IL-12 promotes cellular but not humoral type II collagen-specific Th1-type responses in C57BL/6 and B10.Q mice and fails to induce arthritis

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Keywords: antibody response, autoimmunity, genetic background

Abstract

DBA/1 (H-2^d) and C57BL/6 (H-2^b) mice develop an intermediate immune response when immunized with chicken type II collagen (CII) emulsified with Incomplete Freund's adjuvant (IFA). Only a few animals develop a mild form of arthritis. As reported before and confirmed herein, administration of IL-12 to DBA/1 mice immunized with CII in IFA strongly enhances the cellular and humoral (auto)immune response to CII and induces severe destructive joint disease with an incidence of 80-100%. In contrast, the same treatment did not promote joint disease in C57BL/6 mice. Characterization of the IL-12 effect on the CII-specific immune response of C57BL/6 mice revealed that IL-12 promoted the development of CII-specific T cells producing IFN-γ in DBA/1 and C57BL/6 mice equally well. However, whereas treatment with IL-12 in DBA/1 mice strongly up-regulated the synthesis of CII-specific antibodies, especially of the IgG2a and IgG2b subclasses, it rather slightly down-regulated the CII-specific IgG2a and IgG2b synthesis in C57BL/6 mice. This may indicate that the effect of IL-12 on the CII-specific antibody synthesis is of crucial importance in the pathogenesis of type II collagen-induced arthritis (CIA). The failure of IL-12 to up-regulate IgG2a and IgG2b synthesis in C57BL/6 mice is specific for CII as antigen and not a general property of this strain because the keyhole limpet hemacyanin-specific antibody response is up-regulated by IL-12 in C57BL/6 mice. Furthermore, it is not the H-2^b haplotype of C57BL/6 mice but rather the genetic background (DBA/1 versus BL/6 or BL/10) that limits the effect of IL-12 on the CII-specific antibody response because IL-12 treatment of CII-immunized B10.Q (H-2^d) mice also failed to induce arthritis and to enhance CII-specific IgG2a and IgG2b synthesis. However, as in the two other strains, injection of IL-12 promoted the development of splenic T cells producing IFN-γ upon activation with CII. These results indicate that an enhancement of the cellular and humoral anti-CII response by IL-12 is required for inducing arthritis.

Introduction

Type II collagen-induced arthritis (CIA) is a disease influenced by MHC class II haplotypes as well as non-MHC genes (1-5). Mice bearing the H-2^d haplotype on the DBA/1 background are highly susceptible. The importance of the MHC for CIA was clearly demonstrated by an elegant study of Brunsberg et al. (6). I-A^q molecules (associated with susceptibility) differ from I-A^p molecules (associated with resistance) by only four amino acids in the I-A_b chain. Immunization of transgenic mice expressing a modified A_p molecule, where the relevant positions were exchanged to resemble A_q, with type II collagen (CII) results in an enhanced autoimmune response and in the development of arthritis. Thus, MHC class II polymorphism can exert a dominant influence on the susceptibility to CIA. However, the relative resistance of C57BL/6 (H-2^b) and B10.Q (H-2^d) mice regarding the induction of arthritis by immunization with CII in complete Freund's adjuvant (CFA) appears to be due to the influence of as yet unidentified non-MHC background genes (3,4).

Usually, mice are immunized with CII emulsified with CFA, that contains killed mycobacteria as adjuvant, to induce CIA.
The animals were intradermally immunized (into both auriculae with an equal volume of IFA. The total volume per mouse was 100 µl emulsion. Booster immunizations were performed i.p. with 100 µg CII in PBS in a total volume of 200 µl. Some groups of mice additionally received IL-12 (i.p., 200 µl each mouse) at various doses (dissolved in PBS containing 1% syngeneic normal mouse serum) for 5 days (starting 1 day before the CII immunization). Control mice received 200 µl PBS + 1% normal mouse serum for 5 days instead. Beginning 2 weeks after the priming immunization, the animals were visually inspected at regular intervals for signs of arthritis. Mice were scored positive for CIA if erythema and swelling of at least one digit and/or paw was observed.

**CII-specific immune response as well as on the development of arthritis in 'CIA-resistant' mouse strains.**

**Methods**

**Mice**

DBA/1 mice were originally obtained from Charles River (Sulzfeld, Germany), C57BL/6 mice from the Zentralinstitut für Versuchstierforschung (Hannover, Germany) and B10.Q mice from Bomolice (Bomholtgard, Denmark). Animals of these strains as well as (DBA/1×C57BL/6)F1 mice were bred at the local animal facility. Female and male mice were used in the experiments at 8–12 weeks of age.

**Reagents and antibodies**

Streptavidin–peroxidase was purchased from Boehhringer-Mannheim (Mannheim, Germany). Aluminum hydroxide (2 mg/ml Al(OH)₃) was bought from Eurobio (Raunheim, Germany). The peroxidase substrate 2,2-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), keyhole limpet hemocyanin (KLH) and chicken CII were obtained from Sigma (Munich, Germany). CII was dissolved in 0.01 N acetic acid at a concentration of 4 mg/ml. Before using CII in the stimulation of spleen cells, it was denatured by heat (30 min, 60°C). IFA was obtained from Difco (Detroit, MI). Purified recombinant murine rIL-12 (>95% pure IL-12, as assessed by SDS–PAGE; <5 endotoxin U/mg, as determined by the Limulus amebocyte assay) was prepared from supernatant of transfected CHO cells (10). A pair of mAb recognizing different epitopes of IFN-γ (mAb R46A2 and biotinylated mAb AN-18.17.24) was used to determine IFN-γ by ELISA as described previously (11).

**Immunization of mice and determination of arthritis**

The animals were intradermally immunized (into both auriculae and one site on the back) with 100 µg of chicken CII emulsified with an equal volume of IFA. The total volume per mouse was 100 µl emulsion. Booster immunizations were performed i.p. with 100 µg CII in PBS in a total volume of 200 µl. Some groups of mice additionally received IL-12 (i.p., 200 µl each mouse) at various doses (dissolved in PBS containing 1% syngeneic normal mouse serum) for 5 days (starting 1 day before the CII immunization). Control mice received 200 µl PBS + 1% normal mouse serum for 5 days instead. Beginning 2 weeks after the priming immunization, the animals were visually inspected at regular intervals for signs of arthritis.
IL-12 fails to promote arthritis in mice with C57BL background

Table 1. Time-course of CIA in CII-immunized DBA/1 and C57BL/6 mice ± IL-12 treatment

<table>
<thead>
<tr>
<th>Day</th>
<th>IL-12 (ng/day)</th>
<th>0</th>
<th>1000</th>
<th>200</th>
<th>40</th>
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</thead>
<tbody>
<tr>
<td>(A) Arthritic DBA/1 mice/total number of DBA/1 mice</td>
<td>41</td>
<td>0/11</td>
<td>ND</td>
<td>3/9</td>
<td>ND</td>
</tr>
<tr>
<td>70</td>
<td>0/11</td>
<td>ND</td>
<td>7/8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>0/11</td>
<td>ND</td>
<td>7/8</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>(B) Arthritic C57BL/6 mice/total number of C57BL/6 mice</td>
<td>39</td>
<td>2/10</td>
<td>0/10</td>
<td>1/10</td>
<td>2/10</td>
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<td>61</td>
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<td>1/10</td>
<td>2/10</td>
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<td>0/10</td>
<td>0/10</td>
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</tr>
</tbody>
</table>

Male DBA/1 or C57BL/6 mice were intradermally primed with 100 μg CII in IFA. All animals were boosted again by injecting 100 μg of soluble CII i.p. after 3, 6 and 9 weeks. Some groups of mice were additionally treated with IL-12 (day -1 to +3) at the indicated concentrations. All mice were inspected for clinical signs of arthritis (erythema, swelling of digits or paws) ND, not done.

(five female and five male mice/group) were immunized with CII in IFA. In some groups, mice additionally received injections of IL-12 at three different doses (1000, 200 and 40 ng/daily). For reasons of comparison, two groups of DBA/1 mice were immunized with CII in IFA and the animals of one group received in addition an optimal dose of IL-12 (200 ng/daily). As reported earlier (7) and observed again (Table 1A), DBA/1 mice immunized with CII in IFA did not develop clinical signs of arthritis during a long period of observation (Table 1A, day 41 to 100: CIA incidence 0/11) even after repeated immunizations with CII [this reflects the low incidence (0–20%) of spontaneous arthritis by mice of the local colony]. Co-injection of IL-12 promoted the development of joint disease in DBA/1 mice. At day 41 the CIA incidence was three out of nine mice and increased to seven out of eight mice at day 70 and day 100 (Table 1A). Most of the seven arthritic mice had developed a severe arthritis with massive swelling and deformity of at least one paw (not shown). In striking contrast, administration of IL-12 failed to promote CIA in C57BL/6 mice. In the group immunized with CII in IFA alone, two mice showed erythema and slight swelling of some paws at day 39 after the first immunization (Table 1B). However, these clinical signs of disease disappeared at later time points and the animals were free of symptoms between day 61 and 130. Likewise, only a few animals (3/30) of the IL-12-treated groups expressed symptoms of arthritis at an early time point that were no longer observed at the end of the experiment (Table 1B). Thus, in contrast to the results with DBA/1 mice, injection of IL-12 did not result in an up-regulation of the incidence of CIA in C57BL/6 mice.

We next characterized and compared the CII-specific humoral and cellular immune response of DBA/1 and C57BL/6 mice. When immunized with CII in IFA alone, two strains developed an intermediate CII-specific total Ig response (Fig. 1A). Injection of IL-12 resulted in an up-regulation of the CII-specific antibody response to high Ig levels in DBA/1 mice, whereas total Ig synthesis remained unchanged in C57BL/6 mice. Intermediate levels of CII-specific IgG1 were produced in both strains that were slightly up-regulated in IL-12-treated mice (Fig. 1B). The most striking effect of IL-12 treatment was observed in DBA/1 mice where the relatively low IgG2a and IgG2b synthesis, observed in the absence of IL-12, was up-regulated to high or intermediate levels respectively (Fig. 1C and D). In contrast, a similar treatment of C57BL/6 mice slightly reduced (although statistically not significant) the CII-specific IgG2a and IgG2b response which was, however, higher, intermediate levels, as compared with low levels in DBA/1 mice) in CII-immunized C57BL/6 than in DBA/1 mice in the absence of IL-12 treatment (Fig. 1C and D). Taken together, these data demonstrate that administration of IL-12 has qualitatively opposite effects on the synthesis of CII-specific IgG2a and IgG2b production in DBA/1 and C57BL/6 mice immunized with CII in IFA.

Interestingly, the effect of IL-12 on the development of IFN-γ-producing T cells was comparable in both strains (Fig. 2). Spleen cells of DBA/1 and C57BL/6 mice, immunized with CII in IFA ± IL-12 treatment, were prepared at the end of the arthritis experiments (DBA/1: day 100; C57BL/6: day 130)
IL-12 fails to promote arthritis in mice with C57/BL background

Fig. 2. Synthesis of IFN-γ by spleen cells of DBA/1 and C57BL/6 mice immunized with CII in IFA ± IL-12 treatment. Spleen cells from DBA/1 (n = 4/each group) and C57BL/6 (n = 3/each group) mice were prepared at day 100 or 130 respectively. Mice of both strains were immunized with either CII in IFA alone or additionally treated with IL-12 (200 ng/day). Single cell suspensions were prepared and cultured in 48-well plates with or without heat-denatured CII (100 μg/ml) for 72 h. The stimulation supernatants were collected and assayed for IFN-γ by ELISA (for details see Methods). The mean of IFN-γ synthesis (U/ml) including SD of similarly activated spleen cell cultures is shown.

and single cell suspensions cultured in vitro in the absence or presence of CII. IFN-γ synthesis was low in the absence of CII. Addition of CII to spleen cell cultures of either DBA/1 or C57BL/6 mice that had been immunized with CII in IFA alone induced some IFN-γ production. About 10-fold higher levels of IFN-γ were detected in the supernatants of spleen cell cultures of both DBA/1 (Fig. 2A) and C57BL/6 (Fig. 2B) mice immunized before with CII in IFA and simultaneously injected with IL-12. Thus, administration of IL-12 promoted the generation of IFN-γ-producing, CII-specific T cells in both strains equally well.

Injection of IL-12 promotes arthritis and up-regulates CII-specific IgG2a and IgG2b production in (DBA/1×C57BL/6)F1 mice immunized with CII in IFA

In order to find out whether the relative resistance of C57BL/6 mice or the susceptibility of DBA/1 mice to CIA promoted by IL-12 is dominant, (DBA/1×C57BL/6)F1 mice were immunized with CII in IFA and either left untreated or treated with three different doses of IL-12 (Table 2). Injections of IL-12 at all doses accelerated the onset of arthritis (Table 2, day 55) and increased the incidence of CIA as compared with the group that did not receive IL-12. In particular, at a daily dose of 200 ng IL-12 promoted a very severe form of joint disease with high incidence (Table 2 and unpublished). Determination of the CII-specific IgG2a and IgG2b levels revealed that IL-12 (200 ng/day) treatment up-regulated the synthesis of both isotypes from intermediate (in the absence of IL-12) to high levels in (DBA/1×C57BL/6)F1 mice (Fig. 3). Administration of IL-12 also resulted in enhanced IFN-γ synthesis by spleen cells stimulated ex vivo with antigen (data not shown). Thus, regarding the effect of IL-12 on the synthesis of IgG2a and IgG2b antibodies as well as on the course of joint disease, (DBA/1×C57BL/6)F1 mice resemble DBA/1 mice and are highly susceptible to CIA.

B10.Q mice, which have the same MHC haplotype as DBA/1 mice, are resistant to the CIA-promoting effect of IL-12

C57BL/6 mice differ from DBA/1 mice regarding the MHC haplotype (H-2b versus H-2d) as well as to their non-MHC genetic background. To address the question whether MHC or non-MHC genes regulate the influence of IL-12 on the CIA-
IL-12 fails to promote arthritis in mice with C57/BL background

Specific humoral immune response, four groups of B10.Q mice, which express the same MHC haplotype as DBA/1 mice, were immunized with CII in IFA, and three groups were injected with various doses of IL-12 (1000, 200 and 40 ng/day). The animals were inspected for clinical signs of arthritis and tested for their humoral and cellular immune response. None of the animals of any of the groups developed erythema or swelling during the whole observation period (day 21 to 118). Similar to C57BL/6 mice but in contrast to DBA/1 mice, injections of IL-12 (200 ng/day) into CII-immunized B10.Q mice slightly down-regulated the serum levels of CII-specific IgG2a and IgG2b antibodies (Fig. 4). However, as observed in all strains of mice immunized with CII in IFA so far, administration of IL-12 resulted in enhanced (5.4-fold higher) IFN-γ synthesis by splenocytes activated ex vivo with CII as compared with splenocytes from mice not treated with IL-12 (Fig. 5). Taken together, the results described so far indicate that the genetic background of C57BL/10 and C57BL/6 mice is probably responsible for the failure of IL-12 to enhance the humoral anti-CII response and to promote arthritis in these strains of mice despite the development of a strong T

Administration of IL-12 to C57BL/6 mice immunized with KLH adsorbed to aluminum hydroxide profoundly enhances the KLH-specific IgG2a and IgG2b response

The results obtained with CII-immunized C57BL/6 and B10.Q mice raised the question of whether injection of IL-12 can generally not enhance antigen-specific IgG2a/2b responses in these strains of mice. We have recently reported that IL-12 can strongly up-regulate KLH-specific antibody responses in CBA/J mice (12). Thus, we immunized C57BL/6 mice i.p. with KLH adsorbed to aluminum hydroxide. The developing humoral immune response was characterized by high levels of IgG1 (not shown), intermediate levels of IgG2b and very low levels of IgG2a (Fig. 6). Treatment of KLH-immunized C57BL/6 mice with IL-12 caused a strong enhancement of serum levels of IgG2a (400-fold) and IgG2b (40-fold). This demonstrates that the effects of IL-12 treatment on humoral immune responses in C57BL/6 mice are dependent on the antigen studied. In the case of KLH, IL-12 is a very potent
adjuvant for KLH-specific IgG2a and IgG2b synthesis, whereas it fails to do so with CII as antigen (Fig. 1C and D).

The differential effect of IL-12 on the KLH- or CII-specific antibody response of C57BL/6 mice is indeed due to the antigen and not a consequence of the mode of immunization, since injections of IL-12 up-regulated the KLH-specific IgG2a synthesis of C57BL/6 mice immunized i.d. with KLH i.p. about 100-fold, whereas IL-12 did not augment the CII-specific antibody response of C57BL/6 mice immunized i.p. with CII adsorbed to aluminum hydroxide (unpublished observations).

Discussion

In murine CIA, an animal model for human rheumatoid arthritis (13), disease susceptibility is linked to the H-2d haplotype expressed on the DBA/1 background (1–4). When immunized under the appropriate conditions (CII in CFA or CII in IFA + IL-12), DBA/1 mice mount a strong cellular and humoral anti-CII immune response and develop a destructive arthritis in peripheral joints. Mice bearing the H-2d or H-2h haplotypes respond to CII in CFA, yet do not (or with low frequency) develop arthritis (1,3,4). The purpose of this study was to test whether IL-12 would be able to up-regulate the CII-specific immune response and to promote arthritis in ‘CIA-resistant’ mouse strains immunized with CII in IFA. The CII-specific antibody response of C57BL/6 and DBA/1 mice immunized with CII in IFA is similar in magnitude and IgG subclass distribution. Simultaneous IL-12 treatment supported the generation of CII-specific T\text{h}1 cells equally well in both strains. However, injections of IL-12 strongly up-regulated CII-specific antibody (IgG2a, IgG2b) synthesis and caused arthritis only in DBA/1 but not in C57BL/6 mice. Likewise, IL-12 treatment did neither enhance the CII-specific antibody response nor trigger arthritis in C57BL/10 mice (data not shown). It appears to be the non-MHC background rather than the MHC haplotype that is responsible for this behaviour. Immunization of congenic C57BL/10.Q (H-2d) mice with CII/IFA ± IL-12 treatment again revealed that IL-12 failed to augment CII-specific IgG2a production and to promote arthritis. Since B10.Q mice bear the same MHC as DBA/1 mice, this indicates that as yet unidentified non-MHC background genes modify the effect of IL-12 on antibody synthesis in vivo. Those of the DBA/1 background appear to be dominant because administration of IL-12 to (DBA/1 × C57BL/6)\text{F}_1 mice immunized with CII/IFA augmented IgG synthesis and induced arthritis with relatively high incidence.

IL-12 is considered to be a cytokine promoting cellular immunity (14) and is a key mediator in the induction of T\text{h}1-type responses (11,15,16). It also appears to be critically involved in the pathogenesis of several organ-specific autoimmune diseases (17). Cellular immunity is frequently associated with T cell responsiveness and low or absent antibody synthesis (18–20). The initial reports about the influence of IL-12 on Ig synthesis described mainly inhibitory effects (21–23). In contrast, we recently reported that IL-12 can up-regulate the synthesis of all IgG subclasses in CBA/J mice immunized with protein antigens adsorbed to aluminum hydroxide (12). The effect was most pronounced for antigen-specific IgG2a which was up-regulated 100- to 1000-fold. Likewise, the KLH-specific IgG2a response of C57BL/6 (Fig. 6) mice was up-regulated 100- to 1000-fold after two immunizations with KLH and simultaneous IL-12 treatment. Moreover, Jelinek and Braaten (24) showed that IL-12 can support IgG synthesis by purified human B cells stimulated in vitro with Staphylococcus aureus Cowan. Thus, IL-12 can enhance Ig synthesis by direct and indirect effects. In the context with CIA, it is intriguing that arthritis is only observed under those conditions where IL-12 exerted profound effects on the synthesis of the complement-fixing IgG2a and IgG2b antibody subclasses. This reflects the importance of the humoral anti-CII response (1,2,7) and the synergy between CII-specific antibodies and CII-specific T\text{cells} (25,26) in the pathogenesis of CIA. In C57BL/6 or B10.Q mice, where IL-12 failed to promote CIA, IL-12 did not exert any stimulatory effect on the synthesis of CII-specific IgG2a and IgG2b after two (see Figs 1C, 1D and 4) or even four (unpublished observation) immunizations with antigen. However, injections of IL-12 promoted the development of IFN-\gamma-producing T\text{h}1-type cells equally well in DBA/1, C57BL/6 or B10.Q mice immunized with CII in IFA. But only the DBA/1 T\text{h}1 cells provided efficient help to B cells resulting in increased CII-specific IgG synthesis and arthritis. This may reflect the heterogeneity of T\text{h}1 cell clones regarding their ability to induce antibody responses in vivo (27,28). Whatever the mechanism is, the fact that the presence of CII-specific T\text{h}1-type cells in combination with an intermediate CII-specific antibody response with relatively high levels of IgG1 does not (or with low incidence) lead to joint disease in IL-12-treated C57BL/6 or B10.Q mice indicates a functional heterogeneity within T\text{h}1-type autoimmune responses. Thus, precisely defining the immunological conditions under which IL-12 augments antibody synthesis might be useful for the understanding of the pathogenesis of CIA and probably other autoimmune diseases.

In conclusion, our results show that IL-12 induces arthritis only in DBA/1 but not in C57BL/6 or B10.Q mice immunized with CII in IFA, although it promotes the development of T cells producing IFN-\gamma in all three strains. It is the intermediate CII-specific antibody response of C57BL/6 or B10.Q mice that is not up-regulated by IL-12, due to the influence of so far unidentified non-MHC background genes, that correlates with resistance to CIA.

Acknowledgements

The authors thank Frank J. Podlaski and Maurice K. Gately, Hoffmann-LaRoche Inc., Nutley, NJ for the preparation and generous gift of mouse recombinant IL-12. We also thank Maurice K. Gately for critically reviewing this manuscript. J S was supported by a grant from the DAAD (Deutscher Akademischer Austauschdienst) This work was supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 311.

Abbreviations

CFA complete Freund’s adjuvant
CIA collagen-induced arthritis
CII type II collagen
IFA incomplete Freund’s adjuvant
KLH keyhole limpet hemocyanin
References


