Immunization with a Toll-Like Receptor 7 and/or 8 Agonist Vaccine Adjuvant Increases Protective Immunity against *Leishmania major* in BALB/c Mice

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Activation of Toll-like receptors (TLRs) on antigen-presenting cells of the innate immune system initiates, amplifies, and directs the antigen-specific acquired immune response. Ligands that stimulate TLRs therefore represent potential vaccine adjuvants. In the present study, we determined whether imiquimod and its related compound R848, which are TLR7 and/or TLR8 agonists, represent potential vaccine adjuvants when delivered topically, subcutaneously, or intramuscularly. Using the *Leishmania major* infection model in BALB/c mice, vaccination with crude *Leishmania* antigen was not protective against subsequent challenge infection unless it was administered with R848 or a topical application of imiquimod containing cream on the skin. Subcutaneous vaccination with these adjuvants mediated a TH1 response against *L. major* antigen, which appeared to suppress the TH2 response following a challenge infection. Protective immunity was generated following subcutaneous vaccination but not intramuscular vaccination. These observations suggest that topically administered imiquimod or subcutaneously injected R848 represent potential vaccine adjuvants to enhance the TH1 response, which can be used with existing or new vaccine formulations.

Most vaccines currently in use have been developed by an empirical approach. However, more rational strategies are now needed to develop vaccines against more complex pathogens including *Leishmania*. Future vaccines may be generated on the basis of recent advances in our understanding of how the innate immune response directs the acquired immune response and immunological memory (16). The innate immune response can be activated by diverse pathogen-associated molecular patterns via the Toll-like receptor (TLR) family of receptors, and this plays an important role in directing the acquired immune response (7). Among the 11 mammalian TLRs, TLRs 3, 7, 8, and 9 are present in the endosomes of cells and detect nucleic acids of intracellular DNA and RNA pathogens (23, 26).

Upon the recognition of a pathogen or its components via TLRs, immature antigen-presenting cells such as dendritic cells (DCs) undergo maturation, exit the site of infection, and migrate to the draining lymph node. The mature DCs present their “acquired” antigens and stimulate antigen-specific T cells, leading to antigen-specific acquired immunity along with immunological memory. The type of acquired immunity developed may be influenced by which TLR(s) is activated as well as the particular subset of DC activated (7). The use of TLR agonists as vaccine adjuvants may represent an effective strategy for the development of vaccines with improved protective immunity.

Although there is a plethora of natural pathogen-derived products capable of activating innate immune mechanisms, efforts have focused on developing small synthetic molecules for use in humans that may have advantages with respect to consistent manufacturing as well as with administration by certain routes. This is an important consideration with respect to the development of safe vaccine adjuvants. Imiquimod (R837) is primarily a TLR7 agonist, while R848 (also known as resiquimod) is capable of activating both TLRs 7 and 8 in humans (9). Although mice express TLR8, it appears that it is not activated by human TLR8 agonists except under limited circumstances (5). Topical imiquimod is currently approved as a topical treatment for cutaneous infection with human papillomavirus as well as for actinic keratoses and basal cell carcinoma (11, 21, 24). Because imiquimod is licensed for topical application in humans and is a TLR agonist, we have investigated its potential as a vaccine adjuvant using a well-established live infection model, *Leishmania major* infection in BALB/c mice.

*L. major* is the causative agent of zoonotic cutaneous leishmaniasis, the most common form of human leishmaniasis in the Old World, and is transmitted by the bite of an infected sandfly (15). Rodents are the natural reservoir for *L. major*, and humans are the incidental host. Susceptibility or resistance to *L. major* infection in mice correlates with a T-helper type 2 (TH2) immune response that causes susceptibility and disease or a TH1 response that promotes parasite clearance and resistance to infection (19, 20). Likewise, in humans, there is a good correlation between TH1 responses and resistance to cutaneous leishmaniasis caused by *L. major* (4, 12). In previous studies, vaccination of mice with heat-killed (autoclaved) *L. major* did not provide protection against infection unless an appropriate adjuvant was included (18). Therefore, mice represent an excellent animal model system to study *L. major* infection, immunity, and vaccine adjuvant development.

In the present study, we have investigated the vaccine adju-
vant properties of the TLR7 and/or TLR8 agonists imiquimod and R848 using a live infection model where the immune responses can be determined before and after challenge infection. Vaccination was carried out with autolaved L. major (ALM) in combination with 5% imiquimod cream (Aldara) applied topically over the immunization site to evaluate its ability to mediate a TH1 immune response and provide protective immunity against a challenge infection with live L major promastigotes in BALB/c mice. Vaccination with ALM in conjunction with R848 administered subcutaneously and intramuscularly was also evaluated. BALB/c mice are highly susceptible to L. major; therefore, prophylactic vaccine protection in this animal model represents an important outcome.

MATERIALS AND METHODS

Mice. Female BALB/c mice weighing 15 to 20 g were purchased from Charles River Breeding Laboratories and maintained in the animal care facility under pathogen-free conditions.

Imiquimod and R848. Imiquimod (5% imiquimod cream) and R848 were kindly provided by 3M Pharmaceuticals, London, Ontario, Canada.

Immunization. ALM was prepared from whole-cell, heat-killed L. major promastigotes as previously described (10). Briefly, L. major promastigotes were harvested from stationary cultures and washed twice in phosphate-buffered saline (PBS), resuspended in PBS, and autoclaved at 121°C for 15 min. The protein concentration of ALM was then determined with a Bio-Rad protein assay. ALM (50 μg/BALB/c mouse) was administered alone or with R848 or topical imiquimod cream. R848 was prepared as a sterile stock solution (20 mg/ml) in PBS, and 20 μg was administered in the vaccine preparation with each immunization. Each injection was suspended in sterile PBS and given in a volume of 40 μl. Imiquimod cream (5%) was applied on the injection site (footpad) immediately before the subcutaneous vaccination; a second dose was applied 1 week later. In some experiments, BALB/c mice were also vaccinated by intramuscular injection. For booster immunization, mice received the same immunization at the same site 2 weeks after the primary immunization.

Infectious challenge. L. major Friedlin V9 promastigotes were grown as previously described (27, 28). BALB/c mice were challenged with 5 × 10^6 stationary promastigotes 2 or 10 weeks after the last immunization. Parasites were injected subcutaneously into the footpad contralateral from the site of vaccination in a volume of 40 μl. Weekly measurements of footpad swelling were recorded using a metric caliper. Parasite loads in the footpads of infected mice were determined by limiting dilution as previously described (27, 28). The number of viable parasites in the footpad was determined from the highest dilution of promastigotes that could be grown out after 10 days of culture at 27°C.

Cell cultures and cytokine measurement. Lymphatic tissue (the spleen or the popliteal and inguinal lymph nodes that drained the footpad in mice) was harvested from duplicate mice of each experimental group 2 weeks after the booster immunization or at day 16 postinfection. Cell suspensions were adjusted to 2 × 10^6 cells/ml in Dulbecco’s modified Eagle’s medium containing 10% heat-inactivated fetal calf serum and 5 × 10^-5 M 2-mercaptoethanol (Sigma). Cells were stimulated in vitro (37°C in 5% CO2) with 50 μg/ml of soluble L. major antigen (22), live L. major promastigotes (2 × 10^6 promastigotes/ml), or medium alone. Supernatants were collected after 72 h of culture, and gamma interferon (IFN-γ), interleukin-4 (IL-4), and IL-10 levels were measured by enzyme-linked immunosorbent assay according to the manufacturer’s instructions (eBioscience).

Statistical analyses. The statistical difference among groups was determined by a Student’s t test (for two groups) or by single-factor analysis of variance (for three or more groups). A P value of <0.05 was considered to be significant. Five to 10 mice were included in each experimental group. All experiments were performed a minimum of two or three times with similar results, with the exception of the experiment depicted in Fig. 2, which was performed once but is largely a repeat of the experiment depicted in Fig. 1.

RESULTS

Vaccination against L. major using imiquimod and R848 as adjuvants. In the first set of experiments, BALB/c mice were immunized subcutaneously with ALM alone, ALM including R848, or ALM with imiquimod-containing cream applied topically to the skin. Two weeks following immunization, mice were challenged with 5 × 10^6 L. major promastigotes, and the level of infection was monitored for 10 weeks by measuring footpad swelling. As shown in Fig. 1A, immunization with ALM in the absence of an adjuvant provided no protection against infection, resulting in infection levels similar to those of control mice injected only with saline solution (PBS). In comparison, some decrease in footpad swelling was observed after immunization with ALM along with the topical application of imiquimod or coinjection with R848.

Since mice immunized with ALM in the presence of R848 or topical imiquimod were partially protected with a single immunization, we next determined whether two immunizations might provide better protection. Mice were therefore immunized twice, 2 weeks apart. In this experiment, a group of mice was also immunized intramuscularly with ALM and R848. As shown in Fig. 1B, the two subcutaneous immunizations with ALM along with topical imiquimod or subcutaneous R848 provided a significant level of protection against infection compared to that with PBS or ALM without adjuvant. Interestingly, the progression of infection after immunization with ALM and R848 intramuscularly did not appear to be any better than that with ALM without adjuvant and might have been slightly worse.

In these experiments, footpad thickness was used as a continuous surrogate for infection levels since the mice can be monitored for several weeks without being sacrificed. As shown in Fig. 1C, the level of parasites in the footpad at 6 weeks following challenge infection mirrored the footpad thickness results supporting that the observations shown in Fig. 1B represent the extent of infection in these mice.

The results depicted in Fig. 1 argued that ALM together with topical imiquimod or subcutaneous R848 provided a partial but significant level of protection against infection. However, the possibility remained that the observed protective immunity was entirely due to the TLR agonist. It was therefore necessary to repeat the experiment with two additional groups of mice that received adjuvant alone (topical imiquimod alone or subcutaneous R848 alone). As shown in Fig. 2, mice treated with topical imiquimod alone or subcutaneous R848 alone were not protected against subsequent challenge infection since infection in these groups progressed with the same kinetics as that in mice immunized with ALM alone or PBS. In comparison, mice immunized with ALM in the presence of R848 or topical imiquimod were partially protected, similarly to what is shown Fig. 1. This ruled out the possibility that the activation of TLRs with imiquimod or R848 alone induced protective innate responses against Leishmania infection. Moreover, as shown in Fig. 1, the site of immunization with ALM plus R848 was pivotal since immunization in the muscle may exacerbate the infection following challenge, whereas immunization subcutaneously resulted in a significant level of protection against infection.

Immune response in vaccinated mice before challenge infection. The above-described results demonstrated that the combination of antigen and TLR adjuvant was needed to induce the observed partial protective immunity. It was therefore necessary to characterize the antigen-specific immune response mediated by the TLR agonists. It was previously established that a TH1 cell-mediated immune response is necessary...
for protection and clearance of *Leishmania* infection in mice and humans (4, 19, 20). BALB/c mice, however, develop a nonprotective and disease-promoting TH2 response (19, 20). We therefore examined the type of immune response generated against ALM in the different vaccination groups as determined by measuring cytokines produced by spleen cells stimulated in vitro with soluble *Leishmania* antigen or live *L. major* promastigotes. IFN-γ is the hallmark cytokine produced by TH1 cells, and IL-4 and IL-10 are hallmark cytokines produced by TH2 cells.

As shown in Fig. 3, IFN-γ was detected in significant levels in antigen-stimulated spleen cell suspensions from mice vaccinated with ALM administered subcutaneously plus topical imiquimod or subcutaneous R848. Intramuscular immunization with ALM plus R848 did not, however, result in increased levels of IFN-γ production. In contrast, IL-4 and IL-10 were not detectable in spleen cells following antigen stimulation for all groups of mice (data not shown). The specific production of IFN-γ from spleen cells in response to stimulation with *L. major* antigen in vitro argues that a TH1 immune response was generated against *L. major* antigen specifically in mice immunized with ALM plus imiquimod or ALM plus subcutaneous R848. In contrast, IL-4 and IL-10 were not detectable in spleen cells following antigen stimulation for all groups of mice (data not shown). The specific production of IFN-γ from spleen cells in response to stimulation with *L. major* antigen in vitro argues that a TH1 immune response was generated against *L. major* antigen specifically in mice immunized with ALM plus imiquimod or ALM plus subcutaneous R848.
nized subcutaneously with ALM plus topical imiquimod or subcutaneous R848.

Immunologic response in vaccinated mice after L. major challenge infection. Naive BALB/c mice typically develop a non-protective TH2 response against L. major. It was therefore necessary to examine the immune response generated in the vaccinated BALB/c mice following challenge with live L. major cells to determine whether the protective vaccine overcame this strong TH2 response. As shown in Fig. 4, spleen cell suspensions from infected mice that did not receive a vaccine (PBS control) or that were vaccinated with ALM in the absence of adjuvant all produced antigen-specific IFN-γ, IL-4, and IL-10. Spleen cell suspensions from infected mice vaccinated subcutaneously with ALM plus topical imiquimod or subcutaneous R848, however, had significantly reduced levels of IL-4 and IL-10 production and produced similar or slightly higher levels of IFN-γ than those in infected mice that had not been vaccinated (PBS control). In comparison, infected mice that had received the intramuscular vaccination with ALM plus R848 had decreased levels of IL-4 but not IL-10 production. Since IL-10 mediates susceptibility to L. major infection, this could explain why intramuscular vaccination with ALM plus R848 did not provide protection against infection even though IFN-γ was produced. Taken together, the protective vaccines incorporating ALM plus topical imiquimod or subcutaneous R848 both mediated a higher ratio of TH1 cytokine (IFN-γ) to TH2 cytokine (IL-4 and IL-10) in the spleen that resulted predominantly from a reduction of levels of antigen-specific IL-4 and IL-10 production.

We also examined the antigen-specific immune response in the draining lymph node (DLN) cell suspensions from these infected mice (Fig. 5). It is noteworthy that live promastigotes represented a much better target for the antigen-presenting cells than soluble L. major antigen in these analyses of the DLN. Subcutaneous vaccination with ALM plus topical imiquimod or subcutaneous R848 resulted in a significant reduction in the levels of antigen-specific production of IL-4 and IL-10 in these mice compared to those of control nonvaccinated mice (PBS), mice vaccinated with ALM alone, or mice vaccinated intramuscularly with ALM plus R848. Interestingly, the level of IFN-γ production was also lower in the groups receiving subcutaneous ALM plus topical imiquimod or subcutaneous R848 than in mice vaccinated subcutaneously with ALM alone or intramuscularly with ALM plus R848. The overall observation from the cytokine analysis revealed that the immunological protection associated with subcutaneous vaccination with ALM in the presence of topical imiquimod or subcutaneous R848 following a challenge infection was more closely associated with a reduction in the levels of TH2-associated IL-4 and IL-10 production than with an increase in TH1-associated IFN-γ production.
Protective immunity 10 weeks following vaccination. Long-term protection against infection represents a more stringent criterion for vaccine adjuvant efficacy. We therefore determined whether the protective adjuvant effect observed with topical imiquimod or subcutaneous R848 could continue to provide some protection 10 weeks following vaccination. Mice vaccinated subcutaneously with ALM plus topical imiquimod or subcutaneous R848 showed significant protection compared to that with ALM alone when challenged 10 weeks after vaccination (Fig. 6). Although not as effective as challenging 2 weeks after vaccination, this demonstrates that the antiparasitic immune response generated using R848 or topical imiquimod was present at least 10 weeks following vaccination in these mice.

Discussion

The primary objective of the present study was to determine whether TLR7- and/or TLR8-activating molecules could be effective as vaccine adjuvants when injected or applied topically on the skin. The results from this study demonstrated that ALM vaccination subcutaneously in the presence of topical imiquimod or subcutaneous R848 resulted in significant protection against *L. major* infection in BALB/c mice. The cytokine analysis argued that the subcutaneous vaccines prime the TH1 response as demonstrated from the data shown in Fig. 3. Following the live challenge infection, the primed TH1 response then suppressed the TH2 response, as shown in Fig. 4 and 5. These TLR7 and TLR8 adjuvants therefore altered the quality and not necessarily the magnitude of the immune response. Second, the route of immunization had a major influence on the efficacy of the vaccine. Subcutaneous ALM vaccination with imiquimod or R848 was protective, but vaccination with ALM plus R848 administered intramuscularly was no better and possibly slightly worse than vaccination with control or subcutaneous ALM without adjuvant. This may have wider implications for vaccination in general, many of which are delivered intramuscularly. Third, from a practical consideration, this study shows that the imiquimod vaccine adjuvant can be administered topically on the skin, together with subcutaneous R848. The results are representative of data from two experiments. SLA, soluble *L. major* antigen; Lmpro, *L. major* promastigotes.
design of combination adjuvants that stimulate the necessary immune response for specific pathogens, and the application of topical imiquimod could represent a component of such combinations.

Previous studies have shown that imiquimod and R848 represent potential vaccine adjuvants (8, 25), including a live infection model for herpesvirus infection in guinea pigs (3). This is the first study, however, to investigate the use of imiquimod used topically on the skin and to examine the immune response before and after challenge infection. The use of the L. major infection model in BALB/c mice is well suited to investigate the vaccine adjuvant properties of topical imiquimod and R848 because ALM is not protective against infection in mice unless it is administered with an effective adjuvant. The finding that BALB/c mice are highly susceptible to L. major infection because they mount a nonprotective TH2 immune response (19, 20) makes these data more noteworthy.

We have not investigated the mechanism in which topical imiquimod or subcutaneous R848 mediated the altered balance between TH1 and TH2. However, it was previously reported that R848 injected subcutaneously into the footpads of mice induced the recruitment of natural killer (NK) cells to the draining lymph nodes, and these NK cells provided an early source of IFN-γ necessary for TH1 cell polarization (13). More recently, it was shown that the topical application of imiquimod to the skin of mice mediates the activation of dermis mast cells, resulting in the rapid emigration and activation of Langerhans cells, followed by increased numbers of mature Langerhans cells in the DLN (6). Imiquimod/R848 activation of NK cells, mast cells, and Langerhans cells in the skin could collectively contribute to more effectively bridging the interface between the innate and adaptive immunity producing the vaccine adjuvant properties demonstrated within.

Although subcutaneous vaccination with ALM plus topical imiquimod or subcutaneous R848 provided significant protection against infection, it was surprising that intramuscular immunization with ALM plus R848 provided no protection. It was evident that subcutaneous immunization mediated reduced antigen-specific IL-4 and IL-10 production in the DLN and spleen. In contrast, intramuscular immunization did not result in reduced levels of IL-10 production. It has been argued that IL-10 is as important as IL-4 in the evolution of susceptibility to L. major infection in BALB/c mice (19, 20). Therefore, the failure of intramuscular vaccination with ALM plus R848 was likely due to its failure to suppress antigen-specific IL-10 production. It is possible that the muscle lacks or has fewer of the appropriate cells to respond to R848 or that the populations of responsive antigen-presenting cells resident in the muscle are different from those in the skin, resulting in differences in the amounts of IL-10 production. This result highlights the need to better define the interaction between innate immunity and adaptive immunity originating at different sites used for vaccine delivery.

These results are consistent with results from previous experimental vaccine studies using subcutaneous immunization with ALM or recombinant protein with CpG, a TLR9 agonist (18). That study showed that vaccination with autoclaved subcutaneous L. major plus CpG resulted in an enhancement of a protective TH1 response. It would be interesting to determine whether there is a synergistic effect of adjuvants, since different TLRs such as TLRs 7 and/or 8 and 9 are expressed at different levels in distinct antigen-presenting cells. For example, subcutaneous immunization with CpG may be more effective when combined with topical imiquimod. In addition, there may be a synergistic effect by combining topical imiquimod with subcutaneous injection of ALM plus R848. A vaccine that stimulates complementary TLR pathways may broaden the TH cell response, as recently shown with the yellow fever YF17D vaccine, which activates multiple DC subsets by stimulating TLRs 2, 7, 8, and 9 (17).

The main objective of this study was not to develop the optimum vaccine for leishmaniasis but rather to investigate whether and how the TLR7 and TLR8 agonists may be incorporated into future vaccines. However, the observations reported within are relevant when considering a Leishmania vaccine. First, Leishmania is delivered to the skin by the bite of an infected sandfly during a natural infection. Following the resolution of infection, people are immune for life, demonstrating that protective immunity can be achieved by delivering Leishmania antigen to the skin. Second, we have shown that the treatment of human cutaneous leishmaniasis with topical imiquimod is safe and, in combination with intravenously administered antimony, can enhance the rate of cure of established infections (1, 2, 14). The adjuvant properties of topical imiquimod together with the Leishmania antigen released by antimony killing of the parasite may have enhanced the immunological clearance of infection in these trials. Finally, ALM has been used in human prophylactic leishmaniasis vaccine trials, albeit with limited success (10). As demonstrated within, however, the route of immunization and adjuvant selection have a major influence on the efficacy of vaccination with ALM. This suggests that future vaccine trials for Leishmania or other pathogens may consider including topical imiquimod as an adjuvant either alone or in combination with other adjuvants following subcutaneous immunization.

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