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Murine IL-10 Gene Transfer Inhibits Established Collagen-Induced Arthritis and Reduces Adenovirus-Mediated Inflammatory Responses in Mouse Liver

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The effects of homologous IL-10 administration during an established autoimmune disease are controversial, given its reported immunostimulatory and immunosuppressive properties. Studies of collagen-induced arthritis have shown efficacy with repeated administrations of IL-10; however, when the EBV IL-10 homologue was administered via adenovirus gene transfer technology the results were equivocal. Therefore, the present study was undertaken to elucidate the effects of prolonged homologous IL-10 administration via adenovirus-mediated gene delivery on the progression of established arthritis. Collagen type II (CII)-immunized mice received i.v. injections of $10^7$ or $10^8$ PFU of an E1-deleted adenoviral vector containing the murine IL-10 gene (AdIL-10), after arthritis onset. Mice were monitored for 3 wk for disease progression, and gene transduction was assessed by quantification of serum mIL-10. CII-specific cell-mediated and humoral immune responses were analyzed by lymph node cell proliferation, cytokine production, and anti-CII Ab responses. Furthermore, because adenoviral vectors have been reported to induce organ dysfunction due to cell-mediated immune responses to the viral Ags, we have also evaluated delayed-type hypersensitivity responses and reactive hepatitis to the systemically delivered adenovirus and whether the IL-10 produced could influence those responses. Sustained suppression of autoimmune arthritis and elevated serum levels of IL-10 were achieved in our study. AdIL-10 treatment reduced cell-mediated immune reactivity, but did not affect humoral responses. Furthermore, IL-10 was able to reduce, but not totally abrogate, adenovirus-induced hepatic inflammation. These findings provide further insights into the diverse interplay of immune processes involved in autoimmune inflammation and the mechanism of cytokine immunotherapy.


Interleukin-10 is an immunoregulatory cytokine that has been shown to possess potential therapeutic value for organ-specific autoimmune diseases, as it reduces symptoms of experimental diabetes (1, 2), autoimmune encephalomyelitis (3, 4), inflammatory bowel disease (5), and autoimmune arthritis (6–9).

IL-10 was originally discovered as a factor produced by mouse CD4$^+$ Th2 cells that was capable of inhibiting the synthesis of IFN-$\gamma$ by Th1 T cells (10). Its anti-inflammatory and immunosuppressive properties have emerged from a number of studies showing that IL-10 can inhibit the production of proinflammatory cytokines such as IL-1, IL-12, IL-6, and TNF-$\alpha$ and that it can reduce Ag-specific CD4$^+$ T cell responses through its potent effects on APCs (11). However, IL-10 has shown several stimulatory effects on the immune system. In contrast to its effects on CD4$^+$ T cells, IL-10 enhances the proliferation and cytolytic activity of CD8$^+$ T cells (12); in vitro it may act as a potent stimulator of mouse B lymphocytes and mast cells; it augments B cell differentiation, Ig production, and Ag presentation; and it enhances Ab-dependent cell-mediated cytoxic activity (13–18). In addition, IL-10 is described as a chemotactic factor for CD8$^+$ T cells (19).

Recent studies have suggested that cytokines, and TNF-$\alpha$ in particular, are pivotal to the pathogenesis of rheumatoid arthritis (RA), a chronic disease characterized by immune dysregulation associated with cellular and humoral hypersensitivity, and an imbalance between pro- and anti-inflammatory/immunosuppressive effects probably contributes to the chronicity of this disease (20–22). Several studies performed on sera or synovial samples from patients with RA support an important immunoregulatory role for IL-10 in RA. IL-10 expression was increased in serum and synovial fluid from RA patients compared with that in osteoarthritis patients and healthy individuals (23–25), and in our laboratory evaluation of RA synovial tissue demonstrated increased IL-10 expression by immunostaining, ELISA, and RT-PCR analysis. Furthermore, a neutralizing mAb to IL-10 profoundly enhanced TNF-$\alpha$ and IL-1$\beta$ production in RA joint cultures, suggesting a role for IL-10 as a major endogenous immune regulator and, hence, a potential therapeutic agent (26). Indeed, in vivo studies from our laboratory (7) and others (6, 8) have demonstrated the efficacy of repeated systemic administration of IL-10 protein in experimental arthritis. However, these studies showed different results regarding humoral immune responses to collagen type II

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1. Underlying autoantibody expression those responses probably contributes to the chronicity of this disease (20–22).
2. Address correspondence and reprint requests to Prof. Marc Feldmann, Kennedy Institute of Rheumatology Division, Imperial College School of Medicine, 1 Aspenlea Road Hammersmith, London, U.K. W6 6LH. E-mail address: m.feldmann@ic.ac.uk
3. Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; AdIL-10, mouse IL-10 adenovirus; Ad0, empty adenoviral vector; CII, collagen type II; DTH, delayed-type hypersensitivity; mIL-10, murine IL-10.
and 3 mice were given a daily clinical score per limb from 0 to 3: 0 the day that erythema and/or swelling were first observed, and arthritic (Difco, West Molesey, U.K.). The onset of arthritis was considered to be assessed using calipers (Kroeplin, Schluchtern, Germany).

Paw thickness of the first affected hind paw per mouse was itself had no effect on the progression of CIA (39, 40) 108 PFU of the m

DTH reactions

Treatment protocol

For CIA treatment AdIL-10 was injected at doses that have been shown to yield elevated serum levels of the protein encoded (105 and 106 PFU in 100 μl of isotonic saline/mouse) into the tail vein (i.v.) once at the onset of arthritis (day 1), and mice were monitored for disease progression up to day 21. To confirm our previous observation that injection of the viral vector itself had no effect on the progression of CIA (39, 40) 106 PFU of the empty vector Ad0 was also administered. Blood samples were obtained from all treated and control mice, and serum was stored at −80°C until use. To assess the effects of IL-10 delivery via recombinant adenovirus on delayed-type hypersensitivity (DTH) responses to the viral Ags and on the hepatic inflammatory reactions reported when higher doses of adenovirus are administered systemically, 105 PFU/mouse of AdIL-10 and of the empty vector Ad0 were infused i.v. None of the animals that received recombinant adenoviruses showed adverse effects (100% survival rate, normal coat appearance or general activity) compared with untreated controls.

Detection of mIL-10 protein

mIL-10 protein was detected by ELISA in diluted serum samples from the 105 PFU AdIL-10-injected mice. Briefly, microtiter plates were coated with anti-mIL-10-coating mAb (PharMingen, San Diego, CA) dissolved in PBS overnight at 4°C. After blocking for 3 h with 2% BSA, dilutions of sera from AdIL-10-treated and arthritic controls from 1/25 to 1/6250 were applied to the wells and incubated overnight at 4°C. Each plate included a standard curve of recombinant mouse IL-10 (PharMingen). After washing thoroughly, 50 μl of a detection mAb diluted 1/2000 (PharMingen) was added to all wells, and plates were incubated overnight at 4°C. After washing, 50 μl of streptavidin-biotinylated HRP complex at a dilution of 1/6000 (Amersham, Aylesbury, U.K.) was added to the wells and incubated for an additional 1 h at 37°C. The plates were then developed using tetramethylbenzidine dihydrochloride (Kirkegaard & Perry, Gaithersburg, MD) as substrate reagent, and OD was assessed at 450 nm using a spectrophotometer (Labsystems Multiskan, Helsinki, Finland); the sensitivity of the assay was 123 pg/ml.

Assessment of T cell responses by proliferation assay and cytokine production

Inginal and popliteal lymph nodes were aseptically removed from AdIL-10-treated and control arthritic mice 14 days after arthritis onset and start of treatment. Cells (5 × 106) were cultured in 0.2 ml of DMEM supplemented with 2% heat-inactivated FCS, 100 U/ml penicillin 100 μg/ml streptomycin, 2 × 10−5 M 2-ME, and 1% glutamine in a 96-well flat-bottom plate. Cells were cultured in medium alone or in the presence of 50 μg/ml of denatured bovine type II collagen or 10 μg/ml of an anti-C3D mAb (2C11-145) and incubated at 37°C in 5% CO2. To assess T lymphocyte proliferative responses, 72 h later plates were pulsed with 1 μCi/well of [3H]thymidine (Amersham, Aylesbury, U.K.) and incubated at 37°C for an additional 24 h. The plates were then harvested onto glass-fiber filters with a semi-automated cell harvester (Titer-Tek 550; Flow Laboratories, Irvine, CA). Cellular DNA synthesis was assessed by [3H]thymidine incorporation using a liquid scintillation spectrometer (LKB, Bromma, Sweden). Results are expressed as the arithmetic mean counts per minute of triplicate cultures ± SEM. For detection of IFN-γ and IL-4 production, cell culture supernatants were harvested at 48 h and analyzed by standard ELISAs (PharMingen).

Ab determinations

Anti-CII Ab levels were tested in individual sera by ELISA. Microtiter plates were coated with 2 μg/mI of CII dissolved in Tris-buffered saline overnight at 4°C. After blocking for 1 h with 2% BSA, dilutions of sera from 1/50 to 1/6400 were applied to the wells. For isotype quantitation sheep anti-mouse IgG1 and IgG2a linked to alkaline phosphatase (The Binding Site, Birmingham, U.K.) were used at a dilution of 1/5000. The plates were developed using p-nitrophenyl phosphate (Sigma) as substrate, and OD was assessed at 405 nm using a spectrophotometer (Labsystems Multiskan). Each plate included a standard curve of a positive serum used to define arbitrary units of total IgG1 and IgG2a anti-CII Abs.

Materials and Methods

Recombinant adenovirus vectors

The recombinant E1-deleted type 5 adenoviral vectors, encoding a mouse IL-10 homologue, termed viral IL-10. The results show efficacy, mainly as a prophylactic treatment, whereas therapy of established disease has yielded conflicting results. Therefore, the present study was undertaken to assess the therapeutic potential of prolonged homologous mIL-10 administration via systemic adenoviral gene transfer on established autoimmune arthritis.

Treatment protocol

For CIA treatment AdIL-10 was injected at doses that have been shown to yield elevated serum levels of the protein encoded (105 and 106 PFU in 100 μl of isotonic saline/mouse) into the tail vein (i.v.) once at the onset of arthritis (day 1), and mice were monitored for disease progression up to day 21. To confirm our previous observation that injection of the viral vector itself had no effect on the progression of CIA (39, 40) 106 PFU of the empty vector Ad0 was also administered. Blood samples were obtained from all treated and control mice, and serum was stored at −80°C until use. To assess the effects of IL-10 delivery via recombinant adenovirus on de-
Hepatic morphological analyses

Livers from DBA/1 mice injected i.v. with 10^9 PFU of AdIL-10 or the empty vector Ad0 along with livers from naive DBA/1-uninjected mice were analyzed. Paraffin sections were stained with hematoxylin and eosin according to standard procedures and analyzed in a blinded fashion by two pathologists. A custom-made scoring system for this study was designed based on the degree of hepatitis observed in liver sections obtained from four mice 3 wk after the i.v. injection of a high dose (10^10 PFU) of the empty adenoviral vector compared with sections from livers of two un-treated naive mice (see Table I). Each pathologist analyzed the sections independently and was unaware of the treatment details. A preliminary overall inflammation score was given to each section. To align each observer’s threshold, the observations were subsequently compared. Those sections showing discrepancies were reviewed and discussed together using a multilens microscope until final agreement was reached. Four parameters were then assessed individually, including portal inflammation, parenchymal (lobular) inflammation, apoptosis, and mitoses. Portal inflammation was graded as mild if portal tracts were not expanded and contained a few inflammatory cells, moderate if portal tracts were expanded but not packed by inflammatory cells, and severe if portal tracts were expanded and packed with without involvement of the hepatocyte limiting plate. Scoring was obtained from the most inflamed portal tracts. Parenchymal inflammation was graded by counting the number of necroinflammatory foci in a total of 10 circular areas measuring 2 mm in diameter (×200 magnification) and chosen randomly. The overall consensus agreement grades of inflammation were then used to set the limits for three levels of parenchymal inflammation: grade 1 = 1–4 foci, grade 2 = 5–10 foci, and grade 3 = ≥10 foci. Apoptosis and mitotic activity were graded by counting the number of acidophilic bodies and mitotic figures, respectively, in a total of 10 circular areas measuring 2 mm in diameter (×200 magnification) and chosen randomly. The scores were divided into three categories of equal size using the highest score as the maximum: apoptosis grade 1 = 1–5 foci, grade 2 = 6–10 foci, grade 3 = ≥10 foci, mitoses grade 1 = 1–4 foci, grade 2 = 5–8 foci, and grade 3 = ≥8 foci. A final score was then obtained by adding up each individual score of portal and parenchymal inflammation, apoptosis, and mitoses. The highest number was considered the upper limit of each category, as designated by overall consensus agreement. Mild inflammation was considered for scores between 1 and 4, moderate inflammation for scores between 5 and 7, and severe inflammation for scores ≥8. A final score of 1 was considered not relevant for inflammation. On the basis of this novel scoring system, livers from DBA/1 mice injected i.v. with 10^9 PFU of AdIL-10 or the empty vector along with livers from naive uninjected mice were analyzed.

Statistical analysis

For statistical analysis of macroscopic data the Mann-Whitney U test of significance was applied, using a Minitaab statistical software package (Minitab, State College, PA). Differences were considered statistically significant at p < 0.05.

### Results

Suppression of established CIA by adenovirus-mediated mIL-10 gene transfer

We have previously shown that daily i.p. administration of mIL-10 after the onset of macroscopic arthritis over a 10-day period significantly inhibited the progression of mouse CIA (7). To investigate the effects of prolonged mouse IL-10 administration we used a gene transfer technology known to yield in vivo elevated and relatively steady serum levels of a specific protein (27). Other investigators have addressed this question using the EBV viral homologue of IL-10 (AdIL-10), but the results for established arthritis are controversial (34, 36, 41). In the present study a replication-deficient recombinant adenovirus AdIL-10 was administered i.v. once at 10^7 and 10^8 PFU/mouse on the day of arthritis onset (day 1). Controls consisted of mice treated with PBS or 10^8 PFU of the control adenovirus Ad0. As shown in Fig. 1A, at both doses AdIL-10 treatment was very effective in controlling the progression of articular inflammation, statistically significant from day 5 (p = 0.01) until day 21 (p = 0.007), confirming the previous short term studies with injection of mouse IL-10 protein (7). Administration of a control adenoviral vector did not affect the progression of established arthritis (Fig. 1B), as previously observed (39, 40). To assess gene transduction, a time course of mouse IL-10 produced individually by four mice injected with 10^9 PFU of AdIL-10 was performed on diluted serum samples obtained on days 1, 3, 7, 10, 17, and 21 (see Fig. 2). Serum concentrations of IL-10 were undetectable in arthritic controls and on day 1 postadenoviral injection; on day 3 one mouse showed a serum concentration of 30 ng/ml. By day 7 elevated amounts of mIL-10 were measured (mean ± SEM, 104 ± 33.9 ng/ml); these levels peaked on day 10 (mean ± SEM, 431.9 ± 191.8 ng/ml) and declined on day 17 (mean ± SEM, 288.9 ± 85 ng/ml), but were still high on day 21 (mean ± SEM, 151 ± 64.9 ng/ml). AdIL-10 treatment was also effective in reducing articular swelling as assessed by measuring paw thickness throughout the length of the experiments (data not shown).

mIL-10 gene delivery in vivo interferes with Ag-specific T cell responses

It has been demonstrated that the development of CIA is associated with high levels of cell-mediated and humoral immunity to collagen (42). To determine whether Ag-specific T cell responses were

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### Table I. Design of adenovirus-mediated hepatitis scoring system

<table>
<thead>
<tr>
<th>Overall Inflammation</th>
<th>Portal Inflammation</th>
<th>Parenchymal Inflammation</th>
<th>Apoptosis</th>
<th>Mitoses</th>
<th>Overall Inflammation</th>
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<tbody>
<tr>
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<td></td>
<td>Number of foci</td>
<td>Grade</td>
<td>Number of foci</td>
<td>Grade</td>
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<td>Ad0 3 1 13 3</td>
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</table>

a Four naive DBA/1 mice were injected i.v. with 10^9 PFU of Ad0, and liver sections were obtained 3 wk later. The degree of inflammation observed, compared to liver sections from two untreated mice, was used to establish the hepatitis scoring system described in Materials and Methods. Number of foci = foci of parenchymal inflammation, apoptosis, or mitoses observed in 10 circular areas measuring 2 mm and chosen randomly (magnification, ×200).

b Grade 1 = 1–4 foci; grade 2 = 5–10 foci; grade 3 = ≥10 foci.

c Grade 1 = 1–5 foci; grade 2 = 6–10 foci; grade 3 = ≥10 foci.

d Grade 1 = 1–5 foci; grade 2 = 6–10 foci; grade 3 = ≥10 foci.

© Inflammation scored with the novel scoring system: NR (not relevant) = final score 0–1, mild = final score 2–4, moderate = final score 5–7, severe = final score ≥ 8.
affected by AdIL-10 treatment, lymph node cell proliferation assays were performed in animals injected with AdIL-10 at 10^8 PFU/mouse or arthritic controls as specified in Materials and Methods. The mean clinical score is presented as the average clinical score per group ± SEM. Statistically significant amelioration of disease was observed at both doses starting from day 5 (p = 0.01) until the end of the experiment on day 21 (p = 0.007). A representative result from two experiments is shown (A). As a further control, in a subsequent experiment the higher dose of the adenoviral vector administered, 10^9 PFU, but carrying no insert (Ad0), was also administered to a group of mice (n = 8) at the time of arthritis onset, and disease progression was compared with that in controls (n = 8). No statistically significant difference between the two groups was observed (B).

FIGURE 1. mIL-10 gene transfer suppresses established CIA. Three groups of DBA/1 mice were immunized for arthritis with CII and CFA. On the day of arthritis onset (day 1) 10^7 PFU (○, n = 10) or 10^8 PFU (▲, n = 10) of AdIL-10 in 100 μl of saline or saline alone (□, n = 10) was injected i.v. into the tail vein. Mice were examined every other day for 21 days for signs of joint inflammation and were scored as described in Materials and Methods. The mean clinical score is presented as the average clinical score per group ± SEM. Statistically significant amelioration of disease was observed at both doses starting from day 5 (p = 0.01) until the end of the experiment on day 21 (p = 0.007). A representative result from two experiments is shown (A). As a further control, in a subsequent experiment the higher dose of the adenoviral vector administered, 10^9 PFU, but carrying no insert (Ad0), was also administered to a group of mice (n = 8) at the time of arthritis onset, and disease progression was compared with that in controls (n = 8). No statistically significant difference between the two groups was observed (B).

FIGURE 2. Time course of serum expression of the mouse IL-10 protein in DBA/1 mice injected i.v. with AdIL-10. Gene expression in individual animals was determined by periodic serum quantification of the mouse IL-10 protein. Mice injected i.v. with 10^8 PFU of AdIL-10 on the day of arthritis onset were bled on days 1, 3, 7, 10, 17, and 21, and sera were tested for the presence of IL-10 by ELISA as described. The results are expressed as nanograms per milliliter of mIL-10 and represent individual sera from four mice.

FIGURE 3. AdIL-10 treatment reduces in vitro lymphocyte proliferation and IFN-γ production in response to the disease-inducing Ag CII. A. CII immunized mice were treated with 10^8 PFU of AdIL-10 or saline on the day of arthritis onset (day 1) and were sacrificed 14 days later. Inguinal and popliteal lymph nodes were harvested, and single-cell suspensions were prepared. Cells (5 × 10^5/well) were cultured in 0.2 ml of DMEM with (●) or without (□) 50 μg/ml of denatured CII. Proliferative responses were assessed by [3H]thymidine incorporation and expressed as counts per minute ± SEM of four mice. B. Forty-eight-hour supernatants of lymph node cell cultures from arthritic controls (□) and 10^8 PFU AdIL-10 injected mice (●) incubated with denatured CII or an anti-CD3 mAb were assayed for the presence of IFN-γ by ELISA.
Effect of AdIL-10 treatment on Ab responses

The above results suggest that transgenic expression of mouse IL-10, even in the late stage of an in vivo immune response, can diminish Ag-specific T cell responsiveness, thereby suppressing an established autoimmune response. However, in our experiments measurement of anti-CII Ab responses in AdIL-10-treated and same stage arthritic control mice showed no significant differences in the amount of anti-CII IgG1 or IgG2a isotypes (data not shown). This may suggest that IL-10 treatment in CIA, even at the high circulating serum concentrations achieved with the adenoviral gene transfer for a prolonged time, does not amplify anti-CII humoral responses, despite its reported B cell stimulator activities in vitro, for a possible in vivo compensatory mechanism given the significant reduction of CII-specific T cell help to B lymphocytes (see Fig. 3A) also occurring at the same time. Thus, the Ab reduction predictable by IL-10 immunosuppression of CII-specific T cell responses may be counterbalanced by the IL-10-mediated stimulation of B-lymphocytes.

Systemic adenovirus-mediated gene transfer induces mild hepatic inflammation, which is partially overcome by AdIL-10 treatment

Evaluation of adenoviral technology for systemic in vivo gene delivery has revealed several important limitations, particularly related to the hepatic tropism of i.v. administered adenoviruses, which has been shown to cause liver inflammation. Even if the recombinant constructs used lack E1a and E1b early transcriptional regions, newly synthesized adenoviral proteins are nonetheless expressed on the surface of transduced cells, leading to the generation of specific cellular immune responses to the genetically modified hepatocytes, particularly when high doses of the adenoviral vector are administered, which results in liver inflammation (31, 32). To assess whether the adenoviral delivery of IL-10 was capable of reverting this response, groups of three DBA/1 mice were injected into the tail vein with 10⁹ PFU/mouse of either the Ad0 and 10⁹ PFU AdIL-10 and liver sections obtained 9 wk later. The degree of inflammation observed was compared with that of naive mice (Fig. 4A) also occurring at the same time. Thus, the Ab reduction predictable by IL-10 immunosuppression of CII-specific T cell responses may be counterbalanced by the IL-10-mediated stimulation of B-lymphocytes.

Table II. Effect of the i.v. E1-deleted adenovirus administration on livers of DBA/1 micea

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<thead>
<tr>
<th>Overall Inflammation</th>
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a Naive DBA/1 mice were injected i.v. with 10⁹ PFU of Ad0 and 10⁹ PFU AdIL-10 and liver sections obtained 9 wk later. The degree of inflammation observed was compared to liver sections from untreated mice and analyzed as described in Materials and Methods. Number of foci = foci of parenchymal inflammation, apoptosis, or mitoses observed in 10 circular areas measuring 2 mm and chosen randomly (magnification ×200).

b 0 = none; 1 = mild; 2 = moderate; 3 = severe.

Grade 1 = 1–4 foci; grade 2 = 5–10 foci; grade 3 = > 10 foci.

Grade 1 = 1–5 foci; grade 2 = 6–10 foci; grade 3 = > 10 foci.

Grade 1 = 1–4 foci; grade 2 = 5–8 foci; grade 3 = > 8 foci.

b Inflammation scored with the novel scoring system: NR (not relevant) = final score 0–1; mild = final score 2–4; moderate = final score 5–7; severe = final score ≥ 8.
addressed the question of whether IL-10 delivered systemically via an adenoviral vector could influence this response. Two approaches were used: 10⁹ PFU of AdIL-10 (a dose higher than that used for CIA treatment and for assessing IL-10 production) or 10⁹ PFU of the control Ad0 virus was injected i.v., either 3 days before the sensitization phase of DTH induction or 3 days before footpad antigenic challenge. Mice were challenged on day 8 in one footpad with 10⁻⁶ PFU of UV-inactivated Ad0 and in the contralateral footpad with 10⁻⁶ PFU of vehicle alone. DTH responses were assessed at 24 h (because by 48 h the response was minimal), and the resulting mean paw swelling ± SEM for both protocols are shown in Fig. 5. By this protocol of sensitization a strong DTH response was achieved to the adenoviral proteins. Interestingly, 10⁹ PFU of AdIL-10 administered before the sensitization phase of DTH induction suppressed the response (Fig. 5A), whereas its administration between the induction and the elicitation phases did not alter it (Fig. 5B). Experimentally, DTH reactions are generated by sensitization to a foreign Ag that is presented by specialized resident APCs (Langerhans cells) to naive lymphocytes, resulting in Ag-specific CD4⁺ T cell activation, expansion, and differentiation. In the second phase, or challenge, the same Ag is introduced at a peripheral site where different APCs are believed to present it to an expanded population of circulating memory CD4⁺ T cells, which then mediate DTH by secreting proinflammatory cytokines, namely IL-2, IFN-γ, and TNF-α. These results suggest that IL-10 exerts its anti-inflammatory activity by blocking the induction of Ag-specific CD4⁺ T cells when they undergo initial priming. Once an expanded population of memory cells is present, IL-10 is no longer suppressive, probably because a different mechanism of memory cell reactivation or Ag presentation or different APCs are subsequently involved.

Discussion

In this study we demonstrate that the prolonged expression of homologous IL-10 achieved by the systemic administration of relatively low doses of a replication-deficient recombinant adenoviral
vector effectively suppresses an already established immune-mediated inflammatory disease such as CIA in the long term (3 wk), confirming previous short term (10-day) studies with daily injections of the mIL-10 protein (6–8). Elevated serum levels of mouse IL-10 were achieved via the adenovirus-mediated gene transfer technology (see Fig. 2 for the time course of serum levels of IL-10 obtained with one i.v. injection of 10⁸ PFU of AdIL-10). The long period of expression is probably related to the immunosuppressive effects of the mouse IL-10 protein produced. Serum concentrations assayed at early time points (i.e., 24 and 72 h postviral injection) were below the level of detection of our assay, but they are likely to be underestimates of what is being produced, as there would be only high levels in serum after the mouse IL-10 tissue binding sites (IL-10 receptors) in vivo have become saturated. The mechanism of action of IL-10 in this model was analyzed and involves reduction of T cell activation, as we have demonstrated ex vivo the suppression of specific T cell proliferation in response to the disease-inducing Ag CII from 10⁸ PFU AdIL-10-treated mice (see Fig. 3A) along with inhibition of the synthesis of IFN-γ. This was statistically significant only when lymph node cells were restimulated in vitro with the relevant Ag CII, which requires proper processing and Ag presentation by APCs, but not when a polyclonal T cell activator such as soluble anti-CD3 was added to the cell culture (see Fig. 3B). Thus, Ag presentation to CD4⁺ Th1 T lymphocytes, which are pathogenic cells in CIA (42), is suppressed by IL-10 treatment. Our results suggest that IL-10 inhibits T cell activation by acting on APCs rather than directly on the responder T cells, in line with previous in vitro findings, because only the response to CII was blocked, not that elicited by the anti-CD3 Ab. Our data thus support the interpretation that CII-reactive CD4⁺ Th1 T lymphocytes are pathogenic even during the course of established arthritis.

However, IL-10 has also been shown to down-regulate a number of proinflammatory and proarthritic macrophage functions, including the production of IL-1, IL-6, TNF-α, GM-CSF, and G-CSF, as well as the generation of reactive oxygen intermediates, which are all effector molecules involved in the induction and/or progression of experimental arthritis, and this could help account for the prolonged and significant inhibition of disease progression observed in our study. IL-10 has been shown to down-regulate the constitutive and inducible expression of MHC class II Ags on human monocytes (44), but other investigators have shown that in the mouse IL-10 has no effect on the induction of MHC class II expression, but selectively inhibits the induction of B7 expression on murine macrophages (49). We have previously shown that prolonged protein or adenovirus-mediated gene delivery of a CTLA4-Ig fusion protein, blocking CD80 (B7-1) and CD86 (B7-2) molecules on APC, thus inhibiting the costimulatory signal required for a proper T lymphocyte activation, also suppresses established murine CIA (40, 50). Reduction of specific cell-mediated and humoral immune responses to type II collagen was demonstrated even when CTLA4-Ig treatment was administered during the late phase of the immune response, i.e., after the onset of clinical symptoms (40). Thus, another mechanism by which IL-10 contributes to the therapeutic effect in autoimmune arthritis could be due to the down-regulation of B7 expression on activated macrophages. Ding and colleagues (51) have also demonstrated in vitro that the ability of mouse IL-10 to inhibit murine APC function is only observed when cells from the macrophage lineage are used for Ag presentation, as IL-10 failed to inhibit T cell activation when activated mouse B cells, B cell tumor cells, dendritic cells, or fibroblasts were used as APCs. In our study the humoral branch of the immune response, the anti-CII Ab response in particular, was not affected even by prolonged IL-10 treatment, confirming previous observations by our group when mIL-10 protein was injected daily for 10 days in established CIA. This could be explained by the diverse interplay of immunosuppressive as well as immunostimulatory functions possessed by IL-10. It is known to be a potent stimulator of mouse B lymphocytes, and it augments B cell differentiation and Ig production (13, 14, 16, 18), but during an ongoing immune response in vivo, T cell help is also needed for B cell activation, and here we show that IL-10 significantly reduces specific T cell activation (Fig. 3A). In two previous reports of CIA gene therapy using the EBV IL-10 homologue, anti-CII Ab responses were also unaffected (34, 41) by treatment, whereas an earlier report (8) of continuous IL-10 delivery to treat rat CIA using mini-osmotic pumps showed reduction of anti-CII Abs upon systemic IL-10 delivery, but surprisingly higher serum levels when IL-10 was administered locally in the rear paws. Thus, the complexity of IL-10 biological effects makes it difficult to unravel its therapeutic potential in the universe of immune-mediated diseases unless experiments on the relevant animal model are performed, as in vitro assays to assess T or B lymphocyte activity do not necessarily monitor the same cells that mediate disease or consider the influences of regulatory events that may influence in vivo lymphocyte function.

The use of m IL-10 in our study, instead of the EBV homologue previously used in other gene therapy reports (33–36), has allowed us to specifically address the question of what effects persistently elevated circulating serum levels of homologous IL-10 would have in vivo and, more importantly, when administered at a late stage of an immune-mediated inflammatory response, i.e., after disease onset. The results obtained here are in contrast to those of previous studies that were studying prevention (treatment before disease onset) rather than postonset therapy of CIA in most cases, in which IL-10 was not therapeutic (34, 41).

It has been demonstrated that the human adenovirus used to construct the adenoviral vectors is hepatotropic when administered i.v. (28), with expression of the transgene carried by such vectors taking place predominantly in the liver (29, 30). Even if the recombinant constructs currently in use lack E1a and E1b early transcriptional regions, newly synthesized adenoviral proteins are nonetheless expressed on the surface of transduced cells, leading to the generation of specific cellular immune responses and ultimately inflammation (31, 45, 46). MHC class I-restricted CD8⁺ cytolytic T cell responses toward adenovirally transduced hepatocytes have been reported (32), and as IL-10 has also been described as a chemotactic factor for CD8⁺ T cells (19), we have further investigated the effects of virally delivered homologous IL-10 on adenovirus-induced hepatic inflammation. Our results show that adenovirus-induced liver inflammation is clearly dependent on adenovirus dose, but is not nearly as severe as the hepatitis induced by hepatitis viruses. Hence, a new scoring system more suitable to the milder degree of inflammation had to be devised. An obvious hepatitis was observed only when 10¹⁰ PFU of the empty vector AdΔ0 was administered i.v. to assess the highest degree of liver inflammation achievable in this animal system (see Table I), whereas administration of 10⁹ PFU of the empty vector resulted in mild inflammation, and the effect of same dose of the AdIL-10 was almost comparable to that in naive controls (see Table II). Our data thus show that IL-10 is also able to reduce hepatic inflammation, and hence delivery of immunosuppressive/anti-inflammatory agents protects against the potential proinflammatory effect of adenoviruses when administered i.v.

Cell-mediated immune responses to viral Ags, depending on the site and the degree of cell infection achieved by adenoviral vectors, may cause adverse effects, in general, on a specific target organ.
(45, 47). Hence, we also evaluated in the present study DTH responses to the adenosine. We first sensitized the animals with the UV-inactivated empty virus Ad0 at increasing doses; only with the highest sensitizing dose of 10⁸ PFU was a mild edematous response observed. We subsequently administered the virus emulsified in CFA to amplify the immune response, as reported in standard protocols for eliciting DTH responses to proteins (48), and then addressed the question of whether adenosine-delivered IL-10 could affect this response. We found that it could only inhibit DTH when injected before the sensitization phase, not when administered before antigenic challenge. These results suggest that IL-10 exerts its anti-inflammatory activity by blocking priming, i.e., the induction of Ag-specific CD4⁺ T-cells; once an expanded population of memory cells is present, IL-10 is no longer suppressive, probably because a different mechanism of memory cell reactivation or Ag presentation or different APCs are then involved. An alternative interpretation is that if AdIL-10 is administered halfway through the response, there is not enough time for the IL-10 to manifest its effects on an already initiated immune response.

In conclusion, our findings support the therapeutic role of homologous IL-10 in immune-mediated inflammatory disease states, and in arthritis in particular, and highlight the requirement for further comprehensive safety studies of adenosine-mediated gene therapy. This holds considerable promise, but its potential for human therapy still remains to be understood.

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References


