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Pharmacological inhibition of p110δ subunit of PI3K confers protection against experimental leishmaniasis

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Objectives: This study aimed to evaluate the immuno-prophylactic and -therapeutic effect of p110δ-specific pharmacological inhibitors (CAL-101 and IC87114), either alone or in combination with amphotericin B, against experimental cutaneous leishmaniasis (CL) and visceral leishmaniasis (VL).

Methods: Female BALB/c mice were infected intravenously with *Leishmania donovani* or subcutaneously with *Leishmania major*. Prophylactic treatment was initiated 24 h prior to infection, whereas therapeutic treatments with or without amphotericin B were initiated either 1 week or 2 weeks post-infection. At different times post-infection, mice were sacrificed and parasite burden, regulatory T cell (Treg) numbers and cytokine production were assessed in the liver, spleen, draining lymph nodes and footpads. In addition, direct cytolytic effects of the inhibitors on parasite growth in axenic cultures and inside infected and uninfected macrophages were also assessed.

Results: Prophylactic and therapeutic administration of p110δ pharmacological inhibitors significantly reduced cutaneous lesion (in CL) and parasite burdens (in VL and CL) in the spleens, livers and footpads of infected mice. The reduction in parasite burden was associated with a concomitant reduction in Treg numbers and cytokine production by liver, spleen and lymph node cells. Combined low-dose CAL-101 and amphotericin B therapy caused complete clearance of parasites in mice infected with *L. donovani*.

Conclusions: Our studies clearly show a novel therapeutic option for leishmaniasis based on CAL-101 monotherapy or CAL-101 and amphotericin B combination therapy. These observations have important and direct implications for antimicrobial immunotherapy and drug/vaccine development against leishmaniasis.

Introduction

Leishmaniasis is a vector-borne neglected tropical disease caused by an intracellular protozoan of the *Leishmania* genus. The symptoms range from self-healing skin ulcers in cutaneous leishmaniasis (CL) to weight loss, hepatomegaly and splenomegaly in visceral leishmaniasis (VL), which is a severe, deadly disease.[1] According to WHO, ∼0.7–1.3 million cases of CL and 200 000–400 000 cases of VL occur annually worldwide and ∼50 000 VL patients die from the disease every year.[2] The current drugs used for treatment of *Leishmania* infections are hindered by several problems, which include prolonged duration of treatment, toxicity, high costs and disease relapse.[3] In addition, the emergence of resistance to anti-leishmanial drugs suggests that the currently used monotherapy needs to be reviewed and possibly replaced with multidrug/combination therapies. VL in HIV-infected individuals is associated with higher initial leishmaniasis treatment failure, relapse and mortality rates.[4] Therefore, there is an urgent need to identify new drugs and treatment regimens.

Effective immunity against leishmaniasis is dependent on the development of a type-1 immune response that leads to the production of macrophage-activating cytokines, including IFN-γ and TNF. In contrast, susceptibility is usually linked to the production of macrophage-deactivating cytokines, including IL-10.[5] Reports suggest that regulatory T cells (Tregs)[6,7] and B cells[8,9] also play a critical role in determining the outcome of *Leishmania* infection in both mice[6,9] and humans.[7] Recently, we showed that mice with an inactivating knock-in mutation in the p110δ subunit of the phosphatidylinositol 3 kinases (PI3K, termed $p110\delta^{D910A}$ mice) are hyper-resistant to both experimental CL and VL caused by *Leishmania major*[10] and *Leishmania donovani*,[11] respectively. This

resistance was in part, due to impaired expansion of Tregs in p110δ^{D910A} mice.[10,11]

Given the dramatic hyper-resistance observed in p110δ^{D910A} mice infected with *L. donovani*[11] and *L. major*,[10] we speculated that the use of highly specific pharmacological inhibitors of p110δ may be beneficial in the treatment of experimental CL and VL by modulating the host immune response. We also predicted that the immunomodulatory effects of the inhibitors could act synergistically and allow the use of a lower dose of amphotericin B (a conventional leishmaniasis therapy), thereby significantly reducing the duration of the treatment regimen and drug toxicity, leading to improved drug efficacy.

Among the different categories of PI3K inhibitors developed to date,[12] IC87114 and CAL-101, which are p110δ-specific pharmacological inhibitors, have received wide attention and CAL-101 has recently been approved for treatment of different B cell malignancies.[13–15] Here, we demonstrate for the first time that prophylactic and therapeutic administration of IC87114 and CAL-101 reduces lesion size (CL) and parasite burdens (VL and CL) at the infection sites and visceral organs. We further show that as in infected p110δ^{D910A} mice, CAL-101 treatment significantly reduced B cell and Treg numbers and the percentage of IFN-γ-producing CD4+ T cells without affecting macrophage infectivity by the parasites. Importantly, combined low-dose CAL-101 and amphotericin B therapy leads to complete parasite clearance in mice infected with *L. donovani*. These observations have direct implications for immunomodulation and immunotherapy of both CL and VL.

Materials and methods

Ethics

The University of Manitoba Animal Use Ethics Committee approved all studies involving animals, including infection, humane endpoints, euthanasia and collection of samples (Protocol Number 12-072) according to the guidelines stipulated by the Canadian Council for Animal Care.

Mice, infection and parasite quantification

Female BALB/c mice were bred and maintained at the Central Animal Care Services (CACS) under specific pathogen-free conditions. Mice aged 6–8 weeks and weighing 20 g maintained on a 12 h light/dark cycle with free access to food and water, were infected intravenously with 5×10^7 7 day stationary-phase *L. donovani* (strain LV9) or 10^4 *L. major* (strain MHOM/80/Friedlin) promastigotes grown in complete M199 insect culture medium (Invitrogen, NY, USA).[16] At different times post-infection, mice were sacrificed and parasite burden in the liver, spleen and footpad was determined as previously described.[11] Lesion size was also measured in *L. major*-infected footpads using callipers.

Prophylactic, therapeutic and combination therapy with p110δ pharmacological inhibitors

For prophylactic treatment, mice were administered CAL-101 (idelalisib/GS-1101/Zydelig[17]) (0.05 mg/mouse) or IC87114 (0.5 mg/mouse) (both from Selleck Chemicals LLC, TX, USA), intraperitoneally or orally[18] twice a day, 24 h prior to infection. Intraperitoneal or oral injection of CAL-101 and/or IC87114 was continued every 12 h for 2 weeks. For therapeutic treatment, mice were infected with *L. donovani* promastigotes and after 1 week or 2 weeks CAL-101 was administered intraperitoneally every 12 h for an additional period of 2 weeks. For combination therapy, mice were infected with *L. donovani* promastigotes and after 2 weeks were treated intraperitoneally with CAL-101 (0.05 mg/mouse), amphotericin B (Fungizone, Bristol-Myers Squibb, Montreal, Canada; 0.1 mg/kg), combined CAL-101 and amphotericin B or PBS once daily for 5 consecutive days and sacrificed at 1 week or 3 weeks after last treatment. Additionally, 1 week after the last combined CAL-101 and amphotericin B treatment, some mice were treated with anti-IFN-γ MAb (1 mg/mouse intraperitoneally) as previously described.[19,20] Mice were sacrificed 2 weeks post-anti-IFN-γ mAb administration. In all experiments, control mice received DMSO in PBS (vehicle control).

Assessment of B cells, Tregs and cytokine production

At different times, mice were sacrificed and the spleens, livers and lymph nodes were collected. The spleens and lymph nodes were made into single-cell suspensions in complete DMEM (DMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin). The livers were digested with collagenase D (0.125 mg/mL) for 30 min at 37°C and homogenized in complete DMEM, and liver lymphocytes were separated using Percoll gradient centrifugation as previously described.[11,21] Cells were counted and directly stained *ex vivo* for CD3, CD4, CD25 and B220 (extracellular staining) and Foxp3 (intracellular staining using a BD Biosciences Foxp3 Staining Kit) expression. In some experiments, liver, spleen and lymph node lymphocytes were stimulated with 50 ng/mL phorbol 12-myristate 13-acetate, 500 ng/mL ionomycin and 0.1 mg/mL brefeldin A for 4 h, fixed, surface-stained for CD3 and CD4, and stained intracellularly for IFN-γ.[10,11] Samples were acquired on a FACSCanto II cytometer (BD Bioscience, San Diego, CA, USA) and analysed using FlowJo software (Tree Star, Ashland, OR, USA). Spleen, liver and lymph node cells were also resuspended at 4×10^6/mL in complete DMEM, plated at 1 mL/well in 24-well tissue culture plates, stimulated with freeze-thawed *L. donovani* (0.01 mg/mL) or soluble *Leishmania* antigen (0.005 mg/mL) and cultured at 37°C. After 3 days, the supernatant fluids were collected and assayed for cytokines [IFN-γ, IL-10, IL-6, IL-4 and keratinocyte chemoattractant (KC)/human growth-related oncogene (GRO)] using a V-PLEX Plus Proinflammatory Panel 1 (mouse) Kit (Meso Scale Discovery, MD, USA) or ELISA according to the manufacturer-suggested protocols.

Measurement of serum antibody levels

At sacrifice, serum was obtained from infected and treated (CAL-101 prophylactic treatment) mice and used to determine the *Leishmania*-specific antibody titres (IgG, IgM, IgG1 and IgG2a) by ELISA as previously described.[22] Briefly, high protein binding ELISA plates were coated with freeze-thawed *L. donovani* (5×10^7 parasites/ml, 10 μL/well) overnight at 4°C, incubated with blocking buffer (2% BSA) for 2 h at 37°C and then washed. Serum samples and standards were serially diluted, added to the wells and incubated at 37°C for 2 h. Plates were washed and bound antibody levels were detected at 405 nm using biotinylated anti-mouse IgG, IgM, IgG1 or IgG2a (Southern Biotech).

Assessment of Leishmania growth in the presence of p110δ pharmacological inhibitors

L. major or *L. donovani* promastigotes (2.5×10^5) were cultured in 96-well flat-bottom plates at 37°C in the presence of different concentrations (0, 100 nM, 1 μM and 20 μM) of IC87114 and CAL-101, or in the presence of 10 nM amphotericin B. Parasite growth was monitored daily by counting the number of parasites in the wells under light microscopy.

Assessment of CAL-101- and amphotericin-B-induced toxicity in macrophages in vitro

Retrovirus-immortalized bone marrow-derived macrophage (BMDM) cells (ANA-1 cells)[23] were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/mL penicillin and 0.1 mg/mL streptomycin at 37°C for

3 days. The cells were then treated with either CAL-101 or amphotericin B (ranging from 10 nM to 20 μM) for 24 h and assessed for apoptosis by flow cytometry using an Annexin V FITC Apoptosis Detection Kit according to the manufacturer-suggested protocols.[24]

In vitro infection of macrophages in the presence of P110δ pharmacological inhibitors

ANA-1 cells were grown in complete RPMI medium[23] and infected with 7 day stationary-phase *L. donovani* or *L. major* promastigotes at a cell-to-parasite ratio of 1:5 for 5 h. Free parasites were washed away and infected macrophages were further cultured for 24 and 72 h at 37 °C in the presence of IC87114 (0, 1 and 10 μM) or CAL-101 (0, 0.1 and 1 μM) or amphotericin B (10 nM).[25–27] At the end of the cultures, the level of infection was determined by counting haematoxylin/eosin-stained cytospin preparations under a Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope with a ×100 (oil) objective.

Statistical analysis

Student's *t*-test was used to compare the mean and SEM between two groups and non-parametric one-way or two-way analysis of variance (ANOVA) was used to compare the mean and SD of more than two groups using the Prism program (GraphPad Software Inc., CA, USA). Tukey's or Bonferroni post tests were used where there were significant differences in ANOVA. Error bars indicate ±SEM and differences were considered significant when $P < 0.05$.

Results

Prophylactic administration of p110δ pharmacological inhibitors confers protection against VL and CL

We previously showed that mice with an inactivating knock-in mutation in the p110δ isoform of PI3K (p110δ^{D910A}) are hyper-resistant to *L. major*[10,28] and *L. donovani*.[11,16] Because this resistance is independent of parasite species and genetic background, we wanted to assess whether targeting the PI3K signalling pathway with p110δ pharmacological inhibitors may be useful for treatment of both CL and VL. We administered CAL-101 or IC87114 to mice intraperitoneally[18] or orally (CAL-101 only) twice a day, 24 h prior to intravenous or subcutaneous infection with *L. donovani* or *L. major*. Prophylactic administration of CAL-101 resulted in significantly ($P < 0.01$) lower parasite burden in the spleen (Figure 1a) and liver (Figure 1b) of *L. donovani*-infected mice and footpad (Figure 1c) of *L. major*-infected mice. The reduction in parasite burden in the footpads was also associated with significant ($P < 0.01$) reduction in lesion size of *L. major*-infected mice (Figure 1d). Interestingly, there was no significant difference in parasite burden in the spleens and livers *of L. donovani*-infected p110δ^{D910A} mice either treated or not treated with CAL-101, suggesting that intact p110δ signalling is critical for the effects observed in treated mice (Figure 1a and b). Similar results were observed in mice treated with IC87114 (Figure S1a, b and c, available as Supplementary data at *JAC* Online). Thus, as observed in mice with inactive knock-in mutation, pharmacological inhibition of p110δ isoform of PI3K leads to enhanced resistance to experimental visceral and cutaneous leishmaniasis.

Prophylactic administration of p110δ pharmacological inhibitors alters host immune response to Leishmania

Enhanced resistance of p110δ^{D910A} mice to experimental leishmaniasis is associated with reduced immune activation and cytokine responses.[10,11] Therefore, we assessed the quality of immune response in mice treated with CAL-101 in order to determine whether pharmacological inhibitors also affect the immune response in a similar manner. At sacrifice, liver, spleen or lymph node lymphocytes were assessed directly *ex vivo* for the numbers of Foxp3-expressing (Tregs) and IFN-γ-producing CD4+ T cells. As in p110δ^{D910A} mice, prophylactic administration of CAL-101 (both orally and intraperitoneally) resulted in significantly lower Treg numbers in the spleen and liver of *L. donovani*-infected mice (Figure 2a, b and c; $P < 0.01$) and spleens and lymph node of *L. major*-infected mice (Figure 2g, h and i; $P < 0.05$). In addition, CAL-101 treatment also led to significant reduction in the frequency of IFN-γ-producing cells in the spleen and liver of *L. donovani*-infected mice (Figure 2d, e and f; $P < 0.05$) and spleen and lymph nodes of *L. major*-infected mice (Figure 2j, k and l; $P < 0.01$). We further validated the flow cytometry data by showing that the supernatant culture fluids of liver (Figure 3a, b, c and d), spleen (Figure 3e, f, g and h) and lymph node cells (Figure 3i, j and k) from infected mice treated with CAL-101 have significantly ($P < 0.05$–0.001) lower amounts of IFN-γ, IL-10, IL-6, IL-4 and KC/GRO. p110δ^{D910A} mice have reduced numbers of B cells, leading to impaired B cell responses.[29] Consistent with this, prophylactic oral or intraperitoneal administration of CAL-101 led to significant reduction in B cell numbers (B220+ cells) in the liver and spleen of *L. donovani*-infected WT mice (Figure S2a, b and c; $P < 0.01$). This was also associated with significant reduction in serum levels of *Leishmania*-specific antibody titres, including IgG, IgM, IgG1 and IgG2a (Figure S2d, e, f and g). Collectively, these results show that as in p110δ KI mice, blockade of p110δ signalling with pharmacological inhibitors results in dampening of excessive pathology-associated immune responses in *Leishmania*-infected mice.

Treatment with CAL-101 leads to reduction in parasite burden in mice with established disease

Treatment of leishmaniasis is challenging due to prolonged duration of treatment and the fact that most of the current drugs are toxic and have numerous side effects.[30] Because CAL-101 (idelalisib) has recently been approved by the FDA for use in patients with different B cell malignancies,[13,15,31] and given its beneficial prophylactic effects against leishmaniasis (Figure 1), we assessed its therapeutic potential for treatment of established experimental VL.

Mice were infected with *L. donovani* and after 1 week or 2 weeks were treated with CAL-101 every 12 h for a period of 2 weeks. CAL-101 treatment initiated 1 week or 2 weeks after *L. donovani* infection resulted in significantly ($P < 0.05$) lower parasite burden in the spleens (Figure S3a and Figure 4a) and livers (Figure S3b and Figure 4b) of infected mice. The reduced parasite burden in these organs correlated with a significant ($P < 0.01$) reduction in Treg numbers (Figure S3c and Figure 4c) and the numbers of IFN-γ-producing CD4+ T cells (Figure 4d and Figure S3d). In addition, therapeutic administration of CAL-101 significantly ($P < 0.05$-0.01) reduced some cytokine levels in the culture supernatant fluids of spleen (Figure 4e, f, g and h and Figure S3e, f, g and

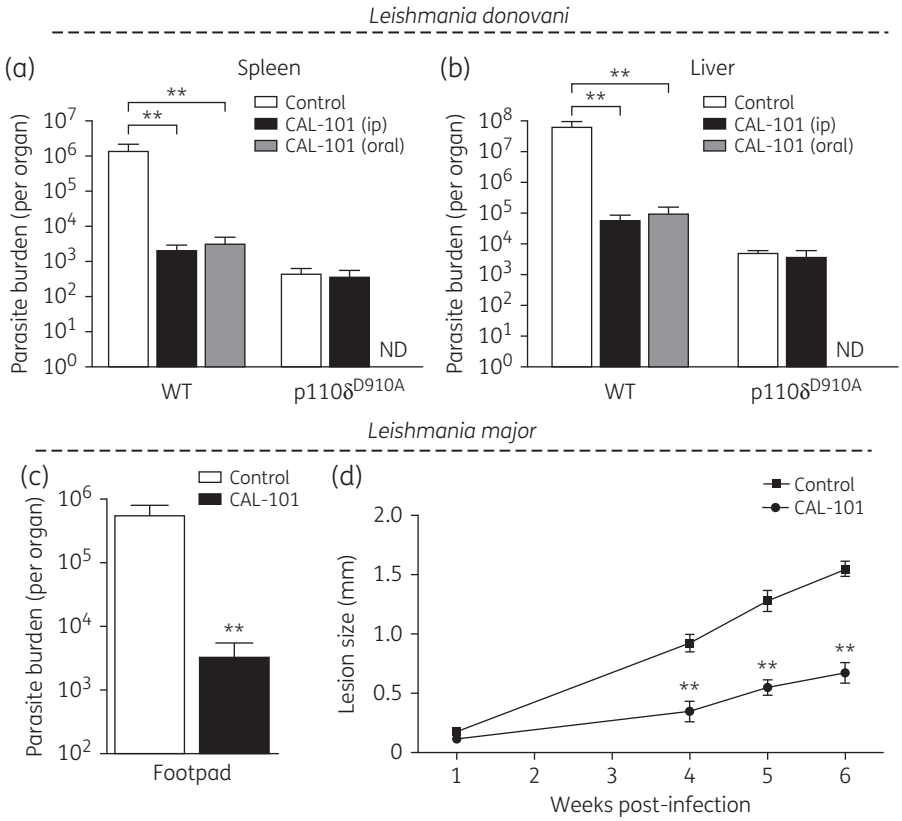

Figure 1. Prophylactic administration of CAL-101 enhances immunity to experimental VL and CL. BALB/c (WT) and p110δ^{D910A} mice were administered CAL-101 (0.05 mg/mouse, twice daily) intraperitoneally or orally 24 h prior to intravenous infection with 5×10^7 *L. donovani* (a and b) or subcutaneous infection with 10^4 *L. major* (c and d). Intraperitoneal or oral administration of CAL-101 was continued every 12 h for an additional 2 weeks and mice were sacrificed (at 2 weeks post-infection). At sacrifice, parasite burden in the spleen (a), liver (b) and footpads (c) was determined by limiting dilution assay. Lesion progression in the infected footpads was monitored weekly with callipers (d). Results are representative of two independent experiments ($n = 4$ mice per group per experiment) with similar results. Error bars, ±SEM. $^{**}P < 0.01$. ND, not done; ip, intraperitoneal.

h) and liver (Figure 4i, j, k and l and Figure S3i, j, k and l) cells from *L. donovani*-infected mice. These findings are consistent with our previous findings indicating that resistance to VL and CL observed in p110δ^{D910A} mice is due to reduced Treg levels and that reduced production of IFN-γ does not affect the outcome of disease. They further show a beneficial therapeutic effect of targeting this pathway in treatment of VL.

Combined CAL-101 and amphotericin B therapy cures experimental VL

The growing resistance to anti-leishmanial drugs and high relapse rates of VL suggest that the currently used monotherapy needs to be reviewed and possibly replaced with multidrug combination and/or immunotherapy. Therefore, we determined whether treatment of infected mice with low-dose CAL-101 and amphotericin B, a commonly used anti-*Leishmania* compound, would lead to better protection that might be associated with minimal toxicity. We treated different groups of *L. donovani*-infected mice with CAL-101 either alone or in combination with amphotericin B (0.01 mg/kg). Remarkably, combination therapy with CAL-101 and amphotericin B (1 week or 3 weeks post-therapy) led to complete clearance of parasites both in the spleen (Figure S4a and Figure 5a) and liver (Figure S4b and Figure 5b) compared with the untreated control groups or those treated with either CAL-101 or amphotericin B alone. CAL-101 and amphotericin B combination therapy led to significant ($P < 0.05$–0.01) reduction in Treg numbers in the spleens and livers of *L. donovani*-infected mice 1 week (Figure S4c and d) or 3 weeks (Figure 5c and d) post-therapy compared with the untreated control group. However, the reduction in Treg numbers was significantly ($P < 0.05$–0.01) greater in the CAL-101-treated group compared with groups that received CAL-101 and amphotericin B combination therapy [1 week (Figure S4c and d) and 3 weeks (Figure 5c and d) post-therapy]. In contrast, while the CAL-101-treated group had lower numbers of IFN-γ-producing cells in their spleens and livers (1 week or 3 weeks post-therapy) and these cells produced lower amounts of IFN-γ in cultures, treatment with either amphotericin B alone or in combination with CAL-101 did not affect the level of IFN-γ production by splenic and hepatic cells from infected mice (Figure S4e and f and Figure 5e and f). Although administration of anti-IFN-γ mAb to mice treated with a combination of CAL-101 and amphotericin-B resulted in significantly ($P < 0.001$) lower IFN-γ at 3 weeks post-therapy, these mice were still resistant to *L. donovani* (Figure 5a and b). These

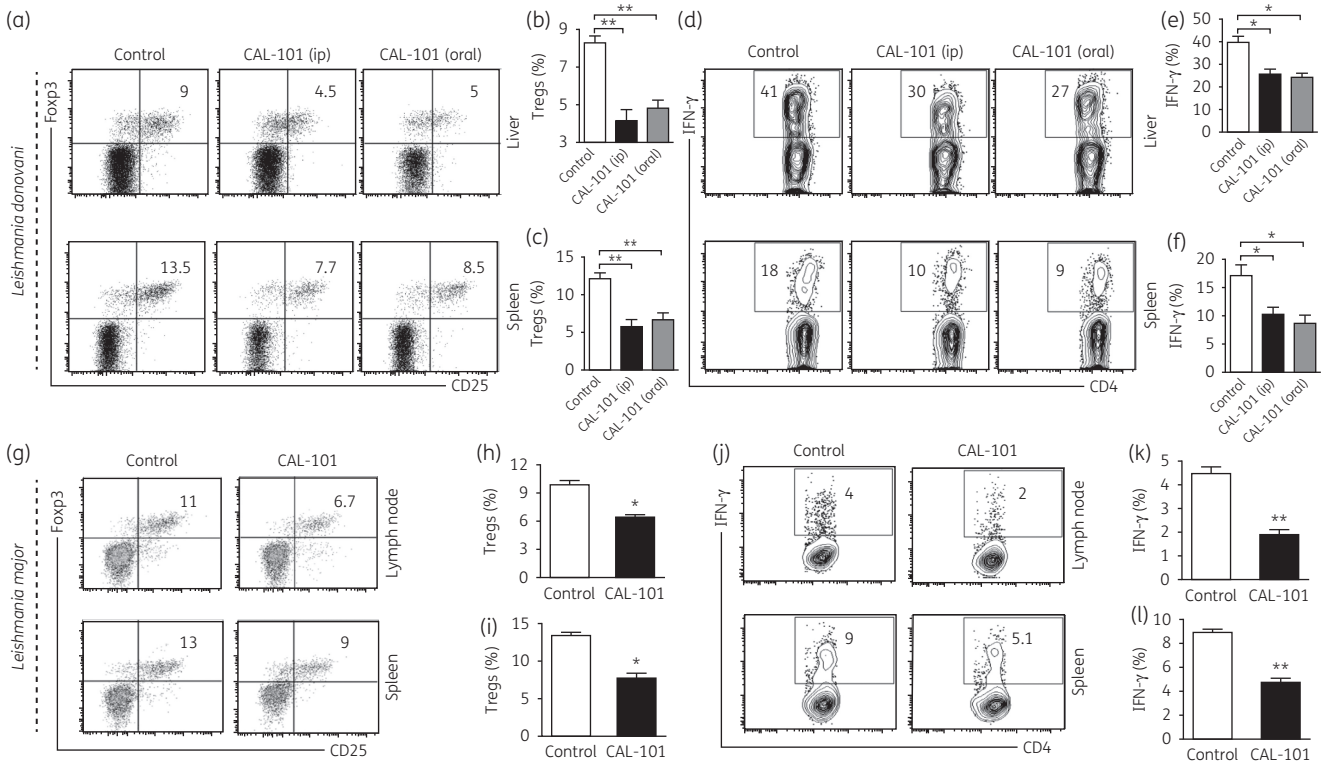

Figure 2. Prophylactic administration of CAL-101 reduces Treg numbers and percentage of IFN-γ-producing CD4+ T cells in spleens, livers and lymph nodes of infected mice. BALB/c mice were administered CAL-101 intraperitoneally or orally twice a day 24 h prior to *L. donovani* or *L. major* infection. Intraperitoneal or oral administration of CAL-101 was continued every 12 h for an additional 2 weeks. Mice were sacrificed at 2 weeks post-CAL-101 treatment and spleen, liver and lymph node cells were directly stained *ex vivo* for Foxp3 and IFN-γ expression (after stimulation with PMA, ionomycin and brefeldin A for 4 h) and assessed by flow cytometry. Shown are percentages of Tregs (a, b, c, g, h and i) and IFN-γ-producing CD4+ T cells (d, e, f, j, k and l) in the spleens and livers of *L. donovani*-infected mice (a, b, c, d, e and f) and spleens and draining lymph nodes of *L. major*-infected mice (g, h, i, j, k and l). Representative dot plots of Tregs (gated on live CD3 + CD4+ cells) (a and g) and IFN-γ-producing CD4+ T cells (gated on live CD3+ cells) (d and j) and their bar graphs (b, c, e, f, h, i, k and l) showing the mean ± SEM are presented. Results are representative of three independent experiments ($n = 4$ mice per group per experiment) with similar results. Error bars, ±SEM. $^{*}P < 0.05$; $^{**}P < 0.01$. ip, intraperitoneal.

observations suggest that the protection in CAL-101 and amphotericin-B-treated mice may be IFN-γ independent and that treated mice were cured of infection.

P110δ pharmacological inhibitors do not directly inhibit Leishmania growth in axenic cultures or in infected macrophages

Because *L. donovani* is known to activate the PI3K/AKT pathway in macrophages,[32] we assessed whether p110δ pharmacological inhibitors influence *Leishmania* growth directly (in axenic cultures) or indirectly (by affecting macrophage activation), leading to parasite death. Therefore, we tested the ability of CAL-101 and IC87114 to directly inhibit proliferation of *L. major* and *L. donovani* promastigotes in axenic cultures. The results were compared with *Leishmania* growth in the presence of amphotericin B. Parasite growth was monitored every day by counting the number of parasites in cultures over 7 days. The results shown in Figure 6(a and b) indicate that the *in vitro* growth and proliferation of these parasites were not affected by any concentration of CAL-101 tested. In contrast and consistent with previous reports, amphotericin B completely inhibited survival and proliferation (Figure 6a and b). Additionally, whereas amphotericin B suppressed parasite proliferation in macrophages, CAL-101 did not affect either infectivity or parasite proliferation inside macrophages *in vitro* (Figure 6c and d). Similar results were also obtained for IC87114 (Figure S5a, b, c and d). We further compared the effects of various concentrations of CAL-101 and amphotericin B on macrophage (ANA-1 cells) apoptosis in order to determine their CC_{50}. The data presented in Figure 6(e and f) show that the CC_{50} of CAL-101 and amphotericin B were 10 μM and 100 nM, respectively. Collectively, these results confirm that the *in vivo* beneficial effects of CAL-101 and IC87114 are not mediated by direct parasite killing but through their ability to modulate the host immune response to *Leishmania*.

Discussion

Leishmaniasis remains an important parasitic disease and is spreading to several non-endemic areas of the world. Importantly, drug resistance and increasing *Leishmania*/HIV coinfections, associated with high failure and relapse rates, pose significant challenges to monotherapy.[33–35] Moreover, the treatment of VL in

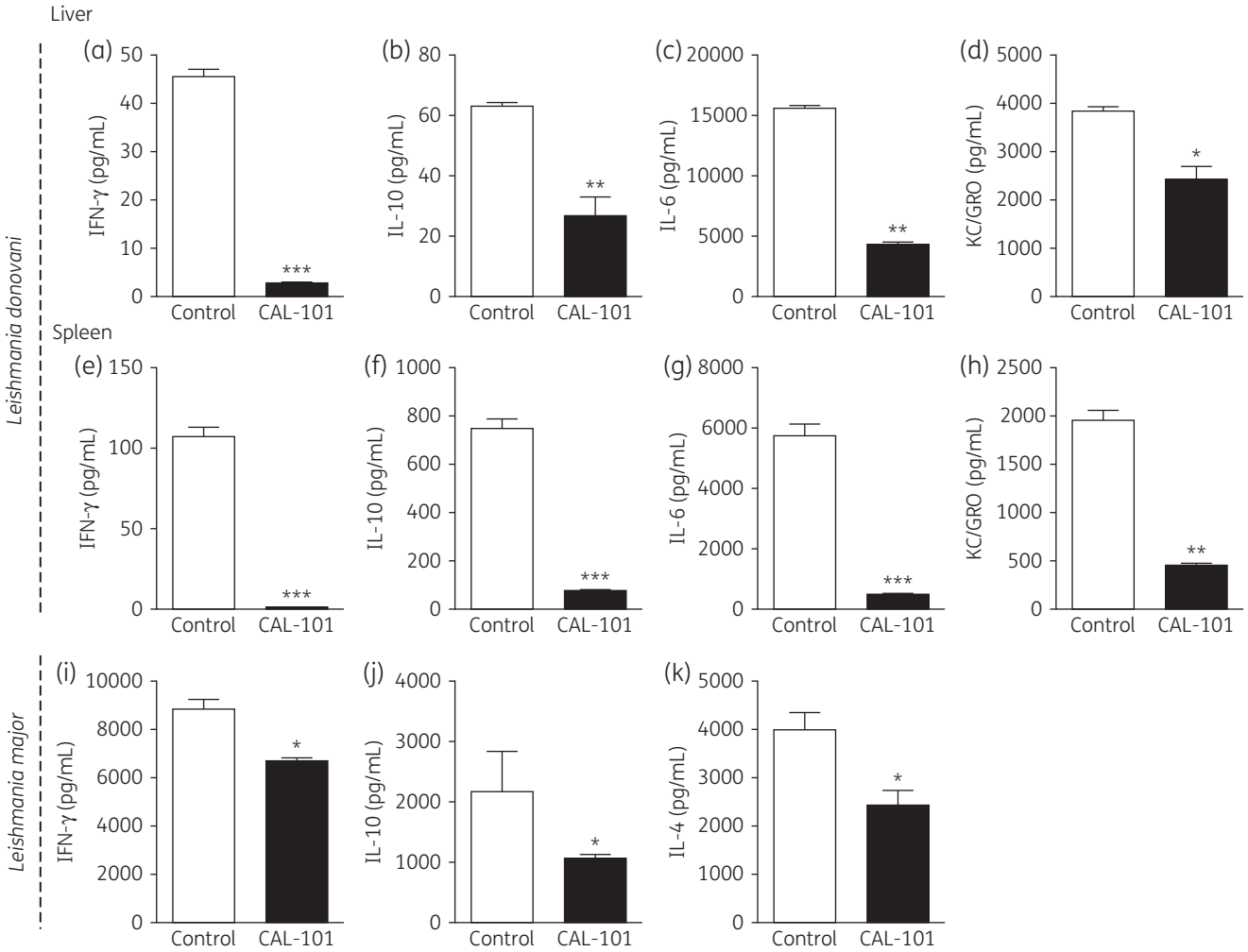

Figure 3. Prophylactic administration of CAL-101 is associated with reduced production of cytokines by different immune cells *in vitro*. BALB/c mice were administered CAL-101 twice a day 24 h prior to *L. donovani* or *L. major* infection. Intraperitoneal administration of CAL-101 was continued every 12 h for an additional 2 weeks. Mice were sacrificed at 2 weeks post-CAL-101 treatment and liver (a, b, c and d), spleen (e, f, g and h) and lymph node (i, j and k) cells were stimulated with freeze-thawed *L. donovani* or soluble *Leishmania* antigen (*L. major*) and cultured for 72 h, and spontaneous cytokine (IFN-γ, IL-10, IL-6, IL-4 and KC/GRO) production in the supernatant fluids was assayed using a V-PLEX Meso Scale Kit (a, b, c, d, e, f, g and h) or ELISA (i, j and k). Results are representative of two independent experiments ($n = 4$ mice per group per experiment) with similar results. Error bars, ±SEM; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

HIV-infected individuals with CD4+ T cell counts <200 cells/mm^3 required even higher doses.[36] Many of the frontline drugs for treating the disease, such as amphotericin B, liposomal amphotericin B and miltefosine, are associated with numerous side effects, including gastrointestinal intolerance and significant toxicity to the liver, kidneys and spleen.[37,38] Therefore, there is urgent need for development of novel therapies, including immunotherapy.

We previously reported that mice with an inactive knock-in mutation in the p110δ subunit of PI3K exhibit enhanced resistance to different experimental forms of leishmaniasis, including CL and VL.[10,11] This resistance was independent of parasite species and mouse genetic background,[10,11] suggesting that targeting this pathway could provide a novel therapeutic approach for treatment of leishmaniasis. Here, we have investigated whether treatment of *Leishmania*-infected mice with highly specific pharmacological inhibitors of p110δ signalling could be beneficial in treating leishmaniasis.

Different categories of PI3K inhibitors, including pan-PI3K, dual-PI3K/mTOR and isoform-specific PI3K inhibitors, have been developed, but most of these have encountered problems as monotherapeutic agents in clinical trials due to limited efficacies and relatively high rates of adverse side effects.[12] Although IC87114 was the first p110δ-specific inhibitor,[39,40] and is still being used in research,[41,42] it has a very high IC_{50} (0.5 μM) compared with CAL-101, which has an IC_{50} of 2.5 nM.[13–15] Therefore, CAL-101 has taken the lead over IC87114 in clinical trials and research applications and has shown great promise in the clinical management of conditions where PI3K inhibitions were thought to be beneficial.[13–15]

We report here for the first time that both CAL-101 and IC87114, when used as prophylactic treatments, can reduce lesion

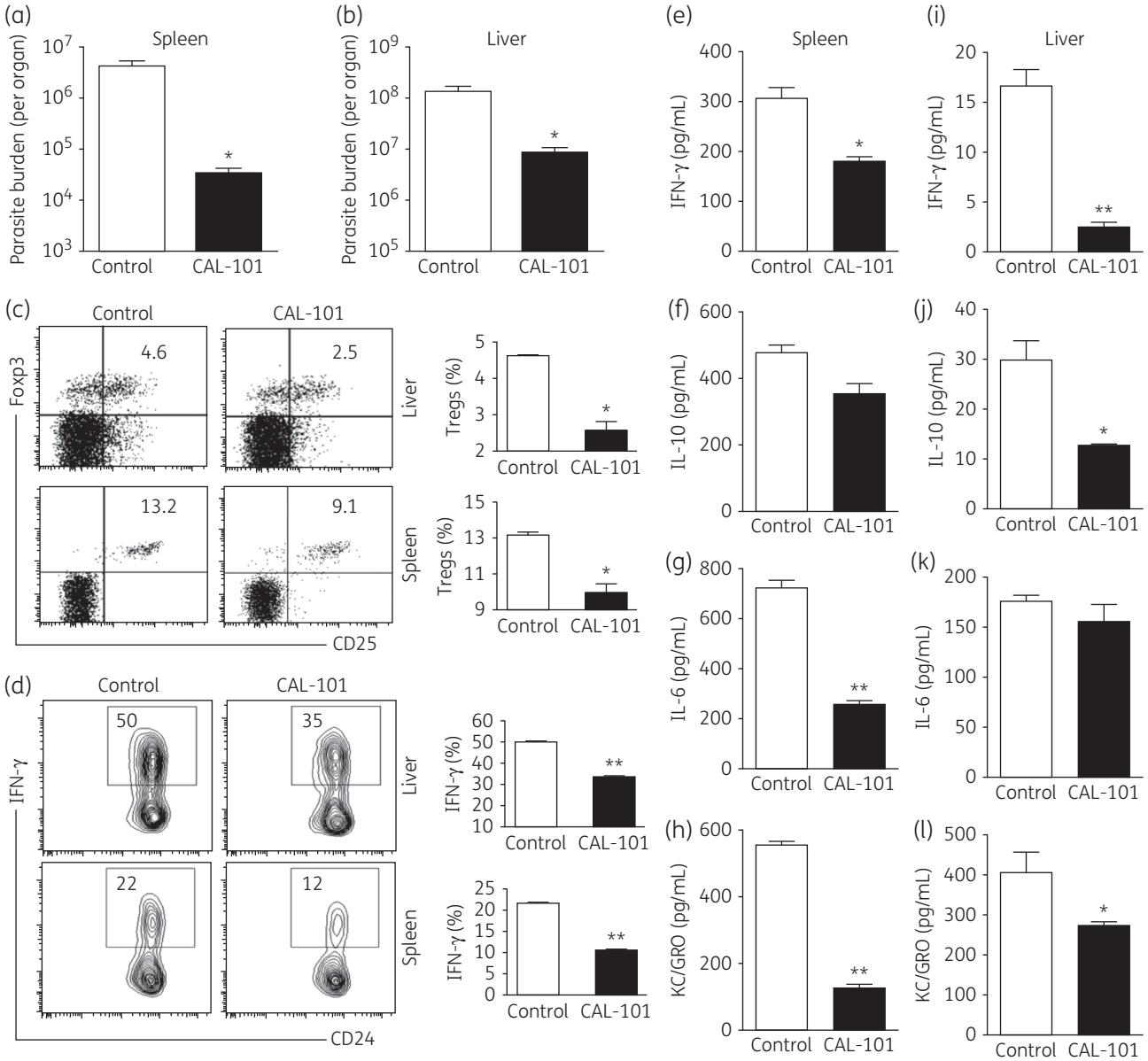

Figure 4. CAL-101 therapy is effective against established *L. donovani* infection. BALB/c mice were infected intravenously with 5×10^7 *L. donovani* promastigotes and after 2 weeks CAL-101 was administered intraperitoneally every 12 h for 2 weeks. At sacrifice, parasite burden in the spleen (a) and liver (b) was determined by limiting dilution assay. Spleen and liver lymphocytes were isolated, directly stained *ex vivo* and the percentages of CD25 + Foxp3+ Tregs (c) were determined by flow cytometry by gating on live CD3 + CD4+ cells. The percentages of IFN-γ-producing CD4+ T cells (d) were also determined after stimulation with PMA, ionomycin and brefeldin A for 4 h after gating on live CD3+ cells. Spleen (e, f, g and h) and liver (i, j, k and l) lymphocytes were also cultured for 72 h in the presence of freeze-thawed *L. donovani* and the levels of cytokines (IFN-γ, IL-10, IL-6 and KC/GRO) in the culture supernatant fluids were determined by V-PLEX Meso Scale. Results are representative of two independent experiments ($n = 4$ mice per group per experiment) with similar results. Error bars, ±SEM. *$P < 0.05$ and **$P < 0.01$.

size (CL) and parasite burdens in the footpads (CL), spleens and livers (VL) of *Leishmania*-infected mice. Also, we demonstrated that CAL-101 has therapeutic effects in VL, as treatment initiated as late as 2 weeks after infection causes a significant reduction in parasite numbers in both the spleens and livers of infected animals. Strikingly, we further demonstrated that combined low-dose CAL-101 and amphotericin B therapy has the ability to cause complete clearance of parasites in *L. donovani*-infected mice, demonstrating the potential benefits of this combination therapy in treatment of VL. This superior effect of CAL-101 and amphotericin B combination therapy offers the advantage of avoiding emergence of drug resistance

Although single- or multiple-dose liposomal formulations of amphotericin B (Fungisome or AmBisome) have been reported to be effective for treatment of VL, prolonged treatment with amphotericin B is still the main treatment regimen in different parts of

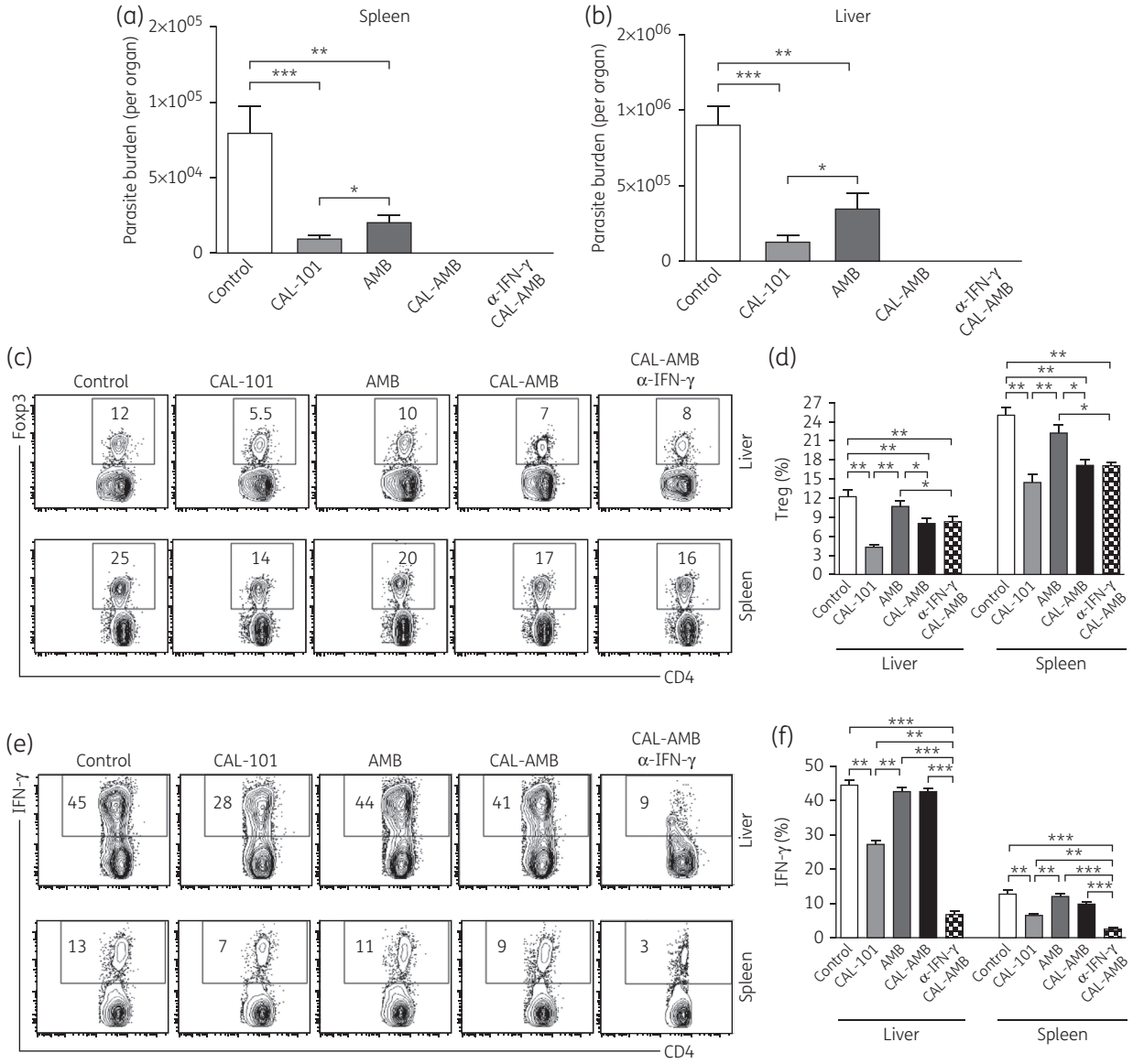

Figure 5. CAL-101 and amphotericin B combination therapy leads to complete parasite clearance in spleens and livers of *L. donovani*-infected mice. BALB/c mice were infected intravenously with 5×10^7 *L. donovani* and, after 2 weeks, treated with CAL-101, amphotericin B or a combination of CAL-101 and amphotericin B once daily for 5 days. In addition, some mice treated with the combined CAL-101 and amphotericin B regimen were treated with anti-IFN-γ mAb at 1 week after the last drug treatment and sacrificed after 2 weeks to determine parasite burden in the spleen (a) and liver (b). At sacrifice, spleen and liver lymphocytes were isolated and directly stained *ex vivo* to assess Foxp3 expression. The dot plot represents Foxp3+ Tregs previously gated on live CD4 + CD25+ cells (c and d). Some cells were stimulated with PMA, ionomycin and brefeldin A for 4 h, and fixed and stained for intracellular IFN-γ expression (e and f). Results are representative of two or three independent experiments ($n = 4$ mice per group per experiment) with similar results. Error bars, ±SEM; $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$. AMB, amphotericin B.

the world. For example, intravenous injections of amphotericin B (1 mg/kg every other day for 15 days and at 15 mg/kg over 30 days) have been reported in human studies.[43] An experimental mouse study found that effective treatment of two strains of *Leishmania infantum* required multiple injections of 0.5–0.8 mg/kg of amphotericin B on various days.[44] Here, we chose to use amphotericin B (Fungizone) at 0.1 mg/kg, 2 weeks after infection, in combination with CAL-101. The results obtained indicate that this combination therapy has the ability to cause complete clearance of parasites both in spleen and liver of *L. donovani*-infected animals, as checked at different timepoints after therapy. Furthermore, parasite load in these mice remained undetectable following administration of anti-IFN-γ MAb, suggesting that combination therapy may have led to complete cure. Thus, CAL-101 and amphotericin B combination therapy dramatically reduced drug dose and duration of treatment, which could potentially reduce the associated toxicity and side effects of the drugs. The combination therapy may also be of particular benefit in the difficult-to-manage VL/HIV coinfection.

The very low IC_{50} associated with CAL-101 allows the administration of relatively higher doses, leading to enhanced target and

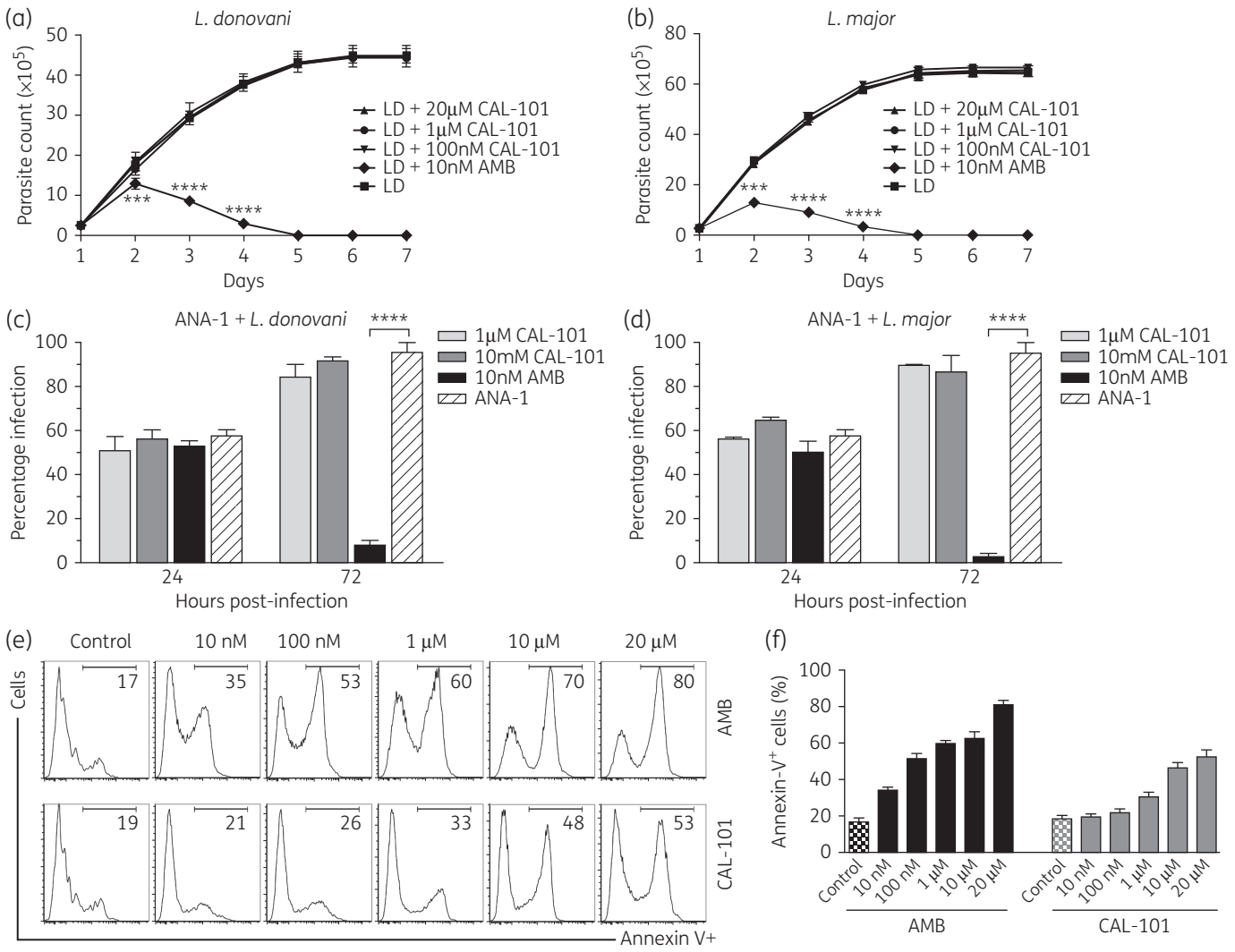

Figure 6. CAL-101 does not directly inhibit *Leishmania* growth in axenic culture or inside infected macrophages *in vitro*. *L. donovani* (a) or *L. major* (b) promastigotes (2.5×10^5) were cultured at 27 °C in the presence of different concentrations of CAL-101 or amphotericin B as indicated. Parasite proliferation was monitored daily by counting under light microscopy. ANA-1 macrophage cell lines were infected with stationary-phase *L. donovani* (c) or *L. major* (d) promastigotes at a cell-to-parasite ratio of 1:5. After 5 h, free parasites were washed away and infected macrophages were cultured at 37 °C in the presence of different concentrations of CAL-101 or amphotericin B (as indicated). At specified times, the number of infected cells per 100 cells (percent infection) was determined by counting haematoxylin/eosin-stained cytospin preparations under a Zeiss Primo Star (Zeiss, Melville, NY, USA) light microscope with a ×100 (oil) objective. ANA-1 cells were treated with different concentrations of CAL-101 or amphotericin B for 24 h and the percentages of apoptotic cells (annexin V+ cells; e and f) were quantified by flow cytometry. Results are representative of two or three independent experiments with similar results. Error bars, ±SEM. ***$P < 0.001$; ****$P < 0.0001$. AMB, amphotericin B.

pathway suppression and efficacy.[31,45] Although in some limited cases CAL-101 has been associated with adverse side effects, such as hepatotoxicity, diarrhoea or colitis, and fatal and serious pneumonia or intestinal perforation,[46,47] the compound remains one of the safest PI3K inhibitors available for clinical practice. Here, we found that the IC_{50} of CAL-101 (2.5 nM) is several orders of magnitude below the CC_{50} (20 μM), confirming that the compound can effectively kill the parasites without causing any adverse effects (e.g. apoptosis of the host cells). In contrast, the IC_{50} of amphotericin B (100–700 nM[48]) is equal to the CC_{50} (100 nM) obtained here, suggesting that the drug would induce host cell apoptosis before killing parasites. This would account for the very high toxicity associated with the current dose of the drug used clinically.[43] Therefore, the very low concentrations of amphotericin B used in this study might have the ability to reduce drug-induced cytotoxicity *in vivo*.

We previously showed that enhanced resistance of p110δ KI mice to CL and VL was paradoxically associated with impaired IFN-γ response by immune cells in different organs of infected mice.[10,11] We also showed that the deficiency of p110δ signalling was associated with impaired Treg expansion and function in infected mice. Extensive analysis further revealed that the enhanced resistance in these mice was related to more efficient effector T cell responses in the face of impaired Treg activities and numbers.[11,28] In line with this, we found here that treatment with CAL-101 was associated with significant

reduction in Treg numbers and cytokine responses in different organs of infected mice, suggesting that the beneficial effects of CAL-101 may be through its immunomodulatory effects. Interestingly, we also found that CAL-101 treatment resulted in reduced numbers of B cells and a concomitant decrease in serum levels of parasite-specific antibodies. This is consistent with previous reports showing that deficiency of p110δ signalling results in defects in B cell receptor (BCR) signal and impaired antibody response.[29] It is conceivable that the impaired B cell response in these mice might contribute to the protection seen in CAL-101-treated mice, given that deficiency of B cells and/or impaired B cell responses have been linked to enhanced anti-*Leishmania* immunity.[8,9] In contrast, amphotericin B therapy alone increased IFN-γ production and had no effect on Treg and B cell numbers in infected mice. This is consistent with reports that demonstrate amphotericin B interacts with both host and parasite membrane cholesterol, thereby effectively disrupting membrane integrity of the cells and parasites.[49] Hence, it could be considered that CAL-101 and amphotericin B combination therapy has two effects. One is targeting the p110δ pathway, which leads to an effective immune response associated with leishmaniasis by reducing B cell and Treg numbers and function, and the other is by directly killing parasites,[50] thereby reducing their entry into macrophages.[49]

Many factors, such as high-dose treatment regimens, toxicity, high costs, drug resistance and poor efficacy, are among the major challenges facing physicians treating patients with leishmaniasis. Therefore, it is critical that the current monotherapy options be enhanced or replaced by developing new drugs or utilizing multi-drug/combination therapy. The combination therapy approach could lower the required drug doses and treatment regimens, reduce drug toxicity, improve drug efficacy, reduce emergence of drug-resistant strains and consequently reduce the chances of disease relapse. CAL-101 (idelalisib) has been approved by the FDA for treatment of several conditions, including B cell malignancies.[13–15] Our studies clearly demonstrate a novel therapeutic option for leishmaniasis based on CAL-101 monotherapy or CAL-101 and amphotericin B combination therapy. These observations have important and direct implications for immunotherapy and drug development against leishmaniasis.

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Transparency declarations

None to declare.

Supplementary data

Figures S1 to S5 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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