Temporal and Lineage-specific Control of T Cell Receptor α/δ Gene Rearrangement by T Cell Receptor α and δ Enhancers

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Summary

To analyze the regulation of gene rearrangement at the T cell receptor (TCR) α/δ locus during T cell development, we generated transgenic mice carrying a human TCR δ gene minilocus. We previously showed that the presence of the TCR δ enhancer (Eδ) within the Jα3-Cδ intron was required to activate a specific step (V-D to J) of transgene rearrangement, and that rearrangement was activated equivalently in the precursors of αβ and γδ T cells. To further explore the role of transcriptional enhancers in establishing the developmental pattern of gene rearrangement at the TCR α/δ locus, we substituted the TCR α enhancer (Eα) in place of Eδ within the transgenic minilocus. We found that V-D-J rearrangement of the Eα + minilocus was restricted to the αβ T cell subset. Further, we found that although V-D-J rearrangement of the Eδ + minilocus was initiated in the fetal thymus by day 14.5, V-D-J rearrangement of the Eα + minilocus did not occur until fetal day 16.5. Finally, whereas V-D-J rearrangement of the Eδ + minilocus is essentially completed within the triple negative population of postnatal thymocytes, V-D-J rearrangement of the Eα + minilocus is only initiated late within this population. Since the properties of minilocus rearrangement under the control of Eδ and Eα parallel the properties of Vα-Dα-Jα and Vα-Jα rearrangement at the endogenous TCR α/δ locus, we conclude that these enhancers play an important role in orchestrating the developmental program of rearrangements at this locus.

Multipotential lymphocyte precursors differentiate into mature T cells via a series of intrathymic steps (1-4). V-(D)-J recombination of TCR gene segments is an essential component of this process. Two distinct types of T lymphocytes, bearing αβ or γδ TCR heterodimers, are generated. These subsets arise independently of each other within the thymus (5-7), and display distinct tissue distributions and appear to perform distinct functions in the periphery (8).

The four TCR genes are organized into three complex genetic loci, with TCR β and TCR γ gene segments unlinked, and TCR δ gene segments nested within TCR α gene segments at the TCR α/δ locus (9). Vγ-Dγ-Jγ and Vα-Jα rearrangements are therefore mutually exclusive on an individual chromosome. Typically, γδ T cells display rearranged TCR γ, δ, and β genes but germline TCR α, whereas αβ T cells display rearranged TCR α, β, and γ genes, with TCR δ deleted on both chromosomes. Thus, the pattern of gene rearrangement at the TCR α/δ locus is a distinguishing feature of αβ and γδ T cells. Furthermore, TCR gene rearrangements occur in an ordered fashion during thymocyte development. TCR δ, β, and γ rearrangements are initiated several days earlier than TCR α rearrangement in the fetal thymus (10-12), and are initiated in less mature phenotypic subsets of T cells than TCR α rearrangement in the postnatal thymus (13, 14).

The mechanisms by which the ordered and lineage-restricted rearrangement of antigen receptor genes are accomplished are of intense interest (15-17). As initially proposed by Blackwell et al. (18) and Yancopoulos and Alt (19), and as demonstrated in numerous studies over the last few years (20-27), transcriptional promoters and enhancers within TCR and Ig gene loci play important roles in controlling the accessibility of the recombinase machinery to chromosomal substrates. Thus, transcriptional promoters and enhancers are cis-acting elements that are likely candidates to impart developmentally unique regulation to V-D-J recombination at different TCR and Ig loci.

The precise mechanism by which these elements regulate accessibility to the recombinase is an open question. There is clearly a correlation between transcriptional and recombinational activity (18, 19, 28, 29). However, it is not clear whether enhancer-induced transcription is causal in promoting locus accessibility, or whether transcription is an independent consequence of enhancer-induced locus accessibility. Recent experiments suggest that rearrangement can in some instances occur in the absence of transcription (24, 26, 30-32),

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and that the core Ig μ enhancer can establish local accessibility in the absence of transcriptional activity (33). These data argue that enhancers can contribute multiple functions that may differentially affect transcription and rearrangement.

We are interested in determining the role of transcriptional enhancers and other cis-acting regulatory elements within the TCR α/β locus in establishing the developmental pattern of V-(D)-J recombination at this locus. To study this issue, we previously generated transgenic mice carrying integrated versions of a human TCR δ gene minilocus (27). Analysis of V-D-J rearrangement of this minilocus revealed the following: First, the only rearrangement intermediate detected was V-D, arguing that the pathway of transgene rearrangement was V to D as a first step, and V-D to J as a second step. Second, the TCR δ enhancer (Eα)¹ (34), located within the J-C intron, was essential for a specific step in transgene rearrangement, V-D to J. It is interesting to note that Eα was unnecessary for V to D rearrangement. This was interpreted to indicate that the minilocus is divided into two discrete regulatory domains, and that Eα controls access to the 3' domain that contains J and C segments, but not to the 5' domain that carries V and D segments. An insulator-like boundary between regulatory domains (35-37) was proposed to exist between D and J segments. Finally, V-D-J rearrangement, although T cell specific, was equivalent in αβ and γδ T cells. This argued that Eα was activated to induce rearrangement in the precursors of both T cell subsets, and was not responsive to a lineage commitment signal that would restrict TCR δ gene rearrangement to γδ T cells.

In the present study we further characterize the role of enhancer elements in orchestrating temporal and lineage-specific V-(D)-J rearrangement within the TCR α/β locus. Specifically, we compared V-D-J rearrangement of the TCR δ gene minilocus under the control of either Eα or the TCR α enhancer (Eα). We find that substitution of Eα for Eα alters both the onset of rearrangement and the lineage specificity of rearrangement in ways that parallel the behavior of the endogenous TCR α/β locus.

Materials and Methods

Transgenic Mice. The production of transgenic mice carrying an Eα⁺ minilocus was previously described (27). To construct the Eα⁺ minilocus, a plasmid carrying the previously described enhancerless (E⁻) minilocus was linearized by digestion with XbaI, treated with the Klenow fragment of Escherichia coli DNA polymerase I to generate blunt ends, and was treated with alkaline phosphatase. A plasmid containing a 1.4-kb KpnI-BamHI fragment of and was cloned into the linearized E- minilocus. In this position Minilocus DNA was purified as described previously, and was E⁺ precisely replaces E⁻ (34) within the J₃-C₃ intron (see Fig. 1).

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¹ Abbreviations used in this paper: E⁺, enhancerless; Eα, TCR α enhancer; Eα, TCR δ enhancer; TN, triple negative.

Results

We previously generated transgenic mice carrying a human TCR δ minilocus (27). This minilocus, referred to here as Eα⁺, is 22.5 kb and contains germline Vα1, Vα2, Dα3, Jα1, Jα3, and Cα gene coding segments, as well as Eα (34) within the Jα3-Cα intron. In this construct the Vα1 and Vα2 gene segments carry mutations that prevent a rearranged transgene from encoding a functional TCR protein and thereby influencing thymic development. For the present study, we generated a new version of the minilocus, referred to as Eα⁺, in which a 1.4-kb KpnI-BamHI fragment carrying the human Eα (38) replaces the 1.4-kb XbaI fragment carrying Eα (Fig. 1). We used this construct to generate transgenic mice, and obtained four founder mice that were bred to produce independent Eα⁺ transgenic lines. Southern blot analysis showed that Eα⁺ line J carries a single copy of the minilocus, lines L and M each carry two to three copies, and line N carries five to six copies (data not shown). Lines J, L, and M were used for the studies described below.

We analyzed rearrangement of these constructs by PCR using primers specific for the Vα1, Vα2, Jα1, and Jα3 gene segments (Fig. 1). As in our previous study (27), PCR using
Figure 1. Schematic representations of germline and rearranged E₆ + and E₆ + TCR δ minilocus constructs. Fragments of 1.4 kb carrying E₆ (•) or E₆ (○) were located within the J₅₃-C₅ intron. The products generated from various V₆₁ rearrangements using V₆₁, J₆₁, and J₆₃ primers are depicted. A similar set of rearranged fragments are generated from V₆₂ rearrangements using V₆₂, J₅₁, and J₅₃ primers.

V and J primers allows detection of V-D-J rearrangements as 0.3-kb products that can be visualized by Southern blotting using V₆₁- and V₆₂-specific probes. PCR using either V primer with the J₆₁ primer allows detection of V-D rearrangements as 1.2-kb products that include the germline segment between D₆₃ and J₆₁. PCR with a pair of C₅ primers and analysis with a C₅ specific probe serves as an internal control for PCR efficiency. We have previously established that our PCR conditions allow quantitative comparisons of rearrangement levels in different samples (27).

Figure 2. Tissue specificity of E₆ + minilocus rearrangement. V₆₁ and J₆₁ primers were used to detect minilocus rearrangement by PCR, using genomic DNA from kidney (K), lung (Lu), brain (Br), liver (L), spleen (S), and thymus (Th) from mouse l7 (16-d-old), spleen(s), purified splenic T cells (T), and purified splenic B cells (B) of mouse L11 (16-d-old), or no DNA (–) as templates. Rearrangement is compared to that in the thymus of E₆ + mouse A-48 (6-wk old). PCR using a pair of C₅ primers served as an internal control. Southern blots of PCR products were probed with radiolabeled V₆₁ and C₅ cDNA fragments.

Figure 3. E₆ + and E₆ + minilocus rearrangement in αβ and γδ thymocytes. Genomic DNA samples from sorted populations of αβ and γδ thymocytes, and a no DNA control (–), were amplified by PCR using the indicated primers. The two E₆ + samples analyzed represent pooled thymocytes from two 12-d-old line A littermates (A, left) and four 17-d-old line A littermates (A, right), two E₆ + samples analyzed represent pooled thymocytes from four 15-d-old line L littermates (L) and two 20-d-old line M littermates (M). Southern blots were probed with radiolabeled V₆₁ and C₅ cDNA fragments.
in E⁺ mice revealed abundant V-D and V-D-J rearrangement in both αβ and γδ T cell samples. Levels of rearrangement, expressed as ratios of V-D-J/C and V-D/C, were very similar in all samples (Table 1). Analysis of E⁺ mice revealed normal levels of V-D rearrangement in both αβ and γδ T cell samples. However, although V-D-J rearrangement was easily detected in the αβ T cell samples, it was virtually undetectable in the γδ T cell samples (Fig. 3 and Table 1). Similar results were also obtained in a third transgenic line carrying an E⁺ minilocus (see Fig. 6). We previously found in mice carrying an E⁻ minilocus that although V-D rearrangement occurred at normal levels, V-D-J rearrangement was severely curtailed (27). Thus, in three different transgenic lines of mice, the E⁺ + minilocus behaves as if it were E⁺ in αβ T cells but E⁻ in γδ cells. E⁺ therefore activates minilocus rearrangement selectively in developing αβ T cells in these mice.

E⁺ and E⁺ Determine the Timing of V-D-J Rearrangement during Ontogeny. Endogenous Vδ-D-Jδ and Vα-Jα rearrangements are activated with distinct kinetics during fetal development (10–12). Since E⁺ and E⁺ appear to play important roles in activating minilocus rearrangement, we sought to determine whether these elements could influence the time at which rearrangements are initiated during ontogeny. We therefore analyzed V-D and V-D-J rearrangement of the E⁺ + and E⁺ + miniloci in series of fetal thymus samples obtained from timed pregnancies.

Analysis of E⁺ line A minilocus rearrangement using Vα1 and Jα1 primers revealed V-D rearrangement at the earliest timepoint examined, fetal day 13.5 (Fig. 4). At this time, V-D-J rearrangement is only barely detectable. However, there is a dramatic increase in V-D-J rearrangement starting at fetal day 14.5, and both V-D and V-D-J rearrangements further increase in abundance through day 17.5. Similar results were also obtained for line B E⁺ mice (data not shown). The observed early onset of rearrangement at the E⁺ + minilocus closely parallels the onset of rearrangement at the endogenous murine TCR δ locus (12). Further, the elevated ratio of V-D/V-D-J rearrangement at early time points supports our previous inference that the predominant pathway of minilocus rearrangement consists of V to D as a first step, and V-D to J as a second step. Consistent with this interpretation, minilocus D-J rearrangement could not be detected at any stage of fetal development by PCR with a 5' D3 and Jα1 or Jα3 oligonucleotides (data not shown).

Similar data for V-D and V-D-J rearrangement was obtained using the primer combinations Vα1-Jα3, Vδ2-Jα1, and Vδ2-Jα3. Very low levels of V-D-J rearrangement were detected with Vα2 and Jα3 primers as fetal day 13.5, but V-D-J rearrangements were readily detected with all primer combinations by fetal day 14.5. Thus, there was not a clear distinction between the onset of minilocus Vα1 and Vδ2 rearrangement during murine fetal thymic development, despite the fact that Vα2 is known to rearrange earlier than Vα1 during human fetal thymic development (39). This apparent deregulation could occur because cis-acting elements that flank the V gene segments are by themselves insufficient to direct ordered rearrangement, or because they are not recognized appropriately in a murine background.

An analogous study was then performed on fetal samples from E⁺ line J. Analysis of V-D and V-D-J rearrangements at fetal days 14.5 through 16.5 revealed that although V-D rearrangement is activated early and is therefore readily detected throughout this time period, activation of the V-D to J step is delayed until fetal day 16.5 (Fig. 5). Analysis of samples from E⁺ line M yielded quite similar results. High levels of V-D rearrangement were detected from fetal day 14.5 onward, whereas V-D-J rearrangement, although first detected at very low levels on fetal day 15.5, was not detected at levels comparable to V-D rearrangement until fetal day 16.5 (data

![Figure 4. Time course of E⁺ minilocus rearrangement in the fetal thymus. Genomic DNA samples from line A fetal thymi isolated on days 13.5 through 17.5 of gestation were amplified by PCR using the indicated primers. Southern blots were probed with radiolabeled Vα1, Vα2, and Cδ cDNA fragments. Due to differences in probe-specific activities and exposure times, the levels of Vα2 and Vα1 rearrangements cannot be directly compared. Note that a technical problem prevented analysis of the control Cδ signal at fetal day 13.5.]

### Table 1. Quantification of Minilocus V-D and V-D-J Rearrangement in αβ and γδ Thymocytes of E⁺⁺ and E⁺⁺⁺ mice

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<td>αβγδ</td>
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<td>V-D-J/C</td>
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Blot hybridization signals from the experiment shown in Fig. 3 were determined using a Betascope. Rearrangement was quantified as a ratio of V-D/C or V-D-J/C for each sample.
Figure 6. Eα+ and Eα+ minilocus rearrangement in postnatal triple negative thymocytes. Genomic DNA samples from total thymocytes (TN) and sorted populations of triple negative (TN), αβ and γδ thymocytes were amplified by PCR using the indicated primers. Thymocytes from 11-d-old Eα+ mice A-449 and A-458 were pooled for sorting and analysis, as were thymocytes from 3-wk-old Eα+ mice J-66 and J-70. Southern blots were probed with radiolabeled Vα1 and Cα cDNA fragments.

Discussion

To analyze the role of transcriptional enhancers in regulating TCR α/δ gene rearrangement during T cell development, we generated transgenic mice carrying a human TCR δ gene minilocus, and studied the rearrangement of this minilocus under the control of either Eα or Eα. We found that the presence of Eα or Eα altered V-D-J rearrangement of the reporter construct, as assessed by the lineage-specificity of rearrangement, the time course of rearrangement during fetal thymocyte development, and the phenotypic stage during which rearrangement occurs during postnatal thymocyte development. Specifically, whereas V-D-J rearrangement of the Eα+ minilocus is equivalent in αβ and γδ T cells, V-D-J rearrangement of the Eα+ minilocus only occurs in αβ T cells. Whereas V-D-J rearrangement of the Eα+ minilocus is initiated by fetal day 14.5, V-D-J rearrangement of the Eα+ minilocus is not initiated until fetal day 16.5. Finally,
whereas V-D-J rearrangement of the Eα+ minilocus is essentially completed within the TN population of postnatal thymocytes, V-D-J rearrangement of the Eα+ minilocus is only initiated late within this population. We conclude that Eα and Eδ play important roles in orchestrating the complex developmental program of V-D-J rearrangement at the endogenous TCR α/δ locus.

We previously showed in mice transgenic for an E- TCR δ minilocus that V to D rearrangement, but not V-D to J rearrangement, could occur independent of the presence of Eα (27). Analysis of early fetal thymocytes and postnatal thymocytes of Eα+ and Eα+ mice in the present study are consistent with this idea. V to D rearrangement is activated very early in both the Eα+ and the Eα+ construct. Although this is very rapidly followed by Eα-dependent V-D to J rearrangement in the Eα+ minilocus, Eδ-dependent V-D to J rearrangement occurs fully 2 days later in the Eα+ minilocus. Further, in the Eα+ minilocus, V to D rearrangement, but not V-D to J rearrangement, occurs in the γδ T cell subset. Our results argue that V to D rearrangement is activated in a very early population of thymocytes. One candidate could be the Fpg1+/CD44lowCD8+CD3- population, which is the earliest precursor population in the thymus, and which contains cells with both T cell and B cell differentiation potential (42). This could account for the low level of minilocus V-D rearrangement that we routinely detect in B cell populations. Although this precursor population is known to carry germline TCR β and TCR γ genes, the configuration of the TCR δ gene has not been examined (43). We note that TCR δ V-D rearrangements are common in human precursor B-ALL cells (44-46).

We previously argued that the TCR δ minilocus studied here is composed of two independent regulatory domains, one containing V and D gene segments, and the other containing J and C gene segments (27). We further argued that Eα selectively influenced J and C segment accessibility within the 3' domain, and that the influence of Eα was limited by the presence of an insulator between D and J segments. It was clear that V to D rearrangement could occur in the presence or absence of Eα, and that the V-D to J step required Eα. If Eα only influenced the second step of the rearrangement process, then the total level of rearrangement (V-D) in E- mice should be identical to the total level of rearrangement (V-D + V-D-J) in E+ mice. However, because the various transgenes studied displayed a range of rearrangement levels that was influenced by the integration sites, it was impossible to evaluate whether the total level of construct rearrangement was influenced by the presence of Eα. In this study, we could compare the properties of the Eα- minilocus in αβ and γδ cells carrying the same integrant. It is clear that V-D to J rearrangement (hence, J accessibility) occurs only in αβ cells. However, it also seems apparent that the total level of construct rearrangement (V-D + V-D-J) is significantly higher in αβ than in γδ cells (see Figs. 3 and 5). This suggests the possibility that activation of Eα can influence the initiation of rearrangement events (i.e., V to D) in the 5' domain of the minilocus. There are two ways in which this result can be rationalized with the two domain model of minilocus accessibility. First, it could be that Eα, because it is much more potent than Eδ, can overcome the influence of the putative insulator between D and J, and thereby influence accessibility in the 5' domain. Second, it could be that under the control of Eα, the length of time that the minilocus is exposed to recombine may be longer in developing αβ cells than in developing γδ cells. Our current data cannot distinguish between these possibilities.

In the human TCR δ gene minilocus studied here, human Eα and Eδ elements differentially activate construct V-D to J rearrangement. While this study was being completed, Capone et al. (47) reported a similar analysis of the role of murine Eα and murine Eδ in V-D-J rearrangement of a hybrid murine TCR β/γδ locus. The regulation of that minilocus is different from ours, in that both D-J and V-D-J rearrangements are enhancer dependent. Despite the differences in minilocus organization and regulation, a comparison of the two studies indicates that human Eα and murine Eα activate rearrangement at an early stage and in both the αβ and γδ T cell subsets, whereas human and murine Eδ activate rearrangement at a later stage and in only the αβ T cell subset. Analogous information is not yet available for TCR γ regulatory elements.

The observation that Eα directs V-D-J rearrangement of the TCR δ minilocus specifically within the αβ T cell compartment could imply that Eα is activated by the binding of trans-acting factors specifically in the precursors of αβ T cells. However, it is possible that lineage-specific activation of Eα need not be invoked to explain the behavior of the Eα+ minilocus. Initial studies of Eα transcriptional activity in transfected cell lines indicated that a core murine Eα fragment did not display αβ lineage specificity; rather, lineage specificity could be conferred by nearby cis-acting silencer elements (48, 49). Consistent with this, Capone et al. (47) found that the murine Eα fragment used in their study, which should not contain silencer elements, did not confer lineage-specific transcriptional activity to their construct. Nevertheless, this Eα fragment did confer lineage-specific rearrangement (47). The human Eα fragment used in our construct was originally reported to display αβ lineage-specific rearrangement activity in transfection experiments (38). However, when we directly compared the ability of Eα and Eδ to activate transcription in the same reporter construct in both αβ and γδ T cell leukemias, we found that although Eα is more potent than Eδ, their relative activities did not vary dramatically between αβ and γδ cell lines (P. Lanzurica, and M.S. Krangel, unpublished observations). Finally, we note that none of the transcription factors that have been shown to interact with Eα are thought to display lineage-specific expression or activity (50).

Thus, core Eα fragments that appear to be transcriptionally active in both αβ and γδ T cells still confer αβ specificity to V-D-J rearrangement. This could reflect a difference between the cis-acting enhancer elements required to activate transcription and those required to activate rearrangement (33). However, it is also possible that the ability of the core Eα fragment to confer αβ specificity to minilocus V-D-J rearrangement could follow as a default from its temporal/stage
specificity, without requiring the involvement of any lineage-specific trans-acting factors that interact with the enhancer. Our experiments with the TCR δ minilocus, and numerous studies of the endogenous TCR α/δ locus, argue that TCR δ rearrangement occurs several days earlier than TCR α rearrangement in developing thymocytes (10-12, 14). TCR γ also initiates rearrangement at a stage prior to TCR α (10, 11). If the core Eα fragment is subject to delayed activation by a developmental clock in maturing thymocytes, and if functional TCR δ and TCR γ rearrangement can result in a shutoff of RAG (51, 52) gene expression, then Eα may become activated too late in developing γδ T cells to have any consequence for TCR gene rearrangement in these cells. The developmental clock could be understood, at least in part, on the basis of the expression characteristics of transcription factors thought to be important for core enhancer function. For example, c-Myb plays an important role in activation of Eδ (53) and is expressed in immature thymocyte subsets and early in ontogeny (54, 55), whereas Ets factors play an important role in activation of Eα (56), and are expressed in more mature thymocyte subsets and late in ontogeny (57).

Several experimental approaches have suggested that T cell lineage is determined before the onset of TCR gene rearrangement and that a lineage commitment step may in fact direct TCR gene rearrangement or expression (49, 58-60). However, it is not yet clear which events at TCR loci represent or respond to regulated lineage commitment steps. If the core Eα fragment activates rearrangement in a lineage-specific fashion by a proactive rather than default mechanism, activation of Eα could be an initial commitment step for the αβ pathway. This could be consistent with recent data arguing that αβ and γδ T cells can both arise from relatively late stage TN cells in the thymus (40). Alternatively, if the core Eα fragment simply responds to a developmental clock, then it cannot mediate an initial lineage commitment decision; rather, it can only function as a default. In this case, it would still be possible for TCR α rearrangement to be lineage determining if TCR α cis-acting elements other than the core Eα fragment (i.e., silencers [49]) are involved. The alternative possibility would be that activation of earlier events, such as TCR δ or TCR γ rearrangement and expression, would be lineage determining. Our data says that activation of TCR δ rearrangement per se is not regulated in a lineage specific fashion. However, TCR δ rearrangement could be prevented in a regulated manner by deletional rearrangement mediated by δ Rec and pseudo-Jα (61), if this event were activated early and in a directed fashion. Further, there is evidence that TCR γ transcription may be subject to lineage-specific control by silencer elements (60), suggesting this as a possible commitment step.

In summary, Eα has the potential to activate TCR δ gene rearrangement, and Eδ has the potential to activate TCR β gene rearrangement, in significant fractions of developing αβ and γδ thymocytes. This argues that control mediated at the level of Eδ and Eα cannot be involved in lineage determination. Regulated activation of δ Rec, TCR γ, and TCR α rearrangement and expression are all candidates that are not yet excluded as lineage determining events. The minilocus experiments show quite clearly that TCR α, β, and δ gene rearrangement events are at a minimum regulated by developmental clock-responsive enhancers. However, given the possibility that the core Eα fragment may mediate lineage-specific rearrangement by default, we note that none of the minilocus data formally excludes the possibility that lineage is determined in a stochastic way, as a consequence of functional TCR gene rearrangements, as initially proposed by Pardali et al. (62).

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