Table 3. *Levels of* tdh$^+$ V. parahaemolyticus *in Thai shellfish samples (log MPN/g).*

Shellfish sample	IS[1] culture[a]		S[2] culture[b]	
	LAMP	PCR	LAMP	PCR
Bloody clam	1.4	1.6	2.0	2.0
Bloody clam	2.4	2.4	2.4	2.4
Bloody clam	2.0	0.6	2.0	2.0
Bloody clam	2.0	0.6	2.9	2.0
Hard clam	1.5	1.5	2.4	2.4
Hard clam	4.0	4.0	4.0	4.0
Hard clam	2.4	2.4	2.6	2.6
Green mussel	4.0	2.9	2.4	2.9
Undulated surf clam	3.2	3.2	3.2	3.2

[a] Culture taken after the first IMS-SPB enrichment step of the IMS-incorporated two-step enrichment (A-IS[1]) procedure.

[b] Culture taken after the second SPB enrichment step of the conventional three-step enrichment (A-S[1]-S[2]) procedure.

Figure 1

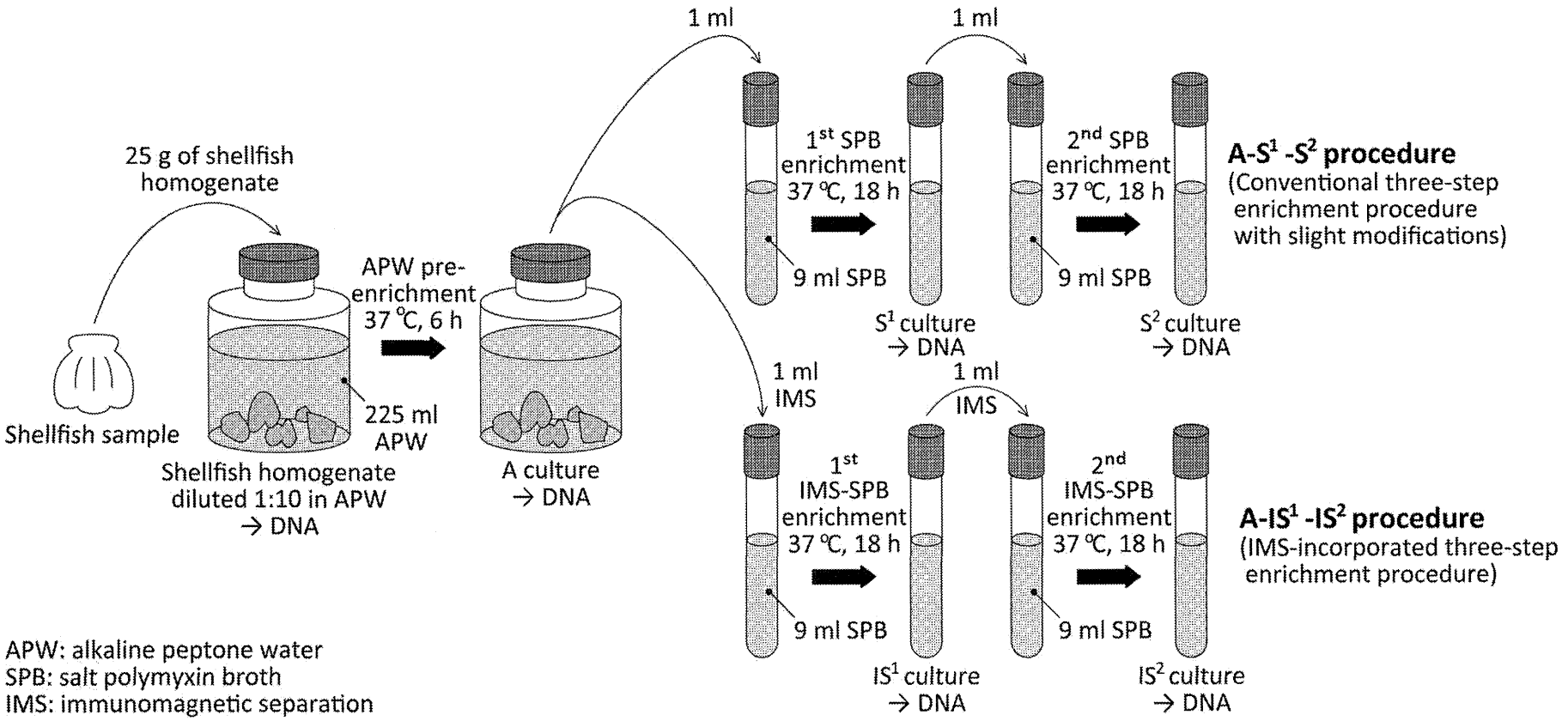
25 g of shellfish homogenate
Shellfish sample
APW pre-enrichment 37 ℃, 6 h
225 ml APW
Shellfish homogenate diluted 1:10 in APW → DNA
A culture → DNA
1 ml
1st SPB enrichment 37 ℃, 18 h
9 ml SPB
S1 culture → DNA
1 ml
2nd SPB enrichment 37 ℃, 18 h
9 ml SPB
S2 culture → DNA
A-S1 -S2 procedure
(Conventional three-step enrichment procedure with slight modifications)
1 ml IMS
1st IMS-SPB enrichment 37 ℃, 18 h
9 ml SPB
IS1 culture → DNA
1 ml IMS
2nd IMS-SPB enrichment 37 ℃, 18 h
9 ml SPB
IS2 culture → DNA
A-IS1 -IS2 procedure
(IMS-incorporated three-step enrichment procedure)
APW: alkaline peptone water
SPB: salt polymyxin broth
IMS: immunomagnetic separation

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Figure 2

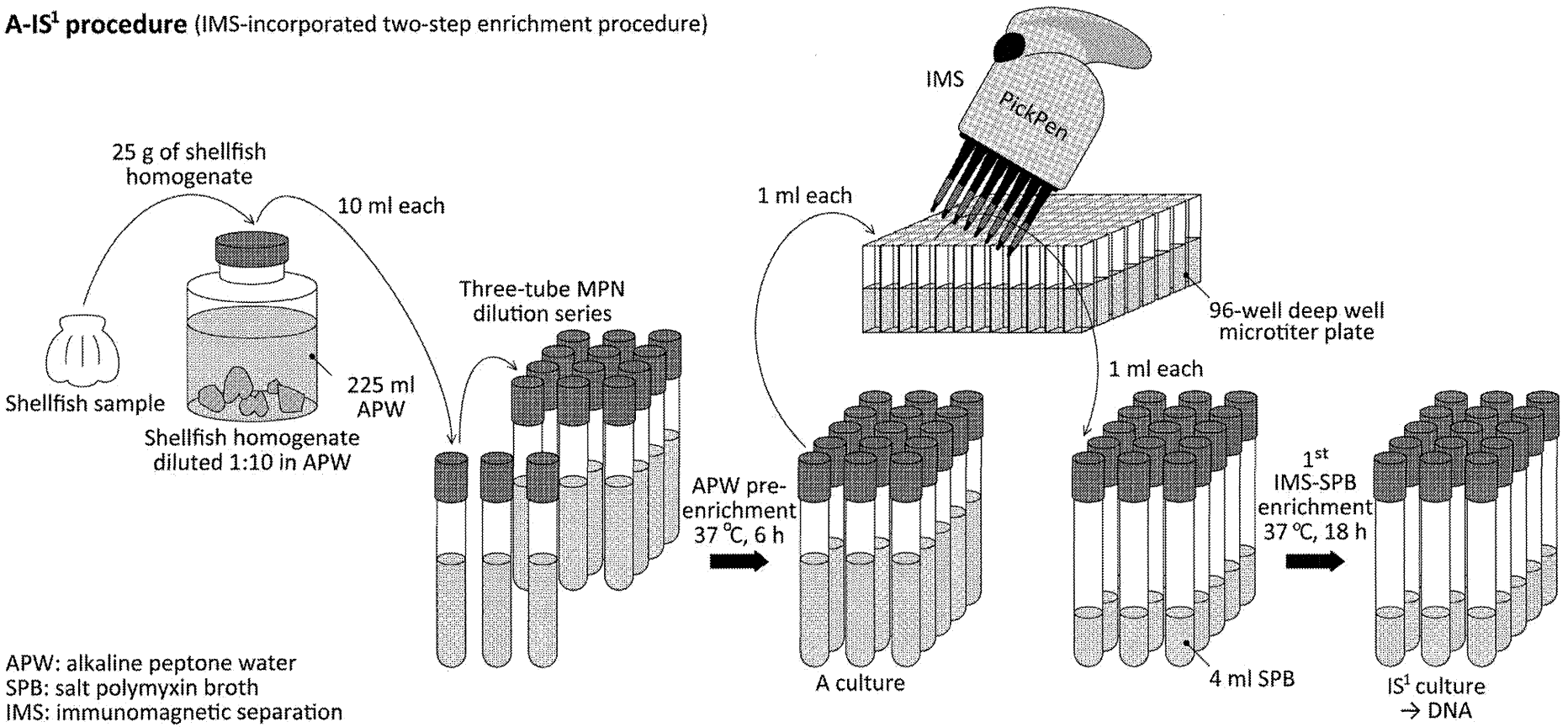
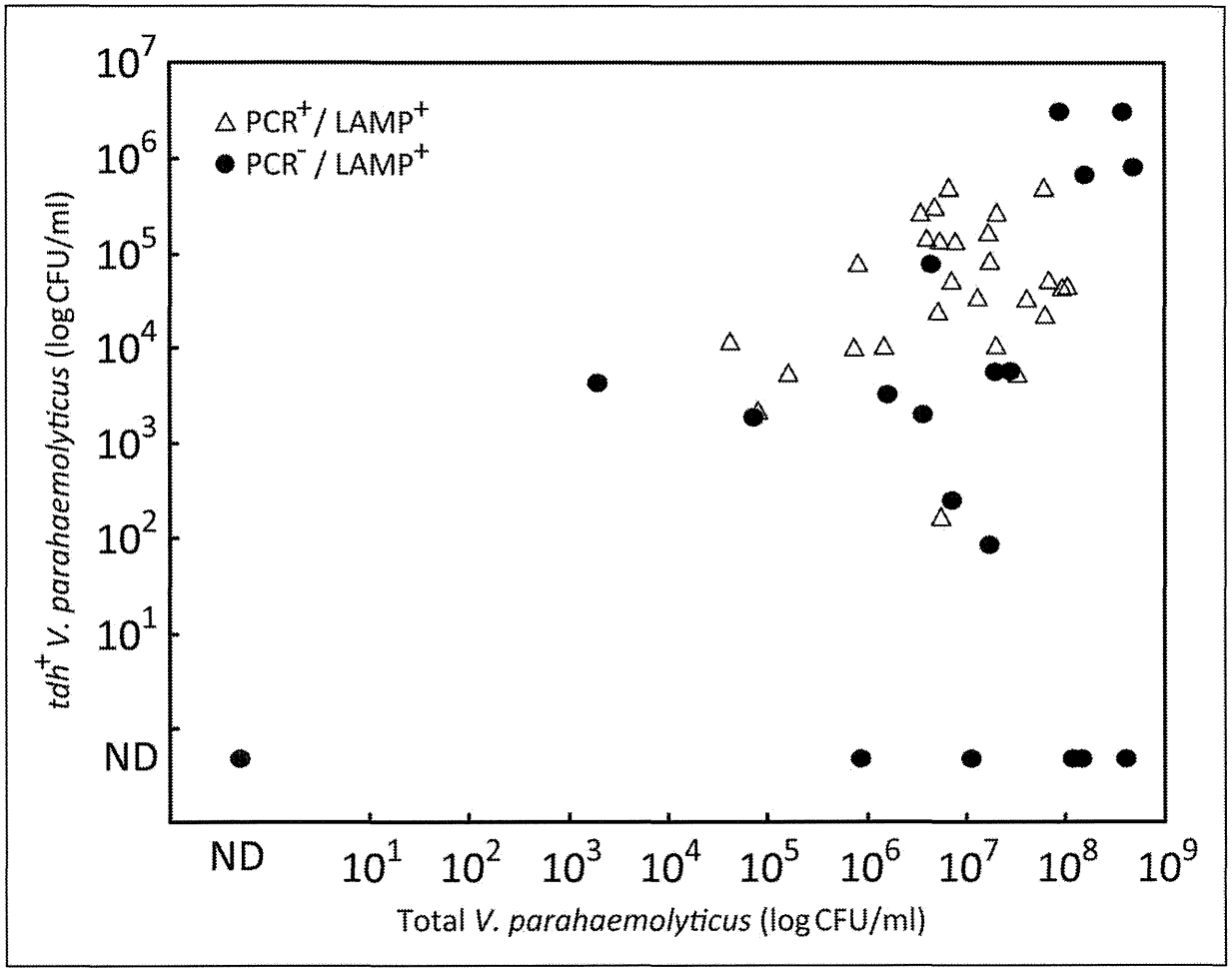

A-IS1 procedure (IMS-incorporated two-step enrichment procedure)
25 g of shellfish homogenate
10 ml each
Shellfish sample
225 ml APW
Shellfish homogenate diluted 1:10 in APW
Three-tube MPN dilution series
APW pre-enrichment 37 ℃, 6 h
A culture
IMS
PickPen
1 ml each
96-well deep well microtiter plate
1 ml each
4 ml SPB
1st IMS-SPB enrichment 37 ℃, 18 h
IS1 culture → DNA
APW: alkaline peptone water
SPB: salt polymyxin broth
IMS: immunomagnetic separation

Figure 3


△ PCR+ / LAMP+
● PCR- / LAMP+
tdh+ V. parahaemolyticus (log CFU/ml)
10^7
10^6
10^5
10^4
10^3
10^2
10^1
ND
ND
10^1
10^2
10^3
10^4
10^5
10^6
10^7
10^8
10^9
Total V. parahaemolyticus (log CFU/ml)

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Clinical and Vaccine Immunology

Efficient Activation of Human T Cells of Both CD4 and CD8 Subsets by Urease-Deficient Recombinant Mycobacterium bovis BCG That Produced a Heat Shock Protein 70-M. tuberculosis-Derived Major Membrane Protein II Fusion Protein

Tetsu Mukai, Yumiko Tsukamoto, Yumi Maeda, Toshiki Tamura and Masahiko Makino
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Efficient Activation of Human T Cells of Both CD4 and CD8 Subsets by Urease-Deficient Recombinant *Mycobacterium bovis* BCG That Produced a Heat Shock Protein 70-*M. tuberculosis*-Derived Major Membrane Protein II Fusion Protein

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For the purpose of obtaining *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) capable of activating human naive T cells, urease-deficient BCG expressing a fusion protein composed of *Mycobacterium tuberculosis*-derived major membrane protein II (MMP-II) and heat shock protein 70 (HSP70) of BCG (BCG-DHTM) was produced. BCG-DHTM secreted the HSP70-MMP-II fusion protein and effectively activated human monocyte-derived dendritic cells (DCs) by inducing phenotypic changes and enhanced cytokine production. BCG-DHTM-infected DCs activated naive T cells of both CD4 and naive CD8 subsets, in an antigen (Ag)-dependent manner. The T cell activation induced by BCG-DHTM was inhibited by the pretreatment of DCs with chloroquine. The naive $CD8^+$ T cell activation was mediated by the transporter associated with antigen presentation (TAP) and the proteosome-dependent cytosolic cross-priming pathway. Memory $CD8^+$ T cells and perforin-producing effector $CD8^+$ T cells were efficiently produced from the naive T cell population by BCG-DHTM stimulation. Single primary infection with BCG-DHTM in C57BL/6 mice efficiently produced T cells responsive to *in vitro* secondary stimulation with HSP70, MMP-II, and *M. tuberculosis*-derived cytosolic protein and inhibited the multiplication of subsequently aerosol-challenged *M. tuberculosis* more efficiently than did vector control BCG. These results indicate that the introduction of MMP-II and HSP70 into urease-deficient BCG may be useful for improving BCG for control of tuberculosis.

Mycobacterium tuberculosis is a causative bacterium of tuberculosis. One-third of the global population is latently infected with *M. tuberculosis*, which is responsible for 1.4 million deaths worldwide each year (1–4). Recently, multidrug-resistant strains of *M. tuberculosis* have emerged and spread worldwide (5), which mandates the development of reliable preventive measures and therapeutic tools. The manifestation of adult lung tuberculosis cannot be prevented by currently used *Mycobacterium bovis* BCG (6); therefore, the development of more-effective single-injection vaccines is strongly desired.

Host defenses against *M. tuberculosis* are conducted largely by type 1 T cells of both CD4 and CD8 subsets (7–9). As one of the most important effector elements, gamma interferon (IFN-γ) is well known (10). IFN-γ can be produced chiefly by $CD4^+$ T cells and $CD8^+$ T cells. $CD8^+$ T cells are also required to differentiate into cytotoxic T lymphocytes capable of killing *M. tuberculosis*-infected macrophages and dendritic cells (DCs) (11, 12). The killing process is via a granule-dependent mechanism involving perforin, which is produced in activated T cells (13, 14). Therefore, both $CD4^+$ T cells and $CD8^+$ T cells, specifically of the memory phenotype capable of responding immediately to *M. tuberculosis*-infected cells, are key elements in host defenses against *M. tuberculosis*, and vaccinating agents are required to have the ability to activate T cells to produce memory subpopulations. BCG activates naive $CD4^+$ T cells substantially but not convincingly and activates naive $CD8^+$ T cells poorly (15, 16). The reasons why BCG cannot prevent the development of tuberculosis have not been elucidated fully, but one of the major reasons is its poor immunostimulatory activities, based on the lack of ability to induce phagosomal maturation (17–19). Therefore, BCG-derived antigens (Ags) cannot be fully processed in the Ag-presenting cells (APCs) and cannot be efficiently presented to $CD4^+$ T cells and $CD8^+$ T cells. These observations indicate that improvement of BCG in terms of activation ability is necessary.

Various molecules, including early secretory antigenic target 6 (ESAT6), the Ag85 family proteins, and polyprotein Mtb72F, have been identified as good candidates for component vaccines against tuberculosis (9, 20–24). However, the development of a fully reliable vaccine using these component molecules has not been successful. Furthermore, the strategy that is necessary to improve BCG is still not fully determined, although some candidate recombinant BCG (rBCG) is already available (17–19). Previously, Grode et al. produced urease-deficient rBCG that produced acidic phagosomes due to lack of ammonium production and effectively translocated into lysosomes (18). However, the urease depletion alone potentiated the immunostimulatory activity of BCG but did not effectively inhibit the multiplication of *M. tuberculosis* in lung. This requires secretion of another foreign Ag, listeriolysin. We independently produced urease-deficient rBCG (BCG-ΔUT-11-3) by depleting the *ureC* gene, which encodes urease, from parent BCG (19). BCG-ΔUT-11-3 strongly activated naive human $CD4^+$ T cells to produce IFN-γ but failed to stimu-

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late naive human $CD8^+$ T cells, which indicates that another modification of BCG is necessary.

Similarly, a new reliable vaccine is needed for prevention of leprosy, which is caused by infection with *Mycobacterium leprae*. We are currently developing a new rBCG capable of inhibiting the multiplication of *M. leprae in vivo*. First, we identified major membrane protein II (MMP-II) (gene name, *bfrA* or ML2038) as one of the immunodominant Ags of *M. leprae* (25). MMP-II can ligate Toll-like receptor 2 (TLR2) and consequently activates the NF-κB pathway of APCs (25), and MMP-II-pulsed DCs activate both naive $CD4^+$ T cells and naive $CD8^+$ T cells (25, 26). Second, we tried to improve BCG by overexpressing MMP-II. When we introduced the MMP-II gene into BCG extrachromosomally, the rBCG showed enhanced activity to stimulate naive T cells of both CD4 and CD8 subsets (27). The second rBCG that we produced was BCG-70M, having a BCG-derived heat shock protein 70 (HSP70)-MMP-II fusion gene, and subcutaneous single BCG-70M vaccination inhibited the multiplication of *M. leprae* in C57BL/6 mice (28). Therefore, the secretion of the HSP70-MMP-II fusion protein was useful for enhancing the T cell-stimulating activity of BCG.

Overall, these results suggest that the combination of urease depletion and intraphagosomal secretion of antigenic protein is useful for construction of a new rBCG. We found that *M. tuberculosis* has an MMP-II gene (gene name, Rv1876) that is 100% homologous to the MMP-II gene of BCG and 90% homologous to that of *M. leprae* at the amino acid level. Previously, we purified the recombinant MMP-II (rMMP-II) protein of *M. tuberculosis* using *Mycobacterium smegmatis* and evaluated its immunostimulatory activities (29). Similar to *M. leprae*-derived MMP-II, *M. tuberculosis*-derived MMP-II ligates TLR2 and activates DCs, and the MMP-II-pulsed DCs activate both subsets of naive T cells (29). Furthermore, both human DCs and macrophages infected with *M. tuberculosis* strains such as H37Rv and H37Ra expressed MMP-II derivatives on their surfaces (29). These results indicate that the MMP-II of *M. tuberculosis* is highly immunogenic and might be a good candidate for vaccine development. Therefore, in this study, we produced a new rBCG, termed BCG-DHTM, in which urease-deficient BCG-ΔUT-11-3 was introduced with a fusion gene composed of the *M. tuberculosis*-derived MMP-II gene and the HSP70 gene of *M. tuberculosis* (Rv0350), and we evaluated its immunostimulatory activities.

MATERIALS AND METHODS

Preparation of cells and Ags. Peripheral blood samples were obtained from healthy purified protein derivative (PPD)-positive individuals, with informed consent. In Japan, BCG vaccination is compulsory for children (0 to 1 year of age). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as described previously (30). For the preparation of peripheral monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMCs by using immunomagnetic beads coated with anti-CD3 monoclonal antibody (MAb) (Dynabeads 450; Dynal Biotech, Oslo, Norway). The $CD3^-$ PBMC fraction was plated on tissue culture plates, and adherent cells were used as monocytes (31). DCs were differentiated as described previously (30, 32). Briefly, monocytes were cultured in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF) (Pepro Tech EC Ltd., London, England) and 10 ng of recombinant interleukin-4 (rIL-4) (Pepro Tech) per ml (32). On day 4 of culture, immature DCs were infected with rBCG at the indicated multiplicity of infection (MOI) and, on day 6 of culture, DCs were used for further analyses of surface Ag and for mixed lymphocyte assays. Macrophages were differentiated as described previously (33, 34). In brief, monocytes were cultured in the presence of 10 ng of recombinant macrophage colony-stimulating factor (rM-CSF) (R&D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were infected with rBCG at the indicated MOIs and, on day 7 of culture, they were used for further analyses of surface Ag and for mixed lymphocyte assays. The rMMP-II protein was produced as described previously (25, 35). The rHSP70 protein was purchased (Hy Test Ltd., Turku, Finland), and H37Rv-derived cytosolic protein was produced as described previously (35).

Vector construction and preparation of rBCG. The genomic DNAs were obtained from BCG substrain Tokyo and from *M. tuberculosis* strain H37Rv. The oligonucleotide primers used for amplification of the *hsp70* gene were F-Mb70Bal (5′-aaaTGGCCAtggctcgtgcggtcggg-3′) and R-Mb70Eco (5′-aaaGAATTCcttggcctcccggccg-3′). The MMP-II sequence from *M. tuberculosis* genomic DNA was amplified with primers F-MMPTBEco (5′-aattGAATTCatgcaaggtgatcccgatgt-3′) and R-MMPT-BSal (5′-aattGTCGACtcaggtcggtgggcgaga-3′). In all primer sequences, capital letters indicate restriction sites. The amplified products were digested with appropriate restriction enzymes and cloned into the parental pMV261 plasmid. For replacement of the kanamycin resistance gene with the hygromycin resistance cassette, the XbaI-NheI fragment from pYUB854 (36) was cloned into the SpeI-NheI fragment of the plasmid (36). The rBCG in which the *ureC* gene was disrupted (BCG-ΔUT-11) was produced as described previously (19). The hygromycin cassette in BCG-ΔUT-11 was removed by using pYUB870 encoding γδ-resolvase (γδ-*tnpR*) (36). The unmarked BCG was named BCG-ΔUT-11-3. The HSP70-MMP II fusion protein-expressing vector was introduced into BCG-ΔUT-11-3 by electroporation. BCG-ΔUT-11-3 containing pMV-HSP70-MMP-II as an extrachromosomal plasmid is referred to as BCG-DHTM, and BCG-Tokyo containing pMV-261-hygromycin is referred to as BCG-261H (BCG vector control). Recombinant BCGs and *M. tuberculosis* strain H37Rv were grown to log phase and stored at −80°C, at 10^8 CFU/ml. Before infection of DCs and macrophages, BCG levels were counted by the colony assay method. There was no significant difference in *in vitro* culture growth between BCG-261H and BCG-DHTM.

Analysis of cell surface Ags. The expression of cell surface Ags on DCs and lymphocytes was analyzed using a FACSCalibur system (BD Biosciences, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich, St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of cell surface Ags, the following MAbs were used: fluorescein isothiocyanate (FITC)–conjugated MAbs against HLA-ABC (G46-2.6; BD Biosciences), HLA-DR (L243; BD Biosciences), CD86 (FUN-1; BD Biosciences), CD83 (HB15a; Immunotech, Marseille, France), CD62L (Dreg 56; BD Biosciences), CCR7 (clone 150503; R&D Systems), and CD27 (M-T271; BD Biosciences) and phycoerythrin-conjugated MAbs to CD162 (TB5; Exbio, Prague, Czech Republic), CD8 (RPA-T8; BD Biosciences), and CD4 (RPA-T4; BD Biosciences).

The expression of MMP-II on rBCG-infected DCs was determined using the MAb against MMP-II of *M. leprae* (M270-13, IgM, kappa), which may detect MMP-II associated with major histocompatibility complex (MHC) molecules (26), followed by FITC-conjugated anti-mouse immunoglobulin (Ig) MAb (Tago Immunologicals, Camarillo, CA). For inhibition of the intracellular processing of phagocytosed bacteria, DCs were treated with 50 μM chloroquine (Sigma-Aldrich) for 2 h, washed, infected with rBCG, and subjected to analyses of MMP-II surface expression. The intracellular production of perforin was assessed as follows: naive $CD8^+$ T cells were stimulated with rBCG-infected DCs for 5 days in the presence of naive $CD4^+$ T cells, and $CD8^+$ T cells were surface stained with phycoerythrin-labeled MAb to CD8 and fixed in 2% formaldehyde. Subsequently, the cells were permeabilized using permeabilizing solution (BD Biosciences) and were stained with FITC-conjugated MAb to perforin (δG9; BD Biosciences) or FITC-labeled isotype control.

APC functions of DCs. The ability of BCG-infected DCs and macrophages to stimulate T cells was assessed using an autologous APC-T cell coculture, as previously described (32, 37). Purification of $CD4^+$ and $CD8^+$ T cells was conducted by using negative-isolation kits (Dynabeads 450; Dynal Biotech) (32). Naive $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with MAb to CD45RO, followed by beads coated with goat anti-mouse IgG MAb (Dynal Biotech). More than 98% of $CD45RA^+$ T cells were positive for the expression of CCR7 molecules. Memory-type T cells were similarly produced by the treatment of cells with MAb to CD45RA Ag. The purified responder cells (1×10^5 cells per well) were plated in 96-well round-bottom tissue culture plates, and DCs or macrophages infected with rBCG were added to give the indicated APC/T cell ratio. Supernatants of APC-T cell cocultures were collected on day 4, and cytokine levels were determined. In some cases, rBCG-infected DCs and macrophages were treated with MAb to HLA-ABC (W6/32, mouse IgG2a, kappa), HLA-DR (L243, mouse IgG2a, kappa), or CD86 (IT2.2, mouse IgG2b, kappa; BD Biosciences) or normal mouse IgG. The optimal concentrations of the MAbs were determined in advance. Also, in some cases, immature DCs and macrophages were treated with the indicated doses of chloroquine, brefeldin A (Sigma-Aldrich), or lactacystin (Sigma-Aldrich) and subsequently infected with BCG-DHTM. The optimal doses of these reagents were determined in advance.

Measurement of cytokine production. Levels of the following cytokines were measured: IFN-γ produced by $CD4^+$ and $CD8^+$ T cells and interleukin 12p70 (IL-12p70), tumor necrosis factor alpha (TNF-α), IL-1β, and GM-CSF produced by DCs or macrophages stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using enzyme assay kits (Opt EIA human enzyme-linked immunosorbent assay [ELISA] set; BD Biosciences).

Animal studies. For inoculation into mice, rBCG and *M. tuberculosis* strain H37Rv were cultured in Middlebrook 7H9 medium to log phase and stored at −80°C, at 10^8 CFU/ml. Before the aliquots were used for inoculation, the concentrations of viable bacilli were determined by plating on Middlebrook 7H10 agar plates. Three 5-week-old C57BL/6J mice (Clea Japan Inc., Tokyo, Japan) per group were inoculated subcutaneously with 0.1 ml of phosphate-buffered saline (PBS) or PBS containing 1×10^3 or 1×10^4 rBCG. The animals were kept under specific pathogen-free conditions and were supplied with sterilized food and water. Four or 12 weeks after inoculation, the spleens were removed and splenocytes were suspended in culture medium at a concentration of 2×10^6 cells per ml. The splenocytes were stimulated with the indicated concentrations of rMMP-II, rHSP70 (HyTest), or H37Rv-derived cytosolic protein, in triplicate, in 96-well round-bottom microplates (19, 27). The individual culture supernatants were collected 3 to 4 days after stimulation. For observation of the effect of BCG vaccination on *M. tuberculosis* infection, five C57BL/6 mice per group were vaccinated with either BCG-261H or BCG-DHTM, at 1×10^4 CFU/mouse, for 6 weeks and were challenged with H37Rv at 100 CFU/lung by aerosol infection using an automated inhalation exposure apparatus (model 099C A4212; Glas-Col Corp.). Six weeks later, bacterial burdens in the lung and spleen were assessed by mechanical disruption in PBS with 0.5% (vol/vol) Tween 80 and enumerated by colony assay. Animal studies were reviewed and approved by the Animal Research Committee of Experimental Animals of the National Institute of Infectious Diseases and were conducted according to their guidelines.

Statistical analysis. Student's *t* test was applied to determine statistical differences. Throughout the *in vitro* experiments, we included 1 or 2 technical replicates in each individual experiment and used at least 3 separate PBMC donors.

RESULTS

Activation of Ag-presenting cells by BCG-DHTM. The purpose of rBCG production is to activate naive T cells effectively and, consequently, to produce memory-type T cells efficiently. In order to stimulate responder T cells, APCs susceptible to BCG infection should be adequately activated by infection with rBCG. We assessed the ability of newly produced rBCG (BCG-DHTM) to activate APCs with respect to phenotypic changes and cytokine production (Fig. 1). To assess phenotypic changes, we examined the expression of MHC, CD86, and CD83 molecules on DCs (Fig. 1a). Both vector control BCG (BCG-261H) and BCG-DHTM upregulated the expression of these molecules, but BCG-DHTM infection induced upregulation more efficiently than did infection with BCG-261H. We measured the production of cytokines, including IL-12p70, TNF-α, and IL-1β, from DCs with stimulation with rBCGs (Fig. 1b). Significantly higher levels of all of these cytokines were produced by BCG-DHTM stimulation. Further, when M-CSF-dependent macrophages were stimulated with either BCG-261H or BCG-DHTM, BCG-DHTM stimulation induced production of significantly higher levels of TNF-α and GM-CSF (Fig. 1c). In all of these experiments, we used various doses of rBCGs for the assessments, and similar changes were observed (data not shown). These results indicated that BCG-DHTM activated DCs and macrophages more efficiently than did BCG-261H.

Activation of memory-type and naive $CD4^+$ T cells by BCG-DHTM. Previously, we reported that the enhanced activation of both $CD4^+$ T cells and $CD8^+$ T cells induced by rBCG that was introduced with the *M. leprae*-derived MMP-II-HSP70 fusion gene was dependent on secretion of the HSP70-MMP-II fusion protein. Since we also confirmed that newly produced BCG-DHTM secreted the fusion protein composed of *M. tuberculosis*-derived MMP-II and HSP70 (data not shown), we assessed the $CD4^+$ T cell-stimulating ability of BCG-DHTM (Fig. 2). When either BCG-261H or BCG-DHTM was used to infect DCs and was used as a stimulator, autologous memory-type $CD4^+$ T cells produced significantly higher levels of IFN-γ by stimulation of BCG-DHTM-infected DCs than BCG-261H-infected DCs (Fig. 2a); around 300 pg/ml of IFN-γ was secreted by stimulation with very small numbers of rBCG-infected DCs (MOI, 0.063) and with very small numbers of DCs (T cell/DC ratio, 80:1). At different T cell/DC ratios, BCG-DHTM exhibited higher activity (data not shown). In addition to IFN-γ, TNF-α and IL-2 were efficiently produced with BCG-DHTM stimulation (data not shown). Then, we assessed rBCG-infected macrophages as stimulators. Compared to DCs, macrophages needed to be infected with higher doses of rBCGs to stimulate memory-type $CD4^+$ T cells; however, more than 100 pg/ml of IFN-γ could be produced by responder $CD4^+$ T cells when BCG-DHTM was used at an MOI of 0.5 to infect macrophages. Further, a larger quantity of macrophages (T cell/macrophage ratio, 10:1) was needed to activate $CD4^+$ T cells convincingly (Fig. 2b). It should be noted that the BCG vector control could not induce the apparent activation of $CD4^+$ T cells. BCG-DHTM did not induce IFN-γ production from either DCs or macrophages (data not shown). Then, we assessed the activity of BCG-DHTM to stimulate naive $CD4^+$ T cells (Fig. 2c). BCG-DHTM induced the production of significantly higher levels of IFN-γ than did BCG-261H at MOIs of 0.063 to 0.25 (T cell/DC ratio, 20:1). Increasing IFN-γ levels could be produced, depending on the dose of BCG-DHTM used to infect DCs. Also, at different T cell/DC ratios, BCG-DHTM showed greater activity (data not shown). However, BCG-DHTM-infected macrophages failed to induce the production of significant levels of IFN-γ from naive $CD4^+$ T cells or $CD8^+$ T cells (data not shown).

Activation of memory-type and naive $CD8^+$ T cells by BCG-DHTM. The effects of BCG-DHTM-infected DCs on $CD8^+$ T cell activation were examined (Fig. 3). While BCG-261H did not ac-

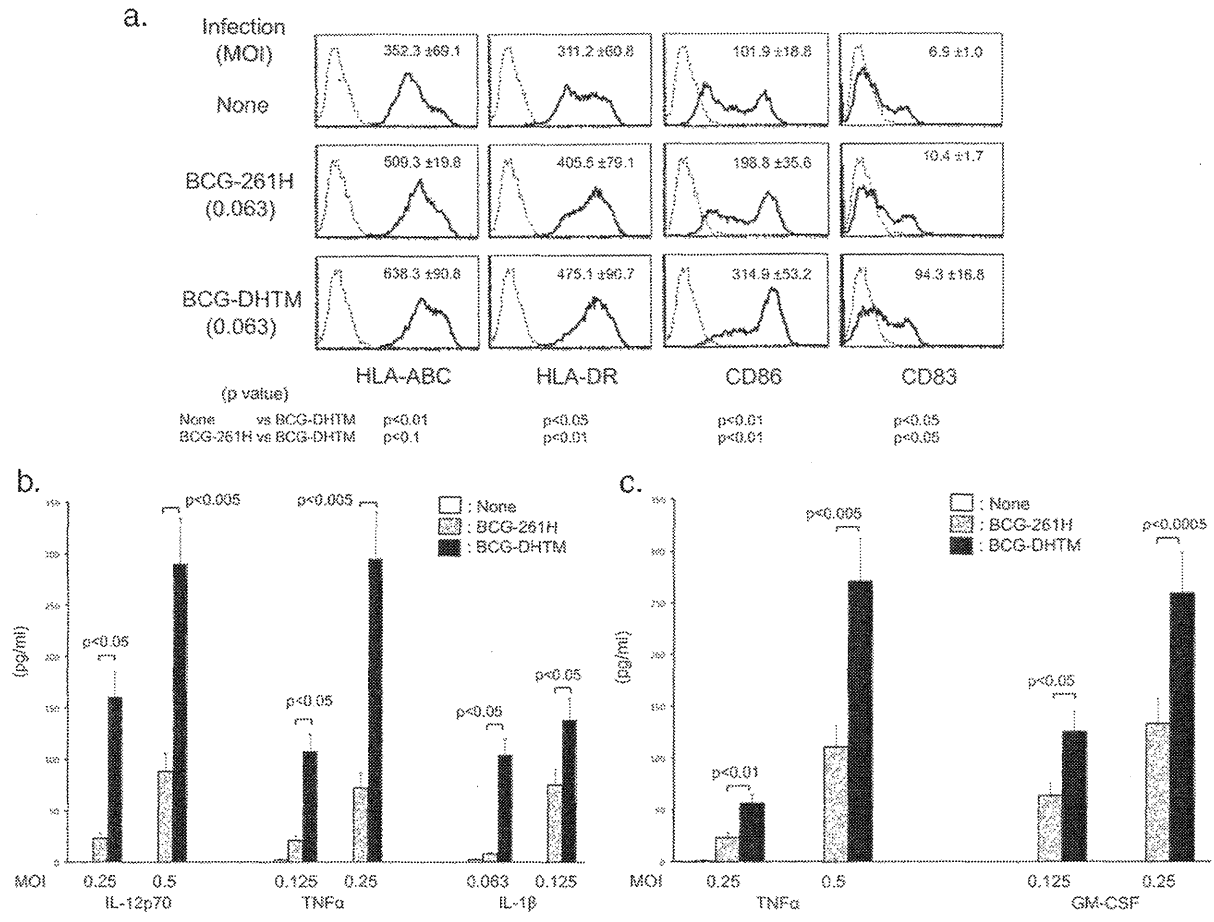

FIG 1 (a) Upregulation of APC-associated molecules and activation markers on DCs by infection with BCG-DHTM. Monocyte-derived immature DCs were infected with either BCG-261H or BCG-DHTM at an MOI of 0.063 and were cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DCs from day 6 of culture were gated and analyzed. Dashed lines, isotype-matched control IgG; solid lines, indicated test MAbs. Representative results of three separate experiments are shown. The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation [SD]) between the control IgG and the test MAb in three independent experiments. (b) Cytokine production from DCs by stimulation with BCG-DHTM. DCs produced using rGM-CSF and rIL-4 were stimulated with either BCG-261H or BCG-DHTM for 24 h. (c) Cytokine production from macrophages by stimulation with BCG-DHTM. Macrophages differentiated from monocytes by using M-CSF were stimulated with either BCG-261H or BCG-DHTM for 24 h. The concentrations of the indicated cytokines were determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test.

tivate memory $CD8^+$ T cells efficiently, BCG-DHTM activated the T cells and induced the production of more than 100 pg/ml of IFN-γ (Fig. 3a). Compared to the dose of rBCG required for activation of memory-type $CD4^+$ T cells, a higher dose of rBCG was needed, which may be based on the fact that parent BCG did not activate naive $CD8^+$ T cells and did not produce BCG-specific memory-type $CD8^+$ T cells efficiently. When autologous naive $CD8^+$ T cells were stimulated by rBCG as a responder population, only BCG-DHTM efficiently activated naive $CD8^+$ cells to produce IFN-γ (Fig. 3b). Efficient concentrations of IFN-γ could be produced from naive $CD8^+$ T cells by stimulation with DCs infected with BCG-DHTM. As observed previously (18, 32), BCG-261H did not activate naive $CD8^+$ T cells. These phenomena were observed consistently under various conditions, including different MOIs and T cell/DC ratios. In order to confirm the activation of naive $CD8^+$ T cells by BCG-DHTM, the expression of activation markers on naive $CD8^+$ T cells was examined (Fig. 3c). When autologous naive $CD8^+$ T cells were stimulated with DCs infected with either BCG-261H or BCG-DHTM in the presence of naive $CD4^+$ T cells, more-efficient downregulation of CD62L expression on $CD8^+$ T cells was induced by BCG-DHTM stimulation. These phenomena were observed at different MOIs and, even at lower MOIs such as 0.031, efficient downregulation was observed in BCG-DHTM-stimulated $CD8^+$ T cells.

Characteristics of BCG-DHTM. Previously, we reported that BCG-70M induced expression of MMP-II on the surface of DCs infected with BCG-70 M (28). Thus, we analyzed the DCs infected with BCG-DHTM in terms of MMP-II expression (Fig. 4a). Whereas both uninfected DCs and DCs infected with BCG-261H did not express MMP-II derivatives on the surface, BCG-DHTM induced expression of MMP-II derivatives (6.8-fold increase in MMP-II expression in BCG-261H-infected DCs). Higher levels of expression were observed when higher MOIs of rBCG were used (data not shown). Further, MMP-II expression was inhibited by the treatment of immature DCs with chloroquine, an inhibitor of phagosomal acidification, prior to infection with BCG-DHTM.

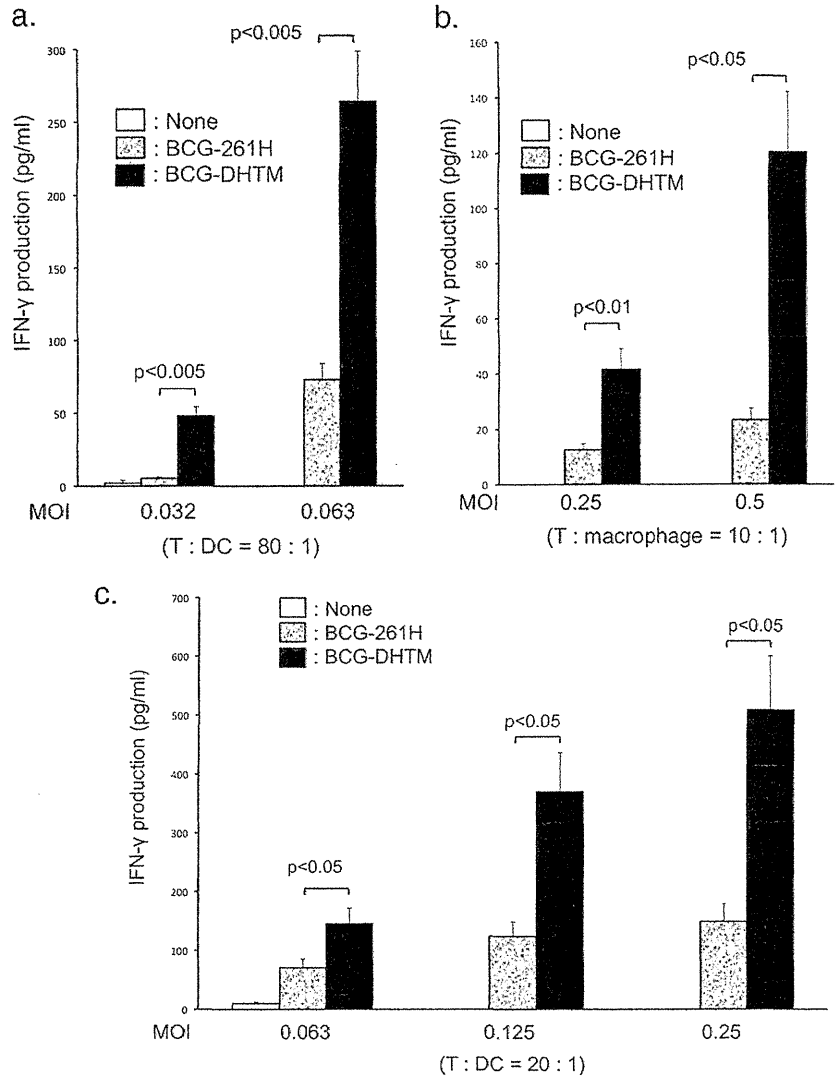

FIG 2 (a) IFN-γ production from memory-type $CD4^+$ T cells by stimulation with BCG-DHTM-infected DCs. Monocyte-derived DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators of memory-type $CD4^+$ T cells in a 4-day culture; 10^5 responder T cells were stimulated with the rBCG-infected DCs at a T cell/DC ratio of 80:1. (b) IFN-γ production from memory-type $CD4^+$ T cells by stimulation with BCG-DHTM-infected macrophages. Macrophages produced by using M-CSF were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators of responder $CD4^+$ T cells in a 4-day culture; 10^5 responder T cells were stimulated with the rBCG-infected macrophages at a T cell/macrophage ratio of 10:1. (c) IFN-γ production from naive $CD4^+$ T cells by stimulation with BCG-DHTM-infected DCs. Monocyte-derived DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators; 10^5 responder naive $CD4^+$ T cells were stimulated with the rBCG-infected DCs at a T cell/DC ratio of 20:1. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test.

According to these results, we analyzed the effect of chloroquine treatment of immature DCs on the activation of T cells by BCG-DHTM (Fig. 4b). IFN-γ production from naive $CD4^+$ T cells and naive $CD8^+$ T cells by stimulation with BCG-DHTM-infected DCs and that from memory-type $CD4^+$ T cells by stimulation with BCG-DHTM-infected macrophages were significantly inhibited by chloroquine treatment of these APCs. These results suggest the possibility that the secreted fusion protein is one of the elements responsible for the activation of both $CD4^+$ T cells and $CD8^+$ T cells. BCG-DHTM-infected DCs or macrophages were treated with MAbs to HLA and CD86 molecules prior to being used as stimulators of responder T cells (Fig. 4c). Treatment of BCG-DHTM-infected APCs with MAbs to HLA-DR, CD86, and MMP-II (data not shown) significantly inhibited IFN-γ production from naive $CD4^+$ T cells and memory-type $CD4^+$ T cells. Also, the treatment of BCG-DHTM-infected DCs with MAbs to HLA-ABC and CD86 molecules significantly inhibited the production of IFN-γ from naive $CD8^+$ T cells. These results suggested that BCG-DHTM activated T cells in an Ag-specific manner, at least partially. In general, for activation of naive $CD8^+$ T cells by bacteria, the activation of cross-presenting pathways in APCs is required. We examined whether BCG-DHTM utilized the cytosolic cross-presentation pathway for the activation of naive $CD8^+$ T cells (Fig. 4d). To this end, we treated immature DCs with either

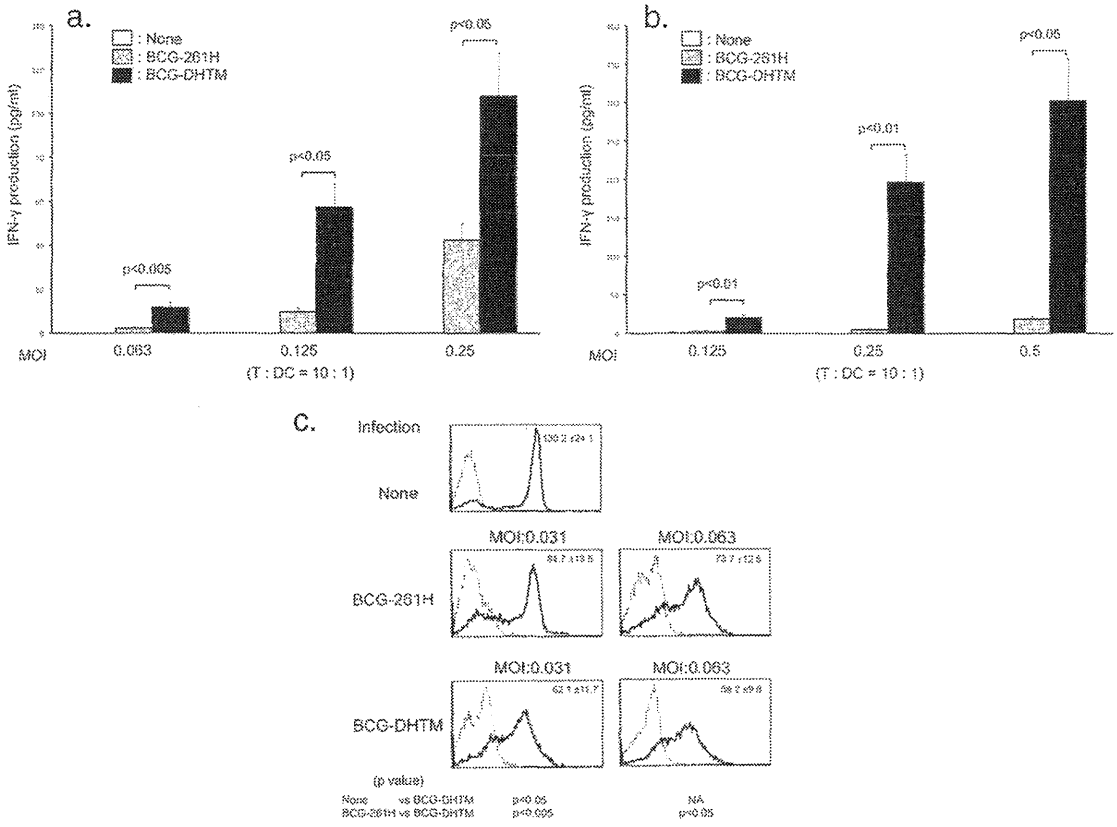

FIG 3 (a) IFN-γ production from memory-type $CD8^+$ T cells by stimulation with BCG-DHTM. Monocyte-derived DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators; 10^5 responder memory-type $CD8^+$ T cells were stimulated for 4 days with rBCG-infected DCs at a T cell/DC ratio of 10:1. (b) IFN-γ production from naive $CD8^+$ T cells by stimulation with BCG-DHTM. DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were used as stimulators; 10^5 responder T cells were stimulated for 4 days with rBCG-infected DCs at a T cell/DC ratio of 10:1. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test. (c) Downregulation of CD62L expression on naive $CD8^+$ T cells by BCG-DHTM stimulation. DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were cocultured with unseparated naive T cells for 5 days at a T cell/DC ratio of 20:1. The stimulated $CD8^+$ T cells were gated and analyzed for expression of CD62L molecules. Dashed lines, isotype-matched control IgG; solid lines, anti-CD62L MAb. The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation) between the control IgG and anti-CD62L MAb in three independent experiments. A representative of three separate experiments is shown.

brefeldin A or lactacystin and subsequently infected them with BCG-DHTM at an MOI of 0.25. These pretreatments of DCs significantly inhibited IFN-γ production from naive $CD8^+$ T cells.

Production of memory and effector T cells from naive $CD8^+$ T cells by BCG-DHTM. Since it is well documented that the production of long-lasting memory $CD8^+$ T cells from naive $CD8^+$ T cells requires help from $CD4^+$ T cells and since BCG-DHTM activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, naive unseparated T cells were stimulated with DCs infected with either BCG-261H or BCG-DHTM and the stimulated $CD8^+$ T cells were gated and analyzed (Fig. 5a). Stimulation with BCG-DHTM more efficiently produced $CD27^{low}$ or $CCR7^{low}$ memory-type T cells from naive T cells. Further, BCG-DHTM produced perforin-producing $CD8^+$ T cells more efficiently than did BCG-261H. Efficient production of these $CD8^+$ T cells was observed with different doses of BCG; however, in the absence of $CD4^+$ T cells, production of memory and effector T cells was not observed (data not shown). Furthermore, it is necessary to produce T cells with high migratory function. Thus, we assessed the expression of CD162 on both $CD4^+$ T cells and $CD8^+$ T cells stimulated with DCs infected with BCG-DHTM (Fig. 5b). BCG-DHTM produced $CD162^{high}$ $CD4^+$ T cells and $CD162^{high}$ $CD8^+$ T cells more efficiently than did BCG-261H. Similar differences between BCG-261H and BCG-DHTM were observed at different MOIs (data not shown).

Production of T cells responsive to secondary stimulation by BCG-DHTM infection *in vivo*. Functional studies using C57BL/6 mice were conducted to examine the ability of BCG-DHTM to produce T cells that were highly responsive to secondary *in vitro* stimulation (Fig. 6). The mice were subcutaneously inoculated with 1×10^5 CFU/mouse of rBCGs 4 weeks (Fig. 6a) or 12 weeks (Fig. 6b) before *in vitro* stimulation. As secondary stimulators, recombinant MMP-II, recombinant HSP70 protein, and H37Rv-derived cytosolic protein were used. Some of these proteins are highly immunogenic and thus they induced substantial IFN-γ production from T cells of uninfected mice; however, splenic T cells from mice inoculated with BCG-DHTM 4 weeks previously produced significantly higher levels of IFN-γ and IL-2 (data not shown) than did T cells from uninoculated mice and those from BCG-261H-infected mice, by responding to all of the secondary stimulators (Fig. 6a). To examine the long-term effects of single inoculations of BCG-DHTM, T cells from C57BL/6 mice similarly

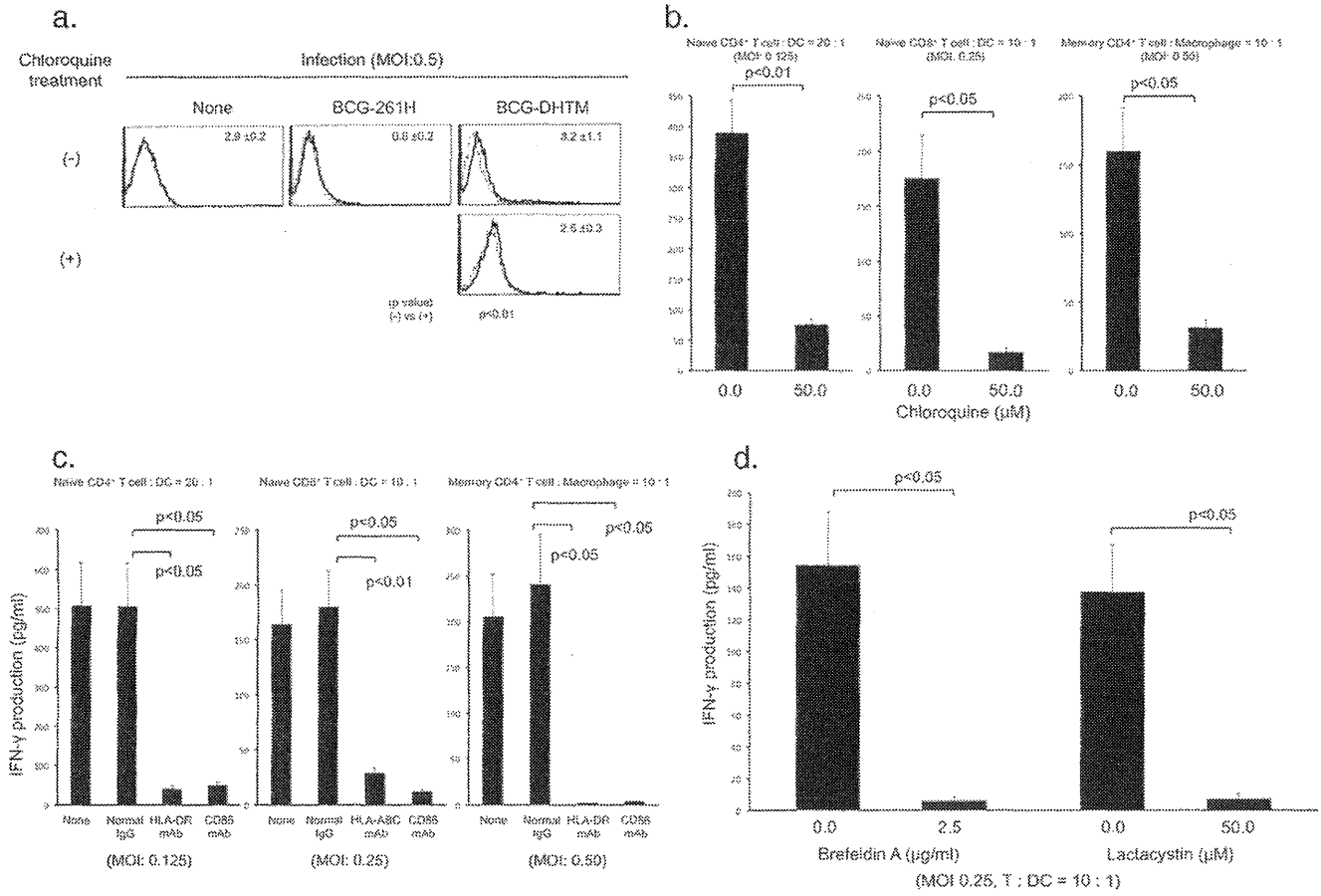

FIG 4 (a) Expression of MMP-II on DCs. Immature DCs were either treated with 50 μM chloroquine for 2 h or not treated and subsequently were infected with either BCG-261H or BCG-DHTM at an MOI of 0.5. After 2 days of culture in the presence of rGM-CSF and rIL-4, DCs were gated and analyzed. Dashed lines, control normal IgM; solid lines, anti-MMP-II MAb (IgM). The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation) between the control IgM and MMP-II MAb in three independent experiments. Representative results of three separate experiments are shown. (b) Effects of chloroquine treatment of DCs and macrophages on the activation of T cells. Immature DCs and macrophages were treated with chloroquine (50 μM for 2 h) or not treated and subsequently were infected with BCG-DHTM at the indicated MOIs. These DCs and macrophages were used as stimulators of the indicated responder T cells at the indicated responder/stimulator ratios. IFN-γ produced by T cells was measured. (c) Inhibition of T cell activation by treatment of BCG-DHTM-infected DCs and macrophages with MAbs. DCs and macrophages were infected with BCG-DHTM at the indicated MOIs and subsequently were treated with 10 μg/ml of the MAb or normal murine IgG. These APCs were used as the stimulators of the indicated responder T cells (1×10^5 cells/well), at the indicated T cell/APC ratios, for 4 days. IFN-γ produced by T cells was measured. (d) Effects of treatment of immature DCs with brefeldin A or lactacystin on the activation of naive $CD8^+$ T cells. Immature DCs from 4 days of culture were treated with either brefeldin A (2.5 μg/ml) or lactacystin (50 μM) or not treated and subsequently were infected with BCG-DHTM at an MOI of 0.25. These DCs were used as stimulators of responder autologous naive $CD8^+$ T cells (1×10^5/well) at a T cell/DC ratio of 10:1. IFN-γ produced by T cells was measured. A representative of three separate experiments is shown. Assays were performed in triplicate, and the results are expressed as mean ± SD. Titers were statistically compared using Student's *t* test.

inoculated with rBCGs 12 weeks previously were examined (Fig. 6b). Again, significantly higher levels of IFN-γ were produced from T cells obtained from mice inoculated with BCG-DHTM with secondary stimulation, although we could not recover BCG from spleen. In a separate experiment, a different dose (1×10^2 CFU/mouse) of BCG was examined, and similar results were obtained (data not shown).

Effect of BCG-DHTM vaccination on the multiplication of H37Rv *in vivo*. C57BL/6 mice vaccinated with either BCG-261H or BCG-DHTM (1×10^4 CFU/mouse) for 6 weeks were challenged with 100 CFU per lung of H37Rv by aerosol infection. Six weeks later, the *M. tuberculosis* recovered from both lungs and spleen was enumerated (Fig. 7). Mice vaccinated with either BCG-261H or BCG-DHTM demonstrated inhibited multiplication of *M. tuberculosis* in the lung, and BCG-DHTM vaccination inhibited *M. tuberculosis* multiplication more strongly than did BCG-261H vaccination (Fig. 7a). Similar results were observed in the spleen (Fig. 7b). Similar protective effects of BCG-DHTM on *M. tuberculosis* recovery were observed in mice 12 weeks after vaccination (data not shown).

DISCUSSION

Studies using the T cell receptor-transgenic *M. tuberculosis* mouse model clearly demonstrated that the most susceptible APCs, including DCs and macrophages, need at least 7 to 10 days to initiate stimulation of $CD4^+$ T cells and $CD8^+$ T cells in regional lymph nodes on aerosol infection with *M. tuberculosis* and the stimulated T cells need 4 to 5 weeks to initiate inhibition of the multiplication of *M. tuberculosis* in lungs (38). The activation of both $CD4^+$ T cells and $CD8^+$ T cells is required for inhibition of the replication

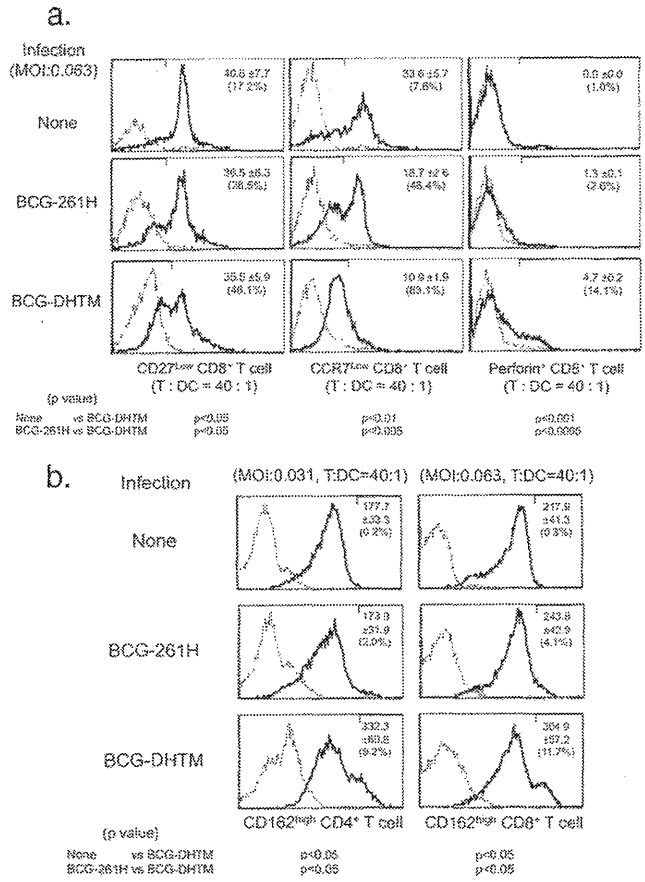

FIG 5 (a) Expression of memory markers and perforin production on naive $CD8^+$ T cells stimulated with DCs infected with BCG-DHTM. DCs were infected with either BCG-261H or BCG-DHTM at an MOI of 0.063 and were cocultured with unseparated naive T cells (T cell/DC ratio of 40:1) for 5 days. The stimulated $CD8^+$ T cells were gated and analyzed for expression of the indicated molecules and for perforin production. (b) Expression of migration markers on naive T cells. DCs were infected with either BCG-261H or BCG-DHTM at the indicated MOIs and were cocultured with naive T cells (T cell/DC ratio of 40:1) for 5 days. Stimulated T cells of the CD4 or CD8 subset were gated and analyzed for expression of CD162 molecules. Dashed lines, isotype-matched control IgG; solid lines, anti-CD162 MAb. The numbers in the top right corners of the panels represent the differences in fluorescence intensity (mean ± standard deviation) between the control IgG and the test MAb in three independent experiments. The numbers in parentheses indicate the percentages of the $CD162^{high}$ T cell population among the indicated T cells. A representative of three separate experiments is shown.

of *M. tuberculosis* or killing of *M. tuberculosis* (7–9), and DCs play a central role in activating T cells (39). It has been reported that $CD4^+$ T cells act at the initial stage of *M. tuberculosis* infection and $CD8^+$ T cells work chiefly at the chronic stage (4). The purpose of vaccination aimed at controlling tuberculosis manifestation is to produce T cells that can immediately respond to antigenic molecules expressed on the surface of *M. tuberculosis*-infected APCs in the regional lymph nodes. BCG is essentially capable of activating naive $CD4^+$ T cells, but its potency is not convincing and is not suitable for stimulation of naive $CD8^+$ T cells (15, 16). Further, macrophages infected with BCG inefficiently activate $CD4^+$ T cells (15). Therefore, BCG is not an excellent vaccine in terms of producing abundant T cells capable of responding to secondary stimulation, and improvement of BCG is necessary.

We have previously made efforts to improve the ability of BCG to stimulate T cells, chiefly aiming to produce better vaccines against leprosy. We used MMP-II protein to improve the function of BCG, as the most important element of vaccines (25), and we found that intraphagosomal secretion of HSP70-MMP-II fusion protein is quite useful to stimulate naive T cells of both CD4 and CD8 subsets. Further, we and others showed that urease-deficient rBCG feasibly translocated into lysosomes, where abundant enzymes are available (18). Although urease-deficient BCG-ΔUT-11-3 activated naive $CD4^+$ T cells, it failed to activate naive $CD8^+$ T cells to produce IFN-γ (19). Furthermore, Grode et al. showed that depletion of urease activity in BCG is not sufficient to inhibit the multiplication of *M. tuberculosis* in lung (18). Therefore, it could be speculated that, in order to overcome fully the intrinsic defect of BCG, that is, a lack of phagosome and lysosome fusion, the combination of urease depletion and intraphagosomal secretion of antigenic molecules would be useful.

Our previous study indicated that the rMMP-II protein of *M. tuberculosis* is highly immunogenic and DCs pulsed with MMP-II proteins activated both naive $CD4^+$ T cells and naive $CD8^+$ T cells, but *M. tuberculosis*-derived MMP-II was superior to *M. leprae*-derived MMP-II in all of these functions (29). Furthermore, individuals who were vaccinated with BCG possessing MMP-II 100% homologous to that of *M. tuberculosis* were assumed to be primed with MMP-II *in vivo* (29). Thus, MMP-II of *M. tuberculosis* was considered to have T cell-stimulating activity, and these activated T cells, which subsequently differentiated into the memory state, may be able to respond to *M. tuberculosis*-infected APCs immediately. Therefore, MMP-II could be a useful candidate as a component of vaccines against tuberculosis.

Based on these previous findings and speculation, we produced a new rBCG termed BCG-DHTM, using the MMP-II of *M. tuberculosis*. BCG-DHTM was produced by introducing the HSP70-MMP-II fusion gene into urease-deficient BCG-ΔUT-11-3. Previously, we reported that urease-deficient rBCG that secretes a fusion protein composed of HSP70 and MMP-II from *M. leprae* was superior to urease-deficient BCG-ΔUT-11-3 and normal BCG that secretes the fusion protein in the activation of APCs and naive T cells and the production of memory-type T cells in mice (40). Therefore, we used only vector control BCG-261H as a control BCG in this study. BCG-DHTM induced enhanced activation of naive $CD4^+$ T cells and convincingly activated naive $CD8^+$ T cells to produce IFN-γ, although the availability of non-BCG-vaccinated naive PBMCs would help to confirm these observations. Naive $CD8^+$ T cell activation was confirmed by the observation of phenotypic changes such as expression of activation markers. The activation of naive $CD8^+$ T cells was induced by using the transporter associated with antigen presentation (TAP) and the proteosome-dependent cytosolic cross-presenting pathway, because IFN-γ production from naive $CD8^+$ T cells was largely inhibited by pretreatment of immature DCs with either brefeldin A, an inhibitor of TAP-dependent transportation, or lactacystin, a proteosomal protein degradation blocker. The activation of naive T cells of the CD4 and CD8 subsets by BCG-DHTM was carried out in an Ag-specific manner, since treatment of BCG-DHTM-infected DCs with MAbs to MHC or CD86 molecules significantly inhibited IFN-γ production from naive T cells. Further, BCG-DHTM could activate $CD4^+$ T cells even when macrophages were used as APCs, the function of which is important, because the parent BCG possesses the solid defect of the

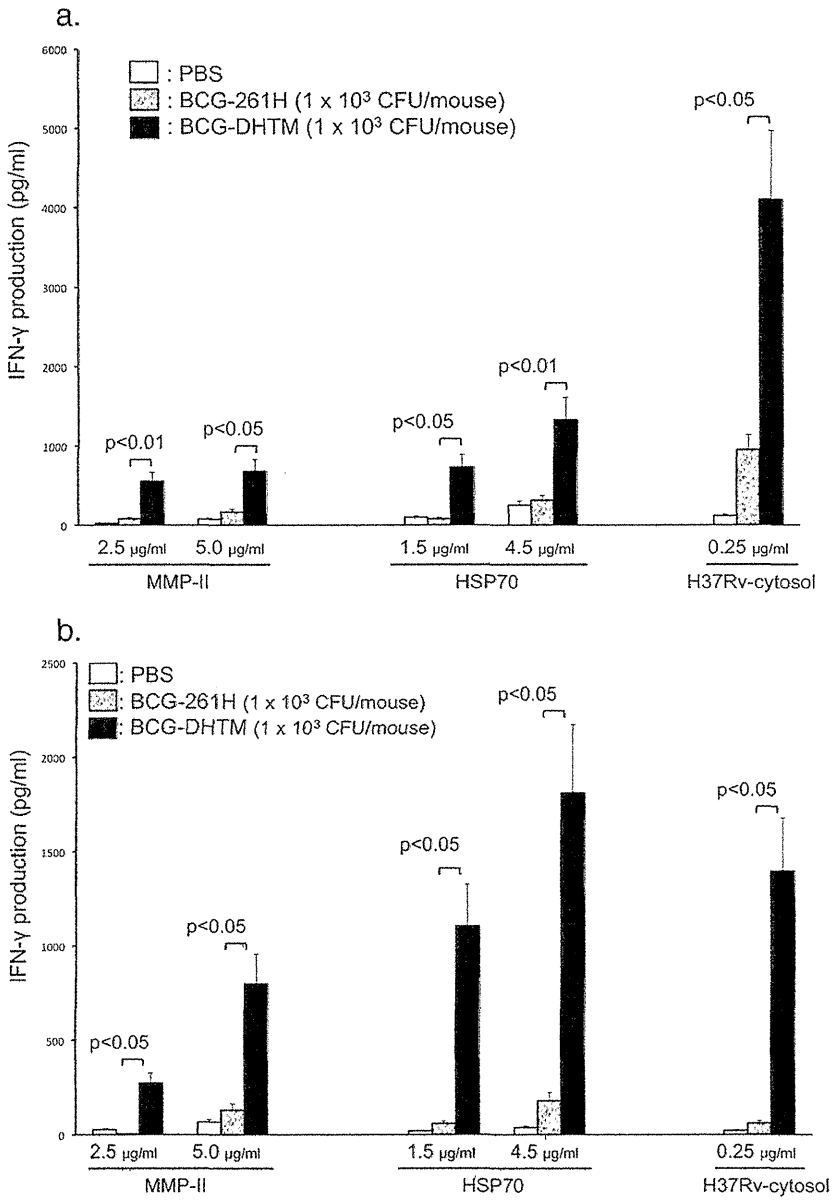

FIG 6 Production of T cells responsive to secondary *in vitro* stimulation in C57BL/6 mice by infection with BCG-DHTM. Three 5-week-old C57BL/6 mice per group were each infected subcutaneously with 1×10^3 CFU of either BCG-261H or BCG-DHTM. Four weeks (a) or 12 weeks (b) after inoculation, splenocytes (2×10^5 cells/well) were stimulated *in vitro* with the indicated stimulators for 4 days, and IFN-γ levels in the cell supernatants were measured. Assays were performed in triplicate for each mouse, and the results for three mice per group are shown as mean ± SD. Representative results of three separate experiments are shown. Concentrations of IFN-γ were statistically compared using Student's *t* test.

inability to activate $CD4^+$ T cells via macrophages. Activation of these naive T cells by DCs and activation of $CD4^+$ T cells by macrophages are closely associated with phagosomal maturation. This conclusion is supported by the observation that pretreatment of DCs and macrophages with chloroquine, an inhibitor of phagosomal acidification, blocked the activation of responder T cells. The HSP70-MMP-II fusion protein secreted in phagosomes can contribute to the activation of naive T cells but, in addition, the fusion protein can be secreted in lysosomes owing to urease deficiency. The protein secreted in lysosomes could be more efficiently degraded into antigenic determinants than that secreted in phagosomes, because lysosomes contain abundant enzymes. The frequency of T cells specific for HSP70-MMP-II fusion protein is low; thus, it seems difficult to inhibit the multiplication of *M. tuberculosis* by the actions of only the fusion protein secreted from normal rBCG. Thus, we need Ag-specific polyclonal T cells. In this respect, urease depletion seems to be useful, because rBCG itself might be processed by the enzyme present in lysosomes. Therefore, BCG-DHTM may be able to activate not only fusion protein-specific T cells but also other T cells polyclonally by using parent BCG-derived Ags. These speculations seem to be supported by animal studies, at least partially. C57BL/6 mice injected with

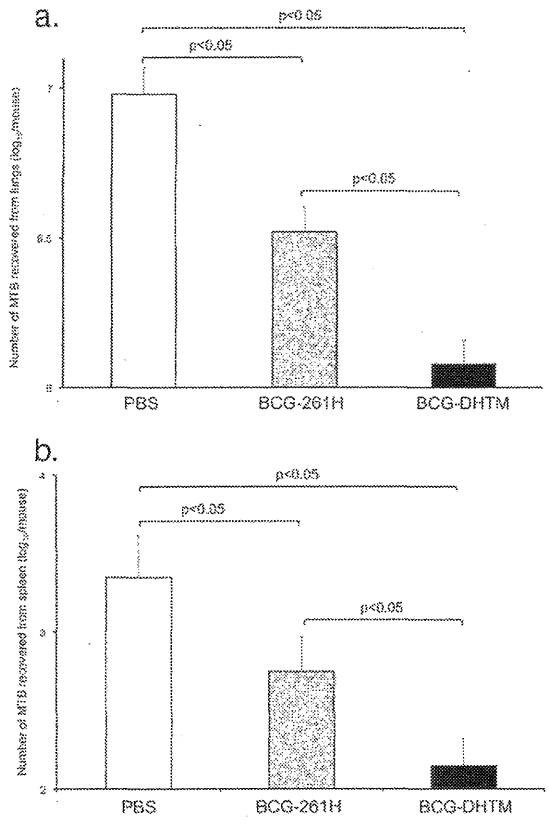

FIG 7 Inhibition of *M. tuberculosis* multiplication by subcutaneous vaccination with BCG-DHTM. Five-week-old C57BL/6 mice (5 mice/group) were subcutaneously vaccinated with either BCG-261H or BCG-DHTM at 1×10^4 CFU/mouse and were challenged with 100 CFU/lung of H37Rv by aerosol infection 6 weeks postvaccination. *M. tuberculosis* (MTB) isolates recovered from the lungs (a) and spleen (b) at 6 weeks postchallenge were enumerated by the colony assay method. Titers were statistically compared using Student's *t* test. A representative of three separate experiments is shown.

BCG-DHTM produced T cells that responded strongly to *in vitro* secondary stimulation with MMP-II and HSP70, as well as *M. tuberculosis*-derived cytosolic protein.

The BCG-DHTM stimulation of naive T cells produced $CD27^{low}$ and $CCR7^{low}$ memory-type $CD8^+$ T cells *in vitro*, and both $CD4^+$ T cells and $CD8^+$ T cells highly expressed migration markers. These observations seem to be important because both subsets of T cells have to migrate immediately and alternatively between lung and regional lymph nodes to react with *M. tuberculosis*-infected APCs efficiently. Production of these T cells may be associated with the partial inhibition of *M. tuberculosis* multiplication in lungs and spleen of mice vaccinated with BCG-DHTM. Although BCG-DHTM showed high immunostimulating activities, it only partially inhibited the growth of *M. tuberculosis* in lungs. There might be several reasons for this unconvincing inhibition. One reason might be the lack of pathogenic features of *M. tuberculosis* in BCG-DHTM, and the second is the relatively small dose of BCG used for vaccination. The third reason may be that we tested bacterial burdens in lungs and spleens at 6 weeks and not 4 weeks, a frequently used time point (41), after *M. tuberculosis* challenge, because 4 to 5 weeks are necessary to reach stable levels of pulmonary bacterial burdens even in naive mice (38). Also, due to BCG strain differences, there may be differences in protective effects in experiments with mice. Therefore, another effort is absolutely required for the production of a more potent BCG to inhibit *M. tuberculosis*. However, the present study may indicate that the HSP70-MMP-II fusion protein could be a candidate vaccine component for the control of tuberculosis.

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An *in vitro* model of *Mycobacterium leprae* induced granuloma formation

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Abstract

Background: Leprosy is a contagious and chronic systemic granulomatous disease caused by *Mycobacterium leprae*. In the pathogenesis of leprosy, granulomas play a key role, however, the mechanisms of the formation and maintenance of *M. leprae* granulomas are still not clearly understood..

Methods: To better understand the molecular physiology of *M. leprae* granulomas and the interaction between the bacilli and human host cells, we developed an *in vitro* model of human granulomas, which mimicked the in vivo granulomas of leprosy. Macrophages were differentiated from human monocytes, and infected with *M. leprae*, and then cultured with autologous human peripheral blood mononuclear cells (PBMCs).

Results: Robust granuloma-like aggregates were obtained only when the *M. leprae* infected macrophages were co-cultured with PBMCs. Histological examination showed *M. leprae* within the cytoplasmic center of the multinucleated giant cells, and these bacilli were metabolically active. Macrophages of both M1 and M2 types co-existed in the granuloma like aggregates. There was a strong relationship between the formation of granulomas and changes in the expression levels of cell surface antigens on macrophages, cytokine production and the macrophage polarization. The viability of *M. leprae* isolated from granulomas indicated that the formation of host cell aggregates benefited the host, but the bacilli also remained metabolically active.

Conclusions: A simple *in vitro* model of human *M. leprae* granulomas was established using human monocyte-derived macrophages and PBMCs. This system may be useful to unravel the mechanisms of disease progression, and subsequently develop methods to control leprosy.

Keywords: Mycobacteria, Leprosy, Granuloma

Background

Leprosy is a chronic mycobacterial infection that presents an extraordinary range of cellular immune responses in humans. Regulation of cell-mediated immunity against *Mycobacterium leprae* through the fine-tuning between cells, cytokines and chemokines continues to be unraveled. Similar to other mycobacterial infections, granulomatous inflammation in the skin lesion defines certain forms of leprosy [1,2]. The bacilli enter and replicate within macrophages, resulting in the production of cytokines and chemokines, which in turn triggers an inflammatory response leading to the recruitment of macrophages and lymphocytes at the infectious site. Granulomas mainly contain macrophages, epithelioid cells (ECs), multinucleated giant cells (MGCs), surrounded by a rim of T lymphocytes [3]. The organization and the cellular constituents of the developing *M. leprae* granulomas vary with the status of the host immune response. Presumptively, granulomatous lesions can be categorized within two polar forms [4]. At one extreme, tuberculoid granulomas are organized as nodular lesions with ECs and MGCs in the lesion center surrounded by a rim of fibrous connective tissue, lymphocytes along the periphery of the granuloma, and acid-fast bacilli are rarely demonstrable in the lesions. At the other extreme, the pathological feature of lepromatous leprosy skin lesions are characterized by a lack of organization of cells, with very high numbers of foamy macrophages containing very large numbers of bacilli, and disorganized lymphocyte infiltration.

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Granulomas have long been believed to benefit the host by containing and restricting the growth of mycobacteria in a localized area, to prevent the spread of the disease to other parts of the tissue or organs [5]. However, some studies in zebra fish infected with *M. marinum and M. tuberculosis* suggested that the granulomas contribute to early bacterial growth and expanding infection [6-10].

The structure, function, and evolution of granulomas have been studied using various animal models [11,12], high-resolution chest computed tomography scans of pulmonary tuberculosis patients [13], and explanted tissues [5,14]. Interestingly, the *in vitro* models of human mycobacterial granulomas have been studied by infection with Bacillus Calmette-Guérin (BCG) or stimulation with antigens such as purified protein derivatives or artificial beads coated with mycobacterial components [15,16]. These studies have identified infected macrophages, ECs, and several types of MGCs, which are thought to play important roles in the formation and maintenance of granulomas. In addition, macrophages demonstrate considerable plasticity that allows them to efficiently respond to environmental signals. These cells are generally classified as M1 (classic) macrophages, which produce proinflammatory cytokines and mediate resistance to pathogens and contribute to tissue destruction, or M2 (alternative) macrophages, that produce anti-inflammatory cytokines and promote tissue repair [17-19]. However, so far, we know little about the relationship between the polarization of macrophages within mycobacterial granulomas.

In this study, we developed an *in vitro* model of *M. leprae* granulomas, which mimicked the human granulomatous skin lesion with progressive recruitment of monocytes around macrophages infected by *M. leprae*, and their differentiation into ECs and MGCs as well as recruitment of activated lymphocytes. This model may be useful for unravelling the mechanisms of disease progression, and find effective strategies to control the spread of bacilli.

Methods

Ethics statement, cell culture and preparation of the bacteria

Peripheral blood was obtained from healthy Japanese individuals with informed consent. The study was approved by the ethics committee of the National Institute of Infectious Diseases (NIID). In Japan, BCG vaccination is compulsory for children aged 0–4 years old. Macrophages were differentiated from monocytes using granulocyte-macrophage colony-stimulating factor (GM-CSF) as described previously [20,21]. Animal experiments were carried out in strict accordance with the recommendations of Japan's Animal Protection Law. The protocol was approved by the Experimental Animal Committee of NIID Tokyo (Permit Number: 211002). *M. leprae* (Thai-53 strain) was propagated in athymic BALB/c-*nu/nu* mice (Clea Co, Tokyo) [22]. At 8–9 months post-infection, mouse footpads were processed to recover *M. leprae* [23]. For all experiments, *M. leprae* was freshly prepared. Human cells without the bacilli were cultured at 37°C but when infected with the bacilli, the cells were cultured at 35°C to maintain the viability of *M. leprae* in host cells.

Culture of macrophages and peripheral blood mononuclear cells for the formation of cellular aggregates

Macrophages, differentiated from monocytes using GM-CSF after 4 days culture in RPMI containing 20% fetal calf serum (FCS) were transferred into 24-well tissue culture plates (Falcon) ($1 \sim 2 \times 10^5$ cells/well). Freshly prepared *M. leprae* were then added to each well. The multiplicity of infection (MOI: 50) was determined based on the assumption that macrophage were equally susceptible to infection with *M. leprae* [24]. After 24 hr, autologous peripheral blood mononuclear cells (PBMCs) were cultured with *M. leprae* infected macrophages at a ratio of 5:1 (PBMCs: macrophages). In some cases, macrophages were infected with *M. leprae* without PBMCs and in others, macrophages and PBMCs were co-cultured and macrophages alone were used as negative controls. The cells were cultured at 35°C for periods from 24 h to 10 days with medium changes every other day. To detach the cells from plates TrypLE Express (Gibco) was used, and then the cells were maintained in medium containing 10%FCS for 30 min, before processing for flow cytometric analyses. In other experiments we have also isolated T lymphocytes and monocytes were isolated using Dynabeads Untouched Human T cells and Dynabeads MyPure Monocyte kit 2 (Invitrogen), and used instead of PBMCs.

Phase-contrast microscopy and fluorescence microscopy

Macrophages grown on a 13-mm coverslip in a 24-well plate, were infected with *M. leprae* for 24 h. Autologous PBMCs were then co-cultured with macrophages for additional 9 days. Macrophages were fixed in 2% paraformaldehyde, or methanol pre-chilled to −20°C, and then observed under a phase-contrast microscope (Olympus CKX41 with × 10 and × 20 objective lenses). Photographs were taken with an Olympus DP50 system. Image acquisition and data processing were performed using DP controller software. In other experiments, cells were stained with May-Grünwald-Giemsa stain (MGG) (Sigma-Aldrich) or by TB Carbolfuchsin ZN stain according to the manufacturer's instructions (BD Biosciences).

Cell imaging was performed using LSM5-Exciter laser scanning microscope equipped with a 568 nm laser (Carl Zeiss). Fixed cells were stained with anti-human CD163 monoclonal antibody (mAb: BioLegend) and the

secondary antibody used was an Alexa Fluor 568-conjugated goat anti-mouse IgG (Invitrogen/Molecular Probes). Nuclei were counterstained with Hoechst 33342 dye (Sigma-Aldrich). *M. leprae* was stained by auramine O (BD Biosciences). Images were obtained under a fluorescence confocal microscope. Data were processed using LSM software ZEN 2007.

Analysis of cell surface antigens on macrophages by flow cytometry and microscopy

Macrophages were collected after time points of 1 and 9 days of co-culture with the PBMCs or *M. leprae* stimulation. The expression of cell surface antigens on macrophages, was analyzed using a FACSCalibur flow cytometer (BD Biosciences). Dead cells were eliminated from the analysis by staining with 7-amino actinomycin D. For the analysis of cell surface antigens, the following mAb were used: FITC-conjugated mAb against CD68 (KP) was purchased from Dako, FITC conjugated TLR4 (HTA125) and CD206 (19.2), and PE conjugated mAb against CD86 (FUN-1) was all purchased from BD Biosciences and PE conjugated mAb to CD14 (HCD14) and CD163 (RM3/1) were from BioLegend. The numbers in the insets indicate the mean fluorescent values of the cells stained with the respective mAbs.

Determination of cytokine levels

The levels of the cytokines: Interferon (IFN)-γ, interleukin (IL)-2, tumor necrosis factor (TNF)-α, IL-12p40, IL-1β and IL-10 in the culture supernatants were quantified using enzyme assay kits, OptEIA Human ELISA Set (BD Biosciences) and processed according to the manufacturer's instructions. IL-4 and IL-13 was purchased from MABTECH AB. Cytokine levels were expressed as pg of protein/ml of protein. Real-time PCR analysis of mRNA extracted using an RNeasy Mini kit (Qiagen), was performed using SYBR Green PCR Master Mix (Applied Biosystems) with specific primers according to the manufacturer's instructions. The instrument used for the detection of the expression of mRNA was StepOnePlus with StepOne software.

Determination of *M. leprae* viability

The viability of *M. leprae* recovered from the macrophages of different groups was detected by radiorespirometry, that measures the oxidation of ^{14}C palmitic acid to $^{14}CO_2$, as described previously [25]. Briefly, the adherent macrophages and granulomas with bacilli were lysed in 300 μl of a 0.1 N NaOH solution to release intracellular *M. leprae*. After neutralization with 0.1 N HCl solution, an equal volume of 2 times concentrated Middlebrook 7H9 broth was added. ^{14}C labeled palmitic acid was added to the lysates of macrophages or granulomas, followed by incubation at 33°C. After 7 days, cumulative amounts of oxidized palmitic acid released as $^{14}CO_2$ by metabolically active *M. leprae* were measured using a Packard 1500 TRI-CARB liquid scintillation analyzer. The unpaired Student's t-test was used to determine the statistical significance of the two data sets.

Results

Granuloma-like aggregates formed by co-culture of *M. leprae* infected macrophages and autologous PBMCs

When PBMCs were incubated with *M. leprae* infected macrophages in a 24-well tissue culture plate, the cells aggregated to form a multilayered granuloma-like aggregates by day 9 as shown in Figure 1A, whereas control groups did not recruit any cells at this stage (Figure 1B, C). We observed formation of a granular ball-like structure caused by some synapses around aggregates. These *in vitro* granulomas exhibited a cellular structure similar to that in histopathological specimens of tuberculoid leprosy lesions showing T lymphocytes surrounding the differentiated, ECs and MGCs that may be involved in cytokine production for intercellular communication. (Figure 1D). Confocal microscopic analysis of *M. leprae*-induced granuloma showed a multilayered structure (about 3–4 cell layers in transverse and straight sections), and some cells were positive for CD163 (red), a macrophage marker (Figure 1E).

Characterization of the cell populations recruited within *in vitro* granuloma-like aggregates

To identify and characterize the different cell types in granuloma-like aggregates, the cells were plated on glass slides and stained on day 9 of co-culture. MGG staining showed that activated macrophages with larger cytoplasm, and MGCs were observed, which resembled those in the granulomas of leprosy (Figure 2B, D). MGCs are thought to be formed as a result of fusion of macrophages, monocytes and ECs (Figures 2A, C). The presence of *M. leprae* in MGCs was confirmed by staining with TB Carbolfuchsin ZN (arrows in Figure 2E, F). In addition, confocal microscopy revealed the presence of MGCs with auramine O stained *M. leprae*, in the cytoplasmic region (Figure 2G, H). To characterize macrophages, ECs and MGCs in the granuloma-like aggregates, we performed immunofluorescence staining for macrophage markers CD68, CD1a and CD163 (data not shown). Both the macrophages and the MGCs could express the CD68 and CD1a marker, but the expression level of CD68 on the macrophages was higher than that on the MGCs. With the increasing number of nuclei in MGCs, lower levels of CD68 was observed (not shown), although there was no significant difference in the expression levels of CD1a between macrophages and MGCs. These data indicate that MGCs belong to the monocyte/macrophage lineage.

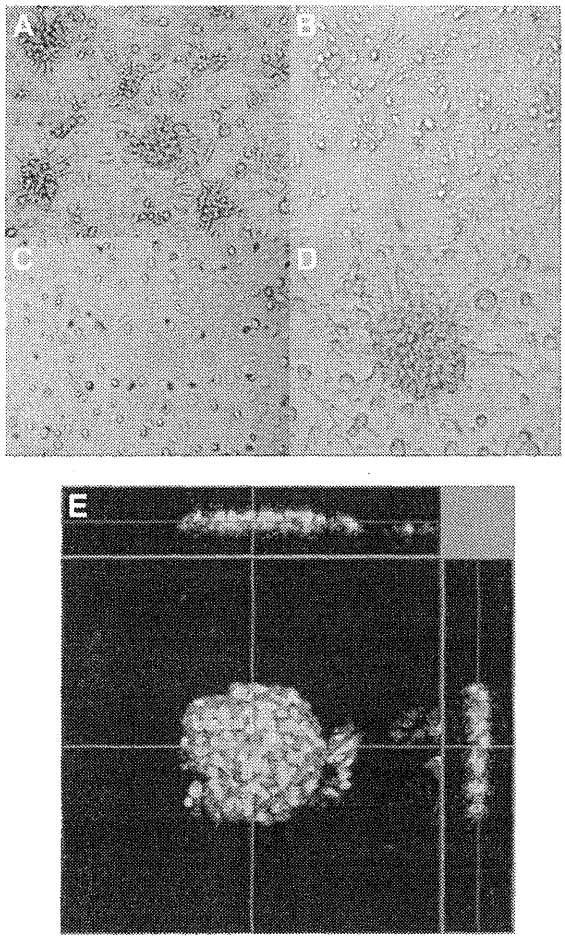

Figure 1 Formation of granuloma-like cellular aggregates by co-culture of PBMCs and macrophages infected with *M. leprae*. (**A**) Co-culture of macrophages (1×10^5), infected with *M. leprae* (MOI:50) and autologous PBMCs (5×10^5) in a 24 well-plate resulted in the formation of granuloma-like aggregates by day 9. (**B**) Culture of macrophages (1×10^5) and autologous PBMCs (5×10^5) for 9 days, without the bacilli. No formation of granuloma-like aggregates was observed. (**C**) Macrophages (1×10^5) infected with *M. leprae* (MOI:50) after 9 days co-culture. (**D**) Higher magnification (2×) of the cell-aggregates in (A). (**E**) Confocal microscopic (LSM5 Exciter) analysis of *M. leprae*-induced granuloma revealed a multilayered structure (about 3–4 cell layers cells in transverse and straight sections). The cells in aggregates were positive for CD163 (red), a marker of macrophages. Nuclei were stained with Hoechst 33343 (blue). Representative data from a single donor are shown.

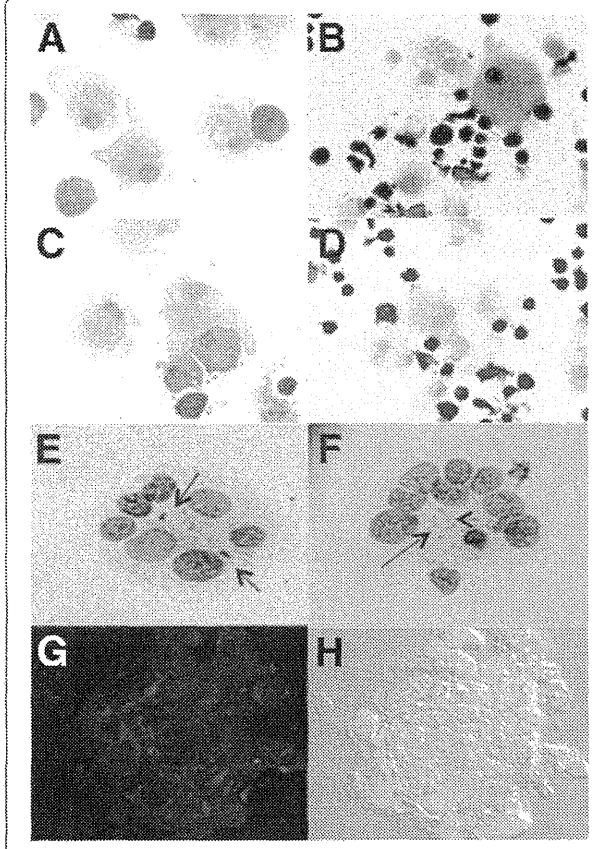

Figure 2 Cell populations in granuloma-like aggregates. May–Grünwald–Giemsa (MGG) staining showed that there are mainly macrophages, ECs (**A**, **D**) and MGCs in the aggregates (**B**, **D**). MGCs were formed by the intercellular fusion and phagocytosis of cells (**C**). *M. leprae* were stained with Ziehl-Neelsen (shown with arrows) and the bacilli were found to be restricted to the central cytoplasmic region of the MGCs (**E**, **F**). Confocal microscopy of MGCs showed *M. leprae* stained with auramine O (green) and the nuclei stained with Hoescht (**G**, **H**).

Expression levels of cell surface antigens on macrophages at different time points

We investigated the expression levels of cell surface antigens on macrophages from different groups at two different time points, day 1 and day 9. On day 1, there was no significant difference in the expression of cell surface antigens on macrophages between groups. Compared with day 1 macrophages, day 9 macrophages, which were infected with *M. leprae* and co-cultured with PBMCs to form granuloma-like aggregates, showed higher expression of CD14 (pattern recognition receptor), CD68 (macrophage marker related to phagocytic activities), CD163 (scavenger receptor) and CD206 (mannose receptor), although the expression of major histocompatibility complex (MHC) class-II, CD86, and toll-like receptor (TLR)-4 did not change (Figure 3). Interestingly, in our long-term culture (9 days) of macrophages infected with *M. leprae*, the expression of CD14, CD68, CD163, TLR4, CD86 and CD206 was significantly lower than that in macrophages infected with *M. leprae* and co-cultured with PBMCs. CD206 expression was the lowest in macrophages co-cultured with PBMCs, although CD163 expression was significantly high (Figure 3). CD163 and CD206 are markers of M2 macrophages, whereas CD86 expression is associated with M1 macrophages. Therefore, the M1 and M2 macrophages appeared to coexist in granulomas.