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# Effects of Castration on Thymocyte Development in Two Different Models of Thymic Involution<sup>1</sup>

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Age-associated thymic involution is accompanied by decreased thymic output. This adversely affects general immune competence and T cell recovery following cytoreductive treatments such as chemotherapy. A causal link between increasing sex steroids and age-related thymic atrophy is well established. Although castration has been demonstrated to regenerate the atrophied thymus, little is known about how this is initiated or the kinetics of thymocyte regeneration. The present study shows that although castration impacts globally across thymocyte development in middle-aged mice, the regenerative effects are initiated in the immature triple-negative compartment and early T lineage progenitors (ETP). Specifically, there was a reduction in number of ETP with age, which was restored following castration. There was, however, no change in ETP reconstitution potential in ETP at this age or following castration. Furthermore, in a chemotherapy-induced model of thymic involution, we demonstrate castration enhances intrathymic proliferation and promotes differentiation through the triple-negative program. Clinically, reversible sex steroid ablation is achieved hormonally, and thus presents a means of ameliorating immune inadequacies, for example, following chemotherapy for bone marrow transplantation. By improving our understanding of the kinetics of thymic recovery, this study will allow more appropriate timing of therapy to achieve maximal reconstitution, especially in the elderly. *The Journal of Immunology*, 2005, 175: 2982–2993.

It is well documented that the adaptive immune system deteriorates with age (1, 2). Much of the decline is associated with changes in T cell populations and function, which, in turn, can be attributed to degeneration of the thymus. Thymic atrophy is characterized by a decrease in size, weight, and cellularity with disruption of structure and function of the organ, culminating in a decrease of emigrants entering the peripheral T cell pool (3). The reduction in naive T cell output from the thymus initiates the homeostatic expansion of pre-existing memory cells (4–6), narrowing the TCR repertoire (7, 8) and decreased responses to new or previously encountered Ags (9).

It has been proposed that the decline of thymic function with age results from either alterations in precursors migrating from the bone marrow (BM)<sup>4</sup> to the thymus or changes in thymocyte development. BM-derived precursors have been shown to be quantitatively and qualitatively compromised with age, with old BM having diminished reconstitution capacity in fetal thymic organ cultures (FTOC) (10, 11) and in BM transplantation (BMT) (12) compared with young BM. This suggests an age-associated defect in either the entry of progenitor cells or the intrathymic prolifer-

ation of these cells. However, transfer of young BM into irradiated aged mice did not restore thymic architecture (13) and produced a smaller thymus than when young thymus was reconstituted with old BM (12). This indicates the involuted thymic microenvironment also has a profound impact on thymocyte development.

BM-derived precursors periodically seed the thymus, maintaining thymic lymphopoiesis throughout adult life (14, 15). The earliest intrathymic precursors are contained in the CD3<sup>-</sup>CD4<sup>-</sup>CD8<sup>-</sup> triple-negative (TN) compartment, which can be subdivided into four populations on the basis of CD44 and CD25 expression (16). The most immature thymocytes are CD44<sup>+</sup>CD25<sup>-</sup> (TN1), which up-regulate CD25 to become CD44<sup>+</sup>CD25<sup>+</sup> (TN2). The transition from TN2 to CD44<sup>-</sup>CD25<sup>+</sup> (TN3) is accompanied by TCR $\beta$ -chain gene rearrangement (16). Cells that have productively rearranged their  $\beta$ -chain commence TCR $\alpha$ -chain gene rearrangement and differentiate into CD44<sup>-</sup>CD25<sup>-</sup> (TN4) (17). This last TN stage is relatively transient with cells differentiating into CD4<sup>+</sup>CD8<sup>+</sup> double-positive (DP) cells, and, in turn, mature CD4<sup>+</sup> or CD8<sup>+</sup> single-positive (SP) thymocytes ready for export.

In aged mice, evidence for a lesion at an early step in thymocyte development stemmed from the finding of a decrease in the number of TN2 and downstream TN cells (18). Because there was no change in number of TN1 cells with age, it suggested a block specifically at the TN1 to TN2 transition rather than a defect in precursor recruitment. However, the TN1 subset is heterogenous and includes cells that do not have T potential (19). Further dissection of TN1 has identified a minute fraction with highly enriched T precursor potential called early T lineage progenitors (ETP) (20). More recently, Min et al. (21) demonstrated age-related developmental defects in ETP, indicating thymic involution initiates at an earlier stage than previously appreciated.

Thymic atrophy becomes most evident from the onset of puberty, predominantly due to elevated sex steroid levels (22, 23). Accordingly, surgical or chemical castration of aged animals

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<sup>4</sup> Abbreviations used in this paper: BM, bone marrow; BMT, BM transplantation; Cx, castrated; Cy, cyclophosphamide; DP, double positive; ETP, early T lineage progenitor; FTOC, fetal thymic organ culture; KGF, keratinocyte growth factor; SP, single positive; TEC, thymic epithelial cell; TN, triple negative.

caused regeneration of the thymus, which was reversed by administration of synthetic sex steroids (24–28). Castrated (Cx) male mice showed enhanced proliferation of CD3<sup>+</sup> thymocytes (29). Conversely, androgen administration to intact female and Cx male mice led to increased thymocyte apoptosis (30). Receptors for estrogen, androgen, and progesterone are found on both thymocytes and thymic stromal cells (31–35). However, a recent study demonstrated chimeric mice with androgen receptor-defective stroma and wild-type thymocytes did not undergo thymic atrophy (31). It therefore appears that the thymic stroma is the main cellular target for sex steroid-, or, at least, androgen-induced thymic atrophy.

The well-documented cellular regeneration that occurs in the aged thymus following sex steroid ablation sets a foundation for rejuvenating thymic function in immunocompromised settings. The present study establishes the kinetics of thymocyte regeneration that is crucial to the design of an appropriate treatment schedule for the aged and/or immunocompromised patient population.

## Materials and Methods

### Animals

Inbred C57BL/6 mice were obtained from the Animal Resources Centre, University of Adelaide, Walter and Eliza Hall Institute, and Baker Institute Precinct Animal Centre. Young mice were 8 wk old, and middle-aged mice were >9 mo old in this study. Mice were maintained at the Monash Medical School animal facility and the Precinct Animal Centre. Mice were allowed to acclimatize for 7 days before experimentation, which was performed according to animal experiment ethics committee guidelines and approval.

### Surgical castration

Mice were anesthetized, and a small incision was made in the scrotum. The testes were exposed, ligated with dissolvable sutures, and removed. The wound was closed with dissolvable sutures or surgical staples. For surgical stress control, sham castration was performed as above, but without removal of the testes.

### Cyclophosphamide (Cy) treatment

Mice were injected i.p. with Cyclophosphamide (Pharmacia) at a dose of 100 mg/kg body weight per day for 2 consecutive days (total dose of 200 mg/kg).

### Flow cytometric analysis

Single cell suspensions of freshly dissected thymii were obtained by mechanical disruption using frosted glass slides. Cells released were washed with cold PBS supplemented with 1% FCS and recovered by centrifugation at  $470 \times g_{\text{max}}$  for 5 min at 4°C. Cell counts were determined by gating viable cells on cell size using an automated cell counter (Beckman Coulter).

To analyze thymocyte subpopulations, cells were labeled with anti- $\beta$ TCR allophycocyanin, CD4 PE, and CD8 PerCP. CD4<sup>+</sup> or CD8<sup>+</sup> SP thymocytes were defined as  $\beta$ TCR<sup>+</sup> CD4<sup>+</sup> or  $\beta$ TCR<sup>+</sup> CD8<sup>+</sup>, respectively. For TN thymocyte analysis, thymocytes were labeled with FITC-conjugated Abs to the lineage markers CD3, CD4, CD8, B220, CD11b, and Gr-1. TN thymocytes were gated on lineage-negative (Lin<sup>-</sup>) cells and detected by labeling with anti-CD44 CyChrome and anti-CD25 allophycocyanin.

For ETP analysis, thymocytes were labeled with PE- or biotin-conjugated Abs to CD3, CD8, CD19, CD11b, CD11c, Gr-1, NK1.1, CD127 (IL-7R $\alpha$ ), and CD25. Biotin-labeled conjugates were detected with streptavidin PE. ETP were gated on Lin<sup>-</sup>/CD127<sup>-</sup>/CD25<sup>-</sup> cells and identified by positive staining with anti-CD44-CyChrome and anti-CD117 (*c-kit*) allophycocyanin.

To detect proliferating thymocytes, cells were surface labeled as above, then fixed with 0.5% w/v paraformaldehyde in PBS and permeabilized using FACS permeabilization solution. Cells were then labeled with anti-Ki67 PE (for TN cells) or anti-Ki67 FITC (for ETP and all other thymocyte subpopulations). To detect apoptosis levels of ETP, cells were washed in annexin V binding buffer and stained with annexin V FITC. Apoptotic ETP were gated on live cells stained positive for annexin V. All buffers and conjugates were purchased from BD Biosciences, except anti-CD127 PE, which was purchased from Chemicon International. All cells were ana-

lyzed on a multiparameter FACSCalibur (BD Biosciences) using CellQuest software (BD Biosciences).

### ETP purification

Collagenase digestion of freshly dissected thymi (10 pooled for each group) was performed, as described previously (36). Thymocytes were incubated with saturating concentrations of anti-CD3 (KT3) and anti-CD8 (53-6.7). Ab-bound cells were removed with magnetic beads conjugated to sheep anti-rat IgG (DynaL Biotech). The negative fractions were then labeled for ETP, as described above, with anti-CD44 CyChrome substituted by anti-CD44 FITC. Cells were sorted on a multiparameter FACStar (BD Biosciences), counted, and analyzed to determine cell recovery and purity.

### FTOC

FTOC were established, as previously described (37). Briefly, E15 thymic lobes were depleted of thymocytes by treatment with 1.35 mM 2-deoxyguanosine (Sigma-Aldrich) for 5–7 days, before seeding with  $10^3$  sorted ETP. Following a 48-h incubation in hanging drop cultures using a Terasaki plate (Nunc), lobes were placed on polyethersulfone membrane disc filters (Pall Gelman Laboratory) supported by Gelfoam gelatin sponges (Pfizer). After an additional 19 days of culture, lobes were individually digested with collagenase, and cells were counted and analyzed.

### Statistical analysis

Statistical analysis was performed with the nonparametric, unpaired Mann-Whitney *U* test using InStat II software. A *p* value of less than or equal to 0.05 was considered statistically significant.

## Results

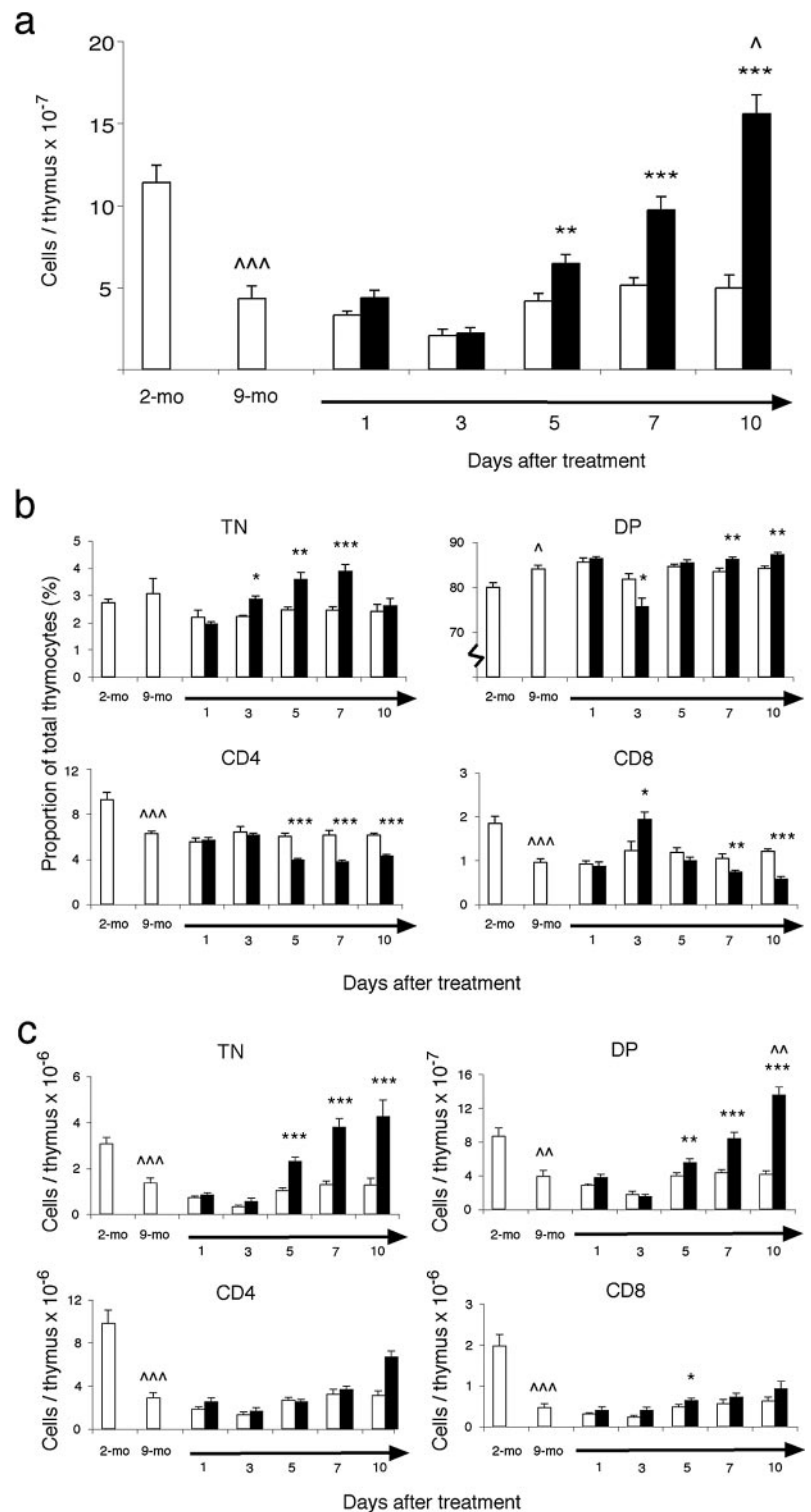
### Rapid reversal of age-related thymic atrophy following castration

To assess the kinetics of thymic regeneration following the sex steroid ablation, middle-aged mice were surgically castrated, which reduces serum testosterone to ~1% of normal levels within 6 h (38), and analyzed at various time points up to 10 days after surgery. The thymic cellularity of untreated middle-aged mice was >2-fold lower than that of 2-mo-old (2-mo) mice (Fig. 1*a*). Castration induced rapid regeneration of the involuted thymus, increasing cellularity beyond 2-mo levels within 10 days (Fig. 1*a*). The thymic cellularity of sham-Cx mice remained similar to that of untreated age-matched controls at all time points examined, indicating the effects of stress caused by surgery were negligible.

Analysis of thymocyte subpopulations in middle-aged mice revealed a proportional skewing, with an increase in CD4<sup>+</sup>CD8<sup>+</sup> DP thymocytes and decrease in mature CD4<sup>+</sup> and CD8<sup>+</sup> SP cells (Fig. 1*b*), while cell number was decreased across all subsets (Fig. 1*c*). Middle-aged mice demonstrated a proportional increase in the immature TN compartment as early as 3 days after castration (Fig. 1*b*). This was evident in cell number by day 5, with Cx mice exhibiting comparable TN cell numbers to untreated 2-mo levels by 7 days (Fig. 1*c*). Although an increase in number of DP thymocytes was also observed at day 5, this did not translate to a proportional increase until day 7. The net result was a decrease in the proportion of mature CD4<sup>+</sup> and CD8<sup>+</sup> SP cells (Fig. 1, *b* and *c*). These data indicate the initial regenerative event occurs in the early TN compartment, which then feeds through to the DP and then SP subpopulations.

### Castration induces proliferation of TN subsets in middle-aged mice

Because castration induced expansion of TN as a whole, changes within the subsets were examined (Fig. 2*a*). There was a numerical reduction in all TN subsets with age (Fig. 2*b*), but the various proportions remained unchanged (Fig. 2*c*), in agreement with Min et al. (21). However, while there was a 4-fold decrease in TN1 with age, there was only a 2-fold decrease in downstream TN subsets (Fig. 2*b*). The increase observed in the TN compartment in Cx

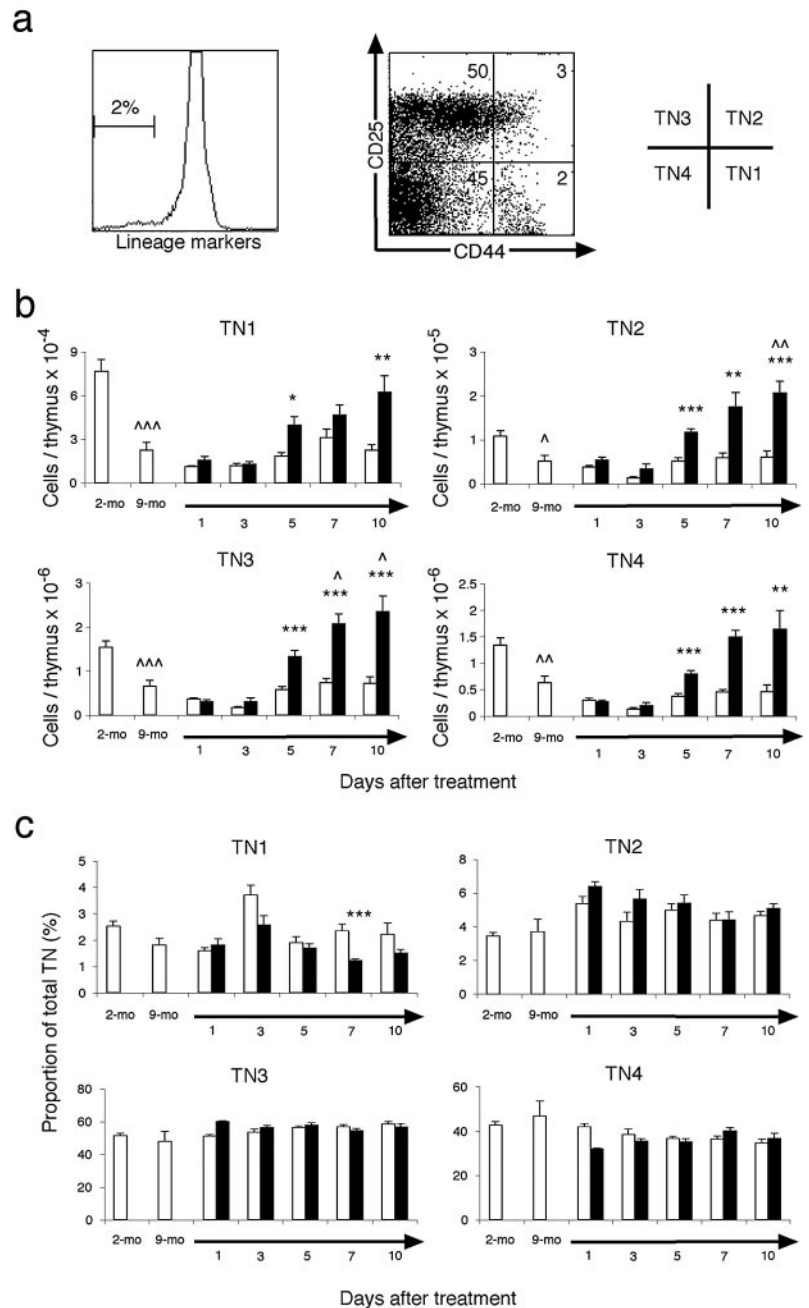


**FIGURE 1.** Castration of middle-aged mice rapidly restored thymic cellularity. *a*, Total thymic cell number of 2-mo, 9-mo, and sham-Cx (□) or Cx (■) 9-mo mice 1, 3, 5, 7, and 10 days after surgery. Total thymic cell number of Cx middle-aged mice was higher than that of sham-Cx mice from day 5, similar to young levels by day 7, and higher by day 10. *b*, Proportion of TN, DP, and mature SP. The proportion of TN cells was higher in Cx mice compared with sham-Cx mice as early as day 3. *c*, Cell number of TN, DP, and SP subsets. Cx mice had higher numbers of TN and DP cells than sham-Cx mice from day 5. Results are expressed as mean  $\pm$  SEM of four to eight mice for each group at each time point from three or more independent experiments. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  compared with sham-Cx 9-mo mice.  $\wedge$ ,  $p \leq 0.05$ ;  $\wedge$ ,  $p \leq 0.01$ ;  $\wedge$ ,  $p \leq 0.001$  compared with 2-mo mice.

mice at 5 days and later was due to an expansion across all four TN subsets (Fig. 2*b*). The reduced proportion of TN1 cells in Cx mice at 7 days reflects the stabilization of TN1, while other TN subsets continued to increase (Fig. 2*c*). The increase in cell numbers of downstream subsets could be derived from the TN1 subset, which itself was maintained by recruitment of BM-derived precursors. Conversely, the increase in downstream subsets may be due to increased proliferation within these subsets, independent of TN1.

To investigate the latter possibility, the TN subsets were analyzed for expression of Ki67, which is expressed in cells of G<sub>1</sub>, S,

G<sub>2</sub>, and M phase (39), and IL-7R $\alpha$ -chain (IL-7R $\alpha$ , CD127) (Fig. 3*a*). There was a decrease in the number of Ki67<sup>+</sup> cells in all TN subsets in middle-aged mice and an increase following castration (Fig. 3*b*). Cx middle-aged mice also showed an increase in the proportion of Ki67<sup>+</sup> cells in TN1, indicating a selective increase in proliferation in this heterogeneous subset (Fig. 3*c*). Additionally, the proportion of TN1 cells expressing IL-7R $\alpha$  decreased with age (Fig. 3*d*). Following castration, there was a decrease in the proportion of TN1 and TN2 cells and an increase in TN3 and TN4 cells bearing IL-7R $\alpha$  (Fig. 3*d*).



**FIGURE 2.** Increase in TN compartment following castration was due to an expansion across all TN subsets. *a*, Representative flow cytometric profile of Lin<sup>+</sup> TN subsets from a 2-mo mouse. *b*, Cell number of individual TN subsets of 2-mo, 9-mo, and sham-Cx (□) or Cx (■) 9-mo mice 1, 3, 5, 7, and 10 days after surgery. Compared with sham-Cx mice, Cx mice had higher cell number in all TN subsets on day 5 and thereafter. *c*, Proportion of individual TN subsets. Cx mice had decreased proportion of TN1 cells compared with sham-Cx mice 7 days after castration. Results are expressed as mean ± SEM of four to eight mice for each group at each time point from three or more independent experiments. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  compared with sham-Cx 9-mo mice. ^,  $p \leq 0.05$ ; ^^  $p \leq 0.01$ ; ^^ $p \leq 0.001$  compared with 2-mo mice.

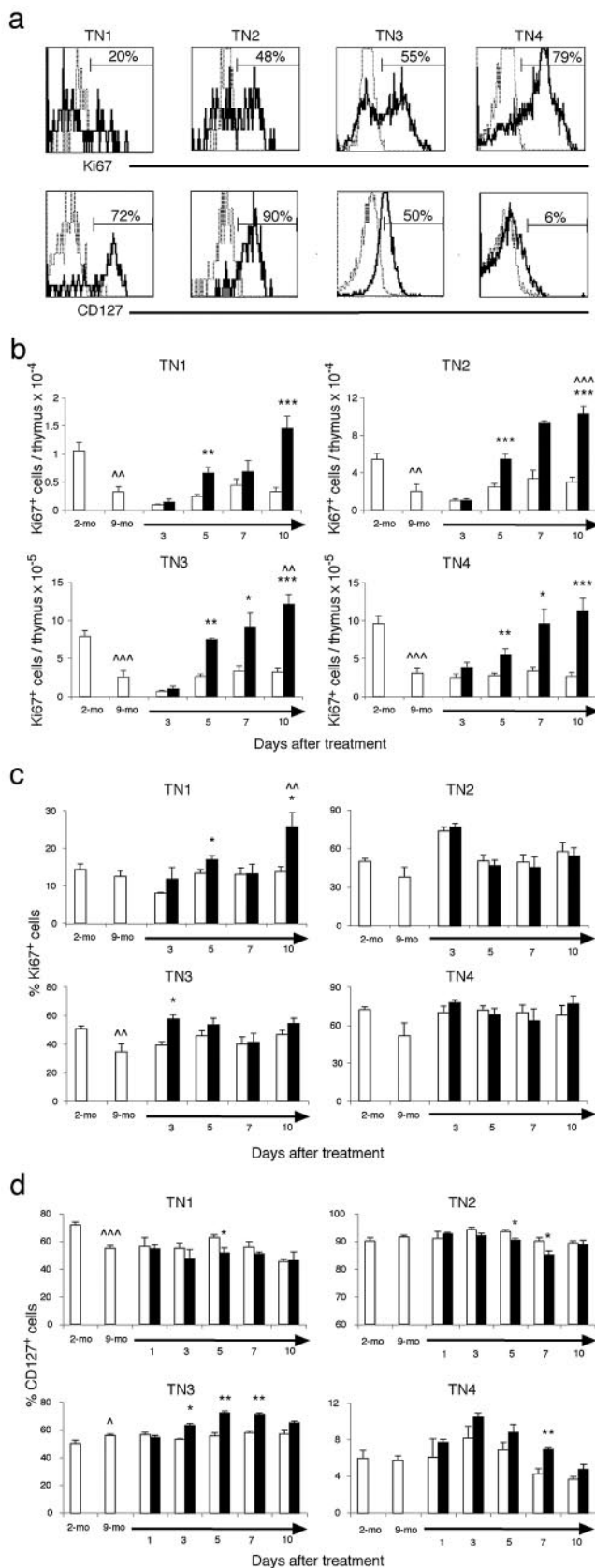
*Increase in ETP in middle-aged mice following castration*

The ETP fraction of TN1 is reportedly reduced with age (21). To investigate the effects of castration on this progenitor population, ETP were identified as Lin<sup>-</sup>/CD127<sup>-</sup>/CD25<sup>-</sup>/CD44<sup>+</sup>/CD117<sup>+</sup>, accounting for <0.01% of total thymocytes, as previously described (Fig. 4a) (20). The proportion of ETP per thymus did not appear to change with age (Fig. 4b). However, within the CD127<sup>-</sup> TN1 compartment, there was a significant decline in ETP proportion with age. This reduction in ETP proportion as well as absolute number was rapidly restored to normal young levels and beyond following castration. Analysis of Ki67 expression showed increased number, but not proportion of Ki67<sup>+</sup> ETP in Cx middle-aged mice (Fig. 4c). This suggests the trigger for thymic regeneration may be at the level of precursor entry rather than enhanced proliferation. Annexin V staining on ETP demonstrated an increase in apoptosis with age, which was restored to young levels

following castration (Fig. 4c). However, middle-aged ETP did not appear to differ from young ETP in their ability to reconstitute thymocyte-depleted fetal thymic lobes, nor did castration influence this (Fig. 4d).

*Castration also increases proliferation of mature SP subsets in middle-aged mice*

Analysis of Ki67 expression on mature CD4<sup>+</sup> and CD8<sup>+</sup> SP subsets showed lower number and proportion of Ki67<sup>+</sup> cells in middle-aged mice compared with 2-mo mice (Fig. 5). Cx middle-aged mice had increased number as well as proportion of Ki67<sup>+</sup> cells in both CD4<sup>+</sup> and CD8<sup>+</sup> populations (Fig. 5). Therefore, there appeared to be an overall effect across both the immature TN and mature SP subsets within 1 wk after castration. As the overall number of SP cells remained relatively constant, it is likely the newly arising cells are dying or leaving the thymus.



**FIGURE 3.** Cx mice showed increased proliferation of TN cells. *a*, Representative flow cytometric profile of Ki67 (*top panel*) and CD127 (IL-7R $\alpha$  chain) (*bottom panel*) (solid line) and isotype control (broken line) labeling of TN subsets from a 2-mo mouse. *b*, Number of Ki67<sup>+</sup> cells in the various TN subsets of 2-mo, 9-mo, and sham-Cx ( $\square$ ) or Cx ( $\blacksquare$ ) 9-mo mice 3, 5, 7, and 10 days after surgery. Cx mice had higher Ki67<sup>+</sup> cell number in all

### Castration enhances thymic recovery following Cy treatment

The regenerative effects induced by castration appear to be initiated within the TN compartment. However, it was difficult to track any initial castration-induced cohort of thymocytes in intact mice as pre-existing cells may mask such effects. Therefore, young mice were treated with the alkylating agent, Cy, which depletes rapidly dividing cells. The sequential appearance of the various thymocyte subsets following this immunoablative treatment thus provided a clear starting point from which to assess thymic regeneration and how this is affected by castration. It also has relevance to the clinical effects of chemotherapy on the thymus.

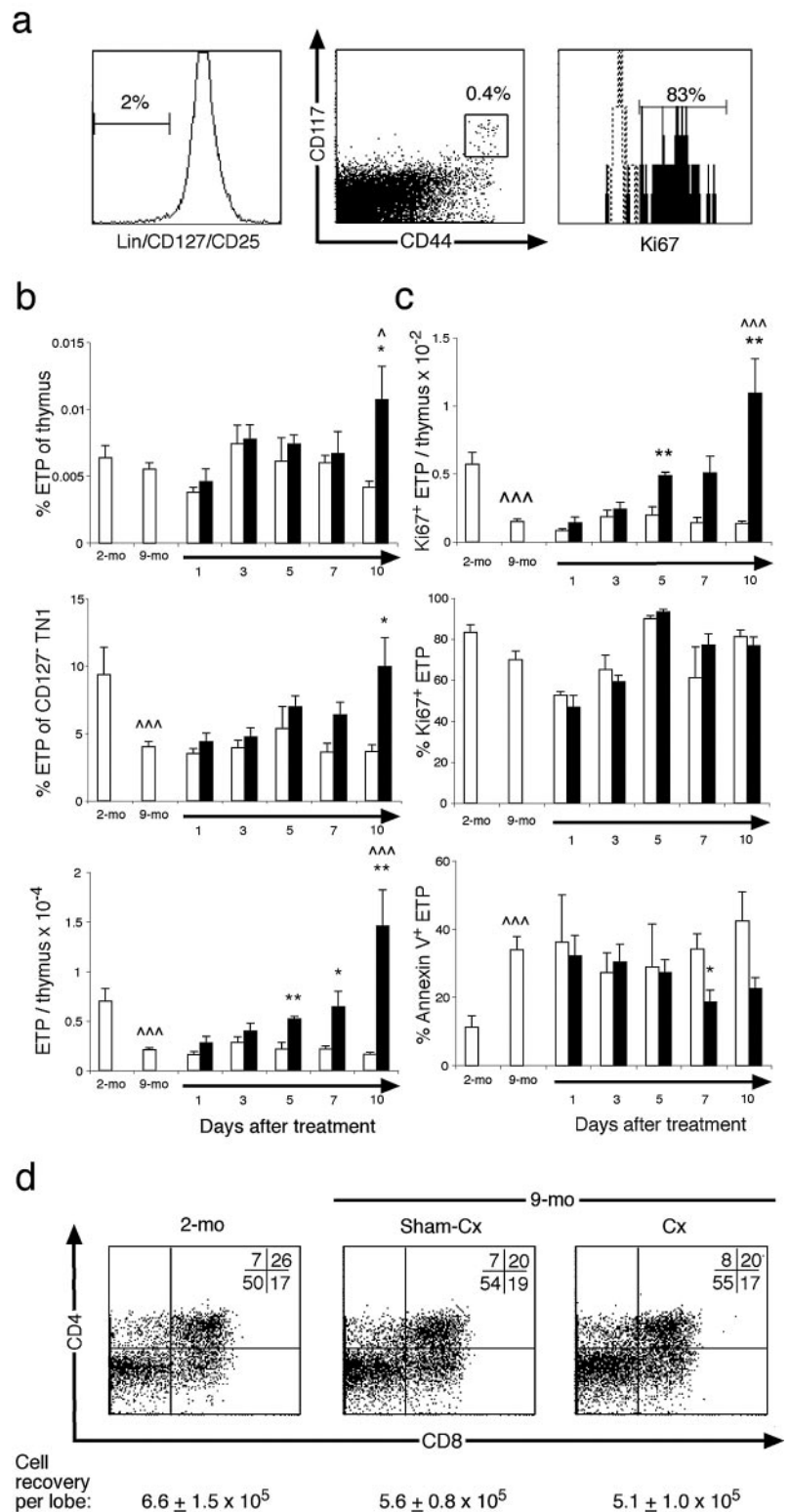
The thymic cellularity of young mice treated with Cy fell to 2% of pretreatment levels 3 days after treatment, with a dramatic decrease in DP cells commensurate with a proportional increase in SP cells (Fig. 6, *a* and *b*). However, even at this early time point, Cx mice had significantly higher total cell numbers than sham-Cx mice. Thereafter, both Cx and sham-Cx groups showed increased thymocyte numbers as the thymii regenerated following the Cy treatment. However, Cx mice exhibited accelerated recovery such that cellularity was more than twice that of sham-Cx controls at day 5. By day 7, absolute cell numbers of castrate thymii were approaching normal young levels, while sham-castrate thymii were still about half that of pretreatment numbers.

Analysis of thymocyte subsets revealed significantly higher number of TN cells in Cx mice compared with sham-Cx controls at all the time points examined (Fig. 6*c*). The increase in TN cell numbers was the only subset contributing to the greater total thymic cellularity in Cx mice up to 5 days after treatment, while an increase in both TN and DP cells in Cx mice accounted for the difference on subsequent days. There appeared to be no difference in SP cell numbers between Cx and sham-Cx mice in the first week, but both mature CD4<sup>+</sup> and CD8<sup>+</sup> SP cell numbers are increased by 2 wk after castration compared with sham-Cx mice in Cy-treated mice (data not shown). These results indicate thymic recovery from Cy treatment can be achieved earlier with castration, and this enhancement is first evident in the TN subset, followed by the DP then SP subsets.

### Accelerated TN development in Cy-treated mice following castration

The results above indicate that the initial changes induced following chemotherapy by castration occur within the TN compartment. Interestingly, 3 days after treatment, Cx mice had increased cell numbers of TN2, TN3, and TN4, but not TN1 (Fig. 7*a*). Analysis of proportional changes in the TN subsets revealed a decreased TN1 frequency concomitant with an increase in TN2 in Cx mice on day 3 after treatment (Fig. 7*b*). Subsequently, on days 4 and 5 after treatment, there was an increase in the proportion of TN3 and TN4 subsets, respectively, in Cx mice. The tracking of this first castration-induced cohort of cells through the various stages of TN development shows the earliest signal in the thymus induced by

TN subsets than sham-Cx mice 7 days after castration. *c*, Proportion of Ki67<sup>+</sup> cells in the various TN subsets, showing an increase in TN1 after castration. Results are expressed as mean  $\pm$  SEM of 4–8 mice for each group at each time point from 3 or more independent experiments. *d*, Proportion of CD127<sup>+</sup> cells in the various TN subsets, showing an increase in TN3 in Cx mice. Results are expressed as mean  $\pm$  SEM of 4–5 mice per sham-Cx and Cx group at each time point, 5 mice for 2 mo, and 23 mice for 9 mo. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  compared with sham-Cx 9-mo mice. ^,  $p \leq 0.05$ ; ^^,  $p \leq 0.01$ ; ^^,  $p \leq 0.001$  compared with 2-mo mice.



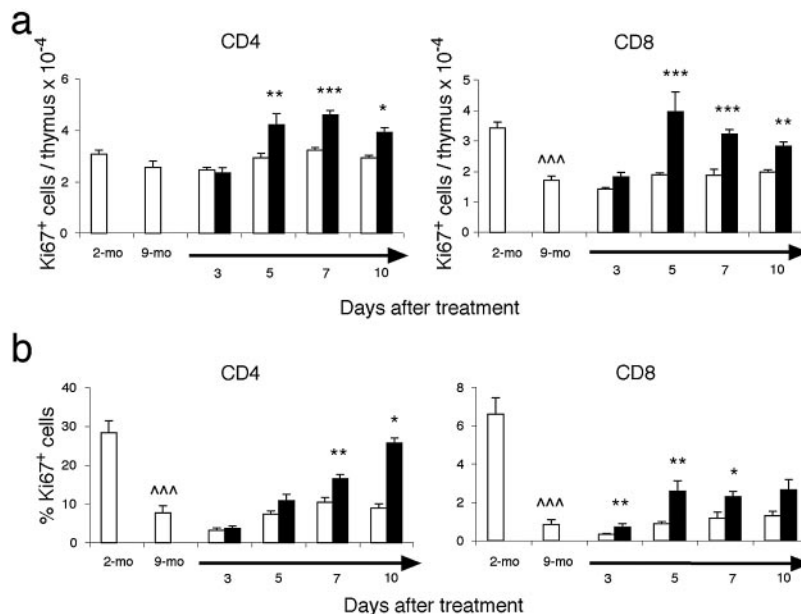
**FIGURE 4.** Decline in ETP was reversed following castration. *a*, Representative flow cytometric profiles showing Ki67 (shaded histogram) and isotype control (unshaded histogram) expression on Lin<sup>-</sup>/CD127<sup>-</sup>/CD25<sup>-</sup>/CD44<sup>+</sup>/CD117<sup>+</sup> ETP in a 2-mo mouse. *b*, Decline in ETP absolute number (*bottom panel*) with age was masked when expressed as a proportion of total thymocytes (*top panel*). This decrease was evident when expressed as a proportion of CD127<sup>-</sup> TN1 (*middle panel*). ETP levels were restored to normal young levels and beyond following castration. *c*, Ki67 expression on ETP, showing a decrease in number (*top panel*), but not proportion (*middle panel*) with age. Cx (■) 9-mo mice exhibited an increase in number of Ki67<sup>+</sup> ETP compared with sham-Cx (□) 9-mo mice. Annexin V staining on ETP shows increased level of apoptosis with age and a decrease with castration (*bottom panel*). Results are expressed as mean ± SEM of 4–5 mice each per sham-Cx and Cx group at each time point, 9–10 mice for 2-mo mice, and 19–25 mice for 9-mo mice. \*, *p* ≤ 0.05; \*\*, *p* ≤ 0.01 compared with sham-Cx 9-mo mice. ^, *p* ≤ 0.05; ^^ *p* ≤ 0.001 compared with 2-mo mice. *d*, Representative flow cytometric profiles of CD4 vs CD8 labeling of thymocytes harvested from fetal thymic lobes seeded with 10<sup>3</sup> ETP sorted from 2-mo, sham-Cx 9-mo, or Cx 9-mo mice, showing no difference in cell recovery and thymocyte differentiation. Mice underwent surgery 7 days before FTOC reconstitution. Results are expressed as mean ± SEM of 8 lobes for 2-mo mice, 5 lobes from sham-Cx 9-mo mice, and 12 lobes from Cx 9-mo mice.

castration facilitated the transition of cells from TN1 to TN2. This could result in the progression of cells from TN1 to subsequent subsets along the maturation pathway while TN1 was constantly being replenished by intrathymic progenitors, causing no overall change in TN1 cell numbers. Alternatively, the increase in TN2, TN3, and TN4 cells could be a result of proliferation within these subsets independently of TN1.

*Enhanced proliferative capacity of TN cells in Cy-treated mice following castration*

Analysis of Ki67<sup>+</sup> cells in Cy-treated mice showed Cx mice had higher number of proliferating cells in each TN subset compared with sham-Cx mice (Fig. 8*a*). Moreover, Cx mice showed increased proportions of Ki67<sup>+</sup> cells at earlier time points, suggesting the

**FIGURE 5.** Castration increased number and proportion of dividing SP cells. *a*, Number of Ki67<sup>+</sup> cells in mature CD4<sup>+</sup> and CD8<sup>+</sup> subsets of 2-mo, 9-mo, and sham-Cx (□) or Cx (■) 9-mo mice 3, 5, 7, and 10 days after surgery. Number of Ki67<sup>+</sup> CD4<sup>+</sup> cells and Ki67<sup>+</sup> CD8<sup>+</sup> cells was increased 5 days and after castration. *b*, Proportion of Ki67<sup>+</sup> SP cells, showing increased proportion of Ki67<sup>+</sup> cells in both SP subsets after castration. Results are expressed as mean ± SEM of four to eight mice for each group at each time point from three or more independent experiments. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  compared with sham-Cx 9-mo mice. ^^^,  $p \leq 0.001$  compared with 2-mo mice.



increase in number of proliferating cells was not just a result of increased cell number in each subset (Fig. 8*b*). This confirms that the increased numbers of TN cells in Cx mice treated with Cy were at least in part due to an enhanced proliferation event induced by the removal of sex steroids. Furthermore, 3 days after Cy treatment, the proportion of Ki67<sup>+</sup> cells in TN1 was higher in Cx mice compared with sham-Cx mice, suggesting TN1 is not a static population despite its decreased proportion, but not overall number at this time point. Additionally, Cx mice demonstrated an initial increase in the proportion of IL-7Rα<sup>+</sup> TN3, but not other TN subsets (Fig. 8*c*). The changes in the TN compartment in this Cy model of thymic depletion and recovery appeared to be independent of ETP, which were undetectable in the time points examined (data not shown).

#### Proliferative effect of castration extends to mature SP subsets

Analysis of mature CD4<sup>+</sup> and CD8<sup>+</sup> SP populations in Cy-treated mice showed no difference in number of Ki67<sup>+</sup> cells for 7 days following castration (Fig. 9*a*). However, Cx mice demonstrated increased proportion of Ki67<sup>+</sup> cells (Fig. 9*b*), despite no alteration in total cell number of either SP subset (Fig. 6*c*). This suggests an expansion of the initial Cy-resistant cohort of mature cells, which were either being exported into the periphery or dying by apoptosis.

## Discussion

There is strong evidence that age-related thymic atrophy is primarily induced by sex steroids, but the underlying mechanisms remain to be defined. Sex steroid ablation, either surgical or chemical, allows thymic regeneration (24–26) and enhanced peripheral T cell function (40). Although androgen receptors, at least, must be expressed on the stromal components for androgen-mediated atrophy (31), how they induce thymic involution and which thymocytes they target are not known. In principle, this age-associated thymic involution may involve either a global reduction across T cell development or specific block points leading to the accumulation of cells at one particular stage and depletion beyond that stage.

Several studies have previously indicated an age-associated lesion in early thymocyte differentiation, specifically a block at the

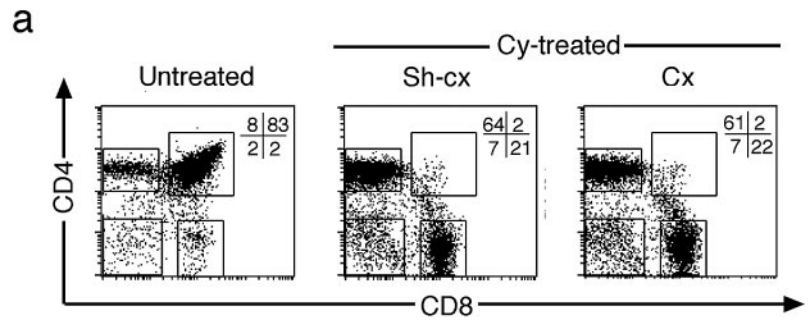
TN1 to TN2 stage (18, 41). However, in agreement with Min et al. (21), the present study identified a reduction in number of all TN subsets with age, particularly TN1. The likely explanation for this disparity is the difference in the lineage markers used to identify TN cells. In addition to CD3, CD4, and CD8 used in previous studies, our analysis excluded cells expressing B220, CD11b, and Gr-1 (which together make up 60–70% of TN1), thereby more accurately focusing on the true thymocyte precursors (21).

Accordingly, the TN fraction was the first thymocyte population to be affected by the removal of sex steroids. Although a simultaneous increase in number of TN and DP cells was observed after castration, a proportional increase in the TN compartment occurred 5 days before that in DP. It has been shown that the number of DP cells is directly proportional to the number of TN cells present in the thymus (42), the size of which is controlled by TN3 and potentially TN2 cells (43). Therefore, the increase in DP cell number observed after castration was likely to be derived from an expansion in the TN compartment.

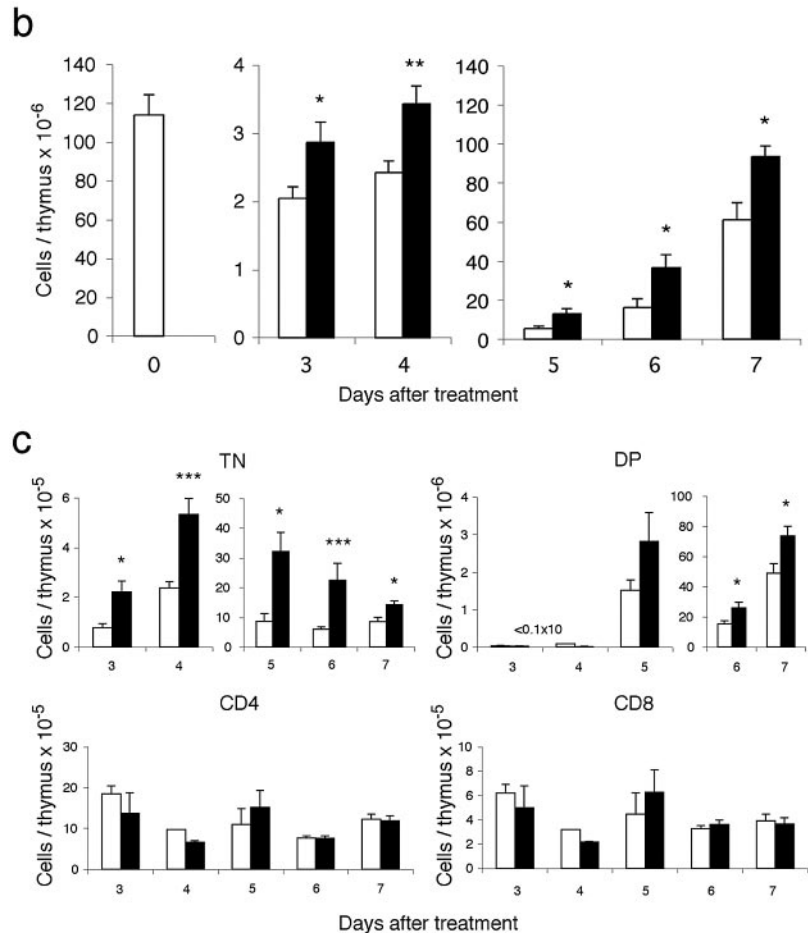
Development through the TN stages is dependent on IL-7, which mediates expansion of TN1 precursors with stem cell factor (44, 45) and maintains survival of thymocytes through Bcl-2 (46–48). IL-7, but not stem cell factor, has been shown to increase TN cellularity of aged mice *in vivo* (49). Therefore, the TN increase induced by castration may be mediated by IL-7. Indeed, Cx mice exhibited an increase in IL-7Rα expression on TN3 cells (demonstrated by mean fluorescence intensity; data not shown). The decrease in the proportion of IL-7Rα<sup>+</sup> TN1 and TN2 cells may reflect accelerated progression of these cells through TN development, resulting in an increase in early (IL-7Rα<sup>+</sup>) TN3 cells. Alternatively, increased levels of IL-7 may have already induced the down-regulation of IL-7Rα, as has been described previously in T cells (50).

Castration induced a numerical expansion in all four TN subsets and, specifically, increased frequency of proliferation in TN1, as determined by Ki67 expression. The heterogeneity of TN1 has been examined in detail recently to define the earliest BM-derived T progenitor (20, 51, 52). The demonstration of an age-related decline in ETP led us to investigate whether castration would reverse this trigger for thymic involution (21). Given their extremely





**FIGURE 6.** Castration enhanced thymic recovery from Cy treatment. *a*, Representative flow cytometric profiles of CD4 vs CD8 labeling of thymocytes from an untreated 2-mo mouse and Cy-treated sham-Cx or Cx 2-mo mouse. There was a dramatic decrease in the DP population of treated mice commensurate with an increase in the proportions of SP cells. *b*, Total thymic cell number of untreated 2-mo mice and Cy-treated sham-Cx (□) or Cx (■) mice 3–7 days after same-day treatment and surgery. Following Cy-induced depletion, thymii from Cx mice showed enhanced recovery of total cell number. *c*, Cell number of TN, DP, and SP subsets. Cx mice had higher number of TN cells at all time points and DP cells 6 and 7 days after treatment. Results are expressed as mean ± SEM of 8–16 mice for each group at each time point from three or more independent experiments. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$ ; \*\*\*,  $p \leq 0.001$  compared with sham-Cx mice.

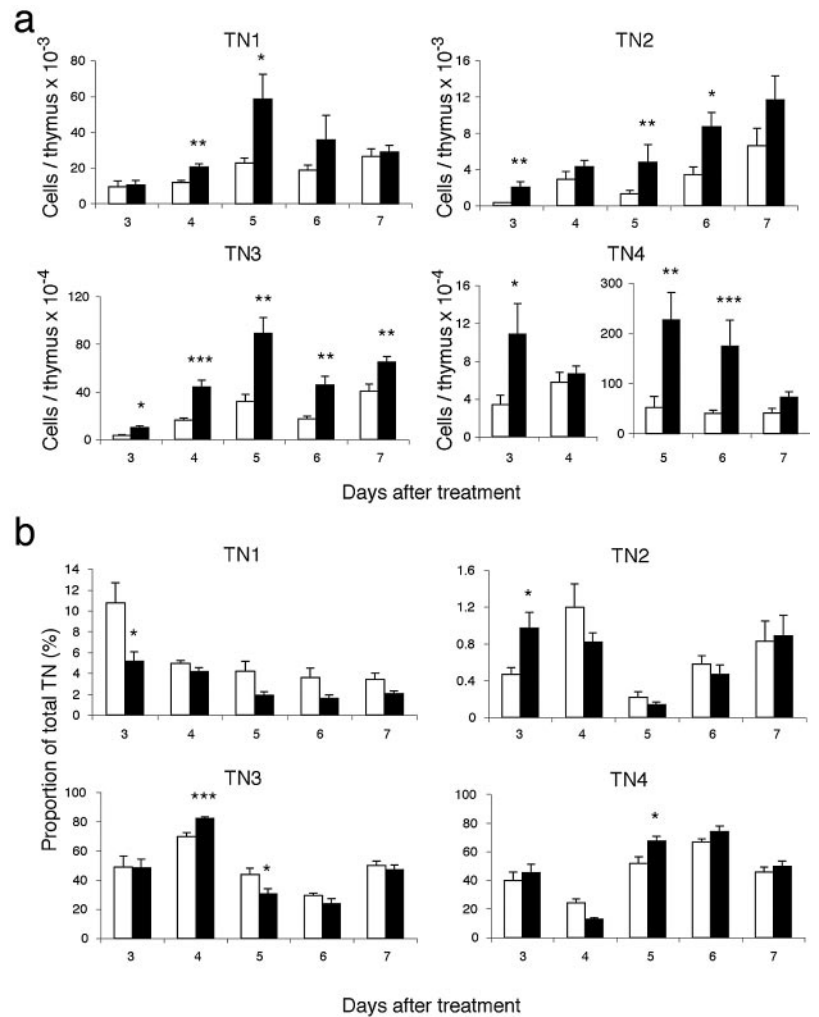


small number, the reduction in ETP as a proportion of total thymus with age was masked by fluctuations in the major thymocyte subsets. When expressed as a proportion of the more immature CD127<sup>-</sup> subpopulation of TN1 (52), the decrease in ETP in middle-aged mice became obvious. Although Min et al. (21) demonstrated a decline in the reconstitution capacity of ETP from 17-mo or older mice, we did not detect this defect in 9-mo mice, suggesting numerical decline occurs before functional defect. We are currently investigating whether castration reverses the reconstitution defect of ETP, as well as BM, observed in older (18- to 24-mo) mice.

In this study, we observed restoration of ETP number and proportion in middle-aged mice following castration. Although there was no change in Ki67 expression, ETP from Cx mice exhibited reduced apoptosis. This Cx-enhanced survival appears to be Bcl-2 independent, as we did not detect a change in intracellular Bcl-2 expression with castration (data not shown). However, as ETP are

too numerous to be the very first thymus-settling progenitors (53), it is possible that the increase in ETP was due to proliferation-induced expansion of an earlier, yet unidentified subset. Additionally, castration could result in increased influx of progenitors. In turn, this may implicate a role for chemokines mediating precursor entry, for example, as CCL21 and CCL25 are involved in recruitment of fetal thymic precursors (54, 55).

In terms of thymocytes downstream of TN cells, the present data show a reduction in cell number across all major thymocyte subsets with age, as previously reported (41). Proportionally, there was an increase in DP and a corresponding decrease in SP. This contrasted with Thoman's (41) findings; however, it suggests that there is a distinct proportional shift in 9-mo mice before that found in 16- and 24-mo mice. The proportional decrease in CD4<sup>+</sup> and CD8<sup>+</sup> SP cells observed with age could be attributed to the reduced fraction of proliferating cells found in the SP compartment. This decline in proliferation was reversed in the absence of sex steroids.



**FIGURE 7.** Accelerated immature thymocyte development in Cx mice at early time points after treatment. *a*, Cell number of individual TN subsets of Cy-treated sham-Cx (□) or Cx (■) 2-mo mice 3–7 days after treatment. *b*, Proportion of individual TN subsets. There was a decrease in the proportion of TN1 alongside an increase in TN2 3 days after castration. Subsequently, there was an increase in the proportions of TN3 and TN4 4 and 5 days after castration, respectively. Results are expressed as mean  $\pm$  SEM of 8–16 mice for each group at each time point from three or more independent experiments. \*,  $p \leq 0.05$ ; \*\*\*,  $p \leq 0.001$  compared with sham-Cx mice.

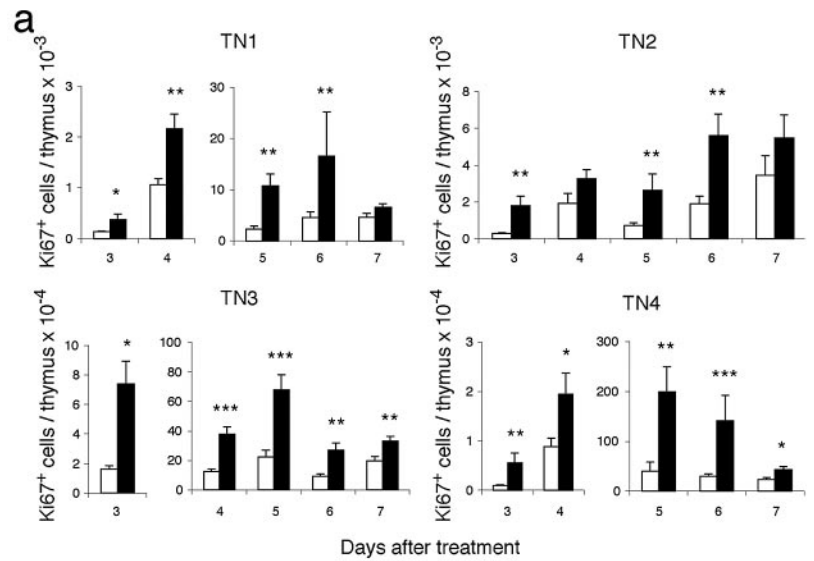
Again, IL-7 could be implicated in this study, as it has been shown to play a role in postpositive selection proliferation in the neonatal thymus (56). The expansion of pre-emigrant thymocytes has also been proposed to involve TCR stimulation (57). The increased fraction of cycling SP cells in the absence of a difference in total SP number in Cx mice could be due to an increase in thymic export, as observed previously (65), or increased apoptosis, for which, however, we have no evidence.

In humans, chronic involution of the thymus is particularly detrimental in situations of immune compromise such as immunoblastic therapy, BMT, and HIV infection (58). This is exemplified in the inverse correlation between patient age and CD4 recovery rate after intensive chemotherapy (59). Moreover, thymic-independent CD8 regeneration results in prolonged T cell subset imbalance following T cell depletion (60). The use of castration in conjunction with Cy treatment in this study thus provides a two-tier strategy: dynamic analysis of the regeneration process of an antecedent cohort of thymocytes and how this process is altered in the absence of sex steroids.

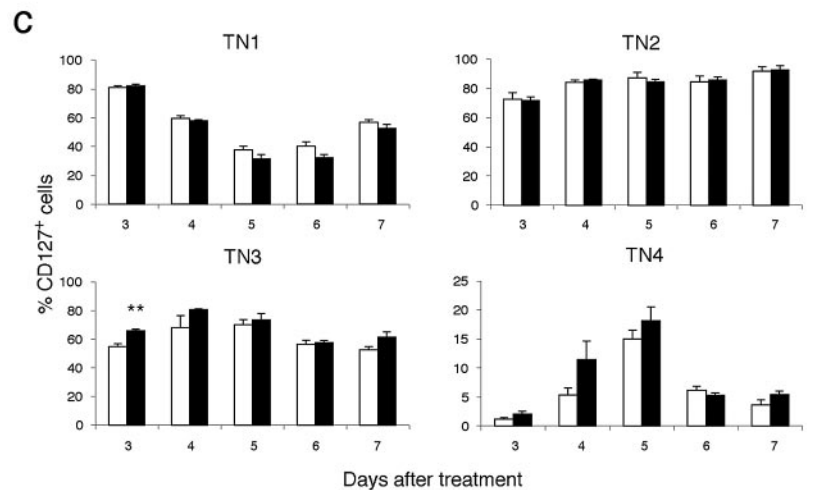
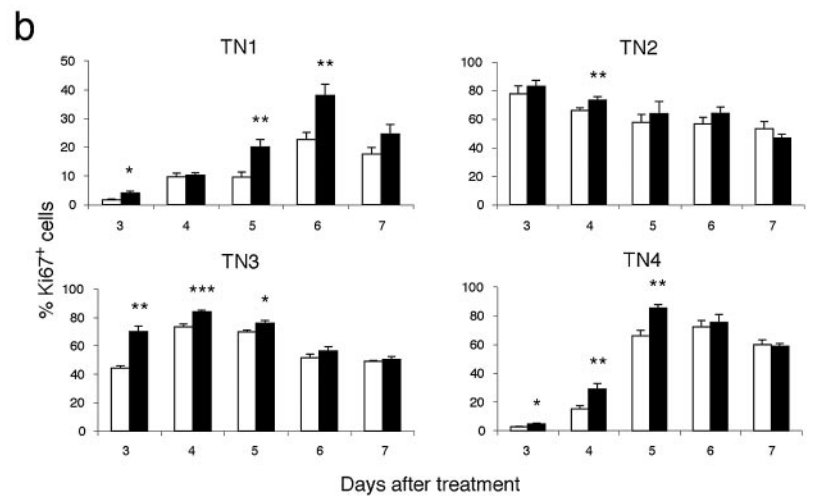
Although this model involved a different mechanism of involution, castration produced similar profound effects on thymocyte regeneration. Cx mice exhibited accelerated thymic recovery following Cy treatment, which could be due to a higher level of resistance to Cy treatment or a greater rate of regeneration in Cx mice. The increase in total thymocyte number was accounted for by a proliferation-induced expansion of TN2, TN3, and TN4. However, despite the lack of change in TN1 cell number, the in-

creased proportion of cycling TN1 cells indicates a more rapid transition from TN1 to TN2 in the absence of sex steroids. Thus, in the face of cytotoxic insult, castration improves thymic recovery by enhancing the proliferative potential and differentiation capacity of immature thymocytes. However, because BM-derived precursors constitute a fraction of the TN1 subset, the possibility of castration reducing a suppressive factor that regulates thymic entry cannot be ruled out. Nonetheless, castration-induced expansion of the TN compartment in Cy-treated mice in the absence of detectable levels of ETP indicates an early intrathymic proliferative event.

The present study shows that castration affects TN and SP development, both of which depend on IL-7. Because thymic stroma mediates androgen-induced thymic atrophy (31), sex steroids may elicit thymic atrophy via down-regulation of IL-7 production on a per cell basis or through loss of IL-7-producing thymic epithelial cells (TECs). However, a recent study demonstrated that transplantation of IL-7-secreting stromal cells into the thymus of old mice increased the proportion of CD25<sup>+</sup> TN cells, but did not restore total thymic cellularity or thymic output, suggesting the involvement of other factors (61). Keratinocyte growth factor (KGF) has been demonstrated to increase the number of IL-7-producing TECs in mice undergoing BMT (62). It is possible that castration induces KGF production by either fibroblasts or thymocytes, which then act on IL-7-producing TECs bearing KGF receptors.



**FIGURE 8.** Castration enhanced proliferation of TN cells in Cy-treated mice. *a*, Number of Ki67<sup>+</sup> cells in the various TN subsets of Cy-treated sham-Cx (□) or Cx (■) 2-mo mice 3–7 days after treatment. Cx mice had higher number of proliferating cells in TN subsets compared with sham-Cx mice at most of the time points examined. *b*, Proportion of Ki67<sup>+</sup> cells in individual TN subsets, showing an increase in each TN subset at earlier time points after castration. Results are expressed as mean ± SEM of 8–16 mice for each group at each time point from three or more independent experiments. *c*, Proportion of CD127<sup>+</sup> cells in the various TN subsets, showing an initial increase in TN3 after castration. Results are expressed as mean ± SEM of 4–5 mice for each group at each time point. \*, *p* ≤ 0.05; \*\*, *p* ≤ 0.01; \*\*\*, *p* ≤ 0.001 compared with sham-Cx mice.

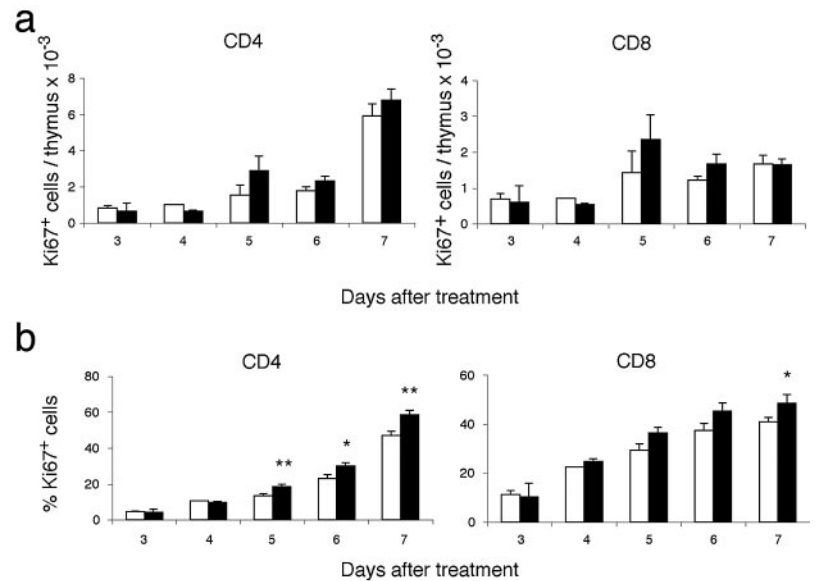


In contrast, sex steroid ablation may reduce suppressive cytokines. It has been shown that TGF-β may exert suppressive effects either directly or via increasing cytokines such as IL-6 and leukemia inhibitory factor that have been reported to increase with age (63, 64). Although analysis of whole CD45<sup>-</sup> thymic stroma from middle-aged mice did not show changes in mRNA expression of IL-7, KGF, and TGF-β with castration,<sup>5</sup> detailed analysis of specific thymic stromal subsets will determine whether the castration-

induced expansion observed is mediated via changes in these cytokines.

Collectively, the data presented provide a context within which the molecular mechanisms of castration-induced thymic regeneration may be determined. More importantly, the kinetics of thymic regeneration documented in this work provides a valuable tool in the design and timing of therapeutic intervention for maximal reconstitution in clinically induced immunodepletion.

**FIGURE 9.** Castration-induced proliferation of SP cells in Cy-treated mice. *a*, Number of Ki67<sup>+</sup> cells in mature CD4<sup>+</sup> and CD8<sup>+</sup> subsets of Cy-treated sham-Cx (□) or Cx (■) mice 3–7 days after treatment. Cx mice had higher number of proliferating CD4<sup>+</sup> and CD8<sup>+</sup> cells compared with sham-Cx mice at the time points examined. *b*, Proportion of Ki67<sup>+</sup> SP cells, showing an increase in both SP subsets after castration. Results are expressed as mean ± SEM of 8–16 mice for each group at each time point from three or more independent experiments. \*,  $p \leq 0.05$ ; \*\*,  $p \leq 0.01$  compared with sham-Cx mice.



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## Disclosures

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