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Cutting Edge: Identification of the Targets of Clonal Deletion in an Unmanipulated Thymus

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Autoreactive thymocytes can be eliminated by clonal deletion during their development in the thymus. The precise developmental stage(s) at which clonal deletion occurs in a normal thymus has been difficult to assess, in large part because of the absence of a specific marker for TCR-mediated apoptosis. In this report, we reveal that Nur77 expression can be used as a specific marker of clonal deletion in an unmanipulated thymus and directly identify TCRintCD4+CD8+ and semimature CD4+CD8− thymocytes as the principal targets of deletion. These data indicate that clonal deletion normally occurs at a relatively late stage of development, as cells mature from CD4+CD8+ thymocytes to single-positive T cells. The Journal of Immunology, 2003, 170: 10–13.
Fort Washington, PA) while agitating (30 min, room temperature (RT)). Fixed cells were washed twice with staining medium, permeabilized with 0.1% Triton X-100 in PBS (15 min, RT), washed three times with internal staining medium (10% FBS, 0.05% NaN3 in PBS), and incubated overnight at 4°C with anti-Nur77 or the isotype control, anti-human CD9. Cells were washed three times, incubated with FITC mAb anti-mouse IgG1 (30 min, RT), washed four times, and resuspended in staining medium. Between 0.6 × 10^6 and 1.0 × 10^6 events were collected for each experiment, and cells were gated on single, live cells (as determined by forward scatter) for analysis on a FACScalibur with CellQuest software (BD Biosciences).

Results

Nur77 expression in thymocytes undergoing clonal deletion can be assessed by flow cytometry

Nur77 expression, an important prelude to clonal deletion in the thymus, has heretofore been assessed by Western blotting (17, 22). To determine whether Nur77 expression could be assessed by flow cytometry, which would permit a close analysis of thymocyte phenotype, we stained purified CD4+CD8+ thymocytes for intracellular Nur77 after TCR-mediated stimulation (Fig. 1). In agreement with previous observations (22), flow cytometric analysis revealed that TCR-mediated apoptotic signals (TCR/CD28 costimulation (10, 11)) induced up-regulation of Nur77 (Fig. 1). Expression kinetics were quite rapid: Nur77 levels peaked between 2 and 3 h after stimulation, then declined to background levels within 5 h. In contrast, Nur77 levels did not change significantly in thymocytes exposed to strong TCR-mediated signals that induce activation and maturation, but not death (TCR/CD2 costimulation (23)) (Fig. 1). Other, non-TCR-mediated apoptotic stimuli, including dexamethasone exposure, also failed to up-regulate Nur77 expression (Ref. 18 and data not shown).

Thymocytes undergoing clonal deletion in vivo express Nur77

The ability to determine the expression of a specific marker of clonal deletion by flow cytometry offered a unique opportunity to directly identify the phenotype of normal thymocytes targeted for clonal deletion. To assess the feasibility of detecting Nur77 expression in unmanipulated thymocytes, we internally stained a freshly isolated suspension of thymocytes for Nur77. Given that very few cells are likely to be undergoing clonal deletion at any one time, we anticipated finding only a very small percentage of Nur77+ thymocytes (24). Indeed, 0.3–0.7% of freshly isolated thymocytes from C57BL/6 mice stained specifically for Nur77 (Fig. 2a).

To determine whether Nur77 expression among this small population of freshly isolated thymocytes was an indicator of clonal deletion events in vivo, we assessed the frequency of cells expressing Nur77 in two models of clonal deletion mediated by 1) self-peptide/MHC in a TCR transgenic and 2) endogenous superantigenics in BALB/c mice.

AND TCR transgenes (Vα11/Vβ3) are derived from a T cell specific to the pigeon cytochrome c/I-Ek complex and provide a model for MHC class II restricted TCR selection (20, 25). AND transgenic are positively selected in the H-2b background (I-A^b) and clonally deleted in the H-2b background (25). When we compared frequencies of Nur77+ thymocytes in AND TCR transgenic thymocytes from both backgrounds, we found that there was a significantly higher frequency of Nur77+ thymocytes developing in negatively selecting AND H-2b/s mice vs wild-type and positively selecting AND H-2a/s mice (p < 0.05) (Fig. 2b, 2c and d).

In BALB/c mice, viral superantigenics delete thymocytes expressing multiple Vβ-chains, including Vβ3+ (26, 27). We

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\text{FIGURE 2. Nur77 expression is associated with clonal deletion in vivo. a, Nur77+ cells in wild-type mice: freshly isolated thymocytes from B6 mice were stained for Nur77 as described. An overlay histogram of control and Nur77 staining, representative of at least six experiments, is displayed, and the percentage of specific staining is indicated. b, Nur77+ cells in AND TCR transgenics: freshly isolated thymocytes from wild-type, H-2b/s, AND TCR transgenic and H-2b/s AND TCR transgenic mice were stained for Vα11 and Nur77 as described. Staining profiles of Vα11 vs isotype control (upper panel) or Nur77 (lower panel) are shown, and the percentage of cells falling into the gates are indicated. The percentage of specific Nur77 staining among Vα11+ AND TCR transgenic thymocytes and total wild-type thymocytes was calculated, and the averages (±SEM) from three experiments are shown as a bar graph (c). The percentage of Nur77+ Vβ11+ cells in AND mice was significantly (p < 0.05) greater than the percentage of Nur77+ cells in both wild-type mice and AND mice. d, Nur77+ cells in BALB/c mice: thymocytes from BALB/c mice were stained for surface Vβ3 or Vβ8 expression and internal Nur77 as described. Vβ3+ thymocytes are specifically deleted by endogenous retrovirus. Staining profiles are displayed with gates used to calculate the percentage of Nur77+Vβ11+ staining (%Nur77+Vβ11+/%Vβ11+), which is indicated. These data are indicative of four individual experiments. The average ratio of cells targeted for deletion over control cells was 4.7 ± 1.6.}
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found that the frequency of Nur77+ thymocytes within the Vβ3+ thymocyte pool was significantly higher than the frequency of Nur77+ thymocytes within the control Vβ8+ pool (Fig. 2d). Together, these data strongly indicate that Nur77 expression is a specific feature of populations undergoing negative and not positive selection in vivo.

*Nur77*+ thymocytes are activated and represent both late-stage CD4+/CD8+ and semimature CD4+CD8+ thymocytes

To directly determine the developmental timing of clonal deletion in an unmanipulated thymus, we stained freshly isolated thymocytes from wild-type C57BL/6 mice for expression of Nur77, CD4, CD8, and one of three markers of T cell maturation: CD69, HSA, or TCR (Fig. 3). Nur77+ thymocytes were phenotypically distinct. Whereas the majority of B6 thymocytes express both CD4 and CD8, Nur77+ thymocytes are roughly divided between the CD4+CD8− and CD4+CD8+ subpopulations (Fig. 3a). Nur77+CD4+CD8+ thymocytes expressed lower levels of CD4 and CD8, a feature of double-positive thymocytes that have recently received a TCR signal (28–30). All Nur77+ thymocytes also expressed relatively high levels of TCR and uniformly high levels of CD69, features of cells that have been selected to mature (28, 30–33) (Fig. 3b). Finally, whereas all CD4+CD8+ thymocytes express HSA, the CD4+CD8− subpopulation can be subdivided into semimature cells that are HSA+ and mature cells that are HSA− (2, 3, 34). Virtually all Nur77+ cells expressed high levels of HSA (CD24) (Fig. 3c, left panels), including the Nur77+CD4+CD8− subpopulation, identifying them as semimature single-positive T cells (Fig. 3c, right panels).

**Discussion**

In this report we show that Nur77 identifies a small but distinct population of thymocytes undergoing clonal deletion in a normal thymus and confirm implications that negative selection occurs at both the CD4+CD8− and semimature CD4+CD8+ stages of development (2, 35). These findings specifically indicate that the principal targets of clonal deletion are relatively mature cells in transit from an activated CD69+TCR+CD4+CD8+ stage to a semimature CD69+HSA+CD4+CD8− stage. Notably, Nur77+CD4+CD8+ cells appear to have already received positive selection signals. Not only do they express lower levels of CD4 and CD8 and high levels of CD69, but they also express increased levels of TCR, the phenotype of cells that have been selected to mature (28, 30–32, 36).

It is important to note that both the rapid kinetics of Nur77 expression and the need for cell fixation to assess Nur77 expression make it difficult to definitively show that all immature thymocytes up-regulating Nur77 are fated to undergo apoptosis. However, our finding that cells undergoing TCR-mediated positive selection (in vivo and in vitro) do not exhibit significant Nur77 expression lends strong support to the view that Nur77 expression in immature thymocytes is induced by TCR signals that specifically result in cell death.

Interestingly, although CD4+CD8− thymocytes are well represented in the Nur77+ population, few CD4−CD8+ thymocytes are found. We favor the possibility that negative selection of thymocytes destined to become CD8+ T cells occurs before their full conversion to the CD4−CD8+ phenotype. Indeed, CD8+ T cells take a relatively circuitous route to maturity and pass through the CD4−CD8low stage before losing CD4 expression (37–39). The absence of Nur77+ cells in populations representing very early developmental stages (CD4−CD8− and TCR−CD4+CD8− cells) is also not surprising. Such early deletion, while well documented in some models (7, 8), depends on the generation of high-avidity TCR interactions in early developmental compartments—a situation most common in transgenic systems (8, 9).

Finally, although the frequency of Nur77+ transgenic thymocytes developing in a negative-selecting background was approximately three times higher than that in wild-type mice, one might have expected even greater frequencies. However, it is important to consider that, despite the abundance of targets for clonal deletion in the transgenic model, the stromal elements that mediate deletion may still be limiting. Therefore, at any

![FIGURE 3.](http://www.jimmunol.org/) The phenotype of wild-type Nur77+ thymocytes. Freshly isolated B6 thymocytes were stained for surface expression of CD4, CD8, and one of three maturation markers: TCR, CD69, or HSA. Cells were subsequently stained for Nur77 as described and analyzed by four-color flow cytometry. CD4/CD8 (a), TCR (b), CD69 (c), and HSA (d) staining profiles for ungated (top) and gated Nur77+ cells (bottom) are displayed. It should be noted that back-gating of data shown in b revealed that the cell population expressing intermediate levels of TCR was CD4+CD8−, and the cell populations expressing high levels of TCR were CD4+CD8− and CD4+CD8+. Also, c shows HSA profiles from both live, ungated cells (left) and gated, CD4+CD8− thymocytes (right). The percentage of cells falling into each indicated gate and their mean channel fluorescence (in italics) are shown in each panel. Similar profiles were observed in three experiments.
one time there may still be few thymocytes receiving negative-selecting signals.

Our results provide the first direct identification of the targets of clonal deletion in a normal thymus. They substantiate the hypothesis that clonal deletion occurs at a relatively late stage of thymocyte development, when cells are in microenvironments (the corticomedullary junction and medulla) rich in the co-stimulatory ligands required for deletion (10, 15, 40). A careful examination of Nur77 expression in situ may further clarify this issue.

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References