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Normal Lymphoid Homeostasis and Lack of Lethal Autoimmunity in Mice Containing Mature T Cells with Severely Impaired IL-2 Receptors[1]

Thomas R. Malek,[2] Brian O. Porter, Elaine K. Codias, Paul Scibelli, and Aixin Yu

The importance of IL-2Rβ function for immune regulation is highlighted by the severe impairment in lymphoid cell function in IL-2Rβ-deficient mice. It has been speculated that failed IL-2/IL-2R signaling in peripheral T cells causes the associated autoimmunity, imbalanced peripheral lymphoid homeostasis, and defective T cell function. This study explored the requirement for IL-2Rβ function in mature T lymphocytes. We show that transgenic thymic expression of the IL-2R β-chain in IL-2Rβ-deficient mice prevents lethal autoimmunity, restores normal production of B lymphocytes, and results in a peripheral T cell compartment that is responsive to triggering through the TCR, but not the IL-2R. The dysfunction of the IL-2R is illustrated by the near complete failure of mature T cells to proliferate to IL-2 in vitro and in vivo, to differentiate into CTL, and to up-regulate IL-2Rα expression. These data indicate that lymphoid homeostasis is largely maintained despite a nonfunctional IL-2R in mature T lymphocytes and suggest that IL-2Rβ provides an essential signal during thymic development to regulate self-reactivity. ***The Journal of Immunology*****, 2000, 164: 2905–2914.**

The IL-2R and the IL-15R are both comprised of three subunits, a unique α-subunit and shared IL-2R β- and γ-chains. This latter subunit is designated γc, as it is also a subunit of the IL-4, IL-7, and IL-9 receptors (reviewed in Ref. 1). The importance of IL-2Rβ function for immune regulation is highlighted by the severe impairment in lymphoid cell function in IL-2Rβ-deficient mice (2). As early as 3 wk of age, these mice exhibit lymphoadenopathy and splenomegaly, the latter accompanied by substantial granulocyte infiltration, and autoimmunity as evident by anti-erythrocyte and anti-DNA Abs. Most of the complex abnormalities associated with IL-2Rβ deficiency appear to be attributable to $CD4^+$ T cells (2). Almost invariably, IL-2R$\beta^{-/-}$ mice die by 12 wk of age. Autoimmunity and peripheral T cell activation have also been noted in IL-$2^{-/-}$ and IL-2R$\alpha^{-/-}$ mice (3–5), but not IL-15Rα-deficient mice (6), suggesting that most of the severe problems manifested by IL-2Rβ-deficient mice are primarily the result of failed IL-2-induced signaling.

Current studies have not unequivocally established the mechanism for this autoimmune syndrome or even the cellular stage normally dependent upon IL-2Rβ function. It has been widely speculated that failed IL-2/IL-2R signaling in peripheral T cells causes this autoimmunity (reviewed in Ref. 7). IL-2 has been shown to efficiently sensitize recently Ag-activated mature T cells to apoptosis upon re-encounter with Ag by Fas/Fas ligand and TNF-α-dependent pathways (8–10). This process has been suggested to limit a specific immune response, to maintain lymphoid homeostasis, and to eliminate self-reactive T cells in the periphery that escaped negative selection in the thymus (7). Nevertheless, a failure in thymic function could also lead to severe autoimmunity. Notably, not only has IL-2Rβ been shown to be expressed in a fraction of pro-T cells, but it also is induced by self recognition within the TCR^{int} $CD8^{low/-}$ subset of thymocytes (11). The functional significance of this latter expression is unknown.

The present study directly explored the extent that a functional IL-2R β-chain in mature T lymphocytes is required to regulate peripheral lymphoid homeostasis, autoimmunity, and T cell function. We developed a transgenic (Tg)[3] mouse model in which the expression of IL-2Rβ was targeted to the thymus of IL-2R$\beta^{-/-}$ mice. The mature T cell compartment of these animals was extremely unresponsive to IL-2 in vitro and in vivo, but, somewhat unexpectedly, lacked autoimmunity and the imbalance in peripheral homeostasis associated with IL-2Rβ deficiency in vivo. These findings demonstrate that the major dysfunction of IL-2R$\beta^{-/-}$ mice is independent of a functional IL-2R β-chain in mature T cells and raise the possibility that the nonredundant function of IL-2/IL-2R may lie primarily at the level of the thymus.

Materials and Methods

Mice

To produce transgenic mice, mouse IL-2Rβ cDNA was blunt end cloned into the *Bam*HI cloning sites of the p1017 vector (12). The purified transgenic expression cassette was microinjected into $(B6 \times SJL)F_2$ oocytes. The resulting founders were identified by Southern blot analysis of *Bam*HI genomic DNA using full-length coding ^{32}P-labeled IL-2Rβ cDNA probe. Transgenic founders were backcrossed to IL-2R$\beta^{+/-}$ mice for three to five generations. In some cases, mice were injected i.p. with 100 μg of purified anti-CD3 in 0.1 ml of PBS.

RNA analysis

Total cellular RNA was isolated using the Trizol reagent (Life Technologies, Grand Island, NY) as described by the manufacturer. RT-PCR was performed using the Gene Amp RNA Core Kit (Perkin-Elmer, Branchburg, NJ) according to the manufacturer's instructions. The PCR primers were IL-2Rβ: 5′-ACA CCT TGG GCA TCT GCA GCA GTG and 5′-GAG CCA CTG CTG TCT CTG CTT GAG; and GAPDH, 5′-TGA TGG GTG

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[3] Abbreviation used in this paper: Tg, transgenic.

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TGA ACC ACG AG and 5′-TCA GTG TAG CCC AAG ATG CC, as previously described (13). The expected sizes of the PCR products were 498 bp for IL-2Rβ and 496 bp for GAPDH. Northern blot analysis was performed as previously described (14), using ^{32}P-labeled cDNA probe to mouse IL-2Rβ, GAPDH, or granzyme B (provided by M. Lichtenheld, University of Miami, Miami, FL).

Abs and FACS analysis

Biotin-anti-CD69, Cy-Chrome-anti-CD8α (53.6.7), biotin-anti-rat Ig (mouse Ig absorbed), anti-IgG1, alkaline phosphatase-IgG1, and PE-streptavidin were purchased from PharMingen (San Diego, CA). FITC-anti-CD4 (GK1.5), biotin-anti-IL-2Rα (7D4), biotin-anti-B220, anti-IL-2 (S4B6) (15), anti-γc (4G3 and 3E12) (16), and anti-IL-2Rβ (5H4) (17) were prepared in our laboratory. FACS analysis was performed as previously described (18) using a Becton Dickinson FACScan and CellQuest software (Mountain View, CA). Typically, 50,000 cells/sample were analyzed. In most experiments IL-2Rβ expression was assessed by three-step staining consisting of an initial incubation with anti-IL-2Rβ, a second incubation with biotin-anti-rat Ig, and a third incubation that first included quenching of unreacted anti-rat Ig sites by addition of rat IgG (1 μg) followed 10 min later by addition of fluorescent-conjugated Abs and PE-streptavidin.

Cell culture

Spleen cells (2×10^5/well) and thymocytes (5×10^5/well) were cultured in 0.2 ml of RPMI 1640 containing 5% FCS, glutamine (30 μg/ml), penicillin (100 U/ml), streptomycin (100 μg/ml), 2-ME (5×10^{-5} M; complete medium) in 96-well flat-bottom culture plates (3595; Costar, Cambridge, MA) with anti-CD3 (5% culture supernatant), PMA (10 ng/ml), cyclosporin A (100 ng/ml; Sigma, St. Louis, MO), anti-IL-2 (10 μg/ml), anti-γc (50 μg/ml each of 4G3 and 3E12), and the indicated cytokines for 48 h. Mouse IL-2 and IL-4 were supernatant fluids from P3×63 and P815 cells transfected with the respective cDNA. Simian IL-15 was provided by Immunex (Seattle, WA), and mouse IL-7 was purchased from PeproTech (Rocky Hill, NJ). Proliferation was measured by the addition of 1 μCi/well of [^{3}H]thymidine (25 Ci/mmol; Amersham, Arlington Heights, IL).

To generate T blasts, spleen cells (2×10^6/well) or thymocytes (5×10^6/well) were cultured with anti-CD3 (5% culture supernatant) in 1 ml of complete medium in 24-well flat-bottom culture plates for 48 h. Cultures of thymocytes were supplemented with 50 U/ml of mouse IL-2 to expand any potentially IL-2-responsive cells. These cells were washed and used for molecular, FACS, or cytokine bioassays. For the cytokine bioassays, the T blasts (2×10^4/well) were cultured in 0.2 ml of complete medium in 96-well flat-bottom culture plates with the indicated cytokines for 24 h, adding [^{3}H]thymidine during the last 4 h of culture. In all proliferation assays, cells were harvested on glass-fiber filters and counted in a beta scintillation counter. Data are reported as the means of duplicate or triplicate values that consistently varied by <10% from each other.

To generate allo-specific CTL, spleen cells (5×10^6/well) were cultured in complete medium with mitomycin C-treated allogeneic BALB/c spleen cells (2×10^6/well) in 24-well flat-bottom tissue culture plates for 4 days. For anti-CD3-induced CTL, spleen cells (2×10^6/well) were cultured in complete medium with anti-CD3 (5% culture supernatant) in 24-well flat-bottom tissue culture plates for 48 h. Allogeneic and anti-CD3-induced redirected CTL activity was performed in a standard 4-h ^{51}Cr release assay with P815 targets as previously described (19).

Anti-nuclear Abs

The assay for anti-nuclear Abs was performed using the Hep2 anti-nuclear Ab IFA assay (Scimedx, Danville, NJ) according to the manufacturer's instructions, except that FITC-anti-mouse Ig (Cappel, Organon Teknika, West Chester, PA) was used.

IL-2 binding assay

The indicated cells (8×10^6/tube) in duplicate were incubated with 50,000 cpm of ^{125}I-labeled human IL-2 (50 μCi/μg) with or without 500 ng of unlabeled IL-2 in 50 μl of complete medium at 37°C for 45 min, a time sufficient for IL-2 binding and internalization, but not degradation. The cells were washed three times with ice-cold PBS, and the cell-associated radioactivity was determined by counting in a gamma scintillation counter.

IgG1 ELISA

Wells were coated with anti-IgG1 (1 μg/ml), blocked with 0.25% gelatin, incubated with a serial dilution of mouse serum or purified IgG1 for 2 h, washed with PBS containing 0.05% Tween-20, and incubated with alkaline phosphatase-anti-IgG1 (1/1000 dilution) for 2 h. After washing, color was developed by incubation with 3 mM *p*-nitrophenyl phosphate (Sigma 104 phosphatase substrate, St. Louis, MO), the OD at 405 nm was determined, and IgG1 levels were calculated by comparison to a standard curve.

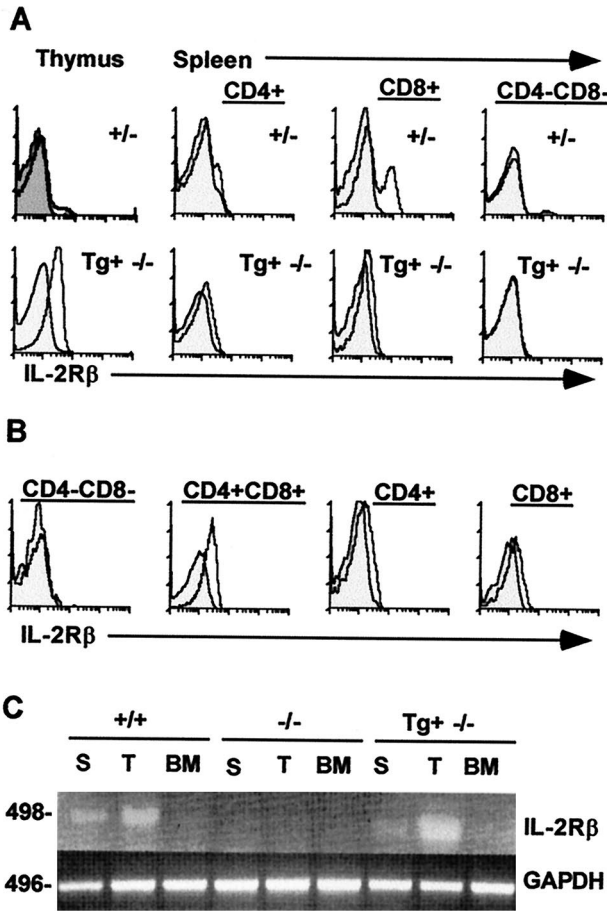

FIGURE 1. Transgenic expression of IL-2Rβ. *A*, Thymus and spleen cells from mice of the indicated genotypes were control stained with biotin-anti-rat Ig/PE-streptavidin (shaded areas) or stained for IL-2Rβ using 5H4 followed by biotin-anti-rat Ig/PE-streptavidin (open areas). For the spleen, cells were gated on the indicated subpopulation and analyzed for IL-2Rβ expression. *B*, IL-2Rβ expression on thymic subsets from Tg$^+$ IL-2Rβ$^{-/-}$ mice. Thymocytes were subjected to three-color FACS for IL-2Rβ, CD4 and CD8 using 5H4 followed by biotin-anti-rat Ig/PE-streptavidin, FITC-anti-CD4, and Cy-Chrome-anti-CD8 and were gated for the indicated subpopulation. *C*, RT-PCR analysis of IL-2Rβ. Total RNA was isolated from the spleen (S), thymus (T), or bone marrow (BM) from mice of the indicated genotypes and subjected to RT-PCR for IL-2Rβ or GAPDH. The data in *A–C* for Tg$^+$ IL-2Rβ$^{-/-}$ mice are from founder line 1.

Results

Development of transgenic mice with thymic expression of IL-2Rβ

Transgenic mice were developed in which mouse IL-2Rβ was expressed under the control of the thymic-specific proximal *lck* promoter. Three independent transgenic founders were bred onto the IL-2Rβ$^{-/-}$ genetic background. The expression and biological activity of transgenic IL-2Rβ were very similar for progeny mice derived from each founder.

The distribution of CD4 and CD8 thymic subsets in Tg$^+$ IL-2Rβ$^{-/-}$ mice was comparable to that in normal C57BL/6 animals or nontransgenic littermates (not shown), indicating that transgenic IL-2Rβ did not obviously alter thymic development. Cell surface IL-2Rβ was readily detected in most thymocytes obtained from Tg$^+$ IL-2Rβ$^{-/-}$ mice (Fig. 1*A*). The highest

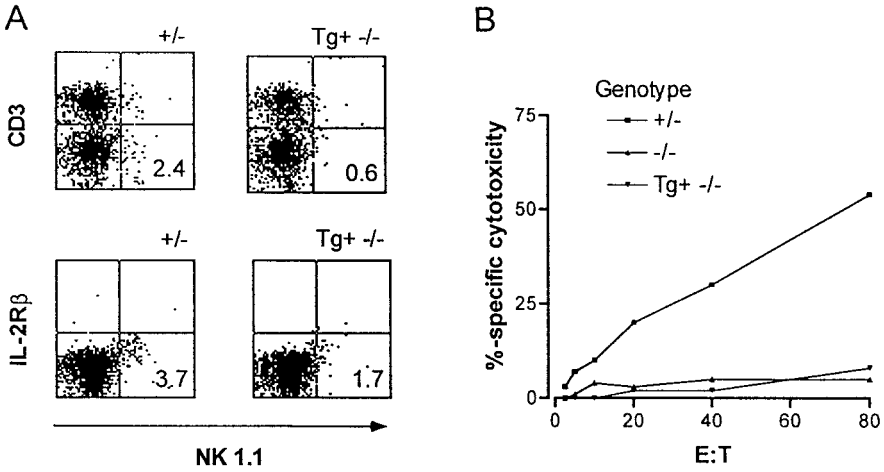

FIGURE 2. NK cell development and function in Tg^+ IL-2R$\beta^{-/-}$ mice. *A*, FACS analysis of NK cells. Spleen cells from mice of the indicated genotypes were stained with biotin-anti-NK1.1 and PE-streptavidin and either FITC-CD3 or FITC-IL-2Rβ. *B*, NK cell activity. Spleen cells of mice of the indicated genotypes from transgenic line 1 were cultured with IL-12 overnight, and NK activity was assessed using ^{51}Cr-labeled YAC-1 target cells. Data shown are representative of four experiments.

expression of transgenic IL-2Rβ was on the $CD4^+CD8^+$ subset, which approaches physiological levels, whereas a lower level was seen on $CD4^+$ and $CD8^+$ mature thymocytes (Fig. 1*B*). Transgenic IL-2Rβ expression was near background levels on the $CD4^-$ $CD8^-$ thymic subset. For the peripheral lymphoid compartment, IL-2Rβ was readily detected on a subset of $CD8^+$ T cells and a small fraction of $CD4^-$ $CD8^-$ spleen cells that corresponds to NK cells in normal mice (20) (see Fig. 2), while very low staining was seen for $CD4^+$ and $CD8^+$ splenic T cells from Tg^+ IL-2R$\beta^{-/-}$ mice (Fig. 1*A*). This low level of staining of the transgenic β-chain by peripheral T cells using this sensitive three-step staining procedure was at least 5- to 10-fold lower than that detected for $CD8^+$ T cells, NK cells, or activated T cells (see Fig. 10*B*) from normal mice.

To further evaluate the extent of transgenic IL-2Rβ expression in the lymphoid compartment, IL-2Rβ mRNA expression was examined by RT-PCR analysis (Fig. 1*C*). As expected, a PCR product was detected for IL-2Rβ mRNA from the spleen and thymus from IL-2R$\beta^{+/+}$ mice, as 2–6% of lymphoid cells in these tissues express IL-2Rβ (Fig. 1*A*). For mRNA from Tg^+ IL-2R$\beta^{-/-}$ mice, an obvious PCR product was amplified from the thymus, consistent with transgenic IL-2Rβ expression in most thymocytes, whereas a minimal band was seen for spleen and perhaps bone marrow. This result further confirms that transgenic IL-2Rβ is minimally expressed in peripheral T cells. Collectively, this pattern of transgenic mRNA and cell surface expression corresponds to the expected tissue-specific activity of the proximal *lck* promoter.

IL-2Rβ is required for the development and function of NK cells (21, 22). The expected number of NK cells was seen for IL-2R$\beta^{+/-}$ littermate spleen cells (Fig. 2*A*), which exhibited typical NK activity as assessed by lysis of YAC-1 targets (Fig. 2*B*). By contrast, splenic $NK1.1^+$ IL-2Rβ^+ NK cells were markedly reduced, and IL-12-induced NK activity was not detected in Tg^+ IL-2R$\beta^{-/-}$ mice. These data indicate that transgenic IL-2Rβ was not expressed at sufficient levels in bone marrow/pro-T cell precursors and/or the spleen to reconstitute NK development and function.

Normal homeostasis and absence of a lethal autoimmune syndrome in Tg^+ IL-2R$\beta^{-/-}$ mice

The wasting and autoimmune syndrome apparent in all young adult IL-2R$\beta^{-/-}$ mice was never seen in Tg^+ IL-2R$\beta^{-/-}$ mice, as reflected by their normal body weight (Fig. 3*A*), general lack of anti-nuclear autoantibodies (Fig. 3*B*), near normal levels of IgG1 (Fig. 3*C*), and unremarkable pathology (not shown). There was also no evidence of autoantibody-mediated hemolytic anemia in Tg^+ IL-2R$\beta^{-/-}$ mice (hematocrit: Tg^+ IL-2R$\beta^{-/-}$, 0.50 ± 0.02, $n = 6$; IL-2R$\beta^{-/-}$, 0.36 ± 0.06, $n = 12$; C57BL/6, 0.48 ± 0.01, $n = 2$).

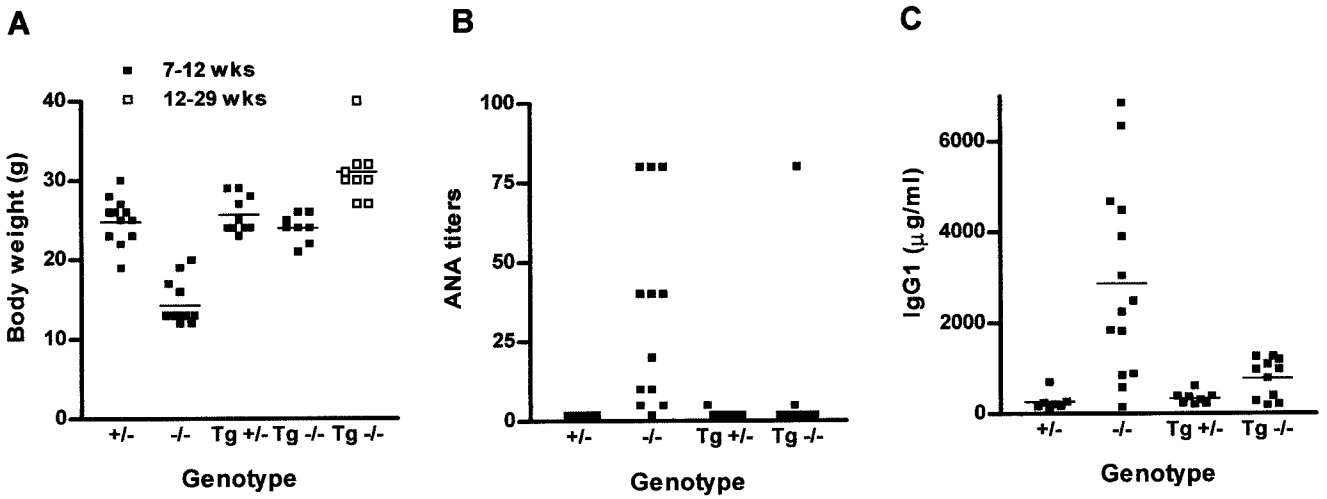

FIGURE 3. Lack of autoimmunity in Tg^+ IL-2R$\beta^{-/-}$ mice. *A*, Total body weight. The first four groups were from 7- to 12-wk-old mice, while the last group was from 12- to 29-wk-old mice. *B*, Autoantibody titers. *C*, Levels of serum IgG1.

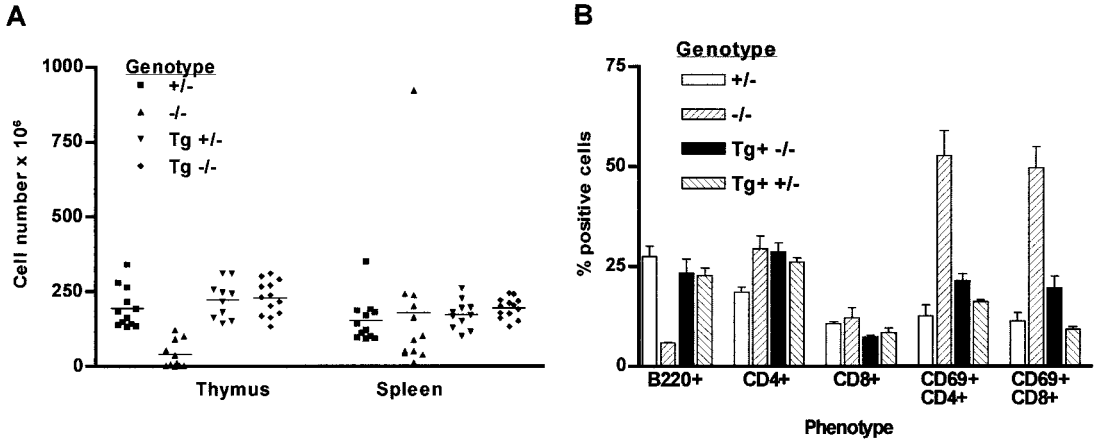

FIGURE 4. Normal lymphoid homeostasis in Tg^+ IL-2R$\beta^{-/-}$ mice. *A*, Viable cell recovery. Spleen and thymus cell number of 6- to 12-wk-old mice was determined by trypan dye exclusion. *B*, Peripheral phenotype of splenic lymphocytes determined by FACS analysis.

Thymus and spleen cellularity was essentially within normal levels for Tg^+ IL-2R$\beta^{+/-}$ and Tg^+ IL-2R$\beta^{-/-}$ mice (Fig. 4*A*). The splenomegaly seen in some IL-2R$\beta^{-/-}$ mice and the hypocellularity of the thymus and spleen observed in others at 8–12 wk of age, when these mice were severely compromised, were not observed in Tg^+ IL-2R$\beta^{-/-}$ mice. Unlike adult spleens of IL-2R$\beta^{-/-}$ mice, which are characterized by a low fraction of B cells and a high fraction of activated ($CD69^+$) T cells, both due to autoreactive T cell (2), the B cell ($B220^+$) and the T cell ($CD4^+$ and $CD8^+$) compositions of the Tg^+ IL-2R$\beta^{-/-}$ mice were essentially normal (Fig. 4*B*). These data indicate that Tg^+ IL-2R$\beta^{-/-}$ mice do not exhibit a major dysfunction in lymphoid homeostasis. Interestingly, Tg^+ IL-2R$\beta^{-/-}$ mice contained a statistically significant ($p < 0.05$) lower fraction of $CD8^+$ T cells compared with IL-2R$\beta^{+/-}$ littermate mice. Furthermore, the CD4:CD8 ratio was 2:1 for IL-2R$\beta^{+/-}$ littermates, 2.9:1 for Tg^+ IL-2R$\beta^{+/-}$ mice, and 3.9:1 for Tg^+ IL-2R$\beta^{-/-}$ mice. As IL-15 has been recently implicated in maintaining the survival of peripheral $CD8^+$ T cells (6, 13), this finding is probably due to lack of IL-2Rβ function, which is a component of the IL-15R, on peripheral $CD8^+$ T cells in Tg^+ IL-2R$\beta^{-/-}$ mice.

Transgenic IL-2Rβ does not support proliferative responses by mature thymocytes and peripheral T cells

One obvious explanation of the reversal in autoimmunity and impaired lymphoid homeostasis was that the low levels of transgenic IL-2Rβ in mature T cells was still sufficient to drive IL-2-dependent functional responses. To address this possibility, proliferation of transgenic thymocytes and peripheral T cells was assayed in vitro. Thymocytes from IL-2R$\beta^{+/-}$ Tg^- littermates readily proliferated when costimulated with either PMA (Fig. 5*A*) or soluble anti-CD3 (Fig. 5*B*) and IL-2 or IL-4. Both $CD4^-$ $CD8^-$ pro-T cells and $CD4^+$ and $CD8^+$ single-positive mature thymocytes are expected to proliferate in response to PMA and IL-2 (23). However, IL-2 should only costimulate the proliferation of mature thymocytes activated with soluble anti-CD3 (24–26). Importantly, the response patterns of IL-2R$\beta^{-/-}$ and Tg^+ IL-2R$\beta^{-/-}$ thymocytes was very comparable and characterized by the selective inability of IL-2 to efficiently costimulate proliferation in response to PMA (Fig. 5*A*) or anti-CD3 (Fig. 5*B*). This failure of IL-2 to costimulate was specific,

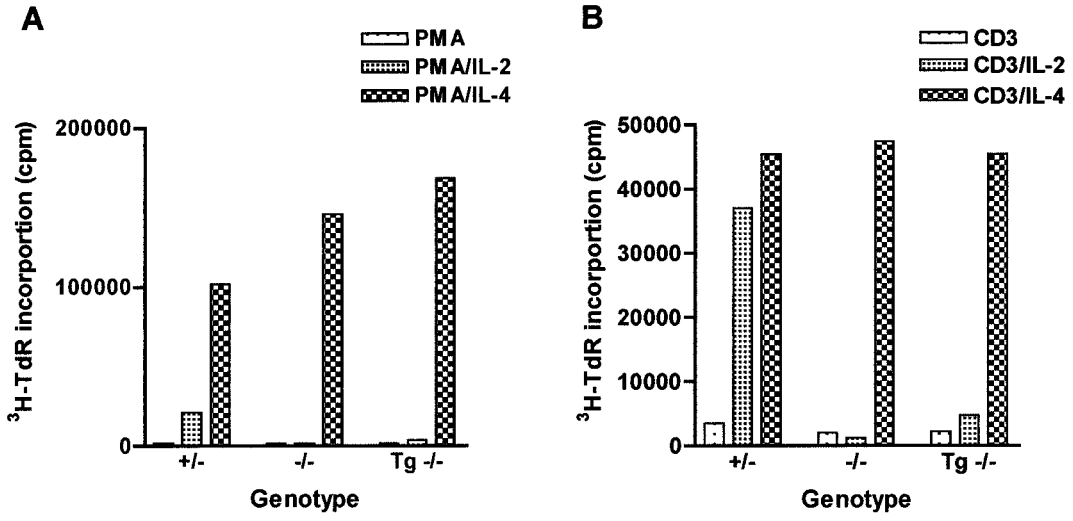

FIGURE 5. Proliferative responses of thymocytes from Tg^+ IL-2R$\beta^{-/-}$ mice. Thymocytes from mice of the indicated genotypes were cultured with PMA (*A*) or anti-CD3 (*B*) and IL-2 (50 U/ml) or IL-4 (50 U/ml). Data shown are representative of four to seven experiments.

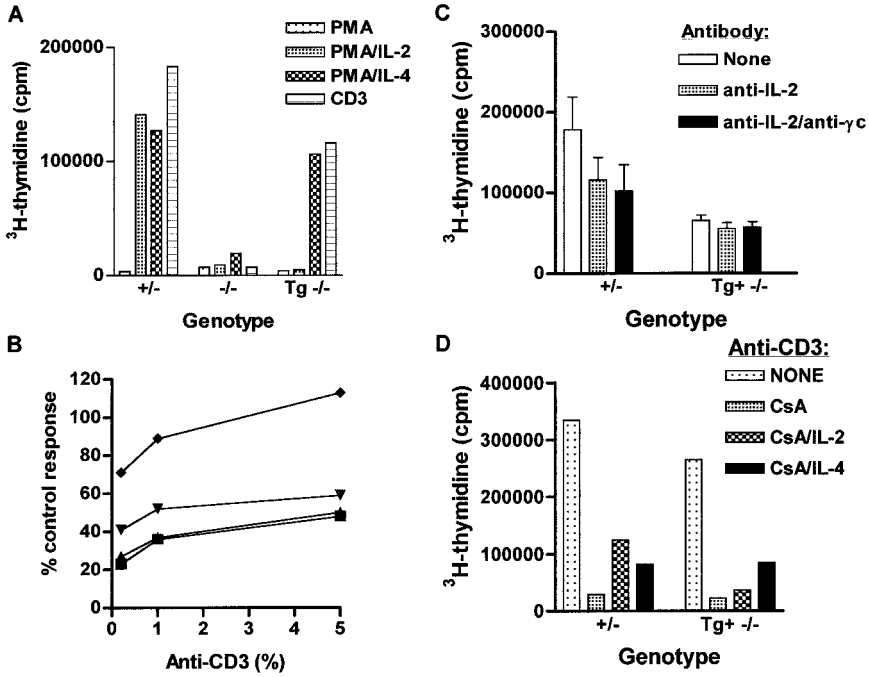

FIGURE 6. Proliferative responses of splenic T cells from Tg$^+$ IL-2R$\beta^{-/-}$ mice. *A*, Response to PMA and cytokine or anti-CD3. Data are representative of eight experiments. *B*, Dose-response to anti-CD3. Shown are the responses of four Tg$^+$ IL-2R$\beta^{-/-}$ mice to dilutions of anti-CD3. The proliferative response of the Tg$^+$ IL-2Rβ spleen cells was compared with that generated by IL-2R$\beta^{+/-}$ littermate cells, and the data are expressed as a percentage of the control response. *C*, Effect of anti-IL-2 on anti-CD3-induced proliferation. Data shown are the proliferation (mean ± SD) by four Tg$^+$ IL-2R$\beta^{-/-}$ and two IL-2R$\beta^{+/-}$ littermate mice. *D*, The effect of cyclosporin A (CsA) on anti-CD3-induced proliferation. Data are representative of three experiments. In all experiments, spleen cells (2×10^5/well) from the indicated mice were cultured for 48 h with anti-CD3 (5%), except in *C* (0.2% anti-CD3). PMA (10 ng/ml), CsA (100 ng/well), IL-2 (50 U/ml), IL-4 (50 U/ml), anti-IL-2 (10 μg/ml), or anti-γc (100 μg/ml each of 4G3 and 3E12) was added as indicated. Data shown are from transgenic line 1.

as IL-4 readily costimulated these responses by Tg$^+$ IL-2R$\beta^{-/-}$ thymocytes.

Peripheral T cells from IL-2R$\beta^{-/-}$ mice are extremely hyporesponsive upon stimulation with anti-CD3 or PMA and IL-4 (Fig. 6*A*) or even to PMA and ionomycin (2). As IL-2R$\beta^{-/-}$ thymocytes readily proliferated in response to PMA and IL-4 (Fig. 5*A*), these low responses by IL-2R$\beta^{-/-}$ T cells may be the result of an intrinsic defect due to chronic autoimmune stimulation rather than a direct failure of IL-2Rβ function. Consistent with this view, Tg$^+$ IL-2R$\beta^{-/-}$ splenic T cells readily responded to CD3 or to PMA and IL-4, albeit at a somewhat reduced level compared with control T cells (Fig. 6*A*). By contrast, IL-2 and PMA failed to costimulate proliferation by Tg$^+$ IL-2R$\beta^{-/-}$ splenic T lymphocytes (Fig. 6*A*). These minimal responses were even lower than those detected after culture of Tg$^+$ IL-2R$\beta^{-/-}$ thymocytes with PMA and IL-2 (Fig. 5*A*).

Dose-response studies of anti-CD3-induced proliferation of spleen cells from four Tg$^+$ IL-2R$\beta^{-/-}$ mice indicate that at a high concentration of anti-CD3 near normal proliferation occasionally occurs, but at a lower concentration the response is always suboptimal (Fig. 6*B*). These suboptimal responses are consistent with a failure in IL-2R signaling, as IL-2 is an important growth factor for TCR-activated T cells. The anti-CD3-induced proliferation by Tg$^+$ IL-2R$\beta^{-/-}$ T cells was not substantially blocked by anti-IL-2 (16.4 ± 6.0% inhibition) or by the mixture of anti-IL-2 and anti-γc (13.3 ± 10.3% inhibition), while these same Abs blocked (anti-IL-2, 35.0 ± 1.1% inhibition; anti-IL-2/anti-γc, 43.9 ± 8.1% inhibition) the more potent anti-CD3-induced proliferation by IL-2R$\beta^{+/-}$ littermate T cells (Fig. 6*C*). We believe it noteworthy that the levels of inhibition by anti-IL-2 and the mixture of anti-IL-2 and anti-γc were similar, as the combination of these Abs is 5- to 10-fold more effective in inhibiting the bioactivity of IL-2 than only anti-IL-2 (data not shown). This finding suggests the residual anti-CD3-induced proliferation is largely independent of IL-2 and is not a failure of the inhibitory Abs. Furthermore, cyclosporin A, a potent inhibitor of T cell cytokine production, effectively inhibited the anti-CD3-induced proliferation by Tg$^+$ IL-2R$\beta^{-/-}$ and control T cells (Fig. 6*D*). The addition of exogenous IL-2 to these cultures only substantially restored the proliferative response by control cells. Exogenous IL-4, on the other hand, was equally effective for both groups of T cells. These latter data also indicate that the anti-CD3-induced proliferation by Tg$^+$ IL-2R$\beta^{-/-}$ T cells was largely independent of IL-2/IL-2R.Thus, this partial restoration of signaling through the TCR represents another abnormality associated with IL-2R$\beta^{-/-}$ mice that was repaired in Tg$^+$ IL-2R$\beta^{-/-}$ mice.

Table I summarizes the proliferative responses of thymocytes and spleen cells from individual Tg$^+$ IL-2R$\beta^{-/-}$ mice from all three founder lines as a percentage of the control response. Founder line 1 showed the lowest responses. These data highlight the consistent inability of IL-2 to efficiently costimulate proliferation from each transgenic line, including young (3-wk-old) and old (15- to 16-mo-old) mice. The lack of IL-2 function by T cells from old mice indicated that impaired IL-2 function is long lasting. These older mice were also outwardly healthy, lacked systemic autoimmunity, and did not exhibit consistent abnormalities in lymphoid homeostasis (not shown). These data indicate that these transgenic animals exhibit a life-long defect in the peripheral T responsiveness to IL-2, which is apparent early in life. Furthermore, the addition of exogenous IL-2 to spleen cells or purified T cells from Tg$^+$ IL-2R$\beta^{-/-}$ mice did not induce proliferation in short term cultures or cause activation and expansion of LAK cells in long term (7- to 10-day) cultures (data not shown). Collectively, all these data indicate that the level of IL-2Rβ on Tg$^+$ IL-2R$\beta^{-/-}$ mature thymocytes and splenic T cells is not sufficient to support proliferative signals by the vast majority of cells. Furthermore, these defects in proliferation cannot simply be attributed to the heightened expression of transgenic IL-2Rβ in the thymus, as these type of proliferative responses by Tg$^+$ IL-2R$\beta^{+/-}$ T cells were always comparable to those by Tg$^-$ IL-2R$\beta^{+/-}$ littermates (data not shown).

Tg$^+$ IL-2R$\beta^{-/-}$ T cells fail to up-regulate IL-2R and express CTL activity

Another and relatively early function of IL-2 is to up-regulate IL-2Rα expression after its initial induction by signaling through

Table I. *Proliferative responses costimulated by IL-2 for Tg^+ IL-2R$\beta^{-/-}$ thymocytes and spleen cells*

Cell Type	Stimulant	Age	% of Control[a] Tg line 1	Tg line 2	Tg line 3
Thymocytes	PMA/IL-2	2–4 mo	3.6 ($n = 7$)	6.0 ($n = 2$)	13.6 ($n = 3$)
		3 wk	11.5 ($n = 2$)		
		15–16 mo	4.2 ($n = 4$)		
	Anti-CD3/IL-2	2–4 mo	6.3 ($n = 4$)		
		3 wk	13.0 ($n = 2$)		
		15–16 mo	5.4 ($n = 4$)		
Spleen	PMA/IL-2	2–4 mo	1.8 ($n = 8$)	2.1 ($n = 3$)	13.0 ($n = 3$)
		3 wk	6.5 ($n = 2$)		
		15–16 mo	1.0 ($n = 4$)		

[a] The % of control responses was calculated by the formula: % control = {[(cpm PMA or anti-CD3 plus IL-2)$_{Tg+IL2R\beta^{-/-}}$ − (cpm PMA or IL-2)$_{Tg+IL2R\beta^{-/-}}$]/[(cpm PMA or anti-CD3 plus IL-2)$_{control}$ − (cpm PMA or IL-2)$_{control}$]} × 100.

the TCR (27). Such IL-2Rα up-regulation was not observed for Tg^+ IL-2R$\beta^{-/-}$ T cells. Compared with control anti-CD3-induced T blasts, they expressed almost 4-fold lower cell surface IL-2Rα, while the levels of CD69 remained equivalent (Fig. 7). Furthermore, compared with T cells from control IL-2R$\beta^{+/-}$ and Tg^+ IL-2R$\beta^{+/-}$ mice, Tg^+ IL-2R$\beta^{-/-}$ T cells showed substantial impairment to generate allo-specific CTL (Fig. 8*A*) or anti-CD3-induced CTL (Fig. 8*B*) as measured by redirected lysis of P815 targets. Northern blot analysis revealed that granzyme B mRNA that functions in CTL was barely induced in Tg^+ IL-2R$\beta^{-/-}$ T cells compared with that in control cells (Fig. 8*C*) These data indicate that mature T lymphocytes in Tg^+ IL-2R$\beta^{-/-}$ mice do not express sufficient levels of IL-2Rβ to support a normal functional response and demonstrate an important role for the IL-2R to induce the granzyme B gene.

IL-2Rβ expression and function by activated Tg^+ IL-2Rβ$^{-/-}$ T cells

We further explored IL-2Rβ expression and function of the resulting anti-CD3-induced T blasts. IL-2Rβ mRNA was not detected for T blasts generated from Tg^+ IL-2R$\beta^{-/-}$ mice by Northern blot analysis in marked contrast to normal mice (Fig. 9*A*). Furthermore, the T cell blasts from Tg^+ IL-2R$\beta^{-/-}$ spleen cells or thymocytes essentially failed to bind ^{125}I-labeled IL-2 compared with control cells (Fig. 9*B*). These data further illustrate the marked impairment of transgenic IL-2Rβ expression in mature T cells, including the lack of cell surface IL-2R. The Tg^+ IL-2R$\beta^{-/-}$ anti-CD3-induced spleen cells minimally proliferated in response to exogenous IL-2

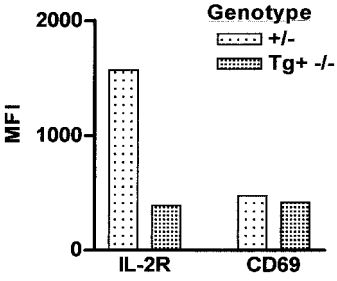

FIGURE 7. IL-2Rα is not up-regulated on Tg^+ IL-2R$\beta^{-/-}$ T cells. Spleen cells of the indicated genotype were stimulated with anti-CD3 for 48 h and subjected to FACS analysis. Data shown are the mean fluorescent intensity (MFI) after staining with biotin-anti-IL-2Rα or biotin-anti-CD69 and PE-streptavidin, which stains essentially all these T blasts. For control stained cells, the MFI was <20. Data shown are representative of three experiments.

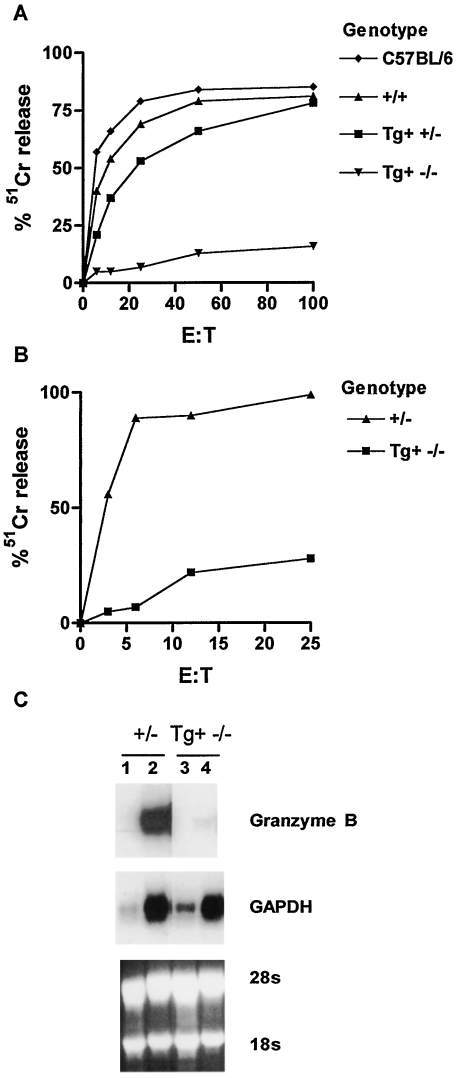

FIGURE 8. CTL activity in vitro by peripheral T cells from Tg^+ IL-2R$\beta^{-/-}$ mice. *A*, Allogeneic CTL responses. A standard 4-h ^{51}Cr assay was performed with P815 ($H2^d$) (shown) or EL4 ($H2^b$) (not shown) targets. No CTL activity was seen on the syngeneic EL4 targets. *B*, Redirected lysis of P815 targets. CTL activity was assessed using ^{51}Cr-labeled P815 targets. *C*, Expression of granzyme B mRNA by anti-CD3-activated T cells. Total RNA was isolated, as indicated, from normal spleen (*lanes 1* and *3*) or anti-CD3-stimulated spleen (*lanes 2* and *4*) and subjected to Northern blotting by probing for granzyme B or GAPDH mRNA. The levels of 28S and 18S ribosomal RNA are shown to further verify levels of mRNA per lane.

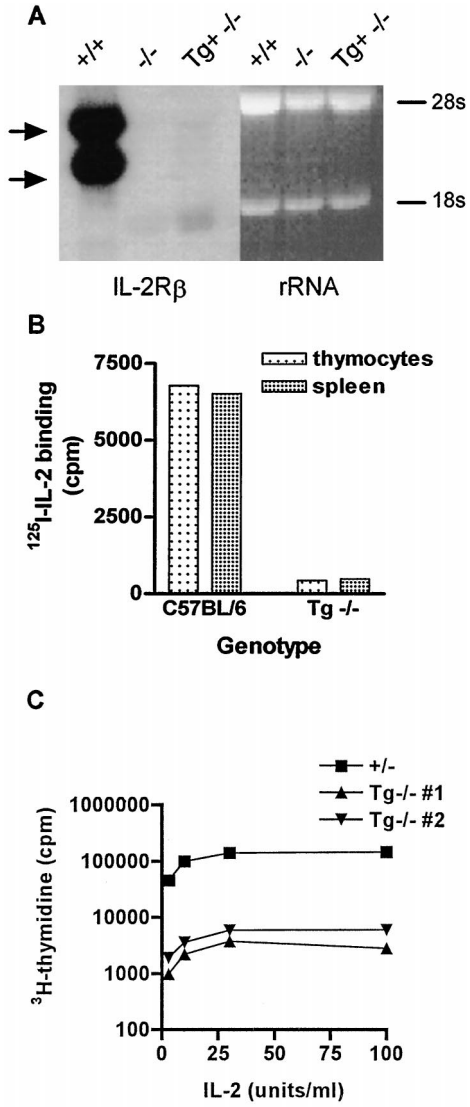

FIGURE 9. IL-2R expression and cytokine-induced functional activity of activated T cells from Tg^+ IL-2R$\beta^{-/-}$ mice. Spleen cells and thymocytes, as shown, were cultured with anti-CD3 for 48 h and then subjected to various assays. *A*, Northern blot analysis of total RNA of splenic T blasts for IL-2Rβ. Assessment of 28S and 18S ribosomal RNA served as controls for equal loading of each lane. *B*, ^{125}I-IL-2 binding to anti-CD3-induced blasts. Specific binding was assessed by cold competition using 500 ng of unlabeled IL-2 and is subtracted from the values shown. Exogenous IL-2 was added during the 48-h culture of thymocytes with anti-CD3. *C*, Response of anti-CD3-induced T blasts to IL-2. Anti-CD3-induced T blasts were washed three times and recultured with IL-2 for 24 h. [^{3}H]Thymidine was added during the last 6 h of culture.

(Fig. 9*C*) and IL-15 (not shown). In seven experiments with Tg line 1, the response to IL-2 was, on the average, 3% that of control cells, which is comparable to results shown in Table I for IL-2-responsive thymocytes and splenic T cells. This minimal response exhibited identical dose-response characteristic as control cells. This finding indicates that the poor proliferation by the Tg^+ IL-2R$\beta^{-/-}$ T blasts is the result of a low number of cells responding normally to IL-2 rather than an abnormal IL-2-induced response.

In vivo activated T cells from Tg^+ IL-2R$\beta^{-/-}$ mice are unresponsive to IL-2

We considered the possibility that the expression of functional IL-2Rβ transgene in vivo might not completely parallel that in vitro, with perhaps more vigorous IL-2 responsiveness by in vivo activated T cells. To that end, we compared the capacity of IL-2 to induce proliferation of T cells after injection of anti-CD3 in vivo (Fig. 10*A*). Compared with control animals, Tg^+ IL-2R$\beta^{-/-}$ lymph node T cells were extremely hyporesponsive to IL-2. As a control for anti-CD3 T cell activation in vivo, normal lymph node cells from untreated C57BL/6 mice generated much more modest proliferative responses to IL-2 or IL-4. Furthermore, anti-CD3 induced relatively high levels of expression of IL-2Rβ on both CD4 and CD8 T cells from IL-2R$\beta^{+/-}$ littermates (Fig. 10*B*) and Tg^+ IL-2R$\beta^{+/-}$ mice (not shown), whereas IL-2Rβ was not detected on cells from Tg^+ IL-2R$\beta^{-/-}$ mice. These data clearly indicate that there is not some compensatory mechanism in vivo that might promote transgenic IL-2Rβ expression and function in peripheral T lymphocytes.

Discussion

Our results indicate that targeting IL-2Rβ expression to the thymus is sufficient to prevent the severe autoimmunity and imbalance in peripheral lymphoid homeostasis that has been associated with IL-2Rβ-deficient mice. This finding raises two fundamental issues with respect to the function of IL-2Rβ in the regulation of the immune system. First, these results are consistent with the idea that the severe imbalance in peripheral lymphoid homeostasis and lethal autoimmunity in IL-2R$\beta^{-/-}$ mice is independent of a functional IL-2R β-chain in mature T lymphocytes. Second, the data reported herein raise the possibility that IL-2R β-chain signaling within the thymus contributes to the regulation of self-reactivity and homeostasis.

The above interpretation of our data is critically dependent upon the extent to which mature T cells in the Tg^+ IL-2R$\beta^{-/-}$ mice are IL-2 nonresponsive. Although the proximal *lck* promoter used to develop this transgenic model is highly active in the thymus, especially at the $CD4^+CD8^+$ stage of development, it sometimes is not entirely shut down in the mature T cell compartment. We observed that transgenic IL-2Rβ expression was readily detectable in $CD4^+CD8^+$ thymocytes in all three transgenic founder lines and at a level comparable to that found on IL-2Rβ+ thymocytes from normal mice. In contrast, minimal IL-2Rβ mRNA and cell surface protein were seen in mature resting and recently activated T cells. Compared with cell populations that normally respond to IL-2, such as $CD8^+$ T cells, NK cells, or activated T lymphocytes, the level of expression of IL-2Rβ by Tg^+ IL-2$\beta^{-/-}$ peripheral T cells was 5- to 10-fold lower. It is noteworthy that IL-2Rβ cell surface expression was detected at a lower level in $CD4^+$ and $CD8^+$ single-positive thymocytes compared with $CD4^+$ $CD8^+$ cells, indicating that the *lck* promoter must be quickly down-regulated during thymic development. This activity in conjunction with the short half-life of ~1–2 h for cell surface IL-2Rβ (28) probably contributes to the establishment of a peripheral T cell pool with minimal IL-2Rβ expression. Most importantly, we must emphasize that this low expression was not functionally relevant. In the Tg^+ IL-2R$\beta^{-/-}$ mice, the defect in T lymphocyte IL-2Rβ function was profound (approximately only 3% of the control responses in the least responsive founder line) and extended to the capacity of IL-2 to costimulate proliferative responses by thymocytes and peripheral T cells, to directly stimulate proliferation by activated T cells primed by anti-CD3 in vitro or in vivo, to up-regulate IL-2Rα expression, to induce CTL, and to regulate the production and activity of NK cells. The minimal induction of granzyme B mRNA by anti-CD3-induced Tg^+ IL-2R$^{-/-}$ is consistent with the lack of cytolytic activity and indicates an important role for IL-2Rβ signaling for the regulation of this mRNA. Thus, the defect in IL-2Rβ

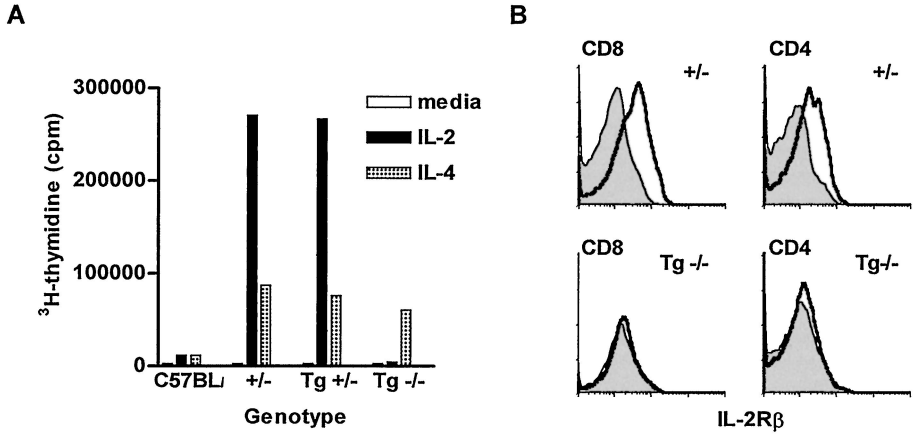

FIGURE 10. IL-2Rβ function and expression after in vivo activation. Mice of the indicated genotype were injected i.p. with 100 μg/ml anti-CD3. Forty-eight hours later, lymph node cells were collected. *A*, IL-2 responsiveness. Cells (2×10^5/well) were cultured with IL-2 or IL-4 for 48 h. [^{3}H]Thymidine was added during the last 6 h of culture. The responses of normal C57BL/6 lymph node cells were from a mouse not treated with anti-CD3. Data are representative of three experiments. *B*, FACS analysis of IL-2Rβ expression. Lymph node cells were subjected to three-color FACS for IL-2Rβ, CD4, and CD8 using 5H4 followed by biotin-anti-rat Ig/PE-streptavidin, FITC-anti-CD4, and Cy-Chrome-anti-CD8 and were gated for the indicated subpopulation. The shaded histogram represents control stained cells.

function extends to multiple activities controlled by the IL-2R. Therefore, it seems highly unlikely that transgenic IL-2Rβ expression prevented autoimmunity due to this minimal expression of IL-2Rβ. Our data, however, cannot rule out the possibility that IL-2R signals were induced in T cells from these transgenic mice that prevented the autoimmune syndrome while being insufficient for IL-2-induced proliferation and differentiation.

It has been hypothesized that the severe autoimmunity and peripheral T cell dysfunction associated with IL-2/IL-2R deficiency are due in part to a breakdown in peripheral tolerance due to the lack of IL-2 signaling (7). Therefore, it was somewhat surprising that Tg^+ IL-2R$\beta^{-/-}$ mice showed minimal dysfunction in peripheral T cell homeostasis and lacked autoantibodies, considering the impaired IL-2 responsiveness by mature T cells in these mice. Based on the current models by which IL-2 mediates activation-induced cell death, it is thought that T cells must be stimulated through the TCR and then respond to IL-2 for several days to be sensitized for apoptosis. Yet, our data show quite clearly that IL-2 was unable to generate this type of a response in vitro and in vivo for T lymphocytes from Tg^+ IL-2R$\beta^{-/-}$ mice. We considered the possibility that autoreactive T cells in Tg^+ IL-2R$\beta^{-/-}$ mice seeded the periphery and were then deleted by an IL-2-mediated process, leaving a peripheral T cell compartment devoid of T cells with a functional IL-2Rβ subunit. Although it is difficult to completely rule out this scenario, it seems highly unlikely for several reasons. First, the peripheral T cells in both young (3-wk-old) and old adult (15- to 16-mo-old) mice were essentially unable to generate biological responses to IL-2. This finding demonstrates a life-long impairment in IL-2Rβ function by peripheral T cells without pathological consequences, while potentially autoreactive T cell are expected to continually emerge in the secondary lymphoid compartment. Second, and more importantly, IL-2 was unable to efficiently costimulate anti-CD3-induced proliferation by mature thymocytes. These data indicate that the functional unresponsiveness to IL-2 occurred before thymic T cell seeding of the peripheral lymphoid tissue.

All Tg^+ IL-2R$\beta^{-/-}$ mice were outwardly healthy and rarely contained detectable autoantibodies. The occasional animal with mild inflammation and autoantibodies might reflect a breakdown in peripheral tolerance. The fact that Tg^+ IL-2R$\beta^{-/-}$ mice have a slight elevation in the fraction of activated $CD69^+$ T cells and a somewhat elevated level of IgG1 is consistent with modest immune stimulation. Nevertheless, the infrequency of Tg^+ IL-2R$\beta^{-/-}$ mice with any pathology suggests that IL-2Rβ function by mature T cells is largely dispensable for peripheral tolerance, perhaps because this function may be redundant with other γc-dependent cytokines. In this regard it is important to note that IL-4 and IL-7 also sensitize mature T cells to apoptosis upon subsequent stimulation through the TCR (29, 30).

Other studies have also employed the proximal *lck* promoter to selectively drive Jak-3 expression in the thymus of Jak-$3^{-/-}$ mice, leading to repair of T cell development while the peripheral T cell compartment exhibited impaired Jak-3 function (31, 32). Unlike our transgenic model, these Jak-3 transgenic mice exhibited a severe impairment in homeostasis of the peripheral lymphoid compartment, even though deficiency in Jak-3 activity was not observed until the mice were 4–5 wk of age. We believe that this finding is noteworthy, as 3-wk-old Tg^+ IL-2R$\beta^{-/-}$ mice already exhibited marked impairment of IL-2 function by peripheral T cells and, therefore, are predicted to exhibit similar problems in homeostasis, as seen in the Jak-3 transgenic mice, if this dysfunction was primarily the result of failed IL-2/IL-2R signaling. Thus, the relatively normal peripheral lymphoid homeostasis in the Tg^+ IL-2R$\beta^{-/-}$ mice may be explained by redundancy of γc-dependent cytokines, which all use Jak-3. Furthermore, mice with selectively impaired Jak-3 in peripheral T lymphocytes were not reported to exhibit lethal autoimmunity. This finding is similar to our IL-2Rβ transgenic model and suggests that the regulation of the autoimmune phenotype is independent of Jak-3-dependent signaling by peripheral T cells.

The signaling pathway by which IL-2Rβ prevents autoimmunity is still not resolved. The imbalance in homeostasis in IL-2R$\beta^{-/-}$ mice was cured after expression of mutant transgenic IL-2R β-chains lacking the A or H domains of the IL-2Rβ cytoplasmic region in their T cells, suggesting that this function of IL-2R may be independent of STAT5, $p56^{lck}$, or any other signaling pathway emerging from these cytoplasmic domains (33). As this study developed transgenic mice using the CD2 promoter, which is highly expressed in the thymus and peripheral T cells, it was not apparent that the prevention of autoimmunity may be independent of IL-2Rβ function by peripheral T cells. Furthermore, IL-2-dependent proliferation was noted in primary T cells that expressed

IL-2Rβ with mutations in either the A or H domain of the cytoplasmic tail (33, 34). Thus, the absence of lethal disease occurred while the mutant IL-2R remained competent to generate proliferative signals. By comparison, peripheral T cells from our Tg^+ IL-2R$\beta^{-/-}$ mice showed an almost absolute defect in IL-2-dependent proliferation while also failing to develop autoimmunity. This observation suggests that the autoimmune syndrome associated with IL-2Rβ-deficient mice is either independent of IL-2R signals, resulting in T cell proliferation, or such signals are stimulated at a cellular level distinct from the mature peripheral T cell compartment.

If the lethal syndrome associated with IL-2R$\beta^{-/-}$ mice is not the result of failed IL-2R function in peripheral T cells, what accounts for the lack of disease? One potential trivial explanation is that transgenic IL-2Rβ is expressed on a much higher fraction of thymocytes, which might alter thymic development such that the peripheral T cell pool is now populated by one that is largely IL-2 nonresponsive and nonautoimmune. This possibility seems extremely remote, as a similar phenotype is then predicted for Tg^+ IL-2R$\beta^{+/-}$ mice, yet these transgenic littermates behaved in virtually all instances in a manner identical with Tg^- IL-2R$\beta^{+/-}$ or C57BL/6 control mice rather than Tg^+ IL-2R$\beta^{-/-}$ mice. The lymphocytes from Tg^+ IL-2R$\beta^{+/-}$ normally proliferated in response to IL-2 in all assays, including those from anti-CD3-treated mice, generated high levels of allo-specific CTL and anti-CD3-induced redirected CTL, and contained functional NK cells. The only abnormality that we have noted to date for the Tg^+ IL-2R$\beta^{+/-}$ mice is a somewhat higher ratio of peripheral CD4:CD8 T cells.

The alternative explanation of our findings is that IL-2Rβ functions within the thymus to regulate the development of at least some $CD4^+$ T cells to prevent self-reactivity and imbalanced peripheral lymphoid homeostasis. We currently favor this hypothesis, as the thymus is the only lymphoid organ where the level of transgenic IL-2Rβ approached normal. Analysis of $CD4^+$ T cells from IL-2R$\beta^{-/-}$ mice indicates that this subset of T cells is primarily responsible for the induction of the severe autoimmunity associated with these mice (2, 21). This function of IL-2Rβ most likely reflects a requirement for IL-2R signaling, as autoimmunity has also been associated with IL-2- and IL-2Rα-deficient mice (3–5), but not IL-15R-deficient animals (6). Furthermore, the severity and onset of autoimmunity in IL-$2^{-/-}$ mice have been shown to largely parallel those described for IL-2Rβ deficiency. This includes a dependency upon thymus-derived T cells and subsequent uncontrolled activation and proliferation of $CD4^+$ cells (4, 35). However, IL-2 is not essential for the production of thymocytes, as the initial thymic cellularity and CD4 and CD8 subset distribution are essentially normal in IL-$2^{-/-}$, IL-2R$\alpha^{-/-}$, and IL-2R$\beta^{-/-}$ mice (2, 5, 36).

Several lines of evidence are consistent with a potential role for IL-2Rβ in thymic function. Self-recognition within the thymus has been shown to induce IL-2Rβ on TCR^{int} $CD8^{low/-}$ $CD4^+$cells, a subset of thymocytes that is undergoing selection and is near maturation to $CD4^+$ or $CD8^+$ thymocytes (11). In addition, thymic negative selection for some endogenous mouse mammary tumor virus superantigens on the BALB/c background was impaired in mice that lacked $\gamma c^{-/-}$ and Jak-$3^{-/-}$ (37, 38), proteins that participate in IL-2Rβ signaling. On the other hand, thymic IL-2Rβ may not be directly involved in thymic selection but, rather, may contribute to the development of a critical regulatory T cell subset, such as the recently described $CD4^+$ T cells that function to inhibit several distinct organ-specific autoimmune diseases (39, 40). Our current work is aimed at distinguishing between these possibilities.

In summary, this study indicates that the severe pathological abnormalities associated with IL-2Rβ-deficient mice are independent of IL-2Rβ function by peripheral T cells. Our data are consistent with the idea that the nonredundant function of IL-2/IL-2R lies at the level of the thymus. Regardless of the precise mechanism by which transgenic expression reversed this disease process, the Tg^+ IL-2R$\beta^{-/-}$ mice represent an animal model to study the function of IL-2Rβ in peripheral T cells without the complication of disease.

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