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Deciphering the unusual HLA-A2/Melan-A/MART-1-specific TCR repertoire in humans

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See accompanying article by Pinto et al.

Abstract

The Melan-A/MART-1_{26-35} antigenic peptide is one of the best studied human tumor-associated antigens. It is expressed in healthy melanocytes and malignant melanoma and is recognized by $CD8^+$ T cells in the context of the MHC class I molecule HLA-A*0201. While an unusually large repertoire of $CD8^+$ T cells specific for this antigen has been documented, the reasons for its generation have remained elusive. In this issue of the *European Journal of Immunology*, Pinto et al. [Eur. J. Immunol. 2014. 44: 2811-2821] uncover one important mechanism by comparing the thymic expression of the *Melan-A* gene to that in the melanocyte lineage. This study shows that medullary thymic epithelial cells (mTECs) dominantly express a truncated *Melan-A* transcript, the product of mis-initiation of transcription. Consequently, the protein product in mTECs lacks the immunodominant epitope spanning residues 26-35, thus precluding central tolerance to this antigen. In contrast, melanocytes and melanoma tumor cells express almost exclusively the full-length *Melan-A* transcript, thus providing the target antigen for efficient recognition by HLA-A2-restricted $CD8^+$ T cells. The frequency of these alternative gene transcription modes may be more common than previously appreciated and may represent an important factor modulating the efficiency of central tolerance induction in the thymus.

One of the first $CD8^+$ T-cell-defined tumor antigens to be identified in humans was the nonapeptide AAGIGILTV, encoded by the Melan-A/MART-1 cDNA [1-3]. This peptide lacks the canonical strong anchor residue at P2 and binds with weak affinity to HLA-A2. Nevertheless, the antigen is strongly immunodominant, as it turned out to be the most frequently recognized peptide by specific $CD8^+$ cytolytic T lymphocytes (CTLs) from tumor-infiltrating lymphocyte (TIL) populations tested from the majority of HLA-$A2^+$ melanoma patients [4,5]. Soon after, it was shown that the decapeptide product, Melan-A_{26-35} (EAAGIGILTV), extended by one residue (Glu) at the amino terminal end, is a more potent antigen than the nonapeptide [6], suggesting that the decapeptide is in fact the optimal length antigenic peptide. This notion was reinforced by the observation that substitution of Ala for Ile at position two of the decapeptide (ELAGIGILTV) leads to a strong increase in both binding to HLA-A2 and efficiency of recognition by CTLs [7]. Intriguingly, the same substitution, when placed at position two of the nonapeptide (ALGIGILTV), while leading to enhanced binding to HLA-A2, as expected, abrogates recognition by specific CTLs but when at position one (LAGIGILTV) both binds well to HLA-A2 and is efficiently recognized by the majority of Melan-A/MART-1-specific clones. The elucidation of the three dimensional structure of the nona- and decapeptide complexes showed that the natural nona- or decapeptide may adopt two different conformations: a stretched out one (nonapeptide), or a bulged-zigzag one (decapeptide). It appears that the Melan-A/MART-1 antigen-specific T-cell repertoire is greatly biased, as T-cell clones from cancer patients exhibit selective specificity for the zigzag conformation, the one favored by the Ala-substituted decapeptide as well as at position one of the nonapeptide [8]. In turn, clones specific for the stretched out conformation are rarely observed and they may be broadly cross reactive with other bound peptide conformations [9].

The identification of the stable HLA-A2 binding Melan-A/MART-1 analog peptide, ELAGIGILTV, that is well recognized by specific CTL clones, allowed the assembly of stable HLA-A2/analog decapeptide tetramers for the direct identification of MART-1-specific T cells [10]. With such a tool it was possible to directly quantify the levels of Melan-A/MART-1-specific $CD8^+$ T cells in advanced melanoma patients. In line with the findings from the pre-tetramer era, it became clear that TILs do contain high frequencies of Melan-A-specific T cells in close to two thirds of melanoma patients examined. Those cells were also regularly found in peripheral blood lymphocytes of melanoma patients, albeit at frequencies that were at least one order of magnitude lower than in TILs. In both cases, the majority of these cells had a typical effector memory phenotype ($CD45RO^+/CD45RA^-$).

The use of HLA-A2/Melan-A/MART-1 peptide tetramers soon revealed that practically all HLA-$A2^+$ healthy subjects surveyed also had a relatively high frequency of tetramer-binding $CD8^+$ T cells in their circulation [11], found to be on average 0.07% in a relatively large screen of HLA-A2 donors without melanoma [12]. Interestingly, tetramer-binding $CD8^+$ T cells are also detectable in HLA-A2-negative healthy subjects at frequencies that are barely detectable ex vivo and approximately one order of magnitude lower than those detected in the HLA-$A2^+$ individuals [13]. In both HLA-$A2^+$ and $A2^-$ healthy donors, the phenotype and functional profile of these tetramer-binding $CD8^+$ T cells are indistinguishable from that of the naïve $CD8^+$ T-cell pool [13]. These findings were surprising and had no precedent in either the human or the mouse immune systems. For most other epitopes of $CD8^+$ and also $CD4^+$ T cells, the precursor frequency of naïve cells is far below the limit of detection of tetramers by ex vivo, multiparameter flow cytometry analyses. The estimates of such frequencies after magnetic bead pull down of $tetramer^+$ T cells have been approximated at one specific T cell per one million T cells [14,15]. In fact, the frequencies of Melan-A/MART-1-specific $CD8^+$T cells in healthy individuals are comparable to those measured of T cells specific for some viral epitopes. In sharp contrast, however, T cells specific for viral epitopes are phenotypically and functionally antigen-experienced memory T cells, corresponding to the previous exposure to the respective antigens [16].

Thus, the question was how such an abundant repertoire of naïve antigen-specific T cells could be generated, at least a hundred times more abundant than most other antigen-specific naïve T-cell precursors measured by tetramer binding assays. Two major reasons have emerged upon careful study of these cells in the human thymus and the composition of their TCR repertoire. It became clear, on the one hand, that a significant proportion of human subjects (more than half) contain detectable Melan-A/MART-1 $tetramer^+$ $CD8^+$ T cells in cord blood lymphocytes. Moreover, these cells are also measurable in single $CD8^+$ thymocytes in thymuses from children. Thus, it appears that a high thymic output is one of the reasons for the high frequency of these cells. This is coupled with a slow in vivo turnover of these cells during adult life, as could be directly estimated by measuring two tell-tale features of proliferative history in human lymphocytes: the length of chromosomal telomeres and the levels of TCR-alpha excision circles [17]. To this day, the remarkable stability of the naïve Melan-A specific T-cell repertoire remains most intriguing. Indeed, the antigen Melan-A is normally expressed by melanocytes and even keratinocytes which receive melanosomes containing the Melan-A/MART-1 polypeptide. It is thus conceivable that enough skin inflammatory events accumulate over a lifetime to provide

Melan-A/MART-1 antigen to activated dendritic cells and prime Melan-A/MART-1-specific $CD8^+$ T-cell responses. Yet, this has been documented in rare cases. The only instance in which priming of these responses has been shown to occur in a consistent manner is in HLA-$A2^+$ individuals with advanced metastatic melanoma [4,10]. On the other hand, a peculiar structural feature also contributes to the abundance of Melan-A $tetramer^+$ $CD8^+$ T cells independently of the expression of the HLA-A2+ presenting allele. The TRAV-12-2 (formerly known as Va2) TCR segment is overrepresented in $CD8^+$ T cells of this specificity so that over 90% of specific T cells express this particular segment, compared to an overall frequency in bulk $CD8^+$ T cells of 6 – 8%.

At this juncture, there were still major questions left open. What is the exact reason for the preferential selection of the TRAV12-2 segment-containing TCR alpha chains? What supports a robust thymic output of Melan-A/MART-1-specific TCRs in the face of detectable expression of the Melan-A gene in mTECs [18]?. Should thymic expression of Melan-A/MART-1 not lead to the negative selection of high-affinity, specific $CD8^+$ T cells? It was assumed that this was probably the case and that the repertoire was devoid of the latter cells. The measurement of specific TCR binding parameters, however, suggested the existence of a large range of avidities [19] (and Rufer et al., data not shown).

In this issue, Sheena Pinto et al. [20] elegantly provide definitive answers for these two questions. First, the authors introduced a single codon mutation, changing glutamine at position 31 of the CDR1 domain encoded in the TRAV12-2 gene segment, and could practically abrogate tetramer binding by T cells made to express the mutant TCR. This experiment nicely confirms and extends the structural data provided earlier by another group on the three dimensional structure of a HLA-A2/Melan-A/TCR pentamolecular complex [21]. Thus, this CDR1□ encoded in the germ line, exhibits selective affinity for the complex HLA-A2/ELAGIGILTV, whereby multiple electrostatic interactions formed between Gln on the CDR1 domain and several amino acid residues, including Glu at P1, on the antigenic peptide provide most of the binding energy. This is also the likely explanation for the high frequency of "allorestricted" tetramer binding $CD8^+$ T cells found in most HLA-$A2^-$ individuals.

Second, the apparent paradox of productive thymic output of self/Melan-A-specific TCRs with a wide range of avidities despite the expression of Melan-A transcripts in the mTECs is now resolved in an unexpected and interesting fashion. Pinto et al. report that the predominant Melan-A transcript that can be found in mTECs is a truncated one, the product of mis-initiation

of transcription [20]. Consequently, the protein product lacks the immunodominant epitope as the first three exons are not transcribed. Thus, the epitope spanning residues 26-35 is not expressed in mTECs and central tolerance is simply not operating in this particular instance (Figure 1). Indeed, the original observation of Melan-A gene expression in mTECs had been done using primers that amplified the transcripts from a 5' sequence in exon 4 [18}. In contrast, melanocytes and melanoma tumor cells express almost exclusively the full length Melan-A transcript thus providing the target antigen for efficient recognition by HLA-A2-restricted $CD8^+$ T cells. These findings illustrate what appears to be a major difference between tissue-restricted gene expression and promiscuous ectopic gene expression in thymic mTECs. According to Pinto et al., the frequency of this alternative gene transcription modes may be more common than previously appreciated and may represent an important source of escape from central tolerance [20].

Taken together, the steady flow of studies on this melanocyte/melanoma tumor antigen makes Melan-A/MART-1 one of the best understood T-cell antigens. The specific TCR repertoire is unique and has provided a useful tool to studying human antigen-specific T cells. There is no instance of such a massive repertoire in the murine immune system. While the generation of TCR transgenic mouse lines has generously paid off in studies of the antigen-driven adaptive immunity, there is one feature of the Melan-A-specific TCR repertoire that remains unmatched by any TCR transgenic experimental model: its polyclonality.

There remain several outstanding questions going forward in the studies on the Melan-A-specific T-cell repertoire. The two most important are perhaps the following: (i) what are the ligands expressed in the thymic cortex that underlie positive selection? (ii) What are the TCR affinity thresholds for thymic selection? A third question follows: (iii) why are A2/Melan-A-specific T cells only rarely activated in the mature immune system, despite the expression of the antigen in melanocytes and keratinocytes?

To speculate on an answer for the first question, it is conceivable that many self peptides participate in the positive selection of reactive TCRs. The Melan-A antigenic peptide is issued from the transmembrane region of Melan-A (itself a type II membrane protein) and display a highly hydrophobic sequence with high sequence homology with transmembrane segments of multiple self proteins [22]. Definitive evidence for this hypothesis remains to be gathered from appropriate humanized mouse systems in which positive thymic selection may be studied. Such studies should at the same time shed light on why the repertoire is so asymmetric: high

frequencies of T cells specific for the zigzag conformation of the deca- and nonapeptides, and very low frequencies against the stretched out conformation of the nonapeptide.

To the second, it is possible that the amount of Melan-A antigen is simply limiting even in repeated inflammatory skin conditions. This is a plausible hypothesis as melanocytes make up only 5% of the skin cell composition. It may be surmised that protein levels are far below the threshold required to ever accomplish successful priming of effector CTL responses. The only situation in which enough antigen and co-stimulatory triggers are finally made available to the immune system for successful priming is that offered by the uncontrolled proliferation and expansion of transformed melanocytes in malignant melanoma. Future studies along these lines should provide valuable insights on the shaping of the T-cell repertoire to this well-known tumor antigen and shed light on the dynamics of homeostatic and tumor antigen-driven T-cell responses directly in humans.

Conflict of interest

The authors declare no financial or commercial conflict of interest.

Figure legend

Figure 1. **T-cell receptor repertoire of functional T cells.** (A) Negative selection in the thymus usually eliminates the majority of positively selected T cells. (B) In contrast, this is much less the case for $CD8^+$ T cells specific for HLA-A2/Melan-A/MART-1. This is due to the failure of negative selection because of the mis-initiation of Melan-A/MART-1 transcription by mTECs, elegantly demonstrated in this issue by Pinto et al. In the periphery, the frequency of both naïve and antigen-primed Melan-A/MART-1-specific T cells is much higher (B) as compared to the vast majority of $CD8^+$ T cells with other specificities (A).

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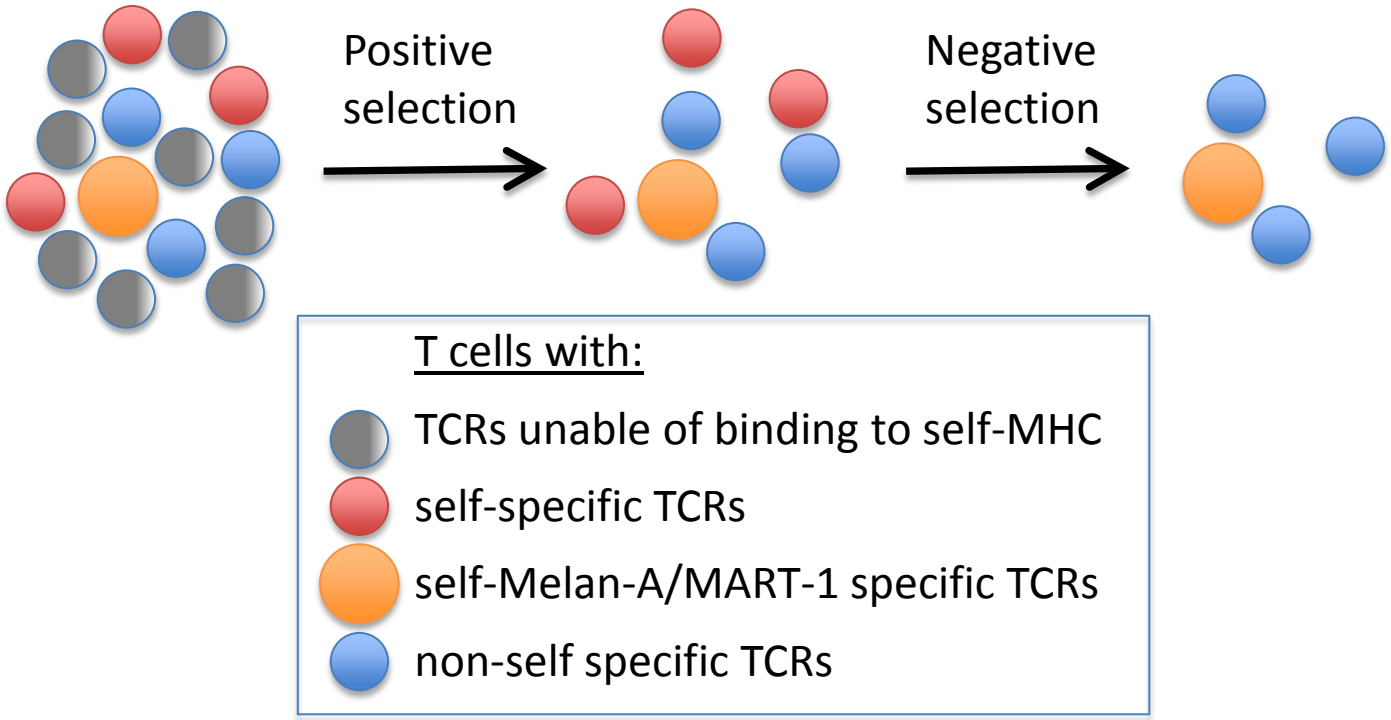
Figure legend

Figure 1. **T-cell receptor (TCR) repertoire before and after thymic selection.** Positive selection eliminates T cells with TCRs that are unable to recognize self-MHC molecules (grey). Negative selection eliminates T cells with TCRs specific for self-peptides (red). This depends on expression and presentation by the respective self-peptides in the thymus. The immunodominant Melan-A/MART-1 peptide (restricted by HLA-A*0201) is not expressed, because of the mis-initiation of Melan-A/MART-1 transcription by mTECs, elegantly demonstrated in this issue by Pinto et al. Therefore, these cells (orange) exit from the thymus, together with the non-self specific (e.g. virus-specific) T cells (blue), establishing the repertoire of mature peripheral T cells. Furthermore, thymic selection depends on TCR affinity (not shown). Finally, the frequencies of antigen-specific T cells may vary, as a result of quantitative differences in thymic positive and negative selection, and peripheral selection and homeostasis (not shown).


Positive
selection
Negative
selection
T cells with:
TCRs unable of binding to self-MHC
self-specific TCRs
self-Melan-A/MART-1 specific TCRs
non-self specific TCRs