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

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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 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 non-replicating dormant state (1). The majority of the disease arises from reactivation of persisting, previously implanted bacteria (2–5).

Bacillus Calmette-Guérin (BCG) is an attenuated live vaccine against tuberculosis and has been given to >2 billion individuals

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* Abbreviations used in this paper: BCG, bacillus Calmette-Guérin; Ag85B, Ag 85 complex B; HLP, histone-like protein; HLPMM, histone-like protein of Mycobacterium tuberculosis; HrpK, 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. Shimjo 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 (HLPm), which is the same molecule as MDP1. Both humoral and lymphoproliferative responses against recombinant HLPm/MDP1 were greater in healthy tuberculin reactors than in nonreactors or tuberculosis patients (25). This suggests that HLPm/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 ~5–10^6 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).

Antigen

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 (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/isooamyl 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; Corixa), 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 with various numbers of times with PBS for 4 min at 37°C and then incubated with 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 of o-phenylenediamine dihydrochloride (Wako Pure Chemicals) was added to the wells, and absorbance at 492 nm was measured by an MTP-300 microplate reader (Corona Electric).

To determine B cell epitopes, overlapping peptides covering the complete 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-phenylenediamine 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^{-3} 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, MDPI, Ag85B, or purified protein derivative (PPD; Kyowa) in a humidified incubator at 37°C under 5% CO_2. IFN-γ of 106 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 GGGGGGGGACGT TGGGGGGGGGGGGGGGGGGGGGGGGGGGGG were purchased from Nisshinbo and designated oligo B. As a control, cytosine-methylated oligo B was synthesized (Mc-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 MDPI and ODNs at final concentrations of 0.5 and 1 μM, respectively. After 10-min incubation at 37°C, the MDPI-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 011B 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 Technie).

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-MDPI IgG production in mice

To explore the antigenicity of MDPI, we first analyzed the humoral immune response to MDPI in mice. BCG was inoculated into three strains of mice, including A/J, BALB/c, and C3H/He. Western blot analysis showed that MDPI elicited a humoral immune response in all strains (Fig. 1A). Sera from nonimmunized mice did not react with MDPI (data not shown). Additionally, anti-MDPI 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 MDPI. Five micrograms of MDPI 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-MDPI IgG (Fig. 1A). MDPI presumably binds to DNA, which includes immunostimulatory CpG motifs (27). Therefore, we tested the simultaneous administration of MDPI and DNA. Five micrograms of MDPI 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 MDPI and DNA elicited MDPI-specific IgG production, whereas MDPI or DNA alone did not (Fig. 1A).

We next determined the optimal dose of DNA that could enhance anti-MDPI IgG production. Using RIB, 5 μg of MDPI 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-MDPI IgG was measured by ELISA (Fig. 1B). The production of IgG was dependent on the amount of DNA; interestingly, 5 ng of DNA most efficiently stimulated IgG production against MDPI. We observed enhanced anti-MDPI IgG production by coadministration of DNA and MDPI in the presence of IFA (Fig. 1C), and the result was similar to that

\[ \text{FIGURE 1. Humoral immune responses to MDPI in mice. A, Western blot analysis. Purified MDPI was blotted onto the membrane and incubated with 200/1 diluted antiserum. Mouse strains are indicated along the top of the panel. The injected Ags, such as BCG, MDPI alone (MDPI1), M. tuberculosis DNA alone (DNA), and MDPI plus DNA (MDPI + DNA) are shown along the bottom. B, DNA dose effects on anti-MDPI IgG production. C3H/He mice were immunized with MDPI (5 μg/mouse) with or without various amounts of DNA (5 μg to 5 ng) emulsified in RIB adjuvant, and levels of anti-MDPI IgG were determined by ELISA. The horizontal axis shows dilution factors of antisera. C, Immunization with MDPI plus DNA emulsified in IFA augmented anti-MDPI IgG production. C3H/He mice were immunized with Ags emulsified in IFA. Immunized Ags are described below the horizontal axis. Cotl, IFA alone. The sera from at least five mice of each experimental group were mixed and diluted to 1/400, and the levels of anti-MDPI IgG were determined by ELISA.} \]
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. 1B). 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. 4C). 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. 5A). 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. 5A). 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. 5B). In C3H/He mice, the level of anti-MDP1 IgG was insignificant when MDP1 alone was used (Fig. 5C). 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. 5C). 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. 5D).

Although the anti-MDP1 Ab titer was higher in BALB/c mice injected with MDP1 plus DNA than in mice immunized with
MDP1 alone, the level of anti-MDP1 IgG against the defined epitope (aa 51–70) was reversed (Fig. 5B). 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. 6A), but not with MDP1-DNA (Fig. 6B), 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. 6C), 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. 7A). We next examined isotypes of anti-MDP1 IgG in BCG-immunized C3H/He mice. BCG inoculation stimulated the production of MDP1-specific IgG1 and IgG2a, but not IgG2b or IgG3 (Fig. 7B). Interestingly, the pattern of IgG isotypes was similar to that observed in the same mouse strain immunized with both
MDP1 and DNA (Fig. 3C). 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. 7C). 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. 7C).

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. 8C). As shown in Fig. 8D, 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.

**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 node cells 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).

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 MDP1. 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
was produced in C3H/He mice challenged with either BCG (Fig. 1A) 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 [3H]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. 5B) 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. 1B). 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. 7A) 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. 7B) (44). Administration of purified MDP1 also expanded the population of IFN-γ-producing cells (Fig. 7C) and stimulated Th1-associated IgG2a production (Fig. 3). Again, simultaneous injection of MDP1 and DNA stimulated adaptive immunity and enhanced IFN-γ production (Fig. 7C). 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 aerosol 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 triggered 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). These 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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