Title: KSAC: the first defined polyprotein vaccine candidate for visceral leishmaniasis

Running title: KSAC vaccine for leishmaniasis

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Abstract

A subunit vaccine using defined antigen(s) may be one of the effective solutions to control leishmaniasis. Because of genetic diversity in target populations, including both dogs and humans, a multiple antigen vaccine will likely be essential. However, cost considerations for a vaccine to be used in developing countries must be considered. We report herein a multi-antigen vaccine candidate comprised of antigens known to be protective in animal models, including dogs, as well as to be recognized by humans immune to visceral leishmaniasis. The polyprotein (KSAC) formulated with monophosphoryl lipid A, a widely used adjuvant in human vaccines, was found to be immunogenic and capable of inducing protection against *Leishmania infantum* responsible for human and canine visceral leishmaniasis as well as against *L. major* responsible for cutaneous leishmaniasis. The results demonstrate the feasibility of producing a practical, cost effective leishmaniasis vaccine capable of protecting both humans and dogs against multiple *Leishmania* species.

Keywords

leishmaniasis; vaccine; polyprotein
Introduction

Leishmaniasis represents a spectrum of diseases caused by protozoan parasites of the genus *Leishmania*. According to the WHO Health Report 2004, the disease is endemic in 88 countries, putting 350 million people at risk and resulting in 12 million cases. Current morbidity and mortality surveys estimate there are 2 million new cases of leishmaniasis and 59,000 deaths annually. Leishmaniasis can be classified into three general types of disease: cutaneous leishmaniasis (CL), mucosal leishmaniasis (ML) and visceral leishmaniasis (VL) based on the clinical manifestations of the disease. These three forms of leishmaniasis are caused by different parasite species, including *Leishmania major* (CL), *Leishmania braziliensis* (ML), and *Leishmania donovani* and *Leishmania infantum* (VL).

Although there is no commercially available vaccine for human leishmaniasis, extensive pre-clinical and promising clinical results indicate that control of the disease by preventative vaccination will likely become a viable approach (10, 32, 33). Evidence that people cured from VL develop protection mediated by Th1-type cellular responses against new infections (8, 19) is also supportive for possibility of a vaccine for disease prevention. First-generation vaccines using either parasite lysates or killed parasites have occasionally been shown to be safe and immunogenic and in some cases exhibited protective efficacy against leishmaniasis (1, 21, 40). However, whole inactivated *Leishmania* parasites will not consistently provide the safe, effective,
stable, and reliable source of antigens that is needed for a large-scale vaccination program against leishmaniasis because of the expense of running both a GMP parasite production facility and the subsequent antigen purification process and because the antigens represented in each batch of parasite culture will be affected by cell culture condition and antigen preparation processes.

Fortunately, there has been progress in characterizing defined *Leishmania* antigens that provide beneficial immune responses (10, 33). Those antigens, which include *Leishmania* homolog of receptors for activated C kinase (LACK), GP63, thiol-specific antigen (TSA), hydrophilic acylated surface protein B (HASPB), sterol 24-c-methyltransferase (SMT), kinetoplastid membrane protein-11 (KMP11), A2, and cysteine proteinase B (CPB), confer protection against disease in different animal models of leishmaniasis (3, 14, 16, 28, 36, 37, 44, 46). Because of the genetic polymorphism in the mammalian immune system, a vaccine composed of multiple antigens rather than a single gene product is more likely to elicit a protective immune response against leishmaniasis in a broad spectrum of individuals. We have previously produced the polyprotein antigen Leish-111f, which is composed of TSA (46), stress inducible protein 1 (LmSTI1) (7, 47) and a homolog to the eukaryotic translation initiation factor eIF4A (LeIF) (41, 42), and have shown that immunization with this polyprotein formulated in an adjuvant protects mice against *L. major* (11). Although the vaccine candidate provides some protection against *L. infantum* infection (9), Leish-111f was originally designed and optimized to...
target CL. In this study we focused on several proteins previously demonstrated to be protective against VL in animal models, including dogs, as well as recognized by humans cured from VL (22). The proteins KMP-11 (3), SMT (16), A2 (13, 14) and CPB (34, 36), were genetically fused to produce a single multi-epitope product developed with the goal of achieving a cost-effective product with maximum efficacy. We evaluated protective efficacy of the resulting polyprotein in two different experimental murine leishmaniases, CL and VL.
**Materials and Methods**

**Animals and parasites.** All mice were maintained in the Infectious Disease Research Institute (IDRI) animal care facility under specific pathogen-free conditions and were treated in accordance with the regulations and guidelines of the IDRI Animal Care and Use Committee. Female BALB/c and C57BL/6 mice (6–8 weeks old) were purchased from Charles River Laboratories (Wilmington, MA). Promastigotes of *L. infantum* (MHOM/BR/82/BA-2) were cultured as previously described (16). The *L. major* Friedlin strain clone V1 was kindly provided by Dr. David Sacks (NIH) and maintained as previously described (38).

**Production of KSAC.** Nucleotide sequences encoding individual components were PCR-amplified using Platinum® *Pfx* DNA Polymerase (Invitrogen) with genomic DNA from either *L. infantum* or *L. donovani* promastigotes. Primers, as listed in Table 1, were designed to amplify nt1-276 of KMP11 (GenBank: XM_001468995.1), nt4-1059 of SMT (GenBank: XM_001469795.1), nt150-779 of A2 (GenBank: S69693.1), and nt738-1687 of CPB (GenBank: AJ420286.1). Each of the sets of primers included unique restriction enzyme sites. After digestion of the amplified DNAs with the corresponding restriction enzymes, products were ligated to create a polyprotein gene construct with the sequence (5’ to 3’) KMP11, SMT, A2 and CPB and inserted into the pET-29 plasmid. The KSAC pET-29 construct was transformed into *E. coli* HMS174 (DE3).
For KSAC protein expression a single colony was inoculated into one liter of 2X yeast extract-tryptone medium containing 30 μg/ml kanamycin and grown at 37°C with shaking (225 rpm) until optical densities reached to 0.4 ~ 0.6. Gene expression was induced with 1 mM isopropyl-β-D-thiogalactopyranoside at 37°C for 3h. Cells were harvested by centrifugation (2,000 g, 15min), and pellets stored at -80°C.

The cell pellet was thawed and resuspended in 40 ml of cold Lysis Buffer (LyB: 50 mM NaCl, 10 mM Tris-HCl, pH8.0) and passed four times through an M-110S microfluidizer (Microfluidics, Newton, MA) set at 40 psi. The lysate was centrifuged at 10,000 g for 30 min, 4°C to pellet the inclusion body (IB). The IB was washed one time with 40ml LyB containing 1% CHAPS and once with 40ml 30% isopropanol with centrifugation (10,000 g for 30 min, 4°C) after each step. The washed IB pellet was stored at -80°C.

The IB material was solubilized in 30 ml Buffer A (8M urea, 20 mM Bis Tris Propane, pH7.0) at ambient temperature for 3h, and insoluble material was removed by a centrifugation at 10,000 g for 30 min, 4°C. Protein purification was carried out using an ÄKTA™ purifier system (GE Lifesciences). The solubilized IB fraction was loaded onto an anion exchange Q Sepharose Fast Flow resin (QFF, Amersham Biosciences) column equilibrated with Buffer A, the column washed with 6 column volumes of Buffer A, and KSAC eluted with 50mM NaCl-Buffer A at a flow rate of 14 ml/min. Those fractions containing KSAC, but having minimal
contamination by *E. coli* proteins (confirmed by western blotting) were pooled and dialyzed at 4°C against Buffer B (20 mM Tris-HCl, pH 7.5). The protein solution was adjusted to 1M NaCl and loaded onto a Macro-Prep Methyl HIC column (Bio-Rad, Hercules, CA) equilibrated with 1M NaCl-Buffer B. After washing the column with five volumes of 1M NaCl-Buffer B and three volumes of 0.75M NaCl-Buffer B, KSAC was eluted with 0.3M NaCl-Buffer B, dialyzed against Buffer B, and used for further studies.

Purity of the protein was assessed by SDS-PAGE and Coomassie blue staining. Protein concentration of the purified protein was calculated using $A_{280nm}$ and the extinction coefficient (1 $A_{280}=0.95$ mg/ml). Endotoxin levels of each purified protein lot were measured by a Limulus amoebocyte lysate test (Cambrex Corporation, East Rutherford, NJ).

**Other protein preparations.** Recombinant proteins of KMP11, SMT, A2 and CPB were produced as previously described (16, 22). L110f, which is an optimized derivative of Leish-111f, was produced as previously described (5). Lysates of *L. infantum*, *L. donovani* and *L. major* were prepared as previously described (15, 17). All the proteins had their endotoxin levels less than 100 EU/mg, determined by the Limulus amoebocyte lysate test.

**Immunization of mice.** Mice were immunized with either 10 μg of recombinant antigen (either SMT, L110f or KSAC) or a mixture of four proteins (2.5 μg each of KMP11, SMT, A2 and CPB).

For these immunizations, 20 μg of MPL-SE (GlaxoSmithKline Biologicals, Rixensart, Belgium)
were used as an adjuvant, the formulated vaccine were given in a volume of 0.1 ml. Control
groups received either saline or MPL-SE alone (20 μg). The mice were immunized three times
subcutaneously at the base of the tail at three week intervals.

**Antibody ELISA.** Mice were bled one week after the last vaccination. Mouse antibody ELISA
was performed as previously described (16). Either Lysate antigen (1 μg/well) or recombinant
protein (200 ng/well) was used for coating the ELISA plate. Reciprocal endpoint titers to
individual antigens were calculated with GraphPad Prism 5 software (GraphPad Software, Inc.,
La Jolla, CA) using the OD of 0.1 as a cutoff value. Endpoint titers of samples were recorded as
<100 if OD values of the samples were lower than the cutoff value at 1:100.

**Cytokine assay.** Spleens were collected two weeks after the last immunization (three mice per
group) and analyzed for antigen-specific IFN-γ production three days after stimulation, as
previously described (15).

**Intracellular staining and flow cytometry.** Single-cell suspensions were prepared from
individual spleens isolated from C57BL/6 mice two weeks after the last immunization. Cytokine
production by the splenocytes were analyzed upon antigen recall, as previously described (16).

**Challenge of mice with *Leishmania* spp.** C57BL/6 mice were challenged with 5 x 10^6
promastigotes of *L. infantum* by intravenous injection into the tail vein three weeks after the last
vaccination, and the liver and spleen parasite burdens were quantified four weeks post infection
by limiting dilution, as previously described (16). The challenge of BALB/c mice with *L. major* was performed by intradermal injection into both the right and left ears three weeks after the last vaccination, measurement of lesion sizes, and determination of ear parasite burdens at eight weeks post infection has been performed as previously described (15).
Results

Construction and immunological characterization of KSAC. The KSAC expression construct was prepared in order to express a fusion of KMP11, SMT, A2 and CPB (Figure 1A). Within the KSAC fusion, KMP11 was full length and the SMT region was missing its N-terminal methionine. A2, on the other hand, lacked its first 26 amino acids; these amino acids were predicted by SignalP 3.0 software (4) to comprise the signal peptide. The partial CPB sequence (a.a. 128-443) in our KSAC construct is the same one shown to be protective in previous studies (34, 36). KSAC was expressed in *E. coli* and purified to homogeneity using two orthogonal chromatographic steps. The apparent molecular mass of the protein (105 kDa by reducing SDS-PAGE) agreed well with the predicted value (Figure 1B). Due to the several Cys residues present in the fusion protein, KSAC has the tendency to form disulfide-bonded multimers in non-reducing conditions with apparent molecular masses at least twice that of the monomer (Fig. 1B). The purified KSAC was free of detectable *E. coli* protein contamination, based on western blotting (data not shown) and had a low endotoxin content (38 EU/mg protein).

To examine the ability of this antigen to elicit antigen-specific immune responses, C57BL/6 mice immunized with KSAC plus MPL-SE were examined for serum antibody titers to KSAC as well as the four component antigens. For comparison, mice immunized with a mixture of the four component antigens (K/S/A/C) plus MPL-SE were also examined. Injection with
either saline alone or MPL-SE alone did not induce antibody responses to KSAC (reciprocal endpoint titers <100). In contrast, mice vaccinated with either KSAC plus MPL-SE or K/S/A/C plus MPL-SE showed high levels of antibodies to KSAC (Figure 2). When responses to individual component antigens were examined, different patterns were observed in these two vaccination groups. When K/S/A/C was used for vaccination, antibody responses to SMT and CPB were dominant compared to responses to KMP11 and A2. In contrast, comparable levels of antibodies were detected to all the four antigens in mice vaccinated with KSAC.

To examine the ability of this antigen to induce the desirable Th1 type of immune response, splenocytes of C57BL/6 mice immunized with KSAC plus MPL-SE were examined for production of the cytokine IFN-γ after ex vivo antigen stimulation (Figure 3A). Injection of saline or MPL-SE alone did not induce any significant antigen-specific responses. Spleen cells from mice receiving either KSAC plus MPL-SE or K/S/A/C plus MPL-SE responded to vaccine antigens with elevated IFN-γ production when stimulated ex vivo with KSAC. These spleen cells were also capable of producing IFN-γ upon recall with lysate antigens of *L. infantum* and *L. donovani*. When individual components of KSAC were used for in vitro recall, SMT and CPB induced high levels of IFN-γ, whereas little, if any, IFN-γ was detected with KMP11 or A2 stimulation. Although IFN-γ response to A2 was higher in the K/S/A/C group than the KSAC group, it was not statistically significant. The general response pattern noted above was not
unique to C57BL/6 mice; a similar pattern was observed in BALB/c mice (Figure 3B). Once again, SMT and CPB were observed to be the dominant antigen components of KSAC.

To further investigate the quality of cellular responses induced by the KSAC/MPL-SE vaccine, splenocytes were analyzed for the frequency of CD4\(^+\) and CD8\(^+\) T-cells producing the Th1-type cytokines TNF-\(\alpha\), IL-2 or IFN-\(\gamma\) in response to incubation with KSAC (Figure 4A). The flow cytometric data show that antigen-specific CD4\(^+\) T-cells, but not CD8\(^+\) T-cells, were induced by KSAC/MPL-SE vaccination (Figure 4B). When the CD4\(^+\) T-cell population was subdivided into categories based on TNF-\(\alpha\), IL-2 and IFN-\(\gamma\) expression (a total of seven distinct populations), many of the Ag-specific T-cells were found to be multifunctional Th1 cells. In fact, that category of Th1 cells simultaneously producing TNF-\(\alpha\), IL-2 and IFN-\(\gamma\) was found to be the largest cell population of the seven (Figure 4C).

The KSAC/MPL-SE vaccine protects against *L. infantum* and *L. major*. The protective efficacy of the KSAC/MPL-SE vaccine against experimental challenge with *Leishmania* spp. was evaluated. To examine the efficacy of the vaccine against VL, immunized C57BL/6 mice were challenged by intravenous injection of 5 x 10\(^6\) promastigotes of *L. infantum*. At this challenge dose, separate experiments showed the peak liver parasite burden in C57BL/6 mice occurred at four weeks of infection (mean = \(\sim\)10\(^7\) amastigotes/liver) and between four and eight weeks in the spleen (\(\sim\)10\(^6\) amastigotes/organ, unpublished data). These kinetics of infection regarding parasite
burdens were consistent with that observed in other inbred mice (23). Thus, we chose four weeks as a time point for determining parasite numbers in spleens and livers of challenged mice. A statistically significant reduction in parasite numbers was seen in the livers of mice immunized with KSAC plus MPL-SE compared with those receiving saline or MPL-SE (Figure 5A). The protection level by vaccination with KSAC plus MPL-SE (93% reduction in parasite burden) was comparable to that with K/S/A/C plus MPL-SE (90% reduction). While not statistically significant, KSAC gave better protection against *L. infantum* infection than the single component SMT (81% reduction) and our first generation polyprotein vaccine antigen L110f (77% reduction). In a separate study, the protection in the spleen was examined for the KSAC/MPL-SE vaccine. Parasite numbers in the spleen of the vaccinated mice were significantly lower than those of unvaccinated mice (66% reduction). In multiple independent experiments, KSAC+MPL-SE consistently protected mice against *L. infantum* infection as well as or better than MPL-SE combined with SMT alone or L110f (Fig. 5 and data not shown).

KSAC/MPL-SE was assessed for the ability to also protect against *L. major* infection. Unimmunized control mice or vaccinated BALB/c mice were challenged with *L. major* promastigotes by intradermal injection into the ear. Control mice developed ear lesions, and the size of the lesions increased over time (Figure 5B, left). The lesions in the KSAC/MPL-SE group were significantly smaller than those of control group mice over the eight week period after
infection. The smaller lesions found in the vaccinated mice were associated with a significantly lower number of parasites (99.7% reduction) at the lesion site (Figure 5B, right). To explore the immune responses associated with vaccine-induced protection against *L. major* infection, unvaccinated mice or those vaccinated with KSAC/MPL-SE were examined for the production of anti-SLA antibodies and the cytokines IFN-γ and IL-4 eight weeks after infection. The IgG1-dominant antibody response to SLA observed in the unvaccinated mice after *L. major* infection was strongly suppressed in mice protected by KSAC/MPL-SE vaccination (Figure 6A), reflecting the observed shift to a Th1 response with vaccination. Analysis of splenic T-cell responses to SLA following infection demonstrated that vaccine-protected mice produced significantly less IL-4 than unvaccinated mice and the ratio of IFN-γ/IL-4 in response to SLA clearly shifted towards Th1 in the vaccinated and protected group (Figure 6B).
Discussion

We herein demonstrated that KSAC/MPL-SE is protective against *L. infantum*, which causes zoonotic VL in humans and dogs. VL is the most severe form of leishmaniasis, and development of a vaccine against VL is a high priority. The primary objective of the present study was to develop a subunit vaccine against VL with high efficacy and broad coverage of human or animal populations. Although Leish-111f/L110, our first generation polyprotein vaccine antigens, are promising, i.e., they are protective against both CL and VL in mouse models (9, 11), show therapeutic vaccine efficacy for canine VL (26, 45), and are showing some promise in human clinical trials (25, 31), the vaccine failed to protect dogs from VL in a field trial (18) and it may be possible to obtain a new construct with improved efficacy. KSAC could be one of such antigens, because it consistently protected as well as or better than Leish-111f/L110 against *L. infantum* infection as shown in this study, and also protected better than L110f against *L. major* infection using a sandfly challenge model (Gomes R et al., unpublished data).

Vaccination with KSAC plus MPL-SE induces antigen-specific multifunctional Th1 cells and protects mice against challenge with *L. infantum*, as well as *L. major*, which causes CL. Darrah et al. have reported that there is a correlation between the induction of multifunctional Th1 cells, which are individually capable of producing more than one Th1 cytokine, and protection against *L. major* infection (12). The three Th1-related cytokines TNF-α, IL-2 and...
IFN-γ are involved in protection against VL (29, 30, 43). As TNF-α synergizes with IFN-γ in killing *Leishmania* parasites (24), induction of Ag-specific T-cells capable of producing multiple cytokines upon Ag recall might be more beneficial for control of *Leishmania* infection than those producing a single cytokine, and such induction may be a good indicator of whether a vaccine composed of Ag and adjuvant is protective against leishmaniasis. The presence of such multifunctional Th1 cells may also have roles in suppressing Th2 responses that are, unless otherwise modulated, generated upon challenge. During *L. major* infection, post-challenge responses in the KSAC/MPL-SE vaccinated mice were associated with an improved Th1/Th2 balance to parasites, as represented by higher IFN-γ/IL-4 ratios and decreased IgG1 responses to SLA. These findings are consistent with previous reports that IL-4, as a Th2 cytokine, is associated with disease progression during *L. major* infection in BALB/c mice (20, 39).

It is not surprising that the polyprotein fusion KSAC is protective against *L. infantum* because individual antigens comprising the fusion proteins were selected based on their protective efficacy against VL (3, 14, 16, 36). Although a number of defined antigens have been identified as protective against leishmaniasis in experimental animal models, any single antigen may not be sufficient to efficiently protect the human population due to differences in their immunological backgrounds: Although inbred mice with uniform genotypes are often used in experimental models, humans and dogs have divergent genotypes. In mouse models, SMT is the
most immunogenic component of KSAC and, in our hands, is the most protective among the four components of KSAC when tested individually (22). In contrast, SMT is not always the strongest antigen in humans cured from VL (22). Thus, it is anticipated that one component may not be sufficient to serve as a protective vaccine antigen for every member of a genetically diverse population. In such cases, it is important to have additional protective components. In the mouse models used in this study, T cells were not as reactive to A2 and KMP11 as they were to SMT and CPB. Although one possibility is that A2 and KMP11 are not properly encoded in the KSAC construct, this can be ruled out based on the antibody ELISA data; mice immunized with KSAC plus MPL-SE showed antibody responses to A2 and KMP11. When the A2 sequence in the KSAC polyprotein was used as a single antigen for immunizing mice, it could induce detectable levels of T-cell responses (data not shown), suggesting the sequence itself is not a problem. A2 or KMP11 antigens were also not strongly recognized by T-cells from humans cured from VL (22), possibly suggesting lower antigenicity/immunogenicity than SMT or CPB in some species. However, these antigens may still be useful anti-VL vaccine antigens in other animals. In fact, protective efficacy of KMP11 delivered as DNA has been shown in a hamster model (3). It is also a fact that KMP11 as a single antigen was not able to induce even antibody response in mice when injected together with MPL-SE, while we could produce polyclonal antibody to this antigen in rabbits by injection with KMP-11 plus Freund’s adjuvant (data not shown). In a related fashion,
the immunogenicity and protective efficacy of A2 have been demonstrated recently in dogs, which are important reservoirs of *Leishmania* parasites in some endemic areas (13). Therefore, it will be intriguing to evaluate this vaccine for VL in variety of affected mammalian hosts including dogs and humans in future studies.

Due to the value of having more than one antigen in a subunit vaccine as a means to circumvent differing immune recognition within a heterogeneous population, a vaccination strategy using multiple antigens has been taken by our group and others as either polyprotein fusions or mixtures of multiple gene products (2, 9, 11, 27, 34). An advantage of a polyprotein over a mixture is the reduced manufacturing cost, provided that protective efficacy are equivalent between the two, as seen in this study. Using KSAC as an example, if the four individual components were produced separately, the cost of producing proteins individually will be much higher than for the KSAC fusion protein, which we have produced and purified on a large scale. Therefore, the use of this fusion strategy may be beneficial over the use of a protein cocktail in terms of simplicity and cost, and would be a practical format in clinical use.

Because some areas are endemic for multiple *Leishmania* species causing different forms of leishmaniasis and because manufacturing and distribution of a single, broadly useful vaccine would be cost effective, vaccines that are protective against multiple *Leishmania* species would be ideal. KSAC/MPL-SE vaccine induced protection not only against *L. infantum*, but also
against *L. major*, which causes CL. This broad protection is likely attributable to observations that, except for *L. donovani* complex-specific antigen A2, the other antigenic components of KSAC are conserved between *Leishmania* species and have been demonstrated to be protective against *L. major* infection (6, 15, 35).

In summary, we produced the polyprotein vaccine candidate designed to target VL and demonstrated its protective efficacy against both *L. infantum* and *L. major*. As vaccine development for leishmaniasis is advancing worldwide with some promise, it is important to have a clinical perspective, even during preclinical vaccine development. Factors relevant to clinical development, manufacturing, and wide-spread use are taken into consideration for the antigens tested in this study, and we believe that this polyprotein, or other *Leishmania* antigens/vaccines developed with these strategies, will be the lead vaccine antigens for leishmaniasis in dogs and humans beginning in the near future.
Acknowledgements

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**Figure 1. Purified recombinant KSAC.** (A) A schematic illustration of the KSAC construct. (B) A Coomassie blue-stained SDS/4-20% polyacrylamide gradient gel loaded with purified proteins (10 μg per lane) in reducing (R) and non-reducing (NR) conditions. Sizes of a protein marker (M) are shown in kDa.

**Figure 2. Induction of antibody responses by KSAC immunization.** C57BL/6 mice immunized three times with either saline, MPL-SE alone, K/S/A/C plus MPL-SE, or KSAC plus MPL-SE were examined for antibody response to KSAC as well as the four component antigens. Mice in saline and MPL-SE alone groups possessed no detectable antibodies to any of the antigens tested (data not shown). Mean and SEM of Reciprocal endpoint titers in K/S/A/C plus MPL-SE and KSAC plus MPL-SE groups are shown.

**Figure 3. Immunogenicity of KSAC in C57BL/6 and BALB/c mice.** C57BL/6 (A) or BALB/c mice (B) were immunized three times with either saline, MPL-SE alone, K/S/A/C plus MPL-SE, or KSAC plus MPL-SE, as described in Materials and Methods. IFN-γ production after ex vivo antigen stimulation of splenocytes from those immunized mice was determined by sandwich ELISA. Mean and SEM of three mice per group are shown.
Figure 4. Induction of multifunctional Th1 cells by the KSAC/MPL-SE vaccine. (A) The flow cytometric gating strategy is shown for CD4⁺ T-cells from the spleens of vaccinated C57BL/6 mice that produce IFN-γ, TNF-α and IL-2 upon antigen recall. (B) Percentage of IFN-γ-producing CD4⁺ or CD8⁺ cells in the spleens of mice immunized with either saline or KSAC/MPL-SE after in vitro culture with or without KSAC. Mean and SEM of three mice per group are shown. (C) Single-cell analysis of CD4⁺ T-cells producing multiple Th1-type cytokines upon recall with KSAC. Mean and SEM of three mice per group are shown.

Figure 5. Immunization with KSAC/MPL-SE protects mice from *L. infantum* and *L. major* challenge. (A) In Experiment 1, C57BL/6 mice were immunized with saline, MPL-SE alone, or the indicated vaccine and challenged with *L. infantum*. The number of parasites in each liver was measured by limiting dilution four weeks after the infection. In Experiment 2, C57BL/6 mice were immunized with saline or KSAC plus MPL-SE and challenged with *L. infantum*. The numbers of parasites in both the liver and spleen were measured by limiting dilution four weeks after the infection. (B) BALB/c mice immunized with saline or KSAC/MPL-SE were challenged with *L. major* intradermally into both the right and left ears. Lesion sizes of mice immunized with saline (○) or KSAC/MPL-SE (●) were measured every week up to 8 wks at which time the
parasite numbers in the ear lesions were determined by limiting dilution. Mean and SEM of five or six mice in each group are shown. *$P<0.05$, **$P<0.01$ and ***$P<0.001$ by unpaired t-test compared with the saline group. These data are representative of at least three independent experiments with similar results.

**Figure 6. Th1/Th2 balance of post-challenge immunity in the vaccinated/protected mice.**

Eight weeks after challenge with *L. major*, BALB/c mice pre-treated with either saline or KSAC/MPL-SE were examined for their immune responses to crude parasite antigen (LmSLA). (A) IgG1 and IgG2a levels to LmSLA were measured by ELISA (OD values are shown). Mean and SEM of five mice are shown. (B) IFN-$\gamma$ and IL-4 produced by spleen cells were analyzed upon antigen recall (open bars: saline placebo; closed bars: KSAC/MPL-SE vaccine). *$P<0.05$ by unpaired t-test is compared with the saline group. This is representative of three independent experiments with similar results.