IL-12p70 production by DC

On day 4, DC were stimulated with the following reagents: LPS, PGN (*Staphylococcus aureus*, Fluka Production GmbH, Buchs, Switzerland), rIL-1β, purified MMP-II, *M. bovis* BCG. After 24 h, IL-12p70 concentrations were measured.

Cytokine production

Levels of the following cytokines were measured; IFN-γ produced by $CD4^+$ T cells, IL-12p70 produced by DC, and IL-1β produced by monocytes and macrophages stimulated for 24 h with *M. leprae* or BCG. The mAb to IL-1β with neutralizing activity was obtained from R & D Systems. The concentrations of IFN-γ, IL-12p70 and IL-1β were quantified using the enzyme assay kits, Opt EIA Human ELISA Set (BD PharMingen International).

Statistical analysis

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

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High-level expression of pseudogenes in *Mycobacterium leprae*

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Abstract

Recent studies have revealed that some RNAs are transcribed from noncoding DNA regions, including pseudogenes, and are functional as riboregulators. We have attempted to assess the gene expression profile throughout the *Mycobacterium leprae* genome using an array technique. Twelve highly expressed gene regions were identified that show an alteration in expression levels upon infection. Six of these were pseudogenes. Although *M. leprae* has an exceptional number and proportion of pseudogenes among species, our results suggest that some of the *M. leprae* pseudogenes are not just 'decayed' genes, but may have a functional role.

Introduction

Sequencing of the *Mycobacterium leprae* genome provided a great deal of information about this bacillus (Cole *et al.*, 2001). The size of the genome was shown to be 3.27 Mb, which is smaller than the 4.4 Mb of *Mycobacterium tuberculosis* (Cole *et al.*, 1998). The predicted number of coding genes is only 1614 (revised number from http://genolist.pasteur.fr/Leproma/), which is much lower than the reported 3959 genes of the *M. tuberculosis* genome. One of the remarkable features of the *M. leprae* genome is the existence of a high number of pseudogenes. Protein-coding genes comprise only 49.5% of the *M. leprae* genome, while 1133 genes (revised number from http://genolist.pasteur.fr/Leproma/) contain recognizable pseudogenes. The remaining 23.5% is thought to contain remnants mutated beyond recognition (Eiglmeier *et al.*, 2001). These data led to the idea that the *M. leprae* genome has fallen into massive gene decay (Cole *et al.*, 2001).

Pseudogenes are defined as nonfunctional copies or close relatives of known genes in which mutations, insertions, deletions and/or frame shifts have occurred. Therefore, despite having DNA sequences similar to those of normal genes, they are regarded as disabled copies of functional genes. Eukaryotes, such as humans, have a large population of 'processed' pseudogenes. They arise from retrotransposition or reverse transcription from mRNA and reinsertion of the cDNA into the genome. After integration, mutations accumulate within the reading frame. In prokaryotes, 'non-processed' pseudogenes are created by the modification of genes during duplication and subsequent disablement. In general, prokaryotic obligate pathogenic bacteria tend to have younger pools of pseudogenes (Liu *et al.*, 2004).

Both the number and proportion of pseudogenes within the *M. leprae* genome is exceptional among both prokaryotic and eukaryotic species (Harrison & Gerstein, 2002; Liu *et al.*, 2004). Several attempts have been made to explain the basis of this exception. The loss of σ factors or of two-component systems have been proposed as possible causes for the increase in pseudogenes in *M. leprae* (Madan Babu, 2003; Tyagi & Saini, 2004). A loss of *dnaQ*-mediated proofreading activities of DNA polymerase III might also contribute to a high mutation rate (Liu *et al.*, 2004). However, because pseudogenes are supposedly eliminated from the genome due to deletion events, their accumulation in *M. leprae* requires further explanation. One group has postulated that because of an intracellular lifestyle, *M. leprae* has a low level of exposure to potentially detrimental DNAs, such as horizontally transferred DNA (e.g. bacteriophages and transposons), resulting in the suppression of chromosomal deletions and the accumulation of pseudogenes (Lawrence *et al.*, 2001). Despite these *in silico* studies, there is no direct evidence showing the actual expression or the possible role of *M. leprae* pseudogenes.

It has been suggested that the gene expression profile of *M. leprae* in the macrophage phagosome differs from that of noninfective cells in the extracellular environment. By extension, genes in these profiles that exhibit differential expression might be important for the pathogenesis and intracellular parasitization of this bacterium. Several attempts have been made to identify bacterial genes that are induced in response to the different environments encountered in infected host cells. Most techniques require *in vivo* expression technology (Mahan *et al.*, 1993) or signature-tagged mutagenesis (Hensel *et al.*, 1995). However, these techniques require gene manipulation and recombinant technology that are not possible to perform with *M. leprae*, due to the lack of an *in vitro* culture system. Other studies on *M. leprae* gene expression have utilized microarray or reverse transcriptase polymerase chain reaction (RT-PCR) techniques (Williams *et al.*, 2004). However, such studies usually target functional genes. It is difficult to establish working array probes or PCR primers for pseudogenes, because transcriptional start and stop sites are variable and such mRNA as exists is thought to be unstable. This study was designed to identify highly expressed RNA copies throughout the *M. leprae* genome, with a particular focus on RNA whose levels alter upon infection.

Materials and methods

Construction of the *M. leprae* cosmid library

Mycobacterium leprae bacilli of the Thai-53 strain were purified from armadillos. Spleens were minced and homogenized in phosphate-buffered saline (PBS) and centrifuged at 3500 *g* for 10 min. The pellet was treated with 0.5 M NaOH, neutralized and resuspended in PBS containing 0.05% Tween 80. This was centrifuged at 100 *g* for 10 min to remove host cell components. The supernatant was then centrifuged at 3500 *g* for 20 min to obtain pure bacterial cells. Genomic DNA of *M. leprae* was purified by mechanical disruption of the bacterial cells as described previously (Dhandayuthapani *et al.*, 1994). DNA fragments of 30–55 kb, randomly sheared during the extraction process, were fractionated and electroeluted from agarose gels using the Takara RECOCHIP (Takara, Kyoto, Japan). The fragments were then rendered blunt-ended using T4 DNA polymerase and dNTPs. Aliquots were ligated into shuttle cosmid vectors for *Escherichia coli* and mycobacteria, pYUB412 (*Xba*I–*Eco*RV and *Eco*RV–*Xba*I) or pYUB415 (*Xba*I–*Bsa*BI and *Bsa*BI–*Xba*I). After *in vitro* packaging using Gigapack III XL extracts (Stratagene, La Jolla, CA), recombinant cosmids were introduced into *E. coli* STBL2 [F-*mcr*AΔ(*mcr*BC-*hsd*RMS-mrr) *end*A1 *rec*A1 lon *gyr*A96 thi *sup*E44 *rel*A1 λ-Δ(*lac-pro*AB)] and stored in 50% glycerol at −80 °C.

Extraction of cosmid DNA and membrane preparation

One hundred and thirty-seven cosmid clones that covered > 98% of the *M. leprae* genome were chosen for analysis. *Escherichia coli* clones were incubated with shaking at 30 °C for 18 h, after which cosmid DNA was extracted using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) according to the manufacturer's protocol. One microgram of DNA from each cosmid was blotted onto nylon filters using a Bio-Dot apparatus (Bio-Rad, Hercules, CA). Membranes were denatured with 0.5 M NaOH followed by UV-cross-linking.

Cell culture and *M. leprae* infection

The murine macrophage cell line J774.1 was obtained from the American Type Culture Collection (ATCC; Manassas, VA) and maintained in Dulbecco's Modified Eagle's Media (DMEM) containing 10% fetal calf serum at 37 °C in 5% CO_2. A total of 4×10^9 *M. leprae* cells were resuspended in DMEM. Half of these cells were added to 2×10^7 J774.1 cells, while the other half were used as a noninfective negative control. The infective and noninfective bacilli were harvested after 4 days of incubation at 37 °C. Briefly, infected J774.1 cells were washed three times with Dulbecco's phosphate-buffered saline (DPBS) at 37 °C to remove free bacilli. Cells were then lysed with DPBS containing 0.1% Tween 80 and subsequently treated with 0.5% trypsin. Intracellular *M. leprae* were collected by centrifugation. Control noninfective *M. leprae* were also collected by pipetting the culture medium followed by centrifugation. The concentration and purity of the bacterial preparation in each step were confirmed by acid-fast staining and microscopic evaluation as described previously (Hashimoto *et al.*, 2002).

RNA purification, cDNA synthesis and amplification

Mycobacterium leprae bacilli were treated with 15 mg mL^{-1} lysozome for 10 min at room temperature and then mechanically disrupted in a tube with glass beads for 1.5 min (× 4). Total RNA was isolated using the RNeasy Protect Bacteria Mini Kit (QIAGEN) according to the manufacturer's protocol as described previously (Suzuki *et al.*, 2002). In order to digest potentially contaminating genomic DNA, total RNA was treated with 50 mU mL^{-1} DNase in the presence of 10 mM $MgCl_2$ at 37 °C for 30 min (Suzuki *et al.*, 1999). Subsequently, mRNA was enriched by removing ribosomal RNA using the MICROBExpress Bacterial mRNA Enrichment Kit (Ambion, Austin, TX) according to the manufacturer's protocol. cDNA was synthesized using the First Strand cDNA Synthesis Kit (Amersham Biosciences, Piscataway, NJ) according to the manufacturer's

protocol as described previously (Suzuki *et al.*, 2002). A random primer containing an *Eco*RI site, 5′-GCCGGAGC TCTGCAGAATTCNNNNNN-3′, was used for both cDNA synthesis and PCR amplification as described previously (Froussard, 1992).

cDNA subtraction

Mycobacterium leprae-derived cDNA from bacilli-infected macrophages and from control fractions was further subjected to subtraction enrichment. Amplified double-stranded (ds) cDNA from the control bacterial cells was digested with *Eco*RI to form cohesive ends, and ligated to the *Eco*RI-digested adaptor DNA fragment bound to a resin on which only one chain of the short dsDNA chain (anchor DNA) was covalently bound at the 5′ end (EASY ANCHOR *Eco*R1-N, Nippon Gene, Tokyo, Japan) (Kato *et al.*, 1998). The other chain can be removed from the resin by exposing it to denaturing conditions. After undergoing denaturation and a wash by centrifugation, the resulting single-stranded (ss) DNA that was anchored to the resin was hybridized with denatured cDNA from *M. leprae*-infected macrophages. Centrifugation produced a supernatant that contained preferentially expressed cDNA derived from *M. leprae*-infected macrophages. The cDNA was further enriched by repeating the above subtraction steps four times. The same procedure was performed to enrich cDNA synthesized from the control bacteria. Finally, enriched cDNA derived from differentially expressed mRNA was amplified by PCR using the same random primer employed to synthesize the original cDNA.

Dot blot hybridization

cDNA was labeled with ^{33}P-dCTP with the BcaBEST Labeling Kit (TakaraBio, Otsu, Japan) as described previously (Suzuki *et al.*, 1998). ^{33}P-dCTP labeled probes were purified using Sephadex G-50 and hybridized overnight at 68 °C to an *M. leprae* DNA library spotted on nylon filters in the presence of QuikHyb hybridization solution (Stratagene) and 100 μg mL^{-1} of sonicated salmon sperm DNA. Stringent washing was carried out three times with 4 × SSPE, 0.5% SDS at 37 °C; three times with 2 × SSPE, 0.1% SDS at 37 °C and once with 1 × SSPE, 0.1% SDS at 65 °C for 30 min (Suzuki *et al.*, 1998). Radioactivity was visualized using a BAS1000 BioImaging analyzer (Fujifilm, Tokyo, Japan), and specific radioactivity from each clone was analyzed using MacBAS software (Fujifilm).

Southern blot analysis

The selected cosmid clones were digested with restriction endonucleases (*Bss*HII, *Bst*XI, *Cla*I, *Eco*RI, *Kpn*I, *Nru*I, *Pst*I and *Pvu*II) and fragments were separated by electrophoresis in a 1% agarose gel. The gels were soaked in denaturing buffer (3 M NaCl, 0.4 M NaOH) for 30 min (× 2) with shaking, and then washed with transfer buffer (3 M NaCl, 8 mM NaOH). After a wash in neutralization buffer (0.5 M Tris-HCl, 1 M NaCl), the membranes were dried and UV crosslinked. Hybridization, washing and detection were carried out in the same manner, and using the same probe, as the dot blot hybridizations.

RT-PCR

cDNA was synthesized using the First Strand cDNA Synthesis Kit (Amersham Biosciences) according to the manufacturer's protocol as described previously (Suzuki *et al.*, 2002). The primer sequences (5′–3′) used for PCR amplification of *M. leprae* genes were: AATGCTAGCATGGTGATGTCG and TATTGACGTTCGTCACTACGG for ML1474, GCAACCATCTGATACATGCG and CATGCTGGACATTGATCACC for ML1475, GCAACCATCTGATACATGCG and CATGCT GGACATTGATCACC for ML1476, GACCTCGTTCTTCTC TTCTTCG and TGATTCTTCGTGACAACCTCG for ML0435, CCAACCATCAGGTCCTTATGG and CATCAACA CGACGTCGTACG for ML2491, CCTCGATACGTGAGTT GTTCC and TGGTCTGATGGTATCGTCACC for ML2492, TGTCGTCAAGCTGTTGACTCC and AACTCTTCAGCCG TGACACC for ML2537, GGTCTGTTGCAGGTGATTCC and ATATCACCACCGAGGTTCTCG for ML1752, ACTGA CCGGAGACAACATCG and CTGACCATTAGCAGTGC TTGC for ML0591, GTTCTGCCTGACATTCATGG and GAGTCGTCGACAACAATCAGC for ML2206, CTATCC GAGAAGCCTTCAGC and ACAACCGAGGTATGTCGACG for ML1624, CAACCGAGTTACTGGATGTGG and GGTT CAGTGGTGTTGTTAGCC for ML1636. Touchdown PCR conditions were as described previously (Suzuki *et al.*, 2002).

Results and discussion

Array preparation and *M. leprae* gene expression profiles within the cosmid clones

A genomic DNA library of *M. leprae* was evaluated by direct sequencing of both ends of the cosmid inserts and comparison to the published genome sequence of *M. leprae* (Cole *et al.*, 2001). Overlapping clones that cover > 98% of the *M. leprae* genome were selected for creation of membrane arrays. Nylon filters were spotted with 1 μg of DNA from 137 selected cosmid clones, control DNA from an empty cosmid and the serially diluted cosmid DNA that encodes ribosomal RNA. Several sets of the array were created in the same batch in order to compare hybridization results.

The array was hybridized with radiolabeled cDNA from *M. leprae* purified from macrophages (Fig. 1a) and from control bacilli (Fig. 1b) after subtractive enrichment. Specific signals were quantified using the BAS1000 analyzer and

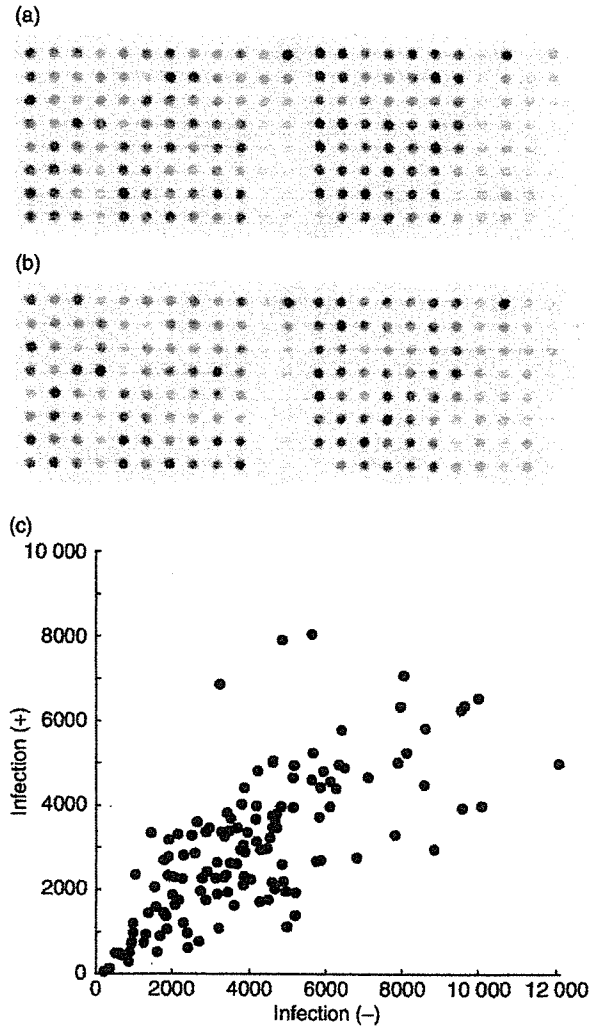

Fig. 1. Hybridization of *Mycobacterium leprae* genome arrays with ^{33}P-dCTP-labeled cDNA probes. *Mycobacterium leprae* DNA covering > 98% of the genome was purified from a cosmid library, spotted on membranes and probed with: (a) cDNA from bacilli-infected macrophages after subtractive enrichment. (b) cDNA from control bacilli after subtractive enrichment. Each experiment was performed twice and typical results are shown. (c) Correlation of signal intensity from arrays hybridized with subtracted cDNA. Densitometric analysis of signal intensity was performed on the array hybridizations and results from the same clones were compared. The *Y*-axis denotes the results from bacilli-infected macrophages, as shown in Fig. 1a. The *X*-axis denotes results from the control bacilli shown in Fig. 1b.

Image Gauge software (Fujifilm). A typical plot of signal intensity derived from subtracted cDNA from infected and noninfective *M. leprae* is shown in Fig. 1c. Although most cosmid clones showed similar expression levels in both assays, the expression of some clones was significantly different. The ratio of signal intensities from corresponding clones was calculated. It was found that five of the 137 cosmid clones contained regions with significantly higher expression (> 2 fold) in infected *M. leprae* than in noninfected controls. There were also 25 clones that showed regions of significantly decreased expression in infected *M. leprae*. These data were further evaluated by a comparison with the results using cDNA probes before subtractive hybridization. The differences in signal intensities of infective and noninfective bacilli were less significant in these arrays (data not shown) compared with those using subtracted cDNA (Fig. 1). However, the data are expected to be more reliable as biases that were possibly introduced during subtractive hybridization and subsequent PCR-based amplification are eliminated. Eight clones that showed differential expression levels in infected and uninfected bacilli in both arrays, and that provided relatively high expression levels over the others, were selected for Southern blot analysis to determine the responsible genes (Table 1). Among these, the expression of one clone (B194) was higher in *M. leprae* from macrophages than in the control bacilli (Table 1). These cosmid clones were expected to contain genes potentially important for the life cycle of *M. leprae*.

Identification of genes within the cosmid clone

The lengths of the *M. leprae* genomic DNA inserts in these eight clones were 36.7–43.2 kb, and each clone contained *c.* 30 genes (Table 1). We next tried to identify the differentially expressed genes within the cosmid clones that contributed to the differences in array signals seen in Fig. 1. To this end, each cosmid was digested with eight different restriction enzymes (Fig. 2a). Southern blots were performed using the same probe that was used for the dot blot hybridizations (Fig. 2b). The results revealed that only a few restriction fragments contributed to the strong signals in the array hybridizations shown in Figs 1a and b, indicating that only some regions within the insert DNA sequences were strongly transcribed.

The location of each restriction fragment within the *M. leprae* genome was analyzed *in silico*, and compared with the sizes of strong bands detected in the Southern hybridizations. These analyses identified 12 genes as contributors to the signals in the array analysis (Table 2). As these genes were identified by array hybridization of cosmid clones and subsequent Southern blot analysis, confirmation of actual mRNA expression in freshly isolated bacilli was needed. Gene expression was determined by RT-PCR using freshly isolated mRNA as a template, rather than subtracted and/or preamplified cDNA. It was confirmed that all 12 genes, including the six pseudogenes, were expressed in freshly isolated *M. leprae* (Fig. 3).

Only one of the 12 genes, ML1752 from clone B194, was upregulated in *M. leprae* after infection. In addition, only one of the identified genes, ML2206 (*purF*), has a known

Table 1. Highly expressed cosmid clones whose expression levels differed between *Mycobacterium leprae* isolated from macrophages and from the noninfective control

Clone	Insert direction	5′-end	3′-end	Insert length (kb)	No. of genes in the insert	Infection (+)/(−) ratio
B7	Forward	1754985	1795100	40.1	31	0.4
B10	Reverse	513018	550924	37.9	35	0.5
B166	Reverse	2959024	2998200	39.2	32	0.3
B185	Forward	2997353	3031966	34.6	30	0.2
B194	Reverse	2090519	2129310	38.9	24	2.3
B197	Forward	691764	733204	41.2	31	0.5
A90	Reverse	2591964	2635201	43.2	38	0.4
A203	Forward	1937742	1974396	36.7	25	0.4

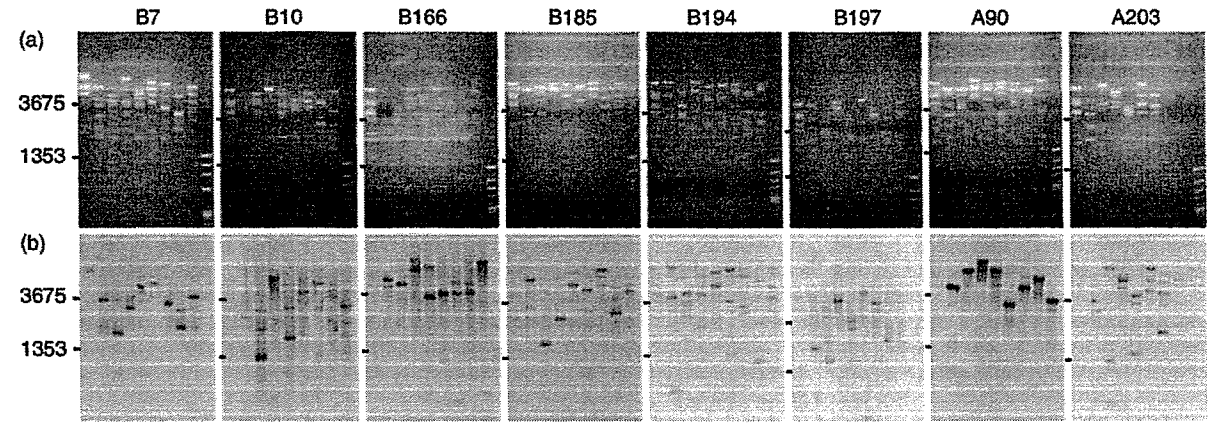

Fig. 2. Identification of fragments containing differentially expressed cDNAs. (a) The eight cosmids listed in Table 1 were digested with *Bss*HII, *Bst*XI, *Cla*I, *Eco*RI, *Kpn*I, *Nru*I, *Pst*I and *Pvu*II and separated on a 1% agarose gel. Samples were applied in this order from the left with a λ*Bst*EII marker in the first lane and a φX174 *Hae*III marker in the last lane. (b) Gels were subsequently transferred to nylon filters and hybridized with the same probes that were used for dot blot hybridization. Specific radioactivities were detected using a BAS1000 BioImaging Analyzer (Fujifilm). Bars shown in each panel indicate DNA sizes of 3675 and 1353 bp.

function (Keer *et al.*, 2001). *purF* encodes phosphoribosylpyrophosphate amidotransferase, an enzyme that is involved in the first committed step of *de novo* purine biosynthesis and has homologues in *M. tuberculosis* and *M. smegmatis* (Keer *et al.*, 2001).

The other 10 genes are conserved hypothetical proteins and/or pseudogenes. Six of the 12 genes were pseudogenes. Three of the six pseudogenes (ML1475c, ML1476c and ML2492) were originally oxidoreductases. All enzymes that catalyze oxidation–reduction reactions belong to this class of enzymes, including oxidases, oxygenases and cytochromes, which are essential for the generation of energy necessary for metabolic processes. There are 76 genes that might relate to oxidoreductase activity in the *M. leprae* genome, 41 (53.9%) of which are pseudogenes (http://genolist.pasteur.fr/Leproma/), while *M. tuberculosis* has 150 functional oxidoreductase genes (http://genolist.pasteur.fr/TubercuList/). Of the oxygenases, eight of 13 are pseudogenes (61.5%) in *M. leprae*, while there are 71 functional oxygenase genes in *M. tuberculosis*. It is of note that the percentage of pseudogenes related to oxidoreductase is much higher than the overall ratio of pseudogenes in the *M. leprae* genome (41.2%). In addition, the overall percentage of oxidoreductase-related genes is lower in *M. leprae* than in *M. tuberculosis* (2% vs. 6%). These observations suggest that metabolic activities are substantially impaired in *M. leprae* compared with *M. tuberculosis*.

Although pseudogenes have generally been considered as 'junk genes' that are transcriptionally and translationally inactive, recent studies have revealed the existence of functional as well as nonfunctional pseudogene transcripts in various organisms including humans, mice, bovines, plants and yeast (Balakirev & Ayala, 2003). For instance, it was shown that *Makorin1-p1*, a mouse pseudogene, is highly expressed, and that its mRNA contributes to the stabilization of *Makorin1*, the functional counterpart gene

Table 2. Highly expressed genes in *Mycobacterium leprae* with significantly different expression levels between bacilli isolated from macrophages and from noninfective bacilli

Clone	Gene	5′-end	3′-end	Description
B7	ML1474c	1776096	1776704	Probable molybdopterin-guanine dinucleotide biosynthesis protein A m*boA* (pseudogene)
	ML1475c	1776713	1777660	Probable oxidoreductase, β subunit (pseudogene)
	ML1476c	1777922	1779435	Probable oxidoreductase α subunit (pseudogene)
B10	ML0435	534769	535414	Probable succinyl-CoA:3-ketoacid-CoA transferase (β subunit) *scoB* (pseudogene)
B166	ML2491	2966115	2967698	Conserved hypothetical protein
	ML2492	2968010	2968791	Probable mono-oxygenase (pseudogene)
B185	ML2537c	3022083	3024005	Conserved hypothetical protein
B194	ML1752c	2121319	2122227	Conserved hypothetical protein
B197	ML0591c	715975	717756	Possible conserved integral membrane protein
A90	ML2206c	2620615	2622285	Amidophosphoribosyltransferase (*purF*)
A203	ML1624	1947637	1949427	Conserved hypothetical protein
	ML1636	1970373	1971813	Conserved hypothetical protein (pseudogene)

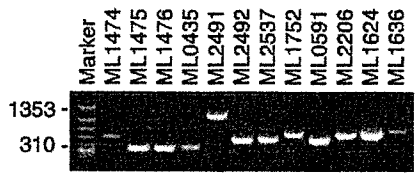

Fig. 3. RT-PCR analysis of freshly isolated *Mycobacterium leprae* mRNA. Total RNA was isolated from freshly prepared *M. leprae* from nude mice footpads. After treatment with DNase, RT-PCR was performed as described in Materials and methods. Bars on the left indicate DNA sizes of 1353 and 310 bp.

(Hirotsune *et al.*, 2003). Disruption of *Makorin1-p1* is lethal in most mice, and the few survivors had severe bone deformities and polycystic kidneys (Hirotsune *et al.*, 2003). These investigators estimated that at least 2–3% of human pseudogenes are expressed (Yano *et al.*, 2004). Expressed pseudogenes are regarded as a class of noncoding RNAs (ncRNAs) (Eddy, 2001; Erdmann *et al.*, 2001). They act as riboregulators, which are important for both transcriptional and posttranscriptional regulation of gene expression. As pseudogenes are functionally less constrained, they have accumulated more mutations than other genes. Assuming that they have functional roles in the regulation of gene expression, this property would allow more rapid functional diversification than is possible with protein-coding genes. However, as it is not possible to cultivate *M. leprae in vitro*, gene manipulation studies cannot be performed to study the function of pseudogenes identified in the present study.

It should be noted that the focus of this study was on cosmid clones with increased expression levels. As each cosmid clone contains ~50 genes, it is possible that a gene whose expression increased following infection coexists with other genes whose expression decreased in the same cosmid clone. Such cosmid clones, if they exist, would not be detected with the method employed in this study. To resolve this issue, we are now analyzing genome-wide expression profiles by use of a whole genome tiling array. Information provided from such studies will shed light on the role of pseudogenes in *M. leprae*, an unusual organism that has an exceptional number of pseudogenes.

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

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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-Guerin (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 *M. leprae* 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 p70 (IL-12p70) through the activation of NF-κB by ligating to Toll-like receptor 2 (TLR2), and MMP-II-pulsed DC activate both naïve and memory $CD4^+$ and $CD8^+$ T cells to produce gamma interferon (IFN-γ) in an MHC molecule-dependent manner (17, 19). In addition, memory-type T-cell subsets from tuberculoid leprosy patients were markedly activated 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

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activation (1, 8). Furthermore, the immunization of mice or guinea pigs with a 30-kDa major secretory protein (α-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-CD3 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 rIL-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 of replication for *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'GAAATCGATTTAACTCGGCGGCCGGGAGA3'. The secretion signal sequence of Ag85A of *M. tuberculosis* was amplified by PCR, using the primers 5'GAAGGATCCAATGCAGCTTGTTGACAGGG3' and 5'CCGGA ATTCTGCCCCCGCGGTCGCCGTG3'. The MMP-II cDNA fragment and Ag85A secretion signal sequence fragment were cloned in frame between the BamHI and ClaI sites of plasmid pMV261 to yield the plasmid pMV-SM. Another plasmid, pMV-PSM, 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-SM 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 10^8 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 culture supernatants of BCG-SM as well as BCG-pMV, cultured for 20 days in Sauton medium, were collected and passed through a 10,000-molecular-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. Polyvinylidene 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 (immunoglobulin G2a [IgG2a], 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. The expression of cell surface Ags on DC 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 Ags, the following MAbs were used: fluorescein isothiocyanate (FITC)-conjugated MAbs 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), CD86 (FUN-1; BD), and CD83 (HB15a; 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 inhibition of the 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 (δG9; 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 FACSCalibur flow cytometry. Naïve $CD4^+$ and $CD8^+$ T cells were produced by further treatment of these T cells with a MAb to CD45RO 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 (1×10^5 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 immunosorbent assay set (BD).

Animal studies. For inoculation into mice, recombinant BCG cells were cultured in Middlebrook 7H9 medium to log phase and stored at 10^8 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 3.3×10^3 or 1×10^4 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 1×10^6 or 2×10^6 cells per ml in culture medium. The splenic T cells (1×10^6 or 2×10^6 cells/ml) were stimulated with the indicated concentration of recombinant MMP-II or recombinant BCG in triplicate in 96-well round-bottomed microplates. The individual culture supernatants were collected 4 days after stimulation, and IFN-γ was measured using an Opt EIA mouse enzyme-linked immunosorbent assay set (BD).

Statistical analysis. Student's *t* test was applied to determine statistical differences.

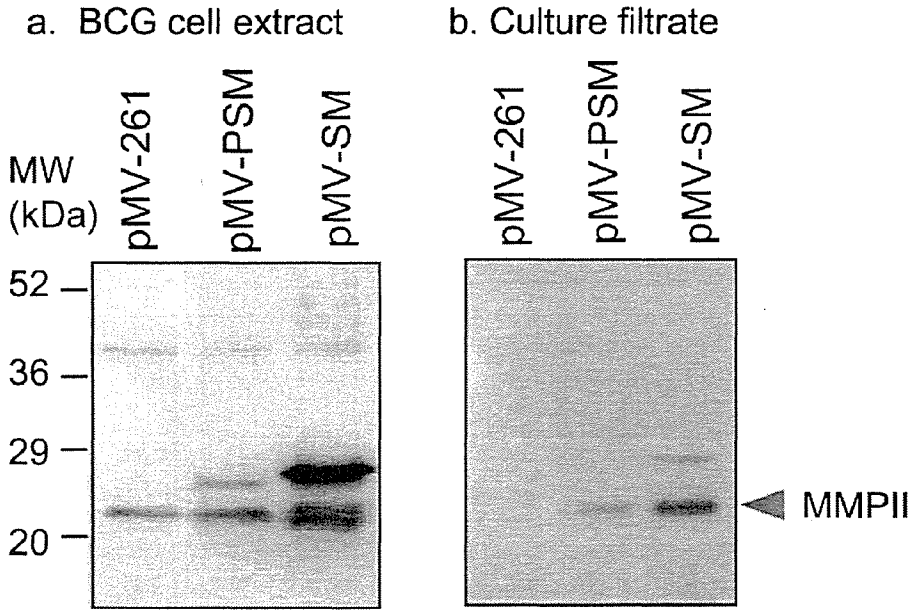

FIG. 1. Western blot analysis of BCG expressing *M. leprae*-derived MMP-II. A MAb to MMP-II (202-3 [IgG2a, kappa chain]) was used to detect MMP-II in BCG cell extracts (a) and culture filtrates (b). BCG was transfected with the following plasmids: lane 1, pMV261; lane 2, pMV-PSM; and lane 3, pMV-SM.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

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 *M. tuberculosis*) in pMV-SM and pMV-PSM, respectively. In both expression vectors, the secretion signal of *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

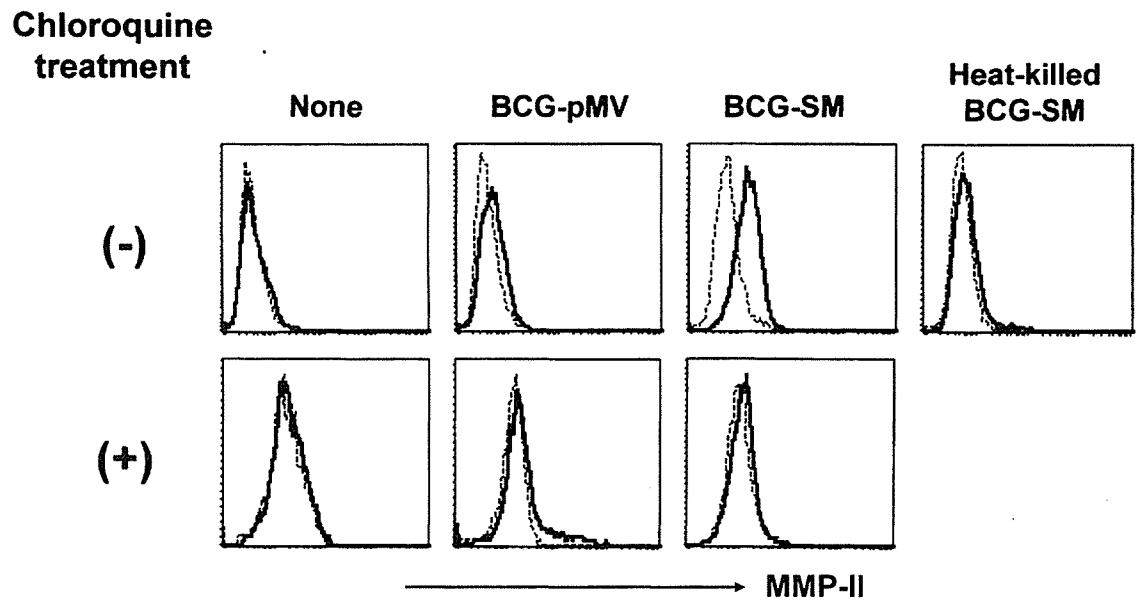

FIG. 2. Surface expression of MMP-II on DC infected with BCG. Immature DC were infected with either BCG-pMV or BCG-SM in the absence or presence of chloroquine (50 μM) or were pulsed with heat-killed BCG-SM at an MOI of 5. After 2 days of culture with rGM-CSF and rIL-4, DC were gated and analyzed for 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.

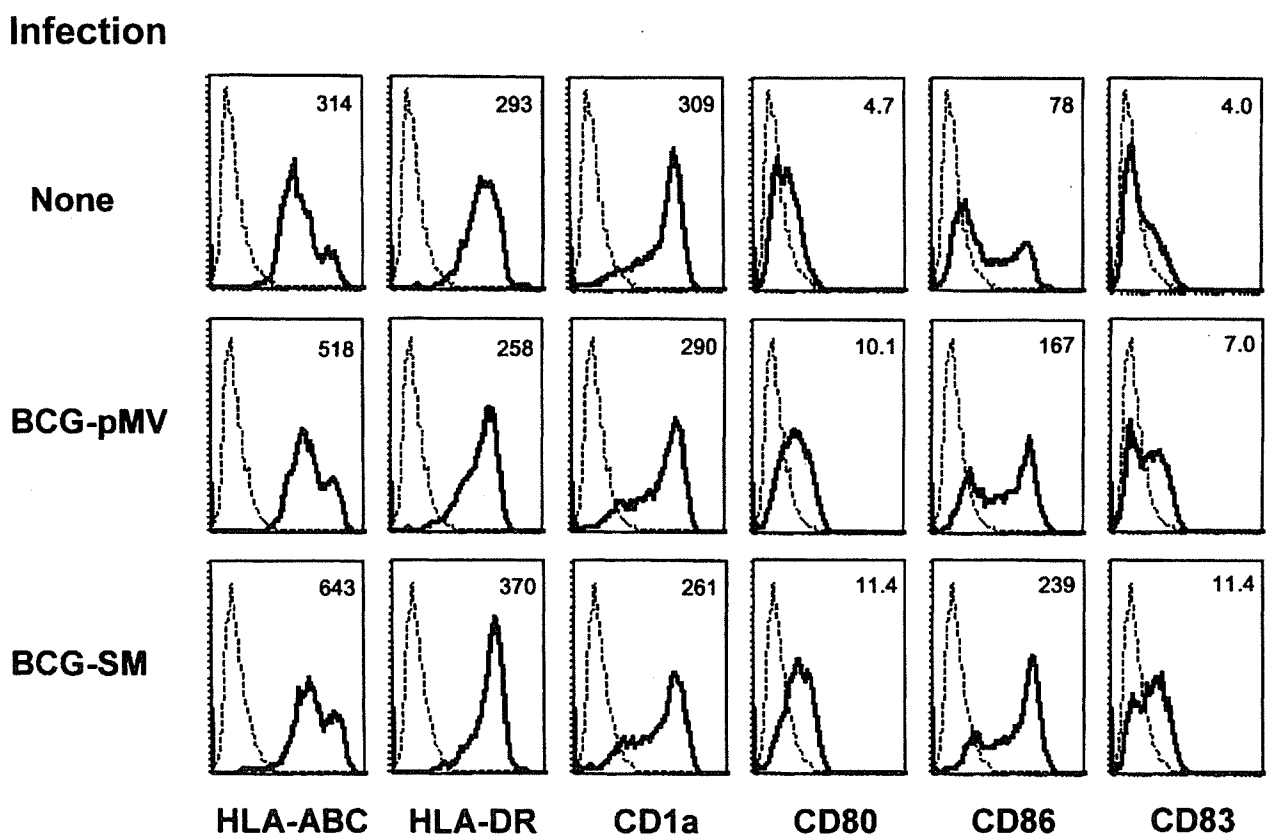

FIG. 3. Expression of various molecules on DC infected with either BCG-pMV or BCG-SM. Monocyte-derived immature DC were infected with BCG at an MOI of 0.125 and were cultured for another 2 days in the presence of rGM-CSF and rIL-4. The DC from day 5 were gated and analyzed. Dotted lines, isotype-matched control IgG; solid lines, the indicated test MAb. The number in the top right corner of each panel represents the difference in mean fluorescence intensities between the control IgG and the test MAb. Representative results for three separate experiments are shown.

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, CD86, 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 naïve $CD4^+$ 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-

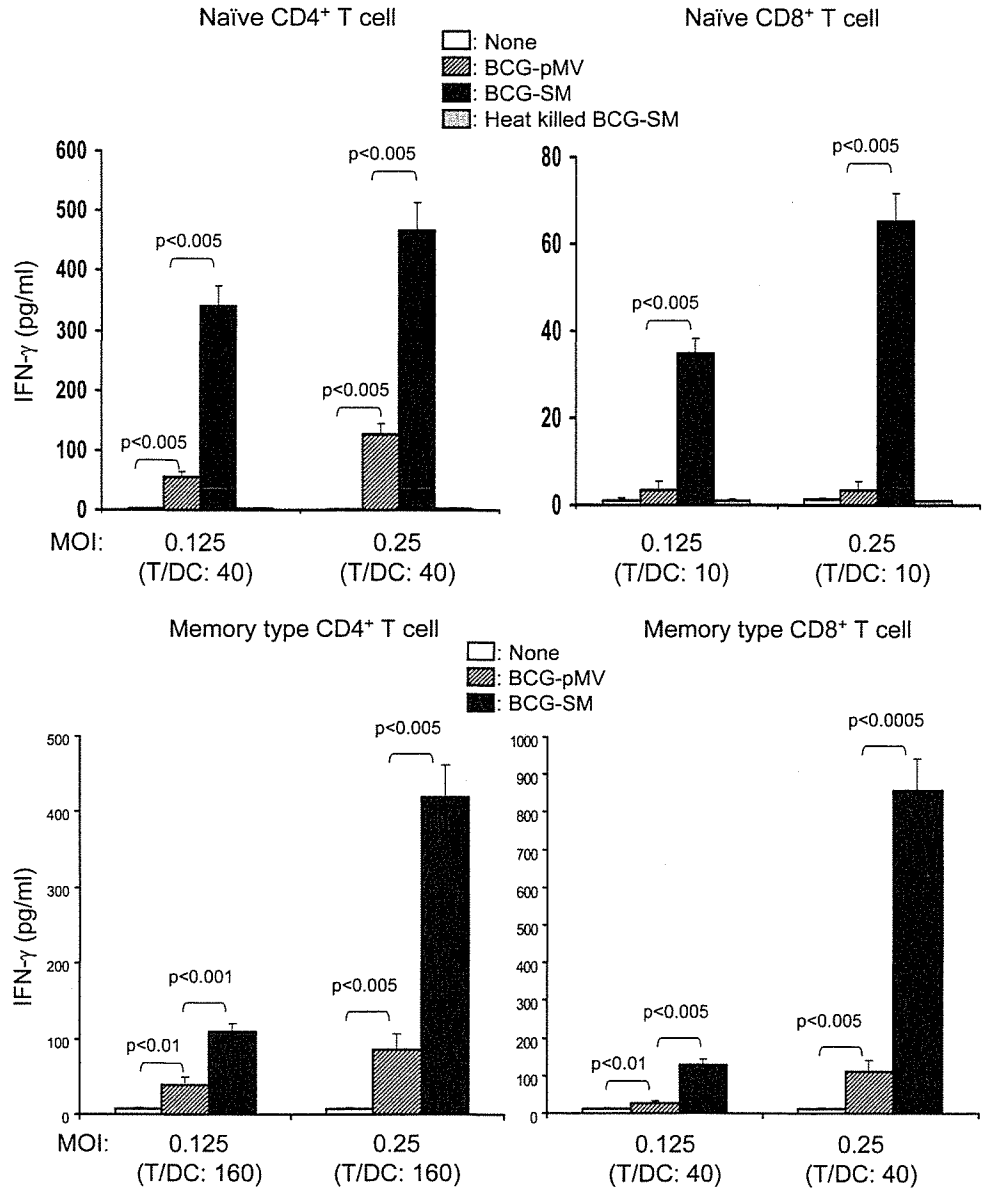

FIG. 4. (Top) Production of IFN-γ from naïve $CD4^+$ and $CD8^+$ T cells. Immature DC, differentiated from monocytes by 3 days of culture with rGM-CSF and rIL-4, were infected with BCG-pMV (vector control BCG) or BCG-SM (recombinant BCG strain secreting MMP-II) at the indicated MOIs or pulsed with heat-killed BCG-SM and then cultured for another 2 days in the presence of rGM-CSF and rIL-4. These DC were used as a stimulator of naïve T cells. $CD45RO^-$ naïve $CD4^+$ T cells (1×10^5 cells/well) were stimulated with 1/40 the number of DC for 4 days, and $CD45RO^-$ naïve $CD8^+$ T cells (1×10^5 cells/well) were stimulated with 1/10 the number of DC for 4 days. (Bottom) Production of IFN-γ from memory-type $CD4^+$ and $CD8^+$ T cells. Immature DC were infected with BCG-pMV or BCG-SM at the indicated MOIs and cultured for another 2 days in the presence of rGM-CSF and rIL-4, as described above. $CD45RA^-$ memory-type $CD4^+$ T cells (1×10^5 cells/well) were stimulated with 1/160 the number of DC for 4 days, and $CD45RA^-$ memory-type $CD8^+$ T cells (1×10^5 cells/well) were stimulated with 1/40 the number of DC for 4 days. Representative results for three separate experiments are shown. Assays were performed in triplicate, and the results are expressed as means ± standard deviations. Titers were statistically compared using Student's *t* test.

tion from the naïve $CD4^+$ T cells. In contrast to $CD4^+$ T cells, a significant production of IFN-γ 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-γ 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

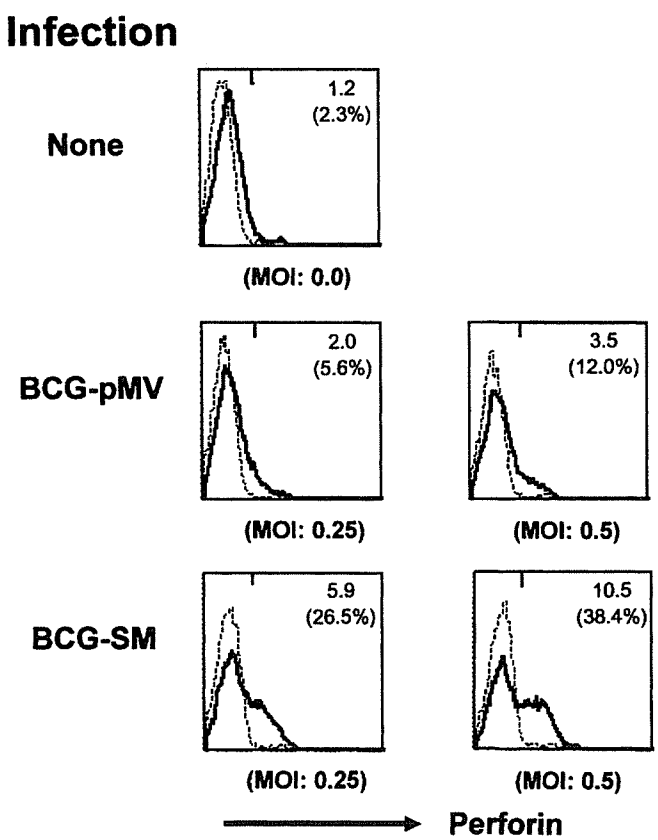

FIG. 5. Intracellular production of perforin in $CD8^+$ T cells stimulated with BCG-infected DC. Immature DC, differentiated from monocytes by 3 days of culture with rGM-CSF and rIL-4, were infected with either BCG-pMV or BCG-SM at the indicated MOIs and cultured for another 2 days in the presence of rGM-CSF and rIL-4. These DC were used as a stimulator of $CD8^+$ T cells. Unseparated purified $CD8^+$ T cells (1×10^5 cells/well) were stimulated with 5×10^3 BCG-infected DC for 5 days and then subjected to analysis of the intracellular perforin production. Dotted lines, isotype-matched control IgG; solid lines, anti-perforin MAb. The number in the top right corner of each panel represents the difference in mean fluorescence intensities between the control IgG and the test MAb. Each number in parentheses is the percentage of positive cells. The results of one experiment, which are representative of those for three separate experiments, are shown.

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 3.3×10^3 or 10×10^3 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 naïve and memory-type $CD4^+$ T cells to produce IFN-γ than did vector control BCG, and furthermore, both naïve 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-

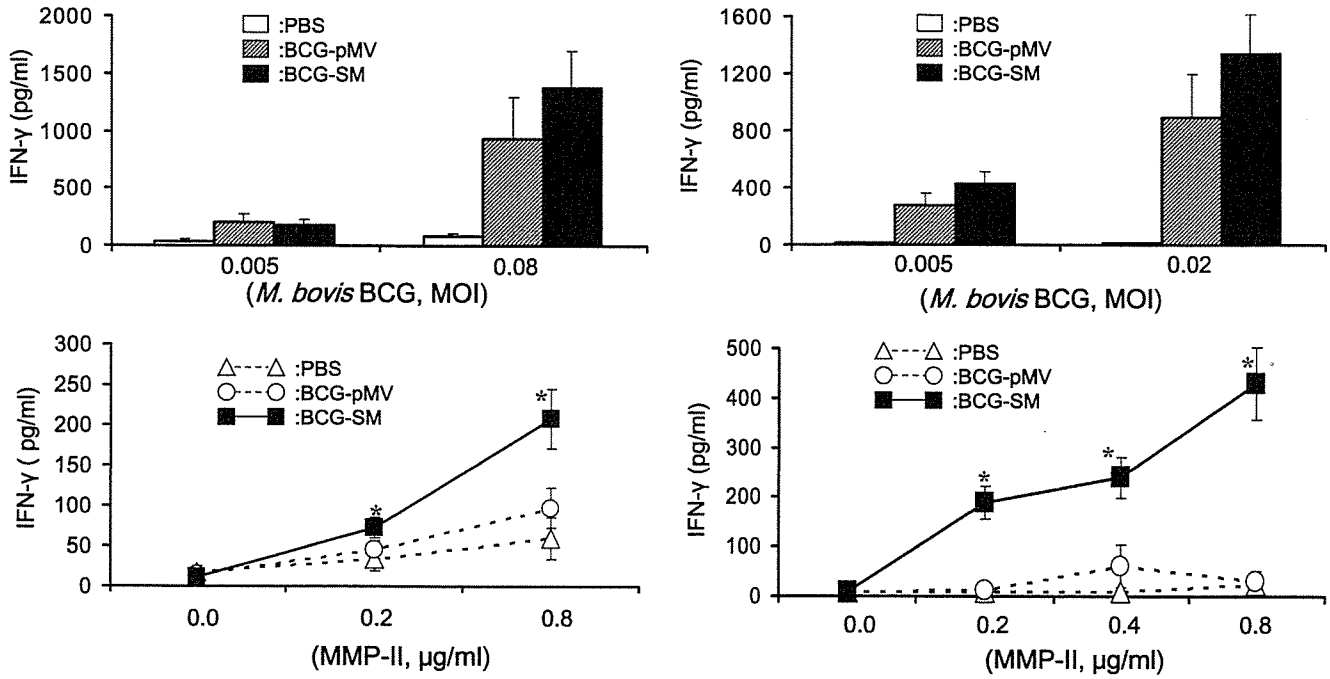

FIG. 6. IFN-γ production by splenic T cells obtained from C57BL/6 mice infected with BCG. Four-week-old C57BL/6 mice were infected with 3.3×10^3 bacilli per head of either BCG-pMV or BCG-SM, and their splenic unseparated T cells were stimulated in vitro with the indicated doses of BCG-pMV or MMP-II for 4 days. For MMP-II stimulation, 2×10^5 splenic T cells were used, and for BCG stimulation, 1×10^5 splenic T cells were used. Assays were performed in triplicate for each mouse, and the results for three mice per group are given, expressed as means ± standard deviations. Representative results for three separate experiments are shown. *, $P < 0.005$ versus BCG-pMV. (Left) C57BL/6 mice infected with BCG 7 weeks before. (Right) C57BL/6 mice infected with BCG 13 weeks before.

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-κB, 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, which are susceptible to BCG, inoculated with BCG-SM produced more IFN-γ 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 tuberculoid 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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Augmented induction of CD8$^+$ cytotoxic T-cell response and antitumour resistance by T helper type 1-inducing peptide

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Summary

The effector CD8$^+$ T cells recognize major histocompatibility complex (MHC) class I binding altered self-peptides expressed in tumour cells. Although the requirement for CD4$^+$ T helper type 1 (Th1) cells in regulating CD8$^+$ T cells has been documented, their target epitopes and functional impact in antitumour responses remain unclear. We examined whether a potent immunogenic peptide of *Mycobacterium tuberculosis* eliciting Th1 immunity contributes to the generation of CD8$^+$ T cells and to protective antitumour immune responses to unrelated tumour-specific antigens. Peptide-25, a major Th epitope of Ag85B from *M. tuberculosis* preferentially induced CD4$^+$ Th1 cells in C57BL/6 mice and had an augmenting effect on Th1 generation for coimmunized unrelated antigenic peptides. Coimmunization of mice with Peptide-25 and ovalbumin (OVA) or Peptide-25 and B16 melanoma peptide [tyrosinase-related protein-2 (TRP-2)] for MHC class I led to a profound increase in CD8$^+$ T cells specific for OVA and TRP-2 peptides, respectively. This heightened response depended on Peptide-25-specific CD4$^+$ T cells and interferon-γ-producing T cells. In tumour protection assays, immunization with Peptide-25 and OVA resulted in the enhancement of CD8$^+$ cytotoxic cell generation specific for OVA and the growth inhibition of EL-4 thymoma expressing OVA peptide leading to the tumour rejection. These phenomena were not achieved by immunization with OVA alone. Peptide-25-reactive Th1 cells counteractivated dendritic cells in the presence of Peptide-25 leading them to activate and present OVA peptide to CD8$^+$ cytotoxic T cells.

Keywords: antigen presentation; cytotoxic T cells; peptide; T helper 1 cells; tumour immunity

Introduction

The identification of tumour antigens has renewed interest in immunotherapy for cancer. There is a body of evidence that tumour-specific T cells recognize tumour-associated antigens on the cancer cells and play an essential role in inhibiting tumour growth and eradicating cancer cells.[1–3] CD8$^+$ cytotoxic T lymphocytes (CTL) from specifically immunized mice are capable of destroying tumour target cells *in vitro*[4] and adoptive transfer of CD8$^+$ T cells from immunized donors confers resistance to tumour transplants on naive mice.[5–7] As CD8$^+$ CTL can lyse tumour cells directly and destroy large tumour masses *in vivo*, much attention has focused on the role of CD8$^+$ T cells in the immunotherapy of cancer. Over the past two decades, a wide range of peptides derived from tumour cells of mice and humans that bind major histocompatibility complex (MHC) class I and are recognized by CD8$^+$ T cells has been defined.[1,8,9] However, in both clinical and animal studies, therapeutic strategies focused on the use of CD8$^+$ T cells and MHC class I-restricted tumour antigens have not been effective in eliminating cancer cells.

There has been a recent reappraisal of the role and importance of CD4$^+$ T helper (Th) cells in antitumour responses, because CD4$^+$ Th cells are required for generating and maintaining potent antitumour immunity.[5,6,10] The role of CD8$^+$ and CD4$^+$ T cells in tumour systems has been the object of intense interest. A major obstacle for the development of optimal cancer vaccines is the lack of effective methods for identifying MHC class

II-restricted tumour antigens that can stimulate $CD4^+$ T cells.[11,12] Identification of such antigens would provide new opportunities for developing effective $CD8^+$ CTL and would improve our understanding of the mechanisms by which $CD4^+$ T cells regulate the host immune system.

A variety of tumour-derived antigens have been defined by immunoglobulin G (IgG) antibodies in sera taken from tumour bearers with serological identification of antigens by recombinant expression cloning (SEREX).[13–16] The SEREX repertoire can be considered a reflection of the $CD4^+$ T-cell repertoire. Shiku and his colleagues reported that coimmunization of mice with plasmids encoding these SEREX-defined wild-type antigens and mutated mitogen-activated protein kinase 2 (mERK2; containing tumour-specific CTL epitope 9m of CMS5) led to a profound increase in $CD8^+$ T cells specific for mERK2.[13] This heightened response depends on $CD4^+$ T cells and on the copresentation of SEREX-defined wild-type antigens and the CTL epitope. Their results indicate the essential role of $CD4^+$ T cells in mediating the increased $CD8^+$ T-cell response and tumour inhibition induced by coimmunization with SEREX-defined antigens.

We have reported that immunization of *Mycobacterium tuberculosis*-primed mice with purified protein derivative (PPD)-modified attenuated X5563 myeloma cells induces an X5563-specific $CD8^+$ CTL response and antitumour immunity.[17–19] We infer from these results that *M. tuberculosis*-derived proteins or peptides may enhance the $CD8^+$ CTL response and antitumour immunity by coimmunization with tumour antigen or neo-tumour antigen. Ag85B, one of the major proteins secreted by *M. tuberculosis*, elicits a strong Th1 response *in vitro* in T cells from both PPD-positive asymptomatic human subjects and Ag85B-primed cells of C57BL/6 ($I\text{-}A^b$) mice. Peptide-25 (amino acids 240–254) of Ag85B, which is the most potent antigen species yet purified for both humans and mice, is a major Th1 cell epitope of Ag85B. Active immunization of C57BL/6 mice with Peptide-25 induces the differentiation of $CD4^+$ T-cell receptor (TCR) $V\beta11^+$ T cells that produce interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α)[20–23].

We investigated whether Th1-inducible Peptide-25 intensifies the $CD8^+$ CTL response to unrelated tumour-specific antigens through stimulation of a $CD4^+$ Th1 cell response leading to the induction of antitumour immunity that is effective in eliminating cancer cells. We also discuss the possible mechanisms of Peptide-25-induced enhancement of the $CD8^+$ CTL response.

Materials and methods

Mice

C57BL/6 mice were purchased from Charles River Japan (Tokyo, Japan). Peptide-25-reactive TCR transgenic (Tg) (P25 TCR-Tg) mice were generated and maintained as described previously.[24] IFN-γ deficient ($IFN\text{-}\gamma^{-/-}$) mice[25] were kindly provided by Dr Y. Iwakura (Institute of Medical Science, University of Tokyo, Tokyo, Japan). Ovalbumin (OVA)-specific TCR-Tg (OT-1) mice were kindly provided by Dr T. Hirano (Osaka University, Suita, Japan). These mice were housed in the animal facility at the Institute of Medical Science, University of Tokyo, under specific pathogen-free conditions, and were used at 8–12 weeks of age.

Antigens and reagents

Peptide-25 (FQDAYNAAGGHNAVF), Peptide-9 (DWY SPACGKAGCQTY), and Peptide-18 (AGGYKAADM WGPSSD) of Ag85B were synthesized by Funakoshi Co., Ltd (Tokyo, Japan). Purified chicken OVA was purchased from Sigma-Aldrich, Co. (St Louis, MO). MHC class I-binding OVA Peptide (SIINFEKL) and B16 melanoma peptide tyrosinase-related protein-2 (TRP-2) (VYDF FVWL)[26] were also synthesized by Funakoshi Co., Ltd.

Culture medium

RPMI-1640 (Gibco BRL, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated fetal calf serum (Sigma-Aldrich, Co.), 50 μM 2-mercaptoethanol, 100 IU/ml penicillin G and 50 μg/ml streptomycin was used as the complete medium for cultures throughout the present experiment.

Cell lines

The murine thymoma line, EL-4 ($H\text{-}2K^b$) was purchased from the American Type Culture Collection (Rockville, MD). EL-4 transfectant of the OVA gene (E.G7 cells) was kindly provided by Dr H. Udono (Nagasaki University School of Medicine, Nagasaki, Japan) and the B16 melanoma cell line was kindly provided by Dr H. Tahara (Institute of Medical Science, University of Tokyo, Tokyo, Japan).

Immunization

Mice were immunized by subcutaneous injection on the abdomen with OVA (10 μg/mouse) emulsified in incomplete Freund's adjuvant (IFA), Peptide-25, or its related peptide (10 μg/mouse) in IFA or a mixture of OVA (10 μg/mouse) and Peptide-25 (10 μg/mouse) in IFA as described previously.[21] In some experiments, mice were immunized with OVA (10 μg/mouse) in IFA on the left-hand side of the abdomen and with Peptide-25 (10 μg/mouse) in IFA on the right-hand side of the abdomen. We also immunized mice with MHC class I-binding TRP-2 peptide (10 μg/mouse) in place of OVA.

In vivo *and* in vitro *T-cell depletion*

$CD4^+$ T cells were depleted *in vivo* by the administration of 0·3 mg monoclonal antibodies (mAbs) against CD4 (GK1.5) on days −13, −12, −11, −6, −5, −4, +1, +2 and +3 relative to immunization. Fluorescence-activated cell sorter (FACS) analysis of blood mononuclear cells from GK1.5-treated mice at the time of immunization confirmed the effectiveness of the $CD4^+$ T-cell depletion. *In vitro* T-cell depletion was achieved by the incubation of spleen cells with either the IgM subclass of mAb against CD4 or CD8 and guinea-pig complement. FACS analysis of the treated spleen cells confirmed the effectiveness of the depletion.

In vitro *CTL induction and* $CD8^+$ *cytotoxic T-cell assay*

In vitro CTL induction and $CD8^+$ CTL assay were carried out according to previously described methods[17,18] with slight modification. Ten days after immunization with OVA in IFA or OVA and Peptide-25 in IFA, spleen cells (1×10^7) were cultured *in vitro* with γ-irradiated (20 000 rad) E.G7 cells (8×10^5). Spleen cells from TRP-2-immunized mice were stimulated *in vitro* with TRP-2 (10 μg/ml). After 5 days in culture, the CTL activity of the resulting effector cells was assayed. Target cells (E.G7, EL-4, and B16 melanoma cells) were labelled with ^{51}Cr (Perkin Elmer Life Science, Boston, MA) at 37° for 40 min. After washing, ^{51}Cr-labelled target cells (1×10^4) were incubated with effector cells at various effector cell to target cell ratios. Release of ^{51}Cr was measured in the supernatants that were harvested after 4 hr incubation. Maximum release was measured by resuspending the target cells in lysis buffer containing 0·1% Triton-X-100. Spontaneous release was obtained from target cells incubated with medium alone and was less than 10% of maximum ^{51}Cr release. The percentage specific lysis was calculated according to the following formula, where c.p.m. represents counts per minute: percentage specific lysis = $[(\text{c.p.m.}_{\text{experimental release}} - \text{c.p.m.}_{\text{spontaneous release}})/(\text{c.p.m.}_{\text{maximum release}} - \text{c.p.m.}_{\text{spontaneous release}})] \times 100$.

A dose–response curve of effector cells was established in all experiments and the number of lytic units (LU) was calculated as previously described.[19] In these calculations 1 LU was arbitrarily defined as the number of spleen cells required to achieve 50% lysis of 1×10^4 ^{51}Cr-labelled target cells during a 4-hr incubation.

Tumour challenge experiments

Three groups of 12 mice were immunized by subcutaneous injection of the abdomen with OVA (10 μg/mouse) in IFA, Peptide-25 (10 μg/mouse) in IFA, or a mixture of OVA (10 μg/mouse) and Peptide-25 (10 μg/mouse) in IFA. Twelve mice were injected with IFA without any protein or peptide to act as a control group. Ten days after the immunization, all mice were challenged by subcutaneous injection with E.G7 (5×10^5 cells/mouse) on their backs. In some experiments, B16 melanoma cells (5×10^5 cells/mouse) were transplanted in TRP-2-immunized mice. Tumour size was assessed using a microcaliper a 2-day to 3-day intervals and was expressed as the square of the smallest diameter of the tumour multiplied by its largest diameter. The survival of the mice was also monitored periodically.

Frequency analysis of OVA-specific CTL

The frequency of OVA-specific CTL in spleen cells after immunization was measured using OVA peptide-loaded H-$2K^b$:Ig protein (BD Biosciences Pharmingen, San Diego, CA) according to the manufacturer's instructions. Spleen cells prepared from mice 10 days after immunization, were stained with 4 μg OVA peptide-loaded H-$2K^b$:Ig protein and incubated for 60 min at 4°. After washing, cells were stained with anti-mouse IgG1 (A85-1)–phycoerythrin (PE; BD Biosciences Pharmingen) and anti-CD8 (53-6.7)–fluorescein isothiocyanate (FITC; BD Biosciences Pharmingen) and incubated for 30 min at 4°. After washing, cells were analysed using FACSCalibur (Becton Dickinson, Mountain View, CA).

Assay for dendritic cell activation

Immature dendritic cells (DCs) were propagated *in vitro* by culturing $CD11c^+$ bone marrow cells with granulocyte–macrophage colony-stimulating factor (GM-CSF) (20 ng/ml) and interleukin-3 (IL-3) (20 ng/ml) for 6 days. To assess the expression of surface molecules and IL-12 production of DCs after Peptide-25 treatment, the cells obtained (5×10^5) were cocultured with Peptide-25 (10 μg/ml) in the presence of $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice for 48 hr. The expression of surface molecules on DCs was analysed by FACS. The IL-12 production was assessed by enzyme-linked immunosorbent assay (ELISA). To assess the antigen-presenting activity of DCs after Peptide-25 treatment, the cells obtained (5×10^5) were cocultured with $CD4^+$ T cells (5×10^5) from P25 TCR-Tg mice and 5-carboxyfluorescein diacetate succinimidyl ester (CFSE)-labelled $CD8^+$ T cells (5×10^5) from OT-1 mice for 96 hr in the presence of Peptide-25 (10 μg/ml) and OVA (10 μg/ml). After the culture, cell division cycles were determined by FACS analysis.[27]

Assay for cytokine production by intracellular cytokine staining and ELISA

For assessment of cytokine production of spleen cells from OVA- or TRP-2-immunized mice, spleen cells