A Single Intramuscular Injection with an Adenovirus-Expressing IL-12 Protects BALB/c Mice Against Leishmania major Infection, While Treatment with an IL-4-Expressing Vector Increases Disease Susceptibility in B10.D2 Mice

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A Single Intramuscular Injection with an Adenovirus-Expressing IL-12 Protects BALB/c Mice Against Leishmania major Infection, While Treatment with an IL-4-Expressing Vector Increases Disease Susceptibility in B10.D2 Mice

Claudia Raja Gabaglia, Brian Pedersen,* Mary Hitt, Nicolas Burdin, Eli E. Sercarz,* Frank L. Graham, Jack Gauldie, and Todd A. Braciak*

Experimental infection of the susceptible BALB/c (H-2k) mouse with the intracellular parasite Leishmania major induces a predominant Th2-type T cell response that eventually leads to death. In contrast, the resistant B10.D2 (H-2b) strain develops Th1 cells that control parasite replication and disease. In this study, we tested the ability of a recombinant adenovirus vector-expressing IL-12 to skew the immune response in a Th1 direction and prevent leishmaniasis in susceptible mice. We report that BALB/c mice treated with the Ad5IL-12 vector on the same day as parasitic challenge are significantly protected against leishmaniasis and acquired long-lasting immunity, because upon rechallenge with L. major parasites they were resistant to disease. The vector-derived IL-12 expression was transient and highly localized to the tissue after i.m. injection; it caused an increase in the number of Ag-specific IFN-γ-secreting lymphocytes and enhanced NK cell activity in the draining popliteal node. In contrast, resistant B10.D2 mice given i.m. injections with a recombinant adenovirus-expressing IL-4 displayed greater susceptibility to disease, and severe lesions were produced in some of the infected animals. These results suggest the potential use of recombinant adenoviruses expressing cytokines as potent immunomodulatory agents for the generation of protective immune responses against intracellular pathogens. The Journal of Immunology, 1999, 162: 753–760.

A paradigm for disease pathogenesis has evolved from studies in murine leishmaniasis that indicates that the cytokines produced against the parasite during the primary infection control differentiation of CD4+ T lymphocytes and the quality of the subsequent immune response. According to the paradigm, CD4+ Th1 (type 1) cell-mediated immunity confers protection against parasitic infection, whereas the development of a CD4+ Th2 (type 2) humoral immune response is associated with disease progression (1). In susceptible BALB/c mice, Leishmania major infection causes a progressive disease that ultimately results in death, with the predominant expression being IL-4 and relatively little IFN-γ production being detected. In contrast, parasitic infection in other strains of mice, such as B10.D2, C3H, C57BL/6, C57BL/10, and 129, produces self-healing lesions associated with a strong Th1 cell-mediated immune response with production of high levels of IFN-γ and other inflammatory cytokines (2). In vivo and in vitro studies concerning the initiation events that cause Th1 or Th2 cell development in L. major-infected mice have demonstrated that IL-4 is the main cytokine inducer of a Th2 response, while IL-12 and IFN-γ promote Th1 cell expansion (3). In fact, therapies directing immune response toward type 1 immunity, including the use of rIL-12 protein as a vaccine adjuvant, have been used successfully to protect BALB/c mice against disease (4–6). However, IL-12 therapies generally require multiple injections, owing to the short half-life of recombinant proteins in vivo. A therapeutic regimen based on a single injection of IL-12 in a suitable vehicle would be a major advance in the prevention of leishmaniasis. Adenoviruses-expressing cytokines are useful agents for delivery of cytokines due to their higher levels of gene expression and their localization at the site of inoculation (7).

Using the Ad5IL-123 vector (a recombinant adenovirus type 5-expressing IL-12), we show that a single ipsilateral-localized i.m. injection of the recombinant vector administered on the same day as L. major parasitic challenge prevented the development of lesions in the footpads of BALB/c mice and the spread of parasitic infection to peripheral organs. The Ad5IL-12 vector treatment induced immune deviation that elicited a protective Th1 immune response against the L. major parasite in the normally Th2-responsive BALB/c strain mice.

Interestingly, in parallel experiments designed to test the effects of vector-derived IL-4 expression, B10.D2 mice (a MHC-matched disease-resistant strain) were rendered susceptible to L. major infection after receiving a single i.m. injection of the Ad5IL-4 vector with parasitic challenge. This is the first report demonstrating that overexpression of IL-4 can increase disease susceptibility in a resistant mouse strain. Taken together, these results suggest that recombinant adenovirus vectors expressing cytokines act as powerful immunomodulatory agents and can serve a useful role as “molecular adjuvants” to generate protective immune responses.

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3 Abbreviations used in this paper: Ad5IL-12, adenovirus type 5-expressing IL-12; Ad5IL-4, adenovirus type 5-expressing IL-4; pfu, plaque-forming units; O/N, overnight; LACK, Leishmania-activated protein kinase C.
Materials and Methods

Adenovirus vectors

All recombinant viruses were propagated on 293 cells and purified by cesium chloride gradient centrifugation. Construction and characterization of the Ad5 vectors expressing IL-12 and IL-4 have previously been described (8, 9). In brief, the Ad5IL-12 vector contains an expression cassette for IL-12 p35 subunit of IL-12 in the E1 region, and the IL-12 p40 subunit in the E3 region. The Ad5IL-4 vector contains an expression cassette for IL-4 in the E1 region. The DL70-3 control virus is an Ad5 variant deleted in the E1 region (10). For treatment, mice were injected with 5 × 10⁶ plaque-forming units (pfu) of recombinant adenovirus in 50 μl PBS into the femoral biceps muscle of the left hind limb on the same day as footpad injection of the L. major promastigotes.

Animals and parasites

BALB/c and B10.D2 mice (6–8 wk of age) were purchased from The Jackson Laboratory (Bar Harbor, ME) and housed in the La Jolla Institute for Allergy and Immunology animal facility. Infections were performed with stationary-phase promastigotes of L. major (strain WHOM/IR/173) grown at 28°C in M199 medium supplemented with 10% FBS, 100 μg/ml penicillin, 100 μg/ml streptomycin, 40 mM HEPES, 0.1 mM adenine, and 5 μg/ml hemin. Animals were injected in the left hind footpad with 2 × 10⁶ stationary-phase L. major promastigotes, and the course of the disease was monitored at weekly intervals by measurement of footpad thickness with a metric caliper. All work was performed in accordance with La Jolla Institute for Allergy and Immunology guidelines for animal use and care.

Parasite burden

Six weeks postinfection, parasite loads were determined by limiting dilution analysis to measure the number of viable L. major parasites in the tissues of infected mice as previously described (11). Briefly, draining lymph nodes and spleens were homogenized in M199 medium with supplements, and serial fivefold dilutions of the homogenates were plated in flat-bottom 96-well microtiter plates and kept at 28°C for 1 week. The wells were assessed for growth of L. major promastigotes microscopically at the highest dilution at which three of eight replicates were positive.

Collection and processing of tissues

At designated periods, blood samples were collected from the retroorbital plexus of mice treated with recombinant adenovirus; or, mice were sacrificed for the preparation of a muscle tissue extract. In addition, draining lymph nodes were collected for cytokine ELISA-spot assays. Frozen tissues were homogenized in PBS (3 ml per muscle) containing 100 μM PMSE, 120 μM aprotinin, and 10 μg/ml aprotinin. The homogenate was freeze-thawed three times and centrifuged at 10,000 × g for 10 min. Aliquots were stored at −20°C until analyzed.

ELISA

IL-12 and IL-4 levels in muscle homogenates and serum were measured by a sandwich ELISA. Briefly, Nunc-ImmuNo Plate MaxiSorp 96 (Nunc, Roskilde, Denmark) were coated with anti-IL-12 (p55/p70; clone Red-T0297-289; PharMingen, San Diego, CA) or anti-IL-4 Abs (purified from 11B11 cell supernatants). After blocking with PBS containing 1% FBS, muscle homogenates or sera were added OD at 4°C. Plates were extensively washed with PBS-Tween and incubated with biotin-conjugated anti-IL-12 (p40/p70; clone C17.8; PharMingen) or anti-IL-4 (PharMingen) Ab. Finally, the plates were washed and developed using avidin-peroxidase and 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) substrate (Sigma, St. Louis, MO). OD values were measured at 405 nm, and the values were determined against a recombinant protein standard.

Cellular ELISA-spot assay

IFN-γ- and IL-4-producing cells were enumerated in recombinant adenovirus-treated mice by a cellular ELISA-spot assay as described (12). Briefly, lymph node cells (5 × 10⁶/ml) were cultured for 48 h in 24-well plates with media alone, paraformaldehyde-fixed L. major parasites, or Leishmania-activated protein kinase C (LACK) peptide 160-173. Millipore HA Nitrocellulose plates (Millipore, Bedford, MA) were coated O/N at 4°C with anti-IFN-γ (purified from R46A2 supernatants) or anti-IL-4 Ab. Plates were blocked, and Ag-stimulated cells were added at different concentrations for 24 h at 37°C. The wells were then incubated with biotin-conjugated anti-IFN-γ (purified from XMG1.2 supernatants) or anti-IL-4 Ab followed by incubation with avidin-peroxidase (Vector, Burlingame, CA). Spots were developed by the addition of 400 μg/ml of 3-aminos-9-ethylcarbazole substrate (Sigma) and enumerated by a computerized image analysis system (Lighthous Research, Encinitas, CA) using the image analyzer program NIH Image 1.61 (National Institutes of Health, Bethesda, MD).

Determination of NK cell activity

NK cytotoxic activity was determined as previously described (4). 51Cr-labeled YAC-1 cells were used as targets in a standard 4-h 51Cr release assay at several E:T ratios for cells isolated from the popliteal lymph node of recombinant adenovirus-treated and control mice. Briefly, 1 × 10⁵ target cells were labeled for 2 h with 500 μCi of sodium chromate in 0.5 ml of RPMI 1640 media (HyClone, Logan, UT) supplemented with 10% FCS, 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 5 × 10⁻⁷ M 2-ME. Samples were recorded in triplicate at each E:T ratio using 5 × 10⁵ YAC-1 target cells per well in V-bottom microtiter plates. The percentage of specific 51Cr release was calculated using the following formula: 100 × [(cpm test sample – cpm medium control)/(cpm 1 N HCl sample – cpm medium control)].

FACS analysis

To characterize the lymphocytes recovered from popliteal nodes in 6-wk recombinant adenovirus vector-post-treated animals, cells were analyzed against a panel of mAbs (PharMingen) specific for CD3, CD4, CD8, CD45RB, Ly49C, DX5, B20, and TCR Vβ8.2, Vρ4, and Vα8 chains using a FACSScan flow cytometer (Becton Dickinson, Mountain View, CA).

Statistical analysis

Data are expressed as the mean ± SEM for each group. Statistical analysis was performed using Statview 4.5 programs from Abacus Concepts (Berkeley, CA). A Student t test was used for the final determination of significance testing the effects of Ad5IL-12 and Ad5IL-4 treatments.

Results

Concomitant i.m. injection with an Ad5IL-12 vector protects BALB/c mice against L. major infection

Because IL-12 had been shown to be an important cytokine in directing Th1 immune responses that are associated with protection against leishmaniasis, we performed experiments to explore the role of the Ad5IL-12 vector vs controls as a way to prevent parasitic lesion development. For this study, BALB/c mice were infected with 2 × 10⁸ L. major promastigotes into the left hind footpad and injected with i.m. with a 5 × 10⁶ pfu dose of recombinant adenovirus on the ipsilateral side. As shown in Fig. 1A, BALB/c mice receiving a single Ad5IL-12 vector i.m. injection were able to effectively control the development of parasitic lesions. In these experiments, 17 of 18 animals were protected and did not develop footpad lesions, whereas all control vector (DL70-3-injected, vector without cytokine insert) and parasite-infected mice developed significant lesions by 6 wk leading to termination of the experiment. The protection afforded by the Ad5IL-12 vector was durable in treated mice because infection remained under control for as long as 1 year (in 12 mice followed). Of these 12 mice, two groups of 4 mice each were rechallenged with an additional parasitic dose of 2 × 10⁸ L. major at either 6 wk or 4 mo after the initial Ad5IL-12 vector treatment. All of these animals were protected against infection after the secondary challenge, indicating the development of immunologic memory to the pathogen (data not shown). To be effective, the IL-12 vector had to be delivered close to the time of L. major challenge. By day 3 post-Ad5IL-12 vector administration, very little protection was provided against leishmaniasis, and if L. major infection was performed 1 wk after Ad5IL12 i.m. injection, protection was absent (Fig. 1B). Simultaneous i.m. administration of Ad5IL-12 vector contralateral to the L. major-infected footpad did not afford any significant protection against disease (data not shown).
Concomitant Ad5IL-4 vector i.m. injection exacerbates L. major infection in B10.D2 mice and allows the spread of parasite to peripheral organs

Because IL-12 expression derived from i.m. injection of the Ad5IL-4 vector blocked the progression of leishmaniasis in susceptible BALB/c mice, we attempted to test if IL-4 vector-derived expression could induce susceptibility in the normally resistant MHC-matched B10.D2 mice. It has been shown that systemic administration of blocking Abs against Th1-type cytokines could divert immune response to the parasite and allow disease to progress in resistant strains (13). However, to date, attempts at inducing disease with rIL-4 protein has failed in resistant mouse strains (14, 15). Here, we report that a single i.m. injection of the Ad5IL-4 vector to resistant B10.D2 mice caused 3 of 11 mice to develop open necrotic lesions (Fig. 2A), with the remaining 8 animals all showing significant increases in footpad swelling compared with the vector-only controls. Fig. 2B shows comparative lesions between “protected” BALB/c and “susceptible” B10.D2 mice after Ad5IL-12 or Ad5IL-4 treatment, respectively, vs DL70–3 vector control. One of the most severely afflicted Ad5IL-4-treated animals had a lesion of 7.3 mm in diameter and was sacrificed at 6 wk. Limiting dilution analysis was performed to determine residual parasite burden on cells isolated from the ipsilateral draining popliteal nodes of the animals at this time point (Fig. 2C). The Ad5IL-4 vector-treated animals had 600-fold (~2.5 log) higher parasite titers in their draining nodes. For one experiment, disease progression was monitored until 15 wk postinfection, when lesions had completely receded in all animals except in one of the Ad5IL-4-treated mice. Quantification of parasite levels in draining lymph nodes at 15 wk postinfection also demonstrated a 125-fold increase in the Ad5IL-4 vector-treated group compared with other groups. Of interest, the most severely afflicted animals from the Ad5IL-4-treated group were found to have L. major promastigotes detectable in the spleen, which were not present in other animals from control groups (data not shown). These data indicate that IL-4 expression not only exacerbates disease locally but renders animals more susceptible to parasitic spread to peripheral organs.

Ad5IL-12 vector i.m. injection in BALB/c mice reduces L. major parasitic burden in the draining popliteal node and prevents the spread of infection to the spleen

In contrast to the enhancing effects of IL-4 expression on parasitic burden in B10.D2 mice, Ad5IL-12 treatment reduced parasitic infection in the BALB/c strain. Mice infected with L. major and receiving additional treatment with Ad5IL-12, adenovirus vectors DL70–3, Ad5IL-4, or no adenovirus treatment were assayed for parasitic burden in lymph node and spleen at 6 wk postinfection (Fig. 3, A and B). At this time point, all control mice had pronounced footpad lesions, with some animals becoming moribund and requiring euthanasia. Meanwhile, all of the experimental Ad5IL-12-treated mice in this experiment were lesion-free and showed no signs of significant morbidity. Parasitic titers in the draining popliteal lymph node of Ad5IL-12-treated mice (Fig. 3A) were dramatically lower than those of control-treated mice (by ~3 log = 3125-fold). The titers detected in the Ad5IL-12-protected BALB/c mice were similar to those that are recovered in healing B10.D2 animals after infection. Gross morphologic analysis of vector-treated mice revealed splenomegaly in controls vs Ad5IL-12-treated BALB/c mice. Limiting dilution analysis revealed that untreated animals with splenomegaly had a 125-fold increased titer of parasites in the spleen compared with Ad5IL-12-treated mice, in which seven of eight animals were parasite free (Fig. 3B). These results indicate that vector-derived IL-12 expression had a significant negative impact on the growth and spread of L. major parasites during the course of infection in susceptible BALB/c mice.

The expression of IL-12 and IL-4 cytokines after i.m. injection of the recombinant adenovirus vector is transient and localized

The cytokine expression patterns of IL-12 in BALB/c and IL-4 in B10.D2 mice were determined following vector administration. For time course studies, expression was followed over a 1-wk period in sera or tissue extracts prepared from recombinant adenovirus-treated animals (Fig. 4). At the 1-day point, IL-12 expression

![Graph A](image1)

**FIGURE 1.** An adenovirus-vector-expressing IL-12 (Ad5IL-12) protects BALB/c mice from leishmaniasis. Data points are expressed as the mean ± SE measurements. (A) BALB/c mice were infected in the left footpad with 2 × 10⁶ stationary-phase L. major promastigotes and treated simultaneously with 5 × 10⁶ pfu of recombinant adenovirus given i.m. Lesion development was monitored at weekly intervals by measurement of footpad thickness with a metric caliper. Ad5IL-12, n = 18 (triangles); Ad5IL-4, n = 10 (circles); DL70–3 control vector, n = 10 (diamonds); and L. major alone n = 18 (squares); pooled from three separate experiments. For 3–6 wk time points, p < 0.01 for Ad5IL-12 values compared with DL70–3 control virus by Student’s t test. (B) The time course for the effect of Ad5IL-12-induced protection in BALB/c mice. BALB/c mice were injected i.m. with 5 × 10⁶ pfu of Ad5IL-12 on the same day or at different time points before infection. Lesion development was then monitored as described. The efficacy of the Ad5IL-12 vector in preventing leishmaniasis was diminished by day 3. For each data point, n = 3. Ad5IL-12 vector given simultaneously (squares), 3 days before (diamonds), 1 wk before (circles), 2 wk before (triangles), and 4 wk before (inverted triangle) L. major infection.
reached 24.9 ± 3.6 ng levels within the muscle tissue in BALB/c mice receiving a 5 × 10^8 pfu dose of the Ad5IL-12 vector (Fig. 4A). IL-12 muscle expression peaked at day 1 followed by a dramatic decline on day 3. This diminished IL-12 expression at the day-3 time point correlates well with the timeframe when reduced protection against parasite infection occurs (Fig. 1B).

Serum levels of IL-12 detected in Ad5IL-12 vector-treated animals were significantly lower and of shorter duration than those achieved at the localized site in muscle; IL-12 serum levels reached 3 ng/ml concentrations by day 1 but were under the limits of detection by day 2. Control animals did not produce detectable levels of this cytokine. Thus, IL-12 expression by the Ad5IL-12 adenosine vector was more localized to the injection site and did not readily disseminate to the peripheral circulation.

The peak level of IL-4 expression in B10.D2 mice, given an i.m. injection of the Ad5IL-4 vector, also occurred at day 1, reaching 2.9 ± 1.1 ng per injected muscle tissue and returning to baseline levels by day 3 (Fig. 4B). As seen for cytokine expression after Ad5IL-12 treatment, the serum levels of IL-4 in B10.D2 mice were significantly lower and of shorter duration than levels achieved at the injected site.

The efficacy of the Ad5IL-12 vector in the prevention of leishmaniasis in BALB/c mice correlates with increasing numbers of IFN-γ-producing cells

Because dramatic differences in disease outcomes were evident after i.m. injection with adenovirus vectors in BALB/c and B10.D2 mice, an ELISA-spot cellular assay was used to study the frequency of Ag-specific IFN-γ and IL-4-secreting lymphocytes recovered from draining lymph nodes postinfection. As shown in Table I, Ad5IL-12 vector treatment had a major impact on the development of Ag-responsive lymphocytes. In both BALB/c and B10.D2 mice, vector-derived IL-12 expression caused a major shift in the background response to L. major and LACK peptide 160–173 toward a Th1 immune profile. The IFN-γ:IL-4 ratio in BALB/c mice shifted from 0.18 to 4.42 in response to fixed L. major parasites and from 0.47 to 16.70 for LACK peptide 160–173. Likewise, the Th1 response in B10.D2 mice was also enhanced after IL-12 vector treatment, IFN-γ:IL-4 ratios to fixed parasites increased from 1.71 to 16.33 and against LACK peptide 160–173 from 0.25 to 9.65. LACK has been reported to be the target Ag in the induction of Th2 immune responses in susceptible BALB/c mice (16). The Th1-induced deviation of response to LACK peptide 160–173 may account for the changes seen in the pathogenicity of the disease.

Interestingly, whereas in BALB/c mice IL-12 expression dramatically altered the cellular cytokine profile, IL-4 vector-derived expression appeared to have little or no impact on the cytokine response to L. major. In B10.D2 mice, IL-4 did not affect the ratio of IFN-γ:IL-4 production significantly from that of the DL70-3 control. However, IL-4 expression had a profound influence over disease progression, with a major change in the disease course occurring at 3 wk (Fig. 2A).

FIGURE 2. An adenovirus-expressing IL-4 (Ad5IL-4) exacerbates L. major infection in B10.D2 mice. (A) B10.D2 mice were infected in the left footpad with 2 × 10^5 stationary-phase L. major promastigotes and treated simultaneously with 5 × 10^8 pfu of recombinant adenovirus given i.m.; lesion development was monitored as described (n = 11 for each data point). Ad5IL-4 (circles); DL70-3 control vector (diamonds) and L. major alone (squares). For 4–6 wk time points, P < 0.01 for Ad5IL-4 values compared with DL70-3 control virus by Student’s t test. (B) Comparative morphology of footpad lesions at 6 wk in BALB/c mice treated with Ad5IL-12 and B10.D2 treated with DL70-3 control vector (straight tails, mice numbers 1 and 2) and BALB/c mice treated with DL70-3 control vector and B10.D2 treated with Ad5IL-4 (curled tails, mice numbers 3 and 4). (C) Ad5IL-4 vector administration in B10.D2 mice exacerbates L. major infestation of the draining lymph nodes. L. major parasitic burden in the draining popliteal lymph nodes of recombinant adenovirus vector-treated B10.D2 mice 6 wk after treatment; mean of each treatment group (bars). Limiting dilution analysis was performed on cells isolated from the draining lymph nodes and cultured in M199 for 1 wk at 28°C in serial fivefold dilutions. Wells were assessed microscopically for L. major growth and were scored as positive when three of eight wells contained parasite (11). Treatment groups included: L. major only, L. major + DL70-3 and L. major + Ad5IL-4, P < 0.02 for Ad5IL-4 values compared with DL70-3 control virus by Student’s t test.
Cytokine stimulation of B cell lymphopoiesis has been demonstrated to aggravate leishmaniasis (17). We are currently investigating whether similar effects are involved for IL-4 expression.

Intramuscular injection of the Ad5IL-12 vector results in increased NK cell activation in the popliteal lymph node

It had been previously demonstrated that rIL-12 protein administration either alone or in conjunction with soluble Leishmania Ag could induce a NK cell response that has been shown to be involved in the protection of BALB/c mice against L. major infection (4). To determine whether i.m. injection with the Ad5IL-12 vector could similarly influence the NK response, we measured NK cell cytotoxic activity recovered in popliteal lymph node cells using 51 Cr-labeled YAC-1 cells as targets from mice that were either naive, Ad5IL-12 vector-treated without parasitic infection, L. major-infected also treated with IL-12 vector control vector (DL70-3), or no adenovirus treatment (Fig. 5). NK activity in Ad5IL-12-treated mice was ~30-fold higher than in untreated mice.
were also injected i.m. with a 5 × 10⁶ L. major parasites alone or were also injected i.m. with a 5 × 10⁶ dose of Ad5IL-12 vector or DL70-3 control vector (an E1 recombinant adenovirus vector without an insert). Noninfected mice, injected with Ad5IL-12 only, and naive uninfected mice were also included as controls. Draining lymph node cells were collected after 1 wk and used as effectors at different ratios against 31Cr-labeled YAC-1 target cells. Ad5IL-12 only (circles), L. major + Ad5IL-12 (triangles), L. major + DL70-3 control virus (diamonds), L. major alone (squares), and uninfected (inverted triangles). Data points are expressed as the mean ± SE measurements of three separate experiments. p < 0.05 for L. major-infected + Ad5IL-12 vector vs L.major-infected + DL70-3 vector at 50:1 and 100:1 E:T ratios by Student’s t test.

FIGURE 5. Ad5IL-12 treatment increases NK cell activity detected in the draining popliteal node of L. major-infected BALB/c mice. BALB/c mice were infected in the footpad with 2 × 10⁶ L. major parasites alone or were also injected i.m. with a 5 × 10⁶ dose of Ad5IL-12 vector or DL70-3 control vector (an E1 recombinant adenovirus vector without an insert). Noninfected mice, injected with Ad5IL-12 only, and naive uninfected mice were also included as controls. Draining lymph node cells were collected after 1 wk and used as effectors at different ratios against ³¹Cr-labeled YAC-1 target cells. Ad5IL-12 only (circles), L. major + Ad5IL-12 (triangles), L. major + DL70-3 control virus (diamonds), L. major alone (squares), and uninfected (inverted triangles). Data points are expressed as the mean ± SE measurements of three separate experiments. p < 0.05 for L. major-infected + Ad5IL-12 vector vs L.major-infected + DL70-3 vector at 50:1 and 100:1 E:T ratios by Student’s t test.

FIGURE 6. Ad5IL-12 i.m. injection prevents the expansion of B220⁺ cells in the draining popliteal node of L. major-infected BALB/c mice. BALB/c mice were infected in the footpad with 2 × 10⁶ L. major parasites and injected i.m. with a 5 × 10⁶ dose of recombinant adenovirus vector. At 6 wk postinfection, an increased number of cells staining for B220 were detected in the draining lymph node in L. major-infected mice compared with Ad5IL-12 vector-treated animals. FACS analysis revealed that 15% of the lymphocytes isolated from mice after Ad5IL-12 treatment stained for CD3. No DX5-positive cells were detected in the analyzed population.

Discussion

Murine leishmaniasis is a well-studied model for evaluating the role of Th1-Th2 choice on disease outcome. The approach of using an adenovirus vector to deliver IL-12 made it possible to prevent disease induction in BALB/c mice after only a single treatment. We also show for the first time that recombinant adenovirus delivery of IL-4 can induce susceptibility to disease in the normally resistant B10.D2 mouse strain.

Long-lasting immunity was conferred on susceptible BALB/c mice treated here with the Ad5IL-12 vector, as was previously demonstrated using DNA vaccination encoding the LACK gene (19). In the vaccination with LACK DNA, mice were reported disease-free for up to 20 wk. In our analysis, Ad5IL-12 vector-treated mice have remained healthy with no evident disease for >1 year. In addition, protected mice have an established immunity to subsequent infection. This efficient generation of a protective immune response by the Ad5IL-12 vector administered at the same time of L. major infection makes it an attractive candidate agent for use as an adjuvant in future vaccines.

Our data suggest that the adenovirus vector-derived IL-12 expression mediates protection at both the innate and adaptive levels of immunity. The early containment of parasites at draining lymph nodes is consistent with the activities previously reported for Ad5IL-12-treated mice. B220 expression in L. major-infected BALB/c mice at 6 wk is expanded approximately twofold compared with Ad5IL-12-treated mice

Although no apparent change in lymph node cellular composition occurred by 1 wk following infection with several phenotype markers studied (data not shown), by 6 wk a clear difference was present. An increase in the proportion of B220 markers studied (data not shown), by 6 wk a clear difference was occurred by 1 wk following infection with several phenotype treated mice L. major

L. major

Recall In Vitro

Table I. The Ad5IL-12 vector increases the IFN-γ/IL-4 ratio in treated animals

<table>
<thead>
<tr>
<th>In Vivo Treatment</th>
<th>Fixed L. major</th>
<th>LACK peptide</th>
<th>Fixed L. major</th>
<th>LACK peptide</th>
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<td>L. major</td>
<td>0.18 (181/984)</td>
<td>0.47 (114/241)</td>
<td>1.71 (251/147)</td>
<td>0.25 (168/654)</td>
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<td>L. major + DL70-3</td>
<td>0.33 (130/384)</td>
<td>0.78 (249/319)</td>
<td>0.86 (851/987)</td>
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<td>L. major + Ad5IL-4</td>
<td>0.50 (322/642)</td>
<td>1.32 (511/385)</td>
<td>1.11 (841/761)</td>
<td>3.25 (530/163)</td>
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<td>4.42 (866/196)</td>
<td>16.70 (167/10)</td>
<td>16.33 (82/254)</td>
<td>9.65 (386/40)</td>
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</tbody>
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α Ratio of IFN-γ/IL-4 spots/10⁶ lymphocytes isolated from the draining popliteal lymph nodes of mice at 1 wk following infection with L. major and i.m. injection with a 5 × 10⁶ pfu dose of recombinant adenovirus vector. Representative data from one of three experiments are shown for BALB/c and B10.D2.

β In parentheses are the absolute values of IFN-γ and IL-4 spots/10⁶ lymphocytes.

γ p < 0.05 by Student’s t test for L. major + Ad5IL-12 vs control treatments recalled in vitro with fixed L. major.

GUIDING IMMUNITY AGAINST LEISHMANIA WITH ADENOVIRUS VECTORS
in the muscle. The fact that simultaneous i.m. administration of IL-12 was enough to prevent disease. Of importance and relevance to future studies using adenovirus vectors to mediate cytokine delivery, differences in the IL-12 expression pattern between this study and our previous one using Ad5IL-12 as an intratumor treatment should be discussed. Intramuscular injection with the Ad5IL-12 vector produced lower levels of cytokine of shorter duration than those previously seen after intratumor injection (34). This may reflect a trivial difference in the detection Ab used (a p40 subunit-specific Ab was used in the previous study), but may be accounted for by differences in cell-specific susceptibility to recombinant adenovirus vector infection that may exist within tissues. One further possibility is that vector-derived cytokine expression was influenced by the tissue microenvironment. IL-10 is known to diminish antigenic display and tumor-mediated immune responses and could account for the higher levels of IL-12 expression detected after intratumor administration. Immune responses generated against adenovirus vectors have already been shown to influence transgene expression (35). The DL70-3 control vector treatment in both BALB/c and B10D2 mice induced some background immune responses. Small protective effects of the control DL70-3 vector were previously seen after direct tumor injection (34).

In conclusion, an increasing number of gene transfer vectors are currently being developed to deliver transgenes including cytokines as therapeutic agents. Of these, the development of short-term, focused special delivery systems in the form of adenovirus vectors holds much promise (36–38). Recombinant adenoviruses have already been used successfully as vaccines in a number of infectious disease models (38–41) and recently in cancer gene therapy (8, 34, 42). In our initial report (7), the first describing adenovirus vectors expressing cytokines, we proposed that these vectors would be ideally suited for delivering cytokines as a transient and localized type of adjuvant therapy, particularly those using cytokine genes that may induce systemic immunotoxities. With respect to leishmaniasis, a previous report demonstrated BALB/c mice infected with L. major were successfully treated with IL-12 and the antimonial drug, Pentostam (43). This also suggests that the adenovirus-mediated deliver of IL-12 might be a useful cotherapy. Adjuvant therapy, delivered by cytokine vectors,
can provide an explosive level of agent, which in the case of a localized tumor, for example, in breast or prostate tissue, could establish a beneficial and rapid therapeutic milieu. These vectors can transduce both quiescent and replicating cells and results in the carriage of the adenovirus genome as episomal DNA, which makes this vector more suitable for delivering effector cytokines owing to its lack of integration. Finally, adenovirus vectors (inherent to DNA viruses) have properties selected by evolution for the delivery of transgenic material to nuclear compartments. In fact, adenovirus vector therapy is essentially a more efficient DNA vaccine. With the recent reported success of DNA vaccine encoding the LACK transgene (19), a direct comparison between DNA vaccines and recombinant adenovirus vectors to evaluate the efficacy of each approach would be of interest. Indeed, it may be advantageous to use naked DNA as a recall for recombinant adenovirus vector-induced responses, to avoid subsequent immunity to the vector. Regardless, our results support the development of an adenovirus vector vaccine incorporating cytokine and Ag that should be a highly efficient alternative to conventional vaccination in the prevention of leishmaniasis; this work is in progress.

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