Published in final edited form as:

Exp Mol Pathol. 2010 October ; 89(2): 126–134. doi:10.1016/j.yexmp.2010.06.003.

Accelerated thymocyte maturation in IL-12R β 2-deficient mice contributes to increased susceptibility to autoimmune inflammatory demyelination

B. Gran^{1,*}, S. Yu¹, G.X. Zhang¹, and A. Rostami¹

S. Yu: shuo.yu@jefferson.edu; G.X. Zhang: guang-xian.zhang@jefferson.edu; A. Rostami: a.m.rostami@jefferson.edu

¹ Department of Neurology, Thomas Jefferson University; 300 JHN Building, 900 Walnut Street, Philadelphia PA 19107, USA

Abstract

IL-12R β 2^{-/-} mice, which are unresponsive to IL-12, develop severe experimental autoimmune encephalomyelitis (EAE). The mechanisms for enhanced autoimmunity are incompletely understood. We report that in IL-12R β 2^{-/-} mice, thymocytes undergo markedly accelerated maturation. This occurs at the transition from double positive (DP) to single positive (SP) phenotype, resulting in higher numbers of CD4 and CD8 SP cells, and to a lesser extent at the transition from double negative (DN) to DP cells. Accelerated maturation is observed in mice injected with anti-CD3 to mimic pre-T cell receptor stimulation, and also in mice immunized with myelin oligodendrocyte glycoprotein (MOG) peptide to induce EAE.

Keywords

Cytokine; Autoimmunity; T cell; Thymus; Experimental autoimmune encephalomyelitis; IL-12 receptor

Introduction

The generation of functional T cells in the thymus requires an ordered sequence of events that lead to mature, single positive (SP) T cells with fully rearranged T cell receptor (TCR) chains. Cells that express functional TCRs are selected through a process that includes both positive and negative events. Positive selection results in CD4/CD8 lineage commitment, TCR gene rearrangement, and self-MHC restriction specificity (Bommhardt et al., 2004; Huseby et al., 2008). This developmental pathway has been delineated in part by the expression of molecules such as CD4, CD8, CD24 (heat-stable antigen, HSA), and the TCR α/β chains. Double negative (DN; CD4⁻CD8⁻) thymocytes include the earliest T cell precursors, which give rise to TCR^{low} then TCR^{hi} double positive (DP) CD4⁺CD8⁺ CD24^{hi} cells, and finally, mature into CD24^{low} single positive (SP) CD4⁺ or CD8⁺ T cells (Jameson et al., 1995). Negative selection

*Corresponding author: Dr Bruno Gran, Division of Clinical Neurology, University of Nottingham; B31 Medical School, Queen's Medical Centre, Nottingham NG7 2UH, United Kingdom, Tel: +44 115 8493363, Fax +44 115 970 9738, bruno.gran@nottingham.ac.uk.

²Present address: Division of Clinical Neurology, University of Nottingham; B31 Medical School, Queen's Medical Centre, Nottingham NG7 2UH, UK.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

results in the elimination of thymocytes with overt self-reactivity (Hakim and Gress, 2007). Within a few days, those SP cells leave the thymus to circulate as mature cells (Starr et al., 2003).

IL-12, a key cytokine in the differentiation and expansion of T-helper type 1 (Th1) CD4⁺ cells in periphery (Kang and Kim, 2006; Trinchieri et al., 2003), is also involved in the development of thymocytes. Thymic APCs and stromal cells produce IL-12 (Foy et al., 1995; Godfrey et al., 1994) and both immature and mature thymocytes express IL-12 receptor (IL-12R) subunits (Kikkawa et al., 2002; Ludviksson et al., 1999). Culture of mouse fetal thymic organ with IL-12 *in vitro* was reported to induce significant cell death in most subsets of thymocytes, while both the percentage and absolute number of TCR $\alpha\beta$ +CD8⁺ cells were increased (Godfrey et al., 1994). Administration of IL-12 induced intrathymic apoptosis, which could be blocked by anti-IL-12 antibodies (Ludviksson et al., 1999; Rodriguez-Galan et al., 2005). In addition, accelerated age-related thymic involution in IL-12p40^{-/-} mice suggests that IL-12 may serve to maintain thymic function and development during aging (Li et al., 2004). However, since mice lacking IL-12p40 are deficient in both IL-12 and IL-23, which possess distinct, and in some cases even opposite functions (Oppmann et al., 2000; Touil et al., 2006; Zhang and Wang, 2008), studies using IL-12p40-deficient mice could not accurately identify the role of IL-12 in thymocyte development. Furthermore, it is not known whether IL-12 influences thymocyte positive selection.

IL-12 exerts its effects through a receptor (IL-12R) composed of two chains, IL-12R β 1 and IL-12R β 2 (Gately et al., 1998). IL-12R β 1 mediates both low and high affinity binding to IL-12, as well as to the IL-12-related cytokine IL-23, whereas IL-12R β 2 is required uniquely for biological responses to IL-12. This makes the IL-12R β 2-deficient model a valuable tool to specifically study the role of IL-12 signals.

We and others have found that mice lacking IL-12p35, therefore IL-12, or lacking IL-12R β 2, therefore IL-12 responsiveness, exhibited significantly more severe clinical and pathological signs of experimental autoimmune encephalomyelitis (EAE), a CD4⁺ T cell-mediated autoimmune disease of the central nervous system (CNS) (Becher et al., 2002; Cua et al., 2003; Gran et al., 2002; Zhang et al., 2003), and other autoimmune diseases like arthritis (Fairweather et al., 2005; Murphy et al., 2003). These findings indicate that IL-12 signaling may play an immunoregulatory role in autoimmune disorders. Consistent with these results, we have found increased numbers of CD4⁺ T cells in the spleen of IL-12R β 2^{-/-} mice with EAE, with marginally decreased apoptosis in CD4⁺ T cells (Zhang et al., 2003). Given the important role of cytokines in thymocyte development, we hypothesized that the increased number of CD4⁺ T cells in the periphery of mice lacking IL-12R β 2 may be due to an accelerated T-cell maturation in the thymus, thus producing increased numbers of mature CD4⁺ T cells. Using mice deficient in IL-12R β 2, we have now tested this hypothesis and discovered an essential role for IL-12 responsiveness in controlling thymocyte maturation and autoimmunity.

Materials and Methods

Mice

Female, eight-week-old (B6 $\times$ 129) mice homozygous for IL-12R β 2 mutation and their wild type controls were purchased from the Jackson Laboratory (Bar Harbor, ME). The IL-12R β 2 gene mutation was created as described and screened by RT-PCR and Southern blot analysis (Wu et al., 2000). Furthermore, we confirmed the genotypes in our laboratory by using sequence-specific primers according to the protocol recommended by the Jackson Laboratory.

In vivo administration of anti-CD3 Abs

Mice were treated with two injections daily for 2 days by the i.p. injection of 25 µg of I45CII anti-CD3ε mAb (BD PharMingen), which can induce thymocyte apoptosis (Freywald et al., 2006; Lamhamedi-Cherradi et al., 2003) or stimulate thymocyte maturation (Boursalian et al., 2004; Porcellini et al., 1999; Schito et al., 2006). Forty-eight hours after the first injection, mice were sacrificed (3–4 mice per group); the thymocytes and splenocytes were prepared and analyzed by flow cytometry. Isotype rat IgG (25 µg) was injected i.p. in parallel as a negative control.

Thymocyte maturation in vitro

A two-step culture system was utilized (Rodriguez-Galan et al., 2005). Briefly, the first cultures were referred to as signaling cultures, and the second cultures were referred to as recovery cultures. For signaling cultures, thymocytes (1×10^6) were cultured for 16 h at 37°C in 5% CO₂–humidified air atmosphere in 1 ml of complete medium on 24-well plates coated overnight with 10 µg/ml anti-TCR (H57-597) and 10 µg/ml anti-CD2 (RM2-5; PharMingen). This culture system promotes the development of CD4⁺8⁺ DP cells into CD4⁺ SP cells (Rodriguez-Galan et al., 2005). To determine the role of IL-7 in the process of T cell maturation, neutralizing anti-IL-7 mAb (R & D) was employed in the culture at 10 µg/ml. Wells with isotype IgG served as control. During the second step, cells from individual signaling cultures were then transferred into uncoated wells for an additional 16 h. Cells then were harvested and analyzed by flow cytometry using CD4, CD8, CD24, and TCR-β antibodies and fluorescently labeled Annexin V.

Flow cytometry

Single cell suspensions were prepared, stained with specific antibodies, and analyzed on a FACSCalibur or FACS Aria (BD) sorter. Dead cells were excluded on the basis of forward and side scatter profiles, and at least 10^5 live-gated events were collected from stained thymus samples. Single cells were labeled with 4–6 fluorochrome-conjugated antibodies. FITC-, PE-, CyChrome- or allophycocyanin (APC)-, PE-Cy7- and APC-Cy7-conjugated mAb specific for CD4, CD8α, CD24, TCRβ, CD44, CD25, CD69, IL-7Rα were from PharMingen. For cell death assay, fluorescently labeled Annexin V and 7-AAD were used following the manufacturer's recommendation (PharMingen). All samples were analyzed using either CellQuest Pro (BD) or FlowJo software (Tree Star Inc., Ashland, OR).

EAE induction

IL-12Rβ2^{−/−} and wild type mice were injected subcutaneously with 100 µg MOG35-55 in CFA containing 4 mg/ml *Mycobacterium tuberculosis* H37Ra (Difco, Detroit, MI) at 2 sites on the back. One hundred ng pertussis toxin was given i.p. on days 0 and 2 post immunization (p.i.). Mice were sacrificed at day 10 p.i., at which time an increased number of splenocytes and decreased number of apoptotic cells had been found in our previous study (Zhang et al., 2003). Thymus and spleen from immunized mice were harvested for flow cytometry analysis. Cells from naïve mice were assayed in parallel as control.

Statistical analysis

The Mann-Whitney U test was used for the comparison of average clinical scores, and the Student's *t* test was used for other parameters among different groups. All tests were two-sided.

Results

Accelerated thymocyte maturation in vivo in IL-12R β 2^{-/-} mice upon anti-CD3 stimulation

To test the hypothesis that the lack of IL-12R β 2 may accelerate T cell maturation in the thymus, we first investigated whether there is any difference in total cell number and percentage of subpopulations in thymocytes between IL-12R β 2^{-/-} and wild type control mice. Mice deficient in IL-12R β 2 developed normally (Wu et al., 2000), and had similar thymi and a comparable total number of thymocytes compared with their IL-12R β 2^{+/+} littermates (Fig. 1a). Flow cytometry analysis of IL-12R β 2^{-/-} mice revealed no abnormal changes in the percentages of CD4⁻CD8⁻ (DN), CD4⁺CD8⁺ (DP), CD4⁺CD8⁻ or CD4⁻CD8⁺ (SP) subpopulations of thymocytes compared to wild type mice (Fig. 1b).

Administration of anti-CD3 mAb, a stimulus inducing thymocyte activation/apoptosis (Boursalian et al., 2004; Lamhamedi-Cherradi et al., 2003; Ludviksson et al., 1999; Porcellini et al., 1999; Rodriguez-Galan et al., 2005), induced a significant decrease of thymocyte numbers in both wild type and IL-12R β 2^{-/-} mice, with a more marked decrease in IL-12R β 2^{-/-} mice compared to mice injected with isotype control mAb (Fig. 1a). The percentage of CD4⁺CD8⁺ immature cells significantly decreased following anti-CD3 injection, while percentages of CD4⁺CD8⁻ and CD4⁻CD8⁺ (SP) mature T cells increased compared with control-Ab-injected mice. This phenomenon was more obvious in IL-12R β 2^{-/-} mice than in wild type mice following CD3 treatment ($P < 0.01$; Fig. 1b and 1c). To determine whether the dramatic decrease of DP cells was due to an anti-CD3 induced apoptosis, we labeled these thymocytes with fluorescently labeled Annexin V and 7-AAD. Annexin V^{pos} 7-AAD^{neg} cells were defined as apoptotic. An increased number of apoptotic cells was found in both wild type and IL-12R β 2^{-/-} mice after anti-CD3 mAb injection at all stages of thymocyte development compared to control-Ab-injected mice. No significant difference for apoptosis was found in the thymus of these two mouse strains after anti-CD3-injection (data not shown). Thus, the significantly lower number of thymic cells in IL-12R β 2^{-/-} mice than in wild type mice may be due to an accelerated maturation followed by migration to the periphery. The increased number of CD4⁺ T cells in the spleen of IL-12R β 2^{-/-} mice supports this possibility (Fig. 3a). Together, these results indicate that anti-CD3 mAb induced enhanced thymocyte maturation in IL-12R β 2^{-/-} mice, mainly at the stage of thymocytes developing from DP immature T cells into CD4 or CD8 SP T cells.

To monitor the influence of IL-12R β 2 deficiency on thymocyte maturation, we determined expression of CD24, TCR $\alpha\beta$, CD44, CD25, and CD69 on thymocyte subpopulations, as these molecules are expressed differentially at various developmental stages (Boursalian and Fink, 2003). We did not find a difference in CD44, CD25, and CD69 expression between IL-12R β 2^{-/-} and wild type mice after anti-CD3 mAb injection. In both mouse groups, anti-CD3 antibodies induced markedly reduced expression of CD24, similar to what is normally observed during T-cell maturation (Boursalian and Fink, 2003). Following anti-CD3 injection, CD24 expression in IL-12R β 2^{-/-} mice was significantly lower than in wild type mice on DN and DP cells, with a tendency to lower CD24 expression at the SP stage as well (Fig. 2a, b). Furthermore, anti-CD3 mAb induced significantly higher TCR $\alpha\beta$ expression at the DP stage in IL-12R β 2^{-/-} mice than in wild type mice (data not shown). Taken together, these data suggest that thymocytes undergo accelerated maturation in IL-12R β 2^{-/-} mice.

Percentages and absolute numbers of T cells in the periphery

We expected that accelerated thymocyte maturation would result in more cells migrating to the periphery. To test this hypothesis, the percentage and absolute numbers of CD4⁺ and CD8⁺ T cells in the spleen were determined in anti-CD3-injected and control mAb-injected mice. Indeed, spleens of IL-12R β 2^{-/-} mice contain significantly higher number of CD4⁺ T

cells than wild type mice in both control mAb-injected and anti-CD3-injected groups (Fig. 3a). The number of spleen CD4⁺ T cells decreased in both IL-12Rβ2^{-/-} and wild type mice after anti-CD3 mAb injection compared to control mAb injection (Fig. 3a), probably resulting from anti-CD3-induced thymic apoptosis. Anti-CD3-induced apoptosis in CD8⁺ T cells in the spleen (Fig. 3b) could also be responsible for the decrease of this population in this organ (Fig. 3a). In addition, it is possible that increased cell proliferation or enhanced survival in the periphery may contribute to increased numbers of spleen CD4⁺ and CD8⁺ T cells in the absence of IL-12Rβ2^{-/-}.

Role of the IL-7/IL-7R system in T-cell maturation of IL-12Rβ2^{-/-} mice

Since IL-7 plays a critical role in thymic T cell development, we then assessed the involvement of the IL-7/IL-7R pathway in accelerated thymocyte maturation in IL-12Rβ2^{-/-} mice. Administration of anti-CD3 antibodies upregulated expression of IL-7Rα in both IL-12Rβ2^{+/+} mice and IL-12Rβ2^{-/-} mice at different stages of thymocyte development. A significantly higher percentage of IL-7Rα expressing cells was found on thymocytes of IL-12Rβ2^{-/-} mice than IL-12Rβ2^{+/+} mice after anti-CD3 injection (Table 1). However, *in vitro* maturation of thymocytes was not blocked by the presence of neutralizing anti-IL-7 antibodies (data not shown).

Accelerated thymocyte maturation in IL-12Rβ2^{-/-} cells in vitro

Because cytokines and hormones released in the periphery may mediate some of the thymocyte maturation in this *in vivo* model, we next investigated thymocyte development in a better-defined *in vitro* system. This system drives CD4⁺CD8⁺ cells into CD4⁺ SP mature T cells (Rodriguez-Galan et al., 2005). We found a significant decrease in percentage and absolute number of CD4⁺CD8⁺ DP cells in IL-12Rβ2^{-/-} thymocytes cultured with anti-CD2 and -TCR mAbs. At the same time, the percentages of CD4 and CD8 SP cells were increased, indicating an accelerated *in vitro* maturation of thymocytes in IL-12Rβ2^{+/+} mice (Fig. 4). Together with the *in vivo* studies, these data provide evidence that IL-12Rβ2 negatively regulates thymocyte maturation both *in vivo* and *in vitro*.

Thymocyte maturation after immunization with MOG_{35–55} in the absence of IL-12Rβ2

The increased clinical and pathological severity of EAE and the increased number of CD4⁺ T cells in the spleen of IL-12Rβ2^{-/-} mice (Zhang et al., 2003) prompted us to investigate thymic maturation in EAE in these mice. First, we immunized IL-12Rβ2^{-/-} and IL-12Rβ2^{+/+} mice with MOG_{35–55} in CFA and evaluated thymocyte development *in vivo*. Mice were sacrificed on day 10 p.i., when a higher number of CD4⁺ T cells is found in the spleen in IL-12Rβ2^{-/-} mice (Zhang et al., 2003). At this time point, IL-12Rβ2^{-/-} may show initial signs of paralysis, whereas no motor deficit is yet apparent in wild type mice (Zhang et al., 2003). Although similar numbers of thymocytes were found in MOG-immunized IL-12Rβ2^{-/-} and wild type control mice, the thymus of IL-12Rβ2^{-/-} mice contained a significantly decreased percentage of CD4⁺CD8⁺ (DP) immature T cells and increased percentages of CD4⁺CD8⁻ and CD4⁻CD8⁺ (SP) mature T cells (Fig. 5). Consistent with these findings, thymocytes of IL-12Rβ2^{-/-} mice showed a significantly lower density of CD24 expression at all stages and a significantly lower percentage of CD24⁺ cells at the SP stage compared to wild type mice (Fig. 6). These data indicate an enhanced T cell maturation in IL-12Rβ2^{-/-} mice upon EAE induction.

Reduced apoptosis in the spleen, but not in the thymus, of IL-12Rβ2^{-/-} mice with EAE

We found that the greater susceptibility of IL-12Rβ2^{-/-} mice to EAE is associated with an increased number of CD4⁺ T cells and decreased apoptotic cells in the spleen of these mice (Zhang et al., 2003). Here we investigated whether apoptosis is also decreased in the thymus

of IL-12R β 2^{-/-} mice in EAE using fluorescently labeled Annexin V. No significant difference was found for apoptotic cells at various stages of thymocytes between IL-12R β 2^{-/-} and IL-12R β 2^{+/+} mice (data not shown). However, the percentage and absolute number of Annexin V⁺ CD4⁺ T cells were decreased in the spleen of IL-12R β 2^{-/-} mice, consistent with increased percentage of CD4⁺ T cells (Fig. 7).

Discussion

Interleukin-12 is considered a growth factor for peripheral T cells and an inducer of Th1 cell differentiation. However, an immunoregulatory effect of IL-12 and its receptor IL-12R β 2 has been described, based on enhanced severity of autoimmune diseases in mice lacking IL-12p35 and IL-12R β 2 (Becher et al., 2002; Cua et al., 2003; Fairweather et al., 2005; Gran et al., 2004; Gran et al., 2002; Murphy et al., 2003; Zhang et al., 2003). We have previously reported that the induction of IFN- γ and the upregulation of the PD-1/PD-L pathway contribute to immunoregulation by IL-12 (Cheng et al., 2007; Gran et al., 2004). However, the mechanisms underlying this phenomenon are not fully understood. An increased number of CD4⁺ T cells had been observed in the periphery of IL-12R β 2^{-/-} mice with enhanced EAE, indicating that IL-12 responsiveness is involved in T-cell generation and/or T-cell death. Indeed, reduced T-cell apoptosis had been described in the periphery of IL-12R β 2^{-/-} mice, as well as increased T-cell proliferation (Zhang et al., 2003). In the current study we tested the hypothesis that IL-12 responsiveness is involved in thymocyte development. Our data show that after anti-CD3 treatment mice lacking IL-12R β 2 undergo accelerated thymocyte maturation, occurring mainly at the developmental stage of transition from DP to SP cells. As a result, more CD4 and CD8 SP cells are produced in the thymus of these mice. Accelerated maturation may also occur at the transition from the DN to the DP thymocyte phenotype, as suggested by the significantly lower expression of CD24, a marker of immature thymocytes (Carl et al., 2008), in these cell subsets from IL-12R β 2-deficient mice. The percentage and absolute number of apoptotic cells in the thymus were similar. Furthermore, an increased absolute number of CD4⁺ T cells in the thymus and in the periphery in MOG-immunized IL-12R β 2^{-/-} mice suggests enhanced positive selection. This occurred in anti-CD3-induced TCR activation, but also in mice with EAE, a model of the human inflammatory demyelinating disease, multiple sclerosis, and more generally of organ-specific autoimmunity. Together, these data provide *in vitro* and *in vivo* evidence that IL-12 signaling via IL-12R β 2 regulates thymocyte development by inhibiting thymocyte maturation, rather than affecting negative selection. Loss of IL-12 responsiveness in the absence of IL-12R β 2 may therefore result in an accelerated maturation and increased propensity to autoimmunity after immunization (Zhang et al., 2003). Conversely, these data are consistent with reduced severity of EAE upon administration of systemic IL-12 (Gran et al., 2004).

It should be noted that we have not ruled out the possibility that increased cell proliferation or enhanced survival in the periphery may also contribute to increased numbers of peripheral CD4⁺ and CD8⁺ T cells in IL-12R β 2^{-/-} mice. Upon stimulation with anti-CD3, we also observed a stronger tendency of CD8⁺ T cells to undergo apoptosis as compared with CD4⁺ T cells, suggesting increased susceptibility of CD8⁺ cells to apoptosis in this experimental paradigm. Since murine CD4⁺ T cells were reported to be more sensitive to FasL-induced apoptosis than CD8⁺ T cells, we speculate that Fas-independent apoptotic pathways may be involved (Suzuki and Fink, 2000).

A number of reports support the view that cytokines such as IL-4, IL-7, and TGF- β , play crucial roles in thymocyte development (Deonarain et al., 2003; MacNeil et al., 1990; Migliorati et al., 1993; Ramanathan et al., 2006; Rezzani et al., 2008; Sandberg et al., 2004; Uckun et al., 1991). In particular IL-7 is non-redundant in thymic T cell development and is critical for early T-cell development, but it also acts on immature and mature T cells (Juntilla and Koretzky,

2008; Laouar et al., 2004). Enhanced IL-7 expression is involved in the inhibition of thymic involution induced by IL-12/IL-23 (Li et al., 2004). IL-7R expression is critically regulated in developing thymocytes; indeed failed positive selection is associated with IL-7R downregulation, whereas cells undergoing positive selection upregulate or maintain their IL-7R expression. Recent data indicate that IL-7 signaling enhances the survival of developing thymocytes and mature T cells (Juntilla and Koretzky, 2008; Ramanathan et al., 2006). We also found that accelerated thymic maturation in IL-12R β 2^{-/-} mice is associated with increased IL-7R expression. Although it has been suggested that APCs (e.g., macrophages and dendritic cells) and epithelial cells in the thymus play an important role in thymic maturation (Boehm et al., 2003; Ernst et al., 1995), we have not found a significant difference in the numbers of these cells between IL-12R β 2^{-/-} and wild type mice, nor in Ia/Ie class II MHC molecules expression on these cells.

If IL-12 responsiveness negatively controls thymic maturation and positive selection, what type of intracellular signals might it generate? Different molecules are involved in distinct thymic developmental stages and in thymic selection. Among these molecules, a sustained ERK activation has been considered important in positive selection (Bhakta and Lewis, 2005; Tsatsanis et al., 2008). IL-12 may be involved in p38/Jnk activation (McNeil et al., 2005), and thus in apoptosis; however, whether IL-12 is involved in a signal transduction pathway for positive selection is not known. By real-time RT-PCR and western blot analysis, we did find increased ERK expression and activation in the thymus of IL-12R β 2^{-/-} mice compared to wild type mice upon *in vivo* injection of anti-CD3 (Zhang et al., unpublished data), providing a potential molecular basis for IL-12-induced thymic maturation. However, the interface between IL-12-induced signals and the molecular pathways involved in thymic maturation needs to be further studied. Whether ERK or other signaling pathways are critically involved, our observation indicates that IL-12R β 2 signal negatively controls thymic maturation and positive selection. The influence of IL-12 responsiveness on the expression and regulation of other molecules involved in thymic maturation, especially from DN to DP, are currently being investigated.

Conclusions

We conclude that IL-12 responsiveness via IL-12R β 2 is an important regulatory factor in thymic maturation, contributing to control the maturation rate and the balance of effector T cells that reach the peripheral immune compartment. The absence of such regulation appears to contribute to enhanced autoimmunity in IL-12R β 2^{-/-} mice. Strengthening regulatory mechanism mediated by IL-12 signaling could be a useful approach in maintaining balanced immune response for prevention and treatment of autoimmune disorders.

Acknowledgments

This study was supported by grants from the NIH and the National Multiple Sclerosis Society.

We thank Katherine Regan for editorial assistance.

Abbreviations

DN	double negative
DP	double positive
SP	single positive
p.i	post-immunization

HSA heat-stable antigen

References

- Becher B, Durell BG, Noelle RJ. Experimental autoimmune encephalitis and inflammation in the absence of interleukin-12. *J Clin Invest* 2002;110:493–497. [PubMed: 12189243]
- Bhakta NR, Lewis RS. Real-time measurement of signaling and motility during T cell development in the thymus. *Semin Immunol* 2005;17:411–420. [PubMed: 16256363]
- Boehm T, Scheu S, Pfeffer, et al. Thymic medullary epithelial cell differentiation, thymocyte emigration, and the control of autoimmunity require lympho-epithelial cross talk via LTbetaR. *J Exp Med* 2003;198:757–769. [PubMed: 12953095]
- Bommhardt U, Beyer M, Hunig T, et al. Molecular and cellular mechanisms of T cell development. *Cell Mol Life Sci* 2004;61:263–280. [PubMed: 14770292]
- Boursalian TE, Fink PJ. Mutation in fas ligand impairs maturation of thymocytes bearing moderate affinity T cell receptors. *J Exp Med* 2003;198:349–360. [PubMed: 12860933]
- Boursalian TE, Golob J, Soper DM, et al. Continued maturation of thymic emigrants in the periphery. *Nat Immunol* 2004;5:418–425. [PubMed: 14991052]
- Carl JW Jr, Liu JQ, Joshi PS, et al. Autoreactive T cells escape clonal deletion in the thymus by a CD24-dependent pathway. *J Immunol* 2008;181:320–328. [PubMed: 18566397]
- Cheng X, Zhao Z, Ventura E, et al. The PD-1/PD-L pathway is up-regulated during IL-12-induced suppression of EAE mediated by IFN-gamma. *J Neuroimmunol* 2007;185:75–86. [PubMed: 17320975]
- Cibotti R, Punt JA, Dash KS, et al. Surface molecules that drive T cell development in vitro in the absence of thymic epithelium and in the absence of lineage-specific signals. *Immunity* 1997;6:245–255. [PubMed: 9075925]
- Cua DJ, Sherlock J, Chen, et al. Interleukin-23 rather than interleukin-12 is the critical cytokine for autoimmune inflammation of the brain. *Nature* 2003;421:744–748. [PubMed: 12610626]
- Deonarain R, Verma A, Porter AC, et al. Critical roles for IFN-beta in lymphoid development, myelopoiesis, and tumor development: links to tumor necrosis factor alpha. *Proc Natl Acad Sci U S A* 2003;100:13453–13458. [PubMed: 14597717]
- Ernst B, Surh CD, Sprent J. Thymic selection and cell division. *J Exp Med* 1995;182:961–971. [PubMed: 7561699]
- Fairweather D, Frisancho-Kiss S, Yuseung SA, et al. IL-12 protects against coxsackievirus B3-induced myocarditis by increasing IFN-gamma and macrophage and neutrophil populations in the heart. *J Immunol* 2005;174:261–269. [PubMed: 15611248]
- Foy TM, Page DM, Waldschmidt TJ, et al. An essential role for gp39, the ligand for CD40, in thymic selection. *J Exp Med* 1995;182:1377–1388. [PubMed: 7595208]
- Freywald A, Sharfe N, Miller CD, et al. EphA receptors inhibit anti-CD3- induced apoptosis in thymocytes. *J Immunol* 2006;176:4066–4074. [PubMed: 16547242]
- Gately MK, Renzetti LM, Magram J, et al. The interleukin-12/interleukin-12-receptor system: role in normal and pathologic immune responses. *Annu Rev Immunol* 1998;16:495–521. [PubMed: 9597139]
- Godfrey DI, Kennedy J, Gately MK, et al. IL-12 influences intrathymic T cell development. *J Immunol* 1994;152:2729–2735. [PubMed: 7511624]
- Gran B, Chu N, Zhang GX, et al. Early administration of IL-12 suppresses EAE through induction of interferon-gamma. *J Neuroimmunol* 2004;156:123–131. [PubMed: 15465603]
- Gran B, Zhang GX, Yu S, et al. IL-12p35-deficient mice are susceptible to experimental autoimmune encephalomyelitis: evidence for redundancy in the IL-12 system in the induction of central nervous system autoimmune demyelination. *J Immunol* 2002;169:7104–7110. [PubMed: 12471147]
- Hakim FT, Gress RE. Thymic involution: implications for self-tolerance. *Methods Mol Biol* 2007;380:377–390. [PubMed: 17876107]

- Huseby ES, Kappler JW, Marrack P. Thymic selection stifles TCR reactivity with the main chain structure of MHC and forces interactions with the peptide side chains. *Mol Immunol* 2008;45:599–606. [PubMed: 17920446]
- Jameson SC, Hogquist KA, Bevan MJ. Positive selection of thymocytes. *Annu Rev Immunol* 1995;13:93–126. [PubMed: 7612239]
- Juntilla MM, Koretzky GA. Critical roles of the PI3K/Akt signaling pathway in T cell development. *Immunol Lett* 2008;116:104–110. [PubMed: 18243340]
- Kang BY, Kim TS. Targeting cytokines of the interleukin-12 family in autoimmunity. *Curr Med Chem* 2006;13:1149–1156. [PubMed: 16719776]
- Kikkawa E, Yamashita M, Kimura M, et al. T(h)1/T(h)2 cell differentiation of developing CD4 single-positive thymocytes. *Int Immunol* 2002;14:943–951. [PubMed: 12147631]
- Lamhamedi-Cherradi SE, Zheng SJ, Maguschak KA, et al. Defective thymocyte apoptosis and accelerated autoimmune diseases in TRAIL^{-/-} mice. *Nat Immunol* 2003;4:255–260. [PubMed: 12577054]
- Laouar Y, Crispe IN, Flavell RA. Overexpression of IL-7R alpha provides a competitive advantage during early T-cell development. *Blood* 2004;103:1985–1994. [PubMed: 14592827]
- Li L, Hsu HC, Stockard CR, et al. IL-12 inhibits thymic involution by enhancing IL-7- and IL-2-induced thymocyte proliferation. *J Immunol* 2004;172:2909–2916. [PubMed: 14978093]
- Ludviksson BR, Ehrhardt RO, Strober W. Role of IL-12 in intrathymic negative selection. *J Immunol* 1999;163:4349–4359. [PubMed: 10510375]
- MacNeil IA, Suda T, Moore KW, et al. IL-10, a novel growth cofactor for mature and immature T cells. *J Immunol* 1990;145:4167–4173. [PubMed: 2124236]
- McNeil LK, Starr TK, Hogquist KA. A requirement for sustained ERK signaling during thymocyte positive selection in vivo. *Proc Natl Acad Sci U S A* 2005;102:13574–13579. [PubMed: 16174747]
- Migliorati G, Nicoletti I, Pagliacci MC, et al. Interleukin-2 induces apoptosis in mouse thymocytes. *Cell Immunol* 1993;146:52–61. [PubMed: 8425230]
- Murphy CA, Langrish CL, Chen Y, et al. Divergent pro- and antiinflammatory roles for IL-23 and IL-12 in joint autoimmune inflammation. *J Exp Med* 2003;198:1951–1957. [PubMed: 14662908]
- Oppmann B, Lesley R, Blom B, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. *Immunity* 2000;13:715–725. [PubMed: 11114383]
- Porcellini S, Panigada M, Grassi F. Molecular and cellular aspects of induced thymus development in recombinate-deficient mice. *Eur J Immunol* 1999;29:2476–2483. [PubMed: 10458761]
- Ramanathan S, Gagnon J, Leblanc C, et al. Suppressor of cytokine signaling 1 stringently regulates distinct functions of IL-7 and IL-15 in vivo during T lymphocyte development and homeostasis. *J Immunol* 2006;176:4029–4041. [PubMed: 16547238]
- Rezzani R, Bonomini F, Rodella LF. Histochemical and molecular overview of the thymus as site for T-cells development. *Prog Histochem Cytochem* 2008;43:73–120. [PubMed: 18555891]
- Rodriguez-Galan MC, Bream JH, Farr, et al. Synergistic effect of IL-2, IL-12, and IL-18 on thymocyte apoptosis and Th1/Th2 cytokine expression. *J Immunol* 2005;174:2796–2804. [PubMed: 15728489]
- Sandberg JK, Stoddart CA, Brilot F, et al. Development of innate CD4⁺ alpha-chain variable gene segment 24 (Valpha24) natural killer T cells in the early human fetal thymus is regulated by IL-7. *Proc Natl Acad Sci U S A* 2004;101:7058–7063. [PubMed: 15118099]
- Schito ML, Demidov ON, Saito S, et al. Wip1 phosphatase-deficient mice exhibit defective T cell maturation due to sustained p53 activation. *J Immunol* 2006;176:4818–4825. [PubMed: 16585576]
- Starr TK, Jameson SC, Hogquist KA. Positive and negative selection of T cells. *Annu Rev Immunol* 2003;21:139–176. [PubMed: 12414722]
- Suzuki I, Fink PJ. The dual functions of Fas ligand in the regulation of peripheral CD8⁺ and CD4⁺ T cells. *Proc Natl Acad Sci USA* 2000;97:1707–1712. [PubMed: 10677522]
- Touil T, Fitzgerald D, Zhang, et al. Pathophysiology of interleukin-23 in experimental autoimmune encephalomyelitis. *Drug News Perspect* 2006;19:77–83. [PubMed: 16628262]
- Trinchieri G, Pflanz S, Kastelein RA. The IL-12 family of heterodimeric cytokines: new players in the regulation of T cell responses. *Immunity* 2003;19:641–644. [PubMed: 14614851]

- Tsatsanis C, Vaporidi K, Zacharioudaki V, et al. Tpl2 and ERK transduce antiproliferative T cell receptor signals and inhibit transformation of chronically stimulated T cells. *Proc Natl Acad Sci U S A* 2008;105:2987–2992. [PubMed: 18287049]
- Uckun FM, Tuel-Ahlgren L, Obuz V, et al. Interleukin 7 receptor engagement stimulates tyrosine phosphorylation, inositol phospholipid turnover, proliferation, and selective differentiation to the CD4 lineage by human fetal thymocytes. *Proc Natl Acad Sci U S A* 1991;88:6323–6327. [PubMed: 2068112]
- Wu C, Wang X, Gadina M, et al. IL-12 receptor beta 2 (IL-12R beta 2)-deficient mice are defective in IL-12-mediated signaling despite the presence of high affinity IL-12 binding sites. *J Immunol* 2000;165:6221–6228. [PubMed: 11086056]
- Zhang GX, Gran B, Yu S, et al. Induction of experimental autoimmune encephalomyelitis in IL-12 receptor-beta 2-deficient mice: IL-12 responsiveness is not required in the pathogenesis of inflammatory demyelination in the central nervous system. *J Immunol* 2003;170:2153–2160. [PubMed: 12574388]
- Zhang S, Wang Q. Factors determining the formation and release of bioactive IL-12: regulatory mechanisms for IL-12p70 synthesis and inhibition. *Biochem Biophys Res Commun* 2008;372:509–512. [PubMed: 18503756]

Fig. 1.

Lack of IL-12Rβ2 alters thymocyte subpopulations *in vivo*. Eight-week-old IL-12Rβ2^{-/-} and IL-12Rβ2^{+/+} C57BL/6 mice (n= 4 in each group) were injected i.p. with 25 μg of I45CII rat anti-mouse CD3ε mAb at 0 and 24 h. Twenty hours after the 2nd injection, mice were sacrificed and their thymocytes were prepared and subjected to flow cytometry analysis. Isotype rat IgG at 25 μg was injected i.p. in parallel as a negative control. (A) The absolute number of thymocytes in both IL-12Rβ2^{-/-} and wild type mice was significantly reduced following anti-CD3 injection. Bars represent mean values ± SD. **P<0.01 for comparisons between IL-12Rβ2^{-/-} and IL-12Rβ2^{+/+} mice. ## P<0.01; ### P<0.001 for comparisons between control Ab- vs. anti-CD3-injected mice at the same strain. (B) Viable thymocytes were gated and the percentage of double-negative, double positive and single positive thymocytes are shown in wild type and IL-12Rβ2^{-/-} mice after *in vivo* administration of anti-CD3 and a control Ab respectively. **P<0.01; ***P<0.001 for comparisons between IL-12Rβ2^{-/-} and IL-12Rβ2^{+/+} mice. (C) CD4/CD8 profiles of thymocytes from IL-12Rβ2^{+/+} (left panels) and IL-12Rβ2^{-/-} (right panels) mice after injection with anti-CD3 (upper panels) or control IgG (lower panels) are compared. Gates designated in the dot plots represent thymocyte subpopulations described in (B), listing percentages of thymocytes in each compartment. Percentages of thymocytes in the indicated quadrants are shown for representative animals. One representative experiments of three is shown.

Fig. 2.

Thymocyte maturation in IL-12R β 2^{-/-} and wild type mice. Thymocytes were prepared as described in Fig. 1 (n= 4 per group) after anti-CD3 treatment. **(A)** Both the MFI of CD24 expression (numbers above the line in the upper left-hand corner of each panel) and the percentage of CD24⁺ cells (numbers below the line in the upper left-hand corner of each panel) in DN and DP thymocytes of IL-12R β 2^{-/-} mice were consistently lower than in IL-12R β 2^{+/+} mice. The MFI of isotype control antibodies was approximately 100. **(B)** Comparison of thymocyte CD24 expression (MFI) between IL-12R β 2^{+/+} mice and IL-12R β 2^{-/-} mice. After anti-CD3 injection, CD24 expression was significantly lower in DN and DP thymocytes of both mice groups compared to control mAb-injected mice. Furthermore,

CD24 expression at DN and DP stages was significantly lower in IL-12R β 2^{-/-} mice compared to IL-12R β 2^{+/+} mice. **P<0.01 refers to comparisons between IL-12R β 2^{-/-} and IL-12R β 2^{+/+} mice. One representative experiments of three is shown.

Fig. 3.

Increased numbers of T cells in spleen of IL-12R β 2^{-/-} mice. Splenocytes of mice described in Fig. 1 (n= 4 per group) were harvested and stained with anti-CD4, CD8, Annexin V and 7-AAD. The absolute number of CD4⁺ and CD8⁺ T cells per spleen (**A**) and percentage of Annexin V⁺7-AAD⁻ T cells (**B**) are shown (n= 4 each group). * refers to the comparison between IL-12R β 2^{-/-} mice and IL-12R β 2^{+/+} mice; # refers to the comparison between mice receiving anti-CD3 and control mAb in each mouse strain. ** and ##, P<0.01; *** and ###, P<0.001. One representative experiments of three is shown.

Fig. 4.

Lack of IL-12R β 2 alters thymocyte subpopulations *in vitro*. A two-step culture system was utilized (Cibotti et al., 1997). Thymocytes (1×10^6) from naïve IL-12R β 2^{-/-} and IL-12R β 2^{+/+} mice (n = 3 per group; see also Fig. 1B, control mAb, and Fig. 1C, lower panels, for the phenotype of starting thymocyte populations) were cultured for 16 h on 24-well plates coated overnight with anti-TCR and anti-CD2. Wells coated with isotype IgG served as control. During the second step, cells from individual signaling cultures were then transferred into uncoated wells for an additional 16 h. Cells were then harvested and analyzed by flow cytometry. Bars represent mean values $\pm$ SD. **P<0.01 for the comparison between IL-12R β 2^{-/-} and IL-12R β 2^{+/+} mice. One representative experiments of three is shown.

Fig. 5.

Lack of IL-12R β 2 alters thymocyte subpopulations in mice immunized with MOG35-55. Eight-week-old IL-12R β 2^{-/-} and IL-12R β 2^{+/+} C57BL/6 mice (n= 4 per group) were immunized with MOG35-55 in CFA. Ten days p.i., mice were sacrificed and their thymocytes were prepared and subjected to flow cytometry analysis. (A) CD4/CD8 profiles of thymocytes from IL-12R β 2^{+/+} (left panels) and IL-12R β 2^{-/-} (right panels) MOG35-55-immunized mice (upper) or naïve mice (lower) are compared. Gates designated in the dot plots represent thymocyte subpopulations described in (B), listing percentages of thymocytes in each compartment. Percentages of thymocytes in the indicated sub-compartments are shown for

individual mice. Bars represent mean values $\pm$ SD. **P<0.01; ***P<0.001 for the comparison between IL-12R β ^{-/-} and IL-12R β ^{+/+} mice. One representative experiments of two is shown.

Fig. 6.

Lack of IL-12Rβ2 alters thymocyte maturation in mice immunized with MOG35-55 in CFA. Thymocytes of immunized mice were isolated at day 10 p.i. (n = 4 per group). Cells were labeled with anti-CD4, CD8, and CD24 mAb. **(A)** Different thymocyte subpopulations of IL-12Rβ2^{-/-} in IL-12Rβ2^{+/+} mice were analyzed by flow cytometry for the percentage (numbers below the line) and the MFI (numbers above the line) of CD24 levels. **(B)** Comparison of CD24 expression (MFI) on thymocyte subpopulations between IL-12Rβ2^{+/+} mice and IL-12Rβ2^{-/-} mice is shown. **P<0.025; ***P<0.001.

Fig. 7.

Increased percentages of CD4⁺ and CD8⁺ T cells in the spleen of IL-12R β 2^{-/-} mice immunized with MOG35-55 in CFA. Splenocytes of immunized mice were isolated at day 10 p.i. Non-immunized mice served as control. The total number of splenocytes was similar between IL-12R β 2^{-/-} and IL-12R β 2^{+/+} mice (data not shown). Percentages of CD4⁺ and CD8⁺ T cells in immunized mice were determined by flow cytometry (A). To study the effect of IL-12R β 2-deficiency in apoptosis, splenocytes were labeled with anti-CD4, CD8, and Annexin V. Fewer apoptotic cells were found in IL-12R β 2^{-/-} mice after immunization with MOG35-55 in CFA (B). * P<0.05; ** P<0.01. One representative experiments of two is shown.

Table 1IL-7Ra expression in the thymus^a

	IL-12Rβ2 ^{+/+}	IL-12Rβ2 ^{-/-}	P values ^b
Anti-CD3 injection			
CD4-8- (DN) cells	49.5 ± 1.4 (**) ^c	53.3 ± 2.3 (**)	0.013
CD4+8+ (DP) cells	41.1 ± 5.1 (**)	52.8 ± 7.6 (**)	0.02
CD4+CD8- (SP) cells	36.4 ± 3.0 (*)	41.5 ± 1.3 (**)	0.009
CD4-CD8+ (SP) cells	34.4 ± 1.5 (*)	37.0 ± 1.4 (**)	0.024
Control Ab injection			
CD4-8- (DN) cells	31.4 ± 0.5	31.9 ± 0.1	NS ^d
CD4+8+ (DP) cells	25.5 ± 0.7	25.5 ± 0.3	NS
CD4+CD8- (SP) cells	32.6 ± 0.1	29.0 ± 1.1	0.03
CD4-CD8+ (SP) cells	30.0 ± 0.2	25.0 ± 2.8	0.01

^a Eight-week-old IL-12Rβ2^{-/-} and IL-12Rβ2^{+/+} C57BL/6 mice (n= 4) were injected i.p. with 25 µg I45CII anti-CD3ε mAb at 0 and 24 h. Control mice were injected i.p. with 25 µg isotype rat IgG. Twenty hours after the second injection, mice were sacrificed and their thymocytes prepared and analyzed by flow cytometry. Values represent percentage of cells for each of the four thymocyte populations defined by the surface markers CD4 and CD8.

^b P values are for comparisons between IL-12Rβ2^{-/-} and IL-12Rβ2^{+/+} mice.

^c Asterisks (*) denote the comparison of each particular subpopulation between anti-CD3-injected mice and control antibody-injected mice.

* P<0.05;

** P<0.01.

^d NS, not significant.