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A Novel Immunostimulator, *N*-[□-*O*-Benzyl-*N*-(acetylmuramyl)-l-alanyl-d- isoglutaminyl]-*N*-*trans*-(*m*-nitrocinnamoyl)-l-lysine, and Its Adjuvancy on the Hepatitis B Surface Antigen

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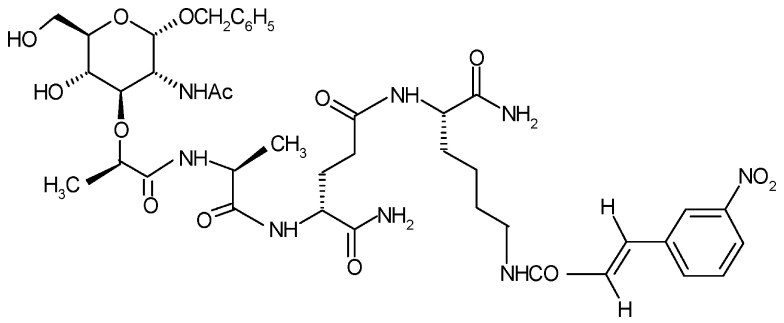

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Articles

A Novel Immunostimulator, N^2-[α-*O*-Benzyl-*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-*trans*-(*m*-nitrocinnamoyl)-L-lysine, and Its Adjuvancy on the Hepatitis B Surface Antigen

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N^2-[α-*O*-Benzyl-*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-*trans*-(*m*-nitrocinnamoyl)-L-lysine (muramyl dipeptide C, or MDP-C) has been synthesized as a novel, nonspecific immunomodulator. The present study shows that MDP-C induces strong cytolytic activity by macrophages on P388 leukemia cells and cytotoxic activity by cytotoxic T lymphocytes (CTLs) on P815 mastocytoma cells. Our results also indicate that MDP-C is an effective stimulator for production of interleukin-2 and interleukin-12 by murine bone morrow derived dendritic cells (BMDCs) and production of interferon-γ by CTLs. Additionally, MDP-C increases the expression levels of several surface molecules, including CD11c, MHC class I, and intercellular adhesion molecule-1 in BMDCs. Moreover, MDP-C remarkably enhances the immune system's responsiveness to hepatitis B surface antigen (HBsAg) in hepatitis B virus transgenic mice for both antibody production and specific HBsAg T-cell responses ex vivo. Our results indicate that MDP-C is an apyrogenic, nonallergenic, and low-toxicity immunostimulator with great potential for diagnostic, immunotherapeutic, and prophylactic applications in diseases such as hepatitis B and cancers.

Introduction

A variety of bacterial cell wall fractions have received more attention as immunoadjuvants for host stimulation against cancer, infectious diseases, and other immunologic disorders in both clinical and experimental situations.[1,2] Muramyl dipeptide (MDP), the minimal bioactive unit of peptidoglycan of Gram-positive bacterial cell walls, is known to have pleiotropic stimulatory effects on host immune systems and to elicit defensive responses.[3] However, MDP showed significant concomitant side effects in vivo, such as pyrogenicity,[4] poor penetration of membranes,[5] and rapid elimination.[6] MDP has been chemically modified[7,8] or conjugated with carriers[9,10] to improve its pharmacological properties. Several MDP derivatives, such as stearoyl-MDP derivatives,[11−16] MTP-PE,[17] FK565, FK156,[18] RP40636,[19] murabutide,[17,20] and threonyl-MDP,[21] have been reported to show strong host-stimulating activities with apyrogenicity.[3,22] The use of immunopotentiators is one of the most promising options for treatment of immunosuppressed hosts such as cancer patients or chronic HBV and HIV patients.[23] A number of clinical studies have indicated that MDP derivatives have great potential for use as vaccine adjuvants,[24] as immunotherapeutic agents for viral infection,[25,26] and in cancer therapeutic regimens.[27−30] In the present study, we present evidence that a chemically modified MDP, namely, N^2-[α-*O*-benzyl-*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-*trans*-(*m*-nitrocinnamoyl)-L-lysine (MDP-C), is a novel and strong immunopotentiator with multiple immunostimulatory activities.

Results

Chemistry. Synthesis of MDP-C. Muramyl dipeptide libraries including MDP-C were previously synthesized by applying a novel and efficient solid-phase synthetic method[31−33] and our "meshed-bag gathered-bunch (MBGB)" combinatorial technology.[34] The key intermediate Fmoc-D-isoGln-OH (**5**) was prepared through a modified synthetic route, saving two chemical steps (Scheme 1). The amino group of glutamatic acid (**1**) was protected in the presence of benzyloxycarbonyl chloride ($C_6H_5CH_2OCOCl$) to yield Cbz-D-Glu-OH (**2**). The α-carboxylic group of **2** was then selectively activated by DCC and further reacted with ammonia gas to directly give Cbz-D-isoGln-OH (**3**). This Cbz group of **3** was then removed by H_2−Pd/C treatment for 14 h at room temperature (**4**). Finally, the Fmoc protecting

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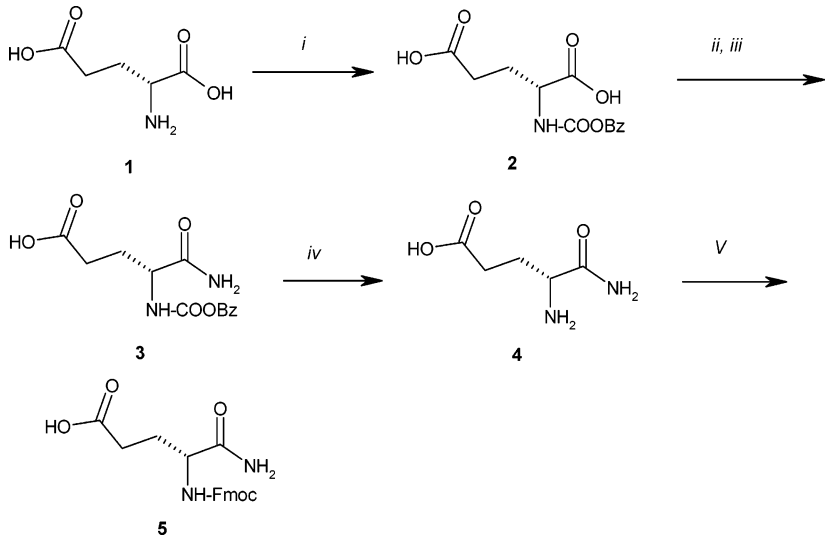
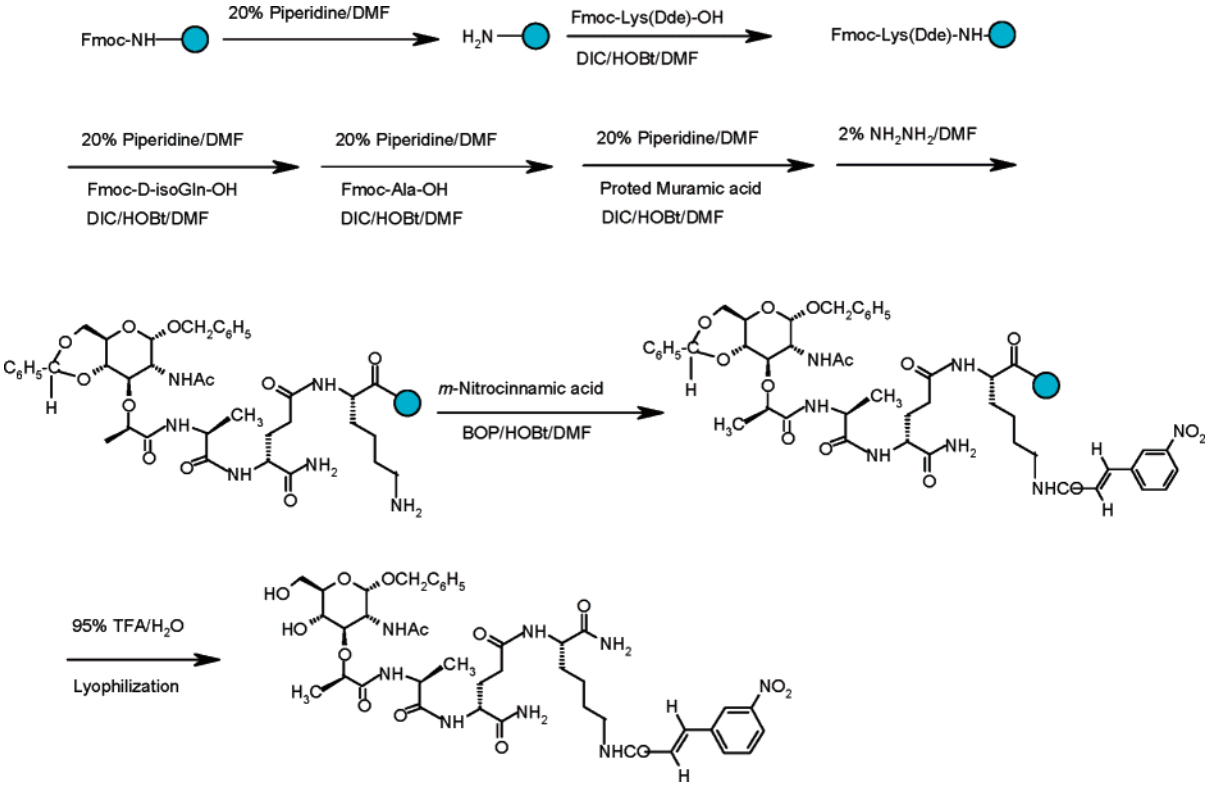
Scheme 1. Modified Synthetic Route to Fmoc-D-isoGln-OH (**5**)[a]



[a] (i) BzOCOCl, NaOH, H_2O; (ii) DCC, THF; (iii) NH_3; (iv) H_2, Pd/C, H_2O; (v) Fmoc-OSu, $NaHCO_3$, acetone, H_2O.

Scheme 2. Solid-Phase Synthesis of MDP-C



group was attached to the amino group of **4** to generate **5**, as described previously.[33]

Protected muramic acid was synthesized on a large scale.[35] The 1-*O*-benzyl group was left on the sugar part because it did not alter the immunomodulating activity of MDP-C when the MDP was modified.[36] The recent solid-phase synthesis of MDP-C (Scheme 2) was carried out according to the standard Fmoc/*t*-Bu protective strategy and BOP/HOBt coupling chemistry on Rink MBHA amide resin.[37] The final products were cleaved off by treatment with 95% TFA/H_2O. The purity and corrected molecular weight of MDP-C were determined by an auto LC−MS/MS system (Thermo Finnigan, LCQ-Advantage) after purification by a semipreparative HPLC on a Vydac C18 reverse-phase column (25 cm × 4.6 μm) to a minimum of 95% purity was attained. The structure was fully characterized by ^{1}H NMR, ^{13}C NMR, IR, and HRFAB MS (detail structural information is in Supporting Information).

Biology. 1. Effects on Tumor Growth. An MDP library consisting of more than 2300 MDP derivatives was synthesized.[34] Using a cell-based assay, we examined the effect of MDP derivatives on killing activities mediated by mature murine macrophages against mice tumor cells (P388). Briefly, macrophages and tumor cells were incubated together with the tested compound for 72 h to evaluate the toxicity of the compound. The number of surviving tumor cells, as evaluated by the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] method, was used to calculate the inhibi-

Table 1. Inhibitory Effects of Muramyl Dipeptide Derivatives on Tumor Growth[a]

sample	OD_{570} (mean ± SD[a])	increased R (%)
control	0.98 ± 0.09	
MDP-C	0.29 ± 0.13	71***,##
romurtide	0.56 ± 0.01	45**

[a] Versus control group: (**) $P < 0.01$; (***) $P < 0.001$. Versus romurtide group: (##) $P < 0.01$. SD means standard deviation.

tory activities of the compounds. We found that romurtide (used as positive control) resulted in an inhibitory ratio (IR) of 44.6% ($P < 0.01$), whereas MDP-C treatment produced an IR of 71.3% ($P < 0.001$) that was much higher than romurtide-induced IR ($P < 0.01$) (Table 1).

2. In Vitro Toxic Effects on Tumor Cells, Macrophages, and BMDCs. To determine any potential toxic effects of MDP-C, the BMDCs, macrophages, and P815 tumor cells were treated with MDP-C, and the cytotoxic effect was determined by the MTT assay. Our results indicated that MDP-C at 1.0×10^{-5} mol/L has no toxic effect on BMDCs, macrophages, or P815 tumor cells (data are in Supporting Information).

3. Immunostimulatory Properties of MDP-C. 3.1. Proliferation of Splenocytes. Splenocytes were treated by MDP-C, with or without 2 μg/mL of concanavalin A (ConA), for 72 h. Proliferation of splenocytes was determined by the MTT method. Both MDP-C (at 1.0×10^{-6} to 1.0×10^{-7} mol/L) and ConA significantly increased the proliferation of nonstimulated, normal murine splenocytes. In addition, ConA-mediated stimulation was synergistically enhanced by MDP-C at 1.0×10^{-6} to 1.0×10^{-7} mol/L. MDP-C at 1.0×10^{-6} mol/L induced higher stimulatory and synergistic effects on murine splenocyte proliferation than the positive control compound romurtide (Table 2).

3.2. Activation of Bone Marrow-Derived Dendritic Cells (BMDCs) (Markers, Expression of Interleukin-2 (IL-2) and Interleukin-12 (IL-12)). As illustrated in Figure 1, as an effective stimulator, lipopolysaccharide (LPS) can remarkably enhance the percentage of $CD11c^+$ dendritic cells (DCs) (Figure 1A), MHC class II^+ DCs (Figure 1B), and ICAM-1^+ DCs (intercellular adhesion molecule-1) (Figure 1C). We found the increased rates of 82.3%, 83.2%, and 10.5%, respectively. This observation agrees with the results of a previous study.[38] Treatment of BMDCs with MDP-C (1 nM, 10 nM, 100 nM, and 1.0 μM) resulted in concentration-dependent increases in the percentage of $CD11c^+$ DCs at 31.5%, 41.3%, 59.3%, and 76.5%, respectively (Figure 1A). Untreated BMDCs presented a 25.6% increase in the percentage of $CD11c^+$ DCs. Similar results were also observed on MHC class II (Figure 1B) and ICAM-1 (Figure 1C) expressed by BMDCs. Additionally, high-level IL-2 and IL-12 synthesized by MDP-C-treated BMDCs were also found. Synergistic effects of MDP-C with LPS were observed on expression of IL-2 and IL-12 (Table 3).

3.3. Allogeneic CTL Response. DCs are potent antigen-presenting cells (APCs) involved in the stimulation of native T cells to initiate primary Th1, Th2, and/or cytotoxic T-lymphocyte (CTL) immune responses.[38] Splenocytes from C57BL/6J mice were cocultured with allogeneic BMDCs preactivated with MDP-C and/or LPS for 24 h and subsequently used as effectors. P815

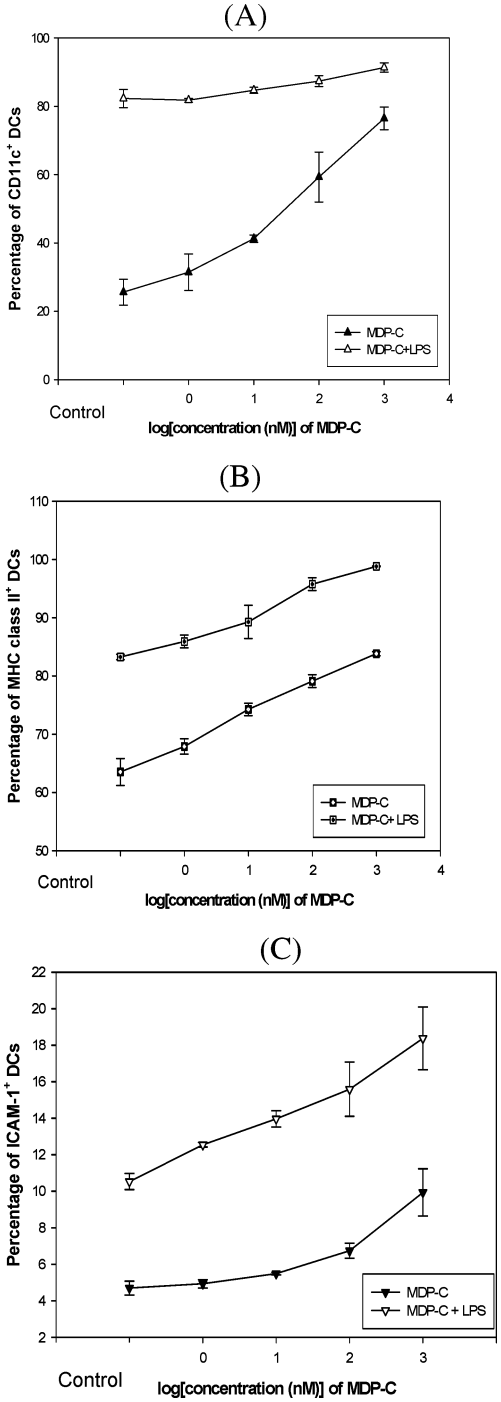


Figure 1. Effects of MDP-C, with or without LPS, on the surface molecule expression by DCs: (A) CD11c expression by DCs; (B) MHC class II expression by DCs; (C) ICAM-1 expression by DCs. DCs were generated from bone marrow cultures and exposed to MDP-C, with or without LPS, for 24 h. Cells were stained using FITC-labeled antimouse CD11c Ab, MHC class II Ab, and ICAM-1 Ab and were gated on living cells. "Control" means the concentration of MDP-C is zero in the "MDP-C" group and contains LPS (1 μg/mL) only in the "MDP-C + LPS" group.

mastocytoma cells were used as target cells. The CTL-mediated cytotoxicity induced by MDP-C-treated BMDCs was measured using the CytoTox 96 non-radioactive cytotoxicity assay kit. MDP-C was found not only to induce a concentration-dependent (1×10^{-8} to 1×10^{-6} mol/L) stimulatory enhancement to the response of

Table 2. Effects of MDP-C on Proliferation of Murine Splenocytes Induced by ConA[a]

	proliferation of murine splenocyte			
	without ConA		with ConA (2.0 μg/mL)	
	OD_{570} (mean ± SD)	increased rate (%)	OD_{570} (mean ± SD)	increased rate (%)
control	0.444 ± 0.003		1.21 ± 0.01	
romurtide (1×10^{-6} mol/L)	0.987 ± 0.037	122***	1.73 ± 0.08	67##
MDP-C (1×10^{-6} mol/L)	1.29 ± 0.03	191***	1.85 ± 0.05	82###
MDP-C (1×10^{-7} mol/L)	0.778 ± 0.007	75**	1.51 ± 0.05	38#
MDP-C (1×10^{-8} mol/L)	0.565 ± 0.031	27.3	1.33 ± 0.05	15
MDP0C (1×10^{-9} mol/L)	0.468 ± 0.035	5.41	1.22 ± 0.06	7.8

[a] Versus control group: (**) $P < 0.01$; (***) $P < 0.001$. Versus ConA group: (#) $P < 0.05$; (##) $P < 0.01$. (###) $P < 0.001$.

Table 3. Secretion of IL-12 and IL-12 by MDP-C-Treated BMDCs[a]

	IL-2 and IL-12 secreted by DCs (mean ± SD, pg/mL)			
	without LPS		with LPS (1.0 μg/mL)	
	IL-2	IL-12	IL-2	IL-12
control	42.3 ± 3.6	556 ± 1	96.7 ± 8.5	793 ± 33
romurtide (1×10^{-6} mol/L)	121 ± 5***	802 ± 16**	130 ± 14##	940 ± 38##
MDP-C (1×10^{-6} mol/L)	103 ± 5***	918 ± 1**	132 ± 6##	988 ± 76###
MDP-C (1×10^{-7} mol/L)	76.0 ± 4.4**	786 ± 7[e]	117 ± 2#	838 ± 8
MDP-C (1×10^{-8} mol/L)	60.0 ± 3.0*	725 ± 10	107 ± 4	819 ± 21
MDP-C (1×10^{-9} mol/L)	43.9 ± 4.6	560 ± 13	94.8 ± 4.9	800 ± 44

[a] Versus control group: (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$. Versus LPS group: (#) $P < 0.05$; (##) $P < 0.01$; (###) $P < 0.001$.

Table 4. Activation of Alloantigen-Specific Cytotoxic T Cells by MDP-C-Treated BMDCs ($n = 3$)[a]

	allogeneic CTL response			
	without LPS		with LPS (1.0 μg/mL)	
	specific release (%)	increased rate (%)	specific release (%)	increased rate (%)
control	11.0 ± 0.1		24.6 ± 0.1	
romurtide (1×10^{-6} mol/L)	31.7 ± 3.4	187***	30.7 ± 0.8	45#
MDP-C (1×10^{-6} mol/L)	37.7 ± 0.1	243***	35.7 ± 0.1	82###
MDP-C (1×10^{-7} mol/L)	23.9 ± 0.1	118***	31.3 ± 0.1	49#
MDP-C (1×10^{-8} mol/L)	15.7 ± 0.1	42.7**	28.5 ± 0.1	28#
MDP-C (1×10^{-9} mol/L)	11.7 ± 0.1	6.4	23.8 ± 0.1	0

[a] Versus control: (**) $P < 0.01$; (***) $P < 0.001$. Versus LPS: (#) $P < 0.05$; (###) $P < 0.001$ vs LPS.

Table 5. Effects of MDP-C on Cyclophosphamide (CY) Treated Mice ($n = 3$)[a]

groups	doses	$CD4^+/CD8^+$ ratio ($\bar{x} \pm s$)
control, PBS		2.63 ± 0.003
MDP-C	0.5 (mg/kg)/day × 7 days	3.18 ± 0.2
MDP-C	2.0 mg/kg)/day × 7 days	4.14 ± 0.2**
MDP-C	10 mg/kg)/day × 7 days	4.47 ± 0.13***
LPS	1.0 mg/kg)/day × 7 days	4.32 ± 0.72**
CY	30 mg/kg)/day × 5 days	2.40 ± 0.14

[a] Versus CY treatment group (30 (mg/kg)/day × 5 days): (**) $P < 0.01$; (***) $P < 0.001$.

CTLs to the P815 cells but also to remarkably increase LPS stimulation (Table 4).

3.4. Immunosuppressed Animal Model. Male C57BL/6J mice were treated with cyclophosphamide (CY, ip) and MDP-C (iv) at the doses indicated in Table 5**.** We collected peripheral blood mononuclear cells (PBMCs) and determined the $CD3^+CD4^+/CD3^+CD8^+$ ratio by flow cytometry. Figure 2 shows the representative flow-cytometric data depicting the counts of $CD3^+$-$CD4^+$ and $CD3^+CD8^+$ in PBMCs from mice treated with CY, LPS + CY, and MDP-C + CY at the doses in Table 5. The results suggested that cyclophosphamide decreased the ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ in PBMCs, whereas MDP-C (at the 2 and 10 mg/kg doses) and LPS (at the 1 mg/kg dose) can significantly recover the ratio induced by CY (Figure 2).

4. Effects of MDP-C on Induction of Hepatitis B Surface Antigen (HBsAg) Specific Response in HBV-Transgenic Mice. 4.1. Generation of Anti Hepatitis B Surface (HBs) Antibodies in Serum. As shown in Table 6, MDP-C at a dose of 50 μg/mL combined with HBsAg (2.5 μg/mL) induced an 83.3% (5/6) serum-positive rate in the immunized mice and a high titer of anti-HBsAb of 131.6 GMT (IU/mL). By comparison, alum at a dose of 125 μg/mL induced a 66.7% serum-positive rate (4/6) and a 73.9 GMT anti-HBsAb level. When the components of vaccines were diluted four times in saline, MDP-C-adjuvanted vaccine still induced a slightly higher serum-positive rate (66.7%) and anti-HBs Ab (81.6 GMT) level compared with alum-adjuvanted vaccine (50% and 71.7 GMT).

4.2. HBsAg-Specific Production of IL-2 in Spleens Induced by Immunization with MDP-C-Adjuvanted Vaccine. To investigate the effect of MDP-C on T-cell response against HBV, HBV-transgenic mice were subcutaneously immunized three times with different vaccine formulations, as indicated in Figure 3.[39] The HBV-transgenic mice were sacrificed 4 weeks after the last immunization. Splenocytes were collected and incubated with or without HBsAg (30 μg/mL) in ELISPOT plates. The difference between the number of IL-2 secreting cells obtained with and without HBsAg stimulation was considered as an indicator of the HBsAg-specific response. MDP-C in combination with

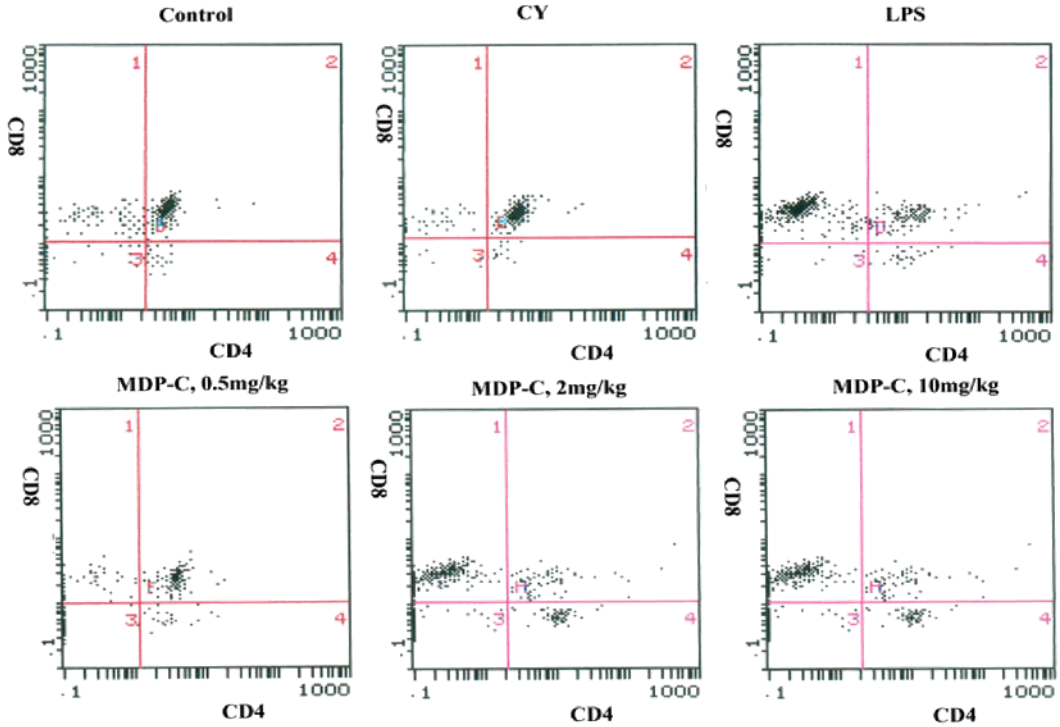


Figure 2. Flow-cytometric analysis of T-cell subsets on the peripheral blood in CY-induced immunosuppressed mice following treatment with MDP-C and LPS. The T cells were stained with CD4-FITC and phycoerythrin-conjugated antimouse CD8 mAb. The cells were analyzed using a flow cytometer. This figure shows representative flow-cytometric data depicting the counts of $CD3^+CD4^+$ and $CD3^+CD8^+$ in the T-cell subset in the PBMC of control, CY-, LPS + CY-, and MDP-C + CY-treated mice with the doses indicated in Table 5.

Table 6. Comparison of MDP-C to Alum as Adjuvants on the Titers of Anti-HBs Ab and Serum-Positive Rate of Immunized HBV-Transgenic Mice ($n = 6$)[a]

components of vaccine	serum-positive rate (%)	titers of anti-HBs Ab (GTM)
MDP-C (50 μg) + HBsAg (2.5μg)	83	132 ± 1*
MDP-C (12.5 μg) + HBsAg (2.5μg)	67	81.6 ± 0.4#
alum (125 μg) + HBsAg (2.5μg)	67	73.9 ± 0.3
alum (31.25 μg) + HBsAg (2.5μg)	50	71.7 ± 0.5

[a] Versus "alum + HBsAg" group: (*) $P < 0.05$; (#) $P > 0.05$.

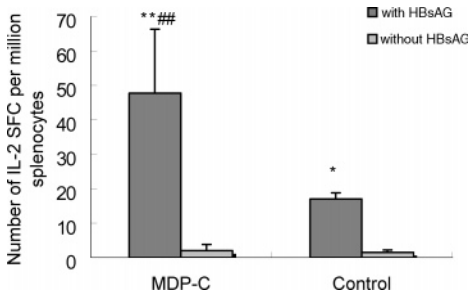


Figure 3. HBsAg-specific IL-2 response in splenocytes isolated from HBV-transgenic mice immunized with MDP-C adjuvanted HBsAg vaccine. HBV-transgenic mice were immunized subcutaneously on day 0 and boosted on days 14, 28, and 56 with HBsAg (2.5 μg in 1 mL of saline) + MDP-C (50 μg in 1 mL of saline), MDP-C alone (50 μg in 1 mL of saline), HBsAg alone (2.5 μg in 1 mL of saline), or saline. Mice were sacrificed 4 weeks after the final boost, and splenocytes were collected. Isolated splenocytes were then cultured for 20 h, with or without HBsAg (30 μg/mL as final concentration). The level of IL-2 expression by splenocytes was determined with an ELISPOT kit. The results are presented as the mean ± SD, $n = 4$: (**) $P < 0.01$, MDP-C + HBsAg treatment compared with HBsAg alone treatment; (*) $P < 0.05$, HBsAg alone treatment compared with no HBsAg treatment; (##) $P < 0.01$, MDP-C + HBsAg treatment compared with HBsAg treatment.

HBsAg significantly increased IL-2 levels whereas HBsAg or MDP-C alone did not, suggesting that MDP-C might function as a potent adjuvant to stimulate specific T-cell response against HBsAg.

4.3. IFN-γ Release by HBsAg-Specific CTLs but Not BMDCs. As illustrated in Figure 4A, the level of IFN-γ in the CTLs culture supernatant was significantly elevated by MDP-C treatment at ≥1 nM. Furthermore, MDP-C (1.0 μM) could induce massive IFN-γ release by CTLs from HBsAg-immunized HBV-transgenic mice (Figure 4B). On the other hand, MDP-C showed a synergistic effect with LPS on potentiating IFN-γ production of CTLs in normal mice (Figure 4A) but not in HBV-transgenic mice (Figure 4B). However, MDP-C did not significantly increase production of IFN-γ by DCs in either normal or HBV-transgenic mice. The level of IFN-γ ranged from 14.84 ± 2.58 to 23.96 ± 2.21 pg/mL (data not shown).

5. Safety Evaluation. 5.1. Pyrogenic Effect in Rabbits. Three New Zealand white rabbits per group were treated with MDP-C at doses of 0.1 and 0.2 mg/kg (iv) in pyrogen-free PBS as described previously.[40] As a result, the rectal temperature of each rabbit was measured at 0, 1, 2, 3, 4, and 24 h. The body temperature of rabbits was not altered ($P > 0.05$), indicating that MDP-C is apyrogenic (data are shown in Supporting Information).

5.2. Effect on Passive Cutaneous Anaphylaxis (PCA) Reaction in Rats. Sera from 10 Wistar rats sensitized with MDP-C (0.1 mg/kg) were pooled, diluted, and injected intradermally (0.1 mL per site) in the shaved dorsal skin of unsensitized Wistar rats. After

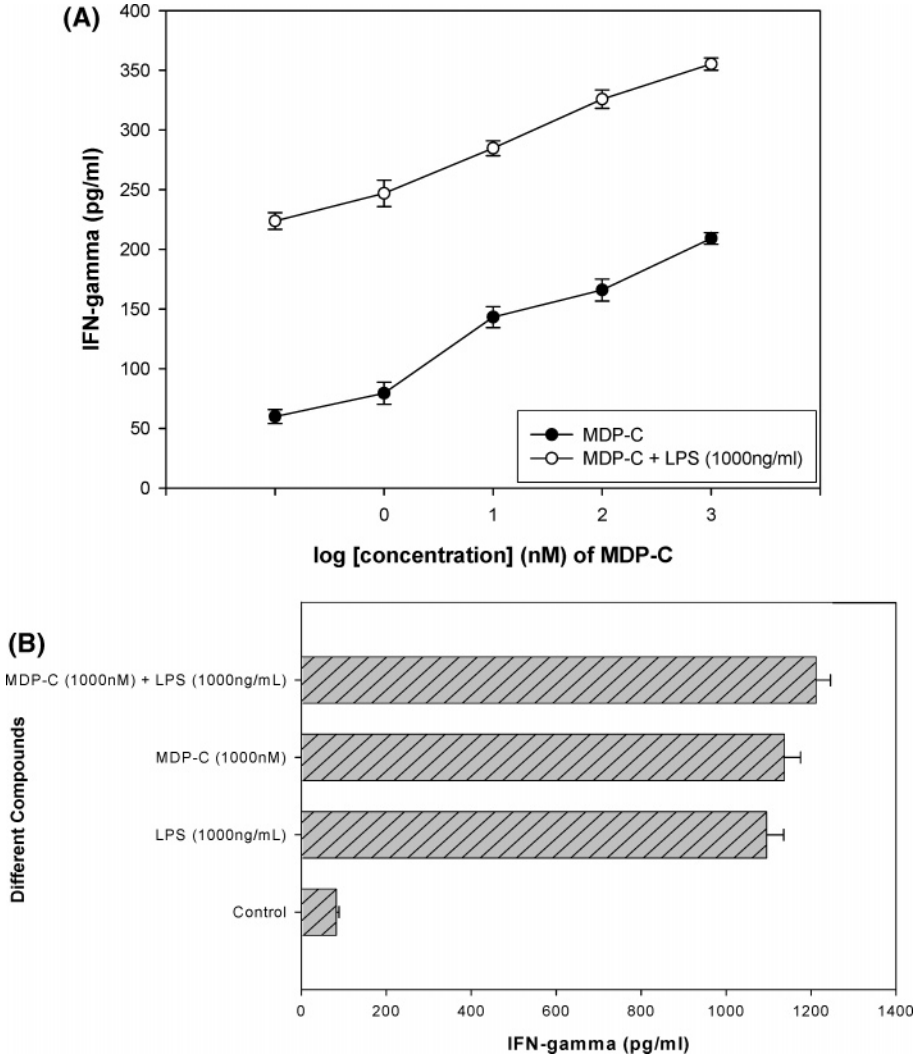


Figure 4. Stimulatory activity and synergistic effects of MDP-C with LPS on IFN-γ production of CTLs from normal mice and HBV-transgenic mice, respectively. (A) IFN-γ release in supernatant of CTLs was assessed in normal mice following the manufacturer's instructions, provided by the murine IFN-γ kit (Diaclone, France). Controls at left dots for two curves represent LPS alone (top) and untreated CTLs by immunostimulators (bottom). (B) IFN-γ production by CTLs from HBV-transgenic mice using the same method as described as above.

24 h, the rats were given 0.1 mL of isotonic saline containing 1 mg/mL of MDP-C (iv) and Evans blue (25 mg in 1 mL saline) (iv). Sixty minutes later, rats were anesthetized and exsanguinated, and their dorsal skin was removed. The diameter of the stain on the inner skin surface was measured. In the present test, MDP-C did not cause blue spots within 20 min after injecting Evans blue. This result indicates that MDP-C has no allergic potential at the dose of 0.1 mg/kg.

5.3. Single Dose Toxicity Studies in Mice. MDP-C was administered (iv) at doses of 500 and 100 mg/kg, respectively. No mouse death was recorded. At a dose of 100 mg/kg, the mass indexes of both spleen and thymus were not significantly changed. At the very high dose of 500 mg/kg, the mass index of spleen was slightly increased. The results showed that MDP-C has very low inherent toxicity (Table 7).

Table 7. Acute Toxicities of MDP-C in Vivo ($n = 6$)[a]

	mass index (mg per 10 g of body weight)			
	spleen		thymus	
	mean ± SD	increased rate (%)	mean ± SD	increased rate (%)[b]
control	56.4 ± 11.5		44.1 ± 5.13	
100 mg/kg MDP-C	59.9 ± 14.6	6	42.8 ± 8.49	−1.4
500 mg/kg MDP-C	86.1 ± 19.1	53*	43. 5 ± 4.35	−2.8

[a] Versus control group: (*) $P < 0.05$. [b] "−" means weak proliferative action.

Discussion

Using our novel synthetic method on solid support[33] and the previously described "MBGB" combinatorial technology,[34] we are able to efficiently synthesize large numbers of MDP analogues and/or MDP mimetics in a short period. The key building block of Fmoc-D-isoGln-OH is prepared by a modified method reported here.

MDP-C was identified as a novel, nonspecific immunostimulating molecule. Like other muramyl dipeptide analogues, MDP-C exerts a targeted effect on APCs such as macrophages and DCs.[41] Macrophage activation plays an important role in immunotherapeutic approaches to viral infectious diseases[42] and cancers.[43] Previous studies have demonstrated that MDP-C could efficiently augment the killing of P388 tumor cells by macrophages by enhancing their antigen-presenting function. Measurements of pyrogenicity, allergicity, and

cytotoxicity demonstrated that MDP-C is apyrogenic and nonallergenic and has very low toxicity, which underscores the promise it holds for studying its pharmacological applications further.

As the most potent APCs and sensors of the immune system, DCs initially recognize danger signals via an array of pattern-recognition molecules for subsequent induction of acquired immunity. The activation of DCs is crucial in the initiation of many adaptive immune responses, especially CTL-mediated immunity.[45,46] Our results show that MDP-C significantly enhances the capability of DCs to induce cytotoxic activity by CTLs together with high-levels of IFN-γ release, which is an indicator of primed memory T cells and an inducer of adaptive immunity.[47] Generally, the differentiation of native CTLs into effector cells is thought to require at least two signals. The first is provided during the engagement of the T-cell receptor (TCR) by the peptide/MHC class II complexes on APCs. The second is provided by the interaction between accessory molecules on the surface of native T cells and the ligands B7-1 or B7-2 on the same APCs.[48] The induction of potent immune responses requires adjuvant to deliver danger/stimulation signals to DCs.[47] DCs, as the dominant cell type involved in the activation of naive T cells,[47] represent a major link between the innate and adaptive immune responses.[49] We have examined herein the contribution of MDP-C-treated DCs for the generation of CTLs responses.

DCs usually exist in a quiescent state in most tissues, but certain stimuli, such as microbial products and inflammatory cytokines, trigger the DCs to become mature and activated.[50] During the maturation and activation process, DCs undergo numerous phenotypic and functional changes: a redistribution of the MHC molecules from intracellular endocytic compartments to the cell surface, an increase in the surface expression of costimulatory molecules, and morphologic changes.[51] The up-regulated expression level of CD11c, the maturation marker for DCs, suggests that MDP-C could efficiently induce maturation of BMDCs. Simultaneously, our results show that MDP-C-treated DCs express MHC class II molecules in high density (murine macrophages have only low-density MHC class II molecules[52]), which is a phenotype known to result in the effective stimulation of primary T-lymphocyte responses, both in allogeneic CTL response (allo-CTLs)[53] and in Ag-specific systems.[54] The expression of ICAM-1, which induces an adhesive interaction between DCs and T cells, was lowered on resting DCs but increased rapidly on MDP-C-treated DCs, which might be a critical finding for generating Th1 responses to prevent viral infection.[54] ICAM-1 on DCs is also required to ensure that a T cell will divide and differentiate into effector cells.[55] Taken together, MDP-C up-regulation of MHC class II molecule and ICAM-1 should contribute to the capability of DCs to process and present specific antigens to naive T cells, to activate them subsequently, and to induce effective immune responses that interfere with infection.

T-cell differentiation from antigen-specific naive T cells, induced by DCs, requires not only prolonged TCR contact with MHC class II molecules/peptide complexes and costimulation molecules but also cytokine secretion to finally polarize the T-cell response.[56,57] IL-2 is known to play an important role in the proliferation of T, B, and NK cells as well as in T-cell activation.[58,59] IL-12 is one of the most important cytokines involved in the activation of Th1-directed responses to intracellular infections.[60] MDP-C was shown to have a capacity to stimulate IL-2 and IL-12 production by BMDCs and also to affect the activation process in CTL cocultures. In addition, significant levels of IFN-γ, which is preferentially considered as a Th1-type cytokine, were detected in the supernatants from MDP-C-stimulated CTL cocultures. Thus, MDP-C is expected to direct DCs toward processing and presenting antigens to cytotoxic T cells, resulting in enhanced killing of infected cells. All of these events are important for host resistance to viral, bacterial, and parasitic infections.[62]

MDP-C shows a synergy with LPS in vitro for the production of cytokines (IL-2 and IL-12 by DCs and IFN-γ by CTLs), as well as for the expression of MHC class II molecules and ICAM-1. Such an adjuvant effect agrees with previous observations of MDP.[63] The adjuvant effect of MDP-C on the immune system's response to HBsAg was investigated in HBV-transgenic mice.[64] The HBV-transgenic mouse model mimics the state of HBV carriers that are immunologically tolerant to HBV-encoded antigens and is therefore useful for evaluating immunotherapy in vivo.[64] The results show that MDP-C enhances the immunogenicity of HBsAg and induces a high serum positive rate in HBV-transgenic mice. MDP-C administered together with HBsAg may contribute to breaking immune tolerance to the HBV antigen, as shown by the synergic induction of IFN-γ and IL-2 ex vivo. This indicates that MDP-C can efficiently regulate dysfunctional antigen-presenting monocytes and transform cellular cytokines that combines with HBsAg to induce dramatically stronger cellular and humoral immunity in vivo.

The above suggests that the MPD-C-stimulated cytokine secretion is instrumental in modulating the HBsAg antigen-processing pathways in APC, resulting in the presentation of immunoepitopes with the potential to strengthen HBsAg-specific cellular immunity and break immune tolerance. It implies that MDP-C has great potential for adjuvancy in prophylactic and immunotherapeutic vaccines.

Conclusion

A novel, nonspecific immunostimulator, MDP-C, was identified from a synthetic muramyl dipeptide library. Our previous data indicate that MDP-C is apyrogenic and nonallergenic and shows low toxicity. MDP-C strongly induces cytolytic activity of macrophages on P388 leukemia cells and cytotoxic activity of cytotoxic T lymphocytes (CTL) on P815 mastocytoma cells. MDP-C is also found to be effective in stimulating the production of IL-2 and IL-12 by BMDCs and the production of IFN-γ by CTLs. MDP-C elevates the expression level of surface molecules, such as CD11c, MHC class II, and ICAM-1 on BMDCs. Most significantly, MDP-C remarkably potentiates the immune system's responsiveness to HBsAg in HBV-transgenic mice for both antibody production and specific HBsAg T-cell responses ex vivo. Taken together, MDP-C shows great potential for

diagnostic, immunotherapeutic, and prophylactic application in diseases including hepatitis B and cancer.

Experimental Section

Reagents. Lipopolysaccharide (LPS, *E. coli* 055:B5), concanavalin A (ConA), and murine-recombined IL-4, GM-CSF, IL-2, and IFN-γ were purchased from Sigma (Milwaukee, WI). A CytoTox 96 nonradioactive cytotoxicity assay kit was purchased from Promega (Madison, WI). ELISA kits for murine-recombined IL-2, IL-12, and IFN-γ were obtained from Diaclone. Rat antimouse FITC-CD11c antibody (Ab), FITC−ICAM-I Ab, and FITC-MHC class II Ab were obtained from PharMingen BD Biosciences (San Diego, CA) and Poly (dI-dC). Poly (dI-dC) was from Amersham Pharmacia Biotech (Piscataway, NJ). MDP was from Sigma. Romurtide was synthesized by our group on solid phase with purity greater than 90% by HPLC.[65]

All chemical reagents and solvents used were of commercial grade unless otherwise specified. Rink MBHA amide resin was purchased from Chem-Impex International, Inc. (Wood Dale, IL). Melting points were measured using a Yanaco MP 500C microscope (Uji-city, Japan) and were uncorrected. IR spectra were recorded on a Nicolet Impact 400 (San Jose, CA). HPLC analysis was performed on a Shimadzu HPLC system equipped with an SPD-10A VP detector, LC-10AT VP pump, and DGU-12A degasser (Columbia). The column employed was a Kromasil C18 column (4.6 μm, 4.6 mm × 50 mm) from DIKMA (Beijing, China). The eluent was a mixture of acetonitrile and water containing 0.05% TFA, with a linear gradient from 5:95 v/v acetonitrile/H_2O to 95:5 v/v acetonitrile/H_2O over 5 min at 1 mL/min flow rate. The UV detection was done at 214 nm wavelength. Auto-HPLC−MS analysis was performed on a Thermo Finnigan, LCQ-Advantage mass spectrometer (San Jose, CA) equipped with a Gilson 322 pump, Gilson UV/vis-152 detector, Gilson 215 liquid handler (Lewis Center), and a fluent splitter. The elution gradient, flow rate, and detection wavelength are the same as above. Five percent of eluent was split into MS system. Mass spectra were recorded in positive ion mode using electrospray ionization. All NMR experiments were carried out on a Varian Mercury 300 MHz or 500 MHz NMR spectrometer (Palo Alto, CA).

Chemistry. Synthesis of *N*-Fmoc-D-isoglutamine (5). To a vigorously stirred solution of D-glutamic acid (60 g, 0.41 mol) in 4 N NaOH (208 mL, 0.83 mol), benzyloxycarbonyl chloride (94 mL, 0.66 mmol) was added dropwise over 30 min in a water−ice cooled bath. This reaction was heated to 60 °C for 15 min. After the mixture was cooled to room temperature, the organic phase was extracted by ethyl ether (350 mL × 4). The water phase was acidified to pH=2 by 1.0 N HCl, then extracted with ethyl acetate (500 mL × 4). After the combined organic layers were dried with $MgSO_4$, the organic solvent was evaporated under reduced pressure. The residue was recrystallized with ethyl acetate and petroleum ether (60−90 °C) to produce a white solid (**2**): yield 110 g (96%).

2 (12.46 g, 44.3 mmol) was dissolved in anhydrous THF (100 mL). DCC (10.98 g, 55.3 mmol) was then added while stirring in a water−ice cooled bath. The reaction was continued for 7 h. The precipitates were filtered. Under protection of argon and machine stirring in a water−ice cooled bath, ammonia gas was bubbled through the reactants for 30 min. The collected white solid was recrystallized with methanol−ethyl ether and produced a colorless solid. This colorless solid was vigorously mixed with ethyl acetate (440 mL) containing 1.0 N HCl (40 mL). The organic layer was then separated and washed with 1.0 N HCl and saturated NaCl solution, respectively. After drying with $MgSO_4$, the organic solvent was evaporated under reduced pressure. The residue was recrystallized to produce a colorless solid (**3**): yield 6.2 g (52.5%), mp 174−176 °C.

To a suspension of **3** (14.0 g, 50 mmol) in water (300 mL), Pd/C (1.0 g) was added. The reactants were continuously hydrogenated for 8 h at room temperature under 3−4 bar until the white solid disappeared. After filtering Pd/C, the filtrate was concentrated to approximate 200 mL at a temperature below 40 °C. This solution was added to $NaHCO_3$ (4.2 g, 50 mmol), Fmoc-OSu (16.85 g, 50 mmol), and acetone (200 mL) and reacted for an additional 3 days at room temperature. The mixture was then cooled in a water−ice bath, and 2.0 N HCl was carefully added until pH 3 was attained. After removal of organic solvent under reduced pressure, the remaining solution was successfully extracted (500 mL × 3) with ethyl acetate, dried over $MgSO_4$, and evaporated in a vacuum. The residue was recrystallized using methanol, resulting in a white solid (**5**): yield 15.35 g (83%), mp 204.3−205.4 °C (literature mp 204−205 °C [66]). ESI-MS *m/z* (%): 369.1 [M + H^+]. ^{1}H NMR (300 MHz, DMSO-d_6): δ 1.75−1.89 (m, 2H, CH_2), 2.24 (t, J = 7.8 Hz, 2H, CH_2), 3.92 (m, 1H, CH), 4.19−4.27 (m, 3H, CH+CH_2), 7.02 (s, 1H, CONH), 7.28−7.88 (m, 10H, 8 ph-H + $CONH_2$), 12.07 (s, 1H, COOH). IR (KBr): 735, 756, 1279, 1319, 1543, 1635, 1685, 1741, 3182, 3313, 3406 cm^{-1}.

Solid-Phase Synthesis of N^2-[α-*O*-Benzyl-*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-*trans*-(*m*-nitrocinnamoyl)-L-lysine (MDP-C). A 1.0 M building block solution in DMF was used for all protected amino acid coupling steps. Coupling reagents were 1.0 M benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate (BOP)/1-hydroxybenzotriazole (HOBt)/*N*-methylmorpholine (NMM) or 1.0 M 1,3-diisopropylcarbodiimide (DIC)/HOBt in redistilled DMF. Fmoc- and Dde-protected groups of lysine were removed by 20% piperidine/DMF for 10 min twice and 2% NH_2NH_2/DMF for 3 min twice under vigorous shaking. Before the coupling reaction, a thorough washing step was performed with DMF, DCM, methanol, and DMF three times for each, respectively (before-coupling washing step). The after-coupling washing step was performed with DMF five times. Coupling time generally was 1.5 h at room temperature, when noted. Coupling efficacy was determined by Kaiser's ninhydrin test.

Next, 50 mg of Fmoc Rink amide resin (1% cross-linked, 0.44 mmol/g) was added to a "MBGB" unit. After removal of Fmoc from the resin and after the before-coupling washing step was performed, Fmoc-Lys(Dde)-OH was attached onto the resin. After thoroughly washing with DMF, the resin was treated again with 20% piperidine/DMF. Repeating the before-coupling washing step, coupling step, and after-coupling washing step as describe above, Fmoc-iso-D-Gln-OH, Fmoc-Ala-OH, and muramic acid were subsequently coupled to resin. After treatment of the resin with 2% NH_2NH_2/DMF for 2 min twice, Dde was completely removed. Repeating the before-coupling washing step again, 1.0 M *m*-nitrocinnamic acid and BOP/HOBt/NMM solution was prepared and then reacted under vigorous shaking for 3 h at room temperature. The excess reaction reagents were washed away by suspension of the "MBGB" unit in DMF, DCM, MeOH, and DMF sequentially. The final compound was cleaved off the resin into a collecting tube using 95% TFA/H_2O after carefully washing the resin three times with DMF, methanol, DMF, and DCM sequentially. TFA was gently evaporated under a slow N_2 flow until the residue was as dry as possible. The residue then was dissolved using 60% acetonitrile/H_2O and purified by using the reverse-phase C18 column and eluents of methanol/H_2O in a gradient with methanol levels ranging from 5% to 75%. The collected fractions were monitored by an LC−MS system using a fast C18 column (5 cm × 4.6 μm) in a gradient in which buffer B went from 5% to 95% in 5 min (buffer A, 0.05% TFA/acetonitrile; buffer B, 0.05% TFA/H_2O). Expected fractions were combined and evaporated under reduced pressure. The residue solution was lyophilized to yield pure N^2-[α-*O*-benzyl-*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-*trans*-(*m*-nitrocinnamoyl)-L-lysine, a white powder.

The molecular weight of MDP-C (ESI-MS) was 886.2 [M + $H]^+$. HRFAB MS *m/z*: [M + $H]^+$ 885.4030 (calcd 885.3994, $C_{41}H_{57}N_8O_{14}$). ^{1}H NMR (500 MHz, DMSO-d_6, Varian Mercury 500 spectrometer): δ 1.22 (d, J = 8.0, 3H, CH_3), 1.23 (d, J = 7.5, 3H, CH_3), 1.77 (s, 3H, $COCH_3$), 1.20−1.52, 1.60−1.71, & 1.94−4.25 (m, 24H), 4.43 and 4.66 (each d, J = 12.5, 2H, OCH_2ph), 4.72 (d, J = 3.0, 1H, CHOBz), 6.79 (d, J = 16.0, 1H, CH=), 6.94 (br, s, 2H, D_2O exchangeable, $CONH_2$), 7.03 (br, s, 2H, D_2O exchangeable, $CONH_2$), 7.27−7.37 (m, 5H, ph-5H), 7.31 (br, s, 1H, D_2O exchangeable, CONH), 7.52 (d, J = 16.0,

1H, CH=), 7.53 (br, s, 1H, D_2O exchangeable, CONH), 7.69 (t, J = 7.8, 1H, ph-H), 7.86 (d, 1H, D_2O exchangeable, CONH), 8.00 (d, J = 8.0, 1H, ph-H), 8.09 (d, 1H, D_2O exchangeable, CONH), 8.13 (d, J = 8.0, 1H, ph-H), 8.17 (d, 1H, D_2O exchangeable, CONH), 8.37 (br, s, 1H, ph-H). ^{13}C NMR and DEPT (125 MHz, DMSO-d_6): δ 18.39 (CH_3), 19.00 (CH_3), 22.63 (CH_3), 23.02 (CH_2), 27.85 (CH_2), 28.82 (CH_2), 31.61 (CH_2), 31.73 (CH_2), 38.72 (CH_2), 48.20 (CH), 52.14 (CH), 52.40 (CH), 52.98 (CH), 60.53 (CH_2), 67.92 (CH_2), 69.52 (CH), 73.20 (CH), 76.50 (CH), 79.16 (CH), 95.98 (CH), 121.54 (CH), 123.68 (CH), 125.25 (CH), 127.54 (CH), 127.63 (2CH), 128.25 (2CH), 130.52 (CH), 133.86 (CH), 136.18 (CH), 136.88 (C), 137.74 (C), 148.33 (C), 164.34 (C=O), 169.67 (C=O), 171.61 (C=O), 172.10 (C=O), 172.71 (C=O), 173.30 (C=O), 173.98 (C=O). IR (KBr): 698, 735, 808, 976, 1045, 1122, 1230, 1352, 1454, 1529, 1658, 2870, 2933, 3288 cm^{-1}.

Biology. Cell Maintenance. The mastocytoma cell line P815 (H-2^d) was a generous gift from Prof. Hong-Yan Liu. The leukemia cell line P388 (maintained in our lab) was cultured in RPMI-1640 medium containing L-glutamine supplemented with 10% heat-inactivated fetal bovine serum (FBS, HyClone), 100 units/mL penicillin (Sigma), and 100 units/mL streptomycin (Sigma). The cells were maintained in a humid 5% CO_2 atmosphere at 37 °C. New batches of frozen cell stock were grown every 2 months, and growth morphology was evaluated.

Animals. Male C57BL-6J mice (H-2^b, 17 g ± 1 g, 6−7 weeks old), BALB/c mice (H-2^d, 17 g ± 1 g, 6−7 weeks old), and Wistar rats (200 g ± 10 g, 5−6 weeks old) were obtained from the Experimental Animal Center, Chinese Academy of Medical Sciences and Peking Union Medical College (SPF, Certificate No. SCXK 11-00-0006). HBV-transgenic [containing the whole HBV (adr subtype) genome] mice (17 g ± 1 g, 6−7 weeks old) were obtained from No. 458 Military Hospital in the city of Guangzhou. All animals were housed in groups under a 12:12 h regime (lights on from 7:00 to 19:00) at 23 ± 2 °C prior to the experiments and were given standard laboratory chow and tap water *ad libitum*.

MTT Assay for Immunostimulatory Activity of MDP Derivatives on Macrophages. Murine peritoneal macrophages were harvested from male C57BL-6J mice 3 days after ip injection of brewer thioglycollate medium (TG, 50 mL·kg^{-1} body weight) by the method set out by Traub et al.[63] Then macrophages, P388 leukemia cells, and test compounds were incubated together for 72 h. P388 leukemia cells incubated with synthetic MDP derivative alone were used as a control to simultaneously evaluate the toxicity of the compound. The macrophage-mediated cytotoxicity to P388 leukemia cells was determined by MTT assay. The optical density (OD) was measured at a test wavelength of 570 nm and a reference wavelength of 630 nm using a Bio-Rad microplate reader (Hercules).

Generation and Activation of BMDCs in Vitro. Mature DCs were generated as described previously,[38] with some modifications. Briefly, bone marrow cells were excised from the femurs and tibias of BALB/c mice. After red blood cells and nonadherent cells were depleted, adherent cells were cultured in complete RPMI 1640 medium supplemented with 10 ng/mL of IL-4 and 10 ng/mL of granulocyte macrophage-colony stimulating factor (GM-CSF). One-half of the medium was replaced with complete RPMI 1640 medium supplemented with cytokines every other day. BMDCs were obtained on day 5 and incubated with different concentrations of MDP-C for 30 min. Cells were then incubated with or without LPS for 48 h and analyzed by flow cytometry. Cell-free supernatant was collected and stored at −80 °C until performance of the ELISA assay.

Determination of Cytokines by ELISA Method. Levels of IL-2, IL-12, and IFN-γ in the culture supernatants were determined using the corresponding ELISA kits (Diaclone, France) according to the manufacturer's instructions. Briefly, the samples of supernatant were incubated on microtiter plates coated with the respective monoclonal antibodies, followed by addition of a biotinylated second antibody. After removal of excess antibodies, color development was finished by enzymatic reaction with streptavidin peroxidase, the intensity of which was directly proportional to the concentration of the respective cytokine in the samples. The amounts of cytokines in the samples were estimated by calibrating the OD values of the samples with the OD values of the standards, supplied with the kits. The lowest levels of cytokines detectable by these kits were the following: IL-2, 15.0 pg/mL; IL-12, 7.8 pg/mL; IFN-γ, 15.0 pg/mL.

Assay of Allogeneic CTL Response Induced by MDP-C-Pulsed-DCs. Splenic lymphocytes (from C57BL/6J mice, H-2^b) were cocultured (in quadruplicate) with allogeneic DCs (from BALB/c mice, H-2^d) that had been pretreated by different concentrations of MDP-C with or without LPS for 24 h. At day 6, living cells acting as effector cells were harvested and adjusted to 5×10^5 cells/mL with complete RPMI medium. A standard CTLs assay was performed with a CytoTox 96 non-radioactive cytotoxicity assay kit. Briefly, effector cells (5×10^5 cells in 100 μL) were cocultured with P815 mastocytoma cells (H-2^d) (1×10^4 cells in 100 μL) for 5 h. Supernatants were then collected, and the OD_{450} value was determined with a Bio-Rad microplate reader. The level of the specific release of LDH (lactate dehydrogenase) from P815 was calculated by $[(E - S)/(M - S)] \times 100$, where E is the average experimental release, S is the average spontaneous release, and M is the average maximum release. The spontaneous release is the LDH activity released by the target cells alone. The maximum release is the LDH activity released after treatment of the target cells with 1% Triton X-100.

Phenotypic Analysis of DCs by Flow Cytometry. DCs (5×10^5 cell/mL) were harvested, washed with PBS, resuspended in buffer (1% BSA, 0.1% sodium azide in PBS, pH 7.4), and stained with FITC-labeled antimouse MHC II (I-Ab), CD11c, and ICAM-1 monoclonal antibodies for 45 min at 4 °C. Stained cells were analyzed using a FACScan flow cytometer (Becton Dickson, Mountain View, CA).

Immunization of Animals and Detection of Anti-HBsAg Ab. HBV-transgenic mice were subcutaneously immunized with HBsAg (2.5 μg in 1 mL saline) adjuvanted by aluminum (alum, 125 μg in 1 mL of saline) or MDP-C (50 μg in 1 mL of saline) on day zero and boosted with 1/4 initial dose on days 14, 28, and 56. Mice were sacrificed 4 weeks after the final immunization. Anti-HBs Ab was determined in pooled sera with an ELISA kit (R&D Systems). The titers of anti-HBs Ab and the serum positive rate (number of mice producing anti-HBs Ab per 10 mice) were obtained. Expression of IL-2 by splenocytes was evaluated using the ELISPOT method described previously.[67] DCs derivated from the bone marrow of immunized mice were cultured as described above.

Assay of Allogeneic CTL Response Induced by DCs Isolated from Immunized Mice. DCs above (from transgenic mice) were cocultured with splenocytes (from C57BL/6J mice) for 6 days. Living cells acted as effector cells with P815 cells as target cells. A standard CTL assay was performed as described previously. IFN-γ production in supernatants by CTL pulsed with the DCs was determined by using a murine IFN-γ ELISA kit.

ELISPOT Assay for IL-2 Release by Splenic Lymphocytes from Immunized HBV-Transgenic Mice ex Vivo. The IL-2 spot forming cell (SFC) was determined using a standard ELISPOT assay, in which the captured anti-IL-2 Ab was previously coated onto PVDF plates (Millipore, Bedford, MA). Briefly, the splenic lymphocytes (3×10^5 cells/well) isolated from immunized HBV-transgenic mice were added to 96-well PVDF plates. The plates were then incubated for 24 h at 37 °C and developed overnight with a biotinylated detection antibody. Color development was finally performed with ELISPOT Blue Color Module (R&D system). Spots of IFN-γ-secreting cells were enumerated using Bio-Reader 4000 (Bio-Sys, Inc., Germany).

Immunostimulatory Effect of MDP-C in Immunosuppressed Mice. Male C57BL/6J mice, ip injected with 30 (mg/kg)/day cyclophosphamide for 5 consecutive days, were divided randomly into five groups. They were iv injected with MDP-C (10, 2, and 0.5 (mg/kg)/day) or PBS (1 (mg/kg)/day) for 7

consecutive days. PBMC samples were then collected on day 8, and the ratio of $CD3^+CD4^+$ to $CD3^+CD8^+$ T cells was determined with a flow cytometer, as described previously.[68]

Pyrogenicity Measurement in Rabbits. Three rabbits were treated with MDP-C at doses of 0.1 and 0.2 mg/kg (sc) in pyrogen-free PBS as described previously.[43] The rectal temperature of each rabbit was measured at 0, 1, 2, 3, 4, and 24 h after administration of MDP-C.

Test for Allergic Activity of MDP-C in Rats. Sera from 10 Wistar rats sensitized with MDP-C (0.1 mg/kg) were pooled, diluted, and injected intradermally (0.1 mL per site) in the shaved dorsal skin of unsensitized Wistar rats. After 24 h, the rats were given 0.1 mL of isotonic saline containing MDP-C (1 mg/mL, iv) and 0.1 mL Evans blue (25 mg in 1 mL saline, iv). Rats then were anesthetized and exsanguinated. The dorsal skin was finally removed. The diameter of the stain on the inner skin surface was measured.

Toxicity Test. MDP-C was administered intravenously into C57BL/6J mice at doses of 100 and 500 mg/kg. Two weeks later, the mice were killed and the corresponding indices, i.e., the mean weight of organs (mg) per 10 g of body weight, were measured.

Statistical Analysis. Data were expressed as the mean ± SD of at least three independent experiments. Differences between groups were inspected using one-way ANOVA, followed by multiple comparisons with Dunnett's test using SPSS 11.0 for Microsoft Windows (Microsoft Inc., Seattle, WA). A value of $P < 0.05$ was considered statistically significant. EC_{50} was derived from dose-dependent effect curves that were fit through "uphill dose−response curves, variable slope" using Prism, GraphPad (version 3.00).

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Appendix

Abbreviations. Ac, acetyl; BOP, benzotriazol-1-yloxytris(dimethylamino)phosphonium hexafluorophosphate; Bz, benzyl; Cbz, carbobenzyloxy; DCC, 1,3-dicyclohexylcarbodiimide; DCM, dichloromethane; Dde, 1-(4,4-dimethyl-2,6-dioxocyclohexylidene)-3-methylbutyl; DIC, 1,3-diisopropylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethyl sulphoxide; Fmoc, 9-fluorenylmethoxycarbonyl; Fmoc-OSu, *N*-(9H-fluoren-2-ylmethoxylcarbonyloxy)succinimide; HOBt, 1-hydroxybenzotriazole; MDP, muramic dipeptide; MTT, 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMM, *N*-methylmorpholine; *t*-Bu, *tert*-butyl; ph, phenyl; TFA, trifluoroacetic acid; THF, tetrahydrofuran.

Supporting Information Available: Tables listing cytotoxic effects of MDP-C and body temperature of MDP-C-treated rabbits and a figure showing the characterization of MDP-C. This material is available free of charge via the Internet at http://pubs.acs.org.

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