An Essential Role for the IL-7 Receptor During Intrathymic Expansion of the Positively Selected Neonatal T Cell Repertoire

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An Essential Role for the IL-7 Receptor During Intrathymic Expansion of the Positively Selected Neonatal T Cell Repertoire

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Intrathymic T cell development is a multistage process involving discrete phases of proliferation as well as differentiation. From studies on IL-7 or IL-7Rα-deficient mice, it is clear that the IL-7 receptor (IL-7R) plays a critical role during the initial stages of intrathymic CD4^+8^- precursor development. In contrast, the role of IL-7R in later stages of thymocyte development are unclear. Here, we have used various approaches to investigate directly the role of the IL-7R in thymocyte positive selection and the recently described phase of postselection proliferation. First, we show that positive selection involves selective up-regulation of IL-7Rα and IL-7Rγ-chains, with the majority of CD4+ and CD8+ cells being IL-7R+. Second, MHC class II+ thymic epithelium—which drives postselection proliferation—expresses IL-7 mRNA. Finally, analysis of positive selection and postselection proliferation in thymocytes from IL-7Rα^-/- neutonates shows that positive selection occurs normally, whereas postselection expansion is drastically reduced. Thus, our data provide the first evidence that, as well as playing a role during early phases of thymic development, IL-7R mediates intrathymic expansion of positively selected thymocytes, which may aid in establishment of the neonatal peripheral T cell pool.


Cells bearing the αβ form of the TCR are generated most efficiently in the thymus, where immature precursors undergo a program of events involving lineage commitment, somatic recombination of Ag receptor genes, and cellular expansion to generate a large cohort of CD4^+8^- thymocytes bearing a random repertoire of TCR specificities (1). Maturation of thymocytes beyond the CD4^+8^- stage relies upon the specificity of the TCRs expressed: cells bearing receptors with no affinity for self peptide/MHC molecules die by neglect, whereas cells bearing TCRs capable of peptide/MHC recognition are subject to two distinct selection events. Thus, negative selection removes cells bearing TCRs with a high affinity for peptide/MHC, whereas positive selection rescues cells making low affinity TCR-peptide/MHC interactions, and triggers their differentiation to the CD4^+8^- and CD4^+8^+ stages (2–4). These cells then emigrate from the thymus to generate the peripheral T cell pool, which recognizes peptides derived from foreign Ags in a self-MHC restricted manner (5).

It is becoming increasingly clear that positive selection is a multistage process, which includes phases of initiation, phenotypic differentiation, acquisition of functional competence, and postselection proliferation (6–11). The initiation phase of positive selection, characterized by expression of the early activation marker CD69 on CD4^+8^- thymocytes, is known to be strictly dependent upon ligation of the TCR complex by peptide/MHC complexes expressed by thymic epithelial cells (9, 12). In contrast, it is now clear that differentiation of CD4^+8^-69^+ cells to the single-positive CD4^+ and CD8^+ stages and the subsequent postselection proliferation, although dependent upon the continued presence of thymic epithelium, does not require ongoing TCR ligation (9).

In this study, we have focused on the molecular mechanisms mediating post positive selection proliferation, which may be an important intrathymic event allowing expansion of newly positively selected thymocytes, thereby aiding establishment of the neonatal peripheral T cell pool. We provide evidence that IL-7, a factor which has previously been shown to play a critical role in development and expansion of early CD4^+8^- thymocyte precursors (13–15), also plays a previously unidentified role in driving the Ag-independent proliferation of positive-negative neonatal thymocytes following positive selection.

Materials and Methods

Mice

BALB/c (H-2^d) mice, used as a source of thymocytes and thymic stromal cells, together with C57BL/6 (H-2^b) mice were bred and maintained at the Biomedical Services Unit, Birmingham University. Neonatal IL-7Rα^-/- (C57BL/6, H-2^b) mice (16) were kindly provided by Dr. Michael Owen (ICRF, Lincoln’s Inn Fields, London, U.K.).

Abs and immunoconjugates

The following Abs were coated onto anti-rat IgG or streptavidin-coated Dynabeads (Dynal, Wirral, U.K.) as appropriate: biotinylated anti-CD69 (clone H1.2F3; PharMingen, San Diego, CA), anti-CD8 (clone YTS169.4; Sera-Lab, Crawley Down, Sussex, U.K.), anti-CD3 (clone KT-3; Serotec, Oxford, U.K.). Abs used for flow cytometric analysis are as follows: PE-conjugated anti-CD4 (clone GK1.5; PharMingen), FITC or APC-conjugated anti-CD8 (clone 53-6.7; Pharmingen), anti-IL-7Rα (clone B12-1; Pharmingen), anti-common γ-chain (clone 4G3; Pharmingen), anti-IL-2Rα (clone 7D4), FITC-conjugated anti-5-bromo-2′-deoxyuridine (BrdU)^3 (clone 3D4; Pharmingen). Unconjugated Abs were detected using sequential incubations in biotinylated anti-rat IgG (Caltag, South San Francisco, CA) and streptavidin APC (PharMingen).

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3 Abbreviations used in this paper: BrdU, 5-bromo-2′-deoxyuridine; RTOC, reaggregate thymic organ culture; TSLP, thymic stromal lymphopoietin; WT, wild type.
Thymocytes. Methods to isolate thymocyte subsets at pre- and intermediate-stages of positive selection have been described in detail elsewhere (6, 7). Briefly, CD4^+ 8^- 69^+ cells were prepared by immunomagnetic selection from neonatal BALB/c mice by isolation of CD69^- cells and then further positive selection using anti-CD8-coated beads, a technique which yields a highly purified population of CD4^+ 8^- 69^+ cells (6). Preselection thymocytes were obtained by depleting newborn suspensions of CD3^- cells, followed by the isolation of CD4^+ 8^- cells, again using anti-CD8-coated beads.

Thymic stromal cells. Enriched preparations of thymic epithelial cells were prepared as described (17), by culturing 15-day gestation BALB/c or C57BL/6 thymus lobes in the presence of 1.35 mM 2-deoxyguanosine for 5–7 days, followed by trypsinization to form a single-cell suspension (7).

Reaggregate thymus organ cultures (RTOCs) RTOCs were prepared from 1:1 mixtures of freshly prepared thymic stromal cells and thymocytes by depositing the combined cell slurry onto the surface of a 0.8-μm Nuclepore (Costar, High Wycombe, U.K.) filter under organ culture conditions (18).

Analysis of post-positive selection expansion To analyze thymocyte proliferation in RTOCs simultaneously with expression of CD4 and CD8, cultures were pulsed with 5 μg/ml BrdU (Sigma, St. Louis, MO) for the final 18 h of a 6-day culture period. RTOCs were subsequently teased apart and viable thymocytes counted. Suspensions were labeled for surface expression of CD4 and CD8, with BrdU incorporation being detected as described by Tough and Sprent (19).

PCR Total RNA was extracted from ~5 × 10^6 cells using TRizol (Life Technologies, Paisley, Scotland) according to the manufacturer’s instructions. RNA samples were treated with Raase-free DNase I (Pharmacia Biotech, Uppsala, Sweden) to remove any contaminating genomic DNA. Reverse transcription was conducted according to Montgomery and Dallman (20). RT-PCR was then performed as described (21) with β-actin as a housekeeping gene to obtain equivalent amounts of cDNA in each sample. The sequences for β-actin, IL-7, and IL-15 oligonucleotides are as follows: β-actin: sense, 5'-GGTACCAACTGGAAGCA-3'; anti-sense, 5'-TG GCCATCTCTTCTGCAGA-3'; IL-7: sense, 5'-ACTACACCACTC CCAGA-3'; IL-15: sense, 5'-GGAGGATACATACATCTGTGC-3'; anti-sense, 3'-GCTGCGGAT CAGTCAG GAC-3'.

Flow cytometry Two- and three-color flow cytometric analyses were performed using a dual laser Coulter Epics Elite machine (Coulter, Hialeah, FL), with forward and side scatter gates set so as to exclude nonviable cells (6, 7).

Results and Discussion Positive selection of thymocytes is accompanied by up-regulation of cytokine receptors, consistent with thymic epithelial cell cytokine production Although the generation of single positive CD4^+ and CD8^+ cells from CD4^+ 8^- precursors does not initially involve cell division, a wave of proliferation that involves multiple cell divisions has been observed in newly formed single positive cells, particularly in the neonatal thymus (22, 23), which may serve in the expansion of the newly generated neonatal repertoire before thymic export. Interestingly, this phase of cellular proliferation is independent of signals through the αβTCR complex (7) but dependent upon thymic epithelial cells (23), suggesting that this event is regulated by additional but as yet undefined signals. Because cytokines are known to be key molecules in both early thymocyte and mature T cell proliferation (24, 25), we hypothesized that particular cytokines may be involved in the regulation of the late phase of intrathymic proliferation. Thus, we conducted an initial series of experiments to analyze expression of various cytokine receptors and associated molecules implicated in mediating signaling by these receptors.

To focus on events following the initiation of positive selection, and so to exclude possible confusion from cytokine receptor expression by proliferating αβTCR CD4^+ 8^- blast cells (26), we analyzed cytokine receptor expression on freshly purified CD4^+ 8^- 69^+ cells, which have been shown to represent developmental intermediates in the positive selection process (23, 27). Analysis of cytokine receptor expression on freshly isolated CD4^+ 8^- 69^+ thymocytes revealed that the majority of cells did not express either the common γ-chain (Fig. 1a) or the IL-7Rα-chain (Fig. 1c). Similarly, only a small proportion of CD4^+ 8^- 69^+ thymocytes were found to express the IL-2Rα-chain (Fig. 1b). Subsequent analysis of cytokine receptor expression during maturation of CD4^+ 8^- 69^+ cells was achieved by culturing these cells with thymic stromal cells in RTOCs for 3 days, during which time cohorts of single positive CD4^+ and CD8^+ thymocytes are generated (Fig. 1d), which subsequently undergo a phase of postselection proliferation (23). Although the cytokine receptors tested were found to be absent from most freshly purified CD4^+ 8^- 69^+ cells (Fig. 1, a–c), notable changes were observed in the pattern of cytokine receptor expression on CD4^+ and CD8^- cells generated from CD4^+ 8^- 69^- precursors in RTOCs. Thus, the vast majority of cells were found to express the common γ-chain (CD132), a component of IL-2, IL-4, IL-7, IL-9, and IL-15 cytokine receptors (Fig. 1, e and h). Interestingly, whereas some CD132 partner chains such as CD25 (IL-2Rα) were found to be absent from most CD4^+ and CD8^- thymocytes (Fig. 1, f and i), the majority were found to express the IL-7Rα-chain (CD127) (Fig. 1, g and j). Notably, these cytokine receptors were absent from virtually all thymocytes that remained at the CD4^+ 8^- stage after 3 days in RTOC. Thus, further maturation of CD4^+ 8^- 69^+ thymocytes to the CD4^- or CD8^- stage appears to involve selective up-regulation of particular cytokine receptors, including the CD132 and CD127 components of the IL-7R complex.

We have previously demonstrated that post-positive selection expansion of thymocytes observed during the neonatal period is dependent upon interactions with MHC class II^- thymic epithelial cells (23), suggesting that these cells provide specific signals driving expansion of newly selected T cells. As the completion of positive selection is accompanied by selective expression of particular cytokine receptors (Fig. 1), we next analyzed cytokine gene expression within this stromal cell compartment using semiquantitative PCR analysis. Of the cytokines analyzed, MHC class II^- thymic epithelial cells were found to express readily detectable levels of mRNA encoding IL-7 (Fig. 2b) and IL-15 (Fig. 2c), but not IL-2, IL-4, or IL-6 (data not shown). Interestingly, as well as pairing with CD132 to form the IL-7R complex, the IL-7Rα-chain has also been shown to pair with another cytokine receptor chain, the thymic stromal lymphopoietin (TSLP) receptor (TSLP-R) (28). TSLP was originally identified as a product of a thymic stromal cell line (29), and appears to have both shared and unique functions, when compared with IL-7 (28, 30). However, whereas TSLP has been shown to play a role in early stages of B cell development (29), unlike the clear demonstration of the importance of IL-7 in IL-7^-/- mice, the role of TSLP in thymocyte development is unclear.

Collectively, these data show that certain cytokines and their receptors are expressed in thymic epithelial cells and positively selected thymocytes, respectively, thereby providing indirect evidence that cytokine-mediated signaling may play a role in the phase of proliferation following positive selection. However, given that IL-7 and IL-15 are both expressed in thymic epithelium, and
that the IL-7R and IL-15R are both $\gamma_c$-containing receptors, our data do not allow direct analysis of the possible involvement of individual cytokines in regulation of postselection expansion.

**IL-7R$^{-/-}$ CD4$^+$ cells show evidence of normal positive selection but severely impaired post-selection proliferation**

Thus, to directly investigate the requirement for specific cytokines in positive selection and related postselection proliferation, we next purified CD4$^+$ thymocytes from neonatal mice deficient in expression of the IL-7R$\alpha$-chain, thereby allowing direct discrimination between IL-7R and IL-15R involvement. Although the role of IL-7/IL-7R interactions during early stages of T cell development are well described (16, 31), analysis of the functional importance of these molecules during positive selection and subsequent postselection events is made difficult by the impaired proliferation and differentiation observed in mice lacking either IL-7 or IL-7R$\alpha$. However, although adult IL-7R$\alpha^{-/-}$ mice show a marked block in thymocyte differentiation, predominantly at the CD4$^-$ stage, some cells do reach the CD4$^+$ stage, particularly in fetal and neonatal mice (31).

Thus, RTOCs were set up combining thymic stromal cells with either wild-type (WT) or IL-7R$\alpha^{-/-}$ CD4$^+$ thymocytes, and, after 5 days in culture, lobes were pulsed with BrdU to allow investigation of proliferation following positive selection. Fig. 3

![Figure 1](http://www.jimmunol.org/)

**FIGURE 1.** Selective up-regulation of the IL-7R complex following positive selection. CD4$^+$69$^+$ thymocytes isolated from WT neonatal mice were analyzed for expression of $\gamma_c$ (a), IL-2R$\alpha$ (b), and IL-7R$\alpha$ (c). A total of $1 \times 10^6$ CD4$^+$69$^+$ thymocytes were cultured with WT thymic stroma as RTOCs. After 3 days, $1.1 \times 10^5$ viable thymocytes were recovered and analyzed for expression of CD4 and CD8 (d), together with expression of $\gamma_c$ (e and h), IL-2R$\alpha$ (f and i), or IL-7R$\alpha$ (g and j). CD4$^+$ and CD4$^+$69$^+$ thymocyte populations were gated on separately to allow analysis of $\gamma_c$, IL-2R$\alpha$, and IL-7R$\alpha$ on either CD4$^+$ cells (e, f, and g) or CD4$^+$69$^+$ cells (h, i, and j).

![Figure 2](http://www.jimmunol.org/)

**FIGURE 2.** Expression of IL-7 and IL-15 mRNA in MHC class II$^+$ thymic epithelial cells. cDNA was synthesized from isolated MHC class II$^+$ thymic epithelial cells and semiquantitative RT-PCR analysis performed to analyze expression of the cytokines IL-7 (b), (every four cycles from 26 to 46) and IL-15 (c) (every four cycles from 24 to 44). Levels of $\beta$-actin were assessed every three cycles from 18 to 33, to ensure equal cDNA loading between samples (a).
shows that cells recovered from RTOCs initiated with either WT or IL-7Rα−/− CD4+8 thymocytes show evidence of positive selection, with cohorts of CD4+8− and CD4+8+ cells observed in both cultures (Fig. 3, a and b). Indeed, when expressed as ratios (Table I), WT and IL-7Rα−/− CD4+8+ thymocytes were found to generate similar proportions of CD4+8+ as compared with CD4+8− cells (2.8:1 WT, 2.2:1 IL-7Rα−/−). In contrast, cell recoveries were reduced overall in IL-7Rα−/− compared with WT cultures, and, in addition, analysis of BrdU incorporation also revealed that at least part of this lower cell recovery was due to differences in the extent of postselection cell division. Thus, both

CD4+8−IL-7Rα−/− and CD4+8−IL-7Rα−/− thymocytes showed considerable reductions in the proportion (Fig. 3, c–f), and absolute cell number (Fig. 4, a and b) of proliferating (BrdU+) cells, as compared with WT controls. Interestingly, proliferation of CD4+8− cells appears to be affected more than that of CD4+8+ cells by lack of IL-7Rα expression, with ratios of 1.9:1 WT and 13:1 IL-7Rα−/− for nondividing CD4+8−:dividing CD4+8− cells, and 0.8:1 WT and 3.5:1 IL-7Rα−/− for nondividing CD4+8+: dividing CD4+8− cells (Table I). Thus, these data suggest that, although positive selection to the CD4+8− and CD4+8+ stage occurs similarly in both WT and IL-7Rα−/− thymocytes, the phase of postpositive selection expansion operating on these newly selected thymocytes is dramatically impaired in IL-7Rα−/− thymocytes, and so highlights a previously unidentified role for the IL-7R in mediating expansion during later stages of thymocyte development. However, as mentioned previously, as both IL-7 and TSLP can utilize surface receptors containing the IL-7Rα-chain (28), it may be the case that both of these cytokines can play a role in this late stage of thymocyte expansion.

Table I. Post-positive selection proliferation of newly generated CD4+8− and CD4+8+ cells is dramatically impaired in IL-7Rα−/− thymocytes

<table>
<thead>
<tr>
<th>Cell Types</th>
<th>WT Thymocytes</th>
<th>IL-7Rα−/− Thymocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total CD4+8−:Total CD4+8+</td>
<td>2.8:1</td>
<td>2.2:1</td>
</tr>
<tr>
<td>CD4+8−BrdU−:CD4+8−BrdU+</td>
<td>1.9:1</td>
<td>13:1</td>
</tr>
<tr>
<td>CD4+8−BrdU−:CD4+8−BrdU+</td>
<td>0.8:1</td>
<td>3.5:1</td>
</tr>
</tbody>
</table>

CD4+8−69 thymocytes from WT or IL-7Rα−/− mice were cultured with WT thymic stroma for 6 days, as described in Fig. 3. Numbers of total CD4+8− and CD4+8− cells, nondividing and dividing CD4+8− cells, and nondividing and dividing CD4+8− cells from both WT and IL-7Rα−/− mice are shown as ratios.

FIGURE 3. Thymocytes deficient in expression of IL-7Rα show normal positive selection but decreased levels of post-positive selection proliferation. A total of 1.3 × 10^6 CD4+8+69 thymocytes from WT or IL-7Rα−/− neonatal mice were cultured as RTOCs with 1.5 × 10^6 WT thymic epithelial cells. After 5 days culture, reaggregates were pulsed with BrdU, and thymocytes harvested 18 h later, on day 6 of culture. A total of 2.8 × 10^6 WT thymocytes (a) and 5 × 10^6 IL-7Rα−/− thymocytes (b) were recovered, and analyzed for expression of CD4 and CD8, together with assessment of cell division by BrdU labeling. Gating upon populations of CD4+8− and CD4+8+ cells allowed analysis of the proliferative status of CD4+8− cells (c and d) and CD4+8+ cells (e and f) from WT (c and e) or IL-7Rα−/− mice (d and f).

FIGURE 4. Reduced levels of proliferation by CD4+8− and CD4+8+ thymocytes from IL-7Rα−/− mice. CD4+8+69 thymocytes from WT or IL-7Rα−/− mice were cultured with WT thymic stroma for 6 days, as described in Fig. 3. Analysis of CD4 and CD8 expression and BrdU labeling allowed calculation of the absolute number of CD4+8− cells (a) and CD4+8+ cells (b), which were dividing, together with the total number of WT or IL-7Rα−/− CD4+8− cells (a) and CD4+8+ cells (b) generated.
Concluding remarks
Recently, we and others have identified a phase of post-positive selection proliferation operating on both CD4^+8^- and CD4^+8^+ thymocytes, which appears to be thymic epithelial cell-dependent but independent of TCR-mediated signaling, because it occurs in the presence of MHC-deficient thymic stromal cells (7). Moreover, as compared with adult thymocytes, postselection proliferation of mature thymocytes is far more evident in neonatal mice (22, 23), where it may act as a mechanism to expand the newly selected neonatal T cell repertoire, thereby aiding in the establishment of the neonatal peripheral T cell pool. Here, we have investigated the mechanism of postselection expansion and provide direct evidence that the IL-7R complex plays a key role in this phase of thymocyte differentiation. First, we have shown that transition from the CD4^+8^-69^- intermediate stage to the CD4^+8^+ and CD4^+8^- stages involves selective up-regulation of both the α- and γ-chains of the IL-7R complex, suggesting that, following the generation of single positive thymocytes by positive selection, these cells may become receptive to IL-7/IL-7R-mediated signals. Second, by directly comparing positive selection and subsequent postselection expansion in WT and IL-7R^α^-/- thymocytes, we show that lack of expression of the IL-7R complex, although not affecting the differentiation of CD4^+8^- into CD4^+8^+ and CD4^+8^- cells, results in a dramatic reduction in post-positive selection proliferation. Collectively then, whereas the cells which regulate postselection expansion—MHC class II^+ thymic epithelial cells—are known to express a variety of cytokines (21), including IL-15 as shown here, our data provide direct evidence for an essential requirement for IL-7/IL-7R interactions during post-positive selection expansion in the neonatal thymus.

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References

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