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Characterization Of Bis-(3', 5')-Cyclic Dimeric Adenosine Monophosphate As A Mucosal Adjuvant And Its Relevance In A Model Of Hookworm Pathogenesis

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1. Abstract

Most pathogens invade their human host or establish infection at mucosal surfaces. In contrast to parenteral vaccines, mucosal immunization has the potential to induce a robust immune response at mucosal surfaces capable of blocking the entry and establishment of many pathogens. Despite these advantages, very few mucosal vaccines are licensed for human use, largely because antigens administered by the mucosal route are either poorly or non-immunogenic. A strategy to overcome this problem is co-administering vaccine antigens with a mucosal adjuvant. Recently, bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) was identified as a potential mucosal adjuvant. To evaluate c-di-AMP as a mucosal adjuvant two studies were conducted: The first characterized the immune responses elicited by c-di-AMP when used as a sublingual and intranasal adjuvant; and the second tested the protective efficacy of c-di-AMP in a vaccine model of hookworm pathogenesis. From our studies, c-di-AMP stimulates strong humoral and cellular immune responses, and induces a predominately Th1/Th17 T cell response pattern, as shown by elevated expression of IgG2a and enhanced secretion of IFN-γ, IL-17, and IL-22. Despite these successes, when tested in a hookworm vaccine model c-di-AMP failed to significantly enhance the protective efficacy of hookworm excretory-secretory (ES) antigens. However, we did discover that ES antigens alone, given intranasally, confer a 73.8% reduction in adult worm burden, and appear to modulate the severity of hookworm-associated anemia and weight loss. We hypothesize that part of this success is due to inherent adjuvant properties of ES, which boost the immunogenicity of vaccine relevant antigens.

2. Introduction

Most infectious agents enter the body, or establish infection, at mucosal surfaces, making the mucosal immune system an important line of defense against human pathogens.[1, 2, 3, 4] However, most vaccines are administered by parenteral injection, which confer protection by stimulating systemic immune responses, such as serum antibodies and in some cases effector T cell responses, but are notoriously inefficient for stimulating primary immune responses in mucosal tissues.[3] In contrast to these vaccines, mucosal vaccinations have the potential to counter mucosa-associated pathogens at the portal of entry by inducing local pathogen-specific immune responses, as well as systemic humoral and cellular responses.[5] Traditionally, the oral and nasal routes have been used for mucosal immunization, but other routes include the rectal, vaginal, sublingual, and transcutaneous routes, all of which share the common goal of strengthening the first line of defense against human pathogens.[4]

The gastrointestinal, respiratory and urogenital tracts, eye conjunctiva, inner ear, and ducts of all the exocrine glands are covered by mucous membranes endowed with powerful mechanical and chemical cleansing mechanisms that repel and degrade most foreign matter.[4] Additionally, they contain a number of other cells of the innate immune system, including phagocytic neutrophils and macrophages, dendritic cells (DCs), natural killer (NK) cells and mast cells. Through a variety of mechanisms these cells contribute significantly to host defense against pathogens and in initiating adaptive mucosal immune responses.[1] The hallmark of adaptive mucosal immunity is the production of secretory immunoglobulin A (IgA) antibodies.[4] Secretory IgA functions not only by preventing attachment to or translocation of pathogens across epithelial cells but also by limiting their transmission from person-to-person.[3] While IgA is the predominant humoral defense

mechanism at mucosal surfaces, locally produced IgM and IgG also contribute to host defense.[1] Additionally, the mucosal immune system contains cytotoxic T lymphocytes (CTLs), crucial for the clearance of many enteric viruses, respiratory viruses and intracellular parasites,[6, 7, 1] and interferon (IFN)-γ-producing $CD4^+$ T cells that have been found to be important for defense for mucosa-associated viral and bacterial infections.[1, 12]

Contrary to parenteral vaccines, mucosal vaccination has the unique ability to leverage these responses to induce 'frontline immunity,' preventing the establishment and dissemination of mucosa-associated pathogens.[8] Additionally, mucosal vaccination offers several logistical advantages over systemic vaccination. For one, mucosal vaccination does not require injection, which makes administration easier (including the possibility of self-administration), reduces the risk needle-stick injuries, and has the potential to increase compliance among people afraid of needles.[9, 5] Furthermore, simplified manufacturing and storage methods, as well as independence from trained medical personnel for delivery, make mucosal vaccines suitable for mass vaccination programs, especially in developing countries and during emergency situations.[8, 4]

Despite the many attractive features of mucosal vaccination, only 7 mucosal vaccines have been licensed for human use, with only 4 approved for use in the US (Table 1).[1, 4] These vaccines target five of the main enteric pathogens — poliovirus, *Vibrio cholerae*, *Salmonella typhi*, rotavirus — and influenza. As shown in Table 1, the majority of these vaccines are based on live attenuated formulations; and with the exception of FluMist®, all of them are orally administered. Typically, attenuation involves propagation of a pathogen under novel conditions so that it becomes less pathogenic to its original host, while still being able to elicit a robust protective immune response to the wild-type strain.

Table 1. Licensed mucosal vaccines

Infection and vaccine(s)	Route	Trade name
Poliovirus		
*Live attenuated polio vaccine (OPV)**	Oral	Many
Vibrio cholerae		
*Cholera toxin B subunit + inactivated V. cholerae 01 whole cells**	Oral	Dukoral®
*Inactivated V. cholerae 01 and 0139 whole cells**	Oral	Shanchol™ and mORCVAX®
Salmonella typhi		
Ty21a live attenuated vaccine	Oral	Vivotif®
Rotavirus		
Monovalent live attenuated vaccine	Oral	RotaRix®
Pentavalent live attenuated vaccine	Oral	RotaTeq®
Influenza		
Live attenuated cold-adapted influenza virus reassortant strains	Nasal	FluMist®

*Not available in the US
Table adapted from Holmgren *et al*[1]

Attenuation, however, is a largely unpredictable process, and on occasion successfully attenuated pathogens may revert back to virulence.[10] The best example of this is the oral polio vaccine (OPV). Introduced in 1955, OPV is the classic oral-mucosal vaccine. Similar to its alternative, the injectable inactivated polio vaccine (IPV), OPV induces systemic antibody production that protects against poliomyelitis by preventing the spread of poliovirus to the nervous system, but superior to IPV, OPV also induces local secretory IgA in the intestinal mucosa capable of blocking colonization, and thus limiting person-to-person transmission through the fecal-oral route.[1] Unfortunately, though, on rare occasions the vaccine strains (i.e., Sabin 1, 2, and 3) have been known to revert back to neurovirulence causing vaccine-associated paralytic poliomyelitis (VAPP), a condition

virtually identical to the wild-type paralytic poliomyelitis. The incidence of VAPP is estimated at 4 cases per 1,000,000 births per year, which has led to the replacement of OPV by IPV in most industrialized countries.[11]

To circumvent this issue, researchers have begun to design vaccines based on a limited number of highly purified recombinant molecules or pathogen subunits.[5, 13] This strategy has greatly improved the overall safety of vaccines, but as a consequence vaccine antigens have lost their inherent immunostimulatory properties and often do not elicit strong immune responses.[5] The problem is even worse when antigens are administrated mucosally where they are forced to contend with the unique characteristics of the mucosal environment, including the presence of mucus, limited antigen uptake, and the possibility of antigen degradation.[5] Moreover, unlike the systemic immune apparatus, which functions in a normally sterile milieu and often responds vigorously to invaders, the mucosa-associated lymphoid tissues guard organs that are replete with foreign matter. Therefore, the mucosal immune system has to economically select appropriate effector mechanisms and regulate their intensity to avoid bystander tissue damage and immunological exhaustion. Consequently, highly purified vaccine antigens are often treated as any other non-pathogenic antigen (e.g., food proteins) and channeled to default pathways usually resulting in tolerance.[1]

Fortunately, to overcome these problems antigens can be co-administered with adjuvants (from *adjuvare,* "to help"), which are molecules, compounds or macromolecular complexes that boost the potency and longevity of specific immune responses to antigens, but cause minimal toxicity or lasting immune effects on their own.[13, 14] In general, adjuvants enhance T and B cell responses by engaging components of the innate immune

system, rather than directly acting on lymphocytes themselves.[13] Microbial detection by the innate immune system relies heavily on pattern recognition receptors (PRRs), which recognize molecular structures common to large groups of microbes (i.e., pathogen-associated molecular patterns, PAMPs). Members of nearly all of the PRR families are potential targets for adjuvants. These include Toll-like receptors (TLRs), NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and C-type lectin receptors (CLRs).[13, 14] By co-administering antigens (which are inefficient at engaging the innate immune system) with adjuvants, these formulations are able to activate innate transcriptional programs that result in the induction of genes encoding cytokines, chemokines, and costimulatory molecules that play a key role in the priming, expansion, and polarization of adaptive immune responses.[14, 13, 15] Thus, adjuvants can provide a crucial link between the innate and adaptive immune systems by providing a proinflammatory environment that favors the development of stronger and broader immune responses, and do not lead to tolerance.[1]

The concept that primary infection leads to immunity against subsequent infections was the cornerstone for the development of first-generation attenuated vaccines against poliomyelitis, measles, and numerous other infections.[16] However, for a number pathogens (e.g., HIV, hepatitis C virus, herpes viruses, mycobacteria, and parasitic infections) natural infection does not confer immunity. For these infections, a central challenge has been developing unique strategies to stimulate artificial immune responses able to elicit protection.[16] In these regards, adjuvants might be particularly useful, since in addition to increasing the magnitude of the adaptive response, they are also able to guide the adaptive immune response to elicit specific types of immune responses. For example, adjuvants have been used in preclinical and clinical studies to elicit functionally appropriate types of

immune responses, such T helper 1 (Th1) versus Th2 versus Th17 responses, $CD8^+$ versus $CD4^+$ T cell responses, and/or specific antibody isotype responses.[13] In this respect, a strategy of mucosally administrating isolated protective antigens with appropriate adjuvants may not only improve vaccine safety, but also offer new strategies for targeting pathogens that have been refractory to vaccination thus far.[13, 2]

Human hookworm infection is an example of a disease that might be particularly suited for such a strategy. Hookworm disease is caused by infection with multiple species of bloodfeeding intestinal parasitic nematodes; the most common being *Ancylostoma duodenale, Ancylostoma ceylanicum,* and *Necator americanus*. Together, these organisms are estimated to infect 500-800 million people worldwide, including approximately 126 million children, causing growth delay (especially in children) and anemia.[17, 18, 19]

Traditional control strategies have focused on community-based treatment of high-risk populations (e.g., school-age children) with benimidazole anthelminthics.[18] However, studies sponsored by the WHO, among other organizations, have shown high rates of hookworm reinfection can occur within a few months following benzimidazole treatments. Presumably, this is the result of the absence of acquired immunity, leaving individuals susceptible to reinfection following exposure to third-stage infective hookworm larvae (L_3) in the soil.[16] Additionally, there is concern about emerging anthelminthic drug resistance among human hookworm populations.[18] Taken together, these issues necessitate a search for alternative or complementary approaches to public health control that do not rely exclusively on anthelminthics.[16]

One approach is the development and use of an anti-hookworm vaccine.[16] The hookworm life cycle begins when eggs excreted in the feces of an infected individual hatch

in the soil, and undergo successive molts to the third larval stage (L_3). Infection is most frequently acquired when L_3 parasites penetrate the skin and enter the circulatory system by invading small blood vessels or lymphatics. From there the larvae are carried passively to the heart, and then the lungs where they traverse alveolar capillaries to enter the respiratory system. After migrating up the trachea, larvae are swallowed and enter the digestive system where they migrate to the small intestine to undergo a final molt into blood-feeding adults.[19] During host stages, the primary interface between the parasite and the host is the excretory-secretory (ES) component, a complex mixture of proteins, carbohydrates, and lipids secreted from the surface or oral openings of the parasite. These products orchestrate a wide range of activities that are thought to be crucial for their survival and propagation, including penetration of the host, tissue invasion, feeding, reproduction, and evasion of the host immune system.[49] Given the importance of the ES component, and the extensive overlap between the hookworm's lifecycle and the host's mucosa, a mucosal vaccine targeting ES antigens might be uniquely poised to confer protective immunity.

As discussed earlier, adjuvants, and specifically mucosal adjuvants, will likely be crucial for the successful development of such a vaccine. Unfortunately, despite assessment of a large number of compounds, only three adjuvants are currently in widespread use in humans: Alum, MF59™, and ASO4, and of these only Alum and ASO4 are approved for human use in the US.[13] Additionally, all of these adjuvants were designed for use in parenteral vaccines, and none of them exhibit activity when administered by the mucosal route. Thus, the lack of effective mucosal adjuvants represents a critical bottleneck in the development of mucosal vaccines.[5, 9]

Recently, though, members of the cyclic di-nucleotide family have emerged as promising mucosal adjuvants. The first molecule explored was bis-(3',5')-cyclic dimeric guanosine monophosphate (c-di-GMP), a small signaling molecule that is thought to act as a danger signal to the host immune system. Using a mouse model, these initial studies showed that c-di-GMP exhibits potent activity as an adjuvant when administered by mucosal and systemic routes.[20] Further studies then identified bis-(3',5')-cyclic dimeric inosine monophosphate (c-di-IMP) as a structurally related but distinct compound, which is also able to enhance antigen-specific immune responses following mucosal immunization of mice.[9] And more recently, bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP) was identified as an additional candidate mucosal adjuvant.[21] C-di-AMP was originally shown to be a bacterial second messenger that signals for DNA integrity in *Bacillus subtilis* during sporulation, however, subsequent studies revealed that it is wide-spread throughout bacterial species, and may play a role in triggering the cytosolic host response of innate immunity.[21] To evaluate c-di-AMP as a mucosal adjuvant two studies were conducted: The first characterized the immune responses elicited by c-di-AMP when used as a sublingual and intranasal adjuvant; and the second tested the protective efficacy of c-di-AMP in a vaccine model of hookworm pathogenesis.

3. Hypothesis & Specific Aims

3.1. Hypothesis

Bis-(3', 5')-cyclic dimeric adenosine monophosphate (c-di-AMP) is a potent mucosal adjuvant that elicits broad immune responses through both sublingual and intranasal routes; and is able to enhance the protective efficacy of hookworm excretory-secretory (ES) antigens.

3.2. Specific Aims

1. Characterize the adjuvant properties elicited by c-di-AMP through intranasal and sublingual immunization of BALB/c mice
2. Test the protective efficacy of c-di-AMP as a mucosal adjuvant in a vaccine model of hookworm pathogenesis

4. Sublingual versus intranasal immunization using c-di-AMP as a mucosal adjuvant †

4.1. Methods

4.1.1. Animals

Immunization studies were conducted in 6-8 week-old female BALB/c mice of the H-2d haplotype (Harlan, Germany). All animal experiments were performed in agreement with the government of Lower Saxony, Germany (No. 33.11.42502-04-017/08).

4.1.2. Bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP)

Previously, c-di-AMP ($C_{20}H_{24}N_{10}O_{12}P_2$) was synthesized by cyclization and purified by reversed phase HPLC, according to established protocols.[21] The lyophilized compound was stored at −20 °C until use when it was resuspended in sterile water for the immunization studies. The presence of LPS was measured using the HEK-Blue-4 reporter cell line and compared with *E. coli* K12 LPS according to manufacturer instructions (HEK Blue™ LPS Detection Kit, InvivoGen, USA).

4.1.3. β-galactosidase (β-Gal)

For all immunization studies, β-Gal (Roche, Germany) was dissolved in sterile water (5 mg/ml) and used as a model antigen. As described above, prepared β-Gal used in the studies was initially tested in a HEK-Blue-4 reporter line and compared with *E. coli* K12 LPS to rule of the possibility of LPS contamination (HEK Blue™ LPS Detection Kit, InvivoGen, USA).

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† This set of experiments was carried out at the Helmholtz Centre for Infection Research, Braunschweig, Germany from May 2011 to August 2011 under the supervision of Thomas Ebensen and Carlos Guzman

4.1.4. Immunization protocols

Female BALB/c mice were immunized intranasally (i.n.) or sublingually (s.l.) on days 0, 14 and 28 with 0.9% NaCl (negative control), 30 μg of β-Gal, or 30 μg of β-Gal co-administered with 10 μg of c-di-AMP (n=5, per group). Vaccines were formulated in 0.9% NaCl and prepared 30 minutes prior to immunization. For intranasal immunization, mice were lightly anesthetized (30-45 seconds) by inhalation of Isoflurane® then given a 20 μl dose of vaccine administered drop-wise to the nostrils. For sublingual immunization, mice were fully anesthetized (30-40 minutes) by intraperitoneal injection of Ketamin with Rompun and given an 8 μl dose of vaccine administered under the tongue. To ensure the dose was given sublingually and not orally, mice were fixed in a vertical position during immunization and returned to their cages in a sitting position with their head lying on their stomach until anesthesia subsided.

4.1.5. Sample collection

Blood was drawn from all animals (through retro-orbital plexus) by on days −1, 13, 27 and 42. Blood samples were centrifuged (10 min at 3000 *g*) to remove red blood cells and the sera were stored at −20 °C further until analysis. Mice were sacrificed on day 42 and nasal (NL) and lung lavages (LL) were collected by flushing the organs with ice-cold PBS supplemented with 50 mM EDTA, 0.1% bovine serum albumin, and 10 mM phenyl-methane-sulfonylfluoride (PMSF).

4.1.6. Detection of β-Gal-specific IgG, IgG1, IgG2a, and IgG2b in serum

Titers of antigen-specific IgG and isotypes (IgG1, IgG2a, IgG2b) to β-Gal were measured in serum by an enzyme-linked immunosorbent assay (ELISA) using microtiter plates (Microlon® 600 High Binding) coated overnight at 4 °C with 100 µl/well of β-Gal (2 µg/ml in 0.05 M carbonate buffer, pH 9.6). Following overnight incubation plates were washed (PBS, 0.1% Tween 20) using an automated plate washer (ELx 405, Bio-Tek) on a 6-cycle setting and blocked (PBS, 3% BSA) for 1 hour at 37 °C. Serial 2-fold dilutions (starting at 1:1,000) of sera in PBS, 3% BSA were then added at 100 µl/well using an automated plate diluter (Precision 2000, Bio-Tek), then incubated for 2 hours at 37 °C. After washing, secondary antibodies were added in PBS, 0.1% Tween 20, 1% BSA according to manufacture recommendations: biotinylated γ-chain-specific goat anti-mouse IgG (1:5,000) or biotinylated goat anti-mouse IgG1 (1:5,000), IgG2a (1:10,000), or IgG2b (1:5,000). Unbound detection antibody was removed with washing before the addition of 100 µl/well of Streptavidin-HRP solution (1:1000 in PBS, 0.1% Tween 20, 1% BSA) and incubation at 37 °C for 1 hour. Following a final wash, reactions were developed using ABTS [2,20-azino-bis (3- ethylbenzthiazoline-6-sulfonic acid)] in 0.1 M citrate–phosphate buffer (pH 4.35) containing 0.01% H_2O_2. Following a 30 minute incubation at room temperature, endpoint titers were expressed as absolute values of the last dilution that gave an optical density at 405 nm that was 2-fold greater than the negative control.

4.1.7. Detection of β-Gal-specific IgA in nasal and lung lavage samples

The amount of antigen-specific IgA to β-Gal per μg of total IgA was measured in nasal and lung lavage samples by ELISA. Briefly, 96-well microtiter plates (Microlon® 600 High Binding) were coated with 100 µl/well of β-Gal (2 µg/ml in 0.05 M carbonate buffer, pH

9.6) and incubated overnight at 4 °C. For detection of total IgA and to generate a standard curve, an additional plate was coated with 100 µl/well of anti-mouse IgA (2 µg/ml in 0.05 M carbonate buffer, pH 9.6) and incubated overnight at 4 °C. For antigen-specific IgA, after blocking (PBS, 3% BSA) for 1 h at 37 °C, the plates were washed (PBS, 0.1% Tween 20) and incubated with 2-fold serially diluted lavage samples (starting at 1:4) for 1 hour at 37 °C. For total IgA and standard, after blocking (PBS, 3% BSA) for 1 h at 37 °C, the plates were washed (PBS, 0.1% Tween 20) and incubated for one hour at 37 °C with either 2-fold serially diluted lavage samples (starting at 1:20) or standardized mouse IgA 2-fold serially diluted in PBS, 3% BSA (starting at 2 µg/ml). After washing, anti-mouse IgA biotinylated detection antibody was added to each well (1:5,000) and incubated for 1 hour at 37 °C. The plates were then washed again and streptavidin-HRP was added (1:1 000) followed by a 1 hour incubation at room temperature. Finally, plates were washed and reactions developed using ABTS in 0.1 M citrate–phosphate buffer (pH 4.35) containing 0.01% H_2O_2. Following a 30 minute incubation at room temperature, a standard curve was generated and endpoint titers for total and antigen-specific IgA were calculated. Values are expressed as antigen-specific titers per µg of total IgA.

4.1.8. Measurement of cellular proliferation

Spleens and cervical lymph nodes of vaccinated mice were aseptically removed, single cell suspensions were prepared and erythrocytes were lysed in ACK buffer (2 minute incubation). Cells were washed twice and adjusted to 2 x 10^6 cells/ml in RPMI complete medium (RPMI, 10% fetal bovine serum, 100 U/ml penicillin, 100 µg/ml streptomycin, 1% glucose). Cells were then seeded at 200 µl/well (5 x 10^5 cells/well) in U-bottomed 96-well

microtitre plates and cultured in quadruplicate for 72 hours (5% CO_2 at 37 °C) in the presence of different concentrations of β-Gal (1, 10, 20, 40 µg/ml), Concanavalin A (5 µg/ml), or medium alone.[22] After 72 hours, 1 µCi of ^{3}H-Thymidine in 50 µl of culture media was added to each well.[23] Then after an additional 18 hour incubation, cells were harvested on Filtermat A filters and the incorporation of thymidine into the DNA of proliferating cells was determined using a scintillation counter (Wallac 1450, Micro-Trilux). The results are expressed as a stimulation index (SI).

Stimulation index (SI) = cpm with β-Gal/cpm without β-Gal

4.1.9. Measurement of cytokine secreting cells by enzyme-linked immune spot technique (ELISPOT)

To determine of the amount of cytokine secreting cells in the spleens and cervical lymph nodes of vaccinated animals murine IFN-γ, IL-2, IL-4 and IL-17 ELISpot kits (BD Pharmingen, USA) were used according to manufacturer instructions. Multiscreen plates (Nunc-Immuno MaxiSorp) were coated with 100 µl/well of capture antibody in PBS and incubated overnight at 4 °C. Plates were then washed and blocked with RPMI complete medium for 2 hours at room temperature (200 µl/well). After washing again, spleen or cervical lymph node cells, prepared as described above, were added in RPMI complete medium (1 x 10^6 cells/well) and incubated (37 °C in 5% CO_2) for 24 hours (IFN-γ) or 48 hours (IL-2, IL-4, IL-17) in the presence or absence of β-Gal (5 µg/ml). All final steps were carried out according to manufacturer recommendations. Briefly, cells were removed and detection antibodies (2 hours at room temperature), avidin-HRP (1 hour at room temperature), and substrates were added to plates, with washing following all steps. After

allowing plates to air-dry overnight, colored spots were counted with an ELISpot reader (C.T.L.) and analyzed using the ImmunoSpot image analyzer (software v3.2). Each spot detected represented a distinct cytokine-secreting cell.

4.1.10. Measurement of cytokine secretion by cytometric bead array

Flow cytometry was used to quantify the amount of cytokines and chemokines secreted by immune cells. Spleen cells from vaccinated animals were seeded in 96-well flat-bottomed plates (5 x 10^5 cells/well) and stimulated with different concentrations of β-Gal (0, 5, 10, 20, 40 µg/ml) in RPMI complete media and incubated at 37 °C in 37% CO_2. Supernatants were then collected after 48, 72, and 96 hours and stored at −80 °C until processing. The amounts of IFN-γ, TNF-α, IL-1α, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IL-17, IL-21, IL-22, and IL-27 were determined using a Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix cytokine array according to the manufacturer's instructions (eBioscience®). Supplied standards were used to generate standard curves, and samples were analyzed by flow cytometry (BD™ LSR II Flow Cytometer).

4.1.11. Statistical analysis of data

Data are expressed as means ± standard errors. For multiple group comparisons, one-way ANOVA was performed followed by Tukey-Kramer Multiple Comparison test (GraphPad Prism 4).

4.2. Results

4.2.1. C-di-AMP and β-Gal were LPS-negative

To determine if potential LPS contamination could contribute to stimulation of TLR-4, β-Gal and c-di-AMP were first tested in a HEK-Blue-4 cell line. Using this cell line it was confirmed that both c-di-AMP and β-Gal tested negative for LPS, showing similar responses to unstimulated cells (0.9% NaCl negative control) (Fig. 1).

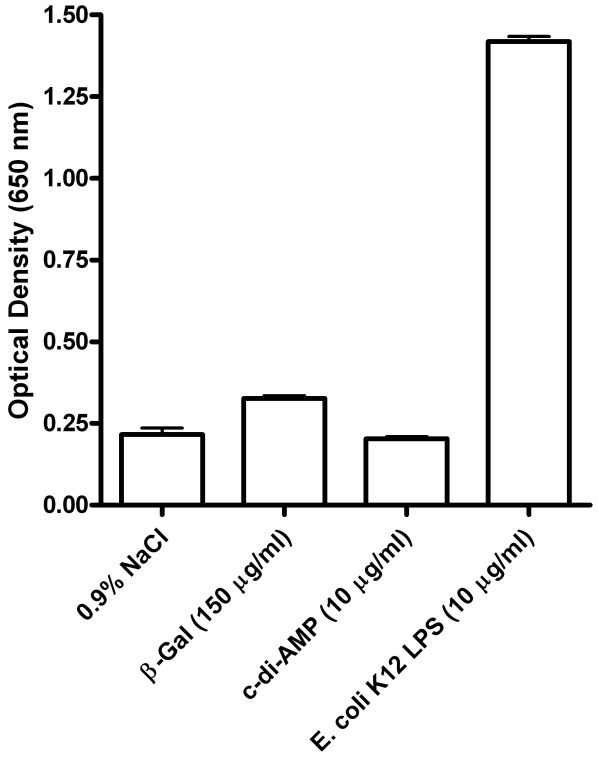

Figure 1. HEK-Blue-4 cells were stimulated with 0.9% NaCl (negative control), β-Gal (150 μg/ml), c-di-AMP (10 μg/ml), or *E. coli* K12 LPS (10 μg/ml) (positive control). After a 20 hour incubation in HEK-Blue Detection Media at 37 °C in 5% CO_2, NF-κβ-induced secreted embryonic alkaline phosphatase (SEAP) activity was assessed by reading the optical density at 650 nm.

4.2.2. Induction of strong humoral immune responses at systemic and mucosal levels after both intranasal and sublingual immunization using c-di-AMP as a mucosal adjuvant

The ability of c-di-AMP to act as a mucosal adjuvant was analyzed *in vivo* by immunizing BALB/c mice with the model antigen β-Gal (30 μg/dose) alone or co-administered with c-di AMP (10 μg/dose) by the intranasal and sublingual routes (n=5, per group), as described in Methods. In comparison to animals vaccinated with β-Gal alone, the use of c-di-AMP significantly increased β-Gal-specific IgG titers following the first boost in both the intranasal and sublingual groups (Fig. 1). Fourteen days after the final immunization animals vaccinated intranasally with β-Gal co-administered with c-di-AMP showed a 2.25-fold (p-value <0.01) increase in β-Gal-specific IgG responses, as compared to the sublingual route (Fig. 2).

The stimulation of mucosal immune responses by c-di-AMP was evaluated by measuring β-Gal-specific IgA in nasal lavage (NL) and lung lavage (LL) samples taken from vaccinated animals. Compared to animals vaccinated with β-Gal alone, the use of c-di-AMP enhanced antigen-specific IgA titers in both mucosal territories, with similar responses observed between intranasal and sublingual groups receiving β-Gal with c-di-AMP (Fig. 3).

4.2.3. c-di-AMP stimulates β-Gal-specific cellular immune responses after both intranasal and sublingual immunization

Lymphoproliferative assays were carried out to evaluate the capacity of c-di-AMP to induce cellular immune responses through intranasal and sublingual vaccination. Spleen cells or cervical lymph node cells isolated 14 days after the final immunization were stimulated *in vitro* with β-Gal. The strongest proliferative responses were observed in cells from mice receiving β-Gal in combination with c-di-AMP. In contrast, no responses were observed in

spleen cells or cervical lymph node cells from mice immunized with the β-Gal alone. Similarly strong proliferative responses were observed in the intranasal and sublingual animals that received β-Gal co-administered with c-di-AMP (Fig. 4A-D).

4.2.4. c-di-AMP stimulates a predominately Th1/Th17 T cell response through both sublingual and intranasal vaccination

To assess the effect of c-di-AMP on T helper responses, β-Gal-specific serum IgG isotypes were first analyzed. In comparison to mice vaccinated with β-Gal alone, intranasal immunization with β-Gal in combination with c-di-AMP significantly enhanced expression of IgG1, IgG2a, and IgG2b. For mice immunized sublingually, c-di-AMP significantly enhanced expression of IgG2a and IgG2b, but not IgG1. In comparison to sublingual immunization, intranasal vaccination with c-di-AMP resulted in a 7-fold (p-value <0.05) and 2.5-fold (p-value <0.05) enhancement of IgG1 and IgG2a, respectively. Taken together, these data suggest c-di-AMP stimulates a predominantly Th1 T helper response, with overall enhancement through intranasal immunization (Fig. 5).

ELISpot was utilized to further investigate the effect of c-di-AMP on T helper responses by identifying populations of β-Gal-specific IFN-γ, IL-2, IL-4 and IL-17 secreting cells. When stimulated with β-Gal *in vitro*, splenocytes from mice immunized intranasally with β-Gal and c-di-AMP exhibited a largely Th1/Th17 phenotype based on their cytokine profile (IFN-γ^{+++}, IL-2^{+}, IL-4^{+}, IL-17^{+++}). Similarly, sublingual immunization resulted in a strongly Th1/Th17 profile (IFN-γ^{+++}, IL-2^{++}, IL-4^{+}, IL-17^{+++}). No statistical difference was observed between intranasal and sublingual animals that received β-Gal co-administered with c-di-AMP (Fig. 6). When cervical lymph node cells from mice immunized intranasally were analyzed their profile was almost exclusively Th1/Th17 (IFN-γ^{++}, IL-2^{+}, IL-4^{-}, IL-17^{++});

and a similar profile was observed in mice immunized sublingually (IFN-γ^{+}, IL-2^{+}, IL-4^{-}, IL-17^{++}). Here, however, intranasal immunization enhanced IFN-γ secretion in the population 3-fold (p-value <0.05) in comparison to sublingual immunization, when β-Gal was co-administered with c-di-AMP (Fig. 7).

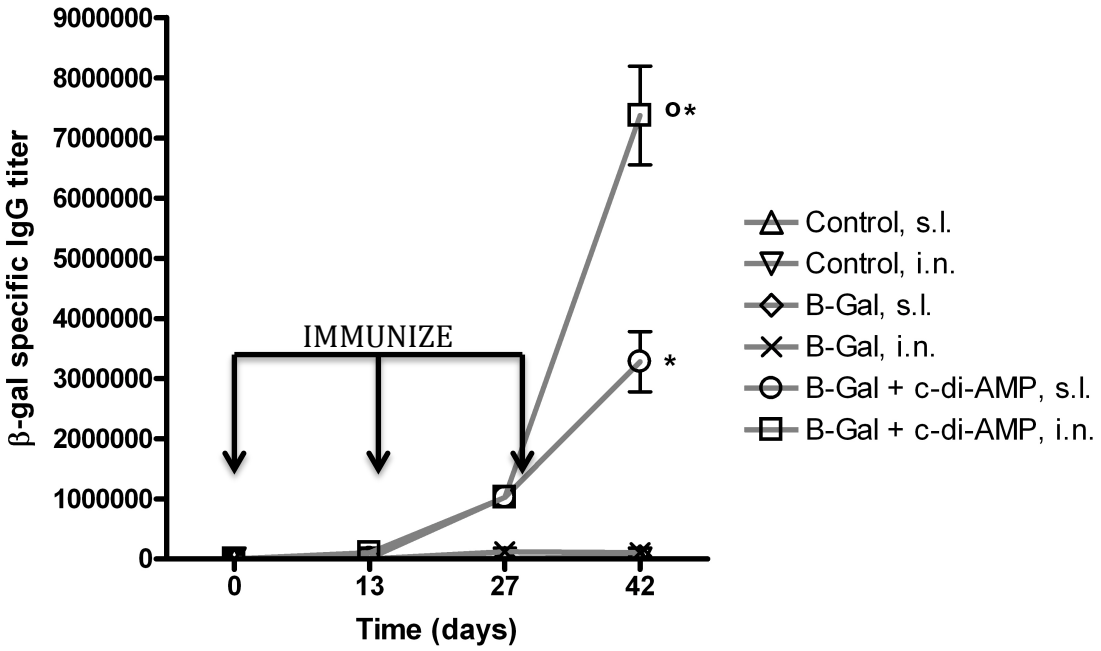

Figure 2. Kinetic analysis of anti-β-Gal IgG responses in sera of BALB/c mice immunized on days 0, 14 and 28 with 0.9% NaCl (control), 30 ug/dose of β-Gal alone, or β-Gal co-administered with 10 ug/dose of c-di-AMP by the sublingual and intranasal routes (n = 5, per group). Sera was collected 2-3 days prior to each immunization and fourteen days after the final immunization and analyzed by ELISA. Significant with respect to mice receiving antigen alone (*, $p < 0.01$). Significant with respect to mice receiving the antigen co-administered with c-di-AMP by the s.l. route (^{o}, $p < 0.01$).

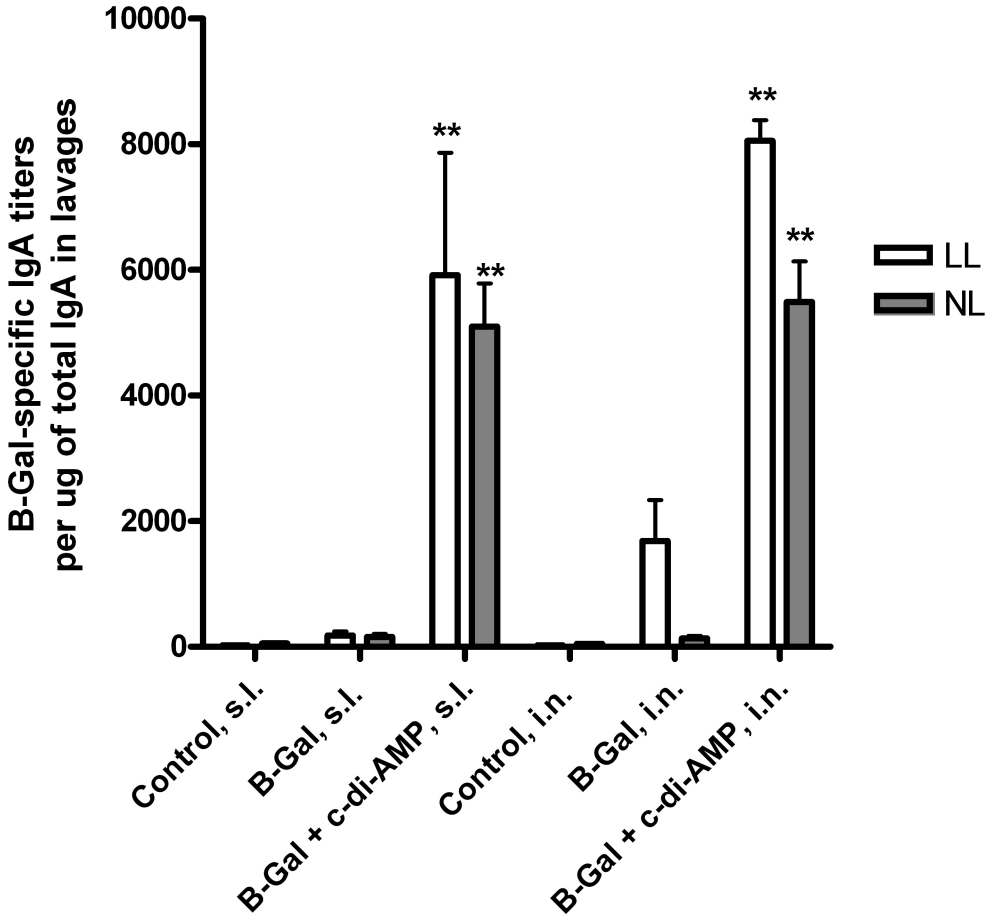

Figure 3. Analysis of anti-β-Gal IgA responses in lavage samples of BALB/c mice immunized on days 0, 14 and 28 with 0.9% NaCl (control), 30 ug/dose of β-Gal alone, or β-Gal co-administered with 10 ug/dose of c-di-AMP by the sublingual and intranasal routes (n = 5, per group). Lavage samples were collected on day 42 and analyzed by ELISA. Significant with respect to mice receiving antigen alone (**, $p < 0.001$).

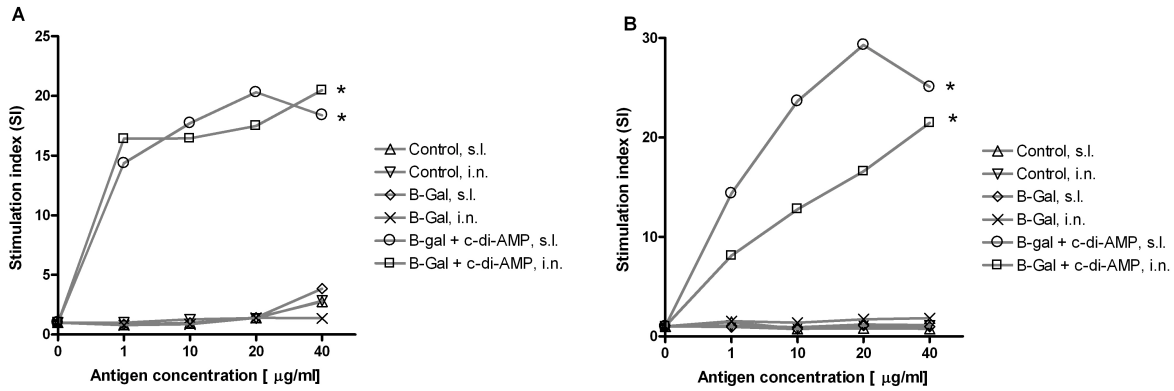

Figure 4. Analysis of cell proliferation. Spleen cells (5 x 10^5) or cervical lymph node cells (5 x 10^5) from vaccinated animals were restimulated with different concentrations of β-Gal (0, 1, 10, 20, 40 μg/ml) for 96 hours. Cellular proliferation was then assessed by determination of [^{3}H] thymidine incorporated into the DNA of replicating cells. (A) Results from spleen cells expressed as a stimulation index (SI). (B) Results from cervical lymph node cells expressed as a stimulation index (SI). All results are the mean value of quadruplicates. Significant with respect to mice receiving antigen alone (*, $p < 0.01$).

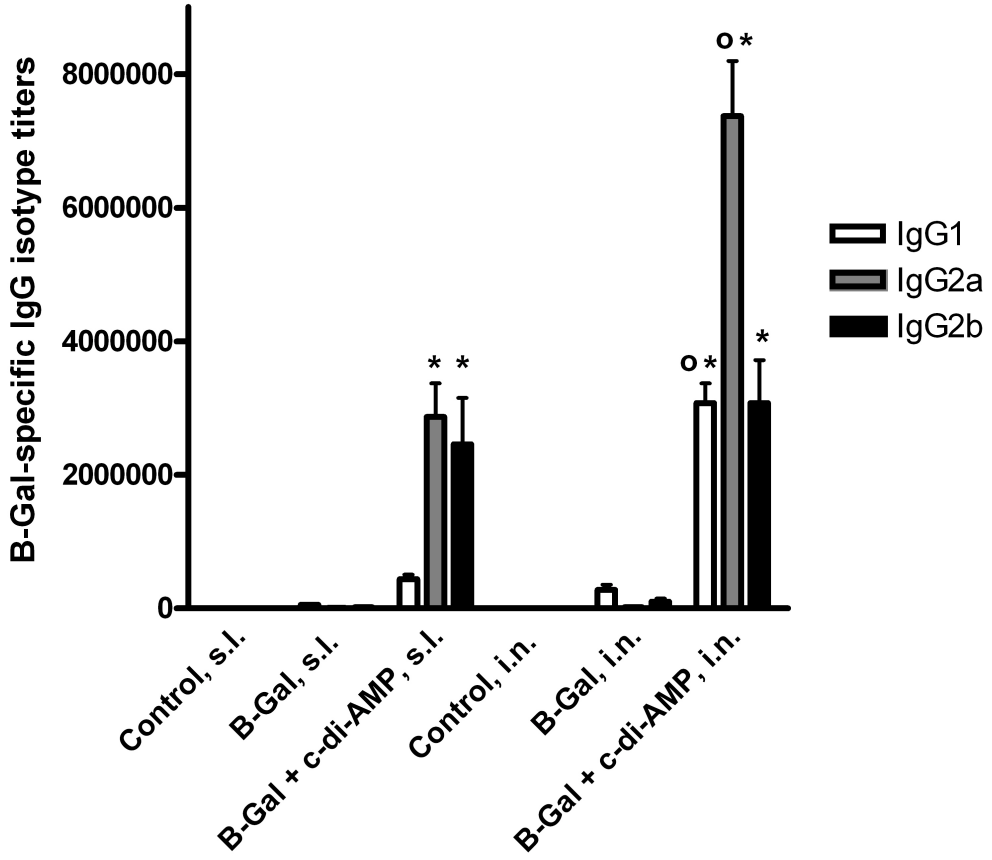

Figure 5. Analysis of β-Gal-specific IgG subclasses (IgG1, IgG2a, IgG2b) in sera of immunized BALB/c mice at day 42 (n = 5, per group). Fourteen days after the final immunization sera samples of immunized mice were analyzed by ELISA. Significant with respect to mice receiving antigen alone (*, $p < 0.01$). Significant with respect to mice receiving the antigen co-administered with c-di-AMP by the s.l. route (^{o}, $p < 0.01$).

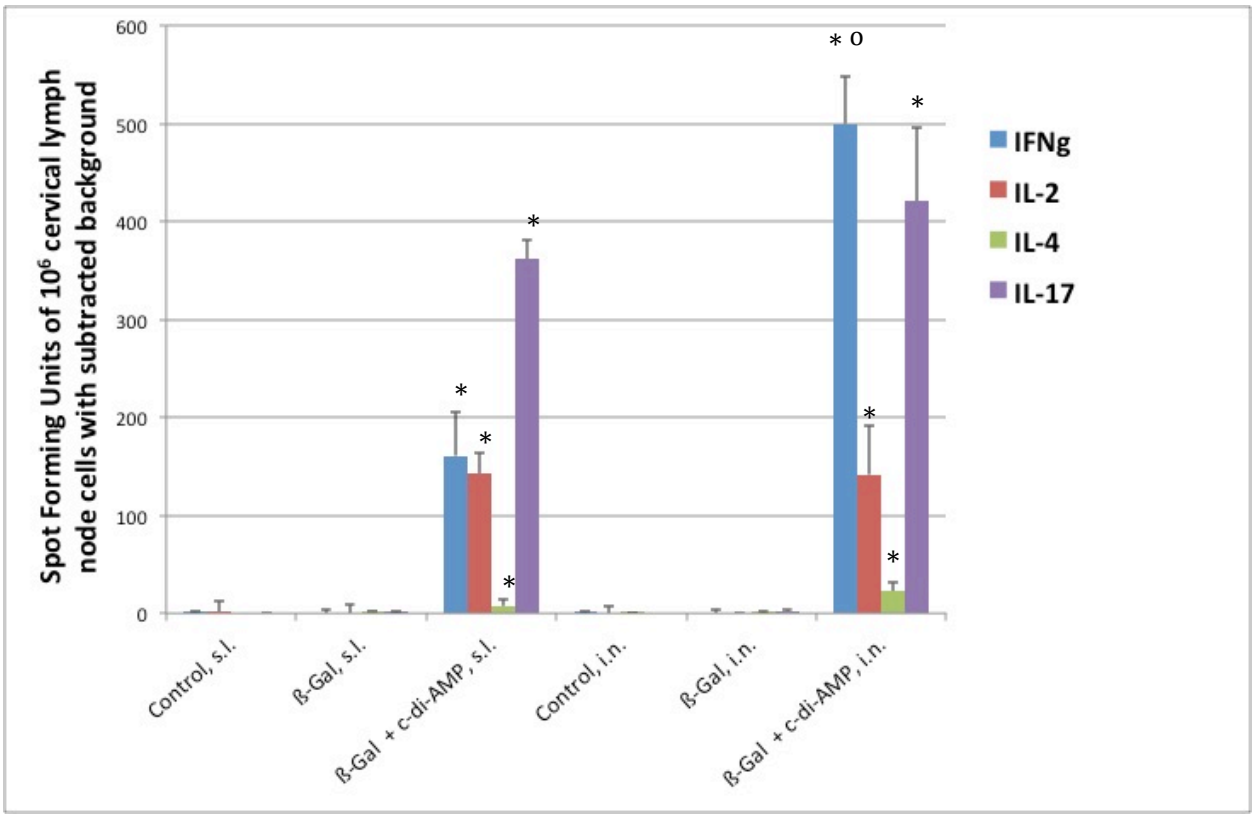

Figure 6. Detection of cytokine secreting cells. Cervical lymph node cells (1 x 10^6) recovered from vaccinated mice were incubated for 24 or 48 h in the presence of B-Gal protein, the number of cytokine secreting cells was then determined by ELISPOT. Results are presented as spot forming units per 1 x 10^6 cells, which were subtracted from the values obtained from non-stimulated cells. Significant with respect to mice receiving antigen alone (*, $p < 0.01$). Significant with respect to mice receiving the antigen co-administered with c-di-AMP by the s.l. route (^{o}, $p < 0.01$).

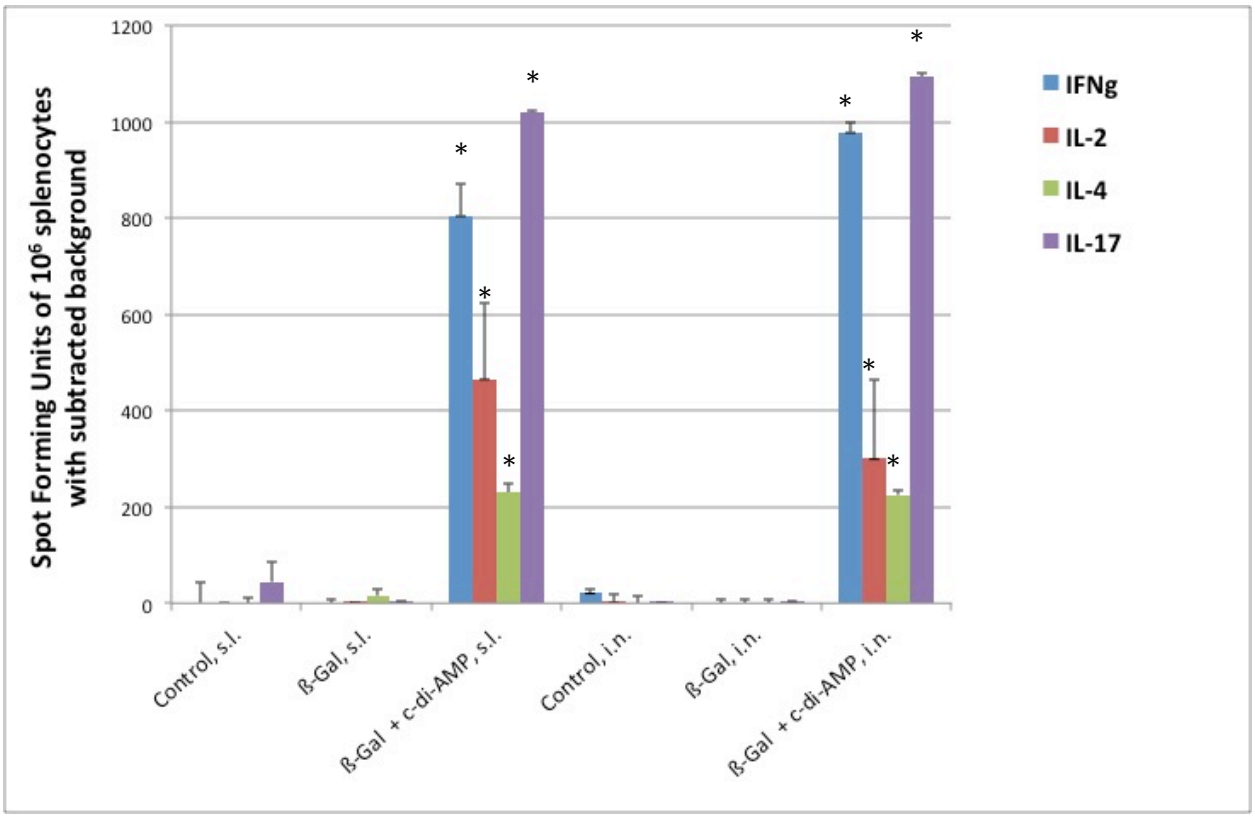

Figure 7. Detection of cytokine secreting cells. Spleen cells (1 x 10^6) recovered from vaccinated mice were incubated for 24 or 48 h in the presence of β-Gal protein, the number of cytokine secreting cells was then determined by ELISPOT. Results are presented as spot forming units per 1 x 10^6 cells, which were subtracted from the values obtained from non-stimulated cells. Significant with respect to mice receiving antigen alone (*, $p < 0.01$).

4.2.5 cytokine secretion

To further investigate the effect of c-di-AMP on cytokine production, spleen cells from vaccinated animals were stimulated *in vitro* with β-Gal and supernatants were evaluated over time using a Mouse Th1/Th2/Th17/Th22 13plex FlowCytomix Multiplex (eBioscience®). Enhanced secretion of IFN-γ, TNF-α, IL-2, IL-13, IL-17, IL-22, and IL-27 was observed in cells from c-di-AMP vaccinated mice with respect to supernatants from cells of animals immunized with β-Gal alone. Overall, trends were very similar between intranasal and sublingual groups; with one noted exception being IL-27 was only observed in the intranasal group (Fig. 8-9).

Looking at concentrations across time, some general trends were observed. For example, IL-2 and TNF-α were strongly produced early after stimulation (48 hours) then decreased thereafter. Conversely, concentrations of IL-22 gradually increased across time; and still further, cytokines such IFN-γ and IL-13, while largely undetectable at 48 hours, stabilized at a high concentration by 72 and 96 hours post-stimulation. Overall, cytokine profiles indicate a mixed, but largely Th1/Th17 T cell response, as evidenced by high concentrations of IFN-γ (Th1), IL-22 (Th17), and IL-17 (Th17); and the absence of Th2 cytokines, such as IL-4 and IL-5. The exception to this was IL-13 (Th2), which was strongly produced at later time-points (i.e., 72 and 96 hours) (Table 8).

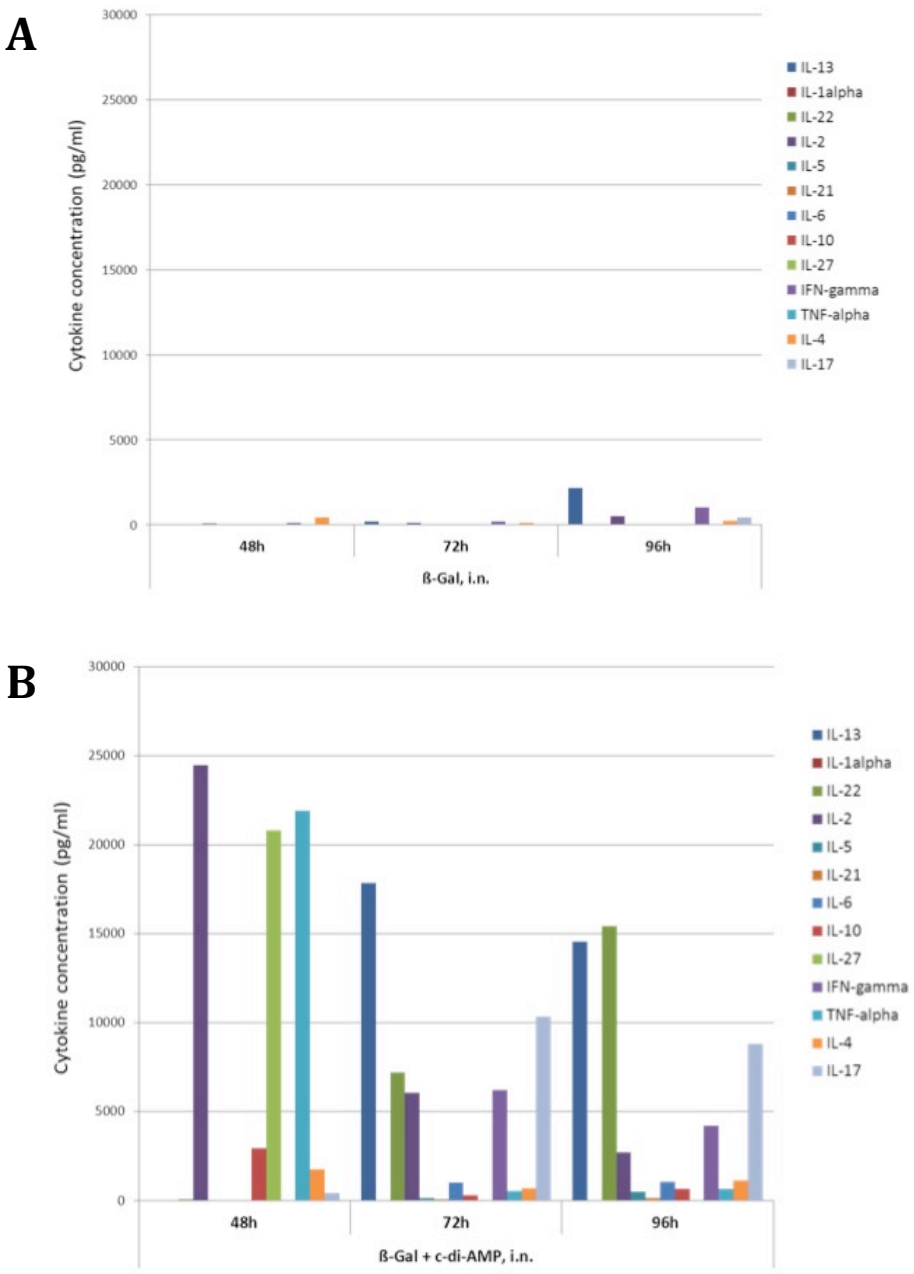

Figure 8. Analysis of cytokines secreted by antigen-restimulated splenocytes of mice immunized by the intranasal route with (A) β-Gal or (B) β-Gal co-administered with c-di-AMP (n = 5). Cells (5 x 10^5) were restimulated in quadruplicates with different concentrations of β-Gal for 48h, 72h, and 96 h. At the end of each time-point, supernatants were pooled from the quadruplicates and the secreted cytokines (IL-13, IL-1alpha, IL-22, IL-2, IL-5, IL-21, IL-6, IL-10, IL-27, IFN-gamma, TNF-alpha, IL-4, IL-17) were analyzed by a Th1/Th2/Th17/Th22 Flowcytomix array. Results are shown from splenocytes restimulated with 40 ug/ml of B-Gal.

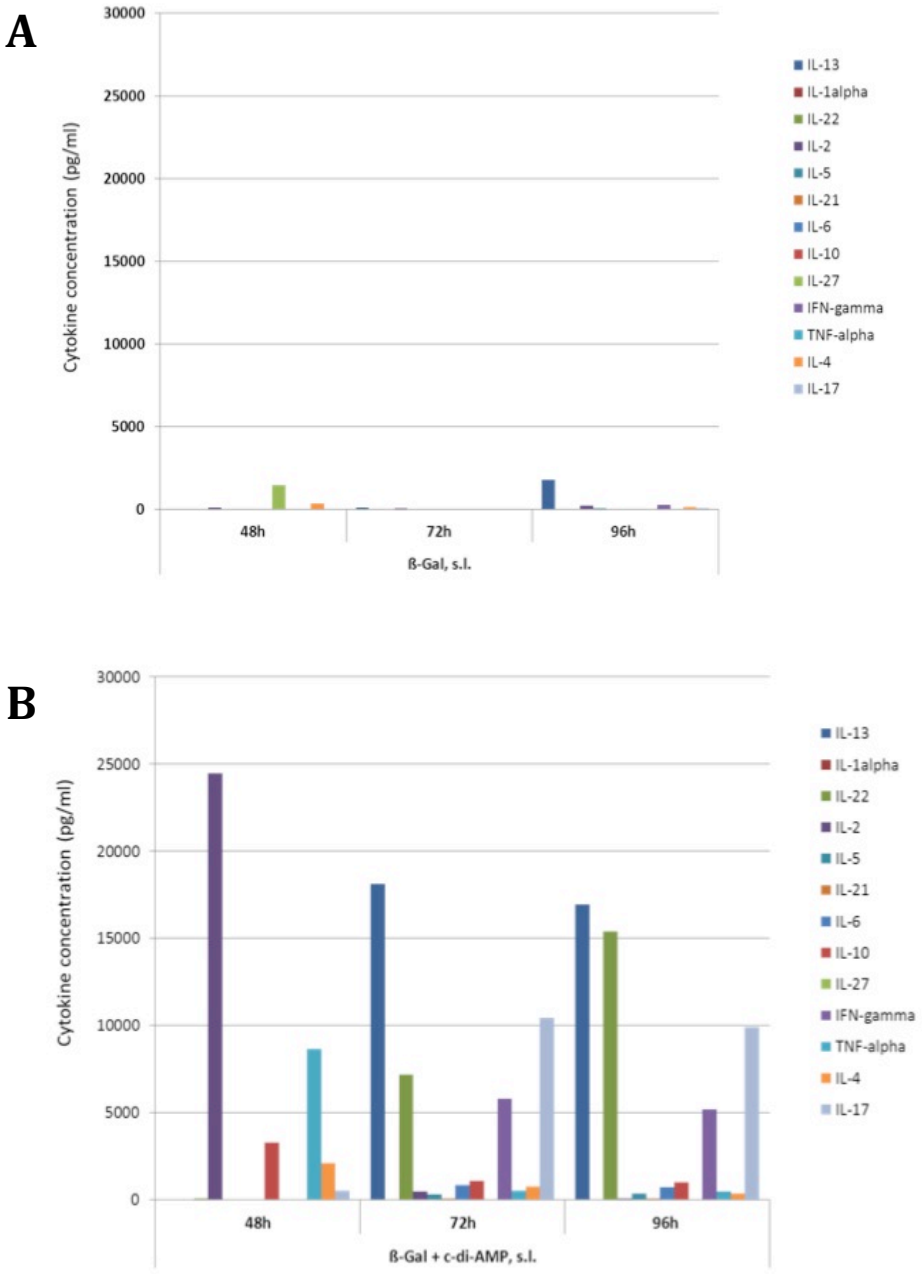

Figure 9. Analysis of cytokines secreted by antigen-restimulated splenocytes of mice immunized by the sublingual route with (A) B-Gal or (B) β-Gal co-administered with c-di-AMP (n = 5). Cells (5 x 10^5) were restimulated in quadruplicates with different concentrations of B-Gal for 48h, 72h, and 96 h. At the end of each time-point, supernatants were pooled from the quadruplicates and the secreted cytokines (IL-13, IL-1alpha, IL-22, IL-2, IL-5, IL-21, IL-6, IL-10, IL-27, IFN-gamma, TNF-alpha, IL-4, IL-17) were analyzed by a Th1/Th2/Th17/Th22 Flowcytomix array. Results are shown from splenocytes restimulated with 40 ug/ml of B-Gal.

	ß-Gal + c-di-AMP, i. n.			ß-Gal + c-di-AMP, s. l.		
	48h	72h	96h	48h	72h	96h
IFN-γ	-	+++++	++++	-	+++++	++++
TNF-α	+++++	++	++	++++	++	++
IL-2	+++++	+++	++	+++++	++	+/-
IL-4	+++	++	+++	++++	++	+++
IL-5	-	+	++	-	+	++
IL-6	-	+++	+++	-	+++	+++
IL-10	+++	++	++	+++	++	++
IL-13	-	+++++	++++	-	+++++	++++
IL-17	+	+++++	+++++	+	+++++	+++++
IL-21	-	+/-	+	-	+/-	+/-
IL-22	+	+++	+++++	+	+++	+++++
IL-1α	-	-	-	-	-	-
IL-27	+++++	-	-	-	-	-

Table 2. Overview of cytokines secreted by antigen-restimulated splenocytes of mice immunized by the sublingual and intranasal routes with B-Gal (30 ug/dose) co-administered with c-di-AMP (10 ug/dose) (n = 5). Cells (5 x 10^5) were restimulated in quadruplicates with different concentrations of B-Gal for 48h, 72h, and 96 h. At the end of each time-point, supernatants were pooled from the quadruplicates and the secreted cytokines were analyzed by a Th1/Th2/Th17/Th22 Flowcytomix array. Results are shown from splenocytes restimulated with 40 ug/ml of B-Gal. Blue indicates Th1 cytokines, red Th2 cytokines, and green a Th17 cytokine.

5. Intranasal immunization with *Ancylostoma ceylanicum* excretory-secretory (ES) proteins co-administered with c-di-AMP†

5.1. Methods

5.1.1. Parasites and hosts

The *Ancylostoma ceylanicum* life cycle was maintained in 3 week-old Syrian golden hamsters of the HsdHan: AURA outbred strain (Harlan, Indianapolis IN), as previously described.[24] Immunization and challenge studies were also conducted in Syrian golden hamsters. Animals were housed at the Yale School of Public Health, and all experiments were performed with the prior approval of the Yale Animal Care and Use Committee.

5*.1.2. Bis-(3',5')-cyclic dimeric adenosine monophosphate (c-di-AMP)*

As described previously, c-di-AMP (LPS negative) was synthesized by cyclization and purified by reversed phase HPLC (100). The lyophilized compound was stored at −20 °C until use when it was resuspended in sterile water for the immunization studies.

5.1.3. A. ceylanicum excretory-secretory (ES) proteins

ES products were prepared as previously described.[25] Briefly, live adult *A. ceylanicum* hookworms were rinsed and incubated in sterile PBS (10 worms per ml) for 6 h at 37°C. The worms were then removed, and the raw ES products were centrifuged (15 mins at 3 300 *g*) before being passed through a 0.45-μm filter to remove insoluble particles. The flow-through was then concentrated using a centrifugal concentrator with a 5-kDa molecular weight cut-off (Millipore Corp., Bedford, MA), and the final concentration of ES

† This set of experiments was carried out at the Yale School of Public Health, New Haven, CT from November 2011 to April 2012 under the supervision of Michael Cappello

protein was determined using the BCA system (Pierce Chemical Co., Rockford, IL) with a bovine serum albumin standard curve. Aliquots of concentrated ES were stored at –80°C until use.

5.1.4. Immunization and challenge infection of hamsters

Male 3-week old Syrian golden hamsters were immunized intranasally (i.n.) on days 0, 14, and 28 with sterile PBS (buffer control), 15 μg of c-di-AMP, 100 μg of ES, or 100 μg of ES co-administered with 15 μg of c-di-AMP (n=5, per group). Vaccines were formulated in sterile PBS and prepared 30 minutes prior to immunization. During intranasal immunization, mice were lightly anesthetized (10-25 seconds) by inhalation of CO_2 then given an 80 μl dose of vaccine administered drop-wise to the nostrils. One week after the final immunization (day 35) immunized groups were infected by oral gavage with 100 third-stage larvae (L_3) of *A. ceylanicum*. An additional group of hamsters (n=5) was left untreated and uninfected that served as a negative control of hookworm pathogenesis.

5.1.5. Sample collection

Blood was drawn from all animals by retro orbital puncture and collected in heparinized capillary tubes (Fisher Scientific) 1 day before all immunizations and twice per week throughout the challenge infection period. For analysis of antigen-specific IgG responses, samples were centrifuged (10 minutes at 3000 *g*) to remove red blood cells and the sera stored at -80°C until further analysis. For measurement of hemoglobin levels, blood was assayed within 4 hours using a Total Hemoglobin assay kit (see below). At 22 days post-infection (dpi), all animals were euthanized and adult worms removed and counted.

Intestinal flush and lung lavage were collected and feces removed from large intestines for individual egg per gram counts, as described below.

For the analysis of mucosal antigen-specific immunoglobulin A (IgA), lung lavage (LL) and intestinal flush (IF) were performed. For lung lavage, a small incision was made in the trachea and 2 ml of ice cold PBS was carefully injected into the lungs and aspirated using a gavage needle (20 Gauge x 1.5”). To remove large debris, lavage samples were centrifuged (15 min at 16 000 *g* [4°C]) and the supernatants collected. For intestinal flush, small intestines were excised from hamsters and flushed with 10 mL PBS using a syringe and 18 gauge needle, manually extruding any remaining mucus. The raw flush was gently agitated for 1 min, centrifuged (15 min at 16 000 *g* [4 °C]) and the supernatant passed through a 0.45-µm syringe filter. For both lung lavage and intestinal flush, samples were stored at -80 °C until further analysis.[25] In all cases, the expression of antigen-specific IgA antibodies was determined in individual animals.

Following intestinal flush, worm burdens for each animal were counted by longitudinally dissecting intestines and repeatedly rinsing them with room temperature PBS. This treatment caused most of the worms to detach from the mucosa, and the remaining worms were then gently removed from the intestinal debris with fine-tipped forceps and the aid of a dissecting microscope. To calculate fecal egg excretion, large intestines were excised from hamsters and feces manually extruded into collection tubes. Fecal samples were stored a 4 °C, and eggs were counted within 4 days of collection (see below). Worm burden and fecal egg counts were determined in individual animals.

5.1.6. Measurement of blood hemoglobin

Blood was drawn from all animals by retro orbital puncture and collected in heparinized capillary tubes (Fisher Scientific) and assayed within 4 hours of collection. Hemoglobin levels were measured using a Total Hemoglobin assay kit (Sigma), as previously described.[26] Briefly, 10 µl of whole blood was mixed into 2.5 mL Drabkin's solution and incubated for 15 min at room temperature. Sample tubes were then vortexed, 200 µl were transferred in duplicate wells to a microtiter plate, and samples, including a hemoglobin standard curve prepared from kit reagents, were read at 530 nm in using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA).

5.1.7. Measurement of fecal egg counts

A modified McMaster technique was used to calculate fecal egg excretion. The McMaster method was based on the modified McMaster described by the Ministry of Agriculture, Fisheries, and Food (1986).[27] Briefly, 1 gram of feces was suspended in 10 ml of saturated salt solution at room temperature. The suspension was then homogenized, and poured through gauze to remove large debris. Then, 0.5 ml aliquots were added to each of the two chambers of a McMaster slide (http://www.mcmaster.co.za), and both chambers were examined under a light microscope using a 100x magnification. The fecal egg counts were obtained by multiplying the total number of eggs by 33, and expressed as eggs per gram (EPG).

5.1.8. Detection of ES-specific IgG in serum

The amount of ES-specific IgG in serum was measured by ELISA. Immulon-2 microtitre plates (Dynex, Chantilly, VA) were coated with 100 µl/well of ES diluted to 2 µg/ml in sterile PBS and incubated overnight at 4 °C. The following day, plates were washed (PBS, 0.05% Tween 20) 4 times and blocked (PBS, 1% Milk) for 1 h at room temperature (RT). For pooled samples, after washing, serial 2-fold dilutions (starting at 1:25) of sera in PBS, 0.05% Tween 20, 1% Milk were added at 100 µl/well, then incubated for 2 hours at 37 °C. For individual samples, sera was diluted 1:4 in PBS, 0.05% Tween, 1% Milk then added to plates in duplicate and incubated for 2 hours at 37 °C. After washing, 100 µl/well of HRP-conjugated goat anti-hamster IgG were added in PBS, 0.05% Tween 20 and incubated at 37 °C for 1 hour. After another wash step, reactions developed with a 30 min incubation in ABTS (1 mg/mL ABTS in 0.1 molar citrate buffer, pH 5.0, 0·03% H_2O_2). For individual samples, the mean optical density at 405 nm was reported after subtraction of background. For pooled samples, antigen-specific titers were calculated by interpolating the dilution giving an optical density of 0.2 after subtraction of background. All values were reported using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA), and normalized to a positive standard (serum from hamsters previously collected 104 days post-infection), which was included in each assay to control for day-to-day variation.

5.1.9. Detection of ES-specific IgA in intestinal flush and lung lavage samples

Similar to IgG, ELISA was used to measure the amount of ES-specific IgA in intestinal flush and lung lavage samples. Briefly, Immulon-2 microtitre plates (Dynex, Chantilly, VA) were coated with 100 µl/well of ES diluted to 2 µg/ml in sterile PBS and incubated overnight at 4 °C. After blocking (PBS, 1% Milk), with wash steps (PBS, 0.05% Tween 20) before and after,

100 µl/well of intestinal flush or lung lavage samples diluted 1:4 in PBS, 0.05% Tween, 1% Milk were added in duplicate and the plates incubated overnight at 4 °C. The following day, plates were washed and 100 µl/well of biotinylated anti-mouse IgA α-chain (1:1 000 in PBS, 0.05% Tween, 1% Milk) were added and plates incubated for 2 h at RT. After washing again, streptavidin-HRP solution (1:5000 in PBS, 0.05% Tween 20) was added and plates were incubated at 37 °C for 1 h. Finally, plates were washed and reactions developed using ABTS (1 mg/mL ABTS in 0.1 molar citrate buffer, pH 5.0, 0·03% H_2O_2). After 30 min incubation at room temperature, the mean optical density at 405 nm was reported using a SpectraMax 190 microplate reader (Molecular Devices, Sunnyvale, CA) after subtraction of background.

5.1.10. Statistical analysis of data

Data are expressed as means ± standard errors. For multiple group comparisons, one-way ANOVA was performed followed by Tukey-Kramer Multiple Comparison test (GraphPad Prism 4).

5.2. Results

5.2.1. Induction of humoral immune responses at systemic and mucosal levels following intranasal immunization with non-adjuvated ES

To evaluate the efficacy of c-di-AMP as a mucosal adjuvant in formulation with hookworm antigens, hamsters were immunized intranasally on days 0, 14, and 28 with PBS (control), 15 μg/dose c-di-AMP (adjuvant), 100 μg/dose *A. ceylanicum* excretory-secretory (ES) proteins, or 100 μg/dose ES co-administered with 15 μg/dose c-di-AMP. One week after the last dose animals were infected with 100 third-stage *A. ceylanicum* larvae (L_3), as described in Methods.

Although results are representative of pooled serum samples, ES-specific IgG responses were similar in ES and ES plus adjuvant treatment groups. In both ES groups responses were similar following the second boost at day 35 when animals were challenged; and although primary infection increased IgG responses in all animals, by 22 days post-infection (dpi) both ES groups showed enhanced ES-specific IgG in comparison to PBS and c-di-AMP treated groups (Fig. 10).

The stimulation of mucosal immune responses by immunization and infection were evaluated by measuring ES-specific IgA in lung lavage (LL) and intestinal flush (IF) samples taken from all animals 22 dpi. Here again, compared to animals vaccinated with ES alone, the use of c-di-AMP did not enhance antigen-specific IgA responses, with similar responses observed between ES groups in both mucosal compartments. In the ES alone group, a significant enhancement in ES-specific IgA was observed in intestinal flush samples, as compared to PBS treated hamsters ($p < 0.05$). Additionally, ES-specific IgA responses were observed in both mucosal compartments of animals immunized with PBS and c-di-AMP,

suggesting primary infection alone elicits local and distal antigen-specific mucosal IgA responses (Fig. 11).

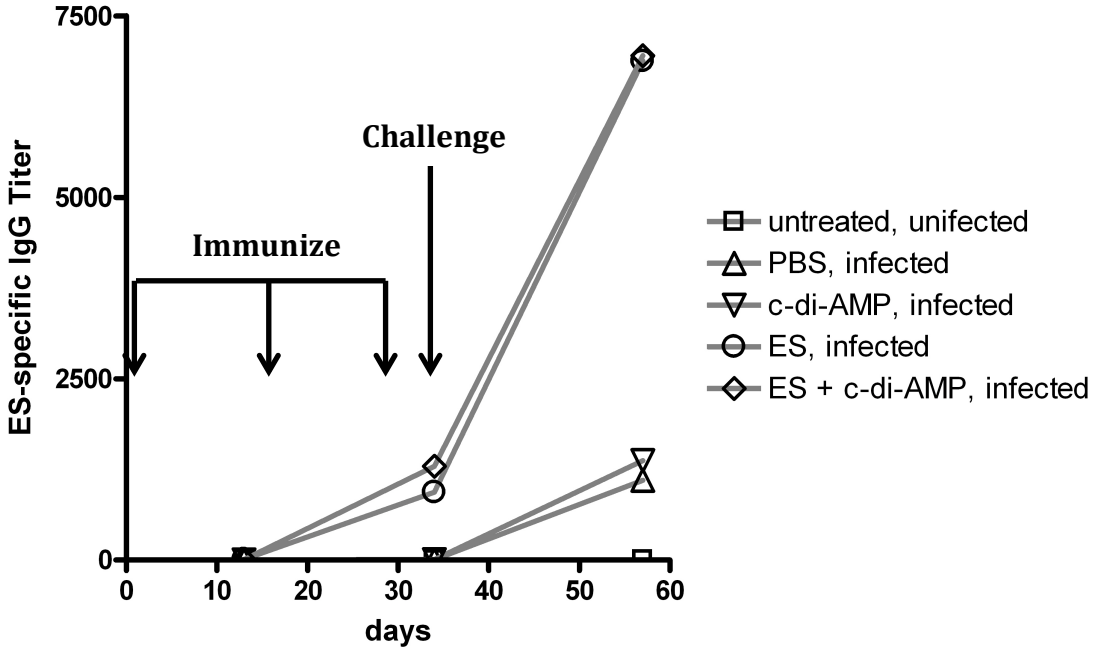

Figure 10. Kinetic analysis of anti-ES IgG responses in sera of Syrian golden hamsters immunized on days 0, 14, and 28 with PBS (control), 15 µg/dose c-di-AMP (adjuvant), 100 µg/dose *A. ceylanicum* excretory-secretory (ES) proteins, or 100 µg/dose ES co-administered with 15 µg/dose c-di-AMP by the intranasal route (n = 5, per group). On day 35 animals were orally infected with 100 third-stage *A. ceylanicum* larvae (L_3). Sera was collected from animals, pooled by group, and analyzed by ELISA.

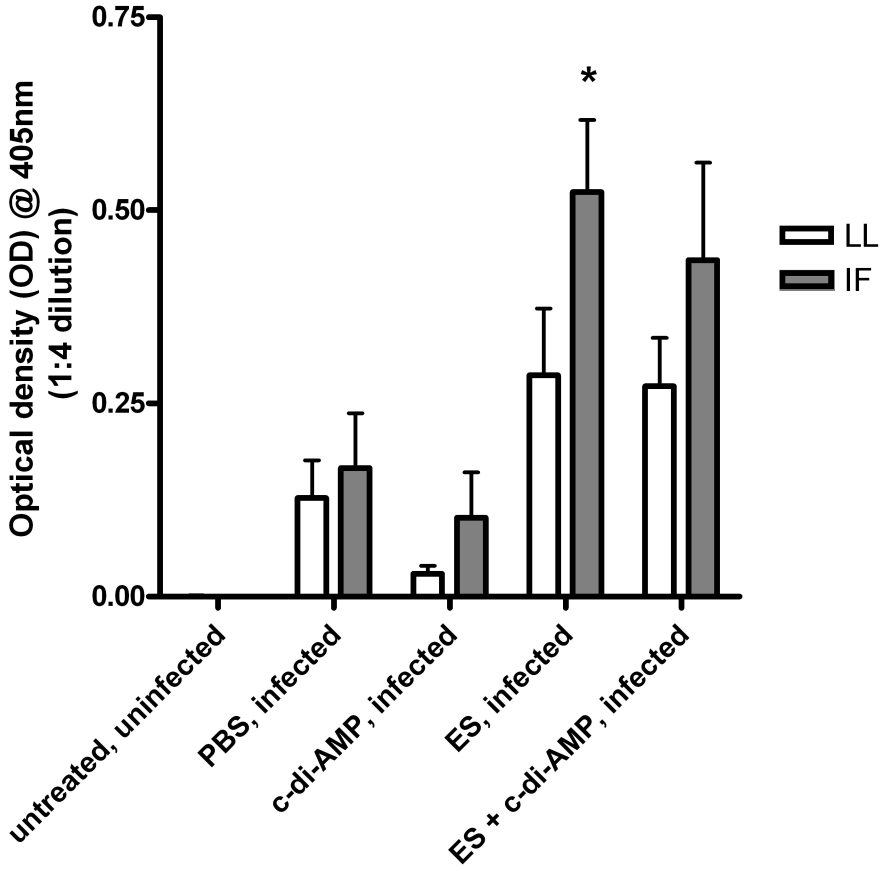

Figure 11. Analysis of mucosal anti-ES IgA responses of Syrian golden hamsters immunized on days 0, 14, and 28 with PBS (control), 15 µg/dose c-di-AMP (adjuvant), 100 µg/dose *A. ceylanicum* excretory-secretory (ES) proteins, or with 100 µg/dose ES co-administered with 15 µg/dose c-di-AMP by the intranasal route (n = 5, per group). On day 35 animals were orally infected with 100 third-stage *A. ceylanicum* larvae (L_3). Upon development of adult worms, 22 days post-infection, the animals were euthanized and lung lavage (LL) and intestinal flush (IF) samples were analyzed by ELISA. Significant with respect to PBS, infected hamsters (*, $p < 0.05$).

5.2.2. Intranasal immunization with unadjuvated ES appears to modulate the severity of hookworm-associated weight loss and anemia

To determine whether immunization with ES plus c-di-AMP could protect animals from hookworm-associated pathology, one week after the final immunization hamsters were challenged with 100 L_3 larvae and observed for 22 days.

As expected, untreated, uninfected control hamsters (mean weight, 108.0 ± 4.9 g at 0 dpi) steadily increased in weight throughout the experiment, reaching a mean of 113.9% of their initial (0 dpi) weight by 22 dpi. For most of the infected groups, by 14 days after challenge weight decline was observed. The most dramatic reductions occurred in the groups without ES (PBS and c-di-AMP alone). In the PBS group (mean weight, 123.5 ± 2.0 g at 0 dpi), weights declined from a mean of 116.6% at 11 dpi to 106.4% of their initial (0 dpi) weight by 22 dpi. Similarly, in the c-di-AMP alone group (mean weight, 112.3 ± 4.3 g at 0 dpi), weights declined from a mean of 113.9% at 11 dpi to 102.5% of their initial weight by 22 dpi. Weight decline was less pronounced in the ES + c-di-AMP group (mean weight, 110.0 ± 6.1 g at 0 dpi), but still apparent. Here, weights declined from a mean of 113.9% at 11 dpi to 107.6% of their initial weight by 22 dpi. Unlike the other groups, the ES alone group (mean weight, 118.0 ± 1.5 g at 0 dpi) appeared relatively refractory to hookworm-associated weight loss, with weights stabilizing around 110% of their initial weight from 11 dpi to 22 dpi, the period the other groups were experiencing profound weight declines (Fig. 12A-B).

Hemoglobin levels in untreated, uninfected control hamsters remained relatively stable. In all the infected groups, however, by 11 days post-challenge blood hemoglobin levels began to steadily drop, declining approximately 40% by 18 dpi. In contrast to the

other infected groups, the ES alone group appeared to be recovering from hookworm-associated anemia by the final time-point (22 dpi). At this time, the ES alone group was only experiencing an approximately 25% reduction from pre-challenge hemoglobin levels, while the other groups remained depressed around 40% (Fig. 12C-D).

5.2.3. Intranasal immunization with unadjuvated ES reduces worm burden and egg excretion

To determine whether response to immunization had an impact on worm development, animals were sacrificed 22 days post-infection and parasites and eggs were counted. Mean intestinal worm burdens were similar in the PBS (45.8 ± 6.1 parasites) and c-di-AMP alone (43.8 ± 4.6 parasites) groups. However, compared to untreated control animals, the ES alone group saw a 73.8% reduction (p-value < 0.01) in mean intestinal worm burden (12.0 ± 4.7 parasites). A non-statistically significant 46.7% reduction in mean worm burden was observed in the ES + c-di-AMP group (24.4 ± 8.6 parasites) (Fig. 13A). Using fecal egg counts as an additional marker of infection intensity; these data largely corroborated the intestinal worm burden results. Here again, counts were highest in the PBS and c-di-AMP alone groups, and lowest in the groups receiving ES. ES alone showed a 67.4% reduction (p-value < 0.001) in fecal eggs per gram (EPG), while the ES + c-di-AMP group showed a 47.4% reduction (p-value < 0.005) in EPG (Fig. 13B).

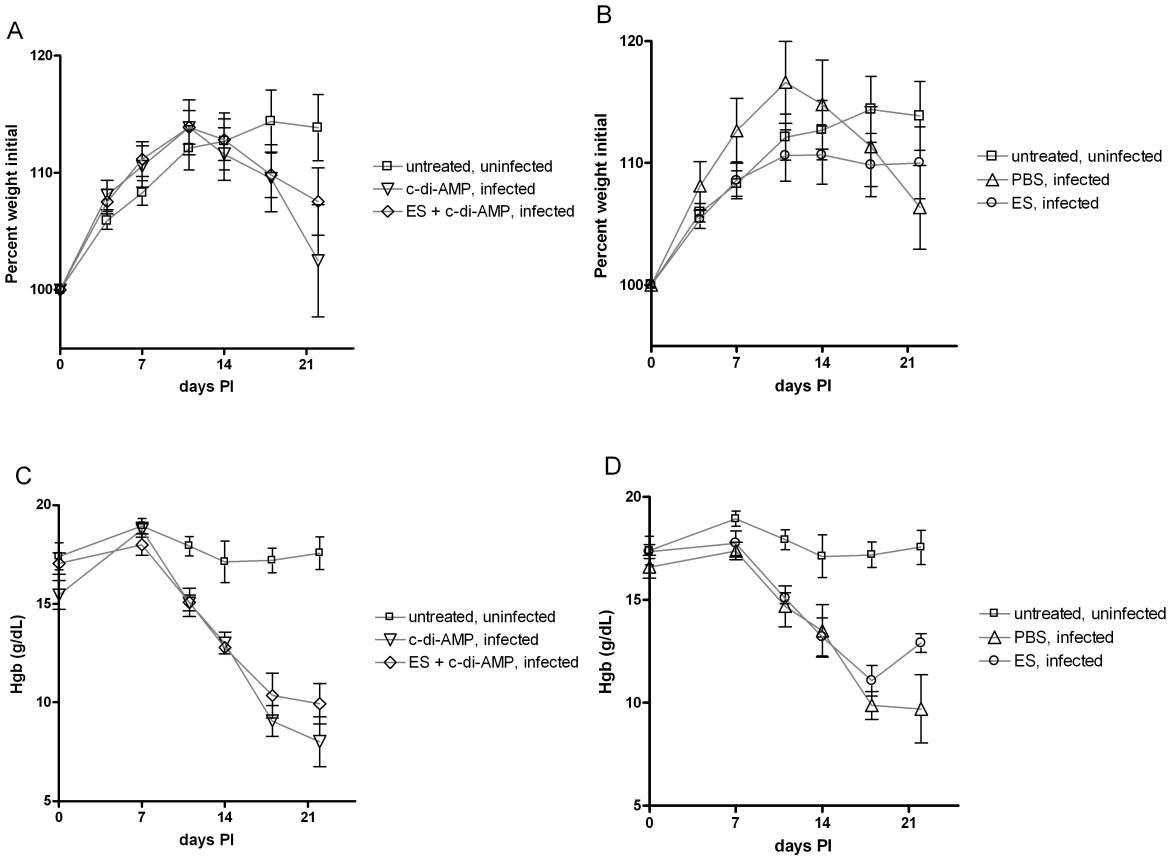

Figure 12. One week following the final immunization animals were orally infected with 100 L_3 *A. ceylanicum* larvae. Post-infection weights with (A) and without (B) adjuvant. Post-infection blood hemoglobin levels with (C) and without (D) adjuvant. All values are means $\pm$ standard errors of the means.

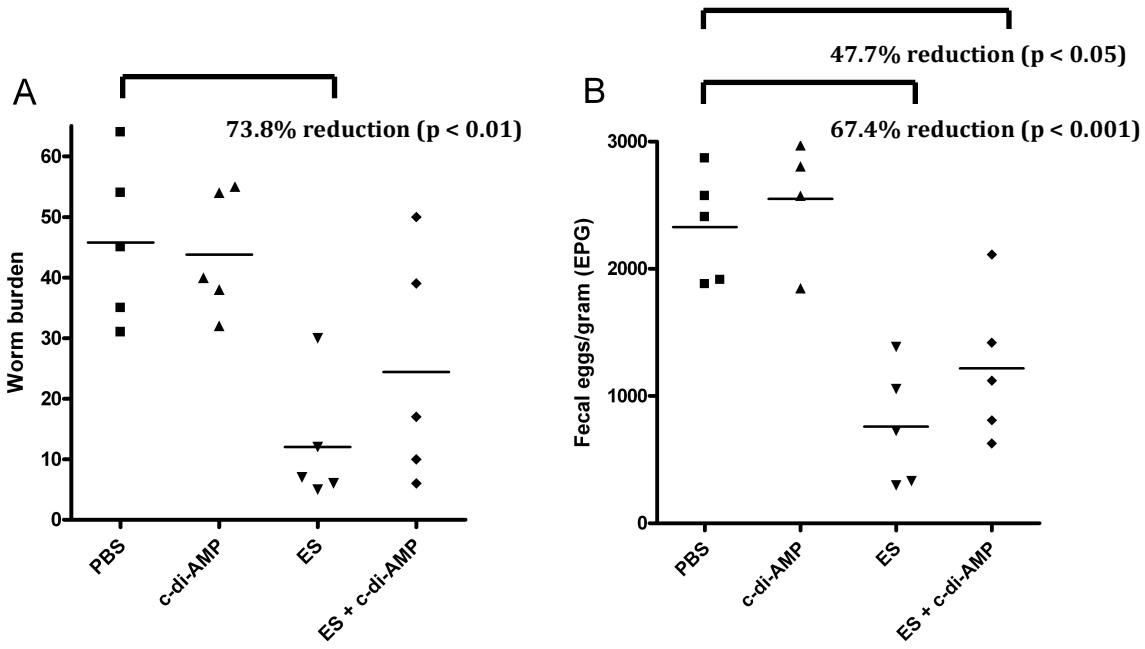

Figure 13. Upon development of adult worms, 22 days post-infection, the animals were euthanized and small and large intestines removed. (A) Intestinal worm burdens for each animal are indicated by a dot, with the group mean indicated by a horizontal line. (B) The McMaster technique was used to calculate fecal egg counts. Fecal eggs per gram (EPG) for each animal are indicated by a dot, with the group mean indicated by a horizontal line.

6. Discussion

Most pathogens invade their human host or establish infection at mucosal surfaces.[1, 2, 3] However, the majority of existing vaccines are administered by parenteral injection. In contrast to these vaccines, mucosal vaccinations have the potential to counter mucosa-associated pathogens at the portal of entry by inducing local pathogen-specific immune responses, as well as systemic humoral and cellular responses. Additionally, targeting mucosal sites also has the potential to induce immune responses at other, remote, mucosal surfaces; and may offer logistical advantages over parenteral vaccination, such as increased patient compliance due to ease of administration.[5]

Despite these advantages, very few mucosal vaccines are licensed for human use, with even fewer available for use in the US (Table 1). This is partially due to the public's increasing demand for safer vaccines, which has led to an increased use of highly purified recombinant molecules or pathogen subunits. While the use of well-defined protective antigens has significantly improved the safety profile of vaccine antigens, these antigens usually lack inherent immunostimulatory properties, and typically do not elicit strong immune responses. The development of effective mucosal vaccines is further challenged by the unique characteristics of the mucosal environment, including the presence of mucus, limited antigen uptake, and the possibility of antigen degradation.[5] As a result of these factors mucosal adjuvants need to be incorporated into the formulation to improve a vaccine's efficacy.[20]

Unfortunately, only a few adjuvants are currently licensed for human use (Alum, AS04, and MF59), with none of them especially effective when delivered mucosally.[14, 9] The lack of effective mucosal adjuvants, therefore, represents a bottleneck in the development

of mucosal vaccines, and thus, is an important priority.[5, 9] As novel candidate adjuvants are investigated, it is important to consider that adjuvants not only enhance the strength of the elicited antigen-specific responses, but also may exhibit intrinsic immunomodulatory properties. In this context, stimulation of the appropriate type of immune response is a key, as eliciting an incorrect type of immune response could lead to immunopathology or even exacerbate disease.[20] In the present studies, we evaluated the mucosal adjuvant properties of c-di-AMP, a member of the cyclic di-nucleotide family, in combination with a single recombinant antigen, and in an infectious disease model using native soluble pathogen proteins.

The mechanism of action of c-di-nucleotides is still unknown; however, it has been speculated that they might act through putative DNA/RNA receptors which activate an intracellular signal transduction cascade. This in turn might regulate the expression of genes promoting a local pro-inflammatory environment that leads to the mobilization, recruitment and activation of antigen presenting cells.[9] From our studies, c-di-AMP stimulates strong humoral (Figs. 2-3) and cellular immune responses (Fig. 4), and induces a mixed Th1/Th2/Th17, but predominately Th1/Th17 T cell response pattern, as shown by elevated expression of IgG2a (Fig. 5) and enhanced secretion of IFN-γ, IL-17, and IL-22 (Fig. 6-9).

We also observed comparable results through the intranasal and sublingual routes. The recent association between an inactivated intranasal influenza vaccine and Bell's palsy has raised concerns of the possibility of retrograde passage of inhaled antigens or adjuvants through the olfactory epithelium, and prompted research into alternative routes of mucosal immunization.[3, 28] Given the comparability of our results, this study adds to a

growing body of literature suggesting that sublingual immunization can result in responses comparable to nasal immunization regarding the magnitude, breadth and anatomic dissemination of induced immune responses.[3]

When investigating the effect of c-di-AMP on the expression of IgG isotypes (IgG1, IgG2a, and IgG2b), co-administration of c-di-AMP with antigen resulted in the greatest enhancement of IgG2a (503-fold [s.l.] and 445-fold [i.n.]); and the least enhancement of IgG1 (8-fold [s.l.] and 11-fold [i.n.]), as compared to antigen alone. These data indicate that by both routes c-di-AMP is predominately associated with a $CD4^+$ T helper 1 (Th1) response, as IgG1 and IgG2a isotypes are broadly associated with Th2 and Th1 T cell responses in mice, respectively.[29] The development of enhanced IgG2a responses is supported by measurements of cytokine secreting cells (Figure 6-7) and cytokine concentrations (Figure 8-9), which showed stronger expression of IFN-γ (Th1) than IL-4 (Th2). A large body of literature suggests that the cytokines IL-4, IFN-γ and TGF-β regulate immunoglobulin class switching through transcriptional regulation.[30, 31, 32] Evidence indicates that the Th2-specific cytokine, IL-4, induces switching to IgG1 and IgE, and inhibits switching to IgG2b. In contrast, the Th1-specific cytokine, IFN-γ, has been shown to inhibit the IL-4-mediated induction of IgG1 and IgE, and to enhance the production of IgG2a.[31] Additionally, TGF-β has the ability to direct class switching to IgA and IgG2b.[32]

In addition to IFN-γ, IL-17 (Figs. 6-9) and IL-22 (Figs. 8-9) were also enhanced through vaccination with c-di-AMP as adjuvant. It is well established that Th1 cells produce IFN-γ and TNF-β, which activate macrophages and are involved in delayed-type hypersensitivity reactions; and that Th2 cells produce IL-4, IL-5, IL-10 and IL-13, which are responsible for strong antibody responses, including IgE production, and the inhibition of

several macrophage functions.[33] Recently, Th17 cells have been identified as a third T helper lineage that regulates inflammation via production of distinct cytokines, such as IL-17, IL-17F, IL-21, and IL-22.[34, 35] There is growing evidence that Th17 cells, and other IL-17-producing cells, are critical for host defense against bacterial, fungal, and viral infections at mucosal surfaces.[36] While the role of IL-17 in vaccine-induced immunity against eukaryotic parasites is not fully defined, there is evidence that induction of IL-17 may be important for protection against parasite pathogens, such as *Leishmania donovani, Eimeria maxima*, and *Nippostrongylus brasiliensis*. As reviewed by Lin *et al* [35], production of IL-17 and IL-22 in human subjects showed a strong and independent association with protection against Kala azar, or visceral leishmaniasis, the disease caused by infection with *L. donovani*. Furthermore, inclusion of IL-17 as an adjuvant along with a purified recombinant protein vaccine was shown to reduce oocyte shedding and induce protection against the parasite *Eimeria acervulina* in chickens. In our study, infection of hamsters with *Acylostoma ceylanicum* was used as model of intestinal hookworm infection; however, infection of mice with the rodent hookworm, *Nippostrongylus brasiliensis*, is another widely used model.[37] In a study by *Liu et al* [38], the authors suggest during migration through the lung *N. brasiliensis* elicits multiple mechanisms to down-regulate local IL-17 and control inflammatory cell infiltration. Together, these studies suggest IL-17 may have an important role in induction of Th17 memory responses and could be a potential target in the design of effective vaccines against parasites.[35]

The encouraging performance of c-di-AMP in these initial studies led us to test it as a mucosal adjuvant in a vaccine model of *A. ceylanicum* pathogenesis. Contrary to our hypothesis, however, c-di-AMP did not significantly enhance the protective efficacy of

hookworm excretory-secretory (ES) antigens. In Figure 12B, we show that hamsters vaccinated intranasally with ES antigens alone are partially protected from hookworm-associated weight loss, and these hamsters exhibited a trend toward recovery from hookworm-associated anemia by 22 days post-infection (Figure 12D). Similar findings were not present in any of the other groups, including the ES plus c-di-AMP group (Figure 12A, C). In figure 13A, we show that ES alone confers a 73.8% reduction (p-value < 0.01) in worm burden, and ES co-administered with c-di-AMP only confers a 46.7% reduction (NS), as compared to animals vaccinated with PBS. Using fecal egg counts as an additional marker of infection intensity, we saw similar levels of reduction, 67.4% (p-value < 0.001) for ES alone versus 47.7% (p-value < 0.05) for ES plus c-di-AMP (Figure 13B), compared to PBS vaccinated controls. The comparability of adult worm burdens and egg counts provides further evidence of the validity of egg counts as a marker of infection intensity.

In the hamster model, our finding of a 73.8% reduction in adult worm burden is similar to a study by Khan *et al* [39] that showed a 67.0% reduction following intramuscular immunization with ES. In that study, the authors immunized male hamsters intramuscularly with three doses of ES (30, 60, 120 μg), using Freund's Complete Adjuvant co-administered with the first dose, and challenged 7 days after the final dose with *A. ceylanicum* (65 ± 10 L_3). The authors reported a pre-challenge ES-specific serum IgG titer of 1:1,600 that correlated with protection; however, a definitive role of antigen-specific serum IgG was not established. In the Khan *et al* study, no data on hook-worm associated pathology were presented; however, a study by Bungiro *et al* [46], showed that 3 subcutaneous immunizations (21-day intervals) with native ES products adsorbed to alum significantly reduced hookworm-associated anemia and weight loss (worm burden not

assessed). Furthermore, we demonstrate enhanced protection to a mucosal immunization study by Fairfax *et al* [40] where the authors cloned and orally immunized hamsters with three doses of *A. ceylanicum* FAR-1 (AceFAR-1), a hookworm orthologue belonging to the nematode fatty acid and retinol (FAR) binding family found in ES. In that study, Fairfax *et al* reported 47% reduction in mean intestinal worm burden 21 days post-infection when 3 doses of AceFAR-1 (100 µg) were co-administered with cholera toxin (10 µg) at 14 day intervals (data on antibody titers was not presented).

As shown in Figure 10, through intranasal immunization we were able to induce a pre-challenge serum antigen-specific IgG titer (1:1,100) that was enhanced following primary infection (1:7,400); similar responses were observed with and without adjuvant. As Figure 11 shows, intranasal immunization with ES also enhanced post-challenge antigen-specific mucosal IgA responses. Responses in animals vaccinated with ES alone showed significant enhancement of intestinal anti-ES IgA responses, as compared to vaccination with PBS; suggesting intranasal administration of antigens primes intestinal antigen-specific IgA responses.

In non-ES vaccinated hamsters, anti-ES serum IgG responses were negative pre-challenge and stimulated during primary infection (1:1,000) (Fig. 10). Additionally, ES-specific IgA responses were detectable in lung and intestinal samples, following primary infection of non-ES vaccinated hamsters (Fig. 11). In our model, hamsters are orally infected with *A. ceylanicum* with the assumption that larvae migrate directly to the small intestine (without migration through the circulatory system and/or lungs) where they mature into blood-feeding, egg-laying adults. In 1972, Ray *et al* [41] provided strong evidence in support of this assumption. Together, this suggests primary infection with *A. ceylanicum*

induces local (i.e., intestinal) and distal (i.e., lung) anti-ES IgA responses. These results are in general agreement and extend findings made by Bungiro *et al* [25] that showed ES-specific IgA responses in naïve hamsters were detectable in fecal and intestinal flush samples 41 days post-infection. To our knowledge, this is the first study to report stimulation of lung-associated antigen-specific IgA following primary infection in this model. Although the relevance of hookworm-specific mucosal IgA to protection has not been established, IgA has been hypothesized to target the parasite by mechanisms such as neutralization of secretory molecules and/or induction of eosinophil degranulation.[25]

In humans, the normal hookworm life-cycle involves migration of L_3 parasites through the lungs; therefore, stimulation of lung-associated immune responses, including antigen-specific IgA, could block parasite migration and the subsequent clinical sequelae. Recently, Harvie *et al* [42] demonstrated this by showing a lung-initiated, $CD4^+$ T-cell-dependent and IL-4- and STAT6-dependent, immune response in mice was sufficient to confer protection against reinfection with *N. brasiliensis*. Furthermore, Tsuji *et al* [43] has reported an intranasal vaccine against *Ascaris suum* that induced lung (antigen-specific IgA) and systemic (antigen-specific IgG and IgE) immune responses that were correlated with reduced larval migration through the lungs.

When given intranasally, ES alone appears to be highly immunogenic and able to induce a protective immune response. A characteristic feature of helminth infection is a Th2-dominated immune response, but stimulation of immunoregulatory cell populations, such as regulatory T cells and alternatively activated macrophages, is also common. Typically, Th1/17 immunity is blocked and productive effector responses are muted, allowing survival of the parasite in a modified Th2 environment.[37] Likely, ES contains

adjuvant properties that modulate the immune response to its vaccine relevant antigens. The ES used in this study was prepared by incubating live adult worms in PBS for 6 hours at 37 °C. As a result, it probably contains known TLR ligands, such as bacterial lipopeptide (TLR-2), lipopolysaccharide (TLR-4), flagellin (TLR-5), CpG DNA (TLR-9) and others, many of which are currently being investigated as adjuvants. Additionally, ES might contain intrinsic molecules able to act as adjuvants. *MacDonald et al* [29] recently produced a recombinant version of an *Onchocerca volvulus* excretory-secretory protein called ASP-1 and demonstrated its ability to augment Th1/Th2 responses. Others have shown the ability of ES proteins of adult *Nippostrongylus brasiliensis* and *Heligmosomoides polygyrus* to act as Th2 adjuvants to bystander proteins.[37, 48] Given the protective phenotype ES seemed to induce in our study, future studies should attempt to characterize the T helper response elicited by ES, as this could be helpful in future vaccine development.

As noted previously, IL-17 might have a protective role in vaccine-induced immunity to parasites. However, IL-17-producing $CD4^+$ T cells have also been implicated in tissue pathology associated with schistosomiasis, demonstrating this sub-population of T helper cells is also capable of driving adverse inflammatory reactions to helminth parasites. Rutitzky *et al* [44, 45] reported that IL-17 exacerbates pathology by recruiting neutrophils to the site of infection contributing to the development of severe schistosome egg-induced immunopathology. In our study, it is possible that high levels of IL-17, stimulated by c-di-AMP, were negatively modulating the protective effect of ES, and causing local inflammation of the small intestine. This is one explanation for why the ES plus adjuvant group developed comparable hookworm-associated weight loss and anemia to the PBS group (Fig. 12), despite having nearly half the worm burden of this group (24.4 ± 8.6

parasites [ES + c-di-AMP] vs. 45.8 ± 6.1 parasites [PBS])(Fig. 13). It was also curious that vaccination with c-di-AMP alone seemed to dampen antigen-specific lung IgA in response to primary infection (Fig. 11). We cannot provide a mechanism for this observation. Previously, *in vivo* experiments were conducted using c-di-AMP alone, in all these studies antigen-specific humoral or cellular immune responses were never detected (data not shown).

In addition to immunomodulatory properties, our study (as well as the work by Khan *et al* and *Bungiro et al*) provides convincing evidence of the availability of protective antigens within ES. In the Bungiro *et al* [46] study mentioned earlier, the authors also cloned and orally immunized hamsters with *Ancylostoma ceylanicum* excretory-secretory protein 2 (AceES-2), a highly immunoreactive protein found in ES. Following challenge infection, they showed a significant reduction in hookworm-associated anemia, but not weight loss (worm burden was not assessed). Interestingly, antigen-specific serum IgG responses were not detected in this group, suggesting serum parasite-specific responses are not absolutely necessary for protection from hookworm associated pathology. Additionally, AceFAR-1 (discussed above) and *Ancylostoma ceylanicum* Kunitz-type inhibitor (AceKI) are two other protective antigens that have been identified and isolated from ES.[40, 47] Conversely to AceES-2, Chu *et al* [47] showed partial protection against hookworm-associated growth delay without a measurable effect on anemia when recombinant AceKI was given subcutaneously (worm burden not assessed). Furthermore, the intranasal vaccine against *A. suum* (mentioned earlier) uses an ES antigen called As16, which the authors have recombinantly expressed in *Escherichia coli.*[43]

In conclusion, c-di-AMP appears to be a promising Th1/Th17 mucosal adjuvant with robust immune responses observed through intranasal, as well as sublingual vaccination. Despite these initial successes, however, c-di-AMP failed to significantly enhance the protective efficacy of excretory-secretory (ES) when tested in a vaccine model of hookworm pathogenesis. Still important was the discovery that ES antigens alone, given intranasally, confers a 73.8% reduction in adult worm burden, and appear to modulate the severity of hookworm-associated anemia and weight loss. We hypothesize that part of this success is due to inherent adjuvant properties of ES, which boost the immunogenicity of vaccine relevant antigens. In these regards, future studies should be conducted to identify both the protective antigens within ES and to characterize the adjuvant properties of ES. Once the relevant protective antigens in ES are identified and isolated, a well-defined profile of the immunomodulatory characteristics of crude ES will be helpful in selection of appropriate adjuvants. Taken together, the inability of c-di-AMP to enhance protective efficacy in a model of hookworm pathogenesis does not rule out its future utility in other models. If anything, these studies highlight the complexity of vaccine biology and underscore the importance of testing in established models of microbial pathogenesis.

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