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Positive and negative selection of T cells in T-cell receptor transgenic mice expressing a *bcl-2* transgene

(T-cell development/tolerance/apoptosis)

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ABSTRACT **To explore the role of *bcl-2* in T-cell development, a *bcl-2* transgene was introduced into mice expressing a T-cell receptor (TCR) transgene encoding reactivity for the mouse male antigen HY presented by the H-2D^b class I antigen of the major histocompatibility complex (MHC). Normal thymic development is contingent on the ability of immature thymocytes to interact with self-MHC molecules presented by thymic stroma (positive selection). Thus, thymocyte numbers are low in female anti-HY TCR transgenic mice with a nonselecting (H-2D^d) background. Expression of *bcl-2* inhibited the death of nonselectable thymocytes since, strikingly, female H-2D^d *bcl-2*/TCR transgenic mice developed normal numbers of CD4$^+$CD8$^+$ thymocytes, although these did not mature further into functional T cells. Hence, TCR–MHC interaction may induce positive selection through two signals, one which saves cells from death by increasing Bcl-2 synthesis and another which promotes maturation. Male H-2D^b anti-HY TCR transgenic mice normally have a very small thymus, due to deletion of the self-reactive T cells. Expression of *bcl-2* reduced the efficiency of deletion, since *bcl-2*/TCR transgenic male mice accumulated 4- to 6-fold more thymocytes than did TCR transgenic male littermates. Anti-HY TCR-expressing cells were also more numerous in the peripheral lymphoid tissues, but these cells expressed abnormally low levels of CD8 coreceptor and were not responsive to the HY antigen. Thus, although *bcl-2* expression hampers the deletion of immature self-reactive cells in the thymus, self-tolerance is maintained.**

Most T lymphocytes developing in the thymus are short-lived (1), because they fail stringent selection criteria (2, 3) which ensure that only cells bearing useful antigen receptors mature for export to the peripheral lymphoid tissues where they regulate immune responses. To survive, immature thymocytes must express T-cell receptors (TCRs) capable of binding to molecules of the major histocompatibility complex (MHC) on thymic epithelial cells (positive selection) (4–7). However, those with receptors which bind with high affinity to self-antigens presented by MHC molecules are induced to die by apoptosis (negative selection), thereby ensuring immunological tolerance (8–12). Selection apparently takes place within the cortex, at the stage when thymocytes express both the CD4 and the CD8 coreceptors (8, 10–13).

The *bcl-2* gene product is an inhibitor of apoptosis in mammalian cells (14, 15) and seems likely to play a role in thymocyte selection, since mature medullary thymocytes contain much more Bcl-2 protein than do immature CD4$^+$CD8$^+$ (abbreviated CD4$^+$8$^+$) cortical cells (16, 17). Furthermore, studies on transgenic mice have shown that constitutive expression of *bcl-2* confers a general survival advantage on T cells, enabling them to resist a variety of cytotoxic influences (18–20), including exposure to antibodies against the CD3 coreceptor, a treatment thought to mimic clonal deletion (21). Despite this survival advantage, T-cell numbers are normal in unmanipulated *bcl-2* transgenic mice, both in the thymus and in the periphery (18).

Previous experiments monitoring the fate of thymocytes with receptors that bind to self-superantigens have led to some disagreement as to whether Bcl-2 inhibits (18, 20) or does not inhibit (19) the deletion of autoreactive thymocytes. We sought a resolution of this question by testing an alternative, non-superantigen-receptor system: TCR transgenic mice expressing a receptor reactive with the male antigen HY presented by class I MHC H-2D^b molecules (for a review see ref. 22). These mice also allowed us to explore the role of *bcl-2* in positive selection.

Our analysis of T-cell development in doubly transgenic *bcl-2*/anti-HY TCR mice of different MHC constitution shows that constitutive expression of *bcl-2* greatly increases the survival of thymocytes in the absence of positive selection. The *bcl-2* transgene also reduces the efficiency of negative selection, but the mature peripheral T cells which appear in increased numbers in doubly transgenic, negatively selecting mice are not functionally autoreactive, despite expressing the transgenic TCR. The implications of these results are that induction of Bcl-2 protein plays a role in positive selection of T cells and that tolerance is safeguarded by a deletion mechanism that can bypass Bcl-2.

MATERIALS AND METHODS

Transgenic Mice. The origin and characteristics of the Eμ-*bcl*-2-25 and -36 strains on a (C57BL/6J × SJL/J)F$_2$ background (18) and the anti-HY TCR$\alpha\beta$ transgenic mouse strain on a C57BL/6J (H-2D$^{b/b}$) or DBA/2J (H-2D$^{d/d}$) background (22) have been described previously. Mice were typed for the *bcl-2* transgenes by DNA assay for a simian virus 40 sequence linked to the human *bcl-2* cDNA (23). Inheritors of the TCR transgene were identified either by immunofluorescence analysis of blood lymphocytes with fluorescein-anti-Thy-1.2 (Becton Dickinson) and biotinyl F23.2 anti-TCRVβ8.2 with phycoerythrin–streptavidin (Caltag, South San Francisco, CA) or by Southern blot hybridization of a TCR Jβ2 probe to tail DNA. H-2D$^{d/d}$ *bcl-2*-36 severe combined immunodeficiency (scid) mice were produced by serial crosses of Eμ-*bcl-2*-36 mice with H-2D$^{d/d}$ BALB/c.B-17-*scid* mice (24). H-2D$^{d/d}$ progeny were selected by H-2 typing and then crossed with (H-2D$^{d/d}$ anti-HY TCR transgenic × C.B-17-scid)F$_1$ mice. Homozygotes for the *scid* mutation were identified by their lack of immunoglobulin-bearing cells after immunofluorescence staining and flow cytometric analysis of peripheral blood. Inheritors of the (hemizygous) *bcl-2* transgene were identified by DNA dot hybridization with a simian virus 40 probe and by the fact that they have 10- to 20-fold

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Abbreviations: TCR, T-cell receptor; MHC, major histocompatibility complex; scid, severe combined immunodeficiency.

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more CD45R(B220)-bearing, surface immunoglobulin-negative peripheral blood B-lineage cells than do littermates lacking the transgene (unpublished observations).

Immunofluorescence Staining and Flow Cytometric Analysis. Dispersed thymocytes, lymph node cells, or spleen cells were stained as previously described (18) with fluorescein-anti-CD8 and phycoerythrin–anti-CD4 (both from Becton Dickinson) and with biotinyl T3.70 (25) (anti-transgenic TCRα chain) plus Tricolor-streptavidin (Caltag); 5000–10,000 viable cells (not stained by propidium iodide) were analyzed in a FACScan flow cytometer (Becton Dickinson). For H-2 typing of mice, blood leukocytes were tested for staining with monoclonal antibodies against H-2^s, H-2^b, and H-2^d (K43, 28-8-6, and KH95 from PharMingen).

***In Vitro* Stimulation of TCR Transgenic CD4$^-$8$^+$ T Cells.** Dispersed lymph node cells from 6- to 10-week-old female and male H-2D^b anti-HY TCR transgenic and *bcl-2*/anti-HY TCR doubly transgenic mice were stained with fluorescein-anti-Thy-1.2 and phycoerythrin-anti-CD4. CD4$^-$8$^+$ T cells were obtained by fluorescence-activated sorting for Thy-1.2$^+$ CD4$^-$ cells with a FACSII (Becton Dickinson). The sorted cells were tested at a range of concentrations for mitogenic responsiveness to irradiated (30 Gy) female or male C57BL/6J-*nu* spleen cells (3×10^6 cells per ml) or, after including irradiated (30 Gy) female C57BL/6J-*nu* spleen cells as filler cells, to T3.70 anti-mouse transgenic TCRα monoclonal antibody (2 μg/ml) or concanavalin A (2 μg/ml). They were cultured in flat-bottom 96-well plates in medium (100 μl per well) supplemented with recombinant mouse interleukin 2 (100 units/ml). Cultures stimulated with T3.70 or concanavalin A were incubated for 3 days, while those stimulated with spleen leukocytes were incubated for 4 days. All cultures were pulsed with [^{3}H]thymidine [1 μCi (37 kBq) per well] during the last 6 hr of incubation.

RESULTS

Accumulation of Autoreactive CD4$^+$8$^+$ Cells in the Thymus. In male H-2D^b anti-HY TCR transgenic mice, developing thymocytes are self-reactive and almost all are therefore eliminated at the CD4$^+$8$^+$ stage of development (9). Expression of the *bcl-2* transgene permitted the mice to accumulate substantial numbers of CD4$^+$8$^+$ thymocytes (Fig. 1*A*-I). On average, male H-2D$^{b/d}$ and H-2D$^{b/b}$ *bcl-2*/anti-HY TCR transgenic mice had 6- to 8-fold more CD4$^+$8$^+$ thymocytes than did male siblings expressing just the TCR transgene (Table 1, groups I and II). Most of these cells expressed the HY-specific TCR (Fig. 1*A*-II and -III; Table 1, groups I and II), which excluded the possibility that they had escaped

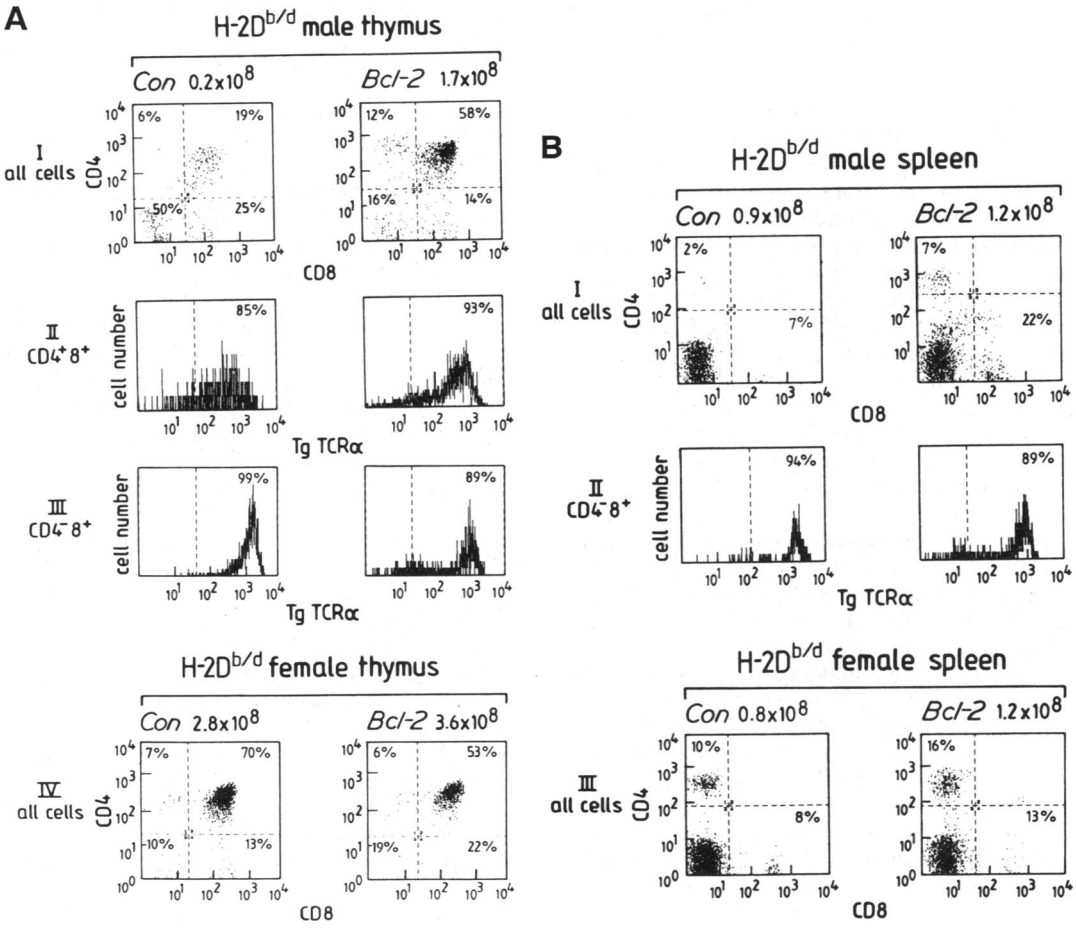

FIG. 1. Constitutive expression of *bcl-2* in anti-HY TCR transgenic mice causes an accumulation of CD4$^+$8$^+$ thymocytes and CD4$^-$8^{low} splenic T cells which express the transgenic TCR. (*A*) Thymocytes from 6-week-old H-2D$^{b/d}$ male (I–III) and female (IV) anti-HY TCR transgenic (*Con, Left*) and *Bcl-2*/anti-HY TCR doubly transgenic mice (*Bcl-2, Right*) were stained with monoclonal antibodies specific for CD4, CD8, and the transgenic (Tg) TCRα chain (T3.70) (25) and characterized by flow cytometric analysis. I and IV show the distribution of CD4 and CD8 among all viable thymocytes; other panels show the expression of the transgenic TCRα chain on CD4$^+$8$^+$ (II) and CD4$^-$8$^+$ (III) thymocytes gated from the cells shown in I. The data shown are from mice derived by crossing Eμ-*bcl-2*-25 (H-2D$^{d/d}$) and anti-HY TCR (H-2D$^{b/b}$) mice. (*B*) Spleen cells from the same 6-week-old male (I and II) and female (III) H-2D$^{b/d}$ anti-HY TCR transgenic (*Con, Left*) and Eμ-*bcl-2*-25/anti-HY TCR doubly transgenic mice (*Bcl-2, Right*) as in *A* were analyzed by immunofluorescence and flow cytometry as above. I and III show CD4 and CD8 expression within the total population of viable cells. II shows the transgenic TCRα chain expression on CD4$^-$8$^+$ cells gated from the total population of I.

Table 1. Influence of *bcl-2* transgene on T-cell selection in anti-HY TCR transgenic mice

Group	*bcl-2*	H2-D	*n*	Thymus: Total cells × 10^{-7}	Thymus: $CD4^+8^+$ Cells × 10^{-7}	Thymus: $CD4^+8^+$ % Tg $TCR\alpha^+$	Spleen $CD4^-8^+$ cells × 10^{-7}
I. Male	−	b/d	3	4.3 ± 2.2	1.4 ± 1.0	91 ± 7	0.9 ± 0.4
	+	b/d	13	17 ± 5	8.0 ± 4.0	81 ± 10	2.8 ± 0.7
II. Male	−	b/b	4	1.6 ± 0.8	0.1 ± 0.1	86 ± 9	0.8 ± 0.3
	+	b/b	3	9 ± 5	0.8 ± 0.4	71 ± 9	1.7 ± 0.4
III. Female	−	b/d	5	38 ± 8	28 ± 7	82 ± 4	0.9 ± 0.4
	+	b/d	9	39 ± 7	23 ± 6	69 ± 8	1.2 ± 0.3
IV. Female	−	d/d	8	14 ± 6	11 ± 5	41 ± 11	1.2 ± 0.2
	+	d/d	7	30 ± 4	22 ± 2	38 ± 5	2.5 ± 0.4
V. Female *scid/scid*	−	d/d	9	6.1 ± 2.6	3.7 ± 1.5	86 ± 4	<0.1
	+	d/d	7	21 ± 11	16 ± 7	87 ± 5	<0.1

Mice of appropriate H-2 haplotypes were produced by backcrossing hemizygous *bcl-2* and anti-HY TCR transgenic (Tg) animals with C57BL/6, DBA/2, or C.B-17-scid strains and by H-2 typing with monoclonal antibodies. These were then intercrossed to produce TCR and TCR/*bcl-2* transgenic mice. The cell number within each subset was calculated from the total thymus or spleen cell number and the percentage of cells within that subset derived from a CD8 versus CD4 flow cytometric dot plot. As no differences were found between Eμ-*bcl-2*-25/anti-HY TCR and Eμ-*bcl-2*-36/anti-HY TCR doubly transgenic mice, those data were pooled. The numbers shown are arithmetic means ± SD. The indicated number of mice (*n*) in each group was analyzed at 4–8 weeks of age. Levels of CD8 expression on $CD4^-CD8^+$ spleen cells, measured as mean fluorescence intensity by flow cytometry, were 344 ± 49 units for group III, 167 ± 44 for group I, and 110 ± 16 for group II mice and were not affected by the *bcl-2* transgene. The frequency of $CD4^-CD8^+$ spleen cells that were $TCR\alpha^+$ was also unaffected by *bcl-2* and was 91% ± 3% for group I, 92% ± 4% for group II, 45% ± 7% for group III, and 44% ± 5% for group IV; the relatively low frequency in females has been reported previously for TCR mice (26); the expansion of clones expressing endogenous TCRα chain presumably represents selection for useful specificities.

death because they expressed endogenous TCR genes instead of the transgene. The transgene-encoded Bcl-2 protein was readily detectable by immunofluorescence and Western blotting using an antibody specific for human Bcl-2 (data not shown) in thymocytes of *bcl-2* transgene-bearing mice.

Clearly, the presence of increased levels of Bcl-2 protein had significantly impeded the deletion of autoreactive thymocytes. Nevertheless, the number of $CD4^+8^+$ thymocytes in male doubly transgenic mice, especially those homozygous for the selecting MHC molecule, $H\text{-}2D^{b/b}$ (Table 1, group II), was very much lower than in female mice (Fig. 1*A*-IV; Table 1, group III). It was thus apparent that the resistance to deletion conferred by Bcl-2 was only partial. Expression of a *bcl-2* transgene has also been found to only partially inhibit deletion of self-superantigen-reactive T cells in normal mice (18, 20) and the deletion of autoreactive B cells in immunoglobulin transgenic mice (27).

Increased Numbers of Nonresponsive $CD4^-8^{low}$ T Cells in the Periphery. Male $H\text{-}2D^b$ anti-HY TCR transgenic mice have been observed to harbor a population of peripheral $CD4^-8^+$ T cells which express the transgenic TCR$\alpha\beta$ heterodimer but have low levels of CD8 compared with normal mature $CD8^+$ T cells (25). We found an equivalent, but 3- to 5-fold more abundant, population in the spleen (Fig. 1*B*) and lymph nodes (data not shown) of $H\text{-}2D^{b/d}$ and $H\text{-}2D^{b/b}$ *bcl-2*/anti-HY TCR transgenic males. The average intensity of CD8 staining on these cells was ½ to ⅓ of that on comparable populations in female anti-HY TCR transgenic mice (see legend to Table 1). Since the cells were small and therefore not cycling, the increased number presumably reflected an extended life span conferred by *bcl-2* expression.

Like the peripheral $CD4^-8^+$ T cells from male $H\text{-}2D^b$ anti-HY TCR mice (25), those from the *bcl-2*/anti-HY TCR transgenic mice were unresponsive to stimulation with male $H\text{-}2D^b$ cells, in contrast to the control cells from corresponding female mice (Fig. 2*B*). The male cells were not inherently anergic because they proliferated normally in response to treatment with concanavalin A or with antibody against the transgenic TCRα chain (ref. 25 and Fig. 2 *C* and *D*). The origin of these T cells is presently unclear. Since the CD8 and CD4 coreceptors play an essential role in signaling via the TCR, the low level of CD8 coreceptor may have enabled them to escape deletion in the thymus (28, 29). Alternatively, they may have developed from an unusual $CD4^-8^-$ population which expresses the TCR but is not subject to negative selection (30, 31).

***bcl-2* Expression Enhances Survival of Thymocytes in the Absence of Positive Selection.** Female mice lack the HY antigen and those of $H\text{-}2D^b$ haplotype provide an appropriate environment for positive selection of thymocytes expressing the anti-HY transgene. Thus, thymocytes expand in number and differentiate in female $H\text{-}2D^b$ anti-HY TCR transgenic mice (4, 22) and, because CD8 binds to class I MHC molecules, mature $CD4^-8^+$ cells predominate over $CD4^+8^-$ cells. In the presence of a nonselecting MHC molecule such as $H\text{-}2D^{d/d}$, however, most of the transgenic thymocytes develop only to the immature $CD4^+8^+$ stage and then die (4, 22). As expected, we found that both immature ($CD4^+8^+$) and mature ($CD4^-8^+$ and $CD4^+8^-$) thymocytes were reduced in number in female $H\text{-}2D^{d/d}$ anti-HY TCR transgenic mice compared with their counterparts with a selecting ($H\text{-}2D^b$) MHC haplotype (Table 1, compare groups III and IV). In striking contrast, $H\text{-}2D^{d/d}$ TCR females harboring a *bcl-2* transgene developed normal numbers of thymocytes (Table 1, group IV). This result suggested that increased Bcl-2 had extended the survival of thymocytes which failed positive selection. A caveat to this conclusion is that a sizeable proportion (≈60%) of the thymocytes in these nonselecting animals lacked transgenic TCRα chains (Table 1, group IV). Instead, they expressed TCR heterodimers containing transgenic β and endogenous α chains (4, 22), presumably because endogenous TCRα gene rearrangement can occur until the receptor interacts with self-MHC molecules (32, 33).

To avoid the complication of endogenous TCRα chain expression, we introduced the TCR and *bcl-2* transgenes into scid mice, creating a situation in which all thymocytes express the anti-HY receptor but are unable to make productive rearrangements of endogenous TCR genes (24). Despite the nonselecting MHC context ($H\text{-}2D^{d/d}$), female scid mice expressing both transgenes had 3- to 4-fold more thymocytes on average than did scid littermates bearing just the TCR transgene (2.1×10^8 versus 6.1×10^7). Indeed, the levels approached those achieved by positive selection in female $H\text{-}2D^{b/d}$ anti-HY TCR transgenic mice (compare

groups III and V in Table 1). The number of precursor ($CD4^-8^-$) thymocytes was essentially unaffected by introduction of the *bcl-2* transgene. The increased cellularity was primarily due to an increase in typical $CD4^+8^+$ cortical thymocytes, most of which expressed the transgenic α and β TCR chains (Fig. 3 *A* and *B*; Table 1, group V) at a lower level than typically displayed by post-positive-selection thymocytes (see Fig. 1). The *bcl-2*/TCR/scid thymocytes were small (Fig. 3*C*), and cell cycle analysis showed that essentially all of them were in G_0/G_1 (data not shown), in contrast to TCR/scid thymocytes, which were large and mitotically active. The latter died within 3 days when placed in tissue culture, while the former could survive for many days (30–60% viability on day 7; data not shown). Taken together, these observations suggest that the increase in thymocytes in the *bcl-2*/anti-HY TCR/scid mice resulted from enhanced longevity *in vivo* of unselected $CD4^+8^+$ cells.

Differentiation Requires More Than a Survival Signal. Mature $CD4^-8^+$ (or $CD4^+8^-$) T cells were not found in significant numbers in female H-2D^d *bcl-2*/TCR scid mice (Fig. 3*A* and data not shown). The small number of $CD4^-8^+$ (and $CD4^+8^-$) cells found in the thymus expressed only low levels of CD8 (or CD4) and the transgenic TCR (Fig. 3) and were probably early developmental intermediates, between the $CD4^-8^-$ and $CD4^+8^+$ stages (34). Thus, enhancing the survival of nonselectable $CD4^+8^+$ thymocytes was not sufficient to permit further differentiation and export to the periphery.

DISCUSSION

By introducing a *bcl-2* transgene into anti-HY TCR transgenic mice and monitoring the development of T lymphocytes in mice of various MHC haplotypes, we have shown here that constitutive expression of *bcl-2* can markedly inhibit the deletion of thymocytes whose antigen receptors cannot bind to self-MHC molecules. This suggests that induction of Bcl-2 may be an important component of the normal mechanism of positive selection of T cells. Consistent with this hypothesis, the mature postselection T cells that populate the thymus medulla and occupy peripheral lymphoid tissues have been found to express considerably more Bcl-2 protein than do the immature cells of the cortex (16, 17). Importantly, the enhanced survival of $CD4^+8^+$ thymocytes in female H-2D^d *bcl-2*/anti-HY TCR/scid mice was not accompanied by the appearance of mature $CD4^-8^+$ (or $CD4^+8^-$) cells. Thus, mere survival is not sufficient for the further differentiation of $CD4^+8^+$ cells. This observation makes the prediction that, during normal T-cell development, the interaction between the TCR and MHC molecules induces at least two responses in thymocytes: a survival signal, postulated to involve Bcl-2, and a differentiation signal (28).

Expression of Bcl-2 reduced the efficiency of negative selection, since substantial numbers of viable thymocytes displaying the transgenic anti-HY TCR were evident in male H-2D^b *bcl-2*/TCR transgenic mice. We have previously noted that our *bcl-2* transgenic mice harbor increased numbers of thymocytes bearing receptors reactive with self-superantigens (18). The *bcl-2* mice analyzed by Siegel *et al.* (20) also contained more thymocytes reactive with self-superantigens, but those studied by Sentman *et al.* (19) apparently did not. The reason for this discrepancy is not clear, but it may relate to the level and/or timing of expression of each transgene in the course of T-cell development.

Despite the clear evidence that *bcl-2* transgene expression hampers the deletion of thymocytes expressing the transgenic TCR, the peripheral T cells in male H-2D^b *bcl-2*/anti-HY TCR transgenic mice were not functionally autoreactive, apparently because CD8 levels were too low. Conceivably, all cells capable of interacting with self-MHC complexes are induced to increase their expression of *bcl-2*, irrespective of whether the MHC molecules are associated with self or nonself antigen. If so, it makes considerable

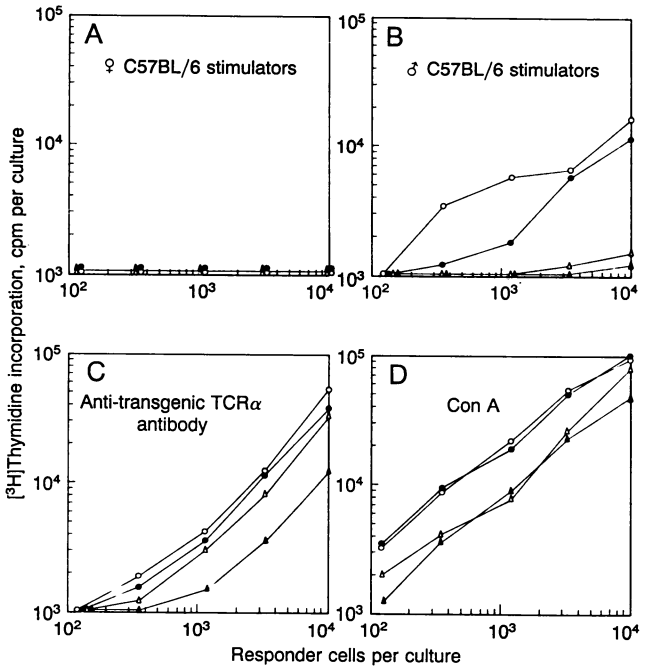

FIG. 2. Male H-2D^b TCR and *bcl-2*/TCR peripheral T cells do not respond *in vitro* to male H-2D^b stimulator cells but proliferate normally in response to concanavalin A and antibody to transgenic TCRα chain. Responder cells: ○, Female anti-HY TCR; ●, female *bcl-2*/anti-HY TCR; △, male anti-HY TCR; ▲, male *bcl-2*/anti-HY TCR. This figure represents one typical experiment of three performed with sorted T cells from individual female and male H-2D^b TCR and *bcl-2*/TCR transgenic mice. Similar results were obtained with unsorted lymph node or spleen cells from H-2D^b female and male TCR and *bcl-2*/TCR transgenic mice.

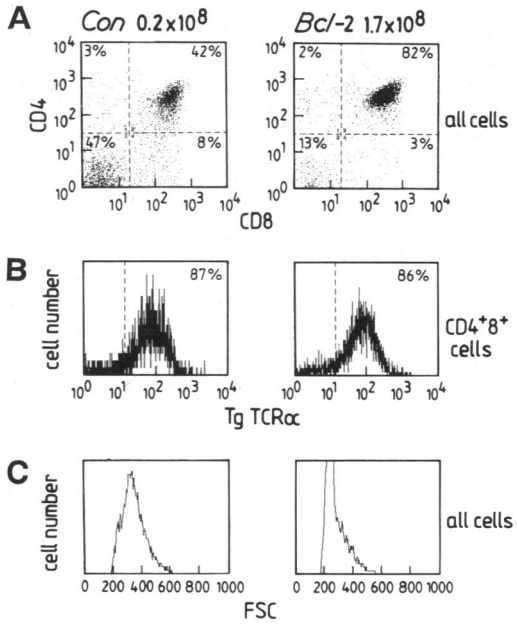

FIG. 3. Constitutive expression of *bcl-2* causes the accumulation of thymocytes in the absence of positive selection. Thymocytes from 5-week-old female H-2D$^{d/d}$ TCR and *bcl-2*/TCR transgenic scid mice were analyzed by immunofluorescence and flow cytometry. (*A*) Distribution of CD4 and CD8 among all viable thymocytes. (*B*) Expression of the transgenic (Tg) TCRα chain on $CD4^+8^+$ thymocytes gated from the cells shown in *A*. (*C*) Forward light scatter (FSC), which correlates with cell size for the viable thymocytes.

biological sense that the *bcl-2* transgene offered only partial protection to autoreactive T cells, since self-tolerance would be in jeopardy if negative selection operated through a mechanism that could be blocked by Bcl-2.

Negative selection is thought to involve induction of increased intracellular calcium in immature $CD4^+8^+$ cells (35). Since *bcl-2* expression can greatly reduce the sensitivity of thymocytes to treatment with the calcium ionophore ionomycin (18, 20), it would appear that the presence of Bcl-2 is ultimately inadequate to prevent the lethal effects of calcium mobilization. Alternatively, and more likely, the deletion mechanism also activates a death pathway that is not blocked by Bcl-2.

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