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Immunology

Bcl-2 expression during T-cell development: Early loss and late return occur at specific stages of commitment to differentiation and survival

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ABSTRACT **During T-cell development $CD3^-CD4^-CD8^-$ (double-negative) thymocytes proliferate and produce an enormous number of $CD3^{lo}CD4^+CD8^+$ (double-positive) thymocytes which are destined to die intrathymically unless rescued by positive selection. Those which survive become mature $CD3^{hi}CD4/8^+$ (single-positive) cells and are the precursor of peripheral blood lymphocytes. The product of the *bcl-2* protooncogene has been implicated in preventing programmed cell death and is required for prolonged lymphocyte survival following maturation. Previously we and others have reported that Bcl-2 protein expression is biphasic, being high in proliferating double-negative stem cells, low in all double-positive thymocytes except for 1–5% of these cells, and restored in mature, single-positive thymocytes. However, it remained unclear which signaling and selection events regulate Bcl-2 during T-cell maturation. Now we have utilized four-color flow cytometry in normal and genetically altered mice for a detailed analysis of Bcl-2 expression as it relates to T-cell receptor (TCR) expression and positive selection. These studies show that (*i*) expression of a transgenic TCR in double-negative thymocytes does not lead to premature loss of Bcl-2; thus, Bcl-2 downregulation is not solely due to TCR expression; (*ii*) Bcl-2 expression is lost at the early transitional $CD3^{-/lo}CD4^-CD8^+$ stage, prior to expression of CD4; (*iii*) the $Bcl\text{-}2^+$ double-positive thymocytes are those which have undergone positive selection; and (*iv*) upregulation of Bcl-2 during positive selection requires participation of the CD4 or CD8 co-receptor. These results demonstrate that Bcl-2 and TCR expression are regulated independently during T-cell development, and suggest a role for the CD4 or CD8 co-receptor in Bcl-2 induction during positive selection.**

Shaping of the mature T-cell repertoire to recognize foreign antigen in a major histocompatibility complex (MHC)-restricted context occurs during maturation in the thymus. This process begins with creation of a unique T-cell antigen receptor (TCR) in each $CD3^-CD4^-CD8^-$ cortical stem cell [double-negative (DN) thymocytes] (1). After TCR gene rearrangement is complete, the TCR/CD3 complex is expressed at low levels on the cell surface and accompanied by CD8 and CD4 expression as cells become $CD3^{lo}CD4^+CD8^+$ [double-positive (DP)] thymocytes (1). To ensure that the mature T-cell repertoire is MHC-restricted, these DP thymocytes are subjected to positive selection and clonal deletion that culminates in the intrathymic death of 95% of these cells (1). Surviving thymocytes have upregulated TCR/CD3 and downregulated either CD4 or CD8 expression to become mature, single-positive (SP) cells ($CD3^+CD4^-CD8^+$ or $CD3^+CD4^+CD8^-$) and are found primarily in the medulla (1).

Whether DP thymocytes successfully differentiate into MHC class I- or class II-restricted mature T cells or die intrathymically is dependent on the MHC specificity of the expressed TCR (1). In general, positive selection and clonal deletion require both TCR–MHC interaction and either CD4 or CD8 binding to nonpolymorphic regions of MHC class I or class II proteins located on thymic stromal cells and/or resident bone marrow-derived cells (2, 3). Recent studies using class I- or class II-deficient mice show that positive selection requires two TCR–MHC interactions in which only the second requires CD4 or CD8 co-receptor participation for survival of lineage-committed cells (4–6).

Despite these advances in understanding selection, the intracellular signals which allow thymocytes to die or survive are largely unknown. A candidate gene which may participate in this process is *bcl-2*, a protooncogene whose expression in a variety of cell lines and tissues prolongs cell survival by inhibiting death via apoptosis (7, 8). Regulation of *bcl-2* is biphasic during normal human and murine T-cell development (9–11): Bcl-2 is highly expressed in immature DN thymocytes, is low in all but a small subgroup of DP thymocytes, and is upregulated in SP thymocytes that have been positively selected and have survived clonal deletion. Cortical thymocytes from mice that expressed *bcl-2* as a transgene targeted to the T-cell lineage were shown to have prolonged survival in simple medium and were resistant to glucocorticoid-induced cell death (7, 12, 13). In *bcl-2*-deficient mice, T-cell development appeared normal, but mature SP thymocytes and T cells had a shortened lifespan when exposed to glucocorticoids *in vitro* or as a result of illness *in vivo* (14, 15). Together, these results suggest that Bcl-2 expression is specifically downregulated in DP thymocytes to facilitate selection but that upregulation of Bcl-2 during positive selection is necessary for prolonged survival.

In this study, we have used four-color flow cytometry to examine Bcl-2 expression relative to TCR/CD3 expression during T-cell development in normal mice, in mice that express a transgenic TCR specific for the male H-Y antigen (H-Y TCR), and in MHC class II-deficient mice. We find that premature expression of a transgenic TCR on DN thymocytes does not lead to loss of Bcl-2. Rather, in both H-Y TCR^+ and normal mice, Bcl-2 expression is lost at the early transitional $CD3^{lo}CD4^-CD8^+$ stage prior to CD4 expression. By examining a variety of parameters to assess positive selection, we observe that the small population of $Bcl\text{-}2^+$ DP thymocytes are those which have already undergone positive selection. Furthermore, studies using MHC class II-deficient mice reveal that upregulation of Bcl-2 during positive selection requires CD4 or CD8 co-receptors. These results define discrete steps in the regulation of Bcl-2 expression that are

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Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; mAb, monoclonal antibody; DN, double negative; DP, double positive; SP, single positive.

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distinct from TCR regulation during T-cell development and suggest a role for the CD4 or CD8 co-receptor in Bcl-2 induction during positive selection.

MATERIALS AND METHODS

Monoclonal Antibodies (mAbs) and Chemicals. The hamster mAb 3F11, specific for mouse Bcl-2, was a gift of S. Korsmeyer (10). The hybridoma that produces the mouse mAb F23.1, specific for the TCR β-chain variable region $V_\beta 8$, was a gift of H. von Boehmer and F23.1 was produced in ascites, purified, and conjugated to fluorescein isothiocyanate (FITC) as described (16, 17). FITC-conjugated anti-mouse CD8, phycoerythrin (PE)-conjugated anti-mouse CD4 and anti-mouse CD8, FITC-conjugated anti-mouse CD69, PE-conjugated anti-mouse Thy-1.2, PE-conjugated anti-mouse H-2^b, FITC-conjugated anti-mouse H-2^d, and biotinylated anti-mouse CD4 mAbs were purchased from PharMingen. Biotinylated goat anti-hamster $F(ab')_2$ was from Caltag (South San Francisco, CA). FITC-conjugated anti-mouse CD3 and RED613-conjugated anti-mouse CD4 were from Life Technologies (Grand Island, NY). FITC- and PE-conjugated rat IgG isotype and hamster Ig controls were from PharMingen. FITC-conjugated mouse IgG isotype control was from Becton Dickinson. $CD4^-$ thymocytes within the single-cell suspension prepared from thymic specimens served as the negative control for RED613-conjugated anti-CD4 mAb staining.

Mice. A breeder pair of mice (H-2^b, B6 background) expressing the α and β transgenes for the H-Y TCR were a gift of H. von Boehmer (2). Mice homozygous for H-Y TCR were identified by Southern blot analysis (18) and were crossed with congenic H-2^d C57BL/6 mice to obtain H-Y TCR on a nonselective background. H-Y TCR^+ H-2^b, H-$2^{b/d}$, and H-2^d were identified by two-color immunofluorescence analysis of peripheral blood leukocytes. MHC class II-deficient mice (C57BL/6 × 129 background) were purchased from GenPharm International (Mountain View, CA), and normal C57BL/6 (H-2^b) and congenic C57BL/6 (H-2^d) mice were purchased from The Jackson Laboratory. All mice used in these studies were 4- to 8-week-old females. For some experiments, mice were given 2 mg of dexamethasone sodium phosphate (American Regent Laboratories, Shirley, NY) intraperitoneally 48 hr prior to sacrifice.

Flow Cytometry. Unseparated thymocytes were stained with a 1% paraformaldehyde fixation/0.3% saponin permeabilization protocol (9, 19). For the analyses that included Bcl-2 staining, cell surface markers were stained first with only directly conjugated mAbs, and then cells were stained for Bcl-2 after fixation. Data were collected on a FACScan flow cytometer (Becton Dickinson) equipped with LYSYS I or LYSYS II software programs or an EPICS Elite flow cytometer (Coulter) equipped with Elite software programs. RED613 emission was detected by using a 610 ± 10-nm bandpass filter (Molecular Products, Portland, OR). For three-color and four-color analyses, 20,000 and 100,000–500,000 stained cells were analyzed, respectively. The results in each figure are representative of at least three experiments.

RESULTS

Independent Regulation of Bcl-2 and TCR/CD3 in Immature Thymocytes. We and others have demonstrated that Bcl-2 is expressed at high levels in $CD3^-CD4^-CD8^-$ thymocytes and is downregulated in DP ($CD3^{lo}CD4^+CD8^+$) thymocytes (9–11). One possibility was that specific downregulation of Bcl-2 was a result of TCR/CD3 surface expression. To test this hypothesis, we examined Bcl-2 expression in DN thymocytes from normal female and H-Y TCR^+ H-2^b female mice by three-color flow cytometry, because this particular transgenic TCR is expressed at high levels in DN thymocytes (20). Despite high levels of TCR/CD3 complex expressed on DN thymocytes from H-Y TCR^+ H-2^b female mice, there was no difference in Bcl-2 expression when compared with DN thymocytes from normal female mice (Fig. 1). No difference was seen in Bcl-2 expression in DP thymocytes between H-Y TCR^+ H-2^b female mice and control mice despite the fact that in H-Y TCR^+ H-2^b female mice, DP thymocytes have significantly higher levels of TCR/CD3 expression (Fig. 1*C* and data not shown). This data demonstrates that Bcl-2 expression is regulated independently of TCR/CD3 expression in immature thymocytes prior to positive selection, suggesting that TCR/CD3 expression is not by itself responsible for the specific reduction of Bcl-2 in DP thymocytes.

Bcl-2 Expression Is Upregulated in $TCR/CD3^{hi}$ Double-Positive Thymocytes. During T-cell development, $TCR/CD3^{lo}CD4^+CD8^+$ thymocytes are subjected to positive selection and clonal deletion (1). Upregulation of the TCR/CD3 complex and resistance to programmed cell death have been considered characteristics of DP thymocytes that have successfully completed positive selection (4, 21). Recent evidence demonstrates that the initial phenotype of these glu-

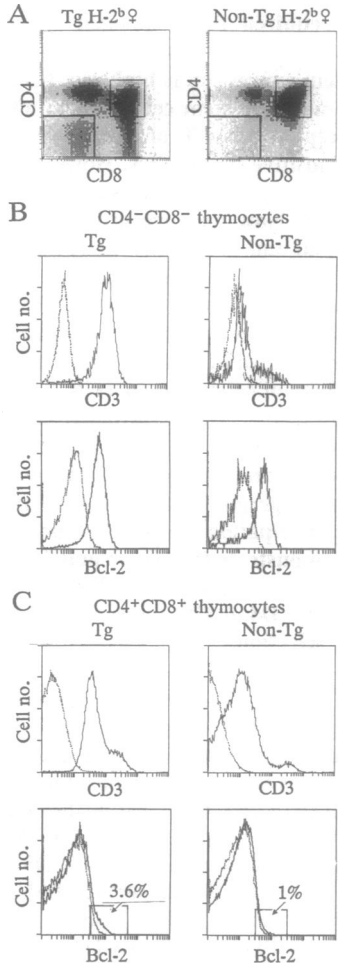

FIG. 1. Effects of TCR transgene expression on Bcl-2 levels in DN and DP thymocytes. Unseparated thymocytes from H-Y TCR^+ (transgenic, Tg) H-2^b and non-Tg H-2^b female mice were stained for CD4, CD8, and either CD3 or Bcl-2 and analyzed by three-color flow cytometry. DN and DP thymocytes from stained samples were gated as shown in *A* and were analyzed for CD3 or Bcl-2. (*B*) DN thymocytes from H-Y TCR^+ H-2^b (*Left*) and normal H-2^b (*Right*) female mice. (*C*) DP thymocytes from H-Y TCR^+ H-2^b (*Left*) and normal H-2^b (*Right*) female mice. The percentage of $Bcl\text{-}2^+$ DP thymocytes from each animal is shown, thus confirming previously reported observations (9, 10).

cocorticoid-resistant, positively selected thymocytes is TCR/CD3^{hi}CD4^{lo}CD8^{+} or TCR/CD3^{hi}CD4^{+}CD8^{lo} (4, 5). We hypothesized that the Bcl-2^{+} DP thymocytes expressed this phenotype. To examine Bcl-2 expression in this subpopulation of DP thymocytes, unseparated thymocytes from normal mice and H-Y TCR^{+} H-2^{b} female mice were stained for CD3, CD4, CD8, and Bcl-2 and analyzed by four-color flow cytometry. We then gated on DP (CD4^{+}CD8^{+}, CD4^{lo}CD8^{+}, and CD4^{+}CD8^{lo}) and SP (CD4^{-}CD8^{+}, CD4^{+}CD8^{-}) thymocytes that were CD3^{lo} or CD3^{hi}, and we displayed Bcl-2 expression as histograms for each subpopulation (Fig. 2).

Fig. 2 *A*, *B*, and *D* are histograms of Bcl-2 content in DP thymocytes from normal mice. When each subpopulation of CD3^{lo} DP thymocytes was analyzed for Bcl-2, all of these cells were Bcl-2$^{-/lo}$ (Fig. 2 *A*, *B*, and *D*, column 3). Low levels of Bcl-2 in these CD3^{lo} DP thymocytes (Fig. 2*A*) are consistent with the phenotype and susceptibility to programmed cell death of this stage of differentiation prior to positive selection (1, 21). In contrast to the CD3^{lo} DP thymocytes, CD3^{hi} DP (CD4^{+}CD8^{+}, CD4^{lo}CD8^{+}, and CD4^{+}CD8^{lo}) thymocytes were Bcl-2^{+} (Fig. 2 *A*, *B*, and *D*, column 4). Identical results were obtained from H-Y TCR^{+} H-2^{b} or H-2$^{b/d}$ mice (data not shown). Thus, Bcl-2 is upregulated in TCR/CD3^{hi} DP thymocytes but not in TCR/CD3^{lo} DP thymocytes. The CD3^{lo}CD4^{lo}CD8^{+} and CD3^{lo}CD4^{+}CD8^{lo} subpopulations of thymocytes may contain those cells that have failed to be positively selected and are destined to die (see below).

Bcl-2 expression was high in the CD3^{hi}CD4^{-}CD8^{+} and CD3^{hi}CD4^{+}CD8^{-} thymocytes, confirming previously published results (9, 10) (Fig. 2 *C* and *E*, column 4). However, in the CD4^{-}CD8^{+} population, there were a substantial number of CD3^{lo} thymocytes from both control H-2^{b} and H-Y TCR^{+}H-2^{b} female mice (Fig. 2*C*, column 3; data not shown). These CD3^{lo} thymocytes were virtually absent from the CD4^{+}CD8^{-} thymocytes (Fig. 2*E*, column 3; data not shown). This suggests that these cells were immature, because DN thymocytes pass through a transitional CD3^{lo}CD4^{-}CD8^{+} phenotype prior to becoming CD3^{lo}CD4^{+}CD8^{+} (22). Notably, these CD3^{lo}CD4^{-}CD8^{+} cells were uniformly Bcl-2$^{-/lo}$. Therefore, these data define further the time of Bcl-2 downregulation during maturation—i.e., after CD8 is fully expressed, but prior to entry into the DP stage of differentiation.

Bcl-2^{+} DP Thymocytes Are Resistant to Glucocorticoid-Induced Cell Death. The previous section described the phenotype of the subpopulations of DP and SP thymocytes that express high levels of Bcl-2. These cells are CD3^{hi}, suggesting that they have completed positive selection. Another characteristic of positively selected DP thymocytes is their relative resistance to glucocorticoid-induced cell death (1, 4). To determine whether these Bcl-2^{+}CD3^{hi} DP thymocytes had this characteristic of mature thymocytes, dexamethasone was administered to normal H-2^{b} female mice, and 48 hr later, unseparated thymocytes were analyzed for CD3, CD4, CD8, and Bcl-2 by four-color flow cytometry. We gated on the same subpopulations of DP and SP thymocytes that were CD3^{lo} or CD3^{hi}, as shown in Fig. 2, and displayed Bcl-2 expression as histograms for each subpopulation (Fig. 3). Within each subpopulation of DP thymocytes, only the CD3^{hi} cells remained after dexamethasone treatment (Fig. 3 *A*, *B*, and *D*), and the majority of these CD3^{hi} DP thymocytes

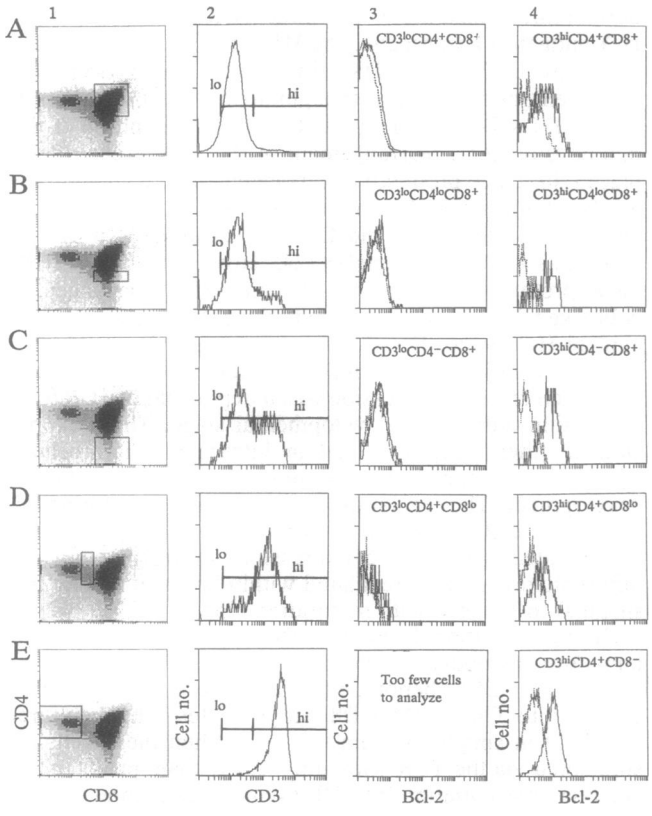

FIG. 2. Analysis of Bcl-2 in DP and SP thymocytes from control H-2^{b} female mice by four-color flow cytometry. Unseparated thymocytes were stained for CD3, CD4, CD8, and Bcl-2. Each subpopulation was gated according to CD4 vs. CD8 expression (column 1) and CD3 expression (column 2). Histograms of Bcl-2 are displayed in columns 3 and 4. Row A, CD4^{+}CD8^{+} thymocytes; row B, CD4^{lo}CD8^{+} thymocytes; row C, CD4^{-}CD8^{+} thymocytes; row D, CD4^{+}CD8^{lo} thymocytes; row E, CD4^{+}CD8^{-} thymocytes.

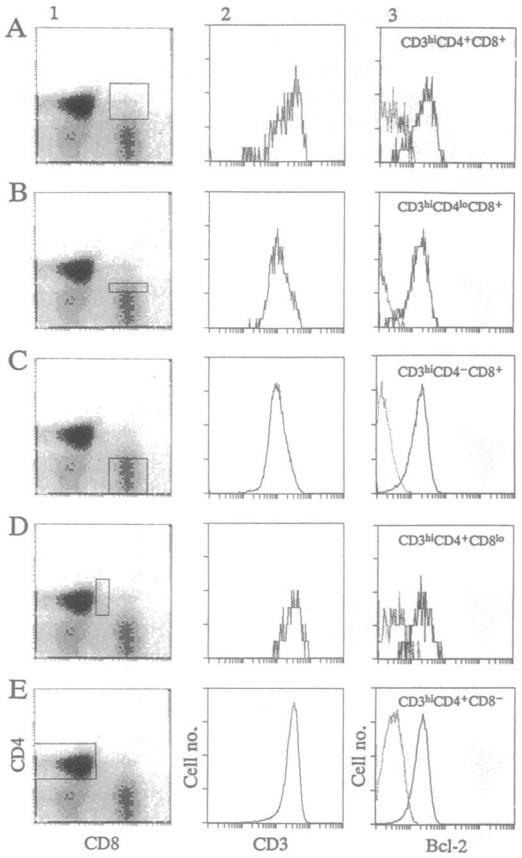

FIG. 3. Only CD3^{hi}Bcl-2^{+} DP and SP thymocytes remain in dexamethasone-treated normal H-2^{b} female mice. Unseparated thymocytes were stained for CD3, CD4, CD8, and Bcl-2 and analyzed by four-color flow cytometry. Each subpopulation was analyzed with the same format as in Fig. 2, except that results are shown only for CD3^{hi} cells.

expressed high levels of Bcl-2. This suggests that the Bcl-2$^+$ DP thymocytes have gained some of the functional characteristics of mature cells that have completed positive selection. As expected, most of the CD3^{hi}CD4$^-$CD8$^+$ and CD3^{hi}CD4$^+$CD8$^-$ thymocytes expressed high levels of Bcl-2 and were resistant to glucocorticoid-induced cell death (Fig. 3 *C* and *E*). Identical results were obtained in H-Y TCR$^+$ H-2^b female mice (data not shown).

Upregulation of Bcl-2 Requires CD4 or CD8 Co-Receptor Participation During Positive Selection. Studies using MHC class I- or class II-deficient mice suggest that positive selection of thymocytes can be a two-step process (4–6). It was proposed that the first step of positive selection does not require CD8 or CD4 co-receptor binding during TCR engagement of MHC class I or class II molecules, respectively, and induces upregulation of TCR/CD3 and CD69 and random downregulation of CD4 or CD8 (4, 5). The second step does require CD8 or CD4 binding to class I or class II MHC molecules for continued maturation and survival of lineage committed cells (4, 5). In MHC class II-deficient mice, maturation of thymocytes that express a MHC class II-restricted TCR stops at the point where CD4 co-receptor participation is required during positive selection (4). To determine in a two-step model at which stage of positive selection Bcl-2 expression was upregulated, we stained unseparated thymocytes from MHC class II-deficient mice.

The distribution of DN, DP, and SP thymocytes from MHC class II-deficient mice was the same as previously described (23). Shown in Fig. 4 *A*, *B*, and *D* is the Bcl-2 content in DP thymocytes. Consistent with data in Fig. 2, all of the CD3^{lo} DP cells were Bcl-2$^{-/lo}$ (Fig. 4 *A*, *B*, and *D*, column 3). However, in contrast to data presented in Fig. 2, the CD3^{hi}CD4^{lo}CD8$^+$ and CD3^{hi}CD4$^-$CD8$^+$ thymocytes (i.e., the cells committed to the CD8 lineage), but not the CD3^{hi}CD4$^+$CD8$^+$ thymocytes, expressed high levels of Bcl-2 (Fig. 4 *B* and *C*, column 4). CD3^{hi}CD4$^+$CD8^{lo} thymocytes (i.e., cells committed to the CD4 lineage) expressed levels of Bcl-2 slightly higher than that seen in CD3^{hi}CD4$^+$CD8$^+$ cells, but clearly lower than was seen in CD8-lineage-committed cells (Fig. 4 *B–D*, column 4). This difference in Bcl-2 expression was quantified by calculating the ratio of mean channel fluorescence (MCF) of Bcl-2 to MCF of control Ig in each subpopulation. There was a slight increase in the MCF ratio from CD3^{hi}CD4$^+$CD8^{lo} cells (MCF ratio = 1.80) compared with CD3^{hi}CD4$^+$CD8$^+$ cells (MCF ratio = 1.47). However, it was substantially lower than the MCF ratio from CD3^{hi}CD4^{lo}CD8$^+$ (3.47) and CD3^{hi}CD4$^-$CD8$^+$ (3.92) thymocytes. To confirm our findings that Bcl-2 upregulation requires co-receptor engagement, we also examined CD69 expression in thymocytes from control and MHC class II-deficient mice by four-color flow cytometry, since CD69, like CD3, is upregulated in DP thymocytes during the first step of positive selection (4). In the MHC class II-deficient mice, Bcl-2 expression was low in CD69^{lo} or CD69^{hi}CD4$^+$CD8^{lo} cells (data not shown). In contrast, CD69^{hi}CD4^{lo}CD8$^+$ and CD69^{hi}CD4$^-$CD8$^+$ cells were Bcl-2$^+$ (data not shown). The results demonstrate that Bcl-2 expression is independent of TCR/CD3 or CD69 expression during positive selection and confirm that Bcl-2 is upregulated when CD4 or CD8 co-receptor participation is required during positive selection.

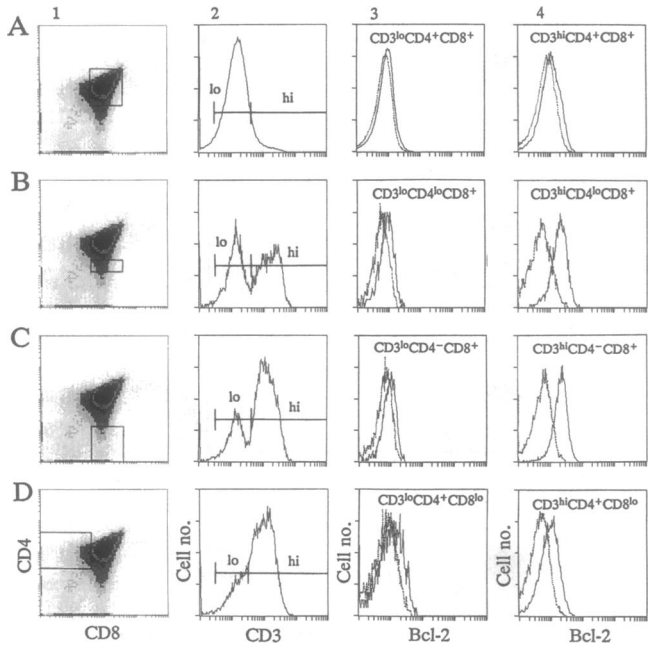

FIG. 4. Bcl-2 expression is not upregulated in CD4$^+$CD8^{lo} thymocytes from MHC class II-deficient mice. Unseparated thymocytes were stained for CD3, CD4, CD8, and Bcl-2. Each subpopulation was analyzed with the same format as in Fig. 2, except that all of the thymocytes committed to the CD4 lineage beyond the CD4$^+$CD8$^+$ phenotype were grouped as CD4$^+$CD8^{lo} (row D).

DISCUSSION

Bcl-2 mRNA and protein expression during human T-cell development is biphasic in that it is high in DN thymocytes, downregulated in DP thymocytes, and upregulated in SP thymocytes (9–11). In this study, to further define the developmental role of Bcl-2 in thymocyte maturation, we have focused on Bcl-2 expression during thymic development in normal mice, mice that express the TCR specific for a male H-Y antigen as a transgene, and MHC class II-deficient mice. Immature CD3$^-$CD4$^-$CD8$^-$ thymocytes are self-renewing stem cells and generally thought to be the precursors to CD3^{lo}CD4$^+$CD8$^+$ thymocytes (1). During this phase of thymic development, rearrangement of the TCR β chain takes place (20). Once productive rearrangement is complete, low levels of the TCR β chain associated with the recently described protein gp33 and CD3 are expressed on the cell surface as a pre-TCR complex (24). However, in H-Y TCR$^+$ mice, productive endogenous β-chain V(D)J rearrangement is blocked (18). When compared with DN thymocytes from control H-2^b mice, DN thymocytes from H-Y TCR$^+$ mice showed no difference in Bcl-2 expression, demonstrating that regulation of Bcl-2 expression was independent of TCR/CD3 expression during this developmental stage. Thus, expression of other receptor(s) and/or intracellular/intercellular signal(s) must regulate Bcl-2 expression during this stage of development. The regulation of Bcl-2 expression during this phase of T-cell maturation is comparable to that of *mcl-1*, a *bcl-2*-related gene whose expression during myeloid cell differentiation is downregulated within hours of receipt of a signal(s) to proceed with maturation (25).

During transition from the DN stage to the DP stage, immature thymocytes have been isolated that express the CD3^{lo}CD4$^-$CD8$^+$ phenotype (22, 26). These cells can differentiate into CD3^{lo}CD4$^+$CD8$^+$ thymocytes when cultured overnight in simple medium (22, 26). When these cells are stimulated via the TCR with anti-TCR β-chain mAbs, spontaneous differentiation into DP thymocytes is inhibited, although, interestingly, these cells do not die (22). Consistent with this commitment to differentiation into the DP stage, our data demonstrate that CD3^{lo}CD4$^-$CD8$^+$ thymocytes from either H-Y TCR$^+$ H-2^b or control H-2^b female mice had already downregulated Bcl-2 expression. This downregulation of Bcl-2 in these cells is curious, given the viability of CD3^{lo}CD4$^-$CD8$^+$ thymocytes demonstrated by other authors (22). Other genes regulating susceptibility to pro-

grammed cell death, such as *bcl-x* (27), *bax* (28), or *nur-77* (29), may be involved.

During the DP stage of T-cell development, it is generally thought that positive selection precedes clonal deletion and the first evidence of positive selection is upregulation of TCR/CD3 (1, 4). Previous work showed that the majority of $CD4^+CD8^+$ thymocytes were $Bcl\text{-}2^{-/lo}$, while a small subpopulation was $Bcl\text{-}2^+$ (9, 10). The data presented here confirm these findings. When $CD4^+CD8^+$ thymocytes from normal and transgenic animals were analyzed for CD3 expression, most but not all of the $CD3^{hi}CD4^+CD8^+$ thymocytes were $Bcl\text{-}2^+$, and these cells were resistant to glucocorticoid-induced cell death. Our results are consistent with the recent findings of Tao *et al.* (30), who have demonstrated that *bcl-2* mRNA is not detectable or is low in $CD3^{lo}CD4^+CD8^+$ thymocytes and is upregulated in $CD3^{hi}CD4^+CD8^+$ thymocytes from H-Y TCR^+ $H\text{-}2^b$ adult female mice. In contrast, Moore *et al.* (31) have shown that *bcl-2* mRNA is detectable in $CD3^{lo}CD4^+CD8^+$ thymocytes from day 18 fetal mice (31). There are several possible reasons for this discrepancy. During T-cell ontogeny, $TCR\alpha\beta^+$ SP thymocytes first appear on day 18, yet these cells express lower levels of the TCR than their adult counterparts (1, 32). Given that upregulation of the TCR is the earliest phenotypic change consistent with positive selection, it is possible that the $CD3^{lo}CD4^+CD8^+$ fetal thymocytes shown by Moore *et al.* (31) to express higher levels of *bcl-2* mRNA have already been positively selected. If so, their findings might in fact be consistent with our results. Alternatively, the difference between our results and those of Moore *et al.* may be secondary to distinct *bcl-2* expression patterns in DP thymocytes at different developmental ages (fetal vs. adult). It also is possible that the discrepancies are attributable to analysis of Bcl-2 protein content versus mRNA content. This seems less likely, given the agreement of our Bcl-2 protein data with the mRNA data of Tao *et al.* (30).

As noted above, our data show that in normal mice a fraction of $CD3^{hi}4^+8^+$ cells, and virtually all apparently more mature cells (i.e., $CD3^{hi}4^+8^{-/lo}$ or $CD3^{hi}4^{-/lo}CD8^+$) express high levels of Bcl-2 (Fig. 2). In contrast, in MHC class II-deficient mice, the failure of CD4-lineage cells to express high levels of Bcl-2 is accompanied by a lack of Bcl-2 upregulation in putatively less mature $CD3^{hi}CD4^+CD8^+$ cells as well. This implies that in normal mice the majority of $Bcl\text{-}2^+CD3^{hi}CD4^+CD8^+$ thymocytes are CD4-lineage-committed cells which have already been through positive selection. As to why so few of these cells are CD8-lineage-committed, we can suggest two possibilities. First, CD8-lineage-committed cells may normally be a small proportion of positively selected DP thymocytes. Their relative higher representation among SP cells and mature T cells could reflect a combination of more efficient maturation vs. CD4-lineage-committed cells or a high rate of loss of CD4-lineage-committed cells due to clonal deletion. Alternatively, it is possible that CD8-committed cells upregulate Bcl-2 at a later stage than CD4-committed cells. This latter possibility seems less likely in our view, since our data on H-Y TCR transgenic mice, where a large percentage of the positively selected cells are CD8-lineage-committed, indicate that the vast majority of $CD3^{hi}CD4^+CD8^+$ cells are $Bcl\text{-}2^+$ (data not shown).

Two models of positive selection, termed selective and instructive, have been proposed (33). Studies using MHC class I- and class II-deficient mice have provided evidence that positive selection may be a two-step process and consistent with the selective model (4–6). This entails two TCR–MHC interactions. The first interaction initiates positive selection, is dependent only on the specificity of the TCR, and leads to "random" downregulation of CD4 or CD8 and upregulation of TCR/CD3 and CD69 (4, 5). The second TCR–MHC interaction completes positive selection and requires coengagement of the appropriate CD4 or CD8 co-receptor for continued maturation as a SP thymocyte and export as a mature MHC-restricted T cell (4). Regardless of whether positive selection proceeds as a two-step or a one-step process, our data show that coengagement of the appropriate CD4 or CD8 co-receptor is required for upregulation of Bcl-2. Our data argue that phenotypic changes of positive selection such as loss of CD4 or CD8 can lag behind intracellular changes such as upregulation of Bcl-2.

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