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Transient Gene Transfer of IL-12 Regulates Chemokine Expression and Disease Severity in Experimental Arthritis

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Murine collagen-induced arthritis (CIA) is characterized by pannus formation, cell infiltration, and cartilage erosion, and shares histologic and immunologic features with rheumatoid arthritis. Numerous cytokines are reportedly associated with RA and/or CIA; however, their mechanistic role is not clear. To determine the role of IL-12 in CIA, DBA/1 LacJ mice were administered 3 × 108 plaque-forming units of mIL-12 i.p. in a nonreplicating adenoviral vector (AdIL-12) on day 25 following primary type II collagen immunization. Our studies demonstrated that systemic transient overexpression of IL-12 accelerated disease progression and augmented the arthritis severity relative to mice expressing a replication-deficient, E1-deleted Ad5 construct. A likely mechanism for this increase in pathology was the increase in the expression of cytokines and chemokines known to play a proinflammatory role in disease. In particular, levels of murine IFN-γ were significantly increased in mice overexpressing AdIL-12 relative to the replication-deficient, E1-deleted Ad5 construct. Interestingly, the C-X-C chemokine murine macrophage inflammatory protein-2, as well as the C-C chemokines murine monocyte chemoattractant protein-1 and murine macrophage inflammatory protein-1α were up-regulated by AdIL-12 relative to controls. In an additional set of studies, neutralization of endogenous IL-12 in CIA mice was shown to delay disease onset and attenuate disease severity. IFN-γ levels in the mice receiving anti-IL-12 were significantly decreased in joint homogenates. These studies demonstrate that IL-12 is an important cytokine involved in controlling the production of chemokines/chemokines leading to the evolution of experimental arthritis. The Journal of Immunology, 1998, 160: 4615–4619.

Rheumatoid arthritis (RA) is a widespread, chronic inflammatory disease that is localized primarily in the joints and has several pathologic features of autoimmune diseases. Although the proliferation of synovial cells and the infiltration of mononuclear leukocytes are fundamental events in the formation of the pannus, it is difficult to longitudinally examine the initiation and maintenance of this pathologic cascade in the development of RA. Therefore, it is necessary to establish and characterize experimental animal models to understand the cellular and molecular events that contribute to the pathogenesis of RA. Collagen-induced arthritis (CIA), an experimental model induced by immunization with type II collagen (CII), shares a number of common clinical, histologic, and immunologic features with RA. CIA is a progressive disease characterized by chronic inflammation of synovial tissue, pannus formation, cartilage destruction, and bone erosion. The joint inflammation is sustained by the perpetuation of Abs to CII. Similar to RA, the pathology of this model in the mouse is characterized by leukocyte elicitation in both the joint space and pannus. Potentially, drug therapy could be directed at the inhibition or stimulation of the mediators of these cell types.

The involvement of a wide array of cytokines and chemotactic cytokines (chemokines) has been reported in RA and/or CIA. Cytokines may be classified according to their production by T cells. Th1 responses are identified by their production of IFN-γ and IL-2, while Th2 cells are characterized by the production of IL-4, IL-5, IL-6, IL-10, and IL-13. The balance of Th1/Th2 type cytokines may play a significant role in the regulation of autoimmune diseases (1, 2). IL-12, a Th1-associated cytokine, is of interest because of its involvement in inflammation and in the arthritic cytokine cascade. In studies of mice that were unable to produce active IL-12, the incidence and magnitude of CIA was greatly reduced, suggesting that this cytokine plays a role in the pathogenesis of experimental arthritis (3). Proteins of the chemokine superfamily are important in inflammation due to their specificity for the movement of certain leukocyte populations. Members of the C-X-C chemokine subfamily are primarily chemotactic for neutrophils, while C-C chemokines are chemotactic for mononuclear leukocytes. The chemokines macrophage inflammatory protein (MIP)-2 (C-X-C) and MIP-1α (C-C) have also been shown by our laboratory to be involved in the cytokine cascade in the arthritic murine joint (4, 5). Likewise, the C-X-C chemokines IL-8, epithelial neutrophil-activating protein-78, and growth-regulated gene product-α, and the C-C chemokines RANTES, monocyte chemoattractant protein (MCP)-1, and MIP-1α have also been identified in human RA samples and may be important for the leukocyte influx into RA joints (6–13). Chemokines induced by cytokine networks may have a significant role in the regulation of arthritis progression.

The advent of gene therapy provided numerous tools for the study of immunopathologic mechanisms. This is especially true

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3 Abbreviations used in this paper: RA, rheumatoid arthritis; CIA, collagen-induced arthritis; CII, type II collagen; m, murine; MIP, macrophage inflammatory protein; MCP, monocyte chemoattractant protein; AdControl, a replication-deficient, E1-deleted Ad5 construct; AdV, adenovirus.

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because transient overexpression can facilitate the study of the biologic functions of cytokines and their mediators in vivo over a given period of time in experimental animal models. Systemic introduction of an adenoviral cytokine construct via the peritoneum (i.p.) was chosen for this study as a means to express systemic levels of IL-12. In the present study, we have analyzed the relationship of IL-12 to disease activity, demonstrating that IL-12 is an important immunomodulator in murine arthritis and can regulate the expression of certain proinflammatory chemokines, including MIP-1α, MIP-2, and MCP-1. These chemokines direct leukocyte migration and are highly relevant to the pathogenesis of RA.

Materials and Methods

Induction and evaluation of murine arthritis

DBA/1 LacJ mice that were 8 to 10 wk old (The Jackson Laboratory, Bar Harbor, ME) were used to generate a murine arthritis model via collagen immunization using previously described methods (14, 15). Chicken CHI (1 ng/ml) (Dr. M. Griffiths, University of Utah, Salt Lake City, Utah) was prepared in 0.1 M acetic acid. For primary immunization, the CHI solution was emulsified with CFA (Sigma, St. Louis, MO). Briefly, two injections were given intradermally near the base of the tail for a total of 100 μg of collagen per mouse on day 0. Mice were immunized 21 days later with an additional 100 μg of CHI. Observations were made on a daily basis for the presence of distal joint swelling and erythema. A mouse was identified as arthritis positive if there was evidence of redness or erythema on any digits or elsewhere on the paws. Severity of disease was assessed by a macroscopic clinical scoring index (0–3) based on degree of erythema, edema, and visible joint distortion for individual hind paws.

rIL-12 adenovirus (AdV)

A double rAdV expressing a functional heterodimeric mIL-12 protein was generated and propagated as previously described (16). The AdmIL-12 vector contains a p19 DNA for the p35 and p40 subunits of IL-12 inserted into the E1 and E3 regions of Ad5 containing the human CMV immediate early promoter and the SV40 polyadenylation signal. Transfection with this replication-defective construct results in the expression of active IL-12 both in vitro and in vivo (16, 17). As a control, a previously described replication-deficient, E1-deleted Ad5 construct (AdControl) was used (18). To determine the role of immunomodulating IL-12 in CIA, DBA/1 LacJ mice were treated systemically (i.p.) with either 3 × 10^9 plaque-forming units of AdIL-12 or AdControl.

Murine cytokine ELISAs and reagents

Extracellular, immunoreactive murine cytokines (IFN-γ, IL-12, MIP-1α, MIP-2, and MCP-1) were quantified using a modified double-ligand procedure of cytokine ELISA. This ELISA method consistently detected cytokine levels from 20 to 50 pg and did not cross-react with other cytokines. Concentrations were recorded as the mean (ng/ml) ± SE, and statistical significance was determined at the p < 0.05 level. We coated 96-well flat-bottom microtiter plates with 50 μl well of rabbit anti-cytokine Abs (1 μg/ml in 0.6 M NaCl, 0.26 M H3BO4, and 0.08 N NaOH (pH 9.6)) for 16 h at 4°C. The plates were then washed in PBS (pH 7.5) and 0.05% Tween-20. Blocking of the nonspecific binding sites was accomplished by incubating plates with PBS containing 2% BSA for 90 min at 37°C. Plates were rinsed thoroughly with wash buffer, and aqueous samples were added. Following a 1-h incubation at 37°C, the plates were washed, and biotinylated rabbit anti-cytokine Ab was added and incubated for 30 min at 37°C. The plates were then washed again, chromagen substrate was added, and plates were subsequently read at 490 nm.

Aqueous hind paw extracts were prepared for ELISA by homogenization of previously frozen hind paw samples in 1 ml homogenization buffer containing 0.5% Nonidet P-40 and PBS on ice followed by centrifugation. Samples were aliquoted and frozen before assays. The total protein of homogenate samples was determined by standard methods in microtiter plates using bicinchoninic acid protein assay reagents (Pierce, Rockford, IL). Protein concentrations did not differ significantly between treatment groups. Blood plasma samples were obtained for AdIL-12 characterization studies in naive mice by retroorbital bleed and were centrifuged and frozen before assay. Similarly, peritoneal lavage samples were collected in 1 ml PBS, centrifuged, and frozen before ELISA.

For passive immunization of CIA mice, specific murine antiserum for IL-12 was raised in our laboratory by immunizing rabbits with mrIL-12. F(ab′)2, fragments of anti-IL-12 and control F(ab′)2, fragments from normal rabbit serum were both generated with an Immunopure kit (Pierce); fragments were given to mice previously immunized with CII, that did not receive AdV, on days 26, 28, 30, and 32 after primary immunization for a total of 7 mg protein per mouse. Mice were sacrificed on day 36, and fluids and tissue were collected for assay. Before commencing the IL-12 neutralization studies in mice, the F(ab′)2, fragments were used in vitro to neutralize IL-12 in murine splenocytes as evidenced by a decrease in IFN-γ production according to ELISA.

Results

Characterization of IL-12 overexpression in naive mice

To characterize the expression levels of mIL-12 via the AdIL-12 vector in DBA/1 LacJ mice, naive, unimmunized animals were given AdIL-12 i.p., and protein levels were assayed at selected time points. Samples were collected from peritoneal lavages, blood, and hind paws at 1, 3, and 7 days after i.p. injection. Following i.p. AdIL-12 administration, significantly elevated levels of mIL-12 were demonstrated by ELISA through day 3. Values were recorded as the mean ± SE.

Augmentation of CIA by IL-12 gene transfer

Mice were examined visually for the appearance of arthritis in the peripheral joints and were scored on a scale of 0 to 3 for individual hind paws following collagen immunization. Experimental murine arthritis develops in >95% of the mice by 32 days after primary CII immunization. The disease that develops is normally gradual and often culminates in severe ankylosis. IL-12 overexpression, which starts at day 25 after primary immunization by employing an adenoviral vector system, demonstrated the ability of IL-12 to significantly exacerbate mCIA (Fig. 2). As previously shown, IL-12 is significantly up-regulated in the blood, peritoneum, and joints for several days after i.p. injection of AdIL-12. In general, the administration of both AdV constructs expedites the date of disease onset by several days; however, with AdControl, the final disease outcome is indistinguishable from CIA mice not receiving AdV.
Chemokine and cytokine regulation in CIA

Cytokines appear to play an important role in inflammation and in the development of arthritis responses. We have demonstrated that IL-12 overexpression by employment of an adenoviral vector system exacerbates mCIA. Accordingly, concentrations of IFN-γ were increased fivefold in mice overexpressing AdIL-12 relative to AdControl (Fig. 3). In addition to the regulation of IFN-γ, which is a Th1 type cytokine, the levels of chemokines were examined. C-X-C chemokine MIP-2, a murine functional homologue of IL-8, was up-regulated approximately twofold in mice transiently expressing mIL-12 (Fig. 4). Levels of MCP-1 were 1.5-fold higher than those seen in AdControl mice. MIP-1α, a C-C chemokine, was elevated 1.6-fold in AdIL-12 mice relative to AdControls. These studies indicate that MIP-1α, MIP-2, and MCP-1 were all expressed in inflamed joints. Our findings also correspond to previous data from our laboratory indicating that chemokines play a role in the exacerbation of joint inflammation.

Passive immunization of endogenous IL-12 in CIA

The previously mentioned observations demonstrate that IL-12 is associated with the enhancement of experimental arthritis and the elevation of certain proinflammatory cytokote protein levels that are likely involved in this disease state. To assess the potential contribution of endogenous IL-12 in this murine arthritis model, CIA mice were passively immunized with neutralizing Abs to mIL-12. Anti-IL-12 or control sera F(ab′)2 fragments were given i.p. to CIA mice just before normal disease onset on day 26. Onset of arthritis normally occurs between days 28 and 32. The F(ab′)2 fragments were used to exclude the possible influence of Fc-dependent activation which could possibly occur during the formation of cytokine/anti-cytokine immune complexes and consequently alter the progression of CIA. Anti-IL-12 was given to mice i.p. every other day for 1 wk. This passive immunization was followed by a delay of disease onset and substantially diminished disease as demonstrated by clinical scoring (Fig. 5). Furthermore, levels of mIFN-γ were assayed following sacrifice on day 36 and were found to be significantly decreased in hind paw homogenates relative to controls, demonstrating the relative efficacy of anti-IL-12 administration in diminishing the production of IFN-γ (Fig. 6). Thus, both overexpression and Ab neutralization data indicate a causal role for IL-12 in CIA.

Discussion

The infiltration and activation of various leukocyte populations in joint tissue are contributing factors to pannus development and the subsequent pathology of RA. The mechanisms involved in eliciting blood born leukocytes into the synovial tissue and fluid remain relatively enigmatic; however, it is known that chemokines are expressed during RA, and that these mediators appear to be important to the perpetuation of joint inflammation. The pathogenesis of CIA is dependent upon the response of the animal to CII challenge and the subsequent generation of Abs that recognize collagen-rich joint tissue. Several studies have found that cytokines appear to direct cell-to-cell communication during the progression of CIA (4, 19–23).

IL-12 is a heterodimeric protein that was originally thought to promote the development of NK and Th1 T cells, enhance the production of IFN-γ from NK and T cells, and increase the cytolytic activity of NK and CD8+ cells (24–26). In addition, however, IL-12 transcripts were detected in RA synovial fluid macrophages, and IL-12 protein was found in synovial fluid from RA and osteoarthritis patients (27, 28). During the immunization phase of CIA, it is known that IL-12 has an IFN-γ-dependent, adjuvant-like stimulatory role in the progression of experimental arthritis according to its actions on CD4+ T cells and IgG2 Ab production (29, 30). s.c. injections of IFN-γ in mice immunized with CII generated more severe arthritis along with a higher disease frequency (20). We have demonstrated significantly increased concentrations of IFN-γ in mice which overexpress AdIL-12 and correspond to enhanced CIA, further demonstrating a role for Th1 type cytokines in CIA (Fig. 3). Studies have demonstrated that treating CIA mice early in the immunization protocol with non-depleting anti-CD4+ mAbs significantly reduced arthritis frequency and diminished IFN-γ levels while elevating IL-4 levels (31). These investigations suggest that the in vivo regulation of CIA may be altered by the cytokine production profile of the T cells. The production of IFN-γ from Th1-type cell responses appears to play an important immunoregulatory role in arthritis progression. In addition, both Th1 and Th2 cells have been shown to have a proinflammatory role in inducible Ag-induced arthritis (32). Moreover, severe arthritis and earlier disease onset were found in mice that were deficient in IFN-γR and immunized with CII, while IL-12-deficient mice had a greatly reduced arthritis incidence (3, 33, 34). Interestingly, high doses of IL-12 (1 μg/day) given for 2...
to 3 wk slowed the onset and severity of CIA in most animals, while therapeutic administration, limited high doses, or lower doses of IL-12 did not diminish disease in a similar manner (35). The role of IL-12 in the development of CIA appears to be dependent upon a number of factors, including timing of administration, dosage of IL-12, and the immunization protocol used (29, 30, 35).

In our study, adenoviral constructs overexpressing mIL-12 or a control vector were given systemically via the peritoneum to assess the immunomodulatory roles of IL-12 on the cytokine/chemokine cascade in this chronic inflammatory model. The use of gene transfer in animal models has been examined by a number of researchers. For example, ex vivo gene transfer to animals in an attempt to antagonize IL-1 has been investigated in normal rabbits by employing retroviral IL-1R antagonist protein constructs and synoviocytes (36, 37). An adenoviral reporter construct was directly expressed in the rabbit knee synovium, demonstrating possible in vivo usage of AdV (38). Furthermore, the expression of retroviral IL-1R antagonist protein by ex vivo methods in mCIA nearly eliminated disease in the transfected knee joints and the draining paws (39). Accordingly, the use of viral systems for gene overexpression, although clinically controversial, has proven useful in the elucidation of experimental arthritis mechanisms.

Direct transient expression of AdIL-12 allowed for the detection of elevated levels of this cytokine in the hind paws, plasma, and peritoneal space, indicating successful systemic administration (Fig. 1). Moreover, passive immunization using F(ab’)_2 fragments was used just before the onset of CIA to establish a contributing role for endogenous IL-12 in the evolution of joint inflammation. This passive immunization with anti-IL-12 had a therapeutic effect on the experimental arthritic disease while down-regulating IFN-γ, demonstrating relevance for the continued examination of the role of IL-12 in the cytokine/chemokine inflammation cascade (Figs. 5 and 6).

Chronic joint inflammation is a multifactorial response that is dependent upon both regulatory cytokines and proinflammatory chemokines. Previously, we have demonstrated that IL-10 has an important regulatory role in CIA. IL-10 increased near the time of arthritis onset, while neutralizing anti-IL-10 Abs hastened arthritis onset and increased disease severity (4). Furthermore, anti-IL-10 administration increased the expression of the chemokines MIP-1α and MIP-2 and increased leukocyte infiltration into the joints. Neutralizing Abs directed against MIP-1α or MIP-2 caused a delay in the onset of the disease and lessened the severity of the arthritis. Our studies, employing an adenoviral construct that transiently

FIGURE 4. Chemokine concentrations in CIA hind paw homogenates. Levels of mMIP-2 (A), mMCP-1 (B), and mMIP-1α (C) were determined by ELISA (n = 8–12 hind paws/treatment group). These chemokines were significantly elevated in mice transfected with AdIL-12 (open bars) compared with AdControl (shaded bars). Values were recorded as the mean ± SE.

FIGURE 5. Neutralization of endogenous IL-12 in collagen-immunized mice. Passive immunization with anti-IL-12 F(ab’)_2 (open circles, n = three mice/treatment group) or control F(ab’)_2 (shaded boxes) had a therapeutic effect on the experimental arthritic disease as determined by a visual scoring index. Mice were passively immunized on days 26, 28, 30, and 32 with a total of 7 mg per mouse.

FIGURE 6. IFN-γ concentrations in CIA mice passively immunized with anti-IL-12. Levels of mIFN-γ were assayed following sacrifice on day 36 and were found to be significantly decreased in the hind paw homogenates of mice receiving anti-IL-12 (open bars) relative to control serum (shaded bars); this observation demonstrates the relative efficacy of the anti-IL-12 administration in diminishing the production of IFN-γ (n = six hind paws/treatment group). Values were recorded as the mean ± SE.
overexpresses IL-12, demonstrated elevated levels of certain che-romekines that corresponded with exacerbated disease. We have shown that RIL-12 delivered by transient gene transfer not only alters disease and inflammation but also regulates cytokine and chemokine levels (Figs. 2, 3, and 4). The C-X-C chemokine MIP-2, a murine functional homologue of IL-8, was up-regulated, as were the C-C chemokines MCP-1 and MIP-1α. There is little doubt that the cytokine-dependent orchestration of immune events is crucial to the pathogenesis of RA, yet molecular mechanisms which dictate joint pathology are still unclear. These observations and others suggest that multiple mechanisms and mediators, including chemokines and cytokines, are likely involved in the pathogenesis of experimental arthritis. As the molecular mechanisms and mediators that dictate pathology in RA become more precisely delineated, the potential of biologic therapeutics that specifi-cally alter these essential pathways early in disease will enable better treatment of arthritic patients.

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