Immunostimulatory Activity of Recombinant *Mycobacterium bovis* BCG That Secretes Major Membrane Protein II of *Mycobacterium leprae*

Masahiko Makino,* Yumi Maeda, and Katsuya Inagaki

Department of Microbiology, Leprosy Research Center, National Institute of Infectious Diseases, 4-2-1 Aobacho, Higashimurayama, Tokyo 189-0002, Japan

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We previously demonstrated that major membrane protein II (MMP-II) is one of the immunodominant antigens (Ags) of *Mycobacterium leprae* capable of activating T cells through Toll-like receptor 2. Based on the observation that *Mycobacterium bovis* BCG secreting a 30-kDa protein offered better protection against tuberculosis, we constructed a recombinant BCG strain (BCG-SM) that secretes MMP-II to improve the potency of BCG against leprosy. The secreted MMP-II protein from BCG-SM stimulated monocyte-derived dendritic cells (DC) to produce interleukin-12. DC infected with BCG-SM expressed MMP-II on their surfaces, and MMP-II expression was suppressed by the pretreatment of DC with chloroquine. These results indicated that secreted MMP-II was processed by DC for higher expression levels on their surfaces. In addition, BCG-SM phenotypically activated DC and induced higher expression levels of major histocompatibility complex, CD86, and CD83 Ags on DC than did vector control BCG (BCG-pMV). The DC infected with BCG-SM more efficiently stimulated naïve and memory CD4⁺ T cells and memory CD8⁺ T cells to produce gamma interferon than did those infected with BCG-pMV. However, naïve CD8⁺ T cells were significantly activated only when they were stimulated with BCG-SM-infected DC. When CD8⁺ T cells were cocultured with BCG-SM-infected DC, the proportion of perforin-producing T cells was significantly higher than that in cells cocultured with BCG-pMV-infected DC. Moreover, MMP-II-specific memory T cells were more efficiently produced in mice inoculated with BCG-SM than in mice inoculated with BCG-pMV. Taken together, these results indicate that BCG capable of secreting the immunodominant Ag is more potent in the stimulation of T cells.

Although *Mycobacterium bovis* bacillus Calmette-Guérin (BCG) carries a risk of inducing disseminated disease in some individuals (3), BCG is the most widely used live attenuated vaccine against pathogenic mycobacterial infections, such as those with *Mycobacterium tuberculosis* and *Mycobacterium leprae*. In some studies, BCG has been shown to partially protect against leprosy, but it was not effective in other studies (15, 22, 25). Thus, there exists no convincing vaccine against leprosy, and as a result, half a million new cases are still detected every year (30). The emergence of multidrug-resistant strains and the complexity of leprosy reactions are also distressing (16). Therefore, the urgent development of a more efficacious leprosy vaccine is desired.

Intracellular bacteria such as BCG remain in the phagosomes of antigen-presenting cells (APCs), such as macrophages and dendritic cells (DC), and primarily stimulate CD4⁺ T cells via antigen (Ag) presentation through the major histocompatibility complex (MHC) class II pathway (10, 14). Furthermore, MHC class I-restricted activation of CD8⁺ T cells, which occur preferentially through cross-priming, is also dependent on the activation of APCs (12). In addition, such APC-mediated activation of both CD4⁺ and CD8⁺ T cells, especially of type 1 cells, plays an important role in the host defense mechanism against *M. leprae* infection (7). In fact, patients with tuberculoid leprosy, a representative clinical leprosy on one pole, enroll DC as APCs to induce the Ag-specific activation of both CD4⁺ and CD8⁺ T cells, leading to the restriction of *M. leprae* in granulomas (13, 26). Therefore, the efficient activation of these T cells is the most important process in suppressing the spread of the bacteria and controlling the multiplication of *M. leprae*. In this process, the expression of immunodominant Ags on the surfaces of DC is thought to be advantageous. We recently identified major membrane protein II (MMP-II) (gene name, *bfrA* or ML2038; also known as bacterioferritin) as one of the immunostimulatory Ags of *M. leprae* (17). MMP-II stimulates DC to produce interleukin-12 (17, 19). On the other hand, T cells from patients with lepromatous leprosy were markedly activated by stimulation with MMP-II (19). In addition, memory-type T-cell subsets from tuberculoid leprosy patients were induced by stimulation with MMP-II (19). On the other hand, T cells from patients with lepromatous leprosy, a representative leprosy on the opposite pole of the clinical spectrum, are sometimes refractory to stimulation with *M. leprae*-derived Ag (5, 23), and thus these T cells need to be stimulated potently to prevent the multiplication of bacilli. However, in previous studies, we showed that the activities of T cells of lepromatous leprosy patients and those of BCG-vaccinated healthy individuals were comparable when stimulated by MMP-II-pulsed DC (19). Therefore, MMP-II could be a promising candidate in terms of activating T cells.

Other reports showed that proteins secreted within host cells from intracellular parasitic pathogens, including *M. tuberculosis* and *Legionella pneumophila*, are potent inducers of T-cell...
activation (1, 8). Furthermore, the immunization of mice or guinea pigs with a 30-kDa major secretory protein (a-antigen or Ag85A) of M. tuberculosis substantially protected them from the development of tuberculosis (9). Also, mice could be protected more efficiently against tuberculosis by vaccination with live BCG rather than killed BCG (6). When the exposure of Ag to host cells is increased through a live vehicle, such as BCG, it is more efficient in the activation of host defense mechanisms (8). Therefore, in this study, we used live BCG as a delivery vehicle for M. leprae-derived MMP-II and constructed a recombinant BCG strain secreting MMP-II. This system would enable better access of MMP-II to the APCs, which may, in turn, stimulate T cells more effectively. We demonstrate that the recombinant BCG strain (BCG-SM) is more potent than the parental strain in the activation of CD4+ and CD8+ T cells, both in vitro and in vivo.

MATERIALS AND METHODS

Preparation of cells. Peripheral blood was obtained from healthy purified protein derivative-positive individuals with their informed consent. In Japan, BCG vaccination is compulsory for children (0 to 4 years old). Peripheral blood mononuclear cells (PBMC) were isolated using Ficoll-Paque Plus (Pharmacia, Uppsala, Sweden) and were cryopreserved in liquid nitrogen until use, as previously described (18). For the preparation of peripheral monocytes, CD3+ T cells were removed from either freshly isolated heparinized blood or cryopreserved PBMC, using immunomagnetic beads coated with anti-CD14 monoclonal antibody (MAB) (Dynabeads 450; Dynal, Oslo, Norway). The CD3+ PBMC fraction was plated on collagen-coated plates, and the nonplastic adherent cells were removed by extensive washing. The remaining adherent cells were used as monocytes (28). Monocyte-derived DC were differentiated as described previously (18, 20). Briefly, monocytes were cultured in the presence of 50 ng of recombinant granulocyte-macrophage colony-stimulating factor (rGM-CSF; Pepro Tech EC Ltd., London, England) and 10 ng of rIF-4 (Pepro Tech) per ml (20). On day 3 of culture, immature DC were infected with recombinant BCG at the indicated multiplicity of infection (MOI), and on day 5 of culture, DC were used for further analyses of surface antigens and for mixed-lymphocyte assays.

Vector construction and preparation of recombinant BCG. For preparation of the recombinant BCG strain secreting MMP-II, plasmid pMV-SM was constructed to have a kanamycin resistance gene and origins for replication of Escherichia coli and mycobacteria. Briefly, the MMP-II-encoding gene was cloned from M. leprae (Thai 53 strain) genomic DNA by PCR, using the forward primer 5’ CAGGAATTCATGCAAGGTGATCCGGATG3’ and the reverse primer 5’ GAAGGATCCAATGCAGCTTGTTGACAGGG3’ (H11032). The secretion signal sequence of Ag85A of M. tuberculosis was amplified by PCR, using the primers 5’ GAAATCGATTTAACTCGGCGGCCGGGAGA3’ (H11002) and 5’ CAGGAATTCATGCAAGGTGATCCGGATG3’ (H11001) to generate pMV-SM. An-other plasmid, pMV-PM, was obtained by switching the Hsp60 promoter sequence of pMV-SM to the Ag85A promoter sequence.

BCG substrain Pasteur was cultured in vitro using Middlebrook 7H9 broth (BD Biosciences Pharmingen, San Jose, CA) supplemented with 0.05% Tween 80 and 10% albumin-dextrose-catalase (BD Biosciences) or Sauton medium containing 0.05% Tween 80. Expression vectors were introduced into BCG by electroporation (27). Transformants were selected on Middlebrook 7H10 agar (BD Biosciences) plates. BCG containing pMV-PM as an extrachromosomal plasmid is referred to as BCG-SM, and that containing pMV-261 is referred to as BCG-pMV (BCG vector control). Recombinant BCG strains were grown to log phase and stored at 106 CFU/ml at –80°C. Before stimulation of DC, BCG cells were counted by the colony assay method and/or the Ziehl-Neelsen staining method. Heat-killed recombinant BCG was prepared by incubating BCG at 60°C for 15 h to kill the mycobacteria completely.

Expression of MMP-II. To verify the secretion of MMP-II from BCG-SM, the cultured supernatant of BCG-SM as well as BCG-pMV, cultured for 20 days in Sauton medium, were collected and washed through a 10,000-weight-
cutoff Amicon Ultra-4 membrane (Millipore, Billerica, MA) after being depleted of cells by centrifugation. The protein fractions of the recombinant BCG strains were prepared as follows. Polyanylidene difluoride-harvested cells were washed with phosphate-buffered saline (PBS) and disrupted with a bead homogenizer. Disrupted cells were centrifuged at 10,000 × g at 4°C for 30 min, and the supernatant was taken as the protein fraction.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and electroblotting were carried out using standard methods (24). Western blotting was performed as follows. Briefly, a membrane with the transferred proteins was blocked in 5% skim milk and then incubated with anti-MMP-II MAb 202-3 (immunglobulin G2a [IgG2a]) and kappa chain. Alkaline phosphatase-conjugated anti-mouse IgG was used as the secondary Ab. Color development was performed by using the nitroblue tetrazolium/5-bromo-4-chloro-3-indolylphosphate (NBT/BCIP) detection reagent (Calbiochem, San Diego, CA).

Analysis of cell surface Ags. Expression of cell surface Ags on DC was analyzed using a FACScan flow cytometer. Dead cells were eliminated from the analysis by staining with propidium iodide (Sigma Chemical Co., St. Louis, MO), and 1 × 105 live cells were analyzed. For the analysis of cell surface Ags, the following MAbs were used: fluorescein isothiocyanate-conjugated (FITC)-conjugated Abs against HLA-ABC (G46-2.6; Pharmingen, San Diego, CA), HLA-DR (L243; BD), CD1a (OKT6; Ortho Diagnostic Systems Inc., Raritan, NJ), CD80 (L307.4; BD), CD96 (FUN-1; BD), and CD83 (HBB15a; Immunotech, Marseille, France).

The expression of MMP-II on recombinant BCG-infected DC was determined using a MAb (M270-13; IgM, kappa chain) against MMP-II, which probably detects MMP-II in complex with MHC molecules on the surfaces of DC (19), followed by FITC-conjugated anti-mouse Ig Ab (Tago Immunologicals, Camarillo, CA). For the in vivo intracellular processing of phagocytosed bacteria, DC were treated with 50 μM of chloroquine (Sigma) for 2 h, washed, infected with BCG, and subjected to analyses of MMP-II surface expression. The intracellular production of perforin and granzyme B was assessed as follows. CD8+ T cells stimulated with BCG-infected DC were surface stained with a phycoerythrin-labeled MAB to CD8 (RPA-T8; BD) and fixed in 2% formaldehyde. Subsequently, they were permeabilized using permeabilizing solution (BD) and stained with FITC-conjugated MABs to perforin (6G9; BD) and granzyme B (GB11; BD).

APC functions of DC. The ability of BCG-infected DC to stimulate T cells was assessed by using an autologous DC–T-cell coculture as previously described (7, 20). Freshly thawed PBMC were depleted of NK cells, MHC class II+ cells, and either CD4+ or CD8+ cells by using magnetic beads coated with MAbs to CD56 Ag, MHC class II, and either CD8 or CD4 Ag (Dynabeads 450; Dynal, respectively) (20). The purity of the CD4+ and CD8+ T cells was >98%, as assessed using FACScan flow cytometry. Naïve CD4+ and CD8+ T cells were produced by further treatment of these T cells with a MAB to CD45RA Ag, which was followed by incubation with beads coated with goat anti-mouse IgG. Memory-type T cells were similarly produced by the treatment of cells with a MAB to CD45RA Ag. The purified responder cells (1 × 105) were per well were plated in 96-well round-bottomed tissue culture plates, and DC were added to give the indicated DC:T-cell ratio. Supernatants of DC–T-cell cocultures were collected on day 4, and the cytokine levels were determined.

Measurement of cytokine production. Levels of the following cytokines were measured: IFN-γ produced by CD4+ and CD8+ T cells and IL-12p70, IL-12p40, tumor necrosis factor alpha, and IL-1β produced by DC stimulated for 24 h with recombinant BCG cells. The concentrations of these cytokines were quantified using the enzyme assay kits in an Opt EIA human enzyme-linked immunosor-bent assay set (BD).

Animal studies. For inoculation into mice, recombinant BCG cells were cultured in Middlebrook 7H9 medium to log phase and stored at 105 CFU/ml at –80°C. Before the aliquots were used for inoculation, the concentration of viable bacilli was determined by plating cells on a Middlebrook 7H10 agar plate. Three 4-week-old C57BL/6J mice per group were inoculated subcutaneously with 0.1 ml of PBS or PBS containing 3.3 × 105 or 1 × 106 recombinant BCG cells. The animals were kept under specific-pathogen-free conditions and were supplied with sterilized food and water. Seven or 13 weeks after injection, the spleens were removed, and the splenic T cells were suspended at a concentration of 1 × 106 or 2 × 106 cells/ml in culture medium. The splenic T cells (1 × 105 or 2 × 106 cells/ml) were stimulated with the indicated DC:T-cell ratio. Supernatants of DC–T-cell cocultures were collected on day 4, and the cytokine levels were determined.

Statistical analysis. Student’s t test was applied to determine statistical differences.
RESULTS

Expression and secretion of MMP-II from recombinant BCG. To express and secrete MMP-II from recombinant BCG, two different plasmids were constructed, as described in Materials and Methods. The expression of the MMP-II gene is driven by the Hsp60 and Ag85A promoters (derived from \textit{M. tuberculosis}) in pMV-SM and pMV-PSM, respectively. In both expression vectors, the secretion signal of \textit{M. tuberculosis} Ag85A was inserted at the 5’ end of the MMP-II-encoding gene. Transformants (BCG-SM and BCG-PSM) were obtained with the BCG Pasteur strain. A putative premature 27-kDa MMP-II with a secretion signal was detected in the cell extracts of BCG-PSM and BCG-SM, in addition to the presumably processed 22-kDa MMP-II, as observed by Western blotting using a MAb to MMP-II (Fig. 1a). When the culture filtrates of BCG transformants were concentrated, the secreted MMP-II was detected in both BCG-PSM and BCG-SM. The Western blot analysis of BCG expressing \textit{M. leprae}-derived MMP-II is shown in Fig. 1b. A MAb to MMP-II was used to detect MMP-II in BCG cell extracts (a) and culture filtrates (b). BCG was transfected with the following plasmids: lane 1, pMV261; lane 2, pMV-PSM; and lane 3, pMV-SM. MW, molecular size markers.

FIG. 1. Western blot analysis of BCG expressing \textit{M. leprae}-derived MMP-II. A MAb to MMP-II (202-3 [IgG2a, kappa chain]) was used to detect MMP-II in BCG cell extracts (a) and culture filtrates (b). BCG was transfected with the following plasmids: lane 1, pMV261; lane 2, pMV-PSM; and lane 3, pMV-SM. MW, molecular size markers.

FIG. 2. Surface expression of MMP-II on DC infected with BCG. Immature DC were infected with either BCG-pMV or BCG-SM in the absence or presence of chloroquine (50 \textmu M) or were pulsed with heat-killed BCG-SM at an MOI of 5. After 2 days of culture with rGM-CSF and rIL-4, DC were gated and analyzed for the surface expression of MMP-II. Dotted lines, control IgM; solid lines, anti-MMP-II MAb (M270-13 [IgM, kappa chain]). Representative results for three separate experiments are shown.

Chloroquine treatment

(-) None BCG-pMV BCG-SM Heat-killed BCG-SM

(+) MMP-II

Ag85A was inserted at the 5’ end of the MMP-II-encoding gene. Transformants (BCG-SM and BCG-PSM) were obtained with the BCG Pasteur strain. A putative premature 27-kDa MMP-II with a secretion signal was detected in the cell extracts of BCG-PSM and BCG-SM, in addition to the presumably processed 22-kDa MMP-II, as observed by Western blotting using a MAb to MMP-II (Fig. 1a). When the culture filtrates of BCG transformants were concentrated, the secreted MMP-II was detected in both BCG-PSM and BCG-SM. The Western blot analysis of BCG expressing \textit{M. leprae}-derived MMP-II is shown in Fig. 1b. A MAb to MMP-II was used to detect MMP-II in BCG cell extracts (a) and culture filtrates (b). BCG was transfected with the following plasmids: lane 1, pMV261; lane 2, pMV-PSM; and lane 3, pMV-SM. MW, molecular size markers.

FIG. 2. Surface expression of MMP-II on DC infected with BCG. Immature DC were infected with either BCG-pMV or BCG-SM in the absence or presence of chloroquine (50 \textmu M) or were pulsed with heat-killed BCG-SM at an MOI of 5. After 2 days of culture with rGM-CSF and rIL-4, DC were gated and analyzed for the surface expression of MMP-II. Dotted lines, control IgM; solid lines, anti-MMP-II MAb (M270-13 [IgM, kappa chain]). Representative results for three separate experiments are shown.
A protein of 22 kDa was clearly seen (Fig. 1b). A lower level of the protein was also detected in the culture filtrate of BCG-PSM than in that of BCG-SM. The MMP-II purified from the BCG-SM culture filtrate stimulated DC and induced a significant level of IL-12p40 production: 1,016 pg/ml of IL-12p40 was produced from DC by stimulation with 0.5 μg/ml of purified MMP-II, while nonstimulated DC produced only a few pg/ml of the cytokine.

Characteristics of BCG-SM. The expression of immunodominant antigenic molecules on the surfaces of DC induces the efficient activation of T cells. To see the intracellular fate of secreted MMP-II from BCG-SM relative to that for MMP-II from the control BCG strain, BCG-pMV, which possesses the plasmid pMV-261, we measured the expression of MMP-II on the surfaces of DC, using an anti-MMP-II MAb. We assessed MMP-II expression by using multiple MOIs of recombinant BCG strains, and significant expression of MMP-II on DC was observed when BCG-SM was used for infection (Fig. 2). Furthermore, the expression of MMP-II was suppressed by the treatment of DC with chloroquine prior to BCG-SM infection and was also deprived by heat treatment of BCG-SM. These results may indicate that MMP-II secreted in the phagosomes of BCG-SM-infected DC was efficiently processed and translocated to the cell surface.

Professional APCs produce various cytokines, which facilitate or suppress T-cell activation. Therefore, we assessed the ability of BCG-SM to produce cytokines, including IL-12, tumor necrosis factor alpha, and IL-1β, from DC. Although these cytokines were induced significantly, there were no significant differences in cytokine production between DC stimulated with BCG-SM- and those stimulated with BCG-pMV (not shown). We then examined the influence of BCG-SM infection on DC from the aspect of the expression of surface Ags (Fig. 3). The expression of most Ags was modulated by BCG infection of DC, but there were apparent differences in the ability to modulate the expression level of Ags on DC between BCG-SM and BCG-pMV. While CD1a expression was down-regulated, the expression of HLA-ABC, HLA-DR, CD80, and CD83 was further up-regulated by infection with BCG-SM compared to the case for DC infected with BCG-pMV. These results indicate that BCG-SM phenotypically activated DC.

T-cell activation by BCG-SM. We assessed the T-cell stimulation activity of BCG-SM-infected DC. Strains BCG-SM and BCG-pMV had similar growth patterns in vitro (not shown). When naive CD4+ T cells from a healthy, BCG-vaccinated donor were stimulated with autologous DC which had been pulsed with either BCG-SM or BCG-pMV, both BCG strains significantly activated the T cells (Fig. 4, top panel). However, BCG-SM induced a significantly higher level of IFN-γ produc-
tion from the naïve CD4\(^+\) T cells. In contrast to CD4\(^+\) T cells, a significant production of IFN-\(\gamma\) from naïve CD8\(^+\) T cells was induced only when the CD8\(^+\) T cells were stimulated with DC pulsed with BCG-SM (Fig. 4, top panel). However, heat-killed BCG-SM lacked the ability to stimulate T cells, and the activation of both T-cell subsets induced by BCG-SM was partially decreased by the treatment of DC with a MAb to MMP-II (not shown). These results indicate that the secretion of MMP-II from BCG enhanced T-cell activation. Next, we examined the responsiveness of CD45RA\(^-\) memory-type T cells to BCG-SM (Fig. 4, bottom panel). Both BCG-SM and BCG-pMV stimulated both CD4\(^+\) and CD8\(^+\) T-cell subsets, and more IFN-\(\gamma\) production was achieved when BCG-SM-pulsed DC were used as a stimulator of the T cells. However, the heat-killed BCG strains did not induce significant T-cell activation (not shown).

The differentiation of CD8\(^+\) T cells to cytotoxic T lymphocytes is required for killing of host cells infected with mycobacteria; thus, we assessed the ability of BCG-SM to produce
in intracellular perforin (Fig. 5). We assessed this point by using multiple MOIs of the BCG strains, and we present the data for maximal expression, where a higher MOI did not change the expression level. Both BCG strains stimulated CD8+ T cells to produce perforin, but BCG-SM produced a larger number of perforin-producing CD8+ T cells than did BCG-pMV. Although we also assessed CD4+ T cells, they did not produce intracellular perforin by stimulation with either BCG strain (not shown). Furthermore, we examined whether BCG-stimulated CD8+ T cells produced intracellular granzyme B, and again, BCG-SM produced a larger number of granzyme B-producing CD8+ T cells than did BCG-pMV (not shown).

**Memory T-cell production by BCG-SM.** Another important aspect to be studied is the induction of Ag-specific memory T cells. To assess this point, we tested the T-cell responses of recombinant BCG-infected mice to MMP-II by using C57BL/6 mice. Splenic T cells obtained from mice infected subcutaneously with either BCG-SM or BCG-pMV were stimulated with MMP-II or parental BCG in vitro. At 7 weeks postinfection with either form of recombinant BCG, T cells responded to both MMP-II and BCG (Fig. 6, left panel). While IFN-γ production from T cells of BCG-infected mice in response to BCG was comparable between the two groups, the T cells from BCG-SM-infected mice responded to MMP-II by producing IFN-γ more vigorously than did those from BCG-pMV-infected mice. C57BL/6 mice were inoculated with either 3.3 × 10^5 or 10 × 10^5 bacteria per mouse, and similar results were obtained in both cases. When the T-cell responses of BCG-infected mice were analyzed at 13 weeks postinfection, again the IFN-γ production by T cells responding to BCG was not significantly different between the two groups of recombinant BCG-infected mice. However, a significantly higher T-cell response to MMP-II was achieved in the mice infected with BCG-SM than in those infected with parental BCG (Fig. 6, right panel).

**DISCUSSION**

The present study describes the immunostimulatory activity of a recombinant BCG strain secreting the antigenic protein MMP-II of *M. leprae* (BCG-SM). We constructed the BCG-SM strain for this study. BCG-SM secreted MMP-II into the culture medium, and the purified MMP-II from the culture filtrate had an IL-12-inducing ability in DC.

The most important characteristic of BCG for protection against subsequent invasion of *M. leprae* is the ability to stimulate the host immune system and to activate Ag-specific type 1 T cells for memory T-cell production. BCG is a potent inducer of CD4+ T cells but is an insufficient stimulator of CD8+ T cells (12). In this respect, BCG-SM more efficiently stimulated naive and memory-type CD4+ T cells to produce IFN-γ than did vector control BCG, and furthermore, both naive and memory CD8+ T cells were significantly activated by BCG-SM (Fig. 4). MMP-II (ML2038), originally identified as bacterioferritin by Pessolani et al. (21), was recently found to be highly immunostimulatory. Recombinant MMP-II stimulates DC to produce IL-12p70 and phenotypically activates DC by ligating to TLR2 (17, 19). Furthermore, MMP-II-pulsed DC efficiently stimulate host CD4+ and CD8+ T cells in an Ag-specific, MHC-dependent manner (17, 19). Therefore, MMP-II secreted from BCG-SM is probably involved in the increased activation of naïve T cells. It is evident that CD8+ T cells need to be differentiated into cytotoxic T cells to kill bacterium-infected target cells and to achieve sustained long-term control of mycobacterial infection (12). Perforin is one of the major cytolytic molecules of cytotoxic T lymphocytes. When we measured the production of intracellular perforin, BCG-SM was superior to BCG-pMV in producing perforin-positive T cells. Again, the secreted MMP-II may facilitate the production of cytotoxic T cells. Although the most susceptible host cells to *M. leprae* are Schwann cells, macrophages are also highly sensitive to *M. leprae* infection, and our data indicate that *M. leprae*-infected macrophages express MMP-II on their surfaces (unpublished data). Effective immunization with the immunodominant Ag can induce a population of T cells that recognize the same immunizing protein when the protein is expressed on macrophages infected with bacilli. Therefore, MMP-II ex-
pressed on the surfaces of macrophages could be a useful target for CD8\(^+\) cytotoxic T lymphocytes. The MMP-II-specific activation of T cells and the production of cytotoxic T lymphocytes could be key factors in regulating the host defense against *M. leprae*.

Another important difference in the features of DC stimulated with BCG-SM or BCG-pMV is that BCG-SM more efficiently activated DC in terms of the surface expression of APC-associated molecules. BCG-SM induced the up-regulation of the expression of MHC molecules, costimulatory molecules, and activation markers. The enhanced expression of these molecules by BCG-SM infection of DC would facilitate the activation of T cells. Although we cannot provide a precise explanation for why BCG-SM activated DC more efficiently, it may be that the secreted MMP-II bound TLR2 in the phagosome and hence activated a transcription factor, such as NF-\(\kappa\)B, more promptly, such as the case for exogenously pulsed recombinant MMP-II, which activates DC through ligation to TLR2 (17).

Although BCG is an excellent live vehicle for Ags and more efficiently immunizes host cells than do recombinant proteins (6), BCG usually resides in the phagosomes of APCs, and only some of the BCG-derived Ags are processed (4). However, BCG-SM expressed MMP-II significantly on the surfaces of DC, and blocking the processing activity of DC with chloroquine totally depleted the expression of MMP-II. Therefore, the most likely explanation for the surface MMP-II expression is that BCG-SM successfully provided soluble antigen in the phagosome, which could feasibly be processed and loaded on MHC molecules. Furthermore, complex formation with MHC molecules was further emphasized by the fact that T-cell activation by DC was largely blocked by the pretreatment of MMP-II-pulsed DC with a MAb to HLA-DR or HLA-ABC (19). Since the DC infected with BCG-SM were phenotypically more activated than those infected with BCG-pMV, the possibility that other BCG-derived Ags besides MMP-II may contribute to the stimulation of T cells cannot be ruled out. However, the display of an MMP-II-derived peptide may be associated most closely with the activation of naïve CD4\(^+\) T cells and with the cross-priming of naïve CD8\(^+\) T cells. Therefore, the secretion of protein by BCG-SM seems to be essential for the better stimulation of T cells.

The contribution of MMP-II in BCG-SM-infected host cells with regard to the production of memory T cells was verified by animal studies. Splenic T cells obtained from C57BL/6 mice infected with BCG-SM produced more IFN-\(\gamma\) by responding to recombinant MMP-II than did those from mice inoculated with BCG-pMV. It has been demonstrated clearly that mycobacteria such as BCG primarily infect macrophages in vivo and that Ags produced by processing mycobacteria or the secreted protein in the cells can be transferred to DC, whereby they are presented to T cells for activation (29). Therefore, DC may contribute to the better activation of MMP-II-specific T cells in vivo for mice infected with BCG-SM, as well as in vitro.

Previously, we demonstrated that recombinant MMP-II equally activated DC from lepromatous leprosy and tuberculoïd leprosy patients and from BCG-immunized healthy do-
nors, although it was reported that some lepromatous leprosy patients have a mutation in the intracellular domain of TLR2 (2, 11). Furthermore, DC pulsed with recombinant MMP-II successfully stimulated T cells from lepromatous leprosy patients to produce IFN-γ to the same level as that produced by healthy donors. Therefore, BCG-SM may be useful for stimulating T cells in lepromatous leprosy patients and for controlling bacterial spread (19). The combination of priming with a more immunostimulatory BCG strain, such as a recombinant BCG strain secreting an immunodominant protein (for example, BCG-SM), and boosting with a recombinant protein, such as MMP-II, may provide more powerful immunostimulatory measures against M. leprae infection. Further study should be pursued to evaluate the protective activity of BCG-SM against leprosy.

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