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The Generation of Mature, Single-Positive Thymocytes In Vivo Is Dysregulated by CD69 Blockade or Overexpression

Toshinori Nakayama, Deborah J. Kasprowicz, Masakatsu Yamashita, Lisa A. Schubert, Geoffrey Gillard, Motoko Kimura, Arnaud Didierlaurent, Haruhioko Koseki, and Steven F. Ziegler

During development in the thymus, mature CD4⁺ or CD8⁺ cells are derived from immature CD4⁺CD8⁺ cells through a series of selection events. One of the hallmarks of this maturation process is the expression of CD69, which first appears on thymocytes as they begin positive selection. We have used blockade and overexpression of CD69 to determine the role of CD69 in thymocyte development. Blockade of CD69 led to a reduction in single-positive cells and a concomitant increase in double-positive cells in the thymus. Overexpression of a CD69 transgene in the thymus resulted in a dramatic increase in both CD8SP and CD4SP cells. Coexpression with a TCR transgene demonstrated that both positive and negative selection were enhanced by the increased levels of CD69 on thymocytes. Finally, mice overexpressing CD69 displayed a sharp reduction in the number of T cells in the spleen and lymph node. Taken as a whole, these data suggest the involvement of CD69 in the process of selection and maturation during the trafficking of thymocytes to the medulla. *The Journal of Immunology*, 2002, 168: 87–94.

In addition to the changes in the expression of cell surface markers, thymocytes traverse through the thymus as they develop. Immature thymocytes reside in the cortex, and as they begin the selection process they traffic from the cortex to the medulla. This transition is marked by the expression of other specific molecules. For example, the chemokine receptor CCR4 is up-regulated in thymocytes as they migrate from the cortex to the medulla (6–8). This in contrast to CCR9, which is expressed on both cortical and medullary thymocytes, but is down-modulated as thymocytes leave the thymus for the periphery (6, 8). These data suggest that chemokine/chemokine receptor interactions are involved in the migration of thymocyte subsets.

In addition to chemokine receptors, other cell surface molecules are expressed in a restricted fashion on developing thymocytes. One of these is the activation marker CD69, which is expressed at high levels on approximately 10–15% of thymocytes. Expression of CD69 is first seen on DP thymocytes as they begin positive selection, and experiments using TCR transgenic mice showed that only those thymocytes being selected express CD69 (9, 10). Several lines of evidence strongly suggest an active role for CD69 in TCR-mediated positive selection of thymocytes. All TCR⁺ thymocytes are CD69⁺, including both TCRlow DP cells and TCRhigh SP cells (5). These CD69⁺ cells can be further subdivided by expression of heat-stable Ag (HSA; HSA−TCRlow, HSA⁺TCRhigh, and HSA−TCRhigh); however, CD69⁺ cells do not express the mature T cell marker Qa-2 (5). Also, using an in vitro reaggregation system, Hare et al. (11) showed that CD69 may regulate an MHC-independent aspect of positive selection, suggesting that CD69 is not merely a marker for cells that have begun the selective process. Thus, CD69-expressing cells represent a population that is undergoing positive selection or has just completed that process.

Despite the compelling circumstantial evidence that CD69 is actively involved in thymocyte development and selection, there is no direct evidence. We have used overexpression and blockade of CD69 to directly test its role in thymocyte development, and demonstrate that CD69 plays a role in thymocyte selection.

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0022-1767/02/$02.00
Materials and Methods

Abs and immunofluorescence

The Abs used in this study were as follows: anti-TCRαβ-FITC (H57-597), anti-CD3ε-FITC (145-2C11), anti-CD69-FITC (H1.2F3), anti-CD25-FITC (7D4), anti-CD8-APC (53-6.72), anti-CD4-PE (GK1.5), anti-CD44-FITC (KM114), anti-Vβ3-biotin (MR9-4), and anti-Qa-2-biotin (1-1-2) from BD PharMingen (La Jolla, CA); and anti-CD62L-FITC (MEL-14), anti-CD45RB-PE (16A), and anti-HSA-FITC (J11d) from Caltag Laboratories (Burlingame, CA). Clone type anti-D011.10 TCR (KJ-26) was a gift from Dr. P. Marrack (National Jewish Hospital, Denver, CO).

For Ab staining, single-cell suspensions were prepared from freshly isolated lymphoid organs from control and experimental animals and suspended in PBS supplemented with 2% FCS and 0.1% sodium azide. In some experiments 2-wk-old C57BL/6 mice (M5/114, BD PharMingen), or an anti-CD69 mAb (H1.2F3, BD PharMingen) were treated daily for 7 days with 500 μg anti-CD69 (three mice per group) for 7 days. Then, the thymocytes were prepared and subjected to FCM analysis.

Generation of CD69 transgenic mice

CD69FL. A cDNA clone encoding the entire coding region of mouse CD69 (13) was subcloned into p1017, which contains the proximal mouse Ick gene promoter and the human growth hormone gene (14). The transgene was excised with NotI and used for pronuclear injections.

CD69 Δcyt. A cDNA construct encoding a cytoplasmic-deletion mutant of mouse CD69 was generated by PCR using the primers 5′-agatctATGGAAAGATCATACCAAGTT-3′ (amino terminus) and 5′-agatctTCAATCTGGAGGGCTTGCTGCA-3′ (carboxyl terminus). The amino terminal primer begins, after the initiating ATG, at codon 34 of mouse CD69. The final construct contains three codons from the cytoplasmic domain of mouse CD69 (the initiating ATG and the final two amino acids of the cytoplasmic domain) preceding the membrane-spanning sequence and extracellular domain. The resulting product was cloned into p1017 and injected as described above.

Double-transgenic mice (CD69FL and either the OT-II or DO11.10 TCR transgene) were generated by crossing the appropriate single-transgenic lines and screening for the individual transgenes. In all cases transgene-positive mice were identified by PCR using primers to the human growth hormone gene as previously described (14).

Results

In vivo treatment with anti-CD69 mAb inhibits the generation of mature thymocytes

The up-regulation of CD69 expression during thymocyte selection suggests a role for CD69 in the generation of mature, SP thymocytes. To begin an analysis of the role of CD69 in thymocyte development, newborn C57BL/6 mice were injected daily for 1 wk with 200 μg anti-CD69 mAb. Control mice were injected with PBS, an irrelevant MHC class II Ab (anti-I-Ak), or the relevant MHC class II Ab (anti-I-Aβ). The latter mice served as a control for the ability to block the generation of CD4SP cells. As shown in Fig. 1 the control mice displayed the anticipated phenotypes. The anti-I-Aβ-treated mice showed a decrease in the number of CD4SP cells relative to the PBS-treated mice, while the anti-I-Aβ-treated mice showed no difference compared with the PBS controls (Fig. 1A). The effect of anti-I-Aβ treatment was specific to CD4SP development as the number of CD8SP cells was unaffected.

The analysis of thymocyte development in the anti-CD69-treated mice showed reductions in both CD4SP (35–50%) and CD8SP (40–50%) cells, suggesting that blockade of CD69 inhibited the generation of SP cells (Fig. 1A). Further characterization of these mice showed that the numbers of Qa-2+ HSA- cells as well as TCRαβ+ HSA- cells, were reduced, consistent with a requirement for CD69 in the generation of mature SP thymocytes.
with a reduction of SP thymocytes (Fig. 1B). In all cases thymic cellularity was unchanged, suggesting that the reduction seen in anti-CD69-treated mice was not due to nonspecific lysis of CD69-expressing cells.

**Overexpression of CD69 in the thymus results in an increase in SP thymocytes**

The data presented above support a role for CD69 in the generation of SP thymocytes. We next determined the effect of CD69 overexpression on thymocyte development. A cDNA encoding full-length mouse CD69 was expressed from the lck-proximal promoter in transgenic animals (these mice will be referred to as CD69FL). Several founder lines were established that expressed varying levels of CD69 in the thymus, as judged by cell surface expression.

As shown in Table I, increased levels of CD69 expression in the thymus tended to skew the developmental profile seen in these mice, with the lines expressing the highest levels of CD69 showing increased numbers of CD4SP and CD8SP cells. Fig. 2 shows the analysis of a representative line, CD69FL-1. Examination of CD69 expression in this line showed that it was markedly increased relative to that in littermate controls, with approximately 99% of thymocytes expressing CD69 (Fig. 2A). When CD3 levels on thymocytes from CD69FL-1 and NLC mice were analyzed, the CD69FL-1 line displayed a higher percentage of CD3^high^ cells (Fig. 2B). Also, there appeared to be two populations of CD3^high^ cells in CD69FL-1 mice, with a small shoulder of cells with slightly lower CD3 levels and a second population with higher CD3 levels (also see Fig. 4). The peak representing the highest level of CD3 expression was CD4SP (gate 1), while the lower peak contained CD8SP cells (gate 2). This pattern of CD3 expression is similar to that seen when CD3 levels are analyzed on mature splenic T cells. We next examined the CD4/CD8 profile of CD69FL-1 and NLC mice. There was a dramatic increase in the number of CD4SP and CD8SP cells and a diminution of CD4/CD8DP cells in CD69FL-1 mice (Fig. 2C), with approximately 37% of the thymocytes from CD69FL-1 mice showing an SP phenotype compared with 17% for the NLC. There was a concomitant decrease in DP cells in CD69FL-1 mice, and overall thymic cellularity was comparable between the two sets of mice, suggesting that an increase in total cell numbers cannot account for this difference in SP cells. We have now examined four CD69FL lines, and all show a similar phenotype, with the number of SP thymocytes proportional to the level of CD69 expression (number of SP cells varies from 25 to 58%; data not shown).

Additional evidence as to the phenotype of the thymocytes from the CD69FL mice came from an analysis of other cell surface markers, including HSA, CD45RB, Qa-2, and CD62L on SP cells. Fig. 3 shows the expression of these markers on CD4SP cells from CD69FL-1 mice and NLC controls.

![Table I. Thymic phenotype of CD69FL transgenic mouse lines](#)

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<tr>
<th>Strain</th>
<th>Cellularity^a^</th>
<th>% CD69^+/MFI^</th>
<th>% CD4 SP</th>
<th>% CD8 SP</th>
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<tr>
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<td>99/1430</td>
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^a Each strain represents an individual CD69FL transgenic line. Normal littermate control (NLC) in each case was age- and sex-matched with CD69FL line (CD69 Tg^+^).

^b Total thymic cellularity, \times 10^8.

^c Percent CD69^+^ thymocytes/Mean fluorescence intensity for the cells in the CD69^+^ gate.
CD69FL-1 and NLC mice. CD4SPs from the control animals had an HSA/H11001 Qa-2/H11002 CD45RB low CD62L low phenotype. In contrast, the cell surface phenotype of the CD4SP cells from CD69FL-1 mice more closely resembles that of mature, peripheral T cells (HSA/H11002 Qa-2/H11001 CD45RB high CD62L high). The CD8SP cells displayed a similar phenotype (data not shown). Finally, thymocytes from the CD69 transgenic mice have a higher proliferative capacity, compared with control mice, when stimulated through the TCR (data not shown). Taken as a whole, these data demonstrate that overexpression of full-length CD69 in the thymus results in the accumulation of SP cells in the thymus that more closely resemble mature, peripheral T cells then SP thymocytes.

Thymocyte development in mice expressing a cytoplasmic deletion mutant of CD69 resembles that seen in mice expressing full-length CD69

To determine whether the phenotype seen in the CD69-overexpressing transgenic animals required CD69-mediated signal transduction, we generated mice expressing a cytoplasmically truncated form of CD69 from the lck-proximal promoter (see Materials and Methods for details of the construct). Several founder lines were generated, and T cell development was studied in four lines (CD69Δcyt-1–4) that displayed CD69 levels roughly equivalent to those seen in the CD69FL lines (Figs. 2A and 4A and data not shown). As was seen with CD69FL-1 mice, CD69Δcyt-1 and littermate control mice displayed similar thymic cellularity. Thymocyte development in CD69Δcyt mice was also very similar to that seen in CD69FL-1 mice. For example, the CD69Δcyt-1 line showed the two populations of CD3high cells seen in CD69FL mice (Figs. 2B and 4B), as well as increases in both CD4SP and CD8SP cells (Fig. 4C), although the CD4SP/CD8SP ratio in CD9Δcyt mice differed from that in CD69FL-1 mice. The significance of this last finding is unclear at this time. Thus, the ability of CD69 to affect the DP to SP transition does not necessarily require a signal through CD69. This suggests that overexpression of the extracellular domain of CD69 is the major cause of the phenotypes seen in the CD69FL and CD69Δcyt transgenic mice, possibly by affecting interactions with its ligand. However, the severity of the phenotype seen in CD69FL mice was greater than that seen in CD69Δcyt mice, demonstrating a possible role for CD69-mediated signals.

Positive and negative selection in CD69FL-1 mice

A possible explanation for the phenotype seen in CD69FL-1 mice is that overexpression of CD69 leads to enhanced positive selection, and thus an increase in the number of SP cells. To directly study the role of CD69 on the positive and negative selection of thymocytes, we crossed CD69FL-1 mice with two TCR transgenic mouse lines, OT-II and DO11.10. Both TCRs are specific for the same chicken OVA peptide (OVA323–332) (15). OT-II-expressing T cells recognize this peptide presented in the context of I-Ab, while DO11.10-expressing T cells see it presented in the context of I-Ak. However, T cells expressing the DO11.10 TCR also are alloreactive against I-Ak (16), and thymocytes bearing the transgenic CD69FL-1 and NLC mice. CD4SPs from the control animals had an HSA´ Qa-2´ CD45RBlow CD62Llow phenotype. In contrast, the cell surface phenotype of the CD4SP cells from CD69FL-1 mice more closely resembles that of mature, peripheral T cells (HSA´ Qa-2` CD45Righb CD62Lhigh). The CD8SP cells displayed a similar phenotype (data not shown). Finally, thymocytes from the CD69 transgenic mice have a higher proliferative capacity, compared with control mice, when stimulated through the TCR (data not shown). Taken as a whole, these data demonstrate that overexpression of full-length CD69 in the thymus results in the accumulation of SP cells in the thymus that more closely resemble mature, peripheral T cells then SP thymocytes.

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TCR are negatively selected in H-2b mice (17). Thus, both positive and negative thymocyte selection in CD69FL-1 mice, which are on the C57BL/6 background, can be examined using these two TCR transgenic lines.

To generate CD69FL/OT-II double-transgenic mice, we crossed the CD69FL-1 line with C57BL/6 mice expressing the OT-II TCR transgene (these mice will be referred to as OT-II). There was no significant difference in thymic cellularity between the two strains (Fig. 5). Thymocyte development was studied in OT-II and CD69FL-1/OT-II F1 mice, using Abs against CD4, CD8, CD3, and Vβ5 (the β-chain of the transgenic TCR). As shown in Fig. 5, the OT-II mice showed a skewing toward CD4SP cells, reflecting positive selection of the OT-II TCR-expressing thymocytes. CD69/OT-II mice displayed a greater skewing toward the development of CDSP cells, with greater than 60% of thymocytes CD4+/Vβ5+ (Fig. 5). We have also examined the ability of CD4SP cells from these mice to respond to Ag in a proliferation assay. CD4+ cells were purified from OT-II and CD69/OT-II mice and cultured with irradiated C57BL/6 splenocytes in the presence of the antigenic peptide. No difference was found between OT-II- and CD69/OT-II-derived cells (data not shown). Taken as a whole, these data show that the process of positive selection proceeds at an enhanced rate in mice overexpressing CD69 in the thymus, and that the selected CD4SP cells respond normally to antigenic stimulation.

We next analyzed negative selection in mice overexpressing CD69. To do this, we took advantage of the fact that thymocytes expressing the DO11.10 TCR as a transgene undergo negative selection on the H-2b background. CD69FL/DO11.10 TCR double-transgenic mice were generated by crossing the CD69FL-1 transgenic line with C57BL/6 mice expressing DO11.10 TCR (these mice are referred to as DO11). Thymocyte development was assessed, using flow cytometry, in F1 mice from this cross that represented the four relevant genotypes (−/−, −/DO11, C69/+−, and CD69/DO11). Thymocytes from the control lines (−/− and CD69/−−) showed the same pattern of development as those shown in Fig. 2, with CD69FL-expressing mice showing an increase in SP cells and a concomitant decrease in DP cells (data not shown).

An analysis of DO11 and CD69/DO11 mice is shown in Fig. 6. DO11 mice displayed a decrease in thymus cellularity (29 × 106 for DO11, compared with 156 × 106 for NLC mice) along with a marked increase in DN cells and a decrease in DP cells.

Initial examination of CD69/DO11 mice showed that they also had a dramatic decrease in thymic cellularity, reduced approximately 83% (27.5 × 106 compared with 156 × 106 for the NLC mouse). Interestingly, the double-transgenic mice displayed features of each single transgene animal, showing both increased SP cells and increased DN cells. In fact, the increase in DN cells was more dramatic than that seen in DO11.10 TCR mice (32% DN cells in CD69/DO11 mice vs 16% in DO11 mice). Also, as was seen in mice expressing either CD69FL or CD69Δcyt, mice expressing both the DO11.10 TCR transgene and the CD69Δcyt transgene displayed the same overall thymic phenotype as CD69/DO11 mice (data not shown).

We next examined the expression of DO11.10 TCR in these mice. As shown in Fig. 6, both lines had similar numbers of CD3+ cells. When the expression of DO11.10 TCR was examined using the clonotypic Ab KJ1-26 (17), both DO11 and CD69/DO11 mice showed a reduction in the number of clonotype-positive cells. DO11.10 TCR expression was then examined in individual thymic subpopulations of both sets of animals. CD4SP cells from both DO11 and CD69/DO11 mice showed a dramatic reduction in KJ1-26 staining, suggesting that the clonotype-positive cells had been negatively selected. However, as mentioned above, CD69/DO11 mice had more CD4SP cells than DO11 mice (Fig. 6). These cells do not express the transgenic TCR and most likely arise through rearrangement of endogenous TCRα genes. This finding is consistent with the increased accumulation of CD4SP cells in CD69FL mice (Fig. 2).

We next examined the expression of the transgenic TCR in the DN compartment in both mice. A majority of DN cells in DO11 mice expressed CD3 and were KJ1-26+ (Fig. 6). Earlier work had shown that these KJ1-26+, CD4−/CD8− cells represented a lineage that bypassed the DP stage (16). In contrast, most of the DN cells from the CD69/DO11 mice were CD3− and KJ1-26−.
FIGURE 6. Coexpression of CD69FL and DO11.10 TCR leads to an increase in negative selection. Cells were isolated from thymi from the indicated mouse lines and analyzed by three-color flow cytometry for CD4, CD8, and either CD3 or KJ1-26 expression. Top panel, CD4/CD8 profiles from each mouse line. The numbers in the upper left corner represent the percentage of cells in each quadrant. Bottom panel, CD3 vs KJ1-26 expression in subpopulations from thymi of DO11 and CD69/DO11 mice. Levels of CD3 and KJ1-26 expression were determined on cells from the indicated cell populations for these two lines. The number refers to the percentage of cells in the gate. Cellularity: DO11, 29 × 10^6; CD69/DO11, 28 × 10^6.

Mice expressing CD69 transgenes had reduced T cells in peripheral lymphoid organs

As shown above, thymi from mice that overexpress CD69, either full-length or containing a cytoplasmic truncation, displayed a dramatic increase in SP cells (Figs. 2 and 4). These cells had the cell surface characteristics of mature T cells (TCR<sup>high</sup>Qa-<sup>2</sup>HSA<sup>-</sup>; Fig. 3 and data not shown). One possible explanation for this phenotype is that following maturation the SP cells are incapable of exiting the thymus. If this explanation were correct, one prediction would be a reduction in the number of peripheral T cells. We tested this hypothesis by examining CD3 levels and CD4/CD8 profiles of spleens and lymph nodes isolated from CD69FL and CD69Δcyt mice. It was apparent that the spleens and lymph nodes from both sets of animals were significantly smaller than those in the control animals, and this observation was supported by CD3 and CD4/CD8 profiles from each animal. As shown in Fig. 7A, CD69FL-1 mice had dramatically reduced numbers of T cells in spleen and almost no T cells in lymph node (data not shown). Concomitantly, the number of CD3<sup>+</sup> cells was severely reduced (data not shown). We also examined T cells in spleens and lymph nodes of CD69Δcyt animals. Similar to what we observed in CD69FL-1 animals, spleens and lymph nodes in CD69Δcyt-1 were smaller than those in littermate control mice. However, CD69Δcyt-1 mice had a 50% reduction of peripheral T cells number (Fig. 7B), a less severe loss than what was seen in CD69FL-1 mice. This finding was consistent with the less severe thymic phenotype seen in these mice (Fig. 4) and suggests a role for CD69 signaling in the phenotypes seen in these two sets of mice.

We also examined peripheral T cells in OT-II and CD69/OT-II mice (Fig. 7C). Splenic cellularity was comparable in the two mice. OT-II mice showed an increase in CD4<sup>+</sup> cells in the spleen, nearly all of which were Vβ5<sup>+</sup> (data not shown). However, spleens from CD69/OT-II mice were nearly devoid of T cells. Subsequent analysis determined that the cellularity in the spleen in these animals was maintained by an increase in the number of B cells (data not shown). The reduction in the number of peripheral T cells in OT-II/CD69 mice was greater than that in CD69FL-1 mice.

Discussion

A great deal of progress has been made defining the role of TCR selection in T cell development in the thymus (reviewed in Refs. 1–3). However, little is known about the roles of cell surface molecules, such as CD69, whose expression is also regulated during thymocyte development. In this report, we have shown that CD69 plays an active role in the development of thymocytes. We have shown that blockade of CD69, through in vivo Ab administration, inhibits development at discreet stages. Ab blockade of CD69 caused a reduction in the number of SP thymocytes and a concomitant increase in the number of DP cells (Fig. 1). While these data strongly suggest that blockade of CD69 inhibits SP development, we cannot rule out that cross-linking CD69 with the mAb results in the deletion of SP thymocytes, or that the lower levels of SP cells reflects phagocytosis of Ab-coated cells. To address the effect of CD69 blockade more directly, we have generated transgenic mice expressing a secreted form of the CD69 extracellular domain. A preliminary analysis of thymocyte development in these mice has suggested that they are similar to what was observed in Ab-treated animals, supporting the hypothesis that CD69 blockade inhibits the development of SP thymocytes (data not shown).

In contrast to what was observed in mice in which CD69 interactions were blocked, mice that overexpressed CD69 in the thymus displayed a dramatic increase in the numbers of CD4SP and CD8SP cells (Figs. 2 and 4 and Table I). In addition, these cells had the phenotype of mature T cells in that they were TCR<sup>high</sup>Qa-
shown by staining with KJ1-26; Fig. 6, bottom panel), suggesting that they arose from cells that rearranged endogenous TCR genes. Those thymocytes expressing the DO11.10 TCR were deleted. These data suggest that, similar to the CD69FL-1 mice, thymocytes in both sets of double transgenic mice were being driven to the SP stage. In the CD69/OT-II mice, these SP cells were selected and accumulated. In CD69/DO11.10 mice those thymocytes that expressed the TCR transgene were deleted, and those that were able to rearrange and express endogenous TCR α-chains accumulated. Consistent with this was our finding that the spleens of DO11 mice contained CD4+ CD8− DO11.10+ cells, as has been previously reported when this TCR transgene is expressed on a H-2b background (16). In CD69/DO11 mice these cells were not present in the periphery, and these mice, similar to CD69FL-1, had a dramatic reduction in the number of peripheral T cells (data not shown).

To assess whether CD69 signal transduction was involved in thymocyte development, we generated mice overexpressing a cytoplasmic mutant of CD69 (CD69Δcyt). In cell culture studies we have shown that expression of this construct in cells inhibited the signaling of endogenous CD69 (data not shown). An analysis of thymocyte development in these mice showed that cell-surface expression of CD69 is sufficient to lead to an increase in SP cells. However, the phenotype in mice overexpressing functional CD69 (CD69FL) is more dramatic than that in mice expressing an equivalent level of CD69Δcyt (compare Figs. 2 and 4). Again, similar to the CD69FL mice, the phenotypes seen in mice expressing the CD69Δcyt transgene correlated with levels of transgene expression. These data, taken together, suggest a model by which a combination of CD69 interaction with an as yet unknown ligand, coupled with CD69-mediated signal transduction, contributes to the generation of SP thymocytes.

There are several possible explanations for the accumulation of thymic SP cells in the CD69 transgenic mice. One possible explanation is that there is greater turnover of those SP cells in the thymus. The fact that overall thymic cellularity is unchanged in these mice argues against this. Also, we have begun to examine apoptosis in these mice, using annexin V staining and TUNEL analysis and found no difference between NLC and CD69 transgenic mice (D. J. Kasprowicz and S. F. Ziegler, unpublished observations). Another possible explanation is that CD69 is involved in the trafficking of thymocytes during their maturation. In this model, unregulated expression of CD69 on thymocytes increases the movement of thymocytes from the cortex to the medulla during differentiation. The inability to down-regulate CD69 expression on these cells causes them to remain in the thymus. Support for this model comes from indirect immunofluorescence of human thymus using anti-CD69 mAb. In this study Jung et al. (18) found that only scattered cells in the cortex expressed CD69, and these cells tended to cluster in the subcapsular region. These may correspond to the CD44+CD25− DN population that we have shown expresses CD69 (bottom panel). For example, double-transgenic mice expressing either positively or negatively selected TCRs had similar cellularity as their single TCR transgenic littersmates. The positively selecting mice (CD69/OT-II) had twice as many CD4SP cells as the OT-II mice, all of which expressed the TCR transgene (Fig. 5, bottom panel). The negatively selecting mice (CD69/DO11) also displayed an increase in CD4SP cells relative to the single TCR transgenic line. However, these CD4SP cells did not express the transgenic TCR (as

2+HSA−. This is the phenotype seen on T cells as they leave the thymus and seed the periphery (5). The fact that this population is present in large numbers in the thymus of CD69 transgenic mice, but not in littermate control mice, suggests that it is the continued expression of CD69 that affects the developmental profile of these cells. Consistent with a more mature phenotype, CD4SP cells from CD69FL mice were more responsive to TCR engagement (D. J. Kasprowicz and S. F. Ziegler, unpublished observation).

The accumulation of SP cells, at the expense of DP cells, in CD69FL mice suggests differences in thymic selection between these animals. We tested this hypothesis by examining the role of CD69 in the selection events using mice expressing transgenic TCRs that were either positively or negatively selected on the C57BL/6 background. The data generated from these double-transgenic mice supports the hypothesis that overall thymic selection is more efficient in mice overexpressing CD69 (Figs. 5 and 6). For example, double-transgenic mice expressing either positively or negatively selected TCRs had similar cellularity as their single TCR transgenic littersmates. The positively selecting mice (CD69/OT-II) had twice as many CD4SP cells as the OT-II mice, all of which expressed the TCR transgene (Fig. 5, bottom panel). The negatively selecting mice (CD69/DO11) also displayed an increase in CD4SP cells relative to the single TCR transgenic line. However, these CD4SP cells did not express the transgenic TCR (as

![Figure 7](http://www.jimmunol.org/)
The finding the mice lacking CD69 have normal thymic development (T. Nakayama, D. J. Kasprowicz, M. Yamashita, and S. F. Ziegler, unpublished results) suggests that there are additional molecules expressed on thymocytes capable of interacting with the same ligand and generating related signals. We have searched the public EST databases and have found three novel C-type lectins that are expressed in the thymus (data not shown). We are currently testing the roles of these molecules in thymocyte development. Also, as mentioned above, our interpretation of the data is predicated on the existence of a ligand for CD69 that is expressed in the thymus. Using purified soluble CD69 we have detected specific binding on cell lines that can serve as APC (data not shown). These data are consistent with a CD69 binding partner being expressed in thymus by cells that regulate selection. We are currently examining thymic stromal cell lines for solCD69 binding.

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