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A long journey coming to fruition: In sight of the pre-selection T-cell repertoire

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Abstract

In addition to MHC restriction and its structural correlate, the recognition of an MHC-peptide complex by the T-cell antigen receptor (TCR), T-cell reactivity is constrained by positive and negative selection in the thymus. While mouse genetic studies have provided compelling evidence for both processes, the actual impact of selection on the mature T-cell repertoire has remained difficult to assess, in particular because it has so far not been possible to follow the intrathymic differentiation of antigen-specific T cells carrying endogenous TCR specificities. In this issue of the *European Journal of Immunology*, Hesnard et al. [*Eur. J. Immunol.* 2016. 46: XXXX-XXXX] report the detection of human antigen-specific immature thymocytes, thereby opening the way to addressing these questions. Here we discuss the implications of this technological advance.

Keywords

MHC restriction; T-cell repertoire; thymocytes; TCR; tetramers

Landmark studies in the early days of T-cell immunology, together with the framework provided by the clonal selection theory, have established the three basic principles that underlie our understanding of T-cell reactivity [1, 2]. First, the T-cell antigen receptor (TCR) recognizes a complex formed between a peptide antigen and an MHC molecule, and both peptide and MHC determinants contribute to TCR antigen recognition [3]. Second, T cells reacting against a particular MHC-peptide complex are selected in the thymus, through TCR triggering by complexes of self peptides with the same MHC molecule, a process called positive selection [4]. Third, T cells with excessive self reactivity are deleted in the thymus, a process called negative selection [5]. Although these principles have long been recognized, how thymic selection shapes the mature T-cell repertoire from the ‘pre-selection’ collection of TCRs generated by Rag-mediated TCR gene rearrangement has remained unclear [6]. In this issue of the *European Journal of Immunology*, Hesnard et al. report the identification of antigen-specific pre-selection thymocytes [7], giving a first glimpse at the pre-selection repertoire. This study opens the door to a quantitative

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assessment of basic tenets of developmental immunology, including the impact and MHC specificity of positive selection signals in the thymus.

The study builds on previous work by the same group assessing the human naïve T-cell repertoire [8]. The authors' approach, based on techniques pioneered by the Davis and Jenkins groups [9, 10], uses flow cytometry to enumerate T cells recognizing a specific MHC-peptide complex (under a tetramerized form to stabilize binding and facilitate detection). When preceded by bead enrichment, tetramers can detect low frequency (down to 10^{-6}) T cells specific for a given MHC-peptide complex [10]. Applied to healthy individuals expressing the MHC class I (MHC-I) molecule HLA-A2 (referred to A2 hereafter), this procedure detects naïve blood CD8⁺ T cells specific for A2-peptide complexes, at frequencies varying between 5×10^{-6} and 5×10^{-3} , depending on the peptide tested, with substantial inter-individual variability [8]. However, and unexpectedly, A2-peptide specific CD8⁺ T cells are also detected in individuals not carrying A2 [8, 11]. The frequency of A2-peptide specific in A2-negative individuals is substantial, 10–20% of that seen in A2-positive counterparts, and well above the detection background. Recognition of A2-peptide complexes is associated with signaling, as cell lines derived from A2-negative individuals have been shown to respond to A2-peptide complexes to the same extent as cell lines derived from A2-positive donors [7, 8]. In contrast, Hesnard et al. show that only a subset of cell lines derived from A2-negative individuals are truly allo-reactive, i.e. reacting against A2 coupled with irrelevant peptides, and none showed reactivity against other MHC alleles [7].

These experiments suggested that T-cell recognition of a given MHC-peptide complex does not strictly require that the cell develops in a thymus expressing the same MHC allele, conflicting with the classical notion of positive selection. This is not unique to MHC-I recognition, as similar observations had been reported earlier for MHC class II (MHC II)-restricted cells, both in humans and mice [12]. However, these previous reports did not address the respective contribution of thymic and post-thymic mechanisms to the generation of such 'promiscuous' T cells (i.e. binding a given MHC-peptide complex although generated in a thymus lacking that particular MHC molecule). In humans, the frequency of 'A2- promiscuous' cells is higher for self than non-self peptides [7, 8]. Thus, even though 'promiscuous' cells have a naïve phenotype, the possibility remained that their high frequency in the blood results from preferential post-thymic amplification, or survival upon self-antigen recognition, of a marginal population generated in the thymus.

Hesnard et al. address this question by following the development of A2-peptide specific cells during human thymic development [7, 13]. Consistent with the previous studies of peripheral blood, A2-peptide specific CD4⁺CD8⁺ (CD8 'single positive', SP) thymocytes, most of which are mature cells, are detected in A2-expressing thymi, with frequencies similar to those seen in the peripheral blood [7]. Quite remarkably, these analyses are sensitive enough to detect tetramer-binding cells among CD4⁺CD8⁺ (double positive, DP) thymocytes, the population in the thymus that carries the 'untouched' pre-selection TCR repertoire, and reflects the diversity generated by the Rag-mediated TCR gene rearrangement process (Figure 1) [4]. For all peptide specificities analyzed by Hesnard et al., the frequency of A2-peptide-specific cells is 3–15 times greater among CD8 SP than DP

thymocytes; such an enrichment indicates positive selection by endogenous A2-peptide complexes.

Having demonstrated they can track antigen-specific thymocytes during positive selection, the authors then examine A2-negative thymi, in which A2-specific cells are not expected to undergo positive selection. Consistent with the idea that immature DP cells are representative of the pre-selection repertoire, the frequency of A2-specific cells among DP thymocytes is similar in A2-negative and A2-positive thymi [7]. Furthermore, positive selection of A2-specific cells is easily detected for most specificities, with a 3–5 fold enrichment in CD8 SP over DP thymocytes. Irrespective of thymic A2 expression, A2-specific CD8 SP thymocytes express surface markers indicative of TCR engagement. Thus, answering the original question, the population of A2-peptide specific T cells in A2-negative individuals results from efficient positive selection in an A2-negative thymus rather than simply from peripheral amplification (although the survival of such cells could be facilitated by the same MHC-peptide that drive their selection in the thymus).

These findings have deep implications for our understanding and future analyses of T-cell development. First, the detection of antigen-specific thymocytes in an unmanipulated thymus represents an important technical advance. In the mouse, this had been achieved in situations where the frequency of antigen-specific cells was enriched in the pool of thymic precursors (by expression of a specific TCR β chain) [14]. However, the low frequency of precursors, together with the low surface TCR expression characteristic of DP thymocytes, have both hampered the identification of unmanipulated antigen-specific pre-selection thymocytes. As a result, the impact of positive selection has so far been studied essentially using TCR $\alpha\beta$ transgenic thymocytes, which all carry the same defined antigen specificity [2]. However, such studies have important limitations, notably linked to premature expression of the TCR $\alpha\beta$ transgene and to the unphysiological over-representation of cells carrying a single TCR specificity [15, 16].

From a conceptual standpoint, the unbiased, even if so far limited, estimate of the pre-selection repertoire suggests a strong correlation between the frequencies of pre-selection DP and post-selection CD8 SP thymocytes. This supports the idea that, for any given peptide, the Rag-mediated TCR rearrangement process is a key limiting factor constraining the depth of the mature T-cell repertoire, in addition to or instead of availability of peptides for positive or negative selection. Future studies will dig deeper into this question, exploring the contribution of each factor to repertoire formation. Given the low frequency of cells specific for some of the peptides studied by Hesnard et al, additional refinements will help to validate the current conclusions. As highlighted by the authors, it remains possible that MHC tetramers do not detect all possible pre-selection thymocytes [7]. Conversely, it will be important to verify the specificity of peptide recognition.

Hesnard et al. show that positive selection is not strictly MHC-allele specific, since A2-peptide reactive cells are generated in A2-negative thymi. While studies in the mouse had hinted at this possibility [17], the new findings by indicate that it is a more general process than previously envisioned, and the concept likely applies to MHC II-restricted cells as well [12]. One key question raised by these findings is the respective part of positive and

negative selection in the enrichment of A2-specific cells in CD8 SP over DP thymocytes. Previous mouse studies [12] suggest that a higher fraction of A2-reactive cells would be deleted in an A2-positive than that in an A2-negative thymus, because of encounters with A2-self peptide complexes. In this perspective, the apparent enrichment of A2-specific cells in A2-positive thymi would underestimate the impact of A2 expression on positive selection. However, it will be interesting to address this issue by further delineating selection intermediates in the human thymus: as discussed below, the intra-thymic deletion of self MHC-peptide reactive cells may not be as frequent as conventionally envisioned.

The molecular bases for the promiscuity in MHC recognition revealed by these studies remain to be explored. It is possible that it simply reflects a high degree of structural similarity between A2 and positively selecting MHC alleles in A2-negative thymi, or, more broadly, a bias of germline-encoded TCR regions towards MHC recognition [18, 19]. Alternatively, it is possible that recognition of A2 and non-A2 peptide complexes by the same TCR involve radically distinct spatial TCR positioning and interaction with the MHC-peptide interface, as has been documented in studies of alloreactive TCRs [20, 21].

One surprising finding of the study by Hesnard et al. is the higher frequency of CD8⁺ T cells specific for self-peptides, relative to non-self [7]; at first glance, this observation is contradictory with the concept of negative selection, which, in its simplest form, predicts that such cells should be deleted in the thymus. However, recent assessments of the impact of negative selection have altered this simple view. Although deletion appears to be a common fate for thymocytes with broad MHC cross-reactivity, whether MHC-I or MHC-II [22], many cells specific for self MHC-peptide, notably for tissue-specific antigens with limited thymic expression, escape deletion in the thymus [23–25]. Current evidence suggests that, when MHC I-restricted, such escapees are functionally inactive, whereas MHC II-restricted escapees preferentially differentiate into regulatory cells, therefore inhibiting auto-immune responses [23–25].

Last, but not least, the study by Hesnard et al., together with previous observations from the same group [8, 26], raises tantalizing questions on the mechanisms of CD4-CD8 lineage differentiation, and their impact on T cell functions. Indeed, a substantial fraction of A2-peptide specific T cells are CD4⁺CD8[−], in both thymus and blood [7, 8]. While it could be conceived that these cells are selected by A2 but ‘misdirected’ towards a CD4⁺ fate during thymic development, two observations support the idea that they are in fact selected by MHC-II molecules. First, the same group previously reported that the frequency of A2-reactive CD4⁺ T cells is not reduced in individuals carrying mutations in the TAP1 peptide transporter, which impair MHC-I expression and selection of MHC I-restricted cells [8]; in contrast, and as expected, TAP1 mutations strongly reduce the size of the A2-reactive CD8⁺ population. Second, Hesnard et al. now show that the frequency of A2-specific CD4 SP thymocytes and T cells is independent of A2 expression [7].

These findings imply that a fraction of the MHC II-restricted repertoire is MHC-I reactive. That is, even though thymocytes with both MHC-I and MHC-II reactivity appear to be prime targets for deletion [22], a subset of them retains the ability to differentiate into CD4⁺ T cells. Are such cross-reactive cells preferentially selected into the CD4⁺ lineage; that is,

are CD4⁺-lineage differentiation signals dominant over those driving CD8⁺-lineage differentiation? Studies in the mouse suggest that this is the case, as very few CD8⁺ T cells recognize MHC II-peptide tetramers [10]. Last, what is the functional significance of such cross-reactive cells? When stimulated by relevant A2-bound peptides, they showed strong cytotoxicity and IFN- γ production [26]. This was the case even for cells recognizing self antigens, suggesting that this A2-specific CD4⁺ repertoire had not been inactivated or directed towards a Treg-cell fate. Whether these cells exhibit similar reactivity towards MHC-II-bound peptides, or, to the contrary, whether MHC-II recognition contributes to maintain them in an unresponsive state in vivo, remains to be determined.

In summary, the preview of the pre-selection T-cell repertoire reported by Hesnard et al. gives us new insights into the impact of selection on T-cell reactivity. The study raises important questions for future analyses, most notably the respective parts of positive and negative selection in shaping the repertoire, and adds a critical new tool to address them.

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Figure 1.

Pre-selection DP thymocytes in an A2-positive thymus (a) express a highly diverse TCR repertoire (colored symbols), which samples the potential diversity achievable by Rag-mediated TCR gene rearrangement. The study by Hesnard et al. [7] offers a direct assessment of the frequency of antigen (A2-peptide)-specific cells (thymocytes with TCRs represented as half-circles, middle panel) among pre-selection thymocytes. Unexpectedly, A2-peptide specific cells also undergo selection in A2-negative thymi (b, other components not repeated for simplicity), indicating that selection is not strictly MHC-allele specific.