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Timing and Casting for Actors of Thymic Negative Selection

Nicole Dautigny, Armelle Le Campion, and Bruno Lucas

We have recently proposed a new model for the differentiation pathway of αβ TCR thymocytes, with the CD4 and CD8 coreceptors undergoing an unexpectedly complex series of expression changes. Taking into account this new insight, we reinvestigated the timing of thymic negative selection. We found that, although endogenous superantigen-driven thymic negative selection could occur at different steps during double-positive/single-positive cell transition, this event was never observed among CD4lowCD8low TCRint CD69+ thymocytes, i.e., within the first subset to be generated upon TCR-mediated activation of immature double-positive cells. We confirm a role for CD40/CD40L interaction, and the absence of involvement of CD28 costimulation, in thymic deletion in vivo. Surprisingly, we found that thymic negative selection was impaired in the absence of Fas, but not FasL, molecule expression. Finally, we show involvement in opposing directions for p59fyn and SHP-1 molecules in signaling for thymic negative selection. The Journal of Immunology, 1999, 162: 1294–1302.

Generation of T lymphocytes takes place in the thymus through an ordered sequence of developmental steps. Functional TCR recognition units are created by somatic rearrangements of gene segments. The end result of these early steps in thymic differentiation is the production of a large number of TCR-bearing immature cells with surface expression of both CD4 and CD8 coreceptors (double-positive (DP) thymocytes) (1). Since TCR segment rearrangements are not directed by the ultimate specificity of the receptor, postarrangement selective mechanisms allow death by neglect of the useless, active elimination of the dangerous, and survival of the useful (2). This selection is accomplished by testing developing T cells in the thymus for reactivity of their αβ TCR with self Ags (short peptides bound to MHC molecules (MHC)) (3). A key aspect of this process involves the death of developing thymocytes with TCR whose interactions with self-peptide:MHC molecule complexes might result in activation of the mature T cell bearing that TCR and the development of a pathologic autoimmune process (thymic negative selection).

Individual specific clones cannot be identified within the whole population of normal thymocytes. To overcome this problem, two experimental systems have been used to study the fate of immature DP thymocytes in vivo, specifically, thymic negative selection. First, experiments with TCR transgenic mice have suggested that deletion can take place early in thymic development (4–6), even before DP thymocyte generation (7, 8). Therefore, such deletion would be driven through interaction of the newly expressed TCR of immature DP thymocytes with self ligands on thymic cortical epithelium (6, 9). A possible bias in this system comes from the properties of TCR transgenic mice. In such mice, TCR density is considerably increased at the surface of immature DP thymocytes (10). Thus, the early deletion observed in these models could directly result from such an unphysiologic early high level of TCR expression, given that maintenance of low levels of TCR at the DP cell surface is an active process whose full significance is still unknown (11–13). The second model came from the discovery of endogenous superantigens that bind to the receptors of all T cells bearing particular Vβ chains and thereby induce deletion of a significant proportion of the T cell repertoire (14). Guidos et al. have shown that thymic negative selection occurs progressively during the DP/mature single-positive (SP) cell transition (15). Subsequent studies have shown that such deletion could happen all along this transition, depending on the avidity of the studied interaction (16–18) and that, in normal mice, the bulk of this deletion took place in the thymic medulla (19, 20). One could argue that the results obtained in these systems do not reflect conventional peptide-mediated thymocyte negative selection but rather are related to the unusual nature of superantigens. Recently, however, many studies have confirmed the role of the thymic medulla in peptide- as well as superantigen-induced negative selection (21–24), suggesting that superantigens rather than TCR transgenic mice would represent a good system to study the timing and mechanisms involved in thymic deletion.

Past studies on this subject have been based on the assumption that extinction of CD4 and CD8 expression by DP thymocytes was a steady, uninterrupted process (15, 25). We have recently shown that, in fact, CD4 and CD8 coreceptors undergo an unexpectedly complex series of expression changes during thymocyte development (26). TCR signaling of CD4+CD8+ thymocytes (initiation of positive selection) induces an extensive down-regulation of both CD4 and CD8 coreceptors, up-regulation of TCR-αβ surface expression, and the expression of the early activation marker CD69, to give rise to CD4lowCD8low TCRint CD69+ thymocytes (26). CD4 reexpression being much faster than CD8 reexpression, these cells become CD4+CD8low TCRint CD69+ cells by the next day. Then, CD4+CD8low TCRint CD69+ thymocytes develop into CD4+CD8 low TCRhi (CD4SP) or CD4+CD8+ TCRhi (CD8SP) cells. Therefore, we decided to reinvestigate the timing of thymic negative selection in the light of this new appreciation of the details of the TCR-αβ thymocyte differentiation pathway.

In the present paper, we have studied the timing of superantigen-mediated negative selection and the role of various molecules in...
this process. We report the absence of detectable superantigen-induced deletion among CD4loCD8lo CD45RBlo CD69+ thymocytes, i.e., within the first subset to be generated upon TCR-mediated activation of immature DP cells. Superantigen-driven thymic negative selection could occur at multiple different subsequent steps during the DP/SP transition. To investigate the role of individual proteins in thymic negative selection, most authors have crossed mice deficient for the expression of this protein with Mtv−/−, I-E− mice, and examined the disappearance of the relevant Vβ families among mature lymphocytes. Such “strong” deletion processes could mask the involvement of the studied protein. Indeed, a role for certain actors in thymic negative selection may become obvious only when the strength of the deletional signal falls below a certain threshold. Therefore, a “weak” deletion model might be more suitable for studying the role of candidate proteins in negative selection. Therefore, we directly assessed thymic deletion of Vβ5-bearing thymocytes in Mtv−/− I-E− mice (C57BL/6 or 129 mouse strains) deficient for the expression of potential actors in thymic negative selection. Using this protocol, we confirm the role of CD40/CD40L interaction in thymic deletion, as well as the absence of involvement of CD28 costimulation in vivo. Surprisingly, we found that thymic deletion was impaired in the absence of Fas, but not FasL, molecule expression. Finally, we show opposite roles for p59fyn and SHP-1 in thymic negative selection.

Materials and Methods

Mice

C57BL/6, 129Sv and C57BL/6 αMT- (27) or CD4- (28) deficient mice were obtained from Centre de Développement des Techniques Avancées pour l’expérimentation animale (Orleans, France). C57BL/6 CD28- (29), B6.129 CD40- (30), B6.129 CD40L- (31), C57BL/6 CD54- (32, 33), B6.129 p59b-, and 129Sv p59b-deficient mice (34, 35) as well as C57BL/6 gld/gld, C57BL/6 ipipip and viable C57BL/6 motheaten (36) mice were purchased from The Jackson Laboratories (Bar Harbor, ME). Mice deficient in I-E class I (ΙKΟ) (37) or class II (ΙIΚΟ) (38) molecule expression were obtained from Taconic (Taconic, NY). All of these mice were studied between 6 and 8 wk of age except for C57BL/6 viable motheaten mice, which were 4 wk old when studied because of the high mortality of such mice after this age.

Cell surface staining and flow cytometry

Thymi and lymph nodes were homogenized on a nylon cell strainer (Falcon, Franklin Lakes, NJ) in PBS, 5% FCS, and 0.2% NaN3 and then distributed into the wells of 96-well U-bottom microplates (2 × 10^6 cells per well). Staining was done on ice for 30 min per step. Flow cytometry was performed on a FACScalibur cytometer (Becton Dickinson, Mountain View, CA). List mode data files were analyzed using Cell Quest software (Becton Dickinson).

For four-color analysis, surface molecules were stained with PE anti-CD4 (clone H1.2F3; Pharmingen, San Diego, CA), FITC anti-CD8 (clone 53-6-7; Pharmingen), and Red613 anti-CD4 (clone H129.19, Life Technologies, Gaithersburg, MD) and biotinylated anti-TCRβ (clone H57-597), anti-Vβ3 (clone KJ25), anti-Vβ5 (clone MR9-4), anti-Vβ6 (clone RR4-7), or anti-Vβ14 (clone 14-2) was revealed by allophycocyanin-streptavidin (Molecular Probes, Eugene, OR). All biotinylated Abs were prepared in our laboratory.

Results

Initiation of positive selection occurs before superantigen-mediated thymic deletion

Thymic subsets were defined based on four-color staining for surface expression of CD4, CD8, TCRβ, and CD69 (Fig. 1) (26). These cells can be labeled as follows: CD4+CD8+ TCRlo CD69−, CD4loCD8lo TCRmi CD69+, CD4loCD8lo TCRhiCD69+, CD4hiCD8+ TCRhiCD69+, and CD4+CD8− TCRhiCD69+ or CD4−CD8+ TCRhiCD69− (CD69+ or CD69−). The Vβ repertoire of these different thymic subpopulations was assessed in CBA/Ca vs CBA/J mice. Mtv-6, -7, and -9 are integrated in the genome of CBA/J mice, leading to thymic deletion of Vβ3-, Vβ6- and Vβ5-bearing cells, respectively, whereas only Vβ5-bearing cells are deleted in CBA/Ca mice (14).

The Vβ repertoire of immature CD4+8+ TCRlo CD69+ thymocytes was indistinguishable in these two strains (Table I), consistent with the accepted concept that these CD69+ cells have not been subject to TCR-mediated signaling and selection events. Therefore, the Vβ distribution among the TCR of such cells reflects only the intrinsic mechanisms of TCRβ gene rearrangements. Unexpectedly, no evidence of superantigen-mediated deletion was observed among the CD4loCD8lo TCRmi CD69− cell subset, which had the same proportion of Vβ3-, Vβ5- and Vβ6-bearing cells in both strains. Vβ3- and Vβ6-bearing thymocytes in CBA/j mice and Vβ5-bearing cells in both strains disappeared only at the CD4+CD8lo TCRhiCD69+ stage of thymic differentiation (Table I). Thus, thymic deletion of such potential autoreactive clones is dramatic and almost completed during the CD4loCD8lo TCRhiCD69−/CD4+CD8lo TCRhiCD69− cell transition. Moreover, an enrichment in Vβ3-bearing cells could be observed within the CD4loCD8lo TCRhiCD69− cell subset in CBA/j mice in comparison with CBA/Ca mice (Table I). Indeed, 1.8% and 1.5% of CD4+CD8lo TCRhiCD69− thymocytes still express Vβ6 and -5, respectively, whereas less than 0.5% of CD4SP as well as of CD8SP thymocytes bear such β-chains (Table I).

It has been described that Vβ14-bearing cells are positively selected in the presence of Mtv-7-encoded superantigen (39). Interestingly, no differences in Vβ14 frequency within CD4loCD8lo TCRmi CD69+ thymocytes could be detected between these two mouse strains. Vβ14 frequency does increase significantly during the CD4loCD8lo TCRhiCD69−/CD4+CD8lo TCRhiCD69− cell transition, and this occurs to a greater extent in CBA/J mice (Table I). This difference may be explained by the deletion of a larger number of other Vβ-bearing thymocytes in CBA/J as compared with CBA/Ca mice. Only the increases of Vβ14 frequency among CD4loCD8lo TCRhiCD69− and more importantly among CD4+CD8+ TCRhiCD69+ thymocytes in CBA/J mice could not be explained by such a compensatory mechanism. The increase of Vβ14+ cell frequency due to Mtv-7-encoded superantigen expression is then a late event in thymic differentiation. A late expansion phase (as recently described by Pénit and Vasseur (40)) or an augmented thymocyte/stromal cell interaction during maturation allowing more Vβ14+ CD8SP cell precursor to complete their differentiation rather than positive selection by itself (i.e., cell rescue from neglect at the immature DP stage of thymic differentiation) could explain such data.

Superantigen-mediated negative selection could occur at different stages of thymic differentiation

Mtv-9-encoded superantigen induces deletion of Vβ5-bearing thymocytes, and this mechanism has been described as depending on...
the expression of the MHC class II molecule I-E (41–43, 14). Furthermore, Bill et al. and Liao et al. have shown that, in all studied I-E

mouse strains, the majority of Vβ5-bearing cells are CD8SP cells and therefore concluded that Vβ5-bearing T cells are positively selected by class I MHC molecules, clonally deleted by class II I-E molecules, and poorly selected by class II I-A molecules (41, 43). None of these studies have considered that the low proportion of Vβ5 cells among CD4SP lymphocytes in I-E2 mice could reflect negative rather than poor positive selection mediated by class II I-A molecules. To investigate this possibility, we decided to analyze Vβ5 frequency changes during T cell differentiation. Several groups have recently demonstrated that CD4lowCD8low TCRint CD69+ as well as CD4lowCD8low TCRint CD4lowCD69+ thymocytes are a mixture of MHC class I- and class II-restricted cells (26, 44–49). Therefore, their repertoire cannot be directly compared with the Vβ frequencies observed within mature SP cell subsets. Then, we choose to investigate the Vβ repertoire of the different thymic cell subsets in the absence of MHC class I or class II molecule surface expression (Table II).

Table I. Initiation of positive selection occurs prior to superantigen-mediated thymic deletion

<table>
<thead>
<tr>
<th>Vβ</th>
<th>Mouse Strain</th>
<th>CD4+8+ TCRlow CD69+</th>
<th>CD4+8+ TCRlow CD69-</th>
<th>CD4+8+ TCRint CD69+</th>
<th>CD4+8+ TCRhigh CD69+</th>
<th>CD4+8+ TCRhigh CD69-</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ3</td>
<td>CBA/Ca</td>
<td>8.6 ± 2.0</td>
<td>3.3 ± 0.5</td>
<td>4.7 ± 0.7</td>
<td>4.4 ± 0.4</td>
<td>3.9 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CBA/J</td>
<td>9.3 ± 1.7</td>
<td>6.7 ± 0.7</td>
<td>0.4 ± 0.1</td>
<td>0.1 ± 0.1</td>
<td>0.2 ± 0.2</td>
</tr>
<tr>
<td>Vβ5</td>
<td>CBA/Ca</td>
<td>ND</td>
<td>10.9 ± 0.6</td>
<td>1.5 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.3 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>CBA/J</td>
<td>ND</td>
<td>13.2 ± 0.6</td>
<td>2.0 ± 0.4</td>
<td>0.4 ± 0.1</td>
<td>1.0 ± 0.5</td>
</tr>
<tr>
<td>Vβ6</td>
<td>CBA/Ca</td>
<td>9.6 ± 2.0</td>
<td>7.9 ± 0.2</td>
<td>8.8 ± 0.7</td>
<td>7.5 ± 0.4</td>
<td>10.9 ± 0.5</td>
</tr>
<tr>
<td></td>
<td>CBA/J</td>
<td>10.1 ± 2.6</td>
<td>8.8 ± 0.6</td>
<td>1.8 ± 0.4</td>
<td>0.2 ± 0.1</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Vβ14</td>
<td>CBA/Ca</td>
<td>4.0 ± 0.5</td>
<td>5.0 ± 0.5</td>
<td>6.4 ± 0.4</td>
<td>6.8 ± 0.6</td>
<td>7.5 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>CBA/J</td>
<td>4.3 ± 0.4</td>
<td>5.1 ± 0.5</td>
<td>8.3 ± 0.3</td>
<td>8.4 ± 0.5</td>
<td>11.7 ± 2.0</td>
</tr>
</tbody>
</table>
also observed in semimature thymocytes (see Fig. 4 and data not shown). These data suggest that, in the absence of I-E molecule expression, deletion of V\(\beta\)5-bearing thymocytes is not abrogated but delayed, occurring at a later stage of thymic differentiation. This decrease in V\(\beta\)5 frequency could hardly be due to an expansion of all cells bearing other V\(\beta\) families resulting from thymocyte proliferation or reentry of peripheral mature T cells into the thymus. Indeed, a twofold decrease of V\(\beta\)5-bearing thymocytes (CD4\(^{1-}\)) was observed in CD4\(^{1-}\) mice whereas CD4\(^{1+}\) cells in the CD8 low TCR int/high CD69 high thymus, it has been shown that such an event is extremely limited (40, 51, 52) and, therefore, could not explain the twofold decrease in V\(\beta\)5 frequency we observe during thymic differentiation. Therefore, a late deletion of V\(\beta\)5-bearing thymocytes during thymic maturation appears likely to be the most suitable interpretation of our results.

Furthermore, CD8SP T cells are also affected by such deletion since only 12.9% of CD8SP thymocytes express V\(\beta\)5 in comparison with 16.3% in IIKO mice in which superantigen-mediated negative selection cannot occur because superantigen presentation requires MHC class II molecule expression. These results suggest that, in I-E- mice, V\(\beta\)5-bearing cell deletion occurs when cells destined to enter both CD4SP and CD8SP cell lineages share a common cell surface phenotype. These target cells have to express CD4 molecules since deletion of V\(\beta\)5-bearing cells is abrogated in CD4- mice whereas V\(\beta\)5-bearing thymocytes of CD4-deficient mice vs 12.9% in control IIKO mice in which superantigen-mediated deletion is not complete, and it is delayed and therefore could be considered as “weak” in comparison with the same deletion observed in the presence of MHC class II I-E molecule expression (or to V\(\beta\)3- and V\(\beta\)6-bearing cell negative selection in I-E+ mouse, respectively; Table I). A “weak” deletion model might allow the visualization of the involvement of new actors in thymic negative selection. V\(\beta\)14 frequencies do not significantly differ within mature thymocyte subsets in control vs MHC molecule-deficient mice (Table III), suggesting that this TCR\(\beta\) chain represents then a good control for additional experiments.

### A casting for actors in thymic negative selection

V\(\beta\)5 frequency among mature CD4SP and CD8SP thymocytes was evaluated in different H-2b mouse strains rendered deficient for the expression of particular proteins by gene targeting (Fig. 2). As already shown by others, CD40/CD40L interaction was found to be of great importance in thymic negative selection (53). Indeed, in the absence of one or the other of these two surface proteins, the frequency of V\(\beta\)5-bearing cells is greatly increased within both CD4SP and CD8SP thymocyte subpopulations (Fig. 2). Similar results were obtained in CD54 (ICAM-1)-deficient mice. The involvement of CD28 signaling in thymic negative selection has been extensively debated and is still controversial (29, 54–62). In our system, no changes were observed in CD28-deficient mice when compared with control C57BL/6 mice (Fig. 2). This result argues against a necessary role of CD28-mediated costimulation in thymic negative selection.

Most studies on lpr/lpr and gld/gld mice have concluded that Fas/FasL interaction is not required for induction of cell death during negative selection (63–68). By contrast, Castro et al. have recently shown that Fas, in conjunction with Ag-specific signals, can modulate apoptosis during negative selection of thymocytes (69, 70). Surprisingly, we have found that V\(\beta\)5 frequencies within mature thymocyte subsets are unchanged in gld/gld mice whereas they are increased in the absence of Fas surface expression (Fig. 2). These changes were not dependent on the age of the mice and, therefore, on the onset and development of the disease that characterizes both strains (see Fig. 4A). These asymmetrical results obtained in lpr/lpr vs gld/gld mice lead us to propose several hypotheses: either an unknown ligand of Fas could exist and be involved in thymic negative selection instead of FasL; or, by simple expression and without any requirement for ligand interaction, Fas could deliver a background signal, and therefore decrease the signaling threshold for inducing cell death within immature thymocytes. Interestingly, the absence of B cells (\(\mu\)MT-deficient mice; Fig. 2) seemed to alter V\(\beta\)5-bearing thymocyte deletion. These results are different from observations made in the same mice by

### Table II. V\(\beta\)5 frequencies within thymocyte subsets

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>MHC</th>
<th>CD41 low CD8 high TCR int CD69+</th>
<th>CD41 low CD8 low TCR int CD69+</th>
<th>CD41 high CD8 high TCR int CD69+</th>
<th>CD41 high CD8 low TCR int CD69+</th>
<th>CD41 high CD8 high TCR int CD69+</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 control</td>
<td>IA1+ IE1- MHC class I+</td>
<td>12.1 ± 0.7</td>
<td>11.9 ± 0.8</td>
<td>4.5 ± 0.2</td>
<td>14.3 ± 0.8</td>
<td>12.9 ± 0.4</td>
</tr>
<tr>
<td>C57BL/6 KO</td>
<td>IA1+ IE1- MHC class I+</td>
<td>7.9 ± 1.4</td>
<td>7.2 ± 0.5</td>
<td>3.4 ± 0.2</td>
<td>11.8 ± 0.5</td>
<td>16.3 ± 1.7</td>
</tr>
<tr>
<td>C57BL/6 IIKO</td>
<td>IA1+ IE1- MHC class I+</td>
<td>16.7 ± 0.4</td>
<td>15.9 ± 0.5</td>
<td>16.8 ± 1.8</td>
<td>16.3 ± 1.7</td>
<td></td>
</tr>
<tr>
<td>CBA/Ca</td>
<td>IA1+ IE1- MHC class I+</td>
<td>10.9 ± 0.6</td>
<td>1.5 ± 0.2</td>
<td>0.2 ± 0.1</td>
<td>0.5 ± 0.3</td>
<td>0.5 ± 0.1</td>
</tr>
</tbody>
</table>

### Table III. V\(\beta\)14 frequencies within thymocyte subsets

<table>
<thead>
<tr>
<th>Mouse Strain</th>
<th>MHC</th>
<th>CD41 low CD8 low TCR high CD69+</th>
<th>CD41 high CD8 low TCR high CD69+</th>
<th>CD41 high CD8 high TCR high CD69+</th>
<th>CD41 low CD8 high TCR high CD69+</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 control</td>
<td>IA1+ IE1- MHC class I+</td>
<td>5.3 ± 0.4</td>
<td>5.2 ± 0.4</td>
<td>8.0 ± 0.3</td>
<td>3.0 ± 0.6</td>
</tr>
<tr>
<td>C57BL/6 KO</td>
<td>IA1+ IE1- MHC class I+</td>
<td>6.6 ± 0.8</td>
<td>7.6 ± 0.7</td>
<td>8.2 ± 0.3</td>
<td>3.5 ± 0.3</td>
</tr>
<tr>
<td>C57BL/6 IIKO</td>
<td>IA1+ IE1- MHC class I+</td>
<td>3.8 ± 0.9</td>
<td>3.5 ± 0.4</td>
<td>3.5 ± 0.3</td>
<td>3.6 ± 0.1</td>
</tr>
<tr>
<td>CBA/Ca</td>
<td>IA1+ IE1- MHC class I+</td>
<td>5.0 ± 0.5</td>
<td>6.4 ± 0.4</td>
<td>6.8 ± 0.6</td>
<td>7.5 ± 0.6</td>
</tr>
</tbody>
</table>
Beutner et al. (27). Differences in the strength of the deletional signal could explain such a discrepancy. In the absence of I-E molecule expression, B cell presentation or production of Mtv-9-encoded superantigen would be required to allow thymic deletion of the matched reactive cells whereas, in its presence, lower levels of superantigen expression by other cells such as dentritic cells may suffice.

The role of intracellular enzymes could also be assessed using the same protocol. Repertoire changes in opposite directions were observed in motheaten mice (me v mice in which only a partially functional form of SHP-1 is produced) vs p59fyn-deficient mice. V\(\beta\)5 frequencies within mature thymocyte subsets were found higher in p59fyn-deficient mice and lower in me v mice than in control C57BL/6 mice (Fig. 2). Thus, thymic deletion seemed to be augmented in the absence of SHP-1 and sharply diminished in p59fyn-deficient mice. No corresponding changes in V\(\beta\)14 cell frequencies were noted in these same mouse strains.

We also investigated the V\(\beta\) repertoire of peripheral CD4SP and CD8SP cells (Fig. 3). In most mouse strains examined, the changes seen in thymic SP cells were not observed among these mature CD4SP cells. In p59fyn-deficient mice only, a significantly higher V\(\beta\)5 frequency than in control mice was seen in the CD4SP peripheral lymphocytes. Even in this case, the difference was smaller than that observed among CD4SP thymocytes. In control mice, the V\(\beta\)5 frequency also diminished with maturation from CD69\(^+\) to CD69\(^-\)CD4SP thymocytes. In p59fyn-deficient mice, since CD4SP CD69\(^+\)thymocytes still contain a high frequency of V\(\beta\)5-bearing cells, V\(\beta\)5 cell deletion completion continues in the periphery (Fig. 4B). Thus, in the absence of this kinase, deletion of autoreactive clones is delayed rather than fully abrogated. Therefore, deletional mechanisms are redundant and still operate after thymic migration to the periphery. In me v mice, V\(\beta\)5-bearing cell deletion is already completed by the time the cells reach the CD4SP CD69\(^+\) cell stage of thymic differentiation (Fig. 4B).

The peripheral CD8SP cell repertoire does not differ from the repertoire observed for CD8SP thymocytes (Figs. 2 and 3). In fact, in the absence of p59fyn, CD40, CD40L, Fas molecule expression, or of B cells, deletion is so delayed that the CD8SP cell precursors do not express then enough CD4 density to be sensitive to the deletion. This result confirms a CD4 coreceptor requirement in V\(\beta\)5 thymocyte negative selection in I-E\(^+\) mice.

Most of the studied mouse strains have a C57BL/6 genetic background, and therefore can be directly compared with control C57BL6 mice. Nevertheless, CD40-, CD40L- and p59fyn-deficient mice have not been fully backcrossed with C57BL/6 mice and are still classified as B6.129 mice. One could argue that the repertoire differences observed in these mice could be due to the 129 background rather than to alterations in thymic negative selection processes due to the loss of the product of the targeted locus. In Table IV, V\(\beta\)5 and -14 frequencies in peripheral CD4SP and CD8SP cells are shown in 129 mice. The frequency of V\(\beta\)5-bearing cells is not increased as observed in CD40-, CD40L-, and p59fyn-deficient mice but, instead, diminished compared with normal C57BL/6 mice and are still classified as B6.129 mice. One could argue that the repertoire differences observed in these mice could be due to the 129 background rather than to alterations in thymic negative selection processes due to the loss of the product of the targeted locus. In Table IV, V\(\beta\)5 and -14 frequencies in peripheral CD4SP and CD8SP cells are shown in 129 mice. The frequency of V\(\beta\)5-bearing cells is not increased as observed in CD40-, CD40L-, and p59fyn-deficient mice but, instead, diminished compared with normal C57BL/6. Therefore differences in genetic background cannot explain the results obtained in CD40-, CD40L-, and p59fyn-deficient mice. Moreover, V\(\beta\)5 frequencies in 129 p59fyn-deficient mice were analyzed, and an ineffective deletion of V\(\beta\)5-bearing cells among mature thymocyte subsets (data not shown) and peripheral CD8SP cells (Table IV) could still be clearly observed.
Discussion

In the present paper, we show that deletion of superantigen-reactive thymocytes cannot be observed within the first subset to be generated upon TCR-mediated activation of immature DP cells, i.e., CD4\textsuperscript{low}CD8\textsuperscript{int} TCR\textsuperscript{int} CD69\textsuperscript{1} thymocytes. In most cases, deletion is abrupt and almost completed during CD4\textsuperscript{low} CD8\textsuperscript{low} TCR\textsuperscript{int} CD69\textsuperscript{1}/CD4\textsuperscript{1} CD8\textsuperscript{low} TCR\textsuperscript{int}/high CD69\textsuperscript{+} cell transition. These results suggest either dramatic changes in the thymic environment with encounter of APCs specialized in negative selection (a change possibly involving thymocyte migration from the thymic cortex to the thymic medulla) or changes in the developmental state of thymocytes induced by positive selection (from resistance to sensitivity to TCR-mediated apoptosis).

It has been proposed that the expression of Mtv-6-encoded superantigen is restricted to dendritic cells (71, 72) and that of Mtv-7 and Mtv-9 to B cells (73, 74). Because both cell types are found predominantly in the thymic medulla, one could argue that our results reflect only the absence of superantigen presentation in the thymic cortex. Several pieces of data argue against such a simple model. First, Beutner et al. have shown that B cells are not essential for presentation of endogenous superantigens in the thymus, because deletion of V\textsubscript{b}5- and V\textsubscript{b}6-bearing thymocytes still occurs in \muMT-deficient mice expressing MHC class II I-E molecules (27). These results suggest that Mtv-7- and Mtv-9-encoded superantigens could be expressed by other cell types than B cells in the thymus (and thus occur in Mtv-6 mediates negative selection). Moreover, it is possible that such cells would be the only thymic APCs to present endogenous superantigens and would explain our results. Such a model is in agreement with recent data showing the importance of the medulla environment in peptide as well as in superantigen-mediated negative selection (6, 21, 75, 76). Furthermore, Foy et al. have shown an important role for CD40L/CD40 interaction in thymic negative selection (53), and CD40 has been found to be primarily expressed by medullary stromal cells (77). Nevertheless, a whole range of cells including cortical epithelial cells has been shown to be capable of inducing negative selection (78–80). Moreover, in TCR transgenic mice, deletion of autoreactive thymocytes often occurs early in T cell development. Indeed, for a given TCR, certain H-2 haplotypes or the injection of high quantities of agonist peptide induces deletion of most immature DP thymocytes (4, 5, 7, and 8). In such cases, negative selection takes place in the thymus cortex (6) and has been shown to be independent of CD40L/CD40 interaction (53). All these data together suggest that, when the Ag is highly expressed and/or when the TCR level is artificially up-regulated at the immature thymocyte cell surface (i.e., when the avidity of the interaction reaches a certain threshold), negative selection does not require dedicated APCs and can take place in the cortex whereas, at physiological peptide concentrations and TCR densities, medullary stromal cells are the most competent APCs for induction of deletion. On the other hand, changes in the sensitivity of thymocytes to TCR-mediated cell death that can influence the timing of negative selection cannot be excluded.
We also show that, depending on the quality of the interaction, thymocytes can be deleted at different time points during the DP/SP cell transition. Indeed, in the absence of MHC class II I-E molecule expression, Vβ5-bearing thymocyte deletion is not only incomplete but delayed. Therefore, as already suggested by others (16–18), a single Ag could induce different degrees of thymocyte elimination at distinct maturational stages as a function of the level of its expression and thus of the strength of the signal delivered.

Using Vβ5 transgenic mice, Fink et al. have shown that the relative expression of Vβ5 in T cell subsets can be influenced by events in the periphery in the absence of MHC class II I-E molecule expression (81). In the present paper, we did not find any differences in VB5 frequencies between the most mature SP thymocytes (CD69−) and peripheral T cells; deletion was thus completed before export to the periphery. Nevertheless, in all mutant mice in which a defect in the intrathymic elimination of Vβ5+ thymocytes was observed, deletion of Vβ5+ CD4SP cells occurred at the peripheral level, suggesting that recent thymic migrants are still sensitive to self Ag-induced deletion.

Table IV. Involvement of p59fyn in Vβ5 deletion

<table>
<thead>
<tr>
<th>Vβ</th>
<th>Mouse Strain</th>
<th>CD4+8− TCRαβ+</th>
<th>CD4+8+ TCRαβ+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vβ5</td>
<td>S129/Sv</td>
<td>2.1 ± 0.1</td>
<td>11.1 ± 0.5</td>
</tr>
<tr>
<td>Vβ5</td>
<td>S129/Sv-p59fyn−/−</td>
<td>2.3 ± 0.1</td>
<td>14.7 ± 0.3</td>
</tr>
<tr>
<td>Vβ14</td>
<td>S129/Sv</td>
<td>9.0 ± 0.2</td>
<td>2.3 ± 0.1</td>
</tr>
<tr>
<td>Vβ14</td>
<td>S129/Sv-p59fyn−/−</td>
<td>9.4 ± 0.2</td>
<td>2.4 ± 0.1</td>
</tr>
</tbody>
</table>
In the absence of MHC class II I-E molecule expression, Vβ5-bearing thymocyte deletion could be considered as “weak”. A weak deletion model is more convenient and sensitive for the analysis of the role of candidate proteins in negative selection. Indeed, using such a system, we clearly show that p59fyn is an important contributor to transduction of the death signal induced by negative selecting ligands, whereas the same molecule appears irrelevant for strong superantigen-mediated negative selection systems (deletion of Vβ6-bearing thymocytes by Mtv-7-encoded superantigen in I-E b mice or injections of bacterial superantigens) (34, 35, 82). p59fyn expression could be implicated in thymic negative selection only when the strength of the deletional signal falls below a certain threshold. In the case of strong deletional signals, other kinases such as p56lck appear fully able to do the job. On the other hand, a preferential involvement of p59fyn in late thymic deletions would agree with its expression among thymocyte subsets. p59fyn expression is nearly null in immature DP cells but increases with maturation (83). It has been reported that p56lck and p59fyn expressions vary in opposite directions during DP/SP cell transition (84, 85). Therefore, one could think that p56lck plays an essential role in early (“strong”) deletions whereas p59fyn would be important for late (“weak”) negative selections. This hypothesis is in agreement with the data reporting a role of p56lck in Vβ6 thymocyte deletion in I-E b Mtv-7+ mice (86, 87). In these reports, the authors have studied the repertoire of mature thymocytes and peripheral cells and concluded that there is a lineage-specific control of superantigen-induced cell death by p56lck because only deletion among CD8SP cells was disturbed. In the present paper, we have also found that, at the peripheral level, in the absence of p59fyn or some other studied proteins, a similar discrepancy can be observed between CD4 and CD8SP cells. A CD4 requirement in Vβ5+ cell deletion easily explained this discrepancy since CD8SP cells are no longer sensitive to negative selection whereas CD4+ cell deletion is only delayed (being eventually completed via redundant pathways). One could imagine that in the absence of p56lck expression, by contrast to normal mice (88), CD4 is required for Vβ6+ thymocyte deletion by Mtv-7-encoded superantigen in I-E b mice, and this would explain the published data (86, 87).

Recent data show that SHP-1 functions as a negative regulator of the TCR and in setting the threshold of activation (89). Using our system, we found that thymic deletion was augmented in the absence of SHP-1 expression. This result suggests that SHP-1 is also implied in setting the threshold of thymic negative selection. In the present paper, we show that endogenous superantigen-mediated negative selection occurs after initiation of positive selection since no deletion could be observed within the first subset to be generated upon TCR-mediated activation of immature double-positive cells. All thymic subsets from this stage up to and including recent thymic migrants are still sensitive to such a mechanism. Vβ5-bearing cell deletion in I-E b mice is a convenient, sensitive, and powerful model for analysis of the role of candidate proteins in negative selection. Indeed, using such a system, we show a role of CD40/CD40L interaction, ICAM-1, and Fas cell surface expression in the efficiency and timing of thymic deletion. Finally, we demonstrate that the threshold of negative selection is decreased in the absence of SHP-1 expression but augmented in the absence of p59fyn expression.

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71. Theofilopoulos, A. N., and V. A. Herzenberg. 1990. Regulation of CD4+ T cell receptor signaling by T cell receptor-induced apoptosis requires a costimulatory signal that can be provided by CD8+ T cells. J. Exp. Med. 176:709.


