Quantitative Impact of Thymic Clonal Deletion on the T Cell Repertoire

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Summary

Interactions between major histocompatibility complex (MHC) molecules expressed on stromal cells and antigen-specific receptors on T cells shape the repertoire of mature T lymphocytes emerging from the thymus. Some thymocytes with appropriate receptors are stimulated to undergo differentiation to the fully mature state (positive selection), whereas others with strongly autoreactive receptors are triggered to undergo programmed cell death before completing this differentiation process (negative selection). The quantitative impact of negative selection on the potentially available repertoire is currently unknown. To address this issue, we have constructed radiation bone marrow chimeras in which MHC molecules are present on radiosensitive thymic epithelial cells (to allow positive selection) but absent from radiosensitive hematopoietic elements responsible for negative selection. In such chimeras, the number of mature thymocytes was increased by twofold as compared with appropriate control chimeras. This increase in steady-state numbers of mature thymocytes was not related to proliferation, increased retention, or recirculation and was accompanied by a similar two- to threefold increase in the de novo rate of generation of mature cells. Taken together, our data indicate that half to two-thirds of the thymocytes able to undergo positive selection die before full maturation due to negative selection.

Abbreviations used in this paper: CD4SP, CD4\(^{+}\)CD8\(^{-}\)TCR\(^{high}\); CD8SP, CD4\(^{-}\)CD8\(^{+}\)TCR\(^{high}\); HSA, heat stable antigen; MHC I/II deficient; MMTV, mouse mammary tumor virus; wt, wild-type.
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Main text:

It remains unclear whether active deletion of thymocytes potentially capable of effective positive selection in the presence of the usual diverse array of peptide-MHC complexes displayed on thymic stromal cells involves a small or large proportion of this population. This is a critical issue for a full understanding of the events involved in intrathymic T cell development, construction of a useful repertoire, and characterization of the ligands involved in these processes (3). In this study, we analyze the quantitative impact of thymic clonal deletion mediated by cells of hematopoietic origin using bone marrow chimeras with different combinations of MHC deficiencies.

Materials and Methods

Mice
Wild-type mice were C57BL/6 (H-2b haplotype) (see Fig. 1); Jackson Laboratories, Bar Harbor, ME; other experiments, Harlan Netherlands, Zeist, The Netherlands. Mice deficient in MHC class I expression (MHC I0) because of a targeted disruption of the β2-microglobulin gene (26) were obtained from Dr. B. Koller and Genpharm (Mountain View, CA). Mice of H-2b haplotype (I-α-high) with an introduced null mutation in the I-αb gene (27), and therefore deficient in MHC class I expression (MHC I0), were obtained from Dr. L. Glimcher and Genpharm and used in the experiment shown in Fig. 1. In all other experiments, mice deficient in MHC class II expression due to an induced disruption of the I-αb gene (28) in C57BL/6 stem cells (I-α-high) were used (provided by Dr. H. Bluethmann, Basel, Switzerland). MHC I0 and MHC I0 animals were interbred in our facilities to obtain MHC I0 Mice. The β2-microglobulin-deficient mice, as well as the I-αb mutants, were crossed at least six generations to C57BL/6 mice, after which intercrossing yielded mice homozygous for the disrupted alleles.

Chimeras
Chimeras were prepared essentially as described previously (29). In brief, hosts were lethally irradiated (1,000 rads) with a Cs137 source and injected the next day i.v. with 10–20 × 10⁶ bone marrow cells depleted of T cells by complement killing using anti-Thy1 antibody J1 (30) and anti-Ly1 antibody C3P0 (31) in the experiment shown in Fig. 1 and anti-Thy1 antibody AT83 (32) in other experiments.

Three-color Flow Cytometry
Single-cell suspensions of thymocytes were incubated on ice with saturating concentrations of the following antibodies: Fig. 1, anti-CD8-FITC, anti-TCR-PE (PharMingen, San Diego, CA), and anti-CD4-Red613 (GIBCO BRL, Gaithersburg, MD; Figs. 2 and 4, anti-TCR-FITC (H57-597), anti-CD4-PE (Boehringer-Mannheim, Mannheim, Federal Republic of Germany), and anti-CD8-Red613 (GIBCO BRL, Gaithersburg, MD). The samples were analyzed on a FACScan® using LYSYS II software (Becton Dickinson, Mountain View, CA).

Four-color Flow Cytometry
Single-cell suspensions of thymocytes were incubated on ice with saturating concentrations of the following antibodies: FITC-labeled anti-CD44 (PGP-1) mAb IM 7.8.1 (33), FITC-labeled anti-heat soluble antigen (HSA) mAb M1/69 (34) or FITC-labeled anti-CD69 mAb H1-2F3 (35), combined with anti-TCR-PE (PharMingen, San Diego, CA), anti-CD8-Red613 (GIBCO BRL, Gaithersburg, MD), and anti-CD4-bio mAb GK1.5 (36) followed by streptavidin-APC. Four-color flow cytometry was performed on a FAC Star Plus® using LYSYS II software (Becton Dickinson, Mountain View, CA).

Kinetics of G1 generation of mature T thymocytes. Chimeras were sub-lethally irradiated (720 rads, Cs137 source) 6 wk after engraftment and thymocytes analyzed 9–13 d later by flow cytometry. CD4 single-positive (CD4SP) cells are CD4+CD8-TCRhigh and CD8SP cells are CD4-CD8-TCRhigh.

Results and Discussion
To determine the quantitative impact of thymic clonal deletion on the T cell repertoire, we have examined thymocyte development in radiation bone marrow chimeras of H-2b haplotype in which MMTV superantigen-mediated deletion is not a significant factor (18, 19). Chimeras in which the donor bone marrow was from wild-type (wt) mice and the lethally irradiated host animals were deficient in both MHC class I and II expression (wt→MHC I0 II0) (37, 38) lacked significant numbers of mature CD48- or CD48-TCRhigh thymocytes (CD4SP and CD8SP, respectively; Fig. 1 A). A quantitative assessment reveals the development of less than 3% of wt levels of CD4SP cells and less than 8% of CD8SPs in such chimeras (Fig. 1 B). These results confirm previous data showing that efficient positive selection depends on expression of MHC molecules on radiosensitive thymic epithelial cells (2, 3) and indicate that in the system used here the previously reported low level of T cell differentiation induced by hematopoietic elements (4–7) plays a negligible role.

Experiments were then conducted to examine the development of mature thymocytes in the absence of the major characterized source of negative selection signals (2). To this end, chimeric mice were produced in which MHC class I was expressed on the radiosensitive thymic epithelium to support positive selection of CD4SP cells, but not on the negatively selecting radiosensitive hematopoietic elements (MHC I0 II0→MHC I0 II0 chimeras). A significant (1.7 ± 0.2-fold) increase in the steady-state percentage of mature CD4SP thymocytes was observed in these chimeras as compared with control (MHC I1→MHC I0) chimeras (Fig. 2). As expected, lack of MHC class I on hematopoietic elements (MHC I0→MHC I0 chimeras) did not result in significantly increased CD4SP percentages (1.1 ± 0.1-fold) as compared with appropriate control (MHC I1→MHC I1) chimeras (Fig. 2). Because thymi from the chimeras had similar total cell numbers (data not shown), these results reflect increases in the absolute number of CD4SP cells. Thus, in the absence of MHC class II expression on hematopoietic elements, a 1.7-fold increase in mature CD4SP cells occurs, suggesting that approximately half of the positively selectable thymocytes with receptors specific for self-MHC class II undergo negative selection on the same MHC molecule.

Several explanations other than the lack of negative selection could account for the increased number of CD4SP cells in MHC I0→MHC I0 chimeras. However, proliferation of mature CD4SP does not account for the increase as cell cycle analysis reveals no augmentation in the number of CD4SP cells in +G2/M phase (Fig. 3 A). A change in their maturation/export rate could result in an increased retention of CD4SP cells in the thymus. CD69 is a molecule transiently expressed during thymocyte development: in vivo, it is first expressed on CD4+CD8+ cells that have...
undergone MHC-mediated activation and is downmodulated only when the thymocytes have already reached the SP stage (39–41, 29). The normal ratio of mature thymocytes from the chimeras expressing high versus low levels of CD69 therefore is an indication of normal thymic maturation (Fig. 3B). Expression of HSA on CD4SP thymocytes is also downmodulated during their final maturation (42, 43). Again, the normal ratio of HSA high versus

**Figure 1.** Positive selection depends on expression of MHC molecules on radioresistant thymic epithelial cells. (A) Thymi from wild-type→wild-type, MHC I I I I →I I I I, and wild-type→I I I I chimeras were analyzed by flow cytometry 6 wk after grafting. Thymocytes were stained with anti-CD8, anti-TCR, and anti-CD4 antibodies and analyzed on a FACScan® using LYSYS II software. Contour plots are 75% logarithmic and TCR histograms are from total thymus and from electronically gated cells using the gates indicated in the figure. (B) Percentages of CD4SP (CD4+CD8–TCR high) and CD8SP cells (CD4–CD8+TCR high) in thymi from the indicated chimeras were determined and depicted as percentage of an age- and sex-matched nonchimeric control mouse ± SD.

HS A low CD4SP cells in the chimeras indicates normal maturation. Taken together, the data on the expression of CD69 and HSA suggest normal thymocyte development and render the possibility of prolonged retention of CD4SP cells in the thymus unlikely. Another possibility would be the recirculation into the thymus of mature peripheral T cells (44–46). Peripheral T lymphocytes do not express significant levels of HSA (42, 43) and the lack of an increased percentage of HSA high CD4SP thymocytes (Fig. 3B) thus is inconsistent with a recirculation model. Reentry into the adult thymus of peripheral T cells is known to be restricted to activated cells (44–46). These cells may be expected to express high levels of the memory/activation marker CD44 (47). No evidence for an increase in CD44 high CD4SP thymocytes was found in the chimeras (Fig. 3B). Collectively, these data strongly suggest that the increased number of CD4SP cells in chimeras lacking MHC class II on hematopoietic elements is not due to proliferation, to increased retention of thymocytes in the thymus, or to recirculation of peripheral T lymphocytes.

Analysis of cell number in the steady-state thymus measures the accumulation rather than the rate of de novo generation of thymocytes. Because the latter parameter more directly addresses the issue of whether additional cells are surviving the selection/differentiation process in the absence of hematopoietic MHC-mediated negative selection, we analyzed the kinetics of development of mature thymocytes in MHC-deficient chimeric mice. Most thymocytes are highly radiosensitive and die upon sublethal irradiation, whereas a small radioresistant CD4–CD8–precursor population will repopulate the thymus upon such treatment. In this model system, CD4–CD8–immature thymocytes appear around day 6 after irradiation and 3–4 d later mature SP cells can be observed (41, 48–52). Using this post-irradiation repopulation model, we studied the kinetics of generation of mature thymocytes in bone marrow chimeras (Fig. 4). In particular, 6 wk after grafting, MHC I I I I→MHC I I I I and control wt→wt chimeras were sub-
The development of CD8SP thymocytes. They reveal a similarly large impact of negative selection on differentiation, homeostasis, or recirculation of these cells. Moreover, the increase in the number of SP cells seen in the steady-state thymus of mice lack-MHC class II on hematopoietic cells is due to the lack of physiological ligands expressed on hematopoietic elements. These data, combined with those on the kinetics of thymocyte development (53, 54), imply that ~90% of developing thymocytes die of neglect and 5% die due to deletion, whereas the remaining 5% survive to populate the periphery. In considering this calculation, one caveat is that the proportion of negatively selected thymocytes could theoretically be larger if some cells are normally deleted despite being unable to be positively selected. However, we observe no increase in the absolute number of CD4-CD8+ thymocytes in deletion-deficient chimeras (data not shown), which is inconsistent with the existence of any appreciable number of such cells. Therefore, when considering the entire thymocyte precursor pool, effective signaling for positive selection places a much greater constraint on the repertoire than negative selection. This conclusion is also consistent with a study by Surh and Sprent (55), which showed that the extent of intrathymic death caused by negative selection is less than that due to neglect, although no quantitative estimate of the extent of deletion could be inferred from these data. However, if one considers only those thymocytes whose interaction with self-peptide-MHC ligands can initiate the positive selection process necessary for contribution to the functional repertoire, the data presented here clearly show that many of these cells never complete maturation, but are eliminated by recognition of the same class of MHC as that responsible for initiating their differentiation.

Two very recent studies bear on the issue of the frequency of positively selected thymocytes that subsequently undergo negative selection. Using transgenic mice expressing a single peptide-MHC class II complex, Ignatowicz et al. (22) found that 65% of T cell hybridomas established from...
positively selected CD4+ cells reacted with wild-type MHC class II molecules presented by APCs. These data were interpreted to mean that 65% of positively selected thymocytes in these transgenic mice would normally have undergone negative selection. However, in another transgenic model in which MHC class II was expressed only on thymic cortical epithelium, Laufer et al. (25) estimated the frequency of positively selected CD4+ cells reactive with MHC class II on APCs to be only 5% by limiting dilution analysis. The apparent discrepancy in the frequency estimates of thymocytes that are potentially subject to negative selection in these two studies could result from differences in the transgenic models and/or assay systems used. Whatever the explanation, it should be emphasized that neither study directly addresses the extent of thymic clonal deletion, because the fate of the MHC-reactive cells in these transgenic systems in vivo could have been either deletion or induction of anergy.

Based on the requirement for epithelial MHC recognition in positive selection, the negative selection events mediated by hematopoietic MHC molecules here appear to occur after initiation of this process. This raises the question of what is different about recognition by partially mature T cells of MHC ligands on bone marrow-derived cells as compared with the recognition of possibly the identical peptide-MHC complex on epithelial cells by immature precursors, such that the former leads to cell death and the latter to promotion of differentiation. Two of the most obvious possibilities are the increased surface expression of TCR by partially mature cells (56, 57, 41), which could change the quantity or even quality of signals received by the cell, and the expression of costimulatory ligands such as CD80/CD86 on the hematopoietic cells. In some models, the latter has been shown to contribute to thymocyte death when combined with TCR signals (58, 59). Whatever the explanation, these data focus attention on the issue of whether peptides unique to epithelial cells make a predominant contribution to repertoire development. Whereas no epithelium-specific peptides could be eluted from class II MHC molecules (60), the existence of such tissue-specific ligands has been demonstrated by transplant experiments (10).

Finally, central (thymic) and peripheral tolerization by means other than deletion have been described (61, 62). In this respect, it will be of considerable interest to determine whether mature T cells from our chimeras that have escaped negative selection will respond when confronted with professional APCs expressing the MHC ligands encountered during positive selection.

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