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On the issue of peptide recognition in T cell development

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CD4-CD8 double positive (DP) thymocytes undergo a differentiation process in the thymus where they are selected based on their ability to recognize peptide antigens presented on self major histocompatibility complex (MHC) molecules. The first stage of this process is positive selection, a quality-control mechanism which ensures that the T cell receptors (TCR) presented on developing thymocytes can transmit signals via peptides presented on either MHC class I (MHC1) or MHC class II (MHC2) molecules. Work over the past decade has revealed that the peptides that drive positive selection of both CD4 and CD8 lineage cells deliver only weak TCR signals. In line with these observations, specialized protein degradation machineries have been discovered in the thymic cortex that presumably generate specialized low-affinity peptide repertoires for presentation on MHC1 and MHC2 molecules. TCR signals transduced through these weak-affinity ligands in the early stages of positive selection alter the kinetics of expression of CD4 and CD8 molecules and play a crucial role in commitment of thymocytes to either the CD4 or CD8 lineages. In this work, we review the experiments that explore the peptide repertoires that are presented to developing thymocytes during positive selection, the observed signaling patterns that lead to CD4 versus CD8 lineage commitment, and speculate about how specialized organization of the signaling machinery in DP thymocytes may allow for efficient transduction of weak signals during the course of positive selection.

Introduction

Before entering the repertoire of mature CD4 or CD8 single-positive (SP) T cells, immature DP thymocytes undergo a series of selective tests.[1] The collective result of these processes is the truncation of the vast TCR repertoire encoded by germline gene segments into a small subset of productive TCRs.[2] The selected few can recognize foreign peptides bound by MHC allelic products—but are largely tolerant toward self peptides presented within the context of the same MHC molecules. Currently, it is commonly accepted that the process of generating such a mature T cell repertoire occurs in two steps. In the first step, TCRs receive signals via low affinity recognition of self peptide loaded MHC molecules presented by epithelial cells in the thymic cortex (cTECs).[3,4] Thymocytes that signal during this stage will mature into SP thymocytes bearing either a CD4 or CD8 coreceptor. For the purposes of this review, we define positive selection as the collective process by which preselection DP thymocytes are signaled and subsequently commit to either the CD4 or CD8 lineage. T cells that do not recognize any self peptide-MHC complexes die by neglect. In the second step, SP thymocytes expressing TCRs which show strong interactions toward host-derived peptide-MHC complexes presented mostly by antigen presenting cells (APCs) in the thymic medulla (mTECs) are negatively selected.[5] Negative selection is thought to be mediated at least in part by promiscuous gene expression of peripheral tissue antigens via the transcription factor AIRE in the medullary epithelium.[6] AIRE is also believed to be a proapoptotic factor for mTECs, such that these antigens are released into the medulla for cross presentation by resident dendritic cells.[7] Recent two-photon imaging studies lend further credence to the view that positive and negative selection are distinct processes which occur in specialized regions of the thymus. While DP thymocytes were confined and migrated randomly within the cortical regions of the thymus, SP thymocytes moved directionally toward the medulla where they again accumulated.[8] Historically, the requirement for recognition of nominally similar repertoires of peptide-MHC complexes for both survival of a select population and the death of others has been referred to as the selection paradox.

Our understanding of how TCR recognizes peptide-MHC complexes has been greatly advanced over the past two decades by studying the interaction between TCR transgenic T cells and their well-defined peptide-MHC complex ligands. We have gleaned much about the kinetics of these interactions from biophysical studies, and attempts have been made to correlate these kinetic parameters with signaling outcomes.[9-11] As we will present, much work has been done to determine the types of peptides that are capable of promoting positive selection of polyclonal T cell populations, but information about the identity of the actual peptides involved in in vivo selection is still limited. Identification of these peptides remains an issue of great interest not only due to their role in facilitating the signaling required for selection processes but also because low-affinity interactions resembling those encountered during positive selection seem to be important for homeostasis of mature T cells in the periphery.[12,13] We propose that sustained TCR signaling

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required for positive selection and lineage commitment is generated by serial, sometimes promiscuous, engagements with low-affinity self peptide MHC-complexes in the thymic cortex. Here we review the experiments done to determine the types of peptide repertoires that promote positive selection, how they are produced in vivo, and provide speculation about the organization of the signaling machinery that may explain the aforementioned patterns of signaling.[70]

CD4 and CD8 in Positive Selection

The kinetic signaling model proposes that all DP thymocytes are preprogrammed to initially downregulate CD8 expression upon transduction of signals through their TCRs. The factor that determines commitment to the CD4 or CD8 lineage after repression of CD8 expression is the ability or inability to sustain signaling through the TCR. Those which continue to signal past CD8 repression in response to MHC2 ligands and continue to maintain high CD4 expression will commit to the CD4 lineage; others will commit to the CD8 lineage. Bosselut and colleagues demonstrated that when both endogenous CD8 and CD8 under the control of the hCD2 promoter are coexpressed in the same thymocytes, only the CD8 controlled by the endogenous promoter is downregulated upon TCR signaling.[14] From these experiments, they concluded that the CD8 gene is transcriptionally repressed by TCR signaling in the DP stage. If the TCR present on the CD4 high, CD8 low thymocytes recognize MHC2 bound peptides, continued signaling through the TCR promotes the continued repression of CD8 expression and upregulation of CD4 transcription. Alternatively, if no signal is relayed through MHC2-bound peptides, coreceptor inversion ensues, and signals transduced through MHC1-bound peptides will effect commitment to the CD8 lineage. When Zap70 expression is confined to the DP stage, TCR signaling past this developmental stage is disrupted, and TCRs that are normally MHC2 restricted mature into the CD8 lineage.[15] In line with these data, Sarafova et al. demonstrated that, when transgenic CD4 molecules are downregulated under the control of a heterologous promoter during DP thymocyte signaling, MHC2 restricted thymocytes are again directed into the CD8 lineage.[16] Thus, continuous TCR signaling is necessary for commitment of DP thymocytes into the CD4 lineage. Evidence does exist, however, that transient downregulation of CD8 expression is not absolutely essential for direction of thymocytes into the CD8 lineage. In the H-Y antigen specific system, Robey et al. demonstrated that thymocytes that constitutively express transgenic CD8αβ along with the H-Y TCR were positively selected at a 5- to 6-fold higher frequency than thymocytes expressing only TCR and endogenous CD8.[17] Could a peptide repertoire that encourages continuous signaling through MHC2 restricted TCRs but frustrates signaling through the MHC1-restricted counterparts explain the observed signaling pattern? The experimental evidence for the types of peptides presented on MHC1 or MHC2 molecules in the thymic cortex appear consistent with this conjecture, as we describe below.

Repertoires for Positive Selection of MHC1-Restricted TCRs

Initial experimental systems used to define the nature of the positively selecting peptide repertoire in the thymus aimed to trim the repertoire to either a single peptide or sparse mixtures of peptides presented either on MHC1 or MHC2. Such systems allowed researchers to question whether special peptides were being generated solely for the purpose of carrying out positive selection. To study MHC1 restricted positive selection, β-2 microglobulin (β-2m)[18] and TAP1[19,20] knockout (KO) mice which effectively abrogated CD8 lineage T cell development were used. In these mice, the deletion of TAP1 or β-2m results in a drastic reduction in surface expression of MHC1. An increase in the level of MHC complexes ensues when exogenous peptides are added to TAP1-deficient cultures. In the case of β-2m deficient mice, the combined addition of recombinant β-2m and exogenous peptide stabilizes surface expression of MHC1 molecules, hence resulting in an increase in MHC1 surface levels.[18] Experiments were conducted in which thymocytes expressing particular MHC1 restricted transgenic TCRs were added into TAP1 and β-2m knockout fetal thymic organ culture (FTOC). Agonists or altered peptide ligands (APLs) for the TCR were then systematically added back to cultures in an effort to determine the identity of peptides capable of selecting them. For the lymphocytic choriomeningitis virus (LCMV) peptide specific TCR clone, P14, low and high concentrations of the LCMV peptide agonist added to the TAP1 KO FTOC caused positive and negative selection, respectively.[21] When the peptide was added back to the TAP1-sufficient FTOC, however, negative selection was induced even at low peptide concentrations. For the same TCR in the β-2m KO system, a qualitatively similar relationship was found where higher concentrations of peptide induced negative selection, while lower amounts induced positive selection.[22] A different strain of mice was bred which displayed L^d at either 35 or 2% of total MHC1 occupancy. These thymi were tested for their ability to positively select the 2C TCR which is normally negatively selected on L^d.[23] Although negative selection proceeded normally in the 35% L^d thymus, the 2C TCR was positively selected in the 2% L^d thymus. Further work in the β-2m KO system showed that an antagonist APL of a TCR specific for OVA peptide presented on K^b supported positive selection of T cells that expressed normal amounts of CD8 and TCR.[24] But when the antagonist was presented in the β-2m (+/-) system, it resulted in negative selection. Work in the F5 TCR model, specific for a peptide derived from the nucleoprotein of Influenza A, demonstrated that an antagonistic APL positively selected two times more thymocytes than the agonist peptide added to TAP1 deficient FTOC at identical concentrations.[25] More recently, mice were generated in which MHC1 molecules containing a single peptide were presented to a polyclonal population of developing thymocytes.[26] In this system, only 10% of the normal number of CD8 lineage cells developed. Although individual TCRs must have been selected by promiscuous recognition of the same peptide, mature T cells taken from these animals after immunization with particular peptides showed strict peptide specificity, as judged by in vitro

lysis of target cells loaded with the peptide used for immunization. Taken together, the body of work indicates that positive selection shows exquisite sensitivity for the strength of signal.

The next step in identification of positively selecting peptides led researchers to begin the difficult task of purifying peptides eluted from MHC1 molecules from wild-type thymi. For the OT-1 transgenic system, Hogquist et al. found seven naturally-occurring peptides which were capable of interacting with the TCR.[27] Capability of interaction with the TCR was gauged by culturing thymocytes with APCs presenting the various eluted peptides to mimic negative selection, which was manifested by "dulling" of coreceptor expression. Using a mass-spectrometry analysis, the identity of one of the peptides was determined, and they showed by FTOC add back experiments that it was capable of supporting positive selection. The sequence of the peptide, however, was only related to the agonist peptide at a few positions near the C terminus. Further work from the group using more sensitive detection techniques combined with bioinformatics tools rediscovered this peptide along with another derived from β-catenin.[28] The latter peptide acts as an antagonist for mature OT-I T cells, shares significant homology with the agonist ligand and is capable of supporting positive selection.

Consistent with the observation that limited TCR signaling during positive selection directs cells into the CD8 lineage and the demonstrated role for TCR antagonists, recent work has now shown that the antigen-processing machinery in the thymic cortex is poised to generate a repertoire of peptide-MHC complexes that is likely inhibitory toward MHC1 restricted TCR signaling.[29-31] A special proteosome, aptly termed the thymoproteosome, has recently been described. It is expressed in 70–80% of cTECs and has not been detected in other tissues. Due to the incorporation of the special subunit, β5t, into the 20s proteolytic site of the traditional proteosome, the thymoproteosome does not cleave polypeptides at hydrophobic amino acid residues. A review of biophysical data of the nearly 1,500 peptides presented by human MHC1 alleles showed that 80% of peptides loaded with high affinity possess hydrophobic amino acids at the C terminus.[30] The N terminal anchor shows less stringent specificity. Neither the thymic architecture nor the level of MHC1 expression are altered in mice deficient in the β5t subunit, but the knockout does result in a reduction in the number of CD8 T cells expressing high levels of TCR.[31] CD4 lineage T cell development in these animals remains unaffected. Others have suggested that MHC1 molecules loaded with low-binding affinity peptides, like those generated by cTECs, imply that two types of MHC ligands which have not been traditionally regarded as ligands for positive selection are likely to be presented on cTECs.[32] These namely include MHC-peptide complexes in which only one side of the peptide is anchored or empty MHC molecules from which peptides have completely disassociated. Antibodies that recognize MHC molecules in a peptide-dependent and independent manner have provided supporting evidence for the presence of such complexes on cortical cells but not within the medulla.[33] The presentation of these types of MHC ligands, then, could be a mechanism that precludes high-affinity interactions between TCR and peptide-MHC1 complexes during positive selection. Since knockout of the β5t subunit does not significantly alter MHC1 expression, its role could be envisioned as diluting the pool of available positively selecting interactions such that signaling through MHC1-restricted TCRs happens only very infrequently, allowing CD8 T cell lineage commitment to occur.

Repertoires for Positive Selection of MHC2 Restricted TCRs

For the study of MHC2 restricted positive selection, model systems were generated in which single peptides were presented to developing polyclonal thymocytes. MHC2 molecules are synthesized and transported to peptide loading compartments associated with the Invariant chain (Ii).[34] The segment of the Ii loaded in the groove of MHC2 is known as the CLIP peptide. At this stage, the Ii is trimmed, resulting in CLIP peptide loaded MHC2 molecules. H-2M then chaperones the loading of other peptides to replace the CLIP peptide. In the H-2M knockout mouse, surface MHC2 molecules are almost exclusively loaded with CLIP.[35,36] This system supported the development of a repertoire 50% of the normal size.[36,37] A similar line of mice was developed in which the Invariant chain was knocked out, and a transgene encoding a mutated Invariant chain was inserted. The Eα peptide replaced the CLIP region of the Invariant chain,[38] and 95% of MHC2 molecules presented the Eα peptide. Normal numbers of CD4 lineage T cells managed to develop. Observations from these systems initially supported the notion that MHC2-dependent positive selection is almost wholly degenerate with respect to peptide recognition. Further experiments, however, in both the H-2M knockout and Ii:pEα mice disputed that conclusion. When MHC2 molecules containing non-CLIP peptides were excluded via antibody blockade in H-2M knockout FTOC, CD4 lineage T cell selection was significantly diminished.[39] Likewise, in the Ii:pEα mice, when H-2M was knocked out, 70% of the T cells which had previously developed were lost.[38] Complementary to this observation, it was shown that 70–80% of TCRs selected on a predominant CLIP epitope were not tolerant and should have been negatively selected as judged by reactivity toward wild-type splenic APCs.[40] In combination, it was possible to conclude that the small fraction of self peptides being presented at very low levels in both of the mice were responsible for effectuating the majority of the positive selection which was occurring.[41]

Experiments were also conducted to determine the ability of specific APLs to induce positive selection of CD4 lineage transgenic T cells. For a pigeon cytochrome *C* (PCC)[43-58] peptide-specific TCR, injections of agonist peptide alone or a mixture of antagonist and agonist peptides were given to Ii, H-2M double KO mice.[42] When injected alone, agonist peptide presumably repopulated MHC molecules in the thymus and supported positive selection of the transgenic T cells that continued for up to 2 weeks. Coinjection of antagonist peptide disrupted the positive selection. With the 2B4 TCR specific for pigeon cytochrome *C* (PCC) peptide, Spain et al. demonstrated via an FTOC approach that antagonist APLs block positive selection, and agonist peptides induce deletion.[43] The differences in outcome achieved with injected agonist peptides may be due to less efficient restoration

of peptide presentation within the thymus. More than a decade passed before experiments were conducted to determine the identity of natural peptides that positively select MHC2 restricted TCRs, which were sought out largely as tools for studying signaling in mature, peripheral T cells in response to peptides that promoted their positive selection. For both the AND and 5C.C7 TCRs, peptides from libraries were screened for their ability to sustain positive selection.[44,45] For the 5C.C7 TCR, using an FTOC approach, a handful of peptides were identified, and the peptide that supported the greatest development of CD4 cells was structurally related to its natural agonist, PCC.[44] A similar library-screening approach was used to identify positively selecting ligands for the AND TCR.[45] Of 95 peptides in the library, only one was shown to support positive selection of thymocytes bearing the AND receptor—gp250, derived from a protein with no relationship to the natural source of the agonist ligand and nonhomologous to the agonist peptide. Both identified ligands for these MHC2-restricted TCRs were able to augment the activation and survival of peripheral CD4 lineage T cells,[44] in seeming contrast to the antagonistic ligands involved in the selection of MHC1 restricted T cells.

In the processing of peptides presented on MHC2 molecules, a proteolytic pathway that operates exclusively in the thymic cortex has been identified.[46,47] Cathepsin L is a lysosomal endoproteinase responsible for degradation of Ii solely in the thymic cortex. Staining with an antibody specific for I-A^b complexed to CLIP showed increased staining of the cortex of cathepsin L KO mice versus wild-type animals, while staining of the medulla was similar in both cases. Flow cytometric analysis performed on peripheral lymphoid cells did not show skewing toward exclusive presentation of the CLIP peptide, furthering the case for altered MHC2 antigen presentation in the thymic cortex.[46] In the KO mouse, a 60–80% reduction in the generation of $CD4^+$ T cells is observed along with a relative increase in the number of CD8 lineage cells. The method by which cathepsin L ultimately mediates presentation of a distinct class of positively selecting peptides still remains unclear.

TCR Microclusters

When T cells interact with APCs, a patterning of interactions takes place in the interface connecting the two cells. This patterning has been described as an immunological synapse, which is characterized by a bull's eye pattern consisting of TCR-peptide-MHC interactions in a central cluster surrounded by a ring of adhesion molecules.[48,49] More recently, a smaller scale clustering of TCRs in response to peptide-MHC has been demonstrated, and these structures have been called TCR microclusters.[50-52] These microclusters are dynamically formed at the periphery of the contact area where they engage antigen and initiate signaling.[53,54] Signaling molecules and kinases, such as Lck, Zap-70, LAT, SLP-76,[50,52,54] and Grb2,[55] are localized to TCR microclusters, which translocate from the periphery of the synapse to the center in an actin dependent manner where signaling is terminated,[51] although the precise function of the cSMAC remains unresolved.[56] TCR microclusters form continuously, thus providing a mechanism for sustained signaling. Interestingly, CD28 interactions with CD80 or CTLA-4 have also been shown to localize to the microclusters.[57,58] Neither the molecular mechanisms nor the physical properties that localize these co-stimulatory interactions to microclusters are known, but they highlight the importance of microclusters as a specialized site for signaling. Quantitative analysis of TCR numbers demonstrated that the ratio of TCR to peptide-MHC complexes within a microcluster is large. Hence, high TCR density seems to be required for TCR microcluster formation, although one report demonstrated that mature CD8 lineage cells displaying 1,000 or less TCRs ($1/20^{th}$ of the normal level) were capable of both proliferation and cytokine production in response to in vivo immunization with agonist peptide.[59] The localization of CD4 or CD8 co-receptors with respect to TCR microclusters is not known, although models of TCR triggering such as the pseudo-dimer model[60] would predict the localization of CD4 to TCR microclusters. As a result, the method by which CD4 or CD8 might contribute to microcluster formation is not known. It is however known that active signaling via Src family kinases is not required for microcluster formation.[50]

Specialized Signaling in DP Thymocytes

While the recognition of self-peptide MHC complexes in the thymus is certainly similar to recognition of antigen in the periphery, the responses of DP thymocytes to the peptide-MHC complexes presented in the thymus are distinct. DP thymocytes probably have a very different distribution of signaling molecules (kinases, phosphatases and adapters) compared to peripheral T cells. This is partially reflected by the differential expression of micro-RNA, mir-181a, that is expressed only in thymocytes and downregulates phosphatase expression in DP cells.[44,61] When thymocytes interact with APCs bearing positively selecting ligands, there is no formation of an immunological synapse.[62] And in direct contrast, transgenic DP thymocytes interacting with negatively selecting ligands form a structure in which multiple TCR-rich foci with extensive tyrosine phosphorylation are present.[63] Two photon imaging of thymocytes interacting with stromal cells in thymic organ culture showed thymocytes displaying both long-lived contacts and transient interactions.[64] Further studies in which thymocytes were visualized in thymic tissue sections revealed that thymocyte stopping correlated with sustained oscillations in the concentration of intracellular calcium,[65] which is reminiscent of the stopping behavior of mature T cells in response to agonist peptides. Additionally, Davey et al. demonstrated that while preselection DP thymocytes, SP thymocytes and mature T cells in the OT-I transgenic system upregulate CD69 equivalently when stimulated with strong agonist ligands, only preselection DP thymocytes show upregulation in response to agonists of weaker affinity.[66] Similarly, in the 5C.C7 system, 90% of DP thymocytes upregulated CD69 in response to a low affinity APL at a peptide dose of 1 μM, while a maximum of 40% of mature T cells showed upregulation at a 100-fold higher peptide dose.[67] And as a further indication of the heightened sensitivity of thymocytes to TCR signals, it has been recently shown that as few as two agonist peptide-MHC complexes could induce

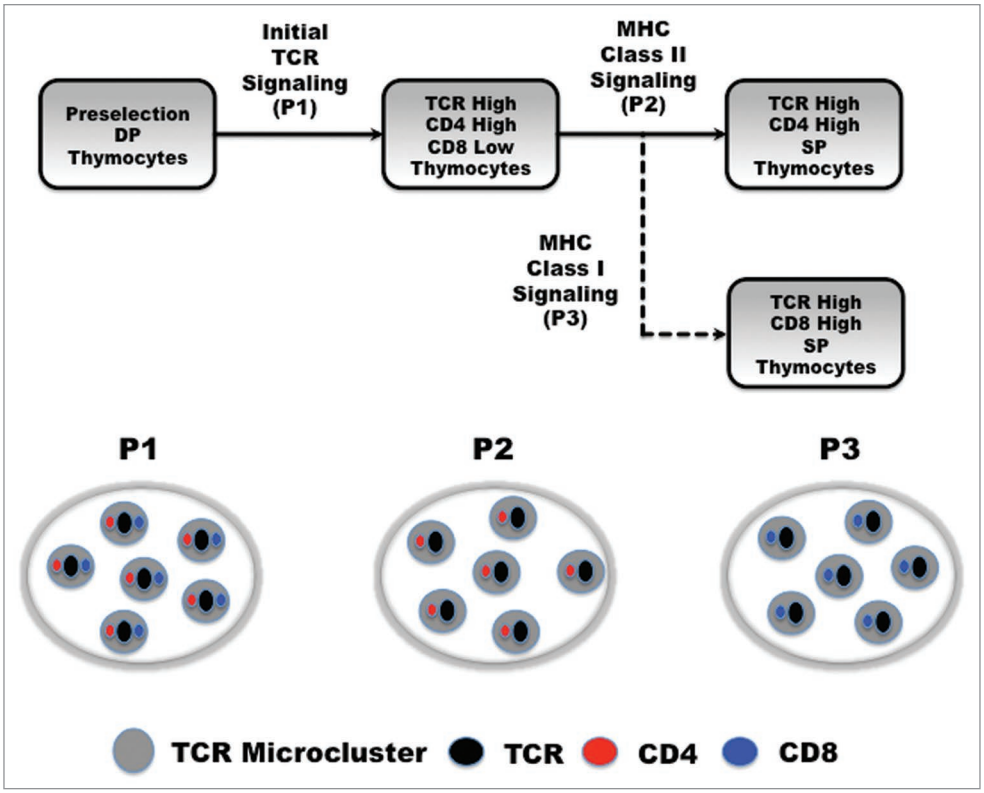

Figure 1. Stage dependent TCR microcluster formation during positive selection. TCR-low preselection DP thymocytes enter the first phase (P1) of positive selection during which transduction of weak signals is facilitated by specialized TCR microclusters (lower) containing both CD4 and CD8. We propose that these microclusters allow for promiscuous recognition of peptides bound to thymic MHC molecules. As TCR signaling continues, Phase 2 begins (P2), TCR and CD4 are upregulated, and CD8 expression is suppressed. At this point, signaling microclusters contain primarily CD4 and TCR, and TCRs must recognize peptides bound to MHC2 molecules with high affinity in order to sustain TCR signaling. If signaling continues through this stage, thymocytes will commit to the CD4 lineage. If not, the lack of TCR signals will cause coreceptor inversion, resulting in microclusters that contain CD8 and TCR alone, Phase 3 (P3). TCRs in this stage which signal through peptides bound to MHC1 molecules will then commit to the CD8 lineage.

apoptosis of DP thymocytes.[62] Thus, by virtue of their distinct phenotype, thymocytes are poised to react differently to the same types of signals received by mature T cells. While mature T cells are singly positive for either CD4 or CD8 and specific toward peptide epitopes presented either by MHC1 or MHC2, preselection thymocytes are doubly positive for both coreceptors and express them at elevated levels. One consequence of high levels of coreceptor expression is that Lck in DP thymocytes can be sequestered to the tails of the CD4 and CD8 coreceptors,[68] perhaps allowing for rapid and highly efficient phosphorylation of ITAMS in response to TCR triggering.

Based on these observations, we propose that the specificity of peptide recognition by thymocytes during T cell development exists as a continuum (**Fig. 1**). During the initial phase of positive selection (**Fig. 1**, P1) when TCR levels are low, the dual presence of both CD4 and CD8 coreceptors serves to stabilize all available interactions between TCR and peptide-MHC complexes. Due to the possible presence of a large proportion of suboptimal ligands, these interactions are likely to dictate microcluster formation in an entirely peptide promiscuous manner. This raises the possibility that when DP thymocytes can recognize self-peptide-MHC complexes and do not die by neglect, they form signaling microclusters containing TCR, CD4 and CD8 that may recognize many self-peptide-MHC complexes. This promiscuous signal transduction would be amplified by the reduction in phosphatase activity in DP thymocytes described previously. As signals are transduced through TCRs on DP cells, upregulation of CD4 transcription ensues, TCR levels begin to rise, and surface expression of CD8 wanes. In this second phase (**Fig. 1**, P2), the patterns of TCR signaling will dictate lineage commitment. Signaling microclusters may now contain only CD4 and TCR, requiring peptide-MHC recognition to become more specific. If the TCR recognizes peptides presented by resident MHC2 complexes with high enough affinity to sustain TCR signals, the cell will commit to the CD4 lineage. If not, CD4 transcription will be silenced, and coreceptor inversion will occur. In this third phase, (**Fig. 1**, P3), TCR microclusters will now predominantly contain CD8 and TCR, and commitment to the CD8 lineage will depend on the ability of the TCR to transmit signals via recognition of peptides presented on MHC1 molecules. An analysis of peptides generated in the cortex suggests that the thymoproteosome exists to limit the presentation of stimulatory peptides by MHC1 molecules; however, this may be compensated for by the relatively high affinity of CD8 for MHC1, which would permit signaling

via low abundance MHC1-peptide complexes in the thymus. In fact, the lack of sialic acid capping of CD8 in thymocytes has been shown to increase the avidity of the CD8-MHC1 interaction.[69] Finally, these lineage-committed SP cells will proceed forward to be tested for reactivity towards specific self peptides during the process of negative selection. Thus, our model for T cell selection contends that thymocytes will recognize peptide-MHC complexes with a spectrum of affinities that is dictated by the dynamics of the organization of TCR microclusters as they proceed through the gauntlet of thymic selection.

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