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Reduction of Inflammatory Cytokines and Prostaglandin E₂ by IL-13 Gene Therapy in Rheumatoid Arthritis Synovium

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The rheumatoid arthritis (RA) joint is characterized by an inflammatory synovial pannus which mediates tissue destruction. IL-13 is a cytokine that inhibits activated monocytes/macrophages from secreting a variety of proinflammatory molecules. The aim of this study was to examine whether gene therapy-delivered IL-13 could reduce the production of key proinflammatory mediators in RA synovial tissue (ST) explants. Adenoviral vectors encoding the genes for human IL-13 (AxCAIL-13) and bacterial β-galactosidase were generated and examined for protein production. Vectors were used to infect RA ST explants and RA synovial fibroblasts, and conditioned medium (CM) was collected at various times for analysis by ELISA and competitive immunoassay. AxCAIL-13 decreased the production of RA ST explant proinflammatory IL-1β by 85% after 24 h. Likewise, TNF-α levels were decreased by 82 and 75% whereas IL-8 levels were reduced 54 and 82% after 24 and 48 h, respectively, in RA ST explant CM. Monocyte chemotactic protein-1 concentrations were decreased by 88% after 72 h in RA ST explant CM. RA ST explant epithelial neutrophil-activating peptide-78 concentrations were decreased 85 and 94% whereas growth-related gene product-α levels were decreased by 77 and 85% at 24 and 48 h, respectively, by AxCAIL-13. Further, IL-13 significantly decreased PGE₂ and macrophage inflammatory protein-1α production. These results demonstrate that increased expression of IL-13 via gene therapy may decrease RA-associated inflammation by reducing secretion of proinflammatory cytokines and PGE₂.


Macrophage-derived cytokines are important mediators of pathophysiologic events and are likely involved in the inflammation and tissue destruction in rheumatoid arthritis (RA) joints (1). The RA synovial tissue (ST) contains a thickened synovial lining layer as well as a subsynovium invaded by a variety of leukocytes (2). In late stage RA, joint inflammation is likely related to an imbalance favoring pro- over antiinflammatory mediators. Many of the conspicuous proinflammatory mediators are monocyte/macrophage derived, whereas some of the low level antiinflammatory proteins are produced by lymphocytes. Because joints are difficult to target by traditional routes of drug delivery, treatment of the RA joint by gene therapy has been proposed and holds promise for regulating monokine-directed cellular ingress (3). Although many candidate therapeutic proteins may aid in the prevention or treatment of RA, a number of lymphocyte-produced Th2 cell products are known to have antiinflammatory effects on activated monocytes. We focus here on increasing the expression of Th2-produced IL-13 by gene therapy to determine whether it may help rebalance the askew synovial cytokine equilibrium.

IL-13 has profound effects on monocytes in vitro, acting not simply to deactivate them but rather in a complex manner to change monocytic morphology, phenotype, function, and cytokine production (4). IL-13 can maintain these antiinflammatory properties in vivo, as demonstrated by suppressing experimental autoimmune encephalomyelitis in rats (5). Perhaps most alluring to those interested in rebalancing the predominant inflammatory cytokine profile of the RA joint is the profound inhibition of IL-13 on the production of proinflammatory cytokines by LPS-stimulated monocytes. In tandem with these properties, which are shared with other Th2-produced cytokines, we have found previously that only low concentrations of IL-13 are present in the synovium and synovial fluids of RA patients (6). In this study, we determined whether IL-13 would have an antiinflammatory effect in a ex vivo setting involving many cell types, a situation closer to the complex reality of inflamed RA synovium. Further, we wished to examine how a gene therapy delivery system would compare with addition of recombinant human (rh) cytokine in a short term study. Therefore, we prepared an adenoviral vector that produced IL-13 and determined its ability to impact the protein concentrations of key inflammatory mediators in a tissue explant model of RA.

Materials and Methods

Patients

RA ST specimens met the revised criteria established by the American College of Rheumatology (7). RA fibroblast studies included cells from one male and five female patients with a mean age of 41.8 years. ST explant studies were performed using tissue from one male and six female RA patients with a mean age of 63 years.
Adenoviral preparation, propagation, purification, and titer determination

Adenoviral vectors containing the lacZ or the human IL-13 genes were prepared via homologous recombination in 293 cells as previously described (8). These genes were under the control of the chicken β-actin promoter and the CMV enhancer of pAXCaWt (9). pAXCaWt is a 45-kb cosmid containing the full length sequence of type 5 adenovirus deleted of E1A, E1B, and E3 regions (10). The cosmid was cotransfected with EcoC2I-digested AdMX-terminal protein complex of AdlX into 293 cells (11). Individual clones were screened by DNA sequence and appropriate protein production (8). Virus from plaques was propagated through successive infection and harvesting of cell lysates. Viral purification was accomplished using cesium chloride density ultracentrifugation and dialysis (12). Viral titer was estimated via the number of PFUs of virus in 293 cells.

TF-1 proliferation assay

Adenoviruses containing the IL-13 gene (AxCaCIL-13)-infected RA ST conditioned medium (CM) was assessed for the ability to induce TF-1 cell proliferation when preincubated with neutralizing anti-IL-13 Ab. TF-1 cells were grown in RPMI 1640 with 10% FBS and 5 ng/ml human granulocyte macrophage-CSF (a gift of Immunex, Seattle, WA). Cells were GM-CSF depleted for 24 h before the assay, and RPMI + 5% FBS was used for all assay dilutions as well as a blank. Cells were washed twice in RPMI containing 5% FBS, resuspended in RPMI + 5% FBS (1 × 10^5 cells/ml). Triplicate wells containing 5000 cells each were incubated for 72 h with various dilutions of CM from RA ST explants infected with AxCaCIL-13. RA ST explant CM were incubated with 20 ng/ml mouse anti-human IL-13 Ab (R&D Systems, Minneapolis, MN) or a mouse IgG1 isotype control for 1 h at 37°C immediately before the proliferation assay.

CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI) was used to determine relative cell concentrations.

RA ST fibroblast isolation and maintenance

ST from RA patients were minced and digested in an enzymatic cocktail for 2 h at 37°C as previously described (13). ST fibroblasts were cultured in the presence of medium with 10% serum and antibiotics. Passage 3–7 cells were used and considered a homogeneous population of fibroblasts. Fifty thousand cells were seeded per well (24-well plates, Nunc, Naperville, IL) in 1 ml RPMI + 10% FBS. At 80% confluence, cells were removed by trypsinization, and their viability was determined by trypan blue exclusion. To determine whether the IL-13 produced by AxCaCIL-13 was biologically active, we used a TF-1 proliferation assay (Fig. 1a).

Fibroblasts were infected with AxCaCIL-13 or adenovirus containing the lacZ gene (AxCaAlacZ) at a multiplicity of infection of 10. Viral infections were performed for 4 h in 0.5 ml RPMI + 5% FBS. After infection, cells were washed in PBS and 2 ml RPMI + 10% FBS were replaced. When indicated, stimulants were added to wells 1 h after the medium was replaced, and the plate was slowly rotated for 2 min to evenly distribute the cytokine. Recombinant human (rh)-IL-13 (Upjohn, Kalamazoo, MI) was used at 600 U/ml (30 ng/ml), and TNF-α (Upjohn) was used at 440 U/ml (34 ng/ml) as we have done previously (14, 15). CM was collected at various time points and frozen at −20°C until inflammatory mediators were assessed by ELISA or competition assay. To investigate whether stimulants may increase adenoviral infectivity, we compared viral DNA from stimulated and nonstimulated RA synovial fibroblast samples. DNA was isolated from cultured synovial fibroblasts using a Qiagen Cell Culture DNA kit (Qiagen, Valencia, CA) under suggested conditions. Briefly, cells were lysed, RNase treated, and bound to anion-exchange resin under low salt and pH conditions. Digested RNA, proteins, and low m.w. impurities were washed away by a medium-salt wash. Genomic and viral DNA was eluted in a high salt buffer, then concentrated and desalted via isopropanol-ethanol precipitations. Stimulated and nonstimulated preparations were analyzed side by side in a 1.0% agarose gel. Analysis of adenoviral DNA bands was confirmed by Southern blotting using a biontin-conjugated adeno-viral probe (Enzo Diagnostics, Farmingdale, NY) in conjunction with a streptavidin–alkaline phosphatase detection system.

RA ST explant isolation, infection, addition of rHL-13, and preparation of CM

RA synovium from patients undergoing joint replacements were processed aseptically. The synovium was minced into 1-mm^3 pieces. ST explants were weighed, placed in a 24-well plate, and infected with adenovirus (1 × 10^8 PFU/well) for 16 h in RPMI + 5% FBS. After infection, tissues were carefully washed with PBS and medium was replaced. In studies that included addition of exogenous cytokine, uninfected cells were washed and medium was replaced including 25 ng/ml rhIL-13. Plates were slowly rotated for 3 min to distribute materials after addition of adenovirus or rhIL-13. Tissues were cultured at a wet tissue weight:medium ratio of 0.25 g tissue/1 ml RPMI + 10% FBS. CM was collected at various time points and frozen at −20°C until assayed.

ELISA

Cytokine quantities were determined using ELISA or immunoassay systems that were commercially available and used in accordance with the procedure of the manufacturer. Quantification of IL-1β, TNF-α, soluble intercellular adhesion molecule-1 (sICAM-1), soluble CD44 (sCD44), and PGE, was performed using kits from Cayman Chemical (Ann Arbor, MI). IL-8, monocyte chemotactic protein-1 (MCP-1), epithelial neutrophil-activating peptide-78 (ENA-78), macrophage-inflammatory protein (MIP)-1α, RANTES, and growth-related gene product α (groα) levels were determined by kits purchased from R&D Systems. IL-13 levels were determined using a kit from Biosource International (Camarillo, CA).

Statistics

High patient-to-patient variability was demonstrated for a number of cytokines. Therefore, the Wilcoxon rank order statistical analysis and a paired Student’s t test were performed. The log-transformed raw values were used to determine significance for all molecules.

Results

Production of biologically active IL-13 by AxCaCIL-13

Infection of RA synovial fibroblasts with AxCaCIL-13 resulted in the production of IL-13 in CM as detected by ELISA (Fig. 1a). Fibroblasts were infected with AxCaCIL-13 or AxCaAlacZ at a multiplicity of infection of 10. CM was collected from 8 to 72 h from fibroblasts that were not stimulated, stimulated with IL-1β, or TNF-α. CM from AxCaCIL-13-infected samples produced appreciable quantities of IL-13 after 24–72 h, whereas AxCaAlacZ-infected samples, regardless of stimulation conditions, did not. Fibroblasts infected with AxCaCIL-13 produced >2 ng/ml after 72 h. However, stimulation with IL-1β significantly increased IL-13 production from RA synovial fibroblasts after 48 and 72 h, by comparison with nonstimulated cells. TNF-α stimulation likewise increased IL-13 production after 24–72 h (Fig. 1a). To determine whether this was the result of increased adenoviral infectivity into these samples, DNA from stimulated and nonstimulated samples were analyzed by Southern blots. A single DNA band was detected by an adenoviral probe, which corresponded with the major DNA band present on the agarose gel. Comparison of stimulated vs nonstimulated AxCaCIL-13-infected RA synovial fibroblast samples showed no difference in the level of adenoviral DNA (data not shown), suggesting that stimulation with IL-1β or TNF-α did not increase adenoviral infectivity.

RA ST explants also produced higher quantities of IL-13 after infection with AxCaCIL-13 than did AxCaAlacZ infection (Fig. 1b). The quantity of IL-13 produced by ST explants was greater than that produced by fibroblasts, exceeding 22 ng/ml at all times. By comparison, the majority of CM from AxCaAlacZ-infected RA ST explants were below the limit of assay detection. Based on these data, later studies that compare addition of exogenous IL-13 with that which is virally produced were performed with 25 ng/ml rhIL-13, because this was the approximate quantity produced by adenovirus at all time points.

To determine whether the IL-13 produced by AxCaCIL-13 was biologically active, we used a TF-1 proliferation assay (Fig. 1c) (16). CM from AxCaCIL-13-infected RA ST explants were compared in the presence of neutralizing anti-IL-13 Ab or an isotype control for their relative ability to induce TF-1 proliferation. A range of dilutions using 72 h CM were assayed. The results demonstrate that CM preincubated with anti-IL-13 failed to induce TF-1 cell proliferation, whereas the same CM incubated with an...
isotype control (mouse IgG1) did (p < 0.05; n = 6). In addition, the effect was dose dependent where the more concentrated CMs induced the greatest levels of TF-1 cell mitogenesis.

**IL-1β and TNF-α levels are reduced in AxCAIL-13-infected RA ST explant CM**

The quantity of IL-1β was measured by ELISA in CM from AxCAIL-13- and AxCAlacZ-infected RA ST explants (Fig. 2A). IL-13 decreased mean IL-1β concentrations by 85% at 24 h (p < 0.05; n = 6) as well as by 77 and 26% at 48 and 72 h, respectively.

TNF-α concentrations were also reduced by exposure to adenovirally produced human IL-13 (Fig. 2B). TNF-α levels in CM from AxCAIL-13-infected RA ST explants were decreased 82 and 75% at 24 and 48 h, respectively (p < 0.05; n = 6). Additionally, TNF-α concentrations were decreased by 81% after 72 h.

**The CXC chemokines IL-8, ENA-78, and groα are all decreased by AxCAIL-13 infection of RA ST explants**

The quantity of the angiogenic CXC chemokine IL-8 was measured by ELISA in CM from AxCAIL-13 and AxCAlacZ-infected RA ST explants (Fig. 3A). Adenoviral human IL-13 decreased the production of IL-8 by 54 and 82% after 24 and 48 h, respectively (p < 0.05; n = 6). Additionally, IL-8 levels were decreased by 74% after 72 h following AxCAIL-13 exposure. An assessment of IL-8 concentrations in IL-1β-stimulated RA synovial fibroblast CM was also performed (Table I). AxCAIL-13 infection did not have significant effects on IL-8 concentrations by comparison with AxCAlacZ in IL-1β-stimulated fibroblasts.

Because IL-13 appeared capable of reducing the production of chemokines that potentially mediate inflammation in the RA synovium, we investigated its effects on ENA-78 and groα. ENA-78 levels in CM from RA ST explants were determined by ELISA.
(Fig. 3B). AxCAIL-13 reduced the secreted quantity of ENA-78 by 85 and 94% at 24 and 48 h, respectively \((p < 0.05; n = 6)\). Further, ENA-78 levels were decreased by 92% after 72 h.

Groα quantities were likewise determined in CM from RA ST explants by ELISA (Fig. 3C). AxCAIL-13 production of IL-13 decreased the levels of groα in CM by 77 and 85% after 24 and 48 h, respectively \((p < 0.05; n = 6)\). These concentrations were reduced by only 43% after 72 h.

The CC chemokines MCP-1 and MIP-1α are decreased by AxCAIL-13 infection of RA ST explants whereas RANTES is not MCP-1, another chemokine implicated in RA, was likewise analyzed in AxCAIL-13- and AxCAlacZ-infected RA ST explant CM (Fig. 4A). In a similar manner, adenovirally delivered IL-13 decreased MCP-1 levels by 65 and 68% after 24 and 48 h, respectively \((n = 7)\). MCP-1 levels were decreased by 88% after 72 h \((n = 7; p < 0.05)\). RA synovial fibroblast CM were also examined using MCP-1 ELISA for an effect of AxCAIL-13 (Table I). In contrast to RA ST explants, adenovirally produced human IL-13 significantly increased MCP-1 at all time points examined \((p < 0.05; n = 7)\) in RA fibroblast CM.

MIP-1α levels were also assessed in RA ST explant CM infected with AxCAIL-13 and AxCAlacZ (Fig. 4B). Similar to MCP-1 concentrations, MIP-1α concentrations were 87, 95, and 68% decreased after 24, 48, and 72 h, respectively \((n = 6; p < 0.05)\).

RA ST explant CM concentrations of the CC chemokine RANTES were also assessed. Mean RANTES levels were reduced by 50 and 74% after 24 and 48 h by AxCAIL-13 (Table II). However, these values were not significantly different, a likely result of high patient-to-patient variability. After 72 h, AxCAIL-13 did not appear to reduce RANTES levels.

AxCAIL-13 has variable effects on concentrations of the soluble adhesion molecules sICAM-1 and sCD44

Soluble adhesion molecules have also been implicated as playing a role in RA pathogenesis. CM concentrations of sICAM-1 and sCD44 were examined by ELISA to determine whether AxCAIL-13 could regulate concentrations of these soluble adhesion molecules (Table II). A similar trend appeared for both molecules. After 24 h, the concentrations of sICAM-1 and sCD44 were increased by an average of 2.1- and 1.9-fold, respectively, in AxCAIL-13-infected RA ST explant CM. After 48 h, the concentrations of both molecules were more comparable in the experimental and control groups. However, after 72 h, AxCAIL-13 had reduced the concentration of sICAM-1 by 49% \((p < 0.05; n = 6)\) and likewise decreased the concentration of sCD44 by 53%. Quantities of other soluble adhesion molecules such as soluble E-selectin and soluble sVCAM-1 could not be detected in RA ST explant CM.

\[ \text{PGE}_2 \text{ concentrations are reduced in AxCAIL-13-infected RA ST explant CM} \]

The concentration of the inflammatory mediator PGE\(_2\) were determined by competition assay in CM from RA ST explants (Fig. 5). AxCAIL-13 significantly decreased PGE\(_2\) concentrations by 66, 80, and 82% at 24, 48, and 72 h, respectively \((p < 0.05; n = 7)\). Nonstimulated RA synovial fibroblast CM were also analyzed for PGE\(_2\) levels (Table I). In accordance with the ST explant findings, PGE\(_2\) levels were decreased by 27 and 24% at 24 and 48 h, respectively \((p < 0.05; n = 6)\).

\[ \text{AxCAIL-13 is more effective at reducing cytokines produced by RA ST explants than rhIL-13} \]

To determine whether virally produced IL-13 would confer a benefit with respect to addition of exogenous IL-13 in a short term
study, we directly compared AxCAIL-13-infected RA ST explants with comparable tissue to which rhIL-13 or PBS was added. CM were analyzed for concentrations of proinflammatory cytokines by ELISA (Fig. 6). Virally produced IL-13 appeared to confer an additional benefit in that mean concentrations of IL-1β (Fig. 6A), TNF-α (Fig. 6B), and IL-8 (Fig. 6C) in CM from AxCAIL-13-infected explants were below those of PBS and rhIL-13 groups at 24, 48, and 72 h. Specifically, when compared with rhIL-13, AxCAIL-13 significantly reduced mean IL-1β concentrations by 85 and 81% after 24 and 48 h, respectively; TNF-α concentrations by 49% after 24 h; and IL-8 concentrations by 69% after 24 h (Fig. 6B; 85 and 81% after 24 and 48 h, respectively; TNF-α concentrations were reduced when compared with those that had rhIL-13 added (data not shown). Interestingly, at some time points, addition of rhIL-13 slightly increased IL-1β or TNF-α concentrations when compared with concentrations produced by PBS controls.

Discussion

Although IL-13 may be best known for its antiinflammatory properties on activated monocytes, this cytokine, like most proteins, demonstrates pleiotropic actions dependent on cell type, cell differentiation, and activation, as well as the cellular microenvironment. When multiple cell types at various stages of activation and growth are involved, the complexity of investigating the cumulative effect of a single protein becomes difficult. Informative studies about the actions of IL-13 on some inflammatory mediators have been investigated using synovial cells as well as infiltrating leukocytes (17, 18). However, isolated cell types may not accurately depict the trends that predominate within the complex multicellular synovium. Therefore, we utilized an ex vivo model of RA ST explants, which retains the synovial architecture and dynamic activation state of component cells (19). The current ex vivo study was designed to indicate whether IL-13 gene therapy should be pursued in an animal model of arthritis. It assesses the potential of virally produced IL-13 on protein levels of multiple cytokines and chemokines and demonstrates that IL-13 is a worthy candidate for further studies in the prevention and/or treatment of arthritis.

The potential of gene therapy for the future treatment of arthritis prompted us to determine whether the ex vivo explant model could be combined with an adenoviral delivery system. We used a gene therapy approach for several reasons. First, we wanted to be certain that adenoviral-related proteins would not interfere with the effects mediated by IL-13. Second, adenoviruses have been shown to be effective in producing their gene products in vitro, as well as in animal models of arthritis (8, 20). Lastly, this method is likely to best represent the potential of IL-13 as delivered by adenovirus in vivo, because it maintained stable IL-13 concentrations over the 72-h time period used. We and others have demonstrated previously that fibroblasts and endothelial cells are readily infected by adenovirus and capable of expressing foreign genes (8, 21, 22). Here, we demonstrate that RA synovial fibroblasts in culture infected with AxCAIL-13 can produce ~2 ng/ml of IL-13 after 72 h (Fig. 1A). When stimulated with IL-1β or TNF-α, the fibroblasts produce significantly more IL-13. This finding suggests that inflamed environments in vivo producing IL-1β or TNF-α may selectively increase production of genes under the control of these regulatory sequences. Increased IL-13 was not the result of increased adenoviral infectivity, as stimulated and nonstimulated RA synovial fibroblasts contained similar quantities of viral DNA by Southern blot analysis. Perhaps stimulation with IL-1β or TNF-α induces an excess of transcription factors, many of which bind the chicken β-actin promoter and CMV enhancer. In addition, ex vivo ST explants produced >20 ng/ml IL-13 at all times examined (Fig. 4).

Table I. IL-8, MCP-1, and PGE₂ concentrations in RA synovial fibroblast CM

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* Values represent the mean ± SE of the indicated molecule (n = 6).

** IL-8 concentrations were determined from RA synovial fibroblasts that were stimulated with 600 U/ml (30 ng/ml) IL-1β. MCP-1 and PGE₂ concentrations represent values from nonstimulated fibroblasts.

*p < 0.05 vs time-matched AxCAIL-13 control by Wilcoxon’s rank order and a paired t test on the log-transformed values.

FIGURE 4. AxCAIL-13 reduces MCP-1 and MIP-1α levels in RA ST explant CM. A, MCP-1 levels in CM from AxCAIL-13- and AxCAIL-13-infected RA ST explants at 24 to 72 h (n = 7; p < 0.05 by a paired t test on the log-transformed values). B, MIP-1α concentrations in CM from AxCAIL-13- and AxCAIL-13-infected RA ST explants at 24 to 72 h (n = 6; p < 0.05 by Wilcoxon’s rank order and a paired t test on the log-transformed values).
IL-1β and TNF-α are major proinflammatory cytokines with a deleterious role in the pathogenesis of RA (1). Several studies suggest that IL-13 can regulate protein levels of these inflammatory mediators. IL-13 inhibits the production of IL-1β by mononuclear cells (MNC) from SF or peripheral blood (PB) of healthy volunteers or inflammatory arthritis patients (17, 18, 23). IL-13 inhibited the synthesis of IL-1β and TNF-α in LPS-treated osteoarthritic (OA) synovial membrane ex vivo cultures (24). Further, Chinese hamster ovary (CHO) fibroblasts secreting murine IL-13 can decrease endogenous and transgenic TNF-α in transgenic mice overexpressing TNF-α (25). Additionally, with the use of the collagen-induced arthritis (CIA) model, the gene for murine IL-13 transfected into CHO fibroblasts (introduced twice s.c.), significantly reduced the arthritic and histologic scores of mice (26). Although this reduction coincided with decreased TNF-α mRNA in the spleens of IL-13-treated animals, regulation of inflammatory mediators within the synovium by IL-13 has not been examined. We demonstrate here that CM from ST explants, representing a variety of RA cell types, secrete decreased quantities of IL-1β and TNF-α in the presence of virally produced human IL-13 (Fig. 2). Therefore, our results suggest that delivery of IL-13 to the synovial microenvironment can significantly reduce the production of two major proinflammatory cytokines.

We examined the effects of IL-13 on the potent polymorphonuclear (PMN) cell chemoattractant and angiogenic factor IL-8. In agreement with a previous report, IL-13 had no effect on IL-8 concentrations secreted by IL-1β-stimulated RA synovial fibroblasts (Table I) (17). However, when RA ST explant CMs were examined following AxCAIL-13 infection, IL-8 concentrations were reduced significantly at two of three time points and reduced by 74% at the remaining time point (Fig. 3A). This effect of IL-13 may be attributable to the effects of IL-13 on MNCs. In PB MNCs from healthy volunteers, IL-8 production was significantly decreased by IL-13 under nonstimulated as well as IL-1β- or TNF-α-stimulated conditions (17).

The regulation of other CXC chemokines, such as ENA-78 and groα, by IL-13 have not been extensively examined. A single report found IL-13 not capable of affecting groα concentrations in CM from HUVECs (27). We demonstrate that IL-13 significantly reduced levels of groα in RA ST explant CM (Fig. 3C). Further, IL-13 was most potent at reducing RA ST explant ENA-78 levels compared with other inflammatory mediators that we examined (Fig. 3B).

The CC chemokine MCP-1 is thought to play a key role in monocyte recruitment and activation in the RA synovium (28–31). IL-13 reduces the production of MCP-1 from LPS-stimulated PB monocytes as well as total MNCs stimulated with IL-1β or TNF-α (17, 32). In contrast, IL-13 selectively induces HUVEC MCP-1 production without up-regulating production of other CC or CXC chemokines (27). Therefore, the predominant effect of IL-13 on synovium containing endothelium and MNCs in addition to other cell types is not easily predicted. We demonstrate here that the net effect is a reduction a MCP-1 protein (Fig. 4A).

The CC chemokine MIP-1α is a product of activated monocyte/macrophages and acts as an important stimulus of T cells, as well as the monocyte/macrophages that produce it. No studies have examined the effects of IL-13 on synoviocyte production of MIP-1α. However, studies on alveolar macrophages, monocytes, and PB PMNs demonstrated that IL-13 inhibits MIP-1α production by these cell types in a dose-dependent manner (33, 34). Similarly, no studies have previously investigated the effects of IL-13 on RANTES protein or mRNA in synoviocytes. In cultured HUVEC or airway smooth muscle cells, IL-13 partially inhibited RANTES production by TNF-α/IFN-γ (35, 36). Here, we demonstrate that IL-13 is capable of decreasing levels of MIP-1α from RA ST explants, whereas the reductions in RANTES levels were not significantly altered.

In this study, we found that AxCAIL-13 infection decreases the levels of PGE2 in RA synovial fibroblast CM (Table I). These results complement those of a previous report that used radiomunoassay to detect PGE1 and PGE2 (17). We demonstrate here that whole ST explants from RA patients mirror the synovial fibroblast findings, showing decreased PGE2 in CM (Fig. 5). This effect of IL-13 may be mediated through action on cyclooxygenase II (COX II). In IL-1α-stimulated long bone cultures, IL-13 inhibits bone resorption by suppressing COX II mRNA expression and consequently PGE2 synthesis (37).

Soluble adhesion molecules may also play an active role in RA (38, 39). We investigated sICAM-1 levels because they are positively correlated with synovial fluid leukocyte counts (39, 40). Also, expression of the cell surface ICAM-1 molecule is inhibited

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* Values represent the mean ± SE of the indicated protein (n = 6).

* p < 0.05 vs time-matched AxCAlacZ control by Wilcoxon’s rank order test.
plants exposed to AxCAIL-13, rhIL-13, or PBS at 24, 48, and 72 h (human IL-13 (Table II). Levels in CM from AxCAlacZ control.

A

![Graph A](attachment:image.png)

**FIGURE 6.** AxCAIL-13 is more effective at reducing proinflammatory cytokines than rhIL-13. A, IL-1β concentrations in CM from RA ST explants exposed to AxCAIL-13, rhIL-13, or PBS at 24, 48, and 72 h (n = 5; p < 0.05 by Wilcoxon’s rank order) as measured by ELISA. B, TNF-α concentrations in CM from RA ST explants exposed to AxCAIL-13, rhIL-13, or PBS at 24, 48, and 72 h (n = 5; p < 0.05 by Wilcoxon’s rank order) as measured by ELISA. C, IL-8 levels in CM from RA ST explants exposed to AxCAIL-13, rhIL-13, or PBS at 24, 48, and 72 h (n = 5; p < 0.05 by Wilcoxon’s rank order) as measured by ELISA.

by IL-13 on OA synovial fibroblasts (41). In addition, IL-13 inhibits CD44 activity by interrupting CD44 ligation to hyaluronan, a key proinflammatory event in MNC adhesion and cytokine production (42). Quantities of sICAM-1 and sCD44 followed a similar trend throughout the time course and exposure to virally produced human IL-13 (Table II). Levels in CM from AxCALacZ control groups consistently increased with time, whereas levels in AxCAIL-13-infected samples started higher and decreased over time. sICAM-1 levels were significantly decreased after 72 h (Table II). Perhaps the ability of IL-13 to decrease levels of sICAM-1 ex vivo give further indication that IL-13 treatment in vivo would be beneficial, because sICAM-1 levels are positively correlated with disease activity.

V irally produced IL-13 appears to hold advantages over addition of exogenous rhIL-13 in its ability to reduce levels of proinflammatory cytokines. If rhIL-13 were losing activity due to its short half-life, it may be anticipated that levels of proinflammatory cytokines would increase over the 72-h time period. This was not the case, because the concentrations of IL-1β, TNF-α, and IL-8 appear consistent over this time frame (Fig. 6). Perhaps the advantage of AxCAIL-13 could be explained by the local concentrations of IL-13 produced by adenovirus relative to the penetration of rhIL-13. For example, we have determined previously that an overnight infection period with slight agitation after addition of an adenovirus producing β-galactosidase appears to fully penetrate a nonminced ST explant after 5-bromo-4-chloro-3-indolyl-6-β-D-galactose staining (data not shown). Therefore, this study used a similar technique in combination with mincing the tissue, which likely assured full adenoviral penetration into the tissue. Addition of rhIL-13 probably penetrated the tissue as well; however, there was likely no concentration gradient, because all cells would be exposed to an equivalent concentration of cytokine. Because a concentration difference of as little as 2% between the front and back of a cell can direct migration (43), such small concentration gradients may also explain the differences here. Because both interior and exterior cells are likely producing IL-13, there is anticipated to be a concentration gradient that is higher at the cell surface where the cytokine is released. Further, 14 to 24% of IL-13-bound receptors are rapidly internalized whereas another 14 to 24% are shed and/or dissociated (44). The consistent removal of IL-13 from the CM may likewise contribute to the difference between AxCAIL-13 and the rhIL-13 groups, because adenovirally infected cells are likely to continue producing IL-13. Therefore, in addition to being a convenient delivery strategy, adenovirally produced proteins may confer benefits toward the reduction of proinflammatory cytokines in RA ST.

We demonstrate herein that there are discrepancies in the effect of AxCAIL-13 on the production of a single cytokine between RA synovial fibroblasts and RA ST explants. We do not believe that this can be accounted for by differences in adenoviral infectivity, because both RA ST and RA synovial fibroblasts appear readily susceptible to adenoviral infection (8, 21, 22, 45). Interestingly, using flow cytometry, Bondeson et al. demonstrated that in addition to synovial fibroblasts and macrophages, RA synovial T cells were surprisingly easy to infect (45). Therefore, differences between the fibroblast and explant studies may represent the complexity whereby IL-13 could have opposite effects by interacting with its receptor on neighboring cells within the RA synovium. This underscores the importance of assessing the cumulative impact of a candidate therapeutic protein on multiple cell types at different stages of activation.

The mechanism whereby IL-13 may mediate levels of the inflammatory cytokines may involve effects on transcription factors. IL-1β and TNF-α as well as other cytokines investigated in this study are under the transcriptional control of NF-κB (46–48). The main form of this transcription factor is a heterodimer of NF-κB1 (p50) and RelA (p65) that is sequestered in the cytoplasm bound by inhibitory proteins of the IκB family (49). IκBα is the most functionally relevant of this family in vitro (50). On inflammatory
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Therefore, in combination with its ability to reduce CIA in vivo (26), IL-13 appears worthy of future consideration as a therapeutical modality for RA.

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References


