A recombinant non-pathogenic *Leishmania* vaccine expressing human immunodeficiency virus 1 (HIV-1) Gag elicits cell-mediated immunity in mice and decreases HIV-1 replication in human tonsillar tissue following exposure to HIV-1 infection

Marie Breton, Chenqi Zhao, Marc Ouellette, Michel J. Tremblay and Barbara Papadopoulou

Research Centre in Infectious Diseases, CHUL Research Centre of Laval University and Department of Medical Biology, Faculty of Medicine, Laval University, QC G1V 4G2, Canada

Live-vector human immunodeficiency virus (HIV) vaccines are an integral part of a number of HIV vaccine regimens currently under evaluation that have yielded promising results in pre-clinical testing. In this report, a non-pathogenic protozoan parasitic vector, *Leishmania tarentolae*, which shares common target cells with HIV-1, was used to express full-length HIV-1 Gag protein. Immunization of BALB/c mice with recombinant *L. tarentolae* led to the expansion of HIV-1 Gag-specific T cells and stimulated CD8+ T cells to produce gamma interferon in response to specific viral Gag epitopes. A booster immunization with recombinant *L. tarentolae* elicited effector memory HIV-1 Gag-specific CD4+ T lymphocytes and increased antibody titres against HIV-1 Gag. Most importantly, immunization of human tonsillar tissue cultured *ex vivo* with Gag-expressing *L. tarentolae* vaccine vector elicited a 75% decrease in virus replication following exposure of the immunized tonsils to HIV-1 infection. These results demonstrated that recombinant *L. tarentolae* is capable of eliciting effective immune responses in mice and human systems, respectively, and suggest that this novel non-pathogenic recombinant vaccine vector shows excellent promise as a vaccination strategy against HIV-1.

**INTRODUCTION**

In recent years, it has become increasingly apparent that cell-mediated immune responses, particularly those involving human immunodeficiency virus 1 (HIV-1)-specific CD8+ cytotoxic T lymphocytes (CTLs), play a key role in control of both acute and chronic HIV-1 infections (reviewed by Letvin, 2005). The ability to elicit potent cellular immune responses has therefore become a priority for HIV-1 vaccine candidates. The goal of T-cell vaccines is to generate long-lived memory CD8 cells capable of recognizing and rapidly expanding to combat viral infection.

The problematic stimulation of neutralizing antibodies in HIV-1 infection (Moore *et al.*, 1995), the recent failure of a recombinant gp120 glycoprotein to demonstrate vaccine efficacy in a phase III human clinical trial (Gilbert *et al.*, 1998) and the inability of traditional vaccine approaches to generate a suitable and safe vaccine for HIV-1 (Baba *et al.*, 1995; Murphey-Corb *et al.*, 1989; Whitney & Ruprecht, 2004) have prompted the quest for the development of novel vaccine strategies. Among these, the most promising are the use of plasmid DNA and live recombinant vectors, either alone or as part of heterologous prime–boost combinations. Although plasmid DNAs have been less immunogenic in early-phase clinical testing in humans than in laboratory animals (MacGregor *et al.*, 1998; McConkey *et al.*, 2003), a number of changes in plasmid DNA constructs have been shown to increase their immunogenicity (Donnelly *et al.*, 2005). Live recombinant vaccines that express HIV-1 immunogens have proven generally to stimulate strong CD4+ and CD8+ T-cell responses and neutralizing antibodies in non-human primates compared with whole killed vaccines, virus-like particles and subunit vaccines (Amara *et al.*, 2001; Barouch *et al.*, 2001; Horton *et al.*, 2002; Seth *et al.*, 2000; reviewed by Letvin *et al.*, 2002; McMichael & Hanke, 2003). Although live recombinant vectors are central to the development of new vaccine strategies against HIV-1, their utilization as vaccine candidates in humans is hampered due to problems related to pre-existing immunity, inefficient antigen delivery or presentation and toxicity.
issues, especially for immunocompromised individuals (Redfield et al., 1987). Therefore, there is an urgent need to develop new live-vaccine vectors that are capable of enhancing antigen presentation and eliciting potent immune responses without the risk of developing disease in humans.

In this study, we describe a novel, non-pathogenic, protozoan parasitic vector, *Leishmania tarentolae*, as a recombinant HIV-1 vaccine candidate to improve the efficacy of HIV vaccine strategies. *L. tarentolae* has several features that make it very attractive as the basis for an effective recombinant HIV vaccine. We have shown recently that *L. tarentolae* efficiently targets antigen-presenting cells (APCs) (e.g. macrophages and dendritic cells) and lymphoid organs and that it activates the process of dendritic cell maturation (Breton et al., 2005). Unlike other pathogenic *Leishmania* strains, *L. tarentolae* lacks the potential to replicate within the targeted APCs and is eliminated after several days from the infected murine host (Breton et al., 2005). Nevertheless, *L. tarentolae* can elicit T-cell proliferation and the production of interferon-γ, skewing the T-cell response towards a Th1-cell phenotype, and it provides inflammatory responses for the APC and acts as an immunostimulatory adjuvant (Breton et al., 2005). In the present study, we developed a recombinant *L. tarentolae* strain expressing high levels of full-length HIV-1 Gag and evaluated its ability to elicit HIV-1 Gag-specific T-cell responses in mice upon HIV antigen stimulation. Moreover, we immunized human lymphoid tissue cultured *ex vivo* that can be infected by both *Leishmania* and HIV-1 (Zhao et al., 2004) to assess the ability of the recombinant *L. tarentolae-Gag* vaccine vector to protect against HIV-1 infection. Such a system has not been used before to evaluate the protective efficacy of vaccine candidates.

**METHODS**

**Parasite growth and transfections.** The *L. tarentolae* Tar II strain (ATCC 30267) was grown in SDM-79 medium (Brun & Schonenberger, 1979) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Multicell) and 5 μg haemin ml⁻¹. *Leishmania* transfections were carried out by electroporation as described elsewhere (Papadopoulou et al., 1992). Approximately 10–15 μg DNA was used for transfections and cells were selected with G-418 (40 μg ml⁻¹; Sigma).

**Plasmid construction and DNA manipulations.** The pNEO-Gag expression vector was made as follows. First, the intergenic region of the α-tubulin gene necessary for mRNA processing was inserted into the XhoI/Xpol sites of pSP72 (Promega). Then, the neomycin phosphotransferase (*neo*) gene with an additional intergenic region of the α-tubulin gene was cloned as a KpnI-EcoRI fragment into the respective vector site. The 1.8 kb RNA gene promoter sequence, described elsewhere (Yan et al., 1999), was cloned in the NdeI site of the above vector. Finally, a 1.6 kb fragment containing the HIV-1 gag gene was amplified using *Pwo* polymerase (Roche) from pNL4.3 (Adachi et al., 1986), a full-length HIV-1 proviral molecular clone, using the following set of primers 5’Gag (Xhol); 5’-GGGGTACCTTATTTGGTACGGG-3’ and 3’Gag (KpnI): 5’-GGGGTACCTTATTTGGTACGGG-3’, and then introduced as an Xhol–KpnI insert between the two α-tubulin intergenic regions to generate vector pNEO-Gag. Southern and Western blot analyses were performed to test for the presence of Gag-expressing vector and to evaluate Gag protein expression using standard procedures.

**Mice and immunizations.** Groups of 8-week-old female BALB/c mice (Charles River Laboratories) were immunized intraperitoneally (i.p.) with 5 × 10⁶ stationary-phase recombinant *L. tarentolae* promastigotes expressing the HIV-1 Gag protein. *L. tarentolae* wild-type promastigotes were also used for comparison. At 2, 4, 6, 8 and 12 weeks post-immunization, mice were sacrificed and blood samples and spleens were collected for immunological analyses. For evaluating T-cell memory responses, groups of five mice were primed with 5 × 10⁶ *L. tarentolae-Gag* cells (i.p. injection) and boosted 1 month later with the same vaccine dose. Six months after the first immunization, splenocytes were isolated, stimulated with 25 pg p24 ml⁻¹ for 24 h in RPMI 1640 and antigen-specific responses were measured by flow cytometry. Approximately 10⁶ spleen cells were stained with FITC-labelled anti-CD4 (H-CAM; BD Biosciences), phycoerythrin (PE)-labelled anti-CD62L (BD Biosciences) or FITC-labelled anti-CD69 (BD Biosciences), PE-labelled anti-CD25 (BD Biosciences) and tri-colour (TC)-labelled anti-CD4 (BD Biosciences). Splenocytes were incubated for 30 min at 4 °C with 500 μl PBS containing 1% FBS and 0.09% NaN₃ containing a saturating amount of each antibody. Cells were then washed and fixed with 2% paraformaldehyde. Flow cytometry was performed using an EPICS Elite ESP (Coulter Electronics) and data were further analysed with WinMDI software.

**Proliferation assays.** Spleen cells were harvested from individual vaccinated mice on day 0 (just prior to immunization) and at 2, 4, 8 and 12 weeks post-immunization and tested in a standard [³H]thymidine incorporation assay. Cells were homogenized, washed and resuspended in 200 μl RPMI 1640 supplemented with 10% FBS (HyClone), 100 U penicillin ml⁻¹, 100 μg streptomycin ml⁻¹, 2 mM L-glutamine, 1 mM sodium pyruvate and 50 mM 2-mercaptoethanol. Cells were cultured in a 96-well flat-bottomed microplate (Corning) (2.5 × 10⁶ splenocytes per well) with different concentrations of p24 (0–50 μg ml⁻¹) for 3 days at 37 °C and 5% CO₂ atmosphere and pulsed with 1 μCi [³H]thymidine (Amersham) per well for 24 h. Cells were harvested on day 3 with a Harvester 96 (Tomtec) and [³H]thymidine incorporation was measured with a 1205 BetaPlate Liquid Scintillation Counter (Amersham). The mean number of c.p.m. for triplicate wells was used to calculate the stimulation index (SI) as follows: SI = c.p.m. with antigen stimulation/c.p.m. with medium alone.

**IFN-γ ELISPOT assay.** To evaluate the frequency of T cells producing IFN-γ, we used an enzyme-linked immunosorbent spot (ELISPOT) assay as described elsewhere (Mashishi & Gray, 2002). Briefly, 96-well multiscreen plates (Millipore) were coated by over-night incubation at 4 °C with rat anti-mouse IFN-γ capture monoclonal antibody (clone R4-6A2; BD Pharmingen) at a concentration of 10 μg ml⁻¹ in PBS. Splenocytes were harvested from mice at 2, 4, 8 and 12 weeks after immunization with recombinant *L. tarentolae*. Cells (1 × 10⁶ per well) were plated in triplicate in a 100 μl final volume with medium alone or stimulated with a pool of overlapping Gag peptides in complete RPMI 1640. HIV-1 Gag peptides of 9, 15 or 20 aa in length were obtained from the AIDS Research and Reference Reagent Program, NIH (Rockville, MD, USA), and used for stimulation at a final concentration of 10 μM. After a 24 h incubation at 37 °C in 5% CO₂ the plates were washed with PBS containing 0.05% Tween (PBST) and incubated for 6 h at room temperature with a secondary biotinylated rat anti-mouse IFN-γ antibody (XMG1.2; BD Pharmingen). Plates were washed six times with PBS and streptavidin–alkaline phosphatase (diluted 1:100000; Sigma) was added. After incubation for 2 h, plates were washed and
developed with NBT/BCIP (Bio-Rad). Plates were air-dried and the spots were counted using a stereomicroscope (Carl Zeiss Canada) at 40× magnification. An ELISPOT assay was also performed on CD4+ T-cell-depleted splenocytes using the panning method (Norton, 1997). The mean number of spots from triplicate wells was calculated for each animal and adjusted to represent the mean number of spots per 106 spleen cells. Data are presented as spot forming units (s.f.u.) per 106 spleen cells from five animals per group.

Anti-p24 antibody titres. Serum was collected from individual mice at 8 weeks post-immunization and anti-p24 antibody titres were determined by ELISA. Ninety-six-well plates (ImmunoPlate Maxisorp; Nunc) were coated by overnight incubation at 4 °C with 50 ng p24 per well. Plates were blocked with PBS plus 5 % BSA for 2 h at room temperature and then washed with PBST. Sera were diluted in PBS from 1:100 to 1:500 and 100 μl peroxidase-conjugated rabbit anti-mouse IgG antibody (Sigma) was added and incubated for 1 h at room temperature. After at least five washes, plates were developed with 3,3′,5,5′-tetramethylbenzidine peroxidase substrate (Research Diagnostics). Reactions were stopped with 2 M H2SO4 and the absorbance was measured at 450 nm using an Organon Teknika Microwell system.

Delivery of L. tarentolae and NL4.3 HIV-1 strain to human tonsillar tissue blocks. Human tonsils were obtained from patients who underwent routine tonsillectomy. Tonsils were washed thoroughly with PBS containing antibiotics, dissected into small pieces of ~2–3 mm3 and cultured in RPMI 1640 supplemented with FBS and antibiotics on collagen sponge gels at the air–liquid interface at 37 °C with 5 % CO2 atmosphere as described previously (Zhao et al., 2004). After 24 h, different concentrations (1 × 103, 1 × 104, 5 × 104, 1 × 105 and 5 × 105 cells) of recombinant L. tarentolae expressing HIV-1 Gag were added on top of the tissue blocks and 3 h later the tonsillar pieces were washed with PBS and kept in culture for up to 2 weeks. At ~3 weeks after immunization with recombinant L. tarentolae, 5 ng NL4.3 HIV-1 strain was applied on top of the tissue blocks. Aliquots of supernatants were harvested 6 days after infective HIV-1 challenge and a sandwich ELISA was performed on supernatants to quantify p24 production and monitor HIV replication. The p24 antibody capture assay has been described previously (Bounou et al., 2004). After 24 h, different concentrations (1 × 103, 1 × 104, 5 × 104, 1 × 105 and 5 × 105 cells) of recombinant L. tarentolae expressing HIV-1 Gag were added on top of the tissue blocks and 3 h later the tonsillar pieces were washed with PBS and kept in culture for up to 2 weeks. At ~3 weeks after immunization with recombinant L. tarentolae, 5 ng NL4.3 HIV-1 strain was applied on top of the tissue blocks. Aliquots of supernatants were harvested 6 days after infective HIV-1 challenge and a sandwich ELISA was performed on supernatants to quantify p24 production and monitor HIV replication. The p24 antibody capture assay has been described previously (Bounou et al., 2002). It should be noted that separate blocks of human tonsillar tissue from six different donors were tested (i.e. a total of eight tissue blocks in four different wells) to normalize for variation in cell number and composition and to allow comparison of immunized and non-immunized tissue.

Statistical analyses. Data were expressed as means ± SEM. The statistical significance of differences between groups was analysed using a paired Student’s t-test. A P value of less than 0.05 was considered significant.

RESULTS

Generation of recombinant L. tarentolae expressing high levels of HIV-1 Gag

To determine whether L. tarentolae could express HIV-1 proteins efficiently, we generated a Gag-encoding expression vector, pNEO-Gag, that was introduced by electroporation into the non-pathogenic protozoan parasite L. tarentolae as described in Methods. In pNEO-Gag, the expression of the HIV-1 gag gene is under the control of the RNA pol I 18S rRNA gene promoter (Fig. 1a), which allows high levels of expression. Western blot analysis using an anti-p24 antibody showed that recombinant L. tarentolae transformed with the HIV-1 Gag expression vector showed high levels of expression of the full-length 55 kDa Gag protein (Fig. 1b). Remarkably, the HIV-1 Gag polyprotein was processed into its other proteolytic products in Leishmania, such as p24, in a similar fashion to HIV-1 Gag from H9 HIV-1-infected cells. These results showed the successful expression of the full-length HIV-1 Gag protein in the non-pathogenic L. tarentolae and suggested that Gag polyprotein can be processed by proteases within the parasite.

Immunization with recombinant L. tarentolae expressing HIV-1 Gag elicits HIV-1-specific cell proliferative responses

To determine whether administration of the recombinant L. tarentolae vaccine vector stimulated T-cell responses, splenocytes harvested from immunized mice were first assessed for antigen-stimulated proliferation. Splenocytes were cultured with various concentrations of a recombinant p24 Gag protein and proliferative responses were measured by [3H]thymidine incorporation. An antigen-specific, dose-dependent proliferation was observed in splenocytes from mice immunized with recombinant L. tarentolae at different time points post-immunization. Proliferation of splenic cells from mice immunized with recombinant L. tarentolae was increased by ~12-fold in response to the highest p24 dose (50 pg ml−1) (Fig. 2a). No p24-specific proliferative responses were observed in splenocytes of mice immunized with wild-type L. tarentolae (Fig. 2a). Interestingly,
antigen-stimulated proliferation was still observed at 12 weeks post-immunization and at slightly increased levels (Fig. 2b).

Immunization with recombinant L. tarentolae elicits HIV-1-specific CD8+ T cells secreting IFN-γ

Vaccine-elicted immune responses were monitored in BALB/c mice using an IFN-γ ELISPOT assay. Splenocytes were harvested from mice at weeks 2, 4, 8 and 12 after immunization with recombinant L. tarentolae and then stimulated with a pool of overlapping peptides representing a processed form of the HIV-1 Gag protein. Our results on total splenocytes indicated an important increase in the number of IFN-γ-producing T cells following stimulation with the Gag peptide pool compared with unstimulated splenocytes (Fig. 3a). The peak response was elicited at 2 weeks post-immunization (386 ± 52.3 s.f.c. per 10^6 splenocytes). However, the number of IFN-γ-producing T cells remained relatively high (293 ± 41.4 s.f.c. per 10^6 splenocytes).
splenocytes), even 12 weeks after immunization with recombinant *L. tarentolae* (Fig. 3a).

To evaluate whether specific HIV-1 Gag epitopes could stimulate T cells to produce IFN-γ, we used five different pools (P1–P5) of 15 overlapping HIV-1 Gag peptides each. These peptides were 9–20 aa long and overlapped by ~8–11 residues. Peptide pool 4 (P4) was capable of inducing a greater stimulation of IFN-γ-producing T cells in splenocytes isolated from BALB/c immunized mice at week 12 post-immunization (Fig. 3b). Peptides in pool P4 cover a region of the HIV-1 Gag protein between aa 281 and 430. This region includes the end of the capsid (p24) and part of the nucleocapsid (p7).

The functional capacity of recombinant *L. tarentolae* to induce HIV-1 Gag-specific CD8⁺ T-cell responses was also determined. CD4⁺ T cells were selectively depleted from the splenocyte T-cell population by the panning approach (Norton, 1997) prior to assaying these cells in an IFN-γ ELISPOT assay. Depletion of CD4⁺ T cells had no significant effect on the Gag-specific-induced IFN-γ response (Fig. 3c), suggesting that recombinant *L. tarentolae*-elicited HIV-1-specific CD8⁺ T cells were capable of secreting IFN-γ in response to HIV-1 Gag peptide stimulation.

### A booster immunization with recombinant *L. tarentolae* stimulates the development of long-lasting immune responses

Protection following viral clearance or successful immunization requires the generation and maintenance of long-lived, antigen-specific CD4⁺ Th cells (reviewed by Kleneman & Hill, 2005). CD4⁺ Th cells are also critical for the development of CD8⁺ T-cell memory (Janssen et al., 2003; Shedlock & Shen, 2003). We first measured the CD4⁺ effector memory response to HIV-1 Gag in mice immunized once with the recombinant *L. tarentolae*-Gag vaccine vector. Six months after immunization, bulk splenocytes were isolated, stimulated with HIV-1 p24 protein and stained with TC-conjugated monoclonal antibodies to CD4, FITC-conjugated antibodies to CD69 and CD44, and PE-labelled antibodies to CD25 and CD62L, and analysed by flow cytometry. CD25 and CD69 are cell-surface markers that are expressed in activated Th1 subpopulations. CD44 is a surface protein required for lymphocyte extravasation to inflammatory sites and its upregulation is a marker for all memory T cells (DeGrendele et al., 1997). CD62L is a lymph node homing receptor that is downregulated upon activation of T-cell populations (Andersson et al., 1994). Upon antigen stimulation, approximately 22% of the CD4⁺ cells were effector cells, CD44High/CD62LLow, compared with 14% in naive mice (Table 1). A significant proportion of the CD4⁺ T cells in vaccinated mice expressed the activation markers CD25 and CD69 (~15% and ~11%, respectively) (Table 1).

To determine whether T-cell responses could be enhanced following a booster immunization, mice were reinjected i.p. with a second dose of recombinant *L. tarentolae*-Gag 1 month after the first immunization. Five months after the second immunization, bulk splenocytes were isolated, stimulated with HIV-1 p24 and stained with specific antibodies to measure antigen-specific CD4⁺ Th cells by FACS analysis as described in Methods. Following antigen stimulation with HIV-1 p24, approximately 46% of the CD4⁺ T cells were effector cells (CD44High/CD62LLow), a 2-fold increase compared with mice immunized with a single i.p. dose of the recombinant vaccine (Table 1). The expression of CD25 and CD69 activation markers on the surface of CD4⁺ T cells was also increased by approximately 2-fold in boosted animals following stimulation with p24 Gag (Table 1). Thus, our data indicated that a second encounter with the HIV-1 Gag antigen stimulates the proliferation of effector memory CD4⁺ T cells.

### Immunization with recombinant *L. tarentolae* expressing HIV-1 Gag elicits low antibody responses against HIV-1 Gag

We evaluated the production of anti-Gag-specific antibodies in the serum of *L. tarentolae*-Gag-immunized mice. Various dilutions (1:100, 1:250, 1:500) were used of sera obtained at 4 and 8 weeks following i.p. immunization with recombinant *L. tarentolae* expressing HIV-1 Gag protein. A p24 ELISA was used to measure the IgG p24-specific immune responses. No antibodies were found in sera after only a single injection with recombinant *L. tarentolae* (Fig. 4 and data not shown). However, we observed a low antibody response (Table 1).

### Table 1. A booster immunization with recombinant *L. tarentolae*-Gag stimulates the proliferation of HIV-1 Gag-specific memory CD4⁺ T cells in BALB/c mice

<table>
<thead>
<tr>
<th>Splenocytes</th>
<th>Cell-surface markers (%)</th>
<th>CD4⁺Naive/CD62LLow</th>
<th>CD25</th>
<th>CD69</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naive</td>
<td></td>
<td>14.04</td>
<td>6.30</td>
<td>8.19</td>
</tr>
<tr>
<td>Primed</td>
<td>Unstimulated</td>
<td>14.77</td>
<td>6.23</td>
<td>5.88</td>
</tr>
<tr>
<td>Primed</td>
<td>p24-stimulated</td>
<td>21.83</td>
<td>14.89</td>
<td>10.75</td>
</tr>
<tr>
<td>Unstimulated</td>
<td>p24-stimulated</td>
<td>22.57</td>
<td>6.23</td>
<td>8.04</td>
</tr>
<tr>
<td></td>
<td>Primed–boosted</td>
<td>45.74</td>
<td>21.14</td>
<td>19.78</td>
</tr>
</tbody>
</table>

Flow cytometry was performed with splenocytes isolated from two groups of vaccinated and naive mice. Ten BALB/c mice were initially immunized i.p. with 5 x 10⁷ *L. tarentolae*-Gag (primed) and half of them (five mice) were boosted 1 month later with the same dose of recombinant *L. tarentolae*-Gag (primed–boosted). Six months after the first immunization, splenocytes isolated from each group of five mice were pooled, stimulated with 25 pg HIV-1 p24 ml⁻¹ and stained with antibodies to CD44, CD62L, CD69 and CD25 as upregulation cell-surface markers for memory T cells and analysed by flow cytometry as indicated in Methods. The percentages shown here were gated on CD4⁺ T cells. Data were analysed using WinMDI software.
response in the 1 : 100 dilution of sera collected 4 weeks after a booster immunization with recombinant \( L. \ tarentolae \) (Fig. 4). Thus, to trigger a specific humoral response against the HIV-1 Gag protein, a secondary encounter with the antigen following a booster immunization with recombinant \( L. \ tarentolae \) is required.

**Immunization of human lymphoid tissue with recombinant \( L. \ tarentolae \) elicits an important decrease in virus replication following exposure to HIV-1**

With the exception of the rhesus macaque model, no other models are being used to evaluate protective immune responses against simian/human immunodeficiency virus infection. Here, we evaluated whether immunization of \( \text{ex vivo} \) human tonsillar tissue with the Gag-expressing \( L. \ tarentolae \) vector could elicit protection following exposure to HIV-1 infection. This system was described initially as a model to study HIV-1 infection (Glushakova et al., 1995). We recently demonstrated that \( Leishmania \) \( donovani \) can infect human lymphoid tissue (Zhao et al., 2004). Here, we showed by both microscopic examination and FACS analysis that non-pathogenic \( L. \ tarentolae \) could be delivered to human lymphoid tissue (Fig. 5a, b). Indeed, \( L. \ tarentolae \) was found in approximately 40 % of the CD14\(^+\) cells (i.e. monocytes and macrophages) within the human tonsils (Fig. 5a). Eight separate blocks of human tonsillar tissue derived from each donor were used to allow comparison of immunized tissue with Gag-expressing \( L. \ tarentolae \) and unimmunized tissue (\( L. \ tarentolae \) wild-type). Three weeks after immunization, tonsillar tissues were infected with the NL4.3 HIV-1 strain. Aliquots of supernatants were harvested 6 days after viral infection. The data shown are means ± SEM of eight tissue blocks in four different wells from one representative culture. Similar data were obtained using replicate wells from six different donors.

---

**Fig. 4.** Serum anti-p24 Gag antibody titres elicited by immunization with recombinant \( L. \ tarentolae \) expressing HIV-1 Gag. Groups of five BALB/c mice were either infected with 5 \( \times \) 10\(^7\) wild-type \( L. \ tarentolae \) cells (▲), primed with 5 \( \times \) 10\(^7\) recombinant \( L. \ tarentolae \) cells (□) or primed and boosted with 5 \( \times \) 10\(^7\) recombinant \( L. \ tarentolae \) cells within a 1 month interval (◆). Eight weeks after the first i.p. immunization or 4 weeks after booster immunization, mice were bled and serial dilutions (1 : 100, 1 : 250 and 1 : 500) from pooled sera from each immunization group were used to determine antibody titres against HIV-1 Gag protein by ELISA. Data represent means ± SEM of five mice per group and each assay was performed in triplicate.

**Fig. 5.** Immunization of human lymphoid tissue cultured \( \text{ex vivo} \) with recombinant \( L. \ tarentolae \) elicited a significant decrease in virus replication following exposure to HIV-1. Different amounts of recombinant \( L. \ tarentolae \) were added on top of small pieces of tonsil as described in Methods. (a) Delivery of \( L. \ tarentolae \) to human tonsillar tissue blocks. FACS analysis was performed on mechanically disrupted tonsil tissue 72 h after the addition of 5 \( \times \) 10\(^5\) recombinant \( L. \ tarentolae \) cells expressing GFP. The left panel represents the percentage of CD14\(^+\) cells (e.g. macrophages, monocytes) within the tonsil tissue. The right panel represents the percentage of phagocytic cells harbouring \( L. \ tarentolae \)–GFP. The grey line represents the isotypic control. (b) Delivery of recombinant \( L. \ tarentolae \) expressing HIV-1 Gag to human tonsillar tissue blocks. Giemsa staining of recombinant \( L. \ tarentolae \) within a tonsil smear 6 days after parasite entry. (c) Approximately 3 weeks after immunization of the human tonsillar tissue with recombinant \( L. \ tarentolae \), 5 ng HIV-1 strain NL4.3 was added on top of each piece and HIV-1 infection was monitored by p24 ELISA 6 days after viral infection. The data shown are means ± SEM of eight tissue blocks in four different wells from one representative culture. Similar data were obtained using replicate wells from six different donors. □, Wild-type \( L. \ tarentolae \); ■, recombinant \( L. \ tarentolae \); ◆, HIV-1.
sandwich was performed to quantify p24 production and to monitor HIV-1 replication. Immunization of human tonsillar tissues with recombinant *L. tarentolae* resulted in an ~75% decrease in virus replication compared with unimmunized tissue (Fig. 5c). Immunization with an increased dose of Gag-expressing *L. tarentolae* (10^7–10^8) resulted in a higher decrease in virus replication (Fig. 5c). Interestingly, highly reproducible results were obtained with blocks of tonsillar tissue from six different donors where a 66–75% decrease in virus replication was observed following immunization of tonsillar tissue blocks with 10^8 *L. tarentolae* cells and subsequent exposure to the NL4.3 HIV-1 strain at 3 weeks post-immunization (data not shown). To rule out the possibility that the parasite could make the tonsils less suitable host cells for HIV replication, by stimulating innate immunity for instance, we incubated the tonsillar material in parallel with wild-type *L. tarentolae* and a recombinant *L. tarentolae* expressing an irrelevant antigen, i.e. green fluorescent protein (GFP). These experiments showed that HIV-1 replication was not affected by the presence of the wild-type parasite (Fig. 5c) or the parasite expressing GFP (data not shown).

The main targets for X4 HIV-1 (NL4.3) are CD4+ T lymphocytes. We therefore tested whether the numbers of these target cells were the same for uninfected, *L. tarentolae*-immunized and *L. tarentolae*-Gag-immunized tissue blocks. Our results indicated that there were no differences in the number of CD4+ T cells between *L. tarentolae*-Gag-immunized and *L. tarentolae*-immunized tissue blocks (data not shown).

**DISCUSSION**

In this study, we demonstrated that a novel, live-vector vaccine of a non-pathogenic protozoan parasite, *L. tarentolae*, expressing full-length HIV-1 Gag protein was immunogenic in mice and, more importantly, that it elicited protective immune responses in human tonsillar tissue cultured *ex vivo* following exposure to HIV-1 infection. *L. tarentolae* is a lizard parasite that has never been associated with any pathology in humans and therefore safety or pre-existing immunity issues in humans are not a major concern. Moreover, the strain has been shown to be non-pathogenic when injected into immunodeficient SCID mice (Breton et al., 2005). Furthermore, recombinant *L. tarentolae* as a non-replicating, live-vector vaccine achieves only one round of infection, which makes it even safer for human use. In addition, *L. tarentolae* has several other features that may make it an effective HIV vaccine vector. *L. tarentolae* is an intracellular parasite that shares common target cells with HIV-1, e.g. macrophages and dendritic cells and secondary lymphoid organs (Breton et al., 2005). Therefore, a recombinant *L. tarentolae* vaccine vector should mimic the processing, maturation and presentation of viral antigens seen during natural infection. A more efficient targeting of HIV-1 immunogens to APCs could enhance MHC/antigen-presentation functions and elicit a more potent stimulation of antigen-specific CD4+ Th cell and CD8+ CTL responses, which are critical for the control of HIV replication (Letvin, 2005). In addition, *L. tarentolae* induces dendritic cell maturation and elicits CD4+ T-cell proliferation and the production of IFN-γ, thereby driving Th1 cell polarization, which can be critical for the secondary expansion of memory CTLs (Breton et al., 2005). Finally, *L. tarentolae* demonstrates a high cloning capacity, is capable of expressing a wide range of multiple foreign antigens and grows rapidly in a cell-free medium at low cost.

The optimal vaccine vector would produce the vaccine antigen in excess of its own proteins (so that the immune response focuses on the target antigen) and would primarily be produced in APCs for induction of CTL responses. The expression of the ribosomal promoter greatly enhances the expression of HIV-1 Gag in *L. tarentolae*. Recombinant *L. tarentolae* elicited HIV-specific CD8+ T-cell responses that were maximal 2 weeks after immunization. This peak immune response has also been described in mice immunized with adenoviral and vaccinia vectors (Barouch et al., 2003; Seaman et al., 2004). CD8+ T-cell activation and IFN-γ production was higher in response to a specific pool of Gag peptides spanning the aa 281–430 region of the Gag protein, which corresponds to the end of the capsid (p24) and part of the nucleocapsid (p7). CD4+ T-cell depletion studies indicated that IFN-γ-producing T cells were mainly of CD8+ type. Diverse evidence supports the importance of the cellular immune response in HIV containment. CTLs have been shown to play a particularly important role in the early control of HIV infection (Betts et al., 2001; Goulder et al., 2001; Jin et al., 1999; Schmitz et al., 1999). Long-term non-progressors also have consistently higher levels of HIV CTLs than progressors (Harrer et al., 1996). In addition, rhesus monkeys lacking CD8+ T cells fail to control simian immunodeficiency virus infection (Schmitz et al., 1999). The ability of *L. tarentolae* to target macrophages and dendritic cells may explain the induction of T-cell activation. In recent years, it has become increasingly apparent that dendritic cells potentially link the innate and adaptive immune systems, coordinating the activation of strong cellular and humoral immunity (Granucci et al., 2003; Melief, 2003).

Interestingly, in mice that have been immunized twice with recombinant *L. tarentolae*, 50% of the HIV-1 Gag-specific CD4+ T cells within spleen cells were effector cells (CD44^*high*/CD62L^*low*). Thus, these data suggest that recombinant *L. tarentolae* is able to elicit HIV-1-specific effector memory *in vivo* after re-encountering the antigen. Memory CD4+ T cells are critical for the development of CD8+ T-cell memory (Janssen et al., 2003; Shedlock & Shen, 2003). In addition to the induction of effector T-cell responses by the recombinant *L. tarentolae* vaccine vector, a booster immunization with this live-vector vaccine elicited Gag-specific humoral responses. The production of antibodies against p24 is probably due to the activation of CD4+ T cells. It is likely that the use of recombinant *L. tarentolae*...
expressing HIV-1 Gag as part of a heterologous prime–boost strategy may be superior for eliciting memory T-cell and recall responses, as all of the secondary responses will focus on the viral immunogen alone and not on internal Leishmania proteins shared between the priming and boosting vectors. Heterologous prime–boost vaccine regimens have been reported to induce more potent T-cell and antibody responses against HIV-1 (Amara et al., 2001).

An important finding of our studies is that immunization of human tonsillar tissue blocks with recombinant L. tarentolae expressing HIV-1 Gag elicited more than a 75% decrease in HIV-1 replication following exposure to HIV-1 infection. The highest decrease in virus replication was observed when an increased inoculum of recombinant L. tarentolae was used for immunization. This can be explained, as non-replicating, live-vector vaccines typically require high doses and booster immunizations to achieve sufficient antigen to drive immune responses. Although further experiments are required to establish the link between the important decrease in virus replication seen only in immunized tonsillar tissue and the development of protective immunity, preliminary data indicated that there was no difference in the number of CD4 T cells between immunized and unimmunized tissue blocks, which suggests that elicitation of HIV-1 Gag-specific immune responses might be responsible for the observed phenotype. Our data support the possibility of using human tonsillar tissue as an ex vivo system to evaluate the protective efficacy of candidate vaccines towards HIV-1 infection. Previous reports have indicated that secondary lymphoid organs constitute preferred anatomical sites for HIV replication and propagation (Grivel et al., 2003). Moreover, the human lymphoid tissue cultured ex vivo has been shown to preserve the general cytoarchitecture found in normal human lymphoid tissue, including a network of follicular dendritic cells, macrophages, CD4+ and CD8+ T lymphocytes and dendritic cells (Glushakova et al., 1995; Margolis et al., 1997), hence permitting evaluation of cellular and humoral immune responses.

In summary, we have described a novel, live-vector vaccine, a recombinant L. tarentolae expressing HIV-1 Gag protein, which is capable of efficient delivery of HIV-1 immunogens to APCs and to lymphoid organs. We showed that this recombinant vaccine vector could induce HIV-1-specific CD8+ T-cell responses and that it stimulated long-lasting immunity by the production of effector memory CD4+ T cells. Moreover, this live-vector vaccine is safe for human use, which makes it an attractive candidate for a vaccination strategy not only against HIV-1 but also against other intracellular pathogens for which T-cell-mediated immunity is required for protection.

ACKNOWLEDGEMENTS

We are grateful to Nicole Bernard for providing us with the HIV-1 Gag overlapping peptides, Ciro Piccirillo for providing us with antibodies and the physician Jacques Leclerc at the ‘Centre Hospitalier de l’Université Laval’ for kindly providing us with human tonsil tissue. This work was supported by Canadian Vaccine Centre of Excellence (CANVAC) and Canadian Institutes of Health Research (CIHR) GR-14500 grants to B.P., M.O. and M.J.T. M.B. was the recipient of a CIHR studentship. B.P. is a Burroughs Wellcome Fund New Investigator in Molecular Parasitology. M.O. is a Burroughs Wellcome Fund Scholar in Molecular Parasitology and holds the Senior Canada Research Chair in Antimicrobial Resistance. M.J.T. is the recipient of the Senior Canada Research Chair in Human Immuno-Retrovirology.

REFERENCES


HIV-1 immunization with a Leishmania-based vaccine


http://vir.sgmjournals.org