

Research article

Collagen-induced arthritis is exacerbated in IL-10-deficient mice

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Abstract

IL-10 is a potent immunoregulatory cytokine attenuating a wide range of immune effector and inflammatory responses. In the present study, we assess whether endogenous levels of IL-10 function to regulate the incidence and severity of collagen-induced arthritis. DBA/1 wildtype (WT), heterozygous (IL-10^{+/-}) and homozygous (IL-10^{-/-}) IL-10-deficient mice were immunized with type II collagen. Development of arthritis was monitored over time, and collagen-specific cytokine production and anticollagen antibodies were assessed. Arthritis developed progressively in mice immunized with collagen, and 100% of the WT, IL-10^{+/-}, and IL-10^{-/-} mice were arthritic at 35 days.

However, the severity of arthritis in the IL-10^{-/-} mice was significantly greater than that in WT or IL-1^{+/-} animals. Disease severity was associated with reduced IFN- γ levels and a dramatic increase in CD11b-positive macrophages. Paradoxically, both the IgG₁ and IgG_{2a} anticollagen antibody responses were also significantly reduced. These data demonstrate that IL-10 is capable of controlling disease severity through a mechanism that involves IFN- γ . Since IL-10 levels are elevated in rheumatoid arthritis synovial fluid, these findings may have relevance to rheumatoid arthritis.

Keywords: antibody, arthritis, autoimmunity, cytokines

Introduction

IL-10 is a potent monocyte/macrophage regulatory cytokine that inhibits expression of proinflammatory mediators [1,2]. Monocytes/macrophages, B cells, murine Th2 cells, and some CD8⁺ cells produce IL-10 [3,4]. Macrophages rapidly produce proinflammatory cytokines such as tumor necrosis factor alpha (TNF- α), IL-1 and IL-12 after activation with lipopolysaccharide or IFN- γ , while production of IL-10 is delayed. Once IL-10 is produced it functions in an autoregulatory fashion to suppress proinflammatory cytokine mRNA expression and protein production [5–7]. In addition, IL-10 suppresses the expression of MHC class II molecules and costimulatory molecules such as intercellular adhesion molecule 1 and B7, leading to reduction in T-cell macrophage interactions [8–10].

The selective suppression of Th1 cell activity is believed to be due to IL-10 inhibition of IL-12, a differentiation factor for Th1 cells [11,12]. The release of reactive oxygen and nitrogen intermediates by macrophages is also suppressed by IL-10 [13,14]. In addition, IL-10 stimulates the production of cytokine inhibitors such as IL-1 receptor antagonist [15]. In patients with rheumatoid arthritis, IL-10 is produced by synovial membrane cells and is found at high levels in the synovial fluid [16,17]. It has been shown that suppression of IL-10 production by synovial cells is associated with increased levels of IL-1 and TNF- α , suggesting that IL-10 plays a suppressive role in rheumatoid arthritis joints [16]. It was also observed that IL-10 directly stimulated proteoglycan synthesis and reversed the cartilage degradation induced by activated mononuclear cells [18]. These immunosuppressive activities indicate that

CFA = complete Freund's adjuvant; CIA = collagen-induced arthritis; ELISA = enzyme-linked immunosorbent assay; FACS = fluorescence-activated cell sorting; FITC = fluorescein isothiocyanate; H & E = hematoxylin and eosin; IFN = interferon; IL = interleukin; MHC = major histocompatibility complex; PCR = polymerase chain reaction; Th cells = T helper cells; TNF- α = tumor necrosis factor alpha; WT = DBA/1 wildtype.

IL-10 is a potential therapeutic approach for autoimmune diseases.

In animal models of arthritis, systemic treatment with IL-10 and adenovirus-mediated transfer of viral IL-10 moderately suppresses the development of arthritis, but is significantly more effective when combined with IL-4 [19–24]. The evidence for the importance of IL-10 is further supported by the fact that *in vivo* anti-IL-10 treatment accelerates disease in collagen-induced arthritis (CIA) [22].

Most studies focused on investigating the role of IL-10 in models of arthritis have involved administration of neutralizing antibodies, large amounts of IL-10, or gene therapy in experimental animals. While these studies are helpful in broadly defining the function of IL-10, it is difficult to determine the cytokine dose and timing by these means. To address the effects of complete elimination of IL-10 *in vivo* on the development of CIA and to understand the mechanism responsible for IL-10 regulation, we examined the development of arthritis in homozygous IL-10^{-/-} IL-10-deficient mice.

Materials and methods

Animals, antigens, and immunization procedure

The IL-10^{-/-} mice were generated as previously described [25]. The original genetic background of these animals was a mixture of the strains 129/Ola and C57BL/6. These IL-10^{-/-} mice were backcrossed to DBA/1 for six generations and further backcrossed for an additional two generations to DBA/1 (Jackson Laboratories, Bar Harbor, MA, USA) in our laboratory.

All mice were typed for the IL-10 mutation by PCR using primer sets that detect either the DBA/1 wildtype (WT) or the mutated IL-10 gene. In addition, splenocytes from IL-10^{-/-} mice activated *in vitro* did not produce IL-10.

The IL-10^{-/-} mice were maintained in sterilized bedding and food with acidified water. Chicken type II collagen was used for generation of arthritis as described elsewhere [26]. Male and female WT, heterozygous IL-10^{+/-} and IL-10^{-/-} mice were immunized once with 100 µg chicken type II collagen emulsified in complete Freund's adjuvant (CFA) (Difco, Detroit, MI, USA) by intradermal injection at the base of the tail.

Assessment of arthritis

Animals were examined for the onset of joint swelling every other day. A standard scoring system based upon redness and swelling of each paw (ranging from 0 to 4 for each paw, thus resulting in a possible maximum severity score of 16) was used for the assessment of disease severity. Histologic studies were performed to determine the extent of joint damage. At the end of the experiment, hind paws were dissected, fixed, and decalcified before

being embedded in paraffin, and were sectioned at 6 µm as previously described [27]. Sagittal sections were stained with H & E.

Assessment of cytokine production by spleen cells *in vitro*

Spleens were obtained at various time points after immunization with collagen. Single cell suspensions were prepared as previously described [28]. Splenocytes (2.0 × 10⁶ cells/ml) were incubated in 24-well Falcon plates (Fisher Scientific, Pittsburgh, PA, USA) in RPMI-1640 media containing 7% fetal bovine serum (Life Technologies, Grand Island, NY, USA), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, 50 mM 2-ME, 1 mM sodium pyruvate, 0.01 mM nonessential amino acids, and 10 mM HEPES. Cells were stimulated in the presence or absence of collagen (100 µg/ml). IFN-γ was measured from day 5 supernatant using the OptEIA mouse cytokine detection system (BD PharMingen, San Diego, CA, USA).

Measurement of immunoglobulin isotypes

An ELISA was used to measure isotype-specific antibodies in serial dilutions (1:500–1:2500) of sera. ELISA plates were coated with 1 µg chicken type II collagen. Collagen-specific IgG isotypes were detected with peroxidase-labeled rabbit anti-mouse IgG₁ or IgG_{2a} (Zymed Laboratories, San Francisco, CA, USA). Titrated concentrations of IgG₁ and IgG_{2a} myeloma proteins were used to generate a standard curve, and the IgGs were detected with the same labeled rabbit anti-mouse IgG₁ or IgG_{2a} antiserum.

Flow cytometry

Flow cytometry was performed on freshly isolated spleen cells. Immunofluorescence staining of cell surface markers was performed using FITC-labeled antibodies against CD3, B220, CD11b, and CD11c (BD PharMingen). FITC-labeled rat IgG isotypes were used as controls. FcRs were blocked using anti-FcR antibody (24G2). Flow cytometric analysis was performed using a FACS Caliburflow cytometer utilizing CELLQuest software (Becton Dickinson, San Jose, CA, USA).

Statistical analysis

Analysis of the arthritis score and disease incidence at different time points were carried out using the nonparametric Mann–Whitney U test. Student's *t* test was used for statistical analysis of all other data. Analyses were performed using SPSS software (SPSS, Chicago, IL, USA).

Results

Augmented CIA in DBA/1 mice lacking IL-10

To determine whether IL-10 functions as an endogenous inhibitor of inflammatory arthritis, we examined the development of disease using the CIA model. Male and female

WT, IL-10^{+/-}, and IL-10^{-/-} littermates were immunized with collagen in CFA intradermally. Data from the male and female mice were pooled because there was no significant difference between the two groups.

All mice succumb to arthritis but the time of onset was somewhat delayed in the WT and IL-10^{+/-} mice compared with that in the IL-10^{-/-} mice (Fig. 1a). Interestingly, the number of arthritic WT and IL-10^{+/-} animals began to recede after day 35, whereas all IL-10^{-/-} mice were still arthritic at the time of termination of the experiments, although with reduced severity. Arthritis severity in IL-10^{-/-} mice was significantly exacerbated in comparison with that of WT or IL-10^{+/-} mice (Fig. 1b).

Inflamed joints showed typical histopathological abnormalities described previously (synovial proliferation, leukocyte infiltration, cartilage and bone erosions) [29,30], which correlated well with the severity of the clinical symptoms.

Taken together, the results described demonstrate that IL-10 is important for controlling disease severity.

Anticollagen antibody is reduced in IL-10-deficient mice

Induction of CIA is dependent on B cells, and high doses of antibodies are pathogenic when transferred to naïve recipients [31]. IL-10 can affect both the viability and the production of immunoglobulin by B cells [32,33]. To determine whether the augmentation in CIA correlated with an alteration in anticollagen antibodies, we collected sera from animals at the time of sacrifice. Interestingly, IL-10^{-/-} mice produced significantly less anticollagen antibody than either WT or IL-10^{+/-} mice (Fig. 2). Both the IgG₁ and the IgG_{2a} isotypes of anticollagen antibodies were substantially reduced.

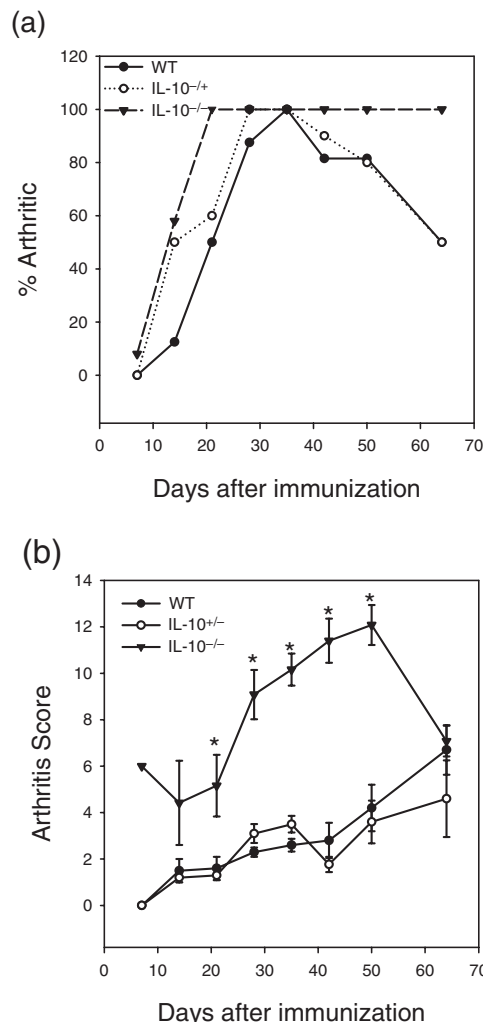
These results suggest that a decrease in anticollagen antibody may be the result of a requirement for IL-10 in B-cell antibody production.

IFN-γ levels are reduced in IL-10-deficient mice

IFN-γ production is observed early in collagen-immunized mice, and progressively increases with the time of clinical manifestation of arthritis. Although the level of IFN-γ correlates with disease, IFN-γ appears to play a dual role in disease activity. Anti-IFN-γ treatment early in the course of disease suppresses arthritis, whereas neutralization of IFN-γ late in disease exacerbates arthritis. In addition, IFN-γ receptor-deficient mice exhibit exacerbated disease [34,35].

We were therefore interested in determining whether the levels of IFN-γ correlated with arthritis in collagen-immunized WT, IL-10^{+/-}, and IL-10^{-/-} mice. Splenocytes were isolated from immunized mice and cultured in the presence and absence of collagen (Fig. 3). IFN-γ levels were

Figure 1



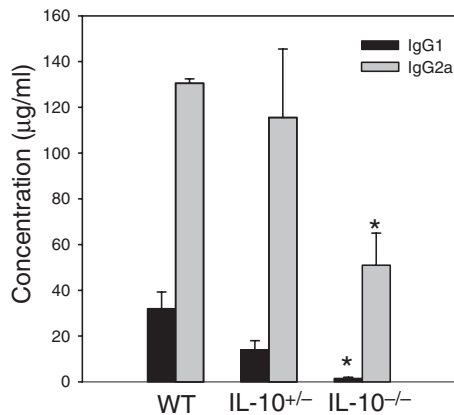
Incidence and severity of collagen-induced arthritis (CIA) in DBA/1 wildtype (WT), IL-10^{+/-}, and IL-10^{-/-} mice. (a) Incidence of CIA, expressed as the percentage of arthritic animals in the WT (n = 17), the IL-10^{+/-} (n = 12), and the IL-10^{-/-} (n = 11) mice groups. (b) Disease severity, expressed as the cumulative arthritis score, in affected animals. Values are presented as the mean ± standard error of the mean, and represent one of two experiments. Severity of arthritis was significantly different between days 20 and 50. * P < 0.05 in comparison with WT mice.

significantly suppressed in the IL-10^{-/-} mice in comparison with either WT or IL-10^{+/-} animals.

These results confirm that reduced levels of IFN-γ were associated with exacerbated arthritis in collagen-immunized animals. These data also suggest that IL-10 positively regulates IFN-γ, either directly or indirectly.

CD11b⁺ expansion correlates with reduced IL-10

Matthys *et al.* recently showed that the enhanced severity of CIA in IFN-γ receptor-deficient mice immunized with

Figure 2

Collagen-specific antibody response is reduced in IL-10^{-/-} mice. DBA/1 wildtype (WT) ($n = 17$), IL-10^{+/-} ($n = 12$) and IL-10^{-/-} ($n = 11$) mice were immunized with collagen, and the serum antibody isotypes to collagen were measured by ELISA. Values are presented as the mean \pm standard error of the mean. * $P < 0.05$ in comparison with WT mice.

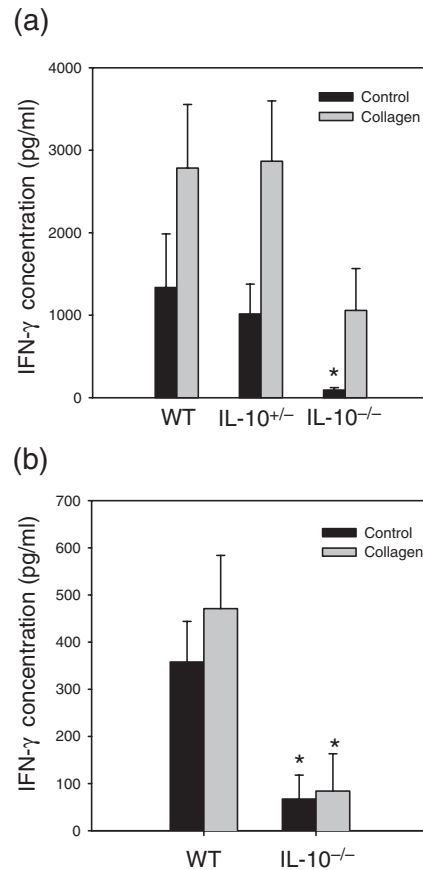
type II collagen in CFA is due to an expansion of the CD11b⁺ cells [36]. Since IL-10^{-/-} mice produce reduced levels of IFN- γ , we were interested in determining whether there was a specific expansion of CD11b⁺ cells.

Although the spleen cell number increased in IL-10^{-/-} mice ($1.42 \times 10^8 \pm 0.17$) in comparison with WT mice ($0.84 \times 10^8 \pm 0.23$), there was a specific expansion of the CD11b⁺ cell population. The spleen from WT mice contained 10.7% CD11b⁺ cells, whereas the spleen of IL-10^{-/-} mice contained 22.5% CD11b⁺ cells (Table 1). When the percentage of cells was corrected for cell number, there was a 3.7-fold increase in CD11b⁺ cells in the spleen of IL-10^{-/-} mice. The net number of T cells, B cells, and dendritic cells was not significantly different.

These data are consistent with the inhibitory effects of IFN- γ on expansion of CD11b⁺ cells. The data suggest that IL-10 is important for controlling IFN- γ and/or other cytokines involved in the process of CD11b⁺ cell expansion.

In an attempt to understand the mechanism responsible for the expansion of CD11b⁺ macrophages in IL-10^{-/-} mice, we examined cytokines associated with inflammation and Th1 cell phenotype. We were unable to detect any difference in the IL-12 or TNF- α levels in IL-10^{-/-} mice in comparison with WT mice. However, IL-1 β was significantly increased in IL-10^{-/-} animals (Fig. 4).

These results suggest the possibility that IL-1 β may play a role in CD11b⁺ cell expansion.

Figure 3

IFN- γ levels are reduced in IL-10^{-/-} mice. Spleens were harvested from collagen-immunized mice and restimulated with collagen *ex vivo*. Supernatants were harvested on day 5 and assayed by ELISA. Values are presented as the mean \pm standard error of the mean of: (a) DBA/1 wildtype (WT) ($n = 17$), IL-10^{+/-} ($n = 12$), and IL-10^{-/-} ($n = 11$) mice; and (b) WT ($n = 13$) and IL-10^{-/-} ($n = 7$) mice. * $P < 0.05$ in comparison with WT mice.

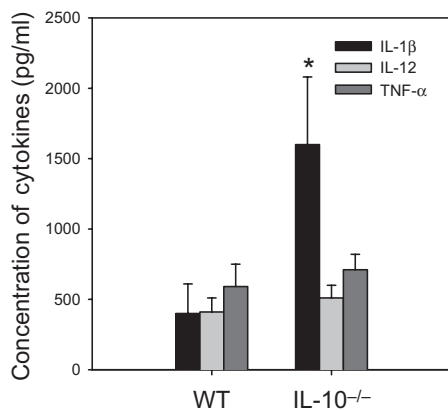
Discussion

IL-10 appears to play an important role in the regulation of several autoimmune disease models. Treatment with recombinant IL-10 in CIA, in proteoglycan-induced arthritis, and in experimental autoimmune encephalomyelitis reduced disease severity, and neutralizing IL-10 with antibodies exacerbated disease [19,20,22,23]. The present data are consistent with previous results and show that a complete absence of IL-10 exacerbates inflammation in CIA [37–39]. The anti-inflammatory properties of IL-10 suggest that endogenous IL-10 may function as a regulator of proinflammatory mediators *in vivo* [39]. It is interesting, however, that disease severity inversely correlates with the levels of IFN- γ in IL-10^{-/-} mice, suggesting that IL-10 may control disease activity via regulating IFN- γ responses.

Table 1**Ratio of cell populations between DBA/1 wildtype spleen and IL-10^{-/-} spleen in IL-10 deficient mice^a**

Cells	DBA/1 wildtype		IL-10 ^{-/-}		Ratio of cell number, IL-10 ^{-/-} /WT
	%	<i>n</i> × 10 ⁷	%	<i>n</i> × 10 ⁷	
CD3	16.4 ± 0.68	1.3 ± 0.4	9.7 ± 3.8	1.4 ± 0.6	1.07
B220	63.7 ± 2.5	5.3 ± 1.6	31.8 ± 3.3	4.5 ± 0.5	0.84
CD11c	4.1 ± 0.4	0.3 ± 0.1	2.33 ± 0.7	0.3 ± 0.1	0.94
CD11b	10.7 ± 2.0	0.9 ± 0.1	22.5 ± 3.7	3.2 ± 0.8*	3.7

^a The percentage of CD3, B220, CD11c, and CD11b cells was determined by flow cytometry as described in Materials and methods, and the exact number of cells calculated based on the total number of splenocytes. Values are presented as the mean ± standard deviation of three mice. The ratio was calculated as the number of IL-10^{-/-} cells divided by the number of DBA/1 wildtype cells. * Significantly different (*P* < 0.05 in comparison with DBA/1 wildtype).

Figure 4

IL-1 β levels are increased in IL-10^{-/-} mice. Supernatants were harvested on day 5 and assayed by ELISA. Values are presented as the mean ± standard error of the mean of DBA/1 wildtype (WT) (*n* = 12) and IL-10^{-/-} (*n* = 7) mice, and represent one of two experiments. * *P* < 0.05 in comparison with WT mice. TNF- α , tumor necrosis factor alpha.

Although CIA is considered a Th1-type disease mediated by IFN- γ , the role of IFN- γ in the pathogenesis of CIA is not clearly understood. IFN- γ appears to have two separate functions, disease promoting as well as disease limiting [40]. Neutralization of IFN- γ with antibodies early in the course of disease exerts a suppressive effect, whereas anti-IFN- γ treatment late in disease enhances arthritis [41]. Also, disease severity in CIA is enhanced in IFN- γ receptor-deficient mice, and loss of the IFN- γ receptor turns mice normally resistant to CIA into an arthritis-susceptible strain [34,35]. IFN- γ thus provides a dominant protective effect in CIA. The reduction in IFN- γ in IL-10-deficient mice is consistent with the disease-limiting properties of IFN- γ . These results suggest that IL-10 plays an unexpected role in regulating IFN- γ production in CIA.

Recent work by Matthys *et al.* demonstrates that the protective effect of IFN- γ is dependent on the presence of the mycobacterial component of the adjuvant [36]. Only when mice are immunized with collagen in CFA is there an increase in disease severity in IFN- γ receptor-deficient mice. Ablation of IFN- γ in these mice is associated with extramedullary hemopoiesis and expansion of CD11b⁺ cells. Consistent with this observation, a similar increase in CD11b⁺ cells was observed in the IL-10^{-/-} mice. These data suggest that IL-10 controls the IFN- γ concentration *in vivo* and that the reduced level of IFN- γ in IL-10^{-/-} mice contributes to expansion of CD11b⁺ cells and increase in disease severity. The increase in IL-1 β we observed in IL-10^{-/-} mice may account for the increase in CD11b⁺ cells as IL-1 β is known for its hematopoietic properties [42].

In addition to the cellular immune response, anticollagen antibodies are required for the development of arthritis. In the studies presented, despite the increase in disease severity in the IL-10^{-/-} mice, anticollagen antibodies are reduced. This reduction in antibody levels may be a direct effect on B cells due to a loss of IL-10 or may be an indirect effect due to downregulation by IFN- γ . The Th1 cytokine IFN- γ is important *in vitro* and *in vivo* for enhancement of IgG_{2a} secretion [43]. It is expected that loss of IFN- γ should result in a reduced collagen-specific IgG_{2a} response, but it is unexpected that the IgG₁ response would also be reduced. These results indicate that IL-10 has a direct effect on maintaining antibody production in CIA. In addition, loss of the anti-inflammatory effect of IL-10 appears to overdrive the requirements for high levels of anticollagen antibodies in CIA.

Conclusion

In summary, these results suggest that IL-10 is an important regulator of inflammation *in vivo*. In CIA, a deficiency in IL-10 leads to an increase in disease severity. The corresponding reduction in IFN- γ levels and the expansion of

CD11b+ cells suggests a potential mechanism for IL-10 regulation of CIA.

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