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Immunization with a Recombinant Stage-Regulated Surface Protein from *Leishmania donovani* Induces Protection Against Visceral Leishmaniasis[1]

Simona Stäger,*† Deborah F. Smith,† and Paul M. Kaye[2]*

Vaccination against visceral leishmaniasis has received limited attention compared with cutaneous leishmaniasis, although the need for an effective vaccine against visceral leishmaniasis is pressing. In this study, we demonstrate for the first time that a recombinant stage-specific hydrophilic surface protein of *Leishmania donovani*, recombinant hydrophilic acylated surface protein B1 (HASPB1), is able to confer protection against experimental challenge. Protection induced by rHASPB1 does not require adjuvant and, unlike soluble *Leishmania* Ag + IL-12, extends to the control of parasite burden in the spleen, an organ in which parasites usually persist and are refractory to a broad range of immunological and chemotherapeutic interventions. Both immunohistochemistry (for IL-12p40) and enzyme-linked immunospot assay (for IL-12p70) indicate that immunization with rHASPB1 results in IL-12 production by dendritic cells, although an analysis of Ab isotype responses to rHASPB1 suggests that this response is not sufficient in magnitude to induce a polarized Th1 response. Although both vaccinated and control-infected mice have equivalent frequencies of rHASPB1-specific $CD4^+$ T cells producing IFN-γ, vaccine-induced protection correlates with the presence of rHASPB1-specific, IFN-γ-producing $CD8^+$ T cells. Thus, we have identified a novel vaccine candidate Ag for visceral leishmaniasis, which appears to operate via a mechanism similar to that previously associated with DNA vaccination. ***The Journal of Immunology*****, 2000, 165: 7064–7071.**

Human infection with *Leishmania donovani* and *Leishmania chagasi*, the causative agents of visceral leishmaniasis (VL),[3] may result in subclinical infection, or progress to a fatal outcome (1, 2). Epidemics of VL continue to exact a significant human toll in developing countries, notably in the Sudan, where overall death rates of 38–57% have been recorded in recent years (2). Although the immunology of VL has received considerable attention (3–7), efforts toward vaccination against leishmaniasis have focused almost exclusively on localized cutaneous disease (8–11). Although a number of candidate Ags, including gp63 (12–15) and LACK (16–19), have shown promise in mice, strong Th1-inducing adjuvants have usually been required, including IL-12 (18), CpG-containing DNA constructs (13, 16, 20), or delivery in recombinant bacteria (14, 15). Progress with these Ags in primate vaccination studies has been limited, requiring the use of both alum and IL-12 to demonstrate immunogenicity and partial protection (21).

Recently, much interest has been stimulated by the observation that protection against cutaneous leishmaniasis induced by protein vaccination is short-lived. Gurunathan et al. (17) demonstrated that protective immunity following immunization with recombinant LACK plus IL-12 waned after 2 wk, in contrast to the sustained (12-wk) protection achieved with a LACK-DNA construct. Furthermore, long-term provision of IL-12, in the form of a DNA/IL-12 construct, was able to induce long-term protection in combination with crude heat-killed *Leishmania major*. While these studies have not formally addressed the question of Ag persistence, they nevertheless suggest that continued presence of IL-12 is a requirement for long-lived vaccine-induced immunity and cast doubt on the utility of protein-based vaccines (17). Surprisingly, given previous data indicating a minimal role for $CD8^+$ T cells in resistance to primary infection (18, 22), $CD8^+$ T cells appear to be required for DNA vaccine-induced protection (16).

We have recently identified a heterogeneous family of acidic surface molecules (named HASPs), expressed only in metacyclic and amastigote stages of the *Leishmania* life cycle (23–25). These proteins share little identity with other polypeptides, but are all modified by dual acylation at their N termini. The HASP lipid anchors have been shown to be essential in intracellular trafficking and export to the parasite surface (26). Although the functions of the HASPs are as yet unknown, recent data have demonstrated the ubiquity of proteins of this type in all *Leishmania* species tested (*L. donovani* (25), *L. chagasi* (27), *L. mexicana*, *L. amazonensis* (S. F. Ma and D. F. Smith, unpublished observations)), suggesting their suitability as candidate Ags in the development of vaccines against the leishmaniases in general. HASPs from *L. donovani* and *L. chagasi* have been shown to be valuable in the immunodiagnosis of visceral leishmaniasis (27, 28).

In this study, we show that recombinant *L. donovani* HASPB1 is highly immunogenic and induces significant protection against challenge infection. rHASPB1 also induces the production of both IL-12p40 and IL-12p70 by splenic dendritic cell (DC). In contrast to immunization with soluble *Leishmania* Ag (SLA) + IL-12, rHASPB1 induces protection in both major target organs of infection. Furthermore, unlike the rHASPB1-specific response in control-infected mice, which is limited to $CD4^+$ T cells, vaccinated

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[3] Abbreviations used in this paper: VL, visceral leishmaniasis; BFA, brefeldin A; DC, dendritic cell; ELISPOT, enzyme-linked immunospot; HASP, hydrophilic acylated surface protein; HASPB1, hydrophilic acylated surface protein B1; LDU, Leishman Donovan units; p.i., postinfection; rm, recombinant murine; SLA, soluble *Leishmania* Ag; STAg, soluble *Toxoplasma* Ag.

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and protected mice have a high frequency of rHASPB1-specific $CD8^+$ T cells, which produce IFN-γ upon in vitro restimulation. Thus, rHASPB1 emerges as a major new candidate Ag for vaccination against visceral leishmaniasis, and the protection induced following rHASPB1 immunization shows characteristics more often associated with DNA vaccination.

Materials and Methods

Mice and parasites

Six-week-old female BALB/c mice were obtained from Tuck and Co. (Battesbridge, U.K.). Animals were kept under conventional conditions with free access to sterile food and water. An Ethiopian strain of *L. donovani* (LV9) was maintained by passage in Syrian hamsters, and amastigotes were isolated as previously described (29). Mice were infected by injecting 2×10^7 amastigotes i.v. via the lateral tail vein. The parasite burden in spleen and liver was determined by examining methanol-fixed, Giemsa-stained tissue imprints. Data are presented as Leishman Donovan units (LDU), in which LDU represents number of amastigotes/1000 host cell nuclei $\times$ organ weight (mg) (30).

Ag preparations

rHASPB1 was expressed as an N-terminal histidine-tagged protein in the pET15b vector (Invitrogen, San Diego, CA) and purified twice to homogeneity by affinity chromatography on a Ni^{2+} resin column (Qiagen, Chatsworth, CA), as described in Alce (25). Briefly, 2 L of logarithmic phase *Escherichia coli* BL21 (DE3) transformed with the pET15b-HASPB1 plasmid was induced for 2 h with 1 mM isopropyl β-D-thiogalactoside. Cells were collected by centrifugation, resuspended in 20 ml binding buffer (10 mM imidazole, 300 mM NaCl, 50 mM $Na_2H_2PO_4$) on ice, and lysed by freeze/thawing twice and sonication (5 times for 30 s on ice) in an ultrasonicator, before a final homogenization through a 25-gauge needle. After application to the column and extensive washing with binding buffer, rHASPB1 was eluted with a linear imidazole gradient starting from 10 mM imidazole up to 250 mM for 135 min, and then from 250 mM to 10 mM for 40 min. Small protein contaminants (<30 kDa) were removed by size exclusion centrifugation (Amicon, Beverly, MA). rHASPB1 was then dialyzed against sterile PBS and subsequently purified on a polymyxin B agarose column (Sigma, Poole, U.K.), to eliminate possible LPS contamination. Before vaccination, batches were tested for functionally relevant LPS contamination, by assaying their ability to synergize with IFN-γ for the induction of inducible NO synthase (31). No activity was detectable in such assays (sensitivity <1 ng/ml LPS; data not shown).

SLA was produced from stationary phase *L. donovani* promastigotes by the method of Scott et al. (32). Promastigotes were harvested from culture, washed three times with sterile PBS, and resuspended in a cocktail of protease inhibitors containing aprotinin (2 μg/ml), *N*-tosyl-L-phenylalanine chloromethyl ketone (100 ng/ml), and EDTA (1 mM). The cells were freeze-thawed twice, and then sonicated at 4°C three times for 30 s in an ultrasonicator. Finally, the suspension was centrifuged at 3000 $\times$ *g* for 20 min, and the pellet was discarded.

Immunization experiments

In the first two vaccination experiments, BALB/c mice ($n = 3$–5 per treatment at each time point) received s.c. immunization with either 1) 10 μg rHASPB1 with 1 μg murine rIL-12 (rmIL-12; Genetics Institute, Cambridge, MA); 2) 10 μg rHASPB1 in saline; 3) 10 μg SLA plus 1 μg rmIL-12; 4) 1 μg rmIL-12; and 5) saline. Three weeks later, mice were boosted with the same schedule, but the IL-12 dose was reduced to 0.5 μg. After an additional 3 wk, a final boost was given omitting IL-12. In the third vaccination experiment, mice ($n = 8$ per treatment at each time point) were immunized three times at 3-wk intervals with 10 μg rHASPB1 or OVA (Sigma). All mice were challenged 3 wk after the last boost with 2×10^7 amastigotes, given i.v. in the lateral tail vein.

Determination of anti-rHASPB1 Ab responses

Sera from immunized and/or infected mice were analyzed by ELISA for the presence of anti-rHASPB1 Abs. Nunc Maxisorp plates (Life Technologies, Paisley, U.K.) were coated overnight with 5 μg/ml rHASPB1 diluted in sodium carbonate-bicarbonate buffer (pH 9.6), and then blocked, after washing with PBS/Tween, with 1% BSA in coating buffer. The plates were then incubated with sera diluted 1/100 in assay buffer (PBS/Tween containing 5% FCS), for 2 h at 37°C. Polyclonal biotinylated rat anti-mouse IgG1 and IgG2a (Serotec, Oxford, U.K.), and streptavidin conjugated to HRP (Serotec) were added consecutively after washing with PBS/Tween. The plates were developed using the ABTS substrate (2,2′-azinobis(3-ethylbenzthiazoline)-6-sulfonic acid; Sigma) and read at 405 nm using an ELISA reader (Molecular Devices, Menlo Park, CA). Data represent the mean value from triplicate determinations of individual mice. Control naive mouse sera gave OD less or equal to zero.

T cell proliferation assays

Spleens from each individual mouse were homogenized through a 20-μm pore size sieve, and erythrocytes were lysed at room temperature using Gey's solution. Splenocytes were washed and resuspended in RPMI medium (RPMI 1640 supplemented with 10% FCS, 2 mM sodium pyruvate, 1 mM L-glutamine, 50 μM 2-ME, 100 U/ml penicillin/streptomycin; Life Technologies, U.K.) to a concentration of 10^6 cells/ml. Cells were then stimulated with rHASPB1, and proliferation was detected on day 4 by [^{3}H]thymidine incorporation. Data represent the mean $\pm$ SE of each group of animals. Culture supernatants were collected from these assays and assayed for IFN-γ and IL-4 using a capture ELISA, as described elsewhere (5).

Cytokine analysis by intracellular flow cytometry

To determine the frequency of T cells producing IFN-γ or IL-4, we used intracellular flow cytometry. Hepatic mononuclear cells were purified by collagenase digestion of perfused livers taken from infected or naive mice, as described in detail elsewhere (6). Hepatic and splenic cell populations (5×10^6/ml) were incubated in vitro for 4 or 18 h with or without 30 μg/ml rHASPB1 and 10 μg/ml rIL-2. Brefeldin A (BFA; 10 μg/ml) was then added to all cultures, and they were incubated for an additional 2 h. Cell suspensions were then recovered, washed in PBS + 0.1% sodium azide, and stained with FITC-labeled anti-$CD4^+$ (clone H129.19; Sigma) and Quantum Red-labeled anti-$CD8^+$ (clone 53-6.7; Sigma). After washing, cells were fixed with 2% paraformaldehyde, permeabilized with 0.1% saponin, and stained with R-PE anti-IFN-γ (clone XMG1.2; PharMingen, San Diego, CA) or biotinylated anti-IL-4 (clone BVD6-24G2; Serotec), followed by R-PE-conjugated streptavidin (Sigma). PE-labeled isotype controls were used to set gates for flow-cytometric analysis, which were performed using a FACScan (Becton Dickinson, Mountain View, CA) and CellQuest software. Ten thousand $CD4^+$ and $CD8^+$ cells were analyzed, and data were collected for individual mice, unless otherwise stated.

IL-12 induction by DC

An in vivo assay (33) was used to determine whether rHASPB1 was capable of eliciting an IL-12p40 response in murine DC. Briefly, mice were injected i.v. with 30 μg rHASPB1, OVA (Sigma), or LPS, or with 2×10^8 *L. donovani* amastigotes. Five and 24 h later, groups of three to five mice were sacrificed and their spleens were processed for the immunohistological detection of IL-12p40 using mAb C17.8. Data represent the frequency of IL-12p40-positive DC per 100 white pulp profiles (visualized by the injection of India ink 1 h before infection (33)). To detect IL-12p70, we used an enzyme-linked immunospot (ELISPOT) assay, as described elsewhere (34). Briefly, spleen cells (10^5/well in complete RPMI with 10% FCS) were seeded into 96-well plates precoated with mAb 9A5 (anti-IL-12p75; 5 μg/ml overnight at 4°C). After 20 h at 37°C, cells were removed by washing in PBS + 0.05% Tween 20, and the plates then incubated with biotinylated mAb C17.8 (anti-IL-12p40; 5 μg/ml overnight at 4°C). Spots were developed using avidin-alkaline phosphatase (Sigma; overnight at 4°C), followed by 5-bromo-4-chloro-3-indolyl phosphate-nitroblue tetrazolium substrate (Sigma). The number of spots per 10^6 spleen cells was calculated from duplicate wells. Each mouse was assayed individually ($n = 3$–5).

Statistical analysis

Statistical analysis was performed using a paired Student *t* test or Wilcoxon test, as appropriate for sample size. $p < 0.05$ was considered significant.

Results

Vaccination with rHASPB1 or SLA + IL-12 reduces hepatic parasite burden following challenge with L. donovani

L. donovani infection in the liver of BALB/c mice is usually self limiting, with granuloma maturation and parasite clearance occurring over a 2–4-mo period (3, 29). This curative response is often regarded as resembling that which occurs during subclinical infection in humans (1). We therefore evaluated the capacity of rHASPB1 to promote this self-curing response in BALB/c mice.

Representative data from a series of independent vaccination experiments, involving over 200 mice, are shown in Fig. 1. In our initial experiments, we wished to compare the vaccine potential of rHASPB1 in the presence or absence of IL-12 as an adjuvant. SLA + IL-12, the benchmark combination for inducing protection against *L. major* (17, 32), was used as a positive vaccine control, even though it has not been previously evaluated for efficacy against *L. donovani*. Our data (Fig. 1*A*) indeed demonstrate that mice receiving SLA + IL-12 acquire significantly enhanced resistance to hepatic infection with *L. donovani* ($p < 0.01$ and $p < 0.05$ at days 28 and 56, respectively), although this does not exceed a 50% reduction in peak parasite burden. Mice vaccinated with rHASPB1 + IL-12 also demonstrated significant levels of protection in the liver (ranging from 49% at day 14 to 78% at day 80, compared with mice receiving IL-12 alone; $p < 0.02$). Unexpectedly, mice immunized with rHASPB1 alone were comparably resistant to those that also received IL-12 (ranging from 31% at day 14 to 91% at day 80, compared with mice receiving saline alone; $p < 0.001$). To confirm that rHASPB1 induced protection in the absence of adjuvant, a further vaccination experiment was performed. Although in this experiment, peak parasite burden was considerably higher (possibly as a result of variations in the infectivity of the amastigotes used), rHASPB1 still induced significant protection at all time points analyzed (Fig. 1*C*; $p < 0.001$; $n = 8$).

rHASPB1, but not SLA + IL-12, induces protection against splenic infection with L. donovani

In contrast to the naturally acquired resistance to hepatic infection, *L. donovani* persists in the spleen of BALB/c mice, with the concomitant development of considerable organ-specific pathology similar to that seen in human kala azar (29). It was therefore important to evaluate the impact of vaccination in this organ. In contrast to its efficacy in the liver, SLA + IL-12 failed to provide any protection against parasite growth in the spleen. In contrast, rHASPB1 demonstrated protection ranging between 70 and 90% at day 80 postinfection (p.i.) (Fig. 1, *B* and *D*; $p < 0.001$). Somewhat surprisingly, rHASPB1 + IL-12, although as effective as rHASPB1 at day 56 p.i., induced intermediate levels of protection at day 80 p.i. However, a single outlying animal in the IL-12 alone control group meant that the protection by rHASPB1 + IL-12 at day 80 was not significant. This notwithstanding, these data indicate that immunization with rHASPB1, in the absence of adjuvant, is able to overcome the natural failure of BALB/c mice to contain parasites in the spleen.

Immunization with rHASPB1 stimulates DC to produce IL-12

DC are now recognized as the dominant APC responsible for T cell priming, and the production of IL-12 by DC may also play a role in subsequent Th1 differentiation (35, 36). Given the protection induced by rHASPB1, and the known importance of IL-12 for natural protection against *L. donovani* (34), we asked whether immunization with rHASPB1 stimulated DC to produce IL-12. To allow us to compare the response to rHASPB1 with other published studies on microbial stimulation, we injected rHASPB1 i.v., to directly target splenic DC populations (33, 37, 38). IL-12 induction was measured in two ways. First, we identified IL-12p40-producing cells in the marginal zone/periarteriolar region of the spleen using immunohistochemistry, and scored their frequency relative to the total number of white pulp profiles examined (33). Within 5 h of rHASPB1 injection, the frequency of IL-12p40-positive cells was increased, compared with both naive mice or mice receiving OVA (118 ± 8 vs 38 ± 8 and 53 ± 8, respectively; $p < 0.001$ and $p < 0.003$). Responding DC were as expected, at a lower frequency than observed following administration of 2×10^8 amastigotes (392 ± 57 IL-12p40$^+$ DC/100 white pulp profile), but IL-12 production was similarly restricted to DC at the borders

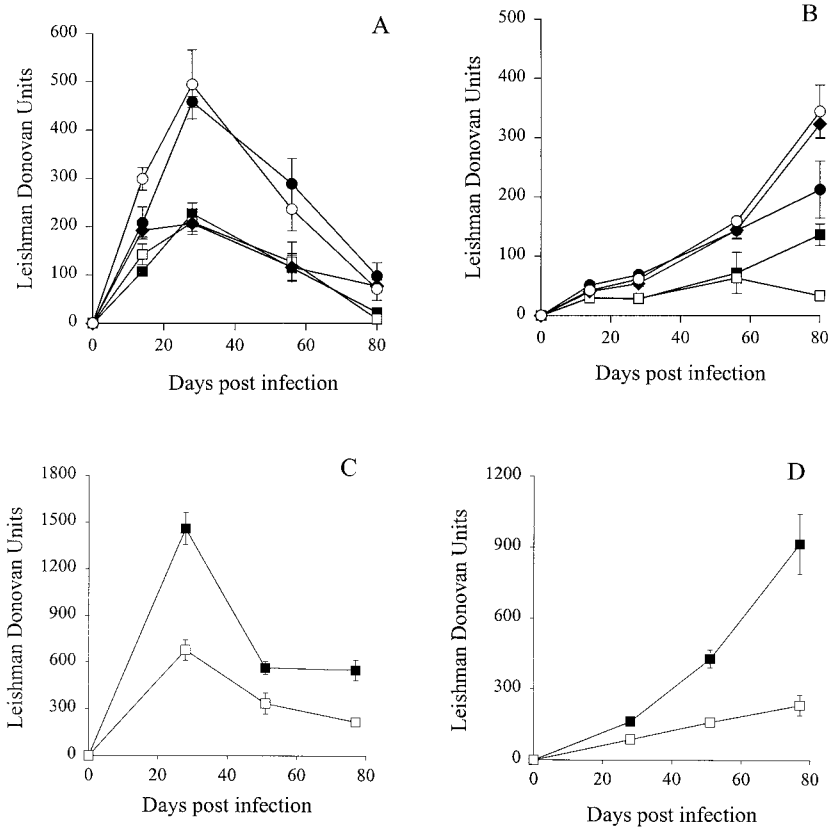

FIGURE 1. rHASPB1 protects BALB/c mice against hepatic infection with *L. donovani.* Mice were immunized with rHASPB1 (□), rHASPB1 + IL-12 (■), or SLA + IL-12 (♦). Control mice received IL-12 alone (●) or saline (○). Three weeks after boosting, they were challenged with 2×10^7 amastigotes of *L. donovani*, and the course of infection determined in the liver (*A*) and spleen (*B*). One of two independent experiments with similar results is shown. In a further experiment, mice were immunized with rHASPB1 (□) or OVA (■), and the course of infection determined in the liver (*C*) and spleen (*D*). Data represent mean LDU ± SE for individual mice ($n = 4$ in *A* and *B*, $n = 8$ in *C* and *D*), determined from stained impression smears.

of the marginal zone and in the periarteriolar lymphocytic sheath. Furthermore, staining was not noted in all white pulp profiles, again as observed following amastigote infection. The production of IL-12p40 was also maintained at comparable levels 24 h after injection (data not shown). In a further control experiment, a similarly purified, His-tagged recombinant protein from *L. major* (the Gene D-encoded surface hydrophilic endoplasmic reticulum-associated protein (Refs. 23 and 24 and E. Knuepfer and D. F. Smith, unpublished observations)) failed to induce significant IL-12p40, ruling out a contribution of the histidine tag or contaminating bacterial products to the bioactivity of rHASPB1 (data not shown). Second, and to demonstrate the production of biologically active IL-12, we used an ELISPOT assay to detect IL-12p70-producing cells. As shown in Fig. 2, rHASPB1 induced IL-12p70 production ($p < 0.04$ and $p < 0.01$ compared with OVA-injected and naive mice, respectively). As expected, infection with amastigotes induced a stronger response than rHASPB1 alone ($p < 0.001$). As DC comprise approximately 5% of the total spleen cell suspension (S. Stäger and L. Dianda, unpublished observations), these data indicate that rHASPB1 specifically induces IL-12p70 in approximately 1% of splenic DC, compared with the 3% stimulated by amastigote infection. These data accord well with the histological evaluation of IL-12p40 (above and (33)). In contrast, 70–90% of lymphoid DC (representing approximately 30–40% of the total DC present in spleen) make IL-12p40 following administration of soluble *Toxoplasma* Ag (Refs. 37 and 38, and L. Dianda, C. R. Engwerda, and P. M. Kaye, unpublished observations). Thus, in vivo injection of rHASPB1 stimulates a restricted number of DC to make biologically active IL-12.

rHASPB1 induces a humoral immune response with Th2 characteristics

Ab isotype profile provides a convenient surrogate marker of Th1 and Th2 $CD4^+$ T cell differentiation (22). We therefore analyzed the response to rHASPB1 following immunization and after challenge infection. We reasoned that preimmunization isotype responses should provide some indication as to the extent to which IL-12 produced by DC in response to rHASPB1 impacted on Th subset development. As shown in Table I, rHASPB1 induced an exclusively IgG1 response, suggesting that in spite of its capacity to trigger IL-12 production by some DC, immunization with this protein stimulates a predominantly Th2 response. The addition of exogenous IL-12 during vaccination promoted a significant decrease in the IgG1 response ($p < 0.001$), with a compensatory trend toward an increased IgG2a response ($p = 0.09$). These data confirm that, as in other systems (18), the presence of sufficient IL-12 is able to skew $CD4^+$ T cell differentiation following rHASPB1 immunization along the Th1 pathway. Mice immunized with SLA + IL-12 failed to make a significant Ab response to rHASPB1 before challenge (Table I), suggesting that in these preparations of SLA, HASPB1 is a minor component.

Following challenge with *L. donovani*, there was little change in the isotype profile in vaccinated mice, although intermouse variability in the response to rHASPB1 was more apparent (Table I). Thus, at day 14 p.i., mice immunized with rHASPB1 made an almost exclusively IgG1 response, compared with that seen in mice immunized with rHASPB1 + IL-12. Abs to rHASPB1 did not appear at significant levels in control unvaccinated mice or mice vaccinated with SLA + IL-12 until day 56 postchallenge and, as described previously, for the response to whole crude *Leishmania* Ags (5), were of mixed isotype. Importantly, these data serve to contrast the mixed isotype response seen in long-term infected control mice with the relatively fixed IgG1 response of rHASPB1-vaccinated mice.

Cellular response to rHASPB1 in vaccinated mice

As a strong IgG1 response was unexpected, we directly examined the production of IFN-γ and IL-4 following restimulation of lymphocytes in vitro. In conventional restimulation assays, spleen cells from mice immunized with rHASPB1, but not control unimmunized or SLA + IL-12-immunized mice, proliferated in response to rHASPB1 (Fig. 3). Analysis of supernatants from these cultures by ELISA failed to detect IL-4 (assay sensitivity <3 U/ml). Surprisingly, given the shift in isotype response, cells from mice immunized with rHASPB1 + IL-12 proliferated to a similar extent and produced comparable levels of IFN-γ to mice immunized with rHASPB1 alone (5.3 ± 1.6 ng/ml vs 8.4 ± 2.2 ng/ml, respectively, at 30 μg/ml rHASPB1). No IFN-γ was detected in cultures from these vaccinated mice in the absence of added Ag (assay sensitivity <0.1 ng/ml).

To more fully characterize the cellular response to rHASPB1, we proceeded to use intracellular cytokine staining followed by

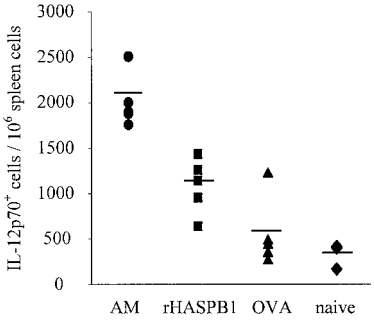

FIGURE 2. Immunization with rHASPB1 induces IL-12p70 production by DC. Mice were injected with 30 μg rHSPB1 or OVA, or infected with 2×10^8 amastigotes (AM) and 5 h later, spleens were removed and assayed by ELISPOT for the production of IL-12p70, as described in *Materials and Methods*. Data represent the frequency of IL-12p70$^+$ cells per 10^6 spleen cells. Each symbol represents an individual mouse ($n = 5$ for AM, rHASPB1, and OVA; $n = 3$ for naive). Mean values are indicated by a bar. Amastigote infection induces a response significantly greater than that of rHASPB1 ($p < 0.001$), and rHASPB1 induces a response significantly greater than seen in OVA-injected mice ($p < 0.04$) and naive mice ($p < 0.01$).

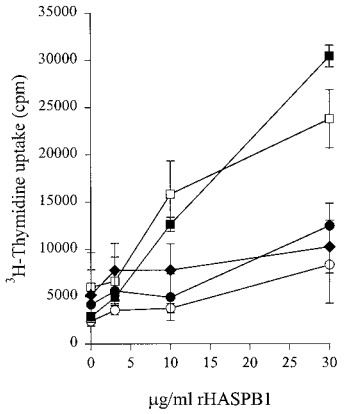

FIGURE 3. T cell responses to rHASPB1 in immunized and control mice. BALB/c mice were immunized as described in *Materials and Methods*, with rHASPB1 (□), rHASPB1 + IL-12 (■), or SLA + IL-12 (◆). Control mice received IL-12 alone (●) or saline (○). Three weeks after the last boost, spleen cells were restimulated in vitro with the indicated doses of rHASPB1, and proliferation determined by thymidine uptake on day 4. Data represent mean ± SE for individual mice ($n = 3$).

Table I. *Isotype-specific responses to rHASPB1 in vaccinated BALB/c mice*

	Anti-rHASPBI Ab Response[a] (mean OD ± SE) in Mice Immunized with				
	rHASPB1	rHASPB1 + IL-12	SLA + IL-12	IL-12	PBS
IgG1					
Prechallenge	1.182 ± 0.080	0.269 ± 0.120	ND[b]	ND	ND
14 days p.i.	0.836 ± 0.205	0.446 ± 0.270	ND	ND	ND
28 days p.i.	0.669 ± 0.300	0.449 ± 0.290	0.080 ± 0.040	0.036 ± 0.017	0.023 ± 0.010
56 days p.i.	0.507 ± 0.140	0.426 ± 0.078	0.511 ± 0.044	0.550 ± 0.042	0.364 ± 0.080
IgG2a					
Prechallenge	0.015 ± 0.004	0.074 ± 0.040	ND	ND	ND
14 days p.i.	0.041 ± 0.038	0.250 ± 0.300	ND	ND	ND
28 days p.i.	0.160 ± 0.234	0.211 ± 0.213	0.010 ± 0.010	ND	ND
56 days p.i.	0.050 ± 0.010	0.100 ± 0.060	0.113 ± 0.050	0.145 ± 0.070	0.260 ± 0.112

[a] Anti-rHASPBI Ab responses were detected using an isotype-specific ELISA, as described in *Materials and Methods.*
[b] ND, Not detectable (OD ≤ 0).

flow cytometry. Pooled spleen cells from immunized or OVA-control immunized mice were restimulated overnight with 30 μg/ml rHASPB1, followed by incubation in BFA for an additional 3 h to allow cytokine accumulation. All cultures were supplemented with IL-2, as this cytokine is known to be an important cofactor for IFN-γ production by $CD8^+$ T cells. The frequency of IFN-γ-producing $CD4^+$ T cells in the absence of Ag was less than 0.25% in both control and immunized mice, and this did not change upon in vitro restimulation with rHASPB1. The results from analyzing IFN-γ production by $CD8^+$ T cells were, however, quite different. Control mice had a very low frequency of $CD8^+$ T cells able to spontaneously make IFN-γ in vitro (<0.1%), and although this was increased by the addition of rHASPB1 (0.23%), the absolute frequency remained low. In contrast, 1% of $CD8^+$ T cells from vaccinated mice produced IFN-γ in the absence of in vitro Ag restimulation (but in the presence of IL-2), and this increased to 2.5% following restimulation with rHASPB1. We were unable to reproducibly detect intracellular IL-4 in these assays, in either the $CD4^+$ or $CD8^+$ T cells. Collectively, these data indicate that the principal recall response to rHASPB1 in vaccinated mice involves IFN-γ production by $CD8^+$ T cells.

Challenge infection expands a rHASPB1-specific $CD8^+$ population in vaccinated but not control mice

Most previous vaccination studies have restricted their analysis of the cellular response to that seen after challenge infection. To analyze the response after challenge infection in our model, we sampled tissues at the times of maximal difference in parasite load between vaccinated and control groups of mice. Figs. 4 and 5 show the results of an analysis of cytokine production by hepatic mononuclear cells derived from vaccinated mice 28 days postchallenge, compared with control OVA-vaccinated mice and naive mice. For technical reasons, we pooled the livers from eight individual mice into four groups of two. These were then restimulated in vitro for 4 h with either rHASPB1 or OVA and analyzed for the production of IL-4 and IFN-γ. Although the frequency of hepatic $CD4^+$ T cells producing IFN-γ was above that of naive mice, as predicted from the presence of an ongoing granulomatous response and from previous ELISPOT data (6, 34), we did not observe any consistent response following rHASPB1 restimulation in vitro (Fig. 4*A*). In contrast, in three of the four pools of hepatic T cells, there was clearly an increase in the frequency of IFN-γ-producing $CD8^+$ T cells following restimulation with rHASPB1 ($p < 0.05$ for the entire group, using Student's *t*; Figs. 4*B* and 5).

The data obtained from day 51 postchallenge spleen cell cultures, in which we were able to examine mice individually ($n = 8$), were even more compelling, and are shown in Fig. 6. A number of important points emerge from this data: 1) naive mice fail to make a significant response to rHASPB1 in vitro; 2) priming of a

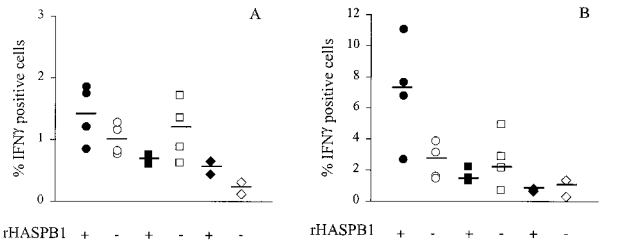

FIGURE 4. IFN-γ production by hepatic lymphocytes in mice immunized with rHASPB1. Hepatic mononuclear cells from naive mice (diamonds), day 28 infected OVA-immunized mice (squares), and rHASPB1-immunized mice (circles) were restimulated in vitro with (closed symbols) or without (open symbols) 30 μg/ml rHASPB1, in the presence of rIL-2. At 4 h, BFA was added, and 2 h later, cells were harvested and stained for CD4, CD8, and IFN-γ, as described. Data represent frequency of $CD4^+$ (*A*) or $CD8^+$ (*B*) cells stained for IFN-γ, and each symbol represents cells pooled from two mice ($n = 4$). Horizontal bar represents the mean value.

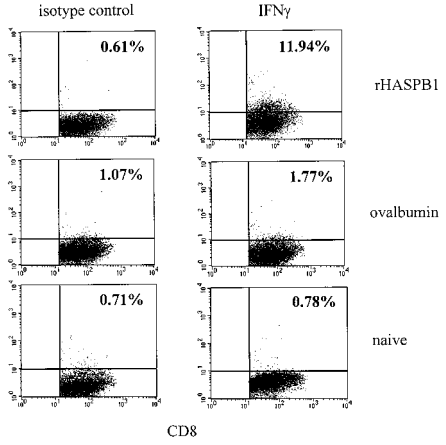

FIGURE 5. IFN-γ production by hepatic $CD8^+$ T cells in rHASPB1-immunized mice. Pooled hepatic mononuclear cells, isolated from pairs of mice immunized with rHASPB1 or OVA, and from two naive mice, were restimulated in vitro with rHASPB1 for 4 h, followed by culture in BFA for 2 h. Gated $CD8^+$ T cells are shown, stained either with a PE-labeled isotype control (*left column*) or with PE-labeled anti-IFN-γ (*right column*). The percentage of positive cells is indicated in the *top right quadrant*. Cells cultured in the absence of rHASPB1 showed little IFN-γ production (see Fig. 4 for full analysis of all mice examined).

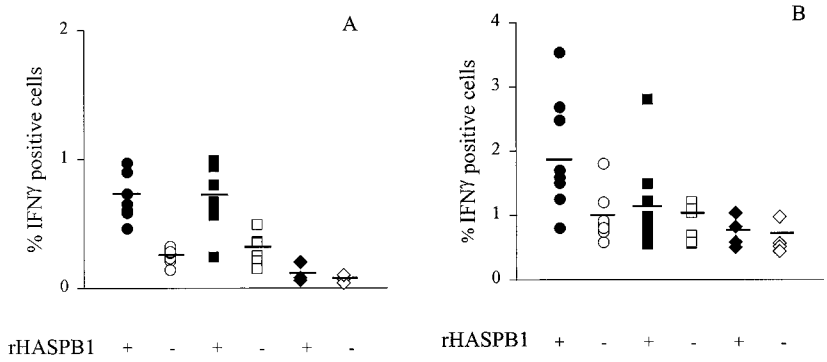

FIGURE 6. IFN-γ production by splenic lymphocytes in mice immunized with rHASPB1. Spleen cells from naive mice (◇, ◆), day 28 infected OVA-immunized mice (□, ■), and rHASPB1-immunized mice (○, ●) were restimulated in vitro with (◆, ■, ●) or without (◇, □, ○) 30 μg/ml rHASPB1, in the presence of rIL-2. At 4 h, BFA was added, and 2 h later, cells were harvested and stained for CD4, CD8, and IFN-γ, as described. Data represent frequency of $CD4^+$ (*A*) or $CD8^+$ (*B*) cells stained for IFN-γ, and each symbol represents an individual mouse ($n = 8$). Horizontal bar represents the mean value.

rHASPB1-specific $CD4^+$ population occurs during natural infection and this population can be restimulated in vitro to produce IFN-γ ($p < 0.01$ vs unstimulated controls); 3) vaccination does not alter the frequency of rHASPB1-specific $CD4^+$ T cells making IFN-γ at this stage of infection. These data are consistent with the detection of similar Ab responses to rHASPB1 in vaccinated and control mice at later times in infection, and again suggest that vaccination has not overtly affected $CD4^+$ Th1 development; and 4) whereas in seven of eight control mice, infection alone fails to prime a rHASPB1-specific $CD8^+$ T cell response, vaccinated and infected mice have a significant $CD8^+$ response following restimulation in vitro with rHASPB1 ($p < 0.025$ vs unstimulated controls). Again, we were unable to detect IL-4 production, by intracellular flow cytometry, in any of these groups of mice following restimulation in vitro (data not shown). Thus, an elevated frequency of Ag-specific, IFN-γ-producing $CD8^+$ T cells is the main correlate of protection in this vaccination model.

Discussion

Although human vaccination against leishmaniasis is currently proceeding with a combination of heat-killed promastigotes and bacillus Calmette-Guérin, there remains an urgent need to identify new candidate Ags for use singly, or as part of a protein or DNA vaccine cocktail. In this study, we have shown that a member of a recently described family of infective stage-specific Ags promotes effective immunity against *L. donovani* challenge. Our data, however, raise a number of important questions regarding the mechanisms of vaccine-induced protection in this and perhaps other models.

First, the finding that rHASPB1 is highly immunogenic and protective, even in the absence of adjuvant, suggested that this molecule may have inherent adjuvant activity, possibly mediated through the induction of IL-12 (36). IL-12 is a key component in the early response to *L. donovani*, and neutralization of IL-12 over the first few days postinfection leads to elevated parasite burdens in both the spleen and liver (34). Recent studies suggest three main routes leading to IL-12 production by DC. The first is typified by bacterial LPS, which induces a rapid but transient IL-12 response (37, 38). In vivo and in vitro, most DC are reported to respond to LPS, and immunohistochemical detection of IL-12p40 highlights a considerable network of DCs in the periarteriolar lymphocytic sheath region of the spleen after LPS administration. Cessation of IL-12 production within 24 h occurs due to apoptotic death of DC exposed to LPS (38).

The second pathway of IL-12 production by DC has been defined with soluble *Toxoplasma* Ag (STAg). Intravenous administration of 30 μg STAg (the dose of rHASPB1 used in our studies) also induces IL-12 with rapid and transient kinetics. Most responsive DC belonging to the $CD8\alpha^+$ lymphoid-derived subset, and up to 90% of such cells are induced to respond in vivo. However, rapid decay in IL-12 production, reaching baseline levels within 24 h of STAg administration, results from the induction of a state of DC paralysis, rather than death (38). Among leishmanial Ags, LeIF has been shown to be a potent inducer of IL-12 from monocyte-derived human DC (39) and from murine macrophages (40) in vitro. It remains to be determined whether LeIF directly stimulates murine DC, either in vitro or in vivo. Notably, LeIF also induces partial protection against *L. major* in BALB/c mice in the absence of added adjuvant (41).

The third pathway for IL-12 production by DC requires cognate interactions with T cells (42–44). This pathway has been best characterized for simple hapten-protein conjugates, and may represent a default response for the production of IL-12 in the absence of a microbial cue. During the response to the hapten (4-hydroxy-3-nitrophenyl)acetyl, DC interactions with naive $CD4^+$ T cells induce a predominantly Th2 response, characterized subsequently by IgG1 subclass switching in cognate B cells (45), and it has been suggested that the levels of IL-12 produced following OX40-OX40L and CD40-CD40L interactions are insufficient to drive Th1 differentiation (46). We have recently shown that following *L. donovani* infection, the production of IL-12 is limited to a small fraction of the total DC pool (<5%), can still be detected at 24 h, and is biased toward the $CD8\alpha^-$ myeloid DC subset (33).[4] Furthermore, IL-12 production by both $CD8\alpha^+$ and $CD8\alpha^-$ DC is abolished by prior depletion of $CD4^+$ T cells.[4] Thus, viable *L. donovani* infection fails to initiate a conventional microbial response by splenic DC. The distribution and frequency of IL-12-producing DC in situ, the kinetics of IL-12 production, and the dominant IgG1 Ab response all suggest that the response to rHASPB1 also results from cognate T cell-DC interactions. Unfortunately, we have not been able to directly demonstrate rHASPB1-specific IL-4-producing cells by intracellular flow cytometry. As our staining techniques are able to detect polyclonal IL-4-producing cells ex vivo from the liver and spleen of infected mice (Sanchez et al., manuscript in preparation), we assume that rHASPB1-specific, IL-4-producing T cells, although potent functionally, are nevertheless present at very low frequency.

A simple default to the Th2 pathway of CD4 differentiation would not, however, explain two other aspects of our data. First, IFN-γ can be detected during in vitro restimulation with rHASPB1, and is equivalent in mice immunized with either rHASPB1 alone or rHASPB1 + IL-12. Furthermore, IL-12 has no additive advantages compared with rHASPB1 alone for the induction of protection in the liver, and in fact was mildly detrimental in the spleen. The Ab isotype profile, however, shifts significantly on addition of IL-12, indicating biological activity of the rIL-12 we

[4] L. Dianda, C. R. Engwerda, and P. M. Kaye. T cell dependence of dendritic cell IL-12 production following *Leishmania donovani* infection. *Submitted for publication.*

used. Second, our data clearly demonstrate that immunization with rHASPB1 induces protection against *L. donovani*, a process believed to require IFN-γ (3, 47). At least a partial explanation for these data may lie in the observation that immunization with rHASPB1 primes $CD8^+$ T cells, which can be subsequently restimulated to produce IFN-γ both before and following challenge infection. IL-12 may be important, both directly and indirectly, for regulating $CD8^+$ T cell function. IL-12 directly promotes low level IFN-γ production by activated $CD4^+$ and $CD8^+$ T cells, but preferentially induces the expression of IL-18R on $CD8^+$ T cells. This facilitates high levels of IFN-γ production by $CD8^+$ T cells in response to IL-18 (48). Thus, if $CD8^+$ T cells are the dominant source of IFN-γ following rHASPB1 immunization, as our data suggest, the inability of exogenous IL-12 to enhance production of this cytokine in vitro, and indeed to promote vaccine-induced protection, may reflect limitations on the production of IL-18 during vaccination and challenge. The observation that anti-IL-12 treatment in this model decreases both IFN-γ and IL-4 levels (34) also suggests that the relatively low level of IL-12 induced by both immunization and infection sustains the development of a broad range of immune responses. Indeed, this may have beneficial consequences, given that early exposure to IL-4 has recently been demonstrated to have an important role in the generation of $CD8^+$ T cell memory (49). Thus, the presence of an excess of exogenous IL-12 during immunization with rHASPB1 may indirectly inhibit $CD8^+$ T cell memory by dampening early Th2 cell development (35), with consequent reduction in long-term protection (Fig. 1). Given these complexities, it will now be important to functionally evaluate the relative contribution of IL-18, as well as potential interactions between $CD8^+$ T cells producing IFN-γ and $CD4^+$ T cells inducing IgG1 (presumptive IL-4-producing Th2 type cells) in this vaccination model. IL-18 has not to date been analyzed during experimental VL, but others have previously provided evidence for a host-protective role for IL-4 (3, 50), and an influence of $CD8^+$ T cells in experimental VL is widely acknowledged (51–53). We are currently evaluating vaccine efficacy in IL-4, IL-4R, and β_2-microglobulin knockout mice, to address some of these issues. The outcome of these studies will be important not only for understanding protection in this model, but also perhaps in ensuring the choice of appropriate strategies for evaluating vaccine efficacy in humans.

$CD8^+$ T cells were recently shown to be critical to the induction and expression of long-term immunity generated by immunization with LACK-DNA (16). Although difficult to directly compare these studies with our own, it is noteworthy that in many of the mice in our study, we detected Ag-specific $CD8^+$ T cells at a greater frequency, but with lower staining intensity, than those reported following LACK-DNA immunization. Whether this reflects methodological differences (e.g., the addition of anti-CD28 mAb (17)), the sampling site, or the relative immunogenicity of LACK and HASPB1 during these infections remains to be determined. The finding that $CD8^+$ T cells are effectively primed by rHASPB1 was unexpected, but the common involvement of $CD8^+$ T cells suggests that rHASPB1 might also induce long-term immunity similar to DNA vaccines. In this regard, preliminary data do indeed indicate that the reduction in peak hepatic parasite burden induced by rHASPB1 remains stable when comparing mice challenged 3 wk or 3 mo after boosting (Stager et al., unpublished). Significantly, no protection is seen in long-term challenged mice following SLA + IL-12 immunization, as also shown for *L. major* (17). In vitro priming of $CD8^+$ T cells by Ag-pulsed DC has been well described, and such Ag-pulsed DC can effectively stimulate effector $CD8^+$ T cell responses upon adoptive transfer (54–58). We are currently evaluating whether rHASPB1-pulsed DC are also able to induce immunity to challenge. This strategy may allow us to further enhance protection mediated by rHASPB1, given the recent finding that IL-12-transfected DC pulsed with SLA induce protection against *L. chagasi* (59).

Although we could detect rHASPB1-specific $CD4^+$ T cell responses in the spleen of immunized mice, we could not detect these in the liver. It has previously been shown that $CD8^+$ T cells predominate in the later stages of hepatic infection with *L. donovani*, and $CD4^+$ T cell numbers begin to decline (60), either by migration or cell death. We cannot exclude that hepatic rHASPB1-specific $CD4^+$ T cells are more sensitive to rapid activation-induced cell death, in the presence of IL-2, than splenic $CD4^+$ T cells. Recent studies[5] do indeed indicate higher levels of apoptosis in the liver than spleen, but this is equally if not more so for $CD8^+$ T cells. Alternatively, and more likely, the APCs in these hepatic mononuclear cell preparations may not support optimal restimulation of IFN-γ-producing $CD4^+$ T cells (61).

Finally, a significant finding in this study is that vaccine-induced immunity, like that following normal infection, is regulated in an organ-specific manner (47). SLA + IL-12, a surrogate for human vaccines currently being tested, was able to induce effective immunity in the liver of mice, to the same degree as that seen in mice vaccinated with rHASPB1. In contrast, vaccination with SLA + IL-12 failed to make any impact on the course of infection in the spleen. Parasites in the spleen are also more resistant to various immunological interventions (5, 6), and to T cell-dependent chemotherapy (62). We have yet to make a formal comparison of the T cell response induced by rHASPB1 vs SLA + IL-12 in these two sites. However, our current data suggest that both the cytokine balance and the cellular source are likely to be important. Whether excess $CD4^+$ T cell-derived IFN-γ (as a result of IL-12 administration) is detrimental to protective mechanisms operating in the spleen, or $CD8^+$ T cells and/or IL-4 (following rHASPB1 immunization) are more protective in this organ will be an important issue to resolve. Our current data, nevertheless, emphasize the need to evaluate vaccines for systemic multiorgan infections in an appropriate selection of tissue sites.

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