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Identification of a Developmentally Regulated Phase of Postselection Expansion Driven by Thymic Epithelium

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To investigate events following the initiation of positive selection, we have used reaggregate organ cultures to follow the maturation of purified CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes; these thymocytes represent a subpopulation of thymocytes which have already received positive selection signals. Using a dilution analysis of an FITC-based membrane-binding dye, 5-(and 6-)carboxyfluorescein diacetate succinimidyl ester, to allow a quantitative measure of proliferation, we show that while newly selected CD4<sup>+</sup> and CD8<sup>+</sup> cells are nondividing, both subsets subsequently undergo a wave of postpositive selection proliferation involving multiple cell divisions. Moreover, in the presence of fetal stromal cells, postselection expansion is more extensive in newborn thymocytes compared with adult thymocytes, suggesting that this phase of expansion is developmentally regulated. We also show that proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells is seen in reaggregates of purified MHC class II<sup>+</sup> thymic epithelial cells, while CD4<sup>+</sup> and CD8<sup>+</sup> cells generated from bcl-2 transgenic CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes in the absence of stromal cell support survive but do not proliferate; this observation indicates that MHC class II<sup>+</sup> thymic epithelial cells are both necessary and sufficient to mediate this wave of cell division. Finally, the maturation of CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes and the subsequent proliferation of CD4<sup>+</sup> and CD8<sup>+</sup> cells occur in the presence of MHC-mismatched thymic stromal cells, suggesting that the later stages of positive selection and the associated postselection events do not depend on interactions with the same peptide/MHC complexes responsible for initiation. The Journal of Immunology, 1998, 160: 3666–3672.

Interactions between immature CD4<sup>+</sup>8<sup>+</sup> thymocytes and thymic epithelial cells result in maturation to the single positive CD4<sup>+</sup> or CD8<sup>+</sup> stages, a process known as positive selection (1, 2). During this process, a variety of phenotypic changes occur, including up-regulation of the αβ TCR complex and transient expression of the early activation marker CD69. Thus, we (3, 4) and others (5–7) have shown that thymocytes of a CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> phenotype contain an intermediate subpopulation of thymocytes that have initiated, but not yet completed, positive selection. In addition to changes in surface phenotype, positive selection also induces maturation from a functionally incompetent to a functionally competent stage, such that while signaling through the TCR complex triggers CD4<sup>+</sup>8<sup>+</sup> thymocytes to undergo apoptosis, CD4<sup>+</sup> and CD8<sup>+</sup> cells are activated and triggered to undergo proliferation (8, 9).

Although a number of studies have investigated the mechanisms regulating the initiation of positive selection of CD4<sup>+</sup>8<sup>+</sup> thymocytes both in terms of the cell interactions required and the subsequent signaling pathways involved (10, 11), events during the later stages of positive selection are less clear. Moreover, little is known about events occurring after the generation of newly selected CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> thymocytes (12). To address these issues, we isolated a subset of CD4<sup>+</sup>8<sup>+</sup> thymocytes on the basis of CD69 expression and followed the development of these CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes under controlled in vitro conditions using reaggregate thymic organ cultures (RTOCs) (2) which closely mimic the requirements for thymocyte-stromal cell contacts in vivo (4, 13–15).

In conjunction with this approach, we have used a method that allows quantitative estimations of cellular proliferation to be made on a per cell basis and identified a phase of postpositive selection expansion involving multiple cell divisions, which involves both CD4<sup>+</sup>8<sup>+</sup> and CD4<sup>+</sup>8<sup>+</sup> subsets. In addition, we show that this phase of expansion is more extensive in neonatal thymocytes compared with adult thymocytes and is dependent on the support of MHC class II<sup>+</sup> thymic epithelial cells. Finally, we show that the development of CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes and the subsequent proliferation of their progeny occur in the presence of MHC-mismatched thymic stromal cells, indicating that the completion of positive selection and associated postselection events is not dependent on continued interaction with peptide/MHC complexes identical to those initiating positive selection.

**Materials and Methods**

**Mice**

H-2<sup>d</sup> BALB/c and H-2<sup>b</sup> C57BL/6 mouse embryos at day 15 of gestation were used as a source of embryonic thymuses for the preparation of thymic stromal cells. The day of detection of the vaginal plug was designated as day 0 of gestation. CD4<sup>+</sup>8<sup>+</sup>69<sup>+</sup> thymocytes were prepared from either BALB/c. β<sub>2</sub>-microglobulin (β<sub>2</sub>-m) knockout (β<sub>2</sub>-m<sup>−/−</sup>) (The Jackson Laboratory, Bar Harbor, ME) or p56<sup>Δ/Δ</sup>bcl-2 transgenic mice (a kind gift of Dr. S. Korsmeyer, provided by Dr. Nick Platt, Sir William Dunn School of Pathology, Oxford, U.K.) as indicated.

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4 Abbreviations used in this paper: RTOC, reaggregate thymic organ culture; BrdU, bromodeoxyuridine; wt, wild-type; CFSE, 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester.
Abs and flow cytometry

The following Abs were used for flow cytometric analysis and immunomagnetic isolation of thymocytes and thymic stromal cells as previously described (13–15): anti-CD4 phycoerythrin (GK1.5), anti-CD8 FITC (53–6.7), anti-CD45 (M1/9; American Type Culture Collection, Rockville, MD), anti-CD69 (H.12F3), anti-IA4 (MK-D6; Becton Dickinson, Mountain View, CA). Multicolor flow cytometry was performed using a Coulter Epics Elite machine (Coulter Electronics, Hialeah, FL), as previously described (13).

Preparation of cell types and reaggregation cultures

CD4^+8^−69^+^ thymocytes. Thymocyte suspensions were prepared from either 4–6-wk-old adult or 0–3-day-old neonatal mice of the indicated strains as appropriate. Cells expressing CD69 were selected from such preparations using streptavidin Dynabeads (Dynal, Wirral, U.K.) coated with biotinylated anti-CD69 Abs, and beads were then removed with Detachabead (Dynal). The isolation of CD4^+8^−69^+^ cells from these CD69^+^ preparations was then achieved by additional selection using anti-rat IgG Dynabeads coated with anti-CD8 (clone YTS 169.4; Sera-Lab, Sussex, U.K.) Abs as previously described (3, 4).

Thymic stromal cells and RTOCs. Mouse embryo thymuses (15 days old) were cultured for 5–7 days in 1.35 mM 2-deoxygluconosine and trypsinized (0.25% trypsin in 0.02% EDTA; Sigma, St. Louis, MO) to form a single-cell suspension. Residual hemopoietic elements were depleted with anti-CD45-coated Dynabeads, and in some cases MHC class II^+^ thymic epithelial cells were further purified from such whole stromal cell preparations using anti-IA^a^-coated Dynabeads as described earlier. RTOCs were prepared by mixing together purified thymocytes and appropriate stromal cells at a ratio of 1:1. Cell mixtures were pelleted by centrifugation, and the resulting cell slurry was deposited on the surface of the 0.8-μm Nuclepore filters as previously described (13–15). Thymocytes were harvested from RTOCs after the indicated culture period by gently teasing them apart with fine knives.

Analysis of cell division using 5- (and 6-) carboxyfluorescein diacetate succinimidyl ester (CFSE)

To allow quantitative analysis of cell division, we used CFSE, an FITC-based lipophilic membrane-binding dye, which has a fluorescence intensity that has been shown to be directly related to cell division (16, 17). Thus, thymocytes were pulsed with 0.1 μM CFSE in PBS for 10 min at 37°C before their incorporation into reaggregate cultures. In some experiments, thymocytes harvested from reaggregate cultures were analyzed for CD4 and CD8 expression together with CFSE content to allow the separate study of CD4^+^8^−^ and CD4^+^8^+^ cells.

Results

CD4^+^8^−^69^+^ thymocytes are developmental intermediates in positive selection: evidence for lineage commitment by this stage

The positive selection of thymocytes involves a commitment step to the CD4^+^8^−^ or CD4^+^8^+^ stage depending upon the ability of the TCR to recognize MHC class II or class I molecules (1, 2). To further underline their status as positive selection intermediates, we compared CD4^+^8^−^69^+^ thymocytes from both wild-type (wt) and β2m^−/−^ mice for evidence of lineage commitment in RTOCs. As β2m^−/−^ mice lack expression of MHC class I Ags (18), and CD69 expression during positive selection is dependent upon TCR-MHC interactions (3), cells of a CD4^+^8^−^69^+^ phenotype present in β2m^−/−^ mice can only be generated as a result of interactions with MHC class II Ags.

Figure 1 shows that after 5 days in RTOCs, as expected, CD4^+^8^−^69^+^ thymocytes from wt mice give rise to large cohorts of both CD4^+^8^−^ and CD4^+^8^+^ cells which express high levels of αβ TCR (data not shown and Refs. 13 and 15), in a ratio of 2:1, in the presence of wt stromal cells. In contrast, CD4^+^8^−^69^+^ thymocytes from β2m^−/−^ mice, while still capable of development in the presence of wt stroma, show a strong skewing toward the production of CD4^+^8^−^ cells, with ~10-fold fewer CD4^+^8^−^ cells seen in comparison with CD4^+^8^−^ cells (Fig. 1). Thus, these data suggest that the majority of CD4^+^8^−^69^+^ thymocytes from β2m^−/−^ mice show evidence of commitment to the CD4^+^8^−^ stage, even when allowed to complete their maturation in the presence of wt thymic stromal cells expressing MHC class I and class II Ags; these data also provide evidence for the status of CD4^+^8^−^69^+^ thymocytes as a subset of thymocytes which have received positive selection signals.

Identification of a developmentally regulated wave of postpositive selection expansion involving multiple cell divisions

To investigate the maturational events following the initiation of positive selection, we analyzed the development and associated cell cycle kinetics of a single population of CD4^+^8^−^69^+^ thymocytes in RTOCs. Using this approach, any proliferation involving prepositive selection cells, such as CD4^+^8^−^69^+^, is avoided. Initially, an analysis of bromodeoxyuridine (BrdU) incorporation (15, 19), is avoided. Initially, an analysis of bromodeoxyuridine (BrdU) incorporation showed that, in agreement with Ernst et al. (20), single positive thymocytes generated from CD4^+^8^−^69^+^ double positive precursors in RTOCs show evidence of cell division (data not shown). However, while BrdU incorporation is a useful parameter of cell division, it does not give an indication of the actual extent of proliferation in terms of the number of cell divisions occurring when it is used in these analyses. Thus, we analyzed the extent of cell division using an FITC-based lipid membrane-binding dye, CFSE, since halving of the fluorescence intensity of these compounds is directly related to a single cell division. Quantitation of the number of cell cycles can be achieved by assessing the dilution...
of CFSE intensity by flow cytometry (16, 17). CD4\(^{+}\)8\(^{+}\)69\(^{+}\) thymocytes were pulsed with 0.1 \(\mu\)M CFSE, reaggregated with thymic stromal cells, and then analyzed for CFSE content by flow cytometry after 3 days of culture. Figure 2b shows the CFSE analysis of cells generated from CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes after 3 days of culture. This dilution profile, seen next to the initial intensity of CFSE labeling (Fig. 2a), shows evidence of an extensive wave of proliferation, with the majority of cells having undergone between one and three divisions and some cells dividing up to six times. Moreover, by analyzing the expression of CD4 and CD8 molecules in association with CFSE labeling, we show that this wave of expansion operates on both single positive CD4\(^{+}\)8\(^{-}\) and CD4\(^{+}\)8\(^{+}\) cells, with a large proportion of each subset having undergone several cell divisions by day 3 of culture (Fig. 2c and d). In addition, we also show that newly selected single positive thymocytes that are generated throughout the 3-day culture period are nondividing but subsequently proliferate (Fig. 3, a–f), indicating that completion of the positive selection process does not involve cell division.

These experiments suggest that thymocytes complete positive selection to the single positive stage; only then do they undergo a phase of cell division. This wave of postselection expansion may be a mechanism to expand the number of cells in the thymus with useful TCR specificity before their export to the periphery, during the stages in development in which the peripheral T cell pool is not yet established. To investigate this, we compared proliferation following positive selection of adult vs neonatal CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes in reaggregate cultures with deoxyguanosine-treated fetal thymic stromal cells, using CFSE as a measure of proliferation. Figure 4 shows that although single positive thymocytes are generated from both adult and neonatal thymocytes in the presence of fetal stromal cells, subsequent postselection expansion is more pronounced in neonatal thymocytes, with the majority of adult cells showing no evidence of cell division after 3 days of culture. These data indicate that the phase of cell division following positive selection shows evidence of developmental regulation, particularly at the level of the thymocyte precursor itself.

**Postselection expansion is dependent upon MHC class II\(^{+}\) thymic epithelial cells but not interactions with the same peptide/MHC complexes responsible for the initiation of positive selection**

Recently, we have shown that CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes from bcl-2 transgenic mice are capable of maturation to the CD4\(^{+}\)8\(^{-}\) and CD4\(^{+}\)8\(^{+}\) stages in a stromal-cell-independent manner (21), and we have also used this approach to investigate the potential requirement for thymic stromal cells in the phase of postselection expansion. Thus, CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes were harvested from neonatal bcl-2 transgenic mice, pulsed with CFSE, and cultured in the absence of stromal cell support for 3 days. Cells were harvested and analyzed for both CD4 and CD8 expression and evidence of proliferation as assessed by CFSE dilution. In agreement with our earlier findings (21), bcl-2 transgenic CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes that are cultured alone acquire a single positive CD4\(^{+}\)8\(^{-}\) or
CD4<sup>−</sup>8<sup>−</sup> phenotype in a manner similar to that seen with wt thymocytes in the presence of stromal cells (Fig. 5a and Fig. 3). However, in marked contrast to cells developing in the presence of thymic stroma (Fig. 3), cells generated from bcl-2 transgenic CD4<sup>18</sup>169<sup>1</sup> thymocytes in the absence of stromal cell support show no evidence of proliferation, with all cells showing a uniform level of CFSE labeling (Fig. 5b). Moreover, in reaggregate cultures in which MHC class II<sup>1</sup> thymic epithelial cells are the only stromal cell type, a similar extent of cell division is seen compared with cultures with whole thymic stromal preparations (Fig. 5c). Thus, these data indicate that interactions with thymic stromal cells, in particular the MHC class II<sup>1</sup> epithelial component, are both necessary and sufficient for the phase of cell division following positive selection.

As MHC class II<sup>1</sup> thymic epithelial cells are the cell type responsible for the initiation of positive selection, which involves interactions between TCR and peptide/MHC complexes (14), we next investigated the role of such interactions in the later stages of positive selection and associated postselection events. Thus, CD4<sup>−</sup>8<sup>−</sup>9<sup>−</sup> thymocytes in the absence of stromal cell support show no evidence of proliferation, with all cells showing a uniform level of CFSE labeling (Fig. 5b). Moreover, in reaggregate cultures in which MHC class II<sup>1</sup> thymic epithelial cells are the only stromal cell type, a similar extent of cell division is seen compared with cultures with whole thymic stromal preparations (Fig. 5c). Thus, these data indicate that interactions with thymic stromal cells, in particular the MHC class II<sup>1</sup> epithelial component, are both necessary and sufficient for the phase of cell division following positive selection.

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![Figure 3](https://www.jimmunol.org/)

**FIGURE 3.** Newly generated single positive thymocytes undergo a phase of postselection expansion involving multiple cell divisions. Three reaggregates, each initially containing 1.3 × 10<sup>6</sup> CD4<sup>−</sup>8<sup>−</sup>9<sup>−</sup> thymocytes, were harvested and analyzed for cell division (CFSE analysis, a–c) and CD4 and CD8 expression (d–f, percentages shown) after 1 day (a and d), 2 days (b and e), and 3 days (c and f) in culture. Yields were 3.5 × 10<sup>5</sup>, 3.2 × 10<sup>5</sup>, and 4 × 10<sup>5</sup> thymocytes for reaggregates after 1, 2, and 3 days in culture, respectively. Note that although single positive cells can be seen from day 1 of culture (d), these cells are nondividing (a), with multiple cell divisions clearly evident by day 3 (c), indicating that the expansion of the newly selected repertoire occurs after the single positive stage has been reached.

![Figure 4](https://www.jimmunol.org/)

**FIGURE 4.** Postpositive selection expansion shows evidence of developmental regulation. Adult or neonatal CD4<sup>−</sup>8<sup>−</sup>9<sup>−</sup> thymocytes (4.4 × 10<sup>5</sup>) were placed in reaggregate cultures with fetal thymic stroma for 3 days. Harvested cells were analyzed for proliferation assessed by CFSE labeling as shown by flow cytometry. In the experiment shown, 5 × 10<sup>4</sup> and 1 × 10<sup>4</sup> cells were recovered from RTOCs of neonatal and adult thymocytes, respectively. These data are representative of three separate experiments.
newly selected CD4\(^{+}\)8\(^{-}\) and CD4\(^{+}\)8\(^{+}\) cells can occur in the presence of peptide/MHC complexes that are different from those which initiated their positive selection.

**Discussion**

While a number of reports have provided evidence that positive selection is a multistage process, the events following its initiation are unclear. In this report, we have purified a subset of CD4\(^{+}\)8\(^{-}\) thymocytes that express the early activation marker CD69. Recently, we (3, 4) and others (5–7) have shown that these thymocytes possess a number of characteristics of cells undergoing the positive selection process. Here, we have further characterized the status of these cells as intermediates in positive selection by showing that CD4\(^{+}\)8\(^{-}\)\text{69}\(^{+}\) cells from b2m\(^{-}\)2 mice that are subsequently cultured with whole stroma or purified MHC class II\(^{+}\) epithelial cells. An input of 6.5 \times 10^5 CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes yielded 0.8 \times 10^5 cells from each reaggregate. A comparison of cell division by CFSE intensity in thymocytes recovered from these two reaggregates reveals that purified epithelium alone is as capable of supporting this wave of expansion as whole stroma.

BrdU incorporation (data not shown). However, since quantitation of cell proliferation was not possible using this method, we have extended these observations using a method that allows the estimation of cell cycle numbers to be made on a per cell basis (16, 17). By analyzing the maturation of CFSE-labeled CD4\(^{+}\)8\(^{-}\)69\(^{+}\) cells over a 3-day period in RTOCs, we show that newly selected CD4\(^{+}\)8\(^{-}\) and CD4\(^{+}\)8\(^{+}\) thymocytes are generated out of cycle, cells of both mature thymocyte lineages undergo a substantial wave of postpositive selection proliferation involving at least six cell divisions (Figs. 2 and 3). The importance of this phase of proliferation, involving newly generated thymocytes which have completed selection processes in the thymus, is unclear. Interestingly however, by comparing the cell cycle kinetics of neonatal vs adult CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes in the presence of a similar source of thymic stromal cells, we show that this wave of postselection expansion, although evident at both developmental stages, is more pronounced in the progeny of neonatal thymocytes. Thus, it may be the case that this proliferation may serve to expand thymocytes that complete positive selection before their export to the periphery, at stages in which the peripheral T cell pool is not yet fully established, as is the case for the neonate.

To study the role of thymic stromal cells in these events, we used CD4\(^{+}\)8\(^{-}\)69\(^{+}\) thymocytes from bcl-2 transgenic mice, which...
are independent of maintenance signals in vitro and so allow discrimination between those signals necessary for cell survival and those required for further differentiation. While bcl-2 transgenic CD4\(^+\) CD8\(^+\) thymocytes (8 \times 10^5) were placed in reaggregates with either MHC-matched (H-2\(^d\)) or MHC-mismatched (H-2\(^b\)) thymic stroma. After 3 days of culture, 1.6 \times 10^5 cells were harvested from the H-2\(^b\) reaggregate, and 1.2 \times 10^5 cells were harvested from the H-2\(^d\) reaggregate. These cells were analyzed for both CD4 and CD8 expression and CFSE dilution. The CD4/CD8 profiles and the percentages of each subset are shown for the MHC-matched (a) and MHC-mismatched (b) reaggregates; a graph showing cell divisions and representing CFSE dilution (c) is also shown.

Finally, by analyzing the continued maturation of CD4\(^+\) 8\(^+\) 69\(^+\) thymocytes that have initiated positive selection on MHC molecules of an H-2\(^d\) haplotype, we find that nontransgenic newly selected thymocytes undergo proliferation in the presence of purified thymic epithelial cells alone (Fig. 5c), indicate that MHC class II\(^+\) thymic epithelial cells are both essential and sufficient to drive the postselection proliferation of newly selected CD4\(^+\) 8\(^+\) and CD4\(^+\) 8\(^+\) thymocytes.

Our findings are in contrast with a recent report (25) in which CD4\(^+\) 8\(^+\) TCR\(^{\text{high}}\) cells isolated from H-Y TCR transgenic mice were shown to be unable to complete maturation when injected into MHC-mismatched thymuses in vivo. The reason for this discrepancy is unclear, although it should be noted that the developmental relationship between CD4\(^+\) 8\(^+\) 69\(^+\) thymocytes from wt mice and CD4\(^+\) 8\(^+\) TCR\(^{\text{high}}\) cells from H-Y TCR transgenic mice is not known. In conclusion, we have identified a wave of cellular expansion, following the positive selection of both CD4\(^+\) 8\(^+\) and CD4\(^+\) 8\(^+\) lineages, that involves multiple cell divisions. This wave of expansion, which appears to be developmentally regulated at the
level of the thymocyte precursor, is dependent on interactions with MHC class II+ thymic epithelial cells. Our current studies are aimed at determining the nature of the signals responsible for post-positive selection proliferation.

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