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Viral IL-10 and Soluble TNF Receptor Act Synergistically to Inhibit Collagen-Induced Arthritis Following Adenovirus-Mediated Gene Transfer[1]

Kwang-Nam Kim, Shohei Watanabe, Yuhe Ma, Sherry Thornton, Edward H. Giannini, and Raphael Hirsch[2]

Viral IL-10 (vIL-10) and soluble TNF receptor (sTNFR) are anti-inflammatory proteins that can suppress collagen-induced arthritis (CIA). These and related proteins have shown efficacy in the treatment of human rheumatoid arthritis; however, neither alone is able to completely suppress disease. Furthermore, they have short half-lives, necessitating frequent administration. To determine the ability of these proteins to act synergistically following gene transfer, arthritis was induced in DBA/1 male mice by immunization with type II collagen on days 0 and 21. Mice were injected i.v. either before disease onset (day 20) or after disease onset (day 28) with 10^{10} particles of adenovirus encoding vIL-10, a soluble TNF receptor-IgG1 fusion protein (sTNFR-Ig), a combination of both vectors, or a control vector lacking a transgene. Significant synergism was observed with the combination of vIL-10 and sTNFR-Ig, with a substantial reduction in both the incidence and severity of disease as well as inhibition of progression of established disease. sTNFR-Ig alone had no effect on CIA. vIL-10 alone inhibited disease when given before disease onset, but had minimal effect on established disease. Both proteins inhibited spleen cell proliferation and IFN-γ secretion in response to stimulation with type II collagen, but only vIL-10 reduced the synovial mRNA levels of the proinflammatory cytokines IL-1β, TNF-α, and IL-6. These findings demonstrate that vIL-10 and sTNFR-Ig act synergistically in suppressing CIA and suggest that gene transfer offers a potential therapeutic modality for the treatment of arthritis. *The Journal of Immunology,* 2000, 164: 1576–1581.

Rheumatoid arthritis (RA)[3] is an autoimmune disease characterized by both cellular and humoral responses. There exists a complex interaction among T cells, monocytes, and fibroblasts in the rheumatoid lesion, leading to perpetuation of the inflammatory process. Proinflammatory, monocyte-derived cytokines, such as IL-6, TNF-α, and IL-1β, are found in the synovium and play a major role in the progression of joint destruction (1, 2). Agents that inhibit secretion of these cytokines, or that can block their binding to cell surface receptors, are increasingly being viewed as potential therapeutic agents that might provide increased specificity compared with traditional drugs.

Delivery of these biologic agents represents particular challenges, including the production of sufficient quantities of purified material suitable for administration to humans. Gene transfer has been suggested as a more efficient means of delivery of these agents. In addition, it might allow one to achieve steady levels of the product, as opposed to the peaks and troughs associated with intermittent protein administration.

Two protein products of particular interest as therapeutic candidates for arthritis include soluble TNF receptor (sTNFR) and IL-10, both of which have undergone clinical studies in RA. These two products act on different components of the inflammatory process. sTNFR directly blocks the tissue destructive effects of TNF, whereas IL-10 indirectly inhibits inflammation by inhibiting T cell proliferation and secretion of proinflammatory cytokines by monocytes.

TNF is found in large quantities in the RA synovium and synovial fluid (1, 2). TNF induces the release of PGE_2 and collagenase by synovial cells, contributes to fibrosis, and facilitates inflammatory cell infiltration by promoting adhesion of neutrophils and lymphocytes to endothelial cells. It can also induce downstream events such as up-regulation of IL-1 and IL-6 secretion and up-regulation of T cell-proliferative responses. A divalent p75 sTNFR-Ig fusion protein (Etanercept; Immunex, Seattle, WA) has shown dramatic efficacy at reducing inflammation in patients with RA (3) and has recently been approved by the Food and Drug Administration for use in RA and juvenile rheumatoid arthritis.

IL-10 is also being studied in clinical trials for RA. IL-10 is a product of Th2 cells, B cells, and macrophages that has mixed immunosuppressive and immunostimulatory properties. It suppresses the production of proinflammatory cytokines by Th1 cells (4, 5) and monocytes (4–7). IL-10 can inhibit collagen-induced arthritis (CIA) (8–10) and is elevated in serum and synovial fluid of patients with RA (7, 11), where its overall effect on the inflammatory process is still debated. Elevated IL-10 levels correlated with rheumatoid factor titers and spontaneous IgM-rheumatoid factor production (11). These immunostimulatory effects may exacerbate disease and bear on its potential efficacy as a therapeutic agent.

Murine IL-10 and human IL-10 cDNA sequences exhibit a strong homology to an open reading frame in the EBV (human herpes virus 4) *Bam*HI C fragment rightward reading frame 1 (BCRF1), termed viral IL-10 (vIL-10) (12, 13). The human and vIL-10 mature protein sequences are 84% identical, with most of their divergence found in the NH_2-terminal 20 amino acids. vIL-10 shares many of the immunosuppressive properties of human IL-10 and murine IL-10, but lacks their immunostimulatory properties (14, 15). Recently, we and others have demonstrated that gene transfer of vIL-10 inhibits CIA (16–18) and can block invasion of cartilage by RA synoviocytes (19, 20). This may be a result of its ability to inhibit the T cell-proliferative response to type II collagen (CII) and to decrease mRNA levels of IL-1β in CIA synovium (16).

Although the antiarthritic properties of vIL-10 and sTNFR-Ig have been extensively studied, neither of them alone can entirely suppress established disease. Given the complexity and redundancy of the inflammatory milieu in the joint, it is unlikely that a single target can achieve complete inhibition of inflammation. The therapeutic effects of simultaneous administration of vIL-10 and sTNFR-Ig has not previously been evaluated. Simultaneous delivery of two or more genes is possible using adenoviral vectors, providing a convenient method for studying the effects of combination therapy for arthritis. To determine whether vIL-10 and sTNFR-Ig can act synergistically in autoimmune arthritis, CIA was induced in mice and the effects of gene transfer of vIL-10 and sTNFR-Ig were determined.

Materials and Methods

Mice

Male DBA/1J mice, 6–10 wk of age, were purchased from The Jackson Laboratory (Bar Harbor, ME), and housed under Institutional Animal Care and Use Committee-approved conditions in the animal resource facility at Children's Hospital Research Foundation (Cincinnati, OH).

Adenovirus constructs

Three recombinant E1a/E3-deleted replication-defective adenovirus type 5 vectors were used in this study. Ad(vIL-10) encodes the BCRF1 cDNA under the control of the elongation factor-1α promoter, the 4F2 heavy chain enhancer, and the bovine growth hormone polyadenylation site. Ad-(*Bgl*II), which was used as a control, lacks the BCRF1 cDNA, the elongation factor-1α promoter, the 4F2 heavy chain enhancer, and the bovine growth hormone polyadenylation site, but is otherwise identical to Ad(vIL-10). These constructs were generously provided by J. A. Bluestone and J. M. Leiden (University of Chicago, Chicago, IL). Ad(sTNFR-Ig) encodes a divalent human p55 sTNFR-Ig/murine IgG1 fusion protein under control of the CMV promoter (21) and was generously provided by B. Beutler (University of Texas, Southwestern, Dallas, TX). Recombinant adenoviruses were produced and propagated in 293 cells and purified by cesium chloride density centrifugation as described previously (22, 23). Viruses were plaque purified three times before the production of seed stocks, and their identities were confirmed by restriction endonuclease and DNA sequence analysis. Viral titers (particles/ml) were determined by $A_{260} \times 10^{12}$ following lysis of viral stocks in 0.1% SDS, 10 mM Tris-HCl (pH 7.4), and 1 mM EDTA at 56°C for 10 min.

Treatment protocols

Arthritis was induced with bovine CII (Elastin Products, Owensville, MO) as described previously (24). Mice were injected intradermally with 100 μg of CII in CFA at the base of the tail on days 0 and 21. Adenovirus was administered on day 20 in 100 μl of buffer containing 10 mM Tris (pH 7.4), 1 mM MgCl$_2$, 10% (v/v) glycerol by i.v. injection as described previously (16). Mice were divided into four groups: control group, 2×10^{10} particles of the control vector Ad(*Bgl*II); sTNFR-Ig group, 10^{10} particles of Ad(sTNFR-Ig) plus 10^{10} particles of Ad(*Bgl*II); vIL-10 group, 10^{10} particles of Ad(vIL-10) plus 10^{10} particles of Ad(*Bgl*II); and sTNFR-Ig/vIL-10 group, 10^{10} particles of Ad(vIL-10) plus 10^{10} particles of Ad(sTNFR-Ig). Mice were evaluated several times a week for arthritis using an established macroscopic scoring system (25) ranging from 0 to 4 (0, no detectable arthritis; 1, swelling and/or redness of paw or one digit; 2, two joints involved; 3, three or four joints involved; and 4, severe arthritis of the entire paw and

digits). The arthritic index for each mouse was calculated by adding the four scores of the individual paws. At various time points, serum samples were collected and kept at −20°C for further analysis. Paws were frozen in liquid nitrogen immediately after harvesting and kept at −80°C for mRNA analysis.

vIL-10 measurement

Titers of vIL-10 were determined by ELISA. ELISA plates were coated overnight at 4°C with 1 μg/ml of the rat anti-mouse IL-10 mAb, JES3–9D7 (PharMingen, San Diego CA), washed (PBS-Tween 20), and blocked by overnight incubation at 4°C with 100 μl of PBS containing 1% BSA. After washing, 50-μl aliquots of the serum samples were added to duplicate wells and incubated at 4°C overnight. After washing, 100 μl of a 1 μg/ml dilution of the biotin-conjugated rat anti-viral IL-10 mAb, JES3-6B11 (PharMingen), was added to each well and incubated at room temperature for 1 h. Plates were washed and developed with peroxidase substrate system 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Kirkegaard & Perry Laboratories, Gaithersburg MD). The plates were read at 410 nm on a kinetic microplate reader (Molecular Devices, Menlo Park, CA). OD readings for the duplicate wells were averaged and subtracted from readings of control serum. The absolute concentration of vIL-10 in serum was calculated using a standard curve generated with dilutions of purified vIL-10.

sTNFR-Ig measurement

Titers of sTNFR-Ig were measured by assaying TNF inhibitory activity as described previously (21). Briefly, samples were serially diluted and 1 μl of each dilution was incubated at 37°C in separate wells of a 96-well plate with murine TNF-α at a final concentration of 1 ng/ml in the presence of 100 μg/ml cycloheximide. After 1 h, 7×10^4 SKMEL-109 cells (sensitive to lysis in the presence of TNF-α) were added to each well, and the incubation was continued overnight. The plates were then washed, stained with crystal violet, washed again, and surviving cells were quantitated by solubilizing the stain in 50% acetic acid and measuring OD at 490 nm. One neutralizing unit was defined as the quantity of inhibitor required to neutralize 150 pg of murine TNF-α. Control sera did not contain any TNF-neutralizing activity.

Anti-CII Ab measurement

The titers of anti-CII Abs in the serum samples were determined by ELISA as described previously (25). All samples were measured in duplicate. Peroxidase-labeled goat anti-mouse IgG and IgM (Kirkegaard & Perry Laboratories) was used to measure CII-specific total Ig. IgG1 and IgG2a were measured using biotinylated rat anti-mouse IgG1 or IgG2a (PharMingen), followed by streptavidin-peroxidase. Plates were developed with peroxidase substrate system 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (Kirkegaard & Perry Laboratories). The plates were read at 410 nm as described above. OD readings for the duplicate wells were averaged. A serum sample from one of the control mice was tested at various dilutions and used as a standard to generate a curve from which relative titers of the other serum samples were calculated.

In vitro T cell function

A total of 5×10^5 spleen cells/well were plated in triplicate at 37°C in 96-well flat-bottom microtiter plates in DMEM containing 0.5% normal DBA/1 mouse serum in the presence of heat-denatured (56°C for 10 min) CII. For IFN-γ assays, supernatants were harvested after 24 h. For proliferation assays, cells were incubated for 72 h, followed by addition of 1 μCi of [^{3}H]TdR for an additional 18 h. Cells were harvested and [^{3}H]TdR incorporation per well was measured and averaged for each triplicate. Background counts from unstimulated cells were subtracted from each group. IFN-γ was measured by ELISA, as described previously (25), using anti-IFN-γ mAbs R46A2 and biotin-labeled XMG1.2 (PharMingen).

mRNA analysis

Paws were homogenized with a Tissue Tearor (Biospec Products, Bartlesville, OK) and RNA was extracted with RNASTAT 60 (Tel-Test, Friendswood, TX). RNase protection assays (RPA) were performed as described previously (26) on 2 μg of RNA utilizing the Riboquant MultiProbe RNase Protection Assay System (PharMingen) following the manufacturer's instructions. Each commercial kit contained a set of cytokine/chemokine templates as well as a template for the housekeeping gene *GAPDH*. The mRNA level of each cytokine was expressed as the ratio of the phosphor imager units (storm 860 and Image Quart software; Molecular Dynamics, Sunnyvale, CA) of the cytokine to those of *GAPDH* from the same RNA sample.
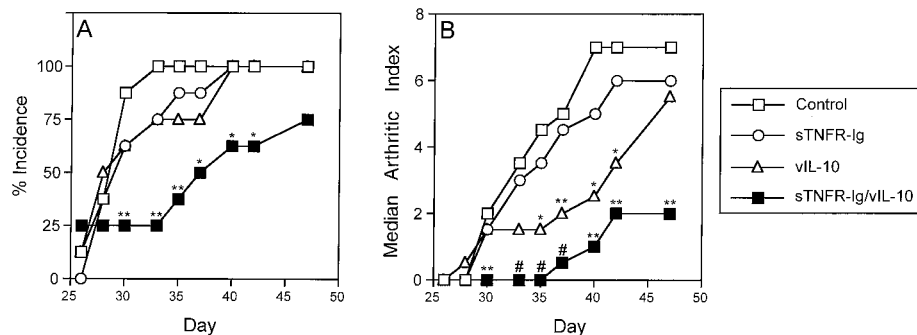
FIGURE 1. Effect of gene transfer of vIL-10 and sTNFR-Ig on prevention of CIA. Mice were immunized with CII as described in *Materials and Methods*. On day 20, 10 mice/group received i.v. injections of one of the following combinations of adenovirus: control (□), Ad(*Bgl*II); sTNFR-Ig (○), Ad(sTNFR-Ig) plus Ad(*Bgl*II); vIL-10 (△), Ad(vIL-10) plus Ad(*Bgl*II); sTNFR-Ig/vIL-10 (■), Ad(vIL-10) plus Ad(sTNFR-Ig). ∗, $p < 0.05$; ∗∗, $p < 0.01$; #, $p < 0.001$.

Statistical analysis

χ^2 with 1 df was used to test categorical data for statistical significance. All comparisons were performed between treated and control mice. Intergroup differences were compared either by the Mann-Whitney U test if the data were nonparametric (arthritic index) or by the two-tailed independent t test if the data were parametric (disease incidence). Comparisons of more than two means were done using the one-way ANOVA. If the F ratio was significant by ANOVA, Tukey's honest significant difference multiple comparison test was used for pairwise comparisons. Uncorrected p values <0.05 were considered to be statistically significant.

Results

Effect of gene transfer of vIL-10 and sTNFR-Ig on CIA

We and others have previously reported that vIL-10 gene transfer can inhibit CIA when given before disease onset (16–18). However, the treatment does not completely prevent CIA or have a significant effect on established disease. To determine whether TNF blockade can act synergistically with vIL-10, mice were treated with adenoviral vectors encoding each cytokine, either alone or in combination. Mice were immunized with CII on days 0 and 21. On day 20, mice received i.v. injections of one of the following combinations of adenovirus: the control vector Ad(*Bgl*II), Ad(sTNFR-Ig) plus Ad(*Bgl*II), Ad(vIL-10) plus Ad(*Bgl*II), or Ad(vIL-10) plus Ad(sTNFR-Ig). Significant synergism was observed with the combination of vIL-10 and sTNFR-Ig on both the incidence and severity of disease (Fig. 1). As we have previously reported (16), vIL-10 alone reduced disease severity; however, the combination of vIL-10 and sTNFR-Ig was more effective. sT-

NFR-Ig alone had no effect on either the incidence or severity of CIA.

To determine the effects on progression of established disease, mice were treated with the same combination of vectors on day 28, when 85% of the mice had developed arthritis (Fig. 2). Again, significant synergism was observed with the combination of vIL-10 and sTNFR-Ig. vIL-10 alone had a mild effect that did not reach statistical significance. sTNFR-Ig alone was ineffective.

To ensure that the observed lack of effect of sTNFR-Ig was not a consequence of insufficient sTNFR-Ig production by vector-transduced cells, serum levels of sTNFR-Ig and vIL-10 were measured following gene transfer. sTNFR-Ig serum levels were found to be quite high and remained so for the duration of the study (Fig. 3). In fact, sTNFR-Ig persisted at stable levels for at least 40 days, as compared with vIL-10, which peaked at day 3 and was undetectable by day 10. The difference in half-lives of the two gene products can be attributed to the more rapid development of neutralizing Abs against vIL-10, as previously reported with these adenoviral vectors (16, 21).

Effect of gene transfer of vIL-10 and sTNFR-Ig on paw mRNA levels of proinflammatory cytokines

One trivial explanation for the observed synergism between vIL-10 and sTNFR-Ig was that sTNFR-Ig might prolong the expression of the vIL-10-encoding virus by inhibiting either the anti-adenovirus or the anti-vIL10 immune response. However, the simultaneous administration of Ad(vIL-10) and Ad(sTNFR-Ig) neither raised the serum level of vIL-10 nor prolonged its half-life (data not shown).
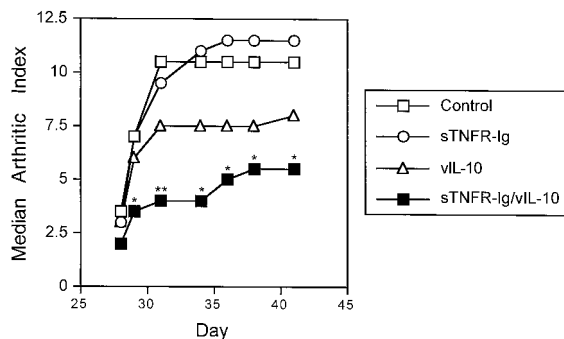

FIGURE 2. Effect of gene transfer of vIL-10 and sTNFR-Ig on established CIA. Mice were immunized with CII as described in *Materials and Methods*. On day 28, when 85% of the mice had developed arthritis, all mice were randomly divided into four groups of 10 and administered adenovirus by i.v. injection. ∗, $p < 0.05$; ∗∗, $p < 0.01$.
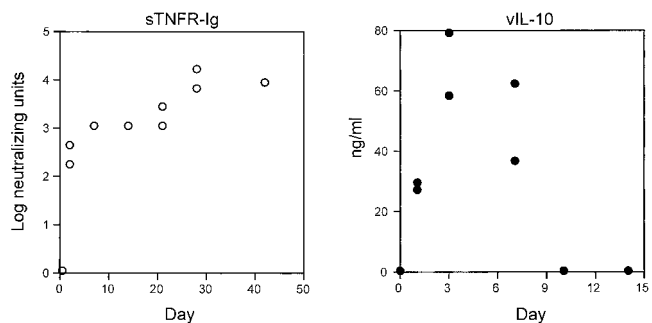

FIGURE 3. Serum levels of vIL-10 and sTNFR-Ig following i.v. administration of Ad(vIL-10) or Ad(sTNFR-Ig). Mice were injected with 10^{10} particles on day 0. Two mice from each group were sacrificed at each time point, and sera were tested as described.
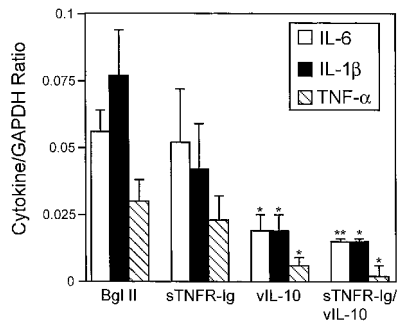
FIGURE 4. Effect of gene transfer of vIL-10 and sTNFR-Ig on mRNA levels of synovial proinflammatory cytokines. Mice were immunized with CII as described in *Materials and Methods*. On day 20, three mice per group received i.v. injections of adenovirus. Mice were sacrificed on day 32 and hind paws were analyzed by RPA. Each bar represents the mean ± SEM of three mice per group. *, $p < 0.05$; **, $p < 0.01$.

To determine the reasons for the ineffectiveness of sTNFR-Ig alone on CIA as well as potential mechanisms of synergy with vIL-10, the synovial tissues of treated mice were analyzed for proinflammatory cytokine mRNA. We have previously demonstrated that vIL-10 reduces mRNA levels of the proinflammatory cytokine IL-1β in the paws of mice with CIA (16). To determine the effects of sTNFR-Ig on mRNA levels of proinflammatory cytokines, paws of treated mice were removed on day 32 and mRNA levels were quantitated by RPA. vIL-10, but not sTNFR-Ig, significantly reduced synovial mRNA levels of IL-1β, TNF-α, and IL-6 (Fig. 4). The combination of[1] sTNFR-Ig and vIL-10 had an additive effect, but this was not statistically significant.
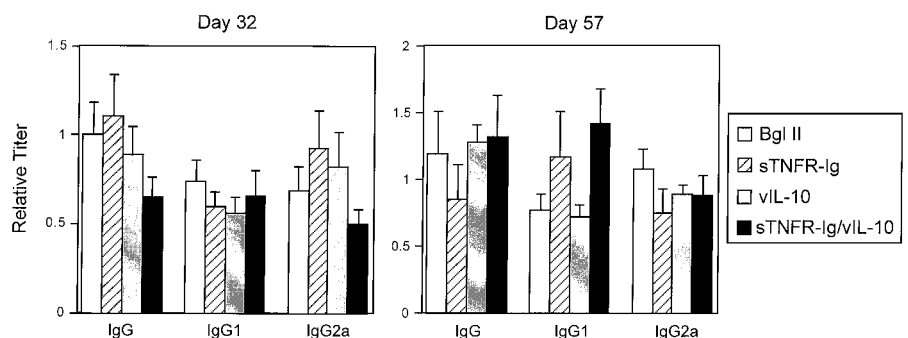
Effect of gene transfer of vIL-10 and sTNFR-Ig on B cell responses

To determine the effects of vIL-10 and sTNFR-Ig on autoimmune responses, humoral and cellular responses to CII were analyzed. To determine the effects on the humoral immune response to CII, mice were bled on days 32 and 57 and sera were analyzed for Abs to CII. Ab levels varied between mice, but no significant differences were observed between the groups in circulating titers of total IgG, IgG1, or IgG2a anti-CII Abs (Fig. 5). There was, however, a trend toward decreased total Ig and IgG2a in the mice receiving Ad(vIL-10) plus Ad(sTNFR-Ig).

Effect of gene transfer of vIL-10 and sTNFR-Ig on T cell responses

We have previously demonstrated that the anti-CII T cell response is inhibited following vIL-10 gene transfer (16). To determine the influence of sTNFR-Ig on T cell responses, DBA/1 mice were immunized with CII on days 0 and 21. Adenovirus was injected on day 20. Three spleens per group were removed on day 33 and

cultured with CII. Both vIL-10 and sTNFR-Ig, as well as the combination, completely inhibited IFN-γ secretion in response to CII (Fig. 6). Proliferation was significantly reduced by vIL-10 and the combination of vIL-10 and sTNFR-Ig, but not by sTNFR-Ig alone.

Discussion

IL-10 and sTNFR-Ig have generated considerable interest as therapeutic agents for RA due to their anti-inflammatory properties. Both products have been tested in clinical trials using s.c. injection. Thus far, sTNFR-Ig appears to be more efficacious and a sTNFR-Ig fusion protein (Etanercept) has been approved for human use. However, IL-10 continues to be of interest as a therapeutic protein because of its potent immunosuppressive properties in vitro and in animal models.

Because neither of these proteins completely inhibits disease, the present study investigated the effects on arthritis of simultaneous administration of sTNFR-Ig and vIL-10 by i.v. gene delivery. Following i.v. injection of adenovirus, transgenes are expressed predominantly in the liver (27, 28), although we have also recently observed low-level expression in the joints (S. Watanabe and R. Hirsch, manuscript in preparation). Given the large number of inflammatory mediators in the arthritic synovium, achieving disease remission by blocking a single early mediator in the inflammatory cascade is probably unrealistic. Therefore, the observed synergism between vIL-10 and sTNFR-Ig is of potential therapeutic interest and suggests that strategies that target multiple proinflammatory pathways may be more effective than targeting a single effector molecule. These findings are reminiscent of the synergism recently observed between the identical sTNFR-Ig-encoding adenoviral construct and IL-1 receptor antagonist in a rabbit model of arthritis (29).

The mechanism of the synergism observed with vIL-10 plus sTNFR-Ig is not entirely clear. One possibility is that vIL-10 alone is so effective at down-regulating the production of proinflammatory cytokines in the joint that the potential additive effect of sTNFR-Ig is difficult to measure, although it may be biologically significant. There was a trend toward greater inhibition of IL-6, IL-1α, and TNF-α with the combination treatment, and, at least for IL-6, the level of significance was substantially greater for the combination ($p = 0.007$) as compared with vIL-10 alone ($p = 0.021$). Also, a trend toward decreased IgG2a anti-CII Abs was observed with combination treatment on day 32, suggesting a shift toward a protective Th2 response.

In the present study, vIL-10 was more effective than sTNFR-Ig at inhibiting murine CIA. This finding is supported by our own earlier studies (16) as well as those of others (17, 18) demonstrating that Ad(vIL-10) can either completely prevent or markedly delay disease onset. The effect of vIL-10 is lost as the serum titer drops secondary to development of anti-vIL-10 Abs (16). We cannot rule out the possibility that neutralizing anti-vIL-10 Abs might

FIGURE 5. Effect of vIL-10 and sTNFR-Ig gene transfer on anti-CII Abs. Mice were immunized with CII as described in *Materials and Methods*. On day 20, eight mice per group received i.v. injections of adenovirus. Titers of anti-CII total Ig, IgG1, and IgG2a Abs were measured by ELISA. Dilutions of a single serum sample from a CII-immunized mouse were used to generate a standard curve from which relative titers of the remaining samples were calculated. Each column represents the mean titers ± SEM of eight individual mice.

FIGURE 6. Effect of gene transfer of vIL-10 and sTNFR-Ig on T cell responses from spleens. Mice were immunized with CII as described in *Materials and Methods*. On day 20, three mice per group received i.v. injections of adenovirus. Mice were sacrificed on day 33, and spleen cells were stimulated with 100 μg/ml of heat-denatured CII. IFN-γ was measured at 24 h. Proliferation was measured at 72 h. The stimulation index represents the proliferation of cells incubated with CII divided by the proliferation from cells incubated in media. Each bar represents the mean $\pm$ SEM of three mice. $*$, $p < 0.05$.
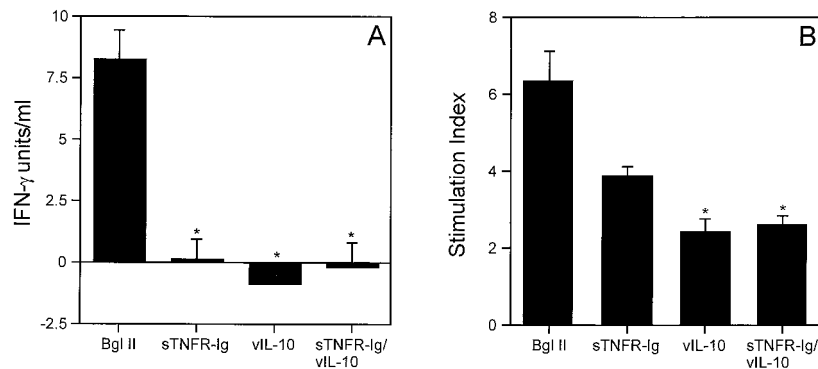
also neutralize protective endogenous murine IL-10. However, this possibility is unlikely, since neither murine IL-10 protein nor mRNA was detected in the sera or the joints of any of the mice, including the controls.

The effects of TNF blockade in murine CIA appear to be more complex. TNF clearly plays a role in the acute phase of CIA, although it might not be as critical a mediator as other cytokines. Analysis of paw mRNA in early CIA demonstrates a preponderance of IL-2, IL-1β, and IL-6, with TNF-α being less dominant (26). Similarly, TNF-α protein is detected in CIA only during the first few days of arthritis (30). The effects of TNF blockade appear to depend both on the timing and duration of blockade as well as the neutralizing agent. For instance, Abs to TNF have been found to either have no effect on established disease (31) or to inhibit disease onset as well as established disease (32). sTNFR also can inhibit CIA. A divalent p75 sTNFR-Ig fusion protein inhibited disease onset as well as established disease (33). A p55 divalent sTNFR-Ig fusion protein similar to the one used in the present study inhibited established disease in one study (34) and disease onset in another study (35). The identical sTNFR-Ig-encoding adenoviral construct used in the present study had a modest inhibitory effect in rat CIA (36).

Our observation that sTNFR-Ig alone could not inhibit disease, despite high TNF-neutralizing titers, correlated with the inability of sTNFR-Ig to block up-regulation of proinflammatory gene products in the synovium. These findings support the recent study of Quattrocchi et al. (37) which utilized the identical p55 sTNFR-Ig-encoding adenoviral vector. In that study, a modest inhibitory effect was observed during the first 10 days of treatment followed by an exacerbation of disease despite continued circulating sTNFR-Ig. This inefficacy was not observed with anti-TNF mAb, raising the intriguing possibility that sTNFR has additional effects beyond TNF neutralization. Our own observations also suggest a mild exacerbation of established disease after day 35, although this did not reach statistical significance (see Fig. 2). Quattrocchi et al. (37) hypothesized that prolonged blockade of TNF is harmful, perhaps by interfering with the up-regulation of homeostatic mechanisms such as production of IL-10, TGF-β, or other mediators that are likely to be involved in limiting inflammation. Thus, prolonged blockade of TNF before disease onset might augment T cell responses, contributing to the observed lack of efficacy of sTNFR-Ig when administered 7–10 days before disease onset.

Depending on the timing and dose, TNF can be either proinflammatory or anti-inflammatory. For instance, it has recently been shown that TNF knockout mice fail to regulate and limit an in vivo inflammatory response (38), and TNF can inhibit T cell responses and suppress autoimmunity in NZBXNZW and NOD mice (39). TNF is also capable of enhancing T cell responses (40). Thus, the inhibitory effects of sTNFR-Ig on the T cell response to CII is not

unexpected. Clearly, the mechanisms of action of these mediators are complex and will require further investigation.

The present study supports earlier observations suggesting that adenoviral vectors can be used to simultaneously deliver multiple therapeutic genes with sufficient efficiency to control arthritis in animals. The observation that stable serum levels of sTNFR-Ig were achieved for over 40 days following gene transfer suggests that even "first-generation" adenoviral vectors, such as those used in the present study, can allow long-term transgene expression, depending on the half-life and immunogenicity of the gene product. Thus, with further modifications, such as removal of all viral genes to reduce the cytotoxic antiadenoviral response, such vectors might ultimately be useful in patients with chronic rheumatic diseases.

Acknowledgments

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