IL-12Rβ2 has a protective role in relapsing–remitting experimental autoimmune encephalomyelitis

Chong Xie [a,b,1], Bogoljub Ciric [a,1], Shuo Yu [a], Guang-Xian Zhang [a], Abdolmohamad Rostami [a,*]

[a] Department of Neurology, Thomas Jefferson University, Philadelphia, PA 19107, USA
[b] Department of Neurology, Changhai Hospital, Second Military Medical University, Shanghai, 200433, China

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ABSTRACT

IL-12Rβ2 is a common receptor subunit of heterodimeric receptors for IL-12 and IL-35, two cytokines that are implicated in immunopathogenesis of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis. We evaluated the role of IL-12Rβ2 in relapsing–remitting EAE (RR-EAE). IL-12Rβ2-deficient SJL/J mice developed markedly more severe clinical EAE, and had greater mortality and more severe relapses compared with wild-type controls. IL-12Rβ2-deficient EAE mice also had more infiltrating mononuclear cells in the CNS, as well as higher T cell proliferative capacity and decreased IFN-γ production at the periphery. These findings demonstrate a protective role of IL-12Rβ2 in RR-EAE.

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1. Introduction

Experimental autoimmune encephalomyelitis (EAE),[2] an animal model of multiple sclerosis (MS), is a CD4$^+$ T cell-mediated autoimmune disease (Noseworthy et al., 2000). In 2003, Cua et al. demonstrated that IL-23, but not IL-12, plays an essential role in EAE development (Cua et al., 2003). This finding dispelled the long-held view that IL-12 is critical to the development of EAE and called for a re-evaluation of its role in autoimmune inflammation of the central nervous system (CNS).

Members of the IL-12 family of cytokines (IL-12, IL-23, IL-27, and IL-35) and their corresponding receptors are both heterodimers that share common cytokine and receptor subunits. IL-12 is a covalent heterodimer of IL-12p40 and IL-12p35 subunits, which form the complete cytokine IL-12p70 (Trinchieri, 1998). IL-23 is comprised of covalently bound IL-12p40 and IL-23p19 (Oppmann et al., 2000); IL-12p40 is therefore a common subunit between IL-12 and IL-23. IL-12 exerts its effects through its receptor (IL-12R), which is comprised of two chains, IL-12Rβ1 and IL-12Rβ2 (Chua et al., 1995). While the IL-12Rβ1 subunit is constitutively expressed by naïve CD4$^+$ T cells

(Chang et al., 1999), the expression of IL-12Rβ2 is upregulated upon activation of naïve T cells that develop into Th1 and Th17 cells (Lexberg et al., 2010; Szabo et al., 1997). In addition to comprising IL-12R, both of its subunits are also involved in the formation of receptors for two other cytokines; IL-12Rβ1 is part of the IL-23 receptor heterodimer (Oppmann et al., 2000), while IL-12Rβ2 also participates in the receptor for IL-35. IL-35 is a non-covalent heterodimer of IL-12p35 and Epstein–Barr-virus-induced gene 3 (EBI3) (Collison et al., 2007), which together with IL-27p28 also forms IL-27 (Pflanz et al., 2002). The molecular composition of the members of the IL-12 family of cytokines and their receptors is schematically shown in Supplemental Fig. 1.

Factors that determine various disease courses in MS and EAE are not well understood, but it is apparent that clinical disease courses in relapsing–remitting (RR) and progressive MS/EAE are governed by different mechanisms. MOG$_{35-55}$-induced EAE in C57BL/6 mice has a chronic disease course, but the majority of MS patients (~85%) develop RR disease (Compston and Coles, 2008), and EAE models that have a RR clinical course are likely better suited for studying immunopathogenic mechanisms of RR-MS. The most commonly used RR-EAE model is in SJL/J mice immunized with PLP$_{139-151}$ peptide (Furlan et al., 2009). Histopathologically and clinically, this model is similar to the RR form of MS.

Given that both IL-12 subunits and both IL-12R subunits are shared with other cytokines and their receptors, it has been difficult to devise an approach that would *in vivo* solely address the role of IL-12, and the same is true for IL-35. The most relevant findings on the role of IL-12Rβ2 in EAE come from a study with IL-12Rβ2$^{-/-}$ C57BL/6 mice.

These mice developed more severe EAE and had a higher mortality rate compared with wild-type (WT) controls. Consistent with severe clinical signs, the IL-12Rβ2$^{-/-}$ C57BL/6 mice had extensive CNS demyelination and inflammation, as well as increased production of pro-inflammatory cytokines. These findings demonstrated that IL-12Rβ2 has a protective role in chronic EAE (Zhang et al., 2003).

In this study, we for the first time demonstrate that IL-12Rβ2 has a pronounced protective role in RR-EAE. IL-12Rβ2$^{-/-}$ SJL/J mice developed markedly more severe EAE characterized by higher mortality and stronger relapses compared with WT counterparts. Given that the effects of IL-12Rβ2 deficiency reflect the roles of both IL-12 and IL-35, we draw conclusions on the role of IL-12Rβ2, without specifically attributing it to either of these two cytokines.

2. Materials and methods

2.1. Mice and EAE induction

IL-12Rβ2$^{-/-}$ SJL/J mice were generated for us by the Jackson Laboratory (Bar Harbor, ME) using the Speed Congenic approach. Mutant IL-12Rβ2 allele was derived from IL-12Rβ2$^{-/-}$ C57BL/6 mice (strain name: B6.129S1-*IL-12Rβ2^{tm1Jm}*/J). IL-12Rβ2$^{-/-}$ SJL/J mice were kept homozygous by brother–sister breeding. WT SJL/J mice were purchased from the Jackson Laboratory. In initial experiments, IL-12Rβ2$^{-/-}$ and WT SJL/J mice were immunized for EAE induction with 100 μg PLP$_{139-151}$ (GenScript) emulsified in complete Freund's adjuvant (CFA) containing 3 mg/ml *Mycobacterium tuberculosis* (H37Ra; Difco Laboratories). In addition, mice were given 200 ng pertussis toxin (List Biologicals Laboratories) intraperitoneally (i.p.) on days 0 and 2 post-immunization (p.i.). In subsequent experiments mice received less PLP$_{139-151}$ peptide (75 μg, or 50 μg) because of the high mortality of mice immunized with 100 μg PLP$_{139-151}$; immunizing conditions are specified in Supplemental Table 1. Mice were graded for clinical manifestations of EAE by the following criteria: 1, tail paralysis; 2, one hind limb paralysis; 3, both hind limbs paralysis; 4, forelimb weakness or paralysis; 5, moribund or dead. A relapse is defined as an increase in at least one clinical grade sustained for at least two consecutive days after animals have previously improved at least a full clinical grade and stabilized. All animal procedures were performed in accordance with the guidelines of the Institutional Animal Care and Use Committee of Thomas Jefferson University.

2.2. Mononuclear cell preparation

Depending on the experiment, mice were sacrificed on various days p.i. After being anesthetized, mice were perfused with 10 ml phosphate-buffered saline. To isolate mononuclear cells (MNCs) from the CNS, spinal cords and brains were digested with 0.5 mg/ml Liberase™ (Roche) for 30 min at 37 °C and then mechanically dissociated through a 70-μm cell strainer. The single-cell suspension was then fractionated on a 70/30% Percoll gradient by centrifugation at 300 ×g for 20 min at room temperature. Cell layer at 70/30 interface was collected and viable cells were counted in 0.4% Trypan blue. For preparation of splenocytes, spleen was dissociated through a 70-μm cell strainer and then red blood cells were lysed with Red Blood Cell Lysis Buffer (BioLegend). Splenocytes were then washed with cold medium and collected for use.

2.3. Splenocyte proliferation assay

Splenocytes were cultured in 96-well plates in 200 μl IMDM medium supplemented with 10% fetal calf serum, L-glutamine (2 mM), penicillin (100 U/ml), and streptomycin (100 μg/ml). In the presence of 20 μg/ml PLP$_{139-151}$ or 1 μg/ml anti-CD3/CD28 antibodies, splenocytes were cultured at a density of 1×10^5 cells/well, while without antigen/mitogen splenocytes were cultured at a density of 4×10^5 cells/well. After 60 h of incubation at 37 °C/5% CO$_2$, cells were pulsed for 12 h with 1 μCi of

[^{3}H]methylthymidine. Cells were then harvested and thymidine incorporation (cpm) was determined using a β-counter.

2.4. Cytokine measurement

Splenocytes of immunized mice were cultured at a density of 2.5×10^6 cells/ml in medium with or without 20 μg/ml PLP$_{139-151}$. Supernatants were collected after 72 h of culturing. ELISA kits for measurement of IFN-γ and IL-17A concentrations were purchased from R&D System. Assays were performed according to the manufacturer's recommendation.

2.5. Flow cytometry

For surface-marker staining, cells were incubated for 30 min on ice with fluorochrome-conjugated antibodies against mouse CD4, CD11b, CD25, and Gr1 (BD Biosciences), or with isotype control antibodies. For intracellular staining, cells were stimulated with PMA and Ionomycin (Sigma-Aldrich) in the presence of GolgiPlug (BD Biosciences) for 4 h and then fixed and stained in the Fix & Perm Medium (Invitrogen); fluorochrome-conjugated antibodies against mouse IFN-γ, IL-17A and Foxp3 were used. Flow cytometric analysis was performed on FACSAria (BD Biosciences) and data were analyzed with FlowJo software (Tree Star).

2.6. Statistics

Clinical scores were analyzed by calculating the area under the curve for each mouse over the clinical period of the experiment. A two-tailed Student's *t*-test was used to analyze differences between groups. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Characterization of IL-12Rβ2$^{-/-}$ SJL/J mice

Congenic IL-12Rβ2$^{-/-}$ SJL/J mouse strain was generated by the "speed congenic" approach, by crossing WT SJL/J mice and IL-12Rβ2$^{-/-}$ C57BL/6 mice. We first confirmed that IL-12Rβ2$^{-/-}$ SJL/J mice are irresponsive to IL-12. IL-12 promotes IFN-γ production through IL-12R signaling (Magram et al., 1996; Trinchieri, 1994; Trinchieri and Scott, 1995); we therefore tested the effect of recombinant IL-12 (rIL-12) on IFN-γ production by splenocytes of naïve mice activated with anti-CD3/CD28 antibodies. rIL-12 significantly increased (~4-fold) IFN-γ concentrations in cell culture supernatants of WT splenocytes, but had no effect in IL-12Rβ2$^{-/-}$ cultures (Supplemental Fig. 2A). These data clearly demonstrate the lack of IL-12R signaling in immune cells of IL-12Rβ2$^{-/-}$ mice.

Next, we characterized basic immunological parameters of IL-12Rβ2$^{-/-}$ SJL/J mice. Comparison of splenocytes from naïve IL-12Rβ2$^{-/-}$ and WT SJL/J mice showed no reproducible differences in the percentages of CD4$^+$, CD8$^+$, NK1.1$^+$, TNFα$^+$, IL-4$^+$, CD4$^+$IFN-γ$^+$, CD4$^+$IL-17A$^+$, and CD4$^+$CD25$^+$FoxP3$^+$ T cells. We also examined other major types of immune cells (NK cells, γδ T cells, and CD3$^+$, CD44$^+$, CD69$^+$, CD11b$^+$, CD11c$^+$ and CD19$^+$ cells) and did not find reproducible differences. However, splenocytes of IL-12Rβ2$^{-/-}$ SJL/J mice had higher percentages of CD11b$^+$Gr1$^+$ cells compared with WT controls (6.9% *vs.* 2.3%, $P < 0.01$). The concentrations of IFN-γ and IL-17A in the supernatants of cultured splenocytes from naïve IL-12Rβ2$^{-/-}$ and WT SJL/J mice also did not significantly differ (Supplemental Fig. 2B). The above findings on IL-12Rβ2$^{-/-}$ SJL mice are in agreement with those made by others in IL-12Rβ2$^{-/-}$ C57BL/6 mice, as similar proportions of CD3$^+$, CD4$^+$, and CD8$^+$ T cells, CD19$^+$ B cells, NK cells, and CD11b$^+$ and CD11c$^+$ cells in splenocytes of IL-12Rβ2$^{-/-}$ and WT C57BL/6 mice were found (Wu et al., 2000). IL-12Rβ2$^{-/-}$ C57BL/6 mice also had normal hemoglobin and hematocrit