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RESEARCH ARTICLE

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Polyclonal activation of naïve T cells by urease deficient-recombinant BCG that produced protein complex composed of heat shock protein 70, CysO and major membrane protein-II

Yumiko Tsukamoto*, Yumi Maeda, Toshiki Tamura, Tetsu Mukai and Masahiko Makino

Abstract

Background: *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) is known to be only partially effective in inhibiting *M. tuberculosis* (MTB) multiplication in human. A new recombinant (r) urease-deficient BCG (BCG-dHCM) that secretes protein composed of heat shock protein (HSP)70, MTB-derived CysO and major membrane protein (MMP)-II was produced for the efficient production of interferon gamma (IFN- γ) which is an essential element for mycobacteriocidal action and inhibition of neutrophil accumulation in lungs.

Methods: Human monocyte-derived dendritic cells (DC) and macrophages were differentiated from human monocytes, infected with BCG and autologous T cells-stimulating activity of different constructs of BCG was assessed. C57BL/6 mice were used to test the effectiveness of BCG for the production of T cells responsive to MTB-derived antigens (Ags).

Results: BCG-dHCM intracellularly secreted HSP70-CysO-MMP-II fusion protein, and activated DC by up-regulating Major Histocompatibility Complex (MHC), CD86 and CD83 molecules and enhanced various cytokines production from DC and macrophages. BCG-dHCM activated naïve T cells of both CD4 and CD8 subsets through DC, and memory type CD4⁺ T cells through macrophages in a manner dependent on MHC and CD86 molecules. These T cell activations were inhibited by the pre-treatment of Ag-presenting cells (APCs) with chloroquine. The single and primary BCG-dHCM-inoculation produced long lasting T cells responsive to *in vitro* secondary stimulation with HSP70, CysO, MMP-II and H37Rv-derived cytosolic protein, and partially inhibited the replication of aerosol-challenged MTB.

Conclusions: The results indicate that introduction of different type of immunogenic molecules into a urease-deficient rBCG is useful for providing polyclonal T cell activating ability to BCG and for production of T cells responsive to secondary stimulation.

Keywords: Tuberculosis, BCG, T cell activation

Background

Tuberculosis is a chronic infectious disease, induced by intracellular infection with MTB and is responsible for around 1.4 million deaths yearly worldwide [1-3], and it is estimated that one-third of the global populations is latently infected with MTB. The emergence and worldwide

spread of multidrug-resistant strains of MTB mandates the development of more effective preventive and therapeutic tools [4]. Studies using T cell receptor transgenic mice specific for Ag85B-derived CD4⁺ T cells epitope which is one of the most useful animal model for understanding host defense mechanisms against MTB clearly demonstrated that the MTB-susceptible APCs including DC and macrophages need 7–10 days to initiate activating type 1 CD4⁺ T cells and CD8⁺ T cells in regional lymph nodes after aerosol MTB infection, and the stimulated T cells need

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4–5 weeks to initiate inhibiting the growth of MTB in primary mice [5,6]. However, after the adequate activation of both subsets of T cells, the number of MTB residing in the lung remains stable [5,6]. These observations clearly demonstrate that type 1 T cells are essential elements in inhibiting the multiplication of MTB and also that lack of memory type T cells capable of reacting to MTB-infected APCs strongly and immediately allows MTB to multiply. In addition to the animal studies, *in vitro* studies using human APCs or T cells reveal that host defense against MTB is conducted chiefly by type 1 CD4⁺ T cells and CD8⁺ T cells [7-9]. Among them, IFN- γ produced from both subsets of T cells is considered as one of the most important element for mycobacteriocidal action [10], and cytotoxic T lymphocytes differentiated from the activated CD8⁺ T cells act chiefly at late stage of MTB infection [1,11,12]. The killing process of MTB-infected APCs is via a granule-dependent mechanism [13,14]. Although BCG has been used as a vaccine against tuberculosis widely, BCG cannot prevent the manifestation of adult lung tuberculosis [15]. The major reason why BCG is not fully functional, remains to be elucidated. BCG, as a vaccine, is not convincing in terms of T cell activation, since BCG activates naïve CD4⁺ T cells substantially, but poorly activates naïve CD8⁺ T cells [16,17]. The reason why BCG cannot activate naïve T cells fully, may be based on the lack of the ability to induce phagosomal maturation [18-20]. Therefore, improvement of T cell-stimulating ability of BCG is strongly required. Presently various new protein vaccine candidates have been selected for clinical trials [18-20]. Actually the vaccine candidates including early secretory antigenic target-6, culture filtrate protein 10, Ag85 family and polyprotein Ag designated Mtb72F and a fusion protein ID93 [9,21-26] are produced based on Ags that are recognized in infected individuals. However, fully reliable new vaccine has not been established yet.

Recently, we have produced recombinant BCG, termed BCG-DHTM which strongly activated human naïve CD4⁺ T cells and naïve CD8⁺ T cells, and, in mice, produced T cells responsive to H37Rv-derived cytosolic protein [27]. In the production of BCG-DHTM, we employed two independent strategies in order to overcome the intrinsic defect of BCG, that is an ability to block phagosome maturation to inhibit processing of Ag and presentation to type 1 T cells. One of the strategies is inactivation of *ureC* gene of BCG, which encodes urease, from BCG [19,20]. The urease produces ammonia from urea and inhibits the phagosomal acidification. The urease depletion facilitates the translocation of BCG to lysosome, and enhanced the ability of BCG to activate human naïve CD4⁺ T cells [19,20]. The other one is the intracellular secretion of antigenic molecule. As the key antigen, we used MMP-II, since MMP-II is recognized by human T cells after infection with *M. leprae* or BCG, and can ligate Toll like

receptor (TLR)2 and consequently activate both DC and macrophages [28-31]. Also we used HSP70, since HSP70 has a chaperon activity and can prime cytotoxic T lymphocytes. The intraphagosomal secretion of HSP70-MMP-II fusion protein induced strong activation of naïve CD4⁺ T cells and CD8⁺ T cells [32]. Since both strategies, that is urease depletion and an intracellular secretion of HSP70-MMP-II fusion protein, worked synergistically in terms of T cell activation, the gene encoding the HSP70-MMP-II fusion protein was introduced into urease-depleted rBCG (BCG- Δ UT-11-3) for production of BCG-DHTM [27].

Although BCG-DHTM activated both subsets of T cells to produce IFN- γ , production of memory T cells capable of responding to MTB-derived molecules which can be induced chiefly in the activating phase of MTB growth, is needed for the tuberculosis protection. To address this point, we selected CysO (Rv1335 or CFP10A) as the target gene, since CysO also participates in cysteine biosynthesis pathway in MTB [33]. MTB has two independent cysteine biosynthesis pathways, namely the conventional pathway and the alternative pathway. CysO is engaged in the alternative pathway. This pathway is more advantageous under the oxidative conditions. Indeed, CysO expression is induced under diamide stress, one of the oxidative stress conditions [34]. This implies that CysO may be essential for MTB to survive within macrophages, since MTB is exposed to oxidative stress in macrophages [35]. Furthermore, CysO expression is repressed in hypoxic condition and induced in reoxygenation condition, indicating that CysO may be essential for MTB in the growth phase [36]. Moreover CysO is categorized under ubiquitin superfamily, and may direct protein towards proteasome degradation pathway, which is essential for many cellular processes [37]. Therefore, the CysO involvement in improved cellular response against MTB within macrophages or in growth phase was considered.

In this study, we introduced CysO gene in combination with HSP70-MMP-II fusion gene into urease-deficient BCG- Δ UT-11-3, and produced new rBCG termed BCG-dHCM, and evaluated its T-cell stimulating activities.

Methods

Preparation of cells and Ags

Peripheral blood was obtained from healthy PPD-positive individuals under informed consent. The study was approved by the ethics committee of the National Institute of Infectious Diseases, Tokyo. In Japan, BCG vaccination is compulsory for children (0~1 year-old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (GE Healthcare, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [38]. The viability of T cells obtained from cryopreserved PBMCs was more than 90% and no selection in terms of functionality was induced in both

monocytes, a precursor of DC and macrophages, and T cells. For the preparation of peripheral monocytes, CD3⁺ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs 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 the non-plastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes [39]. Monocyte-derived DC were differentiated as described previously [38,40]. Briefly, monocytes were cultured in the presence of 50 ng of rGM-CSF (Pepro Tech EC LTD, London, England) and 10 ng of rIL-4 (Pepro Tech) per ml [40]. On day 4 of culture, immature DC were infected with rBCG at an indicated multiplicity of infection (MOI) and, on day 6 of culture, DC were used for further analyses of surface Ag and for mixed lymphocyte assays. Macrophages were differentiated as described previously [41,42]. In brief, monocytes were cultured in the presence of 10 ng of rM-CSF (R & D Systems, Inc., Minneapolis, MN) per ml. On day 5 of culture, macrophages were infected with rBCG at an indicated MOI and, on day 7 of culture, they were used for further analyses of surface Ag and mixed lymphocyte assay. The rMMP-II protein was produced as described previously [28,43], and the CysO protein was produced in an LPS-free condition by using *M. smegmatis*. Polyclonal Ab against CysO was produced by immunizing rabbit with the recombinant protein. The rHSP70 protein was purchased (Hy Test Ltd., Turku, Finland) and H37Rv-derived cytosolic protein was produced as described previously [43].

Vector construction and preparation of rBCG

The genomic DNAs were obtained from BCG substrain Tokyo and from MTB H37Rv strain. The oligonucleotide primers used for the amplification of *hsp70* gene were F-Mb70Bal (5'-aaaTGCCATggctcgtgcggcg-3') and R-Mb70Eco (5'-aaaGAATTCcttgccctcccgccg-3'). MMP-II sequence from MTB genomic DNA was amplified with primers: F-MMP TB Eco (aattGAATTCatgcaaggatgccgatgt) and R-MMP TB Sal (5'-aattGTCGACTcaggtcggtggcgaga). CysO sequence from MTB genomic DNA was amplified with primers: F-CysO (5'-ggccggaggccaagaacgtcacgtatccattcc-3') and R-CysO: (5'-atcggtatcaccttgcccaccggccacggcg-3'). The amplified HSP70 and MMP-II sequence were digested with appropriate restriction enzymes and cloned into parental pMV261H plasmid. For the cloning of CysO sequence into pMV261, In-Fusion HD cloning kit (Clontech laboratories, Mountain View, CA) was used. For replacing kanamycin resistance gene to hygromycin resistance cassette, the *Xba* I-*Nhe* I fragment from pYUB854 [44] was cloned into *Spe* I-*Nhe* I fragment of the plasmid

[44]. The rBCG (BCG-ΔUT-11) of which *ureC* gene was disrupted, was produced as described previously [20]. The hygromycin cassette in the BCG-ΔUT-11 was removed by using pYUB870 encoding $\gamma\delta$ -resolvase ($\gamma\delta$ -*tnpR*) [44]. The unmarked BCG was named BCG-ΔUT-11-3. The HSP70-CysO-MMP II fusion protein expressing vector was introduced into BCG-ΔUT-11-3 by electroporation method. BCG-ΔUT-11-3 containing pMV-HSP70-CysO-MMP-II as an extrachromosomal plasmid is referred to as BCG-dHCM, and that containing pMV-261-hygromycin is referred to as BCG-261H (BCG vector control). Recombinant BCGs and MTB H37Rv strain were grown to log phase, and stored at 10⁸ colony forming unit (CFU)/ml at -80°C. Before infection to DC and macrophages, BCGs were counted by colony assay method. There is no significant difference in the *in vitro* culture growth between BCG-261H and BCG-dHCM.

Analysis of cell surface Ag

The expression of cell surface Ag on DC was analyzed using FACSCalibur (BD Bioscience, San Jose, CA). Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma-Aldrich, St. Louis, MO) and 1 × 10⁴ live cells were analyzed. For the analysis of the cell surface Ag, the following mAbs were used: FITC-conjugated mAb against HLA-ABC (G46-2.6, BD Bioscience), HLA-DR (L243, BD Bioscience), CD86 (FUN-1, BD Bioscience), and CD83 (HB15a, Immunotech, Marseille, France).

APC function of DC

The ability of DC infected with BCG or pulsed with recombinant protein and BCG-infected macrophages to stimulate T cells was assessed using an autologous APC-T cell co-culture as previously described [40,45]. Purification of CD4⁺ and CD8⁺ T cells was conducted by using negative-isolation kits (Dynabeads 450, Dynal Biotech) [40]. The purity of the CD4⁺ and CD8⁺ T cells was more than 95% when assessed using FACSCalibur. Naïve CD4⁺ and CD8⁺ T cells were produced by further treatment of these T cells with mAb to CD45RO, which were followed by beads coated with goat anti-mouse IgGs Ab (Dynal Biotech). The purity of both subsets of naïve T cells was more than 97%. However, there was no contamination of memory type T cells in the naïve T cell preparations. More than 98% of CD45RA⁺ T cells was positive in the expression of CCR7 molecule. Memory type T cells were similarly produced by the treatment of cells with mAb to CD45RA Ag. The purified responder cells (1 × 10⁵ per well) were plated in 96-well round-bottom tissue culture plates, and APCs infected with rBCG or pulsed with protein were added to give the indicated APC: T cell ratio. Supernatants of APC-T cell co-cultures were collected on day 4 and the cytokine levels

were determined. In some cases, rBCG-infected DC and macrophages were treated with mAb to HLA-ABC (W6/32, Mouse IgG2a, kappa), HLA-DR (L243, Mouse IgG2a, kappa), CD86 (IT2.2, Mouse IgG2b, kappa, BD Biosciences) or normal mouse IgG. Also, in some cases, immature DC and macrophages were treated with 50 μ M of chloroquine (Sigma-Aldrich) for 2 h and subsequently infected with BCG-dHCM. The optimal dose of the Abs and reagents was determined in advance.

Measurement of cytokine production

Levels of the following cytokines were measured; IFN- γ produced by CD4⁺ and CD8⁺ T cells, and IL-12p70, IL-12p40, TNF α , IL-1 β and GM-CSF produced by DC and macrophages stimulated for 24 or 48 h with rBCGs. The concentrations of these cytokines were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD Bioscience).

Animal studies

For inoculation into mice, rBCG and MTB H37Rv strain were cultured in Middlebrook 7H9 medium supplemented with Middlebrook ADC enrichment to log phase and stored at 10⁸ CFU/ml at -80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating on Middlebrook 7H10 agar plate supplemented with Middlebrook OADC enrichment. Three 5-week-old C57BL/6 J mice (Clea Japan Inc., Tokyo, Japan) per group were inoculated subcutaneously with 0.1 ml of PBS or PBS containing 1 \times 10³ rBCGs. The animals were kept in specific pathogen free conditions and were supplied with sterilized food and water. Four or 12 weeks after inoculation, the spleens were removed and the splenocytes were suspended at a concentration of 2 \times 10⁶ cells per ml in culture medium. The splenocytes were stimulated with an indicated concentration of rMMP-II, rHSP70 (HyTest), rCysO or H37Rv-derived cytosolic protein in triplicates in 96-well round bottom microplates [20,30]. The individual culture supernatants were collected 3–4 days after stimulation and IFN- γ was measured using Opt EIA Mouse ELISA Set (BD Bioscience). For observing the effect of BCG vaccination on MTB infection, five C57BL/6 mice per group were vaccinated with 1 \times 10³ CFU/mouse either BCG-261H or BCG-dHCM for 6 weeks, and were challenged with 100 CFU/lungs of H37Rv by aerosol infection using an automated inhalation exposure apparatus (Glas-Col Corp., IN, Model 099C A4212). Six weeks later, bacterial burden in lungs and spleen was assessed by mechanical disruption in PBS with 0.05% v/v 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 the statistical differences.

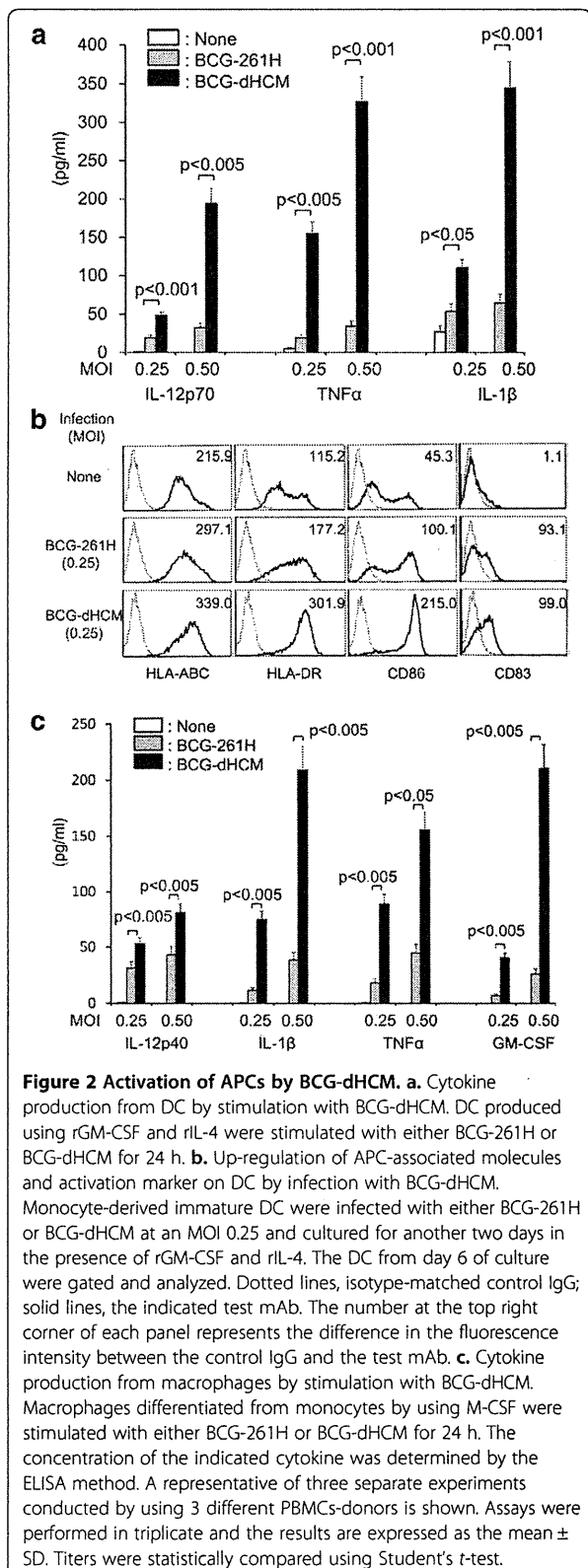
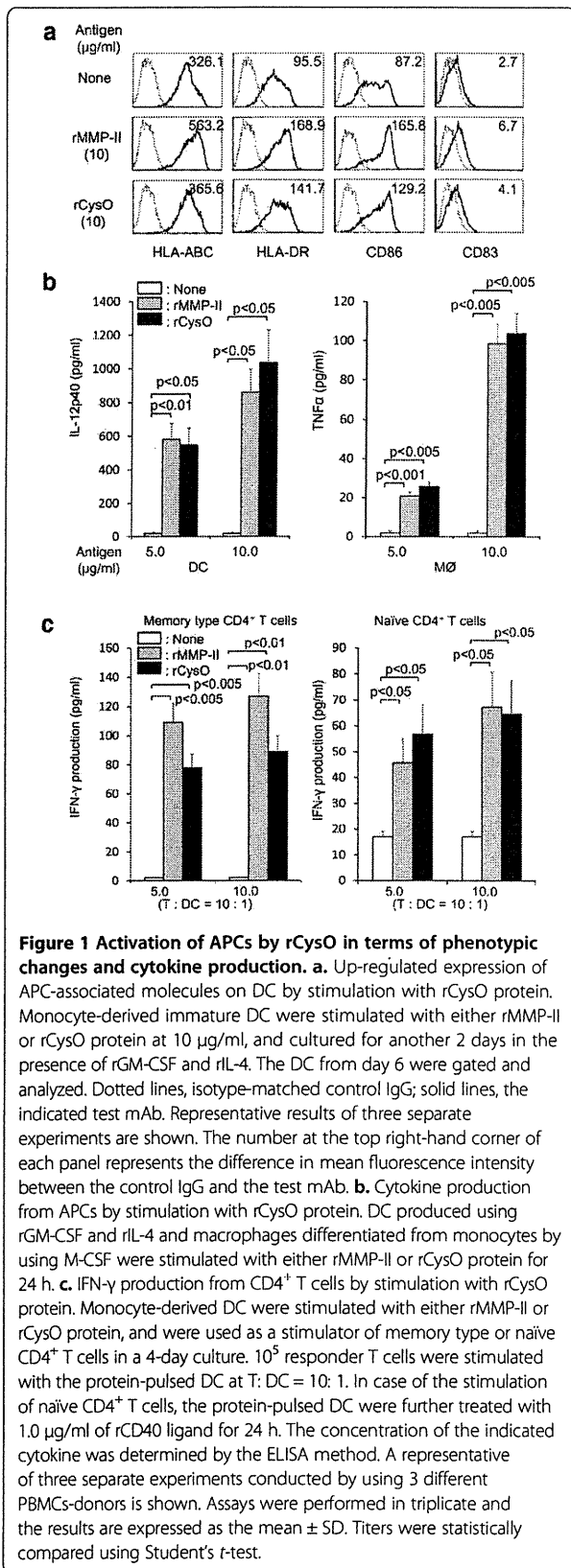
Results

Immunological characterization of rCysO protein

Since rCysO protein produced in *M. smegmatis* revealed a single band on SDS-PAGE electrophoresis and confirmed by Western blot analysis using polyclonal Ab to CysO (not shown), the immunostimulatory activities of rCysO protein were assessed using rMMP-II of MTB as a positive control. We assessed the activation of APCs from the aspect of phenotypic changes and cytokine production (Figure 1). The stimulation of immature DC with rCysO protein up-regulated the expression of MHC molecules, CD86, and CD83 Ags, comparable to rMMP-II protein (Figure 1a). Further, both rMMP-II and rCysO proteins induced IL-12p40 production from DC and TNF α production from macrophages to a similar level (Figure 1b). These results indicate that CysO protein has an ability to activate APCs. When we pulsed immature DC with rMMP-II or rCysO protein and used as a stimulator of autologous CD4⁺ T cells, both proteins induced IFN- γ production from not only memory type CD4⁺ T cells, but also from naïve CD4⁺ T cells with the help of CD40-CD40L interaction. The concentration of IFN- γ released from CD4⁺ T cells by the stimulation with rMMP-II and rCysO was comparable (Figure 1c). Therefore, rCysO protein was found to possess immunostimulatory. We also examined if the newly produced recombinant BCG-dHCM secretes the HSP70-CysO-MMP-II fusion protein by using Western blot analyses. When probed by the Ab to either of HSP70, CysO or MMP-II, BCG-dHCM showed distinct band at 95 kD equivalent to the molecular mass of the fusion protein comprising HSP70, CysO and MMP-II (not shown).

Activation of Ag-presenting cells by BCG-dHCM

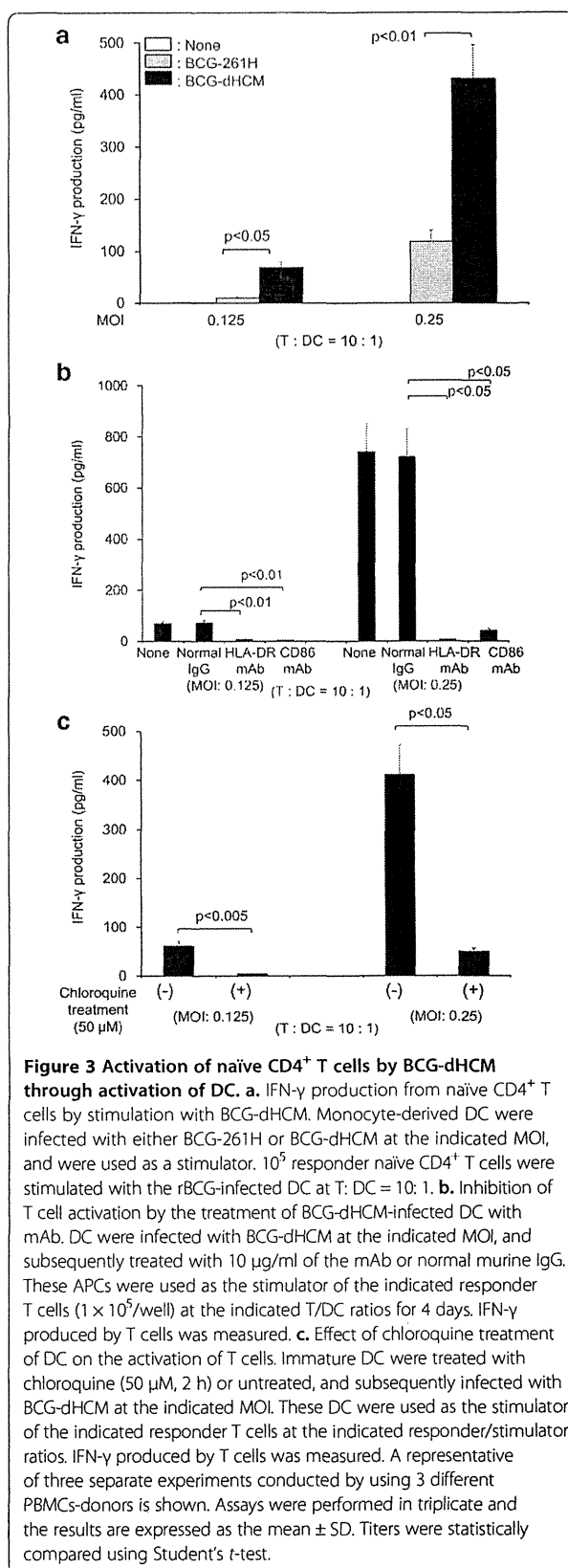
In order to activate naïve T cells and produce memory type T cells efficiently, rBCG should also activate APC adequately. We assessed the activation of DC from the aspects of cytokine production and phenotypic changes (Figure 2). BCG-dHCM stimulated DC to produce IL-12p70, TNF α and IL-1 β more efficiently than BCG-261H at both MOIs: 0.25 and 0.50 (Figure 2a). To assess the phenotypic changes induced by BCG-dHCM infection, we assessed the expression of MHC, CD86 and CD83 molecules on DC (Figure 2b). Both BCG-261H and BCG-dHCM up-regulated the expression of these molecules, but the infection with BCG-dHCM induced higher level of the expression. We used various dose of rBCGs for the assessment, and the similar changes were observed (not shown). Further, the stimulation of macrophages with BCG-dHCM induced the production of



higher level of IL-12p40, IL-1 β , TNF α and GM-CSF than that with BCG-261H at both MOIs: 0.25 and 0.50 (Figure 2c).

Activation of naïve and memory type T cells by BCG-dHCM

The ability of BCG-dHCM to activate naïve CD4⁺ T cells through DC was evaluated using BCG-261H as a control (Figure 3). BCG-dHCM more efficiently activated naïve CD4⁺ T cells than BCG-261H to produce IFN- γ (Figure 3a). The IFN- γ production from naïve CD4⁺ T cells by BCG-dHCM stimulation seems to be induced by an Ag-specific manner, since the treatment of the surface of BCG-dHCM-infected DC with mAb to HLA-DR or CD86 showed 90% inhibition (Figure 3b). Further, pretreatment of immature DC with chloroquine prior to infection with BCG-dHCM, inhibited the production of IFN- γ from naïve CD4⁺ T cells (Figure 3c). These results suggest that the phagosomal maturation is induced by BCG-dHCM infection and is closely associated with the activation of naïve CD4⁺ T cells. Previously, we reported that only when the urease-deficient rBCG, but not normal BCG, was used as a host BCG and introduced with genes encoding antigenic molecules, the rBCG could activate memory type CD4⁺ T cells through M-CSF-dependent macrophages [27]. We checked whether BCG-dHCM activates memory type CD4⁺ T cells, and found that BCG-dHCM induced the significant production of IFN- γ from the responder population (Figure 4a). Again, the activation of memory type CD4⁺ T cells by BCG-dHCM-infected macrophages seems to be dependent on the expression of MHC class II and CD86 Ags, since the treatment of rBCG-infected macrophages with mAb to HLA-DR or CD86 inhibited the IFN- γ production significantly (Figure 4b). Further, the treatment of macrophages with chloroquine prior to BCG-dHCM infection, inhibited IFN- γ production by memory type CD4⁺ T cells (Figure 4c). Next, we assessed whether BCG-dHCM could activate naïve CD8⁺ T cells when DC were enrolled as APC (Figure 5). While vector control BCG did not induce the activation of naïve CD8⁺ T cells as reported [19,20], BCG-dHCM induced significant level of activation of naïve CD8⁺ T cells (Figure 5a). A significant concentration of IFN- γ can be released from naïve CD8⁺ T cells. Also the mAb treatment of BCG-dHCM-infected DC with anti-HLA-ABC Ab or anti-CD86 Ab significantly inhibited the IFN- γ production from naïve CD8⁺ T cells (Figure 5b), and pre-treatment of immature DC with chloroquine, again, inhibited the cytokine production from the responder (Figure 5c). These results suggest that BCG-dHCM can activate not only naïve CD4⁺ T cells, but also naïve CD8⁺ T cells through DC, and also activate memory CD4⁺ T cells through macrophages in an Ag-dependent manner. Further, the phagosomal maturation is closely associated with the T cell activation.



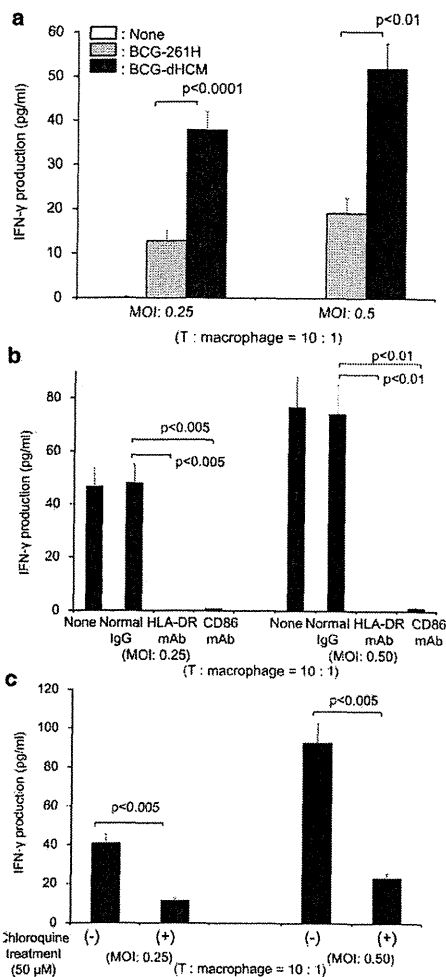


Figure 4 Activation of memory-type $CD4^+$ T cells by BCG-dHCM through activation of macrophages. **a.** IFN- γ production from memory type $CD4^+$ T cells by stimulation with BCG-dHCM. Macrophages differentiated by using M-CSF were infected with either BCG-261H or BCG-dHCM at the indicated MOI, and were used as a stimulator of responder $CD4^+$ T cells in a 4-day culture. 10^5 responder T cells were stimulated with the rBCG-infected macrophages at T: macrophage = 10: 1. **b.** Inhibition of T cell activation by the treatment of BCG-dHCM-infected macrophages with mAb. Macrophages were infected with BCG-dHCM at the indicated MOI, and subsequently treated with 10 μ g/ml of the mAb or normal murine IgG. These APCs were used as the stimulator of the indicated responder T cells (1×10^5 /well) at the indicated T/macrophages ratios for 4 days. IFN- γ produced by T cells was measured. **c.** Effect of chloroquine treatment of macrophages on the activation of T cells. Macrophages were treated with chloroquine (50 μ M, 2 h) or untreated, and subsequently infected with BCG-dHCM at the indicated MOI. These macrophages were used as the stimulator of the indicated responder T cells at the indicated responder/stimulator ratios. IFN- γ produced by T cells was measured. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titers were statistically compared using Student's *t*-test.

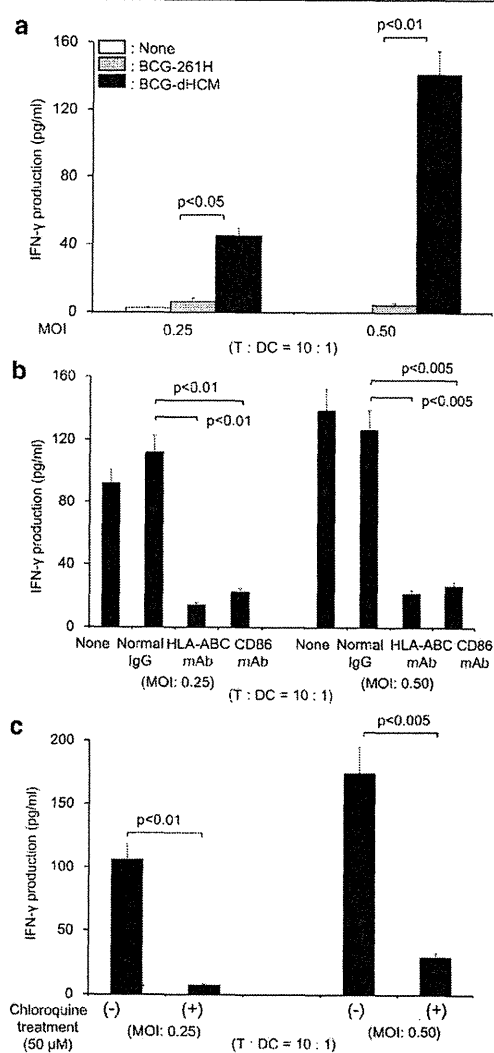


Figure 5 Activation of naive $CD8^+$ T cells by BCG-dHCM through activation of DC. **a.** IFN- γ production from naive $CD8^+$ T cells by stimulation with BCG-dHCM. DC were infected with either BCG-261H or BCG-dHCM at the indicated MOI, and were used as a stimulator. 10^5 responder T cells were stimulated for 4 days with rBCG-infected DC at T: DC = 10: 1. **b.** Inhibition of T cell activation by the treatment of BCG-dHCM-infected DC with mAb. DC were infected with BCG-dHCM at the indicated MOI, and subsequently treated with 10 μ g/ml of the mAb or normal murine IgG. These APCs were used as the stimulator of the indicated responder T cells (1×10^5 /well) at the indicated T/DC ratios for 4 days. IFN- γ produced by T cells was measured. **c.** Effect of chloroquine treatment of DC on the activation of T cells. Immature DC were treated with chloroquine (50 μ M, 2 h) or untreated, and subsequently infected with BCG-dHCM at the indicated MOI. These DC were used as the stimulator of the indicated responder T cells at the indicated responder/stimulator ratios. IFN- γ produced by T cells was measured. A representative of three separate experiments conducted by using 3 different PBMCs-donors is shown. Assays were performed in triplicate and the results are expressed as the mean \pm SD. Titers were statistically compared using Student's *t*-test.

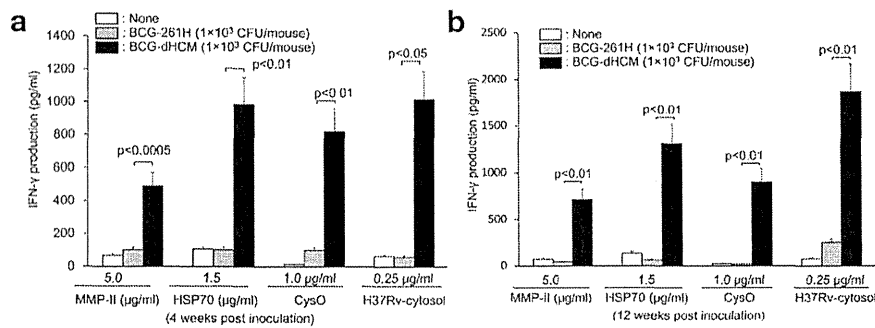


Figure 6 Production of T cells responsive to the secondary stimulation in mice by infection with BCG-dHCM. Three 5-week-old C57BL/6 mice per group were each infected with 1×10^3 CFU of either BCG-261H or BCG-dHCM subcutaneously. Four (a) or 12 (b) weeks after the inoculation, splenocytes (2×10^5 cells/well)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

Production of T cells responsive to the secondary stimulation by BCG-dHCM *in vivo*

The ability of BCG-dHCM to produce T cells highly responsive to the secondary *in vitro* stimulation was examined by *in vivo* functional studies (Figure 6). C57BL/6 mice were subcutaneously inoculated with 1×10^3 CFU/mouse of either BCG-261H or BCG-dHCM 4 weeks before stimulation *in vitro* (Figure 6a). Not only MMP-II, HSP70 and CysO of which encoding genes were introduced into BCG-dHCM, but also H37Rv-derived cytosolic protein were used as a secondary *in vitro* stimulator. The T cells from mice inoculated with BCG-dHCM respond more vigorously to all stimulators and produced higher concentration of IFN- γ (Figure 6a) and IL-2 (not shown) than T cells from mice uninfected or infected with BCG-261H. To examine the long-term effect of the inoculation of BCG-dHCM on the production of responsive T cells, C57BL/6 mice were subcutaneously inoculated with 1×10^3 CFU/mouse of either BCG-261H or BCG-dHCM

12 weeks before restimulation *in vitro* (Figure 6b). Again, a significantly higher concentration of IFN- γ was produced from splenic T cells obtained from mice inoculated with BCG-dHCM by all stimulators than those from mice uninfected or infected with BCG-261H.

Effect of BCG-dHCM vaccination on the multiplication of aerosol challenged *M. tuberculosis*

C57BL/6 mice vaccinated with either BCG-261H or BCG-dHCM (1×10^3 CFU/mouse) for 6 weeks were challenged with 100 CFU per lungs of H37Rv by aerosol infection. Six weeks later, the MTB recovered from both lungs and spleen was enumerated (Figure 7). While the mice vaccinated with BCG-261H minimally inhibit the multiplication of MTB, the vaccination with BCG-dHCM significantly inhibited the growth of H37Rv in lungs (Figure 7a). Similarly, the multiplication of MTB in spleen is significantly inhibited by the vaccination with BCG-dHCM (Figure 7b).

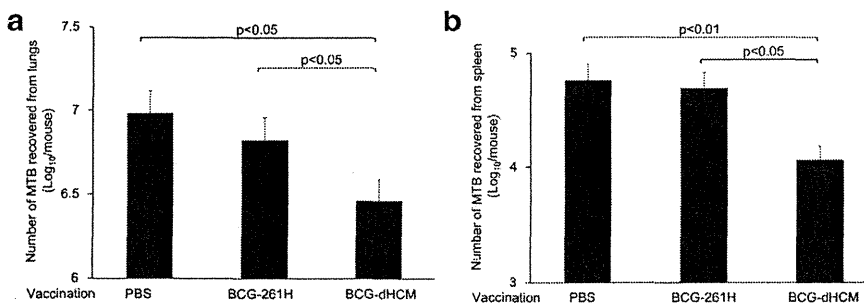


Figure 7 Inhibition of MTB multiplication by subcutaneous vaccination with BCG-dHCM. Five-week-old C57BL/6 mice (5 mice/group) were subcutaneously vaccinated with 1×10^3 CFU/mouse of either BCG-261H or BCG-dHCM and were challenged with 100 CFU/lungs of H37Rv by aerosol infection, at 6 weeks post vaccination. The number of MTB recovered from the lung (a) or spleen (b) at 6 weeks post challenge was enumerated by colony assay method. Representative results of three separate experiments are shown. Titers were statistically compared using Student's t-test.

Discussion

IFN- γ produced by activated type 1 CD4⁺ T cells and CD8⁺ T cells has an essential function in both antimicrobial activity against MTB and limitation of lung inflammation associated with massive accumulation of neutrophils which are recruited by Th17 cells [7-9,46]. Therefore, one of the important aims of vaccination is to produce memory type CD4⁺ T cells as well as memory type CD8⁺ T cells capable of producing abundant dose of IFN- γ by responding rapidly to MTB-infected DC and macrophages. From the studies using T cell receptor-transgenic animal model, it is known that it needs several weeks for host T cells to start inhibiting the multiplication of aerosol infected MTB in primary mice [5,6]. It is unknown whether alveolar APCs could be primed by vaccination, so that time required for APC to move to the regional LN after phagocytosing MTB in lung could be reduced. However, it could certainly reduce the time necessary for T cells to be activated by the interaction with MTB-infected APCs in the LN by differentiating naïve T cells into memory subset by vaccination. BCG has been used widely as a vaccine against tuberculosis, but its effect is quite limited. BCG can only prevent the development of miliary tuberculosis and tuberculosis meningitis in child, but is not effective for prevention of adult lung tuberculosis [15]. However, BCG has many antigenic molecules common to that present in MTB, and moreover, the safety of BCG is well established [16,17]. Thus, we are of the opinion that the improvement of BCG by producing rBCGs would be the fastest route to produce more reliable single injection vaccine against tuberculosis. In this respect, rBCG should have some antigenic molecules which are present in MTB, and also should highly activate not only naïve T cells of both CD4 and CD8 subsets, but also APCs including DC and macrophages. Since both DC and macrophages express MMP-II-related peptide on their surface upon an infection with both H37Ra and H37Rv [31], and also MMP-II can ligate TLR2 and consequently activates NF- κ B pathway of APCs [28-31], MMP-II is considered to be good target that could be used as an active vaccinating agent. In this study, we produced new rBCG termed BCG-dHCM, that is a urease-deficient rBCG that secrete the fusion protein composed of HSP70, CysO and MMP-II. In the production of BCG-dHCM, we used MMP-II as a central component, HSP70 as an adjuvant, and CysO as an element necessary to release Ag in APCs. BCG-dHCM secreted the fusion protein in both lysosome in which abundant enzyme is available and in phagosomes of BCG-susceptible cells. Thus BCG-dHCM strongly activated both subsets of naïve T cells and APCs. The efficient activation of APCs by BCG-dHCM is revealed through up-regulation of APC-associated molecules and by enhanced production of various proinflammatory cytokines including IL-12, IL-1 β and TNF α from DC and

macrophages. The efficient activation of these APCs can be assumed to be directly linked with the effective activation of adaptive immunities. Actually macrophages-infected with BCG-dHCM activated memory type CD4⁺ T cells. Previous rBCG termed BCG-70M that is BCG introduced with HSP70-MMP-II fusion gene failed to activate memory type T cells through macrophages [32], thus, the high immunostimulatory function of BCG-dHCM seems to be owing to the high antigenic load on the surface of BCG-dHCM-infected macrophages, that is due to efficient translocation of BCG-dHCM into lysosome. The effective memory T cells are ones capable of responding to the molecules expressed on the surface of MTB-infected APCs, and are produced by activating naïve T cells in an Ag-specific manner [27,31]. In this respect, the activation of both naïve CD4⁺ T cells and naïve CD8⁺ T cells by BCG-dHCM was dependent on MHC and CD86 molecules expressed on APCs. Thus, BCG-dHCM seems to be stimulating naïve T cells in an Ag-specific fashion. Presumably, APCs infected MTB express various epitopes on their surface, indicating that the presence of clonal diversity of T cells, might provide better control of MTB. Subcutaneous inoculation of BCG-dHCM into C57BL/6 mice produced long lasting T cells responsive to *in vitro* secondary stimulation. Upon stimulation with not only MMP-II, CysO and HSP70, but also H37Rv-derived cytosolic (Figure 6) and membrane (not shown) protein, T cells obtained from mice inoculated with BCG-dHCM were efficiently reactivated and produced high concentration of IFN- γ . Although the exact reason why these T cells were capable of responding to MTB components is not clear, it may be reasonable to speculate that the translocation of BCG-dHCM into lysosome induced the degradation of BCG that leads to production of epitope derived from BCG itself. CysO is engaged in alternative cysteine biosynthesis pathway, which plays an essential role for the MTB survival in the oxidative condition [33,34]. Also, CysO is categorized in ubiquitin superfamily and is possible to direct protein towards proteasome degradation pathway [37]. In fact, in this study, we found that rCysO, produced by using *M. smegmatis* in a LPS-free condition, activated both innate and adaptive cellular responses, since it induced some cytokine production from APCs and phenotypic changes in DC, and also stimulated IFN- γ production from both subsets of T cells. When we introduced CysO gene into urease-deficient rBCG accompanying with HSP70-MMP-II fusion gene, CysO was produced as a part of fusion protein, in fact, the immunogenic function of CysO may be up-regulated because of the chaperone activity of HSP70, that may lead to production of CysO-specific memory T cells *in vivo*, although we could not elucidate the detailed mechanisms of the immunological function of CysO. Actually, mice inoculated with BCG-dHCM produced T cells that

respond to CysO vigorously. These results indicate that CysO protein was certainly secreted from rBCG in APC and was used for T cell activation. CysO expression in MTB is inhibited in hypoxic condition and is induced by re-aeration [34], thus, CysO may be highly expressed in MTB in active phase, and, also, is involved in the survival of MTB in macrophages. Therefore, production of CysO reactive T cells may be advantageous for the induction of host defense reaction against MTB which is re-activated in macrophages. In this study, we used small dose of BCG, one fifth of usual dose [47], and by which the parent BCG cannot inhibit MTB growth in lungs in our hands (not shown), for vaccination of mice in order to elucidate the difference between BCG-261H and BCG-dHCM. As expected BCG-261H did not inhibit the MTB growth, but even a small dose of BCG-dHCM at least partially inhibits multiplication of MTB in both lungs and spleen. In this respect, BCG-dHCM seems to be superior to BCG-261H. The reason why BCG-261H did not inhibit the multiplication of MTB may be because we tested bacterial burden in lung and spleen at 6 wks, but not 4 wks which is a frequently used time point [47], after MTB challenge, because report suggests that 4 ~ 5 wks are necessary to reach the stable level of pulmonary bacterial burden even in naïve mice [6]. Also, due to BCG strain differences, there may be differences in protective effect in mice experiments. However, more detailed studies, for example, the difference between BCG-DHTM and BCG-dHCM, and the relation between the presence of CysO-reactive T cells and MTB replication in macrophages, are absolutely required, to prove the usefulness of CysO involvement for the production of vaccine against tuberculosis.

Conclusions

These results indicate that the secretion of polycomponent antigenic molecules can efficiently produce polyclonal Ag-specific T cells responsive to secondary stimulation *in vivo*, and may provide one possible tool for the development of better vaccine against tuberculosis.

Abbreviations

BCG: *Mycobacterium bovis* BCG; M: *Mycobacterium*; r: Recombinant; MTB: *M. tuberculosis*; HSP: Heat shock protein; MMP: Major membrane protein; IFN- γ : Interferon-gamma; DC: Dendritic cells; Ag: Antigen; MHC: Major histocompatibility complex; APC: Antigen-presenting cell; TLR: Toll-like receptor; PBMC: Peripheral blood mononuclear cell; mAb: Monoclonal antibody; MOI: Multiplicity of infection; CFU: Colony forming unit.

Competing interests

The authors declare that they have no competing interests.

Authors' contribution

YT and MM participated in the design of the study and carried out the cell culture experiments, YM and TT carried out animal studies, YT and TM conducted the construction of recombinant BCG. YT, YM and MM were involved in the preparation of the manuscript. All authors have read and approved the final manuscript.

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Genome-wide re-sequencing of multidrug-resistant *Mycobacterium leprae* Airaku-3

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Abstract

Genotyping and molecular characterization of drug resistance mechanisms in *Mycobacterium leprae* enables disease transmission and drug resistance trends to be monitored. In the present study, we performed genome-wide analysis of Airaku-3, a multidrug-resistant strain with an unknown mechanism of resistance to rifampicin. We identified 12 unique non-synonymous single-nucleotide polymorphisms (SNPs) including two in the transporter-encoding *ctpC* and *ctpI* genes. In addition, two SNPs were found that improve the resolution of SNP-based genotyping, particularly for Venezuelan and South East Asian strains of *M. leprae*.

Keywords: Leprosy, molecular epidemiology, *Mycobacterium leprae*, rifampicin-resistance, single nucleotide polymorphism

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Introduction

Despite a massive decline in leprosy prevalence in the last two decades, more than 200 000 new cases of leprosy are recorded each year globally, indicating active transmission of the infection [1]. Drug resistance to one or more anti-leprosy drugs has been reported but is rare. As the causative agent, *Mycobacterium leprae*, remains uncultivable, molecular drug susceptibility testing offers a practical alternative. This involves PCR-sequencing of the drug-resistance-determining regions of the *rpoB*, *folP1* and *gyrA* genes associated with resistance to rifampicin, dapsone and ofloxacin, respectively [2]. However, a previous study described low-levels of rifampicin resistance in *M. leprae* Airaku-3, isolated from a Japanese patient who relapsed after multidrug therapy [3]. This strain exhibited phenotypic resistance to rifampicin and dapsone in a mouse footpad assay. Although the dapsone-resistance was attributable to the known *folP1* mutation (Thr531Ile), Airaku-3 has a wild-type *rpoB* sequence; hence, an explanation for its rifampicin resistance is not available [2].

Alternative mechanisms of rifampicin resistance have been described in various bacterial species, for example, the *rox* gene mediated mono-oxygenation of rifampicin [4] and duplication of the *rpoB* gene as *rpoB2* in *Nocardia* [5]. There is no orthologue of *rox* in *Mycobacterium tuberculosis* and *M. leprae*. Over-expression of RNA polymerase-binding protein A causes low level rifampicin resistance in *Streptomyces coelicolor* [6]. Its *M. leprae* orthologue *ML1439* and other relevant genes *sigA* (*rpoT*) and *sigE* were all found to be wild-type in our PCR-based analysis. As per the *M. leprae* single nucleotide polymorphism (SNP) -genotyping scheme [7], Airaku-3 belongs to SNP subtype 1D, which is the predominant genotype in many countries like Bangladesh, India, Nepal, Madagascar, Malawi and the French West Indies [7–9] and has a significant representation in Japan [7], Yemen, Venezuela [10] and the USA [11]. However, the genome of only one strain (S11-Inde2 from India) of this subtype has been sequenced [12]. Therefore, the Airaku-3 strain was selected for genome-wide sequencing using the Illumina platform to investigate the genetic basis for resistance to rifampicin and to improve the resolution of the existing SNP-genotyping scheme for *M. leprae*.

In our present study, a bacillary suspension containing $c.10E^{+08}$ cells of *M. leprae* Airaku-3 [2] in 0.1 M NaOH was passed through a 1-mL insulin syringe (0.30-mm needle) three to five times, before DNA extraction [13]. Library preparation was by the TruSeq ChipSeq method (Illumina). Paired-end sequencing, with 101 cycles on a HiSeq2000 instrument provided a total of 174.2 million reads of which 25% could

be aligned with the *M. leprae* TN genome [14], yielding >1000 times coverage, using BOWTIE [15]. *De novo* assembly of these reads was performed using VELVET [16] with *k*-mer size of 91. SNPs were identified as described previously [7,12] by comparison with the other *M. leprae* strains using PCR sequencing or genome comparisons [7,11,12] to identify the unique SNPs of the Airaku-3 genome (see Supporting information, Tables S1, S2). The phylogenetic trees were obtained with SPLITSTREE v.4.13.1 [17] using uncorrected *p* distances, the Neighbour Joining method and 1000 times bootstrapping (Fig. 1).

After *de novo* assembly of the reads the genome of *M. leprae* Airaku-3 comprised 114 contigs (*n*₅₀ = 42 Kb) covering 98.85% of the TN genome. All the gaps corresponded to dispersed repeats. There was no evidence of structural variations compared with the *M. leprae* TN reference genome, thereby ruling out genome rearrangements or gene duplications as an explanation for rifampicin resistance in Airaku-3. Upon comparing the genome sequence of Airaku-3 with the *M. leprae* TN [14] reference genome, a total of 114 SNPs were identified. Of these, 46 SNPs were not present in any other

M. leprae genomes. These included two non-synonymous substitutions in genes with predicted transporter function belonging to the P-type ATPase family: 888973C>T in gene *ctpC* and 3209207G>A in *ctpI*. These two SNPs were also absent in the remaining *M. leprae* strains (Table S2).

Airaku-3 shared 12 SNPs with the other SNP-type ID strain S11-Inde2 (Table S1). We analysed two of these SNPs (953582C>T and 3262657C>T) in 24 strains belonging to SNP subtype ID from different countries (Table S2). SNP 3262657C>T correctly identified all of these ID strains from the remaining 42 strains of other genotypes. Furthermore, the SNP 953582C>G distinguished the Venezuelan ID strains (*n* = 10) from the rest of the ID strains originating from eight different countries (Table S2). The 100 bp flanking region of this SNP in other mycobacterial outgroup species (*M. tuberculosis* complex, *M. avium* complex, *M. kansasii*, *M. marinum* and *M. ulcerans*) revealed a 'C' at the corresponding base, thereby defining the ancestral base. Therefore, Venezuelan ID strains with a 'C' at position 953582 are designated as subtype ID-1 while the remaining ID strains with a derived base 'G' are termed ID-2.

Though our present study does not reveal a clear explanation for the rifampicin resistance phenotype of Airaku-3, it has identified the unique SNPs, including two non-synonymous SNPs in transporter genes, *ctpC* and *ctpI*. However, a functional assay is required to determine whether either of these variant genes/transporters confers any degree of rifampicin resistance. Other transporters, for example, *drrA* (Rv2936), *pstB* (Rv0933) [18] and Rv1258c [19] reportedly confer low-levels of rifampicin resistance in *M. tuberculosis*. The Airaku-3 genome revealed no mutations in its *drrA* (ML2352c) whereas the orthologs of the remaining two genes (*pstB* and *ML1104c*) are pseudogenes in *M. leprae*. Rifampicin-resistant *M. tuberculosis* strains commonly possess compensatory mutations in the *rpoC* gene that restore their fitness [20]. This gene has a wild-type sequence in Airaku-3.

The comparative genomics of ID strains in this study has discovered two useful markers: SNP3262657C>T defines the ID genotype while the SNP953582C>G can further resolve them into ID-1 and ID-2. We have also identified a phylogeographic association of the ID-1 genotype with Venezuela. These markers can be useful for molecular epidemiological studies in many countries where leprosy is endemic and the ID genotype is predominant, and can also provide a possible explanation for disease acquisition in other countries where sporadic cases are reported in the indigenous population or among immigrants or citizens who have lived in areas where leprosy is endemic. Previously, we had successfully resolved the subtype 31 strains into 31-1 and 31-2 using a similar approach [11]. Hence, our present study further

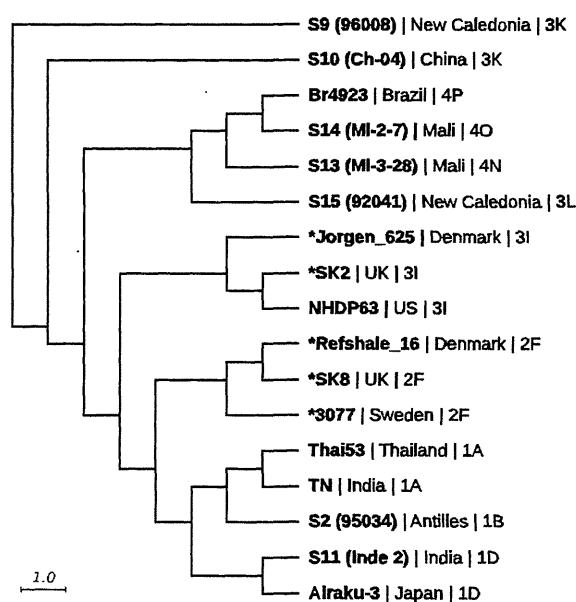


FIG. 1. Phylogenetic relationship of *Mycobacterium leprae* Airaku-3 with other *M. leprae* genomes [12]. For phylogeny, only the genomic positions in which all strains [12] had an unambiguous nucleotide call (674 positions) were considered. *Mycobacterium leprae* Airaku-3 is placed closest to another subtype ID strain, S11-Inde2 from India, both of which share 12 SNPs that were absent in any other SNP genotypes. The geographic origins and the SNP genotypes are indicated against each strain. The ancient strains from Europe are indicated with a *. All bootstrap values are above 87 (average 99).

exemplifies the value of genome-wide comparisons of a few strains to uncover reliable phylogeographic markers that can later be used for rapid PCR-based genotyping.

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Contribution to Authorship

PS and STC designed the study with the help of MK and MM; PS, PB, CP, CA and AP-M did the sample preparation, sequencing and analysis with guidance from KH and STC; AB, SC, JR and PS performed the bioinformatic analysis; PS wrote the manuscript with input from AB, STC and other co-authors. All authors read and approved the final version of the manuscript.

Transparency Declaration

The authors declare no conflicts of interest.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. List of all single nucleotide polymorphisms (SNPs) in *Mycobacterium leprae* Airaku-3 genome compared to the TN genome and their distribution in previously described genomes [7,12]. The unique SNPs and their effect in the Airaku-3 genome are shown in bold, with the SNPs in transporter proteins shown in blue highlight.

Table S2. *Mycobacterium leprae* isolates of various genotypes from different geographic origins, which were tested for the subtype ID specific single nucleotide polymorphisms. The combination of 'T and C' at positions 3262657 and 953582, respectively, is characteristic of the Venezuelan ID-1 genotype whereas a combination of 'T and G' represents the ID-2 genotype.

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Paleopathological Evidence and Detection of *Mycobacterium leprae* DNA from Archaeological Skeletal Remains of *Nabe-kaburi* (Head-Covered with Iron Pots) Burials in Japan

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Abstract

The *Nabe-kaburi* is a unique burial method, the purpose of which is shrouded in mystery. The burials were performed during the 15th to 18th centuries in eastern Japan, and involved covering the heads of the deceased with iron pots or mortars. The identification of leprosy-specific osteological lesions among some of the excavated remains has led to the suggestion that *Nabe-kaburi* burials were a reflection of the social stigma against certain infectious diseases, such as leprosy, tuberculosis or syphilis. However, molecular evidence for the presence of disease has been lacking. The goal of this study was to detect *Mycobacterium leprae* (*M. leprae*) DNA in archaeological human skeletal remains from *Nabe-kaburi* burials. The paleopathological data from three *Nabe-kaburi* burials were re-evaluated before small samples were taken from affected and control areas. DNA was extracted and used as a template to target the *M. leprae*-specific DNA using a combination of whole genome amplification, PCR analysis and DNA sequencing. *M. leprae* DNA fragments were detected in the two sets of skeletal remains that had also shown paleopathological evidence of leprosy. These findings provide definitive evidence that some of the *Nabe-kaburi* burials were performed for people affected by leprosy. Demonstration of the presence of *M. leprae* DNA, combined with archeological and anthropological examinations, will aid in solving the mystery of why *Nabe-kaburi* burials were performed in medieval Japan.

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Introduction

The *Nabe-kaburi* burial was performed during the 15th to 18th centuries in the eastern region of Japan. In Japanese, “*nabe*” means pot and “*kaburi*” means to put on. Thus, the deceased were buried with an iron pot or mortar covering their heads. To date, a total of 105 *Nabe-kaburi* burials have been excavated in Japan [1].

There are two main theories in Japanese folklore as to why the deceased would be buried with iron pots on their heads [2,3]. One is that the iron pots were symbolically “block” spreading particular diseases, such as leprosy, tuberculosis or syphilis, which plagued the deceased when they were alive. The second reason is rather unique and somewhat humorous: *Nabe-kaburi* burials were performed for someone who died during the “Bon” period in Japan. The “Bon” is the Japanese ritual ceremony to welcome the souls of ancestors back from heaven during a 3-day period each summer. Since dying during the festival was considered imprudent, the ancestors beat the head of a descendant when they

encountered each other on the way to and from the next world. Therefore, relatives of the descendant might have been trying to protect the head of the deceased at burial.

Such speculative stories serve to make the *Nabe-kaburi* burial appear more and more mysterious. However, a commonality between these burials and those of leprosy patients was noted as early as the initial study of the *Nabe-kaburi* burials more than 100 years ago. In addition, paleopathological examination of some cases revealed leprosy-specific skeletal changes in excavated specimens. During a time when there was no effective treatment, leprosy would have gradually spread over the entire body and caused specific osteological deformations in the nasal aperture, anterior nasal spine and alveolar process on the premaxilla, cortical areas of the tibia and fibula, distal ends of the metatarsals and diaphyses of the phalanges that may have included both direct and reactive changes [4–10].

To date, 105 *Nabe-kaburi* burials have been found in Japan (Table 1). The oldest were buried in the 15th to 16th centuries,

while the most recent have been dated to the 19th century. About half were excavated from the *Kanto* region of Japan (Figure 1 shaded area). Most others were found in Northeast Japan, with only a few in Midland and Southwest Japan, including *Nagoya*, *Kyoto* and *Osaka*, which were already large cities at that time. Not all of the burial remains have had osteological and paleopathological evaluations, probably because skeletal preservation was poor and trained anthropologists were not always available. According to excavation reports released by the local governments, age was assessed in 25 cases. Age estimates of the excavated remains vary widely, ranging from approximately 10 years to greater than 60 years, but middle age was the most common. Fifteen cases were assessed to be male and seven to be female. Various types of pots were used to cover the heads, including iron pots, earthenware pots and mortars; however, iron pots were most frequently used (Table 2). The fact that such valuable items were used to cover the heads of the deceased suggests a particular importance of this burial style. Paleopathological examinations have been performed for 21 of the 105 burials. Five (24%) displayed osteological signs of leprosy, the most frequently observed of the disorders that were diagnosed based on osteological changes (Table 3). Based on folklore, tuberculosis is also thought to be a basis for *Nabe-kaburi* burials. However, to date, tuberculous lesions have not been found in the skeletal remains that have been subjected to paleopathological evaluation.

In many societies, public stigmatization and exclusion coexist. Leprosy-associated deformities have been responsible for such social stigmatization and discrimination, and in some countries, the stigma is promoted by legislation against patients [10]. This commonality between *Nabe-kaburi* and leprosy burials led to speculation that *Nabe-kaburi* burials could to some extent reflect the discrimination against leprosy during that time period [2,11]. Since 1980, with more excavations and archaeological findings (i.e., the grave goods, the age of the burials and the excavated human skeletal remains), the significance of the *Nabe-kaburi* burial has become an object of discussion not only in folklore but also in Early Modern archaeology. It is believed that study of the *Nabe-kaburi* burials could reveal the reality of social discrimination against particular diseases in a village from the late Middle Ages to the Early Modern period in Japan. Therefore, confirmation that

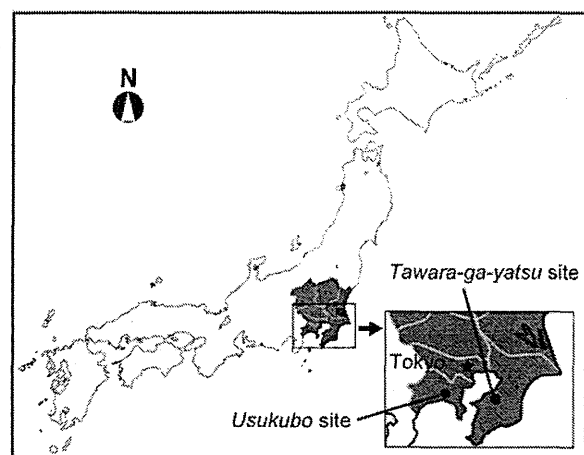


Figure 1. Location of the Tawara-ga-yatsu and the Usukubo sites in Japan. Shaded area denotes the *Kanto* area. Inset illustrates the locations in relation to Tokyo.

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those skeletal remains showing osteological signs of leprosy were actually infected with *M. leprae* while alive became very important.

In the field of palaeomicrobiology, DNA from pathogenic microorganisms can be detected from excavated ancient human skeletons using the polymerase chain reaction (PCR) [12]. PCR is a powerful molecular tool for the discipline of palaeomicrobiology, diagnosing infectious diseases in ancient remains to demonstrate the distribution, spread and genetic evolution of the pathogens [13–15]. It can also be used for the genome-wide comparison of past and modern bacteria when DNA is well preserved [16].

Mycobacterium tuberculosis (*M. tuberculosis*) DNA was first detected in an ancient human skeleton using PCR in 1993 [17]. The next year, *M. leprae* DNA was detected in archaeological skeletal remains [18], which has been followed by several other reports [19–25]. We have recently reported detection of *M. leprae* DNA in excavated human skeletal remains from Japan where preservation of ancient buried skeletons is poor due to humidity and acidic soil from volcanic activity [9]. Maximum sensitivity was achieved with a method that combined whole genome amplification (WGA) and PCR analysis (WGA-PCR) and DNA sequencing [9].

In the present study, we examined three sets of skeletal remains from *Nabe-kaburi* burials. Although paleopathological evidence was briefly documented in reports by local governments, we examined skeletons and re-evaluated the paleopathological findings. We then analyzed small amounts of these samples for the presence of *M. leprae* DNA using WGA-PCR and DNA sequencing.

Materials and Methods

Archeological Sites and Graves of *Nabe-kaburi* Excavations

This study used archaeological skeletal remains excavated from two sites in the *Kanto* area of Japan (Figure 1); the *Tawara-ga-yatsu* and *Usukubo* sites. Below is a brief summary of the excavation sites and graves described in excavation reports obtained from the local governments in Japanese.

The *Tawara-ga-yatsu* site is part of the *Kobama* ruins spreading on hills along the coastline of Tokyo Bay located on the *Boso* Peninsula in Chiba prefecture [26,27]. Perpendicular to the structural remains of roads traversing from south to north are groove-shaped structural remains extending from east to west (Figure 2A). Six grave pits were found annularly arranged on the side of these structural remains. The *Nabe-kaburi* burials were excavated from two of these gravel pits (TK5 and TK6) (Figure 2A). The head of the remains in TK5 was covered with two iron pots and one mortar. The grave goods consisted of *Kan-ei-tsuho* (a metallic currency used in the *Edo* period) and three glass beads (Figure 2B). The corpse in TK6 was covered with one iron pot (head) and one mortar (feet), with grave goods of five *Kan-ei-tsuho* (Figure 2C). Based on the shape of the pots and the presence of the metallic currency, both burials were assessed to be from the early 18th century. TK5 was assessed to have been a middle-aged male, while TK6 was assessed to have been a male in his late 20 s. It was estimated that the rounding deformation seen in the lateral and inferior edges of his nasal aperture resulted from leprosy [28].

The *Usukubo* site refers to ruins located on the lingulate plateau of the plains facing *Sagami* Bay in Kanagawa prefecture, where large-scale colonies dated to 5,000 B.C. (the *Jomon* period) have been excavated [29] (Figure 3A). A bent human skeleton with an iron pot covering the head was excavated from an 18th century grave pit (K48) (Figure 3B). K48 is located near the border of the village next to a ridge traversing the center of the plateau from east to west through farmland. A smaller iron pot, a tobacco pipe and a knife-like iron tablet were also in the pit. The estimated date of this

Table 1. Chronological and geographical distribution of Nabe-kaburi burials in Japan.

Age of burials	Kanto area ^a	Northeast Japan	Midland and Southwest Japan	Total
15 th –16 th century	9	11	2	22
16 th –18 th century	35	22	1	58
19 th century	–	2	–	2
Undetermined	7	14	2	23
Total	51	49	5	105

^aShaded area in Figure 1.
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burial is early to middle 18th century, based on the shape of the tobacco pipe and the properties of the covered soil. The human skeleton excavated from K48 was assessed to be a middle-aged man with collapse of the central facial cranium and bone hypertrophy in most of his limb bones, both of which are suggestive of leprosy.

Sampling of Skeletal Remains

Sampling from three skeletal remains was performed at the Shiomi Storehouse of Board of Education, Kisarazu City, 7-3-7 Shiomi, Kisarazu 292-0834, Japan (for TK5 and TK6) and the National Museum of Nature and Science in Tokyo, 3-23-1 Hyakunincho, Shinjuku 169-0073, Japan (for K48), where no other leprosy materials were stored. Skeletal samples were taken from the affected lesions and from bones with no remarkable changes, as summarized in Table 4, using a small rotating electric saw. Sterile materials were used for sampling to avoid possible contamination. Ethical approval to work with the material was obtained from the review board at the National Institute of Infectious Diseases, Japan. Permission to obtain the sample materials was granted by the Board of Education of Kisarazu City, Kisarazu, Japan and the National Museum of Nature and Science, Tokyo, Japan. All necessary permits were obtained for the described study, which complied with all relevant regulations.

DNA Extraction, whole Genome Amplification (WGA), Polymerase Chain Reaction (PCR), and DNA Sequencing

Genomic DNA was purified using QIAamp DNA Micro Kit (Qiagen, Valencia, CA), and uniformly amplified using the GenomePlex Whole Genome Amplification Kit (Sigma, St. Louis, MO) as reported previously [9]. Eight *M. leprae*-specific primer pairs were used to amplify 1) coding regions: *ML2496c* (*dnaK*; *hsp-70*), *ML2205c* (*purM*) and *ML1309* (*hisE*); 2) pseudogenes: *ML0434* (*scoA*) and *ML0794c* (REP-family protein); and 3) non-coding regions: Nc1593211, Nc2551060 and Nc2664658, where the numbers denote coordinate positions within the *M. leprae* genome [9]. These primers were chosen to generate shorter amplicon sizes, which produce more efficient amplification of degraded samples. Nested (for *ML2496c*) and conventional PCR (remaining regions) were performed using the PCR Thermal Cycler DICE (TaKaRa) [9]. The PCR products were analyzed using 2.0% agarose gel electrophoresis. For DNA sequencing, specific PCR products were cut from an agarose gel and DNA was extracted using the MinElute Gel Extraction Kit (Qiagen). DNA sequencing was performed and analyzed using the ABI PRISM 310 Genetic Analyzer and GeneScan Collection software (Applied Biosystems) [9].

All experiments were performed in a biosafety level 2 (BSL2) facility with secure access; only trained and authorized persons are allowed to enter. To work in a BSL2 laboratory, one must take a one-day lecture course on basic biosafety, pass a multiple choice exam, and have a minimum of 30 hours (depending on previous experience) of actual training in the laboratory under strict

Table 2. Types of pots excavated from the Nabe-kaburi burials in each region.

Types	Kanto area ^a	Northeast Japan	Midland and Southwest Japan	Total
Iron pot with inside loop	7	15	–	22
Earthenware pot with inside loop	4	–	1	5
Iron/copper pot with lifted handles	9	8	1	18
Iron pot with pouring lip	18	9	–	27
Iron pot	4	2	1	7
Mortar	6	5	–	11
Others	3	2	2	7
Undetermined	4	8	–	12
Total	55	49	5	109 ^b

^aShown as a shaded area in Figure 1.
^bTotal number of pots is greater than the number of Nabe-kaburi burials, because some remains were covered with more than two pots.
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Table 3. Paleopathological lesions found in the skeletal remains excavated from the *Nabe-kaburi* burials.

Paleopathological diagnosis	Number of cases
Leprosy	5
Syphilis	3
Inflammatory lesions on the nose	1
Skeletal abnormality	1
No lesions	11
Total	21

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supervision of the laboratory manager. Although *M. leprae*, or its DNA purified from patients or from nude mice foot pads, is handled daily in the laboratory for diagnoses or other basic research, it is handled in a Class II biological safety cabinet in which both inward and outward (exhaust) air are passed through HEPA filters to avoid cross contamination. Only sterile disposable test tubes and other materials such as disposable gloves, masks and labware are used. All waste is packaged in a plastic bag for biohazardous materials, autoclaved for sterilization and disposed

of as medical refuse to avoid contamination. Although the experimental practices of the laboratory may differ somewhat from the guidelines proposed for the detection of aDNA [12,30,31], the laboratory can reliably and consistently detect minute amounts of *M. leprae* DNA from both patient samples and archeological specimens.

Results

Paleopathological Evaluation and Diagnosis

TK5 burial. The skull of TK5 was almost intact and in a good state of preservation (Figure 4A), while most parts of the trunk bones were not preserved. The distal ends of the limb bones were also absent, leaving only the diaphysis. No paleopathological signs were observed in the skull (Figure 4A). Although the area from the glabella to the nasion andinion was in a plane and the profile appeared somewhat unique, no periostitis-induced osteological lesions were observed in the nasal bone or on the edges of the nasal aperture. Extensive hypertrophy was seen in the lower part of diaphysis of the femur, indicating that TK5 was suffering from either periostitis or osteomyelitis (Figure 4B). Although TK5 was excavated from a *Nabe-kaburi* burial and initially suspected of having syphilis of the bone (based on an excavation report provided by the local government), the lesions were limited to the diaphysis of femurs without any syphilis-specific signs. Therefore,

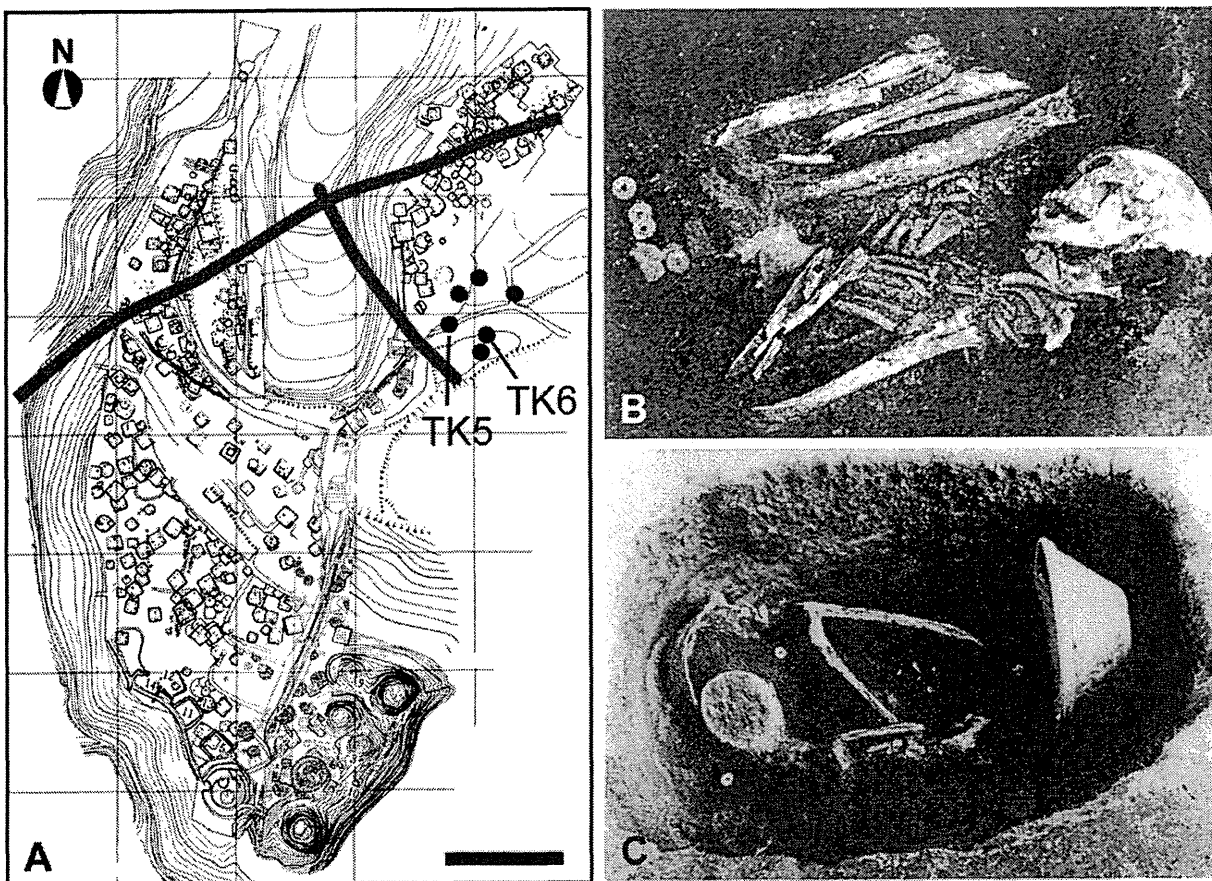


Figure 2. Geographical representation of the *Tawara-ga-yatsu* site. (A) Map of the *kobama* ruins including the *Tawara-ga-yatsu* site where TK5 and TK6 were excavated. Thick lines indicate roads that cross the site. TK5 (B) and TK6 (C) grave pits at excavation. Bar = 50 m. doi:10.1371/journal.pone.0088356.g002