A Requirement for IL-2/IL-2 Receptor Signaling in Intrathymic Negative Selection

Hamid Bassiri and Simon R. Carding

*J Immunol* 2001; 166:5945-5954; doi: 10.4049/jimmunol.166.10.5945

http://www.jimmunol.org/content/166/10/5945

---

**References**
This article cites 49 articles, 27 of which you can access for free at:
http://www.jimmunol.org/content/166/10/5945.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
A Requirement for IL-2/IL-2 Receptor Signaling in Intrathymic Negative Selection

Hamid Bassiri and Simon R. Carding

The nature of the signals that influence thymocyte selection and determine the fate of CD4$^+$8$^+$ (double positive) thymocytes remains unclear. Cytokines produced locally in the thymus may modulate signals delivered by TCR-MHC/peptide interactions and thereby influence the fate of double-positive thymocytes. Because the IL-2/IL-2R signaling pathway has been implicated in thymocyte and peripheral T cell survival, we investigated the possibility that IL-2/IL-2R interactions contribute to the deletion of self-reactive, Ag-specific thymocytes. By using nontransgenic and transgenic IL-2-sufficient and -deficient animal model systems, we have shown that during TCR-mediated thymocyte apoptosis, IL-2 protein is expressed in situ in the thymus, and apoptotic thymocytes up-regulate expression of IL-2Rs. IL-2R$^+$ double-positive and CD4 single-positive thymocytes undergoing activation-induced cell death bind and internalize IL-2. IL-2-deficient thymocytes are resistant to TCR/CD3-mediated apoptotic death, which is overcome by providing exogenous IL-2 to IL-2$^{-/-}$ mice. Furthermore, disruption or blockade of IL-2/IL-2R interactions in vivo during Ag-mediated selection rescues some MHC class II-restricted thymocytes from apoptosis. Collectively, these findings provide evidence for the direct involvement of the IL-2/IL-2R signaling pathway in the deletion of Ag-specific thymocyte populations and suggest that CD4 T cell hyperplasia and autoimmunity in IL-2$^{-/-}$ mice is a consequence of ineffective deletion of self-reactive T cells. The Journal of Immunology, 2001, 166: 5945–5954.

H_thymocytes that mature to the double-positive (DP; CD4$^+$8$^+$) stage and begin to express a productively rearranged TCR$\alpha\beta$ on their cell surface become susceptible to repertoire selection. Of these, thymocytes that bear TCRs that interact weakly with self-MHC/peptide complexes are positively selected, whereas those reacting with high affinity are negatively selected and either die apoptotically in situ (clonal deletion) or are induced into a state of nonresponsiveness (anergy) (1). It is not clear how the strength of these MHC/TCR interactions results in differential death or survival signals. The number of TCRs that are engaged during the intrathymic selection process and the longevity of this interaction are thought to be important factors influencing the outcome of T cell selection (2–4). The possibility that quantitative or qualitative differences in intracellular signaling pathways may influence the fate of DP thymocytes also has received much attention (5). Several studies have identified differentially expressed signaling molecules and pathways in thymocytes undergoing positive vs negative selection (6–10), and abnormal thymocyte selection has been reported in mice with mutations in specific signaling molecules (11–13). By contrast, the extrinsic signals that lead to the initiation of intracellular signaling cascades and ultimately determine the fate of developing thymocytes still remain to be fully elucidated.

One manner in which signals delivered by TCR-MHC/peptide interactions could be modulated is by the action of cytokines produced by the thymic stroma or by thymocytes themselves. Cross-linking of TCRs on DP thymocytes induces the production of various cytokines (14) and numerous studies have shown that cytokines such as IL-7 and IL-2 can profoundly affect thymocyte growth and survival (15). However, the precise role of IL-2/IL-2R signaling in thymocyte development remains controversial due to apparently contradictory studies providing evidence for both the promotion and inhibition of thymocyte growth by this signaling pathway (16). The normal generation of T cells reported in IL-2-deficient (IL-2$^{-/-}$) mice (17) seems to be inconsistent with a direct involvement of IL-2/IL-2R interactions in intrathymic T cell development. However, the presence of dysregulated thymically derived pathogenic T cells (18) and CD4 T cell-mediated autoimmunity (19, 20) in IL-2$^{-/-}$ mice suggests that IL-2 could be involved in thymic selection. Indeed, the lymphoid hyperplasia and autoimmunity present in gnotobiotic (germfree) IL-2$^{-/-}$ mice is consistent with a defect in central tolerance leading to uncontrolled T cell responses directed against self- rather than environmental Ags (21). The impaired ability of thymocytes in IL-2$^{-/-}$ mice to undergo anti-CD3 Ab-mediated apoptosis (22) and the presence of autoimmune disorders and developmental defects in IL-2Ra$^{-/-}$ (CD25$^{-/-}$; Refs. 23 and 24), IL-2Rb$^{-/-}$ (CD122$^{-/-}$; Ref. 25), and Jak3$^{-/-}$ (26–28) mice similar to that seen in IL-2$^{-/-}$ mice agree with this interpretation. The observation that thymic expression of a CD122 transgene in CD122-deficient mice prevents lethal autoimmunity (29) further emphasizes the importance of IL-2/IL-2R interactions in the establishment and maintenance of thymic tolerance. Yet, the finding that IL-2 deficiency has no effect...
on the deletion of self-reactive thymocytes in MHC class I-restricted TCR-transgenic mice (30) suggests that IL-2/IL-2R interactions may be restricted to or used specifically for the selection of MHC class II-restricted thymocytes. Although collectively these observations provide a compelling argument for the involvement of IL-2/IL-2R signaling in T cell development, evidence for IL-2/IL-2R interactions being directly involved in thymocyte selection is lacking.

To address this issue, we have reexamined the involvement of IL-2 in thymocyte development and tested the hypothesis that IL-2/IL-2R interactions play a role in the deletion of self-reactive, Ag-specific thymocytes. By using nontransgenic and transgenic IL-2-sufficient and -deficient animal model systems, our studies show that IL-2 is expressed in situ in response to TCR engagement and is subsequently bound and internalized by apoptotic thymocytes expressing high-affinity IL-2Rs. We also demonstrate that disruption or blockade of IL-2/IL-2R interactions rescues some MHC class II-restricted thymocytes from Ag-induced apoptosis.

**Materials and Methods**

**Mice and treatments**

Mice used in these experiments were maintained as specific pathogen-free (SPF) or germfree colonies at the University of Pennsylvania (Philadelphia, PA). Germfree C57Bl/6-IL-2/- mice were housed in isolator cages within a flexible film isolator in the gnotobiotic facility of the Biology Department, University of Pennsylvania. The germfree status of IL-2/- mice, IL-2/-/-, and IL-2/-/- animals was verified by bacteriologic, histologic, and serologic analysis of tissues and fluids at autopsy as described previously (21). IL-2/-/- mice on the BALB/c background were purchased from The Jackson Laboratory (Bar Harbor, ME) and were mated with H-2b DO11.10 (Jackson Laboratory [Bar Harbor, ME]) and were mated with H-2b H-Y (Jackson Laboratory [Bar Harbor, ME]) mice. Similarly, IL-2/-/- mice on the C57Bl/6 background were mated with H-2k H-Y TCR-transgenic mice to obtain H-Y × IL-2/-/- mice. IL-2/-/- mice were identified from samples of tail DNA by PCR (17). Heterozygote and/or wild-type littermates were used as controls. Presence of TCR transgenes were confirmed by flow cytometric detection of transgene-encoded TCR on PBL, lymph node cells, splenocytes, or thymocytes. Use of laboratory animals conformed to institutional and National Institutes of Health (Bethesda, MD) guidelines.

For the induction of thymocyte apoptosis in vivo, mice were injected i.p. with 50 μg of anti-CD3 Ab (145-2C11, 500/A2, or 29B), 0.1 μg/g of recombinant human IL-2 (Proleukin; Chiron, Emeryville, CA), anti-CD3 plus IL-2, or PBS, and 12 h later, thymus were removed. For corticosteroid-sufficient and -deficient animal model systems, our studies show that IL-2 is expressed in situ in response to TCR engagement and is subsequently bound and internalized by apoptotic thymocytes expressing high-affinity IL-2Rs. We also demonstrate that disruption or blockade of IL-2/IL-2R interactions rescues some MHC class II-restricted thymocytes from Ag-induced apoptosis.

Two million DO11.10 and BALB/c bone marrow cells were mixed at a 1:1 ratio and injected i.v. into lethally irradiated (2 × 550 rad) BALB/c mice. Ten weeks later, chimeras were injected i.p. with PBS or OVA (as described above), and 12 h later, thymus were analyzed for expression of surface Ags and apoptosis by flow cytometry.

**Flow cytometry**

The following Abs and secondary reagents were used for three- and four-color flow cytometry and were purchased from Life Technologies (Grand Island, NY), BD PharMingen (San Diego, CA), or Caltag (San Francisco, CA), or isolated from hybridoma culture supernatants and conjugated to FITC (Sigma), biotin (Sigma), or Cy-5 (Biological Systems, Pittsburgh, PA) in our laboratory: anti-CD4 (H129.19)-Red613 or -PE; anti-CD8α (53-6.7)-FITC, -PE, or -Cy-5; anti-CD3ε (500-A2)-FITC; anti-H-Y-transgenic TCR Vi3.2 (T3.70)-biotin; anti-CD25 (PC61.5.3)-FITC or -Cy-5; anti-CD122 (Tim-3)-biotin, -PE, or -FITC; and anti-DO11.10 clonotype TCR (KJ1-26)-biotin.

All of the following procedures were performed at 4°C in the dark with FACs staining buffer (1× PBS, 2% FCS, and 1% penicillin/streptomycin) unless otherwise specified. Pooled or individual thymus were gently dispersed, the cell suspension was passed through fine nylon mesh to exclude debris, and the resulting single-cell suspension was washed twice. Cells (500,000–2,000,000) were stained in 50 μl of buffer in V-bottom 96-well plates for 20 min with the anti-FcR Ab, 2.A.4.2, and then washed twice. The cells then were stained with primary Abs, washed twice, and incubated for 20 min with appropriate secondary reagents and directly conjugated Abs. Finally, the cells were washed twice and fixed with 200 μl of 1% paraformaldehyde/PBS before analysis on a FACsCan, FACScalibur, or FACStar flow cytometer (Becton Dickinson, San Jose, CA) using CellQuest software (Becton Dickinson).

**Detection of apoptotic cells**

Apoptotic cells were identified in cell suspensions of isolated thymocytes or in situ in frozen sections of thymi by a modified version of a DNA polymerase-mediated dUTP nick translation-labeling (DUNTL) assay (32). Briefly, 5-μm frozen sections were fixed in ice-cold acetone, washed in TBS (10 mM Tris, pH 7.3, 0.15 M NaCl), incubated with labeling buffer (50 mM Tris, pH 7.4, 10 mM MgSO4, 0.1 mM DTT, 1 nmol/ml dATP/CTP/GTP, and 0.7 nmol/ml dTTP) containing 2 μM of DNA polymerase (Promega, Madison, WI) and 40 pmol of dUTP-digoxigenin (Boehringer Mannheim, Indianapolis, IN) and incubated at 37°C for 90 min. As a control, separate sections were incubated with DNA polymerase that had been denatured by heating at 70°C for 10 min in the presence of 2 mM EDTA. Sections then were washed in TBS buffer, incubated with 5 μg/ml alkaline phosphatase-conjugated sheep anti-digoxigenin Fab (Boehringer Mannheim), washed, and then incubated with the alkaline phosphatase substrate, Vector Red (Vector Laboratories, Burlingame, CA). Sections were counterstained with Methyl Green (Vector Laboratories) before mounting and photography.

For flow cytometric detection of apoptotic cells, previously stained and fixed cells were washed in permeabilization buffer (0.1% Triton X-100, 0.1% sodium citrate) and resuspended in labeling buffer containing 2 U of active or inactivated DNA polymerase and 40 pmol of FITC-12-dUTP (Boehringer Mannheim) and incubated at 37°C for 60 min. Cells then were washed in staining buffer and analyzed by flow cytometry. Apoptotic cells were also detected in cell suspension using FITC-annexin V (BD PharMingen).

**Immunohistochemistry**

Frozen thymic sections were incubated with TTBS (TBS with 0.2% Triton X-100) containing mouse IgG (10 μg/ml) and 5% normal rabbit serum to block nonspecific reactivity of primary Abs. Sections then were incubated with TTBS containing a mixture of rat anti-mouse IL-2 Abs (5 μg each of JE56-1A12 and JE56-5H4; BD PharMingen), washed with TTBS, and incubated with biotinylated-F(ab')2 of a rabbit anti-mouse IgG (Vector Laboratories). Bound Ab was visualized with a biotin-avidin-alkaline phosphatase complex and with the Vector Red substrate. Sections were counterstained with Methyl Green before photomicroscopy. As controls, anti-mouse IL-2 Abs were preincubated with recombinant murine IL-2 (BD PharMingen), and isotype-matched rat Abs of irrelevant specificity (Caltag Laboratories) were used.

**IL-2 binding and internalization**

Two million freshly isolated IL-2/-/- thymocytes were incubated on ice with 4 μg of hamster anti-mouse CD3 (2C11-145) for 30 min, washed with cold PBS, and placed in microtiter plates at 37°C for 8 h in the presence of 4 μg of antihamster Ig. During the last 2 h of incubation, cells were added to labeled recombinant human IL-2 (R&D Systems, Minneapolis, MN) that had been preincubated with 0.2 μg of streptavidin PE (Caltag) was added to each well. Cells then were stained with anti-CD4 and anti-CD8 Abs and annexin V and immediately analyzed by FACS. In control cultures, PE-labeled IL-2 was replaced with either 1) PE-labeled control protein (soybean trypsin inhibitor) or 2) PE-labeled proteinase K-degraded m.w. as IL-2; 2) PE-IL-2 mixed with 100-fold excess of recombinant human IL-2, 3) PE-labeled IL-2 preincubated with 40 μg of polyclonal goat anti-human IL-2 antisera, or 4) PE-IL-2 followed 30 min later (chase) by addition of excess rIL-2.
Results

IL-2-deficient thymocytes are resistant to the induction of TCR/CD3-mediated apoptosis unless exogenous IL-2 is supplied

The hypothesis that IL-2 is actively involved in thymocyte apoptosis was tested directly by determining the consequence of IL-2-deficiency on TCR/CD3-mediated thymocyte death in IL-2−/− mice. Although SPF IL-2−/− mice have been used previously to investigate this hypothesis (22, 33), we wanted to exclude any differences in the susceptibility of IL-2−/− thymocytes to activation-induced cell death (AICD) being attributable to thymic pathology and loss of cortical thymocytes that occurs in SPF IL-2−/− mice (34). Consequently, gnotobiotic (germfree) IL-2−/− mice in which the onset of lymphoid disorders is significantly delayed (21)

FIGURE 1. IL-2−/− thymocytes do not undergo apoptosis after anti-CD3-mediated TCR engagement unless IL-2 is supplied exogenously. IL-2−/− mice were injected with anti-CD3 Ab (A), rIL-2 (B), anti-CD3 and IL-2 (C and D), and 12 h later, thymi were removed, sectioned, and analyzed for apoptotic cells with DUNTL. Apoptotic cells appear reddish-brown. As a control, duplicate sections were stained by DUNTL with inactivated DNA polymerase (D). All sections were counterstained with Methyl Green. The results shown are representative of those obtained from five independent experiments. Magnification, ×200.

FIGURE 2. In situ detection of IL-2 protein during thymocyte apoptosis. Sections of thymi from either C57BL/6 mice injected 12 h earlier with anti-CD3 Ab (A, A′, and B) or dexamethasone (C), from DO11.10 TCR-transgenic mice administered OVA (D) or PBS (E) 12 h previously, or from BALB/c mice administered OVA 12 h earlier (F) were stained with anti-IL-2 Abs. Bound Ab was detected by a biotinylated secondary and a tertiary biotin-avidin-alkaline phosphatase conjugate and Vector Red substrate. Sections were counterstained with the nuclear dye Methyl Green before mounting and photomicroscopy. IL-2 protein appears as red staining. Control sections were incubated with anti-IL-2 preincubated with recombinant murine IL-2 (B). Results shown are representative of four independent experiments. Magnifications: A, C, E, and F, ×200; B, ×300; and A′, ×400.
were used for these experiments. Mice of 4–6 wk of age in which thymus cellularity was comparable to that of wild-type littermates and in which thymic pathology is absent (21) were used.

Gnotobiotic wild-type (n = 11) and IL-2–/– mice (n = 9) were injected with anti-CD3 Ab, and 12 h later, thymi were removed. One thymic lobe was used to detect apoptotic cells (Figs. 1 and 2) and IL-2 protein production during thymocyte apoptosis (Fig. 2) by immunohistochemistry. The second lobe was used for flow cytometric analysis of thymocyte apoptosis and IL-2R expression (Fig. 3). Very few apoptotic cells were evident in sections of thymi from anti-CD3-treated IL-2–/– mice (Fig. 1A). By contrast, large numbers of apoptotic cells were readily detected in the thymic cortex of anti-CD3-treated IL-2+/+ littermates (Fig. 2, A and B). The similarity in the cellularity and composition of thymocytes in the germfree IL-2–/– and IL-2+/+ mice excludes the possibility that the induction of thymocyte apoptosis is attributable to abnormalities in the thymocyte subset distributions and loss of DP thymocytes seen in older SPF IL-2–/– mice (34).

The insensitivity of IL-2–/– mice thymocytes to anti-CD3-induced apoptosis was overcome by providing an exogenous source of IL-2 simultaneously with Ab administration (Fig. 1C). The induction of apoptosis required both IL-2 and anti-CD3, because IL-2 alone did not result in an increase in the number of apoptotic cells above that seen in anti-CD3-treated IL-2–/– mice (Fig. 1B). By counting apoptotic clusters in several randomly selected microscopic fields, the level of apoptosis seen in the thymi of IL-2–/– mice administered anti-CD3 and IL-2 was similar to that seen in the thymi of wild-type mice treated with anti-CD3 (Figs. 1C and 2, A and B). Detection of apoptotic cell clusters by DUNTL was specific because no staining was present when DNA polymerase was inactivated before use. However, clusters of apoptotic cells were still visible with the nuclear counterstain Methyl Green (Fig. 1D).

These in situ findings were corroborated by the analysis of apoptotic cells with a flow cytometry-based DUNTL assay. The frequency of apoptotic cells recovered from thymi of anti-CD3-treated IL-2–/– mice (4.1 ± 1.4%) was not significantly different from that seen in thymi of mice treated with PBS alone (3.4 ± 1.2% and 3.1 ± 1.1%, respectively, for IL-2–/– and IL-2+/+ mice). In addition, the frequency of apoptotic cells in the thymi of anti-CD3-treated IL-2+/+ mice (13.3 ± 3.5%) was significantly (p < 0.005) higher than those present in thymi of IL-2–/– mice (4.1 ± 1.4%). Importantly, the combination of IL-2 and anti-CD3 resulted in levels of thymocyte apoptosis (14.0 ± 3.5%) in IL-2–/– mice similar to that seen in anti-CD3-treated IL-2+/+ mice (13.3 ± 3.4%). One implication of these findings is that because IL-2 appears to be necessary for AICD of thymocytes, it might be produced locally in the thymus during this process.

**IL-2 is produced in situ on TCR engagement**

To determine whether IL-2 can be produced in the thymus under conditions that promote thymocyte apoptosis, sections of thymi from C57BL/6-IL-2+/+ mice (n = 6) injected 12 h previously with anti-CD3 Abs (see above) were evaluated immunohistochemically for the presence of IL-2. As noted above, clusters of thymocytes with fragmented and aggregated nuclei were readily identified in the thymic cortex by Methyl Green counterstain (Fig. 2, A and B). Anti-IL-2 Ab staining was detected throughout the cortex of the thymi of anti-CD3-treated mice, with the most intense staining being found in areas adjacent to clusters of apoptotic thymocytes (Fig. 2, A and inset A'). Staining of adjacent thymic sections showed that most of the IL-2 protein staining was in proximity to or within phagocytic F4/80+ and/or Mac-1+ macrophages (data not shown). This pattern of anti-IL-2 staining was specific because no staining was observed with anti-IL-2 Abs previously incubated with excess recombinant murine IL-2 (Fig. 2B). In addition, the anti-IL-2 staining pattern was shown not to be due to nonspecific binding of Ab to dying cells because isotype-matched control Abs of irrelevant specificity gave no staining (data not shown).

Because anti-CD3 engagement of the TCR represents a nonphysiological interaction that bypasses the requirement for MHC/peptide interactions with the TCR, we repeated the anti-IL-2 staining on frozen sections of thymi from adult DO11.10 TCR-transgenic mice that had received a single dose (10 mg) of Ag (OVA). In line with our analyses of IL-2 expression in thymi of anti-CD3-treated wild-type mice, IL-2 protein was found throughout the thymi of OVA-treated DO11.10 mice (Fig. 2D). In contrast to the cortical localization of apoptotic thymocytes in anti-CD3-treated wild-type mice (Fig. 2), apoptotic thymocytes and IL-2 protein were colocalized in the medulla as well as the cortex of OVA-treated DO11.10-transgenic mice. The staining pattern was shown to be specific and attributable to the presence of Ag because administration of PBS to DO11.10 mice (Fig. 3E) or OVA to BALB/c mice (Fig. 2F) failed to reveal any thymocyte death or IL-2 staining. To determine whether the thymic IL-2 production resulted from thymocyte apoptosis mediated by other apoptotic

**FIGURE 3.** Thymocytes dying as a result of TCR engagement express high-affinity IL-2Rs. C57BL/6 mice (n = 5) were treated with anti-CD3 Ab, and 12 h later, thymocytes were stained and processed via DUNTL analysis for detection of viable (dUTP-) and apoptotic (dUTP+) cells (top histogram, filled profile) which were then reanalyzed for expression of CD4, CD8, and IL-2Rs. Control cells were stained with inactivated polymerase (top histogram, open profile). Isotype-matched control Abs for CD122 and CD25 are shown in open profiles on the lower histograms. The values shown in each quadrant of the dot plots and on each histogram represents the percentage of positive cells.
stimuli, wild-type mice were treated with dexamethasone in vivo and their thymi analyzed for IL-2 expression. Although administration of dexamethasone induced rapid thymocyte apoptosis in situ, we were unable to detect any IL-2 protein in these thymi (Fig. 2C). These results suggest that IL-2 expression is not a general byproduct of thymocyte death but is a result of TCR engagement.

**Apoptotic thymocytes dying as a result of TCR engagement display characteristics of IL-2/IL-2R interactions**

Thymic expression of individual chains of the IL-2R complex is either restricted to specific subsets of thymocytes (CD25, CD122), or is expressed at all stages of thymocyte development (CD132). Although thymocytes undergoing spontaneous apoptosis in vitro have also been shown to up-regulate CD25 (35) and CD122 can be induced by self-recognition in the thymus (36), whether or not these receptors are expressed alone and if they are functional is not known. To investigate this further, we established the IL-2R phenotype of thymocytes dying in situ as a result of anti-CD3-induced apoptosis. The majority of apoptotic (dUTP") thymocytes from anti-CD3-treated wild-type mice were DP, with a smaller proportion being CD4 single-positive (SP; Fig. 3). Significantly, dying anti-CD3-treated wild-type mice were DP, with a smaller proportion being CD4 single-positive (SP; Fig. 3). Significantly, dying anti-CD3-treated wild-type mice were DP, with a smaller proportion being CD4 single-positive (SP; Fig. 3).

**Dying thymocytes bind and internalize IL-2**

To confirm the ability of IL-2R-expressing thymocytes to bind and internalize IL-2, an IL-2 binding assay was developed. Thymic expression of individual chains of the IL-2R complex is either restricted to specific subsets of thymocytes (CD25, CD122), or is expressed at all stages of thymocyte development (CD132). Although thymocytes undergoing spontaneous apoptosis in vitro have also been shown to up-regulate CD25 (35) and CD122 can be induced by self-recognition in the thymus (36), whether or not these receptors are expressed alone and if they are functional is not known. To investigate this further, we established the IL-2R phenotype of thymocytes dying in situ as a result of anti-CD3-induced apoptosis. The majority of apoptotic (dUTP") thymocytes from anti-CD3-treated wild-type mice were DP, with a smaller proportion being CD4 single-positive (SP; Fig. 3). Significantly, dying thymocytes uniformly expressed high levels of CD122, with a subset of these cells also expressing CD25. By contrast, very few viable (dUTP") thymocytes expressed detectable levels of CD122 or CD25. Limited analysis of the kinetics of IL-2R expression by thymocytes undergoing apoptosis in vivo indicates that up-regulation of CD25 and CD122 by apoptotic DP thymocytes can be detected within 2–3 h of treatment and that the highest levels of expression are seen after ~16 h (data not shown).

**Dying thymocytes bind and internalize IL-2**

To confirm the ability of IL-2R-expressing thymocytes to bind and internalize IL-2, an IL-2 binding assay was developed. Thymocytes, the binding of IL-2 was more widespread, comprising DN, CD4SP, and DP thymocytes (Fig. 4). Interestingly, virtually no IL-2 binding was seen among CD8SP thymocytes (Fig. 4). The detection of IL-2 binding was specific because a control protein

**FIGURE 4.** Apoptotic thymocytes bind and internalize IL-2. Thymocytes (2 × 10^6) from gnotobiotic C57BL/6 IL-2-deficient mice were incubated at 37°C for 8 h with hamster anti-CD3 and anti-hamster IgG. During the last 2 h of culture, complexes of biotin-labeled human rIL-2 and PE-streptavidin (PE-IL-2) were added either alone (A and B), in combination with 100-fold excess recombinant human IL-2 (C and D), or followed 30 min later (chased) by the addition of excess native IL-2 (E and F). In additional control cultures, PE-IL-2 was replaced with an unrelated PE-labeled protein (G) or PE-IL-2 that previously had been incubated with neutralizing anti-IL-2 Abs (H). Cells were then stained with anti-CD4, anti-CD8, and annexin before four-color flow cytometric analysis. The profiles shown in B, D, and F were obtained by electronic gating of IL-2" annexin" (R1) and IL-2" annexin" (R2) populations identified in A, C, and E, respectively. The values shown in each quadrant of the dot plots represents the percentage of positive cells from the indicated gates. The results shown are representative of those obtained from three independent experiments.
IL-2ability of exogenous IL-2 to overcome the resistance of cortical bound and internalized by dying DP and CD4SP thymocytes. The thymocyte apoptosis, IL-2 is expressed in situ in the thymus and is their known expression of CD25 and CD122 (Fig. 3) suggests that internalization of PE-IL-2 by apoptotic CD4SP and DP cells together with expression by dying thymocytes, the efficient binding and internalization of surface-bound PE-IL-2, resulted in a similar increase in the proportion of thymocyte labeled with PE-IL-2 in the presence (1.7%) vs the absence (1.1%) of native IL-2. Thus, in contrast to 1bated with PE-IL-2 alone, there were fewer IL-2 cells and a higher proportion of IL-2- annexin+ cells (1.9%; Fig. 4E). Interestingly, the inclusion of excess native IL-2 also produced a change in the profile of IL-2- annexin+ thymocytes. Compared with cells incubated with PE-IL-2 alone, there were fewer IL-2- annexin+ DN cells and a higher proportion of IL-2- annexin+ CD4SP and DP cells in cultures containing excess native IL-2. Thus, in contrast to the binding of PE-IL-2 to CD4SP and DP thymocytes binding of PE-IL-2 to apoptotic DN cells can be blocked or reversed. Although we have not directly measured the affinity of IL-2 expressed by dying thymocytes, the efficient binding and internalization of PE-IL-2 by apoptotic CD4SP and DP cells together with their known expression of CD25 and CD122 (Fig. 3) suggests that the binding of PE-IL-2 to at least some of these cells may involve high-affinity IL-2Rs. Collectively, the results presented so far demonstrate that during thymocyte apoptosis, IL-2 is expressed in situ in the thymus and is bound and internalized by dying DP and CD4SP thymocytes. The ability of exogenous IL-2 to overcome the resistance of cortical IL-2-/- thymocytes to TCR/CD3-mediated AICD suggests that IL-2/IL-2R signaling contributes to the inductive phase of thymocyte apoptosis. This was investigated further by determining the consequences of disrupting IL-2/IL-2R interactions on the deletion of Ag-specific thymocytes.

Newborn (<3 days old) DO11.10 mice were administered a mixture (0.5 mg each) of anti-CD25 and anti-CD122 or isotype-matched control Abs on days 1 and 2 followed by two further injections of Abs plus 1 mg of OVA on days 3 and 4, with the thymus and spleen being removed for analysis on day 5. The results in Fig. 5 and Table I show that Ab-mediated disruption of IL-2/IL-2R interactions significantly reduces the deletion of Ag-specific thymocytes as indicated by cellularity and distribution of CD4 and CD8 thymocyte subsets in DO11.10 mice treated with OVA and anti-IL-2R Abs. Administration of Ag alone resulted in the loss of DP thymocytes bearing low to intermediate levels of transgenic TCR (Fig. 5A). By contrast, anti-IL-2R Abs protected a large proportion of TCRlow DP thymocytes from AICD. However, isotype-matched control Abs of irrelevant specificity did not effect the deletion of DP thymocytes.

This system allowed us to address important caveats of the anti-CD3-induced apoptosis experiments with IL-2-/- mice. By demonstrating a role for IL-2/IL-2R interactions in BALB/c-(H-2d) TCR-transgenic mice, we have shown that the involvement of IL-2 in thymocyte death is not solely restricted to C57BL/6 (H-2b) mice. In addition, because anti-CD3 presumably bypasses the requirements for the TCR to interact with MHC/peptide complexes, it was important to show that IL-2/IL-2R interactions occur in response to nominal Ag. Because DO11.10 mice are considered “normal” and do not display any immunological abnormalities, it is unlikely that the involvement of IL-2 in thymocyte death in IL-2-/- mice is attributable to the underlying abnormalities or malignancies that develop in IL-2-/- mice with age. In addition, the use of newborn (<3 days of age) mice that contain few peripheral splenic CD4 T cells (Fig. 5A) allowed us to exclude the possibility that thymocyte death caused by Ag administration was attributable to peripheral T cell activation and the production of glucocorticoids (GCs). Although the finding that intrathymic T cell development proceeds normally in GC receptor (GCR)-/- mice (38) convincingly demonstrates that GCR signaling does not play a central role in thymocyte selection, we attempted to quantitate the level of thymocyte death resulting from Ag- vs non-Ag-specific mechanisms with bone marrow chimeras. Lethally irradiated BALB/c mice were reconstituted with a 1:1 mixture of DO11.10 TCR-transgenic and -nontransgenic BALB/c bone marrow cells. Ten weeks later, groups (n = 5) of chimeric or DO11.10 and BALB/c control animals were injected with OVA, and the level of apoptosis among TCR-transgenic (KJ1-26+) and wild-type (KJ1-26-) thymocytes was assessed. The similarity in the proportion of CD4-expressing thymocytes bearing the transgenic (40%) vs endogenously rearranged (44%) TCR (Fig. 5B) demonstrates true chimerism of the reconstituted hosts. Almost two-thirds of apoptotic thymocytes in OVA-treated mice were

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>Treatment</th>
<th>Total</th>
<th>DN</th>
<th>DP</th>
<th>CD4SP</th>
<th>CD8SP</th>
<th>Total annexin+</th>
</tr>
</thead>
<tbody>
<tr>
<td>DO11.10</td>
<td>PBS</td>
<td>19.4 ± 4.1</td>
<td>3.4 ± 1.8</td>
<td>13.2 ± 1.1</td>
<td>3.6 ± 0.9</td>
<td>0.9 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>DO11.10</td>
<td>OVA + rat IgG</td>
<td>5.5 ± 2.4</td>
<td>1.8 ± 0.7</td>
<td>2.0 ± 1.4</td>
<td>1.9 ± 0.6</td>
<td>0.3 ± 0.1</td>
<td>ND</td>
</tr>
<tr>
<td>DO11.10</td>
<td>OVA + anti-IL-2 Abs</td>
<td>10.8 ± 2.0</td>
<td>1.8 ± 0.6</td>
<td>6.2 ± 1.5</td>
<td>2.8 ± 1.7</td>
<td>0.3 ± 0.2</td>
<td>ND</td>
</tr>
<tr>
<td>DO11.10 × IL-2-/-</td>
<td>OVA</td>
<td>58.2 ± 10.5</td>
<td>2.3 ± 1.1</td>
<td>37.6 ± 9.4</td>
<td>14.2 ± 7.5</td>
<td>8.6 ± 2.4</td>
<td>8.8 ± 2.2</td>
</tr>
<tr>
<td>DO11.10 × IL-2-/-</td>
<td>PBS</td>
<td>68.4 ± 13.4</td>
<td>3.5 ± 1.8</td>
<td>53.2 ± 11.1</td>
<td>10.3 ± 4.6</td>
<td>1.2 ± 0.4</td>
<td>4.4 ± 1.9</td>
</tr>
<tr>
<td>DO11.10 × IL-2-/-</td>
<td>OVA</td>
<td>98.5 ± 18.9</td>
<td>4.7 ± 1.6</td>
<td>78.2 ± 17.5</td>
<td>14.1 ± 2.9</td>
<td>1.1 ± 0.2</td>
<td>7.1 ± 2.3</td>
</tr>
</tbody>
</table>
KJ1-26^+, and the remainder (~30%) were KJ1-26^−, demonstrating that the majority of thymocyte apoptosis after Ag administration to TCR-transgenic mice is a consequence of TCR engagement on thymocytes. However, some non-Ag-specific thymocytes also die as a consequence of peripheral or non-Ag specific effects such as GC and may indirectly involve IL-2. The results from these experiments also corroborate the results of similar experiments investigating mechanisms of thymocyte death with influenza nucleoprotein-reactive F₃ TCR-transgenic mice (39).

In our second approach, DO11.10 mice homozygous for the mutated IL-2 gene (DO11.10 × IL-2^−/−) were obtained from matings of DO11.10 and BALB/c IL-2^+/− mice. At 6 wk of age, CD4 and CD8 thymocyte subset distribution and TCR expression levels were comparable to that of DO11.10 × IL-2^+/− mice (Fig. 6, and data not shown). Interestingly, although BALB/c IL-2^−/− mice display severe anemia and autoimmune disease soon after birth and the majority die within 5 wk of age (20), DO11.10 × IL-2^−/− mice (n = 8) remained disease free (our unpublished
observations). Presumably, the increased lifespan and absence of lymphoid hyperplasia and autoimmunity in these animals is attributable to the skewing of the CD4 T cell repertoire to a single exogenous non-self Ag.

Administration of a single dose (10 mg) of OVA to 6-wk-old DO11.10 x IL-2+/+ mice resulted in an increase in the proportion of apoptotic cells as detected by flow cytometric DUNTL analysis (Fig. 6). In contrast, the level of apoptosis in the thymi of DO11.10 x IL-2−/− mice after OVA treatment was similar to that seen after administration of PBS to DO11.10 x IL-2−/− mice. The resistance of DP thymocytes to AICD in IL-2-deficient DO11.10 mice was confirmed from comparisons of the cellularity and absolute number of thymocyte subsets in these and mock-treated animals (Table I). This result is consistent with the ability of anti-IL-2R Abs to protect Ag-specific DP thymocytes from apoptosis (Fig. 5) and provides further evidence for the direct involvement of the IL-2/IL-2 signaling pathway in thymocyte death and selection.

A different outcome was seen for the deletion of Ag-specific MHC class I-restricted thymocytes in H-Y x IL-2−/− mice. There was no significant difference in the cellularity or thymocyte subset distribution in male H-Y x IL-2+/+ and H-Y x IL-2−/− mice (Fig. 7), and the onset and severity of lymphoid hyperplasia and autoimmune disease was similar to that of C57BL/6 IL-2−/− mice (data not shown). The absence of IL-2 had no obvious effect on the deletion of H-Y-specific CD8+ thymocytes. The cellularity and profile of H-Y-specific thymocytes in the positive selecting environment of the female thymi was similar irrespective of the presence or absence of IL-2.

**FIGURE 6.** Inefficient deletion of IL-2-deficient, Ag-specific MHC class II-restricted thymocytes. Four-week-old DO11.10 x IL-2+/+ and DO11.10 x IL-2−/− mice obtained from the F2 progeny of BALB/c DO11.10 x IL-2−/− matings were injected with a single dose of OVA, and 12 h later, the thymi were removed and the CD4 and CD8 subset distribution and presence of apoptotic (annexin V+) cells was assessed by flow cytometry. Control DO11.10 x IL-2−/− mice received PBS. The values shown on the dot and histogram plots represent the frequency of positive cells.

**FIGURE 7.** Negative selection occurs normally in the thymi of IL-2-deficient MHC class I-restricted TCR-transgenic mice. Male and female HY-TCR-transgenic mice were mated with C57BL/6 IL-2+/+ mice to obtain TCR-transgenic IL-2-deficient male (M) and female (F) mice, the thymi of which were analyzed for CD4 and CD8 subset distribution and expression of the transgenic Vα3.2 TCR recognized by the T3.70 Ab. The percentage values shown on the dot and histogram plots represent the proportion of positive cells of each phenotype. The filled and open profiles shown on each histogram represent the level of staining achieved with the T3.70 and an isotype-matched irrelevant control Ab, respectively. The results shown are representative of those obtained from analysis of two litters of 4-wk-old mice.
Discussion

Thymocytes with self-reactive TCRs die apoptotically in response to signals imparted by high-affinity TCR/MHC interactions. Recent evidence suggests that the intracellular signals that mediate clonal deletion may be different from those that result in positive selection (5). Interactions of thymocytes with stromal adhesion molecules, costimulatory ligands, and cytokines either alone or in combination may result in the divergence of these selection signals. In accordance with such modulation, our results suggest that under certain conditions and in conjunction with TCR-MHC/peptide interactions, IL-2/IL-2R signaling promotes negative selection of Ag-specific thymocytes. Our findings demonstrate that thymocytes dying as a consequence of TCR-mediated activation express IL-2Rs capable of binding and internalizing IL-2. Although IL-2-deficient thymocytes are resistant to TCR/CD3-mediated AICD, this is overcome in the presence of exogenously supplied IL-2. We have also demonstrated the causal nature of the IL-2/IL-2R signaling interactions in the negative selection of a MHC class II-restricted TCR.

It is possible that the thymocyte apoptosis we have observed is mediated by GCs released by the adrenal medulla in response to activation of peripheral T cells (reviewed in Ref. 40). However, the fact that Ag-specific deletion is inhibited by anti-IL-2Rs in neonatal DO11.10-transgenic mice that have few circulating responsive peripheral T cells and that the death of the majority of Ag-specific thymocytes in bone marrow chimeras is a consequence of TCR engagement on thymocytes argues against this being the sole explanation for our observations. Indeed, the recent finding that thymocyte selection proceeds normally in GCR−/− mice (38) convincingly demonstrates that GCR signaling does not play a central role in intrathymic T cell development.

The apparent lack of a requirement for IL-2/IL-2R interactions in deletion of MHC class I-restricted HY-specific thymocytes is consistent with the results from other transgenic mouse model systems in which class I-restricted TCRs specific for endogenous or exogenous Ags have been crossed to IL-2−/− or CD25−/− mouse strains. Ag-mediated selection has been shown to occur normally in F5 nucleoprotein-specific MHC class I-restricted TCR-transgenic mice bred onto the IL-2−/− background (30) and in H-Y TCR-transgenic mice bred onto the CD122−/− (41) and CD132−/− (42, 43) backgrounds. One possible conclusion from these findings is that IL-2/IL-2R-signaling might distinguish between CD4 and CD8 T cell selection. However, caution is warranted in view of unique properties and different mechanisms involved in each experimental model system making it difficult to directly compare the results obtained with different model systems. For example, differences in the properties of the selecting Ag in each model system including its origin (exogenous vs endogenous), nature (free or cell-associated), distribution (cortex vs medulla), concentration, mode, and duration of presentation in the thymus, and the possibility that the involvement of the IL-2/IL-2R signaling pathway is determined by some or all of these factors could account for the different outcomes for CD4 vs CD8 T cell selection in the absence of intrathymic IL-2/IL-2R signaling. Therefore, our findings may be unique to the experimental system and TCR-transgenic mouse strain we have used.

Our data also demonstrate that it is unlikely that IL-2/IL-2R interactions mediate negative selection of all MHC class II-restricted thymocytes. Only a subset of apoptotic Ag-specific or anti-CD3-stimulated thymocytes express functional IL-2Rs, and blocking or disrupting IL-2/IL-2R interactions results in incomplete protection from Ag-specific apoptosis. This may reflect the relative inefficiency of deletion in the experimental systems we have used or that IL-2/IL-2R involvement in thymocyte selection is restricted to specific cell populations and/or specific microenvironmental conditions under which developing T cells are exposed to Ag. The observation that superantigen (Mtv)-mediated deletion of thymocytes is incomplete in CD132−/− (44) and Jak3-mutant mice (28) implies the existence of multiple mechanisms to ensure the escape of as few autoreactive T cell clones as possible from the thymus. In our analyses of H-2b IL-2−/− mice, we too have been unable to detect any abnormalities in deletion of superantigen-reactive thymocyte populations (our unpublished observations).

Collectively, our findings may help explain the underlying basis of the abnormalities described in IL-2−/− (19–21, 34), IL-2R chain−/− (24, 25, 45), and IL-2R signaling molecule-deficient mice (26, 27). Defective negative selection would allow for the escape of autoreactive MHC class II-restricted clones from the thymus that proliferate on encountering self-Ag in the periphery but fail to undergo efficient AICD (23, 24, 46). The occurrence of lymphoid hyperplasia and autoimmune disease in gnotobiotic (germfree) IL-2−/− mice similar to that in SPF IL-2−/− mice (21) demonstrates that these disorders are attributable to a defect in central and/or peripheral tolerance that leads to uncontrolled responses to self and not environmental Ags. The observation that directed expression of CD122 to the thymus in CD122-deficient mice prevents the occurrence of CD4 T cell-mediated autoimmunity (29) is consistent with our hypothesis that IL-2/IL-2R interactions play a critical role in the maintenance of intrathymic central tolerance. Observations regarding defective IL-2/IL-2R-signaling in other unrelated autoimmune diseases such as diabetes (47–49) corroborate this hypothesis.

The source and use of IL-2 within the thymus during negative selection may be autocrine or paracrine in nature. Considering the short half-life and limited range of IL-2 in vivo (50) and the expression of IL-2 protein in close association with clusters of apoptotic cells in situ in the intact thymus, it is likely that IL-2 is produced locally within the thymus. Preliminary studies to identify the cellular source of IL-2 in the thymus during negative selection indicate that nonapoptotic Ag-specific thymocytes produce IL-2 in culture in response to Ag presented by a population of thymic macrophages (our unpublished observations). Moreover, these studies also indicate that this IL-2 production is potentiated by interactions between CD4SP and DP thymocytes and thymic macrophages. These observations underscore the importance of cross-talk between various thymocyte populations and the thymic stroma and suggest the existence of a feedback mechanism by which CD4 T cells could control the level or rate at which unwanted thymocytes are culled from the repertoire.

Our results may help clarify some of the seemingly contradictory findings regarding the role of IL-2 in thymocyte development (reviewed in Ref. 16). IL-2/IL-2R interactions in the thymus, similar to those in the periphery, are capable of both promoting and inhibiting thymocyte survival. These seemingly paradoxical effects may be explained by a developmental-stage-specific responsiveness of thymocytes to the activity of IL-2. Signals generated via the IL-2R may promote thymocyte differentiation by stimulating the proliferation and expansion of TCR+ immature thymocyte populations while they promote the death of TCR+ thymocytes on TCR engagement at the DP and CD4SP selection-susceptible maturation stages. In addition, there may be inherent differences in the production of IL-2 and the mediation of these effects in the presence or absence of thymic APC populations. Indeed, our finding that thymic APC populations are compromised in IL-2−/− mice (34), combined with the requirement for these cells for production of IL-2 might exacerbate the defect in TCR-mediated thymocyte apoptosis in these mice. Finally, some of the controversy...
regarding the role of IL-2 in thymocyte development also may stem from intrinsic differences between fetal and adult thymic stromal cells and T cell precursor populations. Therefore, we cannot exclude the possibility that there may not be a strict requirement for IL-2/IL-2R interactions in negative selection of fetal thymocytes.

In summary, the results of our analyses demonstrate a role for IL-2/IL-2R interaction in the mediation of thymic negative selection of certain CD4 T cells. Therefore, IL-2R-generated signals may represent a unique and important pathway by which negative selection is modulated, and defects in this signaling pathway may result in loss of lymphoid homeostasis and autoimmunity.

References


