Schistosoma mansoni Soluble Egg Antigens Enhance T Cell Responses to a Newly Identified HIV-1 Gag H-2b Epitope

Cac T. Bui,a,b Lisa M. Shollenberger,a,b Yvonne Paterson,c Donald A. Harr,a,b

Department of Infectious Diseasesa and Center for Tropical and Emerging Global Diseases,b University of Georgia, Athens, Georgia, USA; Department of Microbiology, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Schistosomiasis is a helminthic parasitic disease that affects >200 million people worldwide and is listed by the World Health Organization (WHO) as the second leading parasitic disease, after malaria (14). Infection occurs when cercariae that emerge from infected snail intermediate hosts contact and penetrate the skin of the vertebrate host. Larval parasites migrate and mature into adult male and female worms that mate and produce eggs (4, 6). Eggs that become lodged within host tissues are largely responsible for Th2 biasing of the host immune system and induction of anti-inflammatory responses (3, 4, 6, 15, 16).

Immune biasing induced by schistosome infection has been shown to reduce vaccine efficacy in both laboratory and clinical settings (8, 11, 17, 18). Specifically, helminth infection has been shown to suppress immune responses to a Th1-type vaccine and impair the expansion of pathogen-specific cytotoxic CD8+ T cell (cytotoxic T lymphocyte [CTL]) responses (18–20). Furthermore, Da’Dara et al. demonstrated that mice infected with Schistosoma mansoni were unable to mount significant HIV-1 vaccine-specific T cell responses to a plasmid DNA HIV-1 vaccine, even when the vaccine was enhanced (11). Taken together with data from a study by Actor et al. showing suppression of virus-specific immune responses in schistosome-infected mice (7) and a study by Sabin et al. showing suppression of tetanus-specific responses in schistosome-infected children (8), helminth infection may pose a significant problem for the development of virus-specific CTL and Th1-type HIV-1 vaccines (7, 8, 11). One method to overcome helminth-induced vaccine suppression is to identify vaccine vectors that are capable of producing vaccine-specific responses irrespective of helminth infection. In this regard, a Listeria vector HIV vaccine was recently shown to induce potent CD8+ and Th1-type vaccine responses in schistosome-infected mice (12, 13).

Schistosome soluble egg antigens (SEA) are potent inducers of CD4+ Th2-type biasing (4, 6, 9, 21), and in a previous study, we demonstrated that SEA coadministered with a third-party antigen was sufficient to induce vaccine-specific Th2-type cytokine responses (9). Here, we evaluated the influence of SEA on the ability of a Listeria vector HIV-1 vaccine to drive Th1-type and CTL responses in Th1-type C57BL/6 mice. Our first finding was that a known class I epitope for HIV-1 IIIB Gag in C57BL/6 mice, QEVK/H11001, failed to elicit responses. Therefore, we embarked on an epitope mapping study to identify minimal HIV-1 Gag epitopes and successfully identified both minimal class I H-2b and class II I-Aa epitopes for HIV-1 IIIB Gag, and we used these epitopes to
perform our evaluation of the influence of SEA on *Listeria* vector HIV IIIB Gag-induced T cell responses in mice. Consistent with our previous study demonstrating that SEA coadministration increases CTL and Th1 vaccine responses in BALB/c mice (24), we similarly found here that SEA coadministration with the *Listeria* vector HIV vaccine significantly increased gamma interferon (IFN-γ)-producing CTL effector frequency in C57BL/6 mice.

**MATERIALS AND METHODS**

**Biological reagents.** The HIV vaccine, an attenuated strain of *Listeria monocytogenes* expressing the HIV-1 IIIB Gag protein (Lm-Gag) (25), and the control strain, which expresses the E7 oncoprotein of human papillomavirus 16 (Lm-E7) (26), were grown in brain heart infusion (BHI) broth supplemented with streptomycin. *Schistosoma mansoni* (PR strain)-infected Biomphalaria glabrata snails and mice were provided by the NIAID schistosomiasis resource center. Infectious cercariae were obtained by exposing infected snails to direct light. Five- to seven-week-old female C57BL/6 and BALB/c mice were purchased from Harlan or Jackson Laboratories, housed under specific pathogen-free conditions, and allowed to acclimate for 1 week prior to manipulation. All animal work was performed in accordance with institutional policies and approved by the institutional animal care and use committee at the University of Georgia.

**Peptides.** The HIV-1 consensus B Gag (15-mer, 11-amino-acid overlap) peptide library was provided by the NIH AIDS Reference and Research Reagent Program. All other peptides, including additional 15-mer peptides to complement the differences in our vaccine sequence, were synthesized by Biosynthesis, Inc., at >95% purity. All peptides were reconstituted in dimethyl sulfoxide (DMSO) and stored at −20 °C. A list of all the peptides used in this study is provided in Table S1 in the supplemental material.

**Preparation of SEA.** *Schistosoma mansoni* (PR strain)-infected Swiss Webster mice were provided by the NIAID schistosomiasis resource center. Additional female BALB/c mice were infected in the laboratory by intraperitoneal (i.p.) injection of 100 to 150 infectious cercariae of *Schistosoma mansoni*. Seven to eight weeks after infection, parasite eggs were isolated from the livers of infected mice. Eggs isolated from Swiss Webster and BALB/c mice were combined for the production of SEA, as described previously (3, 4, 6, 9).

**Vaccination of mice.** For epitope mapping studies, 6- to 8-week-old female C57BL/6 or BALB/c mice were primed i.p. with 0.2% 50% lethal doses (LD₅₀) (1 × 10³ CFU) of the Lm-Gag vaccine or were left unvaccinated. Mice were boosted 2 weeks after the prime in an identical manner. To study the effect of SEA on vaccine responses, mice were injected i.p. with 30 µg of SEA or left naive. One week after the injection of SEA, mice were primed intravenously (i.v.) with 1 × 10⁸ CFU Lm-Gag vaccine with or without a 30-µg SEA i.p. boost or with the control Lm-E7 vaccine (matched CFU dose) or were left unvaccinated. Mice were boosted 2 weeks after the prime in an identical manner. Mice were sacrificed at 2 weeks post-last vaccination (wplv).

**ELISpot assays.** Splenocytes were harvested at 2 wplv and plated at 150,000, 300,000, or 500,000 cells per well in IFN-γ enzyme-linked immunosorbent spot (ELISpot) plates (BD) in complete medium (RPMI 1640 supplemented with 10% fetal bovine serum [FBS], 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B, 2 mM L-glutamine, 5 µM β-mercaptoethanol, and nonessential amino acids). Cells were stimulated with 20 µM peptide or 1 µg/ml concanavalin A (ConA) (as a positive control) or left unstimulated. After a 20-h incubation, ELISpot assays were performed according to the manufacturer’s instructions, and the spots were enumerated by using an ImmunoSpot analyzer (Cellular Technology Limited).

**T cell purification.** CD8⁺ and CD4⁺ T cells were purified from splenocytes by positive selection using CD8a (Ly-2) and CD4 (L3T4) MicroBeads (Miltenyi Biotec), respectively, according to the manufacturer’s instructions. Purified CD8⁺ and CD4⁺ T cells were fluorescently labeled with anti-CD8ε, anti-CD8α, and anti-CD4 antibodies (BD) and analyzed for purity by flow cytometry. Cell debris was excluded from the analysis based on scatter signals, and the resulting purity was ≥90%, as shown in Fig. S1 in the supplemental material.

**Statistical analysis.** Statistical analyses were performed by using one-way analysis of variance (ANOVA) with a Newman-Keuls or Tukey post hoc test, as detailed in the figure legends (Prism; Graphpad Software, La Jolla, CA).

**RESULTS**

**T cells from vaccinated mice do not respond to the previously reported class I H-2b epitope QEVK.** To validate the vaccine responses in C57BL/6 mice, we vaccinated C57BL/6 mice with Lm-Gag in a prime-boost regimen, 2 weeks apart. At 2 wplv, splenocytes were harvested and analyzed for immune responses by IFN-γ ELISpot assays against the reported H-2b-restricted class I QEVK (22, 23) and class II VHQA (28) epitopes of HIV-1 Gag (Table 1). Stimulation of splenocytes from unvaccinated mice with either peptide did not produce any spots. Stimulation of cells from vaccinated C57BL/6 mice with the VHQA helper peptide generated significant responses compared to the responses of cells from unvaccinated controls (Fig. 1). However, the previously re-

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**TABLE 1** List of abbreviated HIV-1 Gag epitopes in order of discussion

| Epitope     | MHC class, haplotype | Peptide sequence          | Reference(s) |
|-------------|----------------------|---------------------------|--------------|
| QEVK        | I, H-2db             | QEVKNWMTETL               | 22, 23       |
| VHQA (37)   | II, I-Aβ             | VHQAISPRTLN4WV3KVEEK      | 28           |
| CRS-9       | I, H-2b              | CRSLYNVTVA                |              |
| RSLY-9      | I, H-2b              | RSLYNVT4                  |              |
| SLYN-9      | I, H-2b              | SLYNVT4                   | 34, 35       |
| RSLY-8      | I, H-2b              | SLYNVT4                   |              |
| SLYN-8      | I, H-2b              | SLYNVT4                   |              |
| QAIS-11     | II, I-Aβ             | QAISPRTLN4AW             |              |
| AISP-11     | II, I-Aβ             | AISPRTLNW4V               |              |
| AISP-10     | II, I-Aβ             | AISPRTLNW4V               |              |
| AMQM-1      | I, H-2d              | AMQM4LKTET               | 28, 39       |
| NPII        | II, I-Aε             | NPIPPV4GIEYKRWIIGLNK     | 28           |
| EAMS (100)  | I, H-2b              | EAMSQVTNSATIMMQ          | 29–31        |
| KV9         | I, H-2db             | KSLYNVT4VC               | 22           |

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**FIG 1** Lm-Gag-vaccinated C57BL/6 mice do not respond to the previously reported class I HIV Gag peptide. Splenocytes from naive and Lm-Gag-vaccinated mice were harvested at 2 wplv, and immune responses to class I (QEVK) and class II VHQA (28) epitopes of HIV-1 Gag were analyzed by IFN-γ ELISpot assays. Results from individual mice (n = 4) were plotted. Statistical analysis was performed by using one-way ANOVA with a Tukey post hoc test (*, P < 0.05; ns, not significant). SFU, spot-forming units.
ported class I H-2\textsuperscript{b} epitope QEVK failed to induce responses in vaccinated C57BL/6 mice (Fig. 1). Due to our inability to stimulate splenocytes with the known class I epitope, we initiated an epitope mapping study to define the class I H-2\textsuperscript{b} minimal epitope so that we could proceed with our evaluation of the influence of SEA on the Lm-Gag vaccine.

HIV-1 Gag peptide library screening identifies 8 stimulatory peptides. The list of peptides tested in our initial screen included the HIV-1 consensus B Gag peptide library (15-mer, 11-amino-acid overlap), additional synthesized 15-mer peptides to match our vaccine construct, and several control peptides (see Table S1 in the supplemental material). Total splenocytes from vaccinated C57BL/6 mice were stimulated in the presence of peptides or left unstimulated for 20 h, and Gag-specific responses were then measured. Figure 2A shows that of 143 different peptides tested, 8 were found to induce strong responses from splenocytes of HIV IIIB Gag-vaccinated C57BL/6 mice. These stimulating peptides included seven 15-mer peptides (peptides 19, 20, 36, 38, 80, 81, and 82) and the 21-mer positive-control VHQA epitope (peptide 37).

All stimulating peptides were further examined to define their major histocompatibility complex (MHC) restriction. Enriched CD8\textsuperscript{+} T cells were positively selected from pooled splenocytes, with the remaining cells representing CD8\textsuperscript{+}-depleted splenocytes. Both cell fractions were stimulated with 15-mer peptides or left unstimulated and then analyzed by IFN-\gamma ELISpot assays. As shown in Fig. 2B, among the seven 15-mer stimulatory peptides, peptides 19 and 20 stimulated enriched CD8\textsuperscript{+} T cells, suggesting that the class I-restricted epitope is within this region. Peptides 36 and 38 stimulated significant responses from the CD8\textsuperscript{+}-depleted cell fraction (Fig. 2C). This result was expected, as these two peptides overlap the previously identified class II-restricted VHQA epitope of HIV-1 Gag (peptide 37; positive control). Peptides 41 and 70, which were added as internal negative controls, did not induce responses from either enriched CD8\textsuperscript{+} T cells or CD8\textsuperscript{+}-depleted splenocytes (Fig. 2B and C).

Identification of the class I H-2\textsuperscript{b} minimal epitope. Next, we identified the class I H-2\textsuperscript{b} minimal epitope. Nine 9-mer peptides surrounding the overlap region of peptides 19 and 20 were synthesized. We then stimulated total splenocytes, enriched CD8\textsuperscript{+} T cell populations, and enriched CD4\textsuperscript{+} T cell populations with these peptides. Stimulation of cells with peptides 18 and 21 (flanking sequences; negative controls) did not induce any responses from any of the cell populations (Fig. 3A). Peptides 19 and 20 (positive controls) induced significant vaccine responses from total splenocytes, with the level of CD4\textsuperscript{+} T cell responses being higher than the level of CD8\textsuperscript{+} T cell responses. These data suggest that the total vaccine responses against these two 15-mer peptides were primarily from enriched CD4\textsuperscript{+} T cells. Interestingly, responses from the enriched CD4\textsuperscript{+} T cells were significantly abrogated upon stimulation with 9-mer peptides within this region (Fig. 3A). Among the three 9-mer peptides inducing significant responses from total splenocytes and CD8\textsuperscript{+}-enriched T cells, the CRSL-9 and RSLY-9 peptides induced significantly higher-level responses than did SLYN-9 (Fig. 3A). Therefore, we had minimal (8-mer) peptides within this region synthesized and tested them for their ability to induce IFN-\gamma from total splenocytes, enriched CD8\textsuperscript{+} cells, and enriched CD4\textsuperscript{+} cells. Both RSLY-8 and SLYN-8 induced significant responses from total splenocytes, with RSLY-8 driving the highest-level responses from CD8\textsuperscript{+}-enriched T cells (Fig. 3B). Thus, the class I H-2\textsuperscript{b} minimal epitope is the 8-mer peptide RSLY.

FIG 2 Peptide library screening for C57BL/6 class I epitopes. (A) Splenocytes from Lm-Gag-vaccinated mice were harvested at 2 wplv, pooled, and screened for a response against the HIV-1 Gag peptide library (15-mer, 11-amino-acid overlap) and additional peptides by IFN-\gamma ELISpot assays. (B and C) CD8\textsuperscript{+}-enriched T cells (B) and CD8\textsuperscript{+}-depleted splenocytes (C) isolated from vaccinated animals were stimulated with the eight 15-mer stimulatory peptides and analyzed for a response. Splenocytes were pooled from 8 mice and stimulated with peptides in either duplicate (A) or quadruplicate (B and C) wells. Statistical analysis was performed by using one-way ANOVA with a Tukey post hoc test (*, P < 0.05; **, P < 0.01; ***, P < 0.001 [compared to groups without asterisks]).
NTVA (RSLY-8). This minimal peptide was H-2b restricted and not cross-reactive in BALB/c (H-2d) mice (Fig. 3C). However, the response of C57BL/6 splenocytes to this epitope was much lower than the response of BALB/c splenocytes to their cognate H-2d epitope (AMQM) (Fig. 3C).

Response to the class I H-2b minimal epitope was significantly increased by SEA coadministration with the Lm-Gag vaccine. Having identified the minimal class I H-2b epitope, we next evaluated the influence of SEA on the Lm-Gag vaccine in C57BL/6 mice. Four animal groups were immunized and analyzed for vaccine responses, including naive, Lm-E7-vaccinated, Lm-Gag-vaccinated, and SEA- and Lm-Gag-coadministered (SEA/Lm-Gag) groups. At 2 wplv, an IFN-γ ELISPOT assay was performed to measure Gag-specific responses against the newly identified class I RS LNYNTVA (RSLY-8) minimal epitope of HIV-1 Gag. Splenocytes from negative-control groups (naive and Lm-E7-vaccinated mice) did not respond to the RSLY-8 peptide, demonstrating that immunization with the vector does not induce a Gag-specific response (Fig. 3D). Splenocytes from both Lm-Gag- and SEA/Lm-Gag-vaccinated groups responded to the RSLY-8 epitope, with the response of splenocytes from the SEA/Lm-Gag-vaccinated group being significantly greater (Fig. 3D). This finding demonstrates that coadministration of SEA with the Lm-Gag vaccine increases vaccine-specific CTL effector cells in TH1-biased C57BL/6 mice.

Identification of the class II I-Ab minimal epitope. We defined the class II I-Ab minimal epitope for HIV-1 Gag from the amino acid sequence of peptides 36 and 38. Six 11-mer peptides surrounding the overlap region were synthesized and were evaluated for their ability to stimulate total splenocyte, enriched CD8+ T cell, and enriched CD4+ T cell populations from Lm-Gag-vaccinated mice at 2 wplv and analyzed for a response against 9-mer peptides within the 11-amino-acid overlapping region (A) and then against minimal 8-mer peptides within the overlapping region of most stimulatory peptides (B). The identified class I H-2b minimal peptide was tested for cross-reactivity against the class I H-2b peptide in both mouse strains C57BL/6 and BALB/c. (C) Validation of SEA adjuvant activity in C57BL/6 mice by evaluating the vaccine response against the class I epitope using IFN-γ ELISPOT assays. For panels A and D, data were pooled from two independent experiments, and results from pooled splenocytes (A) (n = 7) or individual mice (D) (n = 10) were plotted. Statistical analysis was performed by using one-way ANOVA with a Newman-Keuls post hoc test (*, P < 0.05; **, P < 0.01; ***, P < 0.001 [as indicated or compared to groups without asterisks, within the same population]). For panels B and C, splenocytes were pooled from 12 mice and stimulated with peptides in duplicate wells.

Lastly, we evaluated the ability of SEA to enhance Lm-Gag vaccine-specific responses against the class II AISP-10 minimal epitope. The same animal groups as those described above were immunized and analyzed by IFN-γ ELISPOT assays. Unlike what we observed for the MHC class I response, here we found that splenocytes from Lm-Gag-vaccinated mice had significant T cell
responses to the AISP-10 minimal epitope, and these responses were not enhanced in splenocytes from SEA/Lm-Gag-vaccinated mice (Fig. 4D).

**DISCUSSION**

We initiated this study to determine if SEA, the potent, Th2-biasing saline extract of schistosome eggs, would influence vaccine-specific responses generated by a Th1-type *Listeria* vector HIV-1 Gag vaccine in C57BL/6 mice. In our initial experiments, we were unable to detect vaccine responses against the reported class I epitope (QEVK) of the HIV-1 Gag protein (Fig. 1). The QEVK peptide was previously described as an HIV-1 Gag class I epitope (22, 23). However, this peptide failed to induce class I epitope recall responses in our system. Whether this lack of response is caused by altered expression of the protein by the vector or an aberrant processing response by the host, it necessitated the search for a peptide that could be used to monitor vaccine-specific responses in this system. Therefore, we initiated an epitope mapping study to define the minimal class I H-2b epitope for the Gag protein.

Screening of 143 peptides allowed us to define eight stimulating peptides (Fig. 2A), one of which is the 21-mer positive-control VHQA epitope (peptide 37). Interestingly, the EAMS peptide (peptide 100) was not among the seven 15-mer stimulating epitopes. The EAMS peptide was recently updated in the HIV database and used to analyze Gag-specific CTL responses of C57BL/6 mice against viral vector vaccines (29–31). In these studies, the EAMS peptide sequence did not overlap the viral vector used, suggesting that the induced responses were HIV-1 Gag specific. However, in our study, Lm-Gag-vaccinated mice did not generate any T cells specific for the EAMS peptide (Fig. 2A). The contrast between our results and those of the previous study may be due to significant differences in vectors, viral versus bacterial, which may have influenced antigen presentation and/or antigen restriction and therefore altered the immunodominance of the MHC class I H-2b Gag epitopes. In fact, previous evaluations of a *Listeria* vector expressing the HER-2 antigen suggested that the *Listeria* vaccine vector revealed more epitopes than other vectors (32, 33). In our study, the 15-mer peptides that induced responses in cells from Lm-Gag-vaccinated mice were peptides 19, 20, 36, 38, 80, 81, and 82.

We further evaluated MHC restriction of the seven 15-mer responding peptides by measuring vaccine-specific responses of CD8+ /H11001-enriched and -depleted cells. Bead-based purification of CD8+ /H11001 T cells typically results in 90 to 95% purity (see Fig. S1 in the supplemental material). Thus, there is the possibility of low-level responses from other cell types upon peptide stimulation. However, as shown in Fig. 2B, peptides 19 and 20 induced the highest levels of stimulation of CD8+ /H11001 T cells, suggesting that the MHC class I-restricted epitope is within this region. In addition, CD8+ /H11001-depleted cells responded to several of the 15-mer peptides (Fig. 2C). Here, we noted that the amino acid sequence of the most
stimulatory peptides, peptides 36 and 38, overlapped the class II-restricted VHQCA epitope.

Further screening of shorter peptides successfully identified the two minimal epitopes for MHC class I (RSLY-10) in C57BL/6 mice (Fig. 3 and 4). There is precedent for epitope RSLY-8, as its amino acid sequence is similar to that of the previously defined H-2D\(^b\) K\(\beta\)9 Gag epitope (KSLYNVTVCV) of simian immunodeficiency virus (SIV) (22). RSLY-8 is also a single-amino-acid shift from a well-known human HLA-A*0201-restricted 9-mer epitope, Gag\(_{27-35}\) (SLYNVTATL [SLYN-9]), which was shown previously to be highly reactive in chronically infected patients (34, 35).

Although the panels within Fig. 3 and 4 show different levels of response to the minimal epitope, we note a change in animal vendors between experiments. Nonetheless, our data suggest that RSLY-8 and AISP-10 are indeed the HIV-1 Gag class I and class II minimal epitopes, as they induced the highest levels of CTL effectors and Th1 cells in vaccinated mice, respectively, and are not cross-reactive with BALB/c (H-2\(^d\))- and I-A/E\(^d\)-restricted) epitopes (Fig. 3B and 4B). Overall, the recall responses to peptides in C57BL/6 mice were much lower than those in BALB/c mice (Fig. 3C and 4C), which is not surprising considering that they are genetically disparate mouse strains (36–38).

The MHC restriction of the epitopes defined here is based on selective binding to purified populations of CD4\(^+\) and CD8\(^+\) T cells from 2 inbred mouse strains. Further studies using MHC blocking, tetramer binding, surface plasmon resonance, and transgenic mice can be used to specifically define the MHC restriction.

Evaluation of the influence of SEA on the generation of CD4\(^+\) and CD8\(^+\) T cell responses of Lm-Gag-vaccinated C57BL/6 mice demonstrated that similar to our recent study (24), the administration of SEA with Lm-Gag significantly increased the level of Gag-specific CTL effectors compared to those in mice vaccinated with Lm-Gag alone (Fig. 3D). However, the level of CD4\(^+\) Th1 responses was not influenced by the addition of SEA (Fig. 4D). Thus, our study suggests that for the Lm-Gag vaccine, SEA acts as a CTL adjuvant in C57BL/6 mice, more prone to Th1-type responses, and in BALB/c mice, more prone to Th2-type responses (Fig. 3) (24). We are currently examining if the observed enhancement is simply due to the host immune response to any foreign antigen prior to vaccination with Lm-Gag or is an SEA-specific observation. In addition, we are evaluating other vaccine delivery systems to validate the generality of the SEA adjuvant effect observed for Listeria-delivered Gag.

Together, the results presented here and in our recent study (24) show that SEA functioned counterintuitively when administered with the Lm-Gag vaccine, stimulating CTL responses rather than suppressing them. Although it is well known that Listeria is a potent inducer of CD8\(^+\) immunity, the specific mechanism(s) by which Listeria vector vaccines can both overcome the immunosuppressive environment of chronic helminth infection (12, 13) as well as be adjuvanted by SEA is currently being investigated.

In addition to validating this adjuvant effect in other systems and understanding how Listeria contributes to this effect, we are currently biochemically fractionating SEA in an attempt to define the class(es) of molecules that is responsible for the CTL and Th1 adjuvant effects of SEA when combined with the Lm-Gag vaccine. This may lead to the identification of new Th1 and/or CTL adjuvants that can then be evaluated for adjuvant activity with other vaccines.

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