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Topical Resiquimod Protects against Visceral Infection with *Leishmania infantum chagasi* in Mice

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New prevention and treatment strategies are needed for visceral leishmaniasis, particularly ones that can be deployed simply and inexpensively in areas where leishmaniasis is endemic. Synthetic molecules that activate Toll-like receptor 7 and 8 (TLR7/8) pathways have previously been demonstrated to enhance protection against cutaneous leishmaniasis. We initially sought to determine whether the TLR7/8-activating molecule resiquimod might serve as an effective vaccine adjuvant targeting visceral leishmaniasis caused by infection with *Leishmania infantum chagasi*. Resiquimod was topically applied to the skin of mice either prior to or after systemic infection with *L. infantum chagasi*, and parasite burdens were assessed. Surprisingly, topical resiquimod application alone, in the absence of vaccination, conferred robust resistance to mice against future intravenous challenge with virulent *L. infantum chagasi*. This protection against *L. infantum chagasi* infection persisted as long as 8 weeks after the final topical resiquimod treatment. In addition, in mice with existing infections, therapeutic treatment with topical resiquimod led to significantly lower visceral parasite loads. Resiquimod increased trafficking of leukocytes, including B cells, $CD4^+$ and $CD8^+$ T cells, dendritic cells, macrophages, and granulocytes, in livers and spleens, which are the key target organs of visceralizing infection. We conclude that topical resiquimod leads to systemic immune modulation and confers durable protection against visceralizing *L. infantum chagasi* infection, in both prophylactic and therapeutic settings. These studies support continued studies of TLR-modulating agents to determine mechanisms of protection and also provide a rationale for translational development of a critically needed, novel class of topical, preventative, and therapeutic agents for these lethal infections.

An estimated 12 million people worldwide are infected by the protozoan parasite *Leishmania*, with a growing number (350 million) at risk of infection (1, 2). Clinical syndromes associated with leishmaniasis range from skin and mucous-membrane ulcers (cutaneous leishmaniasis [CL] and mucocutaneous leishmaniasis) to systemic, potentially fatal, disease of the organs (visceral leishmaniasis [VL]). First-line chemotherapy for VL includes antimonial compounds, amphotericin B, paromomycin, and miltefosine, but these options are limited by various factors, including cost, toxicity, and drug resistance (3). Although there are currently no effective vaccines for human use, combining immunotherapeutic approaches with conventional chemotherapy might offer several advantages over treating leishmaniasis with chemotherapy alone (4, 5).

Host immunity is a critical factor that influences the outcome of infection with *Leishmania* species. Toll-like receptors (TLRs) on innate immune cells are critical components of pathways stimulating cellular immunity. TLRs found on antigen-presenting cells (APCs) recognize microbe-associated molecules and trigger APC activation, expression of costimulatory molecules, and cytokine release. Naturally occurring and synthetic TLR ligands have therefore been extensively explored as a means to activate APCs in the context of vaccination. The imidazoquinoline family is comprised of small nucleoside analogs that specifically activate TLR7 and/or TLR8 and stimulate plasmacytoid dendritic cells (pDCs) to mature and produce type I interferons and other cytokines (6, 7). The TLR7 agonist imiquimod is FDA-approved for clinical use against a variety of cutaneous viral infections and neoplasias, including genital warts, actinic keratoses, and superficial basal cell carcinomas (8). Several studies have examined the potential of imiquimod for treating cutaneous *Leishmania* infections (9). In mice, topical imiquimod application reduced infection levels and ulceration in mouse footpads infected with *L. major* (10). Human trials examining the therapeutic benefits of imiquimod against both Old World (11–14) and New World (15–18) CL have demonstrated mixed results, with treatment regimens combining imiquimod with antimonial chemotherapy faring better than imiquimod alone.

Resiquimod (also known as R-848 and S-28463) is a related imidazoquinoline that can activate both TLR7 and TLR8 pathways in humans and is 10- to 100-fold more potent *in vitro* and *in vivo* than imiquimod (7, 19, 20). Although resiquimod has not been as extensively characterized as imiquimod in infectious disease models, both molecules have been studied as vaccine adjuvants against multiple intracellular pathogens and tumors (21–28). We initially sought to determine whether topically administered resiquimod might serve as a useful vaccine adjuvant to enhance immunity against visceral infection with *L. infantum chagasi*. We unexpectedly found that topical resiquimod treatment protected mice from systemic VL infection even in the ab-

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sence of vaccination, suggesting a potency and utility not previously associated with imiquimod. Here we characterize both the prophylactic and therapeutic efficacies of topical resiquimod against VL, as well as the cellular infiltrates in the liver and spleen of resiquimod-treated mice that correlate with reduced parasite burdens.

MATERIALS AND METHODS

Animals, parasites, and reagents. Six- to eight-week-old female BALB/c mice (Jackson Laboratories) were used for all animal experiments. The *L. infantum chagasi* strain MHOM/BR/00/1669 (previously known as *L. chagasi* and now considered synonymous with *L. infantum*) (29), originally isolated from a patient in northeastern Brazil, was used to vaccinate and challenge experimental animals. Parasites were maintained in outbred male golden hamsters, isolated from splenocytes, and passaged in HOMEM medium (30) at 26°C. Cultures enriched for infective metacyclic parasites were obtained by growing freshly subcultured organisms for 5 to 7 days to achieve stationary phase. The imidazoquinoline compounds resiquimod (also known as R-848 or S-28463) and imiquimod were graciously provided by 3M Pharmaceuticals and Graceway Pharmaceuticals (24). 3M Pharmaceuticals prepared the imiquimod in a 5% cream formulation, the resiquimod in 1% cream and 0.2% gel formulations, and control vehicles for both cream and gel. Soluble resiquimod (Sigma-Aldrich) was reconstituted at a concentration of 1 mg/ml in 10% dimethyl sulfoxide (DMSO) and diluted as necessary.

MTT viability assay. Logarithmic-phase *L. infantum chagasi* promastigotes were pelleted at 3,500 rpm for 15 min, washed and resuspended in HOMEM, and placed in a sterile 24-well plate at a concentration of 5×10^6/well with various concentrations of resiquimod or a vehicle (0.5% DMSO) control. Promastigotes were incubated at 26°C for 48 h and then treated with 10 μl of 12 mM MTT (Molecular Probes, CA) according to the manufacturer's protocol. The absorbance was read at 540 nm using a Synergy2 multimode microplate reader to determine the amount of formazan production, which correlates with the relative parasite viability.

Intracellular parasite assay. RAW264.7 mouse macrophages (American Type Culture Collection) growing in complete Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) were harvested, plated in 12-well plates on top of sterile coverslips at a concentration of 2×10^5/well, and allowed to adhere to coverslips overnight. Cells were infected with 10^7 *L. infantum chagasi*/well for 4 h, washed to remove extracellular parasites, and treated with various concentrations of resiquimod. Infection levels were determined after 24 h by microscopic examination of cells, following staining of coverslips using Hema-3 staining reagents (Fisher Scientific).

Prophylactic protection studies. Mice were vaccinated subcutaneously with 10^7 *L. infantum chagasi* promastigotes in the dorsum of the neck, where the parasites remain localized. Resiquimod was applied topically to the area of vaccination on the days described. Mice were euthanized at described time points, and touch preparations of liver sections were made onto glass slides, Giemsa stained, and microscopically analyzed by a blind scorer. Parasites were counted in a minimum of 500 mammalian cells, and the amastigote/cell ratio was multiplied by the liver weight (in mg). As an approximation of the total amastigote load in the liver, this product was multiplied by 2×10^5, a conversion factor that has been previously defined (31).

Therapeutic treatment studies. An inoculum of 10^7 stationary-phase virulent *L. infantum chagasi* promastigotes was intravenously injected into BALB/c mice to establish visceral infection. Starting 3 days after infection, resiquimod (either 1% cream or 0.2% gel) was applied to the shaved backs of mice. Mice were euthanized and organs were isolated for liver touch preps, quantitative PCR (qPCR), or flow cytometric analysis.

qPCR. To determine parasite loads by qPCR, livers and spleens were mechanically homogenized in phosphate-buffered saline (PBS), and total genomic DNA was harvested using an UltraClean tissue DNA isolation kit (MoBio Laboratories). qPCR assays were then performed using a TaqMan system (Life Technologies), with 200 nM primers/probe and genomic DNA template diluted 1:10 following column elution. *L. infantum chagasi* parasite DNA and mouse DNA were detected using primers specific for GP63 and RPLP0 (36B4), respectively. GP63 primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA) with the following sequences: GP63 for, 5′-GTA CGG CTG CGA CAC CTT-3′; GP63 rev, 5′-AGC CGA GGT CCT GGA AGA T-3′; and GP63 probe, 5′-/56-FAM-AGC CCG CAC CGC CCT GGT-36-TAMSp-3′ (where FAM is 6-carboxyfluorescein and TAMSp is 6-carboxytetramethylrhodamine). The RPLP0 TaqMan gene expression assay (Mm00725448_s1) was purchased from Life Technologies. GP63 threshold cycle (C_T) values below 35 were converted to absolute parasite counts using previously determined standard curves and normalized to tissue DNA amounts by RPLP0 C_T values, as previously described (32). Relative parasite loads per cell were calculated by dividing GP63 values by RPLP0 values; relative parasite loads per organ were calculated by multiplying these normalized values by organ weight (in mg). GP63 C_T values above 35 were considered to be below the limit of detection, based on negative controls with no *Leishmania* DNA template.

Preparation of lymph nodes, spleens, and liver leukocytes and flow cytometry. Spleens and livers were isolated and pressed through a nylon filter, and suspensions were centrifuged. Pellets were resuspended in 1× Pharmlyse (BD Biosciences) and incubated at room temperature for 5 min; PBS was then added, and the samples were centrifuged and resuspended in PBS. Aliquots of 10^6 cells from each organ sample were prepared in staining buffer (PBS, 3% FBS, 0.1% sodium azide) with Fc block, incubated with the appropriate antibodies for 15 to 30 min at 4°C, washed and resuspended in staining buffer, and analyzed by flow cytometry on a BD LSR II cytometer. $CD45^+$ leukocytes were gated for further analysis. Total leukocytes in both liver and spleen were counted and multiplied by percentages of each gated subset to quantitate. All antibodies were purchased from BD Pharmingen.

Statistics. Student *t* test was used for all statistical analyses. *P* values < 0.05 were considered significant.

Ethics statement. All animal procedures were conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals and the LA BioMed IACUC.

RESULTS

Topical resiquimod prophylactically protects mice against visceral leishmaniasis in the absence of antigen-specific vaccination. We initially set out to investigate the vaccine adjuvant properties of resiquimod, given the previously reported ability of the related molecule imiquimod to enhance both cellular and humoral immune responses, particular T_H1 responses (33). We used an established method of antileishmanial vaccination (34), by subcutaneously injecting a high inoculum (10^7) of live *L. infantum chagasi* as an antigen source into the dorsum of the neck (these parasites remain localized and do not disseminate). Topical formulations of either imiquimod (included as a control reference) or resiquimod were applied to shaved areas of skin in conjunction with subcutaneous inoculation of the live leishmanial vaccine. A total of three subcutaneous vaccinating doses were delivered, each 2 weeks apart, and each accompanied by three TLR agonist applications (on days −1, 0, and 1 relative to the vaccine injection). As observed by other investigators (34), subcutaneous vaccination with *L. infantum chagasi* alone (with vehicle) led to a reduction in parasite burden (~25%) compared to control (saline-injected) mice at 4 weeks after challenge (Fig. 1A). Vaccination accompanied by imiquimod treatment significantly enhanced protection (~65%), whereas vaccination plus resiquimod conferred even higher levels of protection (>90%). Surprisingly, topical administration of resiquimod to unvaccinated mice also conferred high

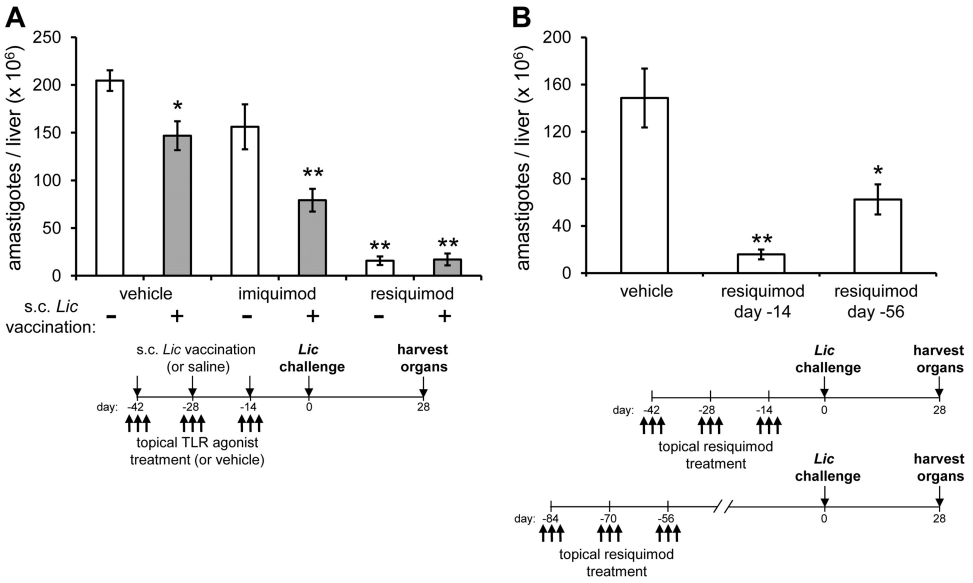

FIG 1 Topical resiquimod treatment prophylactically and durably protects mice against experimental visceral leishmaniasis. (A) Mice were injected subcutaneously with three doses of either saline (no antigen; white bars) or a live *L. infantum chagasi* vaccine (gray bars), each administered 2 weeks apart. Concurrently, either imiquimod (5% cream), resiquimod (1% cream), or a vehicle cream was applied topically to the shaved dorsal area of mice on the day before, the same day, and the day after vaccination injections. At 14 days after the final vaccination, the mice were intravenously challenged with virulent *L. infantum chagasi* and euthanized 4 weeks later to assess average parasite burdens in the liver ($n = 8$ mice per group. (B) Vehicle cream or resiquimod (1% cream) was applied to mice in the absence of vaccination, in three 3-day sets, as before. Mice were challenged with *L. infantum chagasi* either 14 days or 56 days after the last treatment and then euthanized 4 weeks later ($n = 4$ to 5 mice/group for each resiquimod group, $n = 7$ mice/group for vehicle). All results are representative of at least two independent experiments. Error bars denote the standard errors of the means (SEM); all comparisons are to vehicle with no vaccination. *, $P < 0.05$; **, $P < 0.005$.

levels (>90%) of protection. These results demonstrate a potent efficacy of resiquimod compared to imiquimod against intravenous challenge with *L. infantum chagasi* and suggest that the full protective effects of resiquimod do not require prior or concurrent antigen-specific priming.

Short-term induction of cytokine and chemokine expression by resiquimod, as previously shown, could result in protection from infection. To determine whether resiquimod-induced protection was due to short-lived immune responses or persisted over time as a form of durable immunity, we again treated mice over the course of 4 weeks with topical resiquimod alone. We then challenged the mice intravenously with virulent *L. infantum chagasi* either two or 8 weeks after the last resiquimod application. Although protection after 8 weeks was somewhat diminished compared to the protection observed after 2 weeks, the parasite burden remained significantly lower in resiquimod pretreated mice compared to untreated mice in both cases (Fig. 1B), indicating a long-lasting, vaccine-independent protective effect of resiquimod alone against systemic infection.

Topical resiquimod is effective therapy against established experimental murine visceral leishmaniasis. The ability of resiquimod to prophylactically protect mice against subsequent parasite challenge raised the possibility that resiquimod might also have therapeutic efficacy against existing infections. To test whether topical resiquimod treatment would decrease the parasite burden of mice with established VL infections, we injected naive BALB/c mice intravenously with virulent *L. infantum chagasi*. Four days later, we began topical applications of resiquimod either once or three times per week to the shaved healthy skin of infected animals. After 4 weeks of treatment, the animals were euthanized and parasite burdens were quantitated. Mice treated with resiquimod had a significant reduction in liver parasite burdens compared to untreated mice (Fig. 2). Resiquimod treatment once per week displayed equivalent protection levels as treatment three times per week.

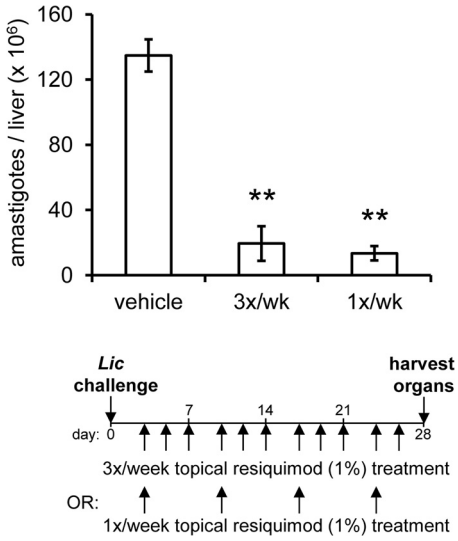

FIG 2 Topical resiquimod treatment leads to reduction of established visceral *Leishmania* infection. (A) Mice were intravenously infected with *L. infantum chagasi*. After 4 days, vehicle cream or resiquimod (1% cream) was applied to shaved dorsal areas either once or three times per week, for 4 weeks. Mice were euthanized 28 days after infection to assess parasite burden in the liver ($n = 5$ mice per group). The results are representative of two independent experiments. Error bars denote the SEM. **, $P < 0.005$.

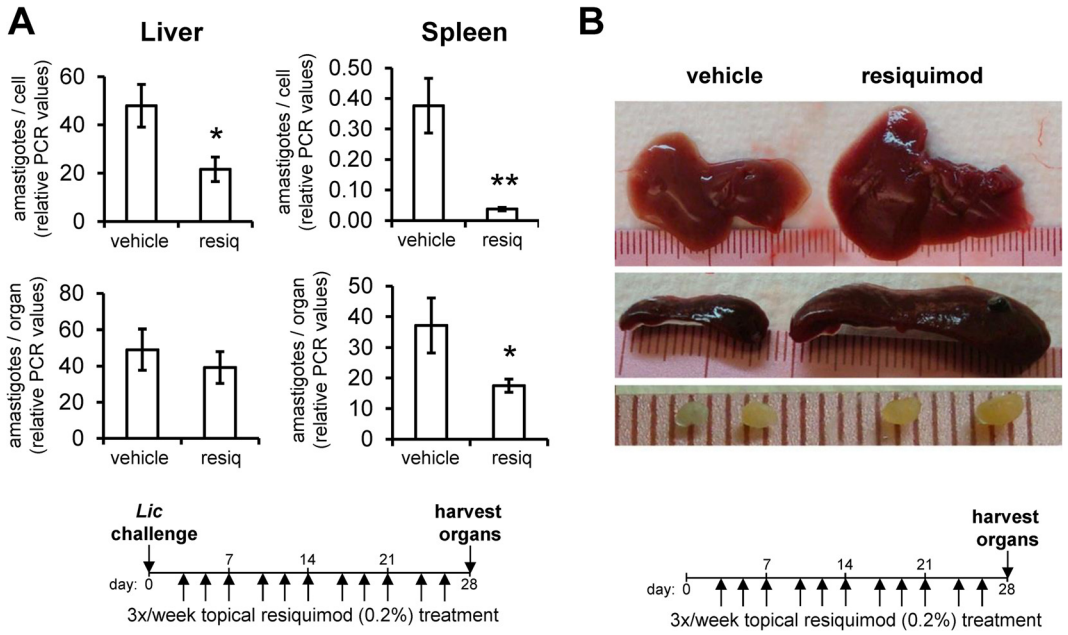

FIG 3 Topical resiquimod treatment leads to organomegaly and reduction of parasite density in liver and spleen. (A) Mice were intravenously infected with *L. infantum chagasi*. After 4 days, vehicle gel or resiquimod (0.2%) was applied to the shaved dorsal areas of mice three times a week for 4 weeks. Mice were euthanized 28 days after infection, and the parasite burdens in the liver and spleen were measured by qPCR. Parasite loads are expressed in relative PCR units as either parasites per cell or parasites per organ ($n = 8$ to 10 mice per group). The results are representative of two independent experiments. Error bars denote the SEM; *, $P < 0.05$; **, $P < 0.005$. (B) Livers, spleens, and lymph nodes from uninfected mice that were treated with resiquimod (0.2% gel, or vehicle gel) for 4 weeks were harvested and weighed. Displayed are representative livers (top), spleens (middle), and a pair of lymph nodes (bottom) from each group. Average weights were calculated from $n = 8$ animals per group.

To determine whether the antileishmanial effects of resiquimod were dose concentration dependent, we next tested the therapeutic efficacy of a less concentrated (0.2%) formulation of resiquimod than has previously been tested in healthy adults (35). As a more sensitive assessment of parasite burden, we utilized qPCR to measure the amount of parasite DNA in organ homogenates, a method which generates similar quantitative results as liver section microscopy and allows for the quantitation of parasite loads in the spleen (which are difficult to assess by microscopy). Topical 0.2% resiquimod treatment over 4 weeks led to a significant parasite reduction in both the liver and spleen on a per-cell basis, as measured by PCR (Fig. 3A). Overall parasite burdens were also significantly reduced in the spleens of resiquimod-treated mice, but not in the liver. As in prior experiments, we observed that resiquimod-treated mice displayed significant increases in organ sizes. Since both *Leishmania* infection and TLR agonist treatment are known to cause organ enlargement (22, 36), we verified that the organ enlargements were primarily due to resiquimod, by topically treating uninfected mice with 0.2% resiquimod as before. Enlargement of livers and spleens were consistently observed in naive, resiquimod-treated mice compared to vehicle-treated mice (Fig. 3B), with an average liver weight of 1.86 g versus 1.13 g ($P = 3.2 \times 10^{-8}$) and an average spleen weight of 480 mg versus 95 mg ($P = 3.5 \times 10^{-15}$). Giemsa-stained liver and spleen sections displayed no differences in gross architecture, despite their enlargement following resiquimod treatment. Due to the resiquimod-induced increased size of the liver, the total numbers of parasites in the liver were not significantly different from controls, even though the density of parasites was reduced by resiquimod (Fig. 3A). Overall, these data demonstrate that topical resiquimod treatment impacts both organ size and parasite density in both the liver and spleen in mice with established infections.

Resiquimod has limited effects on *Leishmania infantum chagasi* promastigotes *in vitro*. We next assessed the direct *in vitro* effects of soluble resiquimod on *L. infantum chagasi* parasites to determine whether the *in vivo* protection could be explained by direct leishmanicidal activity. Log-phase promastigotes were exposed to various concentrations of soluble resiquimod for 48 h, and viability was measured by using an MTT assay. Exposure of promastigotes to resiquimod at various concentrations *in vitro* had no detectable effect on promastigote growth compared to controls (Fig. 4A). Since *Leishmania* parasites predominantly exist in the intracellular amastigote stage during their life cycle following infection, we next determined whether resiquimod could impact amastigote survival within macrophages. RAW264.7 macrophages were infected with *L. infantum chagasi* promastigotes and then treated with increasing doses of resiquimod. Resiquimod treatment for 24 h did not lead to a reduction in the percentage of infected macrophages or the numbers of parasites per cell, with moderate increases (not statistically significant) observed for both measurements (Fig. 4B). We therefore did not detect any significant parasiticidal activity of soluble resiquimod on *Leishmania* growth *in vitro*, either as axenic promastigotes or intracellular amastigotes within macrophages.

Topical resiquimod alters cellular immune profiles in target organs. Toll-like receptor agonist molecules, including imidazoquinolines, alter cell migration (21), induce cytokine production (20), and modulate the distribution of leukocyte subsets (22, 36). To determine the immunological impact of topical resiquimod in the VL model, we assessed the cellular content of livers and spleens

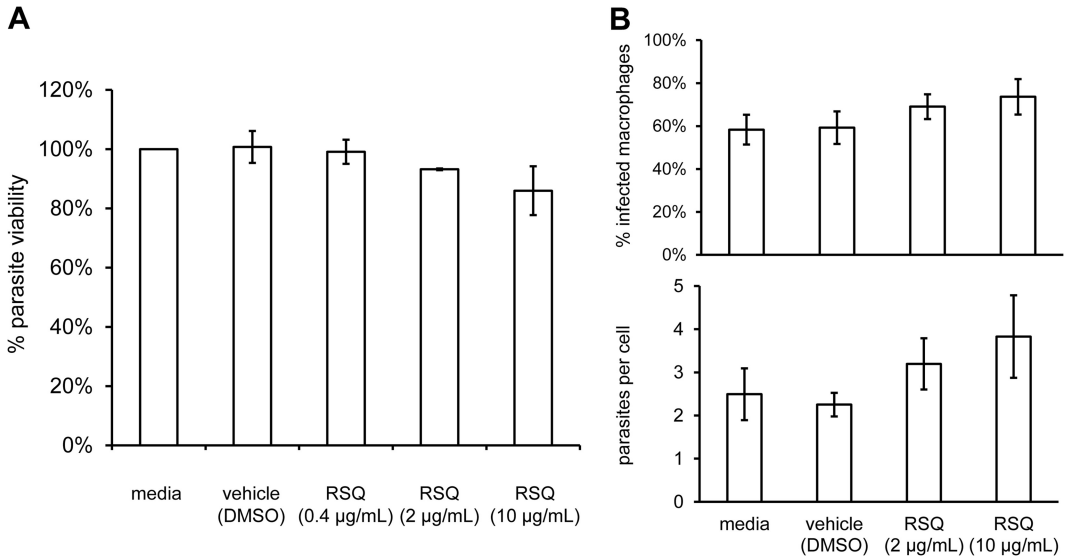

FIG 4 Soluble resiquimod does not impact growth of promastigotes or intracellular amastigotes *in vitro*. (A) Log-phase promastigotes were incubated with increasing concentrations of soluble resiquimod, and metabolic activities (normalized to media alone control) were assessed after 48 h with an MTT assay. Error bars denote the SEM of three combined independent experiments; each experiment was performed in triplicate. (B) RAW264.7 macrophages harboring *L. infantum chagasi* amastigotes were treated with increasing concentrations of soluble resiquimod, and infected cells were assessed by microscopy after 24 h to quantitate the percentage of cells infected and the average number of parasites per cell. Error bars denote the SEM of four independently infected coverslips.

from both infected and uninfected mice after 4 weeks of treatment with 0.2% resiquimod. Consistent with the significant increases in organ sizes observed after resiquimod treatment, there were higher overall numbers of leukocytes in both the livers and the spleens of resiquimod-treated mice compared to mice treated with vehicle gel. Approximately 100×10^6 live splenocytes were recovered from vehicle mice compared to an average of 280×10^6 splenocytes from resiquimod-treated mice, regardless of infection status (Fig. 5). Homogenized liver preparations yielded 6×10^6 live leukocytes compared to an average of approximately 40×10^6 leukocytes from vehicle- and resiquimod-treated mice, respectively, whether naive or *L. infantum chagasi* infected (Fig. 6).

To determine changes in the numbers of specific cellular subsets after 4 weeks of resiquimod treatment, we used flow cytometry and various antibodies to detect cellular markers. Resiquimod-treated mice had higher numbers of $CD19^+$ B cells and cells expressing major histocompatibility complex (MHC) class II on their surface [I-A(d)$^+$] in the spleen (Fig. 5). A significant

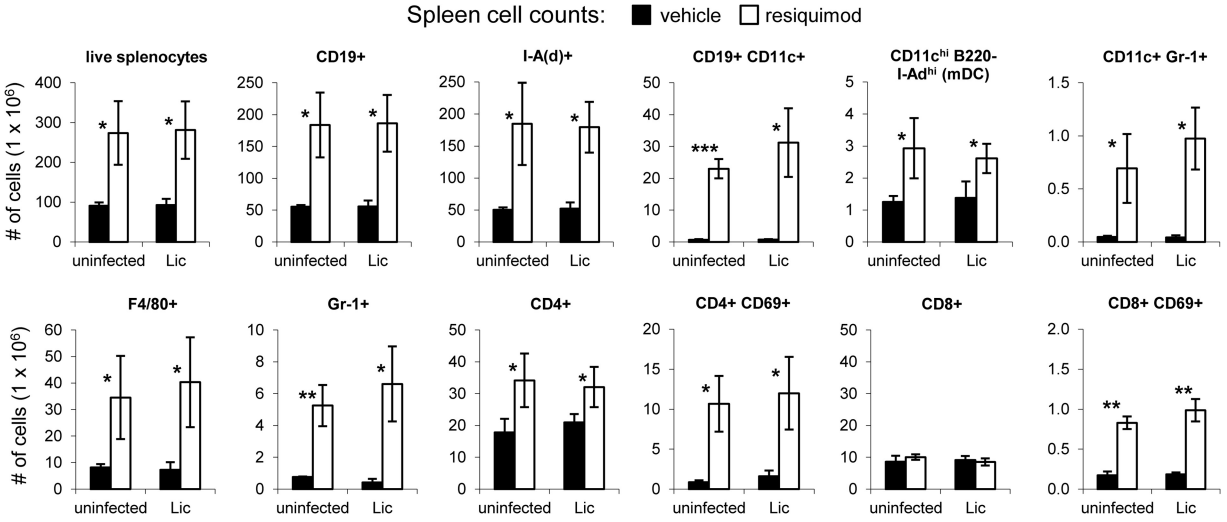

FIG 5 Topical resiquimod treatment leads to increased leukocyte numbers and activation status in spleens of both uninfected and *Leishmania*-infected mice. Mice were intravenously injected with either saline (uninfected) or *L. infantum chagasi*. After 4 days, vehicle gel (black bars) or resiquimod (0.2%; white bars) was applied to the shaved dorsal areas of mice three times a week for 4 weeks (as in Fig. 3). Mice were euthanized 28 days after infection. Single cell suspensions of splenocytes were counted, stained with antibodies against various cell surface markers, and analyzed by flow cytometry. The data are presented in millions of total cells. Error bars represent the SEM ($n = 3$ mice per group). *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$. Vehicle was compared to resiquimod for each comparison.

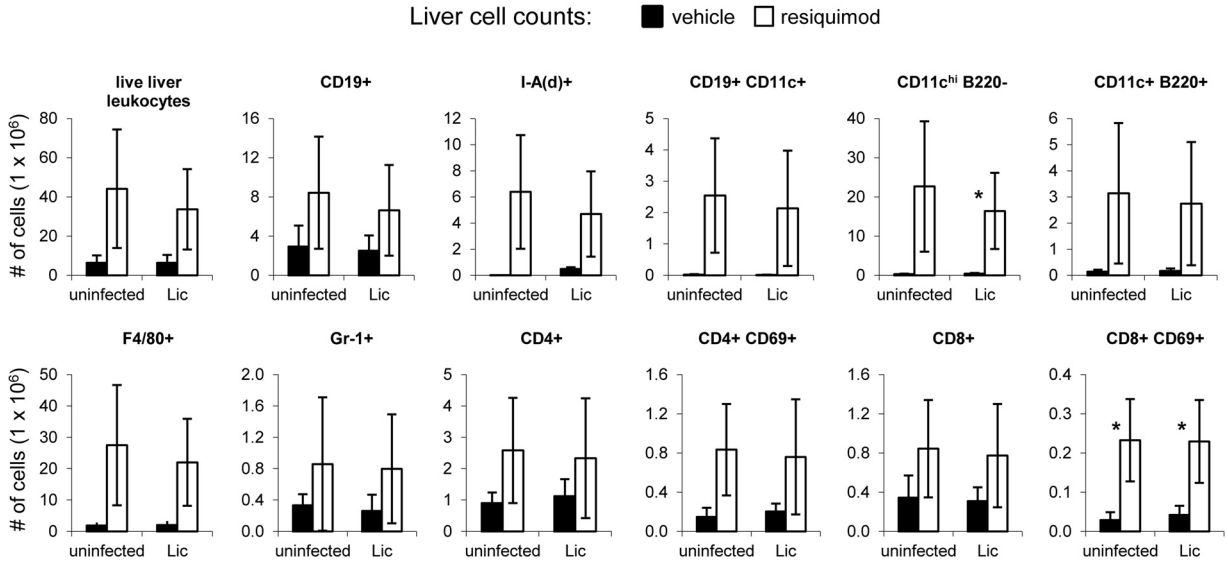

FIG 6 Topical resiquimod treatment leads to increased leukocyte numbers and activation status in livers of both uninfected and *Leishmania*-infected mice. Mice were intravenously injected with either saline (uninfected) or *L. infantum chagasi*. After 4 days, vehicle gel (black bars) or resiquimod (0.2%; white bars) was applied to the shaved dorsal areas of mice three times a week for 4 weeks. Mice were euthanized 28 days after infection. Single cell suspensions of livers were counted, stained with antibodies against various cell surface markers, and analyzed by flow cytometry. The data are presented in millions of total cells. Error bars represent the SEM ($n = 3$ mice per group). *, $P < 0.05$. Vehicle was compared to resiquimod for each comparison.

subset of the resiquimod-induced CD19$^+$ cells were also CD11c$^+$, cells that were not detected in untreated mice. Non-B cell CD11c$^+$ cells that were B220$^-$ but expressed high levels of MHC class II [I-A(d)$^+$] were likely myeloid DCs and were moderately increased in spleens from resiquimod-treated mice. A small population of CD11c$^+$ Gr-1$^+$ pDCs was detected in resiquimod-treated mice (but not untreated mice) as well. Resiquimod treatment also led to significant increases in the numbers of neutrophils (Gr-1$^+$ cells) and macrophages (F4/80$^+$), but not NK cells (data not shown). Resiquimod led to increased expression of the early activation marker CD69 on CD4$^+$ and CD8$^+$ T cells and to higher overall numbers of CD4$^+$ T cells in the spleen. However, the total number of CD8$^+$ cells was not significantly different compared to controls. All resiquimod-induced cellular changes observed were similar in both *L. infantum chagasi*-infected and in uninfected animals. In the liver, resiquimod-induced cellular changes similar to those in the spleen were observed. However, most of these differences were not statistically significant due to high variability in the number of live leukocytes isolated from livers. The numbers of CD19$^+$ B cells, CD11c$^+$ DCs, Gr-1$^+$ neutrophils, F4/80$^+$ macrophages, and activated CD4$^+$ and CD8$^+$ T cells all trended higher in resiquimod-treated livers (Fig. 6) compared to vehicle treatment, similar to the spleen. Unlike the spleen, we were unable to detect any CD11c^{int} Gr-1$^+$ pDCs in the liver. Large increases were seen overall in the number of APCs expressing MHC class II molecules [I-A(d)$^+$]. In sum, these data demonstrate significant increases in specific subsets of lymphocytes, granulocytes, macrophages, and APCs in the liver and spleen, the major organs infected by visceralizing species of *Leishmania*.

DISCUSSION

We initially set out to explore the adjuvant properties of resiquimod compared to imiquimod, hypothesizing that it would enhance prophylactic vaccination in a murine model of VL. Resiquimod applied topically during a 4-week course of subcutaneous vaccination did indeed result in significantly reduced liver parasite burdens following systemic challenge, to a greater extent than imiquimod. Surprisingly, however, resiquimod was directly effective, even in the absence of vaccination. Even when administered up to 8 weeks prior to parasite challenge, resiquimod treatment alone generated significant prophylactic protection compared to controls. The long-lasting nature of this protection was striking and suggested that resiquimod stimulates protective mechanisms that do not require prior priming of *Leishmania* antigen-specific T cells. We hypothesized that other cellular changes resulting from resiquimod-induced cytokine and chemokine production might be potential drivers of the observed protection. The cellular changes in both the liver and the spleen that we report here are a first step in determining the mechanistic basis for these cellular increases and their relationship to the observed long-lasting resiquimod-induced protection against VL.

Because imidazoquinolines are poorly soluble in water, administration of these compounds has focused primarily on delivery via topical formulations. Human trials of topical imiquimod in CL patients, in combination with pentavalent antimony, demonstrated modest improvements over antimony treatment alone (15–18). These studies have focused exclusively on cutaneous disease to which imiquimod could be directly applied, however. Our data in mice suggest that topical administration of the more potent resiquimod has beneficial immunomodulatory effects in cases of systemic, organ-infecting VL. New therapies are needed for these infections, and the ability to apply a topical agent relatively infrequently (e.g., once per week) would be of substantial advantage in deploying the therapy in underdeveloped countries where VL predominates.

Use of a 0.2% resiquimod gel on a small area of shaved mouse skin was sufficient to induce significant cellular changes in the organs and reduction of existing parasite burden, without causing

any severe adverse effects either locally on the skin or systemically. Mild wrinkling and crusting on the skin of mice was observed, and only mild skin irritation has been reported after topical application in humans (35). Other groups have recently delivered resiquimod parenterally to mice by utilizing liposomal (37) or microparticle (38) encapsulation and demonstrated reduced parasite loads of *L. donovani in vivo*. Although the pharmacokinetics of these various forms of delivery will be distinct in humans compared to mouse models, the imidazoquinolines are in general associated with low degrees of toxicity in humans when applied topically, in contrast to many of the currently approved chemotherapies for human VL (35).

In humans, resiquimod stimulates both TLR7 and TLR8, whereas imiquimod binds only TLR7. However, murine TLR8 does not appear to respond to resiquimod (39, 40). The unique ability of resiquimod, but not imiquimod, to generate prophylactic antileishmanial protection in our mouse model of VL infection is therefore probably not attributable to the stimulation of distinct TLR pathways but is perhaps due to differences in the degree of stimulation by the two molecules. Resiquimod induces cytokine production from monocytes and macrophages at doses approximately 10- to 100-fold less than imiquimod (7, 19, 20, 41, 42). Resiquimod, but not imiquimod, is efficient at inducing IgM synthesis and class switching in B cells (43). Further dose-response and mechanistic experiments will be necessary to determine whether the protective efficacy of resiquimod alone against VL is due to its increased potency or to distinct characteristics of the molecule compared to imiquimod.

The cytokine expression profile induced by imidazoquinolines has been well characterized (44). In general, imidazoquinolines induce type I interferons (IFN-α and -β) in pDCs that play an important role in the clearance of many viral infections (45). These cytokines can stimulate cell activation via modulation of the IFN-γ pathway (46, 47), which triggers activation of NO production by macrophages and is generally associated with a protective response in CL (48–50). Early *in vitro* studies of the effects of resiquimod on cytokine expression by immune cells demonstrated high expression levels at concentrations of 1 μg/ml (20). Our *in vitro* results using a macrophage cell line demonstrated that resiquimod concentrations up to 10 μg/ml did not induce macrophage killing of *L. infantum chagasi* intracellular parasites. Soluble resiquimod was previously shown to directly enhance leishmanicidal activity in *L. donovani*-infected macrophages *in vitro*, via induction of iNOS and enhanced release of NO (10). The reasons for this discrepancy are unclear but may reflect different parasite species or different doses of resiquimod used.

This study demonstrates that topical resiquimod treatment leads to systemic immunomodulation, distinct cellular changes in the liver and spleen, and significant protection against experimental murine VL. These results are unique in that topical administration demonstrates efficacy against a systemic (nonlocal) infection and, in the case of prophylactic application, protection occurs in the absence of specific antigen-stimulation prior to challenge. These data support the idea that imidazoquinolines have value for use in direct therapy, as vaccine adjuvants, and/or in combination with conventional chemotherapies, to confer protective or therapeutic responses against protozoan infections (51). Future studies of resiquimod will be necessary to determine the exact mechanisms of this protection and to determine whether these effects are specific to leishmaniasis or whether they can be expanded to other pathogens. Finally, these results demonstrate the potential translational prophylactic and therapeutic benefit of a simple, topical regimen that could be deployed widely even in underdeveloped areas to combat deadly VL infections.

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