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Serological Diagnosis of Leprosy in Patients in Vietnam by Enzyme-Linked Immunosorbent Assay with *Mycobacterium leprae*-Derived Major Membrane Protein II[▽]

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A serological diagnostic test using phenolic glycolipid-I (PGL-I) developed in the 1980s is commercially available, but the method is still inefficient in detecting all forms of leprosy. Therefore, more-specific and -reliable serological methods have been sought. We have characterized major membrane protein II (MMP-II) as a candidate protein for a new serological antigen. In this study, we evaluated the effectiveness of the enzyme-linked immunosorbent assay (ELISA) using the MMP-II antigen (MMP-II ELISA) for detecting antibodies in leprosy patients and patients' contacts in the mid-region of Vietnam and compared to the results to those for the PGL-I method (PGL-I ELISA). The results showed that 85% of multibacillary patients and 48% of paucibacillary patients were positive by MMP-II ELISA. Comparison between the serological tests showed that positivity rates for leprosy patients were higher with MMP-II ELISA than with PGL-I ELISA. Household contacts (HHCs) showed low positivity rates, but medical staff members showed comparatively high positivity rates, with MMP-II ELISA. Furthermore, monitoring of results for leprosy patients and HHCs showed that MMP-II is a better index marker than PGL-I. Overall, the epidemiological study conducted in Vietnam suggests that serological testing with MMP-II would be beneficial in detecting leprosy.

Leprosy is a chronic infectious disease caused by *Mycobacterium leprae* infection, which sometimes leads to progressive peripheral nerve injury and systematic deformity (16, 30). Early detection of *M. leprae* infection and early start of treatment are key in avoiding deformities. Also, in order to decrease the incidence of new cases, it is important to find and treat the sources of the infection as soon as possible. Thus, early detection of these infected individuals who cannot be clinically diagnosed is critical (34). The diagnosis of leprosy is based on microscopic detection of acid-fast bacilli in skin smears or biopsies, along with clinical and histopathological evaluation of suspected patients. Recently, diagnostic methods for leprosy based on *M. leprae* DNA sequences have been developed (10, 20, 25). However, it is difficult to use these methods in developing countries which still have leprosy hot spot areas, because such methods require expensive machines and materials as well as skilled technicians. Although many developing countries have recently established laboratories for DNA-based diagnosis, it is harder to perform DNA tests than serodiagnostic tests. Thus, in countries where leprosy is endemic, diagnosis still relies on clinical observations and easy, inexpensive tests.

Serodiagnosis is generally accepted as the easiest way of diagnosing a disease. For leprosy serodiagnosis, the only antigen currently used is phenolic glycolipid I (PGL-I), which is supposedly specific to *M. leprae* (21, 26, 27). Since the identification of PGL-I in 1981 by Hunter and Brennan (14), a number of serological tools have been developed. Simple assays, such as the Serodia-Leprae method, a dipstick assay, and lateral flow tests based on the PGL-I antigen, have been used to detect leprosy patients in areas where leprosy is endemic (3, 15, 17, 32). However, these tests seem to be insufficient for detection of both multibacillary (MB) and paucibacillary (PB) patients, as well as for early diagnosis, and have not been used as widely as would be expected in field situations (6, 29). Therefore, we have begun the search for a more sensitive antigen. Major membrane protein II (MMP-II; encoded by the ML2038c gene, named *bfrA*, also known as bacterioferritin) was previously identified from the cell membrane fraction of *M. leprae* as an antigenic molecule capable of activating both antigen-presenting cells and T cells (19, 24). A homology search of the mycobacteria nucleotide database revealed that MMP-II is conserved between *M. leprae*, *M. tuberculosis*, and *M. avium*. The amino acid identity is about 86% among the three species. However, we have previously examined the role of MMP-II in the humoral responses of Japanese patients and showed that MMP-II could contribute to the specific serodetection of leprosy patients (18).

In the present study, we performed a serological test using serum samples collected in regions of leprosy endemicity in Vietnam and evaluated the use of MMP-II as an antigen for serodiagnosis of leprosy. We believe that identifying the appropriate antigens for serodiagnosis could facilitate the devel-

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opment of simple diagnostic tests, like dip-stick assays, for use in developing countries.

MATERIALS AND METHODS

Serum samples. A total of 974 serum samples from various individuals, including in- and out-patients of Quyhoa National Leprosy & Dermato-Venereology Hospital (NDH), were obtained under informed consent. The sera were donated by 205 leprosy patients (163 patients undergoing treatment and 42 new patients), 428 household contacts (HHCs), 130 medical staff members, and 211 noncontact healthy individuals. Sera of leprosy patients and their contacts were taken at regional medical centers in the midregion of Vietnam, including those in the Danang, Quangnam, Quangngai, Binhdinh, Phuyen, Khanhhoa, Ninhthuan, Gialai, Kontum, Daklak, and Daknong provinces, where the average prevalence rate is 0.17 (number of cases/10,000 persons) and the average detection rate is 2.13 (number of cases/10,000 persons). Among these provinces, Binhdinh, Ninhthuan, Gialai, and Kontum had hot spot areas. The medical staff members consisted of workers in Quyhoa NDH, including medical doctors, nurses, pharmacists, technicians, and helpers. Only the sera from medical staff members who were not HHCs of leprosy patients were used in this study. Sera were also obtained from healthy persons living in the Binhdinh province (n = 126) and the Longan province (n = 85), which are distantly located from each other. Out of 205 leprosy patients, 121 had MB leprosy and 84 had PB leprosy. We made the initial diagnosis according to the Ridley-Jopling classification system and classified patients as MB and PB types based on the WHO recommendation. In Vietnam, the *M. bovis* bacille Calmette-Guérin (BCG) vaccination against tuberculosis has been undertaken in earnest since 1976. Almost all medical staff personnel who donated their blood for this study were vaccinated with BCG.

MMP-II and PGL-I antigens. The MMP-II gene (ML2038c, or *bfrA*) was expressed in *Escherichia coli* as a fusion construct by using a pMAL-c2X expression vector (New England BioLabs) (18). Synthetic bovine serum albumin-conjugated trisaccharide-phenyl propionate for the detection of PGL-I antibodies was produced by our laboratory. The procedure for synthesis of the antigen is described elsewhere (12).

ELISAs for detection of antibodies. MaxiSorp (Nalge Nunc) microtiter plates were coated with 50 μl antigen solution (MMP-II [0.4 μg/ml] and PGL-I [0.2 μg/ml]) in carbonate-bicarbonate buffer (pH 9.4) and kept at 4°C overnight. The optimal concentrations of these antigens were determined in advance. The enzyme-linked immunosorbent assay (ELISA) protocol was performed as described previously (18). We measured anti-MMP-II immunoglobulin G (IgG) antibodies and anti-PGL-I IgM antibodies. Plate-to-plate variations in optical density (OD) readings were controlled for by using a common standard serum.

Monitoring. One hundred forty-eight leprosy patients have been monitored using MMP-II ELISA and PGL-I ELISA during their multidrug therapy (MDT) treatment since 2001. Twelve-month MDT for MB was carried out, and sampling was performed three to five times. Also, HHCs were monitored once every 3 or 6 months by both the MMP-II and the PGL-I ELISA methods from 2001 to 2004.

Statistics. The data were analyzed using a statistical software package (version 9.3.2.0; MedCalc software). A receiver operator characteristic (ROC) curve was drawn to calculate the cutoff levels (2). Additionally, the statistically significant differences between assays were confirmed by the chi-square test (28).

RESULTS

Comparison of the distribution of ELISA values between MMP-II and PGL-I. We focused on the distribution of ELISA values derived from MB leprosy patients and compared them to those from healthy individuals (Fig. 1). The cutoff OD_{405} value for anti-MMP-II antibody was defined as 0.103 (95% confidence interval, 85.2 to 93.7), and that for anti-PGL-I antibody was defined as 0.452 (95% CI, 85.2 to 93.7), by ROC curve analysis (MedCalc software) using OD titers from 211 healthy individuals and 205 leprosy patients. The distribution pattern of MMP-II ELISA values was quite different from that of PGL-I ELISA for healthy individuals. While the OD values of most healthy individuals were in the low range for MMP-II ELISA (Fig. 1A), the titers obtained by PGL-I ELISA showed a bell-shaped curve which was similar to that of MB leprosy patients (Fig. 1B). The PGL-I ELISA values for PB leprosy patients also showed a similar bell-shaped curve (data not shown).

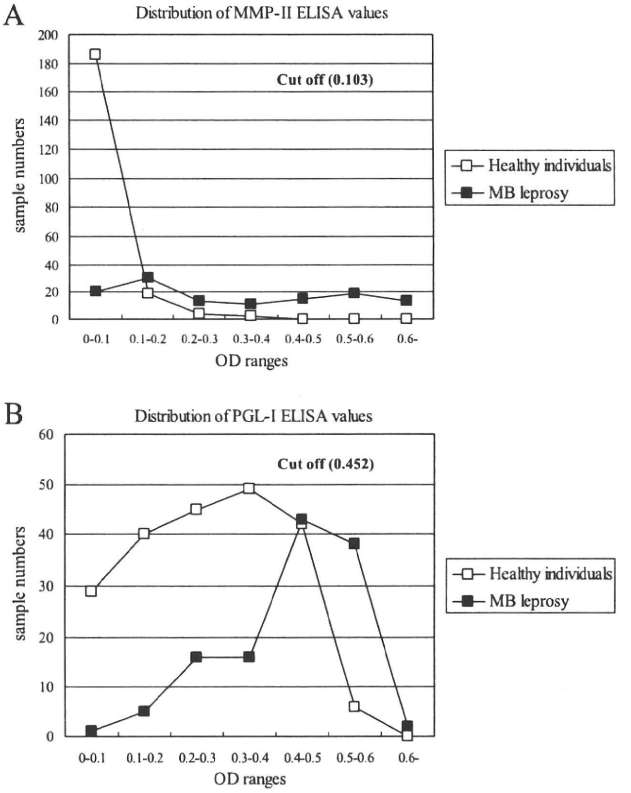

FIG. 1. Comparison of distributions of OD values in MB leprosy patients and normal individuals. (A) Distribution pattern of MMP-II ELISA values in patients and healthy individuals. (B) Distribution pattern of PGL-I ELISA values in patients and healthy individuals. The solid squares show the number of MB leprosy patients in each OD value range, and the open squares show the number of healthy individuals.

Detection rate of antibodies in sera of leprosy patients. Among the MB patients, 85.1% were positive by MMP-II ELISA and 57.0% were positive by PGL-I ELISA; 47.6% of PB patients were positive by MMP-II ELISA, and 20.2% were positive by PGL-I ELISA (Fig. 2). The MMP-II ELISA values for both MB and PB patients were significantly higher than the PGL-I ELISA values ($P < 0.001$) (Fig. 2). Patients undergoing treatment and new cases showed a similar difference (data not shown).

Seropositivity rates of contacts, medical staff members, and healthy volunteers. There was no significant difference in positivity rate between MMP-II ELISA and PGL-I ELISA for healthy individuals and HHCs (Fig. 3). Also, there was no significant difference in positivity rate between MMP-II ELISA and PGL-I ELISA for healthy individuals from different provinces, namely, Binhdinh and Longan (data not shown). In contrast, the medical staff showed a significantly higher rate of positivity by MMP-II ELISA (26.2%) than by PGL-I ELISA. The anti-MMP-II antibody positivity rate for the medical staff

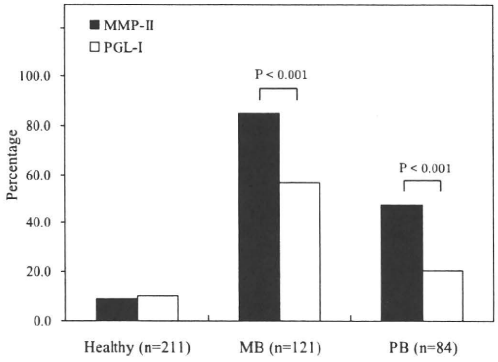

FIG. 2. Comparison of positivity rates of patients as determined by MMP-II and PGL-I ELISA. Black bars show percentages of healthy individuals and patients positive by MMP-II ELISA, and white bars show those for PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as *P* values.

was significantly higher than those for healthy individuals and HHCs.

Monitoring of HHCs. Previous studies suggested the usefulness of PGL-I ELISA in monitoring the effects of leprosy treatment (5, 8, 9, 22). Therefore, we monitored anti-MMP-II antibody titers in patients after treatment and compared them to anti-PGL-I antibody titers. Ninety-two MB and 56 PB patients were monitored. The anti-MMP-II antibody value of approximately 30% of monitored MB patients declined within 1 to 2 years after the start of treatment, in accordance with changes in bacterial index values (data not shown), although approximately 50% of MB patients showed no reduction in ELISA values and 20% of patients showed mild increases in value. Three representative samples of MB patients are shown in Fig. 4. Among PB patients, 18% of the monitored patients had reduced anti-MMP-II antibody titers. On the other hand, anti-PGL-I antibody titers were reduced approximately only 20% in both MB and PB patients during the monitoring period. Therefore, anti-MMP II antibody may reflect the efficacy of treatment similarly to or slightly better than anti-PGL-I antibody in some cases. Furthermore, 9 individuals out of 428

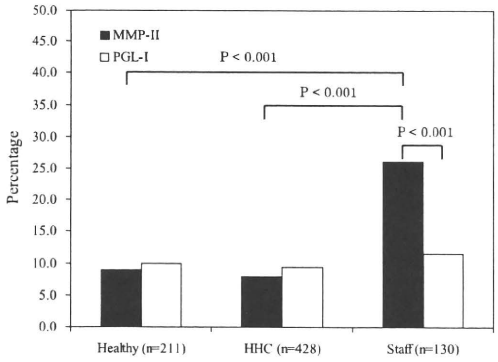

FIG. 3. Positivity rates of HHCs and medical staff members as determined by MMP-II and PGL-I ELISA. Black bars show percentages of HHCs and medical staff members positive by MMP-II ELISA, and white bars show those by PGL-I ELISA. Statistically significant differences were confirmed by the chi-square test and are indicated as *P* values.

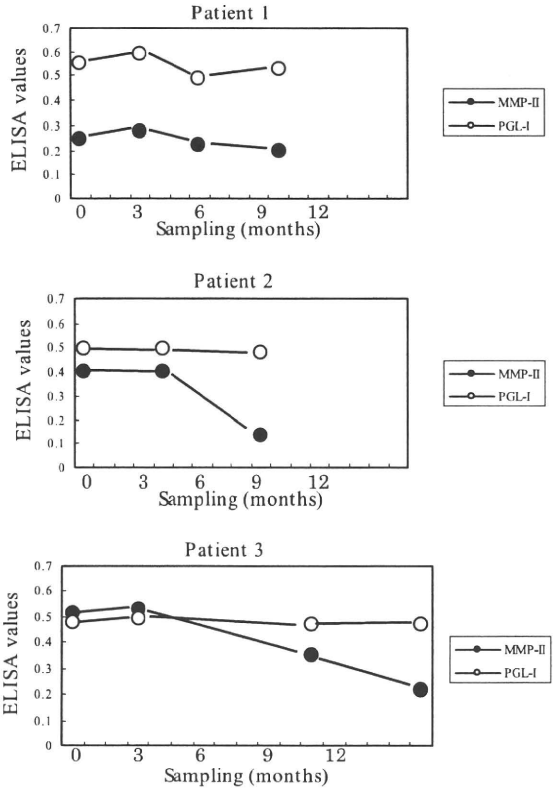

FIG. 4. Monitoring of three MB leprosy patients by MMP-II and PGL-I ELISAs. Three cases of monitored leprosy patients are shown. The closed circles show MMP-II ELISA values, and the open circles show PGL-I ELISA values. Note that the cutoff value for MMP-II is 0.103 and that of PGL-I is 0.452.

HHCs developed leprosy after several years of monitoring. Among the nine cases, two individuals had increasing antibody titers by MMP-II and/or PGL-I ELISA 1 year before manifesting clinical symptoms (data not shown). Patient HHC192 showed a prominent rise in anti-MMP-II antibody values during the asymptomatic period. Both patients developed MB leprosy. The other seven, whose antibody levels did not show an apparent increase during the observation period, developed PB leprosy.

DISCUSSION

Serodiagnosis is the easiest, cheapest, and least invasive diagnostic tool for infectious diseases. Currently, PGL-I is used as a specific antigen for *M. leprae*, but in practice, its sensitivity and specificity are not as high as expected, even though previous studies using stock sera reported that the detection rate for MB patients was more than 80% (1, 3, 4, 7). The present study involving Vietnamese leprosy patients indicated that there is a significant difference between MMP-II ELISA and PGL-I ELISA in detecting both MB and PB leprosy. The positivity rate of anti-MMP-II antibody for MB leprosy was approximately 85%, and that for PB leprosy was 48%; these titers were significantly higher than the titers obtained by PGL-I ELISA (57% and 20%, respectively). The detection rates obtained by

MMP-II ELISA were similar to those for a previous study using stock sera from Japanese leprosy patients (18). However, the positivity rates of anti-PGL-I antibody in the present study were significantly lower than those for the Japanese patients, although the same antigens for both MMP-II and PGL-I were used in the two studies.

There are several possible reasons why the sensitivity of PGL-I ELISA was low in the present study. One reason may be that some healthy Vietnamese individuals have high anti-PGL-I antibody titers. Although we could not conduct further detailed analysis on the subjects, these individuals might be highly exposed to *M. leprae*, and so their B lymphocytes might be repeatedly stimulated with *M. leprae*-derived antigens, including PGL-I. It seems quite difficult to discriminate the healthy individuals from MB or PB leprosy patients by PGL-I ELISA, as shown in Fig. 1. Furthermore, we concluded that a reasonable cutoff point for PGL-I ELISA was an OD_{405} of 0.452, as deduced from Fig. 1 and the ROC values, but this resulted in lower sensitivity. The difference in sensitivity between PGL-I ELISA and MMP-II ELISA may also be due to differences in the biochemical features of the antigens. PGL-I is a glycolipid component, and as such, it might be retained in some infected cells for a long time after the initial exposure (13, 33). This speculation is supported by previous reports showing that healthy individuals residing in areas where leprosy is endemic had high anti-PGL-I antibody titers, and *M. leprae* DNA was recovered by PCR from the nasal swabs of these individuals (31, 32). Also, it has been reported that the usefulness of PGL-I-based tests for early diagnosis is limited, since 7 to 10% of individuals testing positive do not develop the disease (14).

In contrast, MMP-II is a protein antigen and is considered to be one of the immunodominant antigens of *M. leprae* (19). Therefore, in individuals who have been exposed to *M. leprae* but have not developed leprosy, antigen-presenting cells expressing MMP-II might feasibly be eliminated from the body by immune cells such as cytotoxic T lymphocytes and thus lack the ability to produce anti-MMP-II antibodies through antigen-presenting-cell-dependent mechanisms. These speculations seem to be supported by our present observations with sera from patients monitored over time. Anti-MMP-II antibody titers of MB patients declined earlier than PGL-I titers with MDT treatment, indicating the disappearance of MMP-II antigens, while no apparent reduction in PGL-I antigens was observed during the 12 months of observation (Fig. 4). Furthermore, in one case the anti-MMP-II antibody titer increased drastically before manifestation of clinically apparent leprosy (data not shown).

Medical staff members (n = 130) showed a high positivity rate by MMP-II ELISA, compared with healthy individuals or HHCs. These medical staff members were mostly BCG vaccinated, as were the HHCs. Therefore, it seems that BCG vaccination has no effect on anti-MMP-II antibody titers. Although we could not determine a conclusive reason for the high positivity rate, these medical personnel may be repeatedly exposed to *M. leprae* in hospitals. However, we cannot eliminate the possibility that they have produced the antibody in response to exposure to other mycobacteria, since the MMP-II protein is conserved in other pathogenic mycobacterial species, such as *M. tuberculosis* and *M. avium*, though the staff members with high anti-MMP-II antibody titers did not manifest any clinical signs or features indicating infection with other mycobacteria. We tried to perform nested PCR using the *M. leprae*-specific repetitive element for DNA extracted from nasal swabs of some hospital staff members (n = 25). However, because the sampling dates for the serological test and the PCR test were not coordinated, we could not come to a definite conclusion. Nevertheless, we were surprised to find that ≅40% (n = 25) of the nasal swab samples were positive (data not shown). As for tuberculosis, it is said that one-third of the world population is infected with *M. tuberculosis*. The same may be the case with leprosy, although further studies are needed with larger populations, including medical staff members as well as contacts and noncontacts of leprosy.

Taken together, our data indicate that MMP-II ELISA could be useful as a supporting serodiagnostic tool in combination with other clinical diagnostic methods and may also be useful in monitoring disease activity. Furthermore, in this study the correlation between MMP-II and PGL-I was low, with a correlation coefficient among the 205 leprosy patients of only 0.63. If both PGL-I and MMP-II antibodies could be measured simultaneously, the sensitivity of the assay system could be increased. Considering that PGL-I is a sugar antigen (eliciting IgM antibodies) and MMP-II is a protein antigen (eliciting IgG antibodies), assaying for a combination of these antibodies could lead to more-accurate detection of leprosy in the field.

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RESEARCH ARTICLE

$CD4^+$ T-cell activation by antigen-presenting cells infected with urease-deficient recombinant *Mycobacterium bovis* bacillus Calmette-Guérin

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Abstract

We constructed a recombinant *Mycobacterium bovis* bacillus Calmette-Guérin (BCG-ΔUT) that lacks urease, providing acidic intraphagosomal conditions to drive an effective human immune T-cell response. BCG-ΔUT-infected macrophages stimulated autologous $CD4^+$ T cells more efficiently than parent BCG-infected macrophages. For further T-cell activation, BCG-ΔUT-infected macrophages required pretreatment with exogenous recombinant granulocyte-macrophage colony-stimulating factor or costimulation with either CD40 ligand or interferon-γ. By contrast, BCG-ΔUT-infected dendritic cells induced significant activation of naïve $CD4^+$ T cells without costimulating signals. C57BL/6 mice intradermally inoculated with BCG-ΔUT more efficiently produced memory T cells that responded to recall antigen. Therefore, the depletion of urease from BCG is useful for the activation of T cells.

Introduction

Mycobacteria, such as *Mycobacterium leprae* and *Mycobacterium tuberculosis*, are representative parasitic intracellular pathogens. *Mycobacterium leprae* is a causative agent of human leprosy, in cases of which skin lesions and chronic progressive peripheral nerve injury are usually observed (Stoner, 1979; Job, 1989). At present, around one-third of individuals are infected with *M. tuberculosis* and several millions die as result of tuberculosis each year (Dye *et al.*, 2005; World Health Organization, 2006). *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) has been used as a vaccine against leprosy, although its efficacy is quite limited (Andersen & Doherty, 2005; Setia *et al.*, 2006). The emergence of multidrug-resistant strains of these mycobacteria is of concern (Maeda *et al.*, 2001; Kai *et al.*, 2004; Kaufmann, 2005), and therefore the urgent development of a new vaccine, including a more efficacious recombinant BCG, is desired (Kaufmann, 2005).

Among various immunocompetent cells, $CD4^+$ T cells, especially IFN-γ-producing cells, play an extremely important role in inhibiting the multiplication of mycobacteria, killing them in the early stages of infection, and keeping the bacterial load at a stable level (Orme *et al.*, 1993; Dockrell *et al.*, 1996; Hashimoto *et al.*, 2002). $CD4^+$ T cells that can respond quickly to pathogenic mycobacteria and produce IFN-γ are known as memory T cells. The efficient production of such memory T cells needs pre-exposure to antigenic vaccinating molecules, which share their antigenicity with that of pathogenic mycobacteria (Kaufmann, 2006). BCG has been considered a good candidate for a vaccine against *M. leprae* in this respect, however its efficacy is limited in several aspects, including the ability to activate T cells (Kaufmann & McMichael, 2005). BCG resides in the phagosomes of macrophages and thus attenuates the trafficking of antigenic molecules to the macrophage cell surface (Grode *et al.*, 2005). One possible strategy for improving the ability of BCG to stimulate T cells is to enhance its ability to fuse with the lysosomes. To this end, we knocked out the *urease* gene from BCG. The urease-deficient recombinant BCG (BCG-ΔUT) is expected to allow phagosomal acidification in the host cells, and induce efficient phagosome maturation for cytolytic activity of the antigenic molecules of BCG (Schaible *et al.*, 1998; Honerzu Bentrup & Russell, 2001).

In the present study, we evaluated the ability of BCG-ΔUT to activate IFN-γ-producing type 1 $CD4^+$ T cells through

antigen-presenting cells (APCs), and to produce memory CD4$^+$ T cells. When used as a target of BCG-ΔUT, macrophages fully stimulated CD4$^+$ T cells in the presence of costimulatory agents such as CD40 ligand (L) and IFN-γ. In addition, BCG-ΔUT-infected monocyte-derived dendritic cells (DCs) activated type 1 CD4$^+$ T cells more efficiently than parent BCG-infected cells in the absence of these costimulators. Therefore, BCG-ΔUT was found to be a useful T-cell-stimulating agent.

Materials and methods

Preparation of blood cells

Peripheral blood was obtained from healthy purified protein derivative (PPD)-positive individuals with informed consent. PPD-negative individuals provide more information, however, as healthy individuals are PPD-positive, due to compulsory BCG vaccination for children in Japan (0–4 years old). Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described (Makino & Baba, 1997). 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, Oslo, Norway). The CD3$^-$ PBMC fraction was plated on collagen-coated plates and nonadherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (Makino & Baba, 1997). Macrophages were generated by culturing monocytes in the presence of 20% fetal calf serum and recombinant (r) macrophage colony-stimulating factor (M-CSF) (R&D Systems, Abingdom, UK) (Makino *et al.*, 2007). Macrophages were pulsed with rBCGs on day 5 of culture, and were used as a stimulator of T cells on day 7 (Makino *et al.*, 2007). Monocyte-derived DCs were differentiated as described previously (Makino *et al.*, 1999). Briefly, monocytes were cultured in the presence of 50 ng recombinant granulocyte-macrophage colony-stimulating factor (GM-CSF; Pepro Tech EC Ltd, London, UK) and 10 ng of recombinant interleukin (rIL)-4 (Pepro Tech) per millilitre (Makino *et al.*, 1999). On day 3 of culture, immature DCs were infected with rBCGs at the indicated multiplicity of infection (MOI), and on day 5 of culture, DCs were used for further analyses of surface antigens and for mixed-lymphocyte assays.

BCG culture and DNA manipulation

The mycobacterial strain, BCG substrain Tokyo, for DNA manipulation was grown in Middlebrook 7H9 broth (Difco Laboratories) with 0.05% Tween 80 or Middlebrook 7H10 agar (Difco) with 0.5% glycerol, each supplemented with 10% albumin–dextrose–catalase enrichment (Difco). DNA manipulations including isolation of DNA, transformation and PCR, were carried out as described previously (Miyamoto *et al.*, 2004). *Escherichia coli* strain DH5α was used for routine manipulation and the propagation of plasmid DNA. *Escherichia coli* strain STBL4 was used for the construction of plasmid vectors derived from phAE87. Antibiotics were added as required: hygromycin B, 150 μg mL^{-1} for *E. coli* and 75 μg mL^{-1} for *Mycobacterium smegmatis* (mc^{2}155) and *M. bovis* BCG. A recombinant BCG that lacks a *urease* gene was constructed. The sequence of the targeted gene, *ureC* (BCG 1886), was obtained from the BCG list (http://genolist.pasteur.fr/BCGList/). The *ureC* gene was inactivated by inserting a hygromycin-resistance cassette (*hyg*) using a specialized transducing phage system for homologous recombination (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). To construct the disrupted sequence, fragments of around 0.9 kb both upstream and downstream of *ureC* were amplified from BCG-Tokyo genomic DNA using the following two pairs of primers: F UureC and R UureC for upstream of *ureC*, and F DureC and R DureC for downstream of *ureC*. The PCR products were digested with each restriction enzyme and cloned into the corresponding site flanking *hyg* of pYUB854 to give pYUB854-ureC-UD. This plasmid was used for packaging into the phasmid vector phAE87 to construct a specialized transducing mycobacteriophage for gene disruption as described previously (Bardarov *et al.*, 2002; Miyamoto *et al.*, 2006). BCG-Tokyo infected with the mycobacteriophage at an MOI of 50 was incubated at 37 °C for 3 h in 7H9 broth without Tween 80. Harvested bacterial cells were then plated and cultured on 7H10 agar containing hygromycin B (75 μg mL^{-1}) for 3 weeks. The hygromycin B-resistant colonies were selected and evaluated with a conventional urease assay. A change in the color of the assay medium from yellowish to red was scored as urease-positive. Furthermore, genomic DNA obtained from these colonies was subjected to PCR to confirm the disruption of the gene using primers F ureC and R ureC (Fig. 1). The colony which tested negative in the urease assay was named BCG-ΔUT, while the parental BCG substrain Tokyo is referred to as BCG-Tokyo.

Preparation of *M. leprae*

Mycobacterium leprae (Thai-53) was isolated from the footpads of BALB/c-*nu/nu* mice (McDermott-Lancaster *et al.*, 1987). The isolated bacteria were counted by Shepard's method (Charles & Shepard, 1960). The MOI for infection to host cells was determined based on the assumption that macrophages and DCs were equally susceptible to infection with BCG or *M. leprae* (Hashimoto *et al.*, 2002).

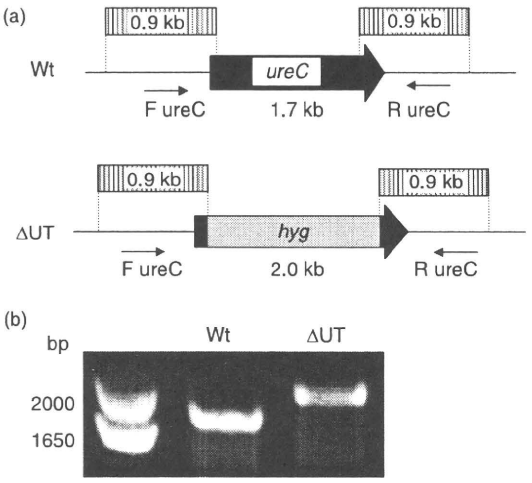

Fig. 1. Disruption of the *ureC* gene. (a) Schematic diagram of the *ureC* region on the chromosome of the wild-type *Mycobacterium bovis* BCG Tokyo strain and its gene disruptant, ΔUT. The shaded boxes indicate the regions included in the recombinant phage for gene disruption. The black arrow represents the coding region of the *ureC* gene. The gray box represents the hygromycin-resistace cassette (*hyg*). The primers used for PCR analysis are indicated by small arrows. (b) PCR analysis of the wild-type and the disruptant using the primers indicated above.

Preparation of mycobacterial antigen

The cytosolic fraction of BCG-Tokyo (BCC) was obtained as described previously (Maeda *et al.*, 2003). Briefly, the mycobacterial suspension containing the protease inhibitors was mixed with zirconium beads at a ratio of *c.* 1 : 1 (v/v) and homogenized using a Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo). The suspension was centrifuged at 10 000 ***g*** to remove the cell-wall fractions. The supernatant was then ultracentrifuged at 100 000 ***g*** and the resulting supernatant was taken as the cytosolic fraction. For preparation of the *M. leprae* membrane (MLM) fraction, *M. leprae* was used instead of BCG and treated similarly. The pellet obtained by ultracentrifugation (100 000 ***g*** for 1 h) was used as a membrane fraction (MLM). The optimal concentration of BCC and MLM for stimulating T cells was determined in advance.

Analysis of cell surface antigens

The expression of cell surface antigens on macrophages and DCs, either untreated or treated with exogenous rIFN-γ (R&D Systems), was analyzed using a FACSCalibur flow cytometer. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 1×10^4 live cells were analyzed. For the analysis of cell surface antigens, the following mAbs were used: fluorescein isothiocyanate (FITC)-conjugated mAbs against HLA-ABC (G46-2.6), HLA-DR (L243), CD14 (M5E2), CD40 (5C3) and CD86 (FUN-1). These mAbs were obtained from BD PharMingen (San Diego, CA).

APC function of rBCG-infected macrophages and DCs

The ability of rBCG-infected macrophages to stimulate T cells was assessed using an autologous mixed-lymphocyte assay as previously described (Wakamatsu *et al.*, 1999; Hashimoto *et al.*, 2002). The responder $CD4^+$ T cells were purified from freshly thawed PBMCs by using a CD4-negative isolation kit (Dynabeads 450; Dynal) (Wakamatsu *et al.*, 1999). The purity of $CD4^+$ T cells was more than 95% as assessed by fluorescence-activated cell sorting (FACS) analysis. Naïve $CD4^+$ T cells were produced by further treatment of $CD4^+$ T cells with an mAb to CD45RO antigen, followed by incubation with beads coated with goat antimouse IgG. Memory-type T cells were similarly produced by the treatment of cells with an mAb to CD45RA antigen. The purified responder cells (1×10^5 well^{-1}) were plated in 96-well round-bottom tissue culture plates and macrophages or DCs were added to give the indicated APC/$CD4^+$ T-cell ratio. Supernatants of the cocultures were collected on day 4 and the concentration of cytokines was determined. In some cases, macrophages were treated with the indicated dose of exogenous rGM-CSF (Pepro Tech) in advance of infection with rBCGs. Further, macrophages were infected with rBCGs in the presence of neutralizing mAb to IL-10 (JES3-9D7; Rat IgG, BD PharMingen) or control normal rat IgG. Macrophages infected with BCGs were further costimulated with either rCD40L (Pepro Tech) or rIFN-γ (R&D Systems), and in some cases, the macrophages were stimulated with rIFN-γ in the presence of anti-IFN-γ receptor α chain (CD119) (GIR-208, mouse IgG1, BD PharMingen) or control normal mouse IgG. In other cases, macrophages infected with BCG-ΔUT in the presence of exogenous rIFN-γ were treated with either mAb to HLA-DR (L243, mouse IgG2a), CD86 (IT2.2, mouse IgG2b, BD PharMingen) or control normal mouse IgG, and subsequently cocultured with responder $CD4^+$ T cells. The concentration of IFN-γ produced by $CD4^+$ T cells was quantified using an enzyme assay kit [OptEIA Human enzyme linked immunosorbent assay (ELISA) Set; BD Biosciences].

Production of IL-12p70 and IL-1β by DCs

The ability of DCs to produce IL-12p70 and IL-1β on stimulation with BCG-Tokyo or BCG-ΔUT was assessed. The DCs were stimulated with BCGs at the indicated MOI for 24 h, and the concentration of these cytokines was quantified using the Opt EIA Human ELISA Set.

Animal studies

For inoculation into mice, BCG-Tokyo and BCG-ΔUT were cultured in Middlebrook 7H9 to log phase and stored at 10^8 CFU mL^{-1} at −80 °C. Before aliquots were used for inoculation, the concentration of viable bacilli was determined by plating cells on the Middlebrook 7H10 agar plate. Three 5-week-old C57BL/6J mice per group were inoculated intradermally with 0.1 mL phosphate-buffered saline (PBS) containing 1×10^2 or 1×10^3 BCG-Tokyo or BCG-ΔUT. The animals were kept under specific pathogen-free conditions and were supplied with sterilized food and water. Four weeks after injection, the spleens were removed, and the splenocytes were suspended at a concentration of 2×10^6 cells mL^{-1} in culture medium, and stimulated with the indicated concentration of BCC or MLM in triplicate in 96-well round-bottomed microplates. The individual culture supernatants were collected 3 days after stimulation, and IFN-γ and IL-2 were measured using an OptEIA mouse ELISA set.

Statistical analysis

The Student's *t*-test was applied to determine statistical differences.

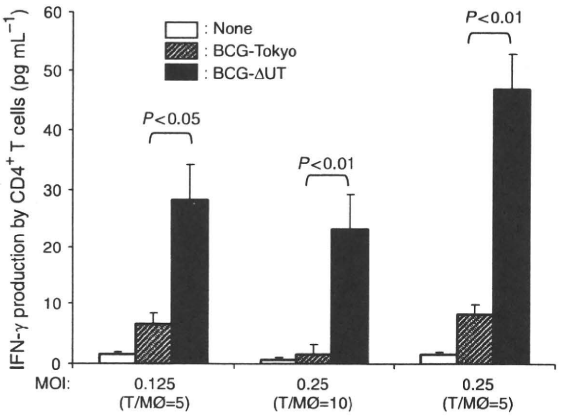

Fig. 2. Production of IFN-γ by CD4$^+$ T cells. Macrophages, differentiated by 5 days of culture with rM-CSF from monocytes, were infected with BCG-Tokyo (parental BCG) or BCG-ΔUT at the indicated MOI, and cultured for another 2 days in the presence of rM-CSF. These macrophages were used as a stimulator of autologous CD4$^+$ T cells (1×10^5 cells well^{-1}) at the indicated T-cell/macrophage ratio in a 4-day culture. A representative example of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

Results

Induction of the fusion of BCG-ΔUT-infected phagosomes with lysosomes

The efficacy with which BCG-ΔUT-infected phagosomes fused with lysosomes in macrophages was examined using confocal microscopy. Lysosomes were stained with anti-LAMP1 mAb after treatment of THP-1 cells with FITC-labeled BCG-Tokyo or BCG-ΔUT for 24 h. The parental BCG colocalized with lysosomes less efficiently than BCG-ΔUT (data not shown). Therefore, BCG-ΔUT may at least partially enhance the ability to induce phagosomal maturation.

T-cell-stimulating activity of BCG-ΔUT

The activity of BCG-ΔUT to stimulate IFN-γ-producing CD4$^+$ T cells, when infected to macrophages, was assessed (Fig. 2). BCG-ΔUT-infected macrophages activated unseparated CD4$^+$ T cells to release IFN-γ substantially more efficiently than parent BCG-infected macrophages. Although BCG-ΔUT-infected macrophages also induced production of IL-2 from CD4$^+$ T cells (data not shown), the extent of IFN-γ (< 50 pg mL^{-1}) and IL-2 production was not as high as expected. Furthermore, BCG-ΔUT did not induce the activation of naïve CD4$^+$ T cells (data not shown). As the activation of T cells is largely influenced by the cytokine milieu, in which T cells and their stimulators are present, we determined the level of cytokines produced from macrophages on stimulation with BCG-ΔUT. BCG-ΔUT produced significantly more cytokines, such as IL-10, GM-CSF, TNFα and IL-1β, than the parental BCG (data not shown). It has been reported that IL-10 inhibits the APC-mediated activation of T cells (Granelli-Piperno *et al.*, 2004) and GM-CSF regulates the function of macrophages (Makino *et al.*, 2007). To examine the role of IL-10 on T-cell activation, macrophages were infected with BCGs in the presence of a neutralizing mAb to IL-10 (Fig. 3a). The IFN-γ production by stimulated CD4$^+$ T cells was not influenced by the treatment of macrophages with control IgG; however, a significantly higher level of IFN-γ was produced on treatment with the neutralizing mAb to IL-10. The up-regulation by IL-10 mAb treatment was observed in both BCG-Tokyo and BCG-ΔUT in a similar fashion. Furthermore, the pretreatment of macrophages with exogenous GM-CSF also significantly upregulated the antigen-presenting function of macrophages, although the effect of GM-CSF was more pronounced in BCG-ΔUT-infected macrophages (Fig. 3b).

Next, we phenotypically assessed the effect of BCG-ΔUT on macrophages (Fig. 4a). BCG-ΔUT induced enhanced expression of both CD14 and CD40 on macrophages compared with BCG-Tokyo. Based on these results, we treated BCG-infected macrophages with CD40L to examine its role as a costimulator of macrophages (Fig. 4b). The CD40L treatment upregulated the T-cell activation by BCG-

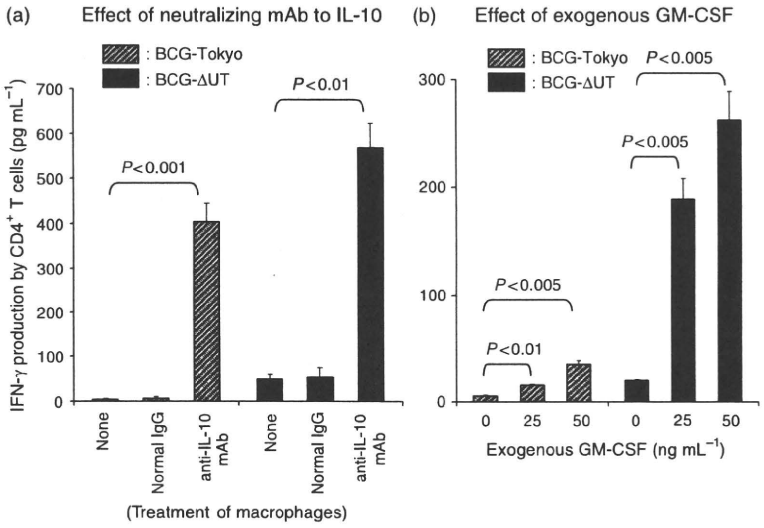

Fig. 3. Effect of IL-10 and GM-CSF on IFN-γ production. (a) Macrophages differentiated from monocytes by using rM-CSF were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 on day 5 of culture and cultured for another 2 days in the presence of rM-CSF. The BCG-infected macrophages were treated with neutralizing mAb to IL-10 or isotype-matched control IgG (10 μg mL^{-1}), and used as a stimulator of CD4$^+$ T cells, at a T-cell/macrophage ratio of 10 : 1, and cultured for another 4 days. The optimal concentration of mAb was determined in advance. (b) Macrophages obtained by 4 days of culture with rM-CSF were treated with the indicated dose of rGM-CSF. The macrophages pretreated with rGM-CSF were infected with BCG-Tokyo or BCG-ΔUT at an MOI of 0.25, cultured for another 2 days in the presence of rM-CSF used as a stimulator of CD4$^+$ T cells on day 8, at a T-cell/macrophage ratio of 10 : 1 (4 days of stimulation). A representative example of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

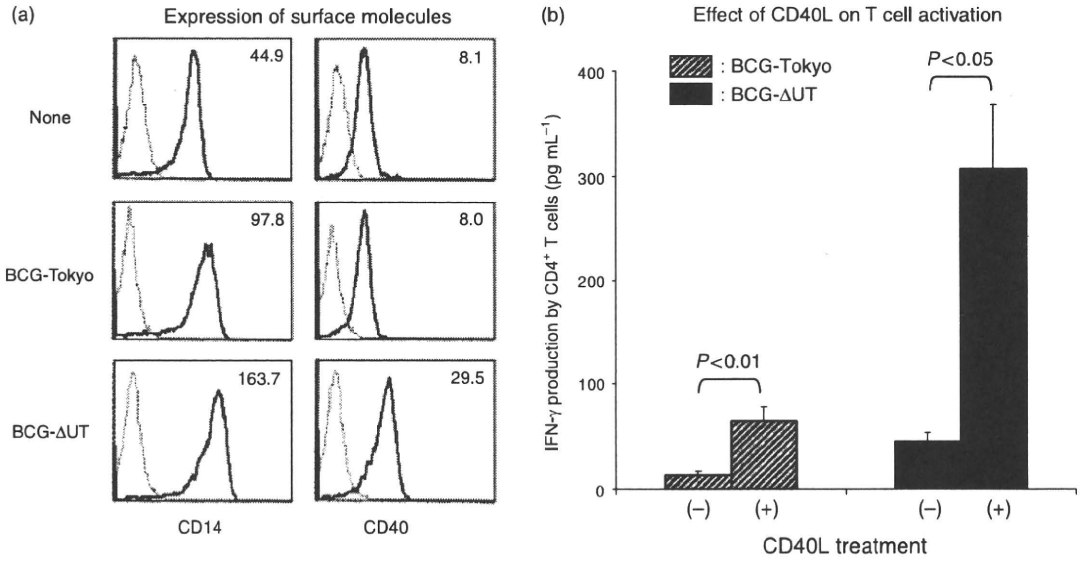

Fig. 4. (a) Expression of CD14 and CD40 molecules on macrophages. Macrophages produced by using rM-CSF were infected with BCGs at an MOI of 0.25, and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (b) IFN-γ production by CD4$^+$ T cells stimulated with BCG-infected macrophages. Macrophages differentiated from monocytes using rM-CSF were infected with BCGs at an MOI of 0.25 on day 5 of culture, further treated with CD40L (1 μg mL^{-1}) on day 6, and used as a stimulator of CD4$^+$ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

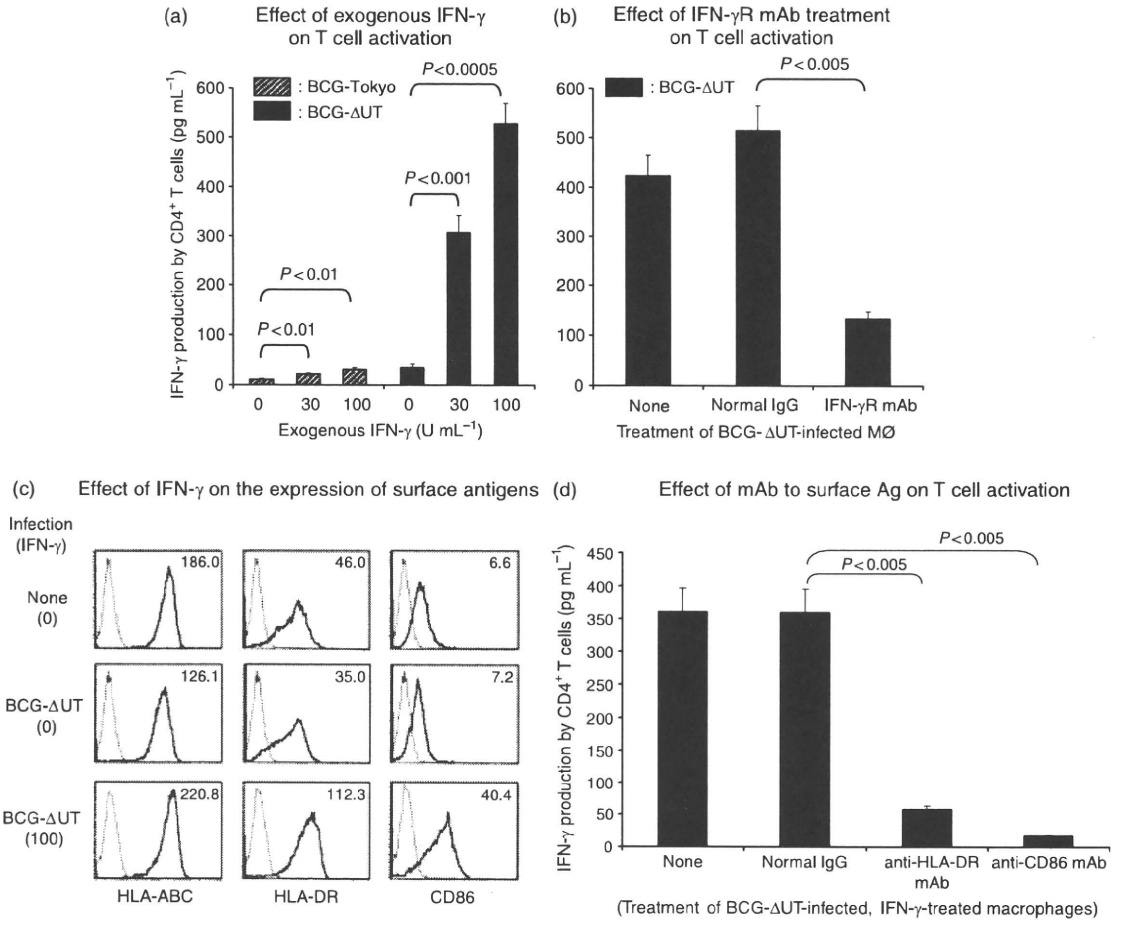

Fig. 5. (a) Effect of exogenous IFN-γ on CD4$^+$ T-cell activation. Macrophages produced by 5 days of culture with rM-CSF from monocytes were infected with BCGs at an MOI of 0.25 and simultaneously treated with the indicated dose of exogenous IFN-γ. The macrophages were used as a stimulator of CD4$^+$ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (b) Involvement of IFN-γ receptor in T-cell activation. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), stimulated with exogenous IFN-γ (100 U mL^{-1}) in the presence of mAb to IFN-γ receptor α-chain (CD119) or isotype matched control IgG (10 μg mL^{-1}), and cultured for another 2 days in the presence of rM-CSF. The macrophages were used as a stimulator of CD4$^+$ T cells (T-cell/macrophage ratio of 10 : 1, 4 days of stimulation). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (c) Surface expression of various molecules on BCG-ΔUT-infected, IFN-γ-treated macrophages. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), stimulated with exogenous IFN-γ (100 U mL^{-1}) and cultured for another 2 days in the presence of rM-CSF. The macrophages on day 7 of culture were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (d) Involvement of surface antigens of BCG-ΔUT-infected, IFN-γ-stimulated macrophages in T-cell activation. Macrophages produced as in (a) were infected with BCG-ΔUT (MOI of 0.25), treated with exogenous IFN-γ (100 U mL^{-1}) and cultured for another 2 days in the presence of rM-CSF. These macrophages were cocultured with autologous CD4$^+$ T cells at a T-cell/macrophage ratio of 10 : 1 in a 4-day culture in the presence of the indicated mAb (10 μg mL^{-1}). A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

infected macrophages, but it more efficiently affected BCG-ΔUT-infected macrophages. Similarly, there was a significant difference between parent BCG and BCG-ΔUT in sensitivity to IFN-γ (Fig. 5a). However, other cytokines such as TNFα and IL-1β did not enhance the T-cell-stimulating activity of rBCG-infected macrophages. The IFN-γ treatment was effective against both BCG-Tokyo- and BCG-ΔUT-infected macrophages; however, more than a 10-fold increase in the production of IFN-γ from T cells was achieved only when BCG-ΔUT-infected macrophages

were stimulated with exogenous IFN-γ. The optimal stimulation of T cells induced the production of more than 500 pg mL^{-1} IFN-γ. The exogenous IFN-γ seems to contribute directly to the enhancement of APC function, as the IFN-γ-mediated enhancement was cancelled out by the pretreatment of BCG-ΔUT-infected macrophages with mAb to IFN-γ receptor α-chain (Fig. 5b). Furthermore, IFN-γ significantly enhanced the expression of HLA-DR and CD86 on BCG-ΔUT-infected macrophages (Fig. 5c), while the phenotypic alteration of BCG-Tokyo-infected macrophages by IFN-γ was minimum (data not shown). When BCG-ΔUT-infected, IFN-γ-treated macrophages were treated with mAb to either HLA-DR or CD86 in advance of being cocultured with $CD4^+$ T cells, IFN-γ production by the T cells was significantly inhibited, while normal murine IgG treatment did not have any effect (Fig. 5d).

$CD4^+$ T-cell activation by BCG-ΔUT-infected DCs

As BCG-ΔUT significantly but less efficiently activated $CD4^+$ T cells through macrophages in the absence of costimulation, the potency of BCG-ΔUT-infected DCs as a T-cell activator was evaluated. Expression of surface molecules on DCs infected with either BCG-Tokyo or BCG-ΔUT was examined (Fig. 6a). Expression of HLA-ABC, HLA-DR, CD86 and CD83 was more significantly upregulated by the infection with BCG-ΔUT than with BCG-Tokyo. Higher levels of IL-12p70 and IL-1β were produced by BCG-ΔUT stimulation (Fig. 6b). Furthermore, we assessed whether BCG-ΔUT activated naïve and memory $CD4^+$ T cells through DCs by using various MOI titers and multiple T/DC ratios (Fig. 6c). IFN-γ levels were significantly higher following stimulation with BCG-ΔUT than with parent BCG in both naïve and memory $CD4^+$ T cells. Also, a higher level of CD40L was expressed on $CD4^+$ T cells after stimulation with BCG-ΔUT-infected DCs (data not shown). These results indicate that the infection of DCs with BCG-ΔUT alone was sufficient, as compared with macrophages which required costimulators to drive a strong T-cell response.

Memory T-cell production by BCG-ΔUT

Another important aspect of using BCG as a vaccine is the production of memory T cells *in vivo*. We examined the response of splenic T cells obtained from BCG-infected C57BL/6 mice to mycobacterial recall antigen (Fig. 7). We used BCC as a recall antigen. At 4 weeks following infection, splenic T cells from BCG-ΔUT-infected mice produced more IFN-γ than those from mice infected with BCG-Tokyo by responding to BCC. The lymphocyte population producing IFN-γ was found to be $CD4^+$ T cells by intracellular staining (data not shown). Furthermore, upon stimulation with MLM, which contains immunodominant antigens of *M. leprae*, $CD4^+$ T cells from BCG-ΔUT-infected mice produced significantly higher levels of IFN-γ than those from uninfected or BCG-Tokyo-infected mice (Fig. 7).

Discussion

To date, BCG is the only suitable vaccine against leprosy; however, its efficacy is quite limited. Overall efficacy in one meta-analysis was reported to be only 26% (Setia *et al.*, 2006). Several reasons might explain why BCG cannot block multiplication of *M. leprae* or inhibit the development of leprosy. The most important defect of BCG is that it is retained in phagosomes of macrophages, avoiding phagosomal acidification and hence interfering in the efficient fusion of BCG-containing phagosomes with lysosomes (Clements *et al.*, 1995; Reyrat *et al.*, 1995; Grode *et al.*, 2005). The lack of phagosome–lysosome fusion inhibits the trafficking of BCG-derived antigens through the major histocompatibility class (MHC) II pathway, which is enrolled for preferential stimulation of $CD4^+$ T cells, the most important cells involved in inhibition of *M. leprae* growth (Sendide *et al.*, 2004). Further, macrophages produce abundant amounts of IL-10 on infection with BCG, which, in turn, inhibits the activation of $CD4^+$ T cells (Mochida-Nishimura *et al.*, 2001; Granelli-Piperno *et al.*, 2004).

In the present study, we constructed a recombinant BCG (BCG-ΔUT) that lacks a *urease* gene through allelic exchange of chromosomal DNA. As urease is involved in the maintenance of intraphagosomal pH at neutral (Grode *et al.*, 2005) or slightly alkaline values (Sendide *et al.*, 2004), lack of this enzyme may contribute to the induction of phagosomal acidification (Sendide *et al.*, 2004), thereby promoting the fusion of BCG-containing phagosomes with lysosomes. The efficient colocalization of BCG-ΔUT with lysosome was observed, leading us to expect an efficient enhancement of T-cell activation by BCG-ΔUT-infected macrophages. Previously, rBCG deficient in urease C was produced by a similar system and found to be superior to parental BCG in producing acidic conditions (pH 4.5–5.5) in BCG-infected phagosomes in murine macrophages (Reyrat *et al.*, 1995; Grode *et al.*, 2005). However, it was not demonstrated whether the rBCG deficient in urease C promoted the MHC class II trafficking pathway and actually activated human $CD4^+$ T cells through APCs. The newly constructed BCG-ΔUT lacked urease activity and *in vitro* studies confirmed that it could not degrade urea to ammonia. When BCG-ΔUT was infected to macrophages, it activated human $CD4^+$ T cells more efficiently than the parental BCG. However, the amount of IFN-γ released from the T cells was not as high as expected (< 50 pg mL^{-1}). These results suggest that

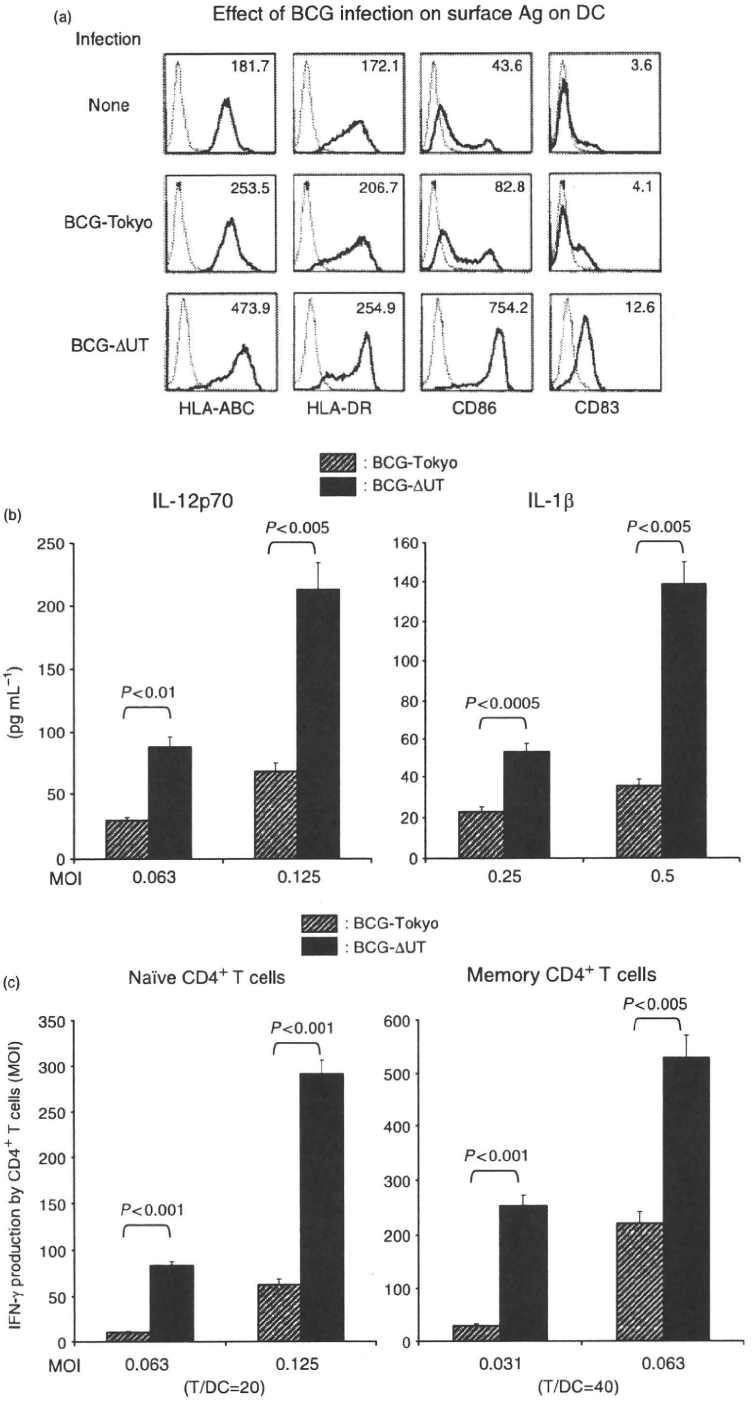

Fig. 6. (a) Expression of various molecules on BCG-infected DCs. Monocyte-derived immature DCs were infected with either BCG-Tokyo or BCG-ΔUT at an MOI of 0.25 and cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DCs from day 5 were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test mAb. The number in the top right-hand corner of each panel represents the difference in mean fluorescence intensity between the control IgG and the test mAb. Representative results of three separate experiments are shown. (b) Cytokine production from DCs stimulated by BCG. Monocyte-derived DCs from 4 days of culture in the presence of rGM-CSF and rIL-4 were stimulated with the indicated dose of either BCG-Tokyo or BCG-ΔUT for 24 h. The concentration of the indicated cytokine was determined by the ELISA method. A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test. (c) IFN-γ production by naïve CD4$^+$ T cells and memory CD4$^+$ T cells. DCs obtained from monocytes infected with either BCG-Tokyo or BCG-ΔUT were used as a stimulator of naïve and memory CD4$^+$ T cells in a 4-day culture. A representative of three separate experiments is shown. Assays were performed in triplicate and the results are expressed as the means ± SD. Titers were statistically compared using Student's *t*-test.

improvement of intraphagosomal pH milieu for efficient phagosome–lysosome fusion was not sufficient for the induction of full T-cell activation as far as macrophages were concerned. Thus, we further searched for factors which might be helpful in inducing full activation of T cells. First, we examined the influence of endogenously produced IL-10, as abundant IL-10 was produced from macrophages by infection with BCG-ΔUT (data not shown). The neutralization of IL-10 from macrophages drastically enhanced T-cell activation (Fig. 3a). Furthermore, pretreatment of

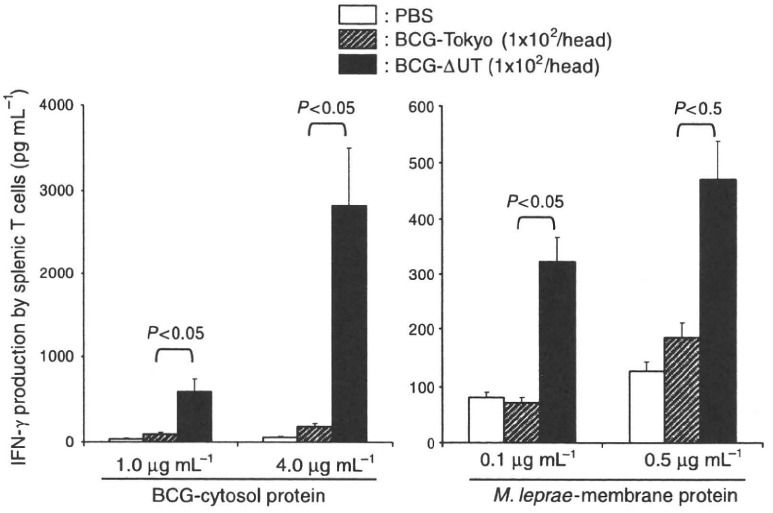

Fig. 7. IFN-γ production by splenic T cells obtained from C57BL/6 mice infected with BCG-Tokyo or BCG-ΔUT. Five-week-old C57BL/6 mice were infected with the indicated dose of BCG intradermally. Four weeks after the inoculation, splenocytes (2×10^5 cells well^{-1}) were stimulated with the indicated dose of either BCG-derived cytosol protein or *Mycobacterium leprae*-derived membrane protein for 4 days. Assays were performed in triplicate for each mouse, and the results for three mice per group are given, expressed as the means ± SD. Representative results for two separate experiments are shown. Titers were statistically compared using Student's *t*-test.

macrophages with GM-CSF, which is normally produced from activated $CD4^+$ T cells, monocytes and macrophages (data not shown), and inhibits IL-10 production (Makino *et al.*, 2007), was also quite efficient in enhancing the BCG-ΔUT-mediated T-cell activity. Therefore, the unexpectedly weak activation of $CD4^+$ T cells by BCG-ΔUT seemed to be at least partly due to the immunosuppressive effect of IL-10. Secondly, we focused on the costimulating factors capable of actively up-regulating the T-cell-stimulating function of macrophages, and found that both CD40L and IFN-γ were quite efficient. It was previously reported that both CD40L and IFN-γ were needed to costimulate macrophages infected with *M. leprae* (Makino *et al.*, 2007); however, in the present study, the sole treatment of BCG-ΔUT-infected macrophages with either CD40L or IFN-γ was enough to confer a sufficient effect (Figs 4 and 5). The high sensitivity of BCG-ΔUT-infected macrophages to CD40L may be due to the ability of rBCG to induce greater expression of CD40 (Fig. 4a). The exogenous IFN-γ may contribute to increased production of IFN-γ from T cells by activating macrophages, as it enhanced the surface expression of HLA-DR and CD86 on BCG-ΔUT-infected macrophages, which facilitated antigen-specific T-cell activation. As reported, *M. leprae* is less sensitive to IFN-γ (Makino *et al.*, 2007), and also parental BCG was found to be clearly less sensitive to IFN-γ than BCG-ΔUT. These results indicate that each mycobacterium may have differential sensitivity to IFN-γ (Verreck *et al.*, 2004). Although the molecular mechanism responsible for the difference in sensitivity remains unexplained, it is well known that IFN-γ facilitates the digestion of intracellular mycobacteria in macrophages, and thus the following speculation may be possible: in the present system, the alteration of the pH milieu of BCG-containing phagosomes caused by the depletion of urease activity may help to establish circumstances where cell activation as well as enhanced trafficking of mycobacterial antigens to the surface by the MHC class II pathway can be induced by IFN-γ treatment. The urease gene of pathogenic mycobacteria may be a good target for combination immunotherapy/chemotherapy as urease depletion downregulates the growth of mycobacteria (data not shown) and upregulates the immunoactivity of intracellular digestion of bacteria in host cells.

In contrast to macrophages, DCs were highly activated by the sole infection with BCG-ΔUT in terms of phenotype and cytokine production, and BCG-ΔUT-infected DCs efficiently activated both naïve and memory $CD4^+$ T cells in the absence of additional costimulation. The activated T cells produced abundant amounts of both IFN-γ (Fig. 5c) and GM-CSF, and induced CD40L expression (data not shown). Therefore, DCs can inherently provide the critical factors needed by BCG-ΔUT-infected macrophages. As BCG infects both macrophages and DCs *in vivo*, we evaluated the efficacy of BCG-ΔUT as a T-cell activator by using C57BL/6 mice. BCG-ΔUT was superior to BCG-Tokyo in the production of murine memory $CD4^+$ T cells, which can respond to BCG-derived recall antigen and also proteins derived from pathogenic *M. leprae*. Just 100 BCG-ΔUT bacilli were sufficient to produce such memory T cells. These findings indicate that BCG-ΔUT convincingly stimulated $CD4^+$ T cells *in vivo*. As the C57BL/6 strain is a T helper (Th)1 response-prone mouse, further study using Th2 response-prone mice would provide further insight into how memory T cells are generated by inoculation with BCG-ΔUT.

Taking our data together, BCG-ΔUT is more potent than the parental BCG in the activation of macrophages, DCs and $CD4^+$ T cells. The depletion of urease from BCG may be useful in upregulating the potency of BCG as an immunostimulator.

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Bacterial Entry to the Splenic White Pulp Initiates Antigen Presentation to CD8$^+$ T Cells

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SUMMARY

The spleen plays an important role in host-protective responses to bacteria. However, the cellular dynamics that lead to pathogen-specific immunity remain poorly understood. Here we examined *Listeria monocytogenes* (Lm) infection in the mouse spleen via in situ fluorescence microscopy. We found that the redistribution of Lm from the marginal zone (MZ) to the periarteriolar lymphoid sheath (PALS) was inhibited by pertussis toxin and required the presence of CD11c$^+$ cells. As early as 9 hr after infection, we detected infected dendritic cells in the peripheral regions of the PALS and clustering of Lm-specific T cells by two-photon microscopy. Pertussis toxin inhibited both Lm entry into the PALS and antigen presentation to CD8$^+$ T cells. Our study suggests that splenic dendritic cells rapidly deliver intracellular bacteria to the T cell areas of the white pulp to initiate CD8$^+$ T cell responses.

INTRODUCTION

The spleen is a highly structured lymphoid organ consisting of three main compartments: the red pulp (RP) where red blood cells are captured and recycled, the marginal zone (MZ) containing marginal zone B cells and phagocytes positioned along the marginal sinus, and the white pulp (WP) where the majority of T cells and B cells reside (Mebius and Kraal, 2005). As microbes enter the marginal sinus of the spleen, they come in contact with a variety of splenic phagocytes, including macrophages, dendritic cells (DCs), and granulocytes (Kraal, 1992). DCs play an important role in carrying antigen from peripheral tissues to draining lymph nodes to trigger the adaptive immune response (Angeli and Randolph, 2006); however, an analogous picture for how antigen transport and presentation takes place in the spleen is lacking. Experiments that use low-dose clodronate-liposome treatment to selectively deplete marginal zone macrophages (MZM) showed that these cells were not required for adaptive immunity to *Listeria monocytogenes* (Lm) (Aichele et al., 2003). An important role for DCs in immunity to Lm infection was more formally demonstrated via the CD11c-DTR system to deplete DCs (Jung et al., 2002; Muraille et al., 2005; Neuenhahn et al., 2006). The issue of whether bacterial antigen transport to the periarteriolar lymphoid sheath (PALS) of the spleen is required for antigen presentation to T cells has been raised by others (Muraille et al., 2005; Kursar et al., 2005) but has not been investigated directly.

To investigate how antigen presentation occurs in the spleen, we used the Lm infection model. Lm is a Gram-positive intracellular bacterium widely used to study innate and adaptive immunity in mice (Mackaness, 1962; Pamer, 2004; Unanue, 1997). After intravenous (i.v.) injection, Lm infects the liver and spleen, proliferates in these tissues for several days, and is eventually cleared by Lm-specific T cell responses. Mice that survive Lm challenge develop long-lasting CD8$^+$ T cell immunity (Busch et al., 1998; Lauvau et al., 2001; Shedlock and Shen, 2003; Sun and Bevan, 2003). Although T cell responses to Lm infection have been studied extensively, only recently has the impact of tissue structure on Lm pathogenesis and development of host responses been examined in situ (Khanna et al., 2007).

Here, we provide evidence that DCs in the spleen transport bacteria from the MZ to the PALS for antigen presentation to T cells, analogous to their role in carrying antigens from peripheral tissues to draining lymph nodes. As early as 9 hr after infection, we observed the clustering of Lm-specific T cells in the PALS by two-photon (2P) microscopy, indicating that antigen recognition occurs rapidly in the spleen. Preventing the entry of Lm into the PALS by pretreating mice with pertussis toxin (PTx) inhibited both the progression of Lm infection and the initiation of antigen presentation to CD8$^+$ T cells.

RESULTS

PTx Treatment Inhibits Lm Entry into PALS

We quantitatively examined the tissue distribution of bacteria during splenic infection. Lm was injected i.v. into BALB/c mice, and at different times after infection, spleen cryosections were stained with antibodies to Lm and B220 and with phalloidin (Muraille et al., 2005) to identify the major spleen compartments (Figure S1 available online). Lm redistributed dramatically from MZ and RP to the PALS over the first 24 hr of infection (Figures 1A and 1B), consistent with previous histological studies (Conlan, 1996; Muraille et al., 2005). Initially, bacteria were found primarily in the MZ (~65%) and RP (~30%) (2 hr, Figures 1A and 1C). Lm was not observed in the PALS before 6 hr but was

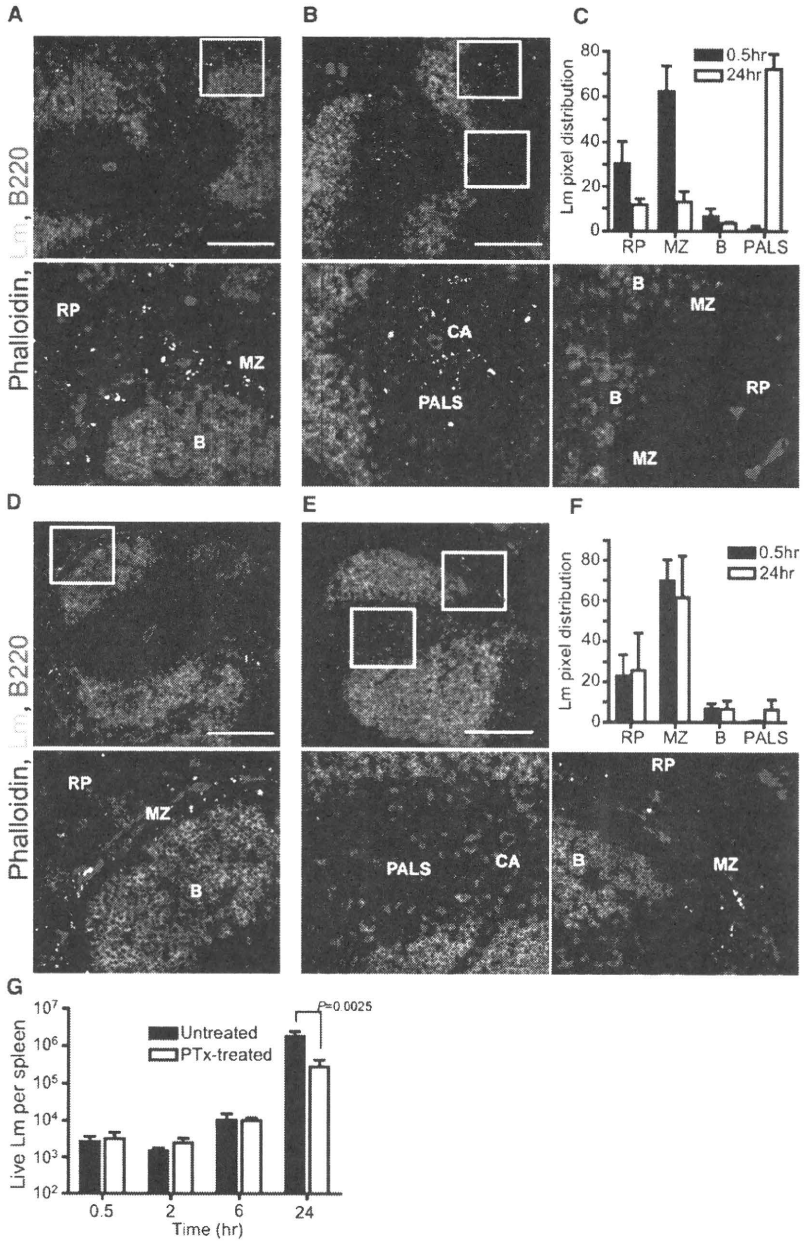

Figure 1. PTx Prevents Lm Translocation to the PALS and Decreases CFUs in the Spleen

(A) Spleen sections of mice 0.5 hr after infection with 10^7 Lm. Cryosections were stained with phalloidin-Alexa350 (blue), Lm antibody (green), and anti-B220-PE (red). Lower panels show magnified regions (white squares in upper panels). Scale bar represents 50 μm.

(B) Spleen sections of mice 24 hr after infection with 10^5 Lm. At 24 hr, many Lm are observed in the PALS area and few in the MZ and RP. Scale bar represents 50 μm.

(C) Quantification of Lm distribution in the spleen at 0.5 hr (black bar) and 24 hr (white bar) after infection (10 pooled nonoverlapping images from 2 mice). The splenic compartments: RP, red pulp; MZ, marginal zone; B, B cell follicle; and PALS, T cell area were separated as described in Figure S1.

(D and E) Spleen sections of PTx pretreated mice at 0.5 hr (D) and 24 hr (E) after infection. Scale bars represent 50 μm. Lower panels show magnified regions (white squares in upper panels).

(F) Lm distribution in the spleen of PTx-pretreated mice at 0.5 hr (black bar) and 24 hr (white bar) after infection (10 pooled nonoverlapping images from 2 mice).

(G) CFU per spleen in PTx-untreated mice (black bar) and PTx-pretreated mice (white bar) at 0.5 hr to 24 hr after infection with Lm (two separate experiments, n = 5 to 7 mice per group). The p value (0.0025) was calculated with a two-tailed Mann-Whitney U-test.

detected in low numbers at 9 hr and 12 hr (data not shown). After 24 hr, the distribution of Lm was reversed and the vast majority of bacteria were located in the PALS of the WP (~70%), with some bacteria (~20%) remaining in the MZ and RP (Figures 1B and 1C).

Soluble substances (~75 kD) can flow directly from the MZ into the white pulp through a conduit network (Mebius and Kraal, 2005). Although bacteria are presumably too large to access the PALS via this conduit network, we examined the possibility that Lm might alter conduit permeability and flow directly into the WP to initiate PALS infection. First, we injected a large number of 1 μm fluorescent beads (~10^8 or 1000 times more than the Lm dose) and analyzed their distribution in the absence of infection. Beads were found exclusively in the MZ and RP at 2 hr and only a few entered the PALS at 24 hr (Figures S2A and S2B). Next we coinjected high molecular weight dextran and Lm to determine whether infection alters access to the WP (Figure S2C). Dextran remained outside the PALS 2 hr after coinjection with Lm, indicating that infection did not markedly affect conduit permeability over this time. We also injected 0.5 μm beads (approximately the diameter of Lm) 4 hr after infection and found that the beads localized to the MZ and RP and did not enter the PALS. Moreover, live Lm in the spleen is insensitive to the cell-impermeable antibiotic gentamicin within minutes of i.v. challenge (Figure S2D), suggesting that bacteria are intracellular and not free to enter the PALS independently.

We postulated that Lm entry into the PALS requires the migration of host cells. To test this hypothesis, we treated mice with 500 ng pertussis toxin (PTx) i.p. and then examined Lm tissue distribution at 0.5 and 24 hr after Lm challenge (Figures 1D and 1F). PTx has been shown to block G protein-mediated (Gαi) chemokine receptor signaling and to inhibit leukocyte trafficking (Ato et al., 2004; Huang et al., 2007; Itano et al., 2003; Okada and Cyster, 2007). PTx treatment did not change the initial tissue

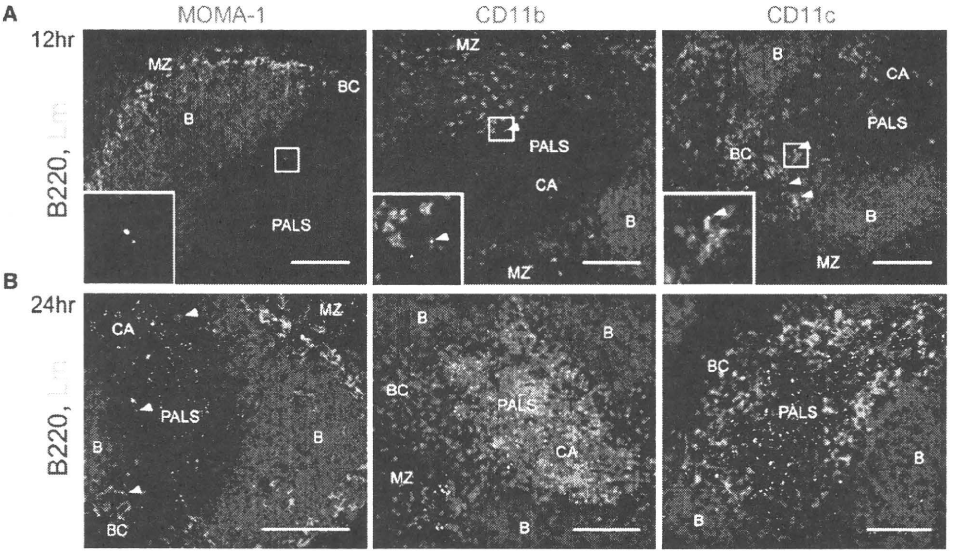

Figure 2. Lm-Infected Host Cells in the PALS

Immunofluorescence images of Lm and various host cell markers in spleen at 12 hr (A) and 24 hr (B) after infection. Cryosections were stained for B220 (blue), Lm (green), and the antibodies to host cell markers as indicated in the figure. Scale bars represent 100 μm. Lm was associated with CD11b$^+$ and CD11c$^+$ cells at 12 hr in the PALS (white arrows) but not with MOMA-1$^+$ metallophilic marginal zone macrophages. At 24 hr after infection, bacteria shown as yellow spots in the image associated with CD11b$^+$ cells and CD11c cells. Lm in MOMA-1$^+$ cells is indicated with white arrows. The lower left inset shows a magnified region in the white square. Images are representative of at least three independent experiments.

distribution or the amount of Lm detected in spleen sections (Figures 1D and 1F); however, it profoundly inhibited Lm entry into the PALS (~99% decrease) 24 hr after challenge (Figures 1E and 1F). The early trapping and growth of Lm appeared normal after PTx treatment because Lm colony-forming units (CFU) in PTx-treated and control mice were similar for up to 6 hr (Figure 1G), and the initial cellular distribution of Lm appeared unaffected by PTx (data not shown). At 24 hr after infection, when Lm proliferation takes place primarily in the PALS, the number of viable bacteria was markedly reduced (~90% decreased) compared with untreated mice (Figure 1G). Thus, the reduced number of bacteria in the PALS after PTx treatment is primarily due to inhibition of bacterial entry and not due to effects on Lm capture or growth during the early stage of infection.

Spleen Remodeling in Response to Lm Infection

We examined the distribution of host cells in the spleen before and after infection to identify the cell types that migrate to the PALS and possibly transport Lm. In uninfected mice, host cell populations were observed in their expected locations in the spleen (Figure S3A). At 12 hr, Lm colocalized with CD11b$^+$ cells in the PALS and CD11c$^+$ cells primarily near the bridging channel, a structure that connects the WP to the RP (Figure 2A; Metlay et al., 1990; Mitchell, 1973). Metallophillic marginal zone macrophages (MOMA-1$^+$) (Figure 2A; Kraal and Janse, 1986) had not entered the PALS in substantial numbers at this time. By 24 hr after infection, the number of MOMA-1$^+$, CD11b$^+$, and CD11c$^+$ cells had increased in the PALS (Figure S3B). In contrast, F4/80$^+$ (RP macrophages), MARCO$^+$ (Elomaa et al., 1995), and ER-TR9$^+$ (MZM) (Dijkstra et al., 1985) remained in their expected locations, similar to uninfected controls (Figures S3A and S3B). At 24 hr, we found extensive Lm growth in the PALS and many CD11b$^+$ cells contained Lm at foci of infection (Figure 2B). Bacteria were also found in MOMA-1$^+$ (Figure 2B, arrowhead) and CD11c$^+$ cells, although in lower numbers than observed for CD11b$^+$ cells.

Lm Entry into PALS Requires CD11c$^+$ Cells

To determine which cells are involved in Lm invasion of the PALS, we took advantage of the different recovery kinetics of splenic macrophages and DCs after clodronate-liposome-mediated depletion (van Rooijen et al., 1989) and looked for an association between host-cell recovery and Lm infection of the PALS.

The injection of clodronate liposomes ablated splenic macrophages by day 2 after treatment and these cells remained depleted for at least 1 week (Figure S4), as reported previously (van Rooijen et al., 1989). In our hands, clodronate liposomes also depleted CD11c$^+$ cells by day 2, but these cells recovered substantially by day 6 (Figure S4). Similar DC recovery kinetics were observed in work with the CD11c-DTR transgenic system (Jung et al., 2002) and is consistent with the high turn-over rate (3 days) reported for splenic CD11c$^+$ cells (Kamath et al., 2000). Notably, CD11b$^+$ cells were not depleted by clodronate liposome treatment and appeared to slightly increase in number by day 2 (Figure S4). The CD11b$^+$ cells identified in our sections are most likely granulocytes (~95% are F4/80$^-$ and Gr-1$^+$, data not shown) and recently recruited inflammatory monocytes or tumor necrosis factor-alpha and inducible nitric oxide synthase-producing DCs (TipDCs) (Serbina et al., 2003).

To determine which cells play a role in the translocation of Lm to PALS, we depleted splenocytes with clodronate liposomes, then infected mice with Lm on either day 2 or 6 after clodronate

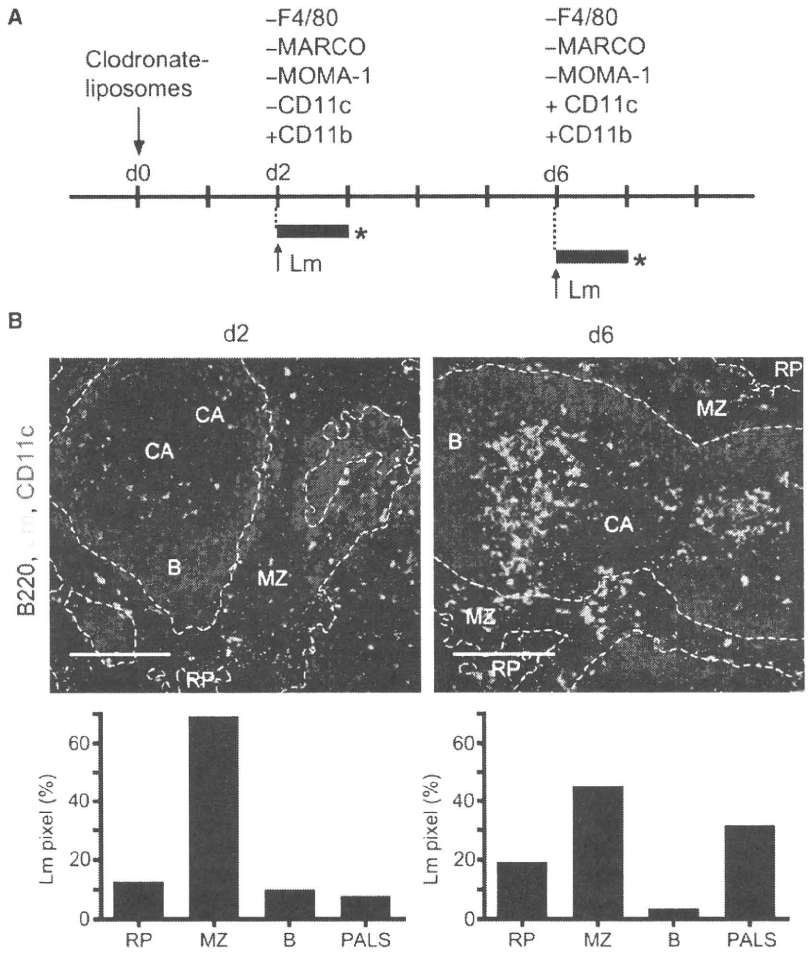

Figure 3. CD11c$^+$ Recovery after Clodronate-Liposome Depletion Correlates with PALS Infection

(A) Outline of the experimental protocol. Clodronate liposomes were prepared as described previously and administered to deplete splenocytes 2 or 6 days prior to infection. Mice were infected with 10^5 Lm and sections were stained for CD11c (red) and Lm (green) to assess the degree of PALS infection 24 hr later (asterisk).

(B) Spleen cryosections from clodronate-treated mice (top) infected on day 2 (left) or day 6 (right). Scale bars represent 200 μm. Dashed lines indicate MZ regions based on phalloidin staining (blue). Quantitative analysis (bottom) of Lm distribution in splenic compartments. Data are representative of two or more independent experiments.

administration. The PALS was examined 24 hr later for the presence of Lm (Figure 3A). In mice infected on day 2, when both macrophages and CD11c cells were depleted, the vast majority of bacteria (~70%) were found in the MZ (Figure 3B, left) and <10% of the total Lm signal was present in the PALS, compared to ~70% in infected mock-depleted controls (Figure 1B). In mice infected on day 6 after clodronate treatment, when CD11c$^+$ cells had recovered but macrophages had not, widespread Lm growth in the PALS was observed (Figure 3B, right), suggesting that DCs play an important role in the progression of Lm infection to the PALS. Because CD11b$^+$ cells are present on day 2 when Lm remains in the MZ, these cells do not play a dominant role in the initial entry of Lm into the PALS, but most likely follow the bacteria into the PALS once it becomes infected. The close association between DC appearance and PALS infection was confirmed by means of CD11c-DTR mice (Jung et al., 2002) and a similar cell depletion and recovery approach (Figure S5).

The Arrival of Infected DCs in the PALS Initiates Antigen Presentation

The precise location of T cell priming in the spleen is unknown. T cells are most abundant in the PALS; however, they are also found in the RP and MZ and antigen presentation could occur in these compartments as well. Based on our depletion and recovery results, we speculated that the entry of infected DC into the PALS triggers antigen presentation to T cells. To test this hypothesis, we used Lm-specific CD8$^+$ T cells from WP11.12 TCR transgenic mice (Mercado et al., 2000; Wong and Pamer, 2003) to detect antigen presentation in situ. BALB/c mice received 4 × 10^6 adoptively transferred CFSE-labeled WP11.12 CD8$^+$ T cells and cells were allowed to home (4–8 hr) before mice were infected with Lm. At various times after infection, spleen cryosections were examined by immunofluorescence microscopy for T cell clustering as evidence of antigen recognition (Figure 4A). In the absence of infection (0 hr), WP11.12 T cells were distributed evenly in the PALS area and appeared bright and small, consistent with a resting phenotype. As early as 9 hr after infection, many WP11.12 T cells clustered in the PALS near the bridging channel (9 hr, inset), indicating that antigen presentation had begun. At 21 hr after infection, clusters had increased in size and moved into the center of the PALS, and by 41 hr, T cells had dissociated and large activated CFSE^{lo} WP11.12 T cells were distributed evenly through out the PALS (41 hr, inset). We could not detect WP11.12 T cell clusters in the MZ or RP over this time frame.

Infected CD11c$^+$ cells were often associated closely with WP11.12 T cell clusters (Figure 4B, CD11c). These Lm-containing cells were MHC II$^+$ and showed characteristic DC morphology (Figure 4B, MHCII). Although CD11b$^+$ cells were found near T cell clusters, they were not MHC II$^+$ and did not localize to the center of clusters (data not shown). Lm was typically observed in the center of T cell clusters in cryosections (Figure 4B) and in spleen explants via GFP-labeled bacteria and 2P microscopy (Figure 4C). We did not find T cell clusters associated with MARCO$^+$ or MOMA-1$^+$ macrophages during the first 41 hr of infection (data not shown). Thus, DCs appear to