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Immunostimulatory activity of major membrane protein-II from *Mycobacterium leprae*

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Abstract

We examined the antigenicity of an immunomodulatory protein, major membrane protein (MMP)-II, from *Mycobacterium leprae*, since host defense against *M. leprae* largely depends on adaptive immunity. Both unprimed and memory T cells from healthy individuals were stimulated by autologous MMP-II-pulsed monocyte-derived dendritic cells (DCs) to produce IFN-γ. The DC-mediated IFN-γ production was dependent on the expression of MHC, CD86, and MMP-II antigens. Memory T cells from paucibacillary (PB) leprosy more extensively responded to MMP-II-pulsed DCs than T cells from healthy individuals, while comparable IFN-γ was produced by unprimed T cells. Memory T cells from multibacillary leprosy, which are normally believed to be anergic, were activated similarly to those from healthy individuals by MMP-II-pulsed DCs. These results suggest that memory T cells from PB leprosy are primed with MMP-II prior to the manifestation of the disease, and MMP-II is highly antigenic in terms of activation of adaptive immunity.

Keywords: *M. leprae*; Leprosy; Major membrane protein-II; T cell; Dendritic cell; IFN-γ; Memory T cell; Paucibacillary leprosy; Host defense

1. Introduction

Mycobacterium leprae is the causative agent of human leprosy, in which a chronic progressive peripheral nerve injury leading to systemic deformity is induced [1,2]. Most individuals infected with *M. leprae* do not manifest leprosy, but a few manifest the disease depending on their immunological status. Leprosy exhibits a wide range of clinical features and therefore, a broad disease spectrum is observed [3]. The representative spectra are the paucibacillary (PB) leprosy and multibacillary (MB)[1] leprosy. In the former disease spectrum, the localized skin and nerve lesions are observed and both $CD4^+$ and $CD8^+$ T cells chiefly act to localize the bacterial spread and, thus, disease lesion [4–6]. In contrast, in the latter disease spectrum, such cell-mediated immune responses are not efficiently evoked, but, rather, T cells show *M. leprae* Ag-specific anergic response [3]. The

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[1] *Abbreviation used:* MMP-II, major membrane protein-II; *M.*, *Mycobacterium*; DC, dendritic cell; MB, multibacillary; IFN, interferon; MHC, major histocompatibility complex; PB, paucibacillary; BCG, *M. bovis* bacillus Calmette-Guérin; Ab, antibody; Ag, antigen; APC, Ag-presenting cell; MDT, multi-drug therapy; TLR, Toll-like receptor; PBMCs, peripheral blood mononuclear cells; m, monoclonal; L, ligand; MLM, *M. leprae*-derived cell membrane; LPS, lipopolysaccharide; LAM, lipoarabinomannan.

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vaccine currently examined for human use against the mycobacterial diseases is *M. bovis* bacillus Calmette-Guérin (BCG). However, its protective effect on *M. leprae* infection is not so convincing. Various efforts have currently been done for the development of new immunostimulatory agents, however, we still do not have an effective anti-leprosy vaccine. Also, in both forms of leprosy, the protective effects of antibody (Ab) in blood could not be observed. Therefore, the identification of the useful bacterial component antigens (Ags) which have immunomodulatory and immunostimulatory activities are desired.

Previously we demonstrated that dendritic cells (DCs), which are the most potent Ag-presenting cell (APC) capable of stimulating both memory and unprimed $CD4^+$ and $CD8^+$ T cell subsets [7–9], played a central role in stimulating T cells of both healthy individuals and PB leprosy patients [10,11]. However, macrophages less efficiently stimulated T cells [12]. Using DCs as APCs, we reported that cell membrane fraction of *M. leprae* was the most T cell stimulatory fraction [11], and therefore we identified major membrane protein-II (MMP-II) from this fraction as an immunomodulatory molecule.

MMP-II was originally identified from *M. leprae* as a major native protein in 1990 and was recognized to be identical to *M. paratuberculosis* bacterioferritin. Purification of MMP-II by reverse-phase chromatography, revealed a large molecular mass of 380 kDa, which has a ferroxidase-center residue. MMP-II contained 1000–4000 atoms of iron per molecule of protein. In the previous study, we showed that purified MMP-II stimulated DCs to produce IL-12 p70, and TNFα through the ligation to toll-like receptor (TLR)-2 [13]. In this study, we evaluated the immunostimulatory activity of purified MMP-II using DCs as APCs, since type 1 T cells response is most closely associated with host defense against *M. leprae* [1,2,14]. Furthermore, we assessed if MMP-II is associated with the activation of T cells in PB leprosy patients.

2. Materials and methods

2.1. Preparation of cells and bacteria

Peripheral blood was obtained under informed consent from healthy volunteers who were PPD-positive due to *M. bovis* BCG vaccination at childhood, and from five cases each of PB and MB leprosy patients. The status of patients used in this study are as follows: PB leprosy: 2 female and 3 male, age range (31–56), and MB leprosy: 1 female and 4 male, age range (21–53). All patients were under multi-drug therapy (MDT) for less than 7 months. We are aware that PPD-negative individuals would help to provide full information for these experiments because *M. leprae* and *M. bovis* BCG share some common Ags. However, in Japan, such individuals are not available for study, because *M. bovis* BCG vaccination was compulsory for children (0–4 year-old) until some years ago. PPD-negative individuals in Japanese population are the ones who do not respond to BCG vaccination; and therefore, it is likely that they may suffer from unknown human disease or immuno-insufficiency. Therefore these individuals cannot be used for our experiments. Peripheral blood mononuclear cells (PBMCs) were isolated using Ficoll–Paque Plus (Pharmacia, Uppsala, Sweden) and cryopreserved in liquid nitrogen until use, as previously described [15]. For preparation of the monocytes, $CD3^+$ T cells were removed from either freshly isolated heparinized blood, or cryopreserved PBMCs using immunomagnetic beads coated with anti-CD3 monoclonal (m) Ab (Dynabeads 450, Dynal, Oslo, Norway). The $CD3^-$ fraction of the PBMCs were plated on collagen-coated plates and cultured for 60 min at 37 °C. The non-plastic-adherent cells were then removed by extensive washing and the remaining adherent cells were used as monocytes and precursors of DCs [14]. Monocyte-derived DCs were differentiated from the plastic-adherent cells as described [15,16]. Briefly, the plastic-adherent cells were cultured in 3 ml of RPMI 1640 medium containing 10% FCS for 5 days in the presence of 50 ng of rGM-CSF (Pepro Tech EC, London, England) and 10 ng of rIL-4 (Pepro Tech) per milliliter. rGM-CSF and rIL-4 were supplied every 2 days and 400 μl of medium was replaced as described previously [16]. In some cases, DCs unpulsed or pulsed with Ags were further treated with a soluble form of CD40 ligand (L) (Pepro Tech) to obtain fully matured DCs capable of efficiently activating T cells. The purity of DCs obtained was 90.5% as judged by the expression of CD1a.

2.2. Purification of whole cell membrane fraction of M. leprae and MMP-II

The whole cell membrane fraction (MLM) was obtained according to previous report [13]. Briefly, the mycobacterial suspension was mixed with Zirconium beads in the presence of protease inhibitors at a ratio of approximately 1:1 (v/v) and homogenized using Beads Homogenizer Model BC-20 (Central Scientific Commerce, Tokyo), at 1500 rpm for 90 s 3–4 times. The beads were separated and the suspension was centrifuged at 10,000*g* for 30 min. The supernatant was then further ultra-centrifuged at 100,000*g* for 1 h. The resulting pellet was suspended in PBS, washed 2 times and taken as the membrane fraction. The *MMP-II* gene was PCR amplified from *M. leprae* chromosomal DNA and cloned into *Escherichia coli* expression vector as described previously [13]. Briefly, the *MMP-II* gene was inserted into the expression plasmid pET28 (Novagen, Madison, WI) and transformed into *E. coli* strain ER2566 (New England

BioLabs, Beverly, MA). The expressed protein was eluted using Whole Gel Eluter (Bio-Rad Laboratories, Hercules, CA) and used for all the experiments conducted in this paper. The amount of lipopolysaccharide (LPS) in the purified MMP-II protein was determined by using Limulus amebocyte lysate assay (Whittaker Bioproducts, Walkersville, MD) and found to be less than 70 pg/mg of MMP-II, of which level did not affect the T cell stimulating activity of DCs as described previously [13].

2.3. Assessment of an antigenicity of MMP-II

The immunostimulatory activity of MMP-II was assessed by using DCs as APCs and autologous T cells as responder population as previously described [10,16]. The Ag-pulsed DCs were treated with 50 μg/ml mitomycin C, washed extensively to remove extracellular Ags, and were used as a stimulator. Freshly thawed PBMCs were depleted of MHC class II^+ cells by using magnetic beads coated with mAb to MHC class II Ag (Dynabeads 450; Dynal) and further treated with beads coated with either CD4 or CD8 mAb to select T cells negatively as previously reported [10]. The purity of $CD4^+$ T cells or $CD8^+$ T cells was more than 98%. Unprimed and memory population of responder T cells was purified from these $CD4^+$ and $CD8^+$ T cells by depleting $CD45RO^+$ memory cells and $CD45RA^+$ naïve cells, respectively. For depletion, mAb to CD45RO or CD45RA (Dako, Glostrup, Denmark) were used, and were followed by immunomagnetic beads coated with mAb to mouse IgG (Dynal). The purified responder cells (1×10^5/well) were plated in 96-well round-bottomed tissue culture plates and DCs were added to give a DC responder $CD4^+$ T cell ratio of 1:10, 20, 40 or 80 and a DC responder $CD8^+$ T cell ratio of 1:5, 10 or 20. The supernatant of stimulator-T cell mixture was collected on day 3 or 4 of co-cultures for assessment of cytokine concentration. To identify molecules restricting T cell activation, the following purified mAbs were used: anti-HLA-ABC Ab (W6/32), anti-HLA-DR (L243), and anti-CD86 Ab (IT2.2, BD PharMingen International). Also, the purified mAb to MMP-II (IgM, k), which were raised in mice [13], was used to mask the MMP-II Ag expressed on the surface of DCs. The optimal concentrations of mAbs were determined in advance. Lipoarabinomannan (LAM) and mAb to LAM were donated by Dr. P.J. Brennan (Colorado State University).

2.4. Assessment of cytokine production

Levels of the following cytokines were measured; IFN-γ and IL-2 produced by $CD4^+$ and $CD8^+$ T cells stimulated for 3 or 4 days with Ag-pulsed DCs. The concentrations of IL-2 and IFN-γ were quantified using the enzyme assay kits Opt EIA Human ELISA Set available from BD PharMingen International.

2.5. Statistical analysis

Student's *t* test was applied to demonstrate statistically significant differences.

3. Results

3.1. Antigenicity of M. leprae-derived MMP-II

The ability of MMP-II to evoke cellular immunity was assessed, since MMP-II stimulated DCs to produce IL-12p70 and MMP-II-pulsed DCs expressed its derivatives on the surface [13]. We examined whether MMP-II-pulsed DCs activated both unprimed and memory T cells by using IL-2 and IFN-γ as a reporter cytokine. When $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells obtained from healthy PPD-positive individuals were stimulated with autologous MMP-II-pulsed DCs, they produced both IL-2 (Table 1) and IFN-γ (Table 2). While 4 μg/ml of MMP-II was required to stimulate DCs for production of significant dose of IL-2 from $CD4^+$ and $CD8^+$ T cells (Table 1), 1 μg/ml of the Ag was sufficient for significant IFN-γ production from both T cells (Table 2). Then, $CD45RA^-$ memory $CD4^+$ and $CD8^+$ T cells, obtained from healthy donors, were stimulated with autologous MMP-II-pulsed DCs (Table 3). In contrast to unprimed T cells, both memory T cell subsets efficiently produced IFN-γ even when they were stimulated with DCs which were pulsed with MMP-II in the absence of maturation factor such as CD40L. MMP-II was found to be more potent than whole membrane fraction (positive control) or lipoarabinomannan (negative control) in the stimulation of both $CD4^+$ and $CD8^+$ T cells. However, no IL-4 was produced from these T cells (not shown). CD40L further up-regulated the IFN-γ production from both T cell subsets. In both cases, $CD4^+$ T cell predominantly produced IFN-γ. The IFN-γ production was induced in a manner dependent on both MMP-II dose (Table 3) and CD40L concentration (not shown). Both unprimed and memory T cells were not stimulated by DCs which were treated with the amount of LPS estimated to be present in 4 μg/ml of MMP-II (not shown).

The role of MHC and co-stimulatory molecules of MMP-II-activated DCs on T cell activation was determined by using mAbs towards these molecules. The IFN-γ production from T cells was suppressed by the treatment of these DCs with mAbs towards HLA-ABC, HLA-DR or CD86 Ags (Table 4). More than 85% of IFN-γ production from $CD4^+$ T cells was suppressed by mAb to HLA-DR and more than 80% of the cytokine production was suppressed by mAb to CD86. However, the cytokine production was not suppressed by mAb to HLA-ABC Ags. On the other hand, the IFN-γ production from $CD8^+$ T cells was suppressed by the DC treatment with mAb to HLA-ABC (85–90% suppression) or

Table 1
IL-2 production from unprimed T cells stimulated with MMP-II-pulsed DCs[a]

MMP-II (μg/ml)	Unprimed $CD4^+$ T cells stimulation by DCs (IL-2, pg/ml)		Unprimed $CD8^+$ T cells stimulation by DCs (IL-2, pg/ml)	
T/DC:	10	20	5	10
0.0	$4.9 \pm 0.8^{b,c}$	$4.4 \pm 1.1^{d,e}$	$1.0 \pm 0.0^{f,g}$	$0.5 \pm 0.0^{h,i}$
1.0	9.3 ± 2.1^{b}	8.7 ± 1.3^{d}	4.1 ± 0.8^{f}	2.9 ± 0.2^{h}
4.0	23.7 ± 3.1^{c}	18.4 ± 1.1^{e}	17.6 ± 1.4^{g}	7.8 ± 1.1^{i}

[a] The responder $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 3 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.
[b] $p < 0.05$.
[c] $p < 0.005$.
[d] $p < 0.001$.
[e] $p < 0.0001$.
[f] $p < 0.05$.
[g] $p < 0.005$.
[h] $p < 0.005$.
[i] $p < 0.01$.

Table 2
IFN-γ production from unprimed T cells stimulated with MMP-II-pulsed DCs[a]

MMP-II (μg/ml)	Unprimed $CD4^+$ T cells stimulation by DCs (IFN-γ, pg/ml)		Unprimed $CD8^+$ T cells stimulation by DCs (IFN-γ, pg/ml)	
T/DC:	10	20	5	10
0.0	$18.5 \pm 2.9^{b,e}$	$14.0 \pm 3.1^{d,e}$	$1.4 \pm 0.2^{f,g}$	$0.9 \pm 0.1^{h,i}$
1.0	66.2 ± 8.7^{b}	44.1 ± 9.3^{d}	10.0 ± 1.8^{f}	7.9 ± 0.9^{h}
4.0	134.3 ± 11.0^{c}	115.9 ± 13.9^{e}	27.7 ± 2.9^{g}	14.3 ± 3.9^{i}

[a] The responder $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.
[b] $p < 0.005$.
[c] $p < 0.005$.
[d] $p < 0.05$.
[e] $p < 0.005$.
[f] $p < 0.05$.
[g] $p < 0.005$.
[h] $p < 0.005$.
[i] $p < 0.05$.

mAb to CD86 Ag (more than 85% suppression). Furthermore, the IFN-γ production was also suppressed by the treatment of MMP-II-pulsed DCs with mAb to MMP-II Ag (Table 4). The percent suppression was ~65% in $CD4^+$ T cells and ~65% in $CD8^+$ T cells. mAb to LAM, used as a negative control Ab, did not suppress the IFN-γ production. No significant suppression was observed on the cytokine production from $CD4^+$ and $CD8^+$ T cells stimulated with DCs unpulsed with any Ags (not shown).

3.2. Efficiency of MMP-II-pulsed DCs in the activation of leprosy T cells

It is known that T cells of PB leprosy have conserved an ability to respond to *M. leprae* infection, but those of MB leprosy are unresponsive towards *M. leprae* Ags [3]. Using T cells from five patients of each disease type, we evaluated the T cells responses to MMP-II-pulsed DCs. First, $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells obtained from PB leprosy were stimulated with autologous MMP-II-pulsed DCs (Table 5). Both subsets of T cells produced a significant dose of IFN-γ and 1 μg/ml of MMP-II was enough to produce the cytokine. However, the amounts of cytokine produced from these T cells were comparable to that produced from T cells obtained from healthy individuals (Table 2). Next, we evaluated the memory $CD4^+$ T cells and $CD8^+$ T cells. The CD45RA-negative T cells were obtained from healthy individuals, PB and MB leprosy patients, and were stimulated with autologous DCs which were pulsed with MMP-II in the absence or presence of CD40L (Fig. 1). Again, T cells from healthy individuals responded to MMP-II-pulsed DCs, but, both $CD4^+$ and $CD8^+$ T cells from PB leprosy produced significantly higher level of IFN-γ than T cells from healthy individuals when stimulated with autologous CD40L-stimulated, MMP-II (0.5 μg/ml)-pulsed DCs. In addition, DCs which were not

Table 3
IFN-γ production from memory T cells stimulated with MMP-II-pulsed DCs[a]

Antigen (μg/ml)	CD40L stimulation of DCs	Memory $CD4^+$ T cells stimulation by DCs (IFN-γ, pg/ml)		Memory $CD8^+$ T cells stimulation by DCs (IFN-γ, pg/ml)	
	T/DC:	40	80	10	20
None	(−)	$8.9 \pm 0.2^{b,c}$	9.6 ± 1.9^{d}	$1.1 \pm 0.1^{e,f}$	0.0 ± 0.0
MMP-II 1.0	(−)	48.7 ± 5.3^{b}	3.3 ± 0.9	7.1 ± 0.9^{e}	2.0 ± 0.0^{g}
MMP-II 4.0	(−)	144.5 ± 4.4^{c}	48.8 ± 2.3^{d}	24.1 ± 3.1^{f}	5.0 ± 0.2^{g}
None	(+)	36.4 ± 1.9	1.5 ± 0.1	6.9 ± 1.3	0.3 ± 0.0
MMP-II 1.0	(+)	117.9 ± 3.6	43.8 ± 1.7	56.6 ± 3.1	13.3 ± 1.0
MMP-II 4.0	(+)	$308.6 \pm 11.3^{h,i}$	$172.8 \pm 4.0^{j,k}$	$153.3 \pm 7.9^{l,m}$	$29.8 \pm 3.4^{n,o}$
MLM 4.0	(+)	79.2 ± 11.3^{h}	39.0 ± 2.1^{j}	35.9 ± 2.8^{l}	12.4 ± 4.8^{n}
LAM 4.0	(+)	32.9 ± 9.8^{i}	2.0 ± 1.2^{k}	10.2 ± 2.0^{m}	2.1 ± 1.0^{o}

[a] The responder $CD45RA^-$ memory type $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Monocyte-derived immature DCs were pulsed with various dose of MMP-II, whole *M. leprae*-derived membrane fraction (MLM) or LAM, untreated or treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.

[b] $p < 0.01$.
[c] $p < 0.0005$.
[d] $p < 0.00005$.
[e] $p < 0.01$.
[f] $p < 0.01$.
[g] $p < 0.005$.
[h] $p < 0.00001$.
[i] $p < 0.00001$.
[j] $p < 0.0001$.
[k] $p < 0.0001$.
[l] $p < 0.001$.
[m] $p < 0.001$.
[n] $p < 0.005$.
[o] $p < 0.005$.

conditioned by CD40L also induced T cell activations, but lesser dose of the cytokine was produced from both T cell subsets. In contrast to PB patient, both subsets of T cells from MB leprosy produced comparable level of cytokine to healthy individuals when they were stimulated with the CD40L-stimulated, MMP-II-pulsed DCs. Little IL-4 was produced from patients' T cells stimulated with MMP-II-pulsed DCs.

4. Discussion

Leprosy represents broad spectrum disease [3]. One representative manifestation is PB leprosy. Studies on the clinical specimens of the skin lesions indicate that the infection is localized, and the spread of *M. leprae* is suppressed by a consequence of activation of both $CD4^+$ and $CD8^+$ T cell subsets [4–6]. On the other hand, MB leprosy usually manifests widespread infection due to the lack of efficient cellular immune response against *M. leprae* components. The mechanisms leading to the broad spectrum are not fully understood yet, but these observations suggest the presence of some host defense associated Ags that trigger the immune responses. However, so far such Ags have not been identified. Previously we evaluated the T cell stimulating function of professional APC and found that DC was superior to macrophage in activating T cells [10,12]. When we examined the antigenicity of subcellular components of *M. leprae* for identification of DC-mediated antigenic molecules, we found that the cell membrane fraction was more adept than other fractions in terms of T cell stimulating ability [11]. Based on these observations, we identified MMP-II from *M. leprae* cell membrane fraction as one of the components, capable of acting as an immunomodulatory agent [13]. In our previous study, we found that purified MMP-II stimulated DCs to produce bioactive IL-12 and TNFα through ligation to TLR-2 on the surface of DCs. However, these DCs did not produce detectable level of IL-10 [13]. As is widely accepted, IL-12 is an important APC-mediated cytokine capable of driving Th1 T cell responses [17], and TLR-2 serves as a bridge to link innate and adaptive immune responses [18,19]. Therefore, in this study, we examined the capability of MMP-II to evoke adaptive immunity, especially in respect to type 1 T cell responses. Consequent to the induction of adaptive immunity, both unprimed and memory T cells are activated to produce type-1 cytokine, such as IFN-γ, which is an extremely important cytokine involved in the host defense, since it can activate macrophages and consequently kill the bacteria [4,14]. When MMP-II-pulsed DCs were evaluated as an inducer of

Table 4
Suppression of IFN-γ production from T cells by treatment of DCs with mAb[a]

Responder	T/DC	IFN-γ production (pg/ml)					
	mAb treatment:	Control	MHC class I	MHC class II	CD86	MMP-II	LAM
$CD4^+$ T cells	40	$392.1 \pm 22.2^{b,c,d}$	360.4 ± 23.1 (8.1%)	51.4 ± 8.8^{b} (86.9%)	74.1 ± 12.7^{c} (81.1%)	101.3 ± 20.1^{d} (74.2%)	401.9 ± 31.4 (0.0%)
	80	$240.9 \pm 20.2^{e,f,g}$	219.3 ± 11.8 (9.0%)	17.8 ± 8.1^{e} (92.7%)	19.1 ± 9.8^{f} (92.1%)	81.3 ± 9.0^{g} (66.3%)	253.2 ± 21.9 (0.0%)
$CD8^+$ T cells	10	$230.8 \pm 21.3^{h,i,j}$	24.1 ± 11.0^{h} (89.6%)	202.4 ± 30.1 (12.3%)	33.7 ± 6.9^{i} (85.4%)	29.5 ± 3.9^{j} (87.2%)	229.8 ± 30.8 (0.4%)
	20	$48.9 \pm 9.6^{k,l,m}$	6.1 ± 2.1^{k} (87.5%)	51.4 ± 3.9 (0.0%)	6.0 ± 1.8^{l} (87.7%)	17.3 ± 4.2^{m} (64.6%)	50.3 ± 5.6 (0.0%)

[a] The responder $CD45RA^-$ memory type $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from healthy individuals, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC), in the presence of various mAb. mAb to LAM was used as negative control Ab. Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate, and results are expressed as mean ± SD. Parenthesis indicates percent suppression by mAb. The optimal concentration of mAb was determined in advance. The titers having the same alphabet were statistically compared by Student's *t* test.
[b] $p < 0.001$.
[c] $p < 0.0005$.
[d] $p < 0.00005$.
[e] $p < 0.001$.
[f] $p < 0.001$.
[g] $p < 0.005$.
[h] $p < 0.001$.
[i] $p < 0.005$.
[j] $p < 0.005$.
[k] $p < 0.05$.
[l] $p < 0.05$.
[m] $p < 0.01$.

Table 5
IFN-γ production from PB leprosy unprimed T cells stimulated with MMP-II-pulsed DCs [a]

MMP-II (μg/ml)	Unprimed $CD4^+$ T cells stimulation by DCs (IFN-γ, pg/ml)		Unprimed $CD8^+$ T cells stimulation by DCs (IFN-γ, pg/ml)	
T/DC:	10	20	5	10
0.0	$20.9 \pm 3.9^{b,c}$	15.3 ± 4.1^{d}	$2.1 \pm 0.4^{e,f}$	$1.9 \pm 0.6^{g,h}$
1.0	73.2 ± 9.2^{b}	22.4 ± 10.3	14.6 ± 2.8^{e}	10.9 ± 1.3^{g}
4.0	173.2 ± 13.0^{c}	60.6 ± 9.9^{d}	33.8 ± 3.1^{f}	20.9 ± 2.7^{h}

[a] The responder $CD45RO^-$ unprimed $CD4^+$ and $CD8^+$ T cells (1×10^5/well), obtained from PB leprosy, were stimulated for 4 days with various dose of autologous DCs to give an indicated T cell:DC ratio (T/DC). Immature DCs were pulsed with MMP-II, treated with CD40L (1.0 μg/ml), and were used as stimulator. Representative of more than three separate experiments is shown. Assays were done in triplicate and results are expressed as means ± SD. The titers having the same alphabet were statistically compared by Student's *t* test.
[b] $p < 0.005$.
[c] $p < 0.005$.
[d] $p < 0.01$.
[e] $p < 0.05$.
[f] $p < 0.005$.
[g] $p < 0.005$.
[h] $p < 0.005$.

adaptive immunity, both $CD4^+$ and $CD8^+$ T cells produced IFN-γ. MMP-II was more potent than whole membrane protein (Table 3) in the stimulation of both $CD4^+$ and $CD8^+$ T cells. Although the detailed process of $CD8^+$ T cell activation by soluble protein like MMP-II is not fully covered, cross priming may be largely involved in activating $CD8^+$ T cells. The cytokine production was restricted by MHC molecules (Table 4), in concordance with the previous report that T cells from PB and MB leprosy were restricted by HLA-DR molecules [20]. Also the IFN-γ production from T cells was suppressed by mAb to CD86, which indicates that the activation of both $CD4^+$ T cells and $CD8^+$ T cells was largely dependent on the expression of CD86 Ag on DCs. Furthermore, the cytokine production was Ag-specific, since more than 60% of IFN-γ production was suppressed by the treatment of the DCs with MMP-II mAb (Table 4). The fact that the activation of both $CD4^+$ and

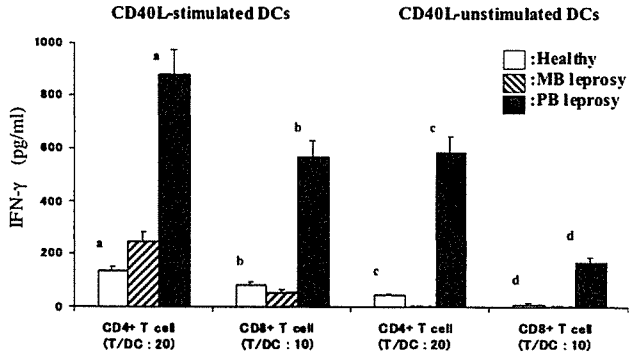

Fig. 1. IFN-γ production of T cells from healthy individuals (PPD-positive), PB and MB leprosy. The responder $CD4^+$ and $CD8^+$ T cells (1×10^5/well) were stimulated for 4 days with autologous MMP-II (0.5 μg/ml)-pulsed DCs at an indicated T cell:DC ratio. The Ag-pulsed DCs were untreated or treated with CD40L (1 μg/ml) for 24 h before T cell stimulation. Representative result obtained from five different patients and healthy individuals are shown. Assays were done in triplicate and results are expressed as mean ± SD of the representative donor. $^a p < 0.001$, $^b p < 0.005$, $^c p < 0.005$, and $^d p < 0.005$.

$CD8^+$ T cells were suppressed by the MMP-II mAb may depend on the immunological feature of the mAb, which should be clarified. In addition to IFN-γ, the MMP-II-pulsed DCs stimulated unprimed $CD4^+$ T cells to produce IL-2 (Table 1). Therefore, it may be deduced that MMP-II is efficient in the activation of DC-mediated adaptive immunity, although other characteristics of MMP-II protein need to be further evaluated.

We further evaluated the potential of MMP-II to activate T cells from leprosy patients. Although DCs from the patients expressed MHC molecules, CD86 and CD80 Ags comparable to the level expressed by healthy individuals (not shown), memory $CD4^+$ and $CD8^+$ T cells from PB patients produced significantly higher dose of IFN-γ than T cells from healthy individuals by stimulation with autologous MMP-II-pulsed DCs (Fig. 1). In addition to CD40L-stimulated MMP-II-pulsed DCs, less matured DCs, which were pulsed with MMP-II in the absence of any additional maturation factor including CD40L, were also quite efficient in the IFN-γ production from both subsets of memory T cells from PB leprosy (Fig. 1). However, in contrast to memory T cells, the IFN-γ production from unprimed T cells obtained from PB leprosy was comparable to that from healthy individuals. We may deduce from these results that peripheral blood T cells of PB leprosy are primed with Ags of *M. leprae* through professional APCs prior to the clinical manifestation of the disease, and one of the Ags responsible for retaining the specific memory T cells could be MMP-II. Therefore, MMP-II can be considered as one of the candidates involved in T cell activation of PB leprosy.

In contrast to PB patient, T cells from MB leprosy responded to MMP-II only when it was pulsed to DCs in the presence of CD40L. Although, the exact reason for the difference between PB and MB leprosy in the requisite form of DCs is not fully covered. However, one possible explanation for the poor cellular immune response in MB leprosy might be associated with recent observation that some MB leprosy has TLR-2 polymorphism [21,22]. We have not examined whether our patients had such polymorphism, but, if so, it might lead to less efficient ligation of MMP-II to the receptor. Another explanation is that T cells cannot be efficiently primed with *M. leprae* derived antigenic components in MB leprosy patients for reasons that are still uncovered. This possibility is more likely, because T cells from MB leprosy produced equivalent dose of IFN-γ by MMP-II stimulation to that produced by healthy individuals T cells.

Taken together, these observations indicate that MMP-II is highly potent in terms of immune stimulation, and is an antigenic element in T cell activation for the control of the growth of the bacilli. Further study should be pursued to evaluate its ability as host defense associated molecule against leprosy.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.cellimm.2005.04.001.

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DNA Augments Antigenicity of Mycobacterial DNA-Binding Protein 1 and Confers Protection against *Mycobacterium tuberculosis* Infection in Mice[1]

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***Mycobacterium* consists up to 7% of mycobacterial DNA-binding protein 1 (MDP1) in total cellular proteins. Host immune responses to MDP1 were studied in mice to explore the antigenic properties of this protein. Anti-MDP1 IgG was produced after infection with either bacillus Calmette-Guérin or *Mycobacterium tuberculosis* in C3H/HeJ mice. However, the level of Ab was remarkably low when purified MDP1 was injected. MDP1 is considered to be associated with DNA in nucleoid, which contains immunostimulatory CpG motif. Therefore, we examined coadministration of MDP1 and DNA derived from *M. tuberculosis*. Consequently, this procedure significantly enhanced the production of MDP1-specific IgG. Five nanograms of DNA was enough to enhance MDP1-specific IgG production in the administration of 5 μg of MDP1 into mice. Strong immune stimulation by such a small amount of DNA is noteworthy, because >1,000- to 100,000-fold doses of CpG DNAs are used for immune activation. A synthetic peptide-based study showed that B cell epitopes were different between mice administered MDP1 alone and those given a mixture of MDP1 and DNA, suggesting that DNA alters the three-dimensional structure of MDP1. Coadministration of DNA also enhanced MDP1-specific IFN-γ production and reduced the bacterial burden of a following challenge of *M. tuberculosis*, showing that MDP1 is a novel vaccine target. Finally, we found that MDP1 remarkably enhanced TLR9-dependent immune stimulation by unmethylated CpG oligo DNA in vitro. To our knowledge, MDP1 is the first protein discovered that remarkably augments the CpG-mediated immune response and is a potential adjuvant for CpG DNA-based immune therapies. *The Journal of Immunology*, 2005, 175: 441–449.**

Tuberculosis is a disease caused by infection with *Mycobacterium tuberculosis* and remains a serious threat to health in the world. Annually, 8 million people contract tuberculosis, and nearly 2 million people die from the disease. Worldwide, 32% of the population is persistently infected with *M. tuberculosis*, and some of these bacteria are thought to be in a nonreplicating dormant state (1). The majority of the disease arises from reactivation of persisting, previously implanted bacteria (2–5).

Bacillus Calmette-Guérin (BCG)[3] is an attenuated live vaccine against tuberculosis and has been given to >2 billion individuals to date. BCG is safe, inexpensive, and effective against both meningitis and miliary tuberculosis in infants, but frequently fails to protect from the most prevalent form of the disease, adult pulmonary tuberculosis (6–9). In addition, there is the possibility of causing opportunistic disease in immunocompromised hosts, such as AIDS patients, because BCG is a live vaccine and can survive in the hosts. Accordingly, there is an urgent need to develop a more effective and safer vaccine than BCG. Extensive studies to date have evaluated possible vaccine candidate proteins, such as a 6-kDa early secretory antigenic target (10); Ag 85 complexes A, B, and C (11); MTB39 and MTB48 (12); and heat shock protein 60 (13).

Mycobacterial DNA-binding protein 1 (MDP1) is produced by the genus *Mycobacterium* and is a major cellular protein, consisting of up to 7% of the total cellular protein (14). MDP1 has nucleic acid-binding activity mediated through interaction with guanine and cytosine residues in DNA (14, 15). Thus, MDP1 is presumed to be a component of the mycobacterial nucleoid and has been shown to localize to the 50S ribosomal subunit and on the bacterial surface (14, 16). The cellular content of MDP1 is increased in the stationary growth phase of mycobacteria relative to the exponential growth phase (14). Dick et al. (17) found that histone-like protein (HLP), the homologue of MDP1, was substantially upregulated in the dormant state of *Mycobacterium smegmatis*. Our previous study showed that MDP1 inhibited macromolecular biosyntheses in vitro and substantially suppressed bacterial growth (18). Taken together, it is conceivable that MDP1 has fundamental

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[3] Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Ag85B, Ag 85 complex B; HLP, histone-like protein; HLP*Mt*, histone-like protein of *Mycobacterium tuberculosis*; HrpA, heat stress-induced ribosome-binding protein A; KO, knockout; LBP-21, laminin-binding protein of 21 kDa; Me-oligo B, synthetic oligo DNA containing methylated CpG sequence; MDP1, mycobacterial DNA-binding protein 1; ODN, synthetic oligodeoxynucleotide; PPD, purified protein derivative; RIB, RIBI adjuvant system; rMDP1, recombinant histidine-tagged MDP1.

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roles in the suppression of growth from both stationary and dormant phases of mycobacteria.

Of interest, MDP1 localizes on the bacterial surface as well as intracellularly (14, 16, 19, 20). During host-bacterium interaction, MDP1 may play a role as an adhesin. Shimoji et al. (20) found that a 21-kDa protein could bind to laminin-2, which is thought to be an *Mycobacterium leprae* receptor involved in attachment to Schwann cells (21). They designated this protein as laminin-binding protein of 21 kDa (LBP-21) and showed it to be a homologue of MDP1 in *M. leprae*, although it was deficient for DNA-binding activity (20). Thus, LBP-21 may have a role in the invasion of *M. leprae* into peripheral nerves, presumably cooperating with another adhesion molecule, phenolic glycolipid-1 (22). In addition to laminin, we recently found that MDP1 binds to glycosaminoglycans (16), which are a major component of the extracellular matrix. Glycosaminoglycans are important in the attachment of mycobacteria, especially in the interaction with nonphagocytic cells such as fibroblasts and epithelial cells (23), which are possible reservoirs of persisting *M. tuberculosis* in healthy humans (24).

Prasad et al. (25) used T cell blot assay to identify an immunodominant protein in healthy contacts with tuberculosis patients. They designated that protein as histone-like protein of *M. tuberculosis* (HLP*Mt*), which is the same molecule as MDP1. Both humoral and lymphoproliferative responses against recombinant HLP*Mt*/MDP1 were greater in healthy tuberculin reactors than in nonreactors or tuberculosis patients (25). This suggests that HLP*Mt*/MDP1 is an immunodominant Ag that may have an important role in host defense.

In this study we report a series of studies that analyze the antigenicity of MDP1 in a mouse model. We show that both humoral and cellular immune responses to MDP1 are stimulated by the presence of bacterial DNA that contains immunostimulatory CpG motifs (26, 27) that initiate immune responses through TLR9 (28). Simultaneous immunization with MDP1 and DNA, but not MDP1 alone, promotes protection against an *M. tuberculosis* challenge. An in vitro study demonstrated that a complex of MDP1 and CpG DNA markedly stimulates the production of proinflammatory cytokines in a TLR9-dependent manner. Proteins produced by pathogenic organisms are major targets of host immune responses that lead to protective immunity. Our data demonstrate that immunostimulatory cellular components that interact with these proteins have significant effects on protein recognition by the host and the subsequent development of protective immunity.

Materials and Methods

Mice

Female A/J, BALB/c, C3H/HeJ, and C57BL/6 mice were purchased from Japan SLC at 5–7 wk of age. TLR9 knockout (KO) mice (B6 129F2 background) were supplied by Dr. S. Akira (Osaka University, Osaka, Japan) (28). All mice were kept under specific pathogen-free conditions.

Bacterial strains and culture

BCG (strain Tokyo) was grown at 37°C in Middlebrook 7H9 media (Difco) supplemented with 10% albumin, dextrose, and catalase enrichment (Difco) and 0.05% Tween 80. When the OD at 630 nm was ~0.5, bacteria were collected by centrifugation and suspended in sterilized water to adjust for an OD of 1.0. Mice were infected i.p. with 5–10 × 10^6 CFU of BCG in 0.2 ml of normal saline. Two weeks later, mice were boosted with the same dose of BCG i.p. The bacterial dose was determined by counting CFUs 3 wk after plating serial 10-fold dilutions of suspension onto Middlebrook 7H11 agar containing oleic acid, dextrose, albumin, and catalase enrichment (Difco; 7H11-OADC agar).

Antigens

Recombinant histidine-tagged MDP1 (rMDP1) was purified from *Escherichia coli* transfected with pET21b$^+$-*mdp1* by methods described previously (16). Native MDP1 was purified from BCG (Tokyo strain) using the method described previously (14). Ag 85 complex B (Ag85B) purified from *M. tuberculosis* H37Rv was a gift from Dr. S. Nagai (29). Heat stress-induced ribosome-binding protein A (HrpA), purified as a recombinant protein (30), was supplied by Drs. N. Ohara and T. Tabira (Nagasaki University, Nagasaki, Japan). Bovine histone H1, histone H2A, and histone H3 were purchased from Roche. Bacterial DNA was purified from *M. tuberculosis* H37Rv by phenol-chloroform extraction (31). Briefly, 5 g of *M. tuberculosis* H37Rv (wet weight) was suspended in 5 ml of 10 mM Tris-HCl and 1 mM EDTA (pH 7.5; TE buffer), mixed with the same volume of chloroform/methanol (2/1), and incubated for 5 min to remove lipids. The suspension was centrifuged at 2,500 × *g* for 20 min, and both organic and aqueous layers were decanted to leave a packed bacterial band. Delipidated bacteria were incubated at 55°C for 20 min to remove traces of organic solvents and were resuspended in 5 ml of TE buffer and 0.5 ml of 1 M Tris-HCl (pH 9.6). Lysozyme (Sigma-Aldrich) was added to a final concentration of 100 μg/ml and incubated for 2 h. Then 0.1 vol of 10% SDS and 0.01 vol of proteinase K (Sigma-Aldrich) were added and additionally incubated overnight. To remove contaminating proteins, the same volume of phenol was added, gently mixed for 20 min, and centrifuged at 12,000 × *g* for 20 min. The aqueous layer was transferred to the fresh tube, and the protein-removing step was repeated again. Then the same volume of chloroform/isoamyl alcohol (24/1) was added and gently mixed for 10 min. The tube was centrifuged at 12,000 × *g* for 10 min, then the supernatant was transferred to new tube. DNA was precipitated by gently mixing after adding 0.1 vol of 3 M sodium acetate (pH 5.2) and 2.5 vol of ethanol. The tube was then centrifuged at 12,000 × *g* for 10 min, and the DNA pellet was rinsed with 70% ethanol. The pellet was resolved in pure water, and the concentration was determined by the absorbance at 260 nm. The endotoxin level of Ags was <50 pg/100 μM, as determined by a *Limulus* test.

Immunization of mice with protein Ags and BCG

Protein Ags were emulsified using the RIBI adjuvant system (RIB; Corixia), which consists of synthetic trehalose dicorynomycolate and monophosphoryl lipid A, or by IFA (Difco). In some cases, Ags were mixed with various amounts of DNA for 10 min at 37°C and then emulsified. Five micrograms of protein with or without DNA was injected i.p. Three weeks later, mice were boosted using the same method as the primary immunization. The same protocol was used for BCG immunization. Five to 10 × 10^6 CFU of BCG was i.p. injected per mouse. Three weeks after the boost, peripheral blood was obtained from the retro-orbital plexus of anesthetized mice, and sera were isolated and stored at −80°C until the assays.

Western blot

One microgram of purified MDP1 was fractionated by SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and reacted with antisera diluted 1/200.

ELISA

Ninety-six-well ELISA plates (Sumitomo) were coated with individual protein Ags, such as MDP1, HrpA, Ag85B, histone H1, histone H2A, and histone H3, by overnight incubation in carbonate buffer (pH 9.6) at 4°C. Wells were then blocked by PBS containing 3% BSA for 2 h at room temperature. Equal volumes of sera from at least five mice were mixed in each experimental group. Sera were serially diluted in PBS containing 1% BSA, added to wells, and incubated overnight at 4°C. The wells were washed four times with PBS containing 0.05% Tween 20, and HRP-conjugated goat anti-mouse IgG (DakoCytomation), IgG1, IgG2a, IgG2b, IgG3 (Santa Cruz Biotechnology), or IgG2c (Bethyl) diluted in PBS containing 1% BSA was added and incubated for 2 h at room temperature. After washing as before, 100 μl of 80 mM citrate-phosphate buffer (pH 5.0) containing 0.4 μg/ml *o*-phenylendiamine dihydrochloride (Wako Pure Chemicals) was added to the wells, and absorbance at 492 nm was measured by an MTP-300 microplate reader (Corona Electronic).

To determine B cell epitopes, overlapping peptides covering the entire sequence of MDP1 were synthesized previously as 20-mer molecules with 10-aa overlaps with the neighboring peptides, with exception of the C-terminal (15). Each peptide was dissolved in PBS at a concentration of 10 μg/ml and immobilized onto type A ELISA plates (Sumitomo) after activation of the wells by 2% glutaraldehyde. Sera diluted 1/200 by PBS containing 0.05% Tween 20 was added and incubated at 4°C overnight. The ELISA procedure described above was performed, and B cell epitopes were defined by color development with *o*-phenylendiamine dihydrochloride.

Lymph node cell culture and stimulation

Mice were killed 3 wk after the booster injection of Ags, and mesenteric lymph node cells were prepared. Cells were cultured in RPMI 1640 medium (Sigma-Aldrich) supplemented with 10% FBS (Sigma-Aldrich), 25 mM HEPES, 2 mM L-glutamine, 5.5×10^{-5} M 2-ME, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete RPMI medium) in the presence or the absence of 10 μg/ml, MDP1, Ag85B, or purified protein derivative (PPD; Kyowa) in a humidified incubator at 37°C under 5% CO_2. IFN-γ in the culture supernatant was measured with ELISA kits (Genzyme Techne).

Prior immunization and challenge with M. tuberculosis

C3H/HeJ or BALB/c mice were s.c. immunized with 5 μg of RIB-emulsified MDP1 with or without 5 ng of *M. tuberculosis* DNA, DNA alone (5 ng), or 5 μg of Ag85B. BCG Tokyo at a dose of 10^6 CFUs was inoculated using the same procedure without emulsification. After 3 wk, mice were boosted i.p. by the same Ags and were challenged 3 wk later i.v. with 1×10^6 CFU of *M. tuberculosis* Kurono strain (ATCC 35812; American Type Culture Collection). On days 14 and 28, lungs were removed and homogenized using an LS-50 homogenizer (Yamato). The lung homogenates were serially diluted and inoculated onto 7H11-OADC agars. Bacterial numbers were calculated and expressed as CFU.

Spleen cell culture and stimulation

Synthetic oligodeoxynucleotides (ODNs) of sequence GGGGGGAACGT TGGGGGGGGGGGGGGGG were purchased from Nisshinbo and designated oligo B. As a control, cytosine-methylated oligo B was synthesized (Me-oligo B). The endotoxin level was <50 pg/100 μM, as determined by a *Limulus* test. Spleens obtained from C57BL/6 and TLR9 KO mice were cut into small pieces and homogenized. These cell suspensions were depleted of erythrocytes using a Ficoll gradient (Lympholyte-M; Cedarlane Laboratories) and centrifuged for 20 min at 1000 × *g* at room temperature. Spleen cells (1×10^6 cells/well) were cultured in the presence or the absence of MDP1 and ODNs at final concentrations of 0.5 and 1 μM, respectively. After 10-min incubation at 37°C, the MDP1-ODN mixture was added to the cell cultures and incubated for 24 h. As a control, cells were also stimulated with LPS derived from *E. coli* 0111 134 (Difco) at concentration of 100 ng/ml. The amounts of TNF-α and IL-6 in the culture supernatants were measured with ELISA kits (Genzyme Techne).

Statistical analyses

Statistical analysis was conducted with a Power Macintosh G4 using StatView 5.0 (SAS Institute). ANOVA was used to determine the significance of differences in means between multiple experimental groups. The significance level of the test was <5%.

Results

Anti-MDP1 IgG production in mice

To explore the antigenicity of MDP1, we first analyzed the humoral immune response to MDP1 in mice. BCG was inoculated into three strains of mice, including A/J, BALB/c, and C3H/He. Western blot analysis showed that MDP1 elicited a humoral immune response in all strains (Fig. 1*A*). Sera from nonimmunized mice did not react with MDP1 (data not shown). Additionally, anti-MDP1 IgG was produced in C3H/He and BALB/c mice challenged with *M. tuberculosis* H37Rv (data not shown).

We next assessed the antigenicity of purified MDP1. Five micrograms of MDP1 was emulsified in RIB and injected into C3H/He mice. In contrast to inoculation of BCG, we could not detect a significant level of anti-MDP1 IgG (Fig. 1*A*). MDP1 presumably binds to DNA, which includes immunostimulatory CpG motifs (27). Therefore, we tested the simultaneous administration of MDP1 and DNA. Five micrograms of MDP1 was incubated with 0.5 μg of DNA derived from *M. tuberculosis* H37Rv, and the mixture was injected into C3H/He mice. Western blot analysis showed that a combination of MDP1 and DNA elicited MDP1-specific IgG production, whereas MDP1 or DNA alone did not (Fig. 1*A*).

We next determined the optimal dose of DNA that could enhance anti-MDP1 IgG production. Using RIB, 5 μg of MDP1 was administered to C3H/He mice with or without 10-fold serial dilutions of DNAs ranging from 5 μg to 5 ng. Three weeks after the booster injection, the level of anti-MDP1 IgG was measured by ELISA (Fig. 1*B*). The production of IgG was dependent on the amount of DNA; interestingly, 5 ng of DNA most efficiently stimulated IgG production against MDP1. We observed enhanced anti-MDP1 IgG production by coadministration of DNA and MDP1 in the presence of IFA (Fig. 1*C*), and the result was similar to that

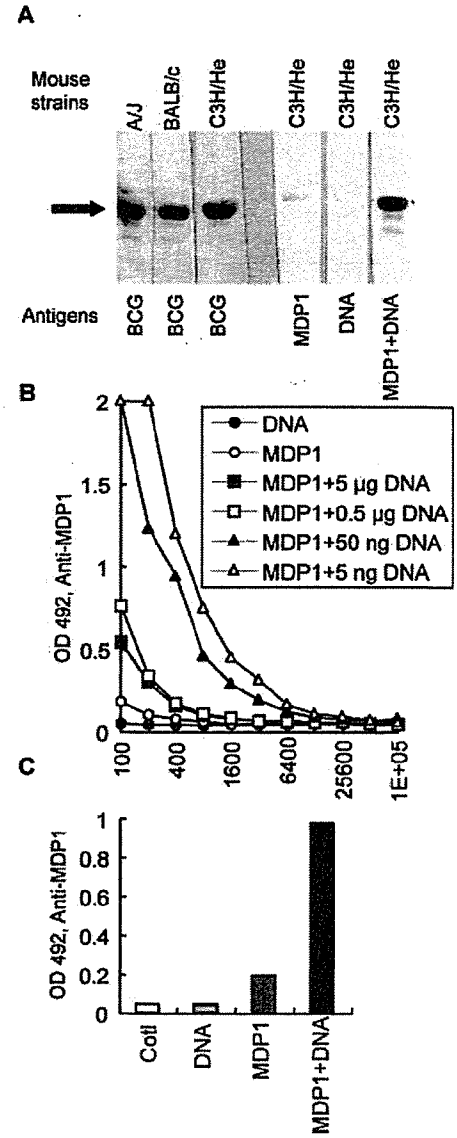

FIGURE 1. Humoral immune responses to MDP1 in mice. *A*, Western blot analysis. Purified MDP1 was blotted onto the membrane and incubated with 200/1 diluted antisera. Mouse strains are indicated along the *top* of the panel. The injected Ags, such as BCG, MDP1 alone (MDP1), *M. tuberculosis* DNA alone (DNA), and MDP1 plus DNA (MDP1+DNA) are shown along the *bottom*. *B*, DNA dose effects on anti-MDP1 IgG production. C3H/He mice were immunized with MDP1 (5 μg/mouse) with or without various amounts of DNA (5 μg to 5 ng) emulsified in RIB adjuvant, and levels of anti-MDP1 IgG were determined by ELISA. The horizontal axis shows dilution factors of antisera. *C*, Immunization with MDP1 plus DNA emulsified in IFA augmented anti-MDP1 IgG production. C3H/He mice were immunized with Ags emulsified in IFA. Immunized Ags are described *below* the horizontal axis. Cotl, IFA alone. The sera from at least five mice of each experimental group were mixed and diluted to 1/400, and the levels of anti-MDP1 IgG were determined by ELISA.

observed using RIB adjuvant, suggesting that the immunostimulatory effect of DNA on anti-MDP1 IgG production is not restricted to RIB adjuvant. The results prompted us to explore whether DNA-dependent IgG production varies between mouse strains. The same immunization procedure using RIB as an adjuvant was performed in other mouse strains, including A/J, BALB/c, and C57BL/6. The results revealed that simultaneous inoculation of MDP1 and DNA augmented the production of IgG against MDP1 in all tested strains (Fig. 2).

To determine whether DNA-mediated enhancement of anti-MDP1 IgG production is restricted to the particular IgG isotype, we analyzed the distribution of subclasses of IgG by ELISA. As shown in Fig. 3, each mouse strain possessed a specific pattern of MDP1-specific IgG isotypes, but DNA enhanced only IgG subclasses produced in mice immunized with MDP1 alone. Thus, a small dose of DNA augments the humoral response to MDP1 without altering the pattern of IgG isotypes.

MDP1-specific, DNA-dependent stimulation of IgG production

Our data showed that a small amount of DNA (5 ng) magnified anti-MDP1 IgG production (Fig. 1*B*). In contrast, 1,000- to 100,000-fold higher amounts (5–500 μg) of bacterial DNA and CpG ODNs have been applied as adjuvants in immunization with foreign Ags (32–34) or immunotherapeutic treatments (34–38). Therefore, we next examined whether 5 ng of DNA stimulated Ab production against other immunogenic mycobacterial proteins such as HrpA (39) and Ag85B (11). These Ags did not bind to DNA, as determined by gel retardation assay (data not shown). Five micrograms of each Ag was injected into BALB/c, C3H/He, and C57BL/6 mice, with or without 5 ng of DNA. We could not detect enhanced Ab production by coadministration of DNA in any of the three mouse strains (Fig. 4, *A* and *B*).

Next we examined whether DNA combined with DNA-binding proteins other than MDP1 stimulates IgG production. Bovine histone H1, histone H2A, and histone H3 were injected into three strains of mouse (BALB/c, C3H/He, and C57BL/6) with or without 5 ng of DNA. We could not detect the production of IgG against both histones H2A and H3 in any of mouse strains tested (data not shown). In contrast, anti-histone H1 Ab was detectable in all mouse strains, but DNA alone did not stimulate anti-histone H1 IgG production (Fig. 4*C*). Although we have not tested all DNA-binding proteins, these results imply that enhanced Ab production by a very small amount of bacterial DNA is a unique feature of MDP1.

DNA alters B cell epitopes of MDP1

To examine humoral immune responses against MDP1 more precisely, we defined the region(s) recognized by anti-MDP1 IgG. B

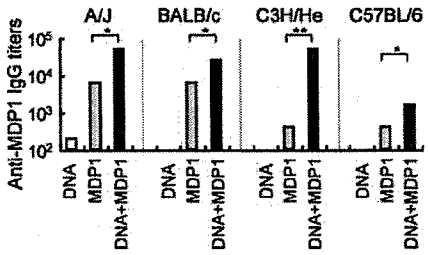

FIGURE 2. DNA stimulates the production of anti-MDP1-IgG in mice. Four strains of mice, including A/J, BALB/c, C3H/He, and C57BL/6, were immunized with DNA alone (□), MDP1 (5 μg/mouse) alone (▨), or MDP1 plus DNA (■). The titer of anti-MDP1-IgG was determined by ELISA. *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

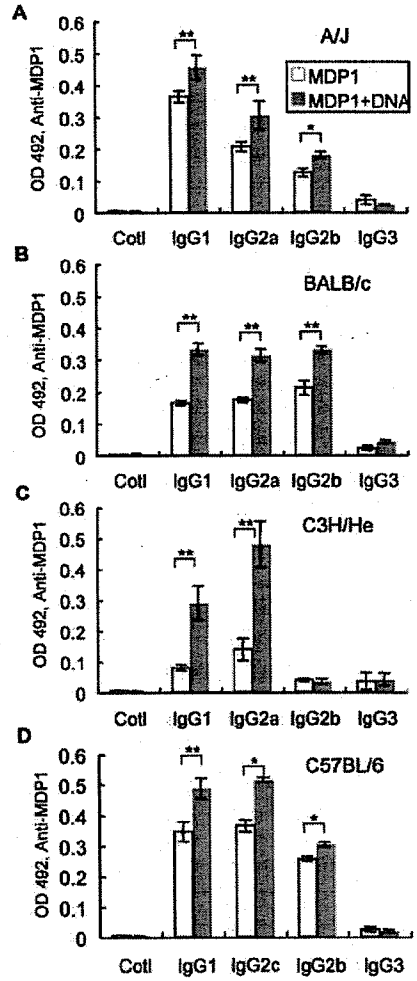

FIGURE 3. Isotypes of anti-MDP1 IgG. The levels of IgG subclasses were measured using isotype-specific Abs against IgG1, IgG2a, IgG2b, IgG2c, and IgG3. Cotl, Controls without secondary Ab. □, Immunization with MDP1 alone; ▨, coadministration of MDP1 and DNA. Antisera were diluted 1/100 (*A–C*) or 1/50 (*D*). *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

cell epitope mapping was conducted by ELISA using synthetic 20-mer peptides covering the entire MDP1 sequence. Antisera were obtained from four strains of mice, including A/J, BALB/c, C3H/He, and C57BL/6, immunized with MDP1 alone or with 5 ng of DNA and were reacted with each peptide. In A/J mice, IgG from animals immunized with MDP1 alone did not react with peptides, although it was bound to MDP1, suggesting that IgG in these mice recognized the conformational structure of MDP1 (Fig. 5*A*). In contrast, two peptides corresponding to aa 61–80 and 71–90 of MDP1 were recognized by anti-MDP1 IgG in mice immunized with MDP1 plus DNA (Fig. 5*A*). In BALB/c mice, anti-MDP1 IgG induced by injection of both MDP1 alone and MDP1 plus DNA reacted with the peptide corresponding to 51–70 of MDP1 (Fig. 5*B*). In C3H/He mice, the level of anti-MDP1 IgG was insignificant when MDP1 alone was used (Fig. 5*C*). In contrast, anti-MDP1 IgG was produced in animals immunized with MDP1 plus DNA and reacted with peptides corresponding to 141–160 and 151–170 (Fig. 5*C*). Thus, the epitope was likely to be the 151–160 region of MDP1. In C57BL/6 mice, Abs from mice immunized with MDP1 alone and MDP1 plus DNA reacted with the 61–80 and 1–20 regions, respectively (Fig. 5*D*).

Although the anti-MDP1 Ab titer was higher in BALB/c mice injected with MDP1 plus DNA than in mice immunized with

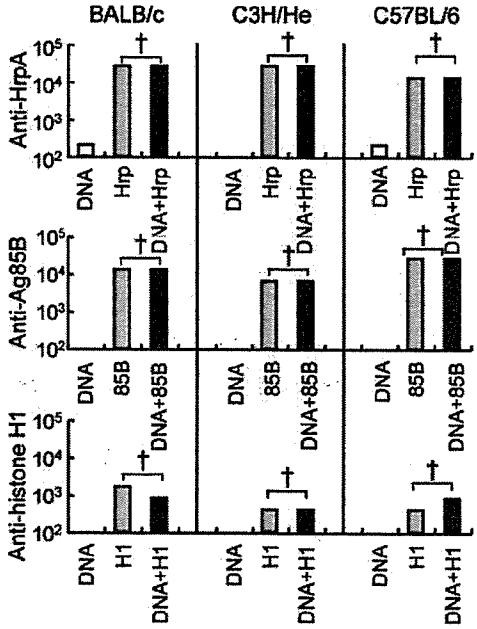

FIGURE 4. DNA fails to stimulate production of IgGs against mycobacterial Ags, HrpA and Ag85B, and a DNA-binding protein, histone H1. BALB/c, C3H/He, and C57BL/6 mice were immunized with 5 μg of HrpA (Hrp), Ag85B (85B), and histone H1 (H1) with (■) or without (▤) 5 ng of DNA. □, Immunization with DNA alone. The titer of Ag-specific IgG was measured by ELISA. †, $p > 0.1$ (by ANOVA).

MDP1 alone, the level of anti-MDP1 IgG against the defined epitope (aa 51–70) was reversed (Fig. 5*B*). This suggests that anti-MDP1 IgG recognizes mainly conformational epitopes in mice immunized with a mixture of MDP1 and DNA. To examine this possibility, inhibition assays were performed. The interaction between MDP1 and IgG from mice immunized with MDP1 alone (Fig. 6*A*), but not with MDP1-DNA (Fig. 6*B*), was inhibited by exogenously added peptide corresponding to aa 51–70 of MDP1 (Fig. 6, *A* and *B*). In contrast, the same molar amount of exogenously added MDP1 alone inhibited both reactions (Fig. 6, *A* and *B*). These data indicate that in BALB/c mice, administration of MDP1 alone produces IgG that recognizes only the 51–70 region. In contrast, administration of MDP1 plus DNA induces anti-MDP1 IgG targeting conformational epitopes on MDP1 in addition to the 51–70 region.

Similar inhibition experiments were conducted using sera from BALB/c mice injected with live BCG. The 51–70 peptide failed to abrogate the IgG-MDP1 interaction (Fig. 6*C*), although MDP1 itself did. This suggests that MDP1 is actually binding to DNA in vivo and is targeted by the host immune response.

MDP1 stimulates IFN-γ production

Protective immunity against *M. tuberculosis* infection is mediated primarily by Th1-type cell-mediated immunity (40, 41). IFN-γ triggers Th1-type cell-mediated immune responses and plays a critical role in host defense against *M. tuberculosis* infection in mice (42, 43). To investigate whether MDP1 participates in BCG-mediated protection against tuberculosis, we examined IFN-γ production induced by MDP1 stimulation. Lymph node cells from C3H/He mice immunized with BCG were cultured in the presence or the absence of MDP1, and the level of IFN-γ in culture supernatants was measured by ELISA. The results show that MDP1 stimulated IFN-γ production in a manner similar to Ag85B and PPD (Fig. 7*A*). We next examined isotypes of anti-MDP1 IgG in

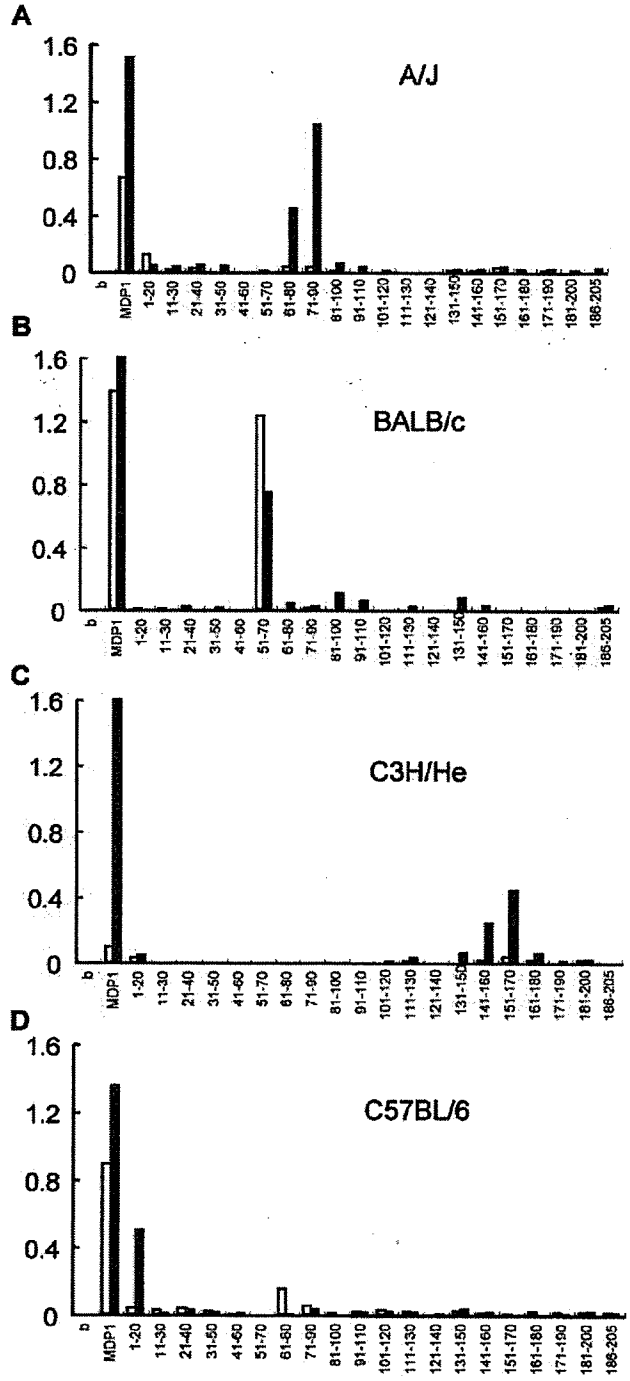

FIGURE 5. B cell epitope mapping of anti-MDP1 IgG. Epitopes of anti-MDP1 IgGs were determined by ELISA. MDP1 or 20-mer synthetic peptides covering the entire MDP1 sequence were coated on the ELISA plate as indicted at the *bottom* of each graph. b, Blank well without Ag coating. The same antisera as those described in Fig. 2 were diluted 1/200 and applied to the wells. *A–D*, Analysis of antisera derived from A/J, BALB/c, C3H/He, and C57BL/6 mice, respectively. □, Antisera from mice inoculated with MDP1 alone; ■, antisera from mice inoculated with MDP1 plus DNA. The ELISA units represent the average of duplicate samples.

BCG-immunized C3H/He mice. BCG inoculation stimulated the production of MDP1-specific IgG1 and IgG2a, but not IgG2b or IgG3 (Fig. 7*B*). Interestingly, the pattern of IgG isotypes was similar to that observed in the same mouse strain immunized with both

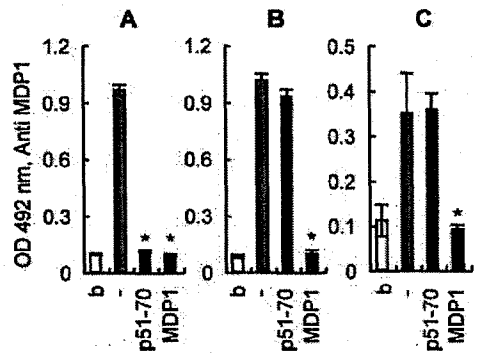

FIGURE 6. Production of anti-MDP1 IgG-targeting conformational epitopes in BALB/c mice immunized with MDP1 plus DNA or BCG, but not with MDP1 alone. Antisera from BALB/c mice immunized with MDP1 alone (*A*), MDP1 plus DNA (*B*), and BCG (*C*) were reacted with immobilized MDP1 on ELISA plates with or without exogenously added peptide corresponding to the 51–70 region of MDP1 (p51–70) or MDP1 (MDP1). b, Blank without Ag coating; −, positive controls without inhibitors. *, $p < 0.05$ (by ANOVA, vs controls without inhibitors (−)).

MDP1 and DNA (Fig. 3*C*). IFN-γ induces IgG2a production (44), whereas both Th1-related and Th2 cytokines stimulate IgG1 production (45, 46). The predominant production of IgG2a implies that the immune response to MDP1 is polarized toward the Th1 type. It is likely that MDP1 is one of the Ags that induce protective immunity after BCG immunization in C3H/He mice.

Next we examined whether the administration of purified MDP1 induces IFN-γ production. C3H/He mice were immunized with MDP1 alone or with MDP1 plus DNA. As controls, RIB and DNA alone were administered to mice as well. Lymph node cells were cultured with or without 10 μg/ml MDP1, and the production of IFN-γ was assessed. The results showed that MDP1 stimulates IFN-γ production (Fig. 7*C*). However, immunization with MDP1 mixed with DNA produced much more IFN-γ than that with MDP1 alone, demonstrating that DNA augments cell-mediated immune responses to MDP1 (Fig. 7*C*).

Simultaneous administration of MDP1 and DNA confers protection against M. tuberculosis *infection in mice*

The ability to produce IFN-γ by MDP1 prompted us to explore whether MDP1 could induce protection in vivo against challenge with a virulent strain of *M. tuberculosis*. C3H/He mice were immunized with MDP1 alone or with MDP1 plus 5 ng of DNA. As controls, mice were given RIB alone, DNA alone, BCG, or Ag85B, which is a major vaccine candidate (11). After a 3-wk interval, mice were boosted with the same Ag; 3 wk later, mice were challenged with *M. tuberculosis* Kurono. After 14 and 28 days, mice were killed, and the numbers of bacteria in the lungs and spleens were determined. These data showed that immunization with Ag85B, DNA, and MDP1 failed to protect (Fig. 8, *A–C*). In contrast, BCG and coadministration of MDP1 and DNA significantly reduced the bacterial load in the lungs ($p < 0.005$ and $p = 0.0119$ on day 14, and $p = 0.008$ and $p = 0.0316$ on day 28, respectively). A protective effect of immunization of MDP1 plus DNA, but not MDP1 alone, was also observed in the spleens ($p = 0.021$; Fig. 8*C*). As shown in Fig. 8*D*, immunization with both MDP1 and DNA resulted in a modest, but significant, decrease in bacterial burden in BALB/c mice as well ($p < 0.005$). Although the effect was less than that of BCG, MDP1 confers substantial protection against *M. tuberculosis* challenge only when it is administered with DNA.

MDP1 augments TLR9-dependent immunostimulation by CpG DNA

Immunostimulatory effects of DNA are dependent on unmethylated CpG motifs (27) that signal via TLR9 (28). Our data revealed that a very small amount of DNA stimulates immune responses against MDP1, in contrast with previous reports (32–34). Therefore, we hypothesized that MDP1 might enhance the immunostimulatory activity of CpG DNA. To test this hypothesis, we evaluated the effect of MDP1 on CpG-ODN-mediated immune activation in vitro. Spleen cells from both C57BL/6 and TLR9 KO C57BL/6 mice were stimulated with oligo B containing CpG DNA sequence in the presence or the absence of rMDP1. Me-oligo B, which has the same structure, except that its cytosine is methylated, and LPS, which signals via TLR4 (47, 48), were used as controls. After 24 h, levels of the proinflammatory cytokines TNF-α and IL-6 in the culture supernatants were determined by ELISA. Oligo B alone (1 μM) did not induce the production of TNF-α (Fig. 9). In contrast, the mixture of rMDP1 and oligo B dramatically stimulated TNF-α production (Fig. 9). This effect was undetectable in splenocytes from TLR9 KO mice or with the combination of Me-oligo B and rMDP1. Similar results were seen for

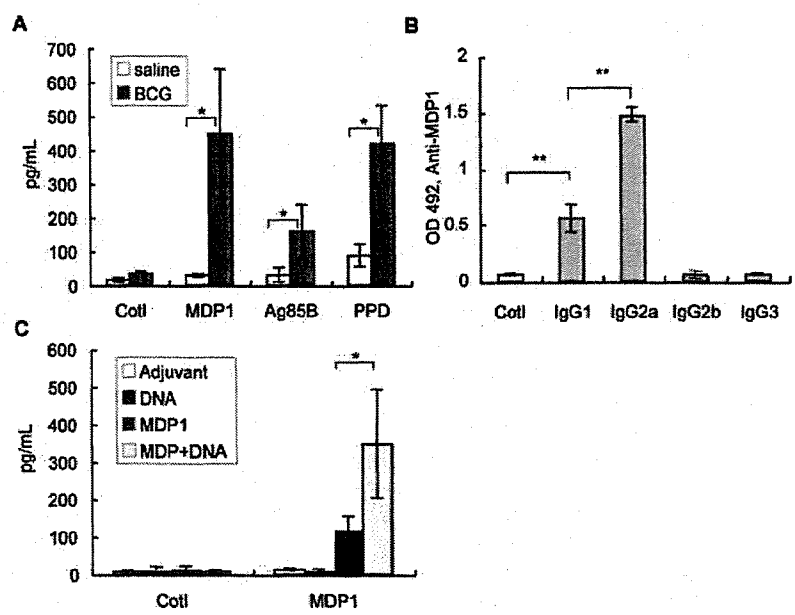

FIGURE 7. Development of Th1-type immune responses against MDP1 after challenge with BCG (*A* and *B*) or MDP1 (*C*). *A*, Amounts of IFN-γ in culture supernatants from lymph node cells were determined by ELISA. Lymph nodes were derived from C3H/He mice immunized with saline (□) or BCG (▨) and incubated for 5 days with 10 μg/ml MDP1, Ag85B, and PPD as indicated. Cotl, without Ag stimulation. The production of IFN-γ was measured by ELISA. *B*, MDP1-specific IgG isotypes (IgG1, IgG2a, IgG2b, and IgG3) in sera of C3H/He mice immunized with BCG detected by ELISA. Cotl, controls without secondary Ab. *C*, Lymph node cells derived from C3H/He mice immunized with adjuvant alone (□), DNA (■), MDP1 (▧), and MDP1 plus DNA (▨) were cultured in the presence (MDP1) or the absence (Cotl) of 10 μg/ml MDP1 for 5 days, and the amounts of IFN-γ in the culture supernatants were determined by ELISA. *, $p < 0.05$; **, $p < 0.01$ (by ANOVA).

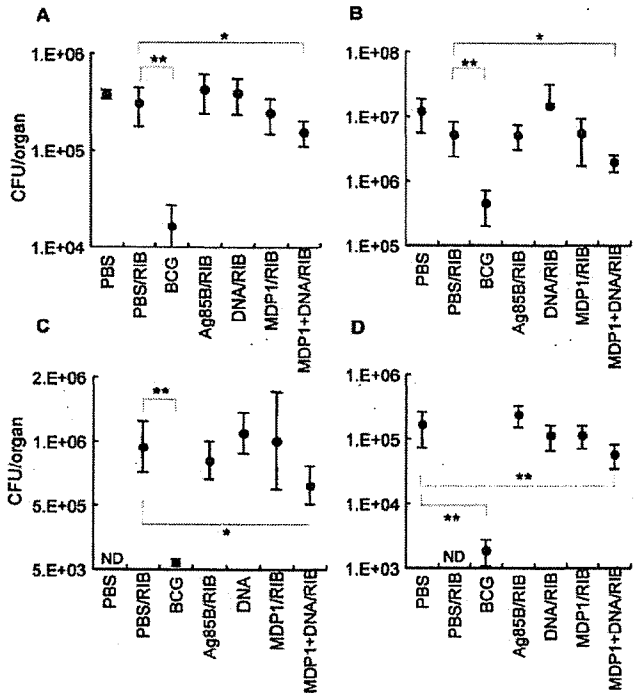

FIGURE 8. Coadministration of MDP1 and DNA confers protection against *M. tuberculosis* challenge. C3H/HeJ (*A–C*) and BALB/c (*D*) mice were immunized with Ags, as indicated below the horizontal axis, and challenged i.v. with 10^6 CFU of *M. tuberculosis* Kurono strain. Fourteen (*A*) and 28 (*B–D*) days after the challenge, bacterial numbers in lung (*A*, *B*, and *D*) and spleen (*C*) were determined by counting CFUs. *, $p < 0.05$; **, $p < 0.005$ (by ANOVA).

IL-6 production, although 0.5 μM rMDP1 itself induced a low level of IL-6 production (Fig. 9). Similar data were obtained when natural MDP1 was used under the same conditions (data not shown). These data clearly demonstrate that MDP1 activates TLR9-dependent immunostimulation by CpG ODN.

Discussion

In the present study we have evaluated the antigenicity of MDP1, a DNA-binding protein specific to mycobacteria. Anti-MDP1 IgG

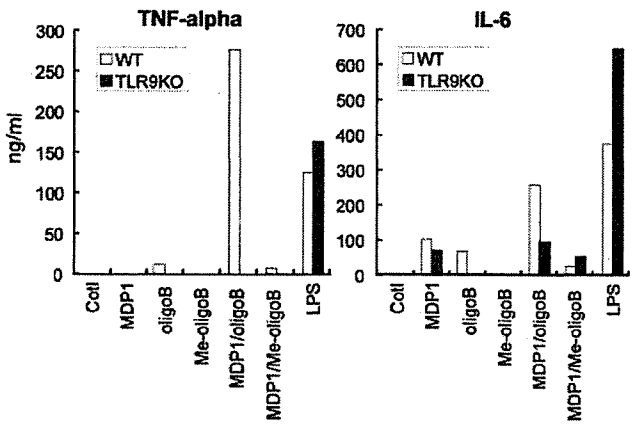

FIGURE 9. MDP1 enhances CpG-mediated production of proinflammatory cytokines in vitro. Splenocytes were stimulated with 0.5 μM MDP1 alone, a mixture of MDP1 and ODNs (1 μM), or *E. coli* LPS (100 ng/ml) for 24 h in vitro. Levels of TNF-α (*right*) and IL-6 (*left*) were determined by ELISA. The ELISA units represent the average of duplicate samples and are representative of two experiments performed.

was produced in C3H/He mice challenged with either BCG (Fig. 1*A*) or *M. tuberculosis* (data not shown). Marked cell proliferation occurred when splenocytes from *M. tuberculosis*-infected mice were stimulated with 10 μg/ml MDP1 in vitro. Uptake of [^{3}H]thymidine was higher compared with stimulation with the gold standard, PPD (our unpublished observations). Thus, in agreement with a human study (25), MDP1 is also highly immunogenic in mice.

However, administration of purified MDP1 failed to produce anti-MDP1-IgG in C3H/He mice (Fig. 1). This lack of antigenicity was reversed by adding mycobacterial DNA when immunizing with MDP1 (Fig. 1). Similarly, DNA enhanced the production of MDP1-specific IgG in other mouse strains (Fig. 2). B cell epitope mapping (Fig. 5*B*) and Ab reaction-inhibition assay (Fig. 6) implied association of MDP1 with genomic DNA in live BCG. These results suggest that the strong immunogenicity of MDP1 in mycobacterial infection is responsible for colocalization of DNA.

Studies to determine the optimal dose of DNA showed that 5 ng of DNA was enough to activate MDP1-specific IgG production (Fig. 1*B*). This dose is unusually low compared with other studies in which 5–500 μg of DNA or ODN/mouse was used for immune activation (32–34, 36–38). We confirmed that 5 ng of DNA did not enhance the production of IgG against other proteins, including DNA-binding proteins (Fig. 4). Thus, a very small amount of DNA-stimulated Ig production appears to be a specific feature of MDP1.

We determined B cell epitopes on MDP1 by using synthetic peptides. B cell epitopes differed among mouse strains. Surprisingly, the epitopes were different when DNA was coadministered with MDP1, even within the same mouse strain (Fig. 5). Thus, DNA not only stimulates MDP1-specific IgG production, but also modifies the recognition site of IgG. This suggests that the three-dimensional structure of MDP1 differs depending on whether DNA is present or absent, and this difference is recognized by the immune system of the host. This conformational change might be involved in the disparate antigenicities of this protein.

To investigate the role of MDP1 in host protection, we examined the activity of MDP1 in the induction of IFN-γ that is critical for host defense against *M. tuberculosis* infection in mice (42, 43). When stimulated in vitro with 10 μg/ml MDP1, lymph node cells derived from BCG-immunized C3H/He (Fig. 7*A*) and C57BL/6 (data not shown) mice produced a significant amount of IFN-γ. Analysis of IgG isotype in BCG-immunized mice revealed the production of MDP1-specific IgG2a, which was indicative of a Th1-type immune response (Fig. 7*B*) (44). Administration of purified MDP1 also expanded the population of IFN-γ-producing cells (Fig. 7*C*) and stimulated Th1-associated IgG2a production (Fig. 3). Again, simultaneous injection of MDP1 and DNA stimulated adaptive immunity and enhanced IFN-γ production (Fig. 7*C*). This was confirmed when mice were infected with *M. tuberculosis*, and MDP1 was found to decrease bacterial load only when coadministered with DNA (Fig. 8). Thus, MDP1 can be a novel vaccine target, although it is effective only when administered simultaneously with DNA. Because *M. tuberculosis* is transmitted by the aerogenic route, future studies are needed to explore the efficacy using the aerosol challenge model.

As discussed above, our data show that MDP1 has a unique feature as an Ag, in that its antigenicity is profoundly enhanced by even a small amount of DNA. This raises an important question as to how this immune stimulation is coordinated. At least six nucleotides are necessary for immune activation by ODN (49). Because DNA is highly sensitive to degradation by DNases, a large amount of DNA is required for immune activation (50). We found that

MDP1 blocks degradation of DNA by DNases in vitro (unpublished observations), and this DNA-protective activity of MDP1 is one possible explanation.

Another possible explanation is the cell-binding activity of MDP1. To exert immunostimulatory activity, CpG DNA must attach to the macrophage surface and be internalized, with subsequent maturation of the phagosome (51). In our preliminary work, biotin-labeled ODN was more quickly bound to the macrophage surface and internalized when it was added with MDP1 (our unpublished observations). We have demonstrated that MDP1 binds to glycosaminoglycans and to A549 human lung epithelial cells through hyaluronic acid (16). After adding 0.5 μM MDP1, >95% of A549 cells became MDP1 positive in 60 min (16). In addition, it has been shown that HupB/MDP1 binds to C3 (52). Complement receptors are major receptors for *M. tuberculosis* on macrophages (53, 54). Collectively, it is reasonable to assume that MDP1 binds to macrophages through surface glycosaminoglycans or complement receptors. This cell-binding activity of MDP1 is advantageous for carrying DNA to/into macrophages, resulting in subsequent immunostimulation.

Immunization with MDP1 plus mycobacterial DNA significantly reduced the bacterial burden compared with treatment with Ag85B (Fig. 8). To develop effective vaccines against tuberculosis, additional studies are necessary to assess vaccine efficacy using MDP1 in conjunction with CpG-ODNs that can induce a Th1 response (32–34). Although the Ag85 complex has been widely studied as a major component of tuberculosis vaccines (11, 55), we did not observe a protective effect (Fig. 8). These conflicting results may be due to the mouse strains used in this experiment, because Ag85A and 85B induce protective immunity against mycobacterial infection in C57BL/6 mice (55, 56). The protective effect of Ag85B is conspicuous in guinea pigs as well (11). Guinea pigs are relatively susceptible to *M. tuberculosis* infection, whereas the mouse has low to moderate susceptibility (57, 58). In addition, guinea pigs, but not mice, develop cavitary lesions and caseous necrosis similar to human tuberculosis. It will be important to examine the protective effect of coadministration of MDP1 and DNA in a guinea pig model.

A key step in initiating adaptive immunity is the presentation of pathogen-derived peptides on class II MHC molecules by APCs. APC functions are up-regulated after recognition of pathogen-associated molecular patterns, including CpG DNA motifs (28). Therefore, we examined the effects of MDP1 on CpG ODN-mediated immune activation. We found that MDP1 magnified CpG-DNA effects, such as the production of the proinflammatory cytokines TNF-α and IL-6 (Fig. 9). As far as we know, MDP1 is the first protein identified that remarkably enhances CpG-mediated immune stimulation. Proinflammatory cytokines are critical for APC activation and promote the maturation of professional APCs. Immunostimulation induced by the interaction between MDP1 and CpG DNA might be involved in inducing strong adaptive immune responses against MDP1, which lead to protection (Fig. 8).

MyD88 is an adaptor molecule critical for the CpG-DNA-TLR9 signaling pathway (59, 60). Recently, it was shown that MyD88 KO mice are highly susceptible to *M. tuberculosis* (61) and *M. avium* (62), although mice with genetic mutations of TLR2 and TLR4 displayed comparable resistance as wild-type mice challenged with *M. tuberculosis* (63) and *M. avium* (62). These studies suggest that resistance to mycobacterial infection is regulated by multiple MyD88-dependent signals in addition to those attributed to TLR2 (64) or TLR4. As we show in this study, MDP1 stimulates TLR9-dependent immune responses by CpG ODN (Fig. 9), and the MDP1-DNA complex can induce protective immunity (Fig. 8). TLR9 signaling stimulated by MDP1-mycobacterial DNA complexes might be involved in MyD88-dependent antimycobacterial immunity.

The immunostimulatory activity of DNA was initially discovered in a DNA-rich fraction derived from BCG, referred to as MY1 (65, 66). Those studies demonstrated that the antitumor activity of MY1 was diminished by DNase treatment. MY1 is a mycobacterial nucleoid (65, 66). It is conceivable that MDP1 is involved in the activity of MY1.

The immunostimulatory activity of DNA has huge potential for immunotherapy against infectious, neoplastic, and allergic diseases (50, 67–69). To our knowledge, MDP1 is the first protein discovered that remarkably augments CpG-mediated immune stimulation (Fig. 9). MDP1 has great potential as an adjuvant for CpG-ODN-based immune interventions.

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Disclosures

The authors have no financial conflict of interest.

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Mycobacterial trehalose 6,6′-dimycolate preferentially induces type 1 helper T cell responses through signal transducer and activator of transcription 4 protein

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Abstract

Mycobacterium tuberculosis is an intracellular pathogen of tuberculosis and its pathogenicity is related to the ability to escape killing by ingested macrophages and induce delayed-type hypersensitivity (DTH). A major component of the cell wall of *M. tuberculosis* is trehalose 6,6′-dimycolate (TDM), which has been implicated as a pathogenetic factor. The expression of DTH and cell-mediated immunity is dependent on the macrophage-cytokine-type 1 helper T (Th1) lymphocyte axis. Cytokines, interleukin-12 (IL-12) and interferon-γ (IFN-γ), play a critical role in the process and IL-12-activated signal transducer and activator of transcription (STAT) 4 is required for the development of fully functional Th1 cells. To clarify host responses to mycobacterial TDM, we have analyzed footpad reaction, histopathology and cytokine profile of experimental granulomatous lesions using STAT4-deficient mice. In the present study, we have demonstrated that mycobacterial TDM selectively induces the Th1 response through the STAT4 signaling pathway, because mice lacking STAT4 protein significantly reduced to develop DTH, hypersensitivity granulomas, and Th1 cytokine responses, when compared to BALB/c mice. These results shed light on the molecular pathogenesis of mycobacterial disease. Taken together with previous studies, TDM is a pleiotropic molecule against the host and participates in the pathogenesis.

Keywords: Trehalose 6,6′-dimycolate (TDM); *Mycobacterium tuberculosis*; STAT4; Granuloma; Delayed-type hypersensitivity (DTH)

1. Introduction

Mycobacterium tuberculosis is one of the most successful human pathogens and infects one-third of world population [1]. The pathogenesis of tuberculosis is concerned with both virulence of the pathogen, *M. tuberculosis*, and the host immune response to the pathogen [2]. *M. tuberculosis* is an intracellular pathogen and its pathogenicity is related to the ability to escape killing by ingested macrophages and induce delayed-type hypersensitivity (DTH) [3]. The macrophage–cytokine–T lymphocyte axis plays a critical role in both disease expression and defense against the infection.

There is much known but we still have a long way to understand the mechanism of *M. tuberculosis* pathogenicity. Mycobacteria are rich in lipids. Lipid components of the mycobacterial cell wall participate in the pathogenesis. Cord factor (trehalose 6,6′-dimycolate; TDM), a surface glycolipid, causes *M. tuberculosis* to grow in serpentine cords in vitro. Injection of cord factor/TDM into experimental animals induces both foreign-body and hypersensitivity granulomas [4,5]. Hypersensitivity lesions are the expression of DTH through induction of interleukin-12 (IL-12) and interferon γ (IFN-γ). TDM can mediate intracellular trafficking events, and influences macrophage

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production of proinflammatory cytokines, such as IL-12 and IFN-γ [6–8]. TDM has also been indirectly implicated in the inhibition of phagosome–lysosome fusion, because it inhibits fusion between phospholipid vesicles in vitro [9]. Collectively, these support a role for TDM not only in macrophage activation, but also in mycobacterial survival within infected macrophages, perhaps as a result of phagosome arrest [10]. Thus, TDM participates in the pathogenicity of *M. tuberculosis*, such as the ability to escape killing by ingested macrophages and induce DTH.

DTH is defined as an immune response in which T cell-dependent macrophage activation and inflammation cause tissue damage. DTH is a common accompaniment of protective cell-mediated immunity against intracellular pathogens including *M. tuberculosis* [11]. The expression of DTH and cell-mediated immunity is dependent on the macrophage–cytokine-type 1 helper T (Th1) lymphocyte axis. Cytokines, IL-12 and IFN-γ play a critical role in the process and IL-12-activated signal transducer and activator of transcription (STAT) 4 are required for the development of fully functional Th1 cells. A transcription factor called T-bet that is induced by IFN-γ also plays an essential role in Th1 cell development. Similarly, IL-4-activated STAT6 is essential for the differentiation of Th2 cells [12,13]. STAT4 is required for promoting Th1 development and for defense against mycobacterial infection with *Mycobacterium avium* [14], *Mycobacterium leprae* [15], and *M. tuberculosis* [16].

To clarify host responses to mycobacterial TDM, including development of DTH and granulomas in association with STAT4 activation, we have analyzed footpad reaction, histopathology and cytokine profile of experimental granulomatous lesions using mice lacking STAT4 protein.

2. Results

2.1. Decreased DTH responses to TDM in mice genetically deficient STAT4 protein

DTH response to TDM was significantly decreased in STAT4-deficient mice immunized with Freund's complete adjuvant (FCA) (1.78 mm ± 0.25), when compared to preimmunized BALB/c mice (2.46 mm ± 0.32) (Fig. 1). However, mice challenged with Freund's incomplete adjuvant (FIA) revealed very mild swelling and no difference was observed in preimmunized BALB/c (0.23 mm ± 0.14) and STAT4-deficient mice (0.23 mm ± 0.09). Histopathologically, mononuclear cells were infiltrated markedly in the dermis, epidermis and perivascular region in BALB/c mice challenged with TDM, whereas mild to moderate infiltration was observed in STAT4-deficient mice (Fig. 2). BALB/c and STAT4-deficient mice challenged with FIA showed negligible tissue response. Thus, DTH response to TDM was depressed in STAT4-deficient mice.

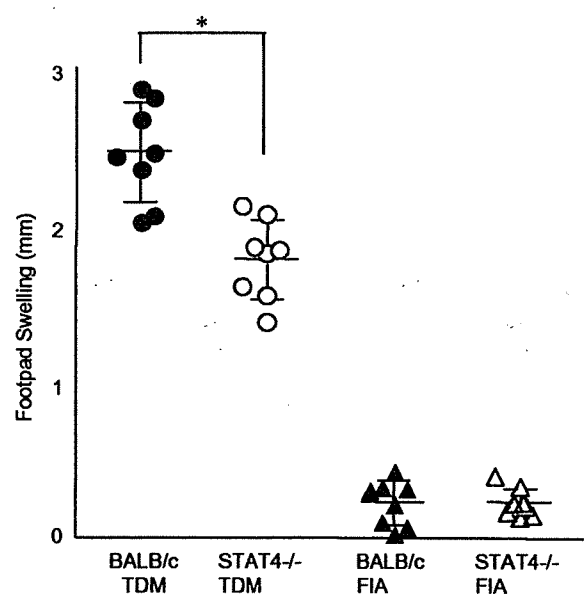

Fig. 1. Footpad DTH responses in BALB/c and STAT4-deficient mice. Mice were immunized with Freund's complete adjuvant (FCA). Eight days after immunization, 20 μl of either TDM (1 mg/ml) or Freund's incomplete adjuvant (FIA) was injected into the hind footpad. The difference of footpad thickness at 24 h after and just before injection was measured. Data represent the mean swelling (mm) ± SD compiled from eight mice per group. The asterisk indicates $P < 0.01$ when compared to BALB/c mice.

2.2. Semiquantitative analysis of granulomatous inflammation of the lungs

It has been demonstrated that w/o/w micelles containing TDM are trapped in alveolar vessels and induce granulomatous inflammation in the lungs [4,5]. To clarify the role of STAT4 in the development of granulomatous inflammation by TDM challenge, 100 μg of TDM was injected intravenously to either BALB/c or STAT4-deficient mice preimmunized with FCA. A significant increase in lung indices and granuloma areas was found in immunized BALB/c mice 3–7 days after the challenge with TDM, compared with STAT4-deficient mice (Fig. 3). A marked increase was found within 3 days, reached the maximum by day 7 in BALB/c mice and by day 3 in STAT4-deficient mice, and gradually declined thereafter. Challenge with w/o/w vehicles alone resulted in the negligible response and no significant difference was observed in BALB/c and STAT4-deficient mice.

2.3. Histopathologic features of the lungs

In both BALB/c and STAT4-deficient mice preimmunized with FCA, mild infiltration of inflammatory cells in alveolar and perivascular areas was seen 1 day after TDM challenge. Three to seven days after the challenge,