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IL-12 Inhibits Thymic Involution by Enhancing IL-7- and IL-2-Induced Thymocyte Proliferation¹

Lina Li,*† Hui-Chen Hsu, † Cecil R. Stockard,* PingAr Yang, † Juling Zhou, † Qi Wu, † William E. Grizzle,* and John D. Mountz‡†‡

IL-12 has been reported to affect thymic T cell selection, but the role of IL-12 in thymic involution has not been studied. We found that in vivo, IL-12b knockout (IL-12b<sup>−/−</sup>) mice exhibited accelerated thymic involution compared with wild-type (WT) B6 mice. This is characterized by an increase in thymocytes with the early development stage phenotype of CD25<sup>−</sup>CD44<sup>−</sup>CD4<sup>−</sup>CD8<sup>−</sup> in aged IL-12b<sup>−/−</sup> mice. Histologically, there were accelerated degeneration of thymic extracellular matrix and blood vessels, a significantly decreased thymic cortex/medulla ratio, and increased apoptotic cells in aged IL-12b<sup>−/−</sup> mice compared with WT mice. There was, however, no apparent defect in thymic structure and thymocyte development in young IL-12−/− mice. These results suggest the importance of IL-12 in maintaining thymic integrity and function during the aging process. Surprisingly, in WT B6 mice, there was no age-related decrease in the levels of IL-12 produced from thymic dendritic cells. Stimulation of thymocytes with IL-12 alone also did not enhance the thymocyte proliferative response in vitro. IL-12, however, provided a strong synergistic effect to augment the IL-7 or IL-2 induced thymocyte proliferative response, especially in aged WT and IL-12b<sup>−/−</sup> mice. Our data strongly support the role of IL-12 as an enhancement cytokine, which acts through its interactions with other cytokines to maintain thymic T cell function and development during aging. The Journal of Immunology, 2004, 172: 2909–2916.

During aging in mammals, there is a gradual decline in thymus integrity and function. The regression in the size, weight, and cellularity of the thymus is known as thymic involution (1–4). Age-related thymic involution is considered an important factor in immune senescence (5–7). However, the molecular mechanisms underlying this phenomenon have not been identified.

We have recently identified a dramatic age-dependent difference in thymic involution in C57BL/6 (B6) compared with DBA/2 (D2) strains of mice (8). In an attempt to map the genetic loci that influence the differential age-related thymic involution rate between these two strains, we analyzed the Con A-induced thymocyte proliferative response of 22-mo-old C57BL/6 × DBA/2 (BXD) recombinant inbred (RI)³ strains of mice (9). The quantitative trait loci (QTLs) regulating the Con A-induced thymocyte proliferative response were mapped to chromosomes (Chr) 1, 3, and 11, and were nearest to 32.1, 5.6, and 18.0 centimorgans (cM), respectively (9). The strongest QTL was on Chr 11 at D11Mit51, and IL-12b, a polymorphic gene between the B6 and D2 strains (10), was considered a candidate gene in the mapped region that may influence age-related thymic involution.

The IL-12b gene encodes the IL-12 p40 subunit. A functional 75-kDa IL-12 heterodimeric glycoprotein is composed of a disulfide-bond by 35- and 40-kDa subunits (11–13). IL-12 is produced primarily by APCs and exerts immunoregulatory effects on T and NK cells (14). It is a potent immunoregulatory cytokine that is crucial for the generation of cell-mediated immunity to intracellular pathogens (13). Although an association between IL-12 and thymic involution has not been described, IL-12 has previously been shown to be secreted by thymic dendritic cells (DC) in mice (15) and humans (16) upon stimulation. Both the p35 and p40 subunits of IL-12 are secreted by thymic epithelial cell lines. mRNAs encoding IL-12 subunits have also been detected in both fetal and adult mouse thymic stromal cells (17). The function of IL-12 in the thymus has been shown to act in the thymocyte negative selection process (18). Godfrey et al. (17) have reported that, in combination with IL-2 and IL-4, the addition of IL-12 resulted in an increase in thymocyte proliferation. More recently, Warrington et al. (19) demonstrated that IL-12 could up-regulate the surface expression of functional CD28 in senescent CD4<sup>+</sup>CD28<sup>−</sup> T cells, suggesting that IL-12 also plays a role in the prevention of immune senescence.

To explore the possibility that IL-12 influences the rate of thymic involution, the present study analyzed the in vivo effects of IL-12b knockout (IL-12b<sup>−/−</sup>) on thymic involution and the in vitro effects of IL-12 on wild-type (WT) B6 thymocytes at different ages. The in vivo study of IL-12b<sup>−/−</sup> mice revealed an accelerated thymic involution in aged, but not young, IL-12b<sup>−/−</sup> mice compared with age-matched WT mice. There was no age-related decline in the production of IL-12b in thymic DCs (TDCs) of WT B6 mice. There was no significant response of thymocytes to IL-12 alone. However, IL-12 enhanced the proliferative response of thymocytes from aged mice to IL-2 and IL-7. Our results suggest that IL-12 acts in an age-dependent fashion to counteract the decline in IL-7 and IL-2 signaling, and thereby inhibits thymic involution in older mice.

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3 Abbreviations used in this paper: RI, recombinant inbred; Chr, chromosome; D2, DBA/2 mouse; DC, dendritic cell; MTS, mouse thymus stroma; QTL, quantitative trait loci; TDC, thymic DC; WT, wild type.
Materials and Methods

Mice

Female B6.129S1-Il12b<tm1/Jm> (IL-12<−/−>) and WT B6 mice were obtained from The Jackson Laboratory (Bar Harbor, ME). The IL-12<−/−> mice had been backcrossed to the B6 background for >10 generations, resulting in a contribution of the B6 background of >99% of the genetic background. The mice were analyzed at different ages, ranging from 2–16 mo. Mice were examined at young (2 mo), middle (5–9 mo), and old (12–16 mo) ages. The mice were aged in the animal facilities of University of Alabama at Birmingham, AL under specific pathogen-free conditions until used for the experiments. Mice were housed in groups of three to five mice per cage and were handled with sterile plastic. All experiments conducted in this study have been reviewed and approved by the institutional animal care and use committee of University of Alabama at Birmingham.

Harvest of the thymus and thymocyte count

The thymus was removed and immediately placed in RPMI 1640 medium. Thymi were photographed using a Cool Pix990 digital camera (Nikon, Tokyo, Japan). After a single-cell suspension was prepared, the mean thymocyte count was determined using a hemocytometer.

Flow cytometry analysis

Single-cell suspensions of thymocytes were analyzed by FACScan flow cytometry (BD Biosciences, Franklin Lakes, NJ) using four-color analysis with a combination of allopheocyanin-conjugated-CD3 (clone 2C-11), PE-anti-CD4 (clone H129.19), PE-anti-CD8 (clone 53-67), FITC-anti-CD25 (clone J Controls), biotin-conjugated anti-CD44 (clone 7D4), FITC-B220 (clone M1/70), allopheocyanin-conjugated anti-CD11c (clone M1/70), allopheocyanin-conjugated anti-CD11b (clone Mac 1), and PE-IL-12 p40/p70 (clone C15.6), which recognizes the IL-12 p40 subunit (BD PharMingen, San Diego, CA). Except for intracellular staining of IL-12 (20), single-cell suspensions of thymocytes (1 × 10⁷) were washed once with FACS buffer (5% FCS and 0.1% sodium azide in PBS), and incubated first with unconjugated anti-CD16/CD3 (Fc Block; BD Pharmingen, San Diego, CA) at room temperature for 20 min. Cells were then incubated with a biotin-conjugated Ab at room temperature for 20 min and washed once with FACS buffer. Second-step incubations were performed at room temperature for 20 min with Cy5-streptavidin and allopheocyanin-, PE-, and FITC-conjugated Abs. After staining, the cells were washed twice with FACS buffer and stored in PBS containing 2% paraformaldehyde until FACS analysis. After cell surface staining, cells were washed twice with FACS buffer and fixed in 1% paraformaldehyde/FACS solution. Cells (30,000/sample) were analyzed by flow cytometry on a FACScan (BD Biosciences). For intracellular staining of IL-12 (20), single-cell suspensions prepared from the thymus were incubated with a protein transport inhibitor, GolgiStop, according to the manufacturer’s protocol (BD Pharmingen) at 37°C for 4 h, then stained with Abs for cell surface Ags as described above and incubated with Cytofix/Cytoperm solution (BD Pharmingen) in the dark for 20 min before incubation with the PE-IL-12 p40/p70 Ab. The histogram analysis was performed using WinMDI software (email address: trotter@scripps.edu). Three to five mice were analyzed for each age group. Forward angle light scatter was used to exclude dead and aggregated cells. For histogram analysis, the results are presented as fluorescence histograms, with the relative number of cells on a linear scale plotted vs the relative fluorescence intensity on a log scale. For dot-plot analysis, the results are presented as relative fluorescence intensity on a log scale plotted against the second-color fluorescence intensity on a log scale.

Histology analysis

The thymi were removed from the mice immediately after sacrifice, fixed in 10% phosphate-buffered formalin (pH 7.0), and embedded in paraffin. Thin tissue sections (4 µm) were cut, deparaffinized, and stained with H&E.

Immunohistochemical staining

Tissue samples of the thymi of sacrificed mice were embedded in Histoprep frozen tissue embedding medium (Fisher Scientific, Fair Lawn, NJ) and snap-frozen on dry ice. Frozen sections of thymic tissue were fixed in neutral-buffered formalin <18 h before immunohistochemical staining. Endogenous peroxidase activity was suppressed by incubating the slides in 3% H₂O₂ for 5 min. Sections were incubated in PBE buffer (PBS containing 1% BSA, 1 mM EDTA, and 0.15 mM Na₂PO₃, pH 7.6) with 1% goat serum for 20 min to reduce nonspecific staining. The sections were then incubated for 60 min with the purified rat anti-mouse thymic extracellular matrix-associated mAb MTS-16 (BD Pharmingen) diluted in PBE buffer.

IL-12 REGULATES AGE-RELATED THYMIC INVOLUTION

The secondary Ab reagent for MTS 16 was biotinylated goat anti-rat IgG (BD PharMingen; 1 µg/ml). The secondary reagents were revealed using streptavidin peroxidase (Signet, Dedham, MA), which was applied for 20 min. The dianaminobenzidine substrate reagent (BioGenex, San Ramon, CA) was prepared immediately before use and applied for 7 min. The stained sections were lightly counterstained with hematoxylin before mounting.

TUNEL staining

TUNEL staining was performed as we previously described (21). Apoptosis was estimated by staining with the ApopTag In Situ Apoptosis Staining kit (Serologicals, Norcross, GA). The tissues were counterstained with hematoxylin before mounting.

TaqMan real-time quantitative RT-PCR analysis

RNA isolated from the thymus of mice at 2, 9, and 16 mo of age (three samples per age group) was used to construct cDNA using a first-strand cDNA synthesis kit (Amersham Pharmacia Biotech, Piscataway, NJ). The probes and primers used to amplify the IL-12p35 and IL-12p40 genes were provided by Applied Biosystems (Foster City, CA). All probes were labeled with the reporter dye 6-FAM at the 5′ end and with a conjugated minor groove binder quencher dye at the 3′ end. All probes were designed to span an intron to prevent amplification of DNA potentially present in the sample.

In each TaqMan run, serial 5-fold dilutions of a single standard cDNA derived from a positive control mouse RNA (Stratagene, La Jolla, CA) were amplified to create a standard curve, and values of unknown samples were estimated relative to this standard curve. Duplicate dilutions of the unknown sample cDNAs from 40 ng of total RNA were combined with a mixture of primers/TaqMan probes, Taq polymerase, and nucleotides (supplied by Applied Biosystems) (22). Each sample (total volume, 25 µl) was assayed in an optical tube designed for the 96-well format for the ABI PRISM 7000 (Applied Biosystems). Each PCR amplification was performed in duplicate using the following conditions: 2 min at 50°C and 10 min at 94°C, followed by a total of 40 cycles (15 s at 94°C and 1 min at 60°C).

Each PCR was run in duplicate. The mean value of the two reactions was defined as representative of the sample. The volumes described above yielded standard curves with a linear relationship between the copy number (defined as 1 ng of 1000 bp DNA = 9.1 × 10¹⁰ molecules) of the original internal standard added and the number of PCR cycles required to exceed a preset threshold according to the method described by Dreskin et al. (23). From these standard curves, the relative amount of cDNA for the 18S ribosomal RNA and the relative amount of cDNA for each gene were determined for each sample.

Thymocyte stimulation and proliferation assay

Single-cell suspensions of thymocytes from the mice were cultured (1 × 10⁶ cells/ml) in 96-well plates in RPMI 1640 medium supplemented with 10% FCS, 50 mM 2-ME, 25 mM HEPES-buffered saline, 2 mM glutamine, 100 U/ml penicillin, and 100 ng/ml streptomycin. Thymocytes from all mice were cultured in triplicate with purified mouse rIL-12 (BioSource International, Camarillo, CA; 0.1–5 ng/ml) or with IL-7 (5 ng/ml) or IL-7 (10 ng/ml; BioSource International). The thymocyte cultures were pulsed with [³H]thymidine (Amersham Pharmacia Biotech., 18 h before harvest. Proliferation was estimated by measuring the incorporation of [³H]thymidine into the cells on days 2, 4, and 6 after stimulation.

Statistical analysis

The values are reported as the mean of triplicate results obtained from three to five mice in each group ± SEM. Statistical analyses of the data were performed using Student’s unpaired two-tailed t test. A value of p<0.05 was considered statistically significant.

Results

Accelerated thymic involution in IL-12<−/−> mice

To study the in vivo effect of IL-12 on thymic involution, IL-12<−/−> mice at different ages were studied for gross and histologic changes. Thymic involution was more extensive in 14-mo-old IL-12<−/−> mice compared with age-matched WT mice (Fig. 1A). Correspondingly, the thymus cell count was significantly lower in old IL-12<−/−> mice compared with age-matched WT mice (Fig. 1B). Histologic staining revealed pronounced structural changes,
including cortical involution and a relative increase in the medulla and fat cells in thymi of 14-mo-old IL-12b−/− mice, compared with age-matched WT mice (Fig. 1C). At 5 mo of age, however, thymus size, thymocyte count, and thymus architecture in IL-12b−/− mice were comparable to those in WT mice. These results suggest that only aged, not young, IL-12b−/− mice show an accelerated thymic involution.

Block of T cell development in old IL-12b−/− mice

The age-related decline in IL-7 has been proposed to result in a block in early stage development (24). To determine whether IL-12b plays a role in early thymocyte development, we analyzed the cell surface expression of CD25 and CD44 in the CD4−CD8− double-negative (DN) thymocyte subpopulation of IL-12b−/− mice. At 2 mo of age, the percentage of the CD44+CD25+ CD4−CD8−CD3dim population of cells was lower in the IL-12b−/− mice compared with age-matched WT mice. There was, however, a gradual increase in this population with age, and at 12 mo of age there was a significantly higher percentage of CD44+CD25+ CD4−CD8−CD3dim thymocytes in IL-12b−/− mice compared with control mice (Fig. 2). These results suggest that T cell development is arrested in the most immature developmental stage in aged, but not young, IL-12b−/− mice compared with B6 mice.

Involvement of the thymus in 12-mo-old IL-12b−/− mice is associated with increased apoptosis

Increased thymocyte apoptosis is another mechanism associated with thymic involution (8, 25–27). We determined whether the accelerated thymic involution in aged IL-12b−/− mice is associated with an increase in thymocyte apoptosis. Apoptosis of thymic cells was determined in situ using TUNEL staining of paraffin sections. The results showed that there was an increase in the percentage of apoptotic cells in both the medulla (Fig. 3A) and cortex regions (Fig. 3B) of 12-mo-old IL-12b−/− mice compared with age-matched WT mice. Quantitation of apoptotic cells in the cortex and medulla showed that the increase in apoptosis in 12-mo-old IL-12b−/− mice was more apparent in the medulla (Fig. 3C) than in the cortex (Fig. 3D). These results suggest that the reduction in the

FIGURE 1. Accelerated-age-related thymic involution in IL-12b−/− mice. IL-12b−/− mice were compared with the same age B6 WT mice at either 5 or 14 mo of age. A, Gross examination of the entire thymus of either WT or IL-12b−/− mice at 5 or 12 mo of age. At least five thymi from either WT or IL-12b−/− mice were evaluated at 5 or 14 mo of age. A representative view of the thymus is shown. B, Thymocyte cell count from either WT B6 or IL-12b−/− mice at 5 and 14 mo of age. A single-cell suspension of thymocytes from these five mice per group was prepared, and the total number of thymocytes was counted. The data represent the mean ± SEM of at least five mice per group. * Significant decrease in thymocyte cell count (p < 0.01). C, Age-related involution of thymus cortex from IL-12b−/− mice. Representative H&E staining was carried in either WT or IL-12b−/− mice at 5 or 12 mo of age. Multiple sections on at least five thymi from each group of mice were stained with H&E.

FIGURE 2. Age-dependent increase in CD25+CD44+ in DN thymocytes of IL-12b−/− mice. A single-cell suspension of thymocytes (10⁶) from WT or IL-12b−/− mice at 2, 5, or 12 mo of age was stained with allophycocyanin-CD3, PE-CD4, PE-CD8, FITC-CD25, and Cy5-avidin against biotin-CD44. To analyze CD4+CD8− DN thymocytes without including B cells, granulocytes, macrophages, and CD3bright cells, thymocytes was first gated using a combination of forward vs side scatter to exclude macrophage and granulocytes. A, DN thymocytes in the gated region were then selected as CD3dim and both CD4− and CD8−negative cells, and the CD25, CD44 population is displayed. The plot represents 20,000 CD4+CD8− thymocytes. B, Percentage of CD44+CD25+ CD4−CD8− DN in total DN cells in IL-12b−/− mice compared with age-matched WT B6 mice. All results represent the percentage of cells (±SEM) from three mice per group.
Figure 3. Increased apoptosis in the thymus of 12-mo-old IL-12b−/− mice. Apoptosis staining was conducted using in situ TUNEL staining. Representative high power views of the thymic medulla (A) and cortex (B) are shown. The number of apoptotic thymocytes was quantitative in the medulla (C) or cortex (D). At least five random fields of view were used, and a number of apoptotic cells were counted using several sections from three to five WT or IL-12b−/− mice. The results represent the mean ± SEM. **, p < 0.05; ***, p < 0.01.

size of the thymus in aged IL-12b−/− mice may be associated with increased levels of apoptosis.

** Changed thymic microenvironment in old IL-12b−/− mice **

The organization of the thymic microenvironment has been shown to be associated with the capability of thymocytes to migrate and undergo proliferation and apoptosis (27–29). To determine whether the altered development of thymocytes in older IL-12b−/− mice was associated with changes in the thymic microenvironment, thymi were stained in situ using the MTS-16 mAb, which labels the thymic extracellular matrix and vasculature (29–31). At young and middle ages, there was no significant difference in the extracellular matrix and vasculature of the thymus of IL-12b−/− mice compared with WT mice. In contrast, at 12 mo of age, extensive changes in thymic architecture and the organization of thymic connective tissues were apparent in IL-12b−/− mice, but not in WT B6 mice (Fig. 4).

** No decrease in IL-12 with age **

IL-12b−/− mice exhibit increased thymic involution only after 5 mo of age, suggesting a role for IL-12 in the maintenance of thymocyte development in late-adult mice. We hypothesize that the age-related thymic involution may be associated with an age-related decrease in IL-12 production by thymic DC. To determine the time course of the expression of both p35 and p40 subunits of IL-12 (IL-12a and IL-12b), thymus mRNA from B6 mice at different ages (2, 9, and 16 mo) was analyzed using a real-time quantitative RT-PCR. To our surprise, there was a progressive, age-related increase in the expression of IL-12p35 and p40 transcript signals in the thymus of B6 mice (Fig. 5A). To determine whether this age-related increase in IL-12 mRNA correlated with its expression at the protein level, the expression of IL-12 protein in the thymus of B6 mice at different ages (2, 9, and 16 mo) was determined using flow cytometric analysis. As IL-12 has been reported to be produced primarily by CD8+CD11c+CD11b+ and B220+CD11c+CD11b+ TDCs (15, 32, 33), intracellular staining was conducted using surface labeling with B220, CD11b, and CD11c, followed by intracellular staining with IL-12. There was an age-related increase in the percentage of CD8+CD11c+CD11b+ and B220+CD11c+CD11b+ TDCs (Fig. 5B). Intracellular staining indicated that within both CD8+CD11c+CD11b+ and B220+CD11c+CD11b+ TDCs, the percentage of IL-12− cells did not increase with age (Fig. 5C). However, as there was an age-related increase in the percentage and absolute number of these TDCs, the absolute number of IL-12-producing thymic DCs also increased with age (Fig. 5D).

IL-12 augments IL-7 and IL-2 signaling in an age-dependent manner

The above results indicate that although IL-12b−/− mice exhibit accelerated late-adult thymic involution, thymic production of IL-12 does not decrease with age. Another explanation for accelerated thymic involution in IL-12b−/− mice is that in WT mice, IL-12 enhances thymocyte signaling by other cytokines that decline with age. We therefore determined the effects of IL-12 by itself or in combination with IL-7, a critical cytokine maintaining thymocyte development, on the thymocyte proliferative response in young (2-mo-old) and aged (14-mo-old) WT B6 mice. As predicted, IL-12 alone did not induce the proliferation of thymocytes from either young or aged B6 mice (Fig. 6A). IL-7 significantly enhanced proliferation in both young and aged B6 mice. However, the IL-7 proliferative response was greatly enhanced in the presence of IL-12 (Fig. 6A). IL-7-induced proliferation of thymocytes was enhanced in an IL-12 dose-dependent manner in both young and aged B6 mice (Fig. 6B). IL-12 enhancement of IL-7 proliferation was greatest 6 days after the addition of both these cytokines in 2- and 14-mo-old mice (Fig. 6, C and D). These results indicate that IL-12 alone does not induce the proliferation of thymocytes from either young or aged mice, but has the greatest effect on augmentation of IL-7-induced proliferation in aged B6 mice.

IL-12 augments proliferation mediated by IL-2

IL-2 has been reported to be low in thymus from aged mice, and the addition of IL-2 can enhance thymocyte proliferation (34, 35).
In the periphery, IL-12 has been shown not only to stimulate, but also to prolong, the expression of IL-2R (34, 35). To determine whether IL-12 can enhance IL-2-induced thymocyte proliferation, single-cell suspensions of thymocytes from young (2-mo-old) or aged (14-mo-old) B6 mice were either unstimulated or stimulated with IL-12, IL-2, or IL-12 plus IL-2 (Fig. 7). IL-12 alone did not induce the proliferation of thymocytes from either young or aged mice, whereas IL-2 stimulated the proliferation of thymocytes in aged mice. The intracellular levels of IL-12p35 expressed on CD8<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup> or B220<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup> thymic DC were determined using four-color FACS analysis as described in Materials and Methods. D, Increased absolute number of IL-12-producing thymic DCs in aged B6 mice. The absolute number of IL-12–producing TDCs was determined by multiplying the total thymocyte count by the percentage of the indicated TDCs by the percentage of each cell type that is positive for the expression of IL-12. For all panels, the arrow bar represents the mean ± SD of three to five different FACS data. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 5. No age-related decline in thymic DC to produce IL-12 in WT B6 mice. A, The expression of IL-12p35 and IL-12p40 in thymus of C57BL6 mice (B6) at the indicated ages was determined using real-time quantitative RT-PCR. B, Increased percentage of the TDCs (CD8<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup> or B220<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup>) during aging determined by FACS analysis in WT B6 mice. C, No difference or increase in IL-12p<sup>+</sup>CD11b<sup>+</sup> thymic DC in aged B6 mice. The intracellular levels of IL-12p12 expressed on CD8<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup> or B220<sup>+</sup>CD11c<sup>+</sup>CD11b<sup>+</sup> thymic DC were determined using four-color FACS analysis as described in Materials and Methods. D, Increased absolute number of IL-12–producing thymic DCs in aged B6 mice. The absolute number of IL-12–producing TDCs was determined by multiplying the total thymocyte count by the percentage of the indicated TDCs by the percentage of each cell type that is positive for the expression of IL-12. For all panels, the arrow bar represents the mean ± SD of three to five different FACS data. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 6. Synergistic effect of IL-12 with IL-7 on thymocyte proliferation. A single-cell suspension of cells (1 × 10<sup>6</sup>/ml) isolated from the thymus of C57BL6 mice (B6) at 2 or 14 mo of age was stimulated with control medium (CM), IL-7, IL-12, or IL-7 plus IL-12. Proliferation was determined by [<sup>3</sup>H]thymidine uptake. A, Synergistic effect of IL-7 and IL-12 on thymocyte proliferation from mice at different ages. Single-cell suspensions of thymocytes were obtained from C57BL6 (B6) mice at age of 2 and 14 mo of age. Thymocyte proliferation was determined on day 6 after incubation with control medium, IL-7 (10 ng/ml), IL-12 (5 ng/ml), or IL-7 plus IL-12 at the same concentrations. *, p < 0.05; **, p < 0.01; ***, p < 0.001. B, A dose-dependent response of IL-12 with or without IL-7 (10 ng/ml). IL-12 was used at the indicated concentration ranging from 0.1–5 ng/ml with or without IL-7 (10 ng/ml) as indicated. Proliferation was determined on day 6 after thymocyte incubation. C and D, Time course of the synergistic effect of IL-7 plus IL-12 on 2-mo-old (C) or 14-mo-old (D) mice. Thymocytes from B6 mice at the indicated ages were cultured with control medium (CM), IL-12 (5 ng/ml), IL-7 (10 ng/ml), or IL-7 plus IL-12 at these concentrations. The proliferative response was determined on days 2, 4, and 6 after culture. The arrow bar represents the mean ± SEM of three separate assays using at least five mice per group.
mice more than in young mice. Cotreatment of thymocytes with both IL-12 and IL-2 resulted in a significantly increased proliferative response compared with IL-2 (Fig. 7A). As with IL-7, IL-12 exhibited a dose-dependent capability to augment the IL-2-induced thymocyte proliferative response (Fig. 7B). This enhanced thymocyte proliferative response to IL-2 in the presence of IL-12 was highest on day 6 after stimulation with both cytokines (Fig. 7, C and D) in thymocytes from both young and aged B6 mice. These results indicate that IL-12 can augment the age-related decline in the proliferative response to both IL-7 and IL-2.

**IL-12 augments IL-7 stimulation of T cells from IL-12b−/− mice**

Our results suggest that IL-12 provides an enhancement signal to augment thymocyte proliferation and cell survival mediated by other cytokines. This hypothesis is further supported by stimulation of thymocytes from IL-12b−/− mice at 2, 9, and 16 mo of age (Fig. 8). Compared with no treatment, there was no significant proliferative response by thymocytes to IL-12 alone in IL-12b−/− mice compared with WT B6 mice in either young (2-mo-old) or old (16-mo-old) animals. This result indicates that supplementation of thymocytes from IL-12b−/− mice with IL-12 does not have a direct stimulatory effect on proliferation. Addition of IL-7 to the culture medium resulted in a significantly higher proliferative response of thymocytes from 9- and 16-mo-old IL-12b−/− mice compared with the response of thymocytes of age-matched WT mice. Interestingly, the addition of IL-12 results in a significantly increased enhancement of the IL-7-induced proliferation. This enhancement is significantly higher in 16-mo-old IL-12b−/− mice compared with WT mice, whereas there is no difference in this enhancement in 2-mo-old IL-12b−/− mice compared with WT mice. These results provide further evidence that IL-12 acts indirectly and in an age-related fashion to sustain thymocytes proliferative response by IL-7 stimulation.

**Discussion**

There was a more rapid thymic involution in aged IL-12b−/− mice compared with WT mice. Interestingly, in young and middle-aged animals, thymic involution was not marked in IL-12b−/− mice. This is in contrast to estrogen receptor α (36) knockout or SMAD3 knockout (37) mice, in which there was an early onset of thymic
involution. *IL-12b−/−* mice exhibited an accumulation of DN thymocytes at the earliest CD44+CD25− development stage, but, again, this defect was not apparent until 12 mo of age. These results suggest that the increased thymic involution in *IL-12b−/−* mice might not be due to a primary defect in IL-12, but is the result of a combined defect of the ability of IL-12 to compensate for a decline in the level or signaling capacity of other cytokines necessary to sustain thymocyte development at an older age. The increased thymic involution in *IL-12b−/−* mice occurs by several mechanisms, including increased thymocyte development block, increased apoptotic thymocytes in the thymic medulla and cortex areas, and altered thymic architecture. Godfrey et al. (17) have shown that IL-12 induced significant proliferation of early CD3−CD4−CD8− triple-negative CD25+CD44+ pro-T cells in combination with stem cell factor. Our results, therefore, strongly suggest a scenario in which the effects of IL-12b promote thymocyte development and function through its interaction with multiple cytokines and factors, including IL-7 and IL-2.

Although *IL-12b−/−* mice exhibited late-age thymic involution, there was no decrease in IL-12 production by thymic DCs in WT B6 in old age. There were comparable or increased intracellular levels of IL-12b in CD8+CD11c−CD11b+ DCs and CD11c+ B220−CD11b− DCs in thymi of aged B6 mice. There was also no decline in IL-12b mRNA with age, as determined by real time-RT-PCR analysis of RNA isolated from total thymic extract. Together, the result indicates that there is no age-related decline in IL-12 production by thymic DC. The addition of IL-12 alone to culture medium also did not induce a thymocyte proliferative response in vitro. These results further support a role for IL-12 to provide a maintenance signal for other cytokines or factors in the thymus that exhibit an age-related decrease.

The present results are the first report showing that IL-12 is necessary, but not sufficient, to prevent thymic involution. IL-12 sustained the ability of IL-7 to stimulate thymocyte proliferation. This enhancement was more prominent in the thymus of aged *IL-12b−/−* mice compared with aged WT or young mice. In addition, IL-12 could greatly sustain the signaling of IL-2. IL-7 and IL-2 are known to be important in thymic involution, and enhanced levels of IL-7 and IL-2 can both act to reverse age-related thymic involution (28, 38–43). Ortmann et al. (44) found that the expression of IL-7 did not decline in BALB/c mice until 7 mo. This relatively late onset of the decline in IL-7 appears to correlate with the effects of IL-12b deficiency in thymic involution in older (at least 12-mo-old), but not younger (<5-mo-old), *IL-12b−/−* mice. In contrast to the decline in IL-7 in thymi from aged mice, IL-7R expression did not decline with age (45). Based on the characteristics of thymic involution (increase in apoptosis and accumulation of CD44+CD25− DN thymocytes) in aged *IL-12b−/−* mice and the role of IL-7 in preventing the development of these phenotypes, we propose that the increase in IL-12 in aged WT mice is to enhance the signaling of IL-7 and IL-2 to compensate for the decrease in these two cytokines in the aged thymus.

The present results are important because they show that IL-12 is an important maintenance factor that can compensate for the age-related decline in IL-7 and IL-2 signaling by augmenting these signaling pathways. This type of cytokine interaction response has previously been shown for IL-10, which may induce a Th2 response by enhancing the ability of IL-13 (46) or inhibiting the secretion of IL-12 (47). The mechanism underlying this enabling effect of one cytokine for another cytokine has been found to be due to both enhanced production of the other cytokine and enhanced receptor signaling by the other cytokine. Indeed, in the periphery, IL-12 has been shown to enhance and prolong the expression of the IL-2R (CD25) and IL-18R signaling pathway (48).

We propose that correction of thymic involution with IL-12, in combination with IL-7 or IL-2, may be due to a similar enhancing mechanism.

Underlying age-related thymic involution, there is a gradual decline in thymus integrity and function. Through comparative analysis of the Con A-induced thymocyte proliferative response in old BXD RI mice, we previously identified a QTL on Chr 11 (*D11Mit51*) that exhibited the strongest influence on the thymocyte proliferative response in aged mice (9). *D11Mit51* on Chr 11 harbors the polymorphic IL-12b gene in different mouse strains, including B6 vs D2 strains of mice (10). Ymer et al. (10) have previously shown that polymorphism of the IL-12b gene was associated with an allele-dependent variation in the expression and function of this gene between strains. Our present study suggests the possibility that polymorphism of this gene may alter the efficiency of IL-12 in sustaining the signal of IL-7 and IL-2, and thus may become an important factor to regulate the thymocyte proliferative response in older BXD RI strains of mice. Our results further suggest the importance of a combined genetic network, but not one single gene, in sustaining thymocyte function in aged mice.

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