Therapeutic Vaccination With Recombinant Adenovirus Reduces Splenic Parasite Burden in Experimental Visceral Leishmaniasis

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Therapeutic vaccines, when used alone or in combination therapy with antileishmanial drugs, may have an important place in the control of a variety of forms of human leishmaniasis. Here, we describe the development of an adenovirus-based vaccine (Ad5-KH) comprising a synthetic haspb gene linked to a kmp11 gene via a viral 2A sequence. In nonvaccinated Leishmania donovani–infected BALB/c mice, HASPB- and KMP11-specific CD8+ T cell responses were undetectable, although IgG1 and IgG2a antibodies were evident. After therapeutic vaccination, antibody responses were boosted, and IFNγ+ CD8+ T cell responses, particularly to HASPB, became apparent. A single vaccination with Ad5-KH inhibited splenic parasite growth by ~66%, a level of efficacy comparable to that observed in early stage testing of clinically approved antileishmanial drugs in this model. These studies indicate the usefulness of adenoviral vectors to deliver leishmanial antigens in a potent and host protective manner to animals with existing L. donovani infection.

The leishmaniases result from infection with protozoan parasites of the genus Leishmania. Spread across 88 countries worldwide, the leishmaniases are regarded as neglected tropical diseases [1, 2], a status reflected in underfunding of drug and vaccine development programs [3]. Antimonial drugs remain the mainstay of chemotherapy, although in the major endemic foci of human visceral leishmaniasis (HVL) in India, drug resistance has made these drugs of limited value. Only amphotericin B, paromomycin, and miltefosine are recognized alternatives [4]. Currently, there are no vaccines licensed for use in humans [5]. Vaccination against leishmaniasis has long been held to be achievable [6]. Self-cure provides long-lasting protection and is the basis of leishmanization. Epidemiological evidence supports a degree of cross-protection between Leishmania species. Prophylactic immunization in rodents, dogs, and primates has been partially successful using killed or attenuated parasites, crude parasite extracts, recombinant proteins, DNA or viral delivery, and peptide immunization [5]. To date, vaccines have had poor efficacy in clinical trials [7], and concerns remain about the ability of experimental models to predict protection against natural, sandfly-transmitted infection [5]. The latter issue is of less significance for therapeutic vaccination and immunotherapy studies, and despite the immunosuppression associated with established disease, both clinical and experimental data confirm the potential of these approaches [8–10]. Therapeutic vaccination may add to the few tools currently available to treat patients, shortening drug regimens and/or reducing dosage...
and reducing relapse rate [11]. In comparison with prophylaxis, it may provide an attractive proposition for vaccine development, given simpler clinical trial design [12].

Here, we report on the proof-of-concept stage of the development of a therapeutic vaccine for HVL. We demonstrate that 2 Leishmania antigens (HASPB and KMP11), delivered by a single dose of a recombinant adenoviral vector, significantly reduce parasite burden in a stringent mouse model. Vaccination was accompanied by newly detectable vaccine antigen-specific CD8+ T cell responses and boosting of pre-existing CD4+ T cell–dependent antibody responses, and vaccine efficacy benefited from the inherent adjuvant activity of the viral vector.

MATERIALS AND METHODS

Mice and Infections
Female BALB/c mice (Charles River) were maintained at the University of York under specific pathogen-free conditions and used at 6–12 weeks of age. L. donovani (MHOM/ET/67/L28/LV9) was maintained in B6.RAG1−/− mice, and amastigotes were isolated as described elsewhere [13]. Mice were infected intravenously with 2–3 × 107 amastigotes and were randomized to receive Ad5-KH (see below) or Ad5-GFP (Vector Bioslabs) in 20–50 μL saline either subcutaneously at the base of the tail or intradermally in the footpad. Mice were killed 10 days after vaccination, and spleens were removed for assessment of parasite burden, as represented by Leishman Donovan Units (LDU; representing the number of amastigotes/1000 host cells × organ weight [13]) and for immunological analysis. Distribution of Ad-GFP was monitored by fluorescent stereomicroscopy [14]. All experiments were approved by the University of York Ethical Review Panel and were performed under UK Home Office license.

Recombinant Adenovirus
A synthetic haspb gene comprising the conserved N and C termini bordering 10 selected HASPB repeats was generated and linked to the coding region of kmp11 by the tetravirus TaV 2A sequence (RAEGRGSLTCDVEENPG; kindly provided by Prof. M. Ryan, St. Andrews, UK). The final sequence was back-translated using Gene Designer DNA2.0 software with codons optimized for human expression and selected to minimize DNA repeat structures. The construct was flanked by Kozak sequence 5’ of the ATG and a SV40-derived polyadenylation sequence to improve translation initiation and allow mRNA processing, respectively. The final synthetic gene, called huKMP11_HASPB_consensus, was synthesized under contract by Geneart. The gene was inserted into an E1/E3 deleted Ad5 viral vector supplied by Vector Biolabs. The viral particle to plaque-forming unit (pfu) ratio of the viruses used was ~20–25:1. Protein expression was confirmed by Western blot using lysates from P815 cells transduced with virus (multiplicity of infection, 100:1) 24–36 hours earlier (data not shown, [15]).

ELISPOT Analysis
A truncated peptide library (PepSet) spanning the huKMP11_HASPB_consensus protein using 11mers with an overlap of 10 and offset of 1 was generated (Mimotopes). The 444 individual peptide sets (each containing a 11mer plus its respective truncated 10, 9, and 8 mers) were used in contiguous pools of 10 for initial epitope mapping studies or individually for fine mapping; 9-mer peptides were custom synthesized by ProImmune. ELISPOT plates (Mabtech) were coated with anti-IFNγ overnight, washed, and then blocked with complete RPMI 1640 (30 minutes at room temperature); 2.5 × 105 spleen cells or 105 purified CD8+ T cells (2 × 107 naive spleen cells) were seeded per well with or without peptides (2 μg/mL). After overnight culture, plates were washed, and numbers of IFN-γ spot-forming cells were detected by conventional methods. The detection limit for epitope-specific responses was conservatively estimated as 20 000 IFNγ+ CD8+ T cells per uninfected spleen and 25 000 IFNγ+ CD8+ T cells per infected spleen.

Flow Cytometry and Cytokine Analysis
Splenocytes (2 × 106 cells/well) were restimulated in vitro for 7 hours at 37°C in the presence of peptides (1 μg/mL) with Brefeldin A (10 μg/mL) added for the last 4 hours. Cells were surface stained (with CD3/CD90-PE-CY7, CD8 APC-Cy7/ APC-H7, and CD4-PE-Cy7), fixed (2% PFA, 10 minutes on ice), washed (FACS buffer + 0.5% saponin), and stained with IFNγ (XMG1.2)-PB, IL-10 (JES5-16E3)-APC, IL-2 (JES6-5H4)-PE, and TNF (MP6-XT22)-FITC. Isotype control antibodies were used to set markers for analysis and determination of the frequency of cytokine producing cells (using a CyAn analyser and Summit software; Beckman Coulter). The number of peptide-specific cells per spleen was calculated from the absolute number of CD3+CD8+ cells per spleen (obtained from count beads) that produced IFNγ after stimulation with KMP11 or HASPB minus the number of IFNγ-producing cells observed in the no antigen control samples.

Generation of Recombinant Proteins
His-tagged recombinant huKMP11 and rhuHASP_B_consensus were purified by sequential nickel chelation chromatography (HisTrap), anion exchange chromatography (HiTrapQ), and gel filtration (Superdex 200 16/60 or Superdex75 16/60, for HASPB and KMP11, respectively) from French Press Escherichia coli lysates clarified by centrifugation and filtration [15]. Purifications were performed on an AKTApurifier100, with GE Healthcare columns. Proteins were analyzed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis and concentrated for storage.

Antibody Responses
Ninety-six–well Maxisorp plates (Nunc) were coated overnight with 10 μg/mL of rhuKMP11 or rhuHASPB in carbonate buffer (pH, 9.6). Plates were washed and blocked with PBS/0.05%
Tween/1% BSA for 2 hours at room temperature, and serum samples were added in 2-fold serial dilutions (2 hours, room temperature). Bound antibody was detected with peroxidase goat anti-mouse IgG1 or IgG2a (2 hours, room temperature) and quantified at 410 nm using ABTS substrate [13].

**Statistical Analysis**

With 8 mice per group, we calculated a 90% power to detect an inhibition of parasite burden of 50%, with a significance level of 0.05 (GraphPad Software). Data were analyzed by 1-way analysis of variance and Tukey’s Multiple Comparison Test, by paired Student’s t test or by χ² test, as appropriate.

**RESULTS**

**Development of a Synthetic Vaccine Gene**

HASPB contains multiple polymorphic 11–14 AA repeats [15–17], and DNA sequencing has identified isolate-specific HASPB variants, with different numbers, composition, and relative order of repeats (Table 1). We designed a synthetic haspb gene (HASPB_consent) encoding the conserved N and C termini bordering 10 of the repeats identified to date. To include also a conserved protein, we produced a synthetic gene with kmpl1 (LinJ.35.2260/2270; Database version TriTrypDB 04.05.2011) and HASPB_consent linked by a 2A sequence derived from the tetravirus Thosea asigna virus (TaV) [18] (Figure 1A). The fully synthetic gene with humanized codon use was cloned into an E1/E3-deficient adenovirus serotype 5 (Ad5) to generate the recombinant viral vaccine Ad5-huKM-P11_HASPB_consent (referred to as Ad5-KH).

**Immunogenicity and CD8⁺ T Cell Epitope Mapping of the Response to Ad5-KH**

To determine immunogenicity and map CD8⁺ T cell epitopes, we used synthetic peptides that spanned the KH sequence in 20 AA segments. Uninfected BALB/c mice were vaccinated subcutaneously with 10⁶ pfu Ad5-KH, and after 10 days, ELISPOT revealed a limited number of CD8⁺ T cell epitopes (Figure 1B). In HASPB, recognition was targeted to one 20mer region in the conserved N-terminus: one spanning the last repeat region and the conserved C-terminus and one spanning 2 repeats. For KMP11, recognition was limited to a 30 amino acid stretch (KMP1138-67). The location of these CD8⁺ T cell epitopes is indicated in Figure 1A and 1C.

We next evaluated whether route and dose influenced the breadth and magnitude of the response (Figure 2). Increasing the dose by 10-fold had little effect on epitope selection. However, whereas immunogenicity of the HASPB epitopes increased by 2- and 4-fold, this was not the case for KMP11 responses (Figure 2A and 2B). Altering the route of vaccination also

| HASPB Amino Acid Repeats | Leishmania donovani Indian Isolates | Leishmania chagasi | L. infantum |
|--------------------------|-------------------------------------|-------------------|-----------|
|                          | BHU3c34 | BHU3c32-6 | BHU5c65 | AG83c1 | AG83c6 | AG83c3 | LV9⁻¹⁰⁸| LV9⁻¹¹⁸⁰⁹ | LV9⁻¹¹⁸¹⁰ | K26 | LJ23V3-1220 |
| PKEDHTQKNDDGGG            | 0       | 1        | 1       | 1 |
| PKEDHTQONDDGGG            | 1       | 1        | 1       | 1 |
| PKEDDHAHNDGGGG            | 2       | 6        | 5       | 5 | 2       | 6       | 1 |
| PKEDHTQKNDDGG             | 3       | 2        | 2       | 1       | 5       | 2       | 22       | 3       | 3 |
| PKEDDHAHNDGGG             | 4       | 3        | 2       | 3       | 2       |
| PKEDDHAHNDNGG             | 5       | 1        |
| PKEDDHAHNDGGG             | 6       | 1        |
| PEEDHTQKNDDGG             | 7       | 1        |
| PKEDDHAHSDGGG             | 8       | 1        | 1 |
| PKEDGHKQKNDGA             | 9       | 1        | 1 |
| PKEDDQRTQNDGGG            | 10      | 1        | 4       | 4 |
| PKEDGRTQNDGGG             | 11      | 1        |
| PKEDGHTQND                | 12      | 1        |
| PKKDHTQKNDDGG             | 13      | 1        | 1 |
| PKEDDHTQKNDDGG            | 14      | 1        | 1 |
| PKKDDHAHNDGGG             | 15      | 1        |
| PKEDGHKQKNDGG             | 16      | 1        |
| PKEDGRTQKNDGG             | 17      | 1        |
| PKEDGRTQKNNDGG            | 18      | 1        |
| PKEDGRTQKNDGGG            | 19      | 1        |

* LV9: MHOM/ET/67/L28/LV9 laboratory challenge strain.
affected epitope selection and immunogenicity. The response to the HASPB C-terminal epitope was 2-fold greater when 10⁸ pfu were administered in the footpad instead of subcutaneously (Figure 2C). Conversely, the response to the HASPB N-terminal epitope and to KMP11 was selectively absent (Figure 2C). Surprisingly, 10⁸ pfu administered in the footpad gave the broadest epitope coverage, although at the expense of potency of individual epitopes (Figure 2D). Epitope selection was not influenced to any appreciable extent by the use of a polyprotein construct, because when KMP11 and HASPB were independently expressed in Ad5 and used for vaccination, similar epitopes were identified (data not shown). Thus, epitope selection is subtly influenced by both dose and route of administration, with combined responses of 2000 IFNγ⁺ CD8⁺ T cells per 10⁶ spleen cells induced by single dose vaccination.

We next evaluated the IgG subclass response in vaccinated mice (Figure 2E–H), as indirect evidence for an underlying CD4⁺ T cell response. Footpad vaccination induced clear and dose-dependent IgG1 and IgG2a responses, but we failed to detect any response after subcutaneous vaccination, again pointing to route of administration as a determinant of the breadth of the host response. To establish whether differential virus distribution might account for these diverse responses, we evaluated the distribution of Ad5-GFP. After subcutaneous injection, GFP⁺ cells were readily detected in inguinal lymph nodes (LNs). In contrast, after footpad injection, intense GFP expression was observed in popliteal LNs, with GFP⁺ cells also detected in inguinal LNs and spleen (Supplementary Figure 1). Although qualitative, these data suggest that footpad immunization may lead to greater viral infection and systemic spread.

To more readily monitor CD8⁺ T cell responses, we fine-mapped the HASPB C terminus and the KMP11 epitopes (data not shown). We identified only one candidate epitope in each region, HASPBGGPKEGENL (where positions 1 and 2 derive from repeat 17 and positions 3–9 are derived from the C...
Figure 2. Response to vaccination with Ad5-KH is dose and route dependent. **A–D**, Naive BALB/c mice were vaccinated either subcutaneously (s.c.; **A, B**) or into the footpad (f.p.; **C, D**) with $10^9$ pfu (**A, C**) or $10^8$ pfu (**B, D**) Ad5-KH. At 10 days after vaccination splenocytes were prepared and restimulated with pooled pepsets and assayed by IFNγ ELISPOT assay. One of 2 experiments giving similar results is shown. **E–H**, IgG1 (**E, F**) and IgG2a (**G, H**) responses to recombinant KMP11 (**E, G**) and HASPB (**F, H**) were determined by ELISA in serum from mice vaccinated with $10^9$ pfu f.p. (open circle), $10^9$ pfu s.c (open square), $10^8$ pfu f.p. (closed circle), or $10^8$ pfu s.c. (closed triangle). Data represent mean ± standard error of the mean derived from 5 mice per group.

Terminus) and KMP11HYEKFERMI (Figure 1C). Synthesized single peptides recalled responses similar to the pools from which they were identified (Figure 3A). The response to HASPBGGPKEGENL was associated with a greater intensity of IFNγ secretion detected by flow cytometry than that stimulated by KMP11HYEKFERMI, and we confirmed the poly-functionality.
and KMP11 epitopes. After 3 h, Brefeldin A was added for an additional 4 h. Cells were surface-stained for CD3 and CD8 and then stained for intracellular IFNγ and IL-10. The percentage of cytokine-positive cells is indicated in each quadrant. CD8+ T cells from mice vaccinated as above were restimulated with HASPB peptide, surface-stained for CD3 and CD8, and stained for intracellular IFNγ and CD8+ T cell responses to these antigens. However, no CD8+ T cell responses could be observed in infected mice (Figure 4A). We studied 2 possible mechanisms that might account for this lack of recognition. First, in the dominant HASPBGGPKEGENL epitope, a HASPB repeat 17 provides po-
sibility of stimulation of the respective peptides (Figure 4A; data not shown). These mice also failed to recognize the respective peptides (Figure 4C; data not shown). These data suggest that HASPB and KMP11 either fail to prime detectable numbers of CD8+ T cells or induce a very rapidly exhausted response during normal infection.

Therapeutic Vaccination Using Ad5-KH
To evaluate the therapeutic efficacy of Ad5-KH, mice infected for 21 days were vaccinated and splenic parasite burden was assessed 10 days later. In preliminary studies, we failed to observe any significant protection using Ad5-KH administered subcutaneously (data not shown) and, therefore, focused on vaccination with 10^9 pfu Ad5-KH administered in the footpad. Splenic parasite burden in control nonvaccinated mice increased from day 21 to day 31 after infection, as expected [13, 21], whereas in vaccinated mice parasite burden at day 31 was significantly lower (P < .001; n = 23) (Figure 5A). Compared with unvaccinated mice, the increase in spleen parasite burden from day 21 to day 31 was inhibited by 66% ± 8% in mice vaccinated with Ad5-KH (vs 29% ± 12% in Ad5-GFP vaccinated mice; P = .013) (Figure 5B). With use of the lower value of the 95% confidence interval of the mean parasite burden of unvaccinated mice as a defining threshold, 91% (21/23) of Ad5-KH vaccinated mice were defined as protected. Mice vaccinated with Ad5-GFP had significantly higher parasite burdens than those vaccinated with Ad5-KH (P < .05) (Figure 5A), which were not significantly different from unvaccinated mice. However, Ad5-GFP vaccinated mice did show a trend indicative of a degree of nonspecific protection compared with unvaccinated mice, at least in some mice (65% responders; P < .03 vs. Ad5-KH vac-
cinated) (Figure 5A). Thus, Ad5-KH constitutes an effective therapeutic intervention in this experimental model of infection, with a component of protection likely being attributable to nonspecific effects of the viral vector.

We then asked whether therapeutic vaccination stimulated HASPB- and/or KMP11-specific CD8+ T cells in infected mice. Similar to nonvaccinated controls, infected mice vaccinated with Ad5-GFP did not respond to any peptides tested, indicating that nonspecific immune stimulation could not recover potentially underlying CD8+ T cell responses (Figure 6A and Figure 4A). In contrast, Ad5-KH–vaccinated mice responded to HASPB with the same breadth of response seen in uninfected, vaccinated
mice (Figure 6A and Figure 2C), with an estimated 200,000 IFNγ+ CD8+ T cells per infected spleen. As also noted in uninfected mice, we detected no response to KMP11 in vaccinated infected mice by ELISPOT (Figure 6A). However, by intracellular flow cytometry, a significant although weak response against KMPHYEKFERMI could be identified in vaccinated mice (Figure 6B). This equated to ~100,000 KMPHYEKFERMI-specific IFNγ+ CD8+ T cells per spleen, compared with ~400,000 IFNγ+ CD8+ T cells responding to HASPBGGPKEGENL (Figure 6C). The discrepancy between the magnitude of the response determined by ELISPOT and flow cytometry is likely attributable to different thresholds for scoring positive responses (electronic gating vs. manual scoring based on spot size).

The poor recognition of HASPB and KMP11 observed in nonvaccinated L. donovani–infected mice did not extend to the CD4+ T cell and B cell compartment, because IgG1 and IgG2a responses to both antigens were readily detectable. These responses were boosted by therapeutic vaccination with Ad5-KH but not by Ad5-GFP (Figure 6D–G). Together, these data indicate that successful therapeutic vaccination with Ad5-KH was accompanied by the appearance of detectable vaccine antigen-specific CD8+ T cell responses, with bias toward HASPB and boosting of antibody responses to both vaccine antigens.

**DISCUSSION**

This study demonstrates that an adenovirus-based vaccine can provide significant therapeutic effects in mice infected with *L. donovani*. With the caveat of the sensitivity of in vitro recall assays, both of HASPB and KMP11 appear to be examples of cryptic antigens in terms of CD8+ T cell recognition (ie, they failed to stimulate detectable responses after infection, but are nevertheless immunogenic when used for vaccination) [23].

Our strategy to develop a therapeutic vaccine was driven by a 5-point product profile. First, we sought to show efficacy in a selected animal model. We chose inhibition of spleen parasite growth in mice, because this organ fails to spontaneously clear infection and is relatively refractory to drug treatment (reviewed in [24]). Furthermore, we studied the impact of therapeutic vaccination when parasite burden was increasing at its maximal rate and when tissue breakdown and immunosuppression were established [14, 20, 25]. Although it is unlikely that a therapeutic vaccine would be used alone [11], we set our criterion for success as inhibition of parasite growth of 50%. This level of response equates favorably to that seen following single dose therapy using 4 mg/kg Ambisome [26] or administration of sodium stibogluconate [27], although better responses are achievable with longer chemotherapy [26, 28]. Second, for clinical practicality, we aimed for efficacy using a single vaccination. Adenoviral vectors have been reported to provide single dose protection against *Plasmodium berghei* in
Figure 6. Immune responses induced by vaccination with Ad5-KH in Leishmania donovani–infected mice. A, CD8+ T cell responses evaluated by ELISPOT in mice vaccinated with Ad5-GFP (top) or Ad5-KH (bottom). B and C, Peptide-specific IFN-γ responses to KMP11HYEKFERMI and HASPBGGPKEGENL were evaluated by flow cytometry and presented as frequency of responding CD8+ T cells (B) and as absolute number of peptide specific CD8+ T cells per spleen (C). Values of individual mice are plotted. Data are derived from one of the 3 experiments included in Figure 5 (in which inhibition of parasite growth induced by Ad5-KH was 96±6% and by Ad5-GFP was 56±10%; n = 8; P < .01). D–G, IgG1 (D and E) and IgG2a (F and G) antibody responses to KMP11 (D and F) and HASPB (E and G) were determined by ELISA using serum from unvaccinated infected mice (circles) and infected mice vaccinated with Ad5-GFP (squares) or Ad5-KH (triangles). Data represent mean ± standard error of the mean derived from 6–8 mice per group from one experiment.
mice [29]. We have not yet studied heterologous prime-boost regimens, although greater efficacy might be anticipated [30–32]. Third, we chose a delivery system that would benefit from inherent adjuvant activity, because a degree of protection due to nonspecific proinflammatory responses is well documented [33–36]. In this respect, marked similarities in host gene expression signatures induced by adenovirus [37] and L. donovani [38] have been noted. It remains to be determined whether this adjuvant effect operates directly [38] or is secondary to a reduction in parasite burden (as might also be anticipated in sequential chemo-immunotherapy). Fourth, with limited resources for clinical trials, we sought to develop a vaccine based on a platform with established safety and immunogenicity data. Adenoviruses are widely accepted as one of the most potent means to induce CD8⁺ T cell priming, along with CD4⁺ T cell and antibody responses [40]. Although some caution remains about the use of Ad5 in humans [41], new generation simian adenoviruses appear to be safe and to circumvent existing anti-Ad5 immunity [42]. Fifth, adenovirus can be stored in a thermostable manner [43], facilitating delivery in countries where the disease is endemic.

The selection of HASPB was based on prophylactic vaccination studies indicating good protection associated with the development of CD8⁺ T-cell responses in mice [13, 21] and immunogenicity in dogs [44]. Although HASPB in our laboratory strain of L. donovani has multiple identical repeats, field isolates show extensive divergence in repeat sequence and arrangement. Such repeat diversity may suggest immune selective pressure [45]. We therefore developed a synthetic HASPB gene in which the sequence and composition of 10 repeat units was chosen (1) to preserve the relative order of repeats that were present in >1 clinical isolate, (2) to conserve some of the repeat reiteration found in natural isolates (eg, strings of repeat 2), and (3) to maintain the overall protein length to that found in natural isolates. Our data showing that, at least in BALB/c mice, CD8⁺ T cell recognition is largely focused on the N and C termini of HASPB and that HASPB is weakly if at all recognized by CD8⁺ T cells during normal infection argue that repeat variation may not be under CD8⁺ T cell selective immune pressure. The IFNγ⁺ CD8⁺ T cell responses against HASPB induced in infected mice by vaccination (~400,000 sfc per spleen for HASPBKGPKEGENL alone) were also similar or better than those reported for single dose vaccination in other systems, including Ad5-L. donovani A2 [29, 46]. Neither published data nor the current study have directly addressed whether CD8⁺ T cells alone are sufficient for protection, and indeed antibody and CD4⁺ T cell responses are present under all conditions examined. Nevertheless, it is notable that adoptive transfer of only 10⁶ CD8⁺ T cells recognizing an ovalbumin epitope expressed in transgenic L. donovani afforded therapeutic protection in a similar model [47].

KMP11 has been shown to be protective in murine L. donovani infection when administered as a DNA vaccine [48], and multiple human epitopes have been mapped [49], suggesting that it would be immunogenic in humans. Under the immunization regimen chosen for studies of protection here, we did not observe an appreciable ELISPOT response to KMP11 after vaccination of either uninfected or infected wild-type mice, although a weak response was detectable by flow cytometry. This limited recognition was vaccine dose- and route-dependent, arguing against problems in KMP11 recognition because of expression as a self-cleaving polyprotein or inherent lack of immunogenicity. The basis for this observation is under further study. Nevertheless, our data clearly demonstrate how subtle alterations in vaccine delivery alter epitope selection and indirectly highlight the difficulties in extrapolating from epitope recognition in vaccinated mice to vaccinated humans.

Finally, although CD8⁺ T cell responses to HASPB and KMP11 have yet to be studied in patients with any form of leishmaniasis, it is of interest that neither HASPB nor KMP11 have proved to be particularly good at inducing recall CD4⁺ T cells responses in patients recovered from HVL [50], although HASPB antibody responses have been shown to have diagnostic potential [51]. Our data suggest that there may be no a priori requirement for an antigen to be well recognized during natural infection for it to be effective when used for therapeutic (and perhaps prophylactic) vaccination. Only clinical trial will determine whether huKMP11 HASPB consensus expressed in an adenoviral vector is immunogenic and effective as a tool for combined immunotherapy against HVL or other forms of leishmaniasis.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://www.oxfordjournals.org/our_journals/jid/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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