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Vaccination with TAT-Antigen Fusion Protein Induces Protective, CD8⁺ T Cell-Mediated Immunity Against *Leishmania Major*

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Abstract

In murine leishmaniasis, healing is mediated by IFN- γ -producing CD4⁺ and CD8⁺ T cells. Thus, an efficacious vaccine should induce Th1 and Tc1 cells. Dendritic cells (DCs) pulsed with exogenous proteins primarily induce strong CD4-dependent immunity; induction of CD8 responses has proven to be difficult. We evaluated the immunogenicity of fusion proteins comprising the protein transduction domain of HIV-1 TAT and the *Leishmania antigen* LACK (*Leishmania* homolog of receptors for activated C kinase), as TAT-fusion proteins facilitate major histocompatibility complex class I-dependent antigen presentation. *In vitro*, TAT-LACK-pulsed DCs induced stronger proliferation of *Leishmania*-specific CD8⁺ T cells compared with DCs incubated with LACK alone. Vaccination with TAT-LACK-pulsed DCs or fusion proteins plus adjuvant *in vivo* significantly improved disease outcome in *Leishmania major*-infected mice and was superior to vaccination with DCs treated with LACK alone. Vaccination with DC + TAT-LACK resulted in stronger proliferation of CD8⁺ T cells when compared with immunization with DC + LACK. Upon depletion of CD4⁺ or CD8⁺ T cells, TAT-LACK-mediated protection was lost. TAT-LACK-pulsed IL-12p40-deficient DCs did not promote protection *in vivo*. In summary, these data show that TAT-fusion proteins are superior in activating *Leishmania*-specific Tc1 cells when compared with antigen alone and suggest that IL-12-dependent preferential induction of antigen-specific CD8⁺ cells promotes significant protection against this important human pathogen.

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CONFLICT OF INTEREST

The authors state no conflict of interest.

INTRODUCTION

Infections with *Leishmania spp.* are among the most frequent infectious diseases worldwide with more than 12 million people affected (Sacks and Noben-Trauth, 2002; Murray *et al.*, 2005). Although 350 million people are at risk of infection every day, an efficacious vaccine does not exist (Murray *et al.*, 2005). The important role of IFN- γ -producing, *Leishmania*-specific CD4⁺ T cells in protective immunity against *Leishmania major* in mice is well established (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). IFN- γ activates infected host macrophages to produce NO⁻ and to efficiently eliminate parasites. Control of disease and long-lasting immunity in mice is thus associated with Th1-predominant immunity, whereas development of Th2 responses, as observed in BALB/c mice, leads to progressive disease (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). Infections of C57BL/6 mice serve as a surrogate model for self-healing cutaneous leishmaniasis of humans. Although mixed antigen-specific Th1/Th2 immune responses are observed in the peripheral blood of humans, lesional cytokine profiles clearly revealed that IFN- γ is associated with lesion resolution, whereas elevated IL-4/IL-10 levels were found in more progressive, nonhealing disease (Kharazmi *et al.*, 1999; van Weyenbergh *et al.*, 2004; Saha *et al.*, 2006; Geiger *et al.*, 2010). Several studies using the physiologically relevant murine low-dose model have indicated that IFN- γ release by CD8⁺ *L. major*-specific T cells (Tc1) also promotes the development of protective immunity (Muller *et al.*, 1991; Belkaid *et al.*, 2002; Uzonna *et al.*, 2004).

Development of Th1/Tc1 immunity requires IL-12, which is released by infected (e.g., skin-derived) dendritic cells (DCs) (Flohe *et al.*, 1998; von Stebut *et al.*, 1998, 2000; Ahuja *et al.*, 1999; Ritter and Osterloh, 2007; von Stebut, 2007). Upon infection, DCs become activated and efficiently present *Leishmania* antigen to CD4 and CD8 cells (Sacks and Noben-Trauth, 2002). Interestingly, restimulation of *Leishmania*-specific CD8⁺ T cells was possible only when *L. major*-containing DCs and not other antigen-presenting cells (i.e., infected macrophages) were used (Belkaid *et al.*, 2002). In addition, infection studies with ovalbumin (OVA)-transgenic parasites showed that proteins released by *L. major* in infected DCs are a major source of peptides for the generation of parasite-specific CD8⁺ T cells (Bertholet *et al.*, 2005). Thus, DCs infected with viable parasites present antigen in the context of both the major histocompatibility complex (MHC) class I and II and promote induction of Th1/Tc1-dependent protective immunity.

The development of an effective anti-*Leishmania* vaccine has yet to be realized. Vaccination approaches include whole-killed or subunit vaccines with adjuvants, genetically attenuated *Leishmania* parasites, *Leishmania* peptides expressed in attenuated organisms (e.g., Bacillus Calmette Guérin), DNA vaccines, and DC agonists (CpG motifs, Flt-3 (FMS-like tyrosine kinase 3) ligand) (Vanloubbeeck and Jones, 2004). Most of these approaches have shown some efficacy in murine models, but may be difficult to implement in humans for different reasons (ethical, non-effectiveness in humans, side effects, and so on). Utilization of recombinant peptides/proteins or DNA encoding these antigens represent promising approaches, as in the case of live parasites the risk of reactivation in immunosuppressed patients (e.g., those coinfecting with strains inducing visceral disease and HIV) is problematic. Some studies have suggested that persisting viable parasites (perhaps residing

in DCs) are required for long-lasting immunity (Moll *et al.*, 1995; Belkaid *et al.*, 2001). Previously, however, it was shown that although effector cells are lost upon complete eradication of parasites, central memory T cells mediate long-term immunity to *L. major* in the absence of persistent parasites (Zaph *et al.*, 2004). These results support continued emphasis on development of a protein/peptide-based vaccine.

Several *Leishmania* vaccines have used DCs pulsed with parasite lysates (Flohe *et al.*, 1998; Ahuja *et al.*, 1999; Ramirez-Pineda *et al.*, 2004) or recombinant parasitic proteins (Berberich *et al.*, 2003). However, protein-pulsed DCs were not as efficient in promoting protection as were DCs infected with viable parasites (von Stebut *et al.*, 2000). We previously purified recombinant model tumor-associated antigens containing the HIV TAT protein transduction domain (PTD) (Shibagaki and Udey, 2002, 2003). The PTD promotes accumulation of protein in the cytosol of cells independent of endocytosis (Wadia and Dowdy, 2005). This fusion protein efficiently transduced DCs and was processed by proteasomes for MHC class I-dependent presentation to cytotoxic T lymphocytes (Shibagaki and Udey, 2002, 2003). In addition, TAT-containing antigen was also presented to CD4 T cells as efficiently as native antigen. Finally, TAT-antigen-transduced DCs induced antigen-specific cytotoxic T lymphocytes *in vivo* and vaccinated against tumors (Shibagaki and Udey, 2002, 2003).

We have now translated this vaccination strategy into a vaccine against leishmaniasis in C57BL/6 mice. We used the *Leishmania*-specific antigen LACK (*Leishmania* homolog of receptors for activated C kinase), which is highly conserved among related *Leishmania* species and is expressed in both promastigote and amastigote forms of the parasite (Mougeon *et al.*, 1995). When compared with LACK alone, TAT-LACK fusion protein was superior in inducing parasite-specific CD8⁺ T cells and in vaccinating against murine *L. major* infections.

RESULTS

Generation of fusion proteins comprising the HIV-1 TAT and LACK

Plasmids encoding TAT PTD-containing and control proteins (analogous to those generated previously using OVA; Shibagaki and Udey, 2002) were engineered, and recombinant proteins were expressed in *E. coli*, purified using Ni²⁺ affinity chromatography, dialyzed against phosphate-buffered saline (PBS), and separated using 4–12% SDS-PAGE gels (Figure 1a). The purified LACK protein was smaller than TAT-LACK as expected.

Generation and protein transduction of DCs

Bone marrow-derived DCs (BMDCs) have previously been used for vaccination against *Leishmania* infections (Ahuja *et al.*, 1999; Berberich *et al.*, 2003; Ghosh *et al.*, 2003; Ramirez-Pineda *et al.*, 2004). We generated DCs by culturing bone marrow of C57BL/6 mice with GM-CSF and IL-4 (Inaba *et al.*, 1992). One representative flow cytometric analysis showing the phenotype and purity of the immature DCs harvested on day 6 is provided in Figure 1b. DCs strongly expressed MHC class II and CD11c as well as intermediate and low levels of CD86 and CD40, respectively, consistent with their immature state.

Recombinant fusion proteins were treated with polymyxin B before addition to DCs. Protein transduction was performed by incubating 2×10^5 DCs with various concentrations (0.3–3 μM) of fusion or control proteins as described (Shibagaki and Udey, 2002). Cytosolic distribution of TAT–LACK when compared with LACK was confirmed using confocal microscopy (Shibagaki and Udey, 2002). Protein transduction itself did not lead to significant alterations of MHC class II/costimulatory molecules when compared with untreated DCs or induction of IL-12 release (Figure 1c).

DCs pulsed with TAT-containing fusion protein efficiently prime and restimulate antigen-specific CD8⁺ T cells

To assess the potential of fusion protein-pulsed DCs to restimulate antigen-specific primed T cells, we first isolated Thy1.2⁺ T cells (purity >84%) from mice infected for ≥ 6 weeks with *L. major*. BMDCs were either pulsed with antigen (2 μM protein) or were infected with *L. major* (5:1 amastigote/DC ratio; mean infection rate $38 \pm 2\%$) for 18 hours. DCs were subsequently irradiated and cocultured with carboxyfluorescein diacetate succinimidyl ester (CFSE)-labeled T cells for 5 days. Antigen-specific expansion was quantified by determining the percentage of proliferating T cells after subtracting baseline proliferation induced with unpulsed DCs (Figure 1d). Results combined from two to three independent experiments are shown. TAT–LACK- and LACK-pulsed DCs induced proliferation of CD4⁺ T cells, but less vigorously than DCs infected with live parasites. Differences between TAT–LACK and LACK in CD4⁺ cells were not statistically significant. In contrast, more efficient restimulation of CD8⁺ T cells was achieved with TAT–LACK-pulsed DCs when compared with LACK-pulsed DCs (8.3 ± 1.6 vs. $2.3 \pm 0.9\%$, respectively, $n \geq 3$, $P \leq 0.05$).

In addition to T-cell expansion, we assessed the cytokine profile of CD4 and CD8 T cells after restimulation of TAT–LACK- or LACK-pulsed DCs (Table 1). CD4 or CD8 T cells were isolated from infected mice. T cells were plated at 10^6 per 200 μl and restimulated with irradiated, untreated, protein-pulsed, or infected DCs (10^5 per 200 μl). Cytokine release from T cells was assessed after 48 hours using ELISA specific for IFN- γ and IL-4. Irradiated DCs did not release IFN- γ , IL-4, or IL-10 (data not shown). When compared with unpulsed DCs, infected DCs and DC + TAT–LACK induced significant IFN- γ release from CD4 cells. The IFN- γ /IL-4 ratios revealed that infected and TAT–LACK-pulsed DCs both induced an IFN- γ -predominant Th1 cytokine profile. Interestingly, LACK-pulsed DCs did not promote IFN- γ release from primed CD4 cells, confirming previous data that LACK-responsive CD4 cells primarily produce IL-4 (Mougneau *et al.*, 1995). In parallel, CD8 cells restimulated with infected DCs or DC + TAT–LACK released higher amounts of IFN- γ when compared with cells stimulated with unpulsed DCs or DCs pulsed with LACK alone. Significant differences in IL-10 levels in the supernatants were not observed.

We next sought to determine whether preferential induction of antigen-specific CD8⁺ T cells (in addition to CD4 cells) could be achieved by *in vivo* vaccination of mice with antigen-pulsed DCs (Figure 1e). BMDCs were pulsed overnight with 333 μM of TAT–LACK or LACK in the presence of polymyxin B or infected with amastigotes of *L. major* (ratio 1:5, mean infection rates after 18 hours $27 \pm 3\%$). Cells were harvested, washed, counted, and injected intradermally into the ear skin of naive mice (2×10^5 cells per 50 μl per ear). After

1 week, lymph node (LN) cells were harvested, labeled with CFSE, and restimulated with soluble *Leishmania* antigen (SLA, 25 $\mu\text{g ml}^{-1}$) or staphylococcal enterotoxin B (10 $\mu\text{g ml}^{-1}$) as a control (data not shown). Antigen-specific expansion of CD4⁺ and CD8⁺ T cells was assessed 5 days after restimulation. Impaired proliferation of T cells was not found in any of the vaccination groups as assessed by stimulation with superantigen. Both DC + LACK and DC + TAT–LACK induced priming of antigen-specific CD4⁺ T cells to a similar degree, suggesting that the level of LACK uptake by DCs *per se* was similar. Protein-pulsed DCs did not induce responses to the extent observed with DCs infected with live parasites. DC + TAT–LACK induced clear CD8 priming as did infected DCs. Most importantly, TAT–LACK-pulsed DCs more efficiently primed CD8⁺ T cells *in vivo* when compared with DCs pulsed with LACK alone (4.5 ± 1.5 vs. 0%, respectively, $n \geq 4$, $P \leq 0.05$).

Vaccination with TAT–LACK-pulsed DCs is superior to DCs pulsed with LACK alone

We next investigated the vaccination potential of fusion protein-pulsed DCs in *Leishmania*-resistant C57BL/6 mice *in vivo*. LACK reactivity of naive CD4⁺ T cells has been detected in C57BL/6 mice (Sacks and Noben-Trauth, 2002). Data generated with *Leishmania*-resistant mice might be most relevant to a clinical setting because the course of disease in C57BL/6 mice most closely mimics natural *L. major* infections of humans.

At 1 week before infection, C57BL/6 mice were vaccinated with 2×10^5 DCs pulsed with fusion protein, LACK alone, or irrelevant controls (DC alone, DC plus TAT- β -galactosidase (βgal), or βgal ; Figure 2). Mice were infected into contralateral ears with either high-dose (2×10^5 parasites, Figure 2a) or low-dose inocula (Figure 2b–f), and lesion development was assessed weekly. In both high- and low-dose infections, vaccination with TAT–LACK-pulsed DCs was more efficacious than vaccination with DCs pulsed with LACK alone. This effect was most pronounced in low-dose infections (Figure 2b). Vaccination with irrelevant controls or unpulsed DCs did not have an effect on lesion development when compared with unvaccinated mice (Figure 2c). Smaller lesion sizes in DC + TAT–LACK-vaccinated mice paralleled significantly decreased lesional parasite burdens (Figure 2d). In week 7 after infection, ears of mice vaccinated with TAT–LACK contained $4.5 \pm 0.9 \times 10^2$ parasites when compared with LACK-DC-treated groups ($5.1 \pm 1.1 \times 10^3$ parasites, $n \geq 6$, $P \leq 0.005$).

In a previous report, pretreatment of antigen-pulsed DCs with CpG oligonucleotides (ODNs) further promoted protection (independent of DC-derived IL-12 production; Ramirez-Pineda *et al.*, 2004). In our model, cotreatment with 10 μg CpG ODN 1826 injected into vaccination sites did not have an adjuvant effect on disease outcome (data not shown).

Successful vaccination with fusion-protein-pulsed DCs is IL-12 dependent

Induction of Th1/Tc1-dependent protection against *Leishmania* infections is critically dependent on the release of IL-12 from infected DCs (Sacks and Noben-Trauth, 2002). We thus determined whether release of IL-12 from protein-pulsed DCs is important for the efficacy of our vaccination approach. BMDCs were generated from C57BL/6 IL-12p40^{-/-} or wild-type controls. Characterization of IL-12p40^{-/-} and wild-type DCs revealed no differences in expression of CD11c, MHC class II, as well as the costimulatory molecules CD40 and CD86, as determined by flow cytometry (data not shown). DCs were pulsed

overnight with antigen. Similar to previous experiments, mice were vaccinated and infections were initiated 1 week later by inoculation of 10^3 metacyclic promastigotes into contralateral ears (Figure 2 e and f). TAT–LACK-pulsed IL-12p40-deficient DCs failed to protect vaccinated mice from *L. major* infection (Figure 2f). Thus, release of IL-12p40 from DCs is required for TAT–LACK-mediated immunity against *L. major*.

Utilization of TAT–LACK for direct *in vivo* vaccination

Generation/isolation of DCs *ex vivo* from humans is laborious and expensive and will not be feasible in *Leishmania*-endemic countries. We thus initiated studies analyzing the potential of a TAT–LACK fusion protein vaccine directly *in vivo* to circumvent *ex vivo* protein transduction of DCs.

First, the ability of the direct protein vaccine to induce antigen-specific T-cell responses *in vivo* was assessed. Mice were vaccinated with TAT–LACK, LACK, irrelevant TAT-OVA, or SLA on days –7 and –6. Subsequently, 10 μg of CpG ODN 1826 was administered into the same ears on day –5. On day 0, LN cells were isolated, labeled with CFSE, and restimulated with 25 $\mu\text{g ml}^{-1}$ SLA. Antigen-specific expansion of CD4⁺ and CD8⁺ T cells was assessed 4 days after restimulation (Figure 3a and b). Despite the fact that the induction of antigen-specific T cells was weaker with protein when compared with the DC-based vaccine (compare Figure 1e) as expected, TAT–LACK-mediated T-cell priming was stronger than that by LACK alone for both CD4 and CD8 cells.

We next attempted to vaccinate with TAT–LACK fusion proteins directly *in vivo*. TAT–LACK and LACK were administered intradermally into ear skin for two consecutive days (10 μg per ear), and infections with 10^3 metacyclic promastigotes were initiated 2 weeks later. When compared with PBS-treated control mice, no protective effect was observed (data not shown). Next, recombinant fusion proteins were administered for 2 days followed by a single injection of CpG ODN 1826 (10 μg per ear; Zimmermann *et al.*, 1998; Stacey and Blackwell, 1999; Walker *et al.*, 1999) as adjuvant on day –12 (Figure 3c). Irrelevant (fusion) proteins plus CpG ODN 1826 were included as controls (data not shown). Interestingly, only TAT–LACK + CpG ODN significantly prevented disease progression of infected C57BL/6 mice, whereas controls were ineffective. As shown for the DC-based vaccination approach, TAT–LACK-mediated protection was always superior to LACK alone (e.g., 4 ± 0.6 vs. 13 ± 2.6 mm³ in week 7 after infection, respectively, $n \geq 12$, $P \leq 0.002$).

The vaccination efficacy of TAT–LACK fusion protein is dependent on both CD4 and CD8 cells

To assess the dependence of the efficacy of TAT–LACK as a vaccine on CD4 and CD8 cells, we performed depletion experiments *in vivo*. To ensure that T cells were depleted only during vaccination, extensive dose-response experiments were performed. To achieve sufficient depletion, 125 μg of anti-CD4 mAb (clone GK1.5) or 50 μg of anti-CD8 (clone 2.43) were injected 2 days before vaccination. As a control, some mice were treated with an irrelevant isotype control antibody. The T-cell depletion efficacy was determined on the day of vaccination. The frequency of CD4⁺ and CD8⁺ cells in peripheral blood of anti-CD4- and anti-CD8-treated mice was 0.05 and 0.9%, respectively. Full restoration of the T-cell

compartment was confirmed by flow cytometry from peripheral blood before mice were infected with low-dose inocula of *L. major* (day 21 after vaccination). Interestingly, if either CD4 or CD8 T cells were depleted during vaccination, the vaccination success of TAT–LACK was completely abrogated, as manifested by lesion volumes (Figure 3c).

DISCUSSION

Protective immunity against *L. major* is dependent on both Th1- and Tc1-dependent immunity. In this study we have used fusion proteins comprising the 11aa PTD from HIV-1 TAT and LACK. We showed that vaccination with fusion proteins containing TAT was consistently superior in promoting protection against cutaneous leishmaniasis when compared with proteins without TAT. Thus, in these proof-of-principle experiments, we have shown that intentional induction of antigen-specific CD8⁺ T cells using TAT-fusion proteins, in addition to CD4⁺ T cells, is beneficial for protection against this important pathogen.

Although infected (skin-derived) DCs seem to be the critical cells for the induction and maintenance of protective immunity against *L. major* (Flohe *et al.*, 1998; von Stebut *et al.*, 1998, 2000; Ahuja *et al.*, 1999; Sato *et al.*, 2000; Lemos *et al.*, 2004; Iezzi *et al.*, 2006), use of protein-loaded DCs showed only limited results when compared with cells infected with live parasites (Berberich *et al.*, 2003; Ramirez-Pineda *et al.*, 2004). Parasites and protein enter DCs in different ways. Parasite internalization results from FcγRI/III-mediated phagocytosis (Woelbing *et al.*, 2006), whereas protein uptake is the result of endocytosis. Importantly, FcγR-mediated parasite uptake facilitates MHC class I and II antigen processing, whereas protein endocytosis favors MHC class II-predominant antigen presentation (Amigorena, 2002; Belkaid *et al.*, 2002; Woelbing *et al.*, 2006). Thus, inefficient induction of CD8 T cells (rather than suboptimal CD4 priming) may be responsible for the inability of protein-pulsed DCs to promote effective and long-term antiparasite immunity when compared with infected DCs. This concept is supported by our finding that vaccination with DC + LACK induced comparable CD4 priming as did DC + TAT–LACK, whereas CD8 priming was weaker in mice vaccinated with DCs pulsed with LACK protein alone.

The role of antigen-specific CD4⁺ T cells in protective immunity is well established (Reiner and Locksley, 1995; Sacks and Noben-Trauth, 2002). Additional studies have highlighted the important role that *Leishmania*-specific CD8⁺ Tc1 cells have in mediating protection, especially in the physiologically more relevant low-dose infection model mimicking natural transmission of *L. major* by sand flies, but not in high-dose infections (Muller *et al.*, 1991; Erb *et al.*, 1996; Belkaid *et al.*, 2002; Uzonna *et al.*, 2004). In addition, previous experiments using DNA-vaccination approaches induced CD8-dependent protection, which led to efficient protection (Gurunathan *et al.*, 1997). In this study we showed that induction of both LACK-reactive CD4 and CD8 T cells contributed to protection against *L. major* infection after vaccination with TAT–LACK fusion protein. In a previous study, DCs transduced with HIV TAT-PTD-containing whole protein antigens also stimulated both antigen-specific CD8⁺ and CD4⁺ T cells (Mitsui *et al.*, 2006). Thus, the superiority of TAT–LACK-pulsed DCs likely relates to their ability to induce CD8 priming in addition to CD4 priming, which was also achieved by LACK-treated DCs. This confirms the results of previous studies that

showed that CD8⁺ T cells are critical for protection using LACK as the vaccinating antigen (Gurunathan *et al.*, 1997, 2000; Shah *et al.*, 2003; Dondji *et al.*, 2008).

LACK has been found to be the focus of the early immune response directed against the parasites, with most LACK-reactive T cells producing IL-4, but not IFN- γ (Mougneau *et al.*, 1995). LACK-induced preferential expansion of parasite-specific Th2 cells and a low level of IFN- γ production result in progressive infection of BALB/c mice and fatal outcome (Heinzel *et al.*, 1989). It is accepted that C57BL/6 mice are by far the better model than BALB/c mice to investigate vaccines with potential relevance for human infections. However, as a reaction status similar to that in BALB/c mice may occur in some humans, the effects of the vaccination procedure described in this study were also assessed in susceptible BALB/c mice (data not shown). Using TAT-fusion proteins in both DC- and protein-based vaccines, TAT-LACK-treated BALB/c mice were significantly protected against progressive infection with *L. major*, as manifested by clearly reduced lesion volumes and parasite burdens. Similar to the findings reported in this study, TAT-LACK was superior to LACK, and TAT-LACK-mediated protection was dependent on CD4⁺ as well as CD8⁺ T cells. LACK may not be the optimal antigen choice for vaccinations. Previous experiments confirmed that LACK-pulsed DCs did not efficiently promote protection relative to that observed with other *Leishmania*-derived antigens (Berberich *et al.*, 2003). Attempts to modify the immune response to the large LACK protein by using different epitopes have been successful (Jensen *et al.*, 2009). Thus, it is reasonable to predict that if an optimized antigen combination (such as certain LACK peptides or other antigens capable of inducing more Th1/Tc1-prone immunity) was used for TAT-fusion protein-based vaccinations, the results may be even more promising.

In this study, fusion protein as well as LACK alone did not promote protection directly *in vivo*, confirming previous studies using proteins without adjuvant (Mougneau *et al.*, 1995; Berberich *et al.*, 2003; Shah *et al.*, 2003; Ramirez-Pineda *et al.*, 2004). Activation of skin DCs and IL-12 synthesis is required as achieved *in vivo* by coadministration of adjuvants such as CpG motifs or others (Zimmermann *et al.*, 1998; Walker *et al.*, 1999; Shah *et al.*, 2003). In addition, we also showed that the vaccination efficacy of DCs pulsed with TAT-LACK was dependent on the production of IL-12 from the DCs, confirming previous results (Berberich *et al.*, 2003). Independent of exogenous DCs, TAT-LACK fusion protein together with CpG efficiently induced protection against progressive leishmaniasis directly *in vivo*. TAT-LACK was consistently superior to LACK alone. These latter results are important, because DC-based vaccination approaches will not be widely applicable.

In summary, in this proof-of-principle study, we have shown that induction of *Leishmania*-specific CD8⁺ T cells, in addition to CD4⁺ cells, is beneficial for the development of protective immunity. In both DC- and protein-based vaccinations, immunization with TAT-LACK was superior to injection of LACK alone. Thus, TAT-fusion proteins represent promising tools for the induction of protective immunity against not only cancer, but also infectious diseases with intracellular pathogens. Future experiments will evaluate in more detail how this immunization strategy can be improved (e.g., by studying the durability of its efficacy) and how this vaccination approach can be translated into humans.

MATERIALS AND METHODS

Generation and characterization of fusion protein

His₆-tagged TAT-hemagglutinin (HA)-OVA, HA-OVA, TAT-HA-βgal, and HA-βgal were obtained and generated as described previously (Shibagaki and Udey, 2002). Constructs encoding His₆-tagged TATHA-LACK were generated by inserting LACK complementary DNA (+427 to +939, 170 aa; provided by Dr D Sacks, National Institute of Allergy and Infectious Diseases, Bethesda, MD) into multiple-cloning sites (*NcoI/EcoRI*) of pTAT-HA plasmid in-frame. HA-LACK constructs were generated by *Bam*HI digestion and religation of pTAT-HA-LACK plasmids. Plasmids were then transformed into a RosettaBlue (DE3) competent bacterial strain (Novagen, Merck, Darmstadt, Germany). For protein extraction, bacterial cell pellets were sonicated in urea-containing medium. Denatured TAT-HA-LACK, HA-LACK, TAT-HA-OVA, HA-OVA, TAT-HA-βgal, and HA-βgal were then purified by sequential Ni²⁺ nitrilotriacetic acid-agarose chromatography and dialyzed against PBS as described (Shibagaki and Udey, 2002). Proteins were stored at -20 °C in PBS/10% glycerol and thawed immediately before use. SDS-PAGE was performed with NuPAGE 4–12% Bis-Tris gels and MOPS running buffer (Shibagaki and Udey, 2002).

Mice and parasites

C57BL/6 mice, 6–8 weeks old, were purchased from the Central Animal Facility of the University of Mainz. IL-12p40^{-/-} C57BL/6 mice were kindly provided by Professor E Schmitt, Department of Immunology, University of Mainz. All animals were housed in accordance with institutional and federal guidelines. The experiments were undertaken with approved license from the animal care and use committee of the Region Rheinland-Pfalz, Germany. Metacyclic promastigotes or amastigotes of *L. major* clone VI (MHOM/IL/80/Friedlin) were prepared as described previously (Woelbing *et al.*, 2006).

Generation and pulsing of DCs

BMDCs were generated from wild-type or IL-12p40^{-/-} mice in GM-CSF- and IL-4-containing media (32) and harvested on day 6 of cell culture. The characteristics of the cell populations were assessed by flow cytometry using relevant surface markers. The following antibodies were used: anti-I-A^{b,d}/I-E^d (2G9), anti-CD11c (HL3), anti-CD40 (3/23), and anti-CD86 (GL1) (all from BD Biosciences/Becton Dickinson, Heidelberg, Germany). For protein pulsing of DCs, cells were plated at 1 × 10⁶ per ml in RPMI/5% fetal calf serum and recombinant proteins were added at 333 nM up to 2 μM (Shibagaki and Udey, 2002). Recombinant proteins were treated with polymyxin B sulfate (50 μg ml⁻¹, Sigma Aldrich, St Louis, MO) before addition to DCs. After 18 hours of DC pulsing, supernatants were harvested and cytokine production was analyzed using ELISA for IL12p40 (R&D Systems, Wiesbaden, Germany).

Restimulation of *Leishmania*-specific T cells with DCs

T cells were isolated from *L. major*-infected C57BL/6 mice using microbeads coated with anti-CD90 (Thy1.2) (Miltenyi, Bergisch-Gladbach, Germany). T cells (5 × 10⁶ per ml) were labeled with 1 μm CFSE (Invitrogen, Karlsruhe, Germany) and subcultured with irradiated

DCs (400 Gy) in a ratio of 10:1 for additional 5 days (10^6 cells per 200 μ l). Antigen-specific proliferation was assessed after 5 days using flow cytometry. T cells were selected for analysis using mAbs against CD4 (L3T4, RM4–5), CD8 (Ly2, 53–6.7), or isotype control mAb (all from BD Biosciences). For each mouse, the percentage of *Leishmania*-reactive cells compared with nonproliferating cells was calculated as described (Woelbing *et al.*, 2006).

To determine the T-cell cytokine profile after restimulation with TAT–LACK-transduced DCs, *Leishmania*-primed T cells were isolated from infected C57BL/6 mice using anti-CD4 and anti-CD8 microbeads (Miltenyi). T cells and DCs were cocultured in a ratio of 10:1 (1×10^6 cells per 200 μ l). After 48 hours of incubation, supernatants were harvested and cytokine production was assessed using ELISA for IL-4 (BD Biosciences) and IFN- γ (R&D Systems).

Assessing the induction of LACK-reactive T cells *in vivo*

Groups of ≥ 5 C57BL/6 mice were vaccinated *in vivo* by intradermal injection of 2×10^5 protein-pulsed DCs. For the assessment of an induction of LACK-reactive T cells, LN cells were harvested 1 week after vaccination of naive mice, labeled with CFSE as described above, and plated at 10^6 per 200 μ l in 96-well U-bottom plates. Cells were stimulated with SLA or SEB. After 5 days, the frequency of daughter cells of proliferating antigen-reactive compared with nonproliferating LN T cells was estimated using flow cytometry (24). T cells were pre-gated using staining for CD4 (L3T4, RM4–5), CD8 (Ly2, 53–6.7), or isotype control mAb (all from BD Biosciences). The percentage of LACK-reactive cells was calculated separately for each mouse and the data of three independent experiments were pooled and expressed as mean \pm SEM.

Vaccination schedules and *L. major* infection

Groups of ≥ 5 mice were vaccinated with 2×10^5 per 50 μ l protein-pulsed or untreated DCs injected intradermally into the ear skin. In some experiments, mice were vaccinated with fusion proteins dissolved in PBS for two consecutive days (10 μ g per ear) followed by a single injection of CpG ODN 1826 (10 μ g per ear). After 1 to 3 weeks, infections were initiated into contralateral ears by inoculating 2×10^5 or 10^3 metacyclic promastigotes as indicated (Belkaid *et al.*, 2000). In some experiments, anti-CD4 (clone GK1.5, 125 μ g per mouse) or anti-CD8 (clone 2.43, 50 μ g per mouse) were injected intraperitoneally 2 days before vaccination.

Disease outcome was determined by assessing lesion volumes, the number of parasites/lesion, and cytokine profiles of draining LN cells. Lesion development was assessed weekly in three dimensions using a caliper, and lesional volumes were reported (in mm^3) as ellipsoids ($(a/2 \times b/2 \times c/2) 4/3\pi$). Organisms present in lesional tissue were enumerated using limiting dilution assays (Woelbing *et al.*, 2006). For measurement of cytokine production, 10^6 retroauricular LN cells per 200 μ l were added to 96-well plates in the presence of SLA (25 μ g ml^{-1}). Antigen-specific IFN- γ (R&D Systems), IL-4, and IL-10 (BD Biosciences) production was determined after 48 hours using ELISA.

Statistics

Statistical analysis was performed using the unpaired Student's *t*-test.

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Abbreviations:

BMDC	bone marrow-derived DC
CFSE	carboxyfluorescein diacetate succinimidyl ester
DC	dendritic cell
HA	hemagglutinin
LACK	Leishmania homolog of receptors for activated C kinase
LN	lymph node
MHC	major histocompatibility complex
ODN	oligonucleotide
OVA	ovalbumin
PBS	phosphate-buffered saline
PTD	protein transduction domain
SLA	soluble Leishmania antigen

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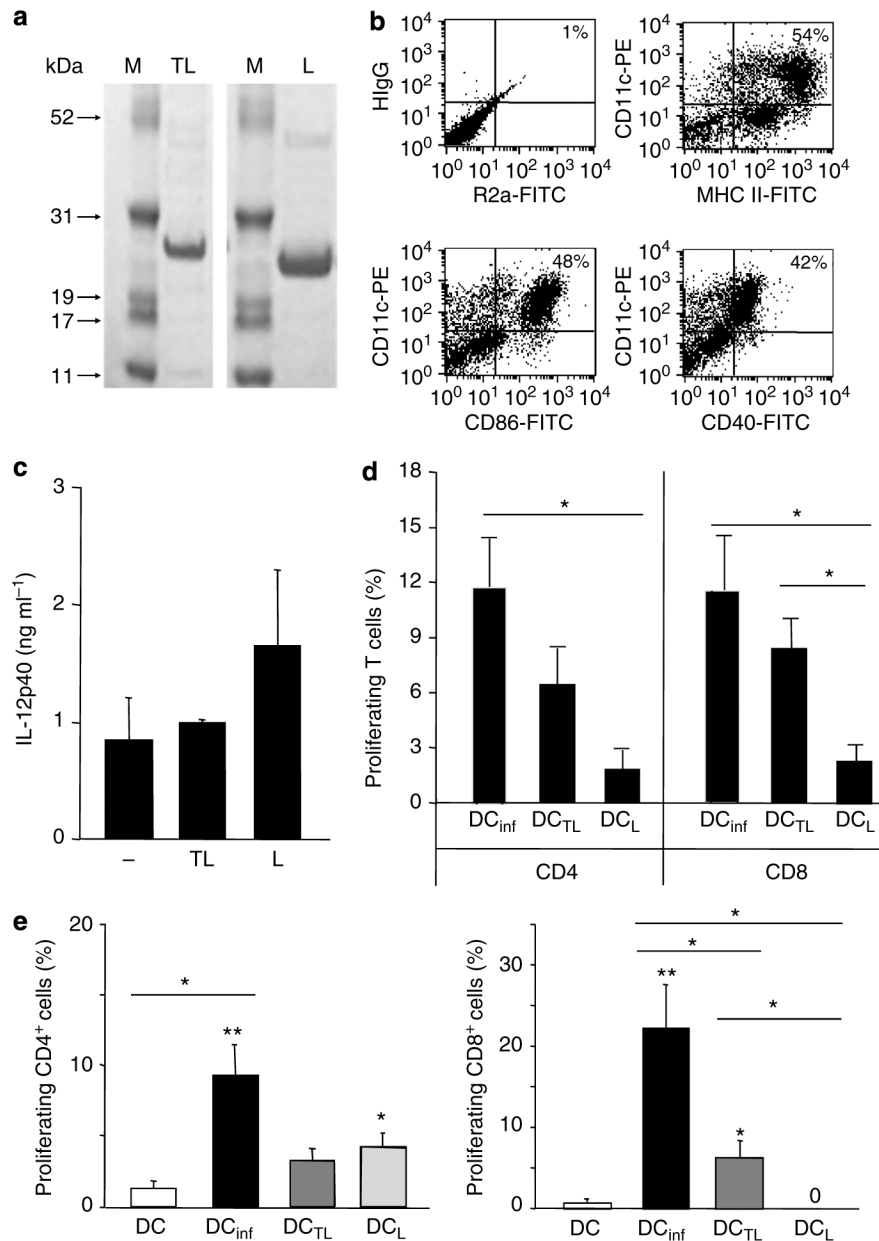


Figure 1. Dendritic cells (DCs) pulsed with TAT-LACK are superior in priming and restimulation of antigen-specific CD8⁺ T cells when compared with LACK DCs.

(a) Coomassie blue-stained 4–12% SDS-PAGE gel in which purified TAT-LACK and LACK were resolved. (b) Bone marrow-derived, immature DCs were generated with rIL-4 and rGM-CSF (10 ng ml⁻¹) and harvested as immature DCs on day 6. Expression of activation markers was analyzed by flow cytometry using monoclonal antibodies against CD40, CD86, and MHC class II as well as rat anti-mouse IgG2a (R2a) and hamster anti-mouse IgG1 (HIgG) as isotype controls. One representative FACS staining is shown. (c) DCs were pulsed for 18 hours with TAT-LACK (TL) or LACK (L), or left untreated. IL-12p40 release was determined by ELISA (mean ± SEM, $n \geq 3$). (d) TAT-LACK (DC_{TL})-treated and LACK (DC_L)-treated DCs were obtained or DCs were infected with *L. major* amastigotes (DC_{inf}, ratio 1:5). T cells were enriched from 6-week *L. major*-infected

C57BL/6 mice using anti-Thy1.2-coated microbeads, labeled with CFSE, and restimulated with irradiated, antigen-pulsed or infected DCs (1:10 ratio of DC/T cells, 1×10^6 per 200 μ l). T-cell proliferation was determined after 5 days using flow cytometry. (e) C57BL/6 mice were vaccinated with 2×10^5 untreated DCs, DC_{inf}, DC_{TL}, or DC_L. After 1 week, lymph node (LN) cells were isolated, labeled with carboxyfluorescein diacetate succinimidyl ester (CFSE), and antigen-specific proliferation was determined by FACS after 5 days of subculture in the presence of soluble *Leishmania* antigen (SLA). T cells were pregated using staining for CD4 or CD8. (d, e) For each mouse, the relative number of *Leishmania*-reactive cells (in percentage of total CD4⁺ or CD8⁺ T cells) was calculated when compared with untreated control cultures (mean \pm SEM, $n \geq 3$, * $P \leq 0.05$, and ** $P \leq 0.005$ when compared with DC alone; *above lines indicate differences between stimulation groups).

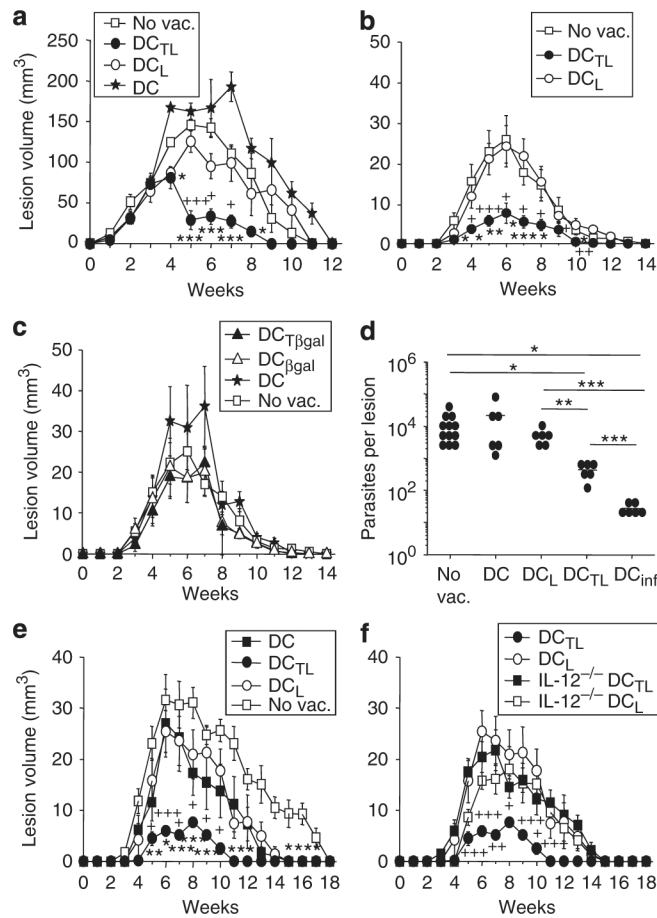


Figure 2. Improved disease outcome after vaccination with fusion protein-pulsed dendritic cells (DCs).

DCs were generated from IL-12p40^{-/-} (f) or wild-type C57BL/6 (a-f) mice and pulsed overnight with TAT-LACK (DC_{TL}), LACK alone (DC_L), TAT-βgal (DC_{Tβgal}), or βgal (DC_{βgal}). Protein-pulsed DCs (2×10^5) were injected intradermally into the ear skin of C57BL/6 mice. After 1 week, mice were infected with 2×10^5 (a) or 10^3 (b-f) metacyclic promastigotes of *L. major*. (a-c, e, f) Lesion volumes were assessed every week using a caliper (mean \pm SEM, $n \geq 4$, statistical differences with unvaccinated groups (*)) and with LACK-pulsed DCs (+) are shown, *, $P \leq 0.05$, **, $P \leq 0.005$, and ***, $P \leq 0.002$. (d) Lesional parasite loads of vaccinated C57BL/6 mice were determined by limiting dilution assays. DCs were infected with amastigotes of *L. major* (parasite/cell ratio 5:1). Each data point represents the number of organisms from one ear and the bars indicate arithmetic means. Pooled data of two independent experiments are shown ($n \geq 6$; * $P \leq 0.05$ and ** $P \leq 0.005$).

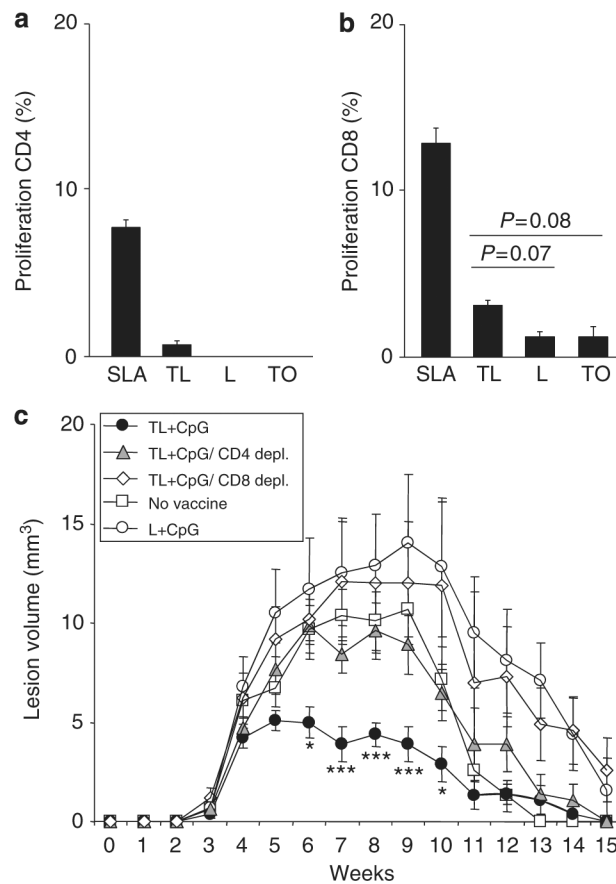


Figure 3. Efficacious vaccination with fusion proteins directly *in vivo* depends on both CD4⁺ and CD8⁺ T cells.

(a, b) C57BL/6 mice were vaccinated with 10 μ g TAT-LACK (TL) or appropriate controls into ears on day -7/-6 followed by 10 μ g CpG 1826 on day -5. On day 0, draining lymph node (LN) cells were harvested, single-cell suspensions were prepared, and cells were stained with carboxyfluorescein diacetate succinimidyl ester (CFSE; 1 μ M). Labeled cells were plated at 1×10^6 cells per 200 μ l and stimulated with antigen. After 4 days, cells were harvested and T-cell proliferation was analyzed by FACS using antibodies against CD4 and CD8. The relative number of *Leishmania*-reactive cells per mouse (%) was calculated when compared with untreated control cultures (mean \pm SEM, $n = 3$). (c) C57BL/6 mice (5 mice per group) were injected with anti-CD4, anti-CD8, or irrelevant control mAb (i.p.) and vaccinated twice with 10 μ g TL or LACK intradermally into the ear skin. CpG oligonucleotide (ODN) 1826 was administered the following day (10 μ g per ear). After 3 weeks, after complete restoration of T cells, mice were infected with 1,000 metacyclic promastigotes of *L. major*. Lesion development was monitored weekly in three dimensions and expressed as mean \pm SEM (pooled data from three independent experiments are shown, $n \geq 9$, statistical differences with unvaccinated groups are shown, * $P \leq 0.05$, and *** $P \leq 0.002$).

Cytokine responses of restimulated T cells after incubation with TAT-LACK- or LACK-pulsed DCs

Table 1.

Restimulation of antigen-specific C57BL/6 T cells w/		DC	DC _{infected}	DC _{TAT/LACK}	DC _{LACK}
<i>CD4</i>					
IFN- γ (pg ml ⁻¹)	20 \pm 20	7,236 \pm 230 ^{***}	973 \pm 370 ^{†,*}	31 \pm 31	
IL-4 (pg ml ⁻¹)	5 \pm 3	107 \pm 58	1 \pm 1	7 \pm 4	
IFN- γ /IL-4	7 \pm 7	317 \pm 163	681 \pm 60 ^{†††,***}	2 \pm 2	
IL-10 (pg ml ⁻¹)	426 \pm 90	631 \pm 287	534 \pm 190	520 \pm 232	
<i>CD8</i>					
IFN- γ (pg ml ⁻¹)	777 \pm 777	4,914 \pm 1,754	1,556 \pm 1,032	360 \pm 360	
IL-4 (pg ml ⁻¹)	2 \pm 1	20 \pm 7 [*]	28 \pm 1	11 \pm 9	
IFN- γ /IL-4	777 \pm 777	225 \pm 126	260 \pm 17 ^{†††}	121 \pm 121	
IL-10 (pg ml ⁻¹)	248 \pm 44	278 \pm 53	308 \pm 109	416 \pm 137	

Abbreviations: DC, dendritic cell; LACK, *Leishmania* homolog of receptors for activated C kinase. DC were incubated with TAT-LACK, LACK, or infected (infection rate 25 \pm 4%, $n \geq 3$) as described in Materials and Methods. CD4⁺ or CD8⁺ cells were isolated from ≥ 5 -week infected mice, plated at 9×10^5 per 200 μ l, and restimulated with DCs (1×10^5) for 48 hours. Cytokine content of supernatants was determined by ELISA (mean \pm SEM; pooled data from three independent experiments; $n \geq 3$ mice per group; statistical comparisons are provided against DC alone (*) or DC/LACK (†)).

^{†,*} $P \leq 0.05$, and

^{†††,***} $P \leq 0.002$.