Protection against Intestinal Amebiasis by a Recombinant Vaccine Is Transferable by T Cells and Mediated by Gamma Interferon

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We have previously shown that vaccination with purified Entamoeba histolytica Gal/GalNAc lectin or recombinant subunits can protect mice from intestinal amebiasis upon intracecal challenge. In this study, we demonstrated with adoptive-transfer experiments that this lectin vaccine protection is mediated by T cells but not serum. The cell-mediated immune (CMI) response was characterized by significant gamma interferon (IFN-γ) interleukin 12 (IL-12), IL-2, IL-10, and IL-17 production. To move toward a human vaccine, we switched to a recombinant protein and tested a range of adjuvants and routes appropriate for humans. We found that subcutaneous delivery of LecA with IDRI’s adjuvant system EN014 elicited a potent Th1-type CMI profile and provided significant protection, as measured by culture negativity (79% efficacy); intranasal immunization with cholera toxin provided 56% efficacy; and alum induced a Th2-type response that protected 62 to 68% of mice. Several antibody and CMI cytokine responses were examined for correlates of protection, and prechallenge IFN-γ+ or IFN-γ-, IL-2-, and tumor necrosis factor alpha-triple-positive CD4 cells in blood were statistically associated with protection. To test the role of IFN-γ in LecA-mediated protection, we neutralized IFN-γ in LecA-immunized mice and found that it abrogated the protection conferred by vaccination. These data demonstrate that CMI for vaccine protection from intestinal amebiasis and reveal an important role for IFN-γ, even in the setting of alum.

The enteric protozoan parasite Entamoeba histolytica is the causal agent of amebic dysentery and liver abscess that affects millions of people worldwide. Bangladeshi children experience a 40% annual incidence of E. histolytica infection (24), and evidence of prior E. histolytica infection can be detected in 8.4% of the general population in Mexico (6). Despite the availability of effective antibiotics, the World Health Organization estimates that up to 100,000 deaths occur annually, highlighting the need for alternate approaches to control amebiasis. One approach is to develop a vaccine to prevent intestinal infection (26).

Several vaccine candidates for amebiasis have been proposed (48), including the serine-rich E. histolytica protein, peroxidoxin, the EhCP112 molecule, and the galactose/N-acetyl-d-galactosamine-inhibitable lectin (Gal/GalNAc lectin). A significant body of work has focused on the latter: vaccination with either parasite-purified Gal/GalNAc lectin (10, 29, 32, 38, 40) or recombinant lectin subunits has provided protection in rodent models against amebic liver abscess and amebic colitis (29, 37, 46, 47, 53). Although these results are encouraging, two limitations remain. First, in most of these vaccine studies, the adjuvants and delivery routes are not compatible with eventual use in humans. Second, the mechanisms of amebiasis vaccine-mediated protection are still not fully understood. For instance, in the intestinal model, there was an association between the presence of an antiparasite lectin fecal immunoglobulin A (IgA) response and subsequent protection, but the association did not extend to the recombinant antigen, and fecal IgA-negative mice remained statistically protected, suggesting that other immune mechanisms exist (29). Indeed, there is increasing evidence for a role of cell-mediated immunity (CMI) in protection from intestinal amebiasis (14, 20, 28). We have found that gamma interferon (IFN-γ), the canonical Th1 cytokine, can clear intestinal amebic infection in CBA mice (21). In this study, we demonstrate for the first time that CMI plays a critical role in lectin-elicited protective immunity to intestinal amebic infection. A variety of vaccine adjuvants and delivery routes were tested for their effectiveness in protection and in eliciting CMI, and the tests demonstrated a clear role for CMI and IFN-γ in lectin-based vaccine protection.

MATERIALS AND METHODS

Antigens and immunizations. Groups of 3- to 4-week-old male CBA/J mice were obtained from the Jackson Laboratory to start immunization studies and were maintained under specific-pathogen-free conditions at the University of Virginia. The production of the two antigens used in trials 1 to 4, native E. histolytica Gal/GalNAc lectin and the recombinant subunit “LecA” (amino acids 578 to 1154 of the lectin heavy chain), have been described previously (29). For trials 5 to 7, LecA was purified using immobilized metal affinity chromatography (IMAC). Briefly, the cells were lysed by sonication and treated with 0.7% NP-40 and 0.7% sodium deoxycholate (Sigma-Aldrich). The isolated inclusion bodies were washed with 0.05% Triton X-100 and denatured in 8 M urea containing 50 mM dithiothreitol. To renature the proteins, the solution was diluted 10-fold and the pH was gradually titrated from 11 to 7.5. IMAC purification was performed...
with a HisPipel 16/10 Fast Flow column (GE Healthcare), which eluted LecA with 300 mM imidazole (Sigma-Aldrich).

Mice were immunized with either native Gal/GalNAc lectin or recombinant LecA with the various regimens specified in Table 1. Briefly, our original proof-of-principle regimen, consisting of three intranasal immunizations and one intraperitoneal immunization at 2-week intervals (29), was administered in trials 1 to 5 (Table 1). In trial 5, LecA was formulated with the following adjuvant(s): IDRI adjuvant system EM014, which consisted of 1 mg/ml of synthetic lipid A, a TLR4 agonist, mixed with 1.25 mg/ml CpG of mouse origin in a stable emulsion; complete (CFA) and incomplete (IFA) Freund adjuvant (Gibco); alum (Rehydragel LV; Reheis, Inc.); or cholera toxin (CT; Sigma-Aldrich). In trial 7, LecA was formulated in the IDRI adjuvant system Al001 (synthetic lipid A bound to the alum) or in LecA alone at 4-week intervals. Formulations with different adjuvants were made as follows: for alum and Freund adjuvants, 1 part of adjuvant was mixed with 1 part of antigen (vol/vol) in a total volume of 100 μl. For EM014 and Al001, 20 μg of LecA was formulated with 20 μg of adjuvant and brought up to 100 μl with phosphate-buffered saline (PBS). For CT, 20 μg of LecA was mixed with 2 μg of adjuvant. Immunizations were administered subcutaneously (EM014, alum, Al001, CFA, and IFA), intranasally (CT) after light isofluorane anesthesia, or intrarectally (CT) after light isofluorane anesthesia, or intraperitoneally (s.c., subcutaneous; i.r., intrarectal).

For single-cell suspensions after lysis of red blood cells, splenocytes (2 × 10^6) were harvested within 1 to 2 weeks after the final immunization and processed to assess the antigen-specific antibody responses, tail vein sera and stool samples were obtained within 1 to 2 weeks after the final immunization (week 7 or 8). The preparation of stool suspensions was described previously (29). Both serum and fecal preparations were stored at desoxysylate (29), both serum and fecal preparations were stored at 4°C overnight and then blocked with 2% bovine serum albumin in PBS for 1 h at 37°C. After three washes with PBS-Tween buffer, the plates were incubated with fecal samples (100 μl per well) diluted 1:4 in PBS-Tween containing 2% bovine serum albumin. Horseradish peroxidase-conjugated goat anti-mouse IgA (Southern Biotechnology Associates) diluted 1:5,000 was added sequentially. Antigen-specific antibodies were detected with 100 μl per well of tetramethylbenzidine microwell peroxidase substrate (Kirkegaard & Perry Laboratories). The reaction was stopped with 100 μl per well of sulfuric acid stop solution, and the absorbance was read at 450 nm. The IgG titer was determined by end-point dilution. The serum IgG subtypes were determined by the ELISA described above, except that 1:10,000-diluted horseradish peroxidase-conjugated goat anti-mouse IgG1 and IgG2a (Southern Biotechnology Associates) were used as secondary antibodies. IgG1 and IgG2a units were determined by use of a standard curve.

To assess antigen-specific CMI, spleens from three or four mice per group were harvested within 1 to 2 weeks after the final immunization and processed for single-cell suspensions after lysis of red blood cells. Splenocytes (2 × 10^6) were cultured in 96-well plates (Costar) in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, and 50 μM beta-mercaptoethanol. Cells were stimulated with 2 μg/ml lectin or LecA for antigen-specific CMI. To analyze the peripheral blood mononuclear cell (PBMC) response, each individual mouse was bled via the tail vein and about 250 μl whole blood was treated with 0.84% NH4Cl buffer to lyse the red blood cells. After two washes, 5 × 10^5 PBMCs were cultured in 24-well plates and stimulated with 2 μg/ml of LecA. One microgram of concanavalin A (ConA) was set up as a positive control, and medium alone was used as a negative control in the CMI studies. Supernatants were collected after 48 h and analyzed by a multiplex suspension array system using Luminex beads (Bio-Rad Laboratories), which included the following cytokines: Th1 (IFN-γ, interleukin-2 [IL-2], IL-12p35, and tumor necrosis factor alpha [TNF-α]), Th2 (IL-4, IL-5, and IL-13), Th17 (IL-17), and IL-10. Samples were run without dilution according to the manufacturer’s protocol and measured in picograms per milliliter of supernatant.

Intracellular cytokine staining of PBMCs. A total of 5 × 10^5 PBMCs were cultured in complete Dulbecco’s modified Eagle medium (Invitrogen) and stimulated with 10 μg/ml of LecA for 22 h. Anti-CD28 and Golgiplug (BD Biosciences) were added in the last 12 h of stimulation. Phorbol myristyl acetate (50 ng/ml) and ionomycin (800 ng/ml) (Sigma-Aldrich) were used as positive controls to assess the cytokine production capacity for each sample.

For intracellular staining, PBMCs were washed and incubated with CD16/CD32 monoclonal antibody (MAb; BD Biosciences) to block Fc binding, followed by surface staining with CD4-phycocerythrin (BD Biosciences). Cells were permeabilized and stained with IFN-γ-fluorescein isothiocyanate (BD Biosciences), IL-2-peridinin chlorophyll protein-Cy5.5 (eBioscience), and TNF-α-

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**Table 1. Vaccine regimens**

<table>
<thead>
<tr>
<th>Trial</th>
<th>Antigen</th>
<th>No. of mice</th>
<th>Scheme</th>
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<tr>
<td></td>
<td></td>
<td>Wk 0</td>
<td>Wk 2</td>
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<tr>
<td>1</td>
<td>Lectin</td>
<td>16</td>
<td>CT (i.n.)</td>
</tr>
<tr>
<td>2</td>
<td>Lectin</td>
<td>20</td>
<td>CT (i.n.)</td>
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<tr>
<td>3</td>
<td>LecA</td>
<td>16</td>
<td>CT (i.n.)</td>
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<tr>
<td>4</td>
<td>LecA</td>
<td>16</td>
<td>CT (i.n.)</td>
</tr>
<tr>
<td>5</td>
<td>LecA</td>
<td>20</td>
<td>CT (i.n.)</td>
</tr>
<tr>
<td></td>
<td>EM014</td>
<td>20</td>
<td>EM014 (s.c.)</td>
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<tr>
<td></td>
<td>Alum</td>
<td>20</td>
<td>Alum (s.c.)</td>
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<tr>
<td></td>
<td>CFA</td>
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<td>CFA (s.c.)</td>
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<td></td>
<td>CT</td>
<td>20</td>
<td>CT (i.n.)</td>
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<tr>
<td></td>
<td>CT</td>
<td>20</td>
<td>CT (i.r.)</td>
</tr>
<tr>
<td>6</td>
<td>LecA</td>
<td>60</td>
<td>EM014 (s.c.)</td>
</tr>
<tr>
<td></td>
<td>Alum</td>
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<td>Alum (s.c.)</td>
</tr>
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</tr>
<tr>
<td></td>
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<td>40</td>
<td>No adjuvant</td>
</tr>
</tbody>
</table>

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a In trials 1 to 3, 10 μg of antigen was delivered per dose; trials 4 to 7 used 20 μg of antigen per dose.

b Sham-immunized mice were administered identical regimens of PBS with adjuvants in each trial.

c Schemes are as shown (route). EM014, synthetic lipid A and CpG mixed with stable emulsion; Al001, synthetic lipid A bound to alum; i.n., intranasal; i.r., intrarectal; s.c., subcutaneous.

d In trials 1 to 6, the last dose of vaccine was given in week 6; in trial 7, the last dose was given in week 8.
allophycocyanin (eBioscience) and run on a BD FACS Calibur (BD Biosciences). The data were analyzed with FlowJo software.

Parasites and intracecal inoculation. Immunoized CBA mice were challenged intracecally with E. histolytica trophozoites 4 weeks after the final boost. Trophozoites were originally derived from laboratory strain HM1:IMSS (ATCC) and were cultured in trypsin-yeast-iron (TYI-S-33) medium supplemented with 2% Diamond vitamins (JRH Biosciences), 13% heat-inactivated bovine serum (Sigma-Aldrich), and 100 U/ml penicillin plus 100 µg/ml streptomycin. For all intracecal inoculations, trophozoites were grown to the log phase and 2 × 10⁶ trophozoites in 150 µl were injected intracecally after laparotomy as described previously (30).

Evaluation of amebic infection. Mice were sacrificed 7 to 11 days after challenge. Half of the cecum was placed in Bouin’s fixative (Sigma-Aldrich) and processed for hematoxylin and eosin staining. Pathology was scored in a blinded manner as described before (30), based on the number of visible ameba and degree of inflammation (scale of 0 to 5). The other half of each cecum was rinsed with PBS and stored in RNAlater (Ambion) for quantitative reverse transcription-PCR analysis. Three hundred microliters of cecal rinse was cultured in complete TYI-S-33 medium with supplemental antibiotics for 5 days at 37°C.

Adoptive-transfer experiments. Mice were immunized with native Gal/GalNAc lectin per trial 2 (Table 1). Two weeks after the final immunization, lectin-immunized and PBS-treated mice were bled for sera and sacrificed to obtain splenocytes and mesenteric lymph node cells. Pooled single-cell suspensions from either lectin- or PBS-immunized donors were enriched for CD3⁺ T-cell populations by negative selection (EasySep mouse T-cell enrichment kit; StemCell Technologies). To determine the efficiency of T-cell enrichment, the donor lymphocytes were stained with phycoerythrin-conjugated anti-B220 (BD Biosciences) and fluorescein isothiocyanate-conjugated anti-CD3 (BD Biosciences) antibodies by flow cytometry before and after T-cell enrichment. For T-cell transfer, 4 × 10⁶ enriched donor cells were injected intravenously into naive CBA mouse recipients. For serum transfer, recipients received 400 µl serum intraperitoneally. Both T-cell and serum recipients were challenged 3 days after transfer and sacrificed 10 days postchallenge. The lectin-specific CMI in recipients was assessed 3 days after transfer to confirm successful reconstitution. In some MAb experiments, monoclonal IgG or IgA was purified from cell bags by the UVA Hybridoma Center and administered intraperitoneally or intracecally at the doses indicated in Results. Some of the serum and MAb transfer experiments utilized C3H/HeJ mice purchased from the Jackson Laboratory.

Cytokine depletion in vivo. For CBA mice that had completed four doses of vaccination, 1 mg of anti-IL-4 (11B11) or anti-IFN-γ (XMG1.2) MAb or a control rat IgG (Lampire Bio lab) was administered intraperitoneally every 3 days, from 3 days before to 6 days after challenge (four doses total). The mice were sacrificed at day 8 for evaluation of the infection and vaccine efficacy.

Statistical analysis. All statistical analyses were performed using GraphPad Prism for Windows, version 5.0 (GraphPad Software). Student's t tests were used to compare values for vaccinated and control groups or protected and unprotected individuals. Two-tailed P values of <0.05 were considered significant.

RESULTS

Gal/GalNAc lectin-mediated protection is transferable by T cells but not by serum. We have previously reported that purified Gal/GalNAc lectin protects the C3H mouse from intestinal amebiasis (29). In this study, we first confirmed the effectiveness of this vaccine in male CBA mice, which offer a more-efficient model since they exhibit a slightly greater susceptibility to infection (3). As expected, native lectin protected CBA mice from amebic infection, as measured by cecal culture, with 71% efficacy (P of <0.001 in comparison with controls) (Fig. 1A). As in previous work, histopathology mirrored the culture results, and there were no significant differences in inflammation scores between infected mice from the sham group and those from the vaccinated group for this or any of the trials (data not shown).

To determine the mechanisms underlying Gal/GalNAc lectin-mediated protection, adoptive-transfer experiments were performed to discern whether Gal/GalNAc lectin-induced protection could be transferred by T cells or serum antibodies. Lymphocytes and sera were obtained from lectin-immunized or PBS-treated mice. The donor lymphocytes were enriched for CD3⁺ T cells from 33% to 95% per the methods described in the legend to Fig. 1B. Purified T cells or sera from lectin-vaccinated or PBS-treated donors were intravenously transferred to naive recipients. Successful transfer was confirmed by documenting the transfer of lectin-specific CMI and serum responses (e.g., IFN-γ production, 298 ± 88 pg/ml in splenocytes of vaccine T-cell recipients versus 7 ± 2 pg/ml in splenocytes from sham T-cell recipients; P < 0.05). Three days after transfer, recipients of either T cells or sera were challenged with E. histolytica and sacrificed at day 10 to assess protection. Mice that received lectin-immunized donor T cells exhibited a lower culture positivity rate than the sham-immunized donor T-cell recipients (Fig. 1C). In contrast, we did not observe protection by passive immunization with serum (Fig. 1C). Of note, recipients of sera from sham-immunized mice exhibited a surprisingly low infection rate, which appeared to be real since we obtained an identical result previously in C3H/HeJ mice (44% culture positivity in immune serum recipients versus 21% in sham serum recipients [n = 16 and 14 mice, respectively]; the P value was not significant), and may reflect a protective effect of nonspecific immunoglobulin or other serum components. In support of the lack of protection by immune sera, we were unable to confer protection using antilectin MAb: transfer of clones 7F4, 1G7, and H85, with 0.33 mg of each per mouse, yielded protection for 8/15 culture-positive mice versus 8/20 mice treated with control IgG (data not shown). Similarly, administration of 500 µg (intraperitoneally) plus 340 µg (intracecally) of a multimeric antilectin monoclonal IgA did not confer protection (observed for 9/13 culture-positive mice that received antilectin IgA versus 6/9 control IgA recipients; data not shown). These adoptive-transfer results indicate that lectin-specific T cells were sufficient for vaccine protection.

Antibody and CMI induced by Gal/GalNAc lectin with the CT-CFA regimen. Lectin vaccination (trial 1) elicited high levels of antigen-specific fecal IgA (Fig. 2A) and serum IgG (data not shown), yet there was no correlation of fecal IgA level with protection (Fig. 2A) (29). Since protection was transferred from an immunized to a naïve mouse by T cells, we focused on the cell-mediated response to vaccination. Splenocytes from vaccinated mice produced higher levels of IFN-γ, IL-12, IL-2, IL-10, and IL-17, but not IL-4, than sham-immunized controls in response to Gal/GalNAc lectin (Fig. 2B). Furthermore, cells from lectin-immunized mice exhibited greater IFN-γ, IL-12, and IL-17 production with ConA stimulation than those from sham-immunized mice, suggesting some intrinsic immunostimulatory properties of native lectin itself. Mesenteric lymph node cells were also assayed and exhibited a CMI pattern similar to that of splenocytes (data not shown).

LecA recapitulated the protection and immunogenicity of lectin with a CT-CFA regimen. Although native Gal/GalNAc lectin has proven effective, its usefulness as a vaccine antigen is limited and therefore we moved our vaccine work toward a bacterially expressed subunit of the Gal/GalNAc lectin, termed “LecA.” LecA was expressed and purified from E. coli using IMAC purification and contained not only the major LecA 75-kDa band, but additional proteins of approximately 250, 150, 60, and 40 kDa (Fig. 3A, Coomassie). Western blot anal-
ysis using native lectin-specific MAbs (3F4 and 7F4) reacted strongly with the major band and also with each minor band, suggesting that these proteins are derived from LecA (Fig. 3A, 3F4 MAb and 7F4 MAb). Based on densitometry scans, the immunoreactive bands comprise >95% of the total protein. Negative-control lysates did not react with any of the bands. Silver stain analysis showed the same protein banding pattern demonstrated by Coomassie blue staining (data not shown), indicating the absence of significant contamination of nonreactive bands. Endotoxin levels of purified LecA ranged from 0.021 EU/g to 0.079 EU/g. In all sham-immunized mice, the same amount of lipopolysaccharide was included as a control.

CBA mice were immunized with LecA with the original CT-CFA regimen and challenged intracecally with *E. histolytica* trophozoites. As shown in Fig. 3B, LecA provided protection in CBA mice (*P* < 0.05 in comparison with controls) with 57% efficacy. LecA also elicited high levels of antigen-specific fecal IgA (Fig. 3C) and serum IgG (with a predominance of IgG2a over IgG1) (data not shown), the levels of which did not correlate with protection. The splenocyte CMI response in LecA-vaccinated mice was similar to that seen with lectin, with significantly higher levels of IFN-γ, IL-12, IL-2, IL-10, and IL-17 than in the sham-immunized controls. The production of the Th2 cytokine IL-4 by vaccinated splenocytes was comparable to that of control cells, without significant elevation (Fig. 3D). Altogether, our data indicated that the LecA vaccination with the original CT-CFA regi-
men recapitulated the protection and immunogenicity profiles of native lectin.

**LecA-mediated protection by different formulations.** LecA administered with the combination of mucosal (CT) and systemic (CFA) adjuvants effectively protected the mice from amebic colitis, but the contribution of mucosal versus systemic routes to protection was not clear. To address the roles of adjuvants and route of immunization in protection, a variety of immunization schemes were evaluated (trial 5) (Table 1). Based on the standard measure of culture positivity upon sacrifice, several schemes were protective, including LecA administered via the original intranasal CT/intraperitoneal CFA regimen, intranasal CT, and subcutaneous immunization with the adjuvants EM014, alum, and Al001 (Fig. 4). There were differences in the calculated efficacy rates (79% for EM014, 62 to 68% for alum), but these differences were not statistically significant at these sample sizes. CFA/IFA administered subcutaneously failed to provide statistically significant protection compared to the level for sham-immunized controls, suggesting that the effect of CT used intranasally in the original protective intranasal CT/intraperitoneal CFA regimen was additive if not critical. As for CT adjuvant alone, the intranasally administered vaccine reduced the infection rate by 56% (P < 0.05), while the intrarectally administered vaccine failed to protect, which could reflect the immunologic superiority of the nasal mucosa for this vaccine or simply technical difficulties in keeping antigen in the lumen of the rectum. Interestingly, though not statistically significant, subcutaneous delivery of LecA alone, without adjuvant, lowered the infection rate (29% efficacy relative to the level for alum/PBS control mice; P = 0.10). Finally, simplification of the immunization scheme to three monthly doses did not diminish the efficacy of LecA plus alum (Fig. 4). Of note, for all experiments illustrated in Fig. 4, the histological ameba scores and inflammation scores obtained were consonant with the culture positivity data and revealed no significant differences in infected mice from the sham and vaccinated groups (data not shown).

**Immunogenicity of different regimens and surrogate markers for protection.** Prechallenge sera, stool, splenocytes, and PBMCs were obtained from mice at 2 to 3 weeks after the final immunization to assess the LecA-specific immunogenicity of these different schemes. For LecA-specific CMI, cytokine re-
sponses were measured in splenocytes, PBMCs, and mesenteric lymph node cells. Across all the trials, the Th1 adjuvant EM014 induced the highest level of IFN-γ release in splenocytes (Fig. 5A) or PBMCs (Fig. 5C); other Th1 cytokines, including IL-12 and TNF-α, were also detected at the highest level in the EM014 group (data not shown). A modest increase in splenocyte-produced IL-4 was observed in the EM014 and alum groups (Fig. 5B); however, alum appeared to induce the most IL-4 secretion by PBMCs (Fig. 5D). We also measured IL-17 production by splenocytes, mesenteric lymph node cells, and PBMCs and found that vaccination with CT via mucosal routes (CT-CFA and CT intranasally and CT intrarectally) induced strong IL-17 production by all three cell populations. CT administered intranasally elicited the highest level of fecal IgA (Fig. 5E). Serum IgG subtype was evaluated as a surrogate marker of Th1/Th2 responses. A predominance of IgG2a over IgG1 was observed in the EM014, CT-CFA, and CT (intranasal) groups, with EM014 exhibiting the most pronounced IgG2a bias. In contrast, alum induced anti-LecA IgG1 without detectable IgG2a, consistent with its Th2 adjuvant nature (4, 5, 45, 52). CFA/IFA elicited comparable levels of the IgG subtypes, and the CT (intrarectal) route failed to induce any detectable serum IgG response (Fig. 5F). When the immunogenicity data were compared between protected and infected mice within each group, none of these measures appeared to offer a clear correlate for protection (Fig. 5C to F). In contrast, the frequency of postvaccination but prechallenge IFN-γ+ CD4 T cells, and IFN-γ+, IL-2+, and TNF-α+ CD4 T cells in blood (Fig. 5G and H), correlated with vaccine protection (P < 0.05).
IFN-γ is important in LecA vaccine-mediated protection. We hypothesized that IFN-γ was playing a crucial role in vaccine-mediated protection, based on vaccine protection being transferred by T cells, the high IFN-γ responses with the high-efficacy EM014 vaccine, and the correlation with IFN-γ + CD4 T-cell frequency. We tested the role of IFN-γ in protection conferred by the different vaccine formulations (Fig. 6). Interestingly, anti-IFN-γ abrogated protection for all of the vaccines. Specifically, EM014- or CT-CFA-immunized mice administered anti-IFN-γ lost the statistically significant protection that the immunized/control IgG-treated mice exhibited. It was not possible to make this conclusion for alum, since the protection in immunized/control IgG-treated mice was not quite statistically significant (as was seen for the non-IgG-treated controls). Yet by a simpler comparison, the culture positivity rate of the immunized/anti-IFN-γ-treated mice was higher than that of the immunized/IgG-treated mice for each of the EM014, CT-CFA, and alum groups, defining an important role for IFN-γ across these three protective vaccine regimens. Not surprisingly, as we previously showed, anti-IL-4 cleared infection in sham-immunized mice (21) and had no additional impact on the vaccinated animals (Fig. 6, anti-IL-4 columns).

DISCUSSION

There are two main findings from this work: T cells are sufficient to provide vaccine protection against intestinal amebiasis, and IFN-γ is required for this protection. The finding that CMI is protective in this model was somewhat surprising, since there was substantial anticipation that the presence of secretory IgA would be critical. In several human studies, fecal antilectin IgA to lectin or its subunits was associated with a decreased risk of amebic reinfection (1, 24, 25, 27, 42). In vitro, antibodies to lectin diminish adherence to colonic mucins (9), providing a putative mechanism for antibody-based protection. However, the vaccines tested here provided protection with no clear correlation between levels of antigen-specific fecal IgA or serum IgG and subsequent protection. Furthermore, passive transfer experiments using either immune serum or monoclonal antilectin IgA/IgG did not demonstrate protection.

The mouse model has increasingly shown that CMI responses can contribute to both protection and disease, in that CD4 cells can promote the severity of colitis in C3H mice (30) and, conversely, can mediate clearance, in that anti-IL-4 treatment clears infection in CBA mice via an IFN-γ-dependent mechanism (21). Human data also suggest that high parasite-specific IFN-γ production by PBMCs is associated with protection against invasive amebiasis (28). In this context, it is not surprising that CMI is protective. Whether the mechanism of CMI protection is through outright prevention of the establishment of infection, or through rapid clearance of established infection, is unknown.

Though we have not established the cytokine requirements of the T cells for protection, all evidence points to IFN-γ. This is best argued from the IFN-γ depletion studies (Fig. 6) and the correlation of protection with vaccine-induced IFN-γ + CD4 T-cell frequency (Fig. 5). Furthermore, several protective vaccines (EM014 plus LecA, CT-CFA plus lectin, and LecA alone), including the CT-CFA plus lectin vaccine that was documented to be CMI transferable, exhibited strong antigen-specific IFN-γ production. The Th1 polarity of synthetic lipid A-based adjuvant systems is well appreciated (34, 39, 50, 51). As for the downstream mechanism of IFN-γ-based protection, there are several possibilities. The simplest explanation is that IFN-γ activates macrophages and neutrophils for E. histolytica killing, which has been shown in vitro (13, 36). Alternately, it is possible that IFN-γ exerts protective effects (e.g., inducing production of chemokines [15, 18] and mucosal defense molecules [16]) on the epithelium, which bone marrow chimera experiments indicate is a critical cell type in the mouse strain-specific resistance to intestinal E. histolytica infection (23).

It is surprising that IFN-γ was required for vaccine protection with alum-based LecA vaccines, since alum is a Th2 type adjuvant and only a marginal level of IFN-γ was detected in our alum-immunized PBMCs or splenocyte supernatants. However, there was a significant frequency of LecA-specific
IFN-γ-positive CD4 cells in peripheral blood postvaccination, and this response correlated with vaccine-induced protection. Moreover there is precedent for IFN-γ in alum-based protection. For instance in an alum-based respiratory syncytial virus peptide vaccine, loss of protection was seen in mice treated with IFN-γ-neutralizing antibody, and purified CD4 T cells from vaccinated IFN-γ knockout donors failed to transfer protection (41). Other vaccine studies employing alum as an adjuvant against *Toxoplasma gondii* (11) and *Streptococcus pneumoniae* (17) infection also suggested a possible role of IFN-γ response in protection.

As for how the IFN-γ was elicited during alum vaccination, this response may be due to the LecA antigen itself. First, IFN-γ production seemed to be promoted by LecA, in that ConA-stimulated cells from our LecA-immunized mice produced more IFN-γ than cells from sham-immunized mice. Furthermore, the frequency of IFN-γ-secreting CD4 T cells in the LecA-only group was significantly higher than that observed for the control. There is a long precedent for the potential Th1 induction of Gal/GalNAc lectin, with several laboratories (8, 31, 43, 44) reporting that native lectin contains epitopes that stimulate IL-12 and TNF-α in macrophages and dendritic cells, particularly amino acids 596 to 1082, which are located within LecA. Such a combination of Th1 stimulus and alum has enhanced the efficacy of vaccines against human immunodeficiency virus (33), *Leishmania major* (22, 35), malaria (49), and *Schistosoma mansoni* (2). Perhaps the LecA afforded a frequency of IFN-γ-secreting CD4 cells in the LecA-alum vaccine

FIG. 5. Immunogenicity across different adjuvants and the correlate markers for protection. Mice were immunized with LecA formulated with EM014, alum, CFA and IFA, CT, or original CT-CFA adjuvants via different routes. The prechallenge immunogenicities of six regimens were assessed for LecA-specific IFN-γ and IL-4 produced by splenocytes (A and B) and PBMCs (C and D). Anti-LecA fecal IgA (E) and the serum IgG2a/G1 ratio (F) were also measured. For LecA-specific IFN-γ-producing CD4 (G) and IFN-γ-IL-2-TNF-α multifunctional CD4 T cells (H), PBMCs were stimulated with LecA for 22 h. The frequency of cytokine-producing cells was determined by flow cytometry on CD4-gated cells. In panel A, the only statistically significant difference in IFN-γ production levels was between EM014 and the control. In panels C to H, vaccinated and infected mice (unprotected) in each group are represented by filled circles, whereas vaccinated and uninfected (protected) mice are represented by open circles. In panels G and H, protected mice in each group were compared to unprotected mice for statistical analysis. *, *P < 0.05; **, *P < 0.01; ***, *P < 0.001. i.n., intranasal; i.r., intrarectal.
that was above a certain threshold for protection (~20%), even though the total quantity detected in the LecA-alum PBMC supernatants was marginal (and not predictive).

The IFN-γ-producing, or triple IFN-γ-, TNF-α-, and IL-2-producing, CD4 T-cell frequency served as the one meaningful marker for the protection observed. The so-called “multifunctional” CD4 T cells that secrete IFN-γ, TNF-α, and IL-2 concordantly have been shown to correlate with protection from *Leishmania major* (12) and *Mycobacterium tuberculosis* (19) better than a single marker alone. We examined them in *E. histolytica* because TNF-α and IFN-γ synergistically activate macrophages for trophozoite killing (36) and IL-2 is associated with resistance to reinfection in amebic liver abscess models (7). The additive value of TNF-α and IL-2 to IFN-γ is unclear, however, since the frequency of triple cytokine production was no more correlative than IFN-γ production alone.

In conclusion, we have demonstrated that protection by Gal/GalNAc lectin-derived vaccines was transferred to naive animals by the transfer of immune T cells and that IFN-γ was essential for protection. The frequency of IFN-γ-producing CD4 T cells in blood stands as a useful surrogate marker for alum-based LecA-mediated protection. The demonstration of CD4 T cells in blood stands as a useful surrogate marker for vaccine-mediated protection by *Amebiasis* vaccine.

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