43. **Sato, M., T. Gotow, Z. You, Y. Komamura-Kohno, Y. Uchiyama, N. Yabuta, H. Nojima, and Y. Ishimi.** 2000. Electron microscopic observation and single-stranded DNA binding activity of the Mcm4,6,7 complex. J Mol Biol **300:**421-31.
44. **Schwacha, A., and S. P. Bell.** 2001. Interactions between two catalytically distinct MCM subgroups are essential for coordinated ATP hydrolysis and DNA replication. Mol Cell **8:**1093-104.
45. **Stillman, B.** 1996. Cell cycle control of DNA replication. Science **274:**1659-64.
46. **Tada, S., A. Li, D. Maiorano, M. Mechali, and J. J. Blow.** 2001. Repression of origin assembly in metaphase depends on inhibition of RLF-B/Cdt1 by geminin. Nat Cell Biol **3:**107-13.
47. **Tanaka, T., D. Knapp, and K. Nasmyth.** 1997. Loading of an Mcm protein onto DNA replication origins is regulated by Cdc6p and CDKs. Cell **90:**649-60.
48. **Tatsumi, Y., S. Ohta, H. Kimura, T. Tsurimoto, and C. Obuse.** 2003. The ORC1 cycle in human cells: I. cell cycle-regulated oscillation of human ORC1. J Biol Chem **278:**41528-34.
49. **Thommes, P., Y. Kubota, H. Takisawa, and J. J. Blow.** 1997. The RLF-M component of the replication licensing system forms complexes containing all six MCM/P1 polypeptides. Embo J **16:**3312-9.
50. **Tye, B. K.** 1999. MCM proteins in DNA replication. Annu Rev Biochem **68:**649-86.
51. **Waga, S., and B. Stillman.** 1998. The DNA replication fork in eukaryotic cells. Annu Rev Biochem **67:**721-51.
52. **Walter, J., and J. Newport.** 2000. Initiation of eukaryotic DNA replication: origin unwinding and sequential chromatin association of Cdc45, RPA, and DNA polymerase alpha. Mol Cell **5:**617-27.
53. **Wiebusch, L., R. Uecker, and C. Hagemeier.** 2003. Human cytomegalovirus prevents replication licensing by inhibiting MCM loading onto chromatin. EMBO Rep **4:**42-6.
54. **Wohlschlegel, J. A., B. T. Dwyer, S. K. Dhar, C. Cvetic, J. C. Walter, and A. Dutta.** 2000. Inhibition of eukaryotic DNA replication by geminin binding to Cdt1. Science **290:**2309-12.
55. **Wuarin, J., V. Buck, P. Nurse, and J. B. Millar.** 2002. Stable association of mitotic cyclin B/Cdc2 to replication origins prevents endoreduplication. Cell **111:**419-31.
56. **You, Z., Y. Komamura, and Y. Ishimi.** 1999. Biochemical analysis of the intrinsic Mcm4-Mcm6-mcm7 DNA helicase activity. Mol Cell Biol **19:**8003-15.
57. **You, Z., and H. Masai.** 2005. DNA binding and helicase actions of mouse MCM4/6/7 helicase. Nucleic Acids Res **33:**3033-47.
58. **Yue, W., E. Gershburg, and J. S. Pagano.** 2005. Hyperphosphorylation of EBNA2 by Epstein-Barr virus protein kinase suppresses transactivation of the LMP1 promoter. J Virol **79:**5880-5.
59. **Zou, L., and B. Stillman.** 2000. Assembly of a complex containing Cdc45p, replication protein A, and Mcm2p at replication origins controlled by S-phase cyclin-dependent kinases and Cdc7p-Dbf4p kinase. Mol Cell Biol **20:**3086-96.

FIGURE LEGENDS

Figure 1. Biochemical analysis of the subcellular distribution of CDC6, MCM4, MCM6, and MCM7 in lytic program-induced Tet-BZLF1/B95-8 cells..

A, Tet-BZLF1/B95-8 cells were cultured in the presence of 2 μg/ml doxycycline and harvested at the indicated times, and subjected to biochemical fractionation as described in the "Materials and Methods". Tet-BZLF1/B95-8 cells were also treated with paclitaxel (20 μM) for 24 hr to arrest cell cycle at the G2/M phase and processed similarly. The relative abundance of each protein in Triton X-100-extractable supernatants (Triton X, S) and extracted nuclear pellets (Triton X, P) was examined by immunoblotting with anti-Cdc6, anti-MCM4, anti- phosphorylated Thr110 of MCM4, anti-MCM6, anti-MCM7, and anti-BZLF1 antibodies, respectively.

B, Subnuclear localizations of MCM4 and MCM7 in lytic program-induced Tet-BZLF1/B95-8 cells. Cells were harvested at 24 h post-induction and treated with 0.5% Triton-X-100-mCSK buffer. Nonionic-detergent extracted cells were fixed with methanol and then immunostained with anti-MCM4 or anti-MCM7, and anti-BMRF1 antibodies. Shown are merged images of MCM4 or MCM7 (red) and BMRF1 (green) proteins.

Figure 2. Phosphorylation of Thr-19 and Thr-110 residues of MCM4 upon induction of EBV lytic replication. Tet-BZLF1/B95-8 and Tet-BZLF1/Akata cells were cultured in the presence of 2 μg/ml doxycycline and harvested at the indicated times. Equal amounts of proteins for each sample (20~50 μg) were applied for

immunoblot analysis with the specific antibodies indicated on the left side of each panel. Anti-CDK2 antibody was used to confirm equal protein loading.

Figure 3. Expression of the EBV-PK encoded by the BGLF4 gene in Hela cells results in phosphorylation of MCM4 at Thr-19 and Thr-110. HeLa cells were transiently transfected with the BGLF4 protein expression vector, pME-BGLF4(F), or a control vector, pME18S, and harvested after 2 days. Whole cell extracts were prepared and equal amounts of proteins for each sample (20 μg) were separated by gradient SDS-PAGE and .applied for immunoblot analysis with the specific antibodies indicated on the left side of each panel. *B*, Effect of expression of the EBV-PK on the proliferation of HeLa cells. HeLa cells (0.6×10^6 cells / 35 mm dish) were transfected with the BGLF4 expression plasmid, pME-BGLF4(F), or the control plasmid, pME18S and were counted with hemocytometer at the indicated times.

Figure 4. EBV-PK phosphorylates Thr-19 and Thr-110 residues on MCM4 of MCM4-6-7 hexamer in vitro. *A*, Purification of MCM4-6-7 hexamers. Sf21 cells were co-infected with recombinant baculoviruses AcMcm4/6 and AcMcm7 and MCM4-6-7 complexes were purified as described in the "Materials and Methods", separated by SDS- 7.5% PAGE, and stained with silver. The positions of the MCM4, Mcm6 and MCM7 proteins were determined as judged by westernblot analyses (data not shown) and indicated by arrows. *B*, Wild-type or kinase-negative GST-BGLF4 proteins were isolated from Sf21 cells infected with AcGST-BGLF4 or AcGST-BGLF4K102I as described in the "Materials and Methods". Human MCM4-6-7 hexamer (100 ng) was incubated with increasing amounts of Wild-type GST-BGLF4, kinase-negative GST-BGLF4K102I or Cyclin A/CDK2. The samples were subjected to

SDS- 7.5% PAGE and analyzed by Western blotting using phospho-specific antibodies against MCM4 at Thr-19 and Thr-110.

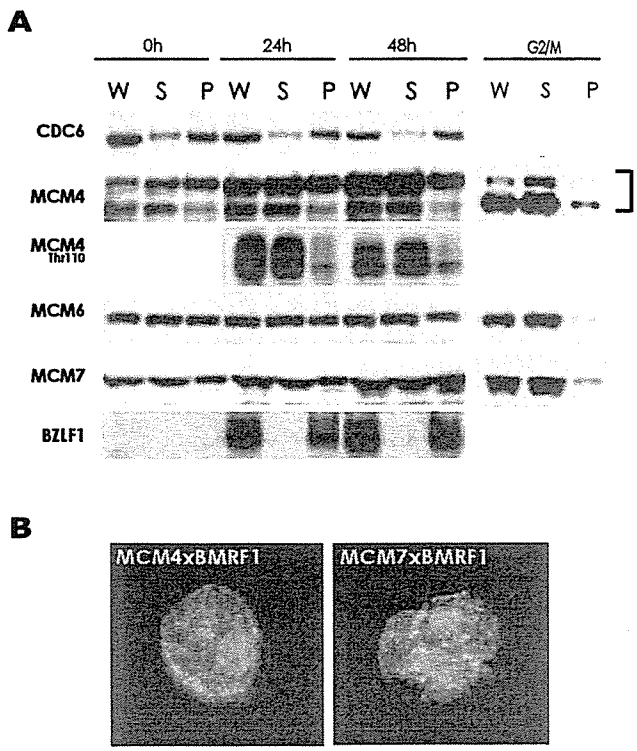
Figure 5. EBV-PK like CDK2/cyclin A inhibits DNA helicase (unwinding) activity associated with the MCM4-6-7 complex.

A, The substrate for DNA helicase assays. A 5 ^{32}P-labelled oligonucleotide (17-mer) annealed to M13 ssDNA is depicted. *B,* DNA helicase assays were performed with 100 fmol of the helicase substrate and hMCM4-6-7 hexamers (100 ng) in the presence of Cyclin A/CDK2 (100 ng), BGLF4 protein (100 and 500 ng) or kinase negative BGLF4K102I protein (200 and 500 ng) as described in the "Materials and Methods". Positions of the DNA substrate (17-mer/M13) and displaced oligonucleotide (17-mer) are indicated by arrows. The left two lanes show results for heat-denatured and native DNA substrate, respectively.

Figure 6. EBV-PK still inhibits helicase activity of MCM4-6-7 hexamers containing mutations in amino N-terminal phosphorylation sites of MCM4 unlike CDK2/cyclin A.

A, MCM4-6-7 hexamers containing mutant MCM4 (*MCM4a*/6/7)(200 ng) or the wild-type (MCM4/6/7)(100 ng) were examined for DNA helicase activity in the presence or absence of BGLF4 protein (500 ng) or Cyclin A/CDK2 (500 ng) as described in the "Materials and Methods". Quantitative analysis of the DNA unwinding activity was shown in a lower panel; The percentages of 17-mer oligonucleotide displaced with each MCM complex in the presence of the BGLF4 EBV-PK or CDK2/cyclin A was calculated from the signal intensity with that in the absence of the

kinase taken as 100 %. Data are means of three independent experiments. *B*, Human MCM4-6-7 or *MCM4a*-6-7 complexes (1 μg) were incubated with indicated amounts of CDK2/cyclin A (*left panel*) or the BGLF4 protein (*right panel*) in a 50 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 100 μM ATP, 2 μCi [gamma-^{32}P]ATP and 0.2 mM sodium orthovanadate. ^{32}P –labeled proteins were separated SDS -7.5 % PAGE, followed by autoradiography. *C*, Human MCM4-6-7 or MCM4a-6-7 complexes (1 μg) were phosphorylated by 500 ng of the BGLF4 protein in a 50 μl reaction mixture containing 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM dithiothreitol, 10 mM $MgCl_2$, 1 mM ATP and 0.2 mM sodium orthovanadate., and products were separated by SDS-7.5 % PAGE. Proteins were analyzed by Western blotting using MCM6 and MCM4 antibodies.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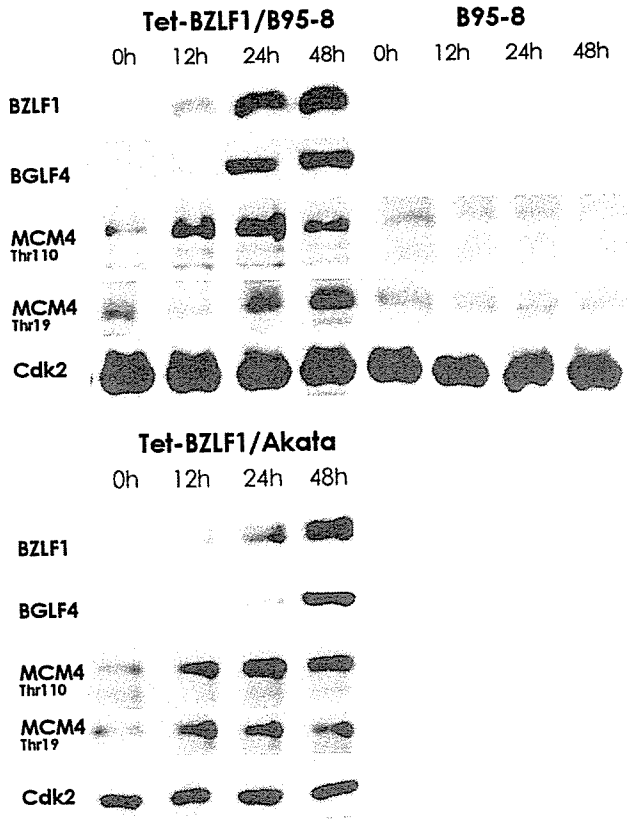
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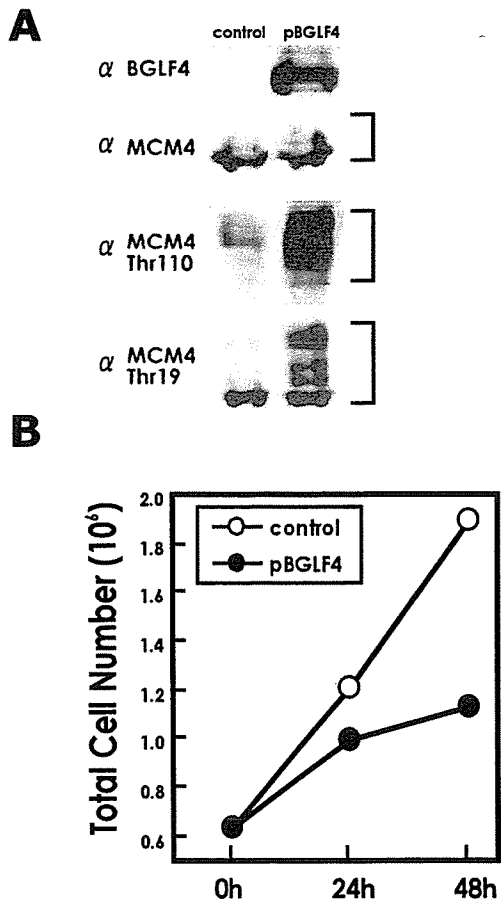
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α BGLF4
α MCM4
α MCM4 Thr110
α MCM4 Thr19
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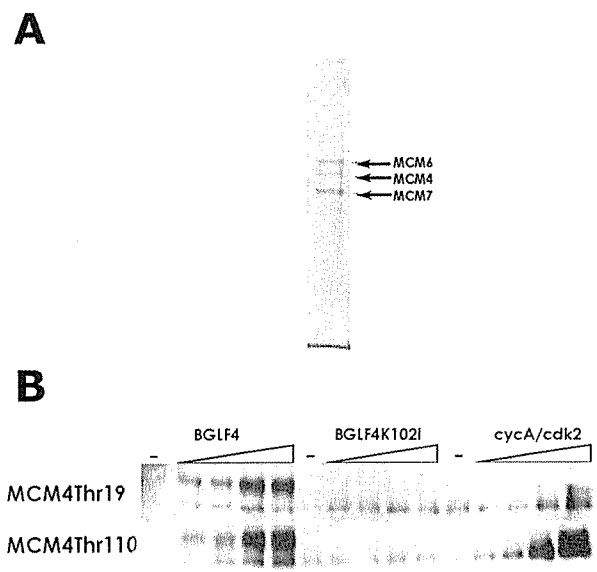
48h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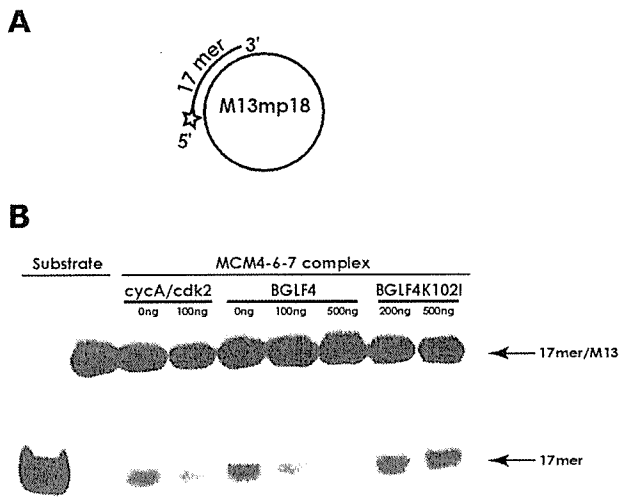
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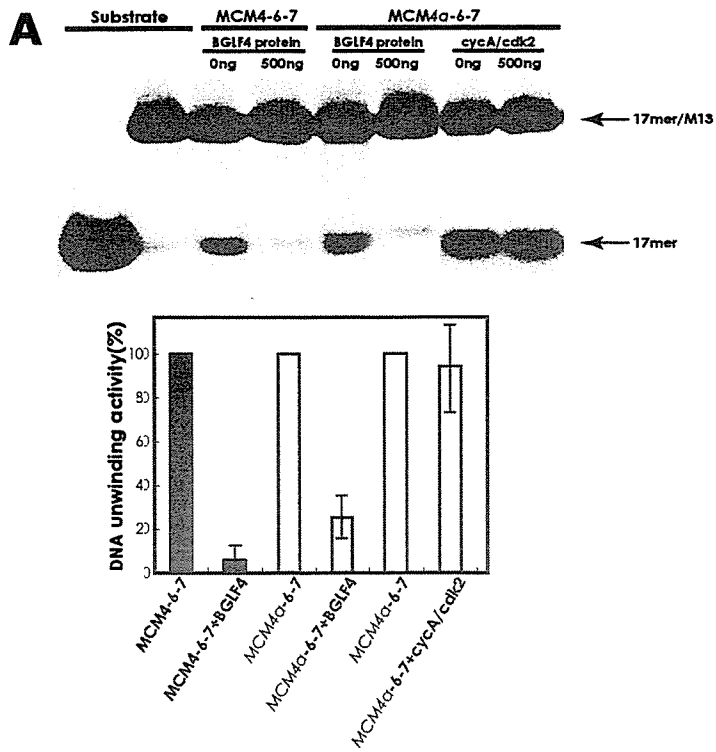
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0ng 500ng
17mer/M13
17mer
DNA unwinding activity(%)
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60
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20
0
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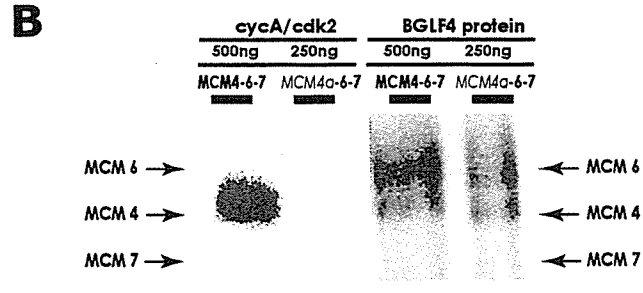
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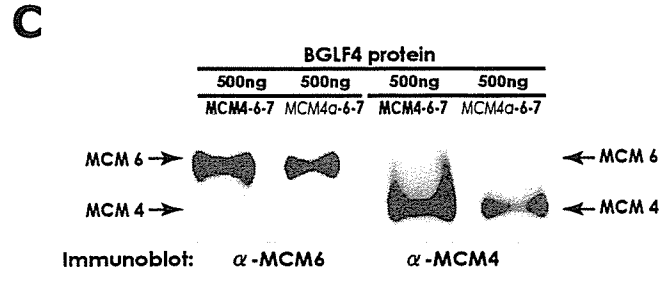
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MCM 4
Immunoblot: α-MCM6 α-MCM4

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BCG vaccine elicits both T-cell mediated and humoral immune responses directed against mycobacterial lipid components

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Abstract

The universe of antigens recognized by αβ T cells has recently been expanded to include not only major histocompatibility complex (MHC)-presented protein antigens but also CD1-presented lipid antigens. The significance of lipid-reactive T cells in host defense has been appreciated, using the guinea pig model of human tuberculosis. Here, we show that immunization with *Mycobacterium bovis* bacillus Calmette-Guerin (BCG), the commonly used anti-tuberculosis vaccine, induces activation of guinea pig cytotoxic T cells recognizing BCG lipids in the context of CD1 molecules. Further, BCG-immunized, but not mock-immunized, guinea pigs mount IgG antibody responses directed against lipoarabinomannnan, an essential cell wall lipid component of mycobacteria. These observations emphasize the ability of BCG to activate the host adaptive immunity to mycobacteria-derived lipids, which could potentially contribute to protection against tuberculosis.

Keywords: BCG; Lipid; CD1; Guinea pigs

1. Introduction

Despite recent advances in the development of specific antimicrobial agents, *Mycobacterium tuberculosis*, a causative microorganism of tuberculosis, is still a tremendous threat to mankind. It is estimated that about one third of the world's population is currently infected with this microorganism and tuberculosis kills more than 2 million people each year [1]. While extensive effort has been made to develop new vaccines that may confer effective immune protection from tuberculosis, bacillus Calmette-Guerin (BCG) is still the only anti-tuberculosis vaccine widely available for humans at present. This live, attenuated vaccine derived from a virulent strain of *Mycobacterium bovis*, has been used over 80 years, and its protective effect has been well appreciated especially against miliary tuberculosis and tuberculous meningitis in the childhood although its ability to confer protective immunity against pulmonary tuberculosis in the adult remains controversial [2].

BCG vaccination prominently elicits activation of MHC class II-restricted, $CD4^+$ T cells that recognize mycobacteria-derived protein antigens, and apparently, a fraction of protein antigen-specific, MHC class II-restricted $CD4^+$ T cells can mediate protection against tuberculosis as evidenced by studies of MHC class II-deficient mice, in which absence of conventional $CD4^+$ T cells is accompanied by increased susceptibility to mycobacterial infection [3,4]. In addition,

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a significant contribution of MHC class I-restricted $CD8^+$ T cells in clearing mycobacterial infection has also been demonstrated in mice, but it has often proved very difficult to identify the $CD8^+$ T cell response in humans [5,6].

Besides protein antigen-specific, MHC-restricted T cells, recent studies have identified a distinct T cell population in humans, but not in mice, which recognizes mycobacteria-derived lipid antigens in the context of group 1 CD1 molecules (CD1a, b, c) [7–12]. Given that these CD1 molecules are expressed on dendritic cells (DCs) and a fraction of activated macrophages, the two major reservoir cell types for mycobacteria, group 1 CD1-dependent activation of specific T cells may occur during the course of mycobacterial infection to mediate protective immunity [13–15]. Indeed, group 1 CD1-restricted $CD8^+$ T cell lines were isolated from healthy subjects as well as patients with mycobacterial infection, and their outstanding ability to detect and lyse mycobacteria-infected $CD1^+$ cells has been noted [16,17]. These observations emphasize the possibility that the human adaptive immunity against mycobacterial infection may involve immune recognition of both protein and lipid antigens derived from mycobacteria.

As a live vaccine, BCG produces not only protein antigens but also lipid antigens, but the ability of the BCG vaccine to induce the lipid-specific immune response remains to be assessed. Thus, we previously set up a study of BCG-immunized human subjects and found that these individuals contained a significant circulating pool of $CD8^+$ T cells that recognized BCG-infected DCs in a CD1-dependent, but MHC-independent, fashion [18]. However, because of limitations in human studies, it was difficult to separate T cell responses induced specifically by BCG vaccination from those potentially resulting from natural infection with other mycobacteria species. To precisely monitor the lipid antigen-specific immune response induced by BCG, we chose guinea pigs as model animals, which, unlike mice and rats, have evolved a set of CD1 proteins that are comparable in function to human group 1 CD1 molecules [19–21]. Using these animals, we show in the present study that the BCG vaccine specifically induces both T-cell mediated and humoral immune responses directed against mycobacterial lipids.

2. Materials and methods

2.1. Animals

Four- to five-week-old female inbred strain 2 guinea pigs were purchased from Japan SLC, Inc. (Shizuoka, Japan), and bred in the animal facilities either at Nippon Medical School or at the Institute for Virus Research of Kyoto University. Animals were housed under specific pathogen-free conditions and all animal experiments were performed according to the Guidelines on Animal Welfare approved by the committee on animal use at the corresponding institutions.

2.2. BCG culture and inoculation

The Tokyo 172 strain of BCG, grown at 37 °C in 7H9 medium, was harvested at its midlog phase growth, washed and suspended in phosphate-buffered saline (PBS). Without prior sonication, the suspension was passed through a 5-μm pore size filter to obtain single-cell bacteria. The viability of bacteria was constantly >90%. For immunization of guinea pigs, 2×10^7 colony forming units were injected intradermally in each upper hind leg.

2.3. Antigens

The total lipid fraction of BCG was obtained by extraction with chloroform/methanol as described previously [22]. Methyl ketomycolate (keto-MA), methyl methoxymycolate (methoxy-MA), methyl α-mycolate (alpha-MA), sulfolipid (SL), trehalose 6,6′-dimycolate (TDM) and lipoarabinomannan (LAM) derived from *M. tuberculosis* (Aoyama-B) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). Mycobacterial culture filtrate proteins (CFP) and *Mycobacterium phlei*-derived glucose monomycolate (GMM) were kindly provided from Drs. John T. Belisle (Colorado State University) and D. Branch Moody (Harvard Medical School), respectively.

2.4. Antibodies

Mouse monoclonal antibodies to guinea pig CD8 (CT6) and CD4 (CT7) were purchased from Serotec (Raleigh, NC). A rat anti-guinea pig CD4 antibody (H155) [23], a rabbit polyclonal antibody specific for LAM [24], a mouse anti-guinea pig CD1 antibody (CD1F2/6B5) [21] as well as a mouse negative control antibody (RPC5.4) [18] have been described elsewhere.

2.5. Isolation of T cells and bone marrow-derived dendritic cells

T cells were enriched from the spleens of immunized or unimmunized guinea pigs by passage through nylon wool fiber columns, and were labeled with either rat anti-guinea pig CD4 antibody (H155) or mouse anti-guinea pig CD8 antibody (CT6). The labeled cells were then removed by two cycles of separation with Dynabeads (Dynal Biotech, Lake Success, NY) coupled with goat antibodies to IgG of the corresponding species to obtain $CD4^+$ T cell-depleted and $CD8^+$ T cell-depleted populations, respectively. Flow cytometric analysis revealed that the percentage of $CD8^+$ T cells contained in the $CD8^+$ T cell-depleted population and the percentage of the $CD4^+$ T cells contained in the $CD4^+$ T cell-depleted population were constantly less than 1.2%.

Bone marrow-derived dendritic cells (BM-DCs) were isolated as described previously [20]. Briefly, guinea pig femurs and tibias were isolated aseptically and flushed by syringe with RPMI 1640 medium (Sigma–Aldrich, St. Louis, MO).

The mononuclear cells in the suspension were obtained by centrifugation at $400 \times g$ on a layer of density gradient medium formulated to a density of 1.107, made by combining 2.5 volumes of Histopaque 1119 (Sigma–Aldrich) and 1 volume of Ficoll Paque Plus (Amersham Biosciences, Piscataway, NJ). The isolated mononuclear cells were cultured in 150-cm^2 flasks in the presence of human granulocyte-macrophage colony stimulating factor (GM-CSF) (40 ng/ml; PeproTech, Rocky Hill, NJ) for 1 day, and non-adherent cells were then transferred to a new flask. The cultures were fed every 2 days by removing half of the medium and replacing it with fresh medium containing GM-CSF. After 10 days, non-adherent cells and loosely adherent cells, representing a population highly enriched for BM-DCs, were collected.

2.6. T cell proliferation assays

Freshly isolated T cells (1×10^5/well) were cultured with irradiated (3000 rad) BM-DCs (2×10^4/well) in triplicate in 96-well U-bottom microtiter plates in the presence or absence of the following antigens: CFP (10 μg/ml), total lipid fraction of BCG (50 μg/ml), keto-MA (2.5 μg/ml), methoxy-MA (2.5 μg/ml), alpha-MA (2.5 μg/ml), SL (2.5 μg/ml), TDM (2.5 μg/ml), GMM (2.5 μg/ml) and LAM (5 μg/ml). Cultures were incubated for 4 days at 37 °C in a 5% CO_2 incubator, pulsed with [^{3}H]thymidine (1 μCi/well; MP Biomedicals, Inc., Irvine, CA) and incubated for an additional 6 h. The plates were harvested on a Tomtec 96-well plate harvester (Wallac, Turku, Finland) and thymidine incorporation was measured with a Betaplate liquid scintillation counter (Wallac).

2.7. Flow cytometry

Cells were incubated with primary antibodies for 30 min on ice, washed twice with FACS buffer (2% heat inactivated fetal calf serum, 0.02% sodium azide in PBS), and then labeled with fluorescein isothiocyanate (FITC)-conjugated goat F(ab′)$_2$ anti-mouse IgG/IgM (BioSource International, Camarillo, CA). Subsequently, the labeled cells were analyzed, using a fluorescence-activated cell sorter (FACSVantage; BD Biosciences, San Jose, CA). Dead cells were excluded by propidium iodide.

2.8. Cytolytic assays

A CD1-negative guinea pig fibroblast cell line (104C1) stably transfected with expression vector constructs encoding either the guinea pig CD1b1, CD1b2, CD1b3 or CD1b4 proteins [25] was labeled with ^{51}Cr for 2 h, and then incubated overnight either with or without the total lipid fraction of BCG (25 μg/ml). The cells were washed extensively and used as target cells in cytolytic assays. For the source of effector T cells for primary cytotoxic T cell responses, splenic T cells from BCG-vaccinated guinea pigs were enriched by nylon wool columns and then cultured for 6 days in the presence of the total lipid fraction of BCG (25 μg/ml) and irradiated (5000 rad) nylon wool-adherent splenic cells. The cytolytic assays were performed as described [25]. The effector:target ratio used in each experiment ranged from 5:1 to 10:1, depending on the yield of the effector T cells.

2.9. Enzyme-linked immunosorbent assays (ELISA) for anti-LAM antibody

Flat-bottom Immulon two microtiter plates (Dynatech Laboratories, Inc., Alexandria, VA) were coated with purified LAM (50 ng/well) and incubated overnight at 4 °C with PBS containing 1% bovine serum albumin (Sigma–Aldrich). The LAM-coated microtiter plates were then incubated with serum samples for 1 h at room temperature, followed by incubation with horse radish peroxidase (HRP)-conjugated F(ab′)$_2$ fragment goat anti-guinea pig IgG antibodies (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). After extensive washing with PBS containing 0.05% Tween-20 (Sigma–Aldrich), color development was performed with the ImmunoPure TMB substrate kit (Pierce, Rockford, IL) according to the manufacturer's instruction, and the absorbance at 450 nm was measured. Rabbit anti-LAM antiserum and HRP-conjugated F(ab′)$_2$ fragment goat anti-rabbit IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.) were used as a positive control. The titer of anti-LAM antibody in each guinea pig sample was expressed either directly as arbitrary units based on the standard curve for the rabbit anti-LAM antiserum.

3. Results

3.1. Protein- and lipid-specific T cell proliferative responses induced by BCG

Inbred strain 2 guinea pigs were either mock treated or immunized with BCG by intradermal injection in each upper hind leg. After 2 weeks, splenic T cells were isolated, and separated into $CD8^+$ T cell depleted and $CD4^+$ T cell depleted populations. Each cell population was cultured with mycobacteria-derived antigens in the presence of MHC class II^+ $CD1^+$ BM-DCs, and antigen-specific responses were assessed by measuring cell proliferation.

The only statistically significant proliferation by the CD8 depleted T cell population was observed when T cells isolated from BCG-vaccinated, but not mock-treated, guinea pigs were cultured with mycobacterial culture filtrate proteins (CFP), indicating that typical MHC class II-dependent, protein antigen-specific T cell responses have been induced by the BCG vaccination protocol used for these studies (Fig. 1A).

In contrast, no significant proliferation by the CD4 depleted T cell population derived from BCG-vaccinated guinea pigs was observed in response to CFP as well as

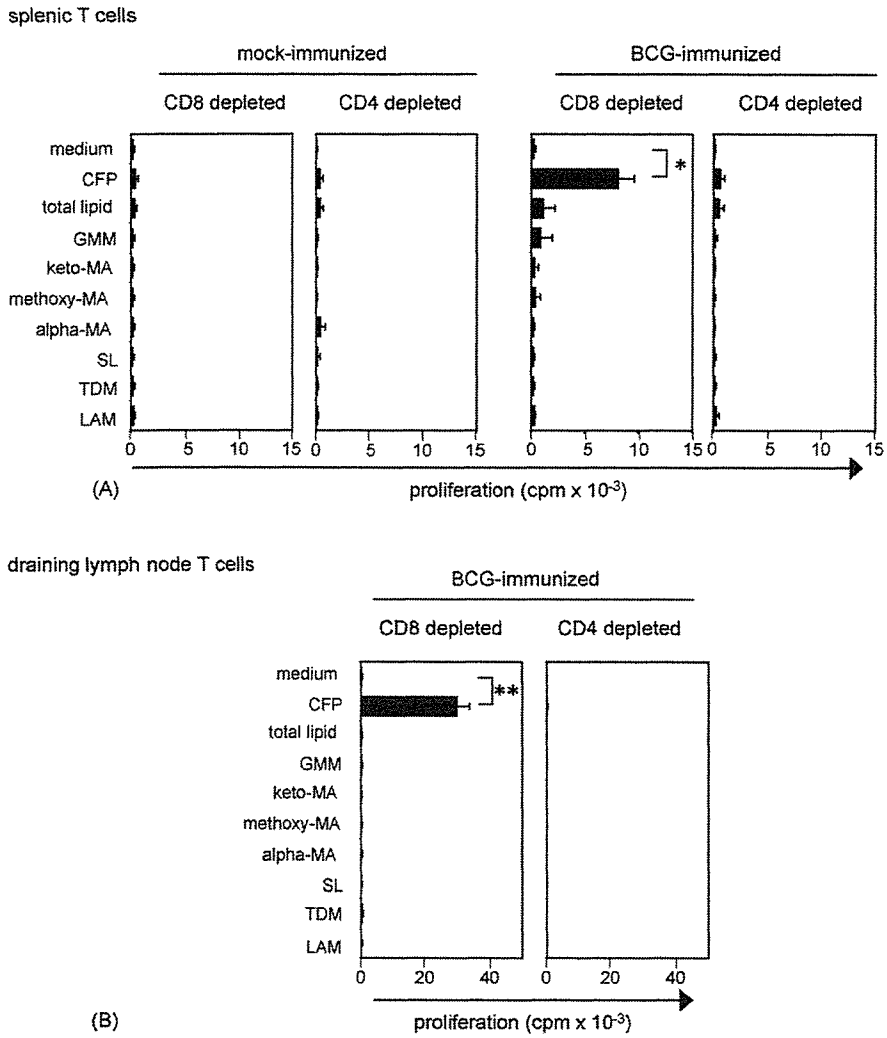

Fig. 1. T-cell proliferation in response to mycobacterial protein and lipid antigens. (A) Guinea pigs were either BCG- or mock-vaccinated ($n = 6$ for each group), and after 2 weeks, the CD8 depleted and the CD4 depleted T cell populations were obtained from the spleen. The cells were then cultured with MHC class II$^+$ CD1$^+$ BM-DCs in the presence or absence of the mycobacterial culture filtrate proteins (CFP), the total lipid fraction of BCG, glucose monomycolate (GMM), methyl ketomycolate (keto-MA), methyl methoxymycolate (methoxy-MA), methyl α-mycolate (alpha-MA), sulfolipid (SL), trehalose 6,6′-dimycolate (TDM) or lipoarabinomannan (LAM), and their ability to respond by proliferation to each antigen was assessed by [^{3}H]thymidine incorporation. The only significant proliferation was observed when the CD8 depleted T cell population was cultured in the presence of CFP ($^*p < 0.01$). Experiments were carried out three times to confirm the results. (B) Draining lymph node T cells were also obtained from three guinea pigs of each group described above, and separated into the CD8 depleted and CD4 depleted T cells. The cells were then cultured with BM-DCs in the presence or absence of each of the indicated antigens, and their proliferative responses were assessed. The only significant proliferation was observed when the CD8 depleted T cell population was cultured in the presence of CFP ($^{**}p < 0.01$). Experiments were carried out twice to confirm the results.

the total lipid fraction of BCG (Fig. 1A). Since the total lipid fraction contained a mixture of low concentrations of specific lipids that might not be sufficient for T cell activation, an array of purified mycobacterial lipid and glycolipid components, including glucose monomycolate and lipoarabinomannan that were known to bind human CD1, was also tested. Even at concentrations high enough for human T cell stimulation, however, splenic T cells (Fig. 1A) and draining lymph node T cells (Fig. 1B) from BCG-vaccinated guinea pigs did not proliferate significantly in response to any of these purified antigens.

3.2. *Lipid-specific cytotoxic T cells activated by the BCG vaccine*

Since it sometimes proves difficult to identify CD8$^+$ T cell responses in cell proliferation assays, we then switched to cytolytic assays. As target cells in these assays, we prepared a strain 2 guinea pig fibroblast cell line (104C1) stably transfected with expression vector constructs encoding the guinea pig CD1b1, CD1b2, CD1b3 or CD1b4 isoforms. Flow cytometric analysis with an antibody (CD1F2/6B5) recognizing all the guinea pig CD1 isoforms showed

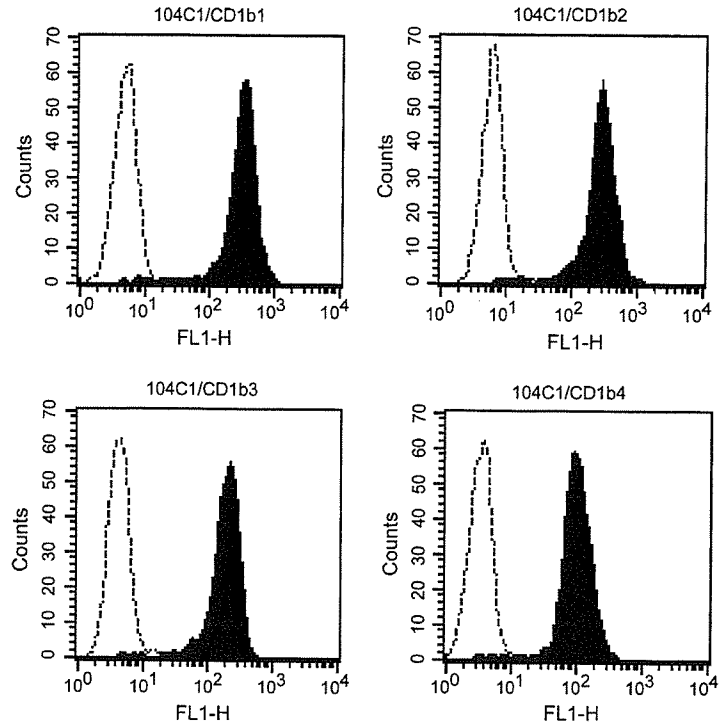

Fig. 2. Flow cytometric analysis of a guinea pig fibroblast cell line (104C1) stably transfected with either CD1b1, CD1b2, CD1b3 or CD1b4 cDNAs. The surface expression of each guinea pig CD1 isoform was detected with the anti-pan CD1 antibody (CD1F2/6B5) that recognized all the known guinea pig CD1 isoforms.

that each transfectant cell line expressed comparable levels of the transfected CD1 isoform on the cell surface (Fig. 2).

Splenic T cells derived from BCG-vaccinated guinea pigs specifically killed 104C1 transfectant cells expressing either CD1b2, CD1b3 or CD1b4 only when these target cells were pulsed with the total lipid fraction of BCG (Fig. 3). In contrast, no specific killing activity was detected against antigen-pulsed 104C1 cells expressing CD1b1. These results indicated that BCG vaccination elicited cytotoxic T cell responses directed against BCG-derived lipid components and that CD1b2, CD1b3 and CD1b4 functioned as restriction elements for these T cell responses. Interestingly, our previous studies detected distinct intracellular distribution patterns between guinea pig CD1b3 and CD1b4 proteins that were similar to those for human CD1a and CD1b isoforms [21,26]. The CD1b3- and CD1b4-restricted T cell responses to BCG lipids in guinea pigs may represent the human CD1a- and CD1b-restricted cytotoxic T cell responses that we detected previously in the circulating $CD8^+$ T cell pool of BCG-vaccinated people [18].

3.3. Lipid-specific humoral immunity induced by BCG

It has been recently suggested that, besides T-cell mediated immune responses, humoral immune responses to mycobacterial lipid components may be elicited upon infection with mycobacteria, which could potentially contribute to the host defense [27,28]. Therefore, we examined the possibility that production of antibodies of the IgG class that were specific for mycobacterial lipid antigens might be induced following BCG vaccination.

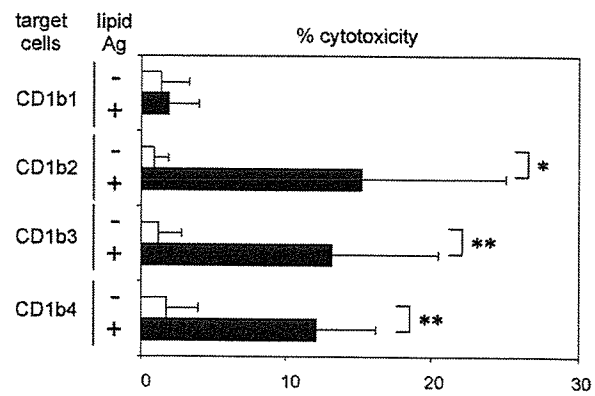

Fig. 3. CD1-dependent recognition of lipid antigen-pulsed target cells by cytotoxic T cells. Six guinea pigs were vaccinated with BCG, and splenocytes obtained after 2 weeks were tested for their ability to lyse 104C1 transfectants expressing either CD1b1, CD1b2, CD1b3 or CD1b4 isoforms in ^{51}Cr release assays. CD1b2-, CD1b3- and CD1b4-expressing cells were specifically killed by T cells only when the target cells were pulsed with BCG-derived lipid antigens ($^*p<0.05$; $^{**}p<0.01$). Experiments were carried out three times to confirm the results.

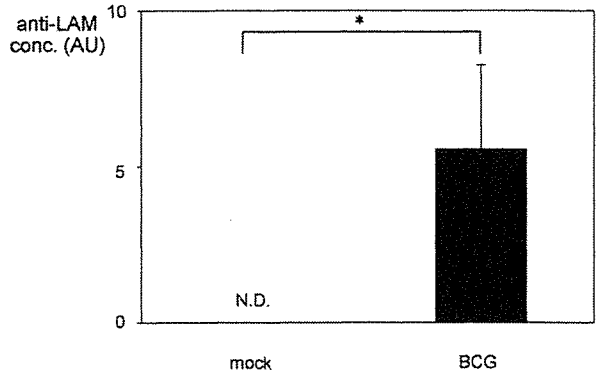

Fig. 4. Induction of IgG anti-LAM antibody production by BCG. Guinea pigs were either BCG- or mock-vaccinated ($n = 5$ for each group), and after 4 weeks, serum samples were collected and tested for IgG anti-LAM antibodies by ELISA. The titers were expressed in arbitrary units (AU) calculated based on the standard curve for the reference anti-LAM antiserum. Note that, whereas no specific IgG antibodies to LAM were detected (N.D.) in sera from any of mock-immunized guinea pigs, BCG vaccinated guinea pigs mounted the IgG antibody response to LAM significantly ($^*p < 0.01$). Experiments were carried out three times to confirm the results.

Guinea pigs were either BCG- or mock-vaccinated, and after 4 weeks, the sera were collected and tested for IgG antibodies against LAM. As shown in Fig. 4, whereas none of the mock-vaccinated guinea pigs mounted detectable levels of IgG responses to LAM, a significant production of the IgG anti-LAM antibodies was detected in all the BCG-vaccinated guinea pigs. These results indicated that BCG vaccination was capable of inducing mycobacterial lipid-specific IgG responses.

4. Discussion

As facultative intracellular pathogens, mycobacteria survive and replicate in the phagosome of macrophages and DCs by inhibiting phagosome acidification and phagosome-lysosome fusion [29,30]. Protein antigens produced in phagosome-resident mycobacteria are unlikely to gain easy access to the cytosol for MHC class I presentation or the lysosome for MHC class II presentation [31]. In contrast, lipid antigens are inserted into the phagosomal membrane and traffic to endocytic compartments which group 1 CD1 molecules sample, enabling efficient monitoring of mycobacteria-infected cells by the CD1 system [26,32–34]. Previously, most studies have examined the group 1 CD1-mediated T cell responses to *M. tuberculosis*. Here, we provide further evidence that responses to lipid antigens are also induced after BCG vaccination in a small animal model that expresses group 1 CD1 molecules. Since these lipid-specific T cell responses are known to be a critical component of the host defense against tuberculosis [35], the present study suggests that the vaccine effect of BCG may be accounted for at least in part by its ability to induce T cell responses directed against mycobacterial lipid antigens.

The cellular pathways for BCG-induced activation of CD1-restricted T cells remain to be determined. Initial studies focusing on $CD1^+$ DCs demonstrated that group 1 CD1-restricted T cell lines were efficiently activated by *M. tuberculosis*-infected DCs [17,36]. This pathway directly triggered by infected DCs may occur following BCG vaccination since a circulating pool of such T cells can be detected in BCG-immunized people [18]. In contrast, macrophages were previously thought not to contribute to activation of lipid-specific T cells because of the apparent lack of group 1 CD1 expression [37]. Recent evidence has suggested, however, that a sizable fraction of BCG-infected macrophages gains the ability to express group 1 CD1 molecules and to mediate stimulation of CD1-restricted T cells [38,39]. Given that macrophages are the major host cell type for mycobacterial infection, this pathway may play a significant role in BCG-induced T cell responses to lipids. Alternatively, some BCG-infected macrophages are prone to undergo apoptosis, and the apoptotic vesicles containing BCG are taken up by $CD1^+$ DCs, resulting in activation of lipid-specific T cells [40,41]. These three distinct pathways may or may not occur simultaneously, depending on the dose of BCG and which cell types are available at the site of immunization.

Besides T-cell mediated immune responses to lipids, the present study indicates that antibody responses to lipids are also elicited by BCG immunization. Previously, antibody responses were not considered effective for protection against intracellular pathogens, such as mycobacteria, but a protective antibody response to LAM has recently been suggested [27,42]. LAM has several immunosuppressive effects that may favor survival of mycobacteria in the host, including downregulation of DC function by interaction with the DC-SIGN receptor. Thus, it may be reasonable to speculate that neutralizing antibodies to LAM could contribute to host defense against mycobacterial infection [43–45].

A fraction of B cells in humans and virtually all B cells in guinea pigs express group 1 CD1 molecules, and double negative CD1-restricted T cells are known to exert helper activity to support IgG class antibody production [21,46]. Further, mycobacteria-derived glycolipid antigens, such as LAM, contain both T-cell and B-cell epitopes [12], suggesting an interesting possibility that production of IgG antibodies to some lipid antigens may be dependent on group 1 CD1 molecules. We speculate that LAM released to the extracellular space from BCG-infected cells may be captured by $CD1^+$ B cells expressing specific surface immunoglobulins and activate LAM-reactive, CD1-restricted T cells, which support class switching to IgG in B cells via cognate interactions.

The successful induction of protein antigen-specific, MHC class II-restricted T cell responses by BCG has been conveniently monitored by the tuberculin test detecting the delayed type hypersensitivity response to purified protein derivatives (PPD). It is considered unlikely, however, that the positive PPD test directly correlates with protective immunity against tuberculosis [47]. The lipid-specific immune responses detected in the present study may be critical for

a complete understanding of BCG-induced protective immunity against tuberculosis.

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References

[1] Dye C, Scheele S, Dolin P, Pathania V, Raviglione MC. Consensus statement. Global burden of tuberculosis: estimated incidence, prevalence, and mortality by country. WHO Global Surveillance and Monitoring Project. JAMA 1999;282(7):677–86.

[2] Roche PW, Triccas JA, Winter N. BCG vaccination against tuberculosis: past disappointments and future hopes. Trends Microbiol 1995;3(10):397–401.

[3] Kaufmann SH. Immunity to intracellular bacteria. Annu Rev Immunol 1993;11:129–63.

[4] Lagranderie MR, Balazuc AM, Deriaud E, Leclerc CD, Gheorghiu M. Comparison of immune responses of mice immunized with five different *Mycobacterium bovis* BCG vaccine strains. Infect Immun 1996;64(1):1–9.

[5] Behar SM, Dascher CC, Grusby MJ, Wang CR, Brenner MB. Susceptibility of mice deficient in CD1D or TAP1 to infection with *Mycobacterium tuberculosis*. J Exp Med 1999;189(12):1973–80.

[6] Flynn JL, Goldstein MM, Triebold KJ, Koller B, Bloom BR. Major histocompatibility complex class I-restricted T cells are required for resistance to *Mycobacterium tuberculosis* infection. Proc Natl Acad Sci USA 1992;89(24):12013–7.

[7] Beckman EM, Porcelli SA, Morita CT, Behar SM, Furlong ST, Brenner MB. Recognition of a lipid antigen by CD1-restricted alpha beta+ T cells. Nature 1994;372(6507):691–4.

[8] Gilleron M, Stenger S, Mazorra Z, Wittke F, Mariotti S, Bohmer G, et al. Diacylated sulfoglycolipids are novel mycobacterial antigens stimulating CD1-restricted T cells during infection with *Mycobacterium tuberculosis*. J Exp Med 2004;199(5):649–59.

[9] Matsunaga I, Bhatt A, Young DC, Cheng TY, Eyles SJ, Besra GS, et al. *Mycobacterium tuberculosis* pks12 produces a novel polyketide presented by CD1c to T cells. J Exp Med 2004;200(12):1559–69.

[10] Moody DB, Reinhold BB, Guy MR, Beckman EM, Frederique DE, Furlong ST, et al. Structural requirements for glycolipid antigen recognition by CD1b-restricted T cells. Science 1997;278(5336):283–6.

[11] Moody DB, Young DC, Cheng TY, Rosat JP, Roura-Mir C, O'Connor PB, et al. T cell activation by lipopeptide antigens. Science 2004;303(5657):527–31.

[12] Sieling PA, Chatterjee D, Porcelli SA, Prigozy TI, Mazzaccaro RJ, Soriano T, et al. CD1-restricted T cell recognition of microbial lipoglycan antigens. Science 1995;269(5221):227–30.

[13] Jiao X, Lo-Man R, Guermonprez P, Fiette L, Deriaud E, Burgaud S, et al. Dendritic cells are host cells for mycobacteria in vivo that trigger innate and acquired immunity. J Immunol 2002;168(3):1294–301.

[14] Moody DB, Ulrichs T, Muhlecker W, Young DC, Gurcha SS, Grant E, et al. CD1c-mediated T-cell recognition of isoprenoid glycolipids in *Mycobacterium tuberculosis* infection. Nature 2000;404(6780):884–8.

[15] Ulrichs T, Moody DB, Grant E, Kaufmann SH, Porcelli SA. T-cell responses to CD1-presented lipid antigens in humans with *Mycobacterium tuberculosis* infection. Infect Immun 2003;71(6):3076–87.

[16] Stenger S, Hanson DA, Teitelbaum R, Dewan P, Niazi KR, Froelich CJ, et al. An antimicrobial activity of cytolytic T cells mediated by granulysin. Science 1998;282(5386):121–5.

[17] Stenger S, Mazzaccaro RJ, Uyemura K, Cho S, Barnes PF, Rosat JP, et al. Differential effects of cytolytic T cell subsets on intracellular infection. Science 1997;276(5319):1684–7.

[18] Kawashima T, Norose Y, Watanabe Y, Enomoto Y, Narazaki H, Watari E, et al. Cutting edge: Major CD8 T cell response to live bacillus Calmette-Guerin is mediated by CD1 molecules. J Immunol 2003;170(11):5345–8.

[19] Dascher CC, Hiromatsu K, Naylor JW, Brauer PP, Brown KA, Storey JR, et al. Conservation of a CD1 multigene family in the guinea pig. J Immunol 1999;163(10):5478–88.

[20] Hiromatsu K, Dascher CC, LeClair KP, Sugita M, Furlong ST, Brenner MB, et al. Induction of CD1-restricted immune responses in guinea pigs by immunization with mycobacterial lipid antigens. J Immunol 2002;169(1):330–9.

[21] Hiromatsu K, Dascher CC, Sugita M, Gingrich-Baker C, Behar SM, LeClair KP, et al. Characterization of guinea-pig group 1 CD1 proteins. Immunology 2002;106(2):159–72.

[22] Grant EP, Degano M, Rosat JP, Stenger S, Modlin RL, Wilson IA, et al. Molecular recognition of lipid antigens by T cell receptors. J Exp Med 1999;189(1):195–205.

[23] Klunner T, Bartels T, Vordermeier M, Burger R, Schafer H. Immune reactions of CD4- and CD8-positive T cell subpopulations in spleen and lymph nodes of guinea pigs after vaccination with Bacillus Calmette Guerin. Vaccine 2001;19(15–16):1968–77.

[24] Dascher CC, Hiromatsu K, Xiong X, Sugita M, Buhlmann JE, Dodge IL, et al. Conservation of CD1 intracellular trafficking patterns between mammalian species. J Immunol 2002;169(12):6951–8.

[25] Hiromatsu K, Dascher CC, Sugita M, Gingrich-Baker C, Behar SM, LeClair KP, et al. Characterization of guinea-pig group 1 CD1 proteins. Immunology 2002;106(2):159–72.

[26] Sugita M, Grant EP, van Donselaar E, Hsu VW, Rogers RA, Peters PJ, et al. Separate pathways for antigen presentation by CD1 molecules. Immunity 1999;11(6):743–52.

[27] Glatman-Freedman A. Advances in antibody-mediated immunity against *Mycobacterium tuberculosis*: implications for a novel vaccine strategy. FEMS Immunol Med Microbiol 2003;39(1):9–16.

[28] Maekura R, Okuda Y, Nakagawa M, Hiraga T, Yokota S, Ito M, et al. Clinical evaluation of anti-tuberculous glycolipid immunoglobulin G antibody assay for rapid serodiagnosis of pulmonary tuberculosis. J Clin Microbiol 2001;39(10):3603–8.

[29] Hart PD, Young MR. Ammonium chloride, an inhibitor of phagosome-lysosome fusion in macrophages, concurrently induces phagosome-endosome fusion, and opens a novel pathway: studies of a pathogenic mycobacterium and a nonpathogenic yeast. J Exp Med 1991;174(4):881–9.

[30] Sturgill-Koszycki S, Schlesinger PH, Chakraborty P, Haddix PL, Collins HL, Fok AK, et al. Lack of acidification in Mycobacterium phagosomes produced by exclusion of the vesicular proton-ATPase. Science 1994;263(5147):678–81.

[31] Ramachandra L, Noss E, Boom WH, Harding CV. Processing of *Mycobacterium tuberculosis* antigen 85B involves intraphagosomal formation of peptide-major histocompatibility complex II complexes and is inhibited by live bacilli that decrease phagosome maturation. J Exp Med 2001;194(10):1421–32.

[32] Sugita M, Cao X, Watts GF, Rogers RA, Bonifacino JS, Brenner MB. Failure of trafficking and antigen presentation by CD1 in AP-3-deficient cells. Immunity 2002;16(5):697–706.

[33] Sugita M, van Der Wel N, Rogers RA, Peters PJ, Brenner MB. CD1c molecules broadly survey the endocytic system. Proc Natl Acad Sci USA 2000;97(15):8445–50.

[34] Xu S, Cooper A, Sturgill-Koszycki S, van Heyningen T, Chatterjee D, Orme I, et al. Intracellular trafficking in *Mycobacterium tuberculosis* and Mycobacterium avium-infected macrophages. J Immunol 1994;153(6):2568–78.

[35] Dascher CC, Hiromatsu K, Xiong X, Morehouse C, Watts G, Liu G, et al. Immunization with a mycobacterial lipid vaccine improves pulmonary pathology in the guinea pig model of tuberculosis. Int Immunol 2003;15(8):915–25.

[36] Jackman RM, Stenger S, Lee A, Moody DB, Rogers RA, Niazi KR, et al. The tyrosine-containing cytoplasmic tail of CD1b is essential for its efficient presentation of bacterial lipid antigens. Immunity 1998;8(3):341–51.

[37] Porcelli S, Morita CT, Brenner MB. CD1b restricts the response of human CD4-8- T lymphocytes to a microbial antigen. Nature 1992;360(6404):593–7.

[38] Krutzik SR, Tan B, Li H, Ochoa MT, Liu PT, Sharfstein SE, et al. TLR activation triggers the rapid differentiation of monocytes into macrophages and dendritic cells. Nat Med 2005;11(6):653–60.

[39] Roura-Mir C, Wang L, Cheng TY, Matsunaga I, Dascher CC, Peng SL, et al. *Mycobacterium tuberculosis* Regulates CD1 Antigen Presentation Pathways through TLR-2. J Immunol 2005;175(3):1758–66.

[40] Keane J, Remold HG, Kornfeld H. Virulent *Mycobacterium tuberculosis* strains evade apoptosis of infected alveolar macrophages. J Immunol 2000;164(4):2016–20.

[41] Schaible UE, Winau F, Sieling PA, Fischer K, Collins HL, Hagens K, et al. Apoptosis facilitates antigen presentation to T lymphocytes through MHC-I and CD1 in tuberculosis. Nat Med 2003;9(8):1039–46.

[42] Teitelbaum R, Glatman-Freedman A, Chen B, Robbins JB, Unanue E, Casadevall A, et al. A mAb recognizing a surface antigen of *Mycobacterium tuberculosis* enhances host survival. Proc Natl Acad Sci USA 1998;95(26):15688–93.

[43] Chatterjee D, Khoo KH. Mycobacterial lipoarabinomannan: an extraordinary lipoheteroglycan with profound physiological effects. Glycobiology 1998;8(2):113–20.

[44] Geijtenbeek TB, Van Vliet SJ, Koppel EA, Sanchez-Hernandez M, Vandenbroucke-Grauls CM, Appelmelk B, et al. Mycobacteria target DC-SIGN to suppress dendritic cell function. J Exp Med 2003;197(1):7–17.

[45] Prinzis S, Chatterjee D, Brennan PJ. Structure and antigenicity of lipoarabinomannan from *Mycobacterium bovis* BCG. J Gen Microbiol 1993;139(11):2649–58.

[46] Sieling PA, Porcelli SA, Duong BT, Spada F, Bloom BR, Diamond B, et al. Human double-negative T cells in systemic lupus erythematosus provide help for IgG and are restricted by CD1c. J Immunol 2000;165(9):5338–44.

[47] Elias D, Akuffo H, Britton S. PPD induced in vitro interferon gamma production is not a reliable correlate of protection against *Mycobacterium tuberculosis*. Trans R Soc Trop Med Hyg 2005;99(5):363–8.

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Implications for Induction of Autoimmunity via Activation of B-1 Cells by *Helicobacter pylori* Urease

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Besides various gastroduodenal diseases, *Helicobacter pylori* infection may be involved in autoimmune disorders like rheumatoid arthritis (RA) or idiopathic thrombocytopenic purpura. Such autoimmune disorders are often associated with autoreactive antibodies produced by B-1 cells, a subpopulation of B lymphocytes. These B-1 cells are mainly located in the pleural cavity or mucosal compartment. The existence of *H. pylori* urease-specific immunoglobulin A (IgA)-producing B cells in the mucosal compartment and of their specific IgM in the sera of acutely infected volunteers suggests the possibility that urease stimulates mucosal innate immune responses. Here, we show for the first time that purified *H. pylori* urease predominantly stimulates the B-1-cell population rather than B-2 cells, which produce antigen-specific conventional antibodies among splenic B220$^+$ B cells. The fact that such stimulation of B-1 cells was not affected by the addition of polymyxin B indicates that the effect of purified *H. pylori* urease was not due to the contamination with bacterial lipopolysaccharide. Furthermore, the production of various B-1-cell-related autoreactive antibodies such as IgM-type rheumatoid factor, anti-single-stranded DNA antibody, and anti-phosphatidyl choline antibody was observed when the splenic B cells were stimulated with purified *H. pylori* urease in vitro. These findings suggest that *H. pylori* components, urease in particular, may be among the environmental triggars that initiate various autoimmune diseases via producing autoreactive antibodies through the activation of B-1 cells. The findings shown here offer important new insights into the pathogenesis of autoimmune disorders related to *H. pylori* infection.

Helicobacter pylori, a gram-negative, spiral-shaped bacterium living in the acidic stomach, causes chronic gastritis and ulcers on the gastroduodenal tract, and it is linked with the development of gastric cancer and mucosa-associated lymphoid tissue (MALT) lymphoma (6, 35). In addition to such gastroduodenal disorders, *H. pylori* infection is associated with various autoimmune diseases such as rheumatoid arthritis (22), Sjögren's syndrome (12), and idiopathic thrombocytopenic purpura (ITP) (17). In the case of ITP, the binding ability of anti-platelet-specific immunoglobulin G (IgG) is enhanced by rheumatoid factors (RFs) that may sequester IgG (26). The marked improvement in platelet counts after *H. pylori* eradication (14) indicates a direct correlation between the pathogenicity of ITP and *H. pylori* infection. Although the precise mechanism by which *H. pylori* infection generates autoimmune disorders remains to be elucidated, the production of RFs seems to be a key event in initiating the autoimmunity.

There are two distinct types of murine B-cell lineages: one is made up of conventional B cells (now called B-2 cells), which reside predominantly in the adult spleen and lymph nodes to form systemic acquired immunity, and the other is made up of CD5$^+$ B cells (now called B-1 cells), which localize mainly in the peritoneal and pleural cavities or the mucosal compartment (23). Several lines of evidence suggest that the B-1 cells generally produce low-affinity and less-mutated antibodies (7). Their repertoire is skewed toward reactivity with T-cell-independent (TI) antigens such as phosphatidyl choline (3) and polyvinyl pyrrolidinone (39), and they dominantly produce IgM and IgG3 antibodies containing little or no somatic mutations caused by gene rearrangements for the establishment of memory and specificity (30). Thus, in contrast to conventional B-2 cells, they do not usually create long-term memory for secondary responses. Moreover, such B-1-cell-derived antibodies are often autoreactive, like the RFs that react with the Fc portion of self-IgG (2). Furthermore, the disappearance of B-1 cells markedly reduces the serum level of IgG3 but not of other IgG subclasses (38), indicating that IgG3 is the dominant subclass of IgG produced by innate B-1 cells.

We have reported previously that the major antigenic component for antibody production against *H. pylori* is its urease (16), and urease-specific IgA antibody is seen in both the sera and gastric juices of *H. pylori*-infected patients (15, 18), indicating that *H. pylori* urease can stimulate mucosal immune responses. We have also observed the close relationship between *H. pylori* urease-specific IgA antibody production and gastric mucosal damage, and such urease-specific IgA-producing B cells are actually found in the mucosal compartment of

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