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Evaluation of Recombinant *Leishmania* Polyprotein Plus Glucopyranosyl Lipid A Stable Emulsion Vaccines against Sand Fly-Transmitted *Leishmania major* in C57BL/6 Mice

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Numerous experimental *Leishmania* vaccines have been developed to prevent the visceral and cutaneous forms of Leishmaniasis, which occur after exposure to the bite of an infected sand fly, yet only one is under evaluation in humans. KSAC and L110f, recombinant *Leishmania* polyproteins delivered in a stable emulsion (SE) with the TLR4 agonists monophosphoryl lipid A or glucopyranosyl lipid A (GLA) have shown protection in animal models. KSAC+GLA-SE protected against cutaneous disease following sand fly transmission of *Leishmania major* in susceptible BALB/c mice. Similar polyprotein adjuvant combinations are the vaccine candidates most likely to see clinical evaluation. We assessed immunity generated by KSAC or L110f vaccination with GLA-SE following challenge with *L. major* by needle or infected sand fly bite in resistant C57BL/6 mice. Polyprotein-vaccinated mice had a 60-fold increase in CD4+IFN-γ+ T cell numbers versus control animals at 2 wk post–needle inoculation of *L. major*, and this correlated with a 100-fold reduction in parasite load. Immunity did not, however, reach levels observed in mice with a healed primary infection. Following challenge by infected sand fly bite, polyprotein-vaccinated animals had comparable parasite loads, greater numbers of neutrophils at the challenge site, and reduced CD4+IFN-γ/IL-17+ ratios versus nonvaccinated controls. In contrast, healed animals had significantly reduced parasite loads and higher CD4+ IFN-γ/IL-17+ ratios. These observations demonstrate that vaccine protection against needle challenge does not necessarily translate to protection following challenge by infected sand fly bite. *The Journal of Immunology*, 2012, 189: 000–000.

*Leishmania* are protozoan parasites that are transmitted to the skin of a mammalian host following the bite of an infected Phlebotomine sand fly. The clinical outcomes of infection with different *Leishmania* spp. is highly diverse, ranging from self-limiting, cutaneous lesions following infection with *L. major* to fatal systemic involvement of the liver, spleen, and bone marrow following infection with *L. donovani* (1). A defining feature of cutaneous disease caused by *L. major* is that dermal lesions eventually heal, but are thought to continue to harbor viable parasites for the lifetime of the individual. This chronic state is associated with the maintenance of a powerful cell-mediated immune response such that individuals with a healed primary infection are protected against reinfection. Consequently, intentional infection with *L. major* parasites at discrete locations on the body has been successfully employed as a live vaccine (2–5). However, issues surrounding the inoculation of viable parasites in immunocompromised individuals and adverse reactions at the site of leishmanization in some individuals have precluded its use as an intervention strategy (5, 6).

Rodents are natural reservoirs of *L. major*, and the study of cutaneous disease in resistant laboratory mice in which lesions heal and parasites establish stable chronic infection is thought to reflect the human experience with this parasite (7, 8). In these murine models, cell-mediated immunity is primarily associated with Th1 CD4+ and CD8+ T cells, most often defined by their ability to produce IFN-γ in response to parasite Ags (8, 9). Therefore, the cell-mediated immune response in humans or mice with a healed primary infection represents the gold standard of protective immunity. Reproducing this protective immune response with protein, DNA-based, or live-attenuated vaccines has been an active area of study because it is assumed this would be the first step toward the development of a safe and effective *Leishmania* vaccine (10–19). However, few experimental vaccines have progressed beyond animal models into human clinical trials due to a lack of consensus regarding their likelihood of success and/or the costs associated with producing a vaccine acceptable for human use (18–20). Of the so-called first-generation whole cell-killed promastigote vaccines that have been used in people, although shown to be safe, none have succeeded in providing the level of protection to natural infection that would justify their further use and development (21, 22).

Recently, several defined polyproteins containing multiple antigenic epitopes from *Leishmania* delivered in a stable emulsion (SE) (water in oil) with monophosphoryl lipid A or glucopyranosyl lipid A (GLA), TLR4 agonists suitable for use in people, have provided protection in susceptible BALB/c and resistant C57BL/6 mice following needle challenge with *Leishmania spp* (23–27). One of these polyproteins, KSAC, containing kinetoplastid membrane protein 11, sterol 24-methyltransferase, A2,
and cysteine proteinase B, was shown to protect BALB/c mice against early lesion development following sand fly transmission of \( L.\) \( majo\), and this was associated with an ~50-fold reduction in parasite load at 5 wk postinfected sand fly challenge (28). These observations are promising because phase I clinical trials have also shown that at least some of these Ag-adjuvant combinations are safe for use in people, and recombinant \( L.\) \( major\) polyproteins lend themselves well to meeting good manufacturing practices and regulatory approval (18, 29–31).

In the resistant C57BL/6 model, we and others have reported that vaccination with autoclaved \( L.\) \( major\) (ALM) delivered with the TLR9 agonist CpG oligodeoxynucleotide, a Th1 adjuvant, results in significant protection following needle challenge (11, 12, 32). However, this protection is significantly weaker than that observed in mice with a healed primary infection and fails to protect following challenge by exposure to the bites of \( L.\) \( major\)-infected sand flies (32). The failure of the ALM+CpG vaccine was not due to a difference in the virulence of inoculated parasites, but rather was associated with the unique immunomodulatory conditions at the site of sand fly inoculation and in particular the prolonged recruitment of neutrophils (32). In the studies reported in this study, the objective was to assess protective immunity generated by polyproteins+GLA-SE vaccination in the context of ALM+CpG-vaccinated and healed mice following needle and infected sand fly challenge in a resistant mouse model of cutaneous Leishmaniasis.

### Materials and Methods

#### Mice

Female C57BL/6 mice were obtained from The Jackson Laboratory. All mice were maintained in the National Institute of Allergy and Infectious Diseases animal care facility under specific pathogen-free conditions.

#### Leishmania cell lines and preparation for needle inoculation

\( L.\) \( major\) Friedlin strain was obtained from the Jordan Valley NIH/FV1 (MHOM/IL/80/Friedlin). \( L.\) \( major\) RYN was obtained from a lesion biopsy of a laboratory worker accidentally exposed to \( Lutzio\) longipalpis sand flies that were experimentally infected with a strain of \( L.\) \( major\) (WR2885) originating in Iraq, as described previously (33). All parasites were grown in vitro as described previously (33). Infective-stage metacyclic promastigotes were isolated from stationary cultures (4–6 d old) by negative selection of noninfective forms using peanut agglutinin (Vector Laboratories) (34).

#### Healed mice and ALM+CpG, KSAC+GLA-SE, and L110f+GLA-SE vaccination

Briefly, healed mice were generated by infection with \( 10^5\) \( L.\) \( major\) (FV1 or RYN) metacyclic promastigotes s.c. in the footpad and used at 23 wk postprimary infection when footpad lesions had completely resolved. ALM plus CpG oligodeoxynucleotide-vaccinated mice were injected s.c. in the footpad with 50 \( \mu\)g ALM plus 50 \( \mu\)g CpG oligodeoxynucleotide sequence 1826 (Coley Pharmaceutical Group), provided by Dr. P. Darragh (Vaccine Research Center, National Institutes of Health), three times, at 2-wk intervals. Additional groups of mice were vaccinated s.c. in the base of the tail with 10 \( \mu\)g Ag (KSAC or L110f) plus 20 \( \mu\)g GLA-SE in 100 \( \mu\)l, three times, at 2-wk intervals (Ags and adjuvant were kindly provided by Dr. S. Reed, Infectious Disease Research Institute). In some experiments, mice were injected with KSAC plus 5 \( \mu\)g GLA-SE, and, where indicated, age-matched control (AMC) mice were injected with saline plus GLA-SE alone. In some experiments, AMC and KSAC+GLA-SE-vaccinated mice were boosted 3 d prior to sand fly challenge with 10 \( \mu\)g KSAC Ag plus 20 \( \mu\)g GLA-SE.

#### Sand fly infection and exposure of mice to infected sand flies

Colonized female \( Phlebotomus\) duboscqi sand flies were infected with \( L.\) \( major\) RYN as described previously (33). After 13 to 14 d, parasite infections within the sand fly midgut were assessed to determine suitability for transmission according to our previously published protocol (33). On the day of challenge, mouse ears were exposed to the bites of four infected flies for 2 to 3 h in the dark at 23°C and 50% humidity. The average number of flies with or without a blood meal was used to assess feeding intensity between treatment groups, and no significant differences were found (data not shown).

#### Determination of lesion size and parasite load

Ear lesion diameters or ear lesion area were measured (in millimeters) weekly as described in the text. Ears with more than one lesion are reported as total lesion diameter or total lesion area per ear. Two to 4 wk following challenge by needle inoculation or 6 wk following challenge by exposure to infected sand fly bites, mice were euthanized, and a single-cell suspension of individual ears was prepared as described previously (32). A portion of each ear was resuspended in phosphate-buffered saline with micromesh, and serial diluted in a 96-well flat-bottom microtiter plate, overlaying 100 \( \mu\)l onto 50 \( \mu\)l NNN medium containing 20% defibrinated rabbit blood. The number of viable parasites in each ear was determined from the highest dilution at which promastigotes could be grown out after 7–10 d of incubation at 26°C.

#### Phenotypic analysis of ear-derived cell populations

Ear-derived cells were incubated with an anti-Fcγ RII/III (CD16/32) receptor Ab (2.4G2; BD Biosciences) in RPMI 1640 without phenol red (Life Technologies) containing 1% FCS for 10 min followed by incubation for 20 min with a combination of six of the following Abs: PE-Cy7 or allophycocyanin anti-CD11b (M1/70; BD Biosciences); PerCP Cy5.5 anti-Ly6C (HK.1.4, eBioscience); FITC or PE anti-Ly6G (1A8, BD Biosciences); allophycocyanin or PE anti-CD11c (HL.3; BD Biosciences); PerCP Cy5.5 anti-CD11c (N418; BioLegend); allophycocyanin anti-F4/80 (MB; eBioscience; FITC anti–F4/80, BD Biosciences); FITC anti–MHC class II (M5/114.15.2, eBioscience). Isotype controls employed were rat IgG1 (R3-34) and rat IgG2b (A95-1 or eB2Ra). Data were collected using FACSDivA software on an FACSCan flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Gating of ear-derived cells was carried out as described previously (55, 56). Cells were analyzed following pooling of ears from mice of the same group as indicated in the text.

#### Restimulation of ear-derived T cells for cytokine analysis by flow cytometry or multiplex ELISA

T cells were restimulated with parasite Ags as described previously (25, 32). Briefly, whole-ear single-cell suspensions were incubated at 37°C in 5% CO\(_2\) for 12–14 h in flat-bottom 48-well plates with 2.5 \( \times 10^5\) bone marrow–derived dendritic cells (BMDCs) or 1 \( \times 10^5\) T cell-depleted (Miltenyi Biotec), irradiated, naïve spleen cells (APCs), with or without 50 \( \mu\)g/ml freeze-thaw \( L.\) \( major\) Ag (L.m.-Ag), 40 \( \mu\)g/ml KSAC, or 40 \( \mu\)g/ml L110f. During the last 4 h of culture, 1 \( \mu\)g/ml brefeldin A (GolgiPlug; BD Biosciences) was added. Following culture, washed cells were labeled with Live/Dead fixable VIOLET or AQUA (Invitrogen) to exclude dead cells and anti-Fc γ II/III (CD16/32) receptor Ab (2.4G2), followed by PE-Cy7 anti–mouse CD4 (RM4-5) for 20 min. Cells were then fixed with BD Cytofix/Cytoperm (BD Biosciences) and stained with a combination of the following anti–mouse Abs: V500, allophycocyanin-Cy7, or PerCP-Cy5.5 anti-CD3 (14-2C11), FITC anti–IFN-γ (XMG1.2), PE or Alexa Fluor-700 anti–TNF-α (MP6-XT22), allophycocyanin anti–IL-2 (JES5-5H4), PerCP-Cy5.5 anti–IL-17A (eBio17B7), or PE anti–IL-10 (JES5-16E3). Intracellular staining was carried out for 45 min on ice. Isotype controls employed were rat IgG1 (R3-34) and rat IgG2b (A95-1 or eB2Ra). All Abs were from eBioscience or BD Biosciences. Data were acquired using FACSDivA software on an FACSCan flow cytometer (BD Biosciences) and analyzed using FlowJo software (Tree Star). Forward-scatter and side-scatter width was employed to exclude cell doublets from analysis. For measurement of chemokines and cytokines in culture supernatants, pooled, single-cell suspensions of ear tissue, prepared as described above, were incubated at 37°C in 5% CO\(_2\) for 12 h in 48-well round-bottom plates with 2.5 \( \times 10^5\) BMDCs with or without L.m.-Ag KSAC, or L110f in a total volume of 1 ml. Following incubation, the concentration of chemokines and cytokines in the culture supernatant was determined by multiplex analysis (Affymetrix).

#### Statistics

Lesion diameters and parasite loads were compared using the Mann–Whitney \( U\) test. All \( p\) values are two-sided. Statistical calculations were done in GraphPad Prism 5.0c (GraphPad; www.graphpad.com). Level of significance is reported as follows: *0.05 \( p\) > 0.005, **0.005 \( p\) > 0.0005, and ***\( p\) < 0.0005.

#### Ethics statement

All animal experiments were performed under an Animal Study Protocol approved by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee using guidelines established by the...
Animal Welfare Act and the Public Health Service Policy on Humane Care and Use of Laboratory Animals.

**Results**

**Vaccination with polyprotein+GLA-SE or ALM+CpG mediates protection against needle challenge with L. major**

C57BL/6 mice were vaccinated with ALM+CpG, KSAC+GLA-SE, or L110f+GLA-SE three times at 2-wk intervals, as described previously (25, 32). Healed mice were used 23 wk after primary infection with $10^7$ live *L. major* metacercaric promastigotes in the footpad. Seventeen weeks following the last vaccine dose, mice were challenged by needle inoculation of the ear dermis with $2 \times 10^5$ purified *L. major* metacercaric promastigotes. Analysis of ear lesion diameters at 2 wk postchallenge revealed that healed and vaccinated mice had significantly larger lesions compared with AMC mice (Fig. 1A), likely due to the recruitment of cells primed by pre-exposure to *L. m.* Ags. By 4 wk postchallenge, the mean lesion diameter of AMC and vaccinated animals was not significantly different. In contrast, at 4 wk postinfection (p.i.), healed mice had significantly reduced lesions in comparison with 2 wk and to the AMC and each of the vaccinated groups. Assessment of parasite numbers per ear at 4 wk p.i. revealed that each of the vaccinated groups significantly reduced the parasite load per ear versus AMC, although not to same degree as healed animals (Fig. 1B). Analysis of cytokine production by ear-derived CD3+CD45+ T cells in response to whole-parasite Ag at 2 and 4 wk post–needle inoculation revealed that vaccinated and healed mice mounted robust immune responses compared with AMC (Fig. 1C). However, only healed mice contained -2-fold greater frequencies and total numbers of IFN-γ+ cells at the earlier, 2-wk time point (Fig. 1C, 1D), confirming our previous observation that the strongest correlate for protection is a robust immune response early after challenge (32). In addition, although the response in healed mice to whole-parasite Ag decreased over time, reflecting the greatly reduced Ag load, responses in each of the other groups increased (Fig. 1C), suggesting that the higher parasite loads in these ears continued to drive T cell expansion. All of the vaccinated groups of mice had significantly reduced lesion size, parasite load, and numbers of Ag-responsive T cells in these animals versus vaccinated mice at 4 wk postchallenge. In contrast, ears of KSAC and L110f vaccinated animals had larger numbers of certain innate cell types, specifically neutrophils, Ly6C+ monocytes, dendritic cells (DCs), and F4/80+ MHC I+ macrophages versus ALM+CpG-vaccinated mice, despite comparable control of parasite numbers (Fig. 2B). Because neutrophils can interfere with primary and secondary CD4+ T cell activation and represented the largest fold increase between polyprotein and either ALM+CpG or healed animals, we wished to investigate the factors that may be influencing their recruitment. Recently, it has been shown that inoculation of *L. major* with the CpG oligonucleotide adjuvant generates IL-17–producing CD4+ T cells and results in IL-17–dependent neutrophil recruitment to the skin (38). IL-17–mediated recruitment of neutrophils has also been implicated in the pathogenesis of *L. major* infection in BALB/c mice (39). Analysis of culture supernatants from ear-derived cells 14 d following challenge revealed that each of the vaccinated groups of mice had elevated levels of IL-17 in response to *L. m.* Ags versus control or healed animals (Fig. 1C). This was most dramatic when cells from L110f-vaccinated animals were stimulated with the L110f protein. IL-17 mediates neutrophil recruitment via induction of neutrophilic chemokines such as CXCL1 and CXCL2 from innate and nonimmune cells (40). We found elevated levels of CXCL1 and CXCL2 in polyprotein+GLA-vaccinated animals, and this was most clear when cells were restimulated with the protein employed for vaccination. These observations suggest that an early IL-17 response may enhance innate cell recruitment in vaccinated animals via induction of CXCL1 and CXCL2. IL-17 production in ALM+CpG-vaccinated mice did not correlate with increased levels of CXCL1 and CXCL2, and this was reflected in lower neutrophil numbers at 4 wk. Because regulatory cytokines such as IL-10 or T cell recruitment can also influence the outcome of infection, we investigated the levels of IL-10 and the Th1-recruiting chemokine CCL5 (RANTES) (Fig. 2C). We found similar levels of IL-10 among vaccinated and healed animals, apart from an increase in KSAC-vaccinated mice restimulated with the KSAC polyprotein. In addition, CCL5 levels were comparable among the vaccinated mice, with slightly higher levels observed in healed animals. Therefore, regulation by IL-10 or lack of Th1 recruiting chemokines does not appear to explain the difference in protection comparing vaccinated and healed animals.

**Polyprotein+GLA-SE vaccination fails to protect against infected sand fly challenge in resistant C57BL/6 mice**

We previously reported that ALM+CpG vaccination protects against needle but not infected sand fly challenge (32). We wished to determine whether polyprotein+GLA-SE vaccination, which
also protects against needle challenge (Fig. 1B), would protect against infected sand fly challenge. Analysis of parasite loads in the ears and draining lymph nodes (DLNs) of polyprotein-vaccinated or healed animals 6 wk following infected sand fly challenge revealed that only animals with a healed primary infection reduced parasite numbers versus control animals, and this pattern was apparent in both the ear and ear DLNs (Fig. 3A, 3B). Surprisingly, vaccination with L110f resulted in significantly increased parasite numbers in the ear versus control or KSAC-vaccinated animals, despite higher or equivalent frequencies of IFN-γ+CD4+ T cells, respectively.
The failure of KSAC-vaccinated mice to reduce parasite loads versus control animals also occurred despite an increase in the frequency of IFN-γ+ cells versus control mice. In contrast, healed mice contained lower total frequencies of IFN-γ+ cells compared with vaccinated mice, likely reflecting the strong reduction in parasite numbers at 6 wk postchallenge in these mice.

Ears of KSAC- and L110f-vaccinated animals contained more neutrophils then control animals (Fig. 3D), and, at least in the case of KSAC-vaccinated mice, this was not attributable to increased parasite loads as assessed at 6 wk. Costaining for IL-17 and IFN-γ or TNF production by CD4+ T cells revealed that IL-17+ cells made up a greater proportion of the secondary immune responses in polyprotein-vaccinated versus healed mice (Fig. 3C). The majority of IL-17+ cells in polyprotein+GLA-vaccinated mice co-produced TNF, whereas the opposite was true in AMC and healed mice. At the 6-wk time point examined, the ratio of CD4+IFN-γ+/CD4+IL-17+ T cells correlated with vaccine success or failure against sand fly challenge, with healed mice having the highest ratio and the L110f mice having a 2.5-fold lower ratio compared with AMC or KSAC-vaccinated animals. The IFN-γ/IL-17 ratio also correlated with the number of neutrophils per ear. These observations demonstrate that challenge by exposure to L. major-

**FIGURE 2.** Analysis of total myeloid populations and cytokine/chemokine production at the site of infection. (A) Representative gating strategy in which subpopulations of CD11b+ myeloid cells are defined by the following markers: Ly6CintLy6G− neutrophils (region 1); Ly6C−Ly6G−CD11c+ MHC II+ inflammatory monocytes (Ly6C+ Mono., region 2); Ly6C−Ly6G−CD11c− MHC II− monocytes/macrophages (Mono./Mac., region 3); Ly6C+Ly6G−CD11c+ MHC II+ monocyte-derived dendritic cells (Mono. Der. DC, region 4); Ly6C−Ly6G−CD11c+ MHC II+ macrophages (II+ Mac, region 6); Ly6C+Ly6G−CD11c− MHC II+ macrophages (II+ Mac, region 7). (B) Relative cell number of the indicated cell types per ear following analysis of pooled ear samples from the mice depicted in Fig. 1 at 4 wk postchallenge. (C) Detection of IL-17, CXCL1 (keratinocyte chemoattractant [KC]), CXCL2 (MIP-2), IL-10, and CCL5 (RANTES) by multiplex analysis following in vitro restimulation of ear-derived cells for 12 h with the indicated Ag at 2 wk postinfection. Error bars represent the SD between replicates of a pooled sample. This experiment was done once.
infected sand flies alters the requirements for protective immunity as compared with challenge by needle inoculation. Lower adjuvant dose, or boosting shortly before challenge, does not enhance the efficacy of KSAC+GLA-SE vaccination against infected sand fly challenge.

In an attempt to improve vaccine efficacy, we focused our attention on the KSAC polyprotein vaccine because L110f resulted in an enhancement of parasite load. To address the possibility that overadministration of the GLA-SE adjuvant was responsible for inducing IL-17–dependent neutrophil recruitment, we included a group of animals vaccinated with a lower dose (5 mg) of GLA-SE. To address the possibility that the vaccination protocol failed to maintain a sufficient number of Leishmania-specific cells available for recall at the time of challenge, we boosted vaccinated and control mice with KSAC+GLA-SE just prior to challenge. Lowering the adjuvant dose did not lead to a reduction in lesion size or parasite load following infected sand fly challenge as compared with mice given the 20-μg dose (Fig. 4A, 4C, 4D). In fact, KSAC-vaccinated mice had increased lesion sizes compared with control mice, and this was attributable to differences in parasite numbers as assessed at 6 wk postchallenge. In contrast, healed mice, which also had an early increase in lesion size versus control animals at 2 wk postchallenge, had smaller lesion sizes compared with vaccinated animals starting at 3 wk (p < 0.01) and significantly fewer parasites per ear compared with all other groups at 6 wk. The relatively flat lesion kinetic in AMC mice shown in Fig. 4A is the result of the wide variation in parasite dose delivered by sand fly bite such that ears receiving low doses of parasites are beginning to present with lesions, whereas ears receiving a high dose are resolving their lesions (41). We also assessed IFN-γ, TNF-α, and IL-17 production by dermal-derived CD4+ T cells following restimulation with APCs alone, APC+L.m.-Ag, or APC+KSAC protein (Fig. 4E, 4F). Vaccination with 5 versus 20 μg GLA in emulsion plus KSAC resulted in lower frequencies of IFN-γ+ cells, but this was also accompanied by a decrease in the frequency of IL-17+ cells, such that the ratio of IFN-γ to IL-17+ CD4 T cells increased from 23 to 37:1 following L.m.-Ag restimulation (Fig. 4G). However, this increased ratio was still lower than the 186:1 observed in mice with a healed primary lesion or the 52:1 ratio observed in control mice. Once again, ears of KSAC-vaccinated animals contained approximately twice the number of neutrophils at the site of challenge versus control or healed mice (Fig. 5). KSAC vaccination with 5 versus 20 μg of GLA-SE did not alter the number of neutrophils at the site of infection, but did trend toward fewer monocyte/macrophages, DCs, and MHC II+ macrophages.

Boosting KSAC+GLA-SE–vaccinated (20 μg) mice 3 d prior to challenge with a fourth dose of KSAC+GLA-SE did not improve the outcome of infected sand fly challenge (Fig. 4B–D). Rather, boosting led to a significant exacerbation of lesion size starting at 3 wk postchallenge (p = 0.029) as compared with KSAC-vaccinated animals that were not boosted (Fig. 4B). In the AMC group that was injected with KSAC+GLA-SE 3 d prior to infected sand fly challenge (AMC-Boost), half of the ears did not present with lesions and did not contain parasites. The most likely explanation for this is that transmission did not occur following exposure to infected sand flies. Analysis of ear lesion size on those AMC-Boost ears that were infected at the time of analysis (open circles in Fig. 4B) revealed these ears had significantly larger lesions at 6 wk posttransmission versus nonboosted AMC (p = 0.014) (Fig. 4B). Boosting AMC or

**FIGURE 3.** Polyprotein+GLA-SE vaccination fails to protect against infected sand fly challenge in resistant C57BL/6 mice. Ears of AMC, healed, KSAC+GLA-SE, or L110f+GLA-SE–vaccinated mice were exposed to the bites of infected sand flies and analyzed 6 wk later. (A) Parasite loads in individual ears. *p < 0.005. (B) Parasite loads in pooled (right plus left [R+L]) ear DLNs. *p < 0.05. (C) Intracellular staining for cytokine-producing CD4+CD3+ T cells after in vitro restimulation of pooled ear-derived cells with APCs plus L.m.-Ag. (D) Number of CD11b+Ly6CintLy6G+ neutrophils per ear. (E) Graphical representation of the mean ratio (n = 2) of CD4+IFN-γ+/CD4+IL-17+ T cells in the ear. This experiment was done once.
Lower adjuvant dose, or boosting shortly before challenge, does not enhance the efficacy of KSAC+GLA-SE vaccination against infected sand fly challenge. Ears of AMC, healed, and KSAC+GLA-SE– (20 μg) or KSAC+GLA-SE–vaccinated (5 μg) mice were exposed to the bites of infected sand flies and analyzed 6 wk later. A separate group of AMC or KSAC+GLA-SE–vaccinated (20 μg) mice were boosted with KSAC+GLA-SE (20 μg) 3 d before exposure to infected sand flies. (A and B) Mean lesion area (± 1 SE) following exposure to infected sand fly bites. Data in (A) and (B) are from the same experiment, but are separated for clarity. (C) Parasite loads in individual ears. **p < 0.005. (D) Parasite loads in pooled (right plus left [R+L]) ear DLNs. Significant differences versus the AMC group, *p < 0.05. (E) Intracellular staining for IFN-γ and/or TNF-α CD4+CD3+ T cells after in vitro restimulation of pooled ear-derived cells with APCs plus L.m.-Ag or KSAC. (F) Frequency of IL-17+ cells among the same cells in (E). (G) Graphical representation of the ratio of CD4+IFN-γ+/CD4+IL-17+ T cells in the ear following APC+L.m.-Ag restimulation. Error bars in (A) and (B) represent the SEM lesion area per ear. This experiment was done once.
KSAC-vaccinated mice led to slight increases in the frequency of IFN-γ+ cells (Fig. 4E). However, this was accompanied by a 2–4-fold increase in the frequency of IL-17-producing cells, such that the ratio of IFN-γ+/IL-17+ cells decreased from 52 to 15 in AMC versus AMC-Boost mice and from 24 to 12 in KSAC versus KSAC-Boost mice (Fig. 4G). This correlated with a 3-fold increase in neutrophil numbers per ear (Fig. 5). Therefore, lowering the adjuvant dose did increase the CD4+IFN-γ+/CD4+IL-17+ ratio, but this increase did not reach the levels seen in control or healed animals and did not reduce neutrophil numbers or lesion size. In addition, although boosting slightly increased the frequencies of IFN-γ-producing T cells, the most dramatic effect was an enhancement of the IL-17 response and increased neutrophil numbers and lesion size, strongly suggesting this is an inappropriate vaccination protocol. Neither alteration to the vaccine protocol led to improved control of parasite numbers.

**Discussion**

The observations reported in this study add to our previous finding that vaccine-induced protection against needle challenge in resistant C57BL/6 mice does not necessarily translate to protection following challenge by exposure to the bites of *L. major*-infected sand flies. In addition, immune responses elicited by *L.m.-Ag* restimulation of cells from the site of challenge in protein plus adjuvant-vaccinated mice again failed to match the early qualitative and quantitative intensity of the response in mice with a healed primary infection. We have argued that a rapid and robust response is critical to deal with the immune modulatory conditions present at the dermal site of infected sand fly bite, which includes the massive and prolonged recruitment of neutrophils (32, 42). *L. major* parasites are not killed by neutrophils, and both CD4+ T cell priming by DCs and IFN-γ production in vaccinated mice are downmodulated in the presence of neutrophils (32, 35, 36). More critically, the efficacy of the whole cell-killed ALM+CpG vaccine against infected sand fly challenge was enhanced in neutrophil-depleted mice (32). Furthermore, the ALM+CpG vaccine still conferred protection against needle challenge with parasites derived from infected sand flies, arguing that the failure of the nonliving vaccines to protect against sand fly challenge is not due to greater virulence of sand fly-transmitted parasites (32). In contrast to infected sand fly challenge, needle challenge results in a transient recruitment of smaller numbers of neutrophils, making needle challenge less likely to require such robust secondary immunity to observe a reduction in parasite load (32). Therefore, the most likely explanation for vaccine failure against infected sand fly challenge is the inability of the vaccine to replicate the early kinetics of the secondary immunity observed in animals with a healed primary infection. So far as we are aware, there does not exist a *Leishmania* vaccine that replicates the protective immune response observed in healed mice.

Following infected sand fly challenge, L110f-vaccinated mice had increased parasite loads versus AMC- and KSAC-vaccinated animals despite comparable levels of IFN-γ+ and multicytokine-producing cells versus the other vaccinated groups. This increased parasite load correlated with the highest frequencies of IL-17+CD4+ T cells and the largest numbers of neutrophils per ear. Previous studies have shown KSAC to provide superior protection against *Leishmania* challenge compared with a vaccine containing L110f (23, 28). In the BALB/c model, Gomes et al. (28) also found that L110f vaccination significantly reduced parasite loads following needle but not infected sand fly challenge, similar to what we have shown in this study employing L110f and KSAC vaccination and published.
previously with the ALM+CpG vaccine (32). Sand fly transmission of *L. mexicana* was also shown to abrogate a delay in lesion progression mediated by soluble *Leishmania* Ag+RIL-12 vaccination following needle challenge of BALB/c mice (43). Lastly, protection conferred by L111f+monophosphoryl lipid A-SE in mice against experimental needle challenge with *L. infantum* did not translate to protection in dogs following natural exposure (26, 44), although it is immunotherapeutic in mild to moderate cases of canine visceral Leishmaniasis (45, 46). Therefore, abrogation of protective immunity following challenge by sand fly bite appears to be a general observation, and this may be largely due to immunomodulation by neutrophils (32, 36).

Our observations employing the KSAC+GLA-SE vaccine should be viewed in the context of those by Gomes et al. (28), in which KSAC vaccination protected otherwise susceptible BALB/c mice against early lesion development and reduced parasite loads ~50-fold following sand fly transmission of the FV1 strain of *L. major*. Protection against lesion development also occurred when infected sand fly challenge was delayed to 12 wk postvaccination and employed the *L. major* WR2885 clinical isolate, a challenge protocol similar to the one employed in this study. There is not a clear explanation for the differences between the protection conferred by the same vaccine in these two different mouse strains. Importantly, this discrepancy is not due to an inability of KSAC vaccination to induce Th1 immunity in C57BL/6 mice, because vaccination protected against needle challenge and Ag-specific IFN-γ*γ* cells were readily detected at the site of challenge. In addition, direct comparison of prechallenge levels of KSAC-specific IFN-γ in C57BL/6 versus BALB/c mice vaccinated with KSAC revealed a trend toward higher levels in C57BL/6 animals (23). However, protection in a resistant mouse requires that the vaccine improve upon what is ultimately a very effective primary response. In comparison, vaccination of BALB/c mice may sufficiently ablate a disease-promoting Th2 response to prevent or slow parasite replication without necessarily inducing the level of Th1 immunity at the site of challenge observed in resistant animals. For example, vaccination with the live, attenuated *L. major* parasite protected BALB/c but not C57BL/6 mice and was associated with a greatly diminished Th2 response rather than a strongly upregulated Th1 response (14, 47). In the studies by Goto et al. (23), the KSAC-vaccinated BALB/c mice that were protected from needle challenge had significantly reduced levels of splenic IL-4 versus control animals, but equivalent levels of IFN-γ. By contrast, the KSAC+GLA-SE–vaccinated BALB/c mice that were protected against sand fly challenge did show significantly increased IFN-γ secretion by splenic cells both pre- and postchallenge; whether their Th2 response was abrogated was not reported (28). The influence of KSAC vaccination on the strength and Th1/Th2 nature of subsequent immune responses to *L. major* transmitted by sand fly bite in these two mouse strains is likely to be in addition to any influence of neutrophils on the expression of this immunity, because sand fly bite is expected to induce neutrophil recruitment in each case.

Recent observations suggest that a sufficient resting period between boosting doses employing the GLA-SE adjuvant may be required for optimal generation of IFN-γ–producing T cells (S. Bertholet, personal communication). Therefore, the 2-wk intervals used in our prime-boost protocol versus the 3-wk intervals used by Gomes et al. (28) as well as others (23) may have been too short and led to less-efficient priming of Th1 immunity than what might otherwise be possible in the C57BL/6 background, though this still would not explain the protection conferred against needle challenge.

The polyprotein+GLA-SE vaccines represent the only defined candidates currently in clinical trial for use as therapeutic or prophylactic vaccines against Leishmaniasis in humans. In the C57BL/6 mouse model employed in this study, vaccination with KSAC or L110f did not provide protection against infected sand fly challenge. The likely explanation for this finding is that vaccination did not sufficiently improve upon the already protective Th1 response mounted in this resistant mouse strain. Lack of protection also seemed to correlate with IL-17 production, increased numbers of neutrophils per ear, and increased lesion size. Vaccine-associated IL-17 production was not limited to sand fly transmitted infections because IL-17 was also detected following needle challenge. The impact of enhanced neutrophil numbers may be especially pronounced in the context of sand fly transmission in which, compared with needle challenge, a stronger and more prolonged period of neutrophil recruitment has been shown to downmodulate secondary immune responses (32). Our observations once again emphasize that mice with a healed primary infection mount the most appropriate secondary response upon challenge, as evidenced by the robust control of parasite numbers following exposure to the bites of infected sand flies. Importantly, and in contrast to protein-plus-adjuvant vaccination, this secondary response is almost completely devoid of IL-17–producing CD4*γ* cells, suggesting that IL-17 does not have a role in the protective secondary response to sand fly-transmitted *L. major* infection.

Despite the observations reported in this study, the current need is for continued evaluation in people, along with a better understanding of the immune response in individuals with a healed primary infection (48). What our data do suggest is that excessive boosting with immune-stimulatory adjuvants may alter an otherwise protective response. An outstanding question is whether boosting, which is intended to expand Leishmania-specific cells, requires the use of an adjuvant once a Th1 response has been primed. Recent findings by Okwar et al. (49) suggest that heat-killed *L.m.-Ag* alone is sufficient to induce a protective response comparable to that observed in healed mice provided the Ag is administered five times. It will be important to test this boosting regimen against infected sand fly challenge.

The observations reported in this study should provide critical information for the further development of a *Leishmania* vaccine, including the factors determining protection following needle versus infected sand fly challenge and the appropriate use of powerful immunostimulatory adjuvants.

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**Disclosures**

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**References**


