

# Positive Selection of $V\beta 8^+ CD4^- 8^-$ Thymocytes by Class I Molecules Expressed by Hematopoietic Cells

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## Summary

A small subset of T cells of mature phenotype express the  $\alpha/\beta$  T cell receptor, but not CD4 and CD8 coreceptors ( $\alpha/\beta$  double-negative [DN] cells). The repertoire of  $V\beta$  usage of  $\alpha/\beta$  DN cells is strongly biased towards  $V\beta 8$  expression, suggesting that the formation of the population is subject to selection. We now report that deficiency of class I expression leads to a strongly depressed frequency of  $V\beta 8^+$  DN cells, but has little effect on  $V\beta 8^-$  DN cells. Studies of hematopoietic chimeras between class I<sup>+</sup> and class I<sup>-</sup> mice demonstrated that expression of class I molecules by hematopoietic cells is necessary and sufficient for selection of most  $V\beta 8$  DN cells. The lack of a role for class I expression by thymic epithelial cells suggests that the mechanism of selection of these cells by class I differs significantly from the mechanism of selection of conventional T cells. Models to explain the selection of these cells as well as their possible function *in vivo* are discussed.

In most strains of mice, ~15–30% of adult thymic  $CD4^- 8^-$  (double-negative [DN]<sup>1</sup>) cells express the TCR- $\alpha/\beta$  (thymic  $\alpha/\beta$  DN cell) (1–3). These cells resemble mature conventional T cells in their pattern of phenotypic markers ( $CD2^{hi}$ ,  $CD5^{hi}$ ,  $Qa-2^+$ ,  $HSA^-$ , and  $pgp-1^{hi}$ ). In addition, they can be stimulated by engagement of their TCRs to proliferate and secrete IL-4 (2, 4, 5). Also, like conventional TCR- $\alpha/\beta^+$  T cells,  $\alpha/\beta^+$  DN thymocytes are susceptible to cyclosporin A-induced arrest during development, suggesting that a TCR engagement event is required for their maturation (6). Unlike conventional T cells,  $\alpha/\beta^+$  DN thymocytes appear rather late in ontogeny (at about the time of birth) (2, 6). It is not known whether  $\alpha/\beta$  DN cells carry out a specific immunological function or, alternatively, represent a byproduct of the developmental process that generates conventional  $CD4^+$  or  $CD8^+$ , TCR<sup>hi</sup> T cells. A possibly related population of TCR- $\alpha/\beta^+$  DN cells is enormously expanded in the peripheral lymphoid organs of mice with the inherited lymphoproliferative disorders conferred by the *lpr* and *gld* mutations.

Approximately 60% of thymic  $\alpha/\beta$  DN cells express a TCR- $V\beta 8$  ( $V\beta 8$ ) chain (1–3, 6, and Fig. 1). In contrast,  $V\beta 8$  is expressed by only ~20% of mature thymic and peripheral T cells. The  $V\beta 8$  family comprises three closely related genes:  $V\beta 8.1$ ,  $V\beta 8.2$ , and  $V\beta 8.3$ . The disproportionately high frequency of thymic  $V\beta 8^+$  DN cells is due primarily to the

overexpression of  $V\beta 8.2$  (1, 6, 7). Among conventional T cells, skewed  $V\beta$  repertoires often indicate biased selection dependent upon self-MHC and/or self-superantigen expression. Such selection can be either negative or positive (8–12). Surveys of different mouse strains have failed to establish a link between  $V\beta 8$  overexpression among thymic  $\alpha/\beta$  DN cells and expression of specific MHC haplotypes (1, 6). On the other hand, MHC and superantigen expression can in some instances result in depressed numbers of thymic  $\alpha/\beta$  DN cells expressing certain  $V\beta$ s, mirroring the effect of superantigens upon the development of conventional T cells. This includes  $V\beta 8.2^+$  DN cells, which are reduced in frequency in mice that are administered the bacterial superantigen SEB at birth (6). Thus, while there is evidence that self-antigen expression can delete thymic  $\alpha/\beta$  DN cells, there is no evidence that interactions with MHC or MHC-like molecules are required to generate these cells.

A rigorous way to assess the role of MHC molecules in shaping the skewed  $V\beta$  repertoire of thymic  $\alpha/\beta$  DN cells is to examine mice deficient in expression of MHC molecules. Mice homozygous for a defective  $\beta_2$ -microglobulin gene ( $\beta_2m^-$ ) are grossly deficient in cell surface expression of class I molecules (13–15). Only very low levels of functional class I molecules have been detected on lymphoid cells of  $\beta_2m^-$  mice (15, 16). However, these levels are insufficient for positive selection of the vast majority of mature  $CD8^+$  T cells, as shown by the 20–50-fold reduction in their frequency in  $\beta_2m^-$  compared with  $\beta_2m^+$  mice (13, 14, 17). Using such mice as well as class II-deficient mice, we have asked what role MHC expression might play in shaping the

<sup>1</sup> Abbreviations used in this paper:  $\beta_2m$ ,  $\beta_2$ -microglobulin; DN, double negative.

V $\beta$  repertoire of thymic  $\alpha/\beta$  DN cells. Our results provide the first indication that class I expression is necessary for the appearance of most V $\beta$ 8<sup>+</sup> DN thymocytes. Surprisingly, however, V $\beta$ 8 overexpression among  $\alpha/\beta$  DN cells required class I expression by hematopoietic cells, rather than thymic epithelial cells. This contrasts sharply with positive selection of CD8<sup>+</sup> or CD4<sup>+</sup> T cells, where thymic epithelial cells and not hematopoietic cells play the major role in directing positive selection. These results suggest that production of V $\beta$ 8<sup>+</sup> DN cells represents a novel thymus selection pathway.

## Materials and Methods

**Mice.** C57BL/6 mice were purchased from The Jackson Laboratory (Bar Harbor, ME).  $\beta_2m^-$  mutant mice (-/-) and wild-type littermates (+/+ and +/-) were bred in a pathogen-free environment at the University of California, Berkeley. In most experiments the  $\beta_2m^-$  mice used were the fifth generation backcross generation of the original 129 strain to C57BL/6. In some experiments (see legend to Fig. 1), third generation backcross of 129 to C57BL/6 and (129  $\times$  C57BL/6)F<sub>2-5</sub> animals were also used.  $\beta_2m^-$  genotype was assayed by Southern blot and PCR (18). All nonchimeric mice were used between 4 and 12 wk of age. Mice deficient for class II expression due to a targeted mutation in the A $\beta^b$  gene (19), and backcrossed five times to B6, were purchased from GenPharm International (Mountain View CA).

**Antibodies.** F23.1 (anti-V $\beta$ 8.1, 8.2, 8.3) (20), F23.2 (anti-V $\beta$ 8.2) (20, 21), and H57.597-2.1 (anti TCR  $\beta$ ) (22) were purified from hybridoma culture supernatants and biotinylated according to standard procedures. Other immunofluorescence staining reagents were obtained from commercial vendors: RM4.4-PE (anti-CD4: Pharmingen, San Diego, CA), 53.67.2-PE (anti-CD8 $\alpha$ : Pharmingen), 53.67.2-FL (Becton Dickinson & Co., Sunnyvale, CA), tricolor-streptavidin (APC-SA) (Caltag Laboratories, South San Francisco, CA), and AF6-88.5-FL (anti-K<sup>b</sup>: Pharmingen).

**Production of Irradiation Fetal Liver Chimeric Mice.** 10<sup>7</sup> fetal liver cells, obtained from embryonic day 16 fetuses, were injected intravenously into groups of mice that had received 980 rad from a <sup>137</sup>Cs source  $\sim$ 2 h earlier. All  $\beta_2m^-$  recipients and  $\beta_2m^-$  fetal liver donors were fifth generation C57BL/6 backcross mice. All  $\beta_2m^+$  mice and  $\beta_2m^+$  fetal liver donors were inbred C57BL/6 mice. Chimeric mice were housed in a pathogen-free environment before being killed for analysis between 14 and 19 wk after reconstitution.

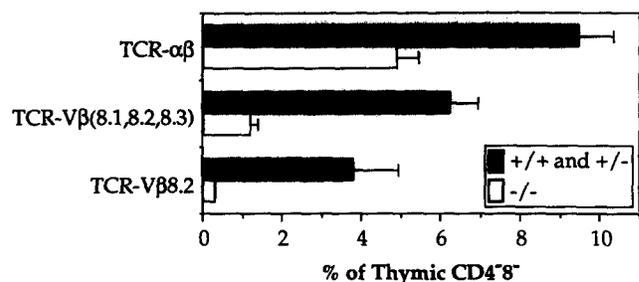
**Antibody plus Complement Depletion of CD4<sup>+</sup> and CD8<sup>+</sup> Cells.** Thymocytes were resuspended at 20–25  $\times$  10<sup>6</sup> cells/ml in 5% FCS, the optimal dilution of anti-CD4 antibody (RL172) and anti-CD8 $\alpha$  antibody (AD4[15]), and a mixture of rabbit and guinea pig complement. After incubation at 37°C for 40 min, viable cells were recovered by passage over a Ficoll gradient and washed several times before further analysis.

**Immunofluorescence Staining and FACS<sup>®</sup> Analysis.** A two-step staining protocol was used to analyze enriched DN thymocytes. In the first step, biotinylated anti-TCR reagent, anti-CD4-PE, and anti-CD8-PE (or anti-CD8-FL) were added to exclude residual CD4<sup>+</sup> and CD8<sup>+</sup> cells; the cytotoxic antibodies used to enrich DN thymocytes do not significantly block staining by the anti-CD4 and anti-CD8 immunofluorescence reagents used (data not shown). The second step consisted of tricolor-streptavidin. To discriminate between donor and host-derived cells when chimeric mice were analyzed, anti-K<sup>b</sup>-FL was added to the cocktail of first-step reagents. Suspensions of 10<sup>5</sup> to 10<sup>6</sup> cells were stained in a final volume of 25  $\mu$ l for 20 min on ice. Staining buffer consisted of

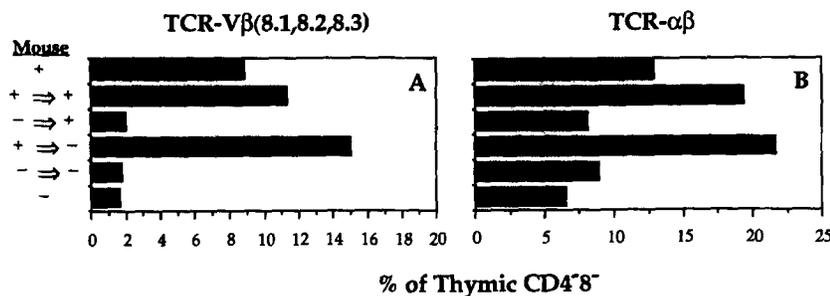
PBS, 5% FCS, 0.02% NaN<sub>3</sub>. Between staining steps, cells were washed two times in 200  $\mu$ l of staining buffer. Before fixation in 200  $\mu$ l of 1% paraformaldehyde, cells were washed two times in staining buffer and one time in PBS. Stained cells were stored, foil wrapped, at 4°C until analysis. Two- and three-color analysis was performed on a FACScan<sup>®</sup> flow cytometer (Becton Dickinson & Co.). Typically, 1–5  $\times$  10<sup>4</sup> events were collected per sample. Dead cells and debris were electronically excluded from analysis by forward and side scatter characteristics.

## Results

**Reduced Frequency of Thymic  $\alpha/\beta$  DN Cells in Class I-deficient Mice.** To examine the frequencies of thymic  $\alpha/\beta$  DN cells, DN thymocytes were prepared by cytotoxic elimination of CD4<sup>+</sup> and CD8<sup>+</sup> cells. The surviving cells were examined by two-color flow cytometric analysis, with TCR-specific antibodies vs. CD4- and CD8-specific antibodies, to exclude any CD4<sup>+</sup> or CD8<sup>+</sup> cells that escaped cytotoxic elimination (see Materials and Methods). The results revealed that the frequency of thymic V $\beta$ 8<sup>+</sup> DN cells in MHC class I-deficient ( $\beta_2m^-$ ) mice was reduced approximately fivefold in comparison with  $\beta_2m^+$  mice (Fig. 1). Most of the V $\beta$ 8<sup>+</sup> cells among thymic  $\alpha/\beta$  DN cells in normal mice are V $\beta$ 8.2<sup>+</sup>, prompting us to examine V $\beta$ 8.2 expression as well. The frequency of thymic DN cells expressing V $\beta$ 8.2 was reduced >10-fold in class I-deficient ( $\beta_2m^-$ ) mice in comparison with  $\beta_2m^+$  mice (Fig. 1). The frequency of thymic DN cells expressing any TCR- $\alpha/\beta$  was reduced only about twofold in  $\beta_2m^-$  mice (Fig. 1). Because V $\beta$ 8<sup>+</sup> cells account for  $\sim$ 50% of  $\alpha/\beta$  DN cells in normal mice, these results suggest the deficit in the  $\beta_2m^-$  mice is primarily in the V $\beta$ 8<sup>+</sup> subset. The sizes of the thymi and the proportion of total DN cells were similar in class I-deficient and normal mice (data not shown). The results demonstrate that



**Figure 1.** Frequency of TCR- $\alpha/\beta$ <sup>+</sup> thymic DN cells is reduced in class I-deficient ( $\beta_2m^-$ ) mice. CD4- and CD8-depleted thymocytes from  $\beta_2m^+$  (+/+ and +/-) or  $\beta_2m^-$  (-/-) mice were analyzed by two-color flow cytometry. Data are pooled results from fifth and third generation C57BL/6 backcross, hybrid (C57BL/6  $\times$  129)F<sub>2-5</sub>, and C57BL/6 mice. A similar pattern was observed when these types of animals were examined separately. The percent of DN cells stained above background (second reagent alone) is shown for anti-TCR- $\alpha/\beta$  ( $\beta_2m^+$ , n = 22;  $\beta_2m^-$ , n = 13), anti-V $\beta$ (8.1, 8.2, 8.3) ( $\beta_2m^+$ , n = 16;  $\beta_2m^-$ , n = 8), and anti-V $\beta$ 8.2 ( $\beta_2m^+$ , n = 10;  $\beta_2m^-$ , n = 3). Simultaneous staining with anti-CD4-PE and anti-CD8-PE allowed electronic exclusion of residual CD4<sup>+</sup> and CD8<sup>+</sup> cells (see Materials and Methods). Bars represent the SEM.



**Figure 2.** Class I expression by thymic hematopoietic cells is necessary and sufficient for the appearance of most Vβ8<sup>+</sup> thymic DN cells. Three-color flow cytometric analysis was used to measure the frequency of TCR-Vβ8<sup>+</sup> (A) and TCR-α/β<sup>+</sup> (B) cells among thymic CD4<sup>+</sup>8<sup>-</sup> cells from irradiation fetal liver chimeras (+ → +, [β<sub>2m</sub><sup>+</sup> fetal liver → β<sub>2m</sub><sup>+</sup>]; + → -, [β<sub>2m</sub><sup>+</sup> fetal liver → β<sub>2m</sub><sup>-</sup>]; - → +, [β<sub>2m</sub><sup>-</sup> fetal liver → β<sub>2m</sub><sup>+</sup>]; - → -, [β<sub>2m</sub><sup>-</sup> fetal liver → β<sub>2m</sub><sup>-</sup>]; +, C57BL/6 mice; and -, fifth generation C57BL/6 back-cross β<sub>2m</sub><sup>-/-</sup> mice). In addition to anti-CD4-PE and anti-CD8-PE, used to electronically exclude residual CD4<sup>+</sup> and CD8<sup>+</sup> thymocytes, anti-K<sup>b</sup>-FL was used to exclude host-derived cells in (+ → -) and (- → +) chimeras. In all cases, >95% of thymocytes were of donor origin. This experiment was repeated two more times with similar results in a separate study where the mice had received intraperitoneal injections of 200 μg of poly(I:C).

normal cell surface expression of class I molecules is required for the appearance of most thymic Vβ8<sup>+</sup> DN cells.

**Role of MHC Expression by Hematopoietic Cells in the Appearance of Thymic α/β DN Cells.** The processes of positive and negative selection involve intercellular interactions between developing thymocytes and thymic stromal cells, of which there are two broad types: bone marrow-derived hematopoietic cells and thymic epithelial cells. Thymic epithelial cells play the major role in directing positive selection (10, 23, 24). Positive selection mediated by bone marrow-derived hematopoietic cells is inefficient, and is observed mainly when positive selection by thymic epithelial cells is prevented (17). Negative selection, in contrast, is directed efficiently by thymic hematopoietic cells (24, 25), and in some instances also by thymic epithelial cells (26, 27) (B. J. Fowlkes, personal communication). We asked whether the requirement for class I molecules exhibited by thymic Vβ8<sup>+</sup> DN cells could be differentially met by expression on hematopoietic cells vs. thymic epithelial cells.

We used irradiation hematopoietic chimeras to target class I expression to specific tissues. β<sub>2m</sub><sup>-</sup> or β<sub>2m</sub><sup>+</sup> fetal liver cells were transferred into groups of lethally irradiated β<sub>2m</sub><sup>+</sup> or β<sub>2m</sub><sup>-</sup> recipients. The thymic epithelial cells in such chimeras are of host origin, whereas almost all the hematopoietic cells are derived from the fetal liver donor. 3–4 mo later the chimeras were killed and the frequencies of donor-derived thymic Vβ8<sup>+</sup> DN cells, TCR-α/β<sup>+</sup> DN cells, and CD8<sup>+</sup> TCR-α/β<sup>+</sup> T cells were determined.

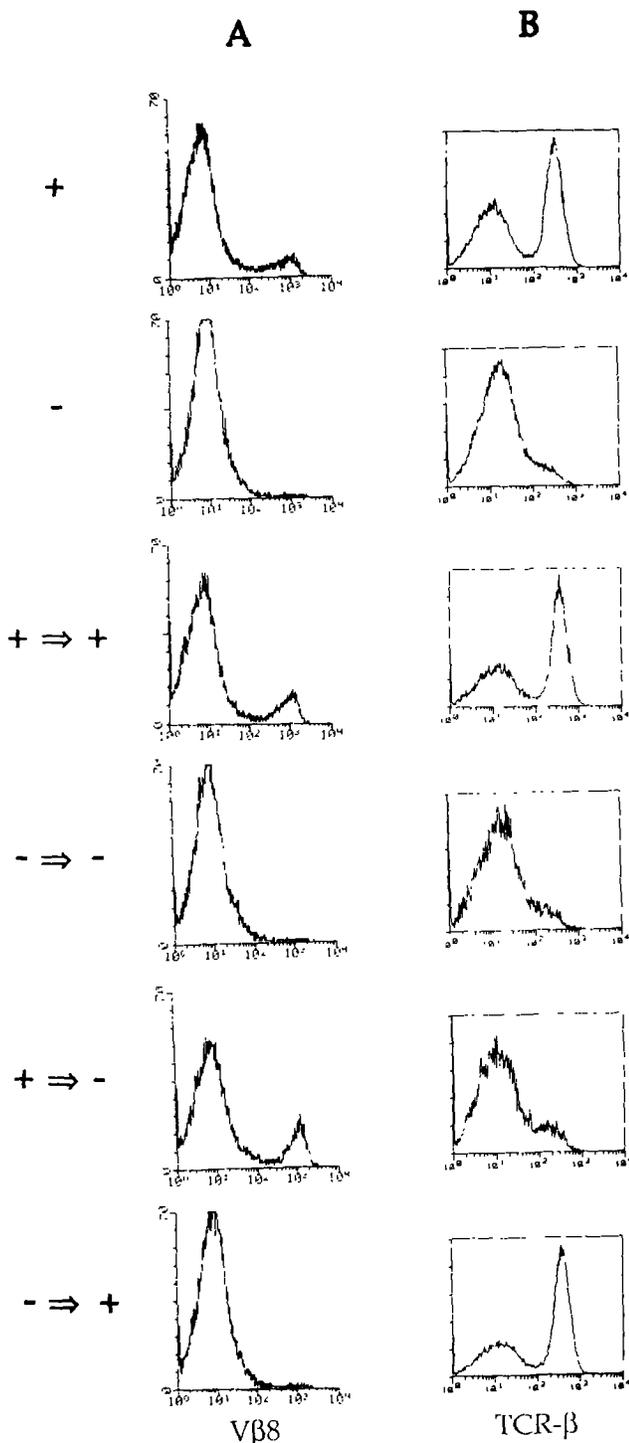
Normal hosts reconstituted with class I<sup>+</sup> fetal liver cells had a frequency of thymic Vβ8<sup>+</sup> DN cells similar to control class I<sup>+</sup> mice, demonstrating that these cells can develop normally in chimeras. However, normal hosts reconstituted with β<sub>2m</sub><sup>-</sup> fetal liver cells displayed a low frequency of thymic Vβ8<sup>+</sup> DN cells, similar to that of unmanipulated β<sub>2m</sub><sup>-</sup> mice (Figs. 2 A and 3 A). These results indicate that class I expression by hematopoietic cells is necessary for the appearance of most thymic Vβ8<sup>+</sup> DN cells. In the same type of chimera, the frequency of CD8<sup>+</sup> TCR-α/β<sup>+</sup> T cells was as high as in normal control animals (Fig. 3 B) (17),

reflecting the lack of a requirement for class I expression by hematopoietic cells for the differentiation of CD8<sup>+</sup> TCR-α/β<sup>+</sup> T cells.

To ask whether class I expression by thymic epithelial cells is also important to direct the elevated frequency of thymic Vβ8<sup>+</sup> DN cells, we analyzed chimeras in which the fetal liver recipients were β<sub>2m</sub><sup>-</sup>. β<sub>2m</sub><sup>-</sup> hosts reconstituted with β<sub>2m</sub><sup>+</sup> fetal liver displayed an elevated frequency of Vβ8<sup>+</sup> DN cells similar to the frequency in control β<sub>2m</sub><sup>+</sup> mice, despite the lack of class I on host thymic epithelial cells (Figs. 2 A and 3 A, and data not shown). By contrast, β<sub>2m</sub><sup>-</sup> hosts reconstituted with β<sub>2m</sub><sup>-</sup> fetal liver cells displayed a low frequency of thymic Vβ8<sup>+</sup> DN cells, similar to that found in control β<sub>2m</sub><sup>-</sup> mice. Development of CD8<sup>+</sup> TCR-α/β<sup>+</sup> cells was impaired in β<sub>2m</sub><sup>-</sup> recipients, regardless of whether the fetal liver cells expressed β<sub>2m</sub>, reflecting a requirement for class I expression by thymic epithelial cells for efficient differentiation of these cells (Fig. 3 B) (17). These results indicate that class I expression by hematopoietic cells is both necessary and sufficient for the appearance of elevated numbers of thymic Vβ8<sup>+</sup> DN cells. Conversely, class I expression by hematopoietic cells is neither necessary nor sufficient for the development of normal numbers of CD8<sup>+</sup> TCR-α/β<sup>+</sup> cells.

The substantial drop in the frequency of Vβ8<sup>+</sup> DN cells in recipients of β<sub>2m</sub><sup>-</sup> fetal liver cells was paralleled in each case by a modest drop in the frequency of total thymic α/β<sup>+</sup> DN cells (Fig. 2 B). As was the case in comparing unmanipulated β<sub>2m</sub><sup>+</sup> and β<sub>2m</sub><sup>-</sup> mice, the magnitude of the reduction in total α/β<sup>+</sup> DN cells was approximately what would be expected if Vβ8<sup>+</sup> cells were primarily affected in the chimeras.

**In Mixed Chimeras of β<sub>2m</sub><sup>+</sup> and β<sub>2m</sub><sup>-</sup> Fetal Liver, High Expression of Vβ8 DN Cells Is Dominant.** There are several possible reasons for the reduced frequency of thymic Vβ8<sup>+</sup> DN cells in animals containing class I<sup>-</sup> hematopoietic cells. Class I molecules may be involved in positively selecting thymic Vβ8<sup>+</sup> DN cells, resulting in an elevated frequency of these cells. Alternatively, considering that thymic Vβ8<sup>+</sup> DN cells are hematopoietic cells, it is possible that the development



**Figure 3.** The frequencies of thymic  $V\beta 8^+$  DN cells and  $CD8^+4^-$  cells show a reciprocal pattern of dependence on class I expressed by thymic epithelial cells vs. thymic hematopoietic cells. (A) One-parameter histograms from three-color analysis showing TCR- $V\beta 8$  staining of  $CD4^-$  and  $CD8^-$  depleted thymocytes electronically gated to exclude residual  $CD4^+$  and  $CD8^+$  cells. Staining with  $K^b$ -FL was used to exclude any residual host-derived cells in  $(+ \rightarrow -)$  and  $(- \rightarrow +)$  chimeras. (B) One-parameter histograms showing staining of total thymocytes, (electronically gated to exclude  $CD4^+$  cells) with anti-TCR- $\beta$  antibody. Cells in A and B are from different animals. Chimeric animal designations are as described in the legend to Fig. 2.

of such cells requires that they themselves express class I molecules. A less likely third possibility is that the absence of class I expression by hematopoietic cells "unmasks" an antigen that deletes or prevents the development of  $V\beta 8^+$  DN cells.

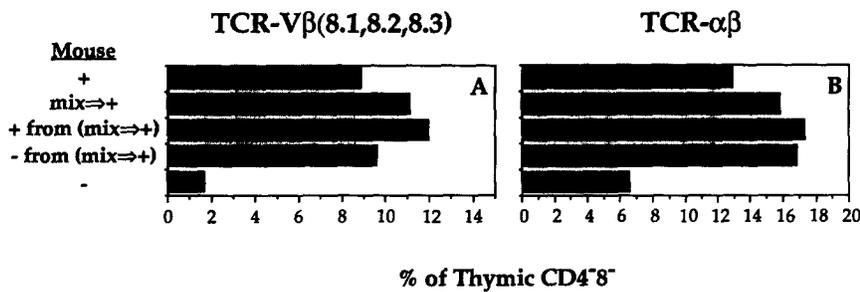
These models can be distinguished by examination of mixed fetal liver chimeras in which both  $\beta 2m^+$  and  $\beta 2m^-$  hematopoietic cells codevelop within the same animal. Therefore, we transferred a mixture containing equal numbers of  $\beta 2m^+$  and  $\beta 2m^-$  fetal liver cells to groups of irradiated  $\beta 2m^+$  recipients. 3–4 mo later, the chimeras were killed and the frequencies of thymic  $V\beta 8^+$  and TCR- $\alpha/\beta^+$  DN cells were determined separately among class  $I^+$  and class  $I^-$  populations, with the use of three-color flow cytometry (see Materials and Methods, and Fig. 4 legend). Both class  $I^+$  and class  $I^-$  populations exhibited an elevated frequency of thymic  $V\beta 8^+$  DN cells comparable to the levels found in control class  $I^+$  animals (Fig. 4 and data not shown). Once again, the overall frequency of thymic TCR- $\alpha/\beta^+$  DN cells reflected the lower frequency of  $V\beta 8^+$  DN cells in these animals. Therefore, class I molecules on one cell can select positively for neighboring  $V\beta 8$  DN cells that do not themselves express class I. These results strongly support a model in which "positive selection" of  $V\beta 8^+$  DN cells requires recognition of class I molecules expressed by hematopoietic cells.

*Class II Mutation Has a Modest Effect on the Frequency of TCR- $\alpha/\beta^+$  DN cells.* We examined the status of TCR- $\alpha/\beta^+$  DN cells in mice deficient for class II MHC expression by virtue of a disrupted  $A\beta$  gene. The frequencies of TCR- $\alpha/\beta^+$ ,  $V\beta 8^+$ , or  $V\beta 8.2^+$  in the thymic DN population were each reduced by  $\sim 40$ –50% in the class II-deficient mice compared with normal mice (Table 1).

## Discussion

*Positive Selection of  $V\beta 8$  DN Cells.* The biased expression of  $V\beta 8$  by  $\alpha/\beta$  DN cells initially suggested that the repertoire of these cells was formed by specific selection processes. Some recent evidence suggests, in fact, that negative selection of some superantigen-specific cells occurs in this population or a progenitor population, since  $V\beta 11^+$  and  $V\beta 17a^+$  DN cells are specifically reduced in the  $IE^+$  strains where superantigen-mediated deletion of conventional T cells bearing these  $V\beta$ s occurs. Not all superantigens mediated deletion in this population, however, since little or no  $Mls-1^+$ -mediated deletion of  $V\beta 6^+$  or  $V\beta 8.1^+$  DN cells was observed (6, 28–30).

In contrast, no previous evidence has been reported that the overall repertoire of thymic DN cells, and in particular the predominance of  $V\beta 8^+$  cells in the population, is determined by positive selection events. Analysis of  $\alpha/\beta$  DN cells in mice expressing  $V\beta 8^+$  TCR transgenes revealed no requirement for positive selection by the known MHC-restricting elements recognized by the receptors, and no negative selection mediated by expression of the nominal antigens recognized by the transgenic receptors (31, 32). Based on the latter results it has been suggested that  $\alpha/\beta$  DN cells are



**Figure 4.** High expression of Vβ8<sup>+</sup> DN cells is dominant in mixed chimeras of β<sub>2</sub>m<sup>+</sup> and β<sub>2</sub>m<sup>-</sup> hematopoietic cells. Analysis was the same as described in the legend to Fig. 2. Three-color flow cytometric analysis was used to measure the frequency of TCR-Vβ8<sup>+</sup> (A) and TCR-α/β<sup>+</sup> (B) cells among thymic CD4<sup>-</sup>8<sup>-</sup> cells from β<sub>2</sub>m<sup>+</sup>/β<sub>2</sub>m<sup>-</sup> mixed fetal liver irradiation chimeras (mix → +, [β<sub>2</sub>m<sup>+</sup> fetal liver plus β<sub>2</sub>m<sup>-</sup> fetal liver → β<sub>2</sub>m<sup>+</sup>], +, C57BL/6 mice; and -, fifth generation C57BL/6 backcross β<sub>2</sub>m<sup>-/-</sup> mice). In addition to anti-CD4-PE and anti-CD8-PE used to electronically gate out residual CD4<sup>+</sup> and

CD8<sup>+</sup> thymocytes, anti-K<sup>b</sup>-FL was used to allow independent analysis of β<sub>2</sub>m<sup>+</sup> (+ from mix → +) or β<sub>2</sub>m<sup>-</sup> (- from mix → +) cells. This experiment was repeated two more times with similar results in a separate study where the mice had received intraperitoneal injections of 200 μg of poly (I:C).

not subject to positive and negative selection (33). In studies of α/β DN cells in normal mice, comparisons of congenic mouse strains expressing different MHC haplotypes revealed no significant changes in Vβ8 use (34).

Despite the previous failures to observe evidence for positive selection events influencing the α/β DN population, we now present direct evidence that formation of the large pool of Vβ8<sup>+</sup> DN cells is dependent upon positive selection events mediated by class I molecules. It will be interesting to determine whether the lymphoproliferation of α/β DN cells in the peripheral lymphoid organs of *lpr* mice is also dependent on class I expression. In contrast to class I deficiency, class II deficiency led to only a modest reduction of α/β DN cells, and the effect was not restricted to Vβ8<sup>+</sup> cells. The modest effect of class II deficiency argues against a general model in which Vβ8 DN cells are selected first on class II molecules and subsequently expanded on class I molecules. However, it remains possible that a fraction of Vβ8<sup>+</sup> (and Vβ8<sup>-</sup>) DN cells require selection by class II molecules. Alternatively, the effect of class II deficiency could be the indirect consequence of the increased CD4 and/or TCR levels observed on CD4<sup>+</sup>CD8<sup>+</sup> double-positive thymocytes in class II-deficient mice (35). Experiments are in progress to distinguish these possibilities.

**Table 1.** Status of TCR-α/β<sup>+</sup> DN Thymocytes in Class II-deficient mice

	B6	Class II deficient
TCR β <sup>+</sup>	8.6 ± 0.3*	5.2 ± 0.5
Vβ8 <sup>+</sup>	5.3 ± 0.1	3.0 ± 0.3
Vβ8.2 <sup>+</sup>	3.8 ± 0.6	1.9 ± 0.2

DN thymocytes were enriched and analyzed for the frequency of TCR<sup>+</sup> cells as described in Materials and Methods. The sizes of the thymi and the proportion of total DN cells were similar in class II-deficient and normal mice (data not shown).

\* Average percentage of DN cells based on three independent determinations with SEM.

The frequency of Vβ8<sup>-</sup> DN cells in the thymus is apparently not influenced by class I expression. Since these cells are only modestly reduced in class II-deficient mice, their appearance may be independent of selection by MHC molecules or they may be selected by either class I or class II molecules.

The failure to observe a requirement for positive selection of Vβ8 DN cells in TCR transgenic mice is still unexplained. One possibility is that cells of this phenotype in transgenic mice are not the counterparts of α/β DN cells in normal mice. In fact, based on phenotypic differences it has been suggested that the transgenic Vβ8 DN cells correspond to γ/δ lineage cells that are precluded from expressing γ/δ receptors due to expression of the TCR-α/β transgenes (B. J. Fowlkes, personal communication, and reference 33). The development of most γ/δ lineage cells is not dependent on class I expression (36). Another possibility is that the transgenic Vβ8 DN cells are selected by nonpolymorphic class I molecules distinct from the restricting class I molecule (see below).

The "positive selection" of Vβ8<sup>+</sup> DN cells differs in at least one striking respect from positive selection events that control formation of the conventional T cell pool. Whereas thymic epithelial class I expression is both necessary and sufficient for efficient positive selection of CD8<sup>+</sup> T cells, it is neither necessary nor sufficient to select high usage of Vβ8<sup>+</sup> by DN cells. Instead, hematopoietic cell class I expression is important for selecting these cells. High usage of Vβ8 among class I<sup>-</sup> α/β<sup>+</sup> DN cells occurs in hematopoietic chimeras containing a mixture of class I<sup>+</sup> and class I<sup>-</sup> cells, demonstrating that the effects of class I expression are mediated by selection rather than by a requirement for Vβ8 DN cells to express class I molecules.

The "positive selection" induced by class I<sup>+</sup> hematopoietic cells may reflect differentiation of the cells from immature precursor cells. Alternatively, it is possible that Vβ8<sup>+</sup> DN cells mature without selection, and "positive selection" in this system reflects class I-mediated activation and expansion of already mature cells. This would account for our finding that hematopoietic cells, which include APC, mediate selection of Vβ8 DN cells by class I molecules. It would also account for the observation that the frequency of Vβ8<sup>+</sup> cells

in the  $\alpha/\beta$  DN population increases for several weeks postnatally (6). Peptides of endogenous or environmental origin bound to class I molecules might drive this cellular expansion.

*Are V $\beta$ 8 DN Cells Positively Selected by Nonpolymorphic Class I Molecules?* The striking difference in cell types mediating positive selection may indicate that the development of V $\beta$ 8 DN cells is governed by a signaling mechanism distinct from that governing the development of conventional T cells, perhaps reflecting a unique biological role for these cells. Recent reports describe human  $\alpha/\beta^+$  DN cell lines with specificity for the class I-like CD1 molecules (37, 38). Some of these CD1-reactive  $\alpha/\beta$  DN T cell lines were autoreactive (37). Mycobacterial antigen-specific, CD1<sup>b</sup>-restricted human  $\alpha/\beta$  DN clones have also recently been reported (38).

These results invite speculation that murine V $\beta$ 8 DN cells recognize and are selected by a specific set of nonclassical class I molecules, such as the class Ib molecules, which map telomeric to H-2, or the class I-like CD1 molecules, which map on chromosome 3 in the mouse (39). Several features of the data concerning V $\beta$ 8 DN cells are consistent with the possibility that they are selected by a specific set of nonclassical class I molecules: (a) most class Ib and CD1 genes display limited or no polymorphism (39), which could explain why the elevated V $\beta$ 8 usage occurs to a similar extent in mouse strains that differ at MHC. (b) Recent evidence suggests that some class Ib molecules present a highly specific set of peptides (40). Specific peptide/MHC complexes have been shown to stimulate T cells with restricted V $\alpha$  or V $\beta$  usage (41, 42). It seems plausible, therefore, that stimulation or selection of  $\alpha/\beta$  DN cells by a specific complex of peptide and nonclassical class I molecule could account for the predominance of V $\beta$ 8<sup>+</sup> cells in the population. (c) The class Ib and CD1 molecules are  $\beta_2m$  associated (39).  $\beta_2m$  is generally required for functional cell surface expression of class I molecules. This would fit with the deficiency of V $\beta$ 8 DN cells in  $\beta_2m$ -deficient mice.

Assuming that V $\beta$ 8 DN cells generally recognize class Ib or CD1 molecules, and are positively selected by them, it is still unclear what their biological role might be. One possibility, originally proposed for various subsets of  $\gamma/\delta$  T cells (43–45), is that  $\alpha/\beta$  DN cells recognize stress-induced autologous antigens bound to class I-like molecules. Alternatively, perhaps one of the nonclassical class I molecules is specialized to present specific antigens, corresponding to a

specific class of pathogen, to  $\alpha/\beta$  DN cells. A precedent is the recent evidence that the H-2M3 class I molecule is specialized to present N-formylated bacterially derived peptides to T cells (40, 46).

*Precursor Cells of V $\beta$ 8 DN Thymocytes.* Our studies are also relevant to the question of the identity of progenitor cells of  $\alpha/\beta$  DN cells. Studies of the methylation patterns of the CD8 gene in thymic  $\alpha/\beta$  DN cells suggest passage of thymic  $\alpha/\beta$  DN cells through an intermediate CD8<sup>+</sup> stage (6, 47). In [ $\beta_2m^+ \rightarrow \beta_2m^-$ ] chimeras, thymic V $\beta$ 8<sup>+</sup> DN cells occur at an elevated frequency while the development of CD4<sup>-</sup>8<sup>+</sup> TCR- $\alpha/\beta^h$  cells is strongly impaired. Conversely, in [ $\beta_2m^- \rightarrow \beta_2m^+$ ] chimeras, most V $\beta$ 8<sup>+</sup> DN cells fail to appear while CD4<sup>-</sup>8<sup>+</sup> TCR- $\alpha/\beta^h$  T cells appear at normal frequencies. The inverse correlation between the appearance of CD4<sup>-</sup>8<sup>+</sup> TCR- $\alpha/\beta^h$  T cells and V $\beta$ 8<sup>+</sup> DN cells argues against a precursor-product relationship between them, and suggests that if V $\beta$ 8<sup>+</sup> DN cells do in fact pass through a CD8<sup>+</sup> intermediate, the transient CD8<sup>+</sup>4<sup>-</sup>TCR<sup>-</sup> stage and/or the CD4<sup>+</sup>8<sup>+</sup>TCR<sup>lo</sup> stage would be likely candidates. Alternatively, it remains possible that the V $\beta$ 8 DN cells arise specifically from a subset of CD8<sup>+</sup>CD4<sup>-</sup>TCR<sup>h</sup> cells that are positively selected by hematopoietic cells (17).

Of interest is the relationship of class I-selected V $\beta$ 8 DN cells to recently described Ly6C<sup>+</sup> thymocytes, which are found in all four thymic subsets defined by expression of CD4 and CD8, and which display a V $\beta$  repertoire skewed toward V $\beta$ 8 (45). We find that in normal B6 mice ~66% of V $\beta$ 8<sup>+</sup> DN thymocytes express Ly6C. In  $\beta_2m^-$  mice the frequencies of both the Ly6C<sup>+</sup>V $\beta$ 8<sup>+</sup> and Ly6C<sup>-</sup>V $\beta$ 8<sup>+</sup> DN cells are reduced, although the reduction is more pronounced in the Ly6C<sup>+</sup>V $\beta$ 8<sup>+</sup> subset (data not shown). These results suggest that both Ly6C<sup>+</sup> and Ly6C<sup>-</sup> subsets of V $\beta$ 8 DN cells are class I dependent.

Obviously, much remains to be learned regarding the origin, selection, and function of  $\alpha/\beta$  DN cells. Analysis of various other mutant mouse strains, including mice deficient for CD4, CD8, or both class I and class II should provide additional clues about the selective events that act on the cells and their lineage precursors. These results will be important in developing a complete understanding of the biological role of these intriguing cells.

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