRSF1B* with a single positive marker SNP were not further analysed.

Sequencing analysis of coding regions of *IL1B* and *IL12RB1* and 3′ UTR of *IL1B* adjacent to the marker SNPs with positive association showed one cSNP in exon 5 of *IL1B* and seven cSNPs in exons 4, 7, 10 and 13, and in 3′UTR of *IL12RB1*. Among them, four cSNPs of *IL12RB1* (641 A/G in exon 7, 1094T/C and 1132C/G in exon 10 and 1573G/A in exon 13) previously reported in Japanese population (Sakai *et al.*, 2001) were non-synonymous and were further analysed for association study (Fig. 1). As shown in Table 2, a significant difference in the genotype and allele frequencies between TB patients and controls was found for *IL12RB1*641 A/G, 1094T/C and 1132C/G SNPs ($P = 0.030$, $P = 0.013$ and $P = 0.013$, respectively). The genotype and allele frequencies of 1132C/G SNP were exactly the same as those of 1094T/C SNP. Genotype and allele frequencies of 1573G/A SNP

 

**Table 1.** List of marker SNPs analysed in this study

| Gene symbol | dbSNP ID[a] | Location | Chromosome position[b] | Minor allele frequency[c] | *P* value[d] |
|---|---|---|---|---|---|
| *SLC11A1(NRAMP1)* | rs4674301 | 5′ upstream | 219,068,367 | 0.20 | 0.854 |
| | rs2290708 | intron | 219,077,882 | 0.08 | 0.096 |
| | rs1059823 | 3′ UTR | 219,085,349 | 0.28 | 0.234 |
| | rs2227255 | 3′ downstream[e] | 219,093,286 | 0.36 | 0.527 |
| *VDR* | rs11608702 | 3′ downstream | 46,515,035 | 0.33 | 0.816 |
| | rs1544410 | intron | 46,526,102 | 0.18 | 0.768 |
| | rs2239183 | intron | 46,530,927 | 0.07 | 0.869 |
| | rs2248098 | intron | 46,539,623 | 0.33 | 0.623 |
| | rs2239180 | intron | 46,542,313 | 0.19 | 0.114 |
| | rs1540339 | intron | 46,543,593 | 0.26 | 0.109 |
| | rs2238138 | intron | 46,550,760 | 0.12 | 0.361 |
| | rs1989969 | intron | 46,564,277 | 0.31 | 0.164 |
| | rs3890733 | intron | 46,575,640 | 0.01 | 0.651 |
| | rs10083198 | intron | 46,582,232 | 0.45 | 0.855 |
| | rs4516035 | 5′ upstream | 46,586,093 | 0.01 | 0.518 |
| | rs7976091 | 5′ upstream | 46,590,819 | 0.37 | 0.694 |
| *IL1B* | rs3917368 | 3′ downstream | 113,299,013 | 0.48 | 0.002* |
| | rs1143643 | intron | 113,304,533 | 0.48 | 0.002* |
| | rs1143629 | intron | 113,309,749 | 0.50 | 0.049* |
| | rs1143623 | 5′ upstream | 113,312,060 | 0.38 | 0.052 |
| | rs13032029 | 5′ upstream | 113,316,646 | 0.45 | 0.055 |
| *IFNG* | rs2193049 | 3′ downstream | 66,833,189 | 0.49 | 0.510 |
| | rs2069718 | intron | 66,836,429 | 0.10 | 0.594 |
| *IFNGR1* | rs11914 | Coding, synonymous | 137,561,281 | 0.07 | 0.159 |
| | rs2234711 | 5′ UTR | 137,582,213 | 0.49 | 0.572 |
| | rs1327474 | 5′ upstream | 137,582,768 | 0.06 | 0.658 |
| | rs608914 | 5′ upstream | 137,588,731 | 0.39 | 0.394 |
| *IFNGR2* | rs2284553 | intron | 33,698,565 | 0.28 | 0.601 |
| | rs2268241 | intron | 33,702,920 | 0.48 | 0.722 |
| | rs9808753 | Coding, non-synonymous | 33,709,182 | 0.47 | 0.784 |
| | rs2834214 | intron | 33,715,576 | 0.18 | 0.640 |
| | rs1532 | intron[f] | 33,726,836 | 0.03 | 0.114 |
| | rs2284556 | intron[g] | 33,728,175 | 0.19 | 0.508 |
| | rs11088252 | 3′ downstream[g] | 33,737,563 | 0.17 | 0.586 |
| | rs7282496 | 3′ downstream[g] | 33,741,452 | 0.19 | 0.594 |
| *IL12A* (p35) | rs2242382 | intron | 161,194,604 | 0.08 | 0.146 |
| | rs668998 | 3′ downstream | 161,198,253 | 0.28 | 0.836 |
| *IL12B* (p40) | rs11135058 | 3′ downstream | 158,667,095 | 0.24 | 0.086 |
| | rs6870828 | 3′ downstream | 158,671,090 | 0.24 | 0.320 |
| | rs2288831 | intron | 158,682,591 | 0.46 | 0.489 |
| *IL12RB1* | C_3057455_10 | 3′ downstream | 18,021,464 | 0.17 | 0.320 |
| | rs404733 | 3′ downstream | 18,030,997 | 0.44 | 0.054 |
| | rs383483 | intron | 18,032,886 | 0.41 | 0.011* |
| | rs2305739 | intron | 18,041,194 | 0.21 | 0.037* |
| | rs2305742 | intron | 18,052,441 | 0.20 | 0.118 |
| | rs436857 | 5′ UTR | 18,058,635 | 0.19 | 0.158 |
| | rs2045387 | 5′ upstream | 18,061,586 | 0.01 | 0.995 |
| | rs7250425 | 5′ upstream | 18,062,757 | 0.30 | 0.441 |
| | rs273504 | 5′ upstream | 18,076,247 | 0.31 | 0.462 |
| *IL12RB2* | rs1546159 | intron | 67,500,447 | 0.22 | 0.875 |
| | rs7518845 | intron | 67,523,001 | 0.24 | 0.557 |
| | rs7535591 | intron | 67,529,168 | 0.23 | 0.709 |
| | rs2252596 | intron | 67,545,522 | 0.22 | 0.680 |
| | rs6685568 | intron | 67,567,318 | 0.23 | 0.671 |
| *STAT1* | rs867637 | 3′ downstream[h] | 191,651,888 | 0.27 | 0.243 |
| | rs12987796 | 3′ downstream | 191,656,372 | 0.23 | 0.148 |
| | rs1914408 | intron | 191,665,482 | 0.31 | 0.561 |
| | rs2280235 | intron | 191,669,336 | 0.41 | 0.680 |
| | rs2280234 | intron | 191,675,605 | 0.18 | 0.004* |
| | rs2280232 | intron | 191,676,272 | 0.20 | 0.653 |
| | rs2066805 | intron | 191,688,407 | 0.05 | 0.093 |
| | rs2066802 | Coding, synonymous | 191,700,173 | 0.22 | 0.585 |
| | rs1467199 | 5′ upstream | 191,706,008 | 0.47 | 0.891 |

 

**Table 1.** *Continued*

| Gene symbol | dbSNP ID[a] | Location | Chromosome position[b] | Minor allele frequency[c] | *P* value[d] |
|---|---|---|---|---|---|
| *IL18* | rs3882891 | intron | 111,519,971 | 0.44 | 0.598 |
| | rs1834481 | intron | 111,529,037 | 0.01 | 0.322 |
| | rs4937113 | intron | 111,534,931 | 0.44 | 0.674 |
| | rs2043055 | 5′ UTR | 111,536,834 | 0.43 | 0.810 |
| | rs360712 | 5′ upstream[i] | 111,545,237 | 0.14 | 0.727 |
| | rs795468 | 5′ upstream[j] | 111,547,407 | 0.14 | 0.761 |
| *IL18R1* | rs1861246 | 5′ upstream[k] | 102,425,301 | 0.42 | 0.148 |
| | rs12999364 | 5′ upstream | 102,432,647 | 0.38 | 0.086 |
| | rs11465567 | 5′ upstream | 102,436,918 | 0.03 | 0.344 |
| | rs1558627 | intron | 102,443,202 | 0.57 | 0.136 |
| | rs1974675 | intron | 102,444,893 | 0.19 | 0.813 |
| | rs2270297 | intron | 102,451,193 | 0.43 | 0.082 |
| | rs3213733 | intron | 102,456,402 | 0.16 | 0.318 |
| | rs2241116 | intron | 102,461,783 | 0.15 | 0.480 |
| | rs2287033 | intron | 102,469,755 | 0.20 | 0.694 |
| | rs3732127 | 3′ UTR | 102,472,268 | 0.16 | 0.371 |
| | rs1420094 | 3′ downstream | 102,474,205 | 0.20 | 0.678 |
| | rs3732124 | 3′ downstream | 102,476,570 | 0.21 | 0.633 |
| *IL23A* | rs2371494 | 5′ upstream | 55,014,267 | 0.06 | 0.635 |
| | rs2066808 | 3′ downstream[l] | 55,024,240 | 0.06 | 1.00 |
| | rs2066807 | 3′ downstream[m] | 55,026,949 | 0.06 | 0.588 |
| *IL23R* | rs1343151 | intron | 67,431,150 | 0.10 | 0.439 |
| | rs10889677 | 3′ UTR | 67,437,141 | 0.28 | 0.922 |
| | rs4655531 | 3′ downstream | 67,439,799 | 0.17 | 0.626 |
| | C_2720245_10 | 3′ downstream | 67,442,774 | 0.12 | 0.678 |
| *IL27*(EBI3, p28) | rs40834 | 3′ downstream | 28,417,894 | 0.28 | 0.767 |
| | rs40835 | 3′ downstream | 28,417,956 | 0.24 | 0.644 |
| | rs181207 | intron | 28,421,031 | 0.13 | 0.183 |
| *IL27RA*(WSX-1) | rs1982632 | 5′ upstream | 14,000,004 | 0.19 | 0.051 |
| | rs2306190 | intron | 14,023,676 | 0.39 | 0.169 |
| | C_1878989_10 | 3′ downstream | 14,033,779 | 0.12 | 0.179 |
| | rs10415758 | 3′ downstream | 14,033,921 | 0.35 | 0.462 |
| *TNF* | rs1800683 | 5′ upstream[n] | 31,648,050 | 0.36 | 0.482 |
| | rs2857713 | 5′ upstream[o] | 31,648,535 | 0.19 | 0.228 |
| | rs1799724 | 5′ upstream | 31,650,461 | 0.22 | 0.522 |
| | rs361525 | 5′ upstream | 31,651,080 | 0.03 | 0.430 |
| | rs769178 | 3′ downstream | 31,655,493 | 0.21 | 0.747 |
| *TNFRSF1A* | rs740841 | 3′ downstream[p] | 6,303,550 | 0.35 | 0.264 |
| | rs2302350 | 3′ downstream[p] | 6,306,014 | 0.29 | 0.132 |
| | rs1860545 | intron | 6,317,038 | 0.18 | 0.369 |
| | rs4149577 | intron | 6,317,783 | 0.46 | 0.159 |
| | rs4149576 | intron | 6,319,376 | 0.19 | 0.295 |
| *TNFRSF1B* | rs590368 | 5′ upstream | 12,157,717 | 0.33 | 0.677 |
| | rs496888 | intron | 12,167,072 | 0.16 | 0.007* |
| | rs976881 | intron | 12,168,020 | 0.15 | 0.294 |
| | rs616645 | intron | 12,175,090 | 0.21 | 0.747 |
| | rs474247 | intron | 12,180,441 | 0.37 | 0.573 |
| | rs653667 | intron | 12,186,074 | 0.31 | 0.111 |
| | rs5746053 | intron | 12,196,564 | 0.16 | 0.127 |
| | rs1061631 | 3′ UTR | 12,202,765 | 0.14 | 0.787 |
| *UBE3A* | rs4906951 | 3′ downstream | 23,126,764 | 0.02 | 0.740 |
| | rs12443207 | intron | 23,141,250 | 0.36 | 0.937 |
| | rs12907375 | intron | 23,151,415 | 0.36 | 0.769 |
| | rs4906708 | intron | 23,169,072 | 0.36 | 0.701 |
| | rs7496951 | 3′ UTR | 23,222,396 | 0.36 | 0.735 |

NOTE: SNP, single nucleotide polymorphism; UTR, untranslated region. [a] When reference SNP (rs) number is not available, assays-on-demand[R] assay ID is shown. [b] chromosome position of SNP is from the DBSNP build 124 in the database of the National Center for Biotechnology Information (http://www.nabi.nlm.nih.gov/). [c] Minor allele frequencies observed in control samples are shown. [d] *P* values of $\chi^2$ test in allele frequency differences are shown. Alternatively, [e] synonymous cSNP of *CTDSP1* gene, [f] SNP in 3′ UTR or [g] SNP in intron of *TMEM50B* gene, [h] SNP in intron of *GLS* gene, [i] synonymous cSNP or [j] SNP in intron of *TEX12* gene, [k] SNP in intron of *IL1RL1* gene, [l] SNP in intron or [m] nonsynonymous cSNP of *STAT2* gene, [n] SNP in 5′ UTR or [o] nonsynonymous cSNP of *LTA* gene, [p] SNP in intron of *PLEKHG6* gene.

 

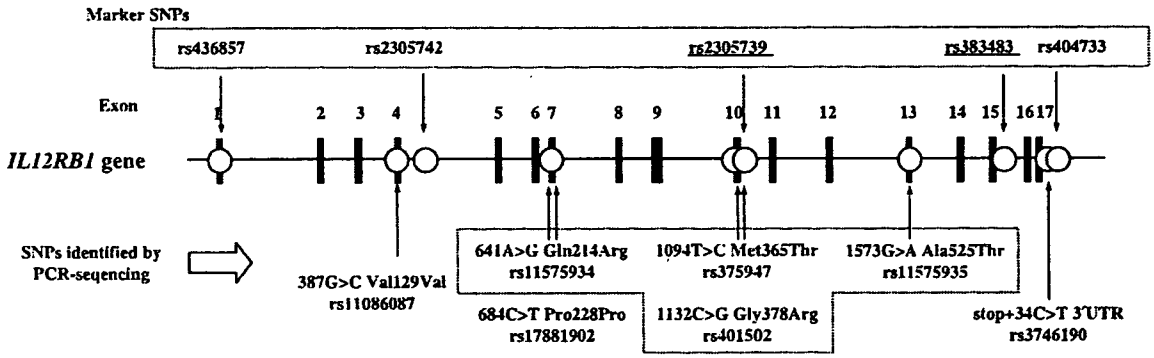


**Figure 1.** Structure of *IL12RB1* gene and location of the marker SNPs and identified cSNPs. SNP, single nucleotide polymorphism; UTR, untranslated region.

**Table 2.** Genotype and allele frequencies of *IL12RB1*641A/G, 1094T/C, 1132C/G and 1573G/A SNPs in TB patients and controls

| *IL12RB1* SNPs | Controls | TB | OR [95%CI] | *P*-value (chi-square) |
|---|---|---|---|---|
| 641A/G | | | | |
| Genotype frequency | | | | |
| AA | 98 (38%) | 23 (27%) | | |
| AG | 120 (47%) | 41 (48%) | 1.46 [0.82–2.59] | 0.20 |
| GG | 37 (15%) | 22 (26%) | 2.53 [1.26–5.08] | 0.0078 |
| Total | 255 | 86 | | |
| Allele frequency | | | | |
| A | 316 (62%) | 87 (51%) | | |
| G | 194 (38%) | 85 (49%) | 1.59 [1.12–2.25] | 0.0087 |
| 1094T/C (1132C/G) | | | | |
| Genotype frequency | | | | |
| TT (GG) | 96 (37%) | 20 (23%) | | |
| TC (GC) | 125 (48%) | 44 (51%) | 1.69 [0.93–3.05] | 0.080 |
| CC (CC) | 39 (15%) | 23 (26%) | 2.83 [1.40–5.73] | 0.0032 |
| Total | 260 | 87 | | |
| Allele frequency | | | | |
| T (G) | 317 (61%) | 84 (48%) | | |
| C | 203 (39%) | 90 (52%) | 1.67 [1.18–2.36] | 0.0034 |
| 1573G/A | | | | |
| Genotype frequency | | | | |
| AA | 1 (0%) | 0 (0%) | | |
| GA | 15 (6%) | 11 (13%) | | |
| GG | 247 (94%) | 76 (87%) | | |
| Total | 263 | 87 | | |
| Allele frequency | | | | |
| A | 17 (3%) | 11 (7%) | 2.23 [0.93–4.40] | 0.071 |
| G | 509 (97%) | 153 (93%) | | |

NOTE: SNP, single nucleotide polymorphism; TB, tuberculosis.

were not significantly different between TB patients and controls (Table 2). When TB patients were divided into two subgroups according to the severity of lung disease, the advanced subgroup (patients with moderately or far advanced lung disease) showed prominent associations with GG genotype ($P = 0.0014$) and G allele ($P = 0.0015$) of 641 A/G SNP, and with CC genotype ($P = 0.00034$) and C allele ($P = 0.00044$) of 1094T/C or 1132C/G SNP (Table 3). There were no significant differences in the genotype and allele distributions of 641 A/G (genotype, $P = 0.48$; allele, $P = 0.36$) and of 1094T/C (1132C/G) (genotype, $P = 0.14$; allele, $P = 0.22$) between men and women of the control group (data not shown). Subsequent LD analysis of the four cSNPs spanning 12 kb of *IL12RB* showed almost complete LD among 641 A/G, 1094T/C and 1132C/G SNPs ($D' = 0.95$–$1.00$) and modest LD between 1573G/A SNP and one of the other three SNPs ($D' = 0.64$–$0.81$) (Table 4). To investigate if a particular haplotype constituted by these cSNPs was associated with the disease, haplotype frequencies were estimated and association analysis was performed. As shown in Table 5, the frequency of GCCC haplotype in TB patients

 

**Table 3.** Genotype and allele frequencies of *IL12RB1* 641A/G, 1094T/C and 1132C/G SNPs in TB patient subgroups classified by disease severity

| *IL12RB1* SNPs | Controls | TB Minimal lung disease | OR [95%CI] | *P*-value (chi-square) | TB Advanced lung disease[a] | OR [95%CI] | *P*-value (chi-square) |
|---|---|---|---|---|---|---|---|
| 641A/G | | | | | | | |
| Genotype frequency | | | | | | | |
| AA | 98 (38%) | 13 (38%) | | | 10 (19%) | | |
| AG | 120 (47%) | 14 (41%) | 0.88 [0.39–1.96] | 0.75 | 27 (52%) | 2.21 [1.02–4.78] | 0.041 |
| GG | 37 (15%) | 7 (21%) | 1.43 [0.53–3.85] | 0.48 | 15 (29%) | 3.97 [1.64–9.63] | 0.0014 |
| Total | 255 | 34 | | | 52 | | |
| Allele frequency | | | | | | | |
| A | 316 (62%) | 40 (59%) | | | 47 (45%) | | |
| G | 194 (38%) | 28 (41%) | 1.14 [0.68–1.91] | 0.62 | 57 (55%) | 1.97 [1.29–3.02] | 0.0015 |
| 1094T/C (1132C/G) | | | | | | | |
| Genotype frequency | | | | | | | |
| TT (GG) | 96 (37%) | 12 (35%) | | | 8 (15%) | | |
| TC (GC) | 125 (48%) | 15 (44%) | 0.96 [0.43–2.15] | 0.92 | 29 (55%) | 2.78 [1.22–6.36] | 0.012 |
| CC (CC) | 39 (15%) | 7 (21%) | 1.44 [0.53–3.92] | 0.48 | 16 (30%) | 4.92 [1.95–12.4] | 0.00034 |
| Total | 260 | 34 | | | 53 | | |
| Allele frequency | | | | | | | |
| T (G) | 317 (61%) | 39 (57%) | | | 45 (42%) | | |
| C | 203 (39%) | 29 (43%) | 1.16 [0.70–1.94] | 0.57 | 61 (58%) | 2.12 [1.39–3.23] | 0.00044 |

NOTE: Comparisons were made between controls and two subgroups of TB patients (minimal lung disease and advanced lung disease), respectively. SNP, single nucleotide polymorphism; TB, tuberculosis; [a], moderately or far advanced lung disease.

**Table 4.** Pairwise linkage disequlibrium analysis for four non-synonymous cSNPs of *IL12RB1* gene

| | 641A/G | 1094T/C | 1132C/G | 1573G/A |
|---|---|---|---|---|
| 641A/G | | 0.95 | 0.95 | 0.64 |
| 1094T/C | | | 1.00 | 0.81 |
| 1132C/G | | | | 0.81 |

NOTE: SNP, single nucleotide polymorphism.
$n = 249$ (control samples), evaluated by absolute D′ static.

**Table 5.** Estimated frequencies of haplotypes constituted by four cSNPs of *IL12RB1* in TB patients and controls

| | Frequency | | | |
|---|---|---|---|---|
| Haplotype[a] | Controls ($n = 249$) | TB ($n = 86$) | chi-square | *P*-value |
| ATGG | 0.598 | 0.483 | 7.46 | 0.0063 |
| GCCA | 0.026 | 0.058 | 3.85 | 0.022 |
| GCCG | 0.339 | 0.436 | 5.23 | 0.050 |
| others[b] | 0.037 | 0.023 | | |

NOTE: SNP, single nucleotide polymorphism; TB, tuberculosis.
[a] Haplotypes constituted by 641A/G, 1094T/C, 1132 G/C and 1573G/A.
[b] Haplotypes with frequencies < 0.03.

was higher than that in controls with a marginal significance ($P = 0.050$), whereas that of ATGG haplotype was significantly lower in TB patients than in controls ($P = 0.0063$).

Because the genotype information of 1094T/C and 1132C/G SNPs in *IL12RB1* was available in the database of International HapMap Project (http://www.hapmap.org), haplotype frequencies of the two loci in different ethnic groups were calculated and compared with those of our subjects. The frequency of CC haplotype of 1094T/C and 1132C/G SNPs in TB group (51.7%) was significantly higher than that in controls (39.0%) (odds ratio = 1.67, $P = 0.0034$), besides the frequencies in HCB (Han Chinese in Beijing, China, 38.7%), CEU (Utah residents with ancestry from Northern and Western Europe, 37.6%), supporting association of this haplotype of *IL12RB1* with TB.

## Discussion

In a gene-based association study on 21 candidate genes for TB susceptibility using SNPs as genetic markers, we demonstrated that three non-synonymous cSNPs of *IL12RB1* were associated with TB in the Japanese population in terms of susceptibility and disease severity. Because direct association analysis using functional variants is limited by incomplete knowledge about functional variation at present, indirect association mapping using marker SNPs has been considered to identify genes conferring susceptibility to common diseases such as myocardial infarction and rheumatoid arthritis (Ozaki *et al.*, 2002; Tokuhiro *et al.*, 2003). We applied gene-based SNPs mapping to screen 21 candidate genes for TB susceptibility in the present study.

Two studies on Japanese population showed the association of *SLC11A1*and *IL12RB1* with TB, respectively. Gao *et al.* (2000) reported that a 5′ promoter $(GT)_n$ polymorphism of *SLC11A1* was associated with active TB in Japanese. On the other hand, Abe *et al.* (2003) found that a SNP in intron 4 (rs3731865) showing strong LD with 5′ promoter $(GT)_n$ did not affect TB susceptibility in Japanese.

 

In the present study, although rs3731865 was not available from Assays-On-Demand™ primer and probe sets, an SNP (rs2290708) with a 2.4-kb distance from it showed no association with TB (Table 1). Akahoshi *et al.* (2003) reported that 641 A/G, 684C/T, 1094T/C and 1132C/G SNPs of *IL12RB1* in almost complete LD were associated with TB and one of the two common haplotypes (GTCC) was significantly associated with TB. The present study demonstrated a similar association as a result of gene-based screening of 21 candidate genes. We performed a haplotype analysis using different combination of SNPs including 1573G/A in exon 13 instead of 684C/T in exon 7. Although the difference in the frequencies of GCCG haplotype between TB patients and controls showed a marginal significance ($P = 0.050$), that of the protective haplotype, ATTG, was significant ($P = 0.0063$), assuring the association between *IL12RB1* and TB susceptibility. Therefore, our study, together with the study by Akahoshi *et al.* (2003), suggested that *IL12RB1* polymorphisms, at least in part, confer genetic susceptibility to TB in Japanese. When TB patients were divided into two subgroups according to the severity of lung disease, advanced subgroup of TB patients showed a prominent association with 641 A/G, 1094T/C and 1132C/G SNPs in the present study. Associations of HLA class II antigens and *SLC11A1* gene with severity of TB have been reported (Brahmajothi *et al.*, 1991; Rajalingam *et al.*, 1996; Kim *et al.*, 2005; Zhang *et al.*, 2005). This is the first report that suggests genetic variants of *IL12RB1* were associated with the progression to advanced forms of TB. In contrast, studies in Morocco and Korea did not demonstrate any association between the same cluster of SNPs and TB susceptibility (Remus *et al.*, 2004; Lee *et al.*, 2005). -2C/T SNP (rs436857), one of the two SNPs reported to be associated with pulmonary TB in the Moroccan study (Remus *et al.*, 2004), was included in the marker SNPs in the screening step of the present study, but no differences in the allele or genotype frequencies were observed between TB patients and controls ($P = 0.157$, Table 1). The difference between Moroccan and Korean studies, and Japanese ones including ours could be partly explained by the hypothesis that distinct environmental and natural selective factors resulted in population-specific immunogenetic adaptations to clinical TB (Stead, 1992; Delgado *et al.*, 2002). It is postulated that, in the area where TB has been endemic for a longer time, survivors were likely to be more resistant individuals. In the present study, the frequency of the resistant allele of 1094T/C (T allele) in controls was 0.61, which was lower than that in Moroccan study (0.74) (Remus *et al.*, 2004) and that in YRI (Yoruba in Ibadan, Nigeria) from the database of International HapMap Project (0.81).

To our knowledge, this is the first comprehensive association study of genes of IL-12/IFN-γ axis for TB susceptibility. IL-12/IFN-γ axis plays a pivotal role in the killing of intracellular mycobacteria. *IL-12RB1* encodes IL-12 Rβ1, one of the two subunits of receptor for IL-12, and is expressed on T and NK cells. Homozygous recessive mutations in *IL12RB1* preclude the surface expression of IL-12Rβ1 and IFN-γ secretion in vitro by otherwise functional T and NK cells (Altare *et al.*, 1998; de Jong *et al.*, 1998). The lack of IL-12-dependent IFN-γ secretion results in susceptibility to weakly virulent mycobacterial species, such as BCG and NTM despite the formation of mature granuloma through IL-12-independent IFN-γ secretion (Dorman & Holland, 2000; Casanova & Abel, 2002). One case of IL-12Rβ1 deficiency associated with the susceptibility to *Mycobacterium avium* complex was reported in Japan (Sakai *et al.*, 2001). The penetrance of IL-12Rβ1 deficiency for the MSMD phenotype is estimated to be less than 40% (Fieschi *et al.*, 2003), suggesting that the remaining patients could show different manifestation caused by related pathogens, such as TB. It is reported that patients with IL-12Rβ1 deficiency developed clinical TB in the absence of any personal or familial history of clinical disease by weakly virulent mycobacterial species (Altare *et al.*, 2001; Caragol *et al.*, 2003; Ozbek *et al.*, 2005). Akahoshi *et al.* (2003) demonstrated that CD2+ lymphocytes from healthy subjects homozygous for 641G, 1094C and 1132C haplotype corresponding to GCCG haplotype in the present study had a lower level of IL-12-induced signaling in vitro. Among the three cSNPs with positive association, 1132C/G (G378R) has been predicted to change the three-dimensional structure of the extracellular domain of IL-12Rβ1 through affecting the length of a predicted sheet (van de Vosse *et al.*, 2003). It is possible that this cluster of cSNPs is associated with functional change of IL-12R and directly affects the susceptibility to TB and progression of the disease in the Japanese population. As for polymorphisms of genes encoding IL–12, no association between rs3212227, an SNP in the 3′UTR of *IL12B*, and TB was demonstrated (Ma *et al.*, 2003). In the present study, both rs6870828 and rs2288831, which are located in the same LD block as rs3212227 based on the database of International HapMap Project, with 4.4 kb and 7.0 kb distance from it, respectively, were not associated with TB (Table 1).

In the screening step, two marker SNPs of *IL1B* in almost complete LD (rs3917368 in 3′ downstream and rs1143643 in intron) showed a significant association ($P = 0.002$), as shown in Table 1. With respect to rs3917368, advanced subgroup of TB patients showed a prominent association with the G allele ($P = 0.004$) and GG genotype ($P = 0.0084$) (data not shown). Although these SNPs are located outside the coding sequence, they might be associated with genetic susceptibility to TB or progression of the disease, through regulating the gene expression and/or alternative splicing, or being in strong LD with other functional SNP(s) in the non-coding region. Further study is needed to examine this possible association.

Limitations in this study include the correction for multiple comparisons and the power of the study to detect significant association, both resulting from a relatively small sample size. When Bonferroni correction was applied to the analysis in the screening step by multiplying cut-off value of 0.05 by 118, $P$ values for the seven positive marker SNPs turned out to be not significant, necessitating confirmation by replication study. However, this study

 

could serve as a replication of the previously observed association between the functional cSNPs and TB in the same ethnic population (Akahoshi *et al.*, 2003). Second, the statistical power to detect an OR of 1.6 at 0.05 significance level using 86 patients and 265 controls was 0.59 when the minor allele frequency in controls was 0.4 (Dupont & Plummer, 1990). Therefore, negative results on SNPs tested in this study do not necessarily exclude their association with TB.

In conclusion, gene-based association study on 21 candidate genes suggested that genetic variants of *IL12RB1*, at least in part, confer genetic susceptibility to TB, and are associated with the progression of the disease in Japanese. It would be warranted to examine whether the same association is observed in other ethnic groups.

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