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

Hyung J. Cho, Samuel G. Edmondson, Arden D. Miller, MacLean Sellars, Shawn T. Alexander, Selin Somersan and Jennifer A. Punt

*J Immunol* 2003; 170:10-13; doi: 10.4049/jimmunol.170.1.10
http://www.jimmunol.org/content/170/1/10

**References**
This article cites 44 articles, 28 of which you can access for free at:
http://www.jimmunol.org/content/170/1/10.full#ref-list-1

**Subscription**
Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**
Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**
Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Cutting Edge: Identification of the Targets of Clonal Deletion in an Unmanipulated Thymus

Hyung J. Cho, Samuel G. Edmondson, Arden D. Miller, MacLean Sellars, Shawn T. Alexander, Selin Somersan, and Jennifer A. Punt

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 TCR\textsuperscript{int}/CD4\textsuperscript{+}CD8\textsuperscript{+} and semimature CD4\textsuperscript{+}CD8\textsuperscript{−} 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\textsuperscript{+}CD8\textsuperscript{+} thymocytes to single-positive T cells. The Journal of Immunology, 2003, 170: 10–13.

Immature T cells (thymocytes) that express autoreactive TCRs can be eliminated in the thymus by clonal deletion. The timing and targets of clonal deletion in a normal thymus remain controversial (1–4). Well-characterized in vivo and in vitro models of thymocyte selection suggest that clonal deletion can occur at multiple developmental stages (3, 5, 6): before development to the CD4\textsuperscript{+}CD8\textsuperscript{−} stage (7, 8), early and late in the CD4\textsuperscript{+}CD8\textsuperscript{+} stage of development (5, 8–11), and even at the CD4 single-positive stage of development (9, 12–15). Although these studies reveal the potential for a variety of thymocyte subpopulations to undergo clonal deletion, they do not identify the actual targets of deletion in a normal thymus. The few thymocytes undergoing TCR-mediated apoptosis are difficult to distinguish from the larger percentage of cells undergoing apoptosis for other reasons, including death by neglect. To directly identify thymocytes undergoing clonal deletion, one needs a marker specific to TCR-mediated thymocyte apoptosis. Nur77, an orphan member of the steroid nuclear receptor superfamily that plays a central role in thymocyte negative selection (16), is an appealing candidate. Dominant negative Nur77 constructs inhibit clonal deletion in a variety of experimental models (17, 18). Notably, Nur77 expression is specifically up-regulated in response to TCR-mediated apoptotic signals in primary T cells and T cell lines (17, 19, 20). Therefore, we examined the possibility that Nur77 expression could be exploited to identify the targets of clonal deletion in an unmanipulated thymus. In this report we demonstrate the value of Nur77 as a marker of clonal deletion and for the first time reveal the phenotype of the targets of clonal deletion in a normal thymus.

Materials and Methods

Antibodies

mAbs anti-CD2 (RM2-5), anti-CD28 (37.51), anti-TCR β-chain (H57-597), biotin anti-TCR β (H57-597), PE anti-CD4 (GK1.5), APC anti-CD4 (RM4-5), FITC anti-CD8 (53-6.7), PE anti-CD8 (53-6.7), biotin anti-CD8 (53-6.7), FITC anti-IgG1 (A85-1), biotin anti-TCR γ/δ (H425), PE anti-CD24/heat-stable Ag (HSA)\textsuperscript{b} (M1/69), PE anti-CD69 (H1.2F3), PE anti-V\textsuperscript{β} (KJ25), PE anti-V\textsuperscript{β}8 (F23.1), anti-Nur77 (12.14), and anti-human CD9 (M-L13) were purchased from BD Biosciences (San Diego, CA).

Mice

C57BL/6, BALB/c, SJL, and AND\textsuperscript{bb} TCR transgenic mice were purchased from Taconic Farms (Germantown, NY) and used at 6–10 wk of age. AND\textsuperscript{bb} and SJL mice were crossed to produce F1 AND\textsuperscript{bb} mice.

Isolation of CD4\textsuperscript{+}CD8\textsuperscript{+} thymocytes

CD4\textsuperscript{+}CD8\textsuperscript{−} thymocytes were purified as previously described (21). Briefly, freshly isolated thymocyte suspensions were plated onto anti-CD8 (83-12-5, 1 μg/ml)-coated petri dishes. After 1 h at 4°C, adherent cells were recovered and plated again. Recovered thymocytes were >95% CD4\textsuperscript{+}CD8\textsuperscript{−}.

Cell stimulation

Twenty-four-well plates were coated with anti-TCR (10 μg/ml), anti-CD2 (10 μg/ml), and anti-CD28 (50 μg/ml), as indicated. For stimulation, 1 × 10\textsuperscript{6} purified thymocytes suspended in culture medium (RPMI 1640, 10% FCS, 2 mM l-glutamine, 1 mM penicillin/streptomycin, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, and 5 × 10\textsuperscript{−3} M 2-ME) were distributed per well and incubated for indicated times (37°C, 5% CO\textsubscript{2}).

Staining

For surface staining, cells (5 × 10\textsuperscript{5} or 5 × 10\textsuperscript{4}) were incubated (30 min, 4°C) with Abs (5–10 μg/ml) in 30 μl or 150 μl staining medium (0.1% BSA, 0.1% NaN\textsubscript{3}, in HBSS (Life Technologies, Rockville, MD)), followed by streptavidin-RED670 (Life Technologies) if necessary. For internal staining, surface-stained cells were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Fort Washington, PA).

Department of Biology, Haverford College, Haverford, PA 19041
Received for publication September 5, 2002. Accepted for publication October 29, 2002.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by the National Science Foundation-Research Undergraduate Institution MCB-009092, the Howard Hughes Medical Institute, the Beckman Foundation, and the Merck Foundation. S.G.E. and S.T.A. are undergraduate Beckman and Howard Hughes Medical Institute Scholars, respectively, and S.S. was an undergraduate Merck Fellow.

2 H.J.C., S.G.E., A.D.M., and M.S. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Jennifer A. Punt, Department of Biology, Haverford College, 370 West Lancaster Avenue, Haverford, PA 19041. E-mail address: jpun@haverford.edu

4 Abbreviations used in this paper: HSA, heat-stable Ag; RT, room temperature.
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 × 10⁶ and 1.0 × 10⁶ 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 superantigens 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-Ab) and clonally deleted in the H-2d 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-2d/b mice vs wild-type and positively selecting AND H-2d/b mice (p < 0.05) (Fig. 2, b and c).

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

FIGURE 1. Negative-selecting signals induce rapid up-regulation of Nur77. Purified CD4+CD8+ thymocytes were stimulated with platebound Abs and stained for anti-Nur77 as described. Overlay histograms of Nur77 expression by unstimulated (dotted line) and stimulated thymocytes (solid line) are displayed. The percentage of cells falling into the indicated gates and their mean channel fluorescence (in italics) are shown. The majority of thymocytes that receive TCR/CD28 costimulatory signals undergo apoptosis during overnight culture (typically >65% vs <30% of cells receiving TCR/CD2 stimulation).

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-2d/b AND TCR transgenic: freshly isolated thymocytes from wild-type, H-2d/b AND TCR transgenic mice were stained for Vα11 and Nur77 as described. Staining profiles of Vα11 vs isotype control (upper panels) or Nur77 (lower panels) 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+ Vα11+ 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+ Vβ+ staining (%Nur77+ Vβ+/%Vβ+) 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.
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+. Virtually all Nur77+ cells expressed high levels of HSA (CD24) (Fig. 3c, left panel), 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+ TCRintCD4+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, very 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 TCRintCD4+CD8-) 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
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 costimulatory ligands required for deletion (10, 15, 40–44). A careful examination of Nur77 expression in situ may further clarify this issue.

Acknowledgments
We thank Dr. Judy Owen, Dr. David Allman, Dr. Terri Lauffer, Kevin Jones, Matt Rivenburgh, and Allison Tan for enlightening discussions and critical review of the manuscript. We also are grateful to Jillian Gallagher and Jessica Nguyen for making many of these studies possible.

References
24. Cho, K., J. A. Punt, G. S. O. Sharrow, and A. Singer. 1996. Lineage commitment in the thymus: only the most differentiated (TCRβ+β+CD8−) subset of CD4+CD8+ thymocytes has selectively terminated CD4 or CD8 synthesis. J. Exp. Med. 184:2391.