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Vaccination with intestinal tract antigens does not induce protective immunity in a permissive model of filariasis

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HIGHLIGHTS

- *L. sigmodontis* intestinal antigens (GutAg) exhibit qualities of hidden antigens.
- Infected mice develop allergy associated immune responses to GutAg.
- Vaccinated mice develop GutAg specific IgG1 and IgG2A antibodies.
- Mice vaccinated with GutAg are not protected from challenge infection.

GRAPHICAL ABSTRACT

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ABSTRACT

Antigens obtained from the intestinal tract of filarial nematodes have been proposed as potential safe and effective vaccine candidates. Because they may be 'hidden' from the immune response during natural infection, yet accessible by antibodies induced by vaccination, intestinal antigens may have a low potential for eliciting allergic responses when vaccinating previously infected individuals. Despite prior promising data, vaccination with intestinal antigens has yet to be tested in a permissive model of filariasis. In this study we investigated the efficacy of vaccination with filarial intestinal antigens in the permissive *Litomosoides sigmodontis* BALB/c model of filariasis, and we evaluated the extent to which these antigens are recognized by the immune system during and after infection. Infected BALB/c mice developed lower IgG antibody responses to soluble intestinal antigens (GutAg) than to soluble antigens of whole worms (LsAg). Similarly, GutAg induced less proliferation and less production of IL-4 and IFN γ from splenocytes of infected mice than LsAg. In contrast to these differences, active infection resulted in equivalent levels of circulating GutAg-specific IgE and LsAg-specific IgE levels. Consistent with this, basophil activation, as assessed by flow cytometric staining of intracellular basophil IL-4 expression, was equivalent in response to GutAg and LsAg. Vaccination with GutAg adsorbed to CpG/alum induced GutAg specific IgG1 and IgG2A production, with GutAg specific IgG titers greater than 5-fold higher than those measured in previously infected animals. Despite this response to GutAg vaccination, vaccinated mice harbored similar parasite burdens 8 weeks post infection when compared to non-vaccinated controls. These studies demonstrate that soluble antigens obtained from the intestinal tracts of *L. sigmodontis* have some qualities of 'hidden' antigens, but they still sensitize mice to allergic reactions and fail to protect against future infection when given as a vaccine adsorbed to alum/CPG.

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1. Introduction¹

Filarial nematodes cause diseases such as African River Blindness and Elephantiasis, debilitating conditions that have severe economic, psychological, and social impacts on affected individuals (Babu et al., 2002, 2006, 2009). Similarly, zoonotic nematodes such as *Dirofilaria immitis* and *Haemonchus contortus* cause severe pathological sequelae in pets and livestock (Getachew et al., 2007; McCall et al., 2008). While there are medications to treat these infections, there are currently no vaccines to prevent them. Filarial vaccines could provide a cost effective tool for aiding in the control of these diseases.

Development of vaccines against parasitic filarial worms is complicated by two major issues. The first challenge is the lack of natural sterilizing immunity. In susceptible hosts, the immune responses that develop during infection are generally inadequate to clear the parasite. Indeed, filarial worms often survive for years within permissive hosts despite the development of robust anti-filarial immune responses (McCall et al., 2008). Furthermore, in many models, these immune responses provide little or no protection against future infection (Denham et al., 1972, 1984; Klei et al., 1990; Kowalski and Ash, 1975).

The second major challenge to filaria vaccine development is the risk that vaccination would elicit allergic responses in exposed individuals. Like other helminths, filariae typically induce a type 2 shifted immune response with production of high levels of parasite specific IgE (Diemert et al., 2012; Maizels et al., 2004). Thus, a vaccine comprised of parasite antigen has the potential to induce allergic reactions in individuals that are currently or have previously been infected with a filarial worm (Diemert et al., 2012).

The use of “hidden” antigens, antigens which are not typically targeted by the immune system during the normal course of infection, could potentially overcome both of these obstacles. Interestingly, prior studies have suggested that intestinal tracts of adult nematodes may contain exactly such antigens. This concept has been best demonstrated with *Haemonchus contortus*, a nematode infection of sheep. Although infection elicits relatively low antibody levels to the intestinal tract of *H. contortus*, vaccination with intestinal antigens induces substantial protection against challenge infection (Cachat et al., 2010; Smith, 1993). Antibodies from vaccinated animals were shown to disrupt the parasite's hemoglobinase activity, suggesting that protection occurs by inhibiting parasite metabolic activity (Ekoja and Smith, 2010). In addition to the work carried out using fractions of intestinal antigens, vaccination with individual digestive tract antigens has also shown protection in many animal models of helminth infection (Acosta et al., 2008; Chlichia et al., 2001, 2003; Dalton et al., 2003; Loukas et al., 2004; Pearson et al., 2009, 2010; Zhou et al., 2010).

In the field of filariasis, vaccination with intestinal antigens has only been tried with the mouse model of *D. immitis* infection. Although dogs infected with *D. immitis* were shown to produce only low level antibody responses to *D. immitis* intestinal antigens, presumably because these antigens are sequestered within the worm (McGonigle et al., 2001), vaccination of mice with *D. immitis* intestinal antigens induced high titer antibody responses to these antigens and resulted in increased protection against challenge infection (McGonigle et al., 2001). While exciting, this study was limited by the use of *D. immitis* challenge into mice. *D. immitis* survives for only a short period of time in mice and has never been shown to complete its lifecycle in this host (Abraham et al., 1988; Zielke, 1977). For this reason, it is not clear if the protection garnered through vaccination was simply the result of an acceler-

ated immune response to *D. immitis*, or if a truly novel protective immune response was produced through vaccination.

The purpose of this study was to investigate the vaccine potential of filarial intestinal antigens using the permissive *Litomosoides sigmodontis* BALB/c model of infection, the only murine model of filariasis in which infective-stage L3 larvae develop into adults that release microfilariae in fully immunocompetent mice (Hoffmann et al., 2000). *L. sigmodontis* is a good model for investigating vaccine approaches for filariasis because the immune responses which develop in infected mice closely mimic those observed in humans (Babayan et al., 2003; Hoffmann et al., 2000; Taylor et al., 2005). Additionally, enumeration of adult worms is reliably complete as over 95% of the adult worms can be recovered from the pleural cavity.

Specific goals of this study were to 1) determine the extent to which intestinal antigens of *L. sigmodontis* (GutAg) are immunologically “hidden” during infection of mice, and 2) to test whether vaccination with GutAg confers protection against challenge infection.

2. Materials and methods

2.1. Infection with *L. sigmodontis*

Infectious larvae of *L. sigmodontis* were obtained by performing a pleural lavage on jirds 4 days after exposure to mites harboring infectious stage larvae as previously described (Hubner et al., 2009). Briefly, jirds were euthanized with CO₂, the peritoneal cavity was opened, and a small incision (0.5 cm) made on the ventral aspect of the diaphragm. A transfer pipette was used to flush the pleural cavity repeatedly with 10 ml warmed (37 °C) RPMI (Mediatech Inc., Manassas, VA, USA). L3 larvae were then counted using a dissecting microscope, aspirated with a 20 µl micropipettor, and placed into a syringe. 40 L3 in 100 µl of RPMI were injected subcutaneously with a 22 gage needle into the nape of the neck of 5–8 week old female BALB/c mice.

2.2. Production of soluble antigen extracts from whole worms (LsAg) and worm intestines (GutAg)

Adult female worms were obtained from the pleural and peritoneal cavities of infected jirds. These were separated and maintained in worm culture media until dissection, up to four days using 12 well culture plates (5 worms in 3 ml/well). Worm culture medium consisted of RPMI (Mediatech Cat 15-040-CV), 1% glucose (Sigma-Aldrich Co. St. Louis, MO, USA), 100 I.U./ml Penicillin (Mediatech Inc., USA), 100 µg/ml streptomycin (Mediatech Inc., USA), and 25 mM HEPES (Mediatech Inc., USA).

Worm dissections were carried out in PBS in a Petri dish using fine tipped forceps and a dissecting microscope. Two sets of forceps were used to grasp the cuticle. Pressure was applied in opposing directions causing the cuticle to tear. The cuticle was then slowly peeled away from the internal structures. The intestinal tract was then separated from the two uterine tubes and placed in ice-cold PBS. The intestines were stored at –20 °C. Soluble GutAg (GutAg) and soluble antigen from adult worms (LsAg) were prepared by placing purified intestinal tracts or whole worms (male and female) into “D” tubes from BioPulverizer System 1 (MP Biomedicals Cat 6750–200) and run on a FastPrep-24 system (MP Biomedicals) at 4.0 M/s² for 20 s. After mechanical homogenization, samples were centrifuged at 16,100g for 1 min, supernatant saved, and the pellet resuspended in another 1 ml PBS. The fast-prep run, centrifugation, and removal of supernatant was repeated on the resuspended pellet. The saved supernatants were then combined, centrifuged at 15,000g for 5 min, and the final supernatant re-

¹ GutAg- Soluble fraction of antigens obtained from the digestive tract of adult *L. sigmodontis*; LsAg- Soluble fraction of antigens obtained from adult *L. sigmodontis* PI-Post Infection.

trieved and saved as either GutAg or LsAg. Protein concentrations were determined using the Pierce BCA Protein Assay Kit (Thermo Scientific Rockford, IL, USA).

2.3. Timepoints of immune responses to GutAg and LsAg in infected mice

As BALB/c mice clear adult worms by 20 weeks post infection (PI), immune responses were evaluated at 8 and 22 weeks post infection (PI) to determine antigen specific immune responses that develop in currently infected and previously infected animals. After CO₂ euthanasia, blood was obtained from mice by cardiac puncture, and plasma was obtained by separation with a plasma separator tube (BD Cat #365958).

2.4. Measurement of GutAg- and LsAg-specific total IgG, IgG1, IgG2a, and IgE antibodies

Prior to running IgE ELISAs, IgG antibodies were first adsorbed from the plasma by overnight incubation with GammaBind Plus Sepharose beads (GE Healthcare). Parasite specific antibody titers were measured by ELISA in 96 well half area EIA/RIA plates (Corning #3690). Wells were coated overnight at 4 °C with 50 µl of 2 µg/ml of either LsAg or GutAg in PBS. Wells were washed in washing buffer (PBS with 0.025% Tween 20 (Acros Organics) using an ELX 405 microplate washer (BioTek Instruments Winooski, VT, USA) and subsequently blocked with 200 µl 5% BSA (Sigma–Aldrich), 0.05% Tween 20 (Acros Organics) in PBS at 37 °C for two hours and washed. Fifty microlitre of serially diluted plasma was added and incubated overnight at 4 °C. Fifty microlitre detection antibody (purified goat anti mouse IgG conjugated to alkaline phosphatase (Mabtech Cincinnati, OH Cat 3310-04)) diluted 1:1000, 2 µg/ml biotinylated rat anti-mouse IgG1 (clone A85-1 BD Cat 50331), 2 µg/ml biotinylated rat anti mouse IgG2a (clone R19-15 BD Cat 550332), or 2 µg/ml biotinylated rat anti mouse IgE (Clone R35-118 BD Cat 553419) was added and incubated 1 h at 37 °C. Fifty microlitre Streptavidin conjugated alkaline phosphatase (Jackson ImmunoResearch #016-050-084) diluted 1:1000 was then added for IgE, IgG1 and IgG2a ELISAs and incubated 1 h at 37 °C. 4-nitrophenyl phosphate disodium salt hexahydrate (Sigma–Aldrich N9389) was then added at a concentration of 1 mg/ml in sodium carbonate buffer (KD Medical Cat CUS-0242) and incubated for 35 min at room temperature. OD values were read at 405 nm with a Victor³ microplate reader (Perkin Elmer). OD values of plasma dilutions were graphed using graphpad, and a four parameter curve was calculated for each mouse. This curve was used to determine the dilution of plasma from each mouse that would provide an OD of twice background.

2.5. Splenocyte isolation

After CO₂ euthanasia of mice, spleens were removed, placed on a 70 µm nylon cell strainer (BD Falcon #352350) in a small petri dish with 3 ml RPMI (Mediatech Inc) and crushed with a rubber tipped plunger from a 3 ml syringe. Splenocytes were washed through the cell strainer with 5 ml of RPMI. Cells were recovered and centrifuged at 400×g for 10 min. Supernatant was removed and cells were placed in 5 ml ACK lysing buffer (Quality Biological #118-156-721) for 5 min. Ten microlitre of IgE media (Iscove's DMEM (Mediatech #15-016-CV) + 10% fetal calf serum (Valley Biomedical #BS3032), 1% L-glutamine (Mediatech #25-005CI), 1% insulin-transferrin-selenium (Mediatech #25-800-CR), and 80 µg/ml Gentamicin (Quality Biological #120-098-661)) was added and the cells were centrifuged again at 400×g for 10 min. Supernatant was removed and the cells were resuspended in 10 ml IgE Media. The number of splenocytes was determined by using a Countess

automated cell counter system (Invitrogen C10227). Cells were then diluted with IgE media to a concentration of 2×10^6 cells/ml.

2.6. Cytokine assays

2×10^6 splenocytes were cultured in 1 ml aliquots in a 48 well plate at 37 °C for 3 days stimulated with either media, 15 µg GutAg, or 15 µg LsAg. After 72 h, plates were centrifuged at 400×g for 10 min and the supernatant was carefully removed and placed in microtiter tubes. These were centrifuged again at 400×g for 10 min to remove any residual splenocytes. The supernatant was saved and stored at –20 °C. Levels of IL-4, IL-10 and IFN-gamma were obtained with the eBioscience, READY SET GO ELISAS (Cat #88-7044-88, #88-7104-88, #88-7314-88) according to their included protocols.

2.7. Proliferation assay

200,000 splenocytes were cultured in a 100 µl aliquots in a 96 well plate at 37 °C for 72 h. Splenocytes were stimulated with either media, 15 µg/ml GutAg or 15 µg/ml LsAg. Cell proliferation was determined using a BRDU Chemiluminescence assay (Roche #11 669 915 001) according to the manufacturer's protocol. BRDU was diluted 1:100 with IgE media to make the BRDU labeling solution. Ten microlitre of BRDU labeling solution was added 72 h after stimulation of splenocytes, and the splenocytes were incubated at 37 °C for 17 more hours. Cells were centrifuged at 300×g for 10 min, and the supernatant was removed. A hair dryer was used to completely dry the plates containing splenocytes. Two hundred microlitre of FixDenat solution was added to each well for 30 min at room temperature. One hundred microlitre anti-BRDU peroxidase (POD) working solution was then added to each well, and plates were incubated for 90 min at room temperature. Wells were then washed three times with 200 µl of the included washing buffer with a soaking time of 1 min each. Finally, 100 µl of substrate solution was added and plates were shaken for 3 min. Luminescence was read with a Victor³ microplate reader (Perkin Elmer). Proliferation indexes were obtained by dividing the luminescence of antigen stimulated wells by those of the media stimulated wells.

2.8. Basophil stimulation

8 week infected BALB/c mice were euthanized with CO₂. Blood was obtained by cardiac puncture with a 22 gage needle, placed in heparinized tubes (Sarstedt #41.1503.105) and centrifuged at 400×g for 10 min. Plasma was removed and cells were washed with RPMI, centrifuged again, and resuspended in a volume of RPMI that corresponded with the amount of plasma removed. Cells were then divided into 100 µl aliquots and stimulated with addition of 100 µl RPMI alone as a control or with 100 µl of 4 µg/ml of GutAg or LsAg in RPMI to obtain final concentrations of 2 µg/ml. Samples were incubated at 37 °C in 5% CO₂. GolgiStop (BD) was added at a final concentration of 0.7 µl/ml after 1 h and cells were then incubated for an additional 2 h. Cells were then washed with PBS and centrifuged at 500×g for 8 min. Red blood cells were lysed and the remaining cells fixed with a whole blood lysing reagent kit (Beckman Coulter). Cells were washed twice with 2 ml PBS and blocked overnight at 4 °C with 1%BSA/PBS. Cells were stained with anti-IgE FITC (BD Clone R35-72), anti-CD4 PerCP (BD clone RM4-5), anti-CD45b/B220 PerCP (BD clone RA3-6B2), and incubated at 4 °C for 30 min. Cells were then permeabilized with 2 ml permeabilization/wash buffer (BD) for 15 min at room temperature in the dark. Cells were centrifuged as before, resuspended in 100 µl BD Perm/wash buffer, and stained with anti-IL-4 APC (BD Clone 11B11) for 30 min at 4 °C in the dark. Cells were washed twice more with PBS and resuspended in 200 µl PBS and

analyzed on an LSR II flow cytometer (BD). Analysis was conducted using BD FACS Diva software. Basophils were identified as IgE high and CD4/B220 low cells. All flow cytometry antibodies were titrated prior to use. Cut-offs for determination of activated basophils on the basis of IL-4 positivity were determined using a fluorescence minus one control.

2.9. Vaccination

GutAg was mixed with CpG (30 BP Thioate), Alum (Thermoscientific), and PBS to create a mixture containing 10 µg of GutAg, 25 µg CpG, 25 µg alum (.64 µl) per 100 µl of fluid. This mixture was shaken at room temperature for 30 min. One hundred microlitre (10 µg of antigen) was injected IP into female BALB/c mice for a total of 3 times at 3 week intervals. Control mice received similar injections of PBS with the same concentration of CpG and Alum, but without antigen. Two hundred microlitre of blood was drawn retro-orbitally 2 weeks after each injection for determination of parasite specific antibody levels. Mice were infected with 40 L3s subcutaneously 3 weeks after their last vaccination.

2.10. Determination of microfilaria levels and adult worm numbers

8 weeks post infection, mice were euthanized with CO₂. Blood was drawn via cardiac puncture with a 22 gage needle. Thirty microlitre of blood was then transferred into 1 ml of ACK lysing Buffer (Quality Biological Gaithersburg, MD, USA) and stored at room temperature for 1–3 days for microfilarial counts. This was centrifuged at 400×g for 10 min, and the supernatant aspirated until about 50 µl remained. The pellet was resuspended in the remaining volume and then placed onto a slide and covered with a cover slip. All microfilariae on the slide were counted.

After cardiac puncture, the peritoneal cavity was opened and all aspects of it visually inspected for adult worms with use of a dissecting probe. A small incision was then made in the ventral aspect of the diaphragm and a large tip transfer pipette was used to lavage the pleural cavity with 10 ml warmed RPMI (Mediatech Inc., USA). The fluid and worms obtained from the lavage were transferred to

a petri dish. After lavage, the diaphragm was cut away and inspected for adult worms along with the rest of the pleural cavity. All remaining adult worms that were found were transferred to a Petri dish using a dissecting probe. Worms were separated using fine tipped forceps, sexed, and counted with the aid of a dissecting microscope.

2.11. Animal use

Mice were housed at the Uniformed Services University of the Health Sciences (USUHS) laboratory animal facility and had access to food and water *ad libitum*. All research was conducted under protocols approved by the USUHS Institutional Animal Care and Use Committee.

2.12. Statistical analysis

Immune responses to GutAg and LsAg in the same mice were compared using the non-parametric Wilcoxon Matched Pairs test. The non-paired, non-parametric Mann–Whitney test was used for immunological and worm burden comparisons between vaccinated and unvaccinated mice. All analyses were conducted using GraphPad Prism software (GraphPad, LaJolla).

3. Results

3.1. Microdissection enables purification of *L. sigmodontis* intestinal tracts

To determine whether dissection of adult female *L. sigmodontis* results in pure preparations of filarial intestines, components of worms obtained during dissection were stained with hematoxylin and eosin and evaluated histologically. As seen in Fig. 1, microdissection enables purification of intestinal tracts from adult female *L. sigmodontis* worms.

Fig. 1. Gross anatomy and histology of adult female *Litomosoides sigmodontis*. Gross anatomy as seen from the dissecting scope prior to (A) and after (B) mechanical rupture of the cuticle (25×). Longitudinal (C) and Cross sectional (D) views of adult female worm prior to dissection (H&E, 400×). (E) Purified intestinal tract from adult female worm with characteristic single layer of cuboidal cells. The intestinal tract is viewed after purification repeatedly folded back on itself (H&E, 400×). (Black arrow = Intestinal tract, Asterisk = Uterine Tube, Blue triangle = Cuticle). (For interpretation of color in this Figure, the reader is referred to the web version of this article).

Fig. 2. Antibody titers to soluble antigens prepared from entire *L. sigmodontis* worms (LsAg) or their digestive tracts (GutAg) in currently infected (8 week) or previously infected (22 week) BALB/c mice. Shown are composite results from two independent experiments, each of which had 5 animals per group. (* = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$).

3.2. Infected mice display lower immune responses to GutAg than to LsAg

To determine the extent to which intestinal antigens are “hidden” from the immune system during *L. sigmodontis* infection, humoral and cellular responses toward parasite antigens were measured in mice with active infections (8 weeks PI) and previous infections (22 weeks PI). IgG antibody titers to either the soluble fraction of the entire worm (LsAg), or the soluble fraction of the worm digestive tract (GutAg) were determined by ELISA. Originally, we hypothesized that currently infected mice would display relatively weak adaptive immune responses to GutAg compared to LsAg because of sequestration of these antigens from the host immune system. We also hypothesized that 22 weeks PI, after worm death and degradation had occurred, that these titers would increase as the immune system may have had an opportunity to interact with antigens from the worm digestive tracts. However, we found that GutAg specific immune responses were low both during and after infection (Fig. 2).

As seen in Fig. 2A, mice developed high titers of LsAg specific IgG antibodies during active infection which dropped after worm clearance had occurred. At both time points, these titers were significantly greater than GutAg specific IgG titers. In contrast to IgG, LsAg and GutAg IgE titers were similar during active infection. In previously infected mice, however, GutAg-specific IgE titers were significantly lower than those against LsAg (Fig. 2B).

Cellular responses were also generally lower against GutAg than LsAg. At both time points evaluated, splenocyte proliferation and production of IL-4 and IFN- γ were lower in response to GutAg than to LsAg (Fig. 3A–C). No differences were observed with respect to splenocyte IL-10 production (Fig. 3D).

3.3. GutAg induces basophil activation in *L. sigmodontis*-infected mice

To assess the allergic potential of GutAg, we assessed the extent to which GutAg activates basophils during infection. As basophils are allergy effector cells that are activated by cross-linking of surface-bound IgE antibodies, basophil activation tests are immunological markers for allergic responses (Hausmann et al., 2009). To measure basophil activation, peripheral blood cells were obtained from 8 week infected mice, stimulated with either GutAg or LsAg, and then evaluated for the production of IL-4 by flow cytometry. As seen in Fig. 4, GutAg stimulation induced equivalent activation of basophils as LsAg (mean % IL-4 + basophils = 33% after media stimulation vs. 60% after both GutAg and LsAg stimulation). This result, along with the development of GutAg-specific IgE antibodies, suggests that vaccination with the soluble fraction of filarial

intestinal antigens may not circumvent the issue of allergic responses in exposed individuals.

3.4. Vaccination results in increased anti-Gut IgG production, and balanced IgG1/IgG2A responses

Since antibody responses to GutAg remained fairly low throughout infection, we tested whether vaccination with GutAg could induce robust anti-GutAg antibody production. Because prior filaria vaccine studies have shown that both type 1 and type 2 responses can have protective effects against filaria infection (Abraham et al., 2001; Hartmann et al., 2006), we chose to use a combination of CpG and Alum as adjuvants to induce a broad-based immune response. As seen in Fig. 5A, mice vaccinated three times with 10 μ g GutAg developed anti-GutAg IgG titers over five fold higher than previously infected mice. Furthermore, vaccinated mice produced a more balanced ratio of IgG subtypes, including both IgG1 and IgG2A. Conversely, previously infected mice produced almost exclusively the TH₂-associated IgG1 subtype which provides very little complement fixation (Klaus et al., 1979; Neuberger and Rajewsky, 1981) (Fig. 5B).

3.5. Vaccination with gut antigen fails to protect against challenge infection

In order to determine the efficacy of a vaccine comprised of GutAg, mice were vaccinated three times with either GutAg adsorbed to CPG/alum or saline with CpG/alum. Three weeks after the last vaccination, mice were infected with 40 infectious larvae. Eight weeks after infection, adult and microfilaria parasite burdens were determined. No protection was garnered from vaccination with GutAg, as vaccinated mice harbored 19.4 worms on average, whereas non-vaccinated mice only harbored an average of 18.3 worms (Fig. 6A). Microfilaria burdens in vaccinated animals were also similar to those of non-vaccinated animals (Fig. 6B).

4. Discussion

Filarial worms cause diverse and debilitating diseases throughout much of the tropics, and a vaccine would aid in both control and possible elimination of these diseases. Vaccine development in filariasis is complicated by a lack of protective immunity during infection and by possible allergic responses to worm antigens after exposure (Denham et al., 1972, 1984; Diemert et al., 2012; Klei et al., 1990; Kowalski and Ash, 1975). Because the digestive tract is sequestered within the worm, and thus may be immunologically “hidden” during the course of normal infection, vaccination against intestinal antigens has been proposed as a possible means of

Fig. 3. Splenocyte responses in 8 week and 22 week infected BALB/c mice when cultured for 3 days at 2 million cells/ml and stimulated with 15 μ g/ml LsAg or GutAg. (A) Splenocyte proliferation as measured by incorporation of BRDU. (B–D) Cytokine levels in the supernatant of splenocytes as measured by ELISA. Shown are composite results from two independent experiments, each of which had 5 animals per group. (* = $p < 0.05$, ** = $p < 0.01$).

overcoming these obstacles (Getachew et al., 2007; Hotez et al., 2010; McGonigle et al., 2001).

Studies using the nematode models of *Haemonchus* and *Diriofilaria* have shown that infection elicits weak antibody responses to parasite intestinal antigens and that vaccination with intestinal antigens induces high antibody responses and protection against infection (McGonigle et al., 2001; Smith, 1993). However, neither of the models used to date have been ideal vaccine models for human filariasis. *Haemonchus* is a non-filarial nematode parasite of the intestinal tract, and the *Diriofilaria* experiments utilized a non-permissive host to determine vaccine efficacy. Thus, in this study we used the permissive *L. sigmodontis* BALB/c model of filariasis to investigate the vaccine potential of filarial intestinal antigens.

Our studies demonstrate that infection, despite inducing lower IgG and cellular responses to GutAg, results in allergic sensitization against filaria intestinal antigens. Further, vaccination with these intestinal antigens fails to protect against challenge infection despite the development of high GutAg-specific antibody titers.

Our first set of experiments evaluated the immunological responses to GutAg in actively infected and previously exposed mice to determine the extent to which intestinal antigens are immunologically 'hidden' during infection. Similar to other models, the IgG responses that developed to GutAg were relatively low. Furthermore, while splenocytes of infected mice responded to GutAg, cytokine production and proliferation of splenocytes were generally lower to these antigens than to LsAg. Overall, the lower humoral and cytokine responses to GutAg when compared to LsAg suggest that a large portion of the antigens contained within the digestive tract of the worm may remain hidden from the immune system throughout infection. It is important to note, though, that the lower antibody and cellular immune responses observed towards GutAg may have also been due to a quantitative phenomenon whereby the more antigenically diverse LsAg induced greater immune responses than GutAg because of a higher number of distinct antigens present in the mixture.

In terms of allergic sensitization, our study clearly shows that GutAg is not immunologically hidden. Both actively infected and previously infected mice produce GutAg-specific IgE. This IgE is functional, as basophil activation assays demonstrated that GutAg was as effective as LsAg in activating basophils of infected mice. These results suggest that even though infection results in lower cellular and IgG responses to GutAg than to LsAg, prior infection still results in allergic sensitization to at least some intestinal antigens. Consequently, future studies investigating vaccine potential of individual intestinal antigens will have to ensure prior infection does not result in allergic sensitization to those antigens.

Vaccination of mice with GutAg adsorbed to CpG and Alum resulted in high titers of GutAg-specific IgG1 and IgG2a antibodies. Despite this, GutAg vaccination failed to protect against infection. This either occurred because the titer of antibodies induced was not high enough to enable protection, or because GutAg specific antibodies simply do not have the capability to protect against infection in this model. One can speculate that GutAg specific antibodies are unable to interact with their specific antigens in this model because of either a lack of antibody ingestion by the parasite or because antibodies are degraded by proteases within the worm digestive tract.

Despite the negative results of this study, we suspect that some intestinal tract antigens do have the potential to function as effective filaria vaccine candidates. First, there is direct evidence that some antigens present in the intestinal tracts of filariae can serve as effective vaccine candidates. Preliminary evidence from a proteomic study we are conducting demonstrates that glutathione-S-transferase and thioredoxin peroxidase are present within the digestive tract of filarial worms. As both of these antigens induce protection when used to vaccinate jirds against filariasis (Dabir et al., 2008; Rathaur et al., 2008; Vanam et al., 2009), it is clear that some antigens located in filarial intestinal tracts can function as effective vaccine antigens. Second, it is unlikely that the lack of protection in this model is due to a lack of worm antibody ingestion as *L. sigmodontis* worms ingest blood between 28 and 56 days

Fig. 4. Basophil responses to LsAg or GutAg in 8 week infected mice. (A) Cells were initially gated by forward and side scatter characteristics (left panel). Basophils were identified by high expression of IgE and low expression of CD4 and B220 (Mid Panel). IL-4 expression in unstimulated (right top) and stimulated basophils (right bottom). (B) Percentages of basophils positive for IL-4 after stimulation with 2 μ g/ml LsAg or GutAg. Shown are composite results from two independent experiments, one which had 4 animals per group and one which had 5 animals per group.

Fig. 5. Anti-GutAg antibody titers in 22 week infected mice and mice vaccinated with GutAg + CpG/Alum as measured by ELISA. (A) Total anti-GutAg IgG titers. (B) IgG1 and IgG2A titers. Shown are composite results from two independent experiments, each of which had 4–5 animals per group. (***) = $p < 0.001$.

PI (Attout et al., 2005), providing some opportunity for antibody ingestion. It is also likely that *L. sigmodontis* ingests antibodies directly from the pleural fluid. Thus, the most likely reason for the lack of protection observed in this study is that no protective antigen within the intestinal fraction was given in a high enough dose to elicit a protective immune response.

Proof of concept demonstrating that individual antigens can function as protective vaccines, even when a mixture of antigens

does not, has been shown with adult filarial worm antigens. In permissive rodent models, vaccination with soluble extracts of adult filarial worms generally does not confer substantial protection. Vaccination of jirds with the soluble fraction of adult *Brugia pahangi* does not elicit any significant decrease in adult worm burden, and vaccination with *Brugia malayi* adult somatic antigen confers only 25% protection against challenge infection (Klei et al., 1982; Vedi et al., 2008). While there are no published reports on the

Fig. 6. Adult worm (A) and microfilaria (B) parasite burden of vaccinated and control mice 8 weeks after infection with *L. sigmodontis*. Vaccinated mice were given 3 IP injections of GutAg + CpG/Alum prior to infection; control mice were given injections of PBS with CpG/Alum. Shown are composite results from two independent experiments, each of which had 4–5 animals per group.

use of LsAg as a vaccine in mice, vaccination of albino rats with LsAg results in no protection against infective larvae or adult stages (Mehta et al., 1981). Despite the lack of efficacy of adult worm antigen extracts, effective vaccination with specific antigens contained in these mixtures has been demonstrated numerous times (Babayan et al., 2012; Li et al., 1993; Vanam et al., 2009; Vedi et al., 2008; Veerapathran et al., 2009). Consequently, the negative results of this study do not rule out the possibility that specific intestinal antigens may be able to confer substantial protection even though a homogenate of intestinal antigens does not.

In conclusion, in this study we demonstrated that the soluble fraction of filarial intestinal antigens elicits allergic sensitization during infection and fails to protect when used as a vaccine adsorbed to alum and CpG. While disappointing in terms of filaria vaccine development, these findings do not rule out the possible existence of individual intestinal antigens that could be both protective and safe. One of the major limitations of this study was obtaining enough GutAg for all of the studies involved. Indeed, it took a single investigator (C.P.M.) more than 350 h to dissect the 3000+ worms used to obtain enough purified filarial intestinal tracts for the experiments done in this study. As such, increasing the concentrations of GutAg used for vaccination or testing various fractions of GutAg are likely not feasible approaches for further investigating the potential of filarial intestinal antigens as vaccine candidates. Consequently, we are currently conducting a proteomic analysis to identify antigens present only within the intestines of filarial worms. Recombinant expression of such antigens or use of DNA vaccines can then be tried in future studies to explore the potential of specific intestinal antigens to function as effective vaccine candidates.

5. Conflict of interest

None of the authors have any commercial or other associations that pose a conflict of interest with the submitted manuscript

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