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Receptors and Signals in Early Thymic Selection

Review

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During differentiation, thymocytes are selected twice for expressing a functional version of the CD3–T cell receptor (TCR). A first round of selection involves the immature $CD4^-CD8^-$ double-negative (DN) cells that express interleukin-2 receptor-α (IL-2Rα). At this stage, thymocytes rearrange their TCRβ genes in a random fashion (Godfrey et al., 1993), leading to a successful rearrangement in approximately 5 of 9 cells (i.e., 56%). Thymocytes that productively rearranged the TCRβ locus are selected by virtue of expressing an immature form of the CD3–TCR, termed pre-TCR, to proliferate, down-regulate IL-2Rα and mature to the $CD4^+CD8^+$ double-positive (DP) stage (Groettrup and von Boehmer, 1993; Levelt and Eichmann, 1993). At the same time, TCRβ locus rearrangement is arrested to achieve allelic exclusion. A second round of selection takes place during the DP stage. At this stage, the TCRα genes are rearranged (Petrie et al., 1993) and thymocytes are subsequently subjected to repertoire selection based on the specificity of the mature $\alpha^+\beta^+$CD3 TCR (Kisielow et al., 1988). DP thymocytes that are positively selected develop into mature $CD4^+$ or $CD8^+$ single-positive (SP) thymocytes (Chan et al., 1993; Davis et al., 1993). This review deals with the first round of selection in T cell development: selection for functional rearrangement and expression of the TCRβ chain.

The important role of the TCRβ chain in thymic development has been brought out first by studies on mice carrying a functionally rearranged TCRβ transgene (von Boehmer, 1990). In normal mice, the TCRβ transgene arrested the rearrangement of the endogenous TCRβ gene loci, reflecting the process of allelic exclusion (Uematsu et al., 1988). In Scid mice (Schuler et al., 1986), in which thymocyte development is arrested at the DN $IL\text{-}2R\alpha^+$ stage (Habu et al., 1987; Shores et al., 1990), a TCRβ transgene restored maturation of thymocytes to the DP stage (von Boehmer, 1990). The pivotal role of the TCRβ chain in early T cell development was further corroborated by the phenotypes of several mutant mouse strains: In mice that cannot produce a TCRβ chain because of a deficiency in the rearrangement machinery (*RAG1*- or *RAG2*-deficient mice) (Mombaerts et al., 1992a; Shinkai et al., 1992a) or because of a mutation in the TCRβ chain itself (Mombaerts et al., 1992b), thymocyte development is blocked at the DN stage. Introduction of a TCRβ transgene in any of these mice restores T cell maturation to the DP stage (Mombaerts et al., 1992b; Shinkai et al., 1992b). In contrast with TCRβ-deficient mice, the thymus of TCRα-deficient mice contains normal numbers of DP thymocytes (Philpott et al., 1992; Mombaerts et al., 1992b), excluding a role for the TCRα chain in early thymic differentiation.

The strong impact of the TCRβ chain on thymocyte development was in good agreement with other reports suggesting that thymocytes are selected on the basis of successful rearrangement of the TCRβ locus. It was shown that more than 75% of all variable–diversity–joined segment (VDJ) rearrangements of the TCRβ locus found in the thymus of TCRα-deficient animals were in frame. This was far higher than the 33% predicted if no selection for functional rearrangement took place, and close to the 71.4% expected if there was selection (Mallick et al., 1993). By flow cytometric analyses of thymocytes for cytoplasmic TCRβ it was demonstrated that expression of the TCRβ chain starts during the $IL\text{-}2R\alpha^+$ stage. Cytoplasmic TCRβ chain was found in virtually all thymocytes on their way to the DP stage and in over 95% of DP thymocytes. Thus, nearly all DP cells have a functionally rearranged TCRβ locus, far more than the 56% expected if there was no selection (Levelt et al., 1993b).

How do thymocytes notice that they have produced a functionally rearranged TCRβ locus? As soon as a TCRβ polypeptide has been synthesized, the TCRβ chain is expressed at the cell surface as the core component of the pre-TCR. Initially, a number of different compositions of the pre-TCR have been reported (Punt et al., 1992; Groettrup et al., 1992, 1993; Shinkai et al., 1992b). The currently accepted version contains the TCRβ chain, disulfide linked to a 33 kDa transmembrane glycoprotein termed pre-TCRα, as well as the γ, δ, and ε components of CD3, perhaps loosely associated with CD3ζ (Figure 1) (Groettrup et al., 1993; Saint-Ruf et al., 1994). The pre-TCRα chain has little homology to the mature TCRα chain. The extracellular domain accommodates only one immunoglobulin-like domain, as opposed to two such domains in the mature TCRα chain. It is speculated that, in parallel to the situation in pre-B cells, a second immunoglobulin-like domain is provided by a putative Vpre-T component (Figure 1). This is supported by the finding that transfection of a $TCR\alpha^-\beta^+$ T cell line with the pre-TCRα gene does not lead to surface expression of the pre-TCR (Saint-Ruf et al., 1994). In addition, the pre-TCRα chain contains an extended cytoplasmic tail, which could have a role in signal transduction through the pre-TCR. However, the absence of significant homology between the cytoplasmic regions of mice and humans has been taken as an argument against an essential signaling function of pre-TCRα (H. von Boehmer, communicated at the Ninth International Congress of Immunology, San Francisco, 1995).

A role for pre-TCRα in signal transduction is further unlikely because signaling through the CD3 complex alone is sufficient for induction of all known pre-TCR dependent differentiation events (Levelt at al., 1993a, 1993c, 1995a, 1995b). Evidence for this came from observing the responses of DN thymocytes to anti-CD3 antibodies. Addition of anti-CD3ε monoclonal antibody (MAb) to fetal thy-

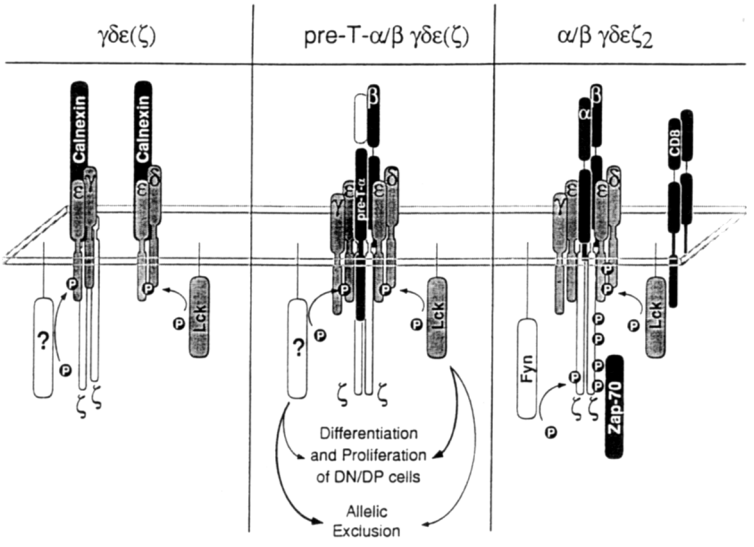

Figure 1. Schematic Representation of the Different (TCR) CD3 Complexes that Are Expressed on the Cell Surface during Thymocyte Development, and their Associated PTKs

The earliest form (left), expressed on DN thymocytes before rearrangement of the TCRβ locus, consists of dimers of CD3εγ, and CD3εδ, complexed with calnexin. CD3ζ has been included on the basis of functional but not of biochemical evidence and may be only weakly associated. The pre-TCR (center) is expressed on late DN cells and on DP tymocytes before rearrangement of the TCRα locus. It consists of a TCRβ chain, pre-TCRα (gp33), and the CD3 complex with possibly weakly associated CD3ζ. The structure drawn next to the TCRβ V region is a hypothetical Vpre-T, a homolog of Vpre-B in B cell development. The mature αβTCR–CD3 complex (right) is expressed on late DP and SP thymocytes. Pre-TCRα has been replaced by a mature TCRα chain. ZAP-70 is important in repertoire selection of DP cells by the mature αβTCR. Fyn has a role in signaling of SP thymocytes. Lck is essential for efficient signaling through all forms of the TCR, with the exception of allelic exclusion, which is almost intact in its absence. Additional PTKs, indicated by question marks, may provide a salvage pathway for allelic exclusion.

mic organ cultures (FTOC) of normal mice, at a timepoint before rearrangement of the TCRβ locus is completed, induced the development of DP thymocytes devoid of TCRβ chain mRNA and protein (Levelt et al., 1993a), owing to blocked V–DJ rearrangement of the TCRβ locus (Levelt et al., 1995a). Moreover, DN thymocytes of *RAG1*-deficient or of TCRβ-deficient mice could be restored to differentiate to the DP stage by treating FTOC from such mice with anti-CD3ε (Levelt et al., 1993c). This suggested that CD3ε is expressed on the surface of DN thymocytes, before and independent of TCRβ. The presence of CD3 complexes without clonotypic TCR chains was demonstrated directly by surface iodination or biotinylation on *RAG*-deficient thymocytes (Jacobs et al., 1994; Shinkai and Alt, 1994), on TCRβ-depleted normal thymocytes (Wiest et al., 1994), and on thymoma cell lines (Mombaerts et al., 1995; Ley et al., 1989). Surface deposition of such CD3 complexes appears to be facilitated by association with calnexin (Figure 1) (Wiest et al., 1995). Their presence at the cell surface may indicate an alternative fashion of assembly and transportation of TCR–CD3 complexes during early thymocyte development. It is unlikely that such early CD3 complexes, even though they are signal-competent, have a signaling function before the appearance of TCRβ, as mice deficient of CD3γ, CD3δ, and CD3ε rearrange their TCRβ genes and proceed in thymocyte development to the IL-2Rα$^+$ stage (Malissen et al., 1995).

The transition of TCRβ to TCRα locus rearrangement is coordinated with the differentiation from the DN to the DP stage. Transcription of the TCRα locus initiates α rearrangement and is first detectable in thymocytes on their way to becoming DP cells (Capone et al., 1993). During the same stage of development, expression of *RAG1* and *RAG2* mRNA is transiently down-regulated (Wilson et al., 1994). In thymocytes of *RAG1*- or *RAG2*-deficient animals, which neither down-regulate the nondeleted *RAG* mRNA nor show significant germline transcription of TCRα, transient shutdown of *RAG1* and *RAG2* mRNAs and the transcription of TCRα could be induced by injection of anti-CD3ε MAb, suggesting that transition from TCRβ to TCRα rearrangement is regulated by the pre-TCR (Levelt et al., 1995a). Support for this notion also comes from mice that are defective in signaling through the pre-TCR (Levin et al., 1993). In such mice, TCRβ rearrangement is not arrested and TCRα rearrangement is not initiated. The reason for the transient down-regulation of *RAG1* and *RAG2* remains unclear. Possibly, it is required to prevent premature rearrangement of the TCRα locus during the proliferative phase. Alternatively, down-regulation of *RAG* may be a necessary step in the allelic exclusion of the TCRβ locus, in parallel with shutdown of *RAG* transcription after positive (repertoire) selection (Turka et al., 1991; Borgulya et al., 1992). Because the *RAG* genes are reexpressed during the DP stage to enable TCRα rearrangement, the permanent arrest of TCRβ rearrangements must be accounted for by additional mechanisms.

The members of the CD3 complex that are likely to be involved in signaling through the pre-TCR are CD3ε and CD3ζ. CD3ζ is expressed as of day 14 in fetal murine thymocytes (Levelt et al., 1993b), but so far no positive biochemical evidence exists that CD3ζ is indeed a component of the pre-TCR (Punt et al., 1992; Groettrup et al., 1992; Wiest et al., 1994). The participation of CD3ζ in immature CD3 complexes as shown in Figure 1 is thus primarily based on the phenotype of mice deficient of this molecule. Such mice have a small thymus, with a 10- to 20-fold reduction in DP thymocytes (Love et al., 1993; Liu et al., 1993; Malissen et al., 1993; Ohno et al., 1993). However, mice that are double-deficient for CD3ζ and *RAG2* can be fully induced to mature to the DP stage by anti-CD3ε MAb, suggesting that CD3ζ does not mediate a specific signaling function in early selection (Levelt et al., 1995b). Furthermore, anti-CD3ε completely blocks rearrangement of the TCRβ loci in CD3ζ-deficient mice

(Wang et al., 1995). One possible function of CD3ζ may therefore be an enhancement of CD3 surface expression, as shown for mature T cells (Weissman et al., 1989). Alternatively, CD3ζ may amplify the signal through the CD3γδε module by additive redundant signaling (Weiss and Littman, 1994). Evidence for either possibility was obtained using various recombinant CD3ζ and CD3ε transgenes: Whereas a complete CD3ζ chain fully restored development of DP thymocytes in CD3ζ-deficient mice, CD3ζ chains lacking the sequences responsible for signal transduction could only partially reconstitute (Shores et al., 1994). Experiments on *RAG2*-deficient mice carrying transgenes encoding the cytoplasmic tail of CD3ε or CD3ζ coupled to the transmembrane and extracellular domains of IL-2Rα suggested that signaling through either CD3ζ or CD3ε, even without CD3γ or CD3δ, is sufficient for early thymocyte maturation (Shinkai et al., 1995). The complete block in early T cell development in CD3γ-, CD3δ-, CD3ε-deficient animals is therefore presumably due to the lack of surface pre-TCR expression, rather than to a specific signaling defect. The redundancy of CD3δ during this maturation step is evident from the finding that DP thymocytes develop normally in mice deficient of this molecule (Kappes and Tonegawa, 1994). CD3γ may not be essential for pre-TCR signaling either, as humans lacking this molecule have peripheral $CD4^+$ and $CD8^+$ T lymphocytes (Timon et al., 1993).

The protein tyrosine kinase (PTK) $p56^{lck}$ (Lck) is so far the only PTK clearly implicated in signaling through the pre-TCR. Mice transgenic for a catalytically active form of *lck* produce DP thymocytes that have blocked rearrangement on both TCRβ loci (Anderson et al., 1992), similar to anti-CD3ε MAb-treated normal thymocytes (Levelt et al., 1993a). The same transgene induces development of DP thymocytes in *RAG1*-deficient animals (Mombaerts et al., 1994). In mice transgenic for a dominant-negative form of *lck*, maturation of DP thymocytes is completely abrogated (Levin et al., 1993). Introduction of a TCRβ transgene in these mice does not block rearrangement of the endogenous TCRβ gene loci (Anderson et al., 1993b). These experiments clearly support an important role for Lck in signaling through the pre-TCR.

Lck may, however, not be equally important for all components of early thymic selection. In mice deficient for Lck the number of DP thymocytes is significantly reduced (Molina et al., 1992). DN thymocytes of mice double-deficient for both *lck* and *RAG1* are poorly induced to mature to the DP stage by anti-CD3 MAb treatment, and down-regulation of IL-2Rα and proliferation of DN and DP thymocytes are significantly compromised (Levelt et al., 1995b). These results are consistent with a pivotal role of Lck in differentiation and proliferation of DN and DP thymocytes. In contrast, allelic exclusion of the TCRβ locus is not detectably compromised in *lck*-deficient mice and introduction of a rearranged TCRβ transgene reduces endogenous TCRβ gene rearrangements by about 90% (Wallace et al., 1995). Thus, it seems that TCRβ locus allelic exclusion is relatively independent of Lck. Alternatively, only a few *lck*-deficient thymocytes succeed in allelic exclusion of the TCRβ locus but, owing to enhanced proliferative capacity, outgrow those that fail in allelic exclusion. The latter possibility is unattractive, however, because an enhanced proliferative capacity of allelically excluded cells would obliterate the need for an efficient induction of allelic exclusion also in normal mice.

It has been speculated that, owing to the design of the knock-out construct used (Molina et al., 1992), the *lck*-deficient mice may express low amounts of a truncated but constitutively active form of Lck. Molina et al. (1992) were unable to detect either Lck protein or activity, but the assays used failed to reveal signals below 3%–5% of the wild-type expression level. A residual Lck activity in these mice is therefore not excluded. Alternatively, the results discussed above point to parallel signal transduction pathways in pre-TCR signaling, involving more than one PTK (Figure 1). Whereas Lck may signal primarily for proliferation and differentiation, allelic exclusion may depend predominantly on another PTK, with a limited capacity of either PTK to carry out the function of the other. This would also account for the differences between Lck-deficient mice and mice overexpressing a dominant-negative form of Lck. Possibly, overexpression of a dominant-negative form of Lck could compete not only with Lck, but also with alternative PTKs with specificities similar to Lck. Moreover, the putative alternative kinase(s) may be overexpressed in Lck-deficient mice. Candidates for such alternative PTKs expressed in immature thymocytes have already been described (Heyek and Berg., 1993; Hu et al., 1995; Sommers et al., 1995). $p59^{fyn}$ is an unlikely candidate, as there is no evidence for a role in pre-TCR signaling (Appleby et al., 1992; Stein et al., 1992). ZAP-70 seems dispensable, too, as in humans and mice lacking this molecule production of DP thymocytes seems normal (Arpaia et al., 1994; Negishi et al., 1995).

The signal through the pre-TCR is not the only factor influencing the generation of DP thymocytes. In normal mice we observed that a small number of thymocytes develop to the DP stage without detectable cytoplasmic TCRβ (Levelt et al., 1993b). Whereas development of DP cells is completely blocked in *RAG*-deficient or TCRβ × TCRδ-deficient animals, mice deficient for either TCRβ or pre-TCRα have a considerable number of DP thymocytes (Mombaerts et al., 1992b; Fehling et al., 1995). The presence of TCR^+ thymocytes may thus have a positive feedback effect on the generation of DP thymocytes. Evidence for this also comes from the finding that Scid mice produce DP thymocytes of Scid origin upon transplantation of bone marrow from normal mice (Shores et al., 1990). A possible common denominator for these findings may be that the enhanced proliferation of DN thymocytes in response to the signal received through the pre-TCR is the critical factor leading to further differentiation. This would be in line with the finding that thymoma cell lines from mice double deficient for *RAG1* and p53 express CD4 and CD8 (Mombaerts et al., 1995). Thus, in normal mice, spontaneous (low level) proliferation may take place without pre-TCR signaling and may lead to development of some DP cells. In the atrophied thymic microenvironment of Scid or *RAG*-deficient mice, spontaneous proliferation may be minimal. The presence of TCR^+ thymocytes has been shown to

improve the thymic microenvironment (Shores et al., 1991; van Ewijk et al., 1994), possibly supporting the development of some DP TCRβ⁻ thymocytes. This would also explain the finding that DP thymocytes appear after sublethal irradiation of *RAG*-deficient mice (Zuñiga-Pflücker et al., 1994; Guidos et al., 1995): Compensatory proliferation of DN thymocytes after radiation-induced cell death of the DN pool may cause their development to the DP stage.

Studies by Anderson et al. (1993a) show that both fibroblasts and MHC class II–positive cortical epithelial cells are necessary for production of DP thymocytes. Addition of supernatants of either cell type cannot compensate its physical presence. The need of two cell types for the production of DP thymocytes indicates that the thymus must provide other factors necessary for early differentiation, such as costimulatory signals involving direct cell to cell contact, or short-range soluble factors. Possibly, one of these cell types provides a ligand for the pre-TCR. As fibroblasts are not expected to express thymus-specific molecules, a possible ligand for the pre-TCR may be expressed on cortical epithelium.

Differentiation of B and T lymphocytes follow surprisingly similar pathways. Upon functional rearrangement of the immunoglobulin heavy (IgH) chain, a pre-B cell receptor is expressed consisting of the IgH chain together with a Vpre-B/λ5 surrogate light chain (Melchers et al., 1993). This complex regulates transition of IgH chain to light (L) chain gene rearrangement and proliferation and maturation of pre-B cells. Elucidation and comparison of signaling pathways and transcriptional control in such related systems may help us understand decision making in the development of the immune system.

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