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*J Immunol* 2001; 167:1584-1591; doi: 10.4049/jimmunol.167.3.1584

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Immunostimulatory DNA-Based Vaccines Elicit Multifaceted Immune Responses Against HIV at Systemic and Mucosal Sites

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Immunostimulatory DNA sequences (ISS, also known as CpG motifs) are pathogen-associated molecular patterns that are potent stimulators of innate immunity. We tested the ability of ISS to act as an immunostimulatory pathogen-associated molecular pattern in a model HIV vaccine using gp120 envelope protein as the Ag. Mice immunized with gp120 and ISS, or a gp120:ISS conjugate, developed gp120-specific immune responses which included: 1) Ab production; 2) a Th1-biased cytokine response; 3) the secretion of β-chemokines, which are known to inhibit the use of the CCR5 coreceptor by HIV; 4) CTL activity; 5) mucosal immune responses; and 6) CD8 T cell responses that were independent of CD4 T cell help. Based on these results, ISS-based immunization holds promise for the development of an effective preventive and therapeutic HIV vaccine. The Journal of Immunology, 2001, 167: 1584–1591.

A protective immune response to infectious pathogens relies on activation of innate and adaptive immunity. It has been postulated that innate immunity has evolved to recognize conserved structural motifs on microbial pathogens that are not present in the mammalian host. These repeated structural motifs on microbial pathogens have been termed pathogen-associated molecular patterns (PAMPs), and their cognate receptors on phagocytic cells have been termed pattern recognition receptors (PRRs). A bacterial cell wall component, LPS, is a prototypical PAMP and is recognized by its cognate membrane-bound PRR, Toll-like receptor 4 (2). Recognition of PAMPs by PRRs leads to: 1) enhanced phagocytosis; 2) induction of signaling events that trigger the secretion of proinflammatory cytokines essential for reduction of infectious load; and 3) orchestration of an adaptive immune response, which includes humoral and cellular immunity against the infectious agent (1, 3, 4). In addition to circulating extracellular factors such as LPS, intracellular infectious products can be recognized by the infected host as PAMPs. For example, dsRNA, a product of viral infections, binds and activates the dsRNA-activated protein kinase, PKR, initiating downstream signaling that inhibits viral replication and up-regulates inflammatory cytokine secretion (5).

Bacterial DNA is also a potent activator of innate immunity, stimulating APCs to produce cytokines (6–8), up-regulate MHC and costimulatory molecules (9–11), and induce cross-presentation of soluble Ag on MHC class I (12). This immunostimulatory activity of bacterial DNA is dependent on immunostimulatory DNA sequences (ISS). ISS are structurally defined by their content of CpG motifs (5′-purine-purine-CpG-pyrimidine-pyrimidine-3′) (7). In vertebrate genomes, there is a diminished frequency of CpG motifs as well as inactivation of them by cytosine methylation (7, 13). Based on their ability to activate innate immunity and their structural differences from vertebrate DNA, ISS contained in bacterial DNA fulfill the definition for PAMPs. The cellular recognition systems for bacterial DNA are just beginning to be elucidated. The potent induction of type I IFNs by ISS and dsRNA but not by LPS (14, 15) suggests that ISS-induced signaling is more analogous to the dsRNA/PKR signaling system than to the LPS recognition system. Recently published work suggests that Toll-like receptor 9 and the intracellular DNA-PK catalytic subunit are essential for ISS-induced activation of innate immunity (16, 17).

Recent studies have emphasized the central role of dendritic cells in initiating adaptive immune responses after exposure to PAMPs in their microenvironment (18). Because the response of cells of the innate immune system, such as dendritic cells, to PAMPs is deeply rooted in evolution, it is likely that PAMPs act as natural adjuvants for the induction of protective immune responses against the pathogens that contain them. This suggests an intriguing strategy for vaccine design: combining a suitable PAMP with pathogen-derived Ags to create better immunogens.

The potent immunostimulatory properties of ISS led us to hypothesize that they could be used as a PAMP candidate for HIV vaccine development. The ability to produce large quantities of synthetic oligodeoxynucleotides (ODNs) containing ISS, and the relatively low toxicity of these synthetic ODNs when compared...
with other PAMPs such as LPS, makes ISS an especially attractive reagent for future clinical use. In the murine studies presented here, we evaluated the immune response elicited by a model HIV-derived Ag (gp120) coadministered with, or directly conjugated to, ISS. We show that these ISS-based immunization schemes elicit potent Ag-specific immune responses, including Ab, Th1 cytokines, CCR5-specific β-chemokines, and CTL activity. These immune responses can be induced systemically, as well as at mucosal sites. Furthermore, ISS-based vaccination elicits these cytokine, chemokine, and CTL responses from CD8 T cells independently of CD4 T cell help. Our findings suggest that ISS-based immunization might also elicit the robust and multifaceted immune responses that will be required for protection against HIV infection and for vaccination in the setting of CD4 T cell deficiency, as seen in AIDS patients.

Materials and Methods

Reagents

HIV gp120 protein from HIV-1,LAI expressed in Chinese hamster ovary cells was obtained from Quality Biological (Gaithersburg, MD). ISS and mutated phosphorothioate oligodeoxynucleotides (mODN) were purchased from Trilink Biotechnologies (San Diego, CA). The sequence of the ISS used in these studies is 5'-TGACTGTGAACGTTCGAGATGA-3'. The mODN has the sequence 5'-TGACTGTGAACCTTAGAGATGA-3'. gp120/ISS and gp120:mODN conjugates were produced in a three-step process as previously described (12, 19). Introduction of maleimido groups onto gp120 molecules was achieved by incubation with a 20 M excess of sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (Pierce Chemicals, Rockford, IL) for 2 h followed by purification on an NAP-25 column (Amersham Pharmacia, Uppsala, Sweden). 5' activation of oligodeoxynucleotides was conducted by incubation with 0.2 M tricarboxyethylphosphine (Pierce Chemicals), and activated ODNs were subsequently purified on an NAP-10 column. Maleimido-modified gp120 and thiol-activated ODNs were then incubated together overnight, and free ODNs were removed by filtration using an Amicon 50 spin column (Amicon, Beverly, MA). The conjugate was analyzed by SDS-PAGE. After electrophoresis, the samples were transferred onto nitrocellulose membranes and visualized by chemiluminescent detection of anti-gp120 Ab (Western blotting), autoradiography after hybridization with complementary [32P]ATP-labeled oligodeoxynucleotides (Southwestern blotting), or UV visualization of shadowing of the oligodeoxynucleotides on TLC membranes (UV shadowing).

Immunization protocols

Female BALB/c mice (The Jackson Laboratory, Bar Harbor, ME) age 4–6 wk were immunized with gp120 (10 μg) alone or with ISS (50 μg) or mODN (50 μg). Alternatively, mice were vaccinated with gp120:ISS or gp120:mODN conjugate (10 μg based on gp120 content). For intradermal (i.d.) immunization, reagents were delivered in 50 μl saline by injection into the base of the tail. For intranasal (i.n.) immunization, reagents were applied topically in 30 μl saline divided equally and delivered to each nare of lightly anesthetized mice. Immunizations were delivered on three occasions spaced 2 wk apart. For CD4 T cell depletion, mice received 1 mg GK 1.5 mAb (Bio Express, West Lebanon, NH) i.p. on three occasions, 4 wk apart. With the use of flow cytometry, we determined that mice receiving GK 1.5 mAb had <1% of the peripheral blood and splenic CD4 T cell counts of untreated mice throughout the course of these experiments. All animal procedures followed the University of California, San Diego, animal care guidelines.

Sample collection and processing

Serum was obtained by serial retro-orbital bleedings until mice were sacriﬁced at week 12. Vaginal washes were obtained during week 12 by lavage with 50 μl PBS. Samples were spun to remove cellular debris and frozen at −70°C until the IgA assay was performed. Feaces were collected at week 12, and IgA was extracted by routine methods. Briefly, three to six pieces of freshly voided feces were collected and subsequently dried in a Speed Vac concentrator. After drying, net dry weights were recorded, and the material was resuspended in PBS with 5% nonfat dry milk and protease inhibitors at a ratio of 20 μg/mg to standardize for variability in the amount of fecal material collected. The solid matter was resuspended by vortexing for 12 h followed by centrifugation at 16,000 × g for 10 min to separate residual solids from the supernatant. Supernatants were frozen at −70°C until the IgA assay was performed.

Splenoocytes (1 × 107/mouse), Peyer’s patch lymphocytes (1 × 107/mouse), and lamina propria lymphocytes (1.5 × 107/mouse) were recovered by routine methods 12 wk after the initiation of immunization (20). Briefly, spleens were harvested and teased to make single-cell suspensions. Intestines were isolated and stripped of mesenteric fat, and the Peyer’s patches were excised. The tissue was washed and incubated in digestion medium (collagenase VIII, 500 U/ml; Sigma, St. Louis, MO; DNase, 1–1.5 μg/ml; Sigma) for 1 h. Single-cell suspensions were obtained by using the digestion mixture over a fine nylon sieve. Cells were subsequently washed, and Peyer’s patch lymphocytes were separated on a 75%/40% Percoll gradient. Lamina propria lymphocytes were isolated by opening residual intestinal tissue longitudinally, washing extensively, cutting intestines into short segments, and incubating in 1 mM EDTA to remove the epithelial layer. After EDTA treatment, the tissue was washed in RPMI (Irvine Scientific, Irvine, CA) supplemented with 10% heat-inactivated FCS (Life Technologies, Gaithersburg, MD), 2 mM l-glutamine (Cellgro, [url]N atham, VA), 100 U/ml penicillin-100 μg/ml streptomycin (Pen/Strep; Cellgro), and Fungizone (Life Technologies). The tissue was poured over a coarse sieve, and residual tissue was incubated with digestion medium. The lamina propria lymphocyte digestion mixture was poured over a fine nylon sieve to obtain a single-cell suspension, and then lymphocytes were purified on a 75%/40% Percoll gradient. These procedures resulted in 40+96% viability of all lymphocyte preparations.

Immunological assays

Antibody levels were determined by routine ELISA techniques (21). Briefly, microtiter plates were coated overnight with 5 μg/ml gp120 in borate-buffered saline (pH 9.2). Plates were then washed and blocked with 1% BSA. Plates were again washed, and serially diluted serum samples were added. After overnight incubation at 4°C, plates were washed, and alkaline phosphatase-labeled goat anti-mouse IgG, IgG2a, or IgA (Southwestern Biotechnology Associates, Birmingham, AL) was added for 2 h. After plates were washed, a solution of p-nitrophenyl phosphate (1 mg/ml; Boehringer Mannheim, Indianapolis, IN) was added and OD405 was read 1–4 h after addition of the substrate. Ab titers were determined by end point titration of the samples and reflect the amount of dilution required to achieve twice-background OD readings. ELISA plates were analyzed using the DeltaSOFT II v. 3.66 program (Biorad Mettler, Princeton, NJ).

For CTL assays, 7 × 10^4 splenocytes, Peyer’s patch lymphocytes, or lamina propria lymphocytes were cultured in supplemented RPMI with 6 × 10^5 mitomycin C-treated naive splenocytes in the presence of recombinant human IL-2 (50 IU/ml; BD PharMingen, San Diego, CA) and an HIV-1 class I (H2d)-restricted gp120 peptide, which has been described in previous publications (p18-I10; RGPGRAFVTI; 4 μg/ml). Ten days, restimulated cells were harvested, and specific lysis of target cells was measured using the Cytoxtron 96 assay kit according to the manufacturer’s instructions (Promega, Madison, WI).

IFN-γ, macrophage-inflammatory protein (MIP) α, and MIPβ responses were evaluated by incubation of splenocytes, Peyer’s patch lymphocytes, or lamina propria lymphocytes at 5 × 10^6 cells/ml in 96-well plates in a final volume of 200 μl supplemented RPMI with gp120 (10 μg/ml or p18-I10 (4 μg/ml). Culture supernatants were harvested at 72 h and analyzed by ELISA for IFN-γ (BD PharMingen), MIPα, MIPβ, or RANTES (R&D Systems, Minneapolis, MN) content, according to the manufacturers’ recommendations. Each culture supernatant was compared with the standard curve on the plate using the DeltaSOFT II v. 3.66 program.

ELISPOT assays were performed using nitrocellulose-backed 96-well plates (Millipore, Bedford, MA). Plates were coated with 50 μl PBS containing rat anti-mouse IFN-γ Ab (BD PharMingen) at 10 μg/ml or goat anti-mouse MIPα Ab (R&D) at 5 μg/ml and incubated overnight at 4°C. Wells were washed with borate-buffered saline-0.05% Tween 20 and then blocked with 200 μl of supplemented RPMI for 1 h at 37°C. Serial dilutions of splenocytes from each mouse starting at 2 × 10^3 cells/well were then plated and incubated in triplicate wells in medium alone or with gp120 (10 μg/ml) or P18-I10 (4 μg/ml). After 24 h, wells were washed, biotinylated anti-IFN-γ (PharMingen) or biotinylated anti-MIPα (R&D Systems) was added to the appropriate wells for 2 h at room temperature. Wells were then washed and HRP-streptavidin conjugate (Zymed, South San Francisco, CA) was added for 1 h at room temperature. Plates were then developed by adding TMB membrane substrate (Kierkegaard & Perry Laboratories, Gaithersburg, MD) to the plates and incubated for 15 min at room temperature. Plates were dried, and spots were counted using a dissecting microscope. The number of peptide-specific cytokine-secreting cells was determined as a frequency of total CD8 T cells by using a correction factor based on the
fraction of CD8 T cells present in spleens of untreated and CD4-depleted mice as determined by flow cytometry (data not shown).

Statistical analyses

Statistical analyses were performed using the GraphPad Prism program (GraphPad Software, San Diego, CA). The significance of differences in means between multiple groups was determined using one-way ANOVA with Bonferroni’s posttest analysis. When only two groups were compared, the significance of differences in means between the two groups was determined by unpaired t test. Significant differences were defined as \( p < 0.05 \).

Results

Synthesis of the gp120:ISS conjugate

Because the optimal antigenic targets for HIV vaccine development have not yet been established, in the present studies gp120 should be considered as a model Ag for the application of ISS-based immunization strategies to generate improved immunity to better HIV target Ags as they are identified. Recent studies in the literature suggest that ISS coadministered with HIV particles can elicit some potentially beneficial immune parameters (24–26). Conjugation of ISS to protein Ags, including gp120, has previously been shown to improve some aspects of their immunogenicity (12, 19, 27, 28). Therefore, we generated the gp120:ISS conjugate to determine whether ISS conjugation might generate an improved immune response to this relatively poorly immunogenic HIV Ag (29). ISS-containing ODN (7.5 kDa) or ODN containing a mutated, nonstimulatory motif (mODN, 7.5 kDa) were conjugated to gp120 protein (120 kDa) as described in Materials and Methods. Coomassie Blue staining after SDS-PAGE of the gp120:ODN conjugates revealed a 140-kDa band, reflecting a protein-ODN ratio of \( \sim 1:3 \) (Fig. 1a). Western blot analysis with anti-gp120 Ab (Fig. 1b), UV shadowing of the oligonucleotides (Fig. 1c), and Southwestern blot analysis with radioactively labeled complementary oligonucleotides (data not shown) confirmed successful conjugation.

Immunization i.d. with ISS-based gp120 vaccines elicits a Th1-biased immune response and chemokine secretion

To determine whether ISS could improve humoral and cytokine responses to gp120, BALB/c mice were immunized i.d. with co-administered gp120 plus ISS or with gp120:ISS conjugate. For comparison, control mice were immunized with gp120 alone, gp120 plus mODN, or gp120:mODN conjugate. In pilot experiments, ISS coadministered with gp120 at a dose similar to that present in the conjugate (1.3 \( \mu \)g ISS per mouse) led to immune responses similar to those seen after immunization with gp120 alone (data not shown). Therefore, in subsequent studies in which unconjugated ISS was codelivered with gp120, a 40-fold higher dose of ISS (50 \( \mu \)g) was used.

To assess the ability of the vaccination reagents under study to augment humoral immune responses, Ag-specific total IgG and IgG2a (which is Th1 dependent) levels from serum collected 12 wk after initiation of immunization were measured (Fig. 2a). Compared with controls, mice immunized i.d. with gp120 plus ISS or gp120:ISS conjugate showed significantly higher levels of total IgG and IgG2a (\( p < 0.001 \)). In addition, both gp120 plus ISS coadministration and gp120:ISS immunization improved IgG1 responses relative to control immunizations (data not shown). Previous studies have shown that gp120 is a poor target for the generation of HIV-neutralizing Abs (29, 30); therefore, neutralization studies were not pursued. Future studies with HIV-related Ags effective at eliciting neutralizing Abs will need to verify the ability of ISS to augment these neutralizing Ab responses.

IFN-\( \gamma \) production is a hallmark feature of Th1-biased immunity and contributes to protection against many viral infections (31, 32). Therefore, the CD4 T cell IFN-\( \gamma \)-response of immunized mice was determined by culture of splenocytes with gp120 and analysis of supernatants by ELISA (Fig. 2b). IFN-\( \gamma \) production was significantly higher for mice immunized with gp120 plus ISS (\( p < 0.05 \)) or gp120:ISS conjugate (\( p < 0.001 \)) when compared with control mice.

FIGURE 2. Immunization i.d. with ISS-based gp120 vaccines elicits Ag-specific Ig and cytokine responses. Female BALB/c mice age 4–6 wk were immunized i.d. on days 0, 14, and 28 with gp120 (10 \( \mu \)g) alone, gp120 plus mODN (50 \( \mu \)g), gp120:mODN conjugate (conj.) (10 \( \mu \)g based on gp120 content), gp120 plus ISS (50 \( \mu \)g), or gp120:ISS conjugate (10 \( \mu \)g based on gp120 content). a, gp120-specific IgG and IgG2a titers in week 12 serum were determined by ELISA. b–d, gp120-specific IFN-\( \gamma \), MIP1\( \alpha \), and MIP1\( \beta \) production from splenocytes restimulated in vitro with gp120 protein was determined by ELISA. Results reflect averages of four mice per group and are representative of three independent experiments. Error bars indicate SEM. Groups with means that are significantly different from gp120-immunized mice are denoted as: \(*\), \( p < 0.05 \); **, \( p < 0.001 \). Groups with means that are significantly higher than gp120 plus ISS-immunized mice are denoted as: #, \( p < 0.05 \); ##, \( p < 0.001 \).
immunized mice. Furthermore, gp120:ISS conjugate was more effective at inducing an IFN-γ response than gp120 plus ISS (p < 0.001).

CCR5 acts as a coreceptor for HIV entry into cells (33–37), and competitive inhibition of this virus-coreceptor interaction by CCR5-specific β-chemokines (MIP1a, MIP1b, and RANTES) may inhibit the intercellular spread of HIV and the natural progression of the infection to AIDS (38). The ability of ISS to induce β-chemokine production from macrophages in an Ag-independent manner (A. A. Horner, S. K. Datta, and E. Raz, unpublished observations) led us to investigate whether ISS-based vaccines could elicit their Ag-specific production. Ag-specific secretion of MIP1α, MIP1β, and RANTES was assessed by ELISA of supernatants from splenocytes cultured with gp120 (Fig. 2, c and d). Mice immunized with gp120:ISS conjugate demonstrated significantly stronger MIP1α and MIP1β responses than controls (p < 0.001) or gp120 plus ISS-immunized mice (p < 0.001 for MIP1α and p < 0.05 for MIP1β). However, although less effective than gp120:ISS conjugate, gp120 plus ISS coadministration also elicited significant MIP1β but not MIP1α production. Interestingly, whereas significant levels of RANTES have been observed upon stimulation of APCs by ISS in vitro, gp120-specific RANTES production was not appreciably induced above background levels in this series of experiments (data not shown).

**Immunization i.n. with ISS-based gp120 vaccines elicits systemic and mucosal immune responses**

Protection against HIV infection is likely to require immunity at mucosal sites because 1) its spread is principally by sexual transmission, 2) the intestinal mucosa represents an important site for the initial replication of the virus, and 3) mucosal immunity is important for protection against mucosal challenge in published models of HIV viral infection (20, 39). As mucosal immunity is best elicited by vaccine delivery to mucosal sites (20, 40), the immunization regents described in the previous sections were administered i.n. to mice at the same doses used for i.d. immunization, and both systemic and mucosal immune parameters were measured. Similar to i.d. immunization, i.n. immunization with gp120 plus ISS or gp120:ISS conjugate elicited significantly higher levels of serum IgG and IgG2a than controls (p < 0.001; Fig. 3a). Furthermore, i.n. immunization with gp120 plus ISS or gp120:ISS conjugate also induced a vigorous secretory IgA response detected in vaginal washes and fecal samples (p < 0.001 vs controls) (Fig. 3b). In contrast, i.d. immunization with these reagents failed to elicit a significant mucosal IgA response. Finally, i.n. immunization with either gp120 plus ISS or gp120:ISS conjugate elicited significantly more gp120-specific IFN-γ, MIP1α, and MIP1β production than control immunizations (p < 0.001 for IFN-γ, p < 0.05 for MIP1α and MIP1β; Fig. 3, c–e).

**ISS-based gp120 vaccines elicit systemic and mucosal CTL activity**

Because an effective CD8 CTL response is important in preventing and controlling HIV infection (41–44), the ability of ISS-based gp120 immunization to elicit Ag-specific CTL activity was determined. Both i.d. (Fig. 4a) and i.n. (Fig. 4b) administration of gp120:ISS conjugate elicited similar levels of high specific lysis in splenic CTL assays. However, although i.d. administration of gp120 plus ISS elicited CTL activity that was similar to the conjugate, i.n. administration of gp120 plus ISS elicited a significantly lower CTL response (p < 0.05). In addition to systemic CTL activity, i.n. gp120:ISS conjugate delivery, and to a much lesser extent gp120 plus ISS coadministration, induced mucosal CTL responses as measured with lamina propria (Fig. 4c) and Peyer’s patch lymphocytes (Fig. 4d). However, consistent with the poor secretory IgA response seen after systemic vaccination, both i.d. gp120 plus ISS and gp120:ISS conjugate immunizations induced only weak CTL responses at these mucosal sites.

**ISS-based gp120 vaccines elicit MHC class I-restricted cytokine and chemokine responses**

The cytokine and chemokine data described in the previous sections include MHC class II-dependent responses, as intact gp120 protein was used to stimulate cells. Cytokine and chemokine secretion by CD8 T cells, in addition to CTL responses, are important for controlling HIV infection, whereas CD4 T cell deficiency is a characteristic feature of AIDS (38, 45). Therefore, the ability of ISS-based vaccines to induce cytokine and chemokine responses from CD8 T cells was investigated. Splenocytes from immunized mice were restimulated in vitro with MHC class I (H2d)-restricted gp120 peptide, and cytokine and chemokine production in culture supernatants was subsequently determined by ELISA.

**FIGURE 3.** Immunization i.n. with ISS-based gp120 vaccines elicits systemic and mucosal Ag-specific Ig and cytokine responses. Mice were immunized i.n. with the same reagents and doses as described in Materials and Methods and Fig. 2. a, gp120-specific IgG and IgG2a titers from week 12 serum were determined by ELISA. b, gp120-specific mucosal (vaginal and fecal) IgA titers at week 12 from mice immunized i.n. or i.d. were determined by ELISA. c–e, gp120-specific splenic IFN-γ, MIP1α, and MIP1β production was determined by ELISA. Data reflect averages of four mice per group and are representative of three independent experiments. Error bars indicate SEM. Statistical significance is denoted as in Fig. 2. conj., Conjugate.
Mice immunized i.d. with either gp120 plus ISS or gp120:ISS conjugate demonstrated significant CD8 T cell production of IFN-γ, MIP1α, and MIP1β compared with control immunized mice (Fig. 5, a–c). Similar results were seen with i.n. gp120 plus ISS and gp120:conjugate vaccination (data not shown).

The class I-restricted cytokine, chemokine, and CTL responses elicited by gp120:ISS immunization are CD4 T cell independent

During the course of HIV infection, CD4 T cells are depleted. Therefore, it would be important for a therapeutic AIDS vaccine to elicit robust immunity in the absence of CD4 T cells. The ability of ISS-based vaccines to elicit cytokine, chemokine, and CTL responses from CD8 T cells led us to investigate whether these responses required CD4 T cell help. Previous investigations have demonstrated that OVA:ISS conjugate vaccination induces equivalent CTL responses in CD4 knockout and wild-type mice, whereas the CTL response in CD4 knockout mice immunized with OVA plus ISS is compromised (12). Therefore, gp120:ISS conjugate was used to i.d. immunize wild-type and CD4 T cell-depleted mice to compare their CD8 T cell responses. Splenocytes from gp120:ISS conjugate-immunized, CD4 T cell-depleted mice that were restimulated with a class I-restricted gp120 peptide demonstrated a retained ability to secrete Ag-specific IFN-γ (Fig. 6a), MIP1α (Fig. 6b), and MIP1β (Fig. 6c) relative to splenocytes from immunized mice that were not CD4 T cell-depleted. Furthermore, by ELISPOT analysis, CD4 T cell-depleted and nondepleted mice immunized with gp120:ISS conjugate had equivalent frequencies of CD8 T cells producing IFN-γ and MIP1α in response to incubation with a class I-restricted gp120 peptide (Fig. 6d). Consistent with these results, Ag-specific CTL activity was also retained in CD4 T cell-depleted mice (Fig. 6e). As expected, restimulation of splenocytes from immunized CD4 T cell-depleted mice with gp120 protein failed to elicit cytokine or chemokine responses (data not shown). Furthermore, CD4 T cell-depleted mice were unable to generate a detectable Ab response after gp120:ISS conjugate immunization despite their development of CD8 T cell immunity (data not shown). Similar to i.d. immunized mice, CD4 T cell-depleted mice immunized i.n. with gp120:ISS conjugate also showed retained CD8 T cell responses, including CTL responses at both systemic and mucosal sites (data not shown).

Discussion

The multifaceted immune response elicited with ISS-based immunization, as described in this paper, highlights the potential for harnessing the immunostimulatory properties of PAMPs to develop more effective HIV vaccines. ISS-based gp120 vaccines are shown to induce robust Ag-specific Ab, Th1 cytokine, and CTL responses. In addition, these investigations highlight the ability of
ISS-based vaccines to stimulate MHC class I- and II-restricted production of CCR5-specific β-chemokines such as MIP1α and MIP1β, which have been shown to protect against cellular invasion by HIV (33–38). Furthermore, these studies establish that ISS-based immunization elicits gp120-specific cytokine, chemokine, and CTL responses from CD8 T cells in CD4 T cell-deficient mice, suggesting that this vaccination strategy could have clinical utility even in CD4 T cell-depleted AIDS patients for whom CD8 T cells are likely to play a crucial role in controlling infection (38, 41–45).

The ability of i.n. ISS-based immunization to induce mucosal immunity, consistent with previously published data using other Ags (46–48), represents another attractive feature of this vaccination strategy. Mucosal immunity is important because HIV is transmitted primarily by mucosal routes and the presence of mucosal IgA in human vaginal secretions has been correlated with resistance to infection in commercial sex workers (49), although mucosal IgA did not provide mucosal protection in models of other viral infections (50, 51). Immunization i.n., especially with the gp120:ISS conjugate, also effectively elicits systemic immune responses, although certain immune parameters are not as robust as with i.d. vaccination. It remains to be seen whether delivery of an ISS-based HIV vaccine by mucosal routes alone is sufficient or whether a combination of systemic and mucosal immunizations is needed to optimize systemic and mucosal immunity.

Similar to previously described results with other Ags (12, 19, 27, 28), the present series of experiments demonstrates that i.d. gp120:ISS conjugate vaccination induces a stronger immune response than coadministration of an equivalent dose of gp120 with 40-fold higher amounts of unconjugated ISS and further establishes the improved immunogenicity of gp120:ISS conjugate for mucosal vaccination. The immunological potency of the gp120:ISS conjugate relative to gp120 mixed with ISS can best be explained by reviewing the known mechanisms of action of ISS. As previously discussed, ISS, like other PAMPs, exert their effects mainly on APCs, such as macrophages and dendritic cells, stimulating them to secrete cytokines and up-regulate Ag processing and costimulatory molecules. ISS-activated APCs are thus primed to instruct lymphocytes and efficiently promote adaptive immune responses (4, 8, 52, 53). The gp120:ISS conjugate physically colocalizes the Ag to these ISS-activated APCs, whereas without ISS conjugation only a fraction of ISS-activated APCs will encounter, process, and present the target Ag for the orchestration of the subsequent adaptive immune response. The present results, as well as previous work (12), suggest that for difficult-to-elicit immune responses, such as the induction of systemic immunity with mucosal immunization or the induction of immunity in the absence of CD4 T cells, gp120:ISS conjugate vaccination offers a distinct advantage over coadministration of gp120 and ISS.

Our previous investigations have shown that OVA:ISS conjugate elicits equivalent systemic CTL responses in CD4 knockout and wild-type mice (12). The present investigations expand on this observation, demonstrating that gp120:ISS conjugate vaccination elicits equivalent systemic and mucosal CD8 T cell effector responses in mice that have been depleted of CD4 T cells compared with nondepleted mice. These CD8 T cell effector responses include cytokine and chemokine secretion as well as CTL responses. The level of cytokines and chemokines secreted by CD8 T cells (Fig. 5) is less than that seen with CD4 T cell responses (Fig. 2), but this CD8 T cell response may be important, especially in states of CD4 depletion such as AIDS. ELISPOT investigations further establish that CD4 T cell depletion does not reduce the frequency of CD8 T cells that are generated in response to gp120:ISS vaccination. These results raise intriguing mechanistic questions about how ISS exert their profound effects on CD8 T cell immunity. The current model for CD8 T cell activation invokes the concept of licensing of APCs (54–56). In this model, CD40-CD40 ligand interaction between APCs and CD4 T cells results in up-regulation of costimulatory molecules and other factors on APCs, which license them to subsequently interact with and activate CD8 T cells. ISS appear to replace the licensing function of CD4 T cells (12), presumably by initiating similar downstream signaling events in APCs (57, 58).

The present studies demonstrate that ISS-based immunization induces CTL activity and other class I-restricted T cell responses against exogenous, soluble Ags. In addition to licensing APCs to become more potent activators of CD8 T cells, ISS appear to allow cross-presentation of Ag, which involves rerouting of phagocytosed, exogenous protein from the MHC class II pathway to the MHC class I pathway. The means by which ISS-mediated cross-presentation occurs have only begun to be elucidated. ISS have been shown to up-regulate expression of MHC class I molecules, as well as other components of the class I Ag processing pathway, such as TAP. Furthermore, studies in TAP knockout mice have demonstrated that induction of CTL activity by ISS-based vaccines...
is dependent on TAP (H. J. Cho and E. Raz, manuscript in preparation). However, the exact mechanisms by which ISS shunt exogenous proteins into the class I Ag processing pathway after phagocytosis require further investigation.

Cytokines induced by ISS, some of which are known to be involved in the Th1-biased immune response seen with ISS-based immunization, probably also play a role in the initiation and maintenance of the CD8 T cell response. For example, IL-15, which is induced by ISS in a type I IFN dependent manner (K. Takabayashi and E. Raz, unpublished observations) has been recently implicated in the proliferation of activated CD8 memory T cells (59, 60). Therefore, in addition to licensing APCs and allowing cross-presentation of Ag, ISS may create an appropriate cytokine milieu for the induction of vigorous CD8 T cell immunity.

The potency of ISS-based immunization demonstrated in this report validates the concept of using PAMPs (i.e., activators of innate immunity) in conjunction with Ags (i.e., activators of adaptive immunity) to elicit robust immune responses. Reports of enhanced immunity to certain microbial Ags delivered with PAMPs derived from other pathogens (61, 62) confirm the viability of this approach for future vaccine development. These studies further demonstrate the potential for harnessing the innate immunostimulatory properties of PAMPs from one pathogen to improve immune responses to Ags from unrelated pathogens.

With respect to the design of vaccines against HIV, our unpublished studies have demonstrated that the HIV genome has only approximately 15% of the ISS predicted by chance, with a genome frequency of 1590 ISS-BASED HIV VACCINE

References


