Interleukin 6 Is Required for the Development of Collagen-induced Arthritis

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Summary

Interleukin-6 (IL-6) is overproduced in the joints of patients with rheumatoid arthritis (RA) and, based on its multiple stimulatory effects on cells of the immune system and on vascular endothelia, osteoclasts, and synovial fibroblasts, is believed to participate in the development and clinical manifestations of this disease. In this study we have analysed the effect of ablating cytokine production in two mouse models of arthritis: collagen-induced arthritis (CIA) in DBA/1J mice and the inflammatory polyarthritis of tumor necrosis factor α (TNF-α) transgenic mice. IL-6 was ablated by intercrossing an IL-6 null mutation into both arthritis-susceptible genetic backgrounds and disease development was monitored by measuring clinical, histological, and biochemical parameters. Two opposite responses were observed: while arthritis in TNF-α transgenic mice was not affected by inactivation of the IL-6 gene, DBA/1J, IL-6−/− mice were completely protected from CIA, accompanied by a reduced antibody response to type II collagen and the absence of inflammatory cells and tissue damage in knee joints. These results are discussed in the light of the present knowledge of cytokine networks in chronic inflammatory disorders and suggest that IL-6 receptor antagonists might be beneficial for the treatment of RA.

Rheumatoid arthritis (RA) is a common human autoimmune disease characterized by chronic inflammation of the synovial joints and by subsequent progressive destruction of articular tissue. Although the etiology and pathogenesis of RA are not yet fully understood, it has become increasingly clear that a series of locally produced cytokines play a central role in disease progression. Indeed, cytokines are responsible both for the mobilization and continuous activation of the inflammatory cell infiltrate and for inducing production of the enzymes that destroy bone and cartilage (for review see reference 1).

The current view of the cytokine network in rheumatoid joints supports the notion that TNF-α activates a cytokine cascade characterized by the simultaneous production of proinflammatory cytokines such as IL-1, IL-6, several chemokines, GM-CSF, and of antiinflammatory factors such as IL-10, IL-1RA, and soluble TNF receptor (for review see reference 2). Disease progression/reactivation or, on the contrary, its silencing, are likely to be due to a dynamic and unstable equilibrium in the production of pro- and antiinflammatory cytokines.

From among these cytokines, IL-6 has been proposed to contribute to the development of arthritis. IL-6 is present at very high levels in serum and synovial fluids of RA and of juvenile RA patients (3–6). Soluble forms of the specific IL-6 receptor subunit α (sIL-6Rα) are elevated (7, 8) and these are known to potentiate IL-6 activity by forming IL-6–sIL-6Rα complexes that bind and homodimerize the signaling-competent transmembrane receptor glycoprotein (gp)130 (9).

Increased IL-6 bioactivity during RA is believed to be responsible for local and systemic effects. IL-6 acts as a stimulator of both B and T cell functions because it promotes proliferation of plasmablastic precursors in the bone marrow and their final stage of maturation into immunoglobulin-producing plasma cells and participates in the activation and proliferation of T cells (for review see reference 10). Moreover, IL-6, in conjunction with sIL-6Rα, has been recently shown to: (a) activate endothelial cell production of a subset of chemokines and adhesion molecules, thus contributing indirectly to recruitment of leukocytes at inflammatory sites (11); and (b) induce synovial fibroblast proliferation (12) and osteoclast formation and activation.

Abbreviations used in this paper: CII, type II collagen; CIA, collagen-induced arthritis; DIL, DBA/1J; IL-6; DIL−/−, DBA/1J mice crossed with IL-6-deficient mice; gp, glycoprotein; RA, rheumatoid arthritis; sIL-6Rα, soluble IL-6 receptor subunit α; TIL, TNF, IL-6; TIL−/−, TNF-α transgenic mice crossed with IL-6-deficient mice.

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IL-6 Gene Inactivation Protects from Arthritis

Materials and Methods

Mice. IL-6–deficient mice (17) were backcrossed into the DBA/1J genetic background (H-2b, Jackson Laboratory, Bar Harbor, ME) for five generations, obtaining DBA/1, IL-6 mice that were named DIL-6 mice. In all experiments only DBA/1, IL-6−/− (DIL-6−/−) and DBA/1, IL-6+/− (DIL-6+/−) littermate mice were used.

TNF, IL-6 mice were obtained by intercrossing TNFα transgenic mice (19) with IL-6−/− mice. Mice carrying the TNFα transgene and heterozygous for the IL-6 null mutation were intercrossed to obtain TNF, IL-6−/− (TIL-6−/−) and TNF, IL-6+/− (TIL-6+/−) mice.

DIL-6 and TIL-6 mice were bred at IRBM (Rome, Italy) in a specific pathogen-free animal facility, whereas the parental DBA/1 strain was obtained from Jackson Laboratory. Mice were maintained in standard conditions under a 12-h light-dark cycle, provided irradiated food (4R F21; Mucedola; Settimo Milanese, Milan, Italy) and chlorinated water ad libitum. Procedures involving animals and their care were conducted in conformity with national and international laws and policies.

Induction of CIA. Antibody Treatment, and Clinical Assessment of Arthritis. Male 8-wk-old DBA/1 and DIL-6 mice were immunized intradermally at the base of the tail with 100 μg of bovine type II collagen (CII; M.M. Griffiths, University of Utah, Salt Lake City, UT) in 0.05 M acetic acid, emulsified with an equal volume of complete Freund’s adjuvant, containing 100 μg of H37RA Mycobacterium tuberculosis (Difco, Detroit, MI). On day 21, mice were boosted by intradermal injection with 100 μg of bovine CII in 0.05 M acetic acid emulsified with an equal volume of incomplete Freund’s adjuvant (Difco).

Starting from time of the CII booster injection, DBA/1 mice were treated subcutaneously once a week for 6 wk with 0.5 or 1 mg/mouse of the following antibodies: (a) the rat mAb 15A7 (20), which neutralizes the murine IL-6 receptor alpha chain, (b) an isotype-matched IgG2b rat mAb against dinitrophenyl hapten (LO-DNP-S7; provided by H. Bazin, University of Louvain, Brussels, Belgium), and (c) total rat IgGs (Sigma Chemical Co., St. Louis, MO)."
To establish possible correlations between IL-6 levels and the severity of arthritis, serum IL-6 levels were evaluated in parallel with disease severity expressed as arthritis index of the affected joints. Mice with macroscopic joint involvement (arthritis index of >1) had serum IL-6 levels (52.2 ± 45.8 U/ml) significantly higher than those of mice without macroscopic involvement (12.5 ± 6.3 U/ml; P = 0.0033) and those of nonimmunized animals (6.3 ± 0.7 U/ml; P = 0.001). In addition, in mice with macroscopic joint involvement (arthritis index of >1) a significant correlation (regression correlation coefficient of Spearman \( R_s = 0.694; P = 0.008 \)) between serum IL-6 levels and the arthritis index was found (Fig. 1), suggesting a direct correlation between IL-6 production and disease severity.

**Figure 1.** Serum levels of IL-6 in DBA/1J mice with CIA correlated with the arthritis index. Type II collagen immunized mice were bled 6 wk after CII immunization. IL-6 activity was measured by hybridoma growth assay and the arthritis index evaluated as described in Materials and Methods. Results were analyzed using the Spearman correlation coefficient. \( R_s = 0.694; P = 0.008. \)

Treatment of CIA with an mAb Neutralizing IL-6 Activity. To investigate the pathogenic role of IL-6 in CIA, we first attempted neutralization of IL-6 in vivo using the mAb 15A7, directed against the murine IL-6 receptor alpha chain (IL-6Ra; reference 20, 23–25). Both 15A7 and control antibodies were administered subcutaneously at weekly intervals starting from the time of the boosting CII injection. In a first experiment the isotype-matched rat mAb LO-DNP-57 (20, 23) was used as a control. Surprisingly, both the 15A7 mAb and the LO-DNP-57 control mAb were able to significantly decrease disease severity, as shown in Fig. 2 A, when used at the dose of 1 mg/mouse but not at the dose of 0.5 mg/mouse. As this result may be due to some unexpected interference with CIA development of the particular antibody used as control, we repeated the treatment using total rat IgGs as control (Fig. 2 B). Again, the 15A7 mAb significantly decreased arthritis development \( (P < 0.02 \) in weeks 9, 10, and 11 after the first CII immunization), although the total IgG treatment also considerably decreased CIA symptoms, albeit with a statistically significant decrease \( (P < 0.04) \) only at week 9. These results do not allow any conclusions to be drawn about the role of IL-6 in CIA.

**Figure 2.** Effects of antibody treatment on arthritis index of CII-immunized DBA/1J mice. (A) Mice \( (n = 9 \) for each group) were treated once per week with 1 mg/mouse (white symbols) or 0.5 mg/mouse (black symbols) of either anti-IL-6Ra antibody 15A7 (circles), anti-dinitrophenyl hapten antibody LO-DNP (triangles), or left untreated (squares). \( *P < 0.05 \) either 15A7 or LO-DNP versus none. (B) Mice were treated with 1 mg/wk/mouse of either anti-IL-6-Ra antibody 15A7 (circles; \( n = 9 \)), rat total IgG antibodies (diamonds; \( n = 8 \)), or left untreated (squares; \( n = 8 \)). \( ^*P < 0.02 \) 15A7 versus none; \( ^*P < 0.04 \) total IgG versus none. Results were reported as mean ± SD. The weeks after the first immunization with CII and the duration of antibody treatment are indicated on the abscissa.

IL-6 Production Is Absolutely Necessary for CIA. An alternative strategy to test the role of IL-6 in CIA is ablation of the IL-6 gene in mice genetically susceptible to CIA. We have therefore introduced an IL-6 null mutation (17) into the CIA-susceptible DBA/1J genetic background. After five consecutive backcrosses both DIL-6\(^{-/-}\) and DIL-6\(^{+/+}\) mice were generated and used for CIA studies. As the
MHC H-2 locus is known to play a major role in the genetic susceptibility to CIA (22), we first confirmed that both DIL-6−/− and DIL-6+/+ mice carried the same H-2 allele (H-2k) as the parental DBA/1J strain (data not shown).

In two independent experiments, a total number of 16 DIL-6+/− and 17 DIL-6+/+ mice were immunized with CII and joint swelling was monitored in the weeks after the injection of the antigen. The results, reported in Fig. 3, show that while DIL-6+/+ mice displayed the expected rate of arthritis development (12/17 animals; 70.6%), DIL-6−/− mice were completely protected (0/16; P <0.0001 by chi-square analysis), suggesting that IL-6 activity is essential for CIA to develop. This conclusion was further supported by histological analysis of the joints. Fig. 4 shows representative sections of six knee joints analyzed for each genotype in which evident inflammatory alterations with proliferating pannus, bone erosion, and mononuclear cell infiltrate were evident only in DIL-6+/+, whereas in DIL-6−/− no pathological alteration was detectable; the joint and the articular discs were normal and no signs of pannus formation and/or inflammatory infiltrate were present.

CIA development has been shown to be dependent on both cellular and humoral immune responses to CII (22). Since IL-6 is a major factor in the growth and differentiation of B cells into antibody-producing cells (10), we measured the level of the total anti-CII IgG. Although DIL-6+/− mice did develop appreciable levels of anticollagen IgGs, they were significantly lower than those found in both DIL-6+/+ mice (Fig. 5A) and in the parental DBA/1J (not shown). Previous studies have also suggested that antibodies of the IgG2a isotype play a major pathogenic role in CIA development (26, 27). We therefore evaluated the anticollagen IgG isotype distribution in both DIL-6+/+ and DIL-6−/− mice (Fig. 5B). Interestingly, although in DIL-6+/+ mice IgG1, IgG2a, and IgG2b were equally present, in DIL-6−/− mice a predominant IgG2a production was observed. Therefore, although the response to CII is quantitatively reduced in the absence of IL-6, it appears qualitatively compatible with disease development.

Inflammatory Polyarthritis in T NF-α T transgenic Mice Is IL-6 Independent. TNF-α transgenic mice overexpress a 3′-untranslated region–modified form of the human TNF-α messenger RNA that is responsible for the development of chronic inflammatory polyarthritis (19). Since TNF-α is known to be an inducing factor of IL-6 synthesis (28), we measured IL-6 levels in vivo to monitor possible correlation with disease development. As shown in Table 1, IL-6 serum levels were constantly elevated in TNF-α transgenic mice independently from their age, and did not increase with the development of the articular disease, in contrast with what we found in the CIA model.

To assess if higher IL-6 production was critical for the development of arthritis, we have generated transgenic TIL-6−/− and TIL-6+/+ mice by genetic intercrosses. A total number of 16 TIL-6−/− and 11 TIL-6+/+ mice were analyzed for timing and severity of arthritis. The results
Discussion

The goal of the present study was to further understand the role played by IL-6 in the pathogenesis of different arthritic diseases by interfering with this cytokine’s activity.

Table 1. IL-6 Serum Levels of TNF-α Transgenic and Wild-type Mice at Different Weeks of Age

<table>
<thead>
<tr>
<th>IL-6*</th>
<th>7 wk</th>
<th>9 wk</th>
<th>11 wk</th>
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<tr>
<td>Wild type (n = 11)</td>
<td>20.93 ± 4.40</td>
<td>20.01 ± 1.44</td>
<td>24.68 ± 2.20</td>
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<tr>
<td>(P &lt; 0.01)</td>
<td>(P &lt; 0.02)</td>
<td>(P &lt; 0.001)</td>
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<tr>
<td>TNF-α (n = 8)</td>
<td>67.65 ± 11.74</td>
<td>56.44 ± 19.14</td>
<td>55.25 ± 5.28</td>
</tr>
<tr>
<td>(P &lt; 0.001)</td>
<td>(P &lt; 0.02)</td>
<td>(P &lt; 0.001)</td>
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*Mean values ± SE. IL-6 activity was measured by hybridoma growth assay and P values were calculated by ANOVA.

Table 2. Human TNF-α Serum Levels of TIL-6 Mice at Different Weeks of Age

<table>
<thead>
<tr>
<th>H human TNF-α (pg/ml)*</th>
<th>8 wk</th>
<th>11 wk</th>
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<tbody>
<tr>
<td>TIL-6-/- (n = 6)</td>
<td>150 ± 56</td>
<td>556 ± 134</td>
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<td>(P &lt; 0.01)</td>
<td>(P &gt; 0.05)</td>
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<tr>
<td>TIL-6+/+ (n = 4)</td>
<td>268 ± 86</td>
<td>647 ± 81</td>
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<tr>
<td>(P &lt; 0.01)</td>
<td>(P &gt; 0.05)</td>
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*Mean values ± SD. Human TNF-α serum levels were measured by ELISA and P values were calculated by ANOVA.
factor, as the continuous influx of activated cells to inflamed caused by IL-6 deficiency (11) may represent an important production and leukocyte recruitment at inflammatory sites cytotoxic activity. In addition, the defective chemokine provoke a decrease in MHC class I–restricted anti-CII in the absence of IL-6. It is therefore likely that the weaker pathogenic polarized Th1 response to collagen still occurs helper 1 response, this finding indirectly suggests that a production of IgG2a isotypes is driven by a polarized T exerts a pathogenic role in CIA (26, 27). In addition, as the absence of inflammatory cell infiltrates in the joints. IL-6 in CIA, we genetically ablated IL-6 activity, and found results did not provide unequivocal evidence of the role of arthritis. It may be argued that the protection from CIA afforded by IL-6 gene inactivation could be at least partly due to a higher presence of non-DBA genes physically linked to the IL-6 locus in the DIL-6/-/- mice. Although this possibility cannot be ruled out, it is extremely unlikely to account for total protection in 100% of the mice. In addition, none of the non-MHC genes that have been previously identified as contributing to genetic susceptibility to CIA map close to the IL-6 locus (22, 40, 41).

The multiple biological activities of IL-6 make it difficult to unravel the mechanism by which absence of this cytokine offers protection from CIA. We have found that although anticollagen IgG production had decreased in DIL-6/-/- mice, it was predominantly the IgG2a subclass that exerts a pathogenic role in CIA (26, 27). In addition, as the production of IgG2a isotypes is driven by a polarized T helper 1 response, this finding indirectly suggests that a pathogenic polarized Th1 response to collagen still occurs in the absence of IL-6. It is therefore likely that the weaker anti-CII response in DIL-6/-/- mice is but one of the factors contributing to protection from the disease, also in the light of results obtained using IL-12 p40/-/- mice (35), which showed impaired anti-CII humoral response but still developed arthritis, albeit at a reduced level. Since IL-6 functions as a late-acting killer helper factor in the differentiation of CTLs (42), and CD8 T cells play an important role in CIA initiation (39, 43), IL-6 ablation may, for example, provoke a decrease in MHC class I–restricted anti-CII cytotoxic activity. In addition, the defective chemokine production and leukocyte recruitment at inflammatory sites caused by IL-6 deficiency (11) may represent an important factor, as the continuous influx of activated cells to inflamed joints is known to be responsible for disease perpetuation through increased cytokine production, tissue destruction, and enhancement of the immune response. IL-6 inactivation may therefore exert its effects on CIA development by acting at several levels. Further studies are needed to clarify this issue.

Ablation of IL-6 production did not, in contrast, affect the development of arthritis in TNF-α transgenic mice. This difference to CIA might be due to a differentiated involvement of the immune system in the two models, as suggested by the finding that TNF-α transgenic mice bearing a RAG null mutation develop arthritis in the absence of functional lymphocytes (44). Moreover, it has been recently shown that most of the TNF-α activity in this model is mediated by its capacity to induce IL-1β and to synergize with it (45), and the constitutive expression of 3’–untranslated region–modified TNF-α may not easily come under the control of regulatory factors, hence leading to an unbalanced cytokine cascade (44). Taken together, our results demonstrate that a given cytokine can have varying relevance in different arthritis models, further corroborating previous observations showing different effects of the neutralization of TNF-α in CIA or streptococcal cell wall–induced arthritis (46), and of the IL-1 receptor antagonist in CIA and antigen-induced arthritis in rabbits or mice (47, 48). The same concept may also be true for human arthritides although anti-TNF-α mAbs markedly ameliorate joint involvement in the majority of patients with RA (49), administration of the same antibody did not affect arthritis in one patient with severe systemic juvenile RA (50).

Our data support the idea that IL-6 blockade could be beneficial for the treatment of human autoimmune arthritis. Administration of an mAb to IL-6 in an open pilot trial of five patients with RA led to clinical and biological improvements (51). An alternative approach is the recently generated human IL-6 receptor superantagonist Sant7, an IL-6 variant that binds the human IL-6Rα receptor chain at high affinity but no longer binds to the gp130 receptor subunit. This molecule has been shown to inhibit the effects of IL-6 with great efficacy on a variety of human cell lines, including myeloma cells (for review see reference 52). In contrast to antibodies directed against IL-6, Sant7 is not expected to prolong IL-6 half-life. Its potential for use in therapy of chronic arthritis awaits further testing in suitable animal models, an approach that so far has not been implemented in the mouse and rat systems due to lack of binding to rodent IL-6Rα (Ciliberto, G., unpublished observation).
References


