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Endogenous IL-12 Is Required for Induction and Expression of Experimental Autoimmune Uveitis

Teresa K. Tarrant,‡ Phyllis B. Silver,* Chi-Chao Chan,* Barbara Wiggert,† and Rachel R. Caspi1*†

Experimental autoimmune uveitis (EAU)2 is a prototypic organ-specific, T cell-mediated disease elicited in rodents and nonhuman primates by immunization with retinal Ags or their fragments that leads to destruction of the neural retina and related tissues. EAU can also be elicited by adoptive transfer of retinal-specific CD4+ T cells between syngeneic rodents (1–4). The pathology of EAU in the mouse model closely resembles that of human uveitis diseases of putative autoimmune etiology and serves as a model for these sight-threatening diseases (2). Therefore, the study of immunologic mechanisms affecting EAU in the mouse can shed light on mechanisms affecting human uveitis as well as other organ-specific, T cell-mediated autoimmune diseases.

IL-12 is a heterodimeric protein composed of two disulfide-linked subunits of 40 kDa (p40) and 35 kDa (p35) (5, 6). APCs secrete IL-12 in response to engagement of the MHC class II and CD40 molecules (7, 8). Following secretion, IL-12 induces the release of IFN-γ from NK cells and T cells and augments cell-mediated immune responses in vitro and in vivo (9–14). IL-12 also regulates Th cell-dependent immune responses by priming T cells for high IFN-γ production and inducing them to differentiate toward the Th1 pathway (15–19).

Recent results from our laboratory suggest that the pathogenesis of EAU is associated with a Th1 response. Pathogenic, but not nonpathogenic, T cell populations produce large amounts of IFN-γ, and susceptible strains of rodents mount a Th1-dominated response to the uveitogen, whereas resistant strains do not (20, 21). However, in apparent contradiction to these findings, mice deficient in IFN-γ are susceptible to EAU and develop the disease in the context of a deviant effector response containing elements of a Th2 response profile (22). Furthermore, administration of IL-12 to EAU-susceptible mice immunized for disease induction aborts development of disease2 (23). We therefore wished to investigate whether endogenous IL-12 production was in fact required for the development of the pathogenic effector cells and expression of disease.

Recently, an IL-12-deficient mouse (12KO) with a disruption at the p40 locus was developed and characterized (24, 25). These mice develop a normal immune system but have impaired Th1 responses (26). These mice were therefore used to study the need for IL-12 and for a Th1 response in the development of EAU. The present data show that IL-12-deficient mice were unable to develop EAU after immunization with a maximal disease-inducing regimen, and their Ag-specific responses to the uveitogenic Ag were Th2-like. However, they were able to develop EAU when infused with their own primed cells that had been incubated with Ag in the presence of IL-12. We therefore conclude that the resistance of 12KO mice to EAU involves an inability to develop a pathogenic Th1 response to the uveitogen and that endogenous IL-12 is required for pathogenesis of EAU.

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1 Address correspondence and reprint requests to Dr. Rachel R. Caspi, Laboratory of Immunology, National Eye Institute, Building 10, Room 10N222, National Institutes of Health, Bethesda, MD 20892. E-mail address: rcaspi@helix.nih.gov

2 Abbreviations used in this paper: EAU, experimental autoimmune uveitis; IRBP, interphotoreceptor retinoid-binding protein; KO, knockout; FTX pertussis toxin; 12KO, IL-12 p40-deficient; wt, wild type; α-MMP, α-methyl-o-mannopyranoside; DTH, delayed-type hypersensitivity.

3 Tarrant, T., P. Silver, L. Rizzo, C. Chan, B. Wiggert, and R. Caspi. Interleukin-12 protects from a Th1-mediated autoimmune disease, experimental autoimmune uveitis, through a mechanism involving IFN-α and NO. Submitted for publication.
Materials and Methods

Animals

C57BL/6 female mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Mice with a targeted disruption of the IL-12, p40 gene (12KO) were developed, screened, and back-crossed for five generations onto the C57BL/6 background. Breeding stock as well as mice for some of the experiments were obtained from Jeanne Magram of Hoffman LaRoche (Nutley, NJ). All animals were housed under conventional conditions, were given water and chow ad libitum, and were used at 1.5 to 6 mos of age. The use of the animals conformed to institutional and National Institutes of Health guidelines.

Reagents

Interphotoreceptor retinoid-binding protein (IRBP) was isolated from bovine retina by Con A-Sepharose affinity chromatography and fast performance liquid chromatography, as described previously (27). BSA, α-methyl-d-nanopyranoside (α-MMP), Con A, pertussis toxin (PTX), CFA, and conalbumin were purchased from Sigma (St. Louis, MO). Horseradish peroxidase (HRPO)-streptavidin was purchased from Southern Biotechnology Associates (Birmingham, AL). Mycobacterium tuberculosis strain H37RA was purchased from Difco (Detroit, MI). Ab pairs for ELISA were purchased from PharMingen (La Jolla, CA) and Southern Biotechnology Associates. Murine TNF-α ELISA detection kits were purchased from Endogen (Cambridge, MA). Purified murine recombinant IL-12 was generously provided by M. K. Gately of Hoffman LaRoche (Nutley, NJ).

Immunization and IL-12 administration

Wild-type C57BL/6 mice were immunized s.c. in the thighs and base of tail with 10 μg IRBP in 0.2 ml emulsion 1:1 v/v with CFA containing 2.5 mg/ml M. tuberculosis and were simultaneously injected i.p. with 1 μg PTX in 0.1 ml as an additional adjuvant. In experiments with 12KO mice, the concentrations of IRBP and PTX were doubled (200 μg and 2 μg respectively) to achieve the maximum disease-inducing conditions. Reconstitution of 12KO mice with IL-12 was done in two ways: 1) During the priming phase, actively immunized 12KO mice were given daily injections of 50 μg IL-12, as specified. 2) During the expression phase, 12KO mice that were adoptively transferred with cultured lymph node cells from IRBP-primed wt donors were given daily injections of IL-12, as specified.

Histopathology and EAU grading

Whole eyes were collected and prepared for histopathologic evaluation on day 21 at the termination of an experiment. The eyes were immersed for 1 h in 4% phosphate-buffered glutaraldehyde and then transferred into 10% phosphate-buffered formaldehyde until processing. Fixed and dehydrated ocular tissue was embedded in methacrylate, and 4- to 6-μm sections were cut through the pupillary-optic nerve plane. Sections were stained by hematoxylin and eosin. An ocular pathologist evaluated the presence or absence of disease in a masked fashion after examining six sections cut at different levels for each eye. Severity of EAU for each eye was scored on a scale of 0 (normal-appearing eyes) to 4 (maximum disease) in half-point increments, according to a semiquantitative system described previously (28). Briefly, the minimal criterion to score an eye as positive by histopathology was inflammatory cell infiltration of the ciliary body, choroid, or retina (EAU grade 0.5). Progressively higher grades were assigned for presence of discrete lesions in the tissue, such as vasculitis, granuloma formation, retinal folding and/or detachment, photoreceptor damage, etc. The grading system takes into account lesion type, size, and number.

Delayed hypersensitivity

Two days before the termination of an experiment, mice received 10 μg of IRBP in 10 μl intradermally into the pinna of one ear. The other ear was injected similarly but with PBS. Ear swelling was measured at the termination of the experiment 48 h later with a spring loaded micrometer. Delayed hypersensitivity results are expressed as Ag-specific swelling, calculated as the difference between the thickness of the IRBP-injected ear and the PBS-injected ear.

Lymphocyte proliferation

Draining lymph nodes (inguinals and iliacs) were collected at the termination of an experiment and pooled within each group. Triplicate cultures of 5 × 10^5 cells/0.2 ml/well were stimulated with 30 μg/ml IRBP in 96-well flat-bottom plates in RPMI 1640 containing 1% naive mouse serum and 20 μg/ml α-MMP. The cultures were incubated for 60 h and were pulsed with [3H]thymidine (1.0 μCi/10 μl/well) for the last 18 h. The data are shown as Ag-specific stimulation index (SI), calculated as mean cpm in cultures stimulated with IRBP divided by mean counts in control cultures with no stimulus.

Determination of lymphokine production in lymphocyte culture supernatants

Draining lymph node cells and spleens were removed at the termination of an experiment and pooled within each group. The cells were cultured in 96-well flat-bottom plates (1 × 10^6 cells/0.2 ml/well) with 50 μg/ml of IRBP in RPMI 1640 medium containing 1% fresh-frozen syngeneic mouse serum and 20 μg/ml α-MMP (to neutralize possible traces of Con A, which is used during the purification of IRBP). Supernatants were collected for cytokine production analysis after 48 h. IFN-γ, IL-4, IL-5, IL-6, and IL-10 were measured by ELISA using Ab pairs from PharMingen, as described previously (29). The murine cytokine ELISA detection kits from Endogen were used to measure TNF-α in all experiments.

Measurement of Ag-specific IgG isotypes

Serum levels of anti-IRBP IgG2a and IgG1 subclasses were determined by ELISA, as described previously for another Ag (30). Briefly, 96-well microtiter plates (Costar, Cambridge, MA) were coated with IRBP (1 μg/ml). After blocking the plates with BSA and an overnight incubation with serum samples, the plates were developed using horseradish peroxidase-conjugated goat anti-IgG subclass-specific Abs (Southern Biotechnology Associates). The concentration of anti-IRBP Ab was estimated using standard curves constructed by coating wells with anti-Ig Ab and by adding polyclonal Ig standards of the pertinent isotype.

Adoptive transfer of EAU

Donor mice were immunized with a uveitogenic regimen of IRBP (12KO donors with 200 μg IRBP + 2 μg PTX; wt donors with 100 μg IRBP + 1 μg PTX). Lymph node cells and spleen cells collected on day 14 after immunization were pooled within the respective groups. The cell suspension was adjusted to 10^7 cells/ml in RPMI 1640 medium supplemented with 10% FCS and 2 mCi/10 μl 3H-thymidine. A portion of the cell suspension was cultured with 50 μg/ml of IRBP in the presence or absence of 50 ng/ml of IL-12, in 75 cm² flasks. To remove excess adherent cells (macrophages), the stimulating cells were transferred into new flasks after 24 h and again after 48 h. After 3 days, the lymphocytes were separated from erythrocytes and debris by discontinuous density gradient centrifugation on Ficoll and counted. Each recipient mouse was injected i.p. with 40 × 10^6 cells. Eyes were collected from the recipients after 10 days and were evaluated for EAU by histopathology.

Reproducibility and statistical analysis

Experiments were repeated at least twice, and usually three or more times. Statistical analysis of EAU scores was by Snedecor and Cochran’s test for linear trend proportions (nonparametric, frequency based) (31). Each mouse (average of both eyes) was treated as one statistical event. Delayed hypersensitivity scores were analyzed by independent t test. Probability values of p < 0.05 were considered significant.

Results

Endogenous IL-12 is required for the pathogenesis of EAU

To examine whether endogenous IL-12 production is required to induce EAU, 12KO and C57BL/6 mice were immunized with 200 μg and 100 μg IRBP, respectively, as described in Materials and Methods. In four separate experiments, 12KO mice were highly resistant to EAU compared with wt C57BL/6 mice (Fig. 1). None of the eleven 12KO mice (0%) developed disease, whereas 15 of 19 C57BL/6 controls (79%) developed EAU, with a mean score of 1.1 ± 0.2. The incidence and severity of EAU in the controls was typical of the C57BL/6 strain, which is moderately susceptible to EAU (32).

Attempts at reconstitution of the ability of immunized 12KO mice to develop EAU, by administering replacement doses of exogenous IL-12, were largely ineffective. Several treatment doses based on findings by Magram et al. (25) were tried, but neither 2, 10 or 50 ng/day of IL-12 given during the first 5 days after immunization to cover the priming period, nor 2 ng/day given five
times per wk for the entire 3-wk duration of the experiment, restored EAU pathology or IFN-γ production by primed lymph node cells of the treated mice (data not shown).

Cellular responses of IL-12-deficient mice
At the termination of each experiment, 48-h DTH responses were measured, and IRBP-specific proliferation of lymph node cultures was assayed, as described in Materials and Methods. The DTH responses of 12KO mice were only marginally reduced compared with controls, and the difference between the groups did not achieve statistical significance (p < 0.11) (Fig. 2). In contrast, in four separate experiments, the IRBP-specific proliferation of 12KO lymph node cells in culture was consistently elevated compared with wt controls (Fig. 3).

IL-12-deficient mice have impaired Th1 and elevated Th2 cytokine responses
Draining lymph node cells and spleen cells were pooled within each group and were stimulated in culture with IRBP. Supernatants were assayed for cytokine content by ELISA as described in Materials and Methods. Lymph node cultures from 12KO mice showed increased production of IL-5 and IL-10, and decreased production of IFN-γ compared with those from controls (Fig. 4). IL-4 was secreted minimally by both groups. The same cytokine production pattern was observed in splenic cultures (Fig. 5). TNF-α and IL-6 were also detected, but there was little difference observed between the two groups of mice (data not shown).

Ab responses of IL-12-deficient mice
Serum was collected from individual mice at the termination of an experiment and was analyzed for IRBP-specific Ab production as described in Materials and Methods. IgG2a and IgG1 isotypes were measured. Ag-specific IgG isotype production did not differ significantly between 12KO mice and C57BL/6 controls. Contrary to expectations, (IFN-γ-dependent) IgG2a isotype Abs were not reduced. If anything, there appeared to be a slight increase in
IgG2a production in the IRBP-immunized 12KO mice compared with wt (Fig. 6).

**IL-12-deficient mice are able to develop EAU when adoptively transferred with polarized Th1 effector cells from wt mice**

As mentioned above, attempts to reconstitute the ability of IRBP-immunized 12KO mice to develop disease through administration of exogenous IL-12 were largely ineffective. Therefore, it was necessary to address the question whether disruption of the IL-12 gene might have rendered the 12KO inherently unable to develop EAU. Primed lymph node cells were isolated from wt mice 2 wk after immunization, were stimulated with Ag in culture, and were infused into naive 12KO or wt recipients. While stimulated lymph node cells of wt mice transferred EAU efficiently to wt recipients, they were extremely poor at transferring EAU to 12KO recipients (Fig. 7a). Addition of IL-12 to the culture up-regulated IFN-γ production by the wt cells approximately sevenfold (from 48 to 346 ng/ml, as measured in 48-h supernatants) and strongly enhanced their ability to transfer EAU to 12KO recipients ($p < 0.004$) (Fig. 7b). Interestingly, reconstitution of the 12KO recipients with 5 ng of IL-12 per day appeared to have the same effect on their ability to develop EAU as culturing the transferred cells with IL-12 (Fig. 7c). This was in contrast to the inefficiency of reconstitution by IL-12 administered to actively immunized 12KO mice (that would have to generate their own effector T cells to express disease).

**Primed lymph node cells of IL-12-deficient mice develop into effector cells and produce IFN-γ when cultured with exogenous IL-12**

We next wanted to address the question whether primed lymphocytes of 12KO mice are able to become uveitogenic effectors if the missing IL-12 is supplied to them exogenously. Draining lymph node cells of 12KO mice were collected 2 wk after immunization, were stimulated with Ag in culture, and were infused into naive 12KO or wt recipients. While stimulated lymph node cells of wt mice transferred EAU efficiently to wt recipients, they were extremely poor at transferring EAU to 12KO recipients (Fig. 7a). Addition of IL-12 to the culture up-regulated IFN-γ production by the wt cells approximately sevenfold (from 48 to 346 ng/ml, as measured in 48-h supernatants) and strongly enhanced their ability to transfer EAU to 12KO recipients ($p < 0.004$) (Fig. 7b). Interestingly, reconstitution of the 12KO recipients with 5 ng of IL-12 per day appeared to have the same effect on their ability to develop EAU as culturing the transferred cells with IL-12 (Fig. 7c). This was in contrast to the inefficiency of reconstitution by IL-12 administered to actively immunized 12KO mice (that would have to generate their own effector T cells to express disease).

**Discussion**

In the present study, we have shown that endogenous production of IL-12 is required for the generation of the uveitogenic effector cell and that it contributes to the pathogenesis of EAU even after that effector cell has been generated. The data show that, in the absence of endogenous IL-12, IRBP-specific IFN-γ production is inhibited and the Ag-specific response deviates toward a nonpathogenic Th2-like phenotype. Nevertheless, the humoral responses of 12KO mice appeared to be relatively unaffected.
12KO mice did not mount a DTH response to wt animals. This is in contrast to findings by Mattner et al., where in our hands the reduction was marginal compared with that 12KO mice develop a reduced Ag-specific DTH response; deficient mice also had enhanced proliferative responses (22). Our produce IL-12, such as nature of the Ag, levels of endogenous immunized 12KO mice. The C57BL/6 strain characteristically produces IL-12 in vitro, and is in line with our previous data showing that IFN-γ-deficient mice also had enhanced proliferative responses (22). Our findings are in agreement with Magram et al. (25), who showed that 12KO mice develop a reduced Ag-specific DTH response; however, in our hands the reduction was marginal compared with wt animals. This is in contrast to findings by Mattner et al., where 12KO mice did not mount a DTH response to Leishmania (26). The discrepancy implies that factors in addition to the ability to produce IL-12, such as nature of the Ag, levels of endogenous IFN-γ production (reduced, but not absent, in 12KO mice), and other genetic factors affect the development of cellular immune responses in these mice.

EAU-resistant 12KO mice produced anti-IRBP Ab responses of similar magnitude and isotype composition to wt. This was unexpected, because the reduced IFN-γ response of these mice should have resulted in lowered Ag-specific IgG2a. The reason for this is unclear and could be influenced by factors such as mouse strain and the nature of the Ag. Our results differ from those of McIntyre et al. in collagen-induced arthritis in 12KO DBA1 mice. These investigators reported decreased titers of anti-collagen type II serum Abs of both IgG2a and IgG1 isotypes in the 12KO mice that paralleled a reduction (but not abrogation) of joint inflammation (33). Pathogenesis of collagen-induced arthritis is known to be highly dependent on Abs (34–37). Our data thus support the notion that Abs play a minimal role in EAU and indicate that in the absence of a pathogenic cellular response, serum Abs by themselves are insufficient to induce pathology.

IRBP-specific proliferative responses were not only present, but were in fact elevated, in 12KO mice, indicating that inadequate Ag priming was not the mechanism of EAU resistance. The cytokine profile that these cells produced, however, differed markedly from the wt (see above). It is likely that decreased IFN-γ production in these cultures contributed to the observed increase in proliferation, because IFN-γ has been shown to exert anti-proliferative effects in vitro, and is in line with our previous data showing that IFN-γ-deficient mice also had enhanced proliferative responses (22). Our findings are in agreement with Magram et al. (25), who showed that 12KO mice develop a reduced Ag-specific DTH response; however, in our hands the reduction was marginal compared with wt animals. This is in contrast to findings by Mattner et al., where 12KO mice did not mount a DTH response to Leishmania (26). The discrepancy implies that factors in addition to the ability to produce IL-12, such as nature of the Ag, levels of endogenous IFN-γ production (reduced, but not absent, in 12KO mice), and other genetic factors affect the development of cellular immune responses in these mice.

IRBP-specific cytokine production was dramatically altered in immunized 12KO mice. The C57BL/6 strain characteristically produces high levels of IFN-γ in response to IRBP and little or no IL-4, which is typical of a dominant Th1 responder to the uveitigen (38). In the IL-12-deficient mouse, lymph node and splenic cultures produced elevated levels of IL-5 and IL-10, and some IL-4 was detectable in the spleen. IFN-γ was reduced up to 10-fold in lymph node cultures and up to 100-fold in the spleen. Our observations thus confirm findings from previous studies that in the absence of endogenous IL-12, the immune response deviates toward the Th2 pathway (26).

Because it proved difficult to reconstitute the ability of actively immunized 12KO mice to develop EAU by administering replacement doses of exogenous IL-12, the question arose whether these mice were able to develop disease under any circumstances. To address this issue, 12KO mice were infused with cultured lymph node cells from wt mice primed with IRBP. These cultures contained Th1-like effector cells, as judged by the fact that they produced IFN-γ in response to IRBP and were capable of adoptively transferring EAU to naive wt recipients. Interestingly, the same cells were able to induce only marginal EAU in 12KO recipients. However, adding IL-12 to the culture resulted in a more potent effector population that not only produced sevenfold more IFN-γ but also was able to induce full-blown EAU in 100% of 12KO recipients. Restoration of the ability to develop EAU was also achieved by reconstituting the 12KO recipients with IL-12. These results led to the conclusion that 12KO mice are not inherently unable to develop EAU and suggested that endogenous IL-12 in the adoptive host has a role in pathogenesis during the expression phase of EAU, after the uveitogenic effector cell has already been primed. Similar conclusions had been reached by Leonard et al. in the model of adoptively transferred experimental autoimmune encephalomyelitis, in which recipient mice were treated with neutralizing Abs to IL-12 (39). The effective reconstitution of the adoptive host was in contrast to the ineffectiveness of reconstitution of the actively immunized animal and is compatible with an interpretation that IL-12 has different roles in the priming stage versus the expression stage of disease. The present results also indicate that whatever the role played by IL-12 during the expression stage of EAU, it can be offset by driving the primed T cells in culture with exogenous IL-12 toward a more polarized Th1 effector phenotype.

The relative ineffectiveness of IL-12 replacement in the actively immunized 12KO mice also raised the question whether 12KO T cells themselves possessed the ability to develop into uveitogenic effector cells. That 12KO T cells could differentiate into pathogenic effectors under the appropriate conditions was confirmed by the finding that, following culture in the presence of exogenous IL-12, primed lymphocytes of 12KO donors were able to produce IFN-γ and to induce EAU in naive 12KO recipients. This indicates that 12KO lymphocytes could be driven to become pathogenic Th1-like effectors under the influence of exogenous IL-12 even after priming.

The cytokine profile produced by 12KO lymphocytes in response to Ag was very reminiscent of the cytokine profile produced by IFN-γ-deficient mice (22). The response of the IFN-γ-deficient animals was also typified by unchanged production of TNF-α, elevated IL-5 and IL-10, and reduced IFN-γ, accompanied by lack of detectable IL-4 production by lymph node cells. Nevertheless, while the IFN-γ-deficient mice were quite susceptible to EAU, 12KO mice were resistant. The exact nature of the uveitogenic effector T cell in IFN-γ-deficient mice remains to be determined; nevertheless, the present data clearly point out that a uveitogenic effector can be generated in the absence of IFN-γ, but not in the absence of IL-12. This further indicates that it is not simply the capacity of IL-12 to up-regulate endogenous IFN-γ production that underlies its role in pathogenesis and points out a role for IL-12 that is independent of IFN-γ. These issues are being currently addressed in IFN-γ-deficient mice treated with neutralizing anti-IL-12 Abs.
In summary, the present study shows that the pathogenesis of EAU is dependent on endogenous IL-12 production not only during the induction, but also during the expression, stage of EAU. Further studies are needed to dissect the IFN-γ-dependent from the IFN-γ-independent actions of IL-12 in the pathogenesis of tissue-specific, cell-mediated autoimmune.

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References


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