EFFECT OF SCHISTOSOME EGG ANTIGENS ON A

LISTERIA VECTOR HIV-1 VACCINE

by

CAC THANH BUI

(Under the Direction of Donald Harn)

ABSTRACT

Vaccines are an important public health measure for prevention and treatment of diseases. In addition to the vaccine immunogen, many vaccines incorporate adjuvants, to stimulate the recipient's immune system and enhance vaccine-specific responses. While vaccine development has advanced from attenuated organism to recombinant protein or use of plasmid DNA, the development of new adjuvants that safely increase immune responses has not kept pace. Previous studies have shown the complex mixture of molecules that comprise saline soluble egg antigens (SEA) from *Schistosoma mansoni* eggs functions to promote $CD4^+$ T helper 2 (Th2) responses. Therefore, we hypothesized that co-administration of SEA with a *Listeria* vector HIV-1 Gag (Lm-Gag) vaccine would suppress host cytotoxic T lymphocyte (CTL) and T helper 1 (Th1) responses to HIV-1 Gag epitopes. Surprisingly, instead of driving HIV-1 Gag-specific responses towards Th2-type, we found that co-administration of SEA with Lm-Gag vaccine significantly increased the frequency of IFNγ producing Gag-specific Th1 and CTL responses over that seen in mice administered Lm-Gag only. Analysis on the functionality and durability of vaccine responses suggested that SEA not only enlarged

different memory T cell compartments but induced functional and long-lasting vaccine-specific responses as well. These results suggest there are components in SEA that can synergize with potent inducers of strong and durable Th1-type responses such as *Listeria*. We hypothesize SEA contains moietie(s) that if defined, can be used to expand type 1, pro-inflammatory responses for use in vaccines.

INDEX WORDS: *Schistosoma mansoni*; soluble egg antigens; adjuvant; vaccine efficacy; HIV vaccine; *Listeria monocytogenes*

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DEDICATION

This dissertation is lovingly dedicated in memory of my grandmother, Ngoc Quyen, an exceptionally wise woman who inspired my life through her strength, courage and boundless love for her family.

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Most of all, the Lord my God for these sustaining words:

"Trust in the Lord with all your heart,

and do not lean on your own understanding,

In all your ways acknowledge Him,

and he will make straight your paths." ~ Proverbs 3:5-6

May the blessings You have been given me throughout this PhD be engraved in my heart always as I continue to walk in faith and follow Your calling.

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CHAPTER 1

INTRODUCTION

I. Vaccines and problems in vaccine research and development

1. Vaccines

Since the first written account of vaccination by Edward Jenner, vaccine development has played an important role in prevention and treatment of diseases caused by infectious agents. Vaccines induce effective pathogen-specific immune responses that lead to protection against infection and or diseases caused by that pathogen. Vaccines have dramatically decreased the mortality of many diseases, including measles, mumps, rubella, hepatitis B, chicken pox, diphtheria, tetanus, pertussis, pneumococcal infection and influenza (1-4). The smallpox vaccine ultimately resulted in eradication of the disease (5-7).

2. Problems in vaccine research and development

i) Problem of complex pathogens

Although vaccine design and development research continues to progress with advanced methodologies for selection of candidate antigens and new technologies for vaccine delivery there are still no fully effective vaccines against many widespread infectious diseases, including HIV-AIDS, malaria, and tuberculosis. These complex pathogens pose difficult problems to vaccine research and development.

The DALYs (Disability-Adjusted Life Year) for HIV, malaria and tuberculosis are daunting. For malaria more than 200 million cases occur each year (8, 9) with an estimated 660,000 deaths worldwide in 2012 (8). For HIV-1, the CDC reported more than 35 million people living with HIV infection in 2012, with an estimated 2.3 million new cases each year and 1.8 million deaths due to AIDS (10, 11). The *Plasmodium* parasite and HIV-1 virus are both complex organisms that evolve through different genetic and immunological stages (12-18). The complexity of the infection/life cycles of these two pathogens makes it difficult to determine when during the pathogen's life cycle to intervene, and how to target vaccine delivery such that protective immune responses are induced (12-18).

When examining immune correlates of protection humoral immunity has been shown to play a role in preventing infection by HIV and can influence certain stages of malaria infection (19-21). However, there is compelling evidence that Th1 cells, $CD8^+$ T cells, or both, also have a critical role in preventing or controlling these infections (22-31). Thus, for malaria, HIV, likely other infectious diseases, as well as for neoplasia, it may be necessary for vaccines to generate potent and perhaps multifunctional T cell responses (32-37).

ii) Problem of target populations

The human immune system matures during early childhood and then begins to senesce around 60 years of age. Therefore, another problem for vaccinology is how to immunize neonates, young children and the elderly. Children younger than 6 months old and preterm infants have immature immune responses (38-42). Similarly, adults over 65

years of age may have an aging immune system that could result in defects in immune response to vaccinations (43-47).

Producing effective vaccines for immune-compromised individuals and /or chronically ill individuals poses similar problems. The immune system can be compromised or suppressed by an infection, or in patients on immune suppressive therapies. In both cases, individuals may not be able to mount effective vaccine responses (48-51). In addition, helminth infections, found in a large percentage of humans, may also suppress immune responses to vaccines (52-54). The unique challenge helminth infection poses for vaccine development will be discussed further in section II.

iii) Adjuvants

a. A lack of FDA approved adjuvants

The word "adjuvant" comes from the Latin word *adiuvare*, which means to aid or help. In immunology, an adjuvant is a substance that acts to stimulate the immune response toward an antigen (55-57). Thus, an adjuvant is often incorporated into a vaccine to accelerate, prolong or enhance vaccine-specific immune responses, in other words, increase vaccine efficacy (55-57).

Adjuvants are used to promote types of immunity not effectively generated by the non-adjuvanted vaccine (58, 59). For example, addition of an adjuvant to a vaccine may be done to enhance mean antibody titers against specific pathogens (58, 59). Adjuvants are also considered to improve seroconversion rates in infants and the elderly. For example, MF59 adjuvant is incorporated into an influenza vaccine to enhance the response of older individuals (60, 61). In addition, incorporation of an adjuvant may

allow for dose-sparing, which means that comparable vaccine responses can be induced using substantially lower amounts of antigen. Dose-sparing is a particularly important consideration for urgent, large-scale vaccinations, where vaccine production is limited. For example, in the emergence of a pandemic influenza strain, it is likely that sufficient vaccine may not be produced rapidly enough to prevent significant morbidity and mortality. Incorporation of an effective adjuvant that allows the use of lower doses of influenza antigen would provide more vaccine doses and thus greater vaccine coverage (62-64). Similarly, incorporation of an adjuvant may reduce the vaccine regimen. The requirement of multiple doses of many vaccines presents not only vaccine supply and compliance issues, in much of the world it is also a significant logistical challenge. Hence, the development of new, safe adjuvants for new generation vaccines is essential and beneficial.

b. Mechanism of action by known adjuvants

Depending on the type or molecular makeup of an adjuvant, the recipient immune system will be activated by various mechanisms. According to the Centers for Disease Control (CDC), aluminum gels or aluminum salts are the only vaccine adjuvants currently licensed for human use in the United States (65). Alums are the most widely studied and clinically used adjuvants and are particulate in nature (66). Alums consist of precipitates of aluminum phosphate and or aluminum hydroxide to which vaccine antigens are adsorbed (67, 68). Alum is used primarily to enhance humoral immunity by increasing antibody production *in vivo* (69). At least three different mechanisms have been proposed to explain the adjuvant activity of aluminum salts. First, post-injection

alum-antigen mixtures generate depots that maintain vaccine antigen(s) at the injection site. The depot is thought to provide slow release of vaccine antigens, so called antigen persistence, to continuously stimulate the immune system (70-72). Second, induction of inflammation, in part via NLRP3 (NOD-like receptor family, pyrin domain containing 3), by alum and antigen(s) mixtures, increases recruitment and activation of antigen presenting cells (APCs) (70-76). The formation of the inflammasome induces production of the pro-inflammatory cytokines IL-1β and IL-18, which establishes an optimal environment for the priming, expansion and polarization of the immune responses (73, 75). However, this hypothesis was challenged by a separate study which showed that alum mediated adjuvant activity was maintained in NALP3 (protein encoded by NLRP3) deficient mice (77). Alum can also trigger necrotic cell death and the release of the endogenous danger signal uric acid (68, 78). The third potential mechanism by which particulate adjuvants like alum function is based on the ability of aluminum salts to bind/adsorb vaccine antigen(s). Alum binding of vaccine antigen(s) leads to formation of multi-molecular aggregates that facilitate uptake by APCs such as macrophages, DCs, and B cells (70-72).

Alum has been added to vaccines to increase antibody production. The ability of Alum to enhance antibody responses has been linked to increased proliferative responses of $CD4^+$ T cells and Th2 cytokine production in both murine and human studies, suggesting that alum boosts humoral immunity by providing Th2 cell help to follicular B cells (70, 79-82). In contrast to enhancing antibody responses, $CD8^+$ CTL responses to a range of polypeptide and protein antigens are poorly induced by alum, possibly because of the lack of cross-presentation of antigens when alum is used (66, 83-85).

Other adjuvants, such as oil-in-water emulsions are considered promising though their mode of action remains unclear. MF59, which was microfluidized (MF) squalene droplets, was licensed for use with human influenza vaccine in Europe (86, 87). Mosca et al. demonstrated that similar to alum, MF59 promoted rapid recruitment of inflammatory cells, including granulocytes monocytes and macrophages to the injection site (88, 89). MF59 also led to an enhanced inflammatory response and upregulated expression of adhesion molecules, establishing a localized immunostimulatory environment (88, 89). In one study, use of MF59 was shown to increase uptake of autologous antigens by DC leading to DC activation and then migration to draining lymph nodes, augmenting B cell responses and increased production of antibodies (90). Antibodies elicited by MF59-adjuvanted influenza HA were shown to have greater avidity than antibodies from recipients immunized with HA alone (91).

A new class of vaccine adjuvants based on targeting the TLR pathways is also emerging as an important element in the design of effective and safe vaccine adjuvants. The concept behind TLR-based adjuvants arises from reports demonstrating adjuvanticity by different TLR agonists (92). Emphasizing the potential and acceptance of these TLR agonists as vaccine adjuvants, two TLR4 agonists are currently approved by the Food and Drug Administration to be incorporated into vaccines for clinical research. TLR4 agonist (3-O-desacyl-4'-monophosphoryl lipid A/MPL) absorbed to alum, known as AS04, is currently approved for use against human papilloma virus (93) and hepatitis B (94). AS04 adjuvanted Ceravix vaccine produced significantly higher neutralizing antibody titers for HPV-16 and increased level of memory B cells at 1 month post last injection (95). Second, synthetic TLR4 agonist Glucopyranosyl Lipid Adjuvant (GLA) absorbed in

stable oil-in-water emulsion (GLA-SE) was also recently approved for use in influenza vaccine (96). GLA-SE adjuvanted vaccine was shown to protect mice and ferrets against a high titer challenge with a H5N1 virus and broaden protective immunity against heterosubtypic viruses (97).

In general, each TLR agonist activates a different innate immune pathway through its specific receptor. The adjuvant AS04, for example, is known to locally activate NF-kB pathway and promote cytokine production, thus providing an optimal environment for APC activation and presentation (98). However, the mechanism of action when these TLR-based adjuvants are incorporated into vaccines remains largely unknown.

c. The need for better adjuvants

Discovery and development of new, safe and effective adjuvants for use in human vaccines is a challenge and a necessity. Due to the reactogenic nature of most adjuvants described to date, the vaccines that incorporate adjuvants have generally used aluminum salts or oil-in-water emulsions. However, for diseases for which effective vaccines have yet to be generated, new adjuvants that drive specific, well-defined immune responses must be identified. CpGs and the new GLA adjuvants fall into this category, by driving TLR9 and TLR4 activation (94-97, 99).

The lack of new, safe adjuvants has limited the potency and efficacy of many existing and new generation vaccines. Though alum has been safely used in childhood vaccines, the current controversies concerning the mechanism of action of alum adjuvants and potential *in vivo* toxicity (68, 78) emphasize the need for better adjuvants.

II. Helminth infection reduces vaccine efficacy

As previously discussed, a neglected challenge to development of vaccines for use in developing countries is helminth infection in the human host. Geographically, the populations with the highest prevalences of malaria, tuberculosis and HIV-1 are in regions of sub-Saharan Africa and South America (100, 101). In 2010, 34 million people were living with HIV-1/AIDS, with 68% of these infected individuals residing in sub-Saharan Africa. Prevalence of HIV-1 exceeds 20% in some southern African countries (8). Unfortunately, these same populations are also often endemic for one or more helminth parasites. Globally, over 2 billion people are infected with at least one species of parasitic worm, with prevalence rates of helminth infection surpassing 50% in some countries (102). Schistosomes are helminth parasites found in sub-Saharan Africa, the Middle East, South America, Asia and the Caribbean (Fig. 1.1). Schistosome infection has been shown to induce strong anti-inflammatory response and in turn, reduce Th1-type vaccine efficacy (52-54, 103).

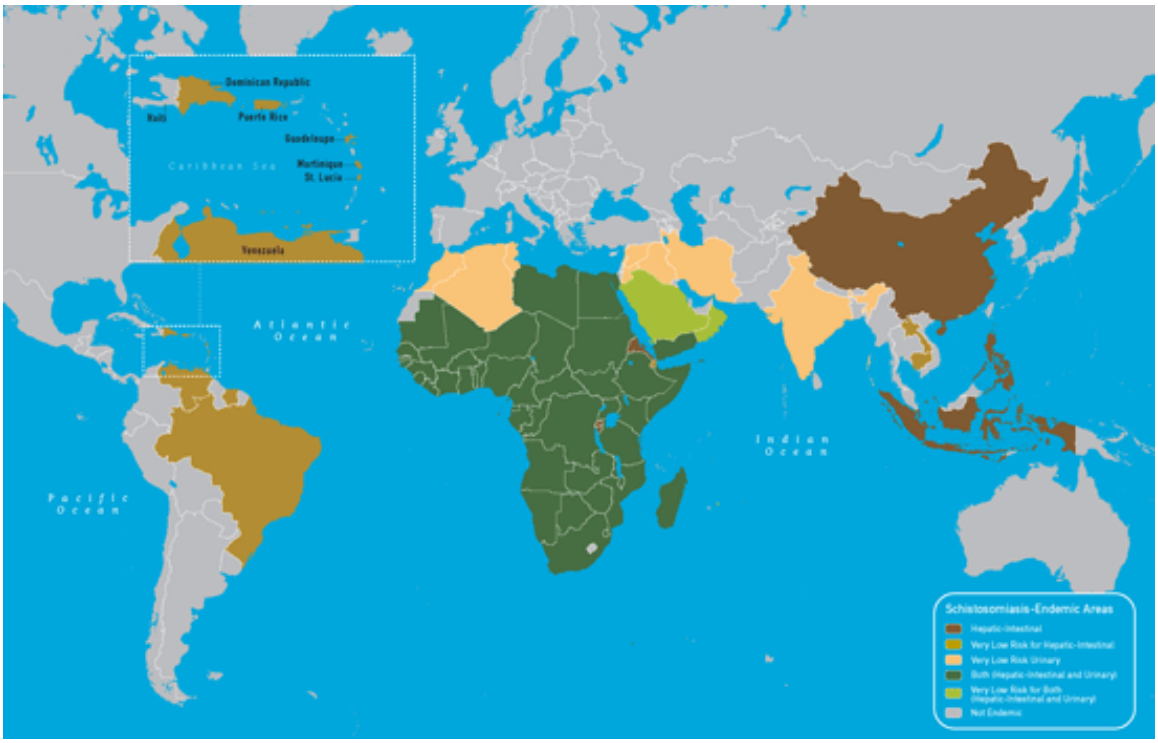


FIG 1.1 Geographic distribution of schistosomiasis. Center for Disease Control and Prevention, <http://wwwnc.cdc.gov/travel/yellowbook/2014/chapter-3-infectious-diseases-related-to-travel/schistosomiasis> (104).

1. Schistosomiasis

Schistosomiasis is a helminth infection caused by parasites of the genus *Schistosoma*. There are three major species that infect man: *Schistosoma haemotobium*, *Schistosoma mansoni* and *Schistosoma japonicum*. Of the 240 million estimated individuals with infection, the estimated mortality of *Schistosoma mansoni* and *Schistosoma haematobium* is 280,000 deaths per year (105, 106). Schistosomes are unique amongst platyhelminth parasites as they are not hermaphroditic. The adult male and female worms mate and the females produce viable eggs. Schistosomes also occupy two rather than three hosts, and the infective larval stage known as cercariae directly

penetrates the skin of humans and then migrate via the vasculature instead of being ingested like other trematode parasites (106).

The *Schistosoma mansoni* life cycle includes multiple developmental stages, both in the snail intermediate host and in the human definitive host (Fig. 1.2). The asexual life cycle is initiated when miracidia hatch from eggs which have been passed from the mammalian host into fresh water. The ciliated miracidia swims around until it encounters the appropriate species of freshwater snail or until it dies (106). Following asexual reproduction of several different developmental stages, thousands of cercariae are produced, which exit the snails in a photoperiodism coinciding with maximal sunlight intensity. Cercariae use their forked-tail to propel themselves to the water surface, and then float down. When cercariae encounter skin, they initiate direct penetration, which is a process involving motion and release of various enzymes to help break down the skin surface (107-110). As cercariae penetrate the skin, the tail breaks off and only the body enters (111, 112). The body is now called a schistosomulum. The outer membranes of the schistosomulum rapidly transform to adjust to life in a saline environment compared with cercariae living in fresh water (111, 112). This involves shedding of the glycocalyx and development of a unique double lipid bilayer that surrounds the entire body as a syncytium rather than individual cells (113, 114). After transforming in the skin, schistosomula find the vasculature and initially arrive in the lungs where they develop a primitive gut. Approximately four to eight days later, the larvae leave and find their way to the liver via the vascular system. In the liver, they grow to young adult male and female worms which pair and mate at 4-5 weeks post-infection, descending into the

mesenteric plexus for *S. mansoni* and *S. japonicum*, and off of the urinary bladder venous system for *S. haematobium* (115).

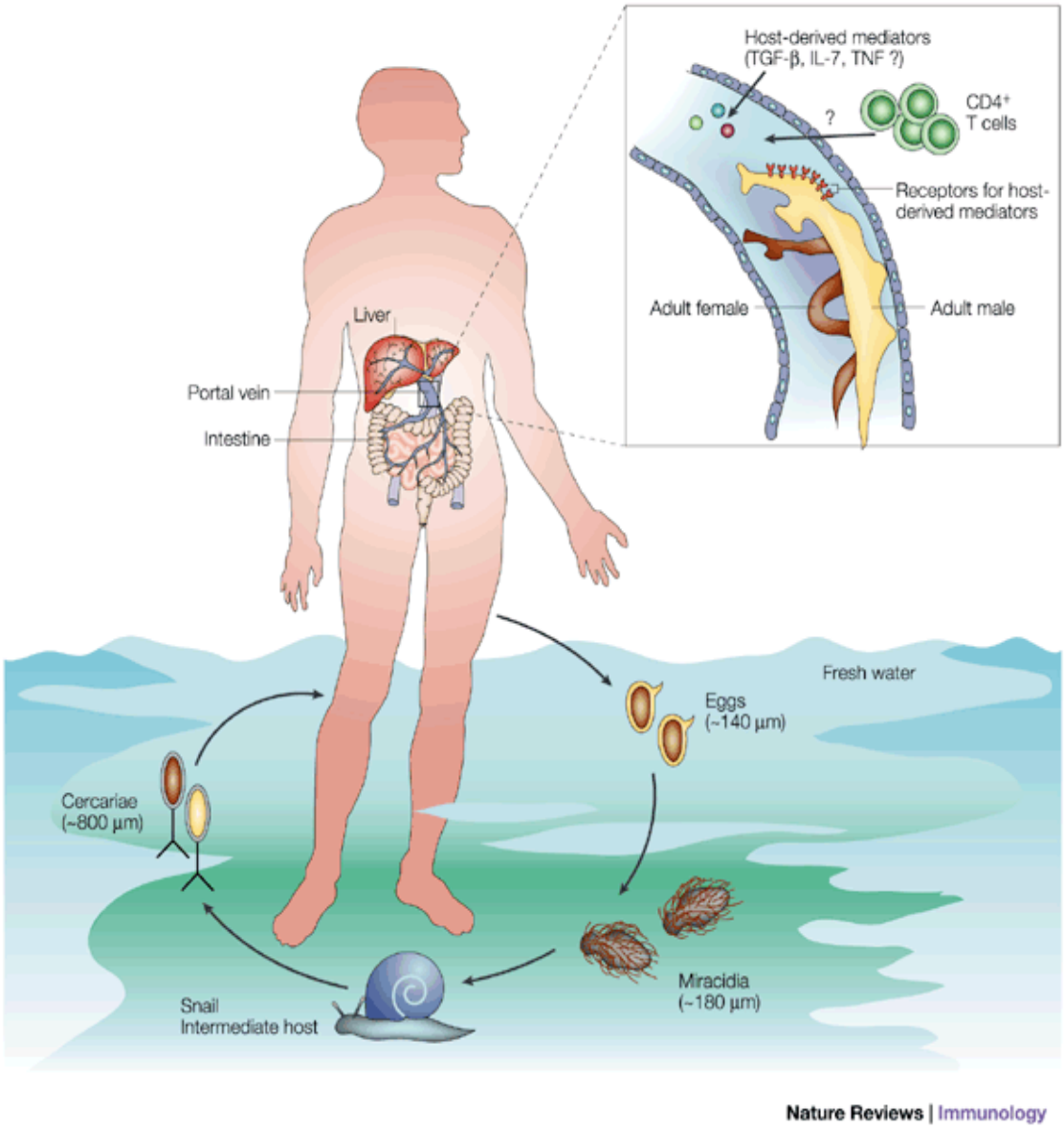


FIG 1.2 The Schistomiasis life cycle. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Immunology* (116), copyright (2002).

Migrating schistosomula drive a T helper (Th) 1 inflammatory response associated with IFNγ, IL-12, and additional pro-inflammatory cytokine production (116). This pro-inflammatory response to schistosome infection continues, even after the initial

deposition of eggs by female worms (Fig. 1.3). *S. mansoni* females release approximately 200-300 eggs per day. These eggs either pass through the intestinal wall and out with the feces in the case of *S. mansoni*, or get caught up in the venous circulation where they become trapped in the tissues, notably the liver (116). The traversing and or death of the eggs can cause severe, pro-inflammatory based pathology, including hepatocyte death. In the livers of infected individuals, eggs lodged in tissue induce formation of granulomas. Pro-inflammatory granulomas are large. Interestingly, within one to two weeks post-initial egg deposition, the immune response of the mammalian host switches from pro-inflammatory to an anti-inflammatory, Th2-$CD4^+$ T cell biased immune response (Fig. 1.3).

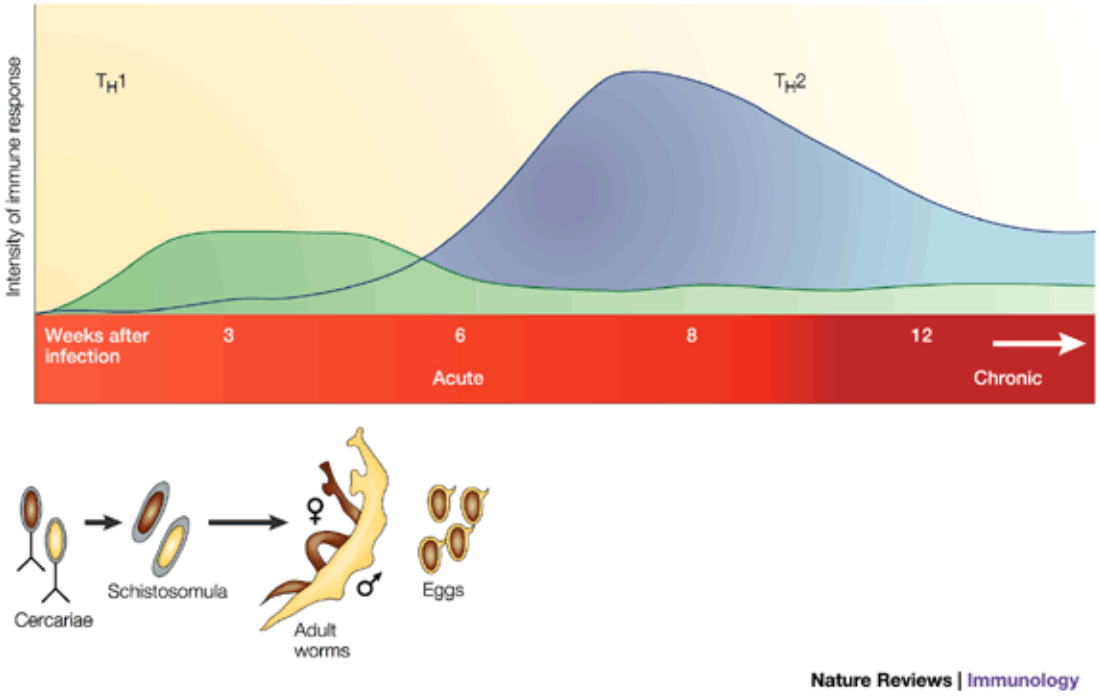


FIG 1.3 The development of immune response in schistosomiasis. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Immunology* (116), copyright (2002).

Schistosome infection can be long-lived, with reports of adult parasites living more than 10 years (117). Schistosome infection can be eliminated by treatment with praziquantel. However, persons living in endemic countries often become re-infected (115). Taken together, some individuals may be infected with egg-laying adult worms for decades. The ability of schistosome infected patients to down-regulate the immune response to schistosome eggs trapped in tissues is essential; as those individuals who cannot down modulate the response to tissue trapped eggs may go on to develop serious hepatosplenic disease as a result of liver fibrosis. The accumulation of fibrotic lesions can eventually cause difficulty in blood flow to the liver. The inability of the liver to perfuse effectively results in hepatotoxic liver damage or steatosis disease (106).

2. Helminth infection reduces cell-mediated immune responses

Helminth infected populations have an impaired ability to respond to infections with other pathogens, especially those that require cytotoxic effector cells (52, 54, 118-123). Similarly, helminth infected populations have impaired vaccine-specific immune responses, especially those designed to drive Th1-type $CD4^+$ and $CD8^+$ T cell responses (52, 54, 118, 121, 124). The ability of schistosome infection to suppress Th1 and cytotoxic $CD8^+$ T cell responses has been amply demonstrated. For example, La Flamme et al. (120) showed that mice co-infected with the protozoan parasite *Leishmania* and *S. mansoni*, had reduced *Leishmania* specific IFNγ, TNFα and nitric oxide production concurrent with increased production of IL-4. In terms of the ability to respond to unrelated antigens, Kullberg et al. (118) demonstrated that schistosome infected mice had significantly reduced production of sperm whale myoglobin-specific IL-2 and IFNγ per

$CD4^+$ T cell coincident with a three-fold increase in $CD4^+$ T cell IL-4 compared to responses from non-infected mice. In order to overcome helminth induced suppression of vaccine specific T cell responses, there has been attempts to use CpG motifs in plasmid constructs to overcome Th2-biasing in schistosome infected mice. For example, Ayash-Rashovsky et al. (125) compared immune responses in mice vaccinated with DNA constructs expressing β-galactosidase to mice vaccinated with recombinant β-galactosidase and obtained mixed results, with both groups of mice producing Th2-type anti-β-galactosidase antibodies, with higher production of recall antigen induced IFNγ production in schistosome infected mice vaccinated with β-galactosidase pCDNA. Unfortunately this study presented no data on Th2-type cytokines, and the conclusions were limited due to initiation of vaccination during the acute stage of schistosome infection where the immune response is still Th0/Th1 biased.

The impact of helminth infection on vaccine induced immune responses was shown in a study by Sabin et al. (54) demonstrating that schistosome infected children had reduced Th1-type responses to tetanus toxin compared to uninfected children from the same endemic area. Importantly, schistosome infected children developed a Th2-type response to tetanus toxin with increased IL-4. In a similar study, Cooper et al. (124) showed that *Ascaris lumbricoides* infected patients had diminished Th1 responses to cholera toxin B subunit, and that elimination of ascarids with albendazole treatment prior to vaccination partially reversed the deficit in IL-2. The study by Elias et al. (126) examined peripheral blood mononuclear cell responses to PPD before and after BCG vaccination in non-infected and helminth positive individuals, and also evaluated the impact of anti-helminthic treatment. Elias et al. showed that elimination of intestinal

worms by albendazole treatment resulted in a significant increase in PBMC PPD-specific IFNγ responses following BCG vaccination compared with the responses in the placebo treated helminth infected group. Taken together, these studies demonstrate that helminth infection impairs vaccine-specific Th1-type responses and that elimination of helminth infection restores these responses.

3. Failure of DNA HIV-1 vaccine to drive Th1-type and cytotoxic $CD8^+$ T cell responses in helminth infected mice.

In terms of the effect of helminth infection on immune responses to HIV-1 antigens, several studies have shown dramatic downregulation of virus-specific cytotxic $CD8^+$ T cell responses in schistosome infected mice. In a study examining immune responses to vaccinia expressing HIV-1 gp160 in schistosome infected vs non-infected mice, Actor et al. (52) showed that there was a significant decrease in the frequency of vaccinia and HIV-1 gp160-specific cytotoxic $CD8^+$ T cells in schistosome infected mice compared to vaccinia infected naïve mice. Da'dara et al. also demonstrated that vaccination of *S. mansoni* infected mice with naked plasmid DNA encoding the TD158 HIV-1 antigens failed to induce significant viral-specific CTL responses (53). The lack of gp160-specific cytotoxic $CD8^+$ T cells and response to the TD158 vaccine in helminth infected vaccine recipients is in agreement with multiple studies suggesting the negative effects of helminth infection on tetanus, BCG and viral vaccines (54, 103, 121, 124, 126).

Fortunately, helminth infections are easily eliminated by drug treatment, praziquantel for schistosomiasis (127-129) and ivermectin or mebendazole for geohelminths (130-132). Depending on the length of time post-treatment, and in the

absence of subsequent re-infection, the host immunity will usually return to a normal mixed Th1-Th2 system within four to six weeks post-treatment, restoring vaccine responses (133, 134). However, as re-infection after treatment is possible and frequent in endemic areas, helminth infection will continue to pose a significant problem for the development of HIV-1 vaccines designed to induce viral-specific Th1- type $CD4^+$ and cytotoxic $CD8^+$ T cell responses.

III. Schistosome soluble egg antigens (SEA) can be used to investigate the negative impact of schistosomiasis on Th1-type vaccine efficacy

1. SEA drives Th2-biasing and immune suppression

The majority of Th2-type immune biasing and immune suppressive properties associated with schistosome infection can be induced with saline soluble extracts of *Schistosoma mansoni* eggs (SEA) (135-137). In murine schistosome infections, SEA induces production of IL-10 and Th2-type cytokines (116, 118, 135-140). Yamashita et al. found SEA also promotes polyclonal B-cell activation *in vitro* (141). In schistosome-infected humans, SEA stimulated peripheral blood mononuclear cells (PBMC) to proliferate and secrete IL-10 (137, 139). Further to this point, one study examining the response of patient PBMC stimulated with glycolipids derived from schistosome worms or schistosoma eggs found that egg glycolipids, but not worm glycolipids, induced increased IL-10 production (142).

SEA is a saline homogenate of *S. mansoni* eggs and therefore contains numerous classes of compounds, including proteins, lipids, glycolipids, nucleic acids and carbohydrates. Many of the proteins in SEA are highly glycosylated (143-149). Many

studies describe the ability of SEA to induce Th2-type and anti-inflammatory responses as being dependent on these carbohydrate components (142, 150-154). Sodium metaperiodate treatment of SEA, a process that alters the structures of glycans but does not remove them, inhibited the ability of SEA to induce nasal lymphocytes to produce IL-4, IL-5, and peritoneal B-1 B cells to expand and produce IL-10 (142, 155). Using monoclonal antibodies against SEA, several immune-stimulatory carbohydrates were identified which contained the Lewis^X (Le^X) trisaccharide, including Lacto-N-fucopentaose III (LNFPIII), Lacto-N-neotetraose (LNnT), Lacdi-Nac and LDN-DF (156, 157). These Le^X structures were able to drive similar overall $CD4^+$ Th2-biased immune responses as SEA (156, 157). The di-fucosylated glycan LDN-DF, which can also be found on schistosomes, has been reported to induce an even stronger response than LNFPIII or LNnT (157).

SEA has also been shown to induce Th2-type responses to unrelated antigens (118, 142, 158-160). An early study by Kullberg et al. demonstrated that schistosome infected mice had significantly reduced production of sperm whale myoglobin-specific IL-2 and IFNγ per $CD4^+$ T cell, coincident with a three-fold increase in $CD4^+$ T cell IL-4 in compared to responses from non-infected mice (118). Previously in our laboratory, Okano et al. found immunization with LNFPIII linked to human serum albumin (HSA) drove robust Th2 immune responses and produced significantly higher levels of HSA-specific IgG, IgG1 and IgE in mice (142, 159). *In vitro* stimulation of nasal lymphocytes with HSA-LNFPIII also produced high levels of IL-4, IL-5 and IL-10, but not IFNγ (142, 159). Taken together, these studies suggest that SEA contains carbohydrate components

functioning as Th2-type adjuvants, which promote Th2-biasing of the immune system, similar to a helminth infection.

2. *Listeria* vector HIV-1 vaccine as Th1 vaccine model

i) *Listeria monocytogenes*

Listeria monocytogenes, a gram-positive, facultative, intracellular anaerobic bacillus, is an opportunistic food-borne pathogen that causes listeriosis in humans (161, 162). The initial entry of the bacteria is through the gastrointestinal tract. *Listeria* is capable of becoming systemic via trafficking of infected monocytes, macrophages and polymorphonuclear leukocytes. Pathogenic *L. monocytogenes* can also infect hepatocytes, endothelial cells, and epithelial cells (162, 163). The intracellular life cycle of *Listeria* involves early escape from the phagocytic vacuole, rapid intracytoplasmic multiplication, bacterially induced actin-based motility, and direct spread to neighboring cells, in which they reinitiate the cycle. The bacterium is phagocytosed by these cells and then secretes a pore-forming toxin called listeriolysin O, which allows the bacterium to escape from the phagosome (161-163). All virulent strains of *L. monocytogenes* synthesize and secrete listeriolysin. Phospholipase A and B are other virulence factors that facilitate escape of *L. monocytogenes* from the phagosome (161-163). Once out of the phagosome, *L. monocytogenes* undergoes rapid division in the cytoplasm, evading the immune response and moving throughout the cytoplasm from cell to cell. *L. monocytogenes* is well known for its ability to propel itself like a rocket through the cell cytoplasm (161-163). *L. monocytogenes* accomplishes such cell motility through a virulence factor called ActA, which initiates polymerization of actin filaments at its tail

end. Actin is arranged in subunits to form microfilaments that are capable of directing cell movement. The rocketing movement of *L. monocytogenes* enables the bacterium to protrude out of one cell and into an adjacent one, evading the immune response (161-163).

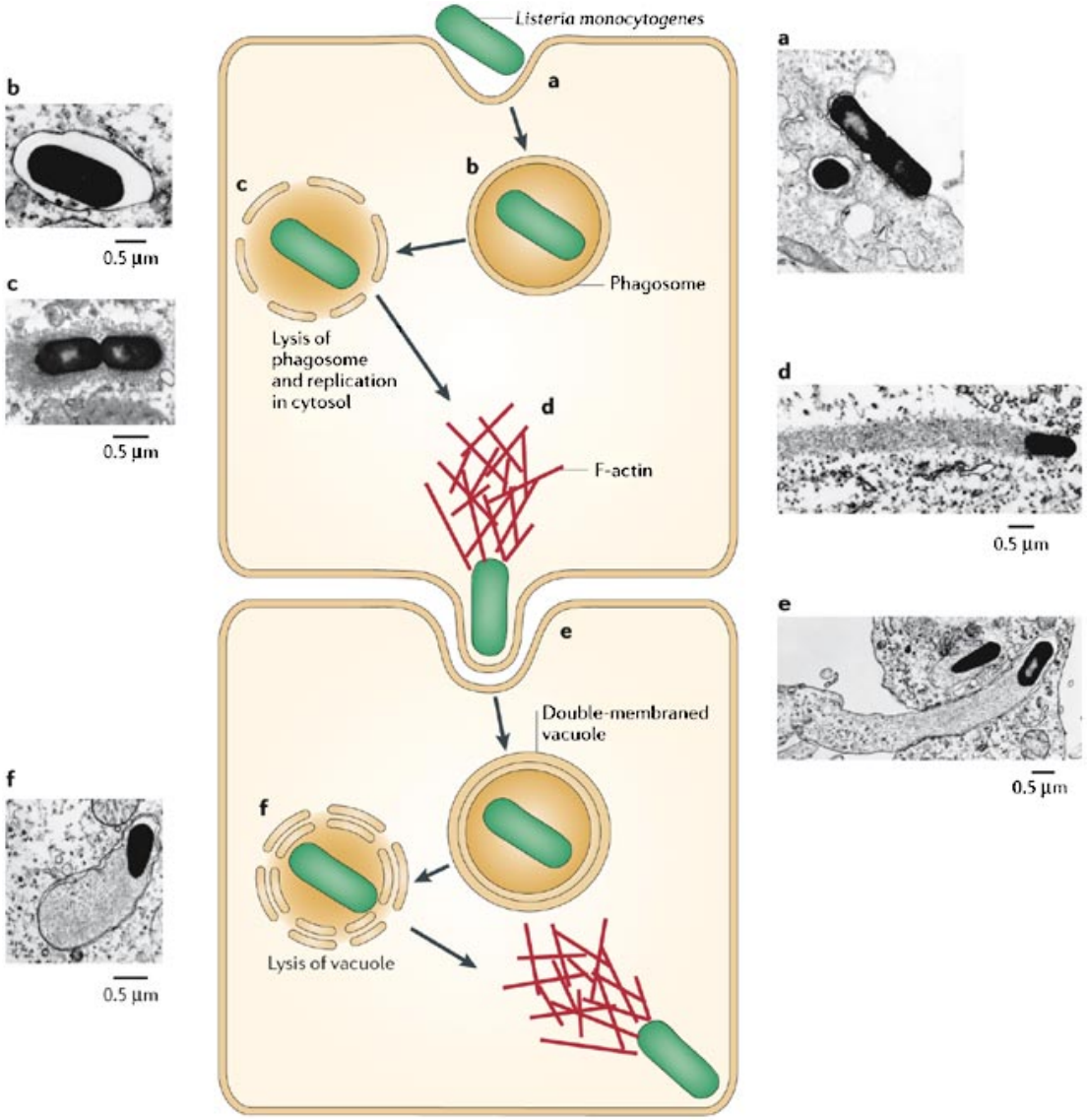


FIG 1.4 Schematic presentation and electron micrograph of the *Listeria monocytogenes* life cycle. Reprinted by permission from Macmillan Publishers Ltd: *Nature Reviews Immunology* (163), copyright (2006).

ii) Advantages of a *Listeria* vaccine vector

Viral and bacterial vectors can induce substantial CTL responses (164). In this regard, *L. monocytogenes* has served as a model pathogen for the study of cell-mediated immunity for many years (162, 165, 166). Thus, information on the effects of infection on the immune system are known. Further, reagents and tools for development of *L. monocytogenes* as vaccine vector are readily available. As a vaccine vector, *L. monocytogenes* has been shown to overcome pre-existing Th2 biasing and reverse vaccine specific immune responses to third party antigens from Th2 to Th1 type (167). *L. monocytogenes* lives within phagosomal and intracytoplasmic compartments, facilitating delivery of vaccine antigens to exogenous and endogenous antigen processing and presentation pathways as well as to the MHCII pathway via cross presentation (168-170). Unlike adenovirus or vaccinia vectors where pre-existing immunity hampers both the priming of immune responses and the possibility to be repeatedly delivered with exogenous antigens (171-174), pre-existing immunity to *Listeria* does not negatively impact the ability of *Listeria* to express pathogen antigens, to be administered repeatedly or prime the immune response to antigens (175-177).

In further support for the use of *Listeria* as a vaccine vector, two separate studies demonstrated that *Listeria* HIV/SIV vaccines induce robust immune responses in macaques, and partially protect against SIV challenge (178, 179). *Listeria* as vaccine vector was used successfully to induce protective immunity to several different pathogens including *Francisella tularensis*, *Herpes simplex type-1*, *Leishmania major* and human papilloma virus (180-183). The use of *Listeria* as vector in these studies resulted in induction of mucosal and protective immune responses. Finally, in regards to the potency

of *Listeria* as a vaccine vector, Seavey et al. demonstrated that vaccination with *Listeria* expressing the "self" antigen Her2/neu- as a fusion protein with *Listeria* listeriolysin O (LLO), was sufficient to induce potent anti-Her2/neu- immune responses which had a significant anti-cancer effect (184). Overall, the use of *Listeria* as a vector in these studies resulted in induction of systemic immune responses. We believe that *Listeria* is an ideal vaccine vector, able to repeatedly boost the $CD4^+$ and $CD8^+$ T cell responses to vaccine antigens.

iii) Limitation of *Listeria* vector vaccines

The use of *Listeria* as vaccine vector has attracted a lot of attention in vaccine development due to its ability to elicit high levels of cell-mediated immunity (178, 179). However, great concerns about the safety of this vaccine vector remain. *L. monocytogenes* produces β-hemolysin, a causative agent of listeriosis, a major public health concern (185-187). Listeriosis as a virulent food-borne pathogen which is well controlled in the United States, though outbreaks and complications still occur (188-190). According to the CDC, the pathogen causes nearly 1600 cases of listeriosis per year and about 260 deaths in the United States (191). Also, listeriosis during pregnancy can lead to multiple complications, including miscarriage and stillbirth (192, 193).

Numerous attempts have been made to attenuate the vaccine vector to confer safety. For example, Chen et al. constructed a *Listeria*-based vaccine expressing multiple foreign antigens for treatment of hepatocellular carcinoma (194). The Lm-MPFG (multiple peptide fusing genes) was developed with the ability to express and secrete hepatocellular carcinoma (HCC)-related tumor-associated antigens. The *Listeria* vector

used in this study was the highly attenuated Lmdd vector (LM ΔdalΔdat), in which essential genes in the D-alanine (d-ala) synthesis pathway, alanine racemase (dal) and d-amino acid aminotransferase (dat), have been deleted (194). As expected, the Lm-MPFG vaccine stimulated T-cell proliferation and enhanced levels of interferon (IFN)-γ *in vitro*. *In vivo* cytolytic activity of IFNγ-producing $CD8^+$ T cells was also significantly increased. In addition, immunization with the vaccine increased the duration of survival of the HCC bearing transgenic mice and retarded the growth of the tumor.

The above studies suggest that *Listeria* vectors can be attenuated and utilized as vaccine vectors to induce strong vaccine-specific cell-mediated responses. However, due to recent outbreaks and health hazards (185, 188-190), the acceptance of this pathogen as a vaccine vector will likely remain a topic of debate.

3. The effects of the Th2 adjuvant, SEA, on the Lm-Gag vaccine

As discussed previously, helminth parasites strongly bias the host immune system towards $CD4^+$ Th2-type (15-16). Further, infection with helminth parasites suppresses host immunity, impairing the expansion of pathogen-specific cytotoxic $CD8^+$ T cells (CTL) and T helper 1 (Th1) cells (52, 54, 136, 138, 140, 195). Thus, helminth infection often renders the host less able to mount an effective immune response to vaccines. One study demonstrated that schistosome infection dramatically impaired the response to an HIV-1 vaccine (28). Interestingly, these same immune biasing and immune suppressive properties have been reported for saline soluble extracts of *Schistosoma mansoni* eggs (SEA) (16). Therefore, this study was initiated to evaluate the effect of SEA on a Th1-type vaccine model, *Listeria* vector HIV-1 Gag (Lm-Gag). Our initial hypothesis was that

co-administration of SEA with Lm-Gag vaccine would suppress the vaccine-specific Th1-type cytotoxic T lymphocyte (CTL) and T helper (Th1) responses to HIV-l Gag.

CHAPTER 2

OPTIMIZING THE ENHANCEMENT OF *LISTERIA* VECTOR HIV-1 VACCINE RESPONSES BY CO-ADMINISTRATION WITH SCHISTOSOME EGG ANTIGENS

I. Introduction

Helminth infection induces strong T helper (Th) 2 polarization of the host immune response and negatively impacts Th1-type vaccines and the host ability to mount strong antiviral T cell responses (195). Helminth infection has been shown to suppress immune responses to Th1-type bacterial vaccines and impair the expansion of pathogen-specific cytotoxic $CD8^+$ T cell (CTL) responses (52, 54, 136, 138, 140, 195). Da' dara et al. 2008, also 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 (53). This observation, taken together with other studies examining virus-specific immune responses in helminth infected mice suggests that helminth infection will pose a significant problem for the development of HIV-1 vaccines designed to induce viral-specific Th1- type $CD4^+$ and CTL responses (52-54).

Interestingly, in schistosomiasis, the induction of $CD4^+$ Th2 biasing and IL-10 mediated immune suppression is primarily due to deposition of parasite eggs into host tissues (135, 196-201). Such anti-inflammatory responses are essential in reducing

hepatic inflammation and contribute to host survival (202-207). Similarly, the saline homogenate of schistosome eggs (SEA) are also known Th2 immune modulators and has been used extensively in research to induce strong $CD4^+$ Th2 responses (135, 202, 203, 208-211). Therefore, we initiated this study to evaluate the effect of SEA on Th1-type vaccines. We hypothesized that addition of SEA to a Th1-type vaccine would reduce generation of $CD4^+$ Th1-type and $CD8^+$ CTL responses and in turn reduce the vaccine efficacy. In order to test this hypothesis, we used a *Listeria* vector HIV-1 Gag vaccine as Th1-type vaccine model and examine the negative effect of SEA on the cell-mediated vaccine responses.

II. Materials & Methods

Biological Materials. The HIV vaccine, an attenuated strain of *Listeria monocytogenes* expressing the HIV-1 IIIB Gag protein (Lm-Gag) (212), and the control strain, which expresses the E7 oncoprotein of the human papillomavirus 16 (Lm-E7) (213), were grown in BHI supplemented with streptomycin. Five to 7 week old female BALB/c mice were purchased from Harlan and Jackson Laboratories, housed in specific pathogen-free conditions and allowed to acclimate for one week prior to manipulation. All animal work was performed in accordance with institutional policy and approved by the institutional animal care and use committee.

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 injection of 100-150 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 production of SEA as described previously (135, 138, 142, 202). The protein concentration of SEA was determined by both the Bradford and bicinchoninic acid (BCA) protein assays (Thermo Scientific).

Vaccination of mice. Six to 8 week old BALB/c mice were injected intraperitoneally with SEA or left unvaccinated. One week after SEA injection, mice were primed with Lm-Gag vaccine, with or without SEA injected intraperitoneally or left unvaccinated. Mice were boosted two weeks after the prime in an identical manner. SEA dose was 50μg unless indicated otherwise. Lm-Gag vaccine was delivered intraperitoneally at $0.2LD_{50}$ or 10^6cfu intravenously as indicated. Mice were sacrificed two weeks post last vaccination (wplv) and analyzed for vaccine-specific responses.

ELISA. Two wplv, splenocytes were harvested and plated at 1.5 million cells per well in 48-well plates in complete media (RPMI-1640 supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B, 2mM L-glutamine, 5μM β-mercaptoethanol and non-essential amino acids). Cells were stimulated with 25μg/ml SEA, 1μg/ml concanavalin A (conA) or left unstimulated for 72 hours, supernatants harvested then analyzed for levels of IFNγ, IL-4 and IL-10 by ELISA (BD).

ELISpot. Splenocytes were harvested, plated at 300K cells per well in IFNγ ELISpot plates (BD) and incubated with complete media in the presence of 20μM specific CTL peptide (H2-Kd-restricted, AMQMLKETI from HIV-1 IIIB Gag protein), 20μM irrelevant peptide (H2-Kd-restricted, TYQRTRALV from influenza A/PR/8/34 nucleoprotein), 20μM specific helper peptide (Class II-restricted, NPPIPVGEIYKRWIILGLNK from HIV-1 IIIB Gag protein), 20μM LLO peptide (H2-

Kd-restricted, GYKDGNEYI from *L. monocytogenes* listeriolysin O), or 1 μg/ml con A (positive control). Peptides were synthesized by Biosynthesis, Inc at greater than 95% purity and reconstituted in DMSO prior to dilution in media. After 20 hours incubation, ELISpots were performed according to manufacturer's instructions and enumerated using an Immunospot analyzer (C.T.L.).

***In vivo* cytotoxic T lymphocyte (CTL) assay.** Assay was performed as described previously (134)**.** Briefly, splenocytes from naïve mice were fluorescently labeled with green (Vybrant CFDA SE Cell Tracer Kit) or violet (CellTrace Violet Cell Proliferation Kit) fluorescent dye, according to manufacturer's instructions (Invitrogen). Green and violet cells were then pulsed with 20 μM specific CTL or irrelevant peptide respectively for 2 hours. Targets were mixed and twelve million cells were injected intravenously per vaccinated animal. After 18 hrs, splenocytes were collected and analyzed for target recovery by flow cytometry. The level of Gag-specific killing was calculated by the formula: % specific killing = $\{1- (r_{naive}/r_{immunized})\}$ x100, where r = % violet cells/% green cells (214).

Sera and Peritoneal lavage fluid. Blood was collected from mice by submandibular venipuncture before sacrifice. Serum was collected after coagulation and centrifugation. Lavage fluid was collected by washing the peritoneal cavity with 200μl of PBS. Lavage fluid was centrifuged at 400xg and supernatant collected to remove peritoneal endothelial cells (PECs). Both sera and peritoneal lavage fluid were stored at -80^{o}C until assayed.

Cytometric bead array (CBA). Cytokine levels in sera and peritoneal lavage fluid from each animal group were measured using a Th1/Th2/Th17 CBA kit according to the manufacturer's instructions (BD).

Liver Enzyme Assays. Levels of Aspartate Aminotransferase (AST) and Alanine Transaminase (ALT) enzyme activities were measured from each animal group using AST and ALT colorimetric assay kits according to the manufacturer's instructions (BioVision).

Endotoxin and Endotoxin Removal Assays. Levels of endotoxin in SEA batches were measured by Toxin Sensor™ LAL Endotoxin kit according to the manufacturer's instructions (Genscript). Endotoxin levels in SEA batches used in these studies was 1EU/30μg. Removal of endotoxin from SEA was done by treatment with Polymyxin B using Toxin Eraser™ Endotoxin Removal kit according to the manufacturer's instructions (Genscript). Level of endotoxin in PMB-treated SEA was reduced 10-fold, resulting in SEA with 0.1EU/30μg.

III. Results & Discussion

1. Immunization strategy using SEA to induce Th2-bias in murine model

In this study, we substituted Th2-driving schistosome soluble egg antigens (SEA), for helminth infection, to investigate the effect of SEA on induction of Lm-Gag vaccine-specific immune responses. In order to induce strong Th2 responses, BALB/c mice were pre-injected with SEA one week prior to vaccination. Naive and SEA primed mice were then immunized intraperitoneally with *Listeria* vector HIV-1 vaccine (Lm-Gag) with or without a simultaneous intraperitoneal injection of SEA. Mice were boosted two weeks after the prime in an identical manner. Two weeks after the boost, the three animal groups, naïve, Lm-Gag vaccinated and SEA co-administered with Lm-Gag (SEA/Lm-

Gag), were sacrificed to evaluate vaccine-specific responses. Experimental timeline presented in Figure 2.1.

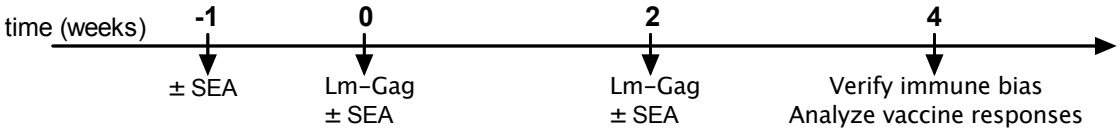


FIG 2.1 Immunization timeline. Mice were primed with SEA or left unvaccinated, each vaccinated with Lm-Gag with or without additional boost of SEA. Immunization protocol is shown.

We verified that administration of SEA using this immunization protocol did induce Th2-biasing of the host immune system. As shown in Figure 2.2, splenocytes from SEA/Lm-Gag group produced significant levels of IL-4 and IL-10 upon stimulation with SEA compared to those of naïve and Lm-Gag alone groups. Interestingly, the level of IFNγ was also highest in this group (Fig. 2.2).

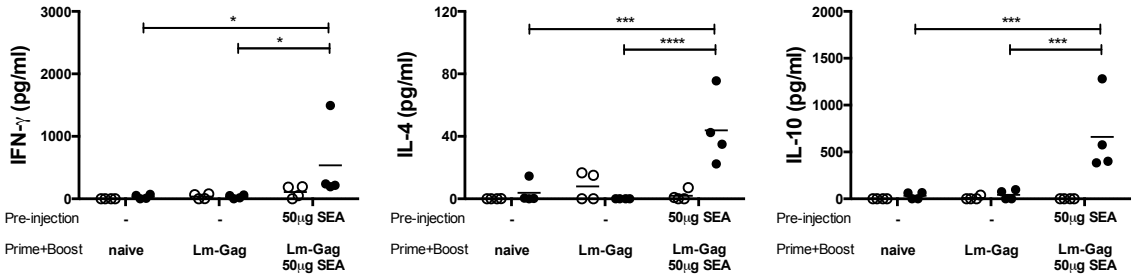


FIG 2.2 Co-administration of SEA drives Th2-bias system. Splenocytes were harvested and stimulated with SEA (filled) or left unstimulated (open). After 72-hour incubation, supernatants were harvested and analyzed for levels of IFNγ, IL-4 and IL-10 by ELISA. Statistical differences were analyzed by two-way ANOVA with Bonferroni post-hoc test, *$p<0.05$, ***$p<0.001$ and ****$p<0.0001$.

2. SEA co-administration increases functional HIV-specific T cell responses despite Th2-biased host immune system

In order to address the effect of SEA on Lm-Gag vaccine efficacy, three animal groups, naïve, Lm-Gag vaccinated and SEA/Lm-Gag were immunized and analyzed for vaccine responses. Two wplv, Gag-specific T cell-mediated responses were measured upon stimulation of splenocytes with immunodominant CTL and helper T cell epitopes of HIV-1 Gag. As expected, cells from naïve mice did not respond to any of the Gag peptides. Lm-Gag vaccinated mice responded to both CTL and helper epitopes in a peptide-specific manner though the responses were quite low (Fig. 2.3). Similar to what we observed with SEA restimulation, co-administration of SEA along with the Lm-Gag vaccine significantly enhanced the numbers of Gag-specific IFNγ producing $CD4^+$ and $CD8^+$ T cells compared to both naïve and Lm-Gag vaccine alone groups. The data show that our initial hypothesis was incorrect. While administration of SEA did polarize the host immune system towards a Th2-type immune response, SEA also functioned to enhance Th1-type HIV-1 vaccine-specific responses.

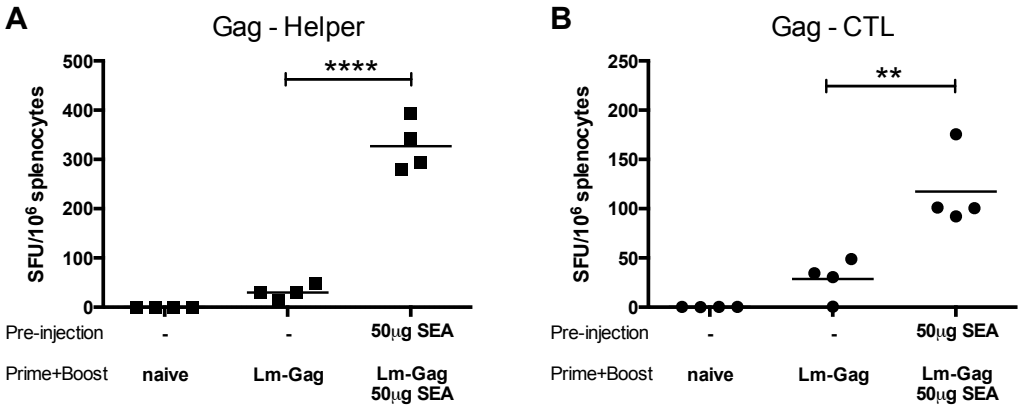


FIG 2.3 Vaccine responses induced by Lm-Gag vaccine were not suppressed by SEA co-administration. Splenocytes from all four animal groups of interest were

harvested and vaccine response to the immunodominant $CD4^+$ helper (**A**) or CTL epitopes (**B**) of HIV-1 Gag were measured by IFNγ ELISpot. Data from two independent experiments were pooled and plotted individually as shown. Statistical differences were analyzed by one-way ANOVA and Tukey post-hoc test, *$p<0.05$ and **$p<0.01$

To validate that the responses measured by ELISpot result from functional CTL effector cells, an *in vivo* cytotoxicity assay was performed to assess Gag-specific $CD8^+$ T cell killing activity *in vivo*. Naïve unvaccinated group, a group vaccinated with vector control Lm-E7 and vaccine alone Lm-Gag group, each with or without SEA co-administration for a total of six animal groups were included in this assay. Vaccination followed the identical immunization timeline (Fig. 2.1). The percent of Gag-specific target population was not significantly different in the four control groups: naïve, SEA only, Lm-E7 vector control and SEA/Lm-E7 groups (Fig. 2.4A), which demonstrates that injection of SEA or vector alone or both do not induce killing of Gag-specific targets. Mice vaccinated with Lm-Gag alone had a modest reduction of Gag-specific targets, whereas the SEA/Lm-Gag mice significantly lysed these targets after 18 hours. The percent of Gag-specific killing of each individual mouse was calculated and plotted (Fig. 2.4B). Percent killing in the Lm-Gag vaccinated group averaged 22% while the SEA/Lm-Gag group ranged from 65% to 96% specific killing. Taken together with previous results, addition of SEA to Lm-Gag vaccine not only enhanced number of Gag-specific IFNγ producing T cells but also increased functional Gag-specific CTL effector cells generated by Lm-Gag vaccine. Based on these results, we reframed our hypothesis to "SEA can function as an adjuvant that enhances Th1-type vaccine responses induced by *Listeria* vector HIV-1 vaccine."

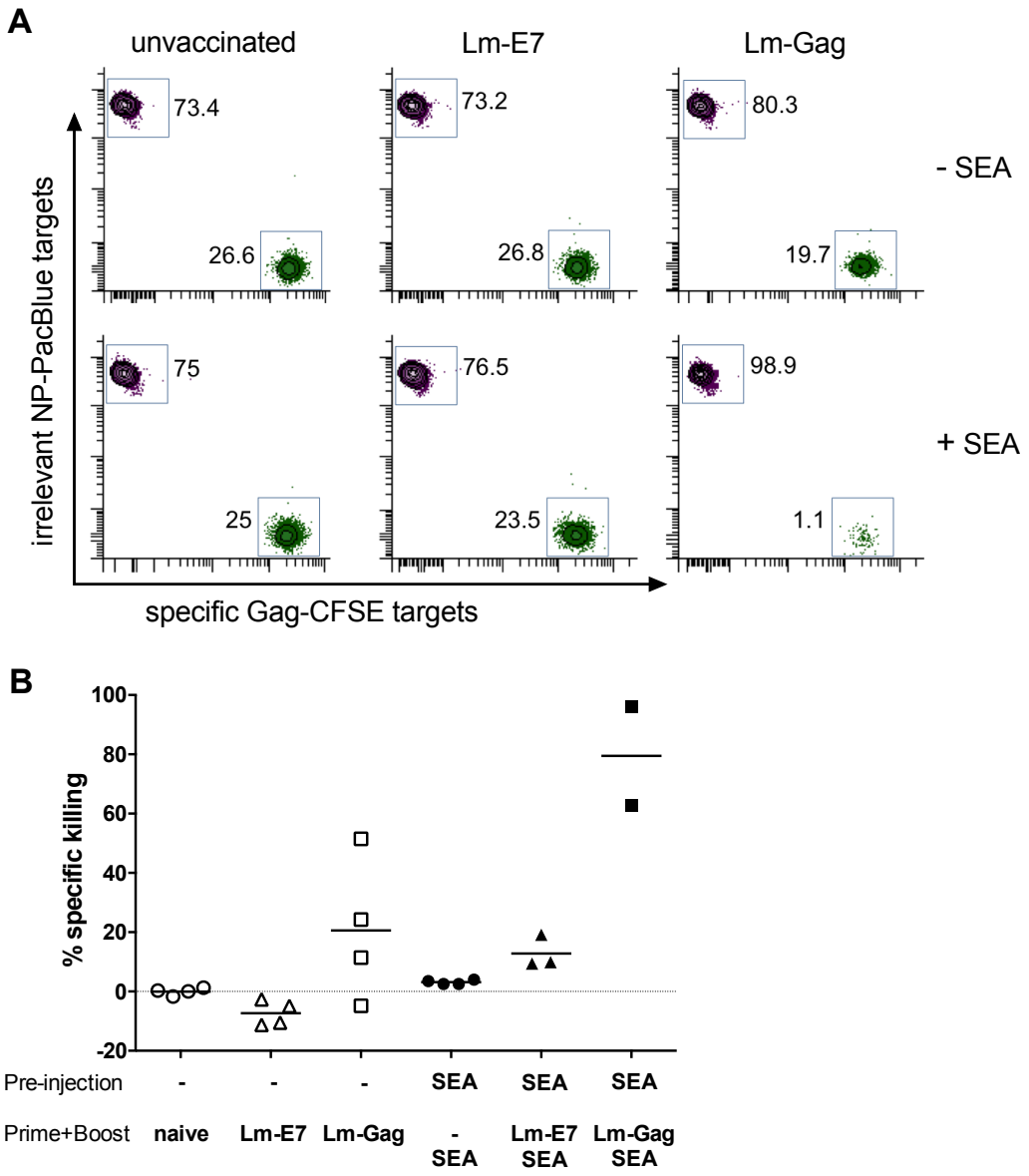


FIG 2.4 SEA co-administration enhances Gag-specific cytotoxicity. In vivo killing assays were conducted in mice that were unvaccinated, Lm-Gag vaccinated or Lm-E7 vaccinated, each with and without SEA co-injection, for a total of six groups. Eighteen hours after introduction of fluorescent-labeled targets, splenocytes from each group were harvested and 10,000 target cells were acquired by flow cytometry. (**A**) Representative data from individual mice are shown. (**B**) Percent killing of targets was determined by flow analysis as shown above and normalized to naïve mice. Data of individual mice were plotted.

3. Administration of SEA can induce death in subsequent Lm-Gag vaccinated mice

In repeats of the *in vivo* cytotoxicity assay, we observed a decrease in survival rate of SEA/Lm-Gag vaccinated animals. Survival for the naïve and Lm-Gag vaccine only groups was 100% whereas the SEA/Lm-Gag group had an average survival rate of only 50% (Fig. 2.5).

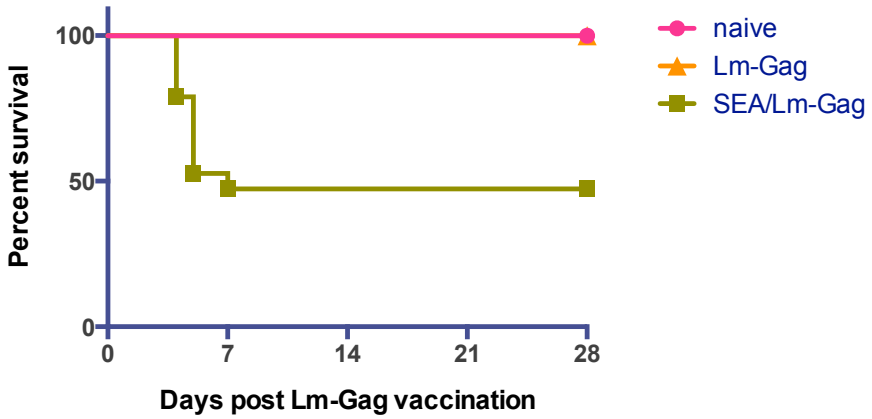


FIG 2.5 Survival rate when mice were immunized with Lm-Gag co-administered with SEA intraperitoneally. Data were pooled from five independent experiments. When SEA primed mice were vaccinated with Lm-Gag vaccine and boosted with SEA intraperitoneally, survival rate was reduced to an average of 50%.

To investigate potential mechanisms of animal death, we examined the levels of cytokines and liver enzymes of individual mice from each animal group. A total of four groups were tested, naïve, Lm-Gag vaccine alone, SEA alone and SEA/Lm-Gag groups. SEA prime was one week prior to vaccination. Post-vaccination, with or without SEA booster, serum and peritoneal lavage fluid were collected and analyzed for cytokine production at different time-points.

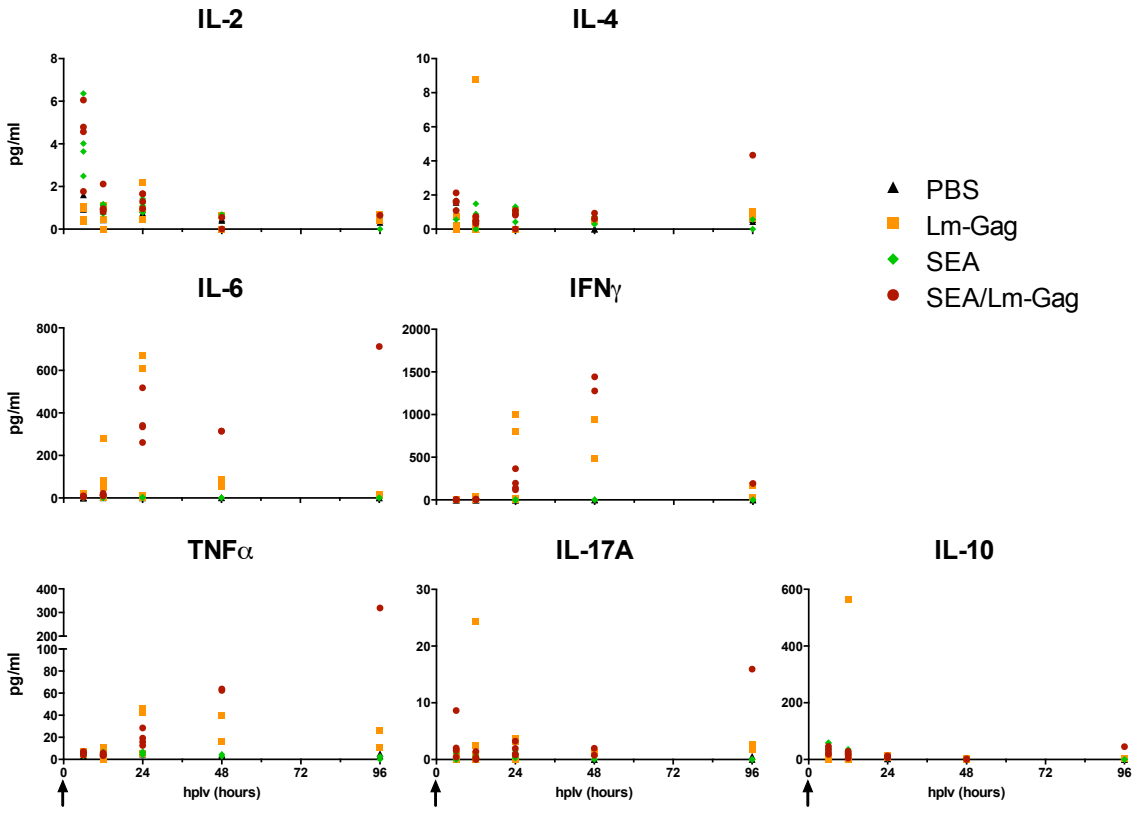


FIG 2.6 SEA/Lm-Gag co-administration induced increased production of pro-inflammatory cytokines in serum of animal host. Animals were primed with PBS or SEA then boosted one week later with or without Lm-Gag vaccination (arrow indicates vaccination timepoint). Sera were collected and analyzed for Th1/Th2/Th17 cytokines at five different timepoints, 6, 12, 24, 48 and 96 hours post last vaccination (hplv).

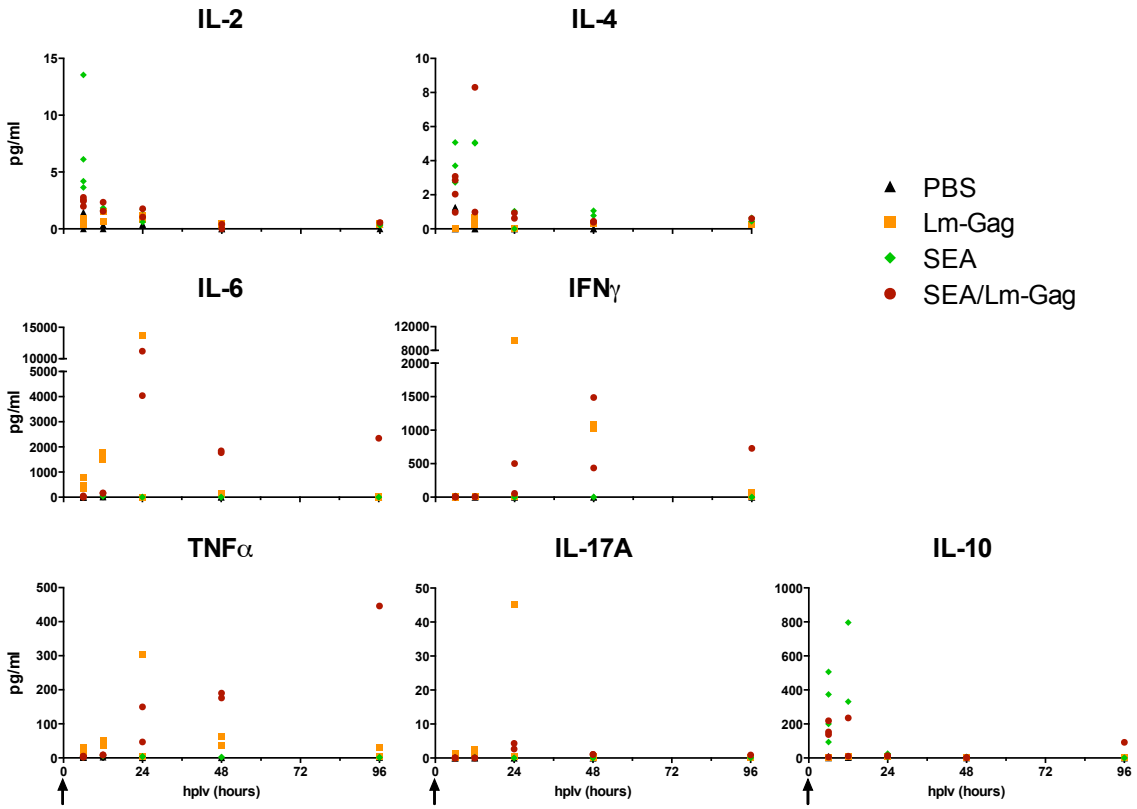


FIG 2.7 SEA/Lm-Gag co-administration induced increased production of pro-inflammatory cytokines in peritoneal lavage fluid of animal host. Animals were primed with PBS or SEA then boosted one week later with or without Lm-Gag vaccination (arrow indicates vaccination timepoint). Peritoneal lavage fluid was collected and analyzed for Th1/Th2/Th17 cytokines at five different timepoints, 6, 12, 24, 48 and 96 hours post last vaccination (hplv).

As shown in Figures 2.6 and 2.7, mice primed with SEA alone produced cytokines as early as 6 hours post last vaccination time (hplv). IL-2 was produced at high levels in both sera and peritoneal cavities. In addition, we also detected IL-4 and IL-10 production in lavage fluid at 6 hplv, confirming the Th2-driving property of SEA, though the levels quickly went down to levels similar to naïve and Lm-Gag alone groups by 24 hplv. Apart from IL-2, IL-4 and IL-10, SEA alone did not induce production of any other cytokines in sera or lavage fluid. Conversely, mice vaccinated with Lm-Gag induced

production of pro-inflammatory cytokines. In Lm-Gag alone group, levels of IL-6 were elevated as early as 12 hplv in sera and lavage fluid, peaking around 24 hplv and dropping significantly at 48 hplv and were undetectable at 96 hplv. Similarly, levels of IFNγ and TNFα in this group increased up to 24 hplv then slowly decreased to levels detected in naive mice at 96 hplv.

SEA co-administered with the Lm-Gag vaccine, induced cytokine profiles in sera and peritoneal lavage fluid that was different than SEA or Lm-Gag alone. Similar to the SEA alone group, SEA/Lm-Gag mice produced IL-2 in sera and IL-2, IL-4 and IL-10 in lavage fluid, though the levels were lower. In addition, levels of IL-6, IFNγ and TNFα cytokines were detected in this group, though the trend was different than that seen in the Lm-Gag alone group. IFNγ levels peaked later, 48 hplv instead of 24 hplv, then reduced to low levels in lavage fluid or to the level of naïve mice in sera. Levels of IL-6 and TNFα also increased in this group. However, instead of slowly reducing to low level after 24 hplv, in some animals we observed continued production of these pro-inflammatory cytokines in both sera (Fig. 2.6) and peritoneal lavage fluid (Fig. 2.7). The continued production of these cytokines correlated with the increased AST and ALT liver enzymes (Fig. 2.8).

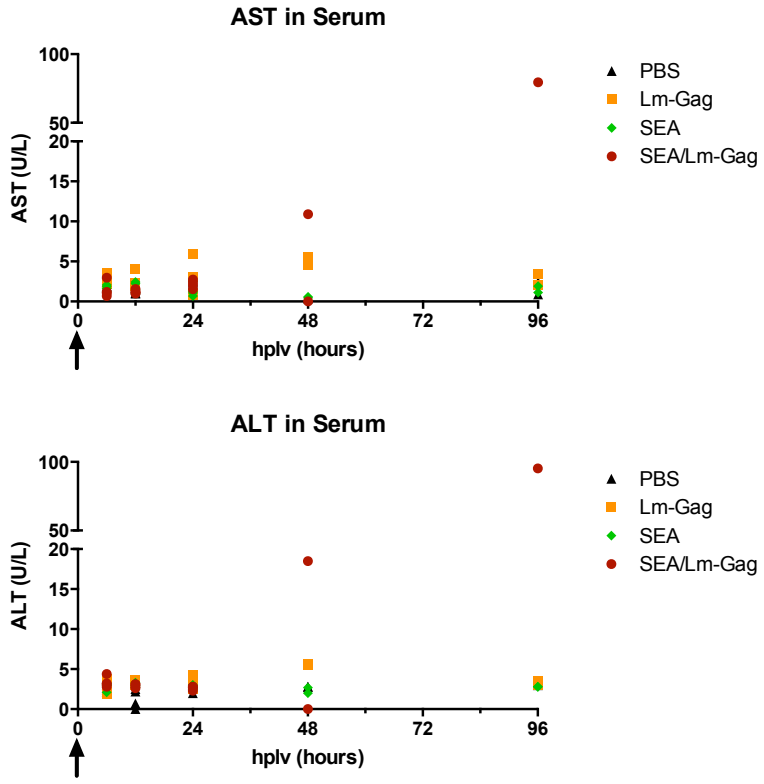


FIG 2.8 SEA/Lm-Gag co-administration increased production of liver enzymes. Animals were primed with PBS or SEA then boosted one week later with or without Lm-Gag vaccination (arrow indicated vaccination timepoint). Sera collected at 6, 12, 24, 48 and 96 hplv were analyzed for levels of Aspartate aminotransferase (AST) and Alanine transaminase (ALT).

Liver enzymes AST and ALT are often used as biomarkers to evaluate hepatic injury and or dysfunction (215-221). Normal ranges of AST and ALT in mouse serum ranges from 47.31-64.84 IU/L and 23.21-34.37 IU/L in 16-week-old mice, respectively (219). Due to the negative effect of freeze-thaw and long-term storage, the AST and ALT enzyme levels were respectively reduced by 20- and 7-fold in our animals. However, the marked increase of AST and ALT in the SEA/Lm-Gag group suggested these mice were suffering hepatocellular damage post vaccination.

Overall, these results suggest that co-administration of SEA with the Lm-Gag vaccine, resulted in an unexpected synergy that induced elevated production of IL-6, TNFα that in turn damaged host liver tissue, indicated by the high level of liver enzymes, and potentially compromised the animals. Therefore, we next focused on optimizing SEA Lm-Gag delivery parameters to balance the adjuvant effect of SEA.

4. Optimization of different parameters for SEA co-administration with Lm-Gag vaccine

i) Schedule with three injections of SEA induces highest level of vaccine responses.

First, we optimized the schedule of vaccination by evaluating vaccine responses when administered without SEA pre-injection and or without vaccine booster. As shown in Figure 2.9, naïve mice and six vaccinated groups of animals were tested, including mice immunized with one or two doses of Lm-Gag vaccine, each with a co-administration of SEA with or without an SEA pre-injection. Two wplv, we did not observe an increase in immune responses to the HIV-1 helper peptide with the Lm-Gag boost group, though, the responses to the Gag CTL epitope were higher compared to the single Lm-Gag vaccination group (Fig. 2.9). When SEA was co-administered with a single dose of Lm-Gag, neither with nor without an SEA pre-injection altered the immune response to CTL and helper epitopes of Gag protein. Co-administration of SEA as pre-injection and with the prime and boost resulted in the highest responses to both helper and CTL epitopes among all tested groups (Fig. 2.9). These results suggest that the original vaccination regimen induces the highest levels of vaccine-specific responses.

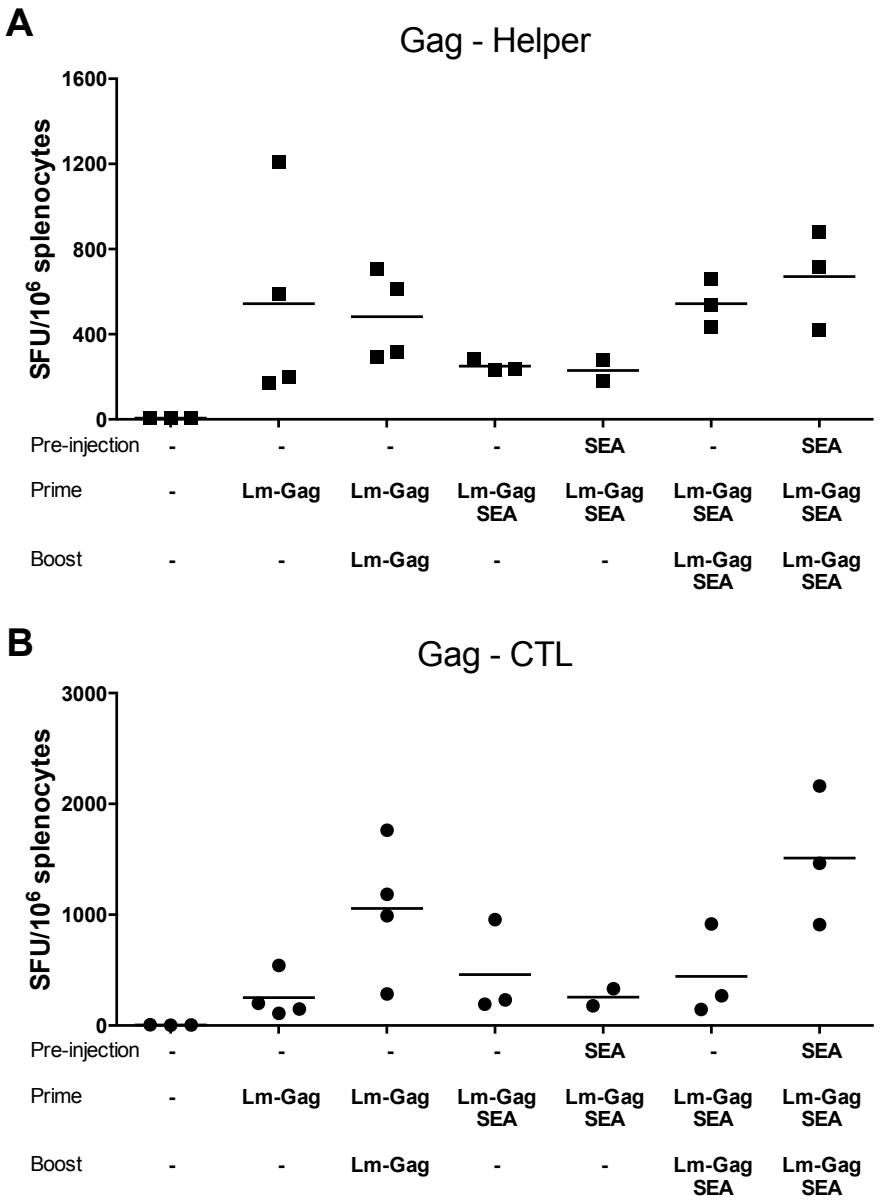


FIG 2.9 Original immunization schedule with three injections of SEA induced highest levels of vaccine responses. Mice were immunized with Lm-Gag with or without SEA using different schedules. Splenocytes from all groups were harvested and immune responses to the immunodominant $CD4^+$ helper (**A**) or CTL peptides (**B**) of HIV-1 Gag were measured by IFNγ ELISpot. Data were plotted individually as shown.

As noted, some mice in the SEA/Lm-Gag groups succumbed to immune activation before we could analyze their responses, indicating the powerful effect of SEA

on the Lm-Gag vaccine. We next compared mice vaccinated using the original schedule with mice primed with a single SEA injection as shown in Figure 2.10. This experiment showed that a single SEA pre-injection was sufficient to increase both the Gag-specific T helper and CTL responses compared to Lm-Gag vaccine alone mice. Further, though not significant, co-administration of SEA with the Lm-Gag vaccine along with SEA pre-injection induced the highest helper and CTL responses.

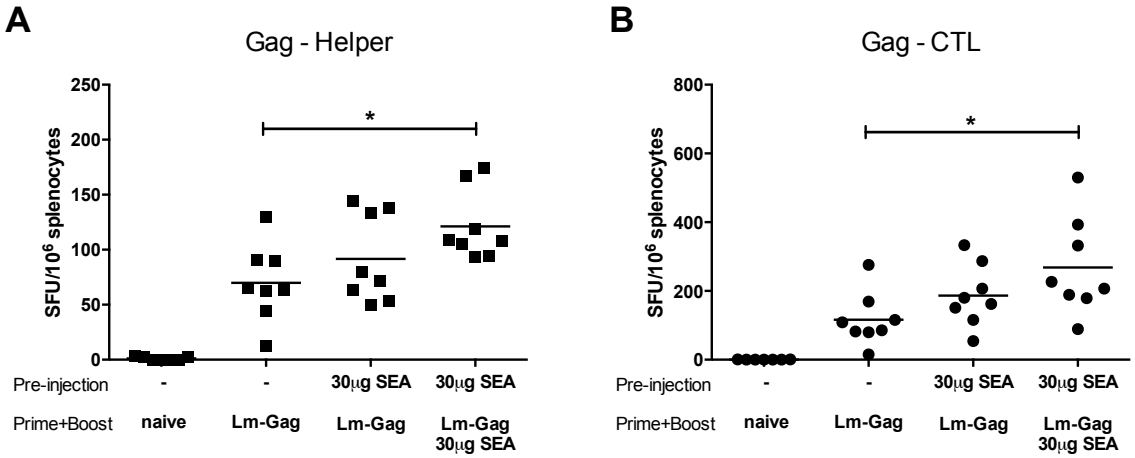


FIG 2.10 Immunization schedule is optimized for delivery of SEA/Lm-Gag. Splenocytes from all groups were harvested and immune responses to the immunodominant $CD4^+$ helper (**A**) or CTL peptides (**B**) of HIV-1 Gag were measured by IFNγ ELISpot. Data were plotted individually as shown. Statistical differences were analyzed using one-way ANOVA and Tukey post-hoc test, *$p<0.05$

ii) Intraperitoneal co-administration of SEA induces highest Lm-Gag vaccine responses and toxicity.

Co-administration of SEA and the Lm-Gag vaccine intraperitoneally induced significant enhancement of Gag-specific CTL and T helper 1 responses (Fig. 2.2), though the immune activation in this group was so high that animals were compromised. Therefore, we decided to optimize the routes and doses for SEA delivery. For SEA route

optimization, seven animal groups were tested, including a naïve group. The Lm-Gag only group and SEA/Lm-Gag groups were evaluated with administration of SEA via five different routes, subcutaneous, intragastric, intranasal, intravenous and intraperitoneal (Fig. 2.11). All vaccinated animals received intraperitoneal vaccination of Lm-Gag at the same dose and schedule as previously described.

As shown in Figure 2.11, administration of SEA via subcutaneous, intragastric or intravenously routes did not induce higher vaccine specific CTL and Th1 responses in the SEA/Lm-Gag mice compared to Lm-Gag vaccinated alone. Interestingly, intranasal delivery of SEA only increased Gag-specific CTL responses compared to the Lm-Gag alone group. Similar to earlier experiments, intraperitoneal co-administration of SEA/Lm-Gag induced the highest level of vaccine responses to both HIV Gag CTL and helper epitopes (Fig. 2.11). These results suggest that intraperitoneal is the optimal route for SEA delivery and should be used for further optimization. Since intranasal injection of SEA also induced enhanced CTL responses in the SEA/Lm-Gag group, it was considered the second best option.

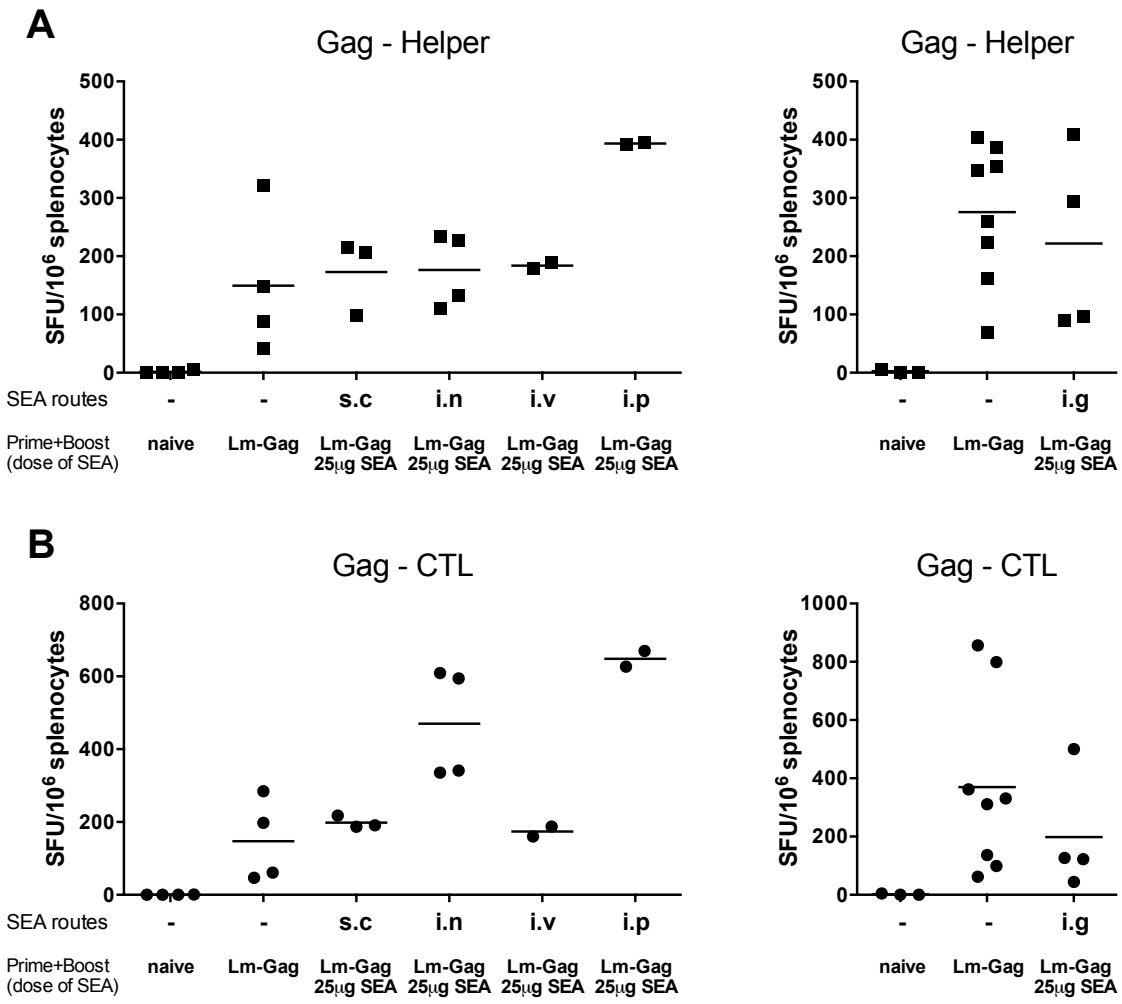


FIG 2.11 Intraperitoneal co-administration of SEA with Lm-Gag induced the highest levels of vaccine responses. Mice were unvaccinated, vaccinated intraperitoneally with Lm-Gag alone or with SEA through five different routes: subcutaneous (s.c), intragastric (i.g), intranasal (i.n), intravenous (i.v) or intraperitoneal (i.p). Splenocytes from all animal groups were harvested and immune response to the immunodominant $CD4^+$ helper (**A**) or CTL peptides (**B**) of HIV-1 Gag were measured by IFNγ ELISpot. Representative data from two independent experiments were plotted individually as shown.

Next, we proceeded to SEA dose optimization. Figure 2.12 summarizes the different route and dose combinations of SEA that we tested with Lm-Gag vaccination. For the two best routes, intraperitoneal and intranasal, we tried multiple SEA doses, ranging from 12.5μg to 50μg (Fig. 2.12).

Lm-gag i.p ($0.2\ LD_{50}$)		SEA route				
		s.c	i.g	i.v	i.n	i.p
SEA dose (ug)	12.5				x	
	15					x
	18				x	x
	20				x	x
	25	x	x	x	x	x
	50	x		x	x	x

FIG 2.12 Matrix of SEA routes and doses tested. SEA was co-administered with the Lm-Gag vaccine using different routes for SEA: subcutaneous (s.c), intragastric (i.g), intranasal (i.n), intravenous (i.v) or intraperitoneal (i.p).

Mice co-administered SEA via intraperitoneal or intranasal routes had increased CTL responses to HIV Gag, similar to our earlier observation (Fig. 2.11). Unfortunately, these groups remained plagued by the synergistic immune activation seen with SEA and Lm-Gag co-administration. As shown in Figure 2.13, 50% or 75% of the mice in the SEA intranasal or intraperitoneal groups respectively, succumbed to immune activation and did not survive these vaccine regimes.

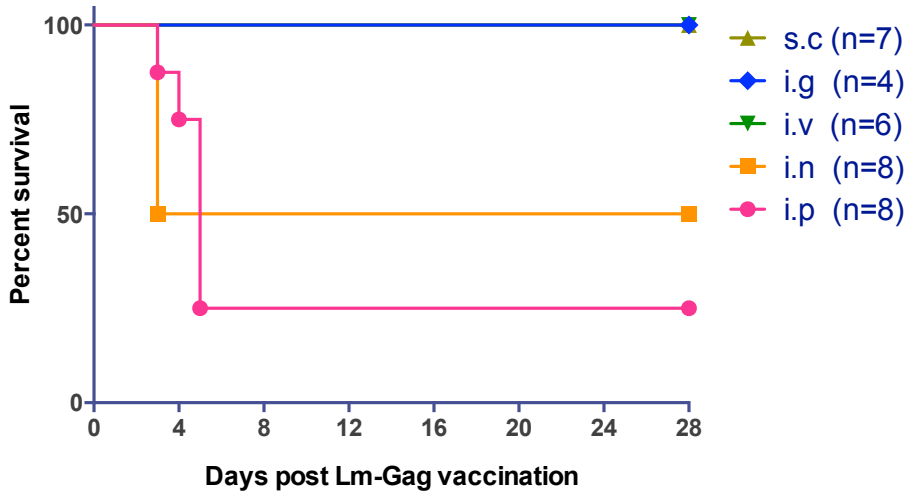


FIG 2.13 Animals co-administered SEA and Lm-Gag via intraperitoneal or intranasal routes succumb to immune activation. Survival curve vs days post-vaccination. Mice were vaccinated intraperitoneally with Lm-Gag vaccine and SEA was co-administered using different routes: subcutaneous (s.c), intragastric (i.g), intranasal (i.n), intravenous (i.v) or intraperitoneal (i.p).

iii) Optimal routes and doses of SEA defined using intravenous vaccination of Lm-Gag

Changing the routes or doses of SEA injections did not alter its powerful effects. Therefore, we changed our focus to the route of Lm-Gag vaccination. We departed from intraperitoneal delivery of Lm-Gag and studied the intravenous route, keeping the intraperitoneal route for SEA. Initially we performed a dose-response study to determine the appropriate dose for intravenous vaccination of Lm-Gag. As shown in Figure 2.14, intravenous immunization with ($0.2LD_{50}$ or 10^6cfu) Lm-Gag resulted in no mice surviving by 7 days post vaccination. Though there was an initial delay, the outcome did not change at 4 fold dose reduction, but at 10 fold dose reduction, all animals survived (Fig. 2.14).

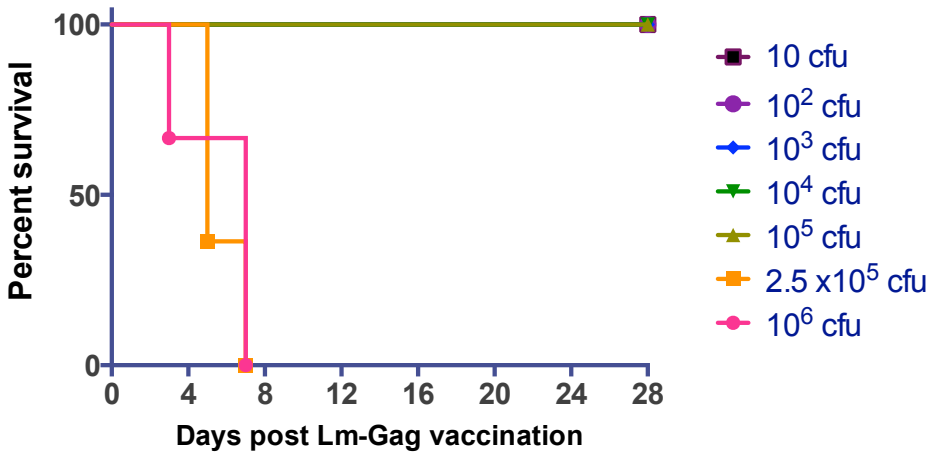


FIG 2.14 Survival curve of intravenous vaccination with *Listeria* vector HIV-1 Gag. Mice survived all doses below $2.5x10^5$ cfu.

In addition to mice surviving the intravenous Lm-Gag vaccination, these mice also responded to the HIV Gag helper and CTL epitopes in a dose-dependent manner (Fig. 2.15).

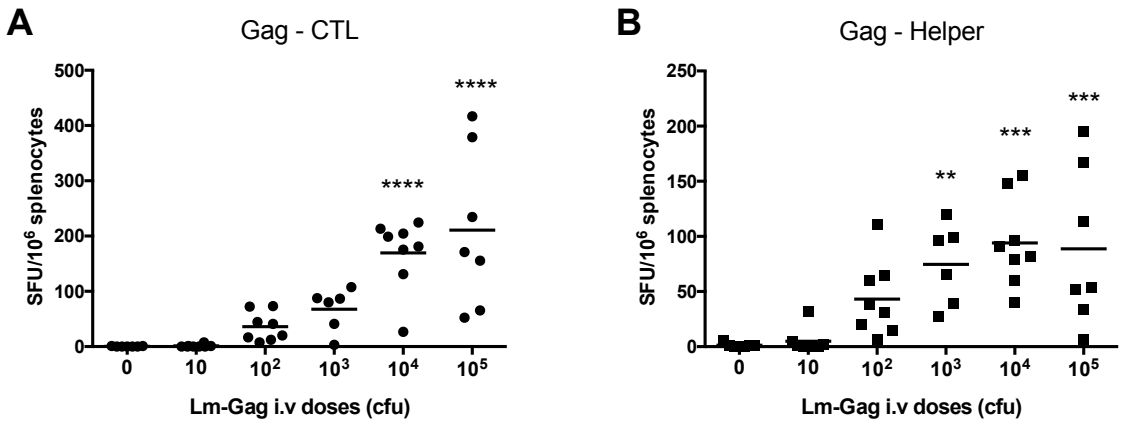


FIG 2.15 Mice immunized with Lm-Gag intravenously induced vaccine responses in a dose-dependent manner. Splenocytes from naïve and vaccinated groups were harvested and immune responses to the immunodominant $CD4^+$ helper (**A**) or CTL peptides (**B**) of HIV-1 Gag were measured by IFNγ ELISpot. Data from two independent experiments were pooled and plotted individually as shown. Differences between

vaccinated and naïve groups were analyzed by one-way ANOVA, Dunnett post hoc test, **p<0.01, ***p<0.001 and ****p<0.0001.

While CTL and or T helper 1 vaccine responses to all three doses, 10^3, 10^4 and 10^5cfu was statistically significant compared to naïve mice, responses generated by 10^5cfu Lm-Gag were saturated and had high variation compared to the other two doses (Fig. 2.15). Thus, only two doses 10^3 and 10^4cfu were used for the optimization with SEA co-administration.

Our previous data suggested intraperitoneal as the optimal route for SEA delivery. Therefore we evaluated the immune responses in mice vaccinated intravenously with 10^3cfu or 10^4cfu, each with or without intraperitoneal SEA co-administration using the schedule in Figure 2.1. As shown in Figure 2.16, all animals survived after vaccination with or without SEA, showing that by employing an appropriate dose by the intravenous route for Lm-Gag, coincident with the intraperitoneal route for SEA, we were able to overcome earlier problems with synergistic hyperimmuneactivation.

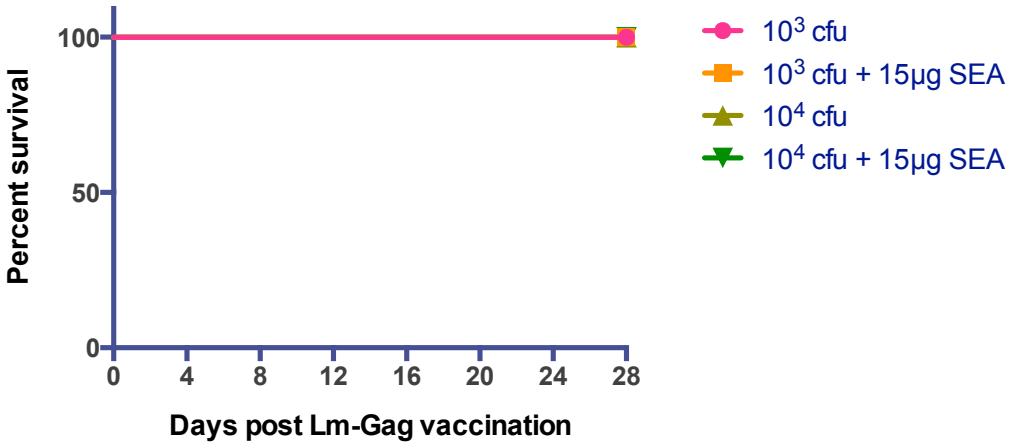


FIG 2.16 Mice survived immunization with Lm-Gag intravenously with SEA administered intraperitoneally. Survival curve when mice were vaccinated with Lm-Gag vaccine intravenously with or without SEA.

As shown in Figure 2.17, intraperitoneal administration of 15µg of SEA along with intravenous administration of 10^3cfu Lm-Gag vaccine significantly increased immune responses to both CTL and T helper 1 epitopes of HIV-Gag compared to vaccine alone. Using the higher dose of Lm-Gag (10^4cfu), both Lm-Gag helper and CTL responses were saturated and therefore we could not detect any differences between the SEA-Lm-Gag and Lm-Gag alone groups (Fig. 2.17). These results suggest that Lm-Gag intravenous dose of 10^3cfu is optimal for further analysis of SEA effect on the Lm-Gag vaccine.

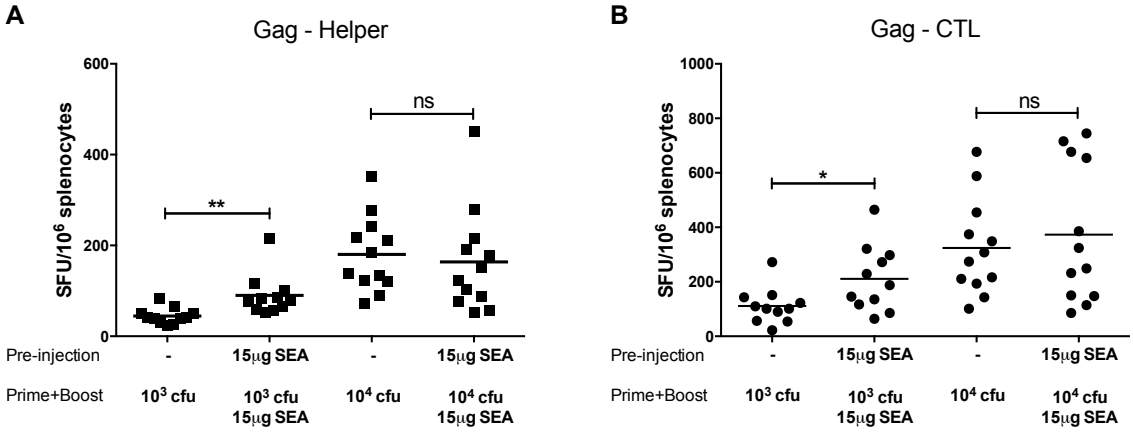


FIG 2.17 Co-administration of SEA increases both helper and CTL responses induced by low dose Lm-Gag. Mice were vaccinated intravenously with Lm-Gag at two doses, 10^3cfu or 10^4cfu, each with and without SEA intraperitoneal co-injection**.** Splenocytes were harvested and immune responses to the immunodominant $CD4^+$ helper (**A**) or CTL peptides (**B**) of HIV-1 Gag were measured by IFNγ ELISpot. Data from two independent experiments were pooled and plotted individually as shown. Statistical differences between groups were analyzed by one-way ANOVA and Tukey post hoc test, *$p<0.05$, **$p<0.01$ and ns-non-significant.

Lastly, we compared how two different doses of SEA (15 and 30μg) would influence the response to the Lm-Gag vaccine. Naïve, unvaccinated mice were controls. In this optimization, we measured the immune response to HIV Gag helper and CTL peptides and to LLO protein.

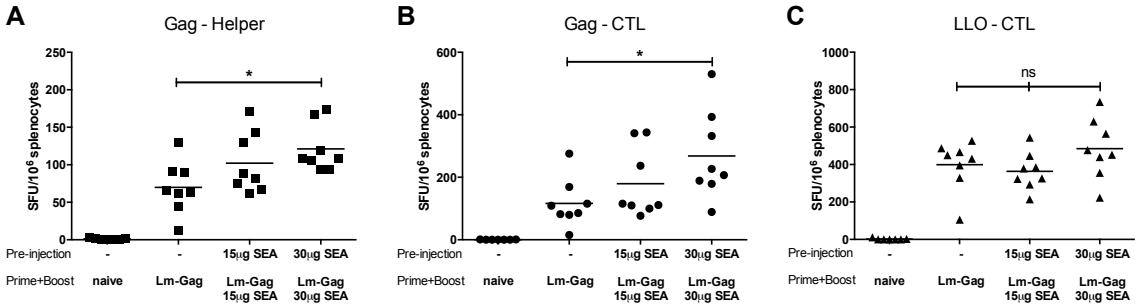


FIG 2.18 Higher dose SEA elevates both helper and CTL responses to Lm-Gag. Mice were primed intraperitoneally with 15 or 30μg of SEA, then vaccinated intravenously with Lm-Gag at two doses, 10^3cfu or 10^4cfu, each with and without SEA intraperitoneal co-injection**.** Splenocytes were harvested and immune responses to the immunodominant HIV-1 Gag $CD4^+$ helper (**A**) or CTL peptides of HIV-1 Gag (**B**) or LLO (**C**) were measured by IFNγ ELISpot. Data from two independent experiments were pooled and plotted as shown. Statistical differences between groups were analyzed by one-way ANOVA and Tukey post hoc test, *$p<0.05$ and ns-non-significant.

As expected, naïve unvaccinated group did not respond to any peptides (Fig. 2.18). Although both SEA/Lm-Gag groups had prominent increases in helper and CTL responses to HIV Gag, only mice vaccinated with 30μg SEA induced significant enhancement of these responses compared to Lm-Gag alone group. All vaccinated groups responded strongly to LLO peptide. However, such responses were not significantly different in the SEA co-administration groups. This was likely due to *Listeria*'s robust induction of LLO-specific cell-mediated responses that were saturated at high levels (Fig.

2.18). The results from these optimization trials provide additional evidence that SEA is able to enhance Th1-type responses to the Lm-Gag vaccine. Further, we determined that the 30μg dose of SEA was required in SEA/Lm-Gag group to gain statistical significance.

5. Adjuvant effect of SEA was not due to endotoxin

Lastly, we performed experiments to determine if endotoxin in SEA were responsible for the observed adjuvant activity. Endotoxin level in SEA was 1EU/30μg SEA or 1EU/dose. After treatment with Polymyxin B (PMB), the level of endotoxin was reduced 10-fold to 0.1EU/SEA dose. Using the optimal vaccination parameters, we compared the vaccine responses of Lm-Gag alone with SEA/Lm-Gag groups in which SEA was either at 1EU or 0.1EU/dose. Similar to earlier observations, SEA/Lm-Gag increased responses to both HIV-1 Gag helper and CTL epitopes (Fig. 2.19).

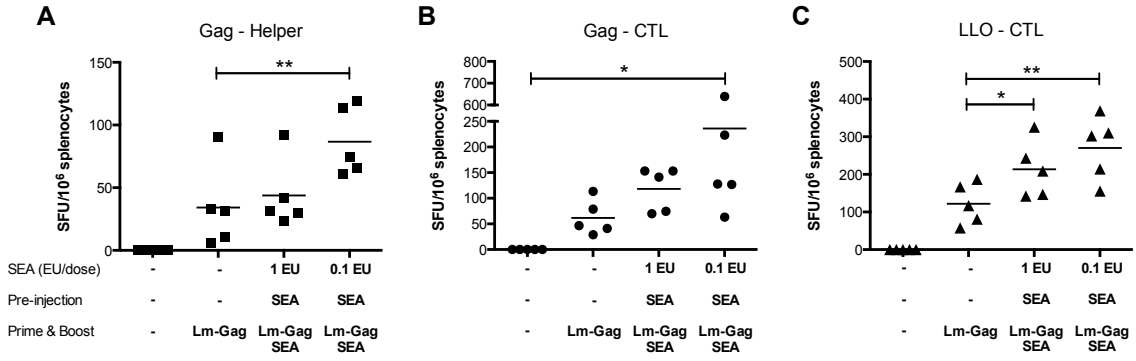


FIG 2.19 Adjuvant effect of SEA on Lm-Gag vaccine was not due to endotoxin. SEA was treated with PMB and limulus assay measured endotoxin levels were reduced 10-fold from 1EU to 0.1EU after treatment. Mice were injected with 1EU and 0.1EU of SEA separately then vaccinated intravenously with Lm-Gag at 10^3cfu, with and without appropriate SEA boost. Splenocytes were harvested and immune response to the immunodominant HIV-1 Gag $CD4^+$ helper (**A**) or CTL peptides of HIV-1 Gag (**B**) or LLO (**C**) were measured by IFNγ ELISpot. Data was plotted individually as shown.

Statistical differences between groups were analyzed by one-way ANOVA and Tukey post hoc test, *$p<0.05$ and **$p<0.01$.

When SEA was treated with PMB to reduce limulus measureable endotoxin to 0.1EU/dose, SEA adjuvant effect on Lm-Gag did not wane but instead enhanced significantly. As shown in Figure 2.19, when 0.1EU SEA was co-administered with Lm-Gag vaccine, vaccine responses to Gag-Helper and LLO peptides were significantly enhanced compared to Lm-Gag alone. Response to the Gag CTL peptide in the SEA/Lm-Gag group was also significantly higher than naïve animals. Overall, our data show that possible endotoxin contamination of SEA was not responsible for the adjuvant effect of SEA. In fact lowering measureable potential endotoxin by PMB treatment actually, increase the SEA adjuvant effects on the Lm-Gag.

IV. Conclusions

Enhancing $CD4^+$ Th1 and $CD8^+$ T cell vaccine specific responses has been a long-term goal of those working to develop vaccines for HIV, tuberculosis, malaria and many other infectious diseases. Schistosome soluble egg antigens (SEA) are known to drive $CD4^+$ Th2-type responses. Thus, we hypothesized that addition of SEA to the strong, pro-inflammatory *Listeria* vector HIV-1 vaccine (Lm-Gag) would reduce generation of $CD4^+$ Th1-type and $CD8^+$ CTL responses. Surprisingly, in contrast to our hypothesis, addition of SEA to the Lm-Gag vaccine enhanced both $CD4^+$ Th1-type and $CD8^+$ CTL vaccine responses (Fig. 2.3). These results led us to revise our hypothesis to propose that there are molecules in SEA with pro-inflammatory properties that can act as a CTL adjuvant and enhance vaccine efficacy.

The pro-inflammatory response induced by co-administration of SEA to the Lm-Gag vaccine was so powerful that it could become lethal to the animal host. We observed a continuous increase in levels of IL-6 and TNFα cytokines in sera and peritoneal cavity of SEA/Lm-Gag group, even after 48 hours post vaccination (Fig. 2.6-7). We postulate that elevated TNFα was partly responsible for the increase in levels of liver AST and ALT (Fig. 2.8), and in turn caused the animals to succumb to complications of immune activation (Fig. 2.5)

After multiple parameter modifications, we successfully optimized the SEA/Lm-Gag delivery system (Fig. 2.1) and immunized mice intravenously with Lm-Gag at 10^3cfu with 30μg SEA co-administration intraperitoneally. Using this refined immunization protocol, we were able to balance the effect between SEA and Lm-Gag vaccine and generate the best vaccine efficacy (Fig. 2.18). The potential presence of endotoxin in SEA was ruled out as the Th1-type driving factor in SEA (Fig. 2.19). Finally, removal of potential endotoxin in SEA by PMB treatment actually improved the adjuvant effects of SEA on the Lm-Gag vaccine (Fig. 2.19).

CHAPTER 3

SCHISTOSOMA MANSONI SOLUBLE EGG ANTIGENS ENHANCE *LISTERIA MONOCYTOGENES* VECTOR HIV-1 VACCINE INDUCTION OF CYTOTOXIC T CELLS[1]

[1] Bui C, Shollenberger LM, Paterson Y, Harn DA. Accepted by *Clinical and Vaccine Immunology*. Print here with permission of the publisher.

I. Abstract

Vaccines are an important public health measure for prevention and treatment of diseases. In addition to the vaccine immunogen, many vaccines incorporate adjuvants, to stimulate the recipient's immune system and enhance vaccine-specific responses. While vaccine development has advanced from attenuated organism to recombinant protein or use of plasmid DNA, the development of new adjuvants that safely increase immune responses has not kept pace. Previous studies have shown the complex mixture of molecules that comprise saline soluble egg antigens (SEA) from *Schistosoma mansoni* eggs functions to promote $CD4^+$ T helper 2 (Th2) responses. Therefore, we hypothesized that co-administration of SEA with a *Listeria* vector HIV-1 Gag (Lm-Gag) vaccine would suppress host cytotoxic T lymphocyte (CTL) and T helper 1 (Th1) responses to HIV-1 Gag epitopes. Surprisingly, instead of driving HIV-1 Gag-specific responses towards Th2-type, we found that co-administration of SEA with Lm-Gag vaccine significantly increased the frequency of IFNγ producing Gag-specific Th1 and CTL responses over that seen in mice administered Lm-Gag only. Analysis on the functionality and durability of vaccine responses suggested that SEA not only enlarged different memory T cell compartments but induced functional and long-lasting vaccine-specific responses as well. These results suggest there are components in SEA that can synergize with potent inducers of strong and durable Th1-type responses such as *Listeria*. We hypothesize SEA contains moietie(s) that if defined, can be used to expand type 1, pro-inflammatory responses for use in vaccines.

II. Introduction

Adjuvants are an integral and critical aspect of many vaccines. In general, adjuvants activate innate immune cells either as a function of their particulate nature, or by ligating pattern recognition receptors on antigen presenting cells, increasing their overall antigen processing capabilities resulting in enhanced vaccine-specific immune responses (58, 59). For immune compromised or low-responder populations, adjuvants in vaccines increase responder rates. For example, the adjuvant MF59 is incorporated into an influenza vaccine to enhance vaccine efficacy in elderly recipients (60, 61). By enhancing vaccine immunogenicity, adjuvants may allow for dose-sparing in vaccines, driving comparable immune responses with lower amounts of antigen. Dose-sparing is a critical consideration when urgent large-scale vaccination is needed and vaccine production limited (62-64). Similarly, use of adjuvants may reduce the number of vaccinations required for any given vaccine, easing compliance issues and, in much of the world, logistical challenges. The lack of new, safe adjuvants has limited the potency and efficacy of many existing and new generation vaccines. Thus, development of functional and safe adjuvants is essential and beneficial in multiple ways.

According to the Centers for Disease Control and Prevention, aluminum gels or aluminum salts are the only adjuvants currently licensed for human use in the United States (65). Alum adjuvants consist of precipitates of aluminum phosphate and or aluminum hydroxide to which vaccine antigens/organisms are adsorbed (67, 68). Aluminum salts enhance humoral immunity (69), and can trigger necrotic cell death and the release of the endogenous danger signal, uric acid (68, 78). Alum has been safely

used for decades, though controversy remains concerning the mechanism of action of alum adjuvants and potential toxicity, emphasizing the need for better adjuvants.

Previously, we demonstrated that infection with the helminth parasite *Schistosoma mansoni* suppressed/eliminated the ability of recipient mice to generate T cell responses to a plasmid DNA HIV-1 vaccine (53, 133). *Schistosomes* induce $CD4^+$ Th2 biasing and IL-10 mediated immune suppression, primarily by deposition of parasite eggs into host tissues (135, 196-201). Schistosome egg induction of anti-inflammatory responses is essential in reducing hepatic inflammation and is key for host survival (202-207). Similar to schistosome eggs, the saline homogenate of schistosome eggs, SEA, also induces strong $CD4^+$ Th2 responses (135, 202, 203, 208-211). In this regard, co-administration of SEA with a third party protein antigen led to an increase in vaccine-specific Th2-type responses (142). Therefore, we hypothesized that addition of SEA to a Th1-driving *Listeria* vector HIV-1 Gag vaccine, would suppress the induction of Type 1, pro-inflammatory T cell responses. Here, we tested this hypothesis and found, unexpectedly, that SEA functioned to enhance Type 1 pro-inflammatory T cell responses, enhancing not only Lm-Gag vaccine efficacy, but also Lm-Gag Th1-type responses and expansion of Lm-Gag specific T cell compartments.

III. Materials & Methods

Biological Materials. The vaccine we tested was comprised of an attenuated strain of *Listeria monocytogenes* expressing the HIV-1 IIIB Gag protein (Lm-Gag) (212). As control vaccine, we used the same *Lm* strain expressing the E7 oncoprotein of the human papillomavirus 16 (Lm-E7) (213). All *Listeria* vector vaccines were grown in BHI

supplemented with streptomycin. Five to 7 week old female BALB/c mice were purchased from Harlan and Jackson Laboratories, housed in specific pathogen-free conditions and allowed to acclimate for one week prior to manipulation. All animal work was performed in accordance with institutional policy and approved by the institutional animal care and use committee.

Preparation of SEA. Approximately seven weeks post-infection, we removed infected livers from *Schistosoma mansoni* (PR strain) infected Swiss Webster mice provided by the NIAID schistosomiasis resource center. In addition, we infected female BALB/c mice by intraperitoneal injection of 100-150 *S. mansoni* cercariae. Parasite eggs were isolated from livers of infected mice and combined from both sources. SEA was prepared as previously described (202). The protein concentration of SEA was determined by both the Bradford and BCA protein assays (Thermo Scientific).

Vaccination of mice. Six to 8 week old BALB/c mice were injected intraperitoneally with 30μg of SEA or left naive. One week later, mice were primed intravenously with 10^3cfu Lm-Gag vaccine, with or without 30μg of SEA injected intraperitoneally, control Lm-E7 vaccine (matched CFU dose) or left unvaccinated. Mice were boosted two weeks after the prime in an identical manner. Mice were sacrificed two weeks post last vaccination (wplv), unless otherwise indicated.

ELISpot. Splenocytes were harvested, plated at 300K cells per well in IFNγ ELISpot plates (BD) and incubated with complete media (RPMI-1640 supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B, 2mM L-glutamine, 5μM β-mercaptoethanol and non-essential amino acids) in the presence of 20μM specific CTL peptide (H2-Kd-restricted, AMQMLKETI from HIV-1 IIIB Gag

protein), 20μM irrelevant peptide (H2-Kd-restricted, TYQRTRALV from influenza A/PR/8/34 nucleoprotein), 20μM specific helper peptide (Class II-restricted, NPPIPVGEIYKRWIILGLNK from HIV-1 IIIB Gag protein), 20μM LLO peptide (H2-Kd-restricted, GYKDGNEYI from *L. monocytogenes* listeriolysin O), or 1 μg/ml con A (positive control). Peptides were synthesized by Biosynthesis, Inc at greater than 95% purity and reconstituted in DMSO prior to dilution in media. After 20 hours incubation, ELISpots were performed according to manufacturer's instructions and enumerated using an Immunospot analyzer (C.T.L.).

Cytometric bead array (CBA). Splenocytes were harvested, plated at 1.5 million cells per well in 48-well plates and stimulated with 20μM peptide, 25μg/ml SEA, 1μg/ml conA or left unstimulated for 72 hours. Cytokine levels in supernatant were measured using a Th1/Th2/Th17 CBA kit according to the manufacturer's instructions (BD).

***In vivo* cytotoxic T lymphocyte (CTL) assay.** To prepare targets, splenocytes from naïve BALB/c mice were fluorescently labeled with 1μM or 10μM green (Vybrant CFDA-SE Cell Tracer Kit) fluorescent dye, according to the manufacturer's instructions (Invitrogen). Cells were then pulsed for 2 hours with 20 μM irrelevant or CTL peptide, respectively. Targets were mixed and twelve million cells were injected intravenously per mouse. After 18 hrs, splenocytes were collected and analyzed for target recovery. The level of Gag-specific killing was calculated by the formula: % specific killing = $\{1-(r_{naive}/r_{immunized})\}$ x100, where r = % $CFSE^{low}$ cells/% $CFSE^{high}$ cells (214).

Flow cytometry. To evaluate different T cell populations, splenocytes were incubated in the presence of PMA, Ionomycin and GolgiStop for 6 hours. Cells were stained with α-CD3ε, α-CD8a and α-CD4 antibodies (BD) then fixed. After membrane

permeabilization, intracellular proteins were stained using α-IFNγ, α-IL-4, α-IL-17A, α-IL-10 or α-FoxP3 antibodies (BD). Live cells (as indicated by LIVE/DEAD fixable dye, Invitrogen) were acquired and analyzed using a BD LSRII flow cytometer running FACS Diva (BD) or Flowjo (TreeStar) software.

Statistical Analysis. Statistical analyses were performed using one-way or two-way ANOVA with Newman Keuls or Bonferroni post-hoc test, as detailed in the figure legends (Prism Graphpad Software, La Jolla, CA).

IV. Results

1. SEA co-administration with *Listeria* vector HIV-1 Gag vaccine enhances pro-inflammatory Gag-specific vaccine responses

To evaluate the effect of SEA on immunogenicity of Lm-Gag vaccine, three animal groups were immunized and analyzed for vaccine-specific responses, including: naïve unvaccinated, Lm-Gag vaccinated (Lm-Gag) and SEA co-administered Lm-Gag (SEA/Lm-Gag) groups. Two wplv, Gag-specific responses were determined by IFNγ ELISpot after stimulation with *Listeria* LLO peptide or HIV Gag-specific CTL and helper peptides. As expected, cells from naïve control mice did not respond to any of the peptides. Meanwhile, SEA co-administration significantly enhanced HIV Gag-specific vaccine responses toward immunodominant CTL (Fig. 3.1A) and helper (Fig. 3.1B) epitopes compared to the Lm-Gag only group. There was no significant difference in response to LLO between *Listeria* vector vaccinated groups (Fig. 3.1C), most likely due to *Listeria*'s robust induction of LLO-specific cell-mediated responses that were saturated at high levels. An IL-4 ELISpot assay was also performed; however, there were minimal

numbers of spots produced for all animal groups (Fig. S3.2), which indicated that Lm-Gag does not induce Th2 type vaccine responses. In preliminary experiments, we injected mice with 50μg of SEA (a higher dose) alone, and this did not induce any IFNγ-producing CTL or T helper 1 responses to HIV-1 Gag (Fig. S3.1A). Further, injection of 50μg SEA alone did not generate CTL effectors capable of Gag-specific killing (Fig. S3.1B). As these results showed SEA itself did not induce Gag-specific responses, we did not include an SEA alone group in the remainder of our studies.

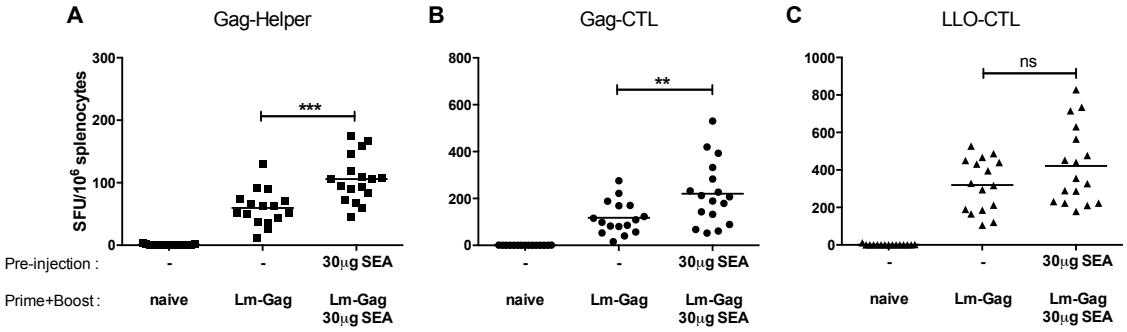


FIG 3.1 SEA enhances vaccine-specific IFNγ-producing Th1 and CTLs. Splenocytes from naive and vaccinated mice were collected 2wplv and immune responses to the immunodominant HIV Gag $CD4^+$ helper (**A**) and CTL epitopes for HIV Gag (**B**) and LLO (**C**) were analyzed by IFNγ ELISpot. Data were pooled from two similar, independent experiments and results from individual mice (n=16) were plotted. Statistical analysis was performed using one-way ANOVA with Newman Keuls post-hoc test, **p<0.01, ***p<0.001 and ns-not significant.

To validate that the responses measured by ELISpot result from functional CTL effector cells, an *in vivo* CTL assay was performed. Lm-E7 vaccinated mice were included as vector control group. As expected, the % measureable CFSE-high, Gag-specific, targets were similar between both the naïve and Lm-E7 control groups (Fig. 3.2A), demonstrating that immunization with the Lm vector does not induce target

killing. Mice vaccinated with Lm-Gag alone modestly reduced the level of Gag-specific targets, whereas the SEA/Lm-Gag group had a significant reduction of Gag-specific targets (Fig. 3.2A). As Gag-specific target recovery was higher than that of irrelevant targets, shown in histogram of naïve mice, the actual level of Gag-specific killing by the SEA/Lm-Gag group was higher than the observed reduction in the histogram. The percentages of Gag specific killing of all individual animals from each group were calculated (Fig. 3.2B). Killing of Gag specific target cells in the Lm-Gag vaccinated group was between 15-20%. The SEA/Lm-Gag group significantly increased specific killing to an average of 40%. These results show that addition of SEA to the Lm-Gag vaccine significantly enhanced not only total Gag-specific IFNg producing T cells, but also increased functional Gag-specific CTL effector cells generated by *Listeria* vector vaccines.

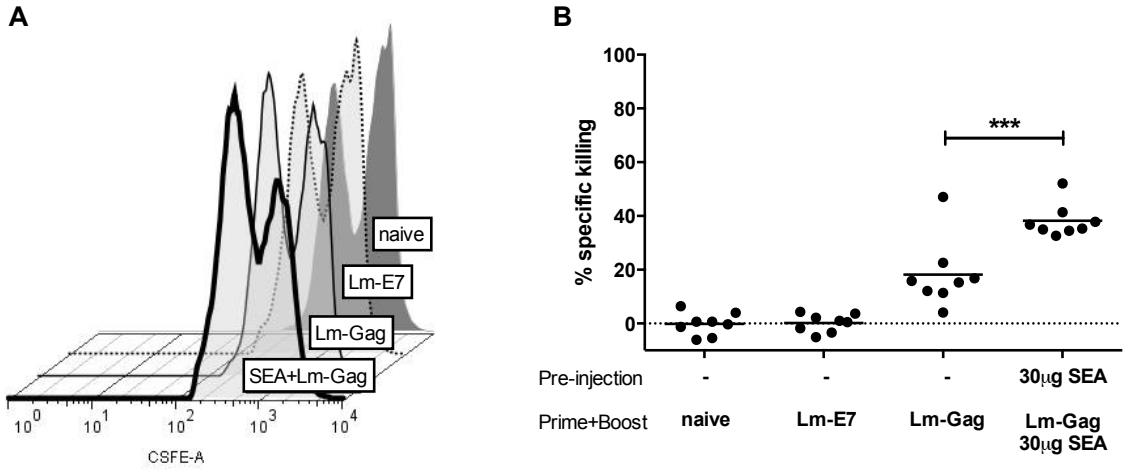


FIG 3.2 SEA augments functional CTL response of vaccinated mice *in vivo*. Naive splenocytes were stained with CFSE at low and high concentrations then pulsed with an irrelevant or immunodominant CTL epitope of HIV Gag, respectively. These targets were injected into naive or vaccinated mice. After 18 hours, splenocytes were collected and analyzed for specific killing of targets using flow cytometry. (**A**) Representative histograms of irrelevant (left peak) and HIV Gag (right peak) targets

recovered from each animal group were shown. (**B**) Representative data from three independent experiments were shown. Levels of specific killing in individual mice (n=8) were plotted and the groups were compared by one-way ANOVA and Newman-Keuls post-hoc test, ***$p<0.001$.

2. SEA co-administration significantly increases multiple Gag-specific T cell populations and induces vaccine-specific Th1-type cytokine production in vaccinated mice

To investigate total T cell compartment composition in Lm-Gag vaccinated mice plus or minus SEA co-administration, splenocytes from naïve and vaccinated groups were harvested 2wplv and analyzed by flow cytometry after polyclonal stimulation with PMA and ionomycin. As shown in Figure 3.3, the percentages of all analyzed T cell populations in mice vaccinated with Lm-Gag only were similar to those of naïve mice. In contrast, addition of SEA to the Lm-Gag vaccine regimen significantly increased the quantity of Th1, Th2 and two different Treg populations compared to both naïve and Lm-Gag only groups (Fig. 3.3A-B,D-E). Th17 cells were unaltered by addition of SEA (Fig. 3.3C).

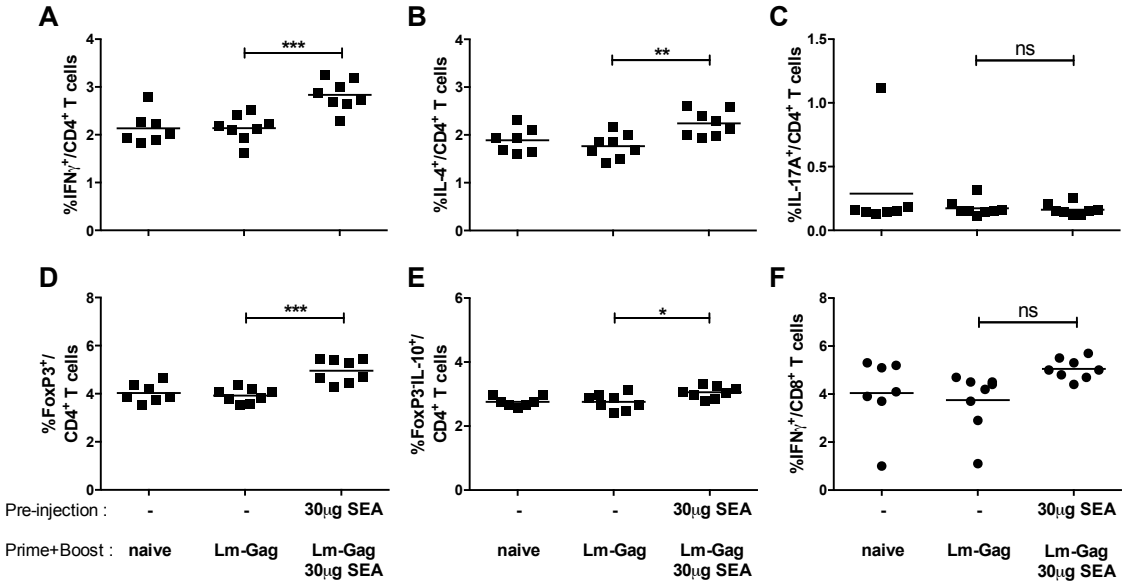


FIG 3.3 SEA increases multiple T cell populations when co-administered with Lm-Gag vaccine. Splenocytes from naïve and vaccinated mice were collected 2wplv and assayed by flow cytometry for Th1 (**A**), Th2 (**B**), Th17 (**C**) and Tregs (**D-E**) or IFNγ-secreting CTLs (**F**). Percentages of each T cell population from individual mice (n=8) were plotted and statistical differences between groups were analyzed by one-way ANOVA and Newman-Keuls post-hoc test, *$p<0.05$, **$p<0.01$, ***$p<0.001$ and ns-not significant.

To further evaluate cytokine production, mouse splenocytes were stimulated with HIV Gag-specific peptides or *Listeria* LLO peptide and different cytokine levels measured. As shown in Figure 3.4, splenocytes from both vaccinated groups (Lm-Gag ± SEA) produced pro-inflammatory cytokines, IL-2, IL-6, IFNγ and TNFα in response to Gag helper peptide. Cells from the SEA/Lm-Gag group had significantly higher levels of these cytokines compared to levels from cells of the naive group (Fig. 3.4). Gag CTL peptide stimulation induced production of IL-6, IFNγ and TNFα but not IL-2 in both the Lm-Gag and SEA/Lm-Gag groups compared to the naïve group. There was an enhancement in IFNγ and TNFα production in cells from the SEA/Lm-Gag group compared to cells from the Lm-Gag only group, though not significant. IL-6, IFNγ and

TNFα were also produced in vaccinated groups stimulated with the LLO peptide, with the SEA/Lm-Gag but not Lm-Gag alone group having significantly enhanced production of these cytokines compared to the naïve group.

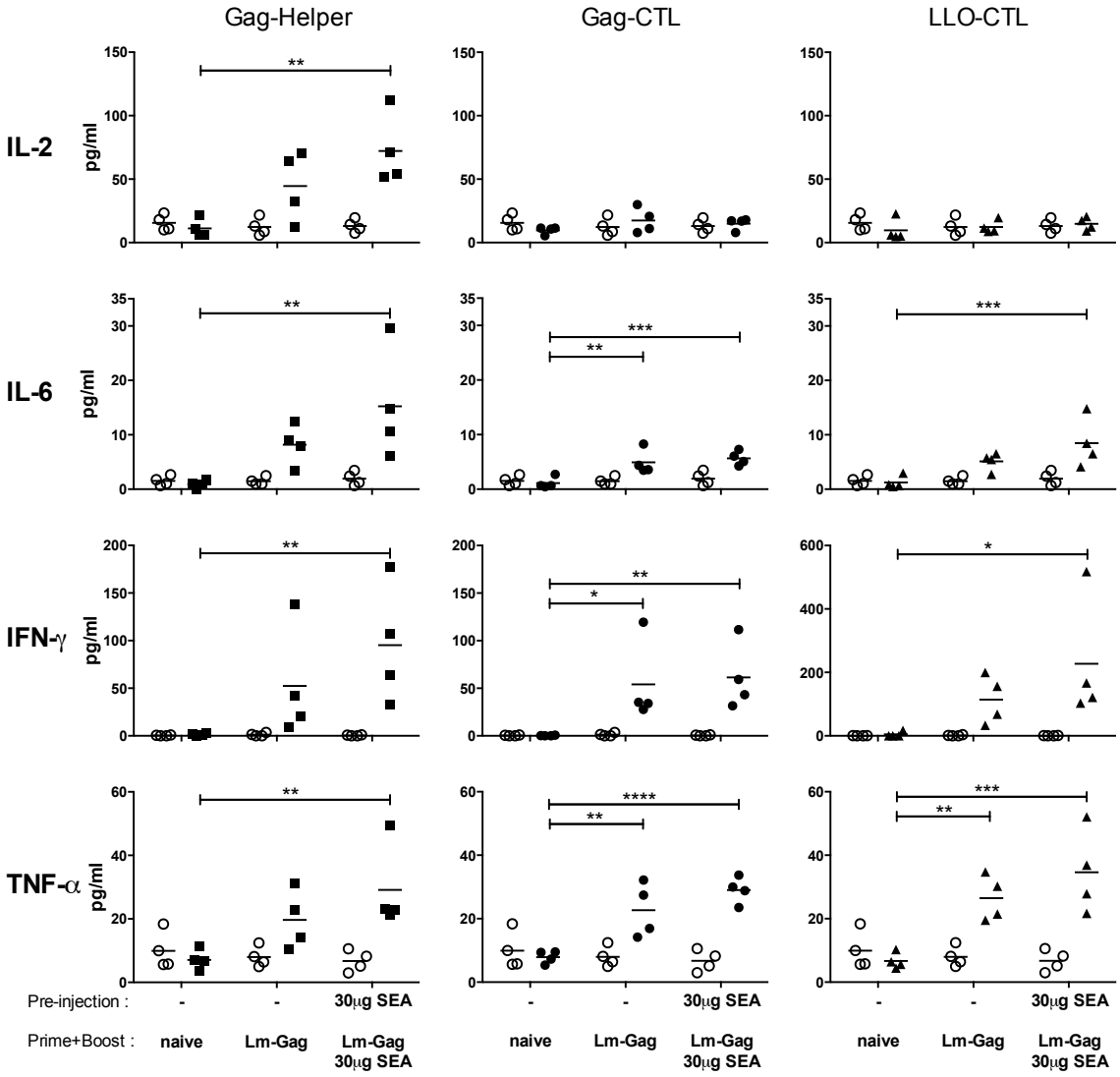


FIG 3.4 SEA increases vaccine-specific pro-inflammatory cytokine production in vaccinated mice. Splenocytes from naive and vaccinated mice were collected 2wplv and stimulated with immunodominant $CD4^+$ helper (square) and CTL epitopes for HIV Gag (circle) and LLO (triangle) for 72 hrs. Unstimulated controls (open circle) were included for each treatment. Cytokine productions in supernatants were quantified by CBA.

Statistical analysis was performed using two-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

As shown in Figure S3.3, stimulation with the HIV Gag peptides or LLO peptide did not induce production of IL-17a, IL-4 or IL-10 in splenocytes from any of the groups. This finding is interesting as SEA induces strong $CD4^+$ Th2 responses (135, 202, 203, 208-211). Thus, we examined cytokine production of naïve and vaccinated splenocytes restimulated with SEA (Fig. S3.4). As expected, SEA restimulation induced significant levels of IL-4 and IL-10 cytokines in the SEA/Lm-Gag group with minimally detectable levels of IL-17A. Interestingly SEA recall significantly increased levels of Th1-type pro-inflammatory cytokines, IL-2, IL-6, IFNγ and TNFα in the SEA/Lm-Gag group compared to naïve and Lm-Gag only groups.

3. Co-administration of SEA induces durable vaccine-specific responses

To evaluate the duration of vaccine-specific cell-mediated immune responses, splenocytes from vaccinated mice were analyzed by IFNγ ELISpot at 12 and 20 wplv. As expected, at both 12 and 20 wplv, cells from the Lm-E7 control vaccinated mice did not respond to HIV Gag epitopes and only responded to the LLO peptide, again demonstrating that the *Listeria* vector does not drive responses to HIV Gag vaccine antigens/peptides (Fig. 3.5). Similar to our earlier observations, cells from SEA/Lm-Gag vaccinated mice had significantly enhanced Gag-specific Th1 and CTL responses at both timepoints compared to cells from Lm-Gag only mice (Fig. 3.5A-B). At 20wplv, the Lm-E7 responses to LLO were significantly higher than the Lm-Gag group; however, no

significant difference in LLO response was observed between Lm-Gag ± SEA vaccinated groups at both time-points (Fig. 3.5C).

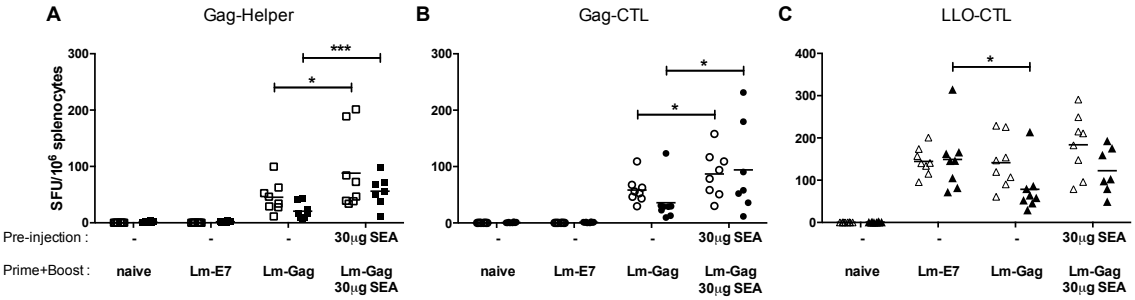


FIG 3.5 SEA improves longevity of vaccine-specific Th1 and CTL responses. Splenocytes from each animal group were collected at 12 (open symbols) and 20wplv (filled symbols) and immune response to the immunodominant $CD4^+$ helper (**A**) and CTL epitopes for HIV Gag (**B**) and LLO (**C**) were assayed by IFNγ ELISpot. Data were pooled from two similar, independent experiments and results from individual mice (n=8) were plotted. Statistical analysis was performed using one-way ANOVA with Newman Keuls post-hoc test, *$p<0.05$ and ***$p<0.001$.

V. Discussion

Our starting hypothesis was that incorporation of schistosome SEA to an Lm-Gag vaccine would suppress vaccine-specific CTL and Th1-type responses generated by the Lm-Gag vaccine. However, results clearly demonstrate that co-administration of SEA with Lm-Gag significantly enhanced responses toward immunodominant HIV Gag-specific CTL and T helper 1 epitopes compared to mice vaccinated with Lm-gag alone (Fig. 3.1). Further analysis of CTL functionality showed that the level of HIV-1 Gag-specific killing was also enhanced in the SEA/Lm-Gag group (Fig. 3.2). SEA co-administration with Lm-Gag also induced long-lasting vaccine-specific responses (Fig. 3.5). Collectively, these results suggest that completely opposite to our expectations, we found that addition of SEA to the Lm-Gag vaccine had a seemingly synergistic effect

with the Lm-Gag vaccine in enhancing Type 1, pro-inflammatory, vaccine-specific T cell responses.

Specifically, we saw a trend of SEA addition in enhancing production of pro-inflammatory cytokines, producing significantly higher levels of IL-6, IFNγ and TNFα compared to cells from naïve mice stimulated with vaccine antigens (Fig. 3.4). These cytokines were likely secreted by CTL and Th1 cells, which were increased in quantity by addition of SEA to the Lm-Gag vaccine (Fig. 3.3A, D). Meanwhile, there was no significant production of vaccine-specific IL-4, IL-17A and IL-10 cytokines in any of the groups (Fig. S3.3). These results strengthen our overall observation that SEA significantly enhanced vaccine-specific Th1-type cytokine production when administered in concert with the Lm-Gag vaccine.

SEA is known to drive $CD4^+$ Th2 biasing (135, 202, 203, 208-211), evidenced in our study by the significant increase in Th2 and Treg populations (Fig. 3.3) associated with significant production of IL-4 and IL-10 in SEA/Lm-Gag mice (Fig. S3.4). However, levels of IL-6, IFNγ and TNFα were also significantly increased, suggesting an ongoing induction of both Th1 and Th2-type responses when SEA is co-administered. Our observation that SEA functions to enhance Lm-Gag efficacy is exciting and unexpected. How Th2-driving SEA functions to enhance vaccine-specific pro-inflammatory T cell responses remains to be discovered, as previous reports demonstrate that addition of SEA to protein vaccines enhances Th2-type responses (135, 138, 142).

While immunoparasitologists categorize SEA as a Th2-type and anti-inflammatory driving mixture of molecules, there is evidence that SEA can also induce pro-inflammatory responses *in vivo*. Immediately post the onset of egg-laying by adult

worms, the host immune responses to eggs are predominantly $CD4^+$ Th1-type coincident with an increase in total numbers of pro-inflammatory, classically activated macrophages (140). Several reports describe the ability of schistosome antigen(s) to ligate and initiate signaling via host pattern recognition receptors including the Toll like receptors (TLRs), driving pro-inflammatory responses (222-226). Recently, SEA was shown to drive production of pro-inflammatory mediators from primary human placental trophoblasts (227, 228). Thus, SEA does contain molecules that stimulate pro-inflammatory responses, and these appear to work in juxtaposition to SEA molecules that drive Th2-type and anti-inflammatory responses. The latter responses occur via SEA molecule ligation of C-type lectins or TLRs (225, 229).

As noted earlier, one possible explanation for our unexpected results is that we are observing a synergy between the pro-inflammatory components in SEA and the pro-inflammatory molecules expressed by the *Listeria* vector used in this study, as Lm-Gag alone did not drive the same levels of vaccine-specific Th1 and CTL responses as seen in mice immunized with SEA/Lm-Gag. Based on the results of our study, we suggest that vaccination with the Th1 vaccine Lm-Gag and additional SEA injections amplify pro-inflammatory vaccine-specific responses, overpowering the Th2-Treg response normally driven by SEA. This allows host vaccine-specific immune responses to polarize toward pro-inflammatory. The pro-inflammatory milieu would amplify classical activation of macrophages, promoting recruitment of immune cells and pro-inflammatory (Th1 and CTL) activation of vaccine-specific $CD4^+$ and $CD8^+$ T cells.

Though SEA has been studied extensively, most of the "immune activating" components are not yet identified. SEA is a saline homogenate of *S. mansoni* eggs and

therefore contains a myriad of different molecules and classes of compounds, including proteins, lipids, glycolipids, carbohydrates and nucleic acids. Numerous studies describe the ability of SEA to induce Th2-type and anti-inflammatory responses as being dependent on carbohydrate components (150, 151, 153, 154, 230). In contrast, reports on schistosome molecules that drive Th1 pro-inflammatory responses are scarce. Aksoy et al. has described double stranded RNAs from schistosome eggs that activate dendritic cells via TLR3 (226), while Duraes et al. found schistosome tegumental molecules that induce IL-12 and TNFα production in dendritic cells (222). The most recently described pro-inflammatory molecule from schistosomes is a glycolipid obtained from extracts of adult worms (224). Thus, our next priority is to chemically fractionate SEA and define the actual class(es) of molecules that are responsible for the pro-inflammatory enhancing activity reported in this study, as well as determining if these observations are limited only to when SEA is co-administered with specific types of vector based vaccines.

VI. Acknowledgement

Infected animals were provided by BRI via the NIAID schistosomiasis resource center under NIH-NIAID Contract No. HHSN272201000005. These materials can be obtained by contacting BEI Resources. The authors would also like to thank the University of Georgia - College of Veterinary Medicine Cytometry Core Facility. This work is supported by grants NIH-AI-071883 and NIH-AI-078787 awarded to Donald A. Harn.

VII. Supplemental

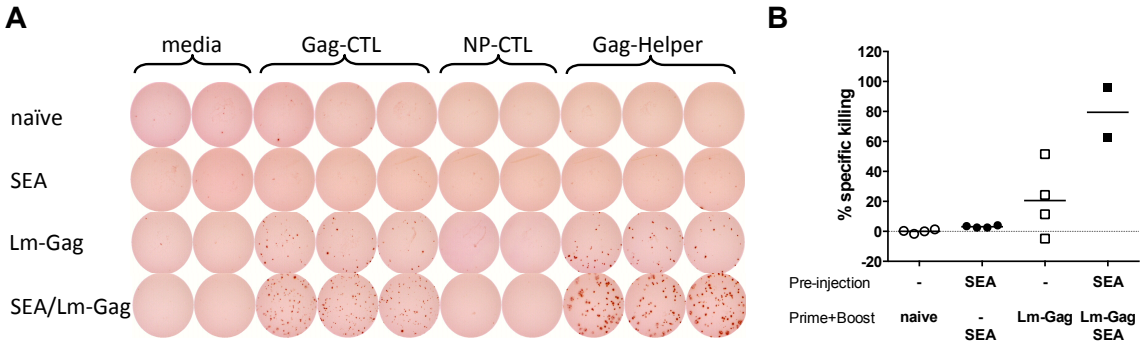


FIG S3.1 SEA alone does not induce Gag-specific CTL or T helper 1 responses. Mice were left naive or vaccinated with Lm-Gag, each with and without co-administration of 50µg SEA. (**A**) Splenocytes from all animal groups were harvested 2wplv and immune response to the immunodominant $CD4^+$ helper and CTL epitopes for HIV Gag were measured by IFNγ ELISpot. Representative ELISpot data from each group were shown. (**B**) Level of Gag-specific killing was measured using *in vivo* CTL assay as described. Data from individual mice (n=2-4) were plotted.

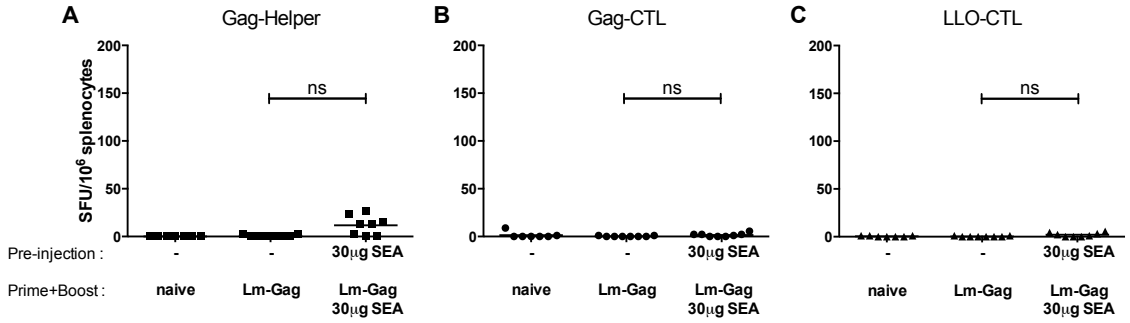


FIG S3.2 SEA does not affect vaccine-specific IL-4 producing Th2 response. Splenocytes from naive and vaccinated mice were harvested 2wplv and immune response to the immunodominant $CD4^+$ helper (**A**) and CTL epitopes for HIV Gag (**B**) and LLO (**C**) were measured by IL-4 ELISpot. Results from individual mice (n=8) were plotted. Statistical analysis was performed using one-way ANOVA with Newman Keuls post-hoc test, ns-not significant.

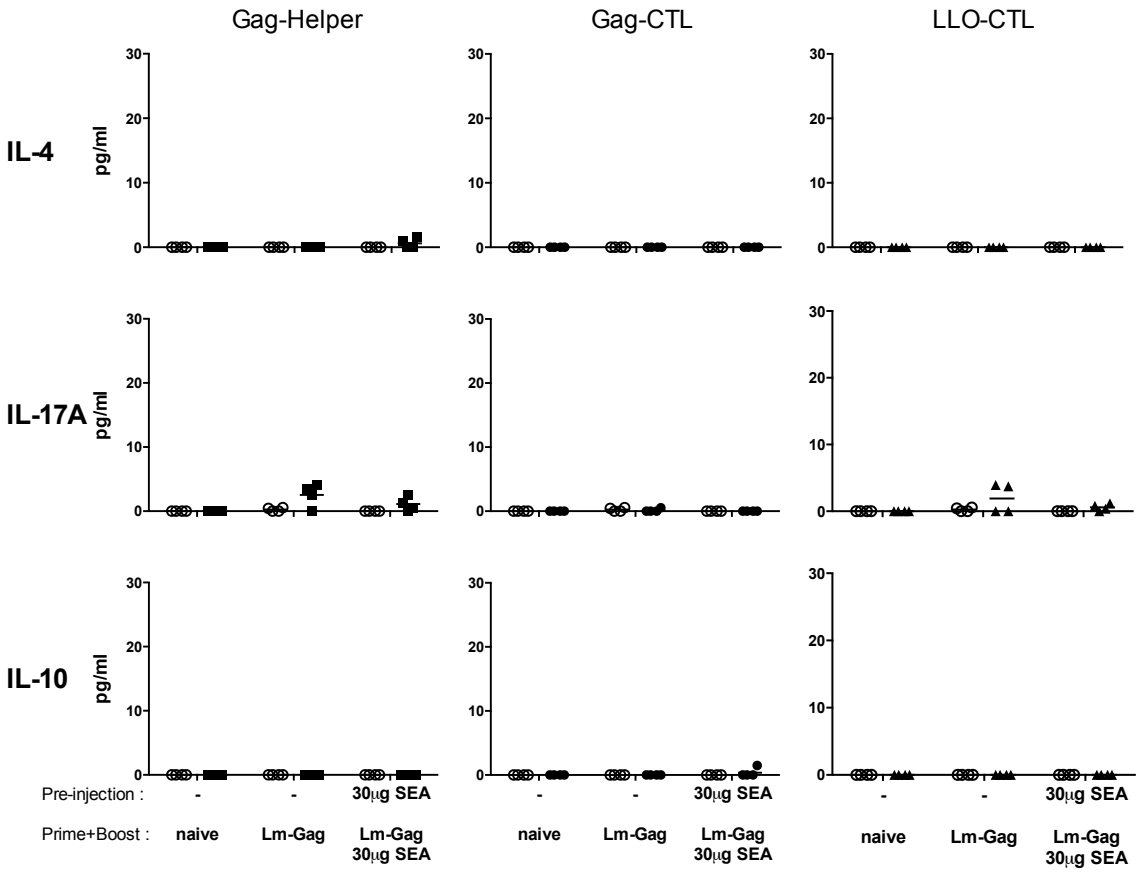


FIG S3.3 SEA co-administration does not induce vaccine-specific Th2 or Th17 cytokine production in mice. Splenocytes from naive and vaccinated mice were collected 2wplv and stimulated with immunodominant $CD4^+$ helper (square) and CTL epitopes for HIV Gag (circle) and LLO (triangle) for 72 hrs. Unstimulated controls (open circle) were included for each treatment. Cytokine productions in supernatants were quantified by CBA. Statistical analysis was performed using two-way ANOVA with Bonferroni post-hoc test.

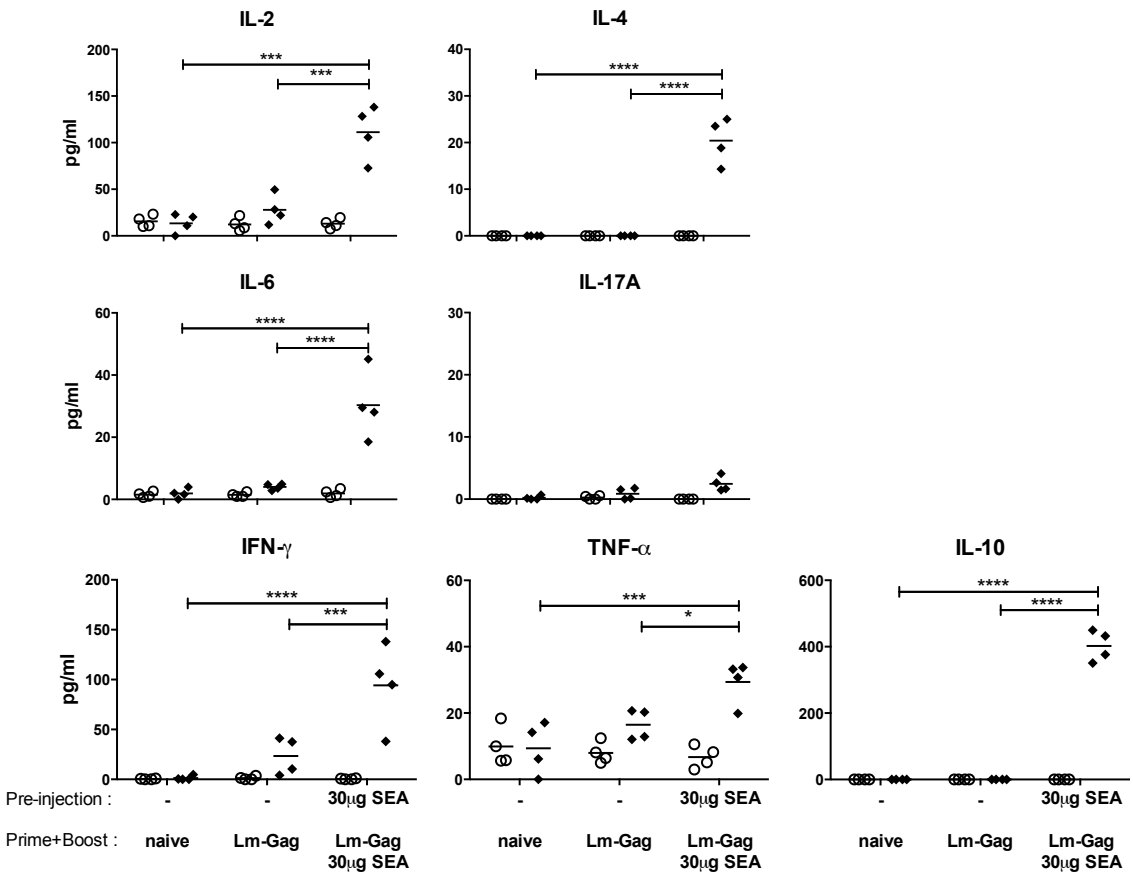


FIG S3.4 SEA/Lm-Gag vaccinated mice promote a mixed Th1/Th2 cytokine profile upon SEA re-stimulation. Splenocytes from naive and vaccinated mice (n=4) were collected 2wplv and stimulated with SEA (diamond) or left unstimulated (open circle) for 72 hrs. Cytokine productions in supernatants were quantified by CBA. Statistical analysis was performed using two-way ANOVA with Bonferroni post-hoc test, *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001.

CHAPTER 4

SCHISTOSOMA MANSONI SOLUBLE EGG ANTIGENS ENHANCE T CELL RESPONSES TO A NEWLY IDENTIFIED HIV-1 GAG H-2^b EPITOPE[2]

[2] Bui C, Shollenberger LM, Paterson Y, Harn DA. To be submitted to *Infection and Immunity*.

I. Abstract

Human schistosomiasis induces significant T helper type-2 (Th2) and anti-inflammatory immune responses and has been shown to negatively impact vaccine efficacy. Our initial goal was to determine if administration of schistosome soluble egg antigens (SEA) would negatively influence the induction of Th1-type T cell responses to an HIV candidate vaccine in the Th1-biased C57BL/6 mouse strain. Initial experiments failed, as we were unable to detect any response to the defined Class I epitope for HIV-1 IIIB Gag. Therefore, we initiated an epitope mapping study to identify C57BL/6 (H-2^b) T cell epitopes in HIV-1 IIIB Gag in order to perform the experiments. We defined two previously unreported minimal Class I H-2^b and Class II I-A^b epitopes for HIV-1 IIIB Gag. The newly defined HIV-1 IIIB Gag epitopes were used to evaluate the influence of SEA on generation of Th1-type HIV-1 IIIB Gag responses. Surprisingly, in contrast to our hypothesis, we observed that co-administration of SEA with the *Listeria* vector expressing HIV-1 IIIB Gag (Lm-Gag), led to significantly increased frequency of IFNγ-producing CTLs in C57BL/6 mice compared to mice immunized with Lm-Gag only. These observations suggest that SEA contains, in addition to Th2-type and immune suppressive molecules, substances that can act with the Lm-Gag vaccine to increase Th1-type vaccine-specific immune responses.

II. Introduction

Vaccines for HIV, tuberculosis and malaria are in development or in clinical trials. The greatest incidence of each of these diseases is in sub-Saharan Africa, where helminth infection is endemic (100, 101). Helminth parasites bias immune responses to $CD4^+$ Th2-type and can be immune suppressive (52, 54, 135, 136, 138, 140, 142, 202).

To evaluate the influence of helminth infection on vaccines, we can evaluate the ability to induce vaccine-specific immune responses in helminth-infected recipients, or in recipients that have been treated with immune-biasing helminth antigens (53, 134, 231).

Schistosomiasis is a helminth parasitic disease that affects more than 200 million people worldwide and is listed by the World Health Organization (WHO) as the second leading parasitic disease, after malaria (232). Infection occurs when the cercarial stage that emerges from the infected snail intermediate host, contacts and penetrates the skin of its vertebrate host. Larval parasites migrate and mature to adult male and female worms that mate and produce eggs (135, 138). Eggs that become lodged within host tissues are largely responsible for Th2-biasing of the host immune system and induction of anti-inflammatory responses (135, 138, 202, 203, 211).

Immune biasing induced by schistosome infection renders some vaccines ineffective in both laboratory and clinical settings (53, 54, 233, 234). 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 (CTL) responses (103, 195, 234). 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 (53). Taken together with the study by Actor et al. showing suppression of virus-specific immune responses in schistosome infected mice (5) and the study by Sabin et al. showing suppression of tetanus-specific responses in schistosome infected children (54), helminth infection poses a significant problem for the development of viral-specific CTL and Th1- type HIV-1 vaccines (52-

54). In this regard, recent studies suggest that a *Listeria* vector HIV vaccine can induce potent $CD8^+$ and Th1-type vaccine responses in schistosome infected mice (134, 231).

Schistosome soluble egg antigens (SEA) are potent inducers of $CD4^+$ Th2-type biasing (135, 138, 142, 208), and in an earlier study, we demonstrated that SEA co-administered with a third party antigen was sufficient to induce vaccine-specific Th2-type cytokine responses (142). 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 the known Class I epitope for HIV-1 IIIB Gag in C57BL/6 mice, QEVK (235, 236), failed to elicit responses. Therefore, we embarked on an epitope mapping study to identify minimal HIV-1 Gag epitopes. We successfully identified both minimal Class I $H\text{-}2^b$ and Class II $I\text{-}A^b$ epitopes for HIV-1 IIIB Gag and used these to perform our evaluation on the influence of SEA on *Listeria* vector HIV IIIB Gag induced T cell responses in mice. Consistent with our earlier study demonstrating that SEA co-administration increases CTL and Th1 vaccine responses in BALB/c mice (237), we similarly found in the current study that SEA co-administration with the *Listeria* vector vaccine significantly increased IFNγ-producing CTL effector frequency in C57BL/6 mice.

III. Materials & Methods

Biological Reagents. The HIV vaccine, an attenuated strain of *Listeria monocytogenes* expressing the HIV-1 IIIB Gag protein (Lm-Gag) (212), and the control strain, which expresses the E7 oncoprotein of the human papillomavirus 16 (Lm-E7) (213), were grown in BHI 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 in specific pathogen-free conditions and allowed to acclimate for one week prior to manipulation. All animal work was performed in accordance with institutional policy and approved by the institutional animal care and use committee.

Peptides. The HIV-1 Consensus B Gag (15-mer, 11 amino acid overlapped) peptide library was provided by the NIH AIDS Research and Reference Reagent Program (238). All other peptides, including additional 15-mer peptides to complement the differences in our vaccine sequence, were synthesized by Biosynthesis, Inc at greater than 95% purity. All peptides were reconstituted in DMSO and stored at -20^{o}C. A list of all the peptides used in this study is provided in table S1.

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 injection of 100-150 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 production of SEA as described previously (135, 138, 142, 202).

Vaccination of mice. For epitope mapping studies, six to eight week old, female C57BL/6 or BALB/c mice were primed intraperitoneally (i.p.) with 0.2 LD_{50} ($1x10^6$ CFU) Lm-Gag vaccine or left unvaccinated. Mice were boosted two weeks after the

prime in an identical manner. To study the effect of SEA on vaccine responses, mice were injected i.p. with 30μg of SEA or left naive. One week after injection of SEA, mice were primed intravenously (i.v.) with $1x10^3$ CFU Lm-Gag vaccine with or without 30μg of SEA i.p. boost, control Lm-E7 vaccine (matched CFU dose) or left unvaccinated. Mice were boosted two weeks after the prime in an identical manner. Mice were sacrificed two weeks post last vaccination (wplv).

ELISpot. Splenocytes were harvested 2wplv and plated at 150K, 300K or 500K cells per well in IFNγ ELISpot plates (BD) in complete media (RPMI-1640 supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B, 2mM L-glutamine, 5μM β-mercaptoethanol and non-essential amino acids). Cells were stimulated with 20μM peptide, 1μg/ml conA (as a positive control) or left unstimulated. After a 20-hour incubation, ELISpots were performed according to the manufacturer's instructions and the spots were enumerated using an Immunospot analyzer (C.T.L.).

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 α-CD3ε, α-CD8a and α-CD4 antibodies (BD) and analyzed for purity by flow cytometry. Cell debris was excluded from the analysis based on scatter signals and resulting purity was ≥ 90%, as shown in Figure S4.1.

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

IV. Results

1. T cells from vaccinated mice do not respond to the published Class I H-2^b epitope, QEVK

To validate the vaccine responses in C57BL/6 mice, we vaccinated C57BL/6 mice with Lm-Gag in a prime-boost regimen, two weeks apart. Two wplv, splenocytes were harvested and analyzed for immune response by IFNγ ELISpot against published H-2^b-restricted Class I QEVK (235, 236) and Class II VHQA (239) epitopes of HIV-1 Gag (Table 4.1).

Table 4.1 List of abbreviated HIV-1 Gag epitopes in order of discussion

Name	MHC / Mouse strain	Peptide sequence	References
QEVK	Class I, H-2^b	QEVKNWMTETL	(235, 236)
VHQA (37)	Class II, I-A^b	VHQAISPRTLNAWVKVVEEK	(239)
CRSL-9	Class I, H-2^b	CRSLYNTVA	
RSLY-9	Class I, H-2^b	RSLYNTVAT	
SLYN-9	Class I, H-2^b	SLYNTVATL	(240, 241)
RSLY-8	Class I, H-2^b	RSLYNTVA	
SLYN-8	Class I, H-2^b	SLYNTVAT	
QAIS-11	Class II, I-A^b	QAISPRTLNAW	
AISP-11	Class II, I-A^b	AISPRTLNAWV	
AISP-10	Class II, I-A^b	AISPRTLNAW	
AMQM	Class I, H-2^d	AMQMLKETI	(239, 242)
NPPI	Class II, I-A/E^d	NPPIPVGEIYKRWIILGLNK	(239)
EAMS (100)	Class I, H-2^b	EAMSQVTNSATIMMQ	(243-245)
KV9	Class I, H-2D^b	KSLYNTVCV	(235)

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 cells from unvaccinated controls (Fig. 4.1). However, the published Class I H-2^b epitope, QEVK failed to induce responses in vaccinated C57BL/6 mice (Fig. 4.1). 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^b minimal epitope, so that we could proceed with our evaluation on the influence of SEA on the Lm-Gag vaccine.

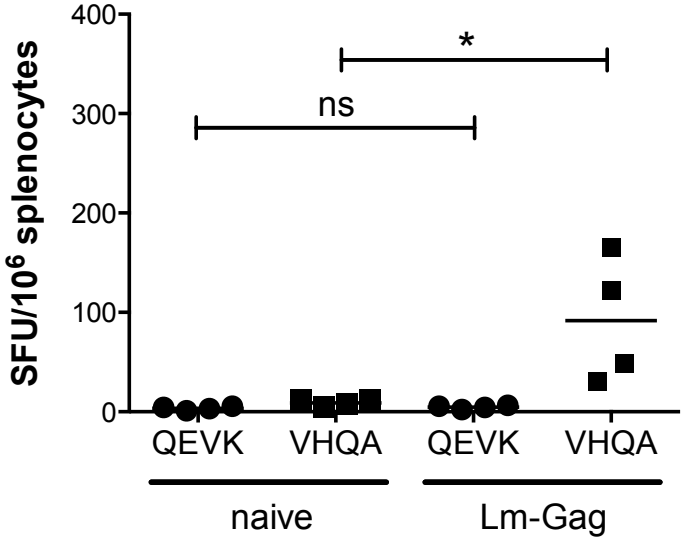


FIG 4.1 Lm-Gag vaccinated C57BL/6 mice do not respond to published Class I HIV Gag peptide. Splenocytes from naive and Lm-Gag vaccinated mice were harvested at 2wplv and immune responses to Class I (QEVK) and Class II (VHQA) were analyzed by IFNγ ELISpot. Results from individual mice (n=4) were plotted. Statistical analysis was performed using one-way ANOVA with Tukey post-hoc test, *$p<0.05$ and ns-not significant.

2. 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 (Table S4.1). Total splenocytes from C57BL/6 vaccinated mice were stimulated in the presence of peptides or left unstimulated for 20 hours then measured for Gag-specific responses. Figure 4.2A shows that of 143 different peptides tested, eight 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 MHC restriction. Enriched $CD8^+$ T cells were positively selected from pooled splenocytes, with the remaining cells representing $CD8^+$-depleted splenocytes. Both cell fractions were stimulated with 15-mer peptides or left unstimulated, then analyzed by IFNγ ELISpot. As shown in Figure 4.2B, among the seven 15-mer stimulatory peptides, peptides 19 and 20 stimulated enriched $CD8^+$ T cells suggesting the Class I-restricted epitope is within this region. Peptides 36 and 38 stimulated significant responses from the, $CD8^+$-depleted cell fraction (Fig. 4.2C). This result is expected as these two peptides overlap with 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 $CD8^+$ T cells or $CD8^+$-depleted splenocytes (Fig. 4.2B-C).

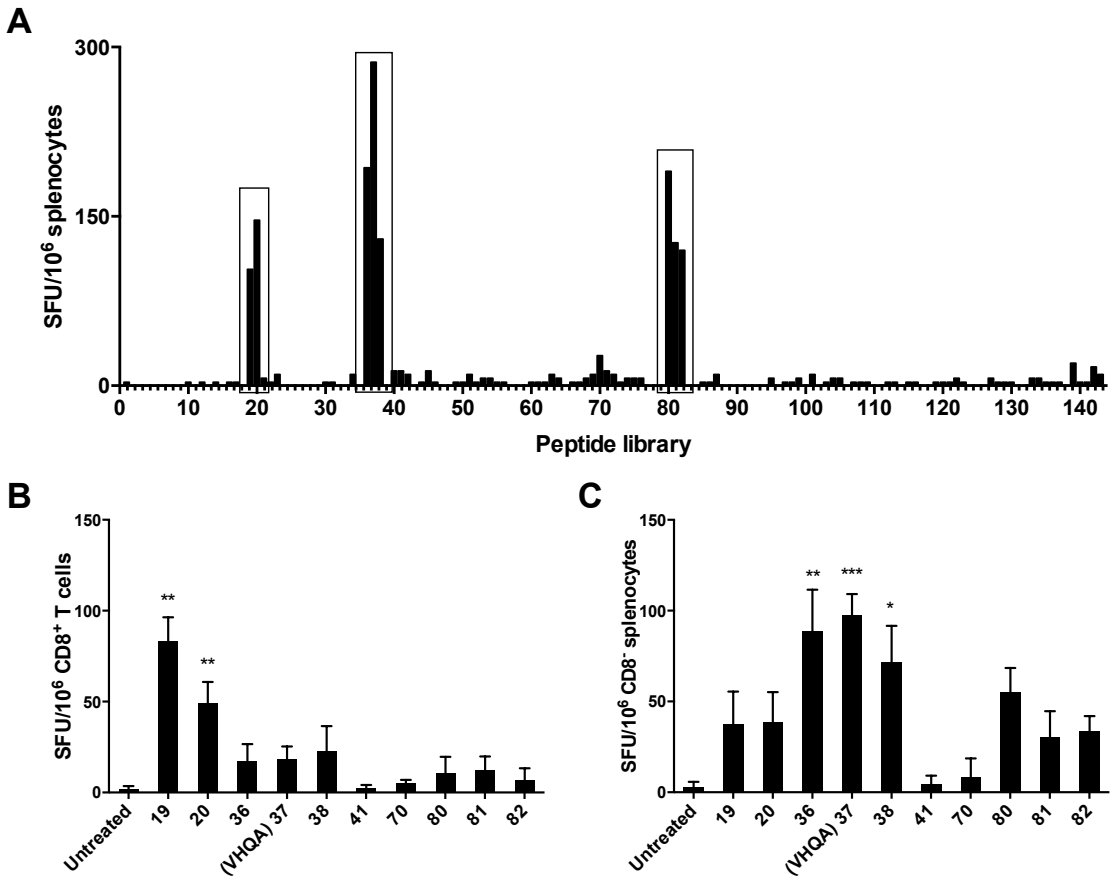


FIG 4.2 Peptide library screening for C57BL/6 Class I epitope. (**A**) Splenocytes from Lm-Gag vaccinated mice were harvested at 2wplv, pooled and screened for response against the HIV-1 Gag peptide library (15-mer, 11amino acid overlapped) and additional peptides by IFNγ ELISpot. $CD8^+$-enriched T cells (**B**) and $CD8^+$-depleted splenocytes (**C**) isolated from vaccinated animals were stimulated with the eight 15-mer responding peptides and analyzed for response. Splenocytes were pooled from 8 mice and stimulated with peptides in either duplicate (**A**) or quadruplicate (**B-C**) wells. Statistical analysis was performed (**B** and **C**) using one-way ANOVA with Tukey post-hoc test, *$p<0.05$, *$p<0.01$ and ***$p<0.001$, compared to groups without asterisk.

3. Class I H-2^b minimal epitope defined

Next, we identified the Class I H-2^b minimal epitope. Nine, 9-mer peptides surrounding the overlap region of peptides 19 and 20 were synthesized. We then stimulated total splenocytes, enriched $CD8^+$ T cell and enriched $CD4^+$ T cell populations

with these peptides. Simulation of cells with peptides 18 and 21 (flanking sequences, negative controls) did not induce any responses from any of the cell populations (Fig. 4.3A). Peptides 19 and 20 (positive controls) induced significant vaccine responses from total splenocytes, with $CD4^+$ T cells responses greater than the level of $CD8^+$ T cells responses. These data suggest that the total vaccine response against these two 15-mer peptides were primarily from enriched $CD4^+$ T cells. Interestingly, responses from the enriched $CD4^+$ T cells were significantly abrogated upon stimulation with 9-mer peptides within that region (Fig. 4.3A). Among the three 9-mer peptides inducing significant responses from total splenocytes and $CD8^+$-enriched T cells, the CRSL-9 and RSLY-9 peptides induced significantly higher responses compared to SLYN-9 (Fig. 4.3A). Therefore, we had minimal (8-mer) peptides within this region synthesized and tested them for their ability to induce IFNγ from total splenocytes, enriched $CD8^+$ cells and enriched $CD4^+$ cells. Both RSLY-8 and SLYN-8 induced significant responses from total splenocytes, with RSLY-8 driving the highest responses from $CD8^+$-enriched T cells (Fig. 4.3B). Thus, the Class I H-2^b minimal epitope is the 8-mer peptide, RSLYNTVA (RSLY-8). This minimal peptide was H-2^b restricted and not cross-reactive in BALB/c (H-2^d) mice (Fig. 4.3C). However, the response from C57BL/6 splenocytes to this epitope is much lower than the responses of BALB/c splenocytes to their cognate H-2^d epitope (AMQM) (Fig. 4.3C).

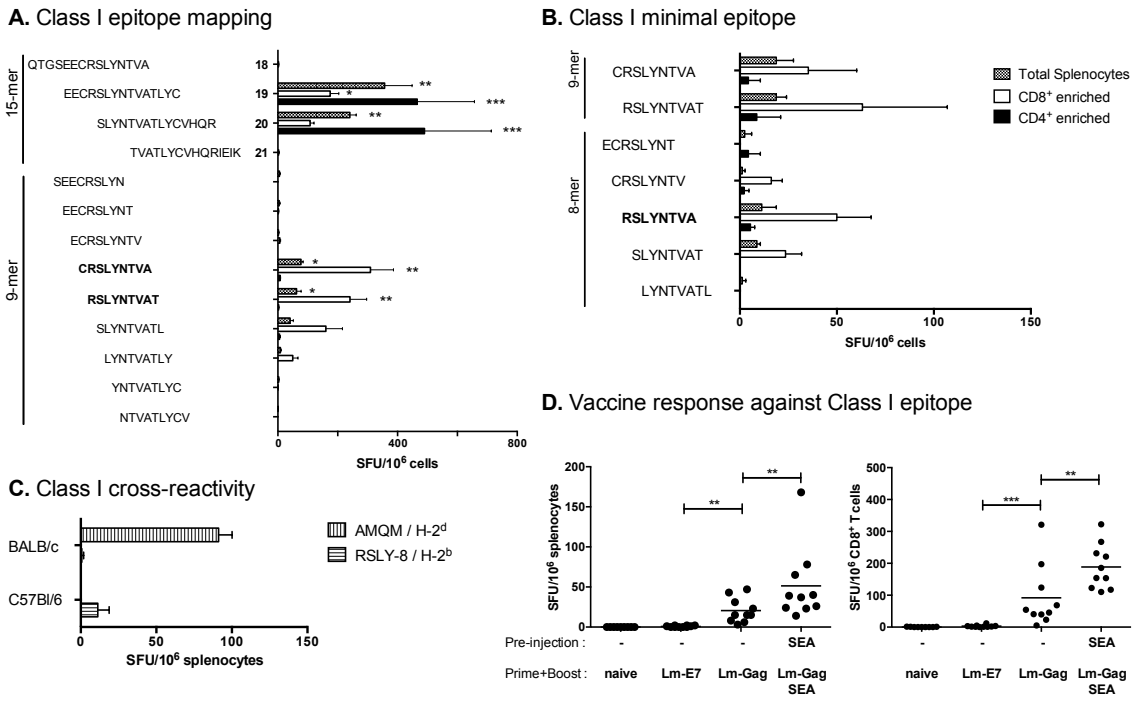


FIG 4.3 Identification of Class I H-2^b minimal epitope. Total splenocyte, enriched CD8$^+$ T cell and enriched CD4$^+$ T cell populations from Lm-Gag vaccinated mice were harvested at 2wplv and analyzed for response against (**A**) 9-mer peptides within the 11-amino acid overlapping region, then (**B**) minimal 8-mer peptides within overlapping region of most stimulatory peptides. (**C**) Identified Class I H-2^b minimal peptide was tested for cross-reactivity against Class I H-2^d peptide in both mouse strains, C57BL/6 and BALB/c. (**D**) Validate SEA adjuvant activity in C57BL/6 mice by evaluating vaccine response against Class I epitope using IFNγ ELISpot. (**A, 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 using one-way ANOVA with Newman Keuls post-hoc test, *p<0.05, **p<0.01 and ***p<0.001, as indicated or compared to groups without asterisk, within the same population. (**B-C**) Splenocytes were pooled from 12 mice and stimulated with peptides in duplicate wells.

4. Response to the Class I H-2^b minimal epitope was significantly increased by SEA co-administration with the Lm-Gag vaccine

Having identified the minimal Class I H-2^b 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 naïve, Lm-E7 vaccinated, Lm-Gag vaccinated and SEA co-administered Lm-Gag (SEA/Lm-Gag) groups. Two wplv, IFNγ ELISpot was performed to measure Gag-specific responses against the newly identified Class I RSLYNTVA (RSLY-8) minimal epitope of HIV-1 Gag. Splenocytes from negative control groups (naïve 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. 4.3D). Splenocytes from both Lm-Gag and SEA/Lm-Gag vaccinated groups responded to the RSLY-8 epitope, with the response from SEA/Lm-Gag splenocytes being significantly higher (Fig. 4.3D). This finding demonstrates that co-administration of SEA with the Lm-Gag vaccine increases vaccine-specific CTL effector cells in Th1-biased C57BL/6 mice.

5. Class II I-A^b minimal epitope identified

We defined the Class II I-A^b 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. The negative control peptides 35 and 39 (flanking sequences) did not induce responses from any of the cell populations, whereas both positive control peptides 36 and 38 induced significant responses from total splenocytes and enriched $CD4^+$ T cells (Fig. 4.4A). The $CD8^+$ T cell response to these two peptides was minimal, suggesting the response is Class II-restricted. The highest responses from the enriched $CD4^+$ T cells were to the QAIS-11 and AISP-11 peptides (Fig. 4.4A). Therefore, we synthesized then analyzed responses to two new 10-mer peptides from this region. The 10-mer AISPRTLNAW peptide (AISP-

10) was identified as the Class II I-A^b minimal epitope of HIV-1 Gag protein due to its significant induction of IFN-γ from total splenocytes and $CD4^+$-enriched T cells (Fig. 4.4B). In addition, the AISP-10 I-A^b minimal peptide did not cross-react with the Class II epitope for BALB/c (I-A^d) mice. However, the response from C57BL/6 splenocytes to AISP-10 is much lower than the responses of BALB/c splenocytes to their cognate I-A/E^d epitope (NPPI) (Fig. 4.4C).

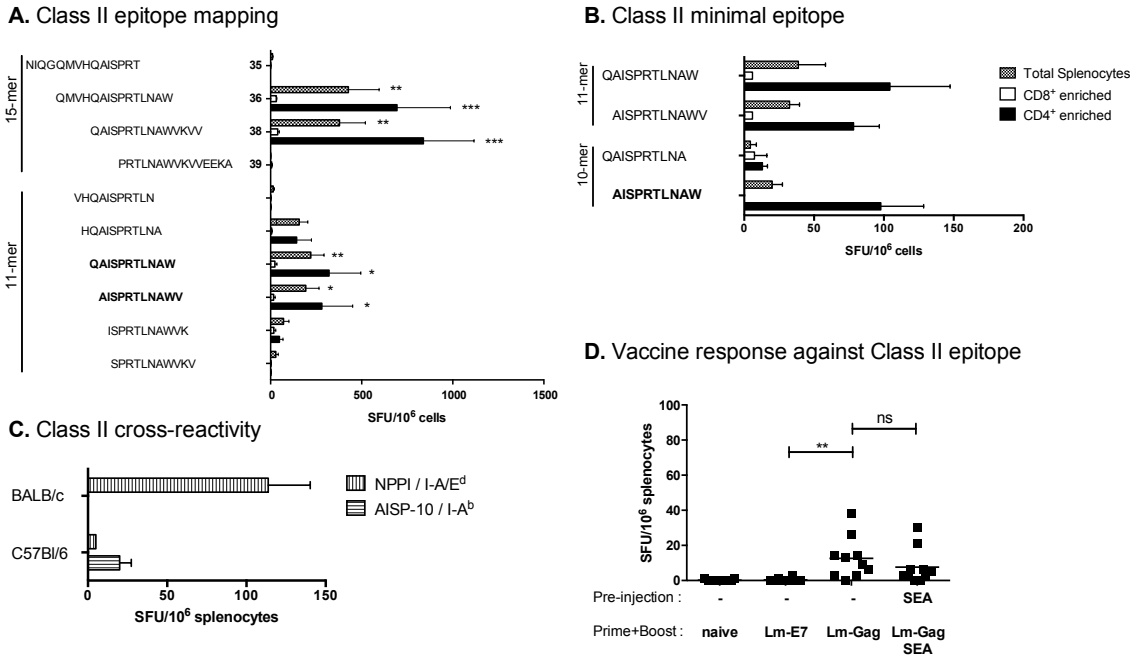


FIG 4.4 Determination of C57BL/6 Class II I-A^b minimal epitope. Total splenocyte, enriched $CD8^+$ T cell and enriched $CD4^+$ T cell populations from Lm-Gag vaccinated mice were harvested at 2wplv and analyzed for response against (**A**) 11-mer peptides surrounding the overlapping stimulatory region, then (**B**) minimal 10-mer peptides within overlapping region of most stimulatory peptides. (**C**) Identified Class II I-A^b minimal peptide was tested for cross-reactivity against Class II I-A/E^b peptide in both mouse strains, C57BL/6 and BALB/c. (**D**) Evaluate SEA adjuvant activity in C57BL/6 mice by analyzing response against Class II epitope using IFNγ ELISpot. (**A, 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 using one-way ANOVA with Newman Keuls post-hoc test, *p<0.05, **p<0.01, ***p<0.001

and ns-not significant, as indicated or compared to groups without asterisk, within the same population. (**B-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 described earlier were immunized and analyzed by IFNγ ELISpot. Unlike what we observed for the MHC Class I response, here we observed that splenocytes from Lm-Gag vaccinated mice had significant T cell responses to the AISP-10 minimal epitope, which were not enhanced in splenocytes from mice vaccinated with SEA/Lm-Gag (Fig. 4.4D).

V. Discussion

We initiated this study to determine if 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 published Class I epitope (QEVK) of HIV-1 Gag protein (Fig. 4.1). The QEVK peptide was previously described as HIV-1 Gag Class I epitope (235, 236). However, this peptide failed to induce Class I epitope recall responses in our system. Therefore, we initiated an epitope mapping study to define the minimal Class I H-2^b epitope for Gag protein.

Screening of 143 peptides allowed us to define eight stimulating peptides (Fig. 4.2A), 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 stimulatory 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 (243-245). In these studies, the EAMS peptide sequence did not overlap with 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. 4.2A). The contrast between our results and those in 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 immunodominance of the MHC Class I H-2^b Gag epitopes. In fact, previous evaluation of a *Listeria* vector expressing the HER-2 antigen suggested the *Listeria* vaccine vector revealed more epitopes than other vectors (246, 247). In our study, the 15-mer peptides that induced responses in cells from Lm-Gag vaccinated mice were numbers 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 $CD8^+$-enriched and -depleted cells. Bead-based purification of $CD8^+$ T cells typically results in 90-95% purity (Fig. S4.1). Thus, there is the possibility of low-level responses from other cell types upon peptide stimulation. However, as shown in Figure 4.2B, peptides 19 and 20 induced the highest levels of stimulation of $CD8^+$-enriched T cells, suggesting the MHC Class I-restricted epitope is within this region. In addition, $CD8^+$-depleted cells responded to several of the 15-mer peptides (Fig. 4.2C). Here, we noted that the amino acid sequence of the most stimulatory peptides 36 and 38, overlapped with the Class II-restricted VHQA epitope.

Further screening of shorter peptides successfully identified the two minimal epitopes for MHC Class I (RSLY-8) and Class II (AISP-10) in C57BL/6 mice (Fig. 4.3,

4.4). There is precedent for epitope RSLY-8, as its amino acid sequence is similar to the previously defined, H-2D^b KV9 Gag epitope (KSLYNTVCV) of simian immunodeficiency virus (SIV) (235). RSLY-8 is also a single amino acid shift from a well-known human HLA-A*0201-restricted 9-mer epitope, Gag_{77-85} (SLYNTVATL, SLYN-9), which was shown to be highly reactive in chronically infected patients (240, 241).

To account for the disparity in level of response between data in Panel A and Panels B-D of Figures 4.3 and 4.4, we note the change in animal vendors between experiments. Nonetheless, our data suggests that RSLY-8 and AISP-10 are indeed the HIV-1 Gag Class I and Class II minimal epitopes, as they induced the highest level 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. 4.3B, 4.4B). Overall, recall responses to peptides in C57BL/6 were much lower than from BALB/c mice (Fig. 4.3C, 4.4C), which is not surprising considering they are genetically disparate mouse strains (248-250).

Evaluation of the influence of SEA on generation of CD4+ and CD8+ T cell responses Lm-Gag vaccinated C57BL/6 mice demonstrated that similar to our recent study (237), administration of SEA with Lm-Gag significantly increased the level of Gag-specific CTL effectors compared to mice vaccinated with Lm-Gag alone (Fig. 4.3D). However, the level of CD4$^+$ Th1 responses was not influenced by the addition of SEA (Fig. 4.4D). Thus, our study suggests that for the Lm-Gag vaccine, SEA acts as a CTL adjuvant and its adjuvant effects do not depend on the immune bias (Th1 vs. Th2) of the host, as SEA co-administration with the Lm-Gag vaccine significantly improved vaccine-

specific CTL responses in both C57BL/6 (Fig. 4.3) and BALB/c strains (237). Together, the results presented here and in our recent study (237), show that SEA functioned counter-intuitively when administered with the Lm-Gag vaccine, stimulating CTL responses rather than suppressing them. To begin to dissect how this occurs, we are currently biochemically fractionating SEA in an attempt to define the class(es) of molecules that are 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.

VI. Acknowledgement

Infected animals were provided by BRI via the NIAID schistosomiasis resource center under NIH-NIAID Contract No. HHSN272201000005. These materials can be obtained by contacting BEI Resources. The HIV-1 Gag peptide library was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: (HIV-1 Consensus B Gag (15-mer) Peptides: cat# 8117). The authors would also like to thank the UGA College of Veterinary Medicine Cytometry Core Facility and Ms. E. Farah Samli for technical assistance. This work was supported by startup funds from the Georgia Research Alliance, the University of Georgia Research Foundation and NIH grants NIH-AI-071883 and NIH-AI-078787 awarded to DAH.

VII. Supplemental

Table S4.1 List of all peptides used in this study. This table includes the HIV-1 Gag peptide library, additional peptides synthesized by Biosynthesis, Inc to complement our vaccine sequence and several controls peptides.

#	Peptide ID	Abbr.	Peptide Sequence	Purity [%]
1	7872		MGARASVLSGGELDR	84.7
2	7873		ASVLSGGELDRWEKI	94.5
3	7874		SGGELDRWEKIRLRP	98.3
4	7875		LDRWEKIRLRPGGKK	97.7
5	7876		EKIRLRPGGKKKYKL	94.2
6	7877		LRPGGKKKYKLKHIV	94.5
7	7878		GKKKYKLKHIVWASR	97.1
8	7879		YKLKHIVWASRELER	86.7
9	7880		HIVWASRELERFAVN	85.4
10	7881		ASRELERFAVNPGLL	97.9
11	7882		LERFAVNPGLLETSE	92.1
12	7883		AVNPGLLETSEGCRQ	90.4
13	7884		GLLETSEGCRQILGQ	83.5
14	7885		TSEGCRQILGQLQPS	92.5
15	7886		CRQILGQLQPSLQTG	86
16	7887		LGQLQPSLQTGSEEL	91.9
17	7888		QPSLQTGSEELRSLY	92.6
18	7889		QTGSEELRSLYNTVA	93
19	7890		EELRSLYNTVATLYC	91.6
20	7891		SLYNTVATLYCVHQR	90.6
21	7892		TVATLYCVHQRIEVK	92.8
22	7893		LYCVHQRIEVKDTKE	93.6
23	7894		HQRIEVKDTKEALEK	95.3
24	7895		EVKDTKEALEKIEEE	95.3
25	7896		TKEALEKIEEEQNKS	97.7
26	7897		LEKIEEEQNKSKKKA	91.3
27	7898		EEEQNKSKKKAQQAA	95.7
28	7899		NKSKKKAQQAAADTG	94.3
29	7900		KKAQQAAADTGNSSQ	90.1
30	7901		QAAADTGNSSQVSQN	90
31	7902		DTGNSSQVSQNYPIV	92
32	7903		SSQVSQNYPIVQNLQ	81.2
33	7904		SQNYPIVQNLQGQMV	95.4
34	7905		PIVQNLQGQMVHQAI	91.9
35	7906		NLQGQMVHQAISPRT	97.3

36	7907		QMVHQAISPRTLNAW	96.4
37	C57-Gag-h	VHQA	VHQAISPRTLNAWVKVVEEK	>95
38	7908		QAISPRTLNAWVKVV	96.5
39	7909		PRTLNAWVKVVEEKA	84.4
40	7910		NAWVKVVEEKAFSPE	97.8
41	7911		KVVEEKAFSPEVIPM	96.4
42	7912		EKAFSPEVIPMFSAL	95.8
43	7913		SPEVIPMFSALSEGA	99
44	7914		IPMFSALSEGATPQD	97.6
45	7915		SALSEGATPQDLNTM	91.5
46	7916		EGATPQDLNTMLNTV	96.8
47	7917		PQDLNTMLNTVGGHQ	93.2
48	7918		NTMLNTVGGHQAAMQ	84.7
49	7919		NTVGGHQAAMQMLKE	89.6
50	7920		GHQAAMQMLKETINE	96.3
51	7921		AMQMLKETINEEAAE	93.2
52	7922		LKETINEEAAEWDRL	92.1
53	7922B		LKETINEEAAEWDRV	>80
54	7923		INEEAAEWDRLHPVH	90.5
55	7923B		INEEAAEWDRVHPVH	>80
56	7924		AAEWDRLHPVHAGPI	97
57	7924B		AAEWDRVHPVHAGPI	>80
58	7925		DRLHPVHAGPIAPGQ	84.2
59	7925B		DRVHPVHAGPIAPGQ	>80
60	7926		PVHAGPIAPGQMREP	88
61	7927		GPIAPGQMREPRGSD	86.5
62	7928		PGQMREPRGSDIAGT	89.8
63	7929		REPRGSDIAGTTSTL	93.7
64	7930		GSDIAGTTSTLQEQI	95.7
65	7931		AGTTSTLQEQIGWMT	93.8
66	7932		STLQEQIGWMTNNPP	80.5
67	7933		EQIGWMTNNPPIPVG	96.3
68	7934		WMTNNPPIPVGEIYK	95.1
69	7935		NPPIPVGEIYKRWII	92
70	7936		PVGEIYKRWIILGLN	88.9
71	7937		IYKRWIILGLNKIVR	98.2
72	7938		WIILGLNKIVRMYSP	90.9
73	7939		GLNKIVRMYSPTSIL	86.5
74	7940		IVRMYSPTSILDIRQ	93.6
75	7941		YSPTSILDIRQGPKE	91.3
76	7942		SILDIRQGPKEPFRD	94.2

77	7943		IRQGPKEPFRDYVDR	88.1
78	7944		PKEPFRDYVDRFYKT	96.1
79	7945		FRDYVDRFYKTLRAE	95.5
80	7946		VDRFYKTLRAEQASQ	91
81	7947		YKTLRAEQASQEVKN	83.9
82	7947B		YKTLRAEQASQDVKN	>80
83	7948		RAEQASQEVKNWMTE	95.8
84	7948B		RAEQASQDVKNWMTE	>80
85	7949		ASQEVKNWMTETLLV	94
86	7949B		ASQDVKNWMTETLLV	>80
87	7950		VKNWMTETLLVQNAN	75.5
88	7951		MTETLLVQNANPDCK	83.3
89	7952		LLVQNANPDCKTILK	96.6
90	7953		NANPDCKTILKALGP	96.4
91	7954		DCKTILKALGPAATL	97.1
92	7955		ILKALGPAATLEEMM	91.6
93	7956		LGPAATLEEMMTACQ	97.4
94	7957		ATLEEMMTACQGVGG	93.5
95	7958		EMMTACQGVGGPGHK	95.4
96	7959		ACQGVGGPGHKARVL	96
97	7960		VGGPGHKARVLAEAM	94.2
98	7961		GHKARVLAEAMSQVT	96.2
99	7962		RVLAEAMSQVTNSAT	95.4
100	7963	EAMS	EAMSQVTNSATIMMQ	85.8
101	7964		QVTNSATIMMQRGNF	90.8
102	7965		SATIMMQRGNFRNQR	91.8
103	7966		MMQRGNFRNQRKTVK	89.7
104	7967		GNFRNQRKTVKCFNC	95.1
105	7968		NQRKTVKCFNCGKEG	89
106	7969		TVKCFNCGKEGHIAK	97.2
107	7970		FNCGKEGHIAKNCRA	97.2
108	7971		KEGHIAKNCRAPRKK	92.5
109	7972		IAKNCRAPRKKGCWK	87.9
110	7973		CRAPRKKGCWKCGKE	91.7
111	7974		RKKGCWKCGKEGHQM	95.6
112	7975		CWKCGKEGHQMKDCT	97.9
113	7976		GKEGHQMKDCTERQA	94.7
114	7977		HQMKDCTERQANFLG	86.2
115	7978		DCTERQANFLGKIWP	94.7
116	7979		RQANFLGKIWPSHKG	97.8
117	7980		FLGKIWPSHKGRPGN	87.3

118	7981		IWPSHKGRPGNFLQS	97.6
119	7982		HKGRPGNFLQSRPEP	98.2
120	7983		PGNFLQSRPEPTAPP	93.1
121	7984		LQSRPEPTAPPEESF	93.4
122	7985		PEPTAPPEESFRFGE	89.7
123	7986		APPEESFRFGEETTT	93.6
124	7987		ESFRFGEETTTPSQK	93.7
125	7988		FGEETTTPSQKQEPI	96.5
126	7989		TTTPSQKQEPIDKEL	95.5
127	7990		SQKQEPIDKELYPLA	98.4
128	7991		EPIDKELYPLASLRS	97
129	7992		KELYPLASLRSLFGN	95.9
130	7993		PLASLRSLFGNDPSS	96.8
131	7994		LRSLFGNDPSSQ	93.9
132	C57-Gag-c	QEVK	QEVKNWMTETL	>95
133	PB1-703-711		SSYRRPVGI	>95
134	PA1 224-233		SSLENFRAYV	>95
135	NP-366-374		ASNENMETM	>95
136	P18		RIQRGPGRAFVTIGK	>95
137	P18-I10		RGPGRAFVTI	>95
138	BALB-Gag-c	AMQM	AMQMLKETI	>95
139	BALB-Gag-h	NPPI	NPPIPVGEIYKRWIILGLNK	>95
140	BALB-Env-c		IGPGRAFYAR	>95
141	BALB-Env-h		QVPVWREATTTLFCASDAKA	>95
142	BALB-NP-c		TYQRTRALV	>95
143	BALB-NP-h		RLIQNSLTIERMVLSAFDERRNK	>95
144	CTL9		SEECRSLYN	>95
145	CTL8		EECRSLYNT	>95
146	CTL7		ECRSLYNTV	>95
147	CTL6	CRSL-9	CRSLYNTVA	>95
148	CTL1	RSLY-9	RSLYNTVAT	>95
149	CTL2	SLYN-9	SLYNTVATL	>95
150	CTL3		LYNTVATLY	>95
151	CTL4		YNTVATLYC	>95
152	CTL5		NTVATLYCV	>95
153	HELP1		VHQAISPRTLN	>95
154	HELP2		HQAISPRTLNA	>95
155	HELP3	QAIS-11	QAISPRTLNAW	>95
156	HELP4	AISP-11	AISPRTLNAWV	>95
157	HELP5		ISPRTLNAWVK	>95
158	HELP6		SPRTLNAWVKV	>95

159	CTL10		ECRSLYNT	>95
160	CTL11		CRSLYNTV	>95
161	CTL12	RSLY-8	RSLYNTVA	>95
162	CTL13	SLYN-8	SLYNTVAT	>95
163	CTL14		LYNTVATL	>95
164	HELP7		QAISPRTLNA	>95
165	HELP8	AISP-10	AISPRTLNAW	>95

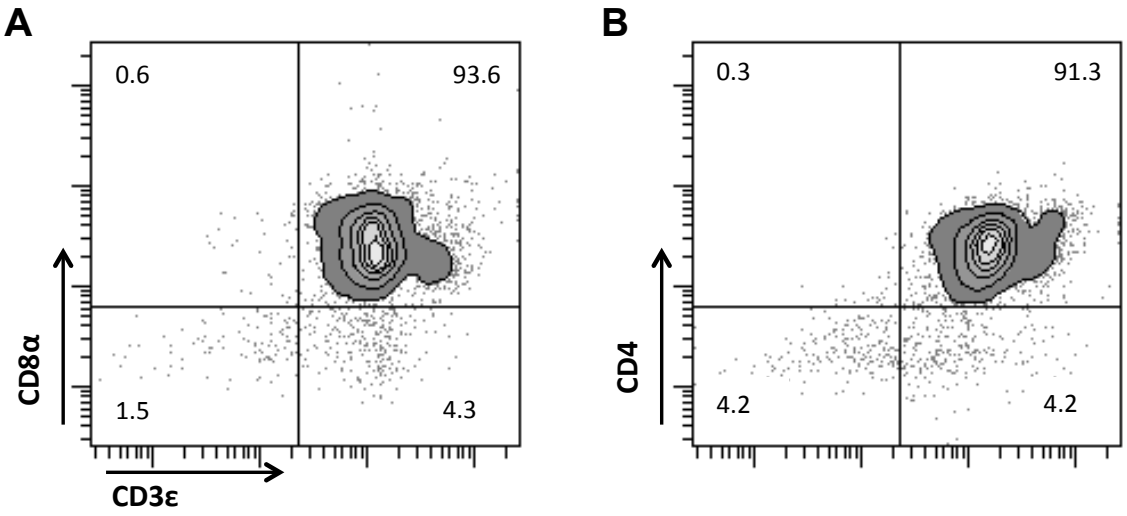


FIG S4.1 MACs purification of T cells resulted in greater than 90% purity. (**A**) $CD8^+$ and (**B**) $CD4^+$ T cells were purified from mouse splenocytes using Microbeads kits according to the manufacturer's instructions. Representative flow data show the phenotypes and purities of the enriched T cells.

CHAPTER 5

ATTEMPT TO DEFINE CLASS(ES) OF MOLECULES IN SCHISTOSOME EGGS THAT HAVE PRO-INFLAMMATORY ADJUVANT PROPERTIES

I. Introduction

Schistosomiasis induces strong Th2 polarization of the host immune response and has a negative influence host response to Th1-type vaccines, suppressing the host's ability to mount strong pathogen-specific T cell responses (52, 54, 136, 138, 140, 195). 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 (53). In addition, multiple studies have shown that helminth infection negatively influences the host's ability to generate virus-specific immune responses (52, 136, 138, 140, 195). Taken together, these studies suggest that helminth infection poses a significant challenge for the development of HIV-1 vaccines designed to induce viral-specific Th1- type $CD4^+$ and cytotoxic $CD8^+$ T cell responses (52, 54, 136, 138, 140, 195).

For schistosome infections, saline soluble egg antigens (SEA) appear to contain the molecules that drive $CD4^+$ Th2 biasing of the host immune response, and induce IL-10 production (135, 138, 142, 208). Thus it was surprising that our study showed that addition of SEA to the Lm-Gag vaccine designed to drive Th1-type and CTL responses to HIV Gag actually enhanced these responses rather than suppressing them (237). Our

results are in contrast to an earlier study demonstrating that co-administration of SEA with a third party antigen induced Th2-type cytokine responses to the antigen (142). The results of our study suggest that SEA co-administration with Lm-Gag not only expanded different T cell populations (Th1, Th2 and Treg) but also increased production of vaccine-specific pro-inflammatory cytokines (237). We hypothesize that the observed enhancement of Lm-Gag vaccine-specific responses was likely due to a synergy between some components of SEA and the Lm-Gag vaccine. Further, it is likely that among the myriad components that comprise SEA, some of them have pro-inflammatory properties and increase the induction of vaccine-specific responses when co-administered with a Th1-type vaccine. In order to test this hypothesis, we chemically fractionated schistosome eggs and then evaluated the ability of these fractions to drive pro-inflammatory responses using various immunological assays.

II. Materials & Methods

Animals. Five to 7 week old female BALB/c mice were purchased from Harlan and Jackson Laboratories, housed in specific pathogen-free conditions and allowed to acclimate for one week prior to manipulation. All animal work was performed in accordance with institutional policy and approved by the institutional animal care and use committee.

Biological Materials. The HIV vaccine, an attenuated strain of *Listeria monocytogenes* expressing the HIV-1 IIIB Gag protein (Lm-Gag) (212), and the control strain, which expresses the E7 oncoprotein of the human papillomavirus 16 (Lm-E7) (213), were grown in BHI supplemented with streptomycin.

Preparation of schistosome eggs and SEA. *Schistosoma mansoni* (PR strain) infected Swiss Webster mice provided by the NIAID schistosomiasis resource center were the primary source for egg isolation. Additional female BALB/c and C57Bl/6 mice were infected by intraperitoneal injection of 100-150 infectious *S. mansoni* cercariae. Parasite eggs were isolated 7-8 weeks post infection from livers of infected mice. Schistosome eggs were sent for biochemical fractionation, or combined from all sources for production of SEA as previously described (202). The protein concentration of SEA was determined by both the Bradford and the BCA protein assays (Thermo Scientific).

Fractionation of schistosome eggs. Schistosome eggs were biochemically fractionated into multiple classes of molecules by three different methods. The fractionation procedures were designed and performed by Drs. Parasto Azadi and Mayumi Ishihara at the Complex Carbohydrate Research Center, UGA. Details of the fractionation methods are shown in Appendix A-B. The following formula was used to estimate fraction dose (x) per 30μg SEA:

x (μg) = 30μg SEA / (SEA/eggs ratio) * (fraction/ eggs ratio); in which SEA/eggs or fraction/eggs ratio is the amount (g) of SEA or fraction recovered from the homogenization of 1g of schistosome eggs.

All fractions were dissolved in DMSO and stored at -80^{o}C.

Cell culture. RAW 264.7 cells (ATCC) were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 100U/ml penicillin, 100 μg/ml streptomycin and 2mM L-glutamate. Cells were plated in 24-well plates at 100K cells/well respectively and then cultured in a humidified incubator at 37°C with 5% CO_2 until they reached 70% confluence.

Bone marrow-derived macrophages (BMDMs) were obtained by flushing bone marrow cells from tibia and femurs with medium and culturing them in DMEM supplemented with 10ng/ml macrophage colony-stimulating factor (M-CSF) as described previously (153). Media was replaced every 2 days with fresh M-CSF medium, and cells were used for treatment on day 6.

Bone marrow-derived dendritic cells (BMDCs) were obtained by flushing bone marrow cells in RPMI 1640 and culturing the cells with 1000U/ml of granulocyte-macrophage colony-stimulating factor (GM-CSF) and 1000U/ml IL-4. On days 2 and 4, fresh medium containing GM-CSF was added to the cells. On day 6, non-adherent cells were used for experiments. Cells were >90% $CD11c^+$ dendritic cells as determined by flow cytometry.

***In vitro* stimulation.** RAW264.7 macrophages were treated with SEA at 1,5,10 or 20μg/ml, LPS at 0.1μg/ml or left unstimulated. Supernatants were collected and analyzed for cytokine production at 24hr and 48hr post treatment.

When egg fractions were used, doses of egg fractions (0.1x, 1x, 5x and 10x) correlated with 0.01, 0.1, 0.5 and 1% (v/v) DMSO. Media, 30μg of SEA, 0.1μg of LPS were prepared in similar DMSO concentrations and included in the analysis.

***Ex vivo* stimulation.** BMDCs and BMDMs were treated with 30μg/ml SEA, 10ng/ml IL-4, 0.1μg/ml CpG, 0.1μg/ml LPS or left unstimulated. Supernatants were collected at 24 hours post treatment and analyzed for inflammatory cytokine production.

Cytometric bead array (CBA). Cytokine levels from supernatants of each treatment group were measured using a Th1/Th2/Th17 or Inflammation CBA kit according to the manufacturer's instructions (BD).

***In vivo* study of peritoneal macrophages.** Six to 8 week old BALB/c mice were injected intraperitoneally with saline, 30μg of SEA, 50μg of CpG, 1x of egg fractions or left naïve. All materials were made to 5% DMSO as indicated.

Flow cytometry. To evaluate different cell populations in the peritoneal cavity, peritoneal exudate cells (PECs) were harvested and stained with α-CD11b, α-F4/80, α-SiglecF and α-Gr-1 antibodies (BD) then fixed. After membrane permeabilization, intracellular proteins were stained using α-RELMα or α-iNOS antibodies (antibodies-online). PECs were also labeled with α-CD11b, α-F4/80, α-CD11c and α-CD86 to verify non-DC phenotype of macrophages. Doublets were removed from the scatter plot. Live cells (as indicated by LIVE/DEAD fixable dye, Invitrogen) were acquired using a BD LSRII flow cytometer and analyzed using Flowjo (TreeStar) software.

Vaccination of mice. Six to 8 week old BALB/c mice were pre-injected intraperitoneally with saline, 30μg of SEA, egg fractions or left naive. 5% (v/v) DMSO was used as indicated for fraction studies. One week after pre-injection, mice were primed intravenously with 10^3cfu Lm-Gag vaccine, with or without booster of 30μg of SEA or egg fractions injected intraperitoneally, or left unvaccinated. 5% (v/v) DMSO was added in similar manner as pre-injection. Mice were boosted two weeks after the prime in an identical manner. Mice were sacrificed two weeks post last vaccination (wplv).

ELISpot. Splenocytes were harvested, plated at 300K cells per well in IFNγ ELISpot plates (BD) and incubated with complete media (RPMI-1640 supplemented with 10% FBS, 100U/ml penicillin, 100μg/ml streptomycin, 0.25μg/ml amphotericin B, 2mM L-glutamine, 5μM β-mercaptoethanol and non-essential amino acids) in the presence of 20μM specific CTL peptide (H2-Kd-restricted, AMQMLKETI from HIV-1 IIIB Gag

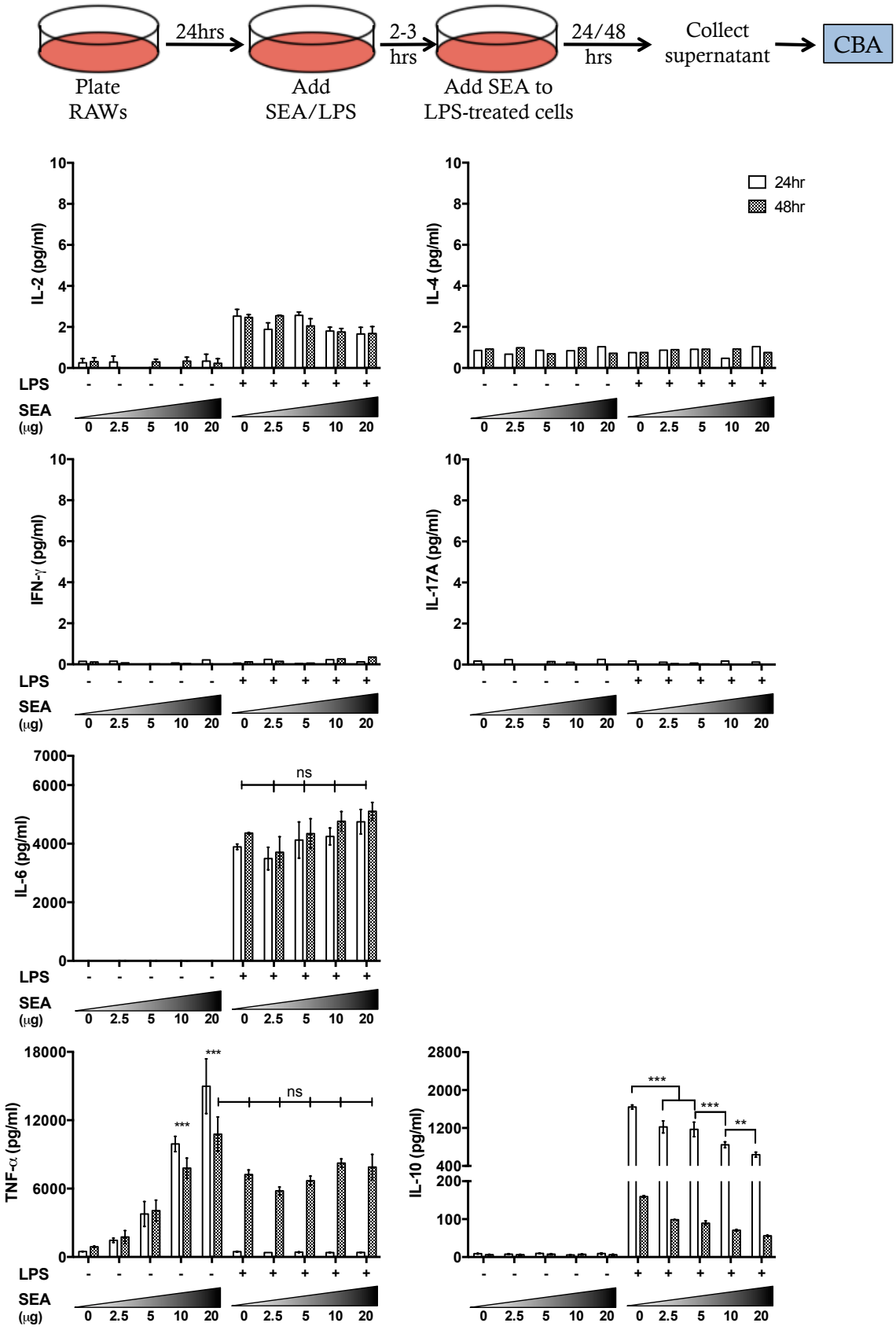
protein), 20μM irrelevant peptide (H2-Kd-restricted, TYQRTRALV from influenza A/PR/8/34 nucleoprotein), 20μM specific helper peptide (Class II-restricted, NPPIPVGEIYKRWIILGLNK from HIV-1 IIIB Gag protein), 20μM LLO peptide (H2-Kd-restricted, GYKDGNEYI from *L. monocytogenes* listeriolysin O), or 1 μg/ml con A (positive control). Peptides were synthesized by Biosynthesis, Inc at greater than 95% purity and reconstituted in DMSO prior to dilution in media. After 20 hours incubation, ELISpots were performed according to manufacturer's instructions and enumerated using an Immunospot analyzer (C.T.L.).

III. Results & Discussion

1. Investigate pro-inflammatory effects of SEA

i. SEA induced production of pro-inflammatory cytokines in RAW264.7 macrophages upon *in vitro* stimulation

We first evaluated the ability of SEA to induce pro-inflammatory responses in RAW264.7 macrophages. RAW264.7 macrophages were treated with several concentrations of SEA, LPS or left untreated for 2-3 hours, after which some of the LPS treated cells were further stimulated with SEA. Supernatants from each treatment group were collected at both 24 and 48 hours post last treatment and analyzed for cytokine production (Fig. 5.1). LPS induced significant production of pro-inflammatory cytokines IL-6 and TNFα starting at 24hr and 48hr post treatment, respectively. LPS stimulation also induced IL-10 production, with levels dropping after 48hr. We did not detect significant levels of IL-2, IL-4, IL-17A and IFNγ from any of the treatment groups.


24hrs
2-3 hrs
24/48 hrs
Collect supernatant
CBA
Plate RAWs
Add SEA/LPS
Add SEA to LPS-treated cells
24hr
48hr
IL-2 (pg/ml)
IL-4 (pg/ml)
IFN-γ (pg/ml)
IL-17A (pg/ml)
IL-6 (pg/ml)
TNF-α (pg/ml)
IL-10 (pg/ml)
LPS
SEA (μg)
0 2.5 5 10 20 0 2.5 5 10 20
ns
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**

FIG 5.1 SEA stimulation induced production of pro-inflammatory cytokine TNFα in RAW264.7 macrophages *in vitro*. RAW264.7 macrophages were stimulated with SEA at different concentrations or with LPS. For LPS stimulated cells, 2-3 hours post-stimulation SEA at various concentrations was added. Supernatants were collected at 24 and 48hr post last treatment and analyzed for cytokine production using Th1/Th2/Th17 CBA kit. Representative data from three independent experiments are shown. Statistical differences were analyzed by one-way ANOVA and Tukey post-hoc test, **p<0.01, ***p<0.001 and ns-not significant.

SEA stimulation of RAW cells induced production of TNFα in a dose dependent manner. The levels of this pro-inflammatory cytokine were maintained until 48hr. Addition of SEA did not alter IL-6 production induced by LPS at either timepoint. However, SEA did significantly inhibit LPS-induced IL-10 production, in a dose-dependent manner at 24hr post last treatment. A similar, not statistically significant trend was observed at 48hr post last treatment. These data suggest that SEA does have some pro-inflammatory properties, (Fig. 5.1). The results also confirm our earlier study demonstrating that the pro-inflammatory effects of SEA are not due to endotoxin, as SEA inhibited LPS-induced IL-10 production (Fig. 5.1). These results demonstrate that *in vitro* stimulation of RAW264.7 macrophages could be used as one read-out to investigate pro-inflammatory component(s) in SEA.

ii. SEA did not induce cytokine production in BMDCs or BMDMs upon *ex vivo* stimulation.

We further examined SEA induction of cytokines in BMDCs and BMDMs. Bone marrow cells were harvested from tibia and femurs of mice and cultured in appropriate cytokines to allow cell differentiation. After 6 days, mature BMDCs or BMDMs were

treated with SEA, IL-4, CpG, LPS or left unstimulated for 24 hours. Supernatants from all five treatments were collected and analyzed for cytokine production.

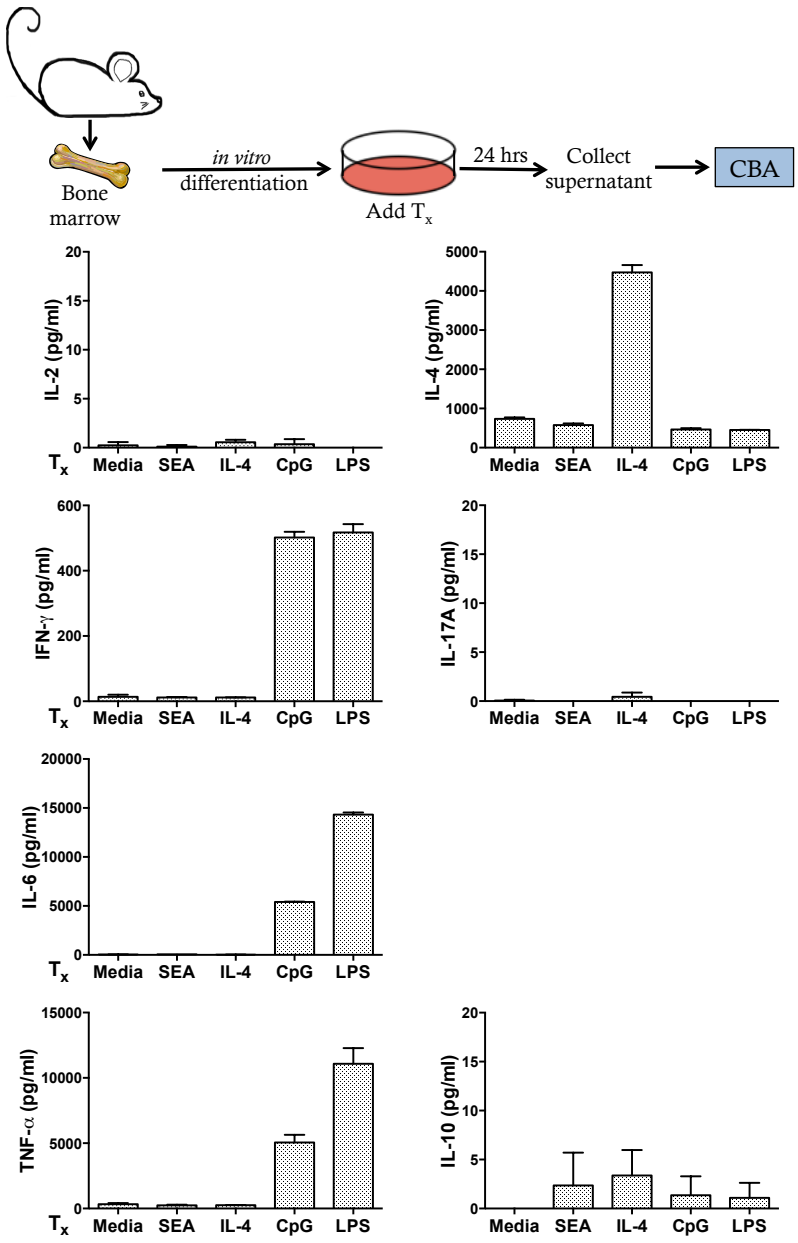

FIG. 5.2 SEA stimulation did not induce cytokine production in BMDCs. Bone marrow was harvested and allowed to differentiate into BMDCs in the presence of cytokines. BMDCs were stimulated with different treatments (T_x) for 24 hours. Supernatants were collected and analyzed for cytokine production using a Th1/Th2/Th17 CBA kit.

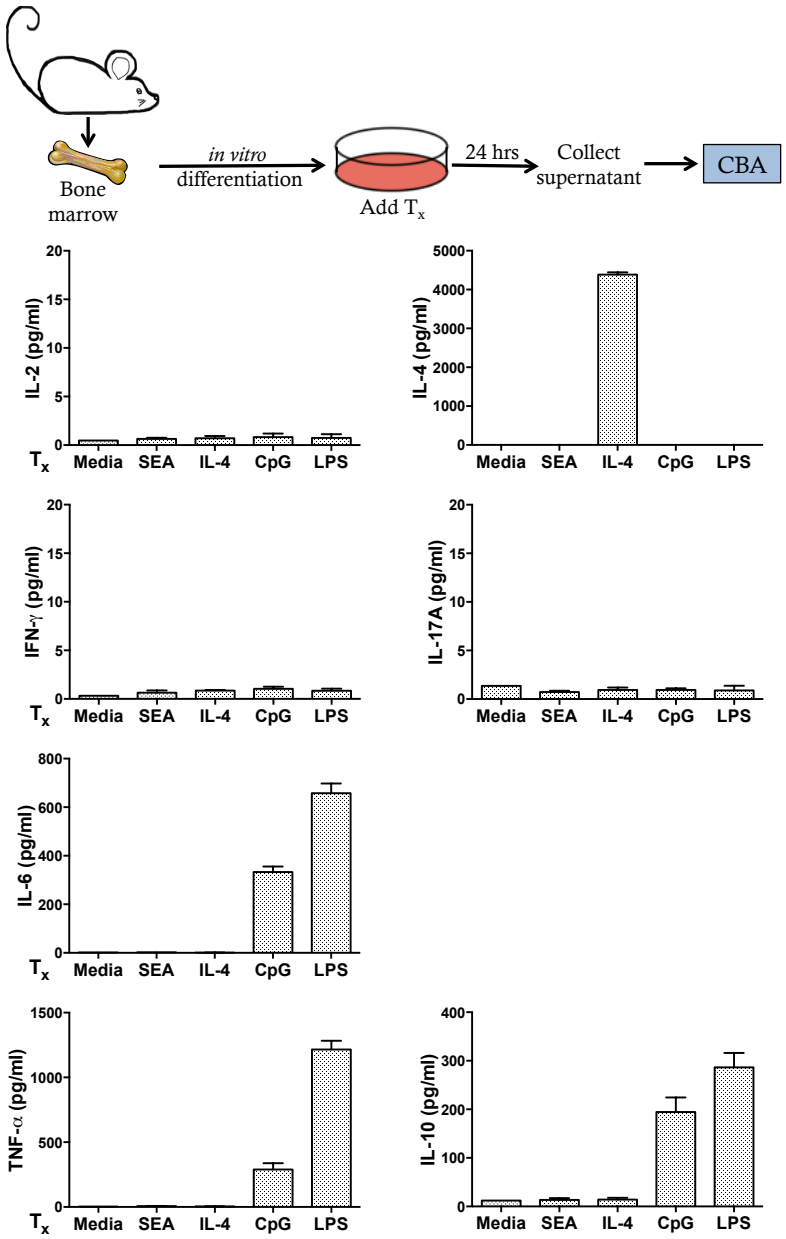

FIG 5.3 SEA stimulation did not induce cytokine production in BMDMs. Bone marrow was harvested and allowed to differentiate into BMDMs in the presence of cytokines. BMDMs were stimulated with different treatments (T_x) for 24 hours. Supernatants were collected 24 hours post last treatment and analyzed for cytokine production using Th1/Th2/Th17 CBA kit.

Similar to LPS, CpGs increased production of pro-inflammatory cytokines IFNγ, IL-6 and TNFα. Significant levels of IL-4 were only detected in the IL-4 treatment group

(Fig. 5.2-3). However, SEA alone did not induce any cytokine production in either BMDCs or BMDMs at this time point (Fig. 5.2-3). Together the data suggest that SEA stimulation functions differently for RAW264.7 cells and BMDCs/BMDMs and that these latter two cell types may need to be stimulated with SEA for longer periods of time to induce pro-inflammatory responses from these bone marrow derived cells.

iii. SEA expanded the small peritoneal macrophage population in the peritoneal cavity

To further evaluate the ability of SEA to induce pro-inflammatory responses, we moved to *in vivo* studies, injecting SEA into the peritoneal cavity. We chose this route because we demonstrated that the most potent adjuvant effect of SEA on the Lm-Gag vaccine occurred when SEA was injected intraperitoneally (i.p). In addition, the i.p. route facilitates collection of cells. BALB/c mice were injected with SEA, CpG or left naïve for about 36 hours. Peritoneal exudate cells were collected by lavage, stained and analyzed by flow cytometry. As shown in Figure 5.4, four distinct leukocytes populations were found in the mouse peritoneal cavity after SEA injection, including polymorphonuclear cells or neutrophils ($CD11b^{int}Gr1^{+}$), eosinophils ($CD11b^{lo}Siglec\text{-}F^{+}$), small and large peritoneal macrophages. These two macrophage populations were previously described as SPM ($CD11b^{int}F4/80^{int}$) and LPM ($CD11b^{hi}F4/80^{hi}$), respectively (251). Phenotypes of all four subsets of peritoneal cells were verified using flow cytometry (Fig. 5.4). Eosinophils expressed high level of Siglec-F while neutrophils expressed high level of Gr-1. LPM and SPM populations were demonstrated not to be DCs by lack of expression of CD11c, a dendritic cell (DC) marker.

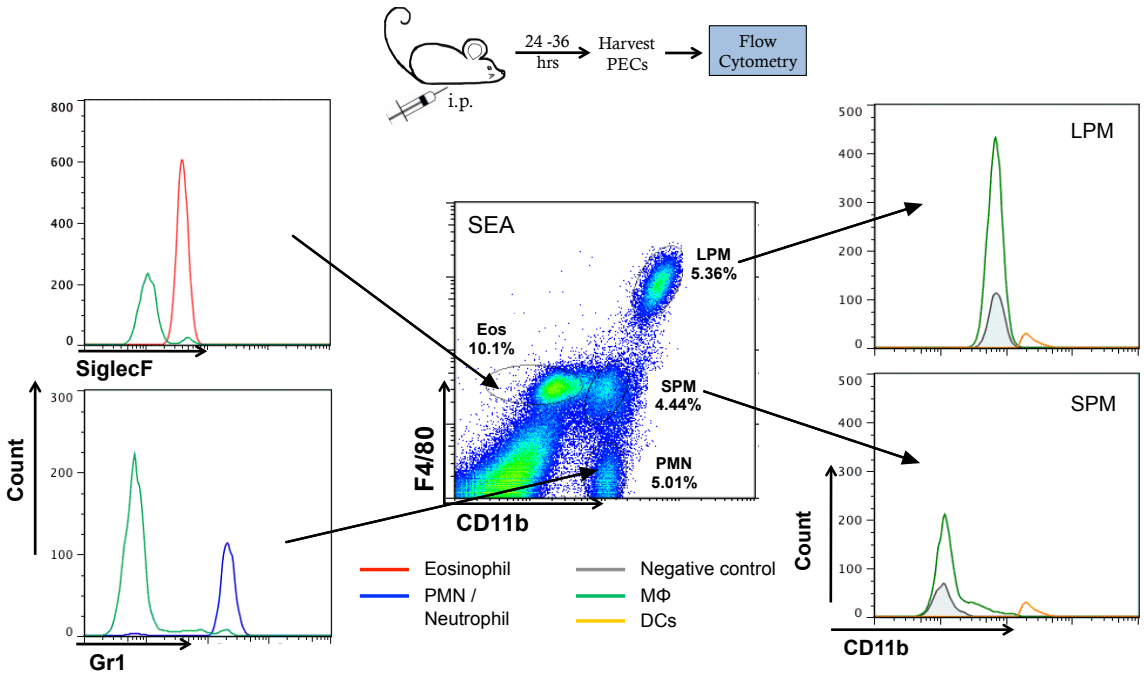

FIG 5.4 Phenotype verification of different peritoneal cell subsets. Peritoneal exudate cells were harvested, fluorescently labeled and analyzed for phenotype using flow cytometry. Macrophage populations were analyzed for expression of the DC surface marker CD11c. Neutrophils and eosinophils were verified by expression of Gr-1 and Siglec-F surface markers respectively. Representative data from an SEA-injected mouse are shown.

In addition to having different sizes, LPM and SPM are functionally different macrophage subsets. LPMs are resident peritoneal macrophages that produce NO in response to stimulation with LPS *in vitro* or *in vivo* (251). The LPM is retained after long-term stimulation (251). Conversely, SPMs originate from blood monocytes that enter the peritoneal cavity after LPS injection, then differentiate into macrophages (251). SPMs also produce NO following LPS stimulation, often at higher levels than LPMs. Due to these characteristics, SPMs are referred to as "inflammatory monocytes" implying their ability to infiltrate into tissues/sites following inflammatory stimuli (251).

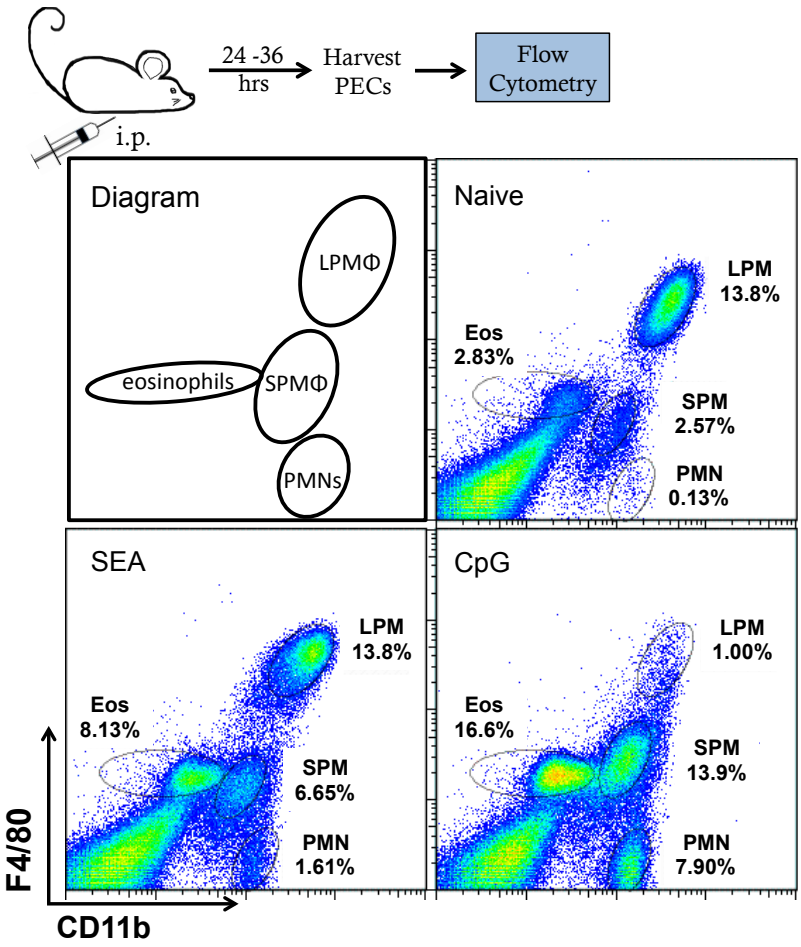

FIG 5.5 SEA injection influences peritoneal cavity cell populations. Mice were injected with SEA, CpG or left naïve. After 36 hours, peritoneal exudate cells were harvested, fluorescently labeled and analyzed for phenotype by flow cytometry. Four distinct cell populations were detected: eosinophils, polymorphonuclear cells (PMNs)/neutrophils, small peritoneal macrophages (SPMs) and large peritoneal macrophages (LPMs). Representative flow data are shown.

LPMs were the most abundant leukocyte subset in the peritoneal cavities of naïve mice. LPMs were at 13.8% while the level of SPMs was 2.6% of total viable PECs (Fig. 5.5). A single injection of CpG expanded the SPM subsets to 13.9% and significantly reduced the LPM subset to 1% (Fig. 5.5). Our results for CpG injected mice are similar to previously reported results for LPS injected mice (251). Interestingly, while SEA injection did not significantly alter the % of LPMs, SEA injection did increase the level

of SPMs to 6.7% of total viable PECs (Fig. 5.4). Levels of neutrophils and eosinophils were both increased after injection with either SEA or CpG adjuvants (Fig. 5.5). Because SPMs are considered as a pro-inflammatory subset, these data suggest that SEA does contain pro-inflammatory component(s), which if identified, will promote expansion of the inflammatory SPM subset (251).

2. Successful fractionation of schistosome eggs into three classes of molecules

To find the pro-inflammatory component(s) in SEA we initiated collaboration with Drs. Azadi and Ishihara at the UGA Complex Carbohydrate Research Center to chemically fractionate schistosome eggs. The initial fractionation scheme used traditional Folch chloroform/methanol lipid extraction method to separate out the total lipid and protein fractions from schistosome egg homogenate (Fig. 5.6).

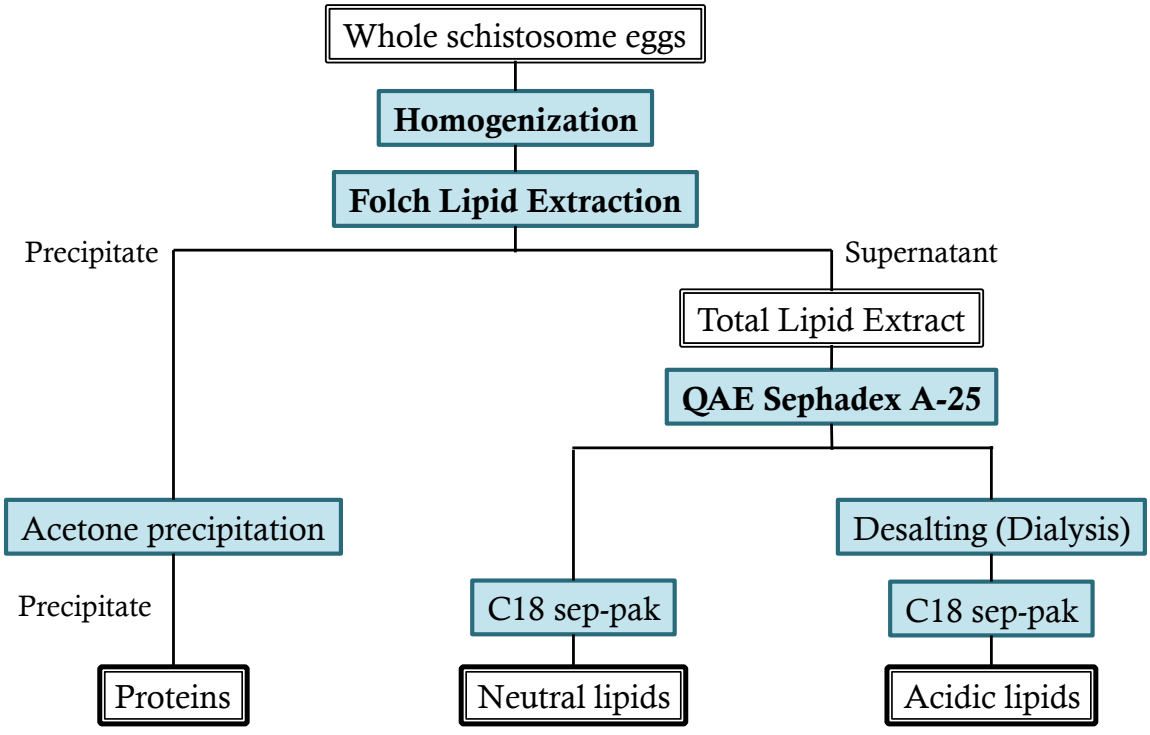

FIG 5.6 Initial scheme for fractionation of schistosome eggs. Fractionation of schistosome eggs yielded three classes of molecules: proteins, neutral lipid and acidic lipid fractions.

Protein/glycoprotein fraction was washed by acetone precipitation while the lipid extract was further separated into neutral lipids and acidic lipids by QAE Sephadex anion exchange chromatography. The acidic lipid fraction (binding fraction) was further desalted by dialysis to facilitate elution from the QAE Sephadex beads. Both lipid fractions were then subjected to C18 sep-pak columns for final wash (Fig. 5.6).

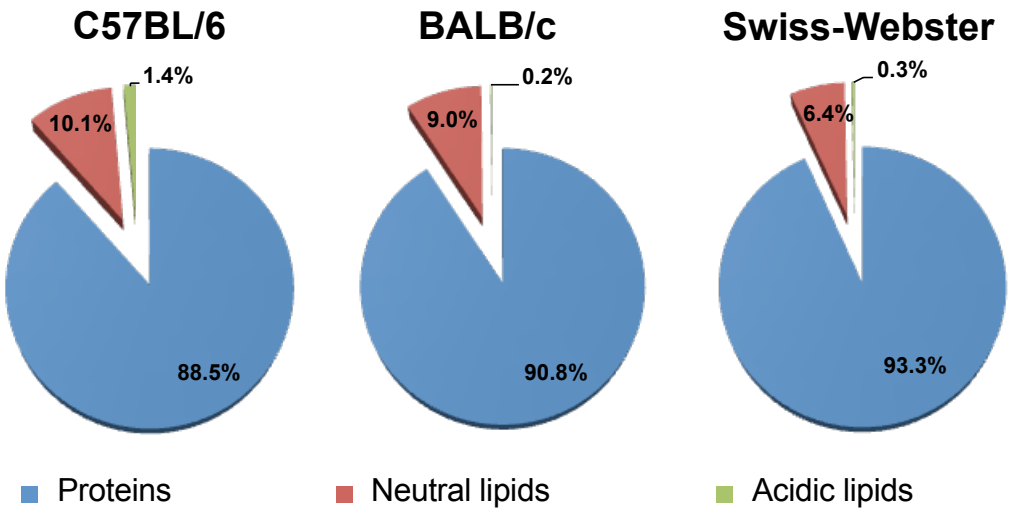

FIG 5.7 Percentages of schistosome egg fractions obtained from schistosome eggs isolated from three different mouse strains. Fractionation of schistosome eggs yielded three fractions, protein (88-93% of total yield), neutral lipid (6-10% of total yield) and acidic lipid (0.2-1.4% of total yield).

Because we isolated schistosome eggs from three different mouse strains, eggs from each strain were separately fractionated then analyzed and compared. As shown in Figure 5.7, the proportion of each egg fraction from eggs of the three strains of mice were similar. The percent of protein/glycoprotein fractions ranged from 88-93% of the total

yield. The neutral lipid fractions ranged from 6-10% of the total yield with the acidic lipid fractions the lowest ranging from 0.2-1.4% of total yield (Fig. 5.7). These percent yields were used to estimate dose (x) of each fraction per 30μg of SEA for future analysis.

3. Attempt to find pro-inflammatory fraction(s) using *in vitro* and *in vivo* assays

i. Protein fraction induces RAW cell production of pro-inflammatory cytokines *in vitro*

We observed that SEA stimulation of RAW264.7 cells induced TNFα production (Fig. 5.1). Therefore, we used RAW264.7 cells to initially evaluate the ability of schistosome egg fractions to induce pro-inflammatory responses. Control groups (media only, SEA and LPS) with the same amount of DMSO were included. Half of the media were removed after 3 hr stimulation and replaced with fresh media not containing DMSO to prevent high-dose-DMSO-induced cell death *in vitro*. Cultures were maintained for an additional 21 hrs then, supernatants were collected and analyzed for inflammatory cytokine productions.

As expected, LPS-treated cells induced significant production of TNFα, IL-6, IL-10 and MCP-1 chemokine, with levels of IL-6 and IL-10 reduced dramatically in the presence of 0.5-1% (v/v) DMSO. SEA treatment also increased production of TNFα compared to media, however, the differences were not statistically significant. Interestingly the protein fraction significantly increased production of both TNFα and MCP-1 compared to the media group (Fig. 5.8).

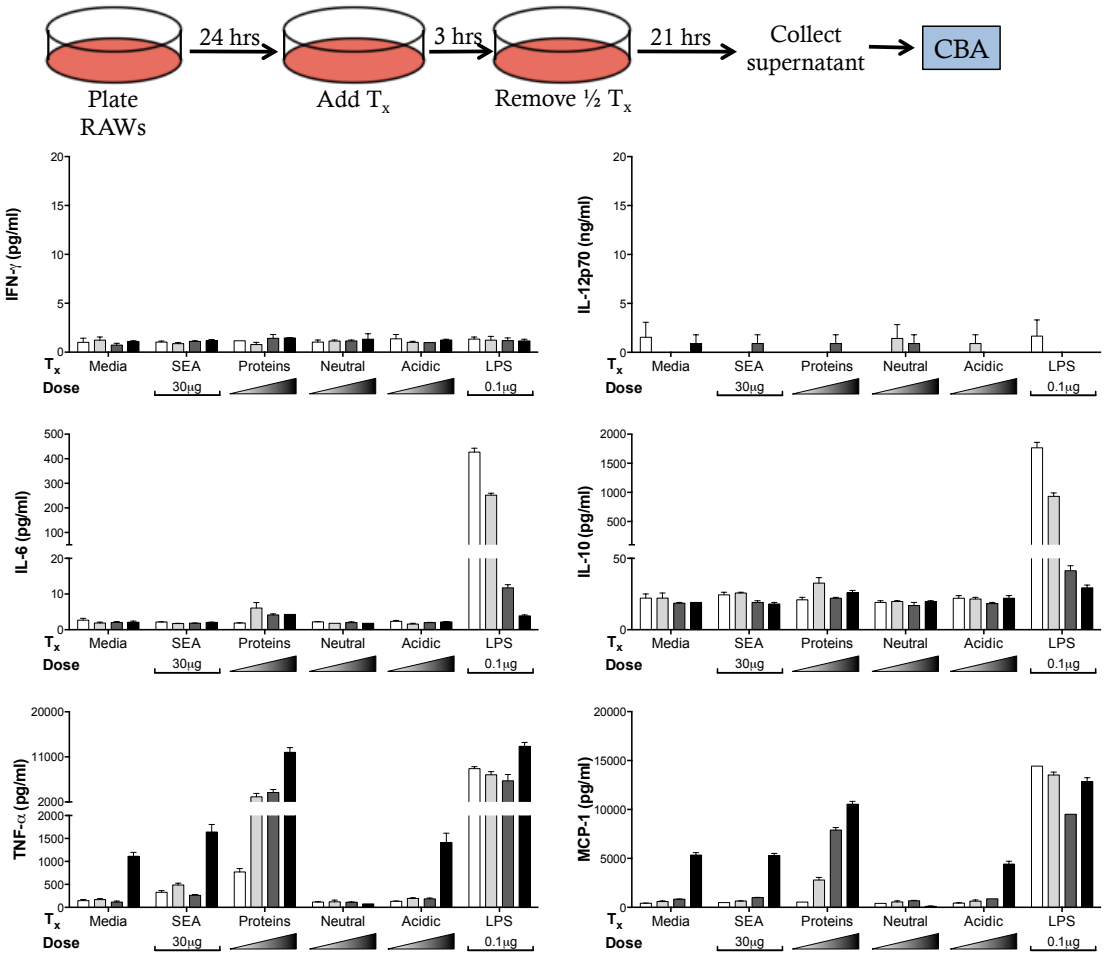

FIG 5.8 Protein fraction of eggs stimulated RAW264.7 cell production of TNFα and MCP-1. RAW264.7 macrophages were stimulated with 30µg SEA, increasing doses (0.1x, 1x, 5x, 10x) of egg fractions (proteins, neutral and acidic lipids) or 0.1µg LPS, in duplicate wells. Light to dark gradient indicates increasing amount of DMSO (0.01-1%, v/v). Three hours post-stimulation, half the media was removed and replaced with media without DMSO. Supernatants were collected at 24hr post treatment and analyzed for cytokine production using Inflammatory CBA kit.

Levels of TNFα and MCP-1 from cells treated with high dose (5x and 10x) protein fraction were comparable to levels induced by LPS (Fig. 5.8). Conversely, neutral and acidic lipid fractions did not induce production of any cytokines (Fig. 5.8). Of note, the neutral lipid fraction, in particular, significantly induced cell death at the high dose (10x) (Fig. 5.9).

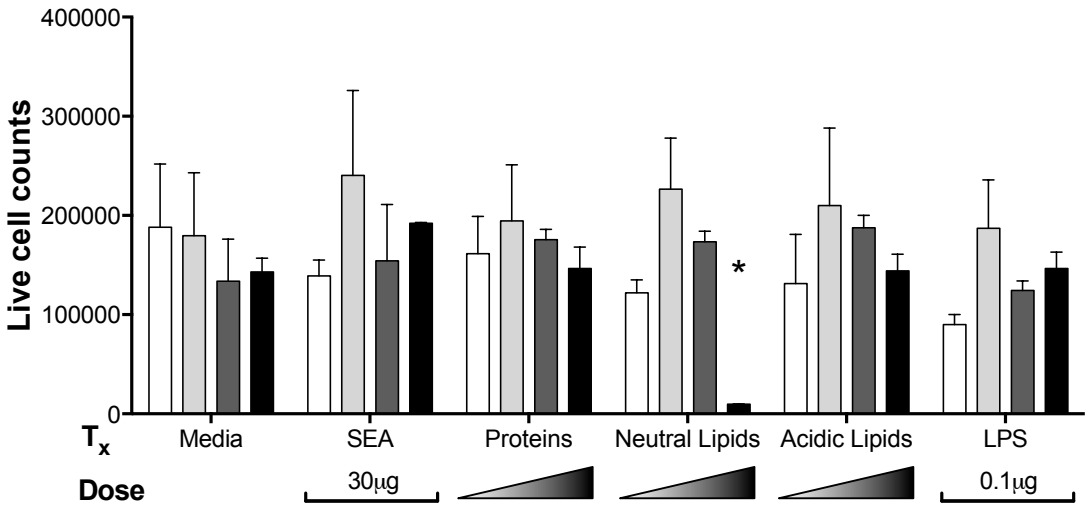

FIG 5.9 The neutral lipid fraction of schistosome eggs reduced cell viability at high doses. RAW264.7 macrophages were stimulated with 30μg SEA, increasing doses (0.1x, 1x, 5x, 10x) of egg fractions or 0.1μg LPS. Light to dark gradient underneath, indicates increasing amounts of DMSO (0.01-1% v/v). Three hrs post stimulation, half the media was removed and replaced with media without DMSO. At 24hrs post treatment, cells were collected from each well and counted to evaluate viability.

These data suggest that the schistosome egg protein fraction contains molecules that induce pro-inflammatory cytokines *in vitro*. However, the neutral lipid fraction, which induced cell death at high concentrations, should be further investigated to determine if this fraction is directly toxic to cells or if this fraction induces high levels of immune or other pathway activation, similar to what we observed in SEA/Lm-Gag vaccinated mice.

ii. Schistosome egg fractions each expand different peritoneal macrophage populations *in vivo*

We next evaluated schistosome egg fractions for adjuvant activity *in vivo*, by examining peritoneal exudate cells following i.p. injection. We initially tested if DMSO

by itself alters peritoneal cavity cell populations. Mice were injected with SEA with or without 5% (v/v) of DMSO. PECs were harvested 36 hours post injection and analyzed by flow cytometry. As shown in Figure 5.10, DMSO did change the numbers of leukocytes, but did not alter the composition of cells in peritoneal cavity. All four cell populations, LPM, SPM, eosinophil and neutrophil were detectable in mice injected with SEA +/- DMSO (Fig. 5.10). The most notable population changes were an approximate doubling of both the eosinophil and neutrophil populations in PECs from SEA+DMSO injected mice over SEA only injected mice. Therefore, we continued to mix fractions with 5% (v/v) DMSO for *in vivo* analysis.

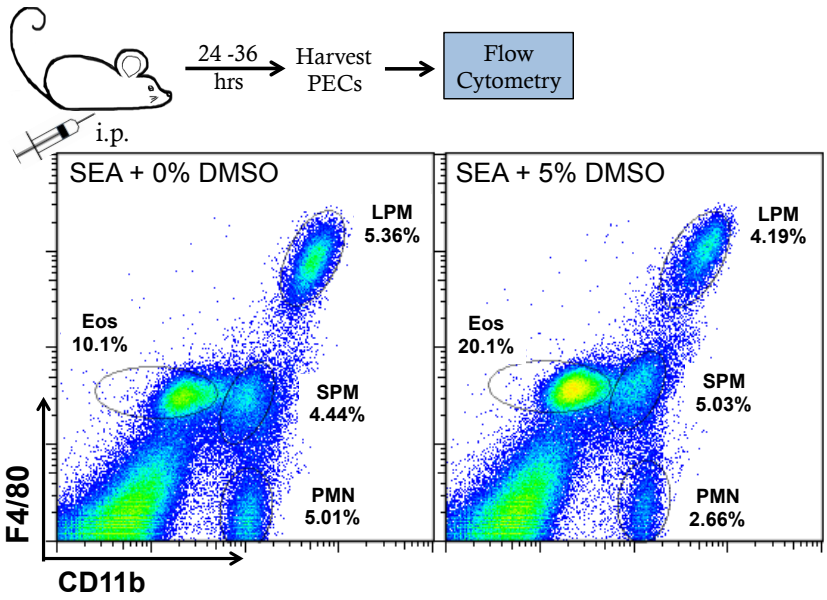

FIG 5.10 DMSO does not significantly alter SEA induced recruitment of LPM and SPM macrophages in the peritoneal cavity. SEA +/- 5.0% DMSO was injected intraperitoneally. Peritoneal exudate cells were harvested 36-hr post-injection, fluorescently labeled and analyzed for phenotype by flow cytometry.

In vivo analysis of the egg fractions utilized the following intraperitoneal injection groups: 1) saline, 2) SEA, 3) CpG, 4) protein, 5) neutral or 6) acidic lipids with 5% (v/v)

DMSO. PECs from each animal group were harvested, stained and analyzed. Neutrophils (PMNs, $CD11b^{int}Gr1^{+}$) were detected and quantified using the first gating between Gr1 and CD11b (Fig. 5.11A). Non-PMN cells were then analyzed for levels of eosinophils and macrophages. CpG injection surprisingly reduced cell viability resulting in low levels of all peritoneal cell populations. Similar to our earlier observation, SEA increased SPM ($CD11b^{int}F4/80^{int}$) and reduced LPMs ($CD11b^{hi}F4/80^{hi}$) populations (Fig. 5.11A).

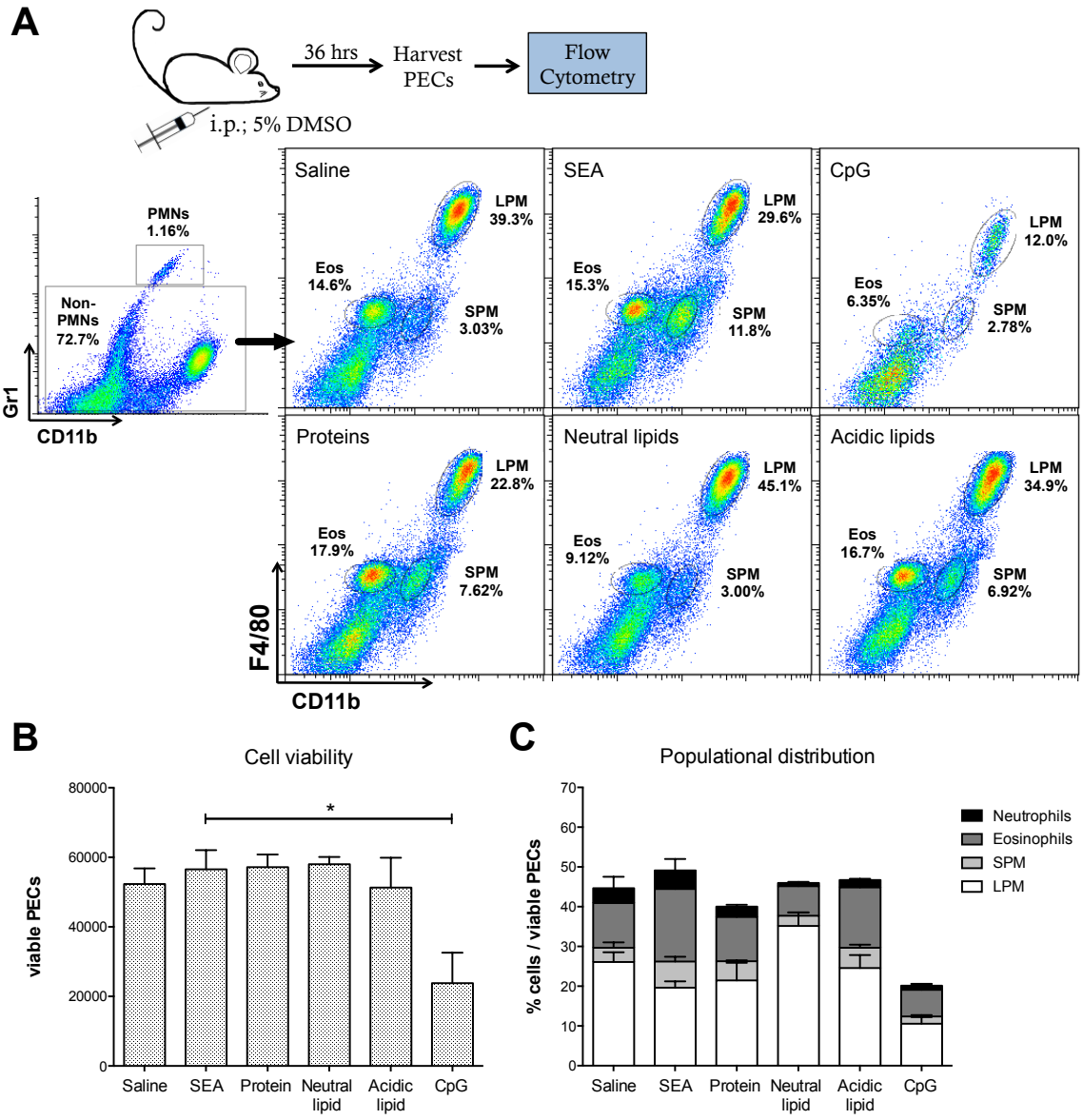

FIG 5.11 Schistosome egg fraction influence different peritoneal cavity cell populations. Thirty six hrs post-injection of controls, SEA or egg fractions, peritoneal exudate cells from each animal group (n=3) were harvested by lavage, fluorescently labeled and analyzed by flow cytometry. (**A**) Representative flow data shows levels of different cell populations in peritoneal cavity post-injection/treatment. (**B**) Cell viability were compared by one-way ANOVA, *$p<0.05$. (**C**) Leukocytes composition of peritoneal cavity after each treatment are presented in stack-bar.

Injection of the three different egg fractions influenced levels of eosinophils, SPM and LPM differently. Similar to SEA, the protein and acidic lipid fractions increased the numbers of both eosinophils and SPMs. However, the protein fraction increased these two populations to a greater extent. In addition, the levels of LPMs were reduced the most in the protein fraction group. The numbers of each of the peritoneal cell types in acidic lipid fraction mice was similar to the saline control group, except for an increase in the SPMs. Conversely, the neutral lipid fraction did not alter SPM levels but did increase the number of LPMs (Fig. 5.11A). These changes in peritoneal cellular composition post injection are also shown in stack-bar presentation (Fig. 5.11C). Based on the increased level of SPM or "inflammatory" macrophages, both the protein and acidic lipid fractions likely contain molecules that induce or upregulate pro-inflammatory responses. Due to the variation in responses to each fraction, we were unable to make a final conclusion.

4. Analysis of egg fractions using ELISpot assay

Though data from our first two experimental attempts were interesting, they did not provide conclusive evidence as to which fraction(s) were most pro-inflammatory. Our first *in vitro* study with RAW macrophages suggested either protein or neutral lipid fractions, whereas the *in vivo* study of peritoneal cells again suggested the protein

fraction, but also the acidic lipid fraction instead of the neutral lipid fraction. Therefore, we co-administered each egg fraction with the Lm-Gag vaccine and monitored for enhancement in Gag-specific T cell responses using ELISpot assay.

To perform this experiment we first needed to demonstrate that the addition of DMSO would not influence the adjuvant effect of SEA on generation of Lm-Gag $CD4^+$ and $CD8^+$ T cell responses. We vaccinated mice with Lm-Gag and SEA/Lm-Gag as previously described (237), each with 5% DMSO. Two wplv, Gag-specific T cell responses were analyzed. ELISpot analysis showed that by adding DMSO to the vaccine regiment we were not able to show any adjuvant effect of SEA on the Lm-Gag vaccine; the frequency of vaccine-specific T helper and CTL effectors were not significantly altered compared to Lm-Gag alone (Fig. 5.12A). Gag-specific CTL response, in particular, was reduced (Fig. 5.12A). To insure that this was not an artifact, we repeated the experiment without the use of DMSO, and again found that SEA enhances Lm-Gag vaccine-specific CTL responses (Fig. 5.12B).

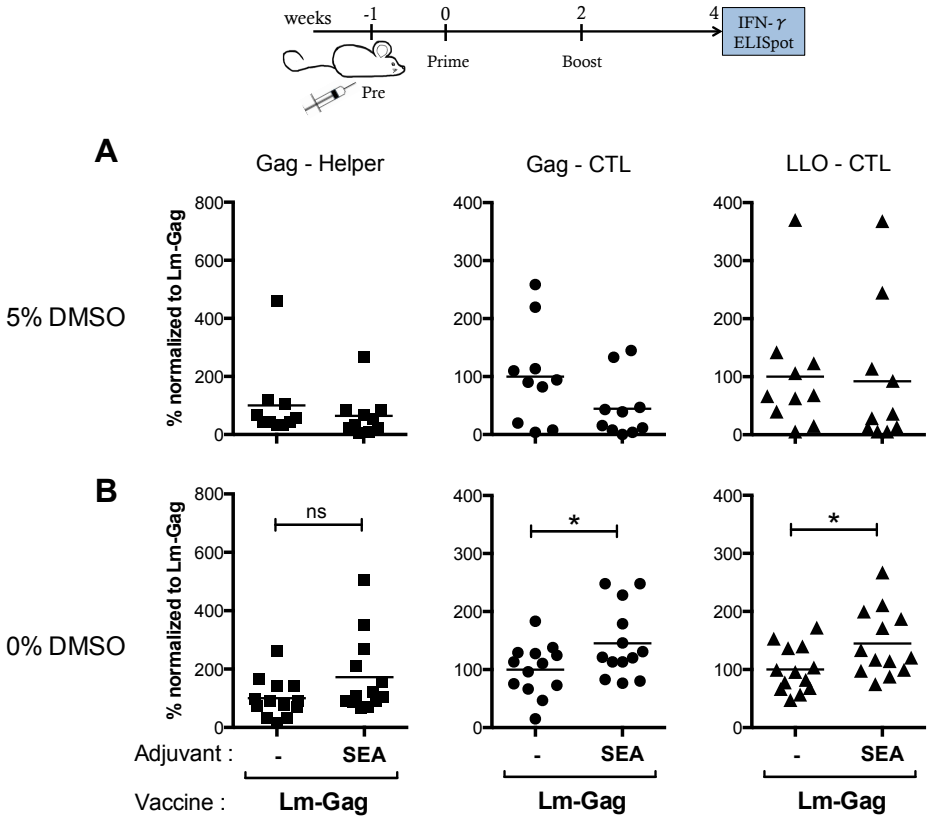

FIG 5.12 Adjuvant effect of SEA inhibited by DMSO. SEA was prepared with (**A**) or without (**B**) 5% DMSO for co-administration with Lm-Gag vaccine. Two wplv splenocytes were harvested and immune responses to the immunodominant HIV-1 Gag $CD4^+$ T helper (square) or CTL peptides of HIV-1 Gag (circle) or LLO (triangle) were measured by IFNγ ELISpot. Raw data from two independent experiments were normalized to Lm-Gag response, pooled and plotted individually as shown. Statistical differences between groups were analyzed by one-way ANOVA and Tukey post hoc test, *$p<0.05$ and ns-non-significant.

These results suggest that addition of DMSO to SEA inhibited the adjuvant effects of SEA when co-administered with the Lm-Gag vaccine. Therefore, the positive control SEA/Lm-Gag group was not included in further analysis. We expect the potential adjuvant fraction/ molecule(s) in schistosome eggs would be potent enough to overcome DMSO inhibition. However, in the case DMSO abrogates the adjuvant effects, we would consider a different solvent for the future fraction studies.

i. Fractionation of Schistosome eggs by new protocol resulted in better yield for lipid fractions and more defined classes of molecules.

Before we proceeded to test the egg fractions for enhancement of Lm-Gag induced vaccine responses, Dr. Ishihara recommended two new fractionation protocols to gain more refined classes of lipids for this study.

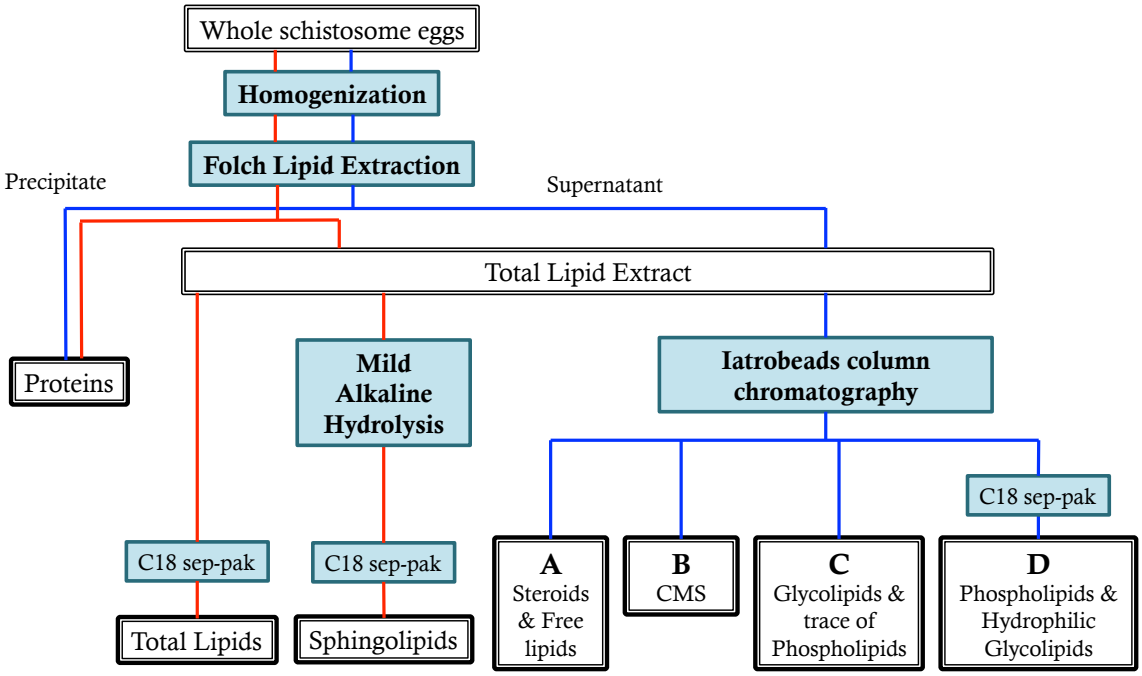

FIG 5.13 Modified protocol for schistosome egg fractionation. Newly modified fractionation scheme resulted in more defined classes of molecules in egg lipid fraction. Lipid extraction was done first on two different batches of schistosome eggs. As protein fractions were separated, mild alkaline hydrolysis was used for the first batch of total lipid extract to separate the alkaline-stable sphingolipid fraction (red). Iatrobeads column chromatography was used on the second batch to gain four different classes of lipids (blue).

Similar to the original fractionation protocol, the protein fraction from two different batches of schistosome eggs (red and blue) were first separated from total lipid extracts by traditional lipid extraction methods (Fig. 5.13). For the red batch of total lipid,

half were used for C18 separation and the other half subjected to milk alkaline hydrolysis to collect the alkaline-stable sphingolipid fraction. Alkaline-labile glycoglycerol-lipids were in the remaining total lipid extract (Fig. 5.13). For the second batch of total lipid (blue), iatrobead column chromatography was used to separate different classes of lipids based on their relative hydrophobicity. This method resulted in about a dozen small fractions (Appendix B), which were too many for us to analyze at once. Therefore, based on their thin-layer chromatography profile, these small fractions were combined into four larger fractions named A, B, C and D (Fig. 5.13). Fraction A contained mainly sterols. Fraction B contained sphingolipids, primarily ceramide monosaccharides. Fraction C was a mixture of different glycolipids with a trace amount of phospholipid and lastly, fraction D contained phospholipids and hydrophilic glycolipids (Fig. 5.13).

The new fractionation protocol resulted in more defined classes of molecules with increased yield of lipid fractions.

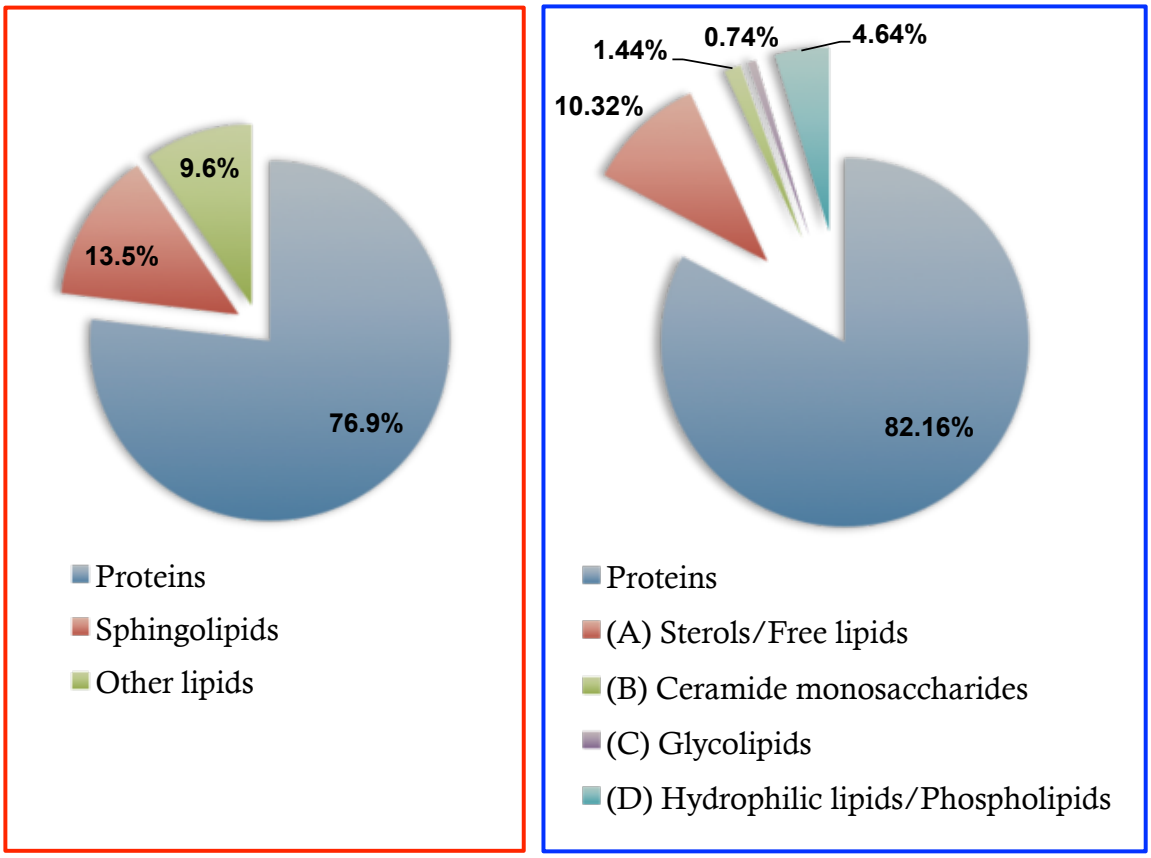

FIG 5.14 Pie charts show the percentages of fractions obtained from schistosome eggs using two new fractionation methods. Fractions obtained from the milk alkaline hydrolysis (red) yielded three classes of molecules: proteins, sphingolipids and other lipids. The iatrobead fractionation of eggs (blue) resulted in five fractions: proteins, A – sterols and free lipids, B – ceramide monosaccharides, C – a mixture of different glycolipids and D – hydrophilic glycolipids and phospholipids.

As shown in the pie-chart presentation, the protein fraction is the largest fraction regardless of the fractionation method used, 76.9% and 82.16% for milk alkaline hydrolysis vs iatrobead fractionation respectively. Using either new fractionation method total lipid content yield increased from 14-23% compared with from 10% using the original protocol (Fig. 5.14). The second largest fraction for the milk alkaline hydrolysis fractionation was the alkaline-stable sphingolipid fraction at 13.5% total yield. Using iatrobead fractionation, we found that fraction A which contained sterols, was second in

terms of total yield at 10.32% (Fig. 5.14). All six lipid fractions and one of the protein fractions were dissolved in DMSO before use.

ii. Lipid fractions significantly increase Lm-Gag vaccine responses compared to unvaccinated and vector control groups

To identify the adjuvant fraction in schistosome eggs, mice were vaccinated with Lm-Gag vaccine and co-administered with each egg fraction using the similar, optimized immunization protocol as for SEA. Unvaccinated and Lm-E7 vaccinated groups were included as negative controls for this analysis. As expected, the Lm-E7 group only induced cellular responses to LLO peptide while unvaccinated mice did not respond to any vaccine-specific peptides (Fig. 5.15).

Among the seven different egg fractions evaluated *in vivo*, we found that co-administration of Lm-Gag with proteins or the total lipid fraction followed by C18 chromatography (Fig. 5.13), did not improve Lm-Gag vaccine responses. The frequencies of Gag-specific T helper 1 and CTL effectors in these groups were slightly reduced compared to Lm-Gag alone. Conversely, co-administration with either sphingolipid or iatrobead fractions (A-D) significantly increased the frequency of Gag-specific T helper 1 and CTL responses compared to unvaccinated, Lm-E7 and Lm-Gag alone groups (Fig. 5.15). Fraction B, in particular, significantly improved effector response against the LLO peptide (Fig. 5.15). These results were exciting since Lm-Gag alone group did not induce such statistical differences in vaccine responses. These data suggest that the adjuvant molecules in SEA are in a lipid fraction.

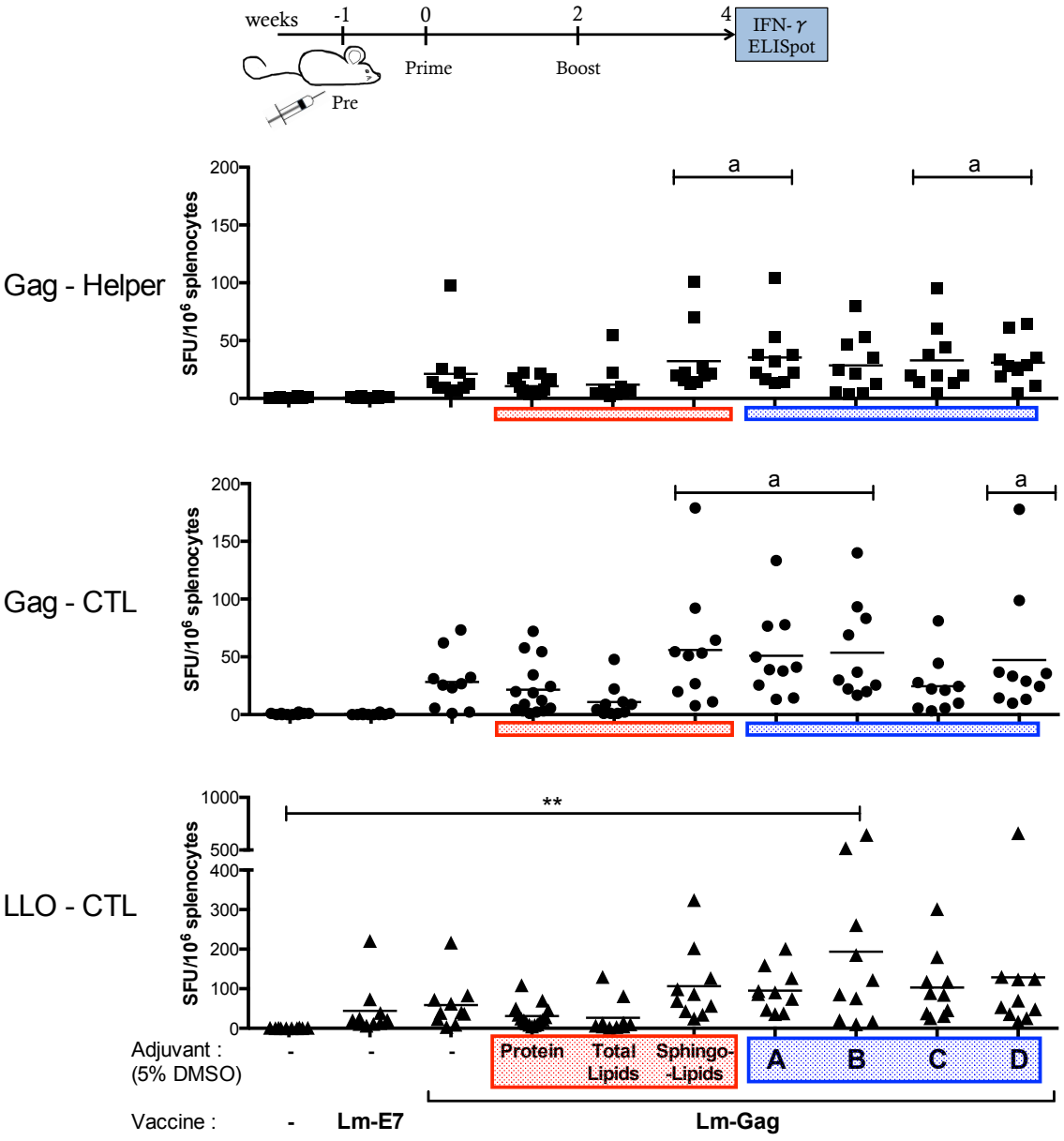

FIG 5.15 Several different egg lipid fractions significantly enhanced Lm-Gag vaccine-specific responses. Two wplv splenocytes were harvested and immune responses to the immunodominant HIV-1 Gag $CD4^+$ T helper (square) or CTL peptides of HIV-1 Gag (circle) or LLO (triangle) were measured by IFNγ ELISpot. Data from two independent experiments were pooled and plotted individually as shown. Statistical differences between groups were analyzed by one-way ANOVA and Tukey post hoc test, **$p<0.01$ and a- significant ($p<0.05$) compared to unvaccinated and Lm-E7 groups.

iii. When mice were co-administered with fraction B, Lm-Gag vaccine responses did not generate dose-dependent T cell responses as expected.

Among the seven egg fractions tested, we decided to further investigate fraction B since it was the only fraction to significantly enhance vaccine-induced LLO responses compared to the unvaccinated group. A small dose-response study was carried out where mice were vaccinated with Lm-Gag and increasing doses (0.2x, 1x and 5x) of fraction B. The frequencies of vaccine-specific T helper 1 and CTL effector responses were then measured by IFNγ ELISpot. Unfortunately, data from this analysis did not show statistical differences between the three vaccinated groups (Fig. 5.16). Though the vaccine responses might have been saturated at the dose as low as 0.2x, this is an unlikely explanation for these results. Therefore, data from this study were inconclusive regarding the adjuvant activity of fraction B (Fig. 5.16).

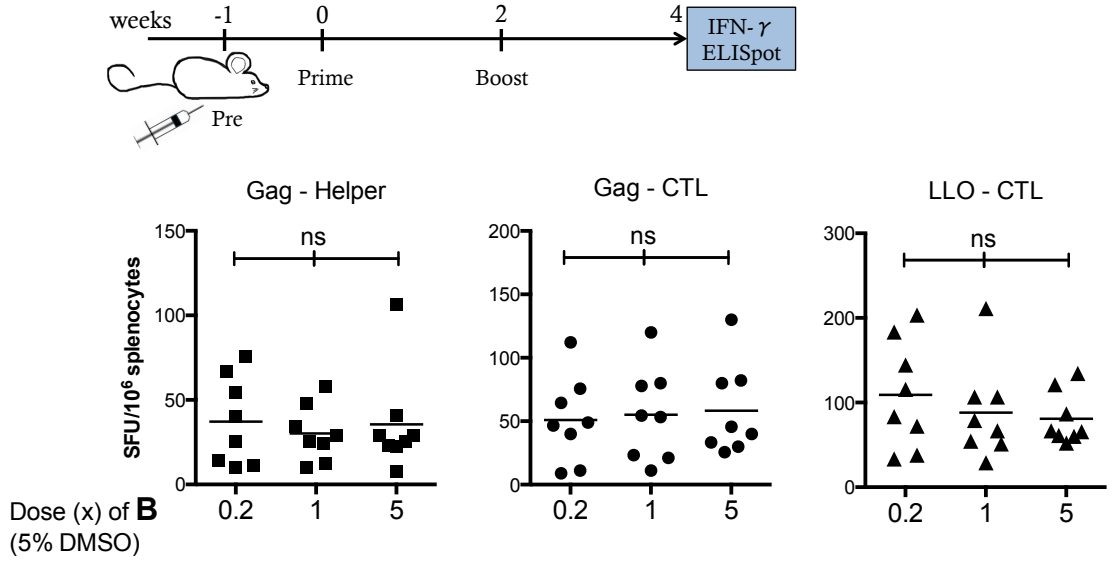

FIG 5.16 Increasing doses of fraction B did not enhance Lm-Gag vaccine responses. Mice were vaccinated with ncreasing doses of fraction B co-administered with Lm-Gag vaccine. Two wplv splenocytes were harvested and immune responses to the

immunodominant HIV-1 Gag $CD4^+$ T helper (square) or CTL peptides of HIV-1 Gag (circle) or LLO (triangle) were measured by IFNγ ELISpot. Data were plotted individually as shown. Statistical differences between groups were analyzed by one-way ANOVA and Tukey post hoc test, ns- not significant.

IV. Conclusions

To begin dissecting the adjuvant characteristics of SEA, we needed to develop viable *in vitro* and *in vivo* assays. For the *in vitro* analysis we demonstrated that RAW264.7 macrophages, stimulated with SEA led to strong production of, TNFα (Fig. 5.1). Thus we had an *in vitro* readout to evaluate egg fractions. We then demonstrated that intraperitoneal injection of SEA expanded the "inflammatory" SPM macrophage population after a single intraperitoneal injection (Fig. 5.5). Based on these results, we proceeded to test three egg fractions, protein, neutral and acidic lipids using these two methods.

In vitro analysis showed that stimulation of RAW264.7 macrophages, with the protein fraction increased production of TNFα. This is similar to the effect of SEA (Fig. 5.1, 5.8). However, we also noted that the neutral lipid fraction compromised cell viability, possibly through robust immune activation, and kept this fraction our "still interesting" list (Fig. 5.9). We proceeded to test the ability of egg fractions to induce the expansion of peritoneal cells. Using this *in vivo* assay we observed that both the protein and acidic lipid fractions expanded the number of "inflammatory" SPMs in the peritoneal cavity (Fig. 5.11). Conversely, the neutral lipid fraction did not alter SPM levels though this fraction did expand the LPM resident macrophage compartment (Fig. 5.11). Overall, these results were exciting since they suggested that schistosome eggs do contain immune-active components. Unfortunately, we were not yet able to conclude whether

these inflammatory effects by either protein or acidic lipid fraction correlated with adjuvant effects. Therefore, we decided to directly test for adjuvant activity *in vivo* using the Lm-Gag vaccine model.

To further define the nature of the types of molecules in SEA that drive pro-inflammatory responses, took the total lipids fraction and subjected them to either of two new fractionation methods, mild alkaline hydrolysis and iatrobead chromatography. The new fractionation methods yielded more defined lipid fractions, including sphingolipids, A – sterols, B – ceramide monosaccharides, C – glycolipids and trace of phospholipids, and D – phospholipids and hydrophilic glycolipids (Fig. 5.13-14). Co-administration of several lipid fractions with the Lm-Gag vaccine significantly enhanced Gag-specific T Helper and CTL responses compared to unvaccinated group (Fig. 5.15). These data suggested that the components in schistosome eggs responsible for enhancing Lm-Gag vaccine specific CTLs are likely lipids.

Both the protein and total lipid fractions slightly reduced Gag-specific vaccine responses (Fig. 5.15). While performing the experiments on SEA and egg fractions we determined that DMSO suppressed SEA enhancement of the Lm-Gag vaccine (Fig. 5.12), DMSO inhibited the CTL adjuvant effects of the otherwise active components and reduced Lm-Gag induced vaccine responses. However, as the active components are purified, or if we use another method to resuspend egg lipid fractions, we might be able to work around DMSO mediated inhibition and confirm which fractions and molecules have adjuvant activity. Therefore, more empirical studies must be done to identify and evaluate the compounds in schistosome eggs that are responsible for enhancement of vaccine induced CTL responses.

CHAPTER 6

DISCUSSION

Tremendous effort continues to go towards development of vaccines that drive potent functional and protective immune responses for numerous infectious diseases. Unfortunately, there still are no fully effective vaccines against many widespread infectious diseases, including HIV-AIDS, malaria, and tuberculosis. Although humoral immunity has a role in preventing infection by HIV and can influence certain stages of malaria infection (19-21), there is compelling evidence that Th1 cells, $CD8^+$ T cells, or both, also have a critical role in preventing or controlling these infections (22-31). For many infectious diseases as well as control/elimination of tumors and cancers, it may be necessary to generate potent and perhaps multifunctional T cell responses in patients who respond poorly to relevant pathogen or tumor antigens (32-37).

While some of the difficulties associated with development of vaccines for these diseases are due to intrinsic aspects of the infecting agent, another challenging task has been development of safe and effective adjuvants to stimulate the recipient's immune system inducing long-lasting protective immunity. In terms of adjuvants, aluminum-salts are the only vaccine adjuvants currently licensed for use in humans in the United States (65). Alum adjuvants consist of precipitates of aluminum phosphate and or aluminum hydroxide to which vaccine antigens are adsorbed (67, 68). Alum is used primarily to enhance humoral immunity (69). However, alum can trigger necrotic cell death and the

release of the endogenous danger signal uric acid (68, 78). The current controversies concerning the mechanism of action of alum adjuvants and potential *in vivo* toxicity emphasize the need for better adjuvants.

Our lab has been investigating molecules derived from the helminth parasite *Schistosoma mansoni* as potential sources of new adjuvants. Helminth parasites including schistosomes strongly bias the host immune system towards $CD4^+$ Th2-type (15-16). Further, infection with helminth parasites suppresses host immunity, impairing the expansion of pathogen-specific cytotoxic $CD8^+$ T cells (CTL) and T helper 1 (Th1) cells (52, 54, 136, 138, 140, 195). Hence, helminth infection may impair the host's ability to mount an effective immune response to vaccines. In this regard, an earlier study demonstrated that schistosome infection dramatically impaired the response to an HIV-1 vaccine (28). For schistosomes, the same immune biasing and immune suppressive properties associated with infection, can also be induced with schistosome eggs, or the saline soluble homogenate of schistosome eggs (SEA) (16). Therefore, our starting point for the identification of new adjuvants are schistosome eggs and SEA.

Our starting hypothesis was that incorporation of SEA to an Lm-Gag vaccine would suppress vaccine-specific CTL and Th1-type responses generated by the Lm-Gag vaccine. However, the results generated here demonstrate just the opposite; co-administration of SEA with Lm-Gag significantly enhanced the HIV Gag-specific CTL and T helper 1 responses compared to mice vaccinated with Lm-Gag alone (237). Surprisingly, we observed the ability of SEA to enhance induction of Gag-specific CTL responses in two immunologically different murine models, BALB/c (237) and C57BL/6 (Fig. 4.3). *In vivo* functional analysis of CTLs showed that the level of HIV-1 Gag-

specific killing was enhanced in mice vaccinated with SEA/Lm-Gag (237). SEA co-administration with Lm-Gag also induced long-lasting vaccine-specific responses (237). Taken together, these results suggest that completely opposite to our expectations, addition of SEA to the Lm-Gag vaccine had a synergistic effect with the Lm-Gag vaccine to enhance Type 1, pro-inflammatory, vaccine-specific T cell responses.

How is it that the homogenate of schistosome eggs SEA, which drives $CD4^+$ Th2 biasing in the host (135, 202, 203, 208-211), is able to elevate CTL and Th1-type responses to an Lm-Gag vaccine? Further to this point, our results show that co-administration of SEA with Lm-Gag did give rise to increased Th2 and Treg populations coincident with significant production of IL-4 and IL-10 (237). To reconcile our observations one only has to examine the evolution of immune responses as a consequence of schistosome infection. For example, from 4-6 weeks post-infection, when adult worms begin laying eggs, host immune responses are predominantly $CD4^+$ Th1-type coincident with an increase in total numbers of pro-inflammatory, classically activated macrophages (140). This fits with multiple reports describing schistosome antigen(s) ligation of host pattern recognition receptors including the Toll like receptors (TLRs), driving pro-inflammatory responses (222-226). Recently, SEA was shown to drive production of pro-inflammatory mediators from primary human placental trophoblasts (227, 228). Thus, SEA contains molecules that stimulate pro-inflammatory responses, and these appear to work in juxtaposition to SEA molecules that drive Th2-type and anti-inflammatory responses via ligation of C-type lectins or TLRs (225, 229).

One possible explanation for our unexpected results is that there is synergy between the pro-inflammatory components in SEA and the pro-inflammatory molecules

expressed by the *Listeria* vector used in this study (Fig. 6.1), as Lm-Gag alone did not drive the same levels of vaccine-specific Th1 and CTL responses as seen in mice immunized with SEA/Lm-Gag. Based on the results of our study, we hypothesize that pre-injection with SEA induces influx/expansion of inflammatory macrophages (SPM) to the peritoneal cavity (Fig. 5.5). Vaccination with the Th1 vaccine Lm-Gag and additional SEA injections further amplify this expansion. The release of pro-inflammatory cytokines and chemokines, such as TNFα and MCP-1 (Fig. 5.1), establishes a strong pro-inflammatory environment that dominates the Th2-Treg responses normally driven by SEA. In the draining lymph node, we would expect host vaccine-specific immune responses to polarize toward pro-inflammatory under these conditions. The pro-inflammatory milieu would amplify classical activation of macrophages, promoting recruitment of more immune cells and enhance pro-inflammatory (Th1 and CTL) activation of $CD4^+$ and $CD8^+$ T cells against HIV-1 Gag (Fig. 6.1).

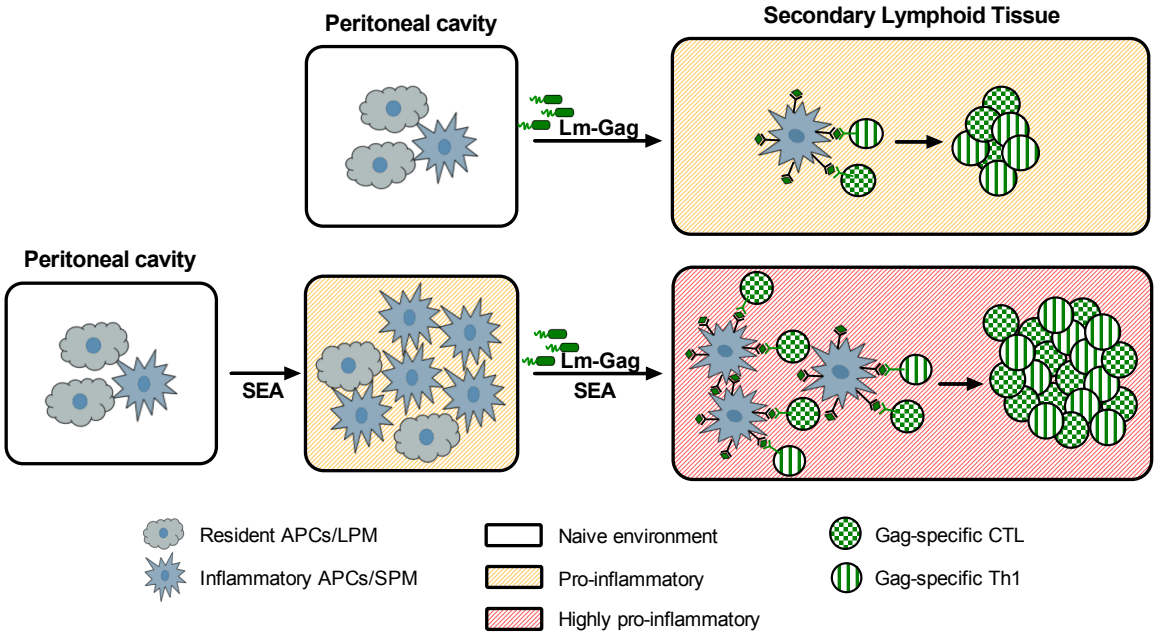

FIG 6.1 Potential mechanism of action by SEA to enhance Gag-specific cell-mediated responses. Vaccination with Lm-Gag vaccine alone induces classical activation

of macrophages and promotes T cell activation against vaccine antigens. SEA co-administration expands the inflammatory macrophage/SPM population, establishing a pro-inflammatory environment that would be further amplified by Lm-Gag vaccination. The synergy between SEA and Lm-Gag significantly enhances pro-inflammatory responses and T cell activation against HIV-1 Gag.

Though SEA has been studied extensively, most of the "immune activating" components are not yet identified. SEA is a saline homogenate of *S. mansoni* eggs and therefore contains numerous classes of compounds, including proteins, lipids, carbohydrates and nucleic acids. Many studies describe the ability of SEA to induce Th2-type and anti-inflammatory responses as being dependent on carbohydrate components (142, 150-154). In contrast, reports on schistosome molecules that drive Th1 pro-inflammatory responses are scarce. Aksoy et al. has described double stranded RNAs from schistosome eggs that activate dendritic cells via TLR3 (226), while Duraes et al. found schistosome tegumental molecules that induce IL-12 and TNFα production in dendritic cells (222). The most recently described pro-inflammatory molecule from schistosomes is a lipid obtained from extracts of adult worms (224). Therefore, in an attempt to identify the adjuvant components in SEA, we collaborated with Drs. Azadi and Ishihara from the UGA Complex Carbohydrate Research Center, to chemically fractionate schistosome eggs. Once fractionated, we then tested the fractions for pro-inflammatory and adjuvant effects.

The initial fractionation protocol resulted in three egg fractions: proteins and neutral and acidic lipids. *In vitro* testing on RAW264.7 macrophages showed the protein fraction significantly induced production of TNFα (Fig. 5.8). This result is similar to what we observed using SEA *in vitro* (Fig. 5.1). The neutral lipid fraction also seemed to have activity as addition to RAW264.7 cells compromised cell viability (Fig. 5.9).

Reduced viability of RAW cells treated with the neutral lipid fraction may have been the result of robust immune activation, or direct toxicity.

We continued our search for SEA pro-inflammatory molecules by examining the expansion of peritoneal cells *in vivo* focusing on examination of peritoneal macrophages. Both protein and acidic lipid fractions expanded the number of "inflammatory" SPMs in the peritoneal cavity (Fig. 5.11). Conversely, the neutral lipid fraction did not alter SPM levels though this fraction expanded the LPM resident macrophage compartment (Fig. 5.11). Overall, these results suggest that schistosome eggs contain pro-inflammatory, immune-activating components. Unfortunately, we were not able to conclude whether the inflammatory effects by the protein or lipid fractions correlated with adjuvant effects. Therefore, we moved to directly test for adjuvant activity *in vivo* using Lm-Gag as a vaccine model.

For the *in vivo* Lm-Gag vaccine studies the fractionation methods were changed from standard Folch separation to Folch followed by either mild alkaline hydrolysis or iatrobead chromatography. The new fractionation scheme yielded the protein fraction as well as total lipids, sphingolipids, sterols, ceramide monosaccharides, glycolipids and trace phospholipids, and phospholipids and hydrophilic glycolipids (Fig. 5.13-14). We noted that co-administration of several lipid fractions with the Lm-Gag vaccine significantly enhanced Gag-specific T helper and CTL responses compared to unvaccinated group (Fig. 5.15). These data suggest that the active components in schistosome eggs responsible for the CTL adjuvant effects will be found in the sphingolipid fraction and or the iatrobeads fractions A-D.

In contrast, both the protein and total lipid fractions reduced Gag-specific vaccine responses (Fig. 5.15). We also observed that addition of DMSO inhibited the SEA adjuvant effect, reducing the Lm-Gag induced vaccine responses (Fig. 5.12). However, we believe that further enrichment/purification of the active components in SEA occur, we may be able to overcome some of the DMSO inhibition issues. Alternatively, we can begin testing other solvents, such as DMF to prevent suppressive effects of dissolving agents.

Overall, the results from our study suggest that SEA contains Th1-type promoting sphingolipids molecules that can enhance functional, vaccine-specific cell mediated immune responses. Enhancing cytotoxic $CD8^+$ T cells and Th1-type vaccine-specific responses has been a long-term goal of those working to develop vaccines for HIV-1, tuberculosis, malaria and many other infectious diseases. Adjuvants that enhance antigen-specific Th1 and $CD8^+$ T cell vaccine responses may also have utility as components of therapeutic vaccines for cancers. Therefore, more empirical studies must be done to identify and evaluate the lipid compounds in schistosome eggs that are responsible for enhancement of vaccine induced CTL responses. Defining new adjuvant(s) that promote CTL responses is novel, and innovative as we started with the polar opposite, Th2-driving compound SEA.

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APPENDIX A

2011 FRACTIONATION ANALYTICAL REPORT

Methods

Whole schistosome eggs were suspended in methanol/water solution and homogenized on ice. Lipids were extracted from the sample by chloroform/methanol/water. Precipitate (protein rich fraction) was further washed with acetone/water and dried. On the other hand, the lipid extract was further fractionated by QAE ion exchange column chromatography. QAE- fractions were further cleaned up by C18- sep pak. An aliquot of each lipid fractions were then examined by thin layer chromatography. Protein -rich powder, QAE-pasing through lipid fraction, QAE-binding lipid fraction thus obtained were then returned to you for further experiment.

Procedure

Preparation of protein rich powder and total lipid extract

Whole schistosome eggs were homogenized and de-lipidated followed by the method of Aoki.et.al (2007). Briefly, eggs were suspended in methanol/water (1:1, by vol.) and homogenized by a dounce homogenizer on ice. Lipids were extracted by adjusting the solvent mixture to give a final ratio of chloroform/methanol/water equal to 4:8:3 (by vol.). The extract was incubated at room temperature with end-over-end agitation. The insoluble proteinaceous material was collected by centrifugation and the

lipids were re-extracted three times. The lipid fraction was dried by a stream of nitrogen. The final pellet of insoluble protein was further washed with cold-acetone/water (4:1, v/v) four times and dried under a stream of nitrogen.

Separation of lipid fraction by QAE sephadex A-25 column chromatography

QAE column chromatography was performed according to the method of Sugita.et al. (1992). Briefly, the lipid extract was reconstituted with C/M/W (30:60:8, by vol.) and loaded to QAE-Sephadex A-25 (OH^- form) column. Neutral glycolipids were washed out with chloroform/methanol/water (30:60:8, by vol.). Thereafter, acidic glycolipids was eluted with 0.5M ammonium acetate in methanol, and then dialysed to remove acetate.

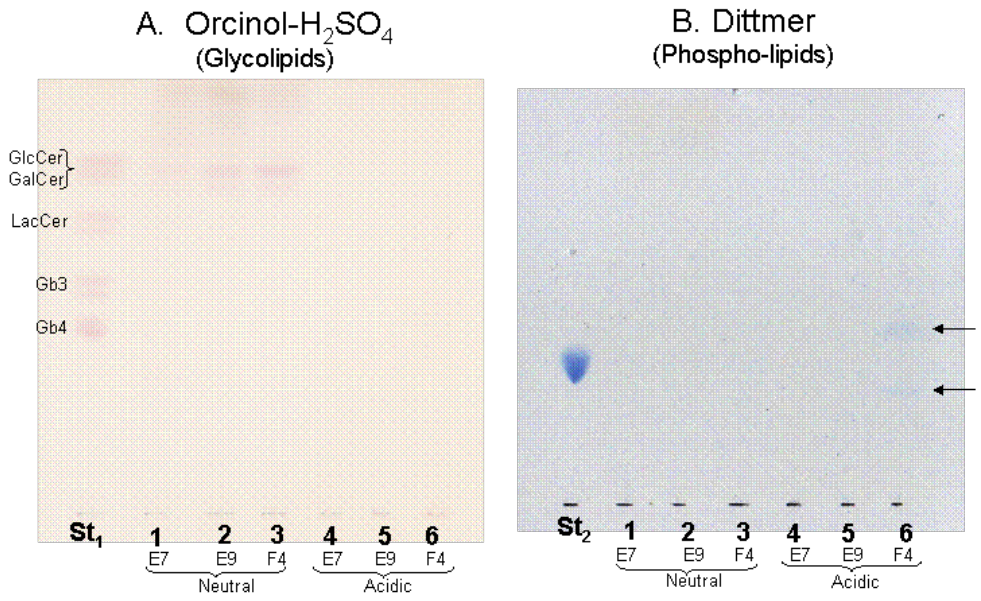
QAE-fractions (passing though- and binding fraction) were further cleaned of contaminants. Briefly, the fractions were reconstituted with 50% methanol in water and loaded into a C18 sep-pak cartridge and then washed with nanopure water. The lipids were eluted with iso-propanol.

Thin layer chromatography (TLC)

TLC was performed using silica gel 60-precoated plates, with a solvent system chloroform/methanol/water (60:40:10, by vol.). Detection was performed by spraying with orcinol/H_2SO_4 reagent for sugars, Dittmer–Lester reagent for phosphorus, ninhydrin reagent for free amino groups, respectively.

Analytical results from TLC

An aliquot of QAE-lipid fractions were examined by thin layer chromatography (TLC) and the results are shown below.



Lane St_1: Neutral glycosphingolipid mixture (standard)
Lane St_2: Sphingomyelin (standard)
Lanes 1~3: QAE-passing through fractions, E7, E9 and F4, respectively
Lanes 4~6: QAE-binding fractions, E7, E9 and F4, respectively

Development: Chloroform/ Methanol/ Water = 60:40:10

Detection: A) Orcinol-H_2SO_4
B) Dittmer- Lester reagent

In QAE-passing through fractions, all three samples (E7,E9 and F4) seems to contain a lipid, which is positive in orcinol detection and shows similar R_f value to ceramide monosaccaride standard. It indicates that all QAE-passing through fractions contain ceramide monosaccharide or something similar to that. The stains, observed above ceramide monosaccharide in orcinol detection are typical R_f value and stain observed from sterols. It indicates all QAE passing-through fractions contain sterols. In QAE-binding fractions, no positive band was observed in orcinol detection, whereas two bands in lane 6 showed positive detection in Dittmer as indicated with arrows. It indicates an existence of phospho-lipids in QAE-binding fraction from F4. No positive band in Dittmer reagent was detected in E7 and E9 samples. It does not mean there is no phospholipid in the sample. E7 and E9 might contain phospholipids as well but the amount of phospholipids in E7 and E9 would be very low (lower than the detection limit of Dittmer reagent).

APPENDIX B

2013 FRACTIONATION ANALYTICAL REPORT

Methods:

The samples were first homogenized by homogenizer on ice with Methanol/water 1:1, followed by lipid extraction by Chroloform/methanol/water (C/M/W). Precipitates were collected by cold centrifugation and further washed with cold-acetone/water, followed by cold-acetone. On the other hands, lipids were harvested by drying down the C/M/W extract. A half of lipid extract from F12 was subjected to mild alkaline hydrolysis to remove glycerolipids and recover sphingolipids. A lipid extract from F13 was separated by iatrobeads column chromatography. A lipid extract (A half of F12 as control), mild alkaline hydrolyzate (the other half of F12), and hydrophilic fraction from iatrobeads column chromatography, were further passed through C18 sep-pak to remove salts, free-oligosaccharides and other contaminants in the fractions.

Procedure:

Preparation of protein rich powder and total lipid extract

Whole schistosome eggs were suspended in methanol/water (1:1, by vol.) and homogenized by a dounce homogenizer on ice. Lipids were extracted by adjusting the solvent mixture to give a final ratio of chloroform/methanol/water (C/M/W) equal to 4:8:3 (by vol.). The extract was incubated at room temperature with end-over-end

agitation. The insoluble proteinaceous material was collected by centrifugation and the lipids were further extracted again with C/M 1:1, 2:1 to C/M/W 4:8:3 gradually by adjusting the solvent ratio. The extraction/centrifugation procedures were repeated total three times. The lipid extract was combined and dried by a stream of nitrogen. The final pellet of insoluble protein was further washed with cold-acetone/water (4:1, v/v) two times, cold-acetone once and dried under a stream of nitrogen and examined by TLC.

Separation of lipid fraction by Iatrobeads column chromatography

The separation of lipids fraction was performed by iatrobeads column chromatography. Iatrobeads (Mitsubishi Kagaku Iatron, Inc.) was packed to a column (bed vol 5mL) and conditioned with Chloroform. The sample (Lipid extract from F13) was reconstituted with C/M 95:5 and loaded to the column. The sample elution was archived by stepwise elution with solvents listed below;

1, C/M 95:5

2, C/M 90:10

3, C/M 80:20

4, C/M/W 60:40:4

5, C/M/W 40:60:6

6, C/M/W 30:60:8

The ratio is described by volume, each solvent was loaded 3 bed volumes (15mL), and 7.5 mL was collected /each fraction manually and each fractions were dried and examined by TLC.

Degradation of glycerolipids by Mild alkaline hydrolysis

A half of F12 lipid extract was subjected to a mild alkaline hydrolysis to degrade glycerolipids and concentrate sphingolipids, which is alkaline-stable. The sample was reconstituted with 500uL of 0.5 M KOH in Methanol and incubated at 37^{o}C overnight.

Desallting, removal of free oligosaccharides and other hydrophilic contaminants by C18 sep-pak cartridge

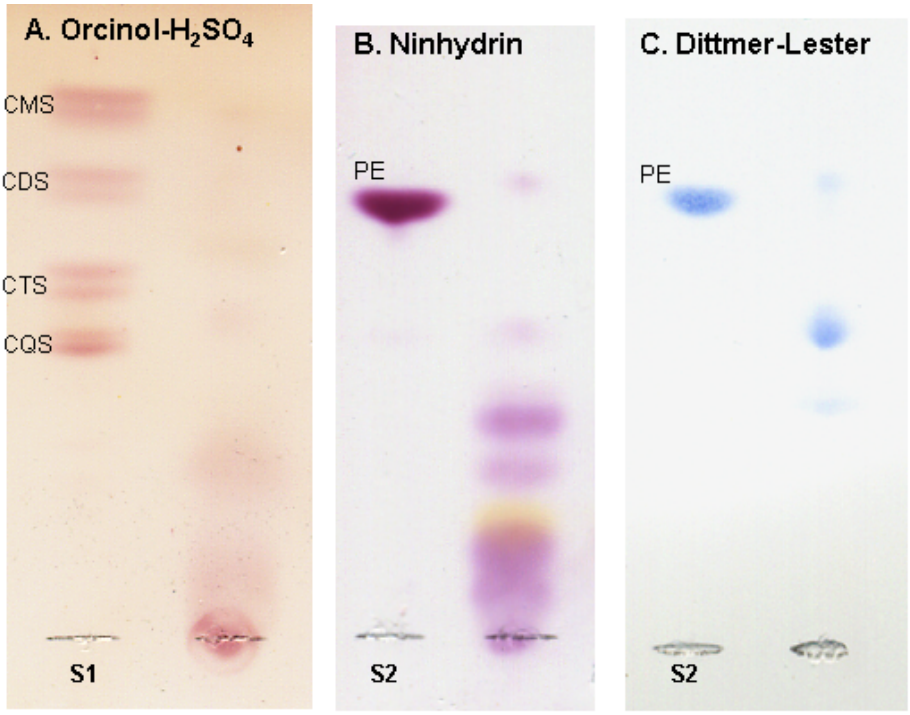
The half of total lipid extract from F12 (as control), the other half of the extract from F12 that is subjected to mild alkaline hydrolysis and the hydrophilic fraction from iatrobeads column chromatography, were further cleaned off contaminants by C18 sep-pak cartridge. Briefly, the fractions were reconstituted with 50% methanol in water and loaded into a C18 sep-pak cartridge and then washed with nanopure water. The lipids were eluted with iso-propanol.

Thin layer chromatography (TLC)

TLC was performed using silica gel 60-precoated plates, with a solvent system chloroform/methanol/water (60:40:10, by vol.). Detection was performed by spraying with orcinol/H_2SO_4 reagent for carbohydrates, Dittmer–Lester reagent for phosphorus, ninhydrin reagent for free amino groups, respectively.

Thin layer chromatogram of lipids at each purification steps

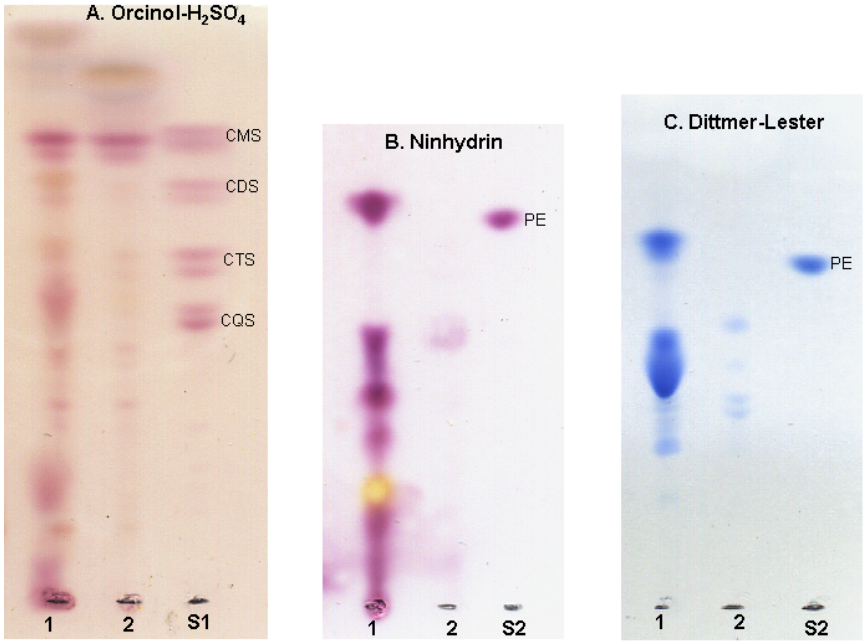
1. **Total lipid extract**



Thin-layer Chromatograms of total lipid extract from *Schistosome* eggs
S1: Neutral glycosphingolipid mixture, ceramide mono- di- tri- and tetra- saccharides. (CMS,CDS,CTS,and CQS, respectively); S2: Phosphatidyl ethanolamine (PE); Development: Chloroform/Methanol/Water 6:4:1 (v:v:v); Detection: A. Orcinol-H_2SO_4 for detection of glyco conjugates; B. Ninhydrin reagent for detection of amino group; C. Dittmer-Lester reagent for phospho-lipids, respectively.

At this point, the lipid extract is still "crude". It is expected to contain salts, free oligosaccharides co-extracted with lipids, also carry-over proteins. Smear bands observed close to the bottom of TLC are most likely free oligosaccharides and proteins. The couple of bands locate at the middle height of the TLC positive in Dittmer and ninhydrin detections, are either phosphatidyl ethanolamine (PE), lysophosphatidyl ethanolamine or other phospholipids that have very similar structure as PE.

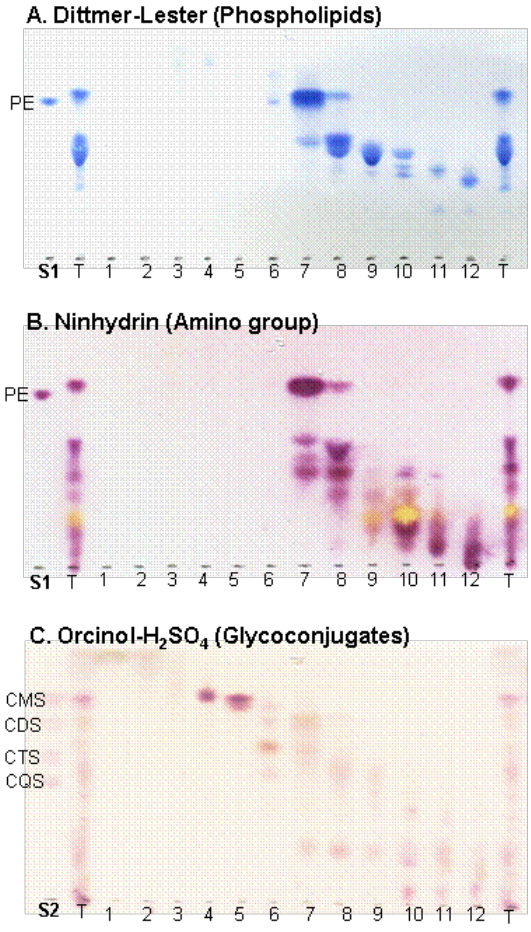
2. Mild alkaline hydrolysis



Thin layer chromatograms of alkaline-stable lipid fraction from *Schistosome* eggs
S1: Neutral glycosphingolipid mixture, ceramide mono- di- tri- and tetra- saccharides. (CMS,CDS,CTS,and CQS, respectively); S2: Phosphatidyl ethanolamine (PE); 1: Total lipid extract; 2: Alkaline-stable lipid fraction. Development: Chloroform/Methanol/Water 6:4:1 (v:v:v); Detection: A. Orcinol-H_2SO_4 for detection of glyco conjugates; B. Ninhydrin reagent for detection of amino group; C. Dittmer-Lester reagent for phospho-lipids, respectively.

Based on the TLC of alkaline-stable lipid fraction, the fraction seems to contain ceramide monosaccharide as a main neutral glycoshingolipid. There are other minor bands positive to orcinol detection indicate there are glycoshingolipids with longer carbohydrate chain and possibly some of them carry carbohydrate and phosphate in the molecules. There are at least four visible bands positive to Dittmer detection indicates the existence of phospho-lipids with ceramide, such as sphingomyelin, ceramide phospho-ethanolamine or/and ceramide phospho-inositol in the fraction.

3. Iatrobeads column chromatography



Iatrobeads column chromatography

S1 – Phosphatidyl ethanolamine as positive standard for Dittmer (A) and Ninhydrin (B) detection

S2 – Neutral glycosphingolipid standard mixture as positive standard for Orcinol-H2SO4 (CMS Ceramide monosaccharide, CDS Ceramide disaccharide, CTS Ceramide trisaccharide, CQS, Ceramide tetra saccharide, respectively.)

T- Total lipid extract from *Schistosoma* eggs

1-12 – Fractions from iatrobeads column chromatography

Development: C/M/W 60:40:10

Detection: Dittmer-Lester (A) for phospholipids, Ninhydrin (B) for amino group, Orcinol-H2SO4 (C) for glycoconjugates, respectively

Frac 1-2 Sterols - A

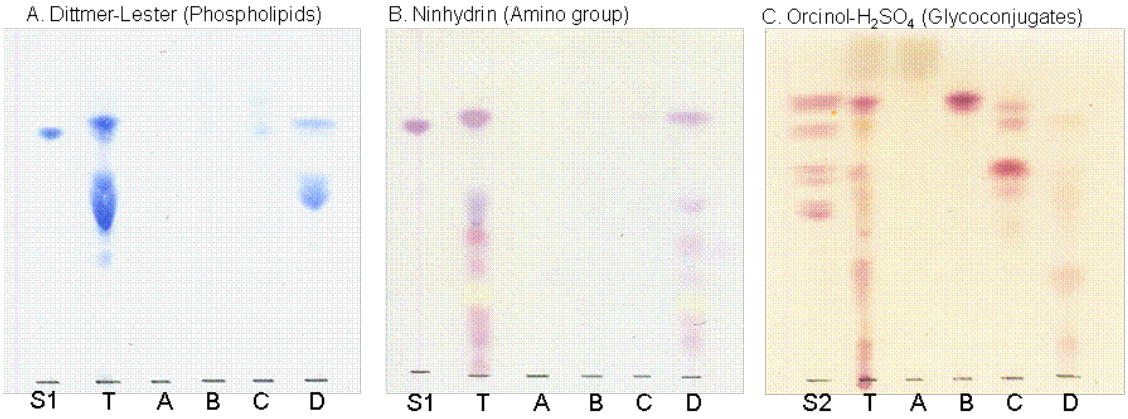
Frac 3-5 Mainly Ceramide monosacchairde – B

Frac 6 glycolipid and a trace amount of phospholipids -C

Frac 7-12 Hydrophilic glycolipids, phospholipids, phospholipids with amino group (such as phosphatidyl ethanol amine) - D

The each fraction from iatrobeads fractions were examined by TLC. The results are shown in Figure 3 above. Fractions 1 and 2 were combined as one fraction includes sterols, and possibly free-fatty acids and acylglycerols (fatty acids, acylglycerols are not visible by the detection methods used for the TLC above but expected to be eluted around this fraction). Fractions 3-5 was combined as one includes ceramide monosaccharides – this fraction seem to be almost singly-purified but contains trace amount of hydrophobic phosholipids. Based on the RF value of the phospholipid in the fraction, which is higher than PE, I would assume this could be cardiolipin or something similar. Fraction 6 contains glycolipids with oligosaccharides and a trace amount of phospholipids could be

phosphatidic acids and PE. The main lipids that are positive to ninhydrin or Dittmer were mostly eluted later in Fractions 7 -12, that are combined as one fraction. Based on the TLC, the fraction seems to contain PE, lyso type PE, other type of phospholipids, and hydrophilic glyco- and possibly phosphoglyco lipids. After combining couple of fractions as indicated above, I examined the fractions again by TLC and the result is shown in Figure 4 below.



. Fractions after iatrobeads column chromatography

S1 – Phosphatidyl ethanolamine as positive standard for Dittmer (A) and Ninhydrin (B) detection

S2 – Neutral glycosphingolipid standard mixture as positive standard for Orcinol-H2SO4 (CMS Ceramide monosaccharide, CDS Ceramide disaccharide, CTS Ceramide trisaccharide, CQS, Ceramide tetra saccharide, respectively.)

T- Total lipid extract from *Schistosoma* eggs

A-D – Final fractions after iatrobeads column chromatography

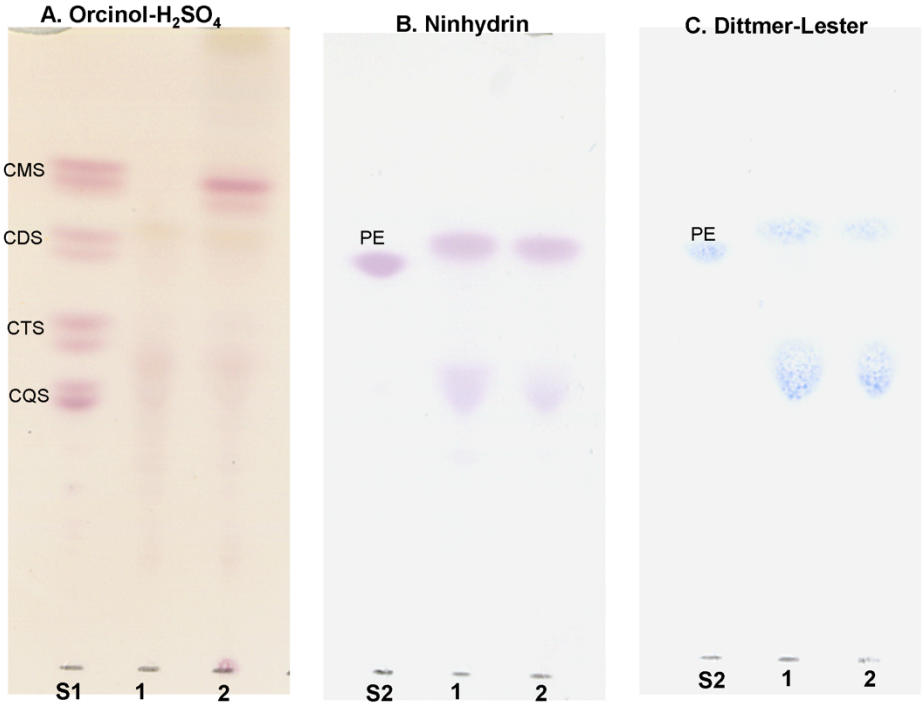
Development: C/M/W 60:40:10

Detection: Dittmer-Lester (A) for phospholipids, Ninhydrin (B) for amino group, Orcinol-H2SO4 (C) for glycoconjugates, respectively

4. Sample cleaning up for total lipid extract and fraction D from iatrobeads by C18-sep-pak cartridge

The lipid extract and the hydrophilic fraction from iatrobeads column chromatography are expected to contain some non-lipid hydrophilic contaminants such as salts (PBS), free-oligosaccharides, carry-over proteins. These samples were passed

through C18-sep-pak, to prevent those contaminants interfere accurate measurement of pro inflammatory activity. After the cleaning, those fractions are examined by TLC and the result is shown in Figure 5.



Thin layer chromatograms of total lipid extract and Fraction D from iatrobeads column after removal of contaminants by C18-sep-pak

S1: Neutral glycosphingolipid mixture, ceramide mono- di- tri- and tetra- saccharides. (CMS,CDS,CTS,and CQS, respectively); S2: Phosphatidyl ethanolamine (PE); 1:Fraction D after C18 cleaning; 2: Total lipid extract after C18 cleaning.

Development: Chloroform/Methanol/Water 6:4:1 (v:v:v); Detection: A. Orcinol-H_2SO_4 for detection of glyco conjugates; B. Ninhydrin reagent for detection of amino group; C. Dittmer-Lester reagent for phospho-lipids, respectively.

Comments:

The recovery from Iatrobeads column chromatography was much higher than that from the anion exchange column chromatography that was previously used for egg sample separation. In previous anion exchange column chromatography, the recovery of the acidic fraction was close to the detection limit of TLC, which gave some concern that the possibility of sample may have degraded during anion exchange. On the other hand, the lipid contents in each fraction in the current batch after Iatrobeads column are clearly visible by TLC. The TLC analysis suggested that high recovery of lipid content, complete removal of non-lipid contaminants, also the sample structures were all retained though the entire Iatrobeads chromatography process.