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Precursor Thymocyte Proliferation and Differentiation Are Controlled by Signals Unrelated to the Pre-TCR

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In-frame rearrangement of the TCR-β locus and expression of the pre-TCR are compulsory for the production of CD4+8+ thymocytes from CD4-8+ precursors. Signals delivered via the pre-TCR are thought to induce the differentiation process as well as the extensive proliferation that accompanies this transition. However, it is equally possible that pre-TCR expression is required for the success of this transition, but does not play a direct role in the inductive process. In the present manuscript we examine this possibility using a variety of normal and genetically modified mouse models. Our evidence shows that differentiation and mitogenesis can both occur independently of pre-TCR expression. However, these processes are absolutely dependent on the presence of normal thymic architecture and cellular composition. These findings are consistent with a checkpoint role for the pre-TCR in regulating the divergence of survival and cell death fates at the CD4-8+ to CD4+8+ transition. Further, our data suggest that precursor thymocyte differentiation is induced by other, probably ubiquitous, mechanisms that require the presence of normal thymic cellularity, composition, and architecture. The Journal of Immunology, 2000, 165: 3094–3098.

During the last decade, much of the emphasis regarding intrathymic T cell differentiation has centered on the role of TCR proteins in this process. The widely held view of TCR (or pre-TCR) as an inductive stimulus in this process is based on two general categories of observations. The first includes studies showing that thymocyte differentiation terminates at precise stages in mice carrying mutations in TCR genes themselves (2, 3) or in genes required for their recombination (4, 5). The second includes experiments showing that TCR gene rearrangements in normal animals correlate with the developmental stages predicted by mutations. For instance, TCR-β-deficient thymocytes arrest at the CD4-8+ (double-negative (DN)) stage of development (2), and TCR-β genes rearrange during the DN stage (6–8). Likewise, TCR-α mutant thymocytes arrest at the CD4-8+ (double-positive (DP)) stage of development (3), and TCR-α gene recombination occurs in DP cells (7). Taken together, these types of studies implicate TCR gene recombination and expression in the induction of thymocyte differentiation.

In addition to a proposed role in differentiation, pre-TCR expression has recently been implicated in the induction of mitogenesis in cells at the DN to DP transition (8–10). In these studies it was shown that the blast component of cells undergoing TCR-β recombination is enriched in cells with in-frame rearrangements, and that this blast population is reduced in cells that cannot express TCR-β. These findings have been interpreted to show that pre-TCR expression induces mitogenesis at the DN to DP transition. However, it is important to note that these findings, as well as those correlating pre-TCR expression with differentiation, can be equally well explained by proposing that expression of this receptor is required for the survival of cells undergoing blast transition at the DP stage, but does not play a direct role in their differentiation or proliferation. In the experiments presented here we test this latter hypothesis. We found that control of mitogenesis and differentiation occur independently of TCR-β gene recombination and pre-TCR expression, suggesting that the pre-TCR serves to mediate a classical cell survival/cell death checkpoint during T cell development.

Materials and Methods

Mice

Wild-type C57BL/6 mice, p53-deficient mice (C57BL/6-Tpr53tm1Tyj), and TCR-β-deficient mice (C57BL/6-Tcrbtm1 Mom) were initially obtained from The Jackson Laboratory (Bar Harbor, ME); RAG-2-deficient mice (B6.129S-yn) were initially obtained from Taconic Farms (Germantown, NY). Mice used in these studies were bred under specific pathogen-free conditions at the Memorial Sloan-Kettering Cancer Center (New York, NY). Mice were generally used at 4–6 wk of age. For the establishment of stable bone marrow chimeras, mice were given a sublethal dose (6 Gy) of irradiation, followed by i.v. administration of a mixture of RAG-2-deficient and normal marrow (4:1 ratio). Chimeras were allowed to recover for 2–3 mo before analysis.

Flow cytometric analysis and cell sorting

Enrichment and cell sorting were performed as previously described (8). Purified thymocyte populations were DN3, CD4-8+24+25+44+ and pre-DP, CD4-8+24+25+44+. Lymph node B cells were identified as being CD24+. Analysis of DNA content was generally performed as previously described (8), except for analysis of proliferative status in bone marrow chimeras. In this case, chimeric thymuses were first depleted of CD4-8+ cells, followed by staining for CD90.2(Thy-1.2)-FITC, CD25-PE, and CD44-CyChrome. Stained cells were fixed in formaldehyde followed by permeabilization with detergent, and DNA content was analyzed using DAPI fluorescence on an LSR two-laser cytometer (Becton Dickinson, Mountain View, CA). Cell cycle distributions were confirmed using two standard algorithms (Watson and Dean-Jett-Fox), and the average values are presented.
Genomic DNA preparation and Southern blotting

Genomic DNA was prepared in low melting point agarose as previously described (7). Purified DNA was digested with ApaLI and SacI, followed by electrophoresis in 0.8% agarose and transfer to nylon membranes. Probe 5’-Dβ1 (460 bp) was synthesized by amplification of C57BL/6 kidney DNA using the following primers (5’-3’); forward, ggggtaccttcgacaggaagt; and reverse, ggggtaccttcgacaggaagt. The DNA loading probe is an intronic sequence located 3’ of the Cα locus as previously described (7). Quantitation of gene rearrangement was performed as previously described (7); in the present case, percent hybridization was calculated by the formula \((\frac{(X_{c}/X_{g})}{X_{p}}) \times 100\), where \(C\) represents the intensity of the DNA loading band, \(X\) represents 5’-Dβ1 intensity, and \(g\) or \(r\) represents germline or thymocyte samples, respectively.

Results

Proliferation in DN cells does not correlate with the extent of TCR-β gene rearrangement

TCR-β recombination initiates in DN3 cells (CD4- 8- 25+ 44low) (6, 7), and only cells that make in-frame TCR-β joints are found in the proliferating DP compartment (pre-DP, CD44low8-25+ 44low) (8, 11, 12). Cells with in-frame TCR-β joints are also enriched in the blast component of DN3 cells (9), leading to the widely held conclusion that TCR-β rearrangement is a prerequisite for proliferation and transition to the DP stage, then the amount of V-Dβ gene rearrangement must be similar in blast DN3 and early DP cells. Previous data from our laboratory suggested that this was not the case (8). However, analysis of the complete sequence of the TCR-β locus (GenBank accession no. AE000663-665) reveals that the excised products of most V-Dβ rearrangements (26 of a possible 28) could not be distinguished from chromosomal DNA using the previous strategy. Because the stability of these excised products (14) could have caused them to influence our measurements, a method was devised to discriminate between target sequences on chromosomal DNA and those on extrachromosomal products of V-Dβ1 recombination. This approach exploits the de novo formation of a novel ApaLI site after religation of recombination signal sequences (RSS) in excised recombination products (15) and is depicted in Fig. 1. A probe hybridizing upstream of Dβ1 (designated 5’Dβ1) recognizes a 4.7-kb fragment after ApaLI/SacI digestion of unarranged (germline) DNA. Partial (D-J) rearrangements between Dβ1 and any member of the Jβ1 cluster (Jβ 1.1-1.6) yields six progressively shorter fragments corresponding to deletion of the intervening DNA, but still flanked by germline ApaLI/SacI sites. Partial rearrangements between Dβ1 and the second Jβ cluster (Jβ 2.1-2.7) yield fragments larger than the germline ApaLI/SacI fragment. Most importantly, rearrangement of any Vβ to Dβ1 results in the production of a nongermline-encoded fragment, flanked by the germline-encoded ApaLI site upstream (ApaLI 151353; Fig. 1) and by a newly formed ApaLI site downstream (ApaLI de novo) of the probe site. Because this band is only present after V-Dβ1 recombination, the levels of unarranged vs rearranged TCR-β loci in DN3 thymocytes can be unambiguously assessed using this assay.

Analysis of recombination in small vs large DN3 as well as in pre-DP thymocytes is shown in Fig. 1. In control cells that do not rearrange TCR-β genes (lymph node B cells), only the 4.7-kb germline band is seen. In DN3 cells, all the predicted products of rearrangement are seen, including the novel 2.0-kb product of V-Dβ1 recombination and reciprocal RSS joining. Pooled analyses of this type show that complete (V-DJ) TCR-β rearrangements are present on 45% of all alleles (30% involving V-Dβ1 (Fig. ID) and an additional 15% involving V-Dβ2). This is ~4-fold higher than our previous estimate of complete rearrangements (8), reflecting the inability of the former approach to discriminate target sequences persisting on extrachromosomal circles, as described above. Nonetheless, large DN3 cells were found to differ substantially from early DP cells that have surpassed the β-selection point (11). Most informative is the clear presence of germline TCR-β genes in both small and large DN3 cells, which becomes nearly undetectable in post-β-selection pre-DP (Fig. 1, C and D). The lack of asymmetry between small and large DN3 and their disparity with cells after the DP transition clearly show that DN3 proliferation does not correlate with complete TCR-β rearrangements. This is consistent with previous findings showing that DN3 cells undergo self-renewing cell division (reviewed in Ref. 16). It should be noted that our findings do not preclude the DN3 blast population from including all cells that have in-frame TCR-β gene rearrangements, as previously shown (9), although our data indicate that these must represent a minority of the DN3 blasts, as discussed in more detail below. However, our findings rule out in-frame TCR-β rearrangement and pre-TCR expression as obligatory differences in both germline TCR-β alleles and excised productions of V-Dβ1 recombination.
Mitogenic activity in DN cells does not correlate with the frequency of pre-TCR expression

To further assess the role of TCR-β in the mitogenic process, we analyzed the proliferative status of thymocytes from mice lacking one or both TCR-β alleles (2). DN3 cells from normal mice (two intact TCR-β alleles) have a 56% probability of generating a productive (in-frame) TCR-β gene (reviewed in Ref. 17), while the probability of success in mice with a single allele (F1 [wild-type × homozygous mutant]) is reduced to 33%. If DN3 proliferation correlates with productive gene recombination (9), a decrease in the proportion of cells with productive rearrangements should lead to a corresponding decrease in the proliferative index. Thus, the DN3 population from mice with a single TCR-β allele should have a 40% reduction in blast cells (33/56%) over that in mice with two alleles. However, the data in Fig. 2 clearly show that proliferative indexes are indistinguishable in DN3 cells from these two types of mice (p > 0.5, by Student’s t test for independent samples). In contrast, homozygous mutant mice (0% probability of successful recombination) show substantially reduced levels of proliferation, consistent with previously published results (8–10). With respect to this latter difference, it is important to note that in addition to the absence of TCR proteins, a large number of other components are absent from homozygous mutant thymuses. For instance, also missing are defined cortical and medullary zones and most of their respective cellular and interstitial elements, including DP and SP cells, the interdependent nonlymphoid stroma (18), and all the factors produced by these cells (cytokines, extracellular matrix proteins, etc.). In virtually all developing tissues and organs, such spatial and contextual interactions are absolutely critical for the success of normal proliferation and differentiation. In the experiments described in the next sections we show that the presence of a normal thymic microenvironment is essential for the regulation of cell proliferation during T cell development, while the pre-TCR is dispensable for this process.

DN proliferation is dependent on the presence of a normal thymic microenvironment

It is intuitive that the presence of a normal thymus is indispensable for normal lymphopoiesis and homeostasis. Consequently, it is also predictable that gross disruption of thymic architecture and composition could result in numerous secondary effects on these processes. Decreased proliferation in TCR-deficient thymocytes is generally interpreted to mean that pre-TCR signals are required to induce proliferation. This conclusion is based on the assumption that the responses to pre-TCR deficiency are completely cell autonomous. However, given the severe disruptions in thymic composition that result from TCR deficiency, it is likely that cells derived from mutant thymuses may experience numerous secondary effects. To distinguish cell autonomous, pre-TCR-mediated influences from the secondary effects of thymic dysplasia, chimeric mice were constructed using RAG-2-deficient bone marrow transplanted into normal Thy-1 congenic recipients (B6.PL-Thy1+) (Fig. 3A). After a 6-wk development period that steady state (>6 wk), thymuses from such mice contained normal numbers and proportions of cells as well as defined cortical and medullary regions (data not shown). A substantial proportion of DN cells from such mice were RAG-deficient cells (Thy-1.2+; Fig. 3A) that had developed in an otherwise normal environment. The presence of DN3 blasts was analyzed in the RAG-deficient thymocytes from such chimeras vs those derived from dysplastic RAG-deficient hosts (Fig. 3B). As previously reported (8–10), DN3 thymocytes from RAG-deficient hosts showed a reduction in the number of blast cells, as well as an overall decrease in cell size, compared with wild-type controls (Fig. 3B, artificial mixture). However, RAG-deficient DN3 cells developing in a normal (chimeric) thymic environment showed essentially no differences in either overall size or blast cell proportion controls (Fig. 3B, chimeric mixture). The proliferative status of RAG-deficient blast cells is further characterized by the data presented in Fig. 3C, which show that cell cycle distribution (DNA content) is restored to nearly normal levels when such cells develop in a normal thymus. Together with the data in Figs. 1 and 2, these findings show that reduced proliferation in TCR-deficient DN3 cells does not directly reflect lack of signaling through the pre-TCR, but, rather, is a secondary effect of disrupted organ development. These findings further imply that the signals that regulate proliferation of DN thymocytes derive from the cellular constituents that comprise a normal thymus, from their organization, or (most likely) from both.

Mitogenic activity is regulated normally in DP cells in the absence of pre-TCR signaling

As outlined in the introduction, pre-TCR-induced signaling late in DN development is thought to induce both differentiation into the DP stage and sustained proliferative expansion of the early DP pool. The data presented in Figs. 1–3 show that DN proliferation does not require pre-TCR expression, but, rather, is induced by non-TCR-mediated influences originating from a normal thymic microenvironment. To determine whether pre-TCR is required for the induction of proliferation in the early DP pool, we evaluated proliferative indexes in thymocytes from RAG-2/p53 double-mutant mice. It has been shown that thymocytes from such mice are able to progress to the DP stage despite the absence of pre-TCR expression, although DP cells do not accumulate in normal numbers (19), suggesting that p53-inducible genes may be involved in the
elimination of cells with sterile TCR-β gene rearrangements. Thus, in addition to showing that the absence of cell death is sufficient to allow DN cells to differentiate into DP, this model provides an opportunity to evaluate mitogenesis at the DN/DP transition in the absence of TCR-mediated signals. The results of such an analysis are shown in Fig. 4. As was the case for normal thymocytes (8), transition from the DN to the DP stage is associated with a 3-fold increase in the mitogenic index in RAG-2/p53-deficient thymocytes. This up-regulation of mitogenic activity was not associated with cellular transformation due to p53 deficiency, because mature DP cells (CD4high 8 high) withdraw from the cell cycle normally in these mice (Fig. 4B). This experiment shows that, similar to our findings with DN cells (Figs. 1–3), pre-TCR expression is not required for proliferative expansion in early DP cells (CD4high 8high). Together, the data presented in Figs. 1–4 show that control of mitogenic activity in DN cells, in DP cells, and at the DN/DP transition occurs independently of TCR-β gene recombination and pre-TCR expression and requires as yet undefined signals from the thymic microenvironment. The inability of RAG-2/p53-deficient DP thymocytes to accumulate in substantial numbers (Fig. 4A) further suggests that although the absence of p53 activity may be sufficient to permit the transient survival of cells at the DN/DP transition, pre-TCR expression is required for prolonged survival at the DP stage of development, consistent with the findings of others (20).

**Discussion**

In the experiments presented in this manuscript we examine whether pre-TCR signaling induces mitogenesis at the DN to DP transition. Much of the rationale for this paradigm is based on a reduction in blast cell proportions in DN3 thymocytes from recombination-deficient mice (8–10). The demonstration that cell size and blast proportions are restored when such cells develop in a normal thymus (Fig. 3) therefore compels a re-evaluation of this concept. Our combined findings show that factors derived from the thymic microenvironment, but unrelated to the pre-TCR, are responsible for the control of cell division and cell cycle progression in precursor thymocytes. It should be noted that our data are not inconsistent with mitogenesis being the immediate fate of cells with complete V-DJβ rearrangements (in-frame or out-of-frame), as proposed by others (9). However, our data indicate that while all cells that have finished V-DJβ rearrangement may be induced to proliferate, not all cells that proliferate have completed V-DJβ rearrangement. Further, in both populations of cells, proliferation is induced by factors unrelated to TCR-β recombination and expression of the pre-TCR.

The uncoupling of proliferation and differentiation from pre-TCR signaling raises the question of the functional role of this molecule. One interpretation that can reconcile the findings presented in this manuscript with all previously published data (2–5, 8–10) is that the pre-TCR fulfills a classical developmental checkpoint role in thymocytes at the DN/DP transition. In this model, DN3...
blasts would include two distinct populations of cells. One population would be dividing, nondifferentiating cells that proliferate to expand the precursor pool (i.e., self-replicating cell division). These may include cells with D-J rearrangements only, as well as cells with a single V-DJ-rearranged allele (in-frame or out-of-frame). The quantitative data presented in Fig. 1D together with the findings of others (reviewed in Ref. 16) suggest that DN3 cells probably only undergo one such self-replicating cell division before differentiating into the DP stage. The remaining DN3 blasts would represent proliferating cells that have been induced to differentiate into the DP stage. At this point, cells that have made productive TCR-β gene rearrangements and express the pre-TCR would possess a full complement of biochemical signals, thus allowing them to survive, continue proliferating, and replenish the DP compartment. Cells with out-of-frame rearrangements would simultaneously receive the same mitogenic and differentiative signals, but would fail to survive this process due to the absence of required (i.e., pre-TCR) gene products. Consequently, DN3 blasts would be enriched for cells with in-frame rearrangements, because cells with two sterile alleles would be eliminated. One appealing aspect of such a model is that it eliminates the need for an active cell fate decision, i.e., cell death vs differentiation into DP. Instead, all cells receive the same stimuli, with proliferation and DP development as the outcome in cells that express the pre-TCR vs inappropriate mitogenesis, failed differentiation, and cell death in its absence. For this purpose, we believe it is revealing to consider nonproductive TCR-β gene rearrangements as what they are in a broader context, i.e., frameshift mutations leading to the production of sterile alleles. At any locus encoding a required gene product, such a mutation would likewise lead to aborted differentiation (cell death). However, it is unlikely that developmental arrest due to mutation of, for instance, a structural gene would be interpreted as evidence that the corresponding protein induces differentiation or proliferation. Rather, the presence of a large variety of essential gene products, including the pre-TCR on immature thymocytes, is required at various stages of development for the differentiation process to be successful.

Characterization of pre-TCR expression as a developmental checkpoint obligates redefinition of the authentic mediators of the differentiative and proliferative processes. Our present data (Fig. 3) show that such signals require the presence of normal thymic anatomy and cellularity, although the precise identities of such signals remain undefined. Potential clues may be derived from the secondary lymphoid system, where the differentiation of naive T cells into functional effectors apparently involves multiple categories of non-TCR-related signals, including cytokines, chemokines, adhesion molecules, and integrins, as well as changes in tissue localization and distribution. Because many such regulatory components display stratified distribution in the thymus (21–30), it is reasonable to postulate that they may play a role in primary lymphoid tissues as well. Further characterization of such molecules and their stage-specific effects on developing thymocytes therefore represents a considerable resource for future studies of the control of thymocyte differentiation and homeostasis.

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