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The Prevention and Treatment of Murine Colitis Using Gene Therapy with Adenoviral Vectors Encoding IL-10

James O. Lindsay, Cathleen J. Ciesielski, Tom Scheinin, Humphrey J. Hodgson, and Fionula M. Brennan

IL-10-deficient (IL-10<sup>−/−</sup>) mice develop colitis with many similarities to Crohn’s disease. Daily IL-10 injections have a short systemic half-life and are unable to induce complete remission in IL-10<sup>−/−</sup> mice with established disease. In this paper, we investigate the duration, potency, and immunogenicity of gene therapy using an adenoviral vector encoding murine IL-10 (AdvmuIL-10). A single systemic injection of AdvmuIL-10 was sufficient not only to prevent the onset of colitis for at least 10 wk but also to induce clinical and histological remission in mice with established disease. In addition, AdvmuIL-10 diminished the systemic manifestations of disease, including elevated acute-phase proteins, as well as the local consequences of inflammation such as raised stool IL-1β concentrations. Both IL-10 protein and the effects of secreted IL-10 were detectable for 10 wk after AdvmuIL-10 injection. Furthermore, the immunoregulatory effect of a single AdvmuIL-10 injection was manifest both by a reduction in TNF-α, IFN-γ, and RANTES release from stimulated splenocyte cultures, and also by a change in the proportion of CD45RB<sup>high</sup>/low lymphocytes in the spleen compared with control mice. The delivery of AdvmuIL-10 resulted in a significantly diminished host antiadenoviral response compared with control adenoviral vectors. Thus, gene therapy strategies using adenoviral vectors encoding immunoregulatory and antiinflammatory cytokines may prove to be a potent approach for the treatment of chronic inflammatory disease. Antiinflammatory cytokine expression protects against immune responses directed at gene vectors. The Journal of Immunology, 2001, 166: 7625–7633.

The chronic gastrointestinal inflammation seen in both Crohn’s disease and a variety of experimental models of colitis is associated with a loss of tolerance to resident enteric bacteria (1), T cell activation (2), and the production of chemokines, proinflammatory monokines, and Th1 cytokines (3). IL-10 plays a crucial role in mucosal immunoregulation, inhibiting aspects of both the innate and cell mediated inflammatory response (4). Gene-targeted IL-10 knockout mice (IL-10<sup>−/−</sup>) spontaneously develop an enterocolitis by 2–3 mo of age that has many similarities to human Crohn’s disease, with multifocal inflammatory lesions throughout the gastrointestinal tract (5). Affected animals also develop several of the systemic manifestations of Crohn’s disease such as anemia, leukocytosis, weight loss, and a marked increase in serum acute-phase proteins such as serum amyloid protein (SAP) (6).

Transfer of specific colonic T cell subsets from the IL-10<sup>−/−</sup> mice into RAG-2<sup>−/−</sup> (lymphocyte-depleted) mice reveal that the colitis is mediated by a subset of CD4<sup>+</sup> T cells that are predominantly of the Th1 phenotype (7). Thus, inflamed colonic tissue secretes increased levels of TNF-α and IFN-γ, but no IL-4 (6). In addition, disease can be abrogated by the administration of anti-IFN-γ (6), anti-IL-12 (8), or anti-TNF-α Abs (T. Scheinin, unpublished observations). Luminal bacteria are essential for disease as IL-10<sup>−/−</sup> mice reared in germ-free conditions remain disease free, while inoculation with specific commensal bacterial strains induces colitis via an Ag-driven Th1 response (9, 10).

Mice given a systemic injection of LPS, a major constituent of luminal Ag, develop endotoxic shock associated with elevated levels of proinflammatory cytokines (11). LPS injection leads to a significantly higher mortality in IL-10<sup>−/−</sup> mice than wild-type controls (12); this effect being mediated by a more profound elevation of serum levels of TNF-α, IL-1, IFN-γ, and IL-12. IL-10 plays a crucial role in regulating the response to LPS, as the excess mortality induced by LPS in IL-10<sup>−/−</sup> mice can be reversed by prior or concurrent administration of recombinant murine IL-10. Likewise, the prior administration of anti-IL-10 Abs to wild-type mice increases the mortality observed with previously sublethal doses of LPS (12).

Further insights into the role of IL-10 in the control of inflammatory mechanisms in colitis come from study of C.B-17 SCID mice repopulated with wild-type CD4<sup>+</sup>CD45RB<sup>high</sup>T cells. These mice develop a colitis that is also dependent on luminal bacteria and driven by a Th1 response (13). Cotransfer of the reciprocal CD45RB<sup>low</sup>CD4<sup>+</sup>T cells with the normally pathogenic CD45RB<sup>high</sup> cells prevents the onset of colitis. IL-10 is essential for the differentiation of this regulatory function, as CD45RB<sup>low</sup>CD4<sup>+</sup> T cells from IL-10<sup>−/−</sup> mice are unable to prevent the colitis induced by wild-type CD45RB<sup>high</sup> cells (14). Furthermore, treatment with an anti-murine IL-10R mAb abrogates the inhibition of colitis mediated by wild-type CD45RB<sup>high</sup> CD4<sup>+</sup> T cells.

The administration of exogenous IL-10 has efficacy in treating experimental colitis in the CD4<sup>+</sup>CD45RB<sup>high</sup> transfer (15) and dextran sodium sulfate models (16). Furthermore, in patients with
Crohn’s disease, daily injection of IL-10 generates a modest clinical response (17, 18), although with lower efficacy than alternative biological therapies such as anti-TNF-α Abs (19). Likewise, while daily injection of weanling IL-10−/− mice with murine IL-10 prevents disease onset, similar treatment once colitis is established is only partially able to suppress disease (6). The dichotomy in therapeutic efficacy in the IL-10−/− mouse may reflect the pharmacodynamics of systemic IL-10 administration, delivery of inadequate IL-10 to inhibit mucosal Th1 responses and proinflammatory cytokine release, or the fact that early exposure to IL-10 is required for the differentiation of immunoregulatory cells.

Alternative strategies for IL-10 therapy that deliver constant high levels of the protein to the gastrointestinal tract may be therapeutic for established disease in the IL-10−/− mouse. Gene therapy using adenoviral vectors encoding IL-10 has been shown to provide high-level protein expression and have therapeutic efficacy in animal models of rheumatoid arthritis (20, 21). The systemic administration of adenoviral vectors encoding β galactosidase (Advβgal) to healthy mice leads to protein expression mainly within the liver and spleen, but also in the colon (22). We hypothesized that i.v. administration of replication-deficient adenoviral vectors encoding murine IL-10 (AdvmuIL-10) would prove efficacious in both the prevention and treatment of colitis in IL-10−/− mice. In this paper, we demonstrate that this approach prevented the spontaneous development of colitis and also reversed the clinical and histological features of established disease. The systemic manifestations of disease were diminished, and the enhanced response to LPS characteristic of IL-10−/− animals was reversed.

Materials and Methods

Reagents

General reagents were of research grade and purchased from Sigma (St. Louis, MO). All reagents used for cell culture were determined to be LPS free using a Limulus amebocyte lysate assay (BioWhittaker, Wokingham, Berkshire, U.K.) as directed by the manufacturer (sensitivity, <10 pg/ml) (23).

Animals

IL-10−/− mice on a C57BL/6 background (purchased from Harlan, Oxon, U.K.) were back-crossed for one generation onto DBA/1 mice to increase breeding vigor and disease expression. Progeny from the interbreeding of DBA1. Briefly, tail samples were digested in 250 μl PCR lysis buffer (120 μg/ml proteinase K, 50 mM KCl, 10 mM Tris·HCl pH 8.8, 0.1 mg/ml gelatin, 714 μM MgCl2, 0.45% Nonidet P-40, 0.45% Tween) at 50°C overnight. The DNA encoding the IL-10 and neomycin regions were amplified by PCR using the following synthetic oligonucleotide murine primer pairs: 5′-TAGGGCAATGTTCCTCC-3′ (IL-10 sense) and 5′-CAGGCAGCAGTACAGTG-3′ (IL-10 antisense); IL-10 sense and 5′-CCTGCGGTCAATCCATATTG-3′ (neomycin). After 35 cycles, the products were separated on 1% agarose gels and visualized by ethidium bromide staining. Bands were observed at 1.5 Kb (neomycin) and 1 Kb (IL-10) under UV light. Mice were maintained in specific pathogen-free (SPF) conditions in a laminar flow hood at all times with free access to food and water. All experiments involving animals were approved by the local ethical review process committee and performed under license from the home office.

Materials and Methods

Experimentation

Animals were examined weekly and given a clinical score that consisted of one point for each of the above signs. Previous work has demonstrated close correlation between the clinical score and the histological severity of colitis (correlation coefficient 0.865; T. Schein, unpublished observation).

Adenoviral vectors

The recombinant El-deleted type 5 adenoviral vectors, encoding murine IL-10 under the transcriptional control of the rous sarcoma virus promoter (AdvmuIL-10), β-galactosidase under the CMV promoter (Advβgal), or empty cassette adenovirus (Adv0), were generously donated by A. P. Byrnes, M. Wood, and H. Charlton (University of Oxford, Oxford, U.K.). Viruses were propagated in the 293 human embryonic kidney cell line (Quantum Biotechnology, Montreal, Canada) and purified by ultracentrifugation through two cesium chloride gradients (Boehringer Mannheim, Lewes, Sussex, U.K.). The titer of adenoviral vectors was determined by plaque assay on 293 cells (24). Viral stocks were aliquoted and stored in 10% glycerol at −80°C until use.

Experimental protocols.

For assessment of the effect of AdvmuIL-10 on the development of colitis, 1 × 106 PFU of viral vector (AdvmuIL-10 or Adv0) in 100 μl PBS or vehicle alone was injected into the tail vein of 4- to 5-week-old IL-10−/− mice under light sedation. To assess the effect of AdvmuIL-10 on established disease, 5 × 106 PFU of viral vector was injected into the tail vein of 10-week-old IL-10−/− mice with clinical signs of colitis. A total of 100 μl PBS was injected into the tail vein of wild-type littermates as a negative control. At weekly intervals, the clinical score of each animal was assessed as previously described, and stool samples were collected. Animals were sacrificed by cervical dislocation at the indicated time points. Serum was collected via cardiac puncture, spleens were harvested, stool samples were collected, and serial segments of colon, cecum, and ileum were fixed in 10% neutral buffered formalin for histological analysis. In addition, 25 age-matched IL-10−/− mice were injected with 1 × 106 PFU AdvmuIL-10 to assess the sites of IL-10 protein expression. Batches of three mice were randomly selected at two weekly intervals and sacrificed. The liver, spleen, and colons of these animals were weighed and homogenized in 5 μl PBS/mg tissue. Supernatants were harvested after centrifugation and stored at −20°C until assay.

Histological analysis.

Samples were routinely processed, sectioned at 5 μm, and stained with hematoxylin and eosin for light microscopic examination. Assessment was performed by an investigator blinded to treatment group. Five segments of colon were examined per mouse and each given a histological score from 0 to 4 as described (6); thus a total score for each mouse from 0 (no change in any segment) to 20 (grade 4 changes in all segments) was obtained. Scores of <5 were deemed to be within normal limits.

Stool samples

Stool samples were collected weekly from all animals and weighed. Samples were emulsified in 500 μl/100 μg stool weight of a solution of 1 mg/ml soy trypsin inhibitor and 1 mg/ml PMSF in PBS. Supernatants were collected after centrifugation at 10,000 × g for 15 min and stored at −20°C.

Spleen cell cultures

After sacrifice, each spleen was placed in RPMI 1640 medium (PAA Laboratories, Yeovil, U.K.) supplemented with 10% FCS, 100 μg/ml penicillin, and 100 μg/ml streptomycin (BioWhittaker). Cell suspensions were obtained by passing tissue through a 200-μm2 nylon mesh. After erythrocyte lysis, cells were washed in HBSS three times before resuspension in medium. Cells were plated at 2 × 105/well in 12-well plates (Falcon; BD Labware, Mountain View, CA) in a final volume of 1 ml of medium with or without 10 μg/ml LPS, 10 ng/ml recombinant murine IL-10 (Schering-Plough, Madison, NJ), 10 μg/ml neutralizing rat anti-murine IL-10 Ab (BD Pharmingen), or 10 ng/ml recombinant mouse IL-10 (Peprotek, CA), or a rat IgGl isotype control (OX20; American Type Culture Collection, Manassas, VA). Cultures were maintained for 24 h before supernatants were harvested and stored at −20°C.

Measurement of serum antienoviral Ab response

The neutralizing antienovirus Ab response was analyzed in serum from untreated or treated IL-10−/− mice 14 days after gene transfer with 5 × 106 PFU of AdvmuIL-10, Advβgal, or Adv0 (25). Serum samples (100 μl) were heat inactivated at 56°C for 30 min and diluted 2-fold in serum-free DMEM. Each dilution was incubated for 90 min at 37°C with 2 × 106 PFU of Advβgal and applied in duplicate to 80% confluent 293 cells on a 96-well plate. After 1 h at 37°C, 50 μl of DMEM containing 10% FCS was added to each well, and cells were cultured for a further 36 h. Cell supernatants were then removed and replaced with 30 μl of 0.25 mM Tris·HCl, pH 7.8. Plates were vortexed, frozen, and then thawed for three cycles to
detach and lyse cells and centrifuged at 1000 rpm for 20 min. Ten microliters of the supernatant from each well was mixed with 90 μl of a β-galactosidase substrate solution containing 1 mg/ml o-nitrophenyl-β-D-galactopyranoside, 1 mM MgCl₂, 45 mM 2-ME, in 0.1 M sodium phosphate buffer, pH 7.5. The enzyme reaction was stopped after 5 min with the addition of 150 μl of 0.1 M Na₂CO₃, and plates were read at 405 nm. The mean OD of serum from four animals for each group was compared at a dilution that resulted in 50% inhibition of the Ab response in Advβgal-treated mice.

**Cytokine analysis**

Cytokine concentrations were measured by sandwich ELISA using paired Abs according to the manufacturers recommendations (IL-10 and TNF-α were purchased from BD PharMingen, San Diego, CA; IFN-γ was purchased from Genzyme Diagnostics, Cambridge, MA; IL-1β and RANTES were purchased from R&D Systems, Abingdon, Oxon, U.K.).

**SAP measurement**

SAP levels were measured in terminal serum samples by ELISA. Briefly, microtiter plates (Maxisorp; Nunc, Roskilde, Denmark) were coated overnight with 4 μg/ml trinitrophenylated keyhole limpet hemocyanin before serial dilutions of sera and standards (murine SAP, Calbiochem-Novabiochem, Nottingham, U.K.) were added. Bound SAP was detected with rabbit anti-mouse SAP (Calbiochem-Novabiochem) followed by peroxidase-labeled donkey anti-rabbit Ig (Amersham Life Sciences, Little Chalfont, U.K.). Samples were developed using the peroxidase substrate system (Kirkgaard and Perry Laboratories, Gaithersburg, MD) and read at 450 nm on a plate reader (Labsystems, Chicago, IL).

**Statistical analysis**

Data that exhibited a normal distribution were analyzed using a two-tailed t test; for comparison of more than two means, a two-way ANOVA was performed. Data that did not exhibit a normal distribution was displayed as the median value.

**Results**

**AdvmuIL-10 administration at 4 wk prevents colitis**

IL-10⁻/⁻ mice spontaneously develop an enteroocolitis with associated systemic features such as weight loss and an elevated acute-phase response (6). Daily i.p. injections of rIL-10 have been shown to prevent disease onset if commenced from 4 wk of age. To investigate the therapeutic efficacy and expression duration of adenoviral vectors encoding IL-10, 4-wk-old IL-10⁻/⁻ mice received a single systemic injection of 1 × 10⁶ PFU AdvmuIL-10, Adv0, or PBS vehicle. Mice that had received PBS or Adv0 developed a progressive colitis associated with failure to thrive, whereas AdvmuIL-10-injected mice did not exhibit clinical signs of colitis and gained weight throughout the 10-wk experiment (Fig. 1; p ≤ 0.001). After sacrifice, histological sections of five regions of each colon were scored for disease activity. The mean histological score of IL-10⁻/⁻ mice treated with AdvmuIL-10 was 2.5 ± 0.8, which was significantly lower than the mean score of mice injected with either Adv0 (8.3 ± 2.3; p ≤ 0.025) or saline (12.1 ± 1.8; p ≤ 0.001).

Analysis of weekly stool samples demonstrated that the colitis observed in Adv0- and PBS-treated mice was associated with the release of significantly higher levels of the proinflammatory cytokine IL-1β than AdvmuIL-10-treated mice (Fig. 1; p ≤ 0.001). On sacrifice, 10 wk after therapy, IL-10⁻/⁻ mice treated with either saline or Adv0 had significantly elevated SAP levels (1742 ± 439 and 1636 ± 369 μg/ml, respectively) compared with AdvmuIL-10-treated mice (280.8 ± 58.65 μg/ml; p ≤ 0.004 cf saline; p ≤ 0.002 cf Adv0; Fig. 2). There were no significant differences in SAP concentration between AdvmuIL-10-treated IL-10⁻/⁻ mice and littermate C57BL/6 × DBA1 mice (280 ± 58.7 and 167.3 ± 51.7 μg/ml, respectively; data not shown).

**AdvmuIL-10 injection at 10 wk ameliorates established colitis**

Daily i/p IL-10 injection to 10-wk-old IL-10⁻/⁻ mice will only partially abrogate established colitis (6). In contrast, a single systemic injection of 5 × 10⁶ PFU AdvmuIL-10 to 10-wk-old IL-10⁻/⁻ mice with overt disease returned clinical scores to that of control mice, while 5 × 10⁶ PFU of Adv0 has no effect on disease.
progression (Fig. 3). Thus the clinical score in AdvmuIL-10-treated mice fell rapidly to baseline over the 4-wk experiment, whereas in Adv0-treated mice it rose from 1.5 ± 0.57 to 2.75 ± 0.5 (p ≤ 0.001). This was reflected in the elevated stool IL-1β concentrations measured in IL-10−/− mice treated with Adv0 compared with both AdvmuIL-10-treated IL-10−/− mice (p ≤ 0.02) and saline-treated C57BL/6 × DBA1 wild-type controls (p ≤ 0.01) (Fig. 3).

Histological analysis of colonic tissue from 10-wk-old IL-10−/− mice demonstrated a marked colitis with a score of 9.4 ± 1.3 (n = 5). The histological score of 14-wk-old IL-10−/− mice treated at 10 wk of age with AdvmuIL-10 was 0.75 ± 0.5 (p ≤ 0.001 cf 10-wk-old littermates), which was not significantly different from age-matched wild-type controls (0.25 ± 0.25; p ≤ 0.39; Fig. 3). In contrast, mice treated with Adv0 demonstrated persistent colitis (histological score of 10.25 ± 3.1; p ≤ 0.02 cf AdvmuIL-10 treatment). Likewise, whereas terminal SAP levels were significantly elevated in Adv0-treated IL-10−/− mice compared with controls (1716 ± 639 µg/ml cf 189 ± 45 µg/ml; p ≤ 0.05), there was no significant difference in acute-phase proteins between AdvmuIL-10-treated IL-10−/− mice and wild-type controls (Fig. 3).

**AdvmuIL-10 treatment inhibits TNF-α and IFN-γ release from LPS-stimulated splenocytes**

IL-10−/− mice release elevated levels of TNF-α and IFN-γ in response to challenge with LPS, a major component of luminal Ag (12). To determine whether a single dose of AdvmuIL-10 resulted in persistent suppression of this inflammatory response, we compared the proinflammatory cytokine release from LPS-stimulated spleen cells harvested from mice used in the above experiments. TNF-α and IFN-γ release from unstimulated splenocytes were below the sensitivity of the ELISA for all animals (<40 pg/ml and <120 pg/ml, respectively). LPS (10 µg/ml) induced a significantly greater TNF-α and IFN-γ response in splenocytes harvested from IL-10−/− mice 10 wk after injection with saline or Adv0 than AdvmuIL-10 or wild-type controls (Fig. 4a, p ≤ 0.005 for TNF-α and p ≤ 0.05 for IFN-γ). AdvmuIL-10 treatment also diminished the LPS response in splenocytes from IL-10−/− mice treated at 10 wk of age and sacrificed 4 wk later, although this did not reach statistical significance (data not shown). The addition of IL-10 (10 ng/ml) decreased both TNF-α and IFN-γ release from all animal groups to undetectable levels (data not shown).

**The effects of IL-10 are detectable in AdvmuIL-10-treated mice for the duration of the 10-wk experiment**

The efficacy of AdvmuIL-10 both on the treatment and prevention of colitis and the long-term suppression of LPS-induced responses from cultured splenocytes may be due to the direct immunosuppressive effect of secreted IL-10 throughout the 10 wk of the experiment. Alternatively, early exposure to IL-10 may redress differences in T cell differentiation that have been reported in the IL-10−/− mouse. Levels of IL-10 protein in both the serum and homogenates of liver, colon, and spleen were determined at 2-wk intervals throughout the experiment.

High levels of IL-10 (17 ± 4.8 ng/ml) could be detected in the serum of AdvmuIL-10-injected IL-10−/− mice for up to 7 days after injection with adenovirus. Serum levels of IL-10 after this time and in all other groups were below the threshold of the ELISA. IL-10 protein was detected in homogenates of the liver, colon, and spleen of IL-10−/− mice for ~10 wk after injection with 1 × 10^8 PFU AdvmuIL-10 (Fig. 5). The biological activity of the IL-10 was determined in splenocyte cultures harvested from both IL-10−/− mice 10 wk after viral injection and C57BL/6 × DBA1 littermates.

The addition of anti murine IL-10 Abs (JES5-2A5) to LPS-stimulated splenocyte cultures harvested from 14-wk-old C57BL/6 × DBA1 littermates led to a 9-fold increase in TNF-α release (Fig. 4b). As expected, neutralizing IL-10 had no enhancing effect on TNF-α release by splenocytes from IL-10−/− mice treated with saline or Adv0 (data not shown); in contrast it led to a 3-fold increase in TNF-α release from splenocytes harvested from IL-10−/− mice treated with AdvmuIL-10 10 wk previously (preliminary experiments determined that the isotype control OX20 had no effect on LPS-stimulated TNF-α release from splenocytes harvested from AdvmuIL-10-treated mice). This demonstrates that at least part of the diminished LPS response of splenocytes from AdvmuIL-10-treated IL-10−/− mice is attributable to secreted IL-10 and confirms the biological activity of the IL-10 detected in splenic homogenates. However, even after secreted IL-10 has been neutralized, TNF-α release from LPS-stimulated splenocytes from AdvmuIL-10-treated IL-10−/− or control C57BL/6 × DBA1 mice is still significantly lower than saline or Adv0-treated IL-10−/− mice, suggesting an immunoregulatory change independent of the contemporaneous presence of IL-10.

Analysis of harvested splenocytes by flow cytometry revealed no differences in either the ratio of CD4+ to CD8+ cells or in the cellular expression of CD62L between groups (data not shown). However, whereas splenocytes from IL-10−/− mice treated with saline or Adv0 at either 4 or 10 wk were predominantly CD45RBlow, those from AdvmuIL-10-treated IL-10−/− mice or control C57BL/6 × DBA1 littermates were predominantly
CD45RB<sup>high</sup> (Fig. 6). In addition, there were marked differences between groups in the level of the T cell-secreted chemokine RANTES, which acts to recruit circulating leukocytes to sites of inflammation. Thus, LPS-stimulated splenocytes harvested from IL-10<sup>−/−</sup> mice treated with AdvmuIL-10 10 wk previously secreted significantly lower levels of RANTES (2.4 ± 0.3 ng/ml) than splenocytes from IL-10<sup>−/−</sup> mice treated with either Adv0 (10.2 ± 0.68 ng/ml; p ≤ 0.0001) or saline (9.7 ± 0.88 ng/ml; p ≤ 0.0013; Fig. 6).

The neutralizing antiadenovirus Ab response is diminished in AdvmuIL-10-injected IL-10<sup>−/−</sup> mice

Previous studies have demonstrated elevated titers of antiadenovirus Abs in mice treated with adenoviral vectors (26). This response can be diminished by interrupting Ag presentation at the time of initial exposure using nondepleting anti-CD4 Abs (27) or coadministration of soluble CTLA4Ig (28). We hypothesized that the neutralizing Ab response to AdvmuIL-10 vectors would be diminished, as the protein encoded for by this virus will act to diminish, as the protein encoded for by this virus will act to suppress T cell activation in response to the presented Ag. A bioassay was used to detect the presence of antiadenoviral Abs in the serum of IL-10<sup>−/−</sup> mice treated 2 wk previously with saline, Adv0, AdvmuIL-10, or Advβgal. A standard curve was generated using 2-fold dilutions of the serum from IL-10<sup>−/−</sup> mice injected with Advβgal. Dilution to 1:16 led to a 50% reduction in the ability of this serum to neutralize the exogenous Advβgal; thus, the mean OD for each group at this dilution was compared. Serum from saline-treated IL-10<sup>−/−</sup> mice gave a mean OD of 1.42 ± 0.01 representing 100% infection of the 293 cells (25). There was no significant difference in the ability of serum from Adv0- and Advβgal-injected mice to neutralize exogenous Advβgal (mean ODs of 0.726 ± 0.001 and 0.74 ± 0.13, respectively; p < 0.9). This suggests that the antiadenoviral Abs target viral Ags and are not specific to the construct inserted into a particular virus. In contrast, a 1:16 dilution of serum from AdvmuIL-10-injected mice had significantly lower ability to neutralize exogenous Advβgal (mean OD 1.2 ± 0.16; p < 0.042 compared with Adv0 and p < 0.044 compared with Advβgal).

Discussion

Mice deficient in IL-10 develop a spontaneous colitis with many similarities to human Crohn’s disease (5). Although systemic administration of rIL-10 is able to prevent disease if given from weaning, it is only partially able to ameliorate established disease (6). We hypothesized that constant high-dose delivery of IL-10 via gene therapy to IL-10<sup>−/−</sup> mice would not only prevent the onset of colitis, but also be therapeutic for established disease; the additional therapeutic effect of gene therapy being due to improved tissue delivery compared with daily systemic administration. Systemic and local IL-10 gene therapy have proven efficacy in the collagen-induced model of arthritis, which is also dependent on IL1CD4<sup>+</sup> T cells (20, 21). Although i.p. administration of AdvmuIL-10 did not demonstrate therapeutic efficacy in the trinitrobenzene sulfonic acid model of colitis in rats, there was some improvement in disease score when AdvmuIL-10 was given before the initiation of colitis (29). In contrast, a recent report demonstrated that daily intragastric administration of the commensal bacteria Lactococcus lactis genetically modified to secrete IL-10 (LL-mIL-10) led to a 50% reduction in the colitis observed in dextran

**FIGURE 3.** AdvmuIL-10 is able to reverse established colitis in the IL-10<sup>−/−</sup> mice. A total of 5 × 10<sup>6</sup> PFU AdvmuIL-10 (n = 4) or Adv0 (n = 4) were injected into the tail vein of sedated 10-wk-old IL-10<sup>−/−</sup> mice that had clinical evidence of colitis; healthy 10-wk-old C57BL/6 × DBA1 wild-type mice were injected with saline vehicle as a negative control (n = 4). Clinical score (a) and stool IL-1β concentrations (b) were measured weekly until sacrifice and are shown as mean ± SE for each group. Results were analyzed by two-way ANOVA to assess the effect of both time and treatment on disease progression (***, p ≤ 0.001; **, p ≤ 0.01 compared with vehicle-treated C57BL/6 × DBA1 controls). Animals were sacrificed at 14 wk (4 wk after injection); histological scores (c) and SAP levels (d) were measured as before (*, p ≤ 0.05 compared with wild-type controls).
sodium sulfate-treated mice and prevented the onset of colitis in IL-10−/− mice (30).

In our study, a single systemic dose of $1 \times 10^8$ PFU AdvmuIL-10 given at 4 wk of age was sufficient to prevent the clinical onset of colitis in IL-10−/− mice throughout the 10 wk of the experiment. In addition, it suppressed the mucosal release of proinflammatory cytokines, diminished the acute-phase response, and led to significantly lower histological scores than Adv0- or saline-treated IL-10−/− mice. Furthermore, in contrast to daily i.p. injections of IL-10, a single i.v. dose of $5 \times 10^6$ PFU AdvmuIL-10 administered to 10-wk-old IL-10−/− mice with clinical and histological signs of colitis completely reversed their disease. Four weeks after injection, the histological score and SAP level was not significantly different to littermate C57BL/6 × DBA1 controls. In controls contrast, 10-wk-old IL-10−/− mice treated with Adv0 developed progressive colitis with elevated histological scores and SAP levels.

The colitis in IL-10−/− mice is dependent on an enhanced Th1 T cell response to the bacterial component of luminal Ag (7). The differentiation of Th1 cells reactive to luminal Ag is dependent on both the cognate interaction between naïve T cells and APCs and the stimulatory effects of cytokines such as IL-12 (31). This effect of APC-derived IL-12 is enhanced by the IFN-γ release from the developing Th1 lymphocyte (32). In the intact gastrointestinal tract, Ag presentation also results in IL-10 release that acts to antagonize the positive feedback loop toward Th1 lymphocyte differentiation. Thus in the IL-10-deficient mouse persistent antigenic stimulation and uncontrolled release of IL-12 and IFN-γ may lead to excessive generation and uncontrolled activation of a set of Th1 T cells reactive to luminal Ag, thus converting protective immunity to a pathological response (33). This hypothesis is supported by increased TNF-α and IFN-γ release in IL-10−/− mice exposed to LPS, a major constituent of luminal Ag (12). In our experiments there was no difference in TNF-α or IFN-γ release from cultured spleen cells isolated from wild-type, IL-10−/−, or IL-10−/− mice treated with AdvmuIL-10. However, the addition of LPS to the
cultures resulted in significantly increased release of TNF-α and IFN-γ from splenocytes isolated from IL-10−/− mice. IL-10−/− mice were injected with 1 × 10^9 PFU (4-wk-old mice) or 5 × 10^9 PFU (10-wk-old mice) of AdvmuIL-10, Adv0, or saline vehicle. Wild-type mice received saline vehicle only at similar time points. Spleens were harvested on sacrifice at 14 wk of age, and single-cell suspensions were prepared. A total of 1 × 10^6 cells were washed in PBS and stained with PE-CD45RB, FITC-CD4, or isotype control and analyzed by flow cytometry. Representative histograms from (a) IL-10−/− mice treated with Adv0, (b) IL-10−/− mice treated with AdvmuIL-10, and (c) wild-type mice are shown. The percentage of cells expressing a CD45RB^high^ phenotype for (d) mice treated at 4 wk and (e) mice treated at 10 wk are also shown (*, p < 0.05 compared with wild-type mice). Only four wild-type animals were studied. A total of 2 × 10^6 cells per well were cultured for 24 h in the presence of 10 μg/ml LPS. Supernatants were harvested and assayed for RANTES by ELISA. Results (f) are expressed as the mean of each group of animals assayed in triplicate (***, p = 0.001 vs Adv0 or saline).

Thus, neutralizing secreted IL-10 with a mAb increased LPS-induced TNF-α release from AdvmuIL-10-treated IL-10−/− mice as well as control C57BL/6 × DBA1, but had no effect on cultures of spleen cells from IL-10−/− mice treated with saline. Therefore, it is possible to detect the sequelae of AdvmuIL-10 therapy for at least 10 wk after a single injection.

Recombinant IL-10 delivery is able to prevent the colitis induced by the transfer of CD45RB^high^CD4^+^ T cells into SCID mice (15). However, this effect is only transient, as colitis will develop after the treatment is stopped. In contrast, long-term protection is achieved by cotransfer of CD45RB^low^CD4^+^ T cells, which are dependent on IL-10 for their formation and both IL-10 and TGF-β for their effect (14). The effectiveness of AdvmuIL-10 therapy could result from immunosuppression due to long-term secretion of IL-10 from infected cells in the liver, spleen, and gastrointestinal tract. Alternatively, IL-10 exposure may result in the differentiation of a group of regulatory T cells that exert their effect by the release of immunoregulatory molecules such as TGF-β.

LPS-stimulated splenocytes isolated from IL-10−/− mice 10 wk after AdvmuIL-10 treatment released significantly less TNF-α and IFN-γ than Adv0- or saline-treated IL-10−/− mice. The addition of anti IL-10 Abs was unable to reverse this effect completely, which
may suggest that there are phenotypic differences between spleen cells harvested from IL-10−/− mice and those harvested from IL-10+/− mice treated with AdvmuIL-10. In support of this hypothesis, Berg et al. have reported that CD4+ cells isolated from IL-10−/− mice express low levels of CD45RB compared with wild-type mice (6). It should be remembered that whereas wild-type CD45RBlowCD4+ T cells protect against colitis in the CD45RBlow transfer to SCID mouse model, CD45RBhigh cells isolated from IL-10−/− mice are in fact pathogenic. We demonstrate that, whereas spleen cells isolated from IL-10−/− mice expressed low levels of CD45RB, those isolated from either AdvmuIL-10-treated IL-10−/− mice or wild-type littermates expressed high levels. In addition, treatment with AdvmuIL-10 reduced the levels of RANTES secreted by splenocytes from IL-10−/− mice. Elevated levels of RANTES, a T cell-derived chemokine that directs infiltrating leukocytes to sites of inflammation, have been found in the mucosa of patients with Crohn’s disease (34, 35). Therefore, in addition to inhibiting proinflammatory cytokine release, AdvmuIL-10 therapy is likely to decrease leukocyte recruitment.

Levels of IL-5, IL-10, and TGF-β release were below the limit of detection of the assays used (data not shown). In this study, it was not possible to confirm whether treatment with AdvmuIL-10 promotes the formation of regulatory T cells.

Studies of adenoviral vectors encoding foreign and immunoregulatory proteins have demonstrated host antivirus immune responses that limit the duration of delivered gene expression and prevent tretertourment. (26, 36, 37). Both cellular and humoral antiviral responses can be diminished by coadministration of non-depleting anti-CD4 Abs or CTLA4Ig (27, 28). It is possible that administration of adenoviral vectors encoding immunoregulatory proteins such as IL-10 will also provoke a diminished host response. Thus, prolonged IL-10 expression from AdvmuIL-10-infected respiratory epithelium has been reported, whereas the expression of the foreign protein β-galactosidase is curtailed by antiadenviral host responses (38). One might predict that IL-10−/− mice would generate enhanced antiviral responses, leading to increased adenviral vector clearance and reduced tissue expression of the delivered protein. However, we have demonstrated that both the expression of IL-10 within tissue homogenates and its immunoregulatory effects persist for at least 10 wk in this model after systemic administration of AdvmuIL-10. Furthermore, we have demonstrated that the prolonged efficacy of AdvmuIL-10 is associated with a diminished antiviral Ab response compared with administration of Adv0 or Advβgal.

Trials of systemic IL-10 in patients with Crohn’s disease have achieved only a modest therapeutic benefit, despite the ability of exogenous IL-10 to inhibit proinflammatory cytokine release by mononuclear cells isolated from Crohn’s disease tissue in vitro (39). In this paper, we have demonstrated the therapeutic advantage of adenviral vectors encoding IL-10 over systemic IL-10 administration in the prevention and treatment of colitis in the IL-10−/− mouse. In addition, the delivery of an immunoregulatory cytokine gene is shown to minimize the host response to the adenviral vector and thus allow long-term IL-10 expression. Whether, using alternative delivery systems such as adenviral vectors, IL-10 could prove effective in patients with Crohn’s disease remains speculative. However, these results highlight the potential of IL-10 gene therapy in the treatment of chronic inflammatory conditions.

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References


