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In favor of the selective model of positive selection

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The mechanisms of thymocyte commitment towards the CD4+ and CD8+ lineage remain unresolved. Two models—one based on instruction, the other on selection—have previously been proposed. The instructional model has been popularly received based on results of earlier studies. However, our data from MHC class II, class I, and double-deficient mice suggest otherwise. There exists a significant population of CD4+ cells that is intermediate in maturity between CD4+CD8+ and fully mature CD4+CD8- thymocytes in class II-deficient animals; an analogous population of CD4-CD8+ cells exists in class I-negative mice. We suggest that a selective model in which two TCR-MHC molecule engagements are required: the first induces a random down-modulation of either CD4 or CD8 and some differentiation; the second, involving the participation of the appropriate coreceptor, permits end-stage differentiation.

Key words: CD4+ thymocyte / lineage commitment / MHC class II-negative / positive selection / selective model

THE IMMUNE SYSTEM develops from pluripotent stem cells to become an orchestrated group of cells which play relatively fixed and unique roles. For T lymphocytes, differentiation takes place largely in the thymus. Eventually mature major histocompatibility complex (MHC) class II-restricted, CD4+ helper and MHC class I-restricted, CD8+ cytotoxic T cells emerge from the thymus into the periphery to perform their assigned functions.

Although T cell differentiation in the thymus is no longer a complete 'black box', certain key issues remain. For example, what events shape the fate of the differentiating thymocyte? What processes, molecular and cellular, ensure that the mature circulating T cell pool is self-tolerant and self-MHC-restricted? Equally important, how and when do thymocytes irreversibly commit to the CD4+ versus CD8+ cell lineage?

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Recent studies have shown that the major lineage of αβ T cell receptor (TCR)-expressing lymphocytes follows a distinct route upon entering the thymus. Precursor T cells which are CD4-CD8-TCR- begin expressing the CD4 and CD8 coreceptors to become CD4+CD8+ double-positive thymocytes.[1] During this time their TCR genes finish rearranging and double-positive cells express between low to intermediate levels of TCR.[2,3] Thanks largely to TCR transgenic (tg) and gene-targeting systems, it is now evident that CD4+CD8+ thymocytes up-regulate their TCR levels following positive selection on appropriate self-MHC molecules expressed by thymic stromal cells and become mature CD4+CD8-TCR^{hi} or CD4-CD8+TCR^{hi} single-positive cells, or face programmed cell death.[4-8]

Thus the thymic microenvironment is crucial during T cell differentiation. Radiation bone marrow chimera experiments,[9-13] work from transgenic animals expressing MHC molecules in a compartmentalized fashion.[7,14,15] and reconstitution assays[16-18] have shown cortical epithelial cells to be the most efficient mediators of positive selection. Radiation-sensitive bone marrow-derived stromal cells, such as macrophages and dendritic cells, are either inefficient (for CD8+ cells) or unable (for CD4+ cells) to fulfil the task.[19,20] In addition, microenvironments within the thymic cortex and medulla, which descend from disparate sites during embryonic development, may exert diverse influences.[21] At present the precise mechanisms by which stromal cells contribute to positive selection and lineage commitment remain largely unidentified.

Instructional versus stochastic/selective commitment

Two basic models were originally raised to explain the mechanism of positive selection from the viewpoint of the immature thymocyte. The instructional model, first proposed by von Boehmer,[22] asserts that positive selection occurs from a precise coupling or recognition of the thymocyte TCR for stromal MHC molecules. This interaction instructs the thymocyte

to become either a CD4+ helper or a CD8+ cytotoxic cell depending on the affinity of the TCR for MHC class I or class II molecules. In other words, a CD4+CD8+ thymocyte bearing a class II-restricted TCR will down-regulate the superfluous CD8 coreceptor upon association with MHC class II molecules; alternatively, a double-positive cell bearing a class I-restricted TCR will down-regulate the CD4 coreceptor upon interaction with MHC class I molecules. In this scenario, only single-positive thymocytes which bear the correctly matched MHC-restricted TCR and coreceptor will be generated.

The stochastic/selective model predicts that CD4+ CD8+ thymocytes down-regulate one coreceptor regardless of TCR specificity to generate CD4+CD8− or CD4−CD8+ cells which express TCR specific for either MHC class I or class II proteins before positive selection.[23] Those single-positive thymocytes which by chance bear the appropriate match of TCR and coreceptor will then be positively selected to emigrate from the thymus. In this model, the mechanism by which the double-positive thymocyte down-modulates one coreceptor is unknown, and need not involve a specific TCR-MHC engagement.

Both models recognize that mature CD4+ T cells will bear class II-restricted TCR while CD8+ T cells will bear class I-restricted TCR. The difference between them is the window of positive selection and its underlying connection to lineage commitment. The instructional model predicts that commitment is concomitant with selection and that this is established at the double-positive stage of differentiation, thus all single-positive thymocytes will bear the correct TCR. In the selective model, commitment and selection are sequential, and each single-positive thymocyte compartment will contain cells that express TCR specific for MHC class I or class II molecules, if transiently.

In support of the instructional model

Early acceptance of the instructional model came from imaginative studies using mice transgenic for the class I-restricted HY TCR α- and β-chains, whose T cells were also forced to express the CD8 coreceptor.[23,24] A stochastic model predicts that CD4+CD8− (CD8tg+) cells would be observed in the CD4+ 'single-positive' thymocyte compartment and CD4−CD8+(CD8tg+) cells would be observed in the CD8+ compartment; the transgenic CD8 coreceptor would 'rescue' would-be CD4+ HY TCR+ cells, which had down-regulated their endogenous CD8 coreceptors, by allowing positive selection through the HY TCR and the transgenic CD8. Yet, as reviewed elsewhere in this issue, only CD4−CD8+(CD8tg+) thymocytes expressing the HY TCR were detected in these animals. On the other hand, rescue of CD4+CD8−(CD8tg+) thymocytes was observed when a transgene-encoded chimeric CD8 molecule was expressed which joined the CD8 extracellular domain to the CD4 transmembrane segment and cytoplasmic tail.[25]

Evidence that double-positive thymocytes are instructed to differentiate into the appropriate single-positive phenotype upon positive selection was also provided by non-transgenic systems. CD4+CD8+ thymocytes were found to up-regulate their TCR levels and certain activation markers only in the presence of the correct MHC ligand.[22] Similarly, *in vitro* culture of CD4+CD8+TCR^{hi} thymocytes from class I-restricted TCR transgenic mice could produce solely CD4−CD8+ cells.[26,27]

CD4+ thymocytes in MHC class II-negative mice

Given the results of the above studies, the detection of a significant population of CD4+ thymocytes (up to 30% of normal numbers) showing high levels of TCR in genetically engineered MHC class II-negative (II^0) mice was unexpected.[28] Exhaustive analyses showed that these cells, which express high levels of CD4 and low levels of CD8, simultaneously display markers associated with immature double-positive and fully mature single positive CD4+ thymocytes. Like CD4+CD8+ cells, the CD4+CD8^{lo} thymocytes are detected only in the cortex; they are dexamethasone-sensitive; they are peanut agglutinin+, 6C10+ and 3C11−. Like positively selected mature CD4+ cells, the CD4+CD8^{lo} population expresses intermediate to high levels of TCR and is CD69-positive; it shows diminished expression of the RAG-1 and TdT genes; and significantly, it has gained functional reactivity to stimulation, such as to phorbol diester and calcium ionophore.[29] The CD4+CD8^{lo} cells also appeared to be generated by the same pathway as typical $\alpha\beta$ T lymphocytes, as defined by their kinetic emergence following bromodeoxyuridine labeling. Thus we concluded that these cells compose a transitory

intermediate population between $CD4^+CD8^+$ cells and end-stage $CD4^+$ thymocytes. A similar population of $CD4^{lo}CD8^+TCR^{hi}$ thymocytes was also detected[29] in β_2-microglobulin-negative (I^0) animals which lack MHC class I molecules.[30]

Curiously, the $CD4^+CD8^{lo}$ thymocytes appear to be positively selected on MHC class I molecules. When II^0 mice were crossed with I^0 animals, the effect was convincingly clear: no MHC molecules, no single-positive thymocytes.[29] The intermediate $CD4^+CD8^{lo}$ population detected in II^0 mice (and the reciprocal $CD4^{lo}CD8^+$ cells in I^0 mice) was essentially not found in double-deficient animals. These data provided evidence that class I molecules can select a population of cells differentiating towards the $CD4^+$ lineage and *vice versa* for class II molecules and thymocytes differentiating towards the $CD8^+$ lineage. The notion that $CD4^+CD8^{lo}$ thymocytes may also be class I-restricted came from further observations that HY TCR transgenic mice generate $CD4^+CD8^{lo}$ thymocytes bearing the class I-restricted transgenic TCR, although they are less evident in class II-positive than in class II-negative animals. In addition, $V\beta5$ expression, which is normally associated more closely with $CD8^+$ cells,[31] is observed in surprisingly large numbers (16-20%) in $CD4^+CD8^{lo}$ thymocytes of II^0 mice.[29]

At this point we would like to address specific concerns regarding our experiments. First, it has been shown that there exists a symbiotic relationship between thymocytes and the thymic stroma;[32] consequently double-positive cells might not mature in MHC I^0II^0 mice because regions of the thymic microenvironments were not properly developed due to a dearth of mature thymocytes. It is a valid point that the medulla in I^0II^0 animals is only a rudiment of the one in normal wild-type or even II^0 mice (unpublished results). However, both the double-positive and the intermediate populations are found in the thymic cortex; the cortical regions in both the II^0 and I^0II^0 animals appear morphologically normal, if enlarged (unpublished results).

Second it was wondered if the $CD4^+CD8^{lo}$ thymocytes might not be a 'dead end' population that was not selected and was down-modulating coreceptors as well as other surface molecules *en route* to programmed cell death. Since these cells exhibited some phenotypic characteristics typical of positively selected thymocytes and more importantly, gain of some function to stimulation, it is highly unlikely that they were unselected. On the other hand, it is correct that they are, in the final result, a population that will die because they will never express the CD8 coreceptor to function with their class I-restricted TCR.

Another view of the selective model

The presence of MHC class I-restricted $CD4^+CD8^{lo}$ thymocytes in II^0 mice contradicts the prediction of the instructional model that there would be no such cells in the absence of class II molecules. Our findings and data from other laboratories,[33,34] reviewed elsewhere in this issue, support the selective model. Nevertheless, MHC molecules are required at the double-positive stage of differentiation, suggesting that a positive signal occurs between the TCR and the MHC protein at this step.

We envision that the selective model proceeds as follows (see Figure 1). There are at least two steps of selection that occur between the maturing thymocyte and stromal cells. The first step takes place at the double-positive stage. $CD4^+CD8^+$ thymocytes are selected on the basis of their TCR affinity for either MHC class I or class II molecules; coreceptors may or may not be involved. Those cells which are positively selected begin to differentiate by down-modulating either the CD4 or CD8 coreceptor independently of their TCR affinity; they increase their TCR and CD69 levels, and gain at least some function. A second engagement is then required at the intermediate $CD4^+CD8^{lo}TCR^{hi}$ or $CD4^{lo}CD8^+TCR^{hi}$ stage. Selection at this step requires a precise interaction between the MHC, TCR, and the appropriate coreceptor, and it appears to be the final checkpoint before end-stage differentiation. Only single-positive thymocytes which express the appropriately matched TCR and coreceptor are allowed to continue maturation. Those cells which bear the wrong coreceptor and TCR combination, like the population of $CD4^+CD8^{lo}$ thymocytes in II^0 mice, will presumably face death from non-selection.

It is unknown from our data whether the two engagements require signals which are separate and distinct, or simply a long continuum of the same signal. Furthermore, if these signals are distinct, we do not know if they are distinct extracellularly, in terms of ligand-receptor interaction or cytokine production which may differ at various junctions of the thymus, or intracellularly, due to the maturing thymocyte's diverging susceptibility to the same extracellular signals. We do know, however, that the TCR-MHC engagement is crucial at the

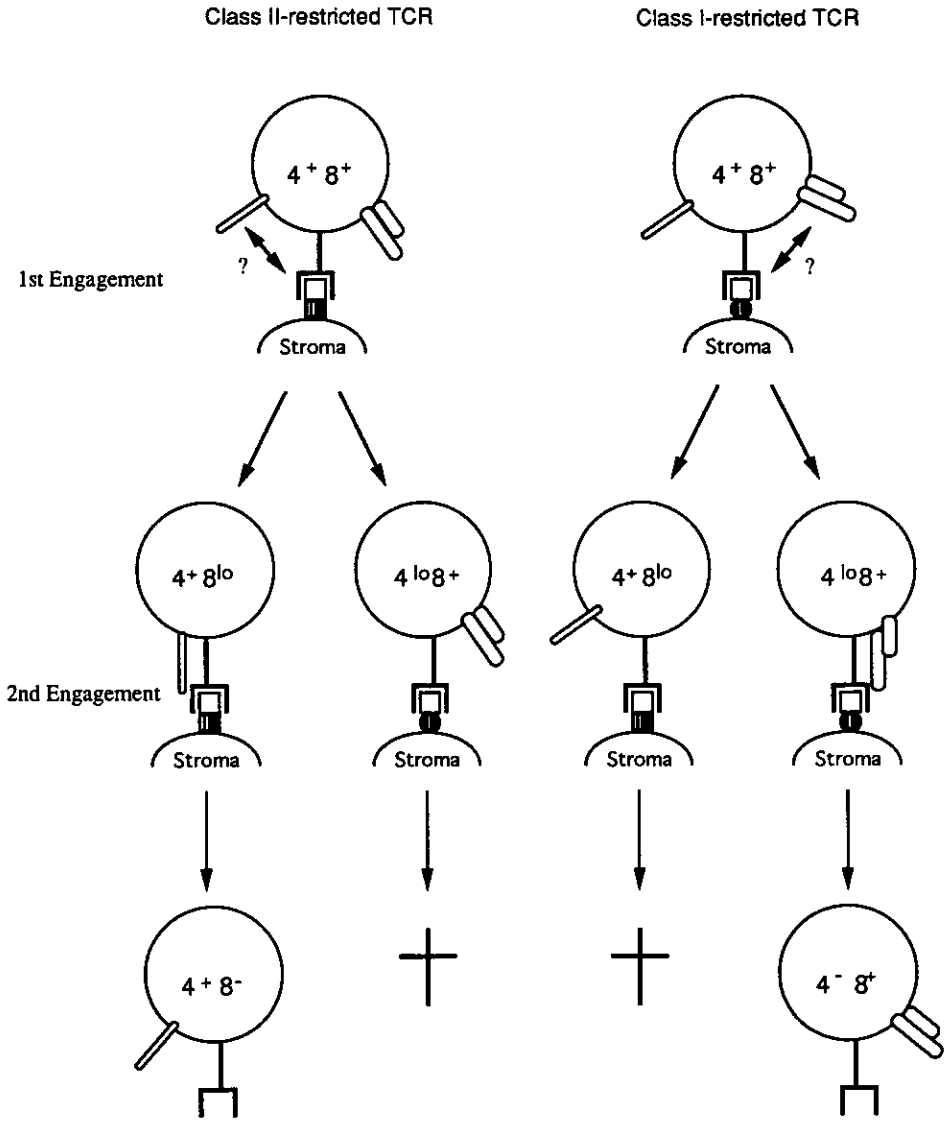

Figure 1. Schematic diagram of the new stochastic/selective model of positive selection. CD4 and CD8 are depicted as protein monomers and heterodimers, respectively. The TCR is depicted in bold. I and II on stroma represent MHC class I and class II molecules. † denotes death.

$CD4^+CD8^+$ stage and that the correct coreceptor is additionally required at the intermediate phase.

If lineage commitment and positive selection are dissociated, when does commitment take place for the individual cell and under what criteria? It is important to note at this juncture that a stochastic selection does not necessarily denote a purely random decision of becoming $CD4^+$ versus $CD8^+$, resulting in odds of 50:50. By this we mean that double-positive thymocytes bearing a class II-specific TCR may not (and in reality do not) have an equal chance of becoming a $CD4^+CD8^{lo}$ or $CD4^{lo}CD8^+$ thymocyte. Indeed there may be a skewing of class II-restricted thymocytes for the $CD4^+$ compartment and a reciprocal skewing of class I-restricted thymocytes towards the $CD8^+$ phenotype. It is unclear how this dichotomy arises. It is also unclear why higher numbers of class I-restricted intermediate $CD4^+CD8^{lo}$ thymocytes exist in II^0 animals than class II-restricted $CD4^{lo}CD8^+$ ones in I^0 mice. Possibilities may include some of the following. Maturing thymocytes may be influenced by microenvironments which

exude unique combinations of cytokines or which stimulate the thymocytes through particular cell surface accessory molecules, and coerce them into a particular path of differentiation. Alternatively, there may be differences in the affinities/avidities of TCR-coreceptor-MHC interactions between thymocytes, leading to different signals to the nuclei. These variations may bias a thymocyte towards one lineage or the other, perhaps by stimulating or changing the expression of transcriptional factors responsible for specific lineage pathways.

Why is it necessary for a thymocyte to receive two positively selecting signals during differentiation? Why waste energy to down-regulate the wrong coreceptor at the double-positive stage after the initial TCR-MHC engagement only to require a second engagement to test the appropriateness of the coreceptor? A plausible explanation for the apparent wastefulness in the stochastic pathway may lie in the implied simplicity of its signaling mechanisms. In an instructional format, the thymocyte must possess the ability to sense the class of MHC molecule that is stimulating the TCR. Is there TCR-MHC engagement? Is the MHC molecule class I or class II? While this capability may be facilitated by coengagement of the appropriate coreceptor, it suggests a differential treatment of CD4-versus CD8-mediated signals. Thus the thymocyte instantaneously becomes faced with three choices—to live or die, to become $CD4^+$, to become $CD8^+$. By contrast, the stochastic/selective system simply necessitates the repetition of a binary decision process—to differentiate or to die. For the $CD4^+CD8^+$ thymocyte, the choice to differentiate or die lies on the quality of the TCR-MHC interaction. For the intermediate $CD4^+CD8^{lo}$ or $CD4^{lo}CD8^+$ cell, the choice to differentiate or die depends on whether the same quality of signal still exists following down-modulation of one coreceptor. Thus the stochastic/selective model requires a simple processing or input data by the maturing thymocyte, a mechanism that is similar to that used by other differentiating cell types in the immune system. Indeed, one could draw a parallel between the instructive/stochastic dichotomy of positive selection models and the older induced fit/clonal selection debate concerning the generation of immunoglobulin repertoire.

Ongoing studies

The instructional model predicts that signals lineage commitment are directly linked to positive selection. In contrast, the selective model predicts that commitment and selection play separate although probably linked roles. More specifically, it is likely that positive selection occurs in multiple steps throughout maturation, as a result of ligand-receptor interaction or cytokine influence. This notion is most perceived in the recent articles which demonstrate that the rearrangement and expression of the TCR β-chain is necessary for the eventual differentiation of the double-negative thymocyte subset to the double-positive one.[35,36] Hence thymocytes demand rescue from cell death at the double-negative, double-positive and single-positive stages. Lineage commitment may or may not be associated with these known steps. Thymocytes may require positive selection only to proceed with their selected program. It was recently proposed that T cell lineage may be decided as early as in the bone marrow.[37] This suggestion has yet to be proven at the single cell level, and there are currently no markers available which define $CD4^+$ versus $CD8^+$ lineages in precursor T cells. Thus, the question becomes, is commitment linked to selection? With these thoughts in mind, one can imagine several lines of experiments to address this issue.

First, are coreceptors involved in the first TCR-MHC engagement? The CD4 and CD8 coreceptors play important roles in influencing the affinity of the TCR for MHC molecules, presumably by directly binding to these proteins.[38-40] In addition, the transmembrane and cytoplasmic tails of CD4 and CD8 have access to signaling mechanisms such as through the lymphocyte-specific tyrosine kinase, $p56^{lck}$.[41-44] Our previous results did not ascertain coreceptor involvement for positive selection at the double-positive stage. However, we now have preliminary data that the CD8 coreceptor is required by $CD4^+CD8^+$ thymocytes to begin differentiation in II^0 mice (unpublished results). If the coreceptor is absent, thymocytes are arrested at this step. Thus the TCR-coreceptor-MHC combination is crucial for the first step of positive selection, but these molecules do not instruct lineage commitment in an obvious way.

Second, if thymocytes can be positively selected into the periphery with mismatched MHC-restricted TCR and coreceptor, what would be the function of these lymphocytes? For example, if the class I-restricted intermediate $CD4^+CD8^{lo}$ cells in II^0 mice were forced to mature, which phenotype would these cells take—helper according to their CD4 expression or cytotoxic according to their MHC

restriction? Rescuing these cells was first attempted by transfer into deoxyguanosine-treated wild-type fetal thymic lobes expressing both classes of MHC molecules, without significant success (unpublished results). Presumably these cells were not only selected on class I molecules, they are also restricted in that they have little or no affinity for class II proteins. Therefore, they should never be effectively rescued after down-regulation of their CD8 coreceptor.

Alternatively, it should be theoretically possible to rescue these cells by forcing CD8 coreceptor expression on them through transgenesis, mirroring Davis *et al*,[33] who showed that pushing very high levels of transgene-encoded CD4 expression on $CD4^{lo}CD8^+$ thymocytes in I^0 mice rescues mature class II-restricted T lymphocytes which are $CD4^-$ $(CD4tg^+)CD8^+$. Nevertheless, forcing low levels of CD8 expression on class I-restricted HY TCR^+ thymocytes has previously failed to rescue $CD4^+CD8^-(CD8tg^+)$ cells into the periphery or even to end-stage maturation in the thymus.[23,24] As noted earlier, we have found that HY TCR^+ thymocytes can be found in the intermediate $CD4^+CD8^{lo}$ population, indicating that double-positive cells expressing this class I-restricted TCR are capable of down-modulating their CD8 coreceptor. One possibility for their lack of rescue may be that higher levels of CD8 are required, similar to the levels of CD4 needed to rescue $CD4^-$ $(CD4tg^+)CD8^+$ cells in I^0 animals.[33] We are currently testing the hypothesis of whether intermediate $CD4^+CD8^{lo}$ thymocytes can be rescued by forcing transgene-encoded CD8 α- and β-chain expression on them, and whether these cells will present the $CD4^+$ or $CD8^+$ phenotype, or both.

Clearly, a detailed analysis at the molecular and biochemical levels will be needed to understand how, where and when $\alpha\beta$ T cell differentiation occurs. This will entail multiple investigations into specific regulatory pathways, from signal transduction at the cell surface, to the activation and suppression of individual genes and promoter elements, to the expression of as yet unknown protein products. A fraction of this body of work has already yielded fruitful results such as: the findings that positive or negative selection brings about the down-regulation of RAG activity to prevent further TCR gene rearrangement;[45,46] the involvement of lymphocyte-specific tyrosine kinases upon TCR crosslinking;[26,41,47] the investigations into regulatory regions and components which may control CD4 and CD8 gene expression;[48-50] and finally, the analyses of minute stages in thymocyte differentiation during which such genes play a critical function, as deduced from the creative matings of 'knock out' mice. This is undoubtedly a beginning. It is equally clear from recent studies regarding positive selection and lineage commitment that much remains to be accomplished.

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