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Effects of MIP-1*α*, MIP-3*α*, and MIP-3*β* on the Induction of HIV Gag-specific Immune Response with DNA Vaccines

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Abstract

Transfection of DNA vaccines with chemokines may recruit dendritic cells (DCs) locally to capture the antigenic genes and their gene products to generate enhanced $CD8^+$ cytotoxic T lymphocytes (CTLs). In this study, we investigated the effects of macrophage inflammatory protein (MIP)-1*α*, MIP-3*α*, and MIP-3*β* on human immunodeficiency virus (HIV) Gag DNA vaccination. The chemokine plasmids markedly enhanced the local infiltration of inflammatory cells and increased the presence of $CD11c^+$ $B7.2^+$-activated DCs. MIP-1*α* and MIP-3*α* were potent adjuvants in augmenting CTLs and afforded strong protection to immunized animals against challenge with vaccinia virus expressing Gag (vv-Gag). However, decreased humoral response was observed. MIP-3*β* plasmid did not dramatically alter immunity. The chemokine inoculation time with respect to DNA vaccine priming was also investigated. The injection of pMIP-3*α* three days before Gag plasmid (pGag) vaccination markedly increased specific CTLs compared with simultaneous injection and led to higher protection against vv-Gag. Immunity was also shifted toward a T-helper type-1 (Th1) response. In contrast, inoculation with pMIP-3*α* three days after pGag vaccination shifted immunity toward a Th2 response. Our data suggest that administration of a chemokine with DNA vaccines offers a valuable strategy to modulate the efficacy and polarization of specific immunity and that chemokine– antigen timing is critical in determining overall biological effects.

INTRODUCTION

Ample evidence exists to support the role of vaccine-induced cytotoxic T lymphocytes (CTLs) in controlling a variety of infectious diseases, including acquired immunodeficiency syndrome.[1,2] Genetic vaccines encoding viral antigens have shown promise in generating specific humoral and cellular immunity via both major histocompatibility complex class I and II pathways. Nevertheless, the eficacy and potency of DNA vaccine remain sub-optimal. Recruitment and activation of antigen-presenting cells, such as dendritic cells (DCs), at the site of vaccination may further improve the immune responses induced.

DCs are the initiators and modulators of adaptive immunity. After detecting infectious agents, immature DCs, derived from bone marrow precursors, are activated and undergo extensive transformations in their phenotype and function. The mature DCs migrate to lymphoid organs and present antigen to lymphocytes, thereby generating specific immunity.[3–5] DCs also control the type of immunity by activating $CD4^+$ T-helper cells to release T-helper type-1 (Th1)

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or Th2 cytokines.[6] Recruitment of DCs to the local site of immunization is a prerequisite for the initiation of specific immunity.

Chemokines play a pivotal role in regulating the trafficking of leukocytes, including DCs. Chemokines tweak adaptive immunity[7] by recruiting and activating lymphocytes at the site of infection, and modulate the subsequent Th1 or Th2 reaction in secondary lymphoid organs.[8–12] Consequently, manipulating the level of chemokines at the site of vaccination is a novel strategy for enhancing immune protection.[13–17] There is growing interest in the exploration of chemokines to modulate the efficacy of specific immunity.[7,18–23] Several groups have examined the ability of chemokines, including macrophage inflammatory protein (MIP)-1*α* and MIP-3*β*, to modulate herpes simplex virus specific immunity.[19,24–26] When the human immunodeficiency virus (HIV) *gp120* gene was fused with macrophage chemo-attractant protein-3, the specific antibody (Ab) response was markedly enhanced.[27]

Immature DCs express CCR6, the only receptor for MIP-3*α* (CCL20).[28–31] MIP-3*α* triggers adaptive immunity by attracting immature DCs to the site of inflammation. Immature DCs also express CCR1, CCR4, and CCR5, which account for chemotactic response to MIP-1*α* (CCL3) and CCL5. After antigen uptake, immature DCs lose their responsiveness to MIP-1*α* and MIP-3*α*, and CCR7 expression is strongly up-regulated. Mature DCs migrate to lymph nodes in response to MIP-3*β* (CCL19), a ligand of CCR7.[32] Because MIP-1*α*, MIP-3*α*, and MIP-3*β* play critical roles in the regulation of DC trafficking, we compare the roles of these chemokines in modulating specific immunity induced by DNA vaccines. We postulate that expression of MIP-1*α* or MIP-3*α* at the site of vaccination, achieved by transfection of MIP-1*α* or MIP-3*α* plasmid with an HIV Gag plasmid (pGag), may induce immature DCs to the site of injection and capture DNA vaccines and their expression products. As a result, the major histocompatibility complex I–restricted CTL is primed by both endogenous and exogenous antigens, which may lead to enhanced T-cell response.[33] In our studies, we investigated whether inoculation of plasmid MIP (pMIP)-1*α*, pMIP-3*α*, or pMIP-3*β* with pGag vaccination could modulate Gag-specific immunity. In addition, we sought to explore the adjuvant mechanism of chemokines by varying the inoculation time of pMIP-3*α* with respect to DNA vaccine priming.

RESULTS

Construction of chemokine expression plasmids and their chemotactic effects

The HIV Gag gene was cloned into pRK expression vector, which has an internal human immunoglobulin G (IgG) intron. The expression of p55 was confirmed in transfected human embryonic kidney 293 (HEK293) cells and African green monkey kidney COS-7 cells by western blotting. The genes encoding MIP-1*α*, MIP-3*α*, and MIP-3*β* were prepared from the complementary DNA of lymphoid tissues of BALB/c mice. The genes were sequenced and cloned into the pRK vector. To verify the bioactivity of the constructs, HEK293 cells were transiently transfected. At day 3 after transfection, an average of 48 ng/ml of MIP-1*α* protein was detected in the supernatants when 1×10^6 cells were transfected with 2 μg pMIP-1*α*. the expression of pMIP-3*α* was confirmed using a western blot (data not shown). The lack of an Ab against MIP-3*β* meant that *in vitro* expression of pMIP-3*β* could not be assessed.

The ability of the expression products from these chemokine constructs to stimulate the recruitment of bone marrow–derived DC was evaluated using the chemotaxis microchamber assay. As shown in Figure 1a, the most potent chemo-attractant activity against immature DCs was detected with the supernatant of HEK293 cells transfected with pMIP-3*α*. Supernatant from pMIP-3*β*-transfected HEK293 cells showed potent chemo-attractant activity against mature DCs (Figure 1b). These data indicate that bone marrow–derived immature DCs are attracted by MIP-3*α*. In contrast, mature DCs of the same origin are recruited by MIP-3*β*.

Although MIP-1*α* did not show significant chemo-attractant activity toward DCs derived from bone marrow, it has been reported to act as a chemo-attractant for macrophages, monocytes, and monocyte-derived immature DCs.[34]

Enhanced recruitment of inflammatory cells by injection of MIP plasmids

We then investigated whether intramuscular inoculation of plasmids encoding DC-specific chemotactic factors would result in enhanced recruitment of inflammatory cells compared with DNA alone. After injection of pMIP-1*α*, pMIP-3*α*, or pMIP-3*β* into BALB/c mice, significant infiltration of inflammatory cells was observed at the site of injection on days 3 and 7, but not in control samples injected with phosphate-buffered saline, pRK vector, or pGag alone (Figure 2a). To identify the type of cells recruited by the MIP constructs, we harvested the injection-site muscle tissue 5 days after vaccination. The inflammatory cells were isolated and stained with anti-CD11c and anti-B7.2. We observed an accumulation of higher levels of $CD11c^+$ DCs expressing the co-stimulatory molecule B7.2 after inoculation with MIP constructs (Figure 2b), especially pMIP-3*α* and pMIP-3*β*, when compared with naïve muscle or muscle injected with phosphate-buffered saline or pGag alone. These results indicate that inoculation with MIP-3*α* not only recruits immature DCs to the site of injection but also enhances DC function by up-regulation of co-stimulatory molecules. The increased levels of $CD11c^+B7.2^+$ cells at the local site after MIP-3*β* injection is more likely due to the recruitment of mature DCs directly.

Immuno-adjuvant effects of pMIP-1*α*, pMIP-3*α*, and pMIP-3*β* on a DNA vaccine

To determine the immuno-adjuvant efficacy of the chemokine plasmids, we immunized BALB/c mice intramuscularly with pGag and one of the three plasmids (pMIP-1*α*, pMIP-3*α*, or pMIP-3*β*) twice at a 2-week interval. Two weeks after vaccination, blood was taken from the animals and sera were collected for enzyme-linked immunosorbent assays. Mouse splenocytes were used for enzyme-linked immunosorbent spot (ELISPOT) and CTL assays. Gag-specific IgG1, IgG2a, and total IgG concentrations from serum samples collected 2 weeks after immunization were measured. Compared with mice immunized with pGag alone, mice immunized with pMIP-3*β* and pGag showed a slightly higher level of specific total IgG and similar levels of IgG1 and IgG2a (Figure 3a). However, mice vaccinated with pMIP-1*α* and pGag showed lower levels of IgG, IgG1, and IgG2a versus mice immunized with pGag alone. The injection of pMIP-3*α* with pGag resulted in an even lower Gag-specific humoral response. To determine the relative sizes of the Th1 cytokine–producing populations of $CD8^+$ cells, we assayed splenocytes using ELISPOT (Figure 3b). More interferon-*γ* (IFN-*γ*)-secreting cells were present after immunization with pMIP-3*α* or pMIP-1*α* and pGag than after pGag injection alone. A negligible number of spots were detected in the absence of p7g peptide. The Gag-specific CTL response was measured in the immunized animals to investigate whether these cytokine-secreting $CD8^+$ T cells were functional in terms of killing target cells. Immunization with pMIP-3*α* and pGag resulted in the greatest enhancement of specific lysis in the CTL assay, with an increase from 27% specific lysis (pGag alone) to 59% at an effector-to-target-cell ratio of 40 (Figure 3c). The injection of pMIP-1*α* and pGag also potently enhanced the CTL response, whereas vaccination with pMIP-3*β* and pGag resulted in a slightly decreased CTL response compared with injection of pGag alone.

In addition, we measured the release of IFN-*γ* and interleukin-4 (IL-4) by stimulated T cells cultured in the presence of IL-2 and p7g peptide. Injection of pMIP-1*α* or pMIP-3*α* and pGag led to IFN-*γ* production two to four times higher than with pGag immunization alone (Figure 3d). Inoculation with pMIP-3*β* and pGag did not enhance IFN-*γ* production. In all the groups, IL-4 production by T cells was minimal (data not shown).

Inoculation of pMIP-3*α* three days ahead of pGag immunization leads to improved T-cell response

To explore the adjuvant mechanism of chemokine plasmids, we correlated the timing of pMIP-3*α* administration with the resulting immune responses. BALB/c mice were immunized twice at a 2-week interval with 50 μg of pGag alone or with 50 μg of pMIP-3*α*, either 3 days before, simultaneously with, or 3 days after pGag vaccination at the same injection site. Animals immunized with vaccinia virus expressing HIV Gag [vv-Gag, 1×10^7 plaque forming units (pfu)] and naïve animals were used as experimental controls.

Among the experimental groups, injection of pMIP-3*α* three days before pGag vaccination resulted in the greatest specific lysis in the CTL assay: 57% specific lysis of the p7g-pulsed target cells at an effector-to-target-cell ratio of 40 (Figure 4a). A potent Gag-specific CTL response was also observed after the simultaneous inoculation of pMIP-3*α* and pGag (48% specific lysis). The injection of pMIP-3*α* three days after pGag vaccination resulted in 29% specific lysis, whereas 14% specific lysis was observed in animals immunized with pGag alone. The administration of pMIP-3*α* three days before pGag vaccination decreased specific Ab response, including total IgG, IgG1, and IgG2a, compared with simultaneous injection of pGag and pMIP-3*α* (Figure 4b). When pMIP-3*α* was administered 3 days after pGag injection, an enhanced Gag-specific Ab response was observed compared with the simultaneous inoculation.

In all the experimental groups, immunization with pMIP-3*α* three days before pGag vaccination led to the highest IFN-*γ* production in the supernatants of cultured T cells. Inoculation with pMIP-3*α* three days after pGag vaccination resulted in decreased IFN-*γ* production compared with the simultaneous administration of pGag and pMIP-3*α* (Figure 4c). IL-4 production by T cells was negligible in all of the groups, except for the vv-Gag experimental control (data not shown).

To determine the relative sizes of the Th1 cytokine–producing populations of $CD8^+$ cells, we assayed the splenocytes isolated from vaccinated animals using ELISPOT and intracellular IFN-*γ* staining. Both methods clearly indicated that larger numbers of IFN-*γ*-secreting cells were present when pMIP-3*α* was injected 3 days before pGag (Figure 5a and b) than when the two plasmids were injected simultaneously, and the numbers dropped when pMIP-3*α* was given 3 days after the DNA vaccination.

Vaccination with pGag and MIPs could protect animals from recombinant vv-Gag

To assess whether vaccination with pGag and MIP constructs would protect animals from challenge with recombinant vv-Gag, unvaccinated mice and mice pre-vaccinated with pGag alone or pGag administered with one of the chemokine constructs were challenged with intraperitoneal injection of vv-Gag 2 weeks after vaccination.[35] The strongest protection was observed in animals immunized with pMIP-3*α* and pGag, with a 1,300-fold reduction in vaccinia virus pfu compared with naïve animals (Figure 6a). Inoculation with pMIP-1*α* and pGag also resulted in strong protection, with a nearly 200-fold reduction in virus pfu. In contrast, injection of pMIP-3*β* with pGag did not increase protection compared with injection of pGag alone. In a control experiment in which recombinant vaccinia virus expressing LacZ instead of vv-Gag was used to challenge the animals, neither the pre-vaccinated groups nor the naïve group demonstrated protection (Figure 6b). These data confirm that immunization with pMIP-3*α* or pMIP-1*α* and pGag generates significantly stronger Gag-specific antiviral T-cell responses compared with pGag vaccination alone. The protection is presumably due to a $CD8^+$ T-cell–mediated response, as Gag is not incorporated into the vaccinia virus. A separate experiment examined whether the timing of pMIP-3*α* injection relative to pGag injection would affect the protection of animals against challenge with vv-Gag. Among all the experimental

groups, the strongest protection was observed in animals immunized with pMIP-3*α* three days before pGag vaccination, with a 1,250-fold reduction in virus pfu compared with naïve animals (Figure 6c). Simultaneous injection of pMIP-3*α* and pGag resulted in a nearly 300-fold reduction in virus pfu, and pMIP-3*α* injection 3 days after pGag vaccination produced a 25-fold reduction. In contrast, no protection was observed in these groups when vaccinia virus expressing LacZ was used to challenge the animals (Figure 6d).

DISCUSSION

Cytokines have been intensively studied as potent immuno-adjuvants. However, increasing evidence points to the equally important role played by the family of chemokines in modulating adaptive immune response. Our findings demonstrate that inoculation with pMIP-1*α* or pMIP-3*α* and a DNA vaccine can markedly shift the response toward Th1 immunity, as evidenced by the increase of specific $CD8^+$ T-cell response, decrease of humoral response, and enhanced IFN-*γ* but not IL-4 production. In contrast, pMIP-3*β* does not significantly alter the polarization or potency of the resultant immune response when used together with pGag DNA vaccine. Administration of pMIP-1*α* or pMIP-3*α* with pGag also provides greater protection against a subsequent challenge with vv-Gag compared with administration of pGag alone, as demonstrated by a significant decrease of virus titers. In addition, the timing of pMIP-3*α* administration has a strong influence on the nature of the resultant specific immunity generated by pGag vaccination. Injecting pMIP-3*α* three days before pGag administration at the same site markedly increases specific $CD8^+$ T-cell response compared with the simultaneous injection of pMIP-3*α* and pGag, as shown by CTL assay, ELISPOT, and intracellular IFN-*γ* staining. The immune response is shifted toward Th1 immunity, as demonstrated by an increase in IFN-*γ*-producing cells and a decrease in Gag-specific humoral response. This approach also leads to augmented protection against challenge with vv-Gag. In comparison, pMIP-3*α* inoculation 3 days after pGag vaccine priming decreases Gag-specific CTLs and shifts the response toward Th2 compared with the simultaneous injection of pMIP-3*α* and pGag.

To date, the adjuvanticity of MIP-3*α* has been less recognized than that of other chemokines such as MIP-1*α*. Complete tumor regression can be achieved when tumor cells are transduced with a retroviral vector carrying the *MIP-3α* gene in a mouse model.[36] In this study, we provide the first direct evidence that MIP-3*α* possesses significant immuno-adjuvant activity when injected with a DNA vaccine. MIP-3*α* is the only chemokine ligand for CCR6, which is expressed on immature DCs,[37,38] memory T cells,[31] and B cells.[39] In addition, the stimulation of epithelial cells induces recruitment of immature DCs through up-regulation of MIP-3*α* expression.[40] Thus, MIP-3*α* triggers the initiation of an adaptive immunity by attracting immature DCs to the site of inflammation. In agreement with these observations, our study demonstrates that pMIP-3*α* is the most potent activator of Gag-specific $CD8^+$ CTLs among the three MIP plasmids examined.

The adjuvant effects of MIP-1*α* have been studied by many groups.[7,15,18,19] MIP-1*α* acts as a chemo-attractant to inflammatory cells involved in antigen presentation such as immature DCs, macrophages, and monocytes.[34] In addition, MIP-1*α* stimulation enhances IFN-*γ* production,[41,42] which is essential for the induction of Th1 immunity. Furthermore, MIP-1*α* knockout mice studies demonstrate the key role of MIP-1*α* in mediating virus-induced inflammation.[43] Our findings agree with these studies in that administration of pMIP-1*α* with pGag markedly enhances specific $CD8^+$ T-cell response, decreases Ab response, and strengthens the resistance to vv-Gag challenge.

The details of the mechanisms by which chemokine plasmids can act as adjuvants to boost specific immune responses remain to be deciphered. Inflammatory cell infiltration was observed at the injection site 3 and 7 days after intramuscular injection of the chemokine

constructs, and some of the accumulated inflammatory cells were $CD11c^{+}B7.2^{+}$-activated DCs, which appeared to mediate the induction of specific immunity. Our hypothesis is that recruitment of immature DCs by MIP-3α or MIP-1α to the site of injection may enable immature DCs to pick up more DNA molecules and lead to enhanced endogenous expression of DNA vaccines and potent major histocompatibility complex class I presentation. In contrast, the recruitment of mature DCs by MIP-3β may not be of significant benefit to $CD8^{+}$ T-cell response induced by DNA vaccination because of the low antigen uptake capacity of mature DCs. However, there may be only a limited number of DNA molecules left by the time immature DCs are recruited to the site of injection. The timing experiment clearly indicates that injection of the chemokine plasmid ahead of the DNA vaccine is beneficial.

It has been shown that Gag-specific immunity can also be enhanced by inoculation of cytokines such as GM-CSF. It would be interesting to investigate the combination of chemokine and cytokine molecules to enhance immunity further. Studies by other groups using cytokine plasmids, such as GM-CSF[44] or IL-2/Ig,[45] demonstrate that immunostimulatory cytokines appear to have stronger effect when administered 2–3 days after priming with antigen. It is tempting to speculate that the potential of DNA vaccines may be optimized when chemokine plasmids are administered before the antigen priming to recruit DCs to the site of immunization, with the subsequent administration of cytokine plasmids to amplify specific response to antigen.

Overall, our studies demonstrate that inoculation with certain chemokines, such as MIP-3α and MIP-1α, is a promising strategy for modulating the efficacy and polarization of antigen-specific immunity. Because the activation of a viral antigen–specific Th1 immune response is vital for HIV protection and therapeutic efficacy, our studies demonstrate that MIP-1α and MIP-3α are promising immuno-adjuvants for the induction of HIV-specific Th1 immunity.

MATERIALS AND METHODS

Animals

Female 6- to 8-week-old BALB/c ($H\text{-}2^{d}$) mice were purchased from Charles River Laboratories (Wilmington, MA) and housed in the animal facilities at the Johns Hopkins University (JHU) School of Medicine (Baltimore, MD). All mice used in this study were kept in accordance with institutional animal welfare guidelines.

Reagents

HIV IIIB Gag p55 protein was kindly provided by the National Institutes of Health AIDS reagent program. HIV Gag p7g (aa 199 AMQMLKETI 207) peptide is an $H\text{-}2^{d}$-restricted peptide and was synthesized at the synthesis and sequencing facility of JHU. Mouse IL-2 was purchased from R&D Systems (Minneapolis, MN). The vaccinia viruses expressing HIV HXB2 Gag p55 or LacZ were the kind gift of Dr. R.F. Siliciano at JHU.

Plasmid construction

Murine *MIP-1α*, *MIP-3α*, and *MIP-3β* genes were amplified by polymerase chain reaction from mouse complementary DNA. Murine total RNA was isolated from lymph nodes and spleen of BALB/c mice using an RNeasy Mini Kit (Qiagen, Valencia, CA), and complementary DNA was generated from the total RNA with a OneStep RT-PCR Kit (Qiagen). The murine MIP-1α expression plasmid, pMIP-1α was constructed by polymerase chain reaction amplification of the complementary DNA using the following primers: 5′-CCCCGAATTCGCCACCATGAAGGTCTCCACCACTGC-3′ and 5′-CCCCA AGCTTTCAGGCATTCAGTTCCAGG-3′ containing the *Hind*III and *Eco*RI restriction sites. The amplification product was cloned into the pRK-KS vector (a kind gift from Dr. P.A.

Beachy, JHU) containing the cytomegalovirus immediate early promoter and a human IgG intron. The MIP-3*α* and MIP-3*β* expression plasmids, pMIP-3*α* and pMIP-3*β*, respectively, were constructed in the same vector with the same restriction sites using primer pairs: 5′-CCCCGAATTCGCCACCATGGCCTGCGGTGGCAAG-3′, 5′-CCCCAAGCTTTTACATCTTCTTGACTCTTAGG-3′ and 5′-CCCCGAATTCGCCACCATGGCCCCCCGTGTGACC-3′, 5′-CCCCAAGCTTTCAAGACACAGGGCTCCTTC-3′, respectively. The constructs were confirmed by DNA sequencing. The HIV HXB2 Gag expression plasmid, pGag, was constructed by cloning a Gag coding region from PGAG plasmid[46] into pRK-SK vector using restriction sites *Xba*I and *Eco*RI. Plasmids were prepared using the Endofree plasmid Giga Kit (Qiagen). The bioactivity of pMIP-1*α* was confirmed by transfection of HEK293 cells with 2 μg of pMIP-1*α* using Lipofectamine (Invitrogen, Carlsbad, CA). The expression of pMIP-1*α* was assayed 3 days after transfection using a Colorimetric Sandwich ELISA kit (R&D Systems, Minneapolis, MN).

Dendritic cell chemotaxis assay

HEK293 cells were seeded at a density of 6×10^4 cells/ml into a 12-well plate and incubated until nearly 70% confluent. The cells were transfected with 2 μg pMIP-1*α*, pMIP-3*α*, or MIP-3*β* using Lipofectamine. On day 3 after transfection, 25 μl of HEK293 cell culture supernatants containing the chemokine gene expression products was added to the lower compartment of a 48-well Micro Chemotaxis chamber (Neuro Probe, Gaithersburg, MD). A polycarbonate nitrate filter of 5-μm pore size was applied to the lower compartment. To the upper compartment was added 50 μl of immature (1.8×10^6 cells/ml) or mature mouse DCs (6×10^5 cells/ml). After incubation for 60 minutes at 37 °C, the number of cells that had migrated to the bottom wells was quantified with light microscopy.

Histological analysis of mouse muscle and inflammatory cell typing study

We injected 10 μg of pMIP-1*α*, pMIP-3*α*, or pMIP-3*β* into tibialis muscle of BALB/c mice. At 3 and 7 days after injection, the muscle was fixed with 4% paraformaldehyde, sectioned, stained with hematoxilineosin, and observed with light microscopy. At 5 days after injection, the muscle was harvested, and the infiltrated inflammatory cells were isolated using a 70-μm cell strainer (Becton Dickinson, Franklin Lakes, NJ). The inflammatory cells were labeled with fluorescein isothiocyanate anti-CD11c Ab and phycoerythrin-labeled anti-CD86 (B7.2) Ab (PharMingen, San Diego, CA) and analyzed with FACScan (Becton Dickinson).

Vaccination of mice

The tibialis muscles of BALB/c mice ($n = 5$) were injected with 50 μg of pGag in the presence or absence of 50 μg of pMIP-1*α*, pMIP-3*α*, or pMIP-3*β* in a total of 50 μl phosphate-buffered saline (Invitrogen) via a 28-gauge needle twice at a 2-week interval.

Enzyme-linked immunosorbent assay for Gag-specific Ab response

Gag-specific Ab response was measured as describedelsewhere.[48] In brief, Gag p55 (2 μg/ml) was adsorbed onto microtiter wells. The plates were then blocked with 4% dehydrated milk and 1% normal goat serum (blocking buffer). Antisera from vaccinated mice were diluted with blocking buffer and then incubated with goat anti-mouse IgG-conjugated horseradish peroxidase. The plates were developed with 3,3′,5,5′-tetramethyl benzidine. The optical density at 450 nm was read on a microplate reader (Bio-Rad, Hercules, CA). Titers were expressed as the reciprocal $\log_2$ value of the final detectable dilution, which is defined as two SDs above the mean optical density of pre-immunized samples. For the determination of relative levels of IgG sub-classes, anti-murine IgG1 and IgG2a conjugated with horseradish peroxidase was used instead of anti-murine IgG-conjugated horseradish peroxidase.

Cytotoxic T lymphocyte assay

Gag-specific CTL response was measured as described elsewhere.[47] In brief, T cells from immunized mice were purified from total splenocytes and incubated in the presence of IL-2 (15 U/ml) and an immuno-dominant major histocompatibility complex class I peptide p7g (2 μg/ml) for 6 days as effector cells. Target P815 cells were prepared by incubation for 2 hours with p7g and 100 μCi of ^{51}Cr. After a standard 4-hour cytolytic assay at the indicated effector-to-target ratios, specific lysis was measured as % specific lysis = (experimental lysis – minimum lysis)/(maximum lysis – minimum lysis) × 100.

ELISPOT assay

Flat-bottomed nitrocellulose plates (Millititer HA; Millipore, Bedford, MA) were coated with IFN-γ mAb (2 μg/ml; PharMingen) in phosphate-buffered saline and incubated overnight at 4 °C. Splenocytes were harvested and serial dilutions starting from 10^6 cells in a total of 200 μl of medium were added to each well with IL-2 (15 U/ml) and with or without p7g peptide (2 μg/ml). After 30 hours' incubation, cells were removed and IFN-γ secretion was visualized using biotinylated Ab against IFN-γ (1 μg/ml; PharMingen), followed by avidin-alkaline phosphatase (1.25 μg/ml; Sigma, St. Louis, MO) and substrate (5-bromo-4-chloro-3-ondolylphosphate toluidinium/nitroblue tetrazolium; Boehringer Mannheim, Mannheim, Germany). The spots were counted using a dissecting microscope.

Intracytoplasmic cytokine staining and flow cytometry analysis

Splenocytes were cultured at 5×10^6 cells/ml with IL-2 (15 U/ml) and with or without p7g peptide (2 μg/ml) for 20 hours and then treated with Golgis-top (PharMingen) for 10 hours before harvesting. Splenocytes were then stained with phycoerythrin-conjugated rat anti-mouse CD8 (PharMingen) for 20 minutes. The cells were subjected to intracellular cytokine staining using the Cytofix/Cytoperm kit (PharMingen) and fluorescein isothiocyanate-conjugated anti-IFN-γ (20 μg/ml). Samples were analyzed with FACScan.

Vaccinia virus titer in the ovaries of challenged mice

BALB/c mice were vaccinated with 50 μg of pGag in the presence or absence of 50 μg of pMIP-1α, pMIP-3α, or pMIP-3β twice at a 2-week interval. Two weeks after the second immunization, control and vaccinated animals were challenged intraperitoneally with 1×10^7 pfu of vv-Gag or LacZ. On day 5 after challenge, the ovaries were harvested, homogenized, sonicated, and assayed for vaccinia virus titer by plating serial tenfold dilutions on a plate of BSC-1 indicator cells. On day 2 after exposure, the media were removed, the BSC-1 cell monolayer was stained with 0.1% crystal violet (Sigma) for 5 minutes, and the number of plaques was counted.

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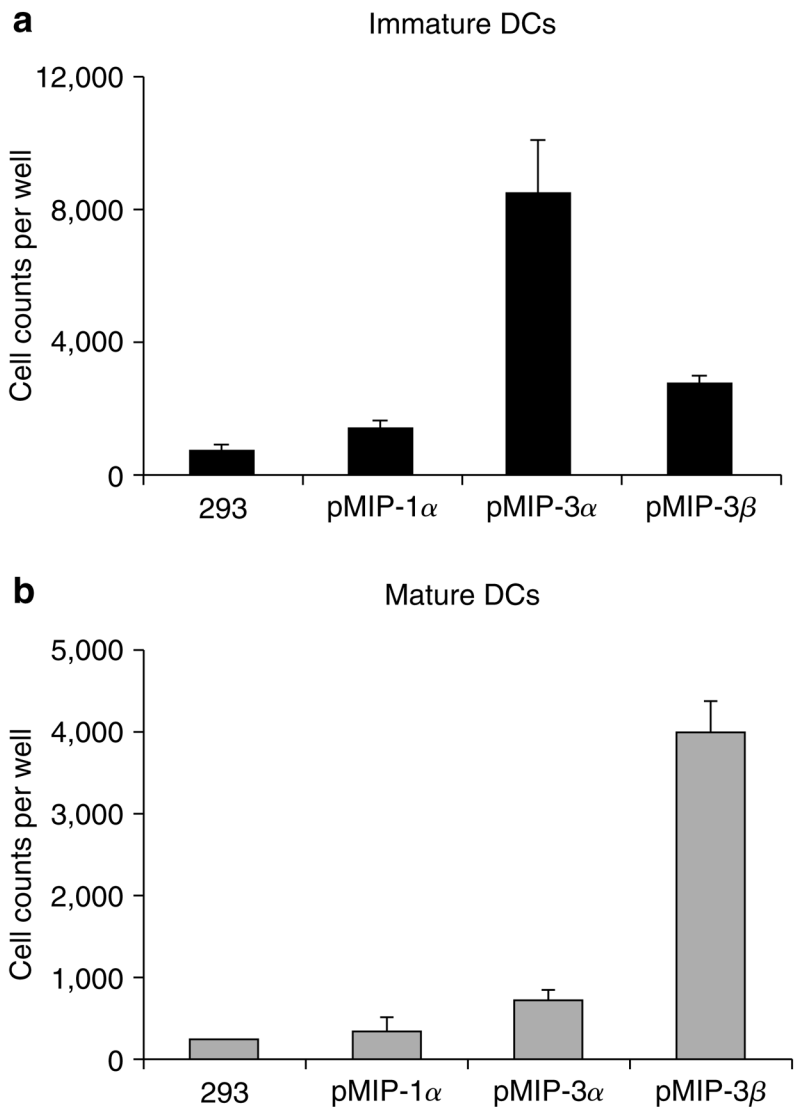

Figure 1. Macrophage inflammatory protein (MIP)-3α and MIP-3β recruit immature and mature dendritic cells (DCs), respectively

The ability of the expression products from plasmid MIP (pMIP)-1*α*, pMIP-3*α*, or pMIP-3*β* to attract (**a**) murine immature DCs and (**b**) murine mature DCs was evaluated in a chemotaxis chamber assay. Results are expressed as the total number of migrated cells in response to the chemo-attractants. Supernatants of untransfected human embryonic kidney 293 cells were used as negative controls.

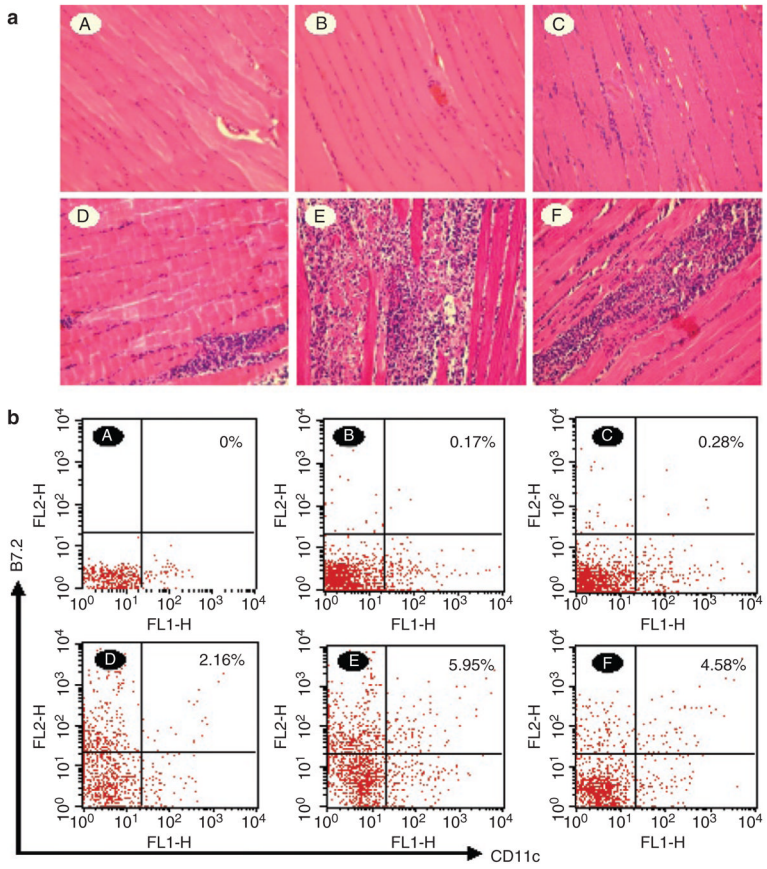

Figure 2. The injection of macrophage inflammatory protein (MIP) plasmids leads to local recruitment of inflammatory cells

(**a**) Hematoxylin and eosin staining was carried out 3 days after injection of the MIP cassettes into mouse tibialis muscle. (**b**) The type of cells was measured at the site of injection 5 days after inoculation: (A) naïve muscle; (B) muscle injected with phosphate-buffered saline; (C) muscle injected with Gag plasmid (pGag); muscle injected with 10 μg of chemokine encoding the constructs (D) plasmid MIP (pMIP)-1*α*, (E) pMIP-3*α*, and (F) pMIP-3*β*. The infiltrated inflammatory cells were isolated from the muscle and stained with fluorescein isothiocyanate–labeled anti-CD11c antibody and phycoerythrin-labeled anti-CD86 (B7.2) antibody, and analyzed by FACScan.

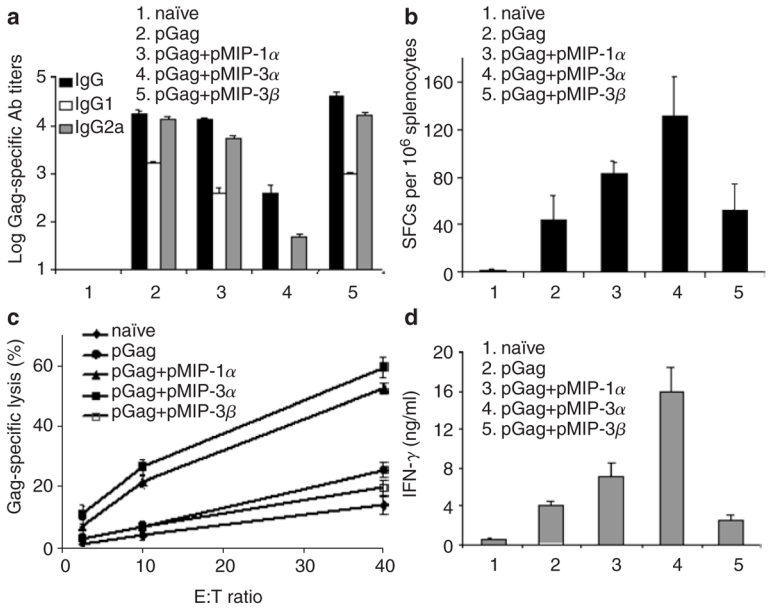

Figure 3. The immuno-adjuvant effects of the macrophage inflammatory protein (MIP) plasmids on Gag plasmid (pGag) vaccination

Determination of (**a**) Gag-specific humoral responses, (**b**) interferon-*γ* (IFN-*γ*) expression by enzyme-linked immunosorbent spot (ELISPOT), (**c**) cytotoxic T lymphocyte (CTL) response, and (**d**) IFN-*γ* production by T cells in immunized BALB/c mice. Mice were intramuscularly immunized twice, at weeks 0 and 2, with 50 μg of pGag alone or in conjunction with 50 μg of one of the MIP plasmids pMIP-1*α*, pMIP-3*α*, or pMIP-3*β*. Animals were killed 2 weeks after the second immunization, and sera and splenocytes were harvested. Gag-specific enzyme-linked immunosorbent assay was used to measure specific immunoglobulin G (IgG), IgG1, and IgG2a levels, and the splenocytes were studied for IFN-*γ* production using standard ELISPOT assay. Enriched T cells of the splenocytes were used for CTL assay. T cells were cultured at 5×10^6 cells/ml in the presence of interleukin-2 (IL-2) and p7g peptide for 5 days. Culture supernatants were collected and assayed for cytokine secretion using enzyme-linked immunosorbent assay kits for mouse IFN-*γ* and IL-4. E:T ratio, effector-to-target-cell ratio.

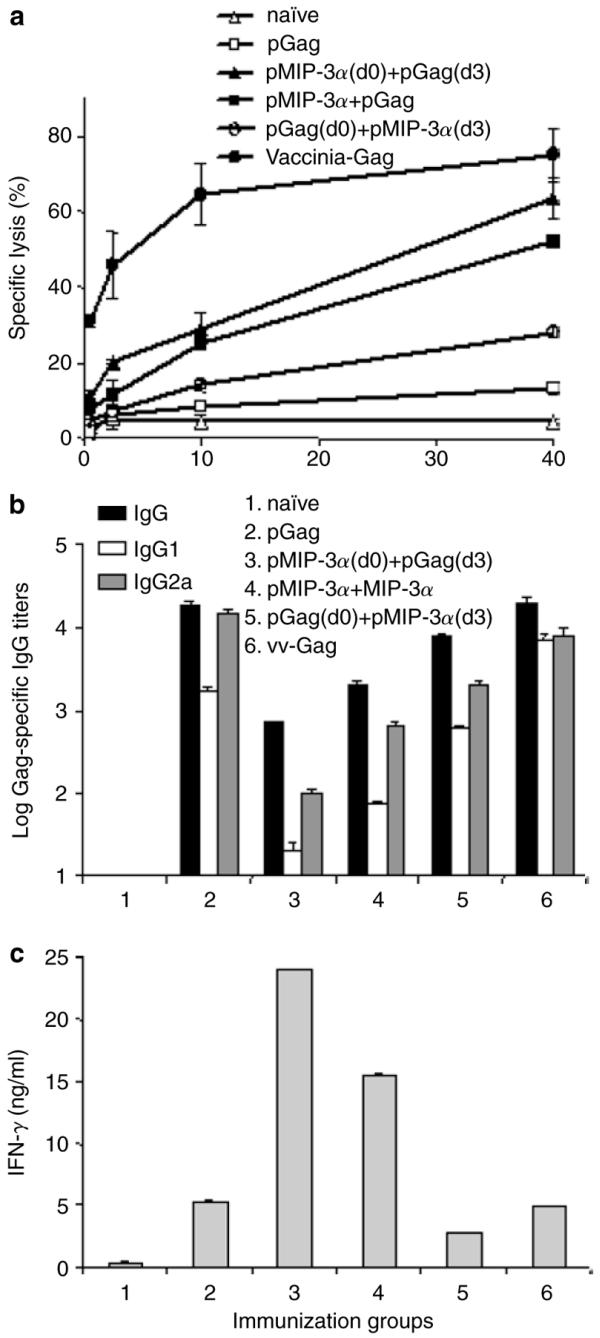

Figure 4. Administration of macrophage inflammatory protein-3*α* plasmid (pMIP-3*α*) 3 days before Gag plasmid (pGag) vaccination leads to enhanced cytotoxic T lymphocyte (CTL) response and interferon-*γ* (IFN-*γ*) production

Determination of (**a**) Gag-specific CTL response, (**b**) Gag-specific humoral response, and (**c**) IFN-*γ* production by T cells in immunized BALB/c mice. Mice were intramuscularly immunized twice, at weeks 0 and 2, with 50 μg of pGag alone or in conjunction with 50 μg of pMIP-3*α* either 3 days before, simultaneous with, or 3 days after pGag vaccination. Sera were collected 2 weeks after the second immunization. An enzyme-linked immunosorbent assay was employed to measure specific immunoglobulin G (IgG), IgG1, and IgG2a concentrations. Animals were killed 2 weeks after the second immunization, and enriched T cells of splenocytes

were studied for CTLs. T cells were cultured at 5×10^6 cells/ml in the presence of interleukin-2 (IL-2) and p7g peptide for 5 days. Culture supernatants were collected and assayed for cytokine secretion using enzyme-linked immunosorbent assay kits for mouse IFN-γ and IL-4.

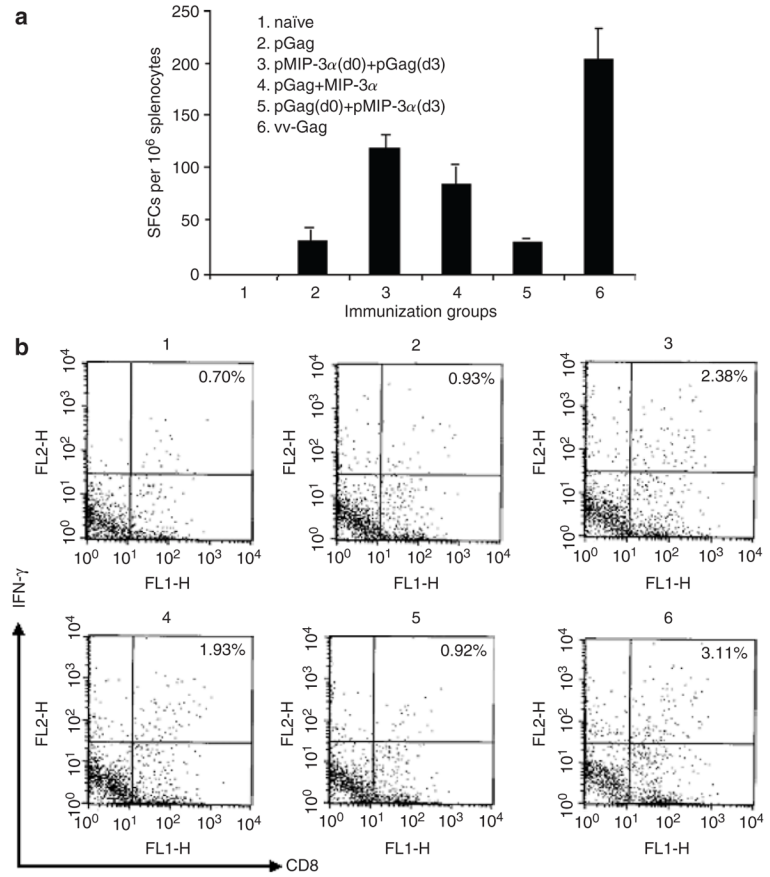

Figure 5. Inoculation with macrophage inflammatory protein-3*α* plasmid (pMIP-3*α*) 3 days ahead of Gag plasmid (pGag) immunization results in improved interferon-*γ* (IFN-*γ*) expression in specific T cells

IFN-*γ* expression in Gag-specific T cells determined by (**a**) enzyme-linked immunosorbent spot (ELISPOT) and (**b**) intracellular staining. Mice were intramuscularly immunized twice, at weeks 0 and 2, with 50 μg of pGag alone or in conjunction with 50 μg of pMIP-3*α*, either 3 days before, simultaneous with, or 3 days after pGag vaccination. Animals were killed 2 weeks after the second immunization, and the splenocytes were harvested. Splenocytes were cultured for 30 hours in the absence or presence of 2 μg/ml of p7g peptide, and cells producing IFN-*γ* were detected using standard ELISPOT assay. For intracellular IFN-*γ* staining, two-color analysis on CD8 and IFN-*γ* was performed on splenocytes that had been previously stimulated at 5×10^6 cells/ml for 30 hours with 2 μg/ml of p7g peptide. vv-Gag, vaccinia virus expressing Gag; SFC, spot-forming cell.

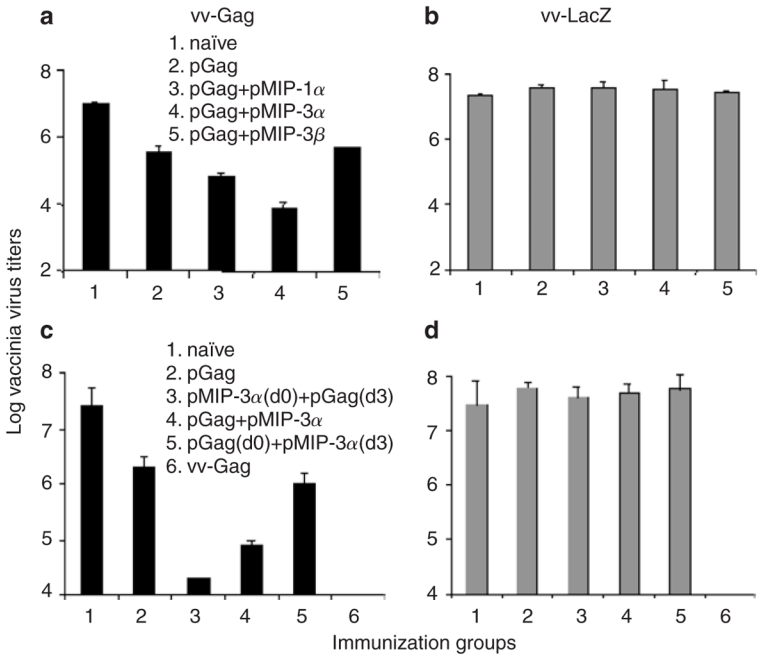

Figure 6. Vaccination with Gag plasmid (pGag) and macrophage inflammatory proteins (MIPs) could protect animals from recombinant vaccinia virus expressing Gag (vv-Gag)

BALB/c mice were immunized twice with 50 μg of pGag alone or in conjunction with 50 μg of plasmid MIP (pMIP)-1*α*, pMIP-3*α*, or pMIP-3*β* (upper panels). Two weeks after the second immunization, mice were challenged with (**a**) vv-Gag or (**b**) vaccinia virus expressing LacZ (vv-LacZ). In a separate experiment, groups of mice were immunized twice with 50 μg of pGag alone or in conjunction with 50 μg of pMIP-3*α* either 3 days before, simultaneous with, or 3 days after pGag vaccination (lower panels). Two weeks after the second immunization, animals were challenged with (**c**) vv-Gag or (**d**) vv-LacZ. The virus titer was determined 5 days after challenge on BSC-1 cells (epithelial cells of African green monkey kidney).