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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

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Experimental infection of the susceptible BALB/c (H-2d) 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-2d) 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.
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 a 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 a Ad5 variant deleted in the E1 region (10). For treatment, mice were injected with 5 × 10^6 plaque-forming units (pfu) of recombinant adenovirus in 50 μl PBS into the femoral bicep muscle of the left hind leg 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/RJ-/73) grown at 28°C in M199 medium supplemented with 10% FBS, 100 U/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 × 10^7 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, 10 mM EDTA, 10 μg/ml aprotinin, and 10 μg/ml streptomycin. The homogenate was freeze-thawed three times and clarified by centrifugation. 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 F96 (Nunc, Roskilde, Denmark) were coated with anti-IL-12 (p55/p70; clone Red-T/O297-289; PharMingen, San Diego, CA) or anti-IL-4 Abs (purchased from BioLegend, San Diego, CA). Plates were labeled for 2 h with 500 Ci of sodium chromate in 0.5 ml of 3-ME. Samples were recorded in triplicate at each E:T ratio using a gamma-counter (Tri-Carb, Packard, Downers Grove, IL). Data are expressed as the mean ± SEM for each group. Statistical analysis was performed using Student’s t test, ANOVA, or the Mann-Whitney test. A p value of less than 0.05 was considered statistically significant.

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 × 10^6 L. major promastigotes into the left hind footpad and injected i.m. with a 5 × 10^6 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 × 10^6 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).

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’s t test was used for the final determination of significance testing the effects of Ad5IL-12 and Ad5IL-4 treatments.
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 i f 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 postinfec tion (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 titters 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
Treatment groups included: B10.D2 mice 6 wk after treatment; mean of each treatment group (bars). 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).

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 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.

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 adenovirus 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.

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 x 10^5 stationary-phase L. major promastigotes and treated simultaneously with 5 x 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 naive mice.

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 51Cr-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).
were also injected i.m. with a 5 × 10⁸ dose of Ad5IL-12 vector alone or were also injected i.m. with a 5 × 10⁴ 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 naïve uninfected mice were also included as controls. Draining lymph node cells were collected after 1 wk and used as effectors at different ratios against 51 Cr-labeled L. major –infected controls. Representative data from one of three experiments are shown for BALB/c and B10.D2.

*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 × 10⁸ pfu dose of recombinant adenovirus vector. Representative data from one of three experiments are shown for BALB/c and B10.D2.

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

**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>BALB/c Fixed L. major</th>
<th>LACK peptide</th>
<th>B10.D2 Fixed L. major</th>
<th>LACK peptide</th>
</tr>
</thead>
<tbody>
<tr>
<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>
</tr>
<tr>
<td>L. major + DL70-3</td>
<td>0.33 (130/384)</td>
<td>0.78 (249/319)</td>
<td>0.86 (851/987)</td>
<td>3.00 (631/210)</td>
</tr>
<tr>
<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>
</tr>
<tr>
<td>L. major + Ad5IL-12</td>
<td>4.42 (866/196)</td>
<td>16.70 (167/10)</td>
<td>16.33 (828/254)</td>
<td>9.65 (386/40)</td>
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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 × 10⁴ 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 naïve uninfected mice were also included as controls. Draining lymph node cells were collected after 1 wk and used as effectors at different ratios against ⁵¹Cr-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.

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⁺ cells (a pan B cell marker) was evident in L. major–infected compared with Ad5IL-12-treated animals (Fig. 6). In Ad5IL-12–treated mice, 15% of the lymph node cells stain for B220 (normal phenotype) compared with 28.5% in the L. major–infected BALB/c. T cells were the predominant population of cells isolated from the popliteal nodes of Ad5IL-12–treated BALB/c mice, accounting for 56% of the total staining population. In comparison, a smaller proportion, 45% of the cells isolated from L. major–infected animals, stained for CD3. No DX5-positive cells were detected in the analyzed population.

**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 × 10⁴ 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 the B220 marker vs 28.5% in L. major–infected controls. Representative data from one of three experiments is shown. p < 0.02 was found for Ad5IL-12 vs control-treated mice.

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 and at the local site of injection is a feature of resistant strains, while in BALB/c mice L. major parasites can be recovered in the spleen, lungs, and bone marrow 24 h after infection (20). It was found that the containment of infection depended on the development of a Th1 population in disease-susceptible BALB/c mice by rIL-12 administration. This cytokine causes T cells and NK cells to proliferate (21) and secrete IFN-γ (22), which helps to create the necessary milieu for Th1 skewing. In our hands, Ad5IL-12 treatment similarly enhanced NK activity in addition to deviating the normal BALB/c Th2 pattern of Ag-specific lymphocyte response toward Th1.

In previous reports describing the prevention of leishmaniasis in BALB/c mice, several administrations of rIL-12 were required before an effect was evident (6). IL-12 protection was possible only in a narrow window of time (between 16 and 48 h after infection), after which Th2 cells are already committed to a type 2 response and treatment is ineffective (16, 23). In our protocol, a single i.m. injection gave rise in 24 h to a local IL-12 level of cytokine that was enough to prevent disease. Of importance and relevance to potential therapy in humans, the adenovirus vector-induced IL-12 expression was found to be highly localized to the site of injection in the muscle. The fact that simultaneous i.m. administration of Ad5IL-12 vector contralateral to the L. major-infected site did not protect against disease indicates that colocalized expression of the cytokine with the Ag is an important parameter in the use of this vector to elicit protection against infection. This effect of targeted and localized transgene expression by recombinant adenovirus vectors is a highly desirable feature for expression of cytokines with potential toxicity. In other studies, systemic administration of rIL-12 had been found to be associated with the induction of anemia, leukopenia, thrombocytopenia, and splenomegaly among other symptoms (24–26). Recently, a phase 1 trial in cancer using rIL-12 had to be temporarily suspended owing to apparent toxicity (27). Unlike these earlier studies, the adenovirus vector-derived IL-12 expression induced no apparent toxicity in our experiments. In fact, the splenomegaly that occurs as a consequence of parasitic spread to peripheral organs during leishmaniasis in BALB/c mice was prevented by Ad5IL-12 treatment.

The role of IL-4 in inducing susceptibility to leishmaniasis has been a topic of much discussion, and its effect is still unclear. IL-4 has been shown to enhance Th2 immune responses, which are generally considered nonprotective against L. major parasites. However, a recent publication demonstrated that C57BL/6 mice (a L. major-resistant strain) had increased levels of IL-4 mRNA in the first week of infection but still controlled disease development (28). Systemic administration of IL-4 had no effect on disease induction in these mice (14). In another resistant strain (C3H/HeN), i.p. injection of rIL-4 was insufficient to generate disease susceptibility, although the normal Th1 response was curtailed (15).

However, a role for IL-4 in the induction of leishmaniasis has been indicated. Intranasal delivery of rIL-4 in BALB/c mice has been shown to exacerbate disease when administered at an early stage (29). Further, administration of anti-IL-4 Abs was shown to prevent lesion development in BALB/c mice. However, previous attempts to render resistant strains, such as B10.D2, susceptible to infection by IL-4 administration have been unsuccessful. Interestingly, administration of the Ad5IL-4 vector simultaneously to infection increased disease susceptibility in B10.D2 mice. This is the first report in which IL-4 overexpression has been described to influence leishmaniasis in resistant strains in such a dramatic fashion. It is possible that in previous studies rIL-4 administration failed to influence leishmaniasis in resistant mouse strains due to intrinsic problems with delivering and sustaining recombinant cytokine levels at the local tissue site. Further, it is possible that the temporal aspects of IL-4 administration may be important. It has recently been described that an early burst of IL-4 expression within 16 h of L. major infection can render BALB/c mice unresponsive to IL-12 (23). This effect may reflect IL-4-mediated down-regulation or modification of the IL-12R β2-chain (30).

Phenotypic cellular analysis at 1 wk following treatment with Ad5IL-12 revealed no dramatic differences in TCR expansion or memory markers vs control BALB/c mice; however, an increase in B220+ cells was present at 6 wk in the L. major-infected control mice. This difference in B220 expression may reflect changes in the number of B cells. B220 has been described to be expressed on NK cells; however, we did not detect DX5-positive cells in the analyzed population. Previous work has indicated a deleterious effect associated with the presence of B cells in murine leishmaniasis, and a pronounced effect for B cells in efficiently activating IL-4 production by T cells in BALB/c mice (31). An important role for B cells in the pathogenesis of disease is in accord with the previous observation that depletion of B cells in BALB/c mice reduced lesion size (32). This effect was confirmed in a scid mouse model where cotransfer of B cells made resistant mice susceptible to L. major infection (33). It is possible that some of the protective effects attributed to Ad5IL-12 vector therapy involve its influence on B cell development during parasitic infection. One of the more intriguing possibilities is that both the IL-4 and IL-12 effects are due in part to either direct or indirect influence on B cell compartments and their activation.

Of 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 vaccination 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.

Acknowledgments

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