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Evidence for a single-niche model of positive selection

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ABSTRACT **Thymocyte maturation depends on interactions with thymic stromal elements expressing major histocompatibility complex (MHC) molecules. Mutant mouse strains lacking MHC class I (β_2-microglobulin-null) or class II (A_β-null) expression fail to generate normal CD8 or CD4 T-cell populations and provide model systems for reconstitution experiments. We have constructed *in vitro* chimeras between normal and MHC-deficient thymi to evaluate the efficiency of positive selection. Unexpectedly, the generation of mature single-positive thymocytes was proportional to the fraction of wild-type (i.e., MHC-expressing) stroma over a wide range of chimerism. Similar results were obtained for the development of T-cell receptor-transgenic thymocytes in graded chimeras expressing selecting and nonselecting MHC alleles. These findings are best explained by hypothesizing that positive selection involves a rate-limiting step at which each thymocyte can interact with only one stromal cell niche.**

T lymphocyte precursors interact via the clonotypic T-cell antigen receptor (TCR) and the accessory molecules CD4 and CD8 with major histocompatibility complex (MHC)–ligand complexes on thymic stromal elements, a process which can either abort T-cell development (negative selection) or allow it to progress beyond the immature $CD4^+CD8^+$, double-positive (DP) stage (positive selection) (1–3).

In normal mice, only about 2% of thymocytes are selected to complete their developmental program (4, 5). In TCR transgenic mice, the conversion rate of DP to $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes is 10-fold higher (5), indicating that the thymus has considerable spare capacity to support T-cell maturation and that in TCR nontransgenic mice, the limiting factor for positive selection is the occurrence of suitable TCR specificities. Nevertheless, there seems to be an upper limit to the number of microenvironments, or "niches," competent to mediate positive selection, since even in TCR transgenic mice, not more than one in five thymocytes do mature (5). This limitation may not be imposed by the abundance of suitable MHC–ligand complexes as such, since *in situ* hybridization studies in several TCR transgenic systems showed that the majority of cortical thymocytes had fewer Rag-1 transcripts on the appropriate (selecting) MHC haplotype (ref. 6; V. Kouskoff, S. Gilfillan, C.B., and D.M., unpublished results). Because TCR engagement can downregulate Rag (7), this result may suggest that most if not all thymocytes have access to MHC–ligand complexes. To investigate the relationship between the expression of selecting ligands and the efficiency of thymocyte selection, we have devised an *in vitro* system based on graded chimeras between normal and MHC-deficient thymi.

MATERIALS AND METHODS

Animals. Mice homozygous for the null allele of the A_β locus ($Ab^{o/o}$) (8) and for the null allele of the β_2-microglobulin (β_2m) locus ($B2m^{o/o}$) (9) (C57BL/6 background) and mice transgenic for the 2B4 $\alpha\beta$ TCR (10) (C57BL/6 or B10.BR background) or the HY $\alpha\beta$ TCR (3) (C57BL/6 or CBA background) were typed by immunofluorescence staining (see below). C57BL/6, SJL/J, B10.BR, and CBA mice were obtained from Iffa Credo or Harlan Olac (Bichester, U.K.), and C57BL/6 *Thy-1.1* congenic B6.PL mice were the gift of H. Mossmann (Max-Planck Institut für Immunbiologie, Freiburg, Germany).

Immunofluorescence Staining and Flow Cytometry. Cryostat sections and cell suspensions were stained as described (8). Stromal cell suspensions (prepared as detailed below) were stained for A^b with the monoclonal antibody (mAb) 2A2 (11) coupled to fluorescein isothiocyanate (FITC) or for K^b with the mAb K9.178 (12) followed by goat anti-mouse IgG-FITC (Caltag, South San Francisco, CA) and analyzed on a FACScan (Becton Dickinson).

Aggregation Chimeras. Fetal thymi were from timed matings between $Ab^{+/o} \times Ab^{o/o}$ and/or $B2m^{+/o} \times B2m^{o/o}$ mice or between homozygous MHC-deficient and wild-type mice set up in parallel [either $(C57BL/6 \times SJL/J)F_2$ or B6.PL, Thy-1.1)]. Embryonic day 0 (E0) designates the day of the vaginal plug. Thymus suspensions were prepared (13) by incubation in phosphate-buffered saline/0.5 mM EDTA/0.25% trypsin for 30 min at 37°C. Where required, littermates were typed for their MHC status by staining aliquots for A^b (11) or K^b (12). B10.BR or C57BL/6 females were mated with $H\text{-}2^k$ or $H\text{-}2^b$ males transgenic for the (independently segregating) 2B4 TCR α and β chains, or B10.BR or CBA females were mated with $H\text{-}2^k$ or $H\text{-}2^b$ males transgenic for the HY TCR. Thymi (E15 or E16) were dissociated with collagenase (Sigma) at 0.5 mg/ml for 30 min at 37°C and typed for TCR expression by flow cytometry using mAbs A2B4-2 (for the 2B4 α chain), KJ25 (for the $V_\beta3$ family employed by the 2B4 β chain), or T3.70 (for the HY α chain) (3, 10). Embryos were sexed by the morphology of their internal sexual organs.

Aggregation chimeras were made as described (14) by mixing aliquots of dissociated thymi at the indicated ratios and culture as hanging drops ($5–10 \times 10^4$ cells). After 48 hr, aggregates were transferred to Nuclepore filters (0.8 μm; Costar) floating on Iscove's modified Dulbecco's medium with 10% heat-inactivated fetal bovine serum or 1% β_2m° mouse serum, 20 μM 2-mercaptoethanol, and gentamicin at 50 μg/ml. Cells were cultured for 7–14 (typically 10) days prior to analysis.

RESULTS

Thymocyte Maturation in MHC Class II Chimeras. We aimed to quantitate the fraction of class II-expressing cells required to restore $CD4^+$ T-cell development in A_β° thymi, intrigued by *in vivo* observations that $CD4^+$ T-cell develop-

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Abbreviations: β_2m, β_2-microglobulin; DP, double positive; E*n*, embryonic day *n*; FITC, fluorescein isothiocyanate; mAb, monoclonal antibody; MHC, major histocompatibility complex; PE, phycoerythrin; SP, single positive; TCR, T-cell antigen receptor.

ment in $A_\beta°$ mice reconstituted with class II-E transgenes correlates closely with the fraction of class II-expressing thymic cortical epithelial cells (M.M., unpublished work). To avoid the variations of MHC expression levels inherent in this *in vivo* system, and the poor predictability of *in vivo* chimeras, we chose an *in vitro* approach based on fetal thymic organ culture. This system supports T-cell development from E13–E15 intrathymic precursors, which are largely devoid of CD4, CD8, and $\alpha\beta$ TCR expression (15). Our strategy to produce chimeras between normal and MHC-deficient thymi exploited observations that fetal thymic stroma can be enzymatically dissociated, manipulated, and reaggregated to form structures with normal morphology and function (13, 14). It is outlined in Fig. 1.

As demonstrated by immunofluorescence staining of frozen sections (Fig. 2), class II-expressing stromal elements were evenly distributed throughout $A_\beta^+/A_\beta°$ chimeras, presumably readily accessible to developing thymocytes. Flow cytometric analysis of chimeras showed that the fraction of class II-expressing stromal cells after reaggregation and culture reflected the input ratio of $A_\beta°$ and A_β^+ thymi (Fig. 3 *a* and *b*). As expected from the analysis of $A_\beta°$ mice (8) and organ culture of fetal $A_\beta°$ thymi (data not shown), $A_\beta°$ aggregates generated few, and not fully mature, CD4 SP thymocytes, expressing low levels of $\alpha\beta$ TCR only (Fig. 3*d*; gated area shown in Fig. 3*c*). $CD4^+$ SP development was restored in a dose-dependent fashion by adding A_β^+ thymic digests to $A_\beta°$ preparations (Fig. 3 *c* and *d*, left to right). Data for 12 chimeras per titration point are summarized in Fig. 4. While total thymocyte numbers and the frequency of mature CD8 SP thymocytes varied only slightly with chimera composition, a nearly linear relationship was seen between increasing chimerism for A_β^+ and the frequency of mature $CD4^+$ SP cells (defined by high levels of $\alpha\beta$ TCR and low levels of heat-stable antigen, HSA). $CD4^+$ SP thymocyte development was proportional to the A_β^+ contribution over the entire range tested, suggesting that microenvironments or niches formed by class II-expressing stromal cells were rate-limiting for thymocyte maturation.

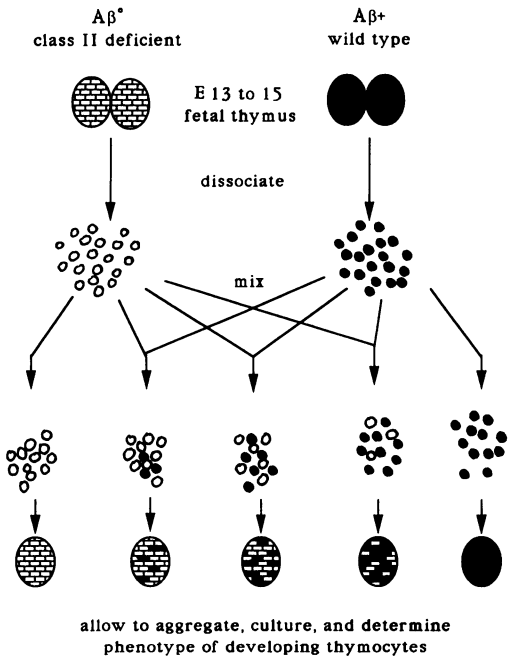

FIG. 1. Construction of thymic chimeras. Normal (A_β^+) or MHC class II-deficient ($A_\beta°$) fetal thymi were dissociated by trypsin treatment. The resulting cell suspensions were mixed at the desired ratios and allowed to reaggregate. The chimeras were cultured, and the maturation of endogenous thymocyte precursors was monitored.

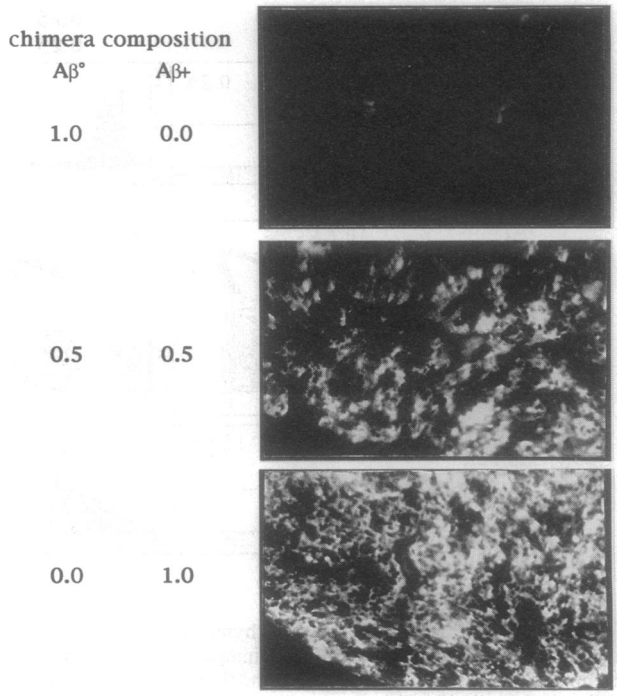

FIG. 2. Distribution of MHC class II-expressing cells in $A_\beta^+/A_\beta°$ thymus chimeras as visualized on frozen sections with 2A2-FITC (11).

The possible effects of MHC class I expression on thymocyte–microenvironment interactions were addressed in graded $A_\beta^+/A_\beta°$ chimeras on a class I-deficient background (β_2m°). $CD4^+$ SP thymocyte maturation showed a roughly linear relationship to the A_β^+ fraction also in class I-deficient thymi (Fig. 5).

The described chimeras were established from whole thymic digests (including stromal cells and T-cell precursors), so it is worth noting that $A_\beta°$ precursors will generate $CD4^+$ T cells when placed in class II-expressing stromal cell environments. This was shown *in vivo* by transgenic (18) and transplantation (19) approaches and confirmed *in vitro* with Thy-1 congenic chimeras (data not shown). As expected, T-cell maturation in our system is controlled by stromal components and not T-cell precursors.

Thymocyte Maturation in $\beta_2m^+/\beta_2m°$ Chimeras. We wished to complement the above results by a study of $CD8^+$ T-cell development because earlier work on the efficiency of positive selection had concentrated on this T-cell subset (5), and previous reports had suggested that $CD4^+$ and $CD8^+$ T cells may differ in their requirements for maturation (19–21). Also, our dissociation/reaggregation approach favors $CD8^+$ over $CD4^+$ SP development when compared with unmanipulated cultures (14). In graded $\beta_2m^+/\beta_2m°$ chimeras, mature CD8 SP frequencies titrated with the β_2m^+ fraction. A large wild-type contribution was required for optimal $CD8^+$ SP maturation (Fig. 6*a*), but, in contrast to $CD4^+$ T-cell development, maximal $CD8^+$ SP frequencies were typically obtained in 75% wild-type chimeras (this was confirmed in additional experiments not shown here). Flow cytometric analysis of MHC class I expression is consistent with the possibility that in chimeras with a large wild-type contribution some genetically $\beta_2m°$ cells may express low levels of K^b, apparently resulting in a favorable environment for $CD8^+$ thymocyte maturation (compare Figs. 6*b* and 3*b*).

Generic Versus Allele-Specific Positive Selection. Using MHC-deficient thymi, we investigated positive selection of immature thymocytes in a very general sense—namely, as the need for interactions with MHC molecules of the appro-

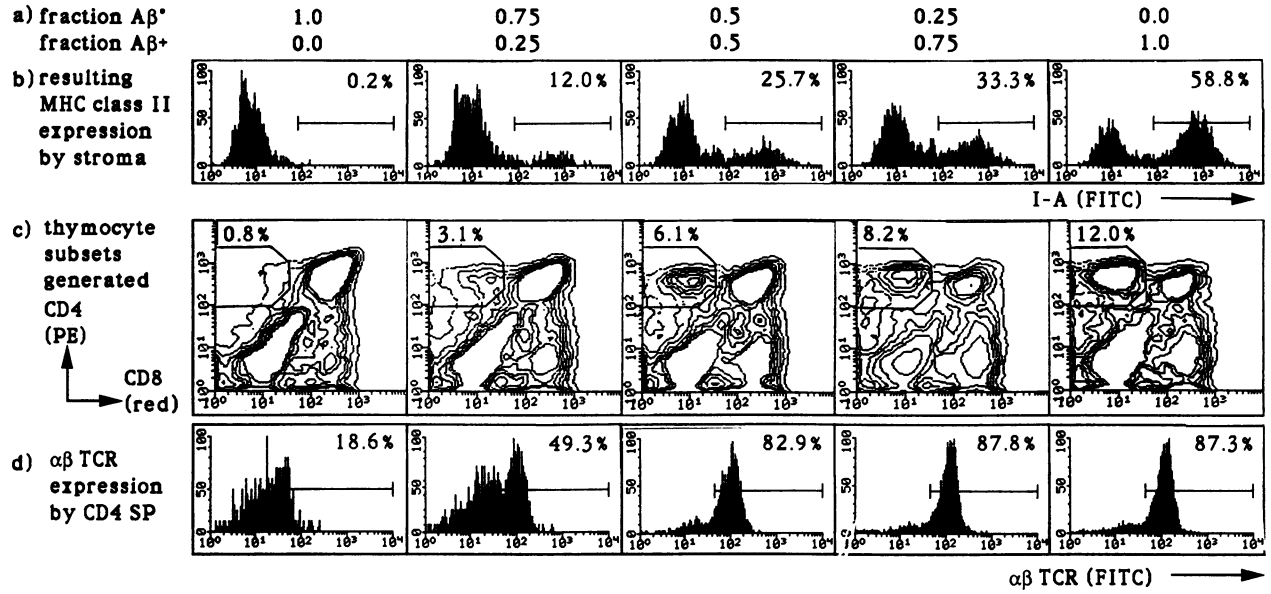

FIG. 3. Generation of CD4+ SP thymocytes in A_β^+/A_β° chimeras. (*a*) Chimeras were prepared as described in Fig. 1. (*b*) Following aggregation and 10 days in culture, chimeric thymi were once more dissociated with trypsin. Class II expression by stromal cells was assayed by immunofluorescence staining (11) and flow cytometry. (*c* and *d*) Thymocytes were released mechanically and stained with CD4-phycoerythrin (PE), CD8-biotin/SA-Tricolor, and the TCR β-chain-specific mAb H57-597-FITC (16). Probability plots of CD4 and CD8 expression are shown in *c*, where a box shows the gate for histograms of αβ TCR expression by CD4+ SP thymocytes (*d*).

priate class. A second powerful system is provided by transgenic mice expressing TCR of defined specificity and MHC restriction. In this case, thymocyte maturation beyond the DP stage requires the presence of *specific* MHC alleles (1, 3, 10, 22). To compare generic and allele-specific positive selection, we employed mice transgenic for the E^k-restricted 2B4 TCR (10). *In vivo*, clonotype-expressing CD4+ T cells emerge preferentially in the "selecting" H-2^k haplotype, whereas H-2^b is considered "neutral" (10). Graded chimeras expressing H-2^k and H-2^b products were made to determine the H-2^k fraction required for optimal CD4+ T-cell maturation. The frequency of A2B4-2-expressing CD4+ SP thymocytes was proportional to the H-2^k fraction and was maximal in aggregates containing only H-2^k cells (Fig. 7). Cultured 2B4 transgenic thymi also generated numerous A2B4-2-expressing CD8+ SP thymocytes (23), but this was independent of the MHC haplotype (data not shown).

Negative Selection. To address the formal possibility that our *in vitro* system might artificially sequester thymocytes from selecting stromal cells, we analyzed negative selection, a process considered to be highly efficient (24). We used thymocytes transgenic for the HY TCR, specific for a male-specific antigen in the context of the MHC class I molecule D^b (3), to make chimeras in which all stromal cells carried selecting MHC products but only a fraction expressed nominal antigen. The percentage of DP thymocytes declined

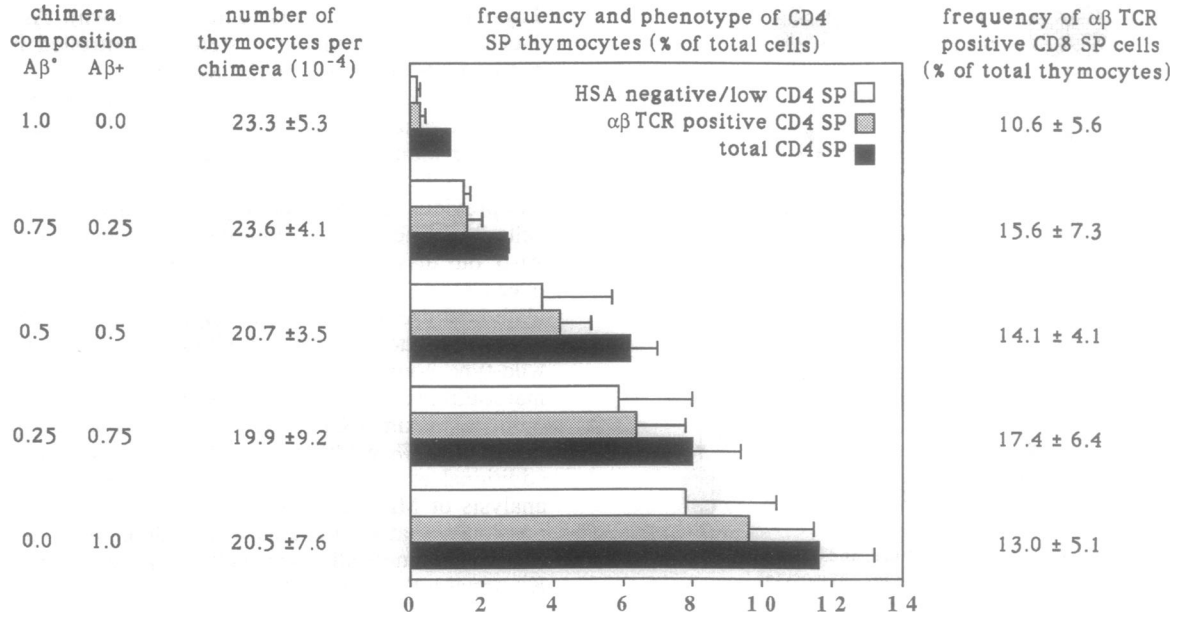

FIG. 4. The class II-positive fraction of *in vitro* chimeras specifies the frequency of mature CD4+ SP thymocytes. Thymocytes were recovered from aggregation chimeras (12 per titration point) after 7–14 days in culture, counted, and stained for CD4, CD8, and αβ TCR (see Fig. 3). Means and standard deviations are shown. Expression of heat-stable antigen (HSA) was determined with CD4-PE, CD8-FITC, and biotinylated M1/69 (17), followed by SA-Tricolor.

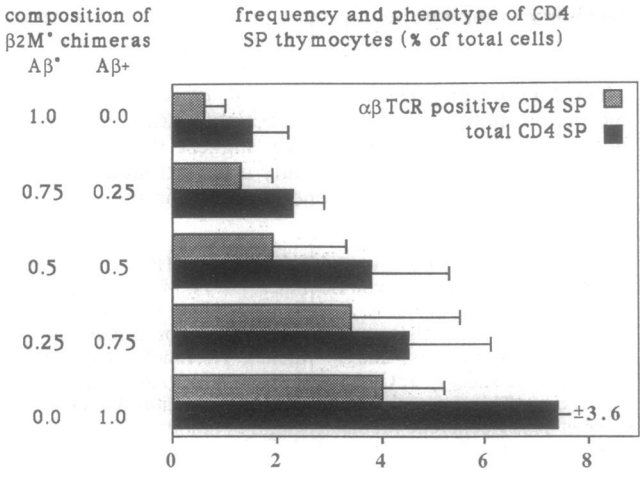

FIG. 5. CD4+ SP thymocyte maturation in A_β^+/A_β°, β_2m°-deficient chimeras. Aggregates were constructed from β_2m° A_β° and β_2m° A_β^+ littermate thymi at the indicated ratios. The frequencies of total (black bars) or $\alpha\beta$ TCR^{high} (stippled bars) CD4+ SP thymocytes were determined in six experiments. Comparable results were obtained in 10% fetal bovine serum or 1% β_2m° mouse serum, excluding *in vitro* reconstitution of class I expression by serum-derived β_2m, (not shown).

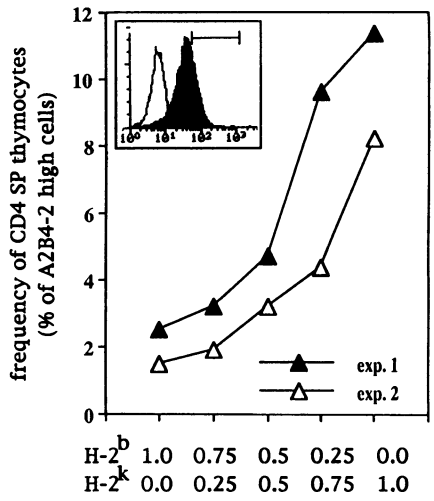

FIG. 7. Maturation of TCR transgenic thymocytes in graded chimeras expressing "selecting" and "neutral" class II products. The maturation of CD4+ SP thymocytes expressing the 2B4 transgenic TCR was assessed by staining with CD4-PE, CD8-biotin/SA-Tricolor, and A2B4-2 [specific for the transgenic α chain (10)] followed by goat anti-mouse IgG-FITC (Caltag) in H-2^b/H-2^k chimeras. Percentages represent CD4+ SP thymocytes in two different experiments gated on A2B4-2^{high} cells. (*Inset*) A2B4-2 staining of normal (open histogram) or 2B4 TCR transgenic (filled histogram) thymocytes, and the arbitrary gate defining the A2B4-2^{high} population. More than 85% of CD4+ SP thymocytes expressed $V_\beta3$ (data not shown).

sharply with the fraction of D^b-expressing male cells (Fig. 8), demonstrating free access of DP thymocytes to selecting stromal cells or vice versa. Male cells of the neutral H-2^k haplotype did not deplete DP thymocytes, confirming that the observed effect was MHC-restricted and not due to the transfer of male antigen to adjacent female cells.

DISCUSSION

To investigate T-cell maturation under conditions where only a fraction of thymic stromal cells express selecting ligands, we have devised an *in vitro* system in which the stromal cell composition can be varied experimentally without affecting the level of MHC expression per cell. CD4+ T-cell development in A_β°/A_β^+ chimeric thymi was proportional to the A_β^+ stromal cell fraction over the entire range tested, either in the presence or in the absence of MHC class I. Strikingly, CD4+ SP maturation was not saturated even at a ratio of A_β^+ to A_β° stroma as high as 3:1. Similarly, CD8+ SP maturation titrated with the β_2m^+ fraction in $\beta_2m^+/\beta_2m^\circ$ chimeras, but maximal CD8 SP frequencies were typically obtained in 75% β_2m^+ chimeras. This may be due to different requirements for CD4+ versus CD8+ T-cell development [e.g., transfer of normal bone marrow into MHC-deficient mice partially restores the selection of CD8+ T cells but not CD4+ T cells (19–21)] or to the "leaky" phenotype of the β_2m° mutation (25).

T-cell maturation in TCR transgenic mice requires particular MHC alleles (3, 10), and allele-specific positive selection was assayed as the development of 2B4 TCR transgenic thymocytes (10) in graded chimeras between selecting (H-2^k) and nonselecting (H-2^b) thymi. The generation of A2B4-expressing CD4+ SP thymocytes was proportional to the H-2^k fraction. Assuming that H-2^b is truly "neutral," it seems that the "wrong" class II is essentially equivalent to no class II at all and that specific TCR/MHC–ligand interactions in

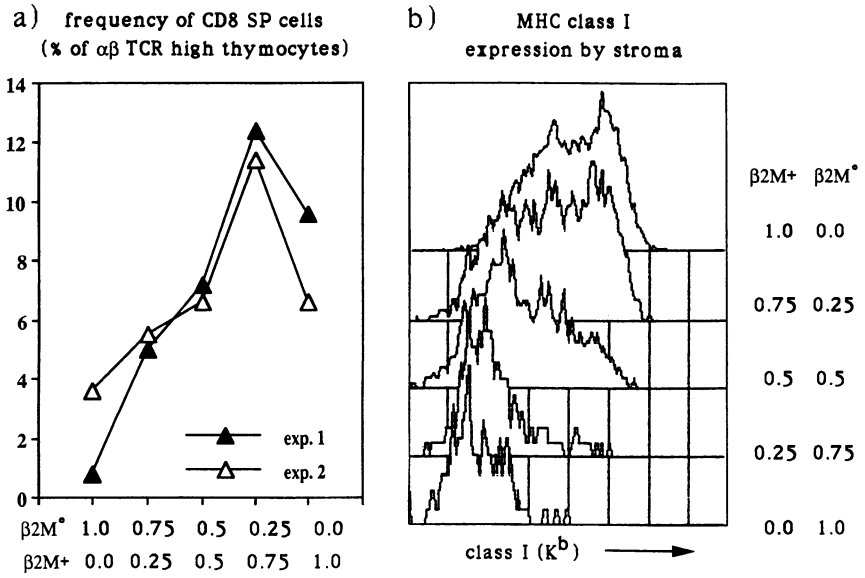

FIG. 6. CD8+ SP maturation in $\beta_2m^+/\beta_2m^\circ$ chimeras. Thymic cell suspensions from β_2m^+ and β_2m° littermates were mixed at the indicated ratios (all thymi were A_β^+). (*a*) Percentages of CD8 SP thymocytes in two different experiments are displayed gated on $\alpha\beta$ TCR^{high} cells (as shown in Fig. 5*d*). (*b*) Cultured chimeras were trypsinized and stained for K^b expression (12). The histograms displayed are gated on stromal-cell light scatter characteristics (14).

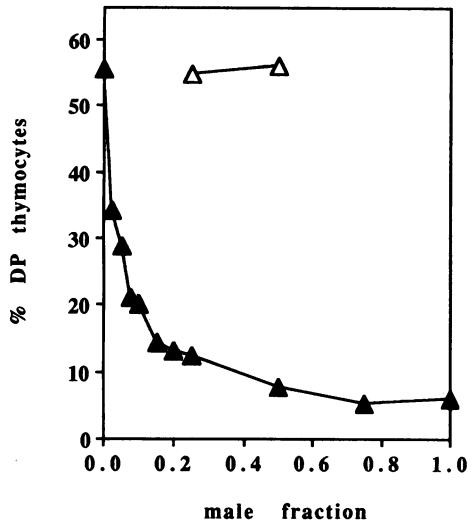

FIG. 8. DP thymocytes in thymus chimeras are readily accessible to negatively selecting stromal cells. Dissociated E15 HY TCR transgenic female or male thymi expressing the selecting haplotype (H-2^b, ▲) or male thymi expressing a neutral haplotype (H-2^k, △) were titrated. After reaggregation and 7 days in culture, thymocyte subsets were stained with anti-CD4, anti-CD8, and T3.70, a mAb to the transgenic TCR α chain (3). Shown is the fraction of DP cells among thymocytes expressing the transgenic TCR.

positive selection are not aided by allele-nonspecific MHC interactions—e.g., between class II molecules and CD4.

We conclude that a large fraction of relevant stromal elements must express selecting ligands to allow the efficient generation of mature thymocytes *in vitro*. In contrast, studies by Huesmann *et al.* (5) had indicated that the thymus has considerable (≈10-fold) excess capacity to support T-cell maturation (see Introduction). Why, then, is T-cell maturation not fully restored by a moderate fraction of wild-type stroma? We can exclude two trivial reasons: (*i*) selecting and nonselecting stromal cells do not segregate but are evenly mixed in thymus chimeras; (*ii*) the efficient deletion of DP thymocytes shows that thymocytes are not artificially sequestered from selecting stromal cells. Our favored explanation is therefore that thymocytes do not migrate between microenvironments during positive selection. In this scenario, only thymocytes located in a class II-expressing microenvironment, but not those "stuck" in a class II-deficient niche, have a chance to become mature $CD4^+$ T lymphocytes. Similarly, thymocytes in a β_2m° niche lose the option to mature into $CD8^+$ T cells. According to this interpretation, T-cell precursors do not select suitable thymic microenvironments on the basis of either class I or class II expression. If thymocytes were to ignore stromal cells devoid of MHC expression, $CD4^+$ T-cell development would be relatively independent of the $A_\beta{}^+/A_\beta{}^\circ$ stromal cell fraction in β_2m° chimeras, because precursors would avoid getting trapped in $A_\beta{}^\circ$ niches lacking class I. Thymocyte–stroma interactions are therefore probably mediated by other cues, such as adhesion molecules or matrix-bound cytokines. The suggested single-niche model does not imply that positive selection is a "single-hit" event: there is good evidence that thymocytes may sequentially rearrange and display more than one TCR α chain (26).

The physiological basis of our results could be that immature hemopoietic cells are highly dependent on continued stromal cell contact at certain developmental stages (27). It may also explain observations by Kodama *et al.* (28), who isolated variants of a stromal cell line, PA6, which had lost the ability to support hemopoietic precursors *in vitro*. Mixed stromal cell layers consisting of competent and incompetent cells yielded intermediate levels of hematopoiesis (28).

The data presented here establish the availability of thymic microenvironments displaying selecting ligands as a limiting factor for T-cell maturation. We suggest that in positive selection, thymocytes contact only a few stromal elements, possibly reflecting their dependence on continued stromal cell contact at this stage.

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