Vaccination with Plasmid DNA Encoding TSA/LmSTI1 Leishmanial Fusion Proteins Confers Protection against *Leishmania major* Infection in Susceptible BALB/c Mice

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We have recently shown that a cocktail containing two leishmanial recombinant antigens (LmSTI1 and TSA) and interleukin-12 (IL-12) as an adjuvant induces solid protection in both a murine and a nonhuman primate model of cutaneous leishmaniasis. However, because IL-12 is difficult to prepare, is expensive, and does not have the stability required for a vaccine product, we have investigated the possibility of using DNA as an alternative means of inducing protective immunity. Here, we present evidence that the antigens TSA and LmSTI1 delivered in a plasmid DNA format either as single genes or in a tandem digene construct induce equally solid protection against *Leishmania major* infection in susceptible BALB/c mice. Immunization of mice with either TSA DNA or LmSTI1 DNA induced specific CD4⁺-T-cell responses of the Th1 phenotype without a requirement for specific adjuvant. CD8 responses, as measured by cytotoxic-T-lymphocyte activity, were generated after immunization with TSA DNA but not LmSTI1 DNA. Interestingly, vaccination of mice with TSA DNA consistently induced protection to a much greater extent than LmSTI1 DNA, thus supporting the notion that CD8 responses might be an important accessory arm of the immune response for acquired resistance against leishmaniasis. Moreover, the protection induced by DNA immunization was specific for infection with *Leishmania*, i.e., the immunization had no effect on the course of infection of the mice challenged with an unrelated intracellular pathogen such as *Mycobacterium tuberculosis*. Conversely, immunization of BALB/c mice with a plasmid DNA that is protective against challenge with *M. tuberculosis* had no effect on the course of infection of these mice with *L. major*. Together, these results indicate that the protection observed with the leishmanial DNA is mediated by acquired specific immune response rather than by the activation of nonspecific innate immune mechanisms. In addition, a plasmid DNA containing a fusion construct of the two genes was also tested. Similarly to the plasmids encoding individual proteins, the fusion construct induced both specific immune responses to the individual antigens and protection against challenge with *L. major*. These results confirm previous observations about the possibility of DNA immunization against leishmaniasis and lend support to the idea of using a single polygenic plasmid DNA construct to achieve polyspecific immune responses to several distinct parasite antigens.

Leishmaniasis is an endemic disease in 88 countries on all continents of the world except Australia. A total of 350 million people, including adults and children, are at risk of acquiring the disease, and it is estimated that there are at least 12 million cases of the various forms of leishmaniasis worldwide (<http://www.who.org>). In addition, approximately 1.5 to 2 million new cases are believed to occur annually. Moreover, *Leishmania*-human immunodeficiency virus coinfection is emerging as an extremely serious new disease, particularly in Southern Europe and South America (1, 9, 34, 41). These alarming figures are believed to be due primarily to the lack of both an efficacious vaccine and safe and efficient methods to control the various vectors that transmit the diseases. In addition, domesticated and wild dogs represent an important reservoir of the disease in many areas; thus, the development of a vaccine for veterinary use represents an important potential control measure. We have recently presented strong evidence that a cock-

tail containing two leishmanial recombinant antigens (LmSTI1 [the *Leishmania major* recombinant protein homologue to eukaryotic stress-inducible protein] and TSA [the *L. major* recombinant protein homologue to eukaryotic thiol-specific-antioxidant protein]) induce solid protection in both murine and nonhuman primate models of cutaneous leishmaniasis (5). In these experiments, the cytokine interleukin-12 (IL-12) was used as an adjuvant. However, despite the fact that IL-12 has proven to be one of the most powerful and consistent adjuvants to polarize the host immune response to a Th1 phenotype, the type of response necessary for protection against leishmaniasis, problems related to toxicity, price, and availability preclude the use of IL-12 as an adjuvant in humans as well as dogs. Therefore, practical adjuvants or new forms of antigen delivery are in high demand, not only for antileishmaniasis vaccines but also for many other diseases in which polarization of the immune response (Th1 or Th2) needs to be induced. Over the past few years, much progress has been made using antigen-encoding plasmid DNA as a practical and efficient means of antigen delivery (2, 11, 53). This system has been particularly successful in vaccine experiments against human immunodeficiency virus, tuberculosis, and leishmaniasis (6, 15, 17, 19, 30, 32, 38, 39, 43,

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46, 52). It is believed that this form of antigen delivery can induce both CD4 (Th1)- and CD8-positive responses to the immunizing antigen (11, 33, 45). Moreover, it has been suggested that DNA immunization, in contrast to protein immunization, induces long-lasting immunity (18, 49).

In this communication, we present evidence that the antigens TSA and LmSTI1 delivered in a plasmid DNA format either as single genes or in a tandem digene construct induce solid protection against *L. major* infection in susceptible BALB/c mice.

MATERIALS AND METHODS

Animals. BALB/c and C57BL/6 mice were obtained from Charles River Laboratories (Wilmington, Mass.). The mice were maintained under pathogen-free conditions and used at 8 to 12 weeks of age.

Microorganisms, infections, and immunizations. *L. major* (Friedlin strain) was maintained *in vivo* in BALB/c mice. For most experiments, mice were infected in the rear footpad with 10^4 amastigote forms of the parasite freshly obtained from the lesions of previously infected mice. Amastigotes were prepared (enriched) by differential centrifugation. Virulent *Mycobacterium tuberculosis* strain H37Rv (ATCC 35718) was suspended in phosphate-buffered saline (PBS)-Tween 80 (0.05%) and pushed through a 26-gauge needle six times. Mice were immunized intramuscularly (i.m.) three times, 1 month apart, with 100 μ g of plasmid DNA containing the gene of interest or with DNA alone (empty vector). Thirty days after the last immunization, the mice were challenged with 10^4 amastigote forms of *L. major* freshly obtained from infected mice. In some experiments, mice were also challenged with 2×10^5 promastigote forms of *L. major* that had been cultured only once after the isolation of the parasites from infected mice. *M. tuberculosis* H37Rv was delivered intravenously at 2×10^5 CFU per mouse. After infection, organ (spleen and lung) homogenates in PBS-Tween 80 (0.05%) were prepared and plated at 5- or 10-fold serial dilution on Middlebrook 7H11 Bacto Agar (Becton Dickinson Microbiology Systems, Cockeysville, Md.). CFU were enumerated 3 weeks later.

IgG isotype ELISA. Mice were bled 3 weeks after the last DNA immunization, and the sera were stored at -20°C until use. The specific serum immunoglobulin G (IgG) isotype antibody response was measured by conventional enzyme-linked immunoadsorbent assay (ELISA). Immulon-4 96-well ELISA plates were coated with recombinant antigens at a concentration of 200 ng/well. Sera were added at twofold serial dilutions, followed by washes and addition of biotinylated isotype-specific secondary antibodies (rabbit anti-mouse IgG1 or IgG2a; Zymed Laboratories Inc., San Francisco, Calif.). The wells were then washed and incubated with streptavidin-conjugated horseradish peroxidase (Zymed), after which the substrate and chromogen were added and absorbance was read on an ELISA plate reader (Dynatech, Chantilly, Va.) at 450 nm.

Cytokine assays. Spleen cells were obtained by conventional procedures and then centrifuged over Ficoll-Hypaque to remove the red cells. Mononuclear cells were cultured at 37°C and 5% CO_2 in the presence of either medium only (RPMI medium containing 10% fetal bovine serum, 50 μM 2- β -mercaptoethanol, and 50 μg of gentamicin/ml) or medium plus recombinant antigen. For cytokine analysis, spleen cells at 10^6 /well (in 24-well tissue culture plates) were cultured in the presence of anti-IL-4 receptor monoclonal antibody (MAb) (Immunex Corp.) with or without antigens for 72 h. The addition of anti-IL-4 receptor MAb in these assays prevents the utilization of the cytokine and consequently allows its accumulation and detection in culture supernatants (54). The supernatants were harvested and analyzed for gamma interferon (IFN- γ) and IL-4 by a double sandwich ELISA using specific MAb (PharMingen, San Diego, Calif.) as previously described (4).

DNA vaccines. The full-length coding sequences of TSA and LmSTI1 were PCR amplified using specific primer pairs containing sequences derived from the 5' and 3' coding portions and devoid of their stop codons. The oligonucleotide primers also contained restriction endonuclease cleavage sites used in both the ligation of the two open reading frames and the subsequent cloning into the eukaryotic expression vector pcDNA3.

Western blot analyses. To confirm that the various DNA constructs were functional and to determine the efficiency of protein expression, HEK-293T cells were transfected with 1 μg of each plasmid using the transfecting reagent Eugene (Roche) according to the manufacturer's protocol. Briefly, HEK-293T cells were maintained in six-well tissue culture plates in Dulbecco's modified Eagle's medium plus 10% fetal calf serum and were transfected when they reached approximately 50 to 75% confluence. Seventy-two hours posttransfection, the cells were

harvested, washed three times with ice-cold PBS, and immediately lysed by addition of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample loading buffer. Lysates derived from equivalent numbers of cells were resolved by SDS-PAGE and transferred to nitrocellulose, and the blots were probed with rabbit antiserum raised against either TSA or LmSTI1 as previously described (46).

CTL assay. The target cells were P815 cells retrovirally transduced with either TSA or LmSTI1 genes essentially as described previously (6). Briefly, the retroviral construct was used in transfections of Phoenix-Ampho, an amphotropic retroviral packaging line. Approximately 48 h posttransfection, supernatants containing recombinant virus were harvested and used to transduce P815 cells. Transduction efficiency was measured by fluorescence-activated cell sorting using P815 transduced with pBIB-EGFP (enhanced green fluorescent protein) viral supernatants as a positive control. All transfectants were selected with blasticidin-S (Calbiochem, San Diego, Calif.) at a concentration of 10 $\mu\text{g}/\text{ml}$ and cloned twice by limiting dilution. These cells were then used as targets for standard ^{51}Cr release cytotoxic-T-lymphocyte (CTL) assays using mononuclear spleen cells isolated from mice immunized with either TSA DNA or LmSTI1 DNA as effector cells.

RESULTS

Immunogenicity of naked DNA formulations. We have recently shown that the leishmanial recombinant proteins TSA and LmSTI1 induced protection in both BALB/c mice and rhesus monkeys challenged with *L. major*. In these experiments, the TSA and LmSTI1 proteins were administered with IL-12 as an adjuvant. To test alternative means of antigen delivery, experiments were designed to evaluate the protection potential of these two antigens delivered in a DNA format. This format of antigen delivery has recently been successfully used with several leishmanial antigens (6, 15, 17, 19, 32, 38, 39, 43, 46, 52). To test this system, TSA and LmSTI1 genes were subcloned into the eukaryotic expression vector pcDNA3 for expression under the control of a strong cytomegalovirus (CMV) promoter. Because expression of recombinant proteins by mammalian cells transfected with bacterial plasmid DNA is a critical condition for the stimulation of the immune system, the expression of the TSA and LmSTI1 proteins was initially assessed in HEK-293T cells transfected with these constructs. Transfected cells were cultured for 3 days and washed, and expression of the recombinant proteins was assessed by SDS-PAGE and Western blot analyses. Cells transfected with either TSA DNA or LmSTI1 DNA produced high levels of the recombinant protein that could be clearly detected by Western blotting (Fig. 1). The additional bands present in lysates of LmSTI1-transfected cells represent breakdown products of LmSTI1 that are often observed after transfection of eukaryotic cells with the LmSTI1 construct. LmSTI1 seems to be to some extent an inherently unstable protein. Indeed, breakdown of LmSTI1 is observed even in lysates of *Leishmania* promastigotes (55). In addition, it is unlikely that these bands are contaminating products because they were not detected by the anti-LmSTI1 antiserum in lysates of untransfected cells. Although the *in vivo* instability of LmSTI1 may have biological significance, its investigation is beyond the scope of the present study.

For the immunogenicity studies, BALB/c mice were immunized i.m. three times, 1 month apart, with 100 μg of the individual plasmids or with a mixture of both plasmids (100 μg of each). Anti-LmSTI1 and anti-TSA antibody responses and T-cell responses (CD4^+ and CD8^+) were evaluated 3 weeks after the last immunization. IgG1 and IgG2a antibody re-

FIG. 1. Expression of TSA and LmSTI1 in HEK 293T cells. HEK 293T cells growing in six-well plates were transfected with DNA constructs (1 μ g/well) encoding the *L. major* antigen TSA or LmSTI1 under the control of a constitutive CMV promoter. Seventy-two hours posttransfection, the cells were harvested, lysed, and analyzed by Western blotting using anti-TSA or anti-LmSTI1 polyclonal antiserum to determine the levels of protein expression. Lysates prepared from untransfected HEK 293T cells and bacterially expressed recombinant protein containing an N-terminal six-His tag were used as negative and positive controls, respectively, for antibody specificity. Both constructs directed high-level expression of proteins of the correct size, although TSA expression tended to be stronger than that of LmSTI1 (arrows).

sponses were evaluated by ELISA using specific anti-mouse isotype antibodies. The results indicated that the mice immunized with either LmSTI1 DNA or TSA DNA developed high titers of IgG2a-specific antibody. However, IgG1-specific antibodies were also present, and at higher titers, for LmSTI1 (Fig. 2). No anti-LmSTI1 or -TSA antibody response was detected in the sera of mice immunized with the vector control (not shown). Similar responses were also detected in mice immunized with a mixture of both DNAs, thus indicating that no antigenic competition occurred between these antigens (not shown).

To measure the CD4⁺-T-cell response, splenic mononuclear cells were obtained 3 weeks after the last DNA immunization and were stimulated in vitro with the recombinant proteins. After 3 days of incubation, the supernatants were harvested and assayed for both IFN- γ and IL-4. To increase the sensitivity of the IL-4 assay, a monoclonal anti-IL-4 receptor antibody was added to the cultures. The results are depicted in Fig. 3 and indicate that both LmSTI1 and TSA specifically stimulated the production of large quantities of IFN- γ by the mononuclear spleen cells of immunized mice. Similar to the humoral

response, cells from mice immunized with the mixture of the two DNAs responded equally to recombinant TSA (rTSA) and rLmSTI1 as measured by IFN- γ production. In contrast, no IL-4 could be detected in the supernatants of any of the cultures stimulated with either TSA or LmSTI1 (not shown). These results are in synchrony with the high titers of IgG2a antibody response and suggest that the CD4⁺-T-cell response elicited by the DNA immunization with these two genes is preferentially of the Th1 phenotype.

To measure the CD8⁺-T-cell response in the DNA-immunized mice, mononuclear spleen cells were stimulated for 5 days in vitro with irradiated P815 cells transduced with either LmSTI1 or TSA genes. The stimulated cells were washed and tested for cytotoxicity (⁵¹Cr release assay) against P815-EGFP-, P815-LmSTI1-, and P815-TSA-transfected targets. Figure 4 shows that immunization of mice with the TSA DNA construct results in the generation of CTL activity specific for P815 cells transfected with the TSA gene but not against cells transfected with the vector alone. In contrast, no CTL activity could be detected for spleen cells of mice immunized with the LmSTI1 gene.

FIG. 2. IgG isotype antibody response to the recombinant proteins TSA and LmSTI1 in BALB/c mice immunized with TSA DNA and LmSTI1 DNA. Mice (three per group) were immunized three times i.m. (1-month intervals) with 100 μ g of either TSA DNA or LmSTI1 DNA. One month after the last immunization, the animals were bled. Sera were obtained and tested by ELISA for specific anti-TSA (A) and -LmSTI1 (B) antibody responses of both IgG1 and IgG2a isotypes. This is one representative experiment of four separate experiments with virtually the same results.

Induction of protection against challenge with *L. major* in mice immunized with LmSTI1 DNA and TSA DNA. Given that the immunogenicity experiments revealed that DNA immunization resulted in the induction of strong antigen-specific CD4 and CD8 responses, we next assessed whether this form of antigen delivery could provide protection against infection of mice with *L. major*. For these purpose, BALB/c mice were immunized i.m. three times as described above, 1 month apart, with 100 μ g of either LmSTI1 or TSA DNA or with a mixture containing 100 μ g of each DNA. As negative controls, groups of mice were also immunized with DNA alone (empty vector) or injected with saline only. Thirty days after the last DNA immunization, the mice were challenged in the right footpad with 10^4 amastigote forms of *L. major*. Footpad swelling was then measured weekly. The results are shown in Fig. 5A and clearly indicated that mice immunized with naked DNA containing the LmSTI1 gene were partially protected whereas mice immunized with either TSA DNA alone or with the mixture containing both TSA and LmSTI1 genes were highly protected against infection with *L. major*. No protective effect was seen when mice were immunized with the control vector.

A parallel experiment using 2×10^5 stationary promastigote forms of *L. major* resulted in the same pattern of protection induced by both DNAs as for the mice challenged with the amastigote forms, i.e., greater protection induced by TSA DNA than by LmSTI1 DNA (Fig. 5B). It is interesting that none of the two visual macroscopic cardinal signs of inflammation that can be observed in mice, i.e., redness and swelling, were present in mice immunized with either TSA DNA or TSA DNA plus LmSTI1 DNA. In contrast, all mice from the two control groups (saline and empty vector) developed redness, notable swelling, scabies, and, not rarely, ulcerations 4 to 5 weeks after challenge (not shown).

Specificity of DNA-induced protection. Because DNA immunization can activate several arms of the innate immune system, including the production of IL-12 and nitric oxide, which might promote resistance to leishmaniasis in a nonspecific manner (12, 16, 22, 23, 25, 31, 42, 48), it became important to determine if the protection observed in the present studies was mediated by innate or acquired immune response mechanisms. To investigate this possibility, mice were immunized with either TSA DNA or Mtb8.4 pcDNA3. The latter construct

FIG. 3. Cytokine production by spleen cells of BALB/c mice immunized with TSA DNA and LmSTI1 DNA. Mice (three per group) were immunized three times i.m. (1-month intervals) with 100 μ g of either TSA DNA or LmSTI1 DNA. One month after the last immunization, the animals were sacrificed, and their spleen cells were obtained and cultured in vitro for 3 days in the presence of medium or TSA or LmSTI1 protein. The supernatants were harvested and assayed by ELISA for both IFN- γ and IL-4. No IL-4 could be detected in any supernatant (not shown). This is one representative experiment of two separate experiments with virtually the same results.

FIG. 4. Induction of CTL activity in BALB/c mice immunized with TSA DNA and LmSTII DNA. Mice (three per group) were immunized three times i.m. (1-month intervals) with 100 μ g of either TSA DNA or LmSTII DNA. One month after the last immunization, the animals were sacrificed, and their spleen cells were obtained and stimulated for 6 days with either TSA DNA- or LmSTII DNA-transduced P815 cells. The stimulated cells were washed and tested for cytotoxicity in a 4-h 51 Cr release assay against control-transduced P815 cells (EGPF), against TSA-transduced P815 cells, and against LmSTII-transduced P815 cells. The results are expressed as percent (with standard deviation) specific cytotoxicity against the respective transduced cell targets. This is one representative experiment of two separate experiments with virtually the same results.

encodes an *M. tuberculosis* protein that induces protection against tuberculosis in the murine model of this disease when delivered in DNA format (6). In this model, protection against tuberculosis is mediated by the same type of immune response that mediates resistance against leishmaniasis. Therefore,

these two plasmid DNAs are highly appropriate for studies concerning the specificity of the protection induced by this format of immunization. Mice (BALB/c for challenge with *L. major* and C57BL/6 for challenge with *M. tuberculosis*) were immunized with either TSA DNA or Mtb8.4-pcDNA3 and then challenged separately with *L. major* or *M. tuberculosis*. More specifically, BALB/c mice immunized with either TSA DNA or Mtb8.4 DNA were challenged with *L. major*, and C57BL/6 mice immunized with either Mtb8.4-pcDNA3 or TSA DNA were challenged with *M. tuberculosis*. Before challenge, the mice were bled and the antibody response to the recombinant proteins was tested by ELISA. Mice immunized with TSA DNA developed specific IgG1 and IgG2a antibodies to rTSA only, i.e., no anti-Mtb8.4 antibody response could be detected in the sera of these animals. Conversely, mice immunized with Mtb8.4-pcDNA3 developed only anti Mtb8.4 antibody (IgG1 and IgG2a) responses (not shown). Protection against leishmaniasis was assessed by measurement of footpad swelling as described for Fig. 5. For tuberculosis, protection was assessed by enumerating the bacteriological burden in the spleens and lungs of infected animals 3 weeks postchallenge. Figure 6 illustrates the results and clearly indicates that the protection induced by these DNA immunizations is specific, i.e., mice immunized with TSA DNA were protected against *L. major* but not against *M. tuberculosis* whereas mice immunized with Mtb8.4-pcDNA3 were protected against *M. tuberculosis* but not against *L. major* infection. Thus, these results indicate that although DNA immunization may stimulate the innate immune system, the protections induced with TSA-pcDNA3 and Mtb8.4-pcDNA3 are mediated by acquired or specific immune responses.

Protection induced by a TSA/LmSTII pcDNA3 chimera. In view of the fact that both TSA DNA and LmSTII DNA induced protection when used individually or mixed together and

FIG. 5. Vaccination of BALB/c mice against *L. major* infection with TSA DNA and LmSTII DNA. Mice (five per group) were immunized three times i.m. (1-month intervals) with saline; 100 μ g of control DNA (pcDNA3) (Empty vector), TSA DNA, or LmSTII DNA; or a mixture containing 100 μ g of TSA DNA and LmSTII DNA (100 μ g of each). One month after the last immunization, the animals were infected in the right footpad with either 10⁴ amastigote forms (A) or 2 × 10⁵ promastigote (metacyclic) forms (B) of *L. major*, and footpad swelling was measured weekly thereafter. Standard errors for all points in A and B were less than 15%. This is one representative experiment of five separate experiments with virtually the same results.

FIG. 6. Specificity of protection induced by DNA vaccination. Mice (five per group) were immunized three times i.m. (1-month intervals) with either saline or 100 μ g of control DNA (pcDNA3) (Empty vector), TSA DNA, or Mtb8.4 DNA. One month after the last immunization, one set of mice was infected in the right footpad with 10^4 amastigote forms of *L. major*, and footpad swelling was measured weekly thereafter (A). The other set of mice was challenged intravenously with 2×10^5 viable *M. tuberculosis* organisms, and CFU in the spleen and lungs were enumerated 3 weeks later (B). This is one representative experiment of three separate experiments with virtually the same results. The bars indicate standard deviations.

that no antigenic competition occurred between them, we next evaluated the immunogenicity and protective capacity of an engineered gene chimera composed of both of these DNAs. Such a chimera is an attractive molecule, as it contains the genetic information for both TSA and LmSTI1 epitopes in a single product, thus constituting a much simpler vaccine to manufacture. Before immunization, the expression of the fusion protein by mammalian cells was evaluated. Transfected HEK-293T cells were lysed, and the expression of both TSA and LmSTI1 was evaluated by Western blot analyses. Figure 7 shows the results, which confirmed that the cells transfected with the chimerical DNA expressed a fusion protein of the expected size that was recognized by both the anti-TSA and anti-LmSTI1 polyclonal antisera. BALB/c mice were then immunized with the chimera as described for the individual DNAs and challenged with *L. major*. Similar to the immunization with the mixture of TSA and LmSTI1 DNAs, immunization with the chimerical DNA resulted in specific antibody and T-cell responses against both recombinant proteins (not shown). More importantly, immunization with the chimerical DNA induced solid protection (Fig. 8) in BALB/c mice challenged with *L. major*.

DISCUSSION

Successful immunization that induces protection against leishmaniasis is highly dependent on adjuvants that preferentially stimulate the Th1 phenotype of immune response. For example, immunization of BALB/c mice with the recombinant leishmanial antigen LmSTI1 in conjunction with IL-12 results in the generation of a strong and specific Th1 response to the immunizing antigen, and the mice mount excellent protection against challenge with virulent *L. major* (5). In contrast, when BALB/c mice are immunized with the same antigen formulated with alum as an adjuvant, the outcome of the vaccination is rather different. Thus, the mice develop a strong Th2 polarized specific immune response to LmSTI1 and show no resistance whatsoever when challenged with *L. major* (unpublished observations). Unfortunately, IL-12 is difficult to prepare, is expensive, and does not have the stability required for a vaccine product. Several alternatives for antigen delivery have

been tested over the past decade, and plasmid DNA is one of the most interesting.

The data presented here show that immunization of BALB/c mice with the plasmid pcDNA3 containing the leishmanial TSA and LmSTI1 genes induces unambiguous protection against challenge of these mice with virulent *L. major*. Protection was observed against challenge with infective metacyclic promastigote forms of *L. major* and with freshly isolated amastigotes, the relevant form of the parasite that ultimately causes pathology in the host. As expected, DNA immunization resulted in the development of an immune response to these two antigens that recapitulated the Th1 response observed in mice immunized with recombinant antigens mixed with IL-12. Both TSA DNA and LmSTI1 DNA stimulated high titers of specific IgG2a antibody, a phenotypic marker of Th1 response. Interestingly, immunization with LmSTI1 DNA also resulted in the generation of high titers of IgG1 antibody, an isotype of immunoglobulin traditionally associated with a Th2 response. However, recent evidence (13) has demonstrated that IgG1 antibodies are divided into two distinct families of molecules, one that is dependent on IL-4 (Th2 associated) and another that is dependent on IL-12 and IFN- γ (Th1 associated). Therefore, the presence of high titers of anti-LmSTI1 IgG1 is not necessarily an indication of a Th2 response to the recombinant antigen after DNA immunization. Indeed, the in vitro recall experiments indicated that immunizations of mice with either TSA DNA or LmSTI1 DNA induce typical Th1 responses. This conclusion is based on the fact that spleen cells from these mice, when stimulated in vitro with the corresponding antigens, produced only IFN- γ and no detectable IL-4 even in the presence of anti-IL-4 receptor antibody in the tissue culture, a condition that favors the detection of this cytokine (54). Also it is interesting that immunization of mice with a mixture containing both TSA DNA and LmSTI1 DNA resulted in the development of immune responses (humoral and cellular) to the individual recombinant proteins that were essentially the same as those observed after immunization with the single genes, clearly showing that no antigenic competition exists between these two molecules (not shown).

FIG. 7. Expression of a TSA/LmSTI1 fusion protein in HEK 293T cells. HEK 293T cells growing in six-well plates were transfected with DNA constructs (1 μ g/well) encoding the *L. major* antigen TSA or LmSTI1 or a TSA/LmSTI1 fusion protein under the control of a constitutive CMV promoter. As described in the legend to Fig 1, lysates from transfected and untransfected cells were analyzed by Western blotting using anti-TSA or anti-LmSTI1 polyclonal antiserum to determine levels of protein expression. Lysates prepared from untransfected HEK 293T cells and bacterially expressed recombinant protein containing an N-terminal six-His tag were used as negative and positive controls, respectively, for antibody specificity. The fusion construct directed high-level expression of a TSA/LmSTI1 fusion protein of the correct size that was reactive with both the anti-TSA and anti-LmSTI1 polyclonal antisera (arrows).

In contrast to conventional immunization that results in stimulating primarily CD4⁺-T-cell responses, DNA immunization has been shown to stimulate both CD4⁺- and CD8⁺-T-cell responses (11, 33, 45). This property of stimulating CD8⁺ T cells is highly interesting because resistance to several intracellular pathogens, including *Leishmania*, is believed to be dependent on both CD4⁺ and CD8⁺ T cells (7, 8, 14, 21, 24, 36, 37, 40). The experiments delineated to investigate the induction of CD8⁺-T-cell response (CTL activity) in the mice immunized with TSA DNA and LmSTI1 DNA indicated that only TSA DNA stimulated CTL activity. At this point, we do not have an explanation for these results. Both plasmid DNA preparations, when transfected into eukaryotic cells, expressed the encoded proteins. However, it is possible that TSA and LmSTI1 differ in their intracellular trafficking, i.e., TSA is transported to both major histocompatibility complex class I and class II pathways of the antigen-processing machinery and LmSTI1 is transported only to the class II pathway. Regardless

of the mechanisms that explain this difference, the fact that only TSA DNA induces CTLs is an attractive finding to support the proposed role of this cell population in immunity against leishmaniasis (18, 49). Thus, both TSA DNA and LmSTI1 DNA induce strong CD4⁺-T-cell response of the Th1 phenotype, and only TSA DNA induces CTL activity. Coincidentally, when delivered individually, TSA DNA induces solid protection and LmSTI1 DNA induces only partial protection, suggesting that the presence of a CTL response upon immunization with TSA DNA may be an important accessory arm of the immune system for the development of acquired resistance against leishmaniasis.

In addition to inducing a specific immune response, DNA immunization can result in a potent nonspecific activation of the innate immune system. This property has been shown to be mediated by certain oligodeoxynucleotides containing specific motifs centered on CpG dinucleotide (CpG ODN) sequences (27–29, 35, 44, 56). Thus, it was observed that plasmid DNA

FIG. 8. Vaccination of BALB/c mice against *L. major* infection with a digene plasmid DNA construct. Mice (five per group) were immunized three times i.m. (1-month intervals) with either saline or 100 μ g of control DNA (pcDNA3) (Empty vector) or with a pcDNA3 construct containing in tandem the genes for TSA and LmSTI1 (Di-gene-DNA). One month after the last immunization, the animals were infected in the right footpad with 10^4 amastigote forms of *L. major*, and footpad swelling was measured weekly thereafter. This is one representative experiment of five separate experiments with virtually the same results.

containing such sequences could act as a polyclonal activator of B cells, stimulate up-regulation of costimulatory molecules, and activate macrophages for the production of IL-12 and inflammatory cytokine, as well as the production of oxidant radicals such as nitric oxide. The molecular mechanisms of the immunostimulation caused by these molecules have not yet been fully elucidated, but a member of the Toll-like receptor family (TLR9) has been implicated in mediating these responses (3, 20, 50). However, preclinical studies have shown that CpG ODN can enhance innate immunity against a variety of infectious organisms and act as an immunomodulatory adjuvant as well (10, 26, 47, 51, 57). Indeed, several recent studies have shown that injection of BALB/c mice with CpG ODN without antigens induces a state of partial resistance in these animals for up to 5 weeks against challenge with *L. major*. In the experiments described here, it is unlikely that possible immunostimulatory CpG sequences present in the leishmanial genes are responsible for the acquired resistance induced by the DNA immunization. This interpretation is supported by the results of cross protection experiments in which it was shown that immunization of mice with TSA DNA had no effect on the resistance of these animals when they were challenged with *M. tuberculosis*. It is well known that this intracellular pathogen, similarly to *Leishmania*, is highly susceptible to various arms of the innate mechanisms of defense that are induced by the CpG ODN. Therefore, it seems that immunization of mice with TSA DNA has no detectable effect on the in vivo activation of the innate immune system.

Finally, one important aspect of vaccine development is the manufacture of the final product. A vaccine composed of several antigens is often more difficult to standardize and also more expensive than a single-product vaccine. For these reasons, a plasmid DNA containing a fusion construct of both TSA and LmSTI1 genes was prepared and tested. This single product, when transfected into a eukaryotic cell, was capable of

producing a recombinant fusion protein containing epitopes of both TSA and LmSTI1. Moreover, immunization of mice with this construct resulted in immune responses to both proteins that were essentially identical to the humoral and cellular immune responses induced by the individual plasmid DNAs. More importantly, this digene construct induced excellent protection against challenge of BALB/c mice with *L. major*. Despite the fact that TSA DNA as a single molecule induces unambiguous protection and could, perhaps, in itself constitute a vaccine against leishmaniasis, a cocktail composed of TSA and LmSTI1 is conceivably a better vaccine because specific immunity will be generated against an increased number of parasite epitopes. This is a desirable condition, because a vaccine containing a broad range of different protective epitopes is unlikely to suffer from major histocompatibility complex-related unresponsiveness in heterogeneous outbred populations, such as humans and dogs.

In conclusion, these results confirm previous observations of the validity of DNA immunization against leishmaniasis and lend support to the idea of using a multiepitope polygenic plasmid DNA to achieve broadly specific immune response to several defined recombinant antigens.

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