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Commentary

Immunoproteasomes edit tumors, which then escapes immune recognition

Sebastian Joyce

Veterans Administration Tennessee Valley Healthcare System and the Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, Nashville, TN, USA

In 1985, John Monaco—the discoverer of LMP-2 and -7, the inducible components of the immunoproteasome—asked his advanced immunology class as to why the MHC region contained not only structural genes, but several others as well, whose functions were then unknown. As we drew a blank, he quipped: perchance because many of the MHC genes are induced by IFN-γ! The ensuing three decades have witnessed the unveiling of the profound fundamental and clinical implications of that classroom tête–à–tête. Amongst its multitudinous effects, IFN-γ induces genes enhancing antigen processing and presentation to T cells; such as those encoding cellular proteases and activators of proteases. In this issue, Keller et al. [Eur. J. Immunol. 2015. 45: 3257–3268] demonstrate that the limited success of MART-1/Melan-A-targeted immunotherapy in melanoma patients could be due to inefficient MART-$1_{26—35}$ presentation, owing to the proteolytic activities of IFN-γ-inducible β2i/MECL-1, proteasome activator 28 (PA28), and endoplasmic reticulum-associated aminopeptidase-associated with antigen processing (ERAP). Specifically, whilst β2i and PA28 impede MART-$1_{26—35}$ liberation from its precursor protein, ERAP-1 degrades this epitope. Hence, critical to effective cancer immunotherapy is deep knowledge of T-cell-targeted tumor antigens and how cellular proteases generate protective epitope(s) from them, or destroy them.

Keywords: Antigen processing · Cellular cytotoxicity · ER-aminopeptidase · Immunoroteasome subunit · Melanoma · Melanoma antigen recognized by T cells/melanoma antigen A · PA28 · Standard proteasome subunit

See accompanying article by Keller et al.

T-cell-targeted immunotherapeutics have recently emerged as a new arsenal against cancers. This arsenal includes weapons such as MHC-restricted antigens—including tumor-specific antigens (reviewed in [1]), e.g. the melanocyte lineage-specific antigen MART-1/Melan-A (henceforth MART-1), and neoantigens [2]—checkpoint inhibition with the aid of specific monoclonal antibodies—e.g. anti-CTLA4 or anti-PD-1 and anti-PD-L1, et cetera; [3, 4]—and tumor-infiltrating antigen-specific T cells (TILs) [3]). MART-1 has been used in clinical trials with limited efficacy even though it contains the immunodominant HLA-A*02;01-restricted $CD8^+$ T-cell epitope MART-$1_{26(27)—35}$ [1, 5]. Studies have shown that interference with antigen processing perhaps underlies the noted poor clinical efficacy of MART-1 immunotherapy. MART-1 contains two A*02;01-restricted epitopes—the MART-$1_{26–35}$ 10mer and the MART-$1_{27\text{-}35}$ 9mer—yet only the 9mer epitope is naturally processed and presented at the surface of melanoma cells [6]. The generation of these epitopes is dependent on the standard, constitutively expressed,

Correspondence: Dr. Sebastian Joyce
e-mail: sebastian.joyce@vanderbilt.edu

proteasomes [7, 8]. IFN-γ, which in many instances enhances antigen presentation and T-cell recognition—e.g. generation of $TRP2_{360–368}$ from the melanoma antigen tyrosinase-related protein 2 [9], interferes with efficient generation of MART-$1_{26–35}$ and other epitopes [7, 10–12]. In this issue, Keller et al. [13] tease out how IFN-γ interferes with MART-$1_{26—35}$ presentation.

$CD8^+$ cytotoxic T lymphocytes (hereinafter $CD8^+$ T cells) play critical roles in tumor immune surveillance. T-cell functions are controlled in a process termed MHC restriction. MHC restriction entails the processing of proteins to short peptides and their presentation at the cell surface by MHC-encoded class I and class II molecules for an appraisal by self-peptide (p)/MHC-tolerant T cells. $CD8^+$ T-cell functions are controlled by MHC class I molecules, which in humans are encoded by HLA-A, -B, and -C loci. Antigen presentation by HLA class I molecules requires proteolytic processing of proteins to short peptides of 9—13 amino acid residues in the cytosplasm. Such proteolysis within the cytoplasm is accomplished by the proteasomes. Proteasomal products—short and long, i.e. longer than the typical 9—13 amino acid residue-peptides that bind to class I molecules—are further transported from the cytoplasm into the ER lumen by TAP. Within the ER, peptides are made available to peptide-receptive class I molecules within the peptide loading complex (PLC). Those that have class I-binding motifs assemble with class I molecules; but those that contain the motif but are longer than can fit into the antigen-binding groove are further trimmed to size by the ER amino-peptidases-associated with antigen processing (ERAP)-1 and 2. Once fully assembled with a bound peptide, the PLC releases class I molecules, which then egress from the ER, negotiate the Golgi apparatus and arrive at the cell surface (reviewed in [14]).

Proteasomes are multicatalytic endoproteinase complexes composed of four rings in which each ring is made of seven related subunits (Fig. 1A). The two outer rings, composed of α subunits, sandwich the two inner catalytic rings of β subunits. This quartet of heptameric rings, forming the core 20S proteasome, assembles in such a way that they form an interior chamber. The N-terminal residues of the α rings gate the catalytic rings, the opening of which is controlled by the regulatory cap made up of the 11S proteasome activators (PA) and/or the AAA+ ATPase-containing 19S unit. The N-terminus of β1, β2, and β5 subunits is exposed to the interior chamber and contains the proteolytic active sites (reviewed in [15, 16]).

IFN-γ enhances MHC-restricted antigen presentation by inducing the expression of multiple structural and regulatory genes, including HLA class I, β1i/LMP (low molecular mass polypeptide)-2, β2i/MECL (multicatalytic endopeptidase complex-like)-1, β5i/LMP7, the regulatory cap PA28 and ERAP, among others, especially within immune cells (Fig. 1B) [17, 18]. The induced proteasomal components occupy the place of the homologous component within the constitutive, standard proteasome (Fig. 1A), creating the immunoproteasome (Fig. 1B). Immunoproteasome formation is a highly ordered process: β2i requires β1i for efficient incorporation into preproteasomes, and preproteasomes containing β1i and β2i require pre-β5i for efficient maturation and, thereby, ensures the assembly of homogeneous immunoproteasomes for efficient generation of peptides presented by class I molecules [15, 19–21].

Melanomas are known to express certain, if not all, inducible components of the immunoproteasome, such as β1i, β2i, β5i, and PA28α and PA28β (see Fig. 1A in ref. [13]). Keller et al. [13] identify an A*02;01 and MART-1 positive melanoma line—UKRV-Mel-15a, which contains very-low-to-undetectable levels of immunoproteasome components and ERAP-1, but upregulates them in response to IFN-γ. Unexpectedly, treatment of this melanoma with IFN-γ reduced the activation of a MART-$1_{26—35}$-reactive $CD8^+$ T-cell clone to secrete TNF-α. This result, in conjunction with past findings alluded to above [7, 10, 11], suggested to the investigators that one or more IFN-γ-induced components of the immunoproteasome affects MART-$1_{26—35}$ generation. To determine which IFN-γ-inducible components are responsible for preventing MART-$1_{26—35}$ generation, a HeLa cell line previously made to express A*02;01 was transduced with genes coding for individual immunoproteasome components or combinations thereof [13]. As HeLa cells constitutively express basal levels of β5i and low basal levels of β1i, proteasome assembly containing single, double, or triple inducible components in transduced cells was accomplished [19–22]). The resulting lines modeled intermediate proteasomes similar to those observed in some melanomas [23].

To set the stage, cleavage studies of the precursor polypeptide MART-1_{15-40} encompassing the $CD8^+$ T-cell epitope MART-$1_{26–35}$ (bold letters in Fig. 1) were initially performed using standard proteasomes and immunoproteasomes isolated from nonstimulated or IFN-γ-stimulated HeLa cells, respectively. The data revealed that the immunoproteasomes were unable to generate MART-$1_{26–35}$, owing to inefficient cleavage at the carboxyl-terminus of MART-$1_{26–35}$ compared to the standard proteasomes. As well, the immunoproteasome in the UKRV-Mel-15a melanoma line did not cleave the MART-$1_{15–40}$ substrate between residue 25 and 26 to generate the epitope's amino-terminus (Fig. 1B, right). In additional experiments, Keller et al. [13] found that β1i- and/or β2i-containing HeLa cells poorly activated MART-$1_{26—35}$-specific $CD8^+$ T cells. Accordingly, 20S proteasomes isolated from these cells yielded low-to-no MART-$1_{26—35}$ epitope, owing to poor liberation of the carboxyl-terminus by β2i, as well as the amino-terminus by both β1i and β2i (Fig. 1C, D). The role of β2i in MART-$1_{26—35}$ generation was confirmed by the T1A mutant β2i, which had lost its proteolyic activity (see β2i* in Fig. 1D).

IFN-γ also induces PA28α and PA28β, which are required together for the assembly of the hetero-heptameric PA28 ring [24]. Forced overexpression of PA28α and PA28β resulted in the loss of MART-$1_{26—35}$ epitope recognition by specific $CD8^+$ T cells. Accordingly, RNA interference-mediated suppression of the gene encoding PA28α or PA28β therefore enhanced MART-$1_{26—35}$ epitope recognition by specific $CD8^+$ T cells [13]. Furthermore, PA28-containing proteasomes do not generate the MART-$1_{26—35}$ epitope, owing to poor liberation of the carboxyl-terminus and the inability to cleave between residues 25 and 26 of the MART-$1_{15—40}$ substrate while inducing a lethal cleavage between the penultimate (V34) and ultimate (I35) residue of the MART-$1_{26—35}$ epitope (Fig. 1E).

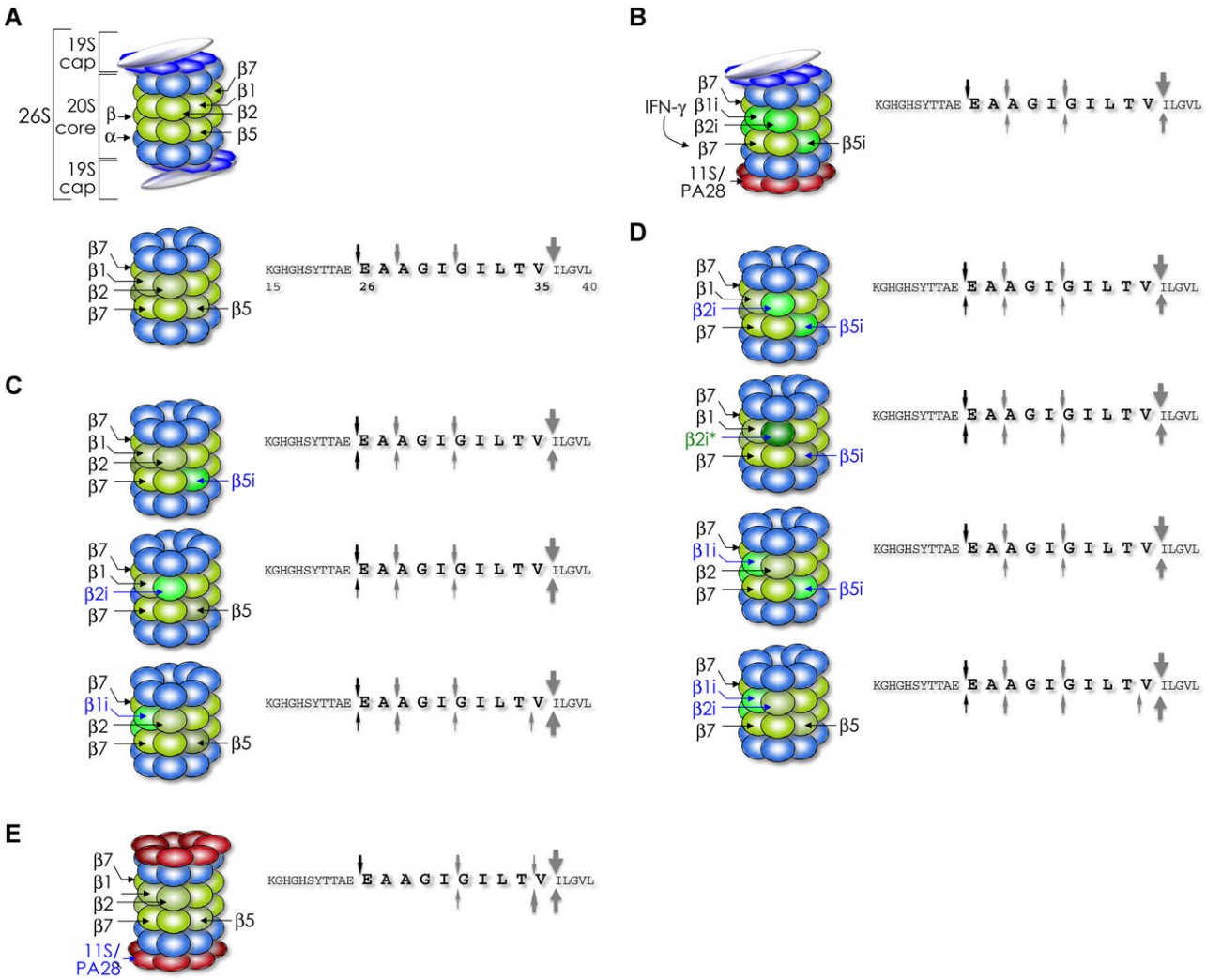

Figure 1. The making and breaking of a tumor epitope for surveillance—MART-$1_{26—35}$ in focus. (A) Anatomy of a 26S proteasome; it is made of a 20S core plus one or two 19S regulatory cap(s) (top structure). (B) Immunoproteasomes have replaced the β1, β2, and β5 subunits of the 20S core with β1i, β2i, and β5i, respectively that are bound with one or two 19S cap(s) or one or two 11S αβ heteromeric PA28. (A) Proteolytic activity of the standard proteasome (20S structure) on the MART-$1_{15—40}$ substrate. In Keller et al. [13], this activity is compared with that of (B) the immunoproteasome induced by IFN-γ or (C–E) of proteasomes containing individual or combinations of the immunoproteasome components. Arrows pointing down, cleavage sites of the standard 20S proteasome isolated from HeLa cells; upward pointing arrows, cleavage sites of immunoproteasomes, or proteasomes containing one or two components of the immunoproteasomes; arrow thickness, cleavage sites based on yields of products identified by mass analysis; capital letters, amino acid sequence of the proteasome substrate used in the study; numbers, amino acid position; bold letters, HLA-A*02;01-restricted MART-$1_{26—35}$ epitope; β2i*, catalytically inactive MECL-1 mutant. Figure is an adaptation of Fig. 3B and 4D of Keller et al. [13].

Normally, the amino-terminus of class I-binding peptides can be custom generated in the ER lumen from longer substrates by the action of ERAP-1 [25–27]—another IFN-γ-induced product in the class I antigen processing pathway. Incidentally, chemical inhibition of ERAP-1 in UKRV-Mel-15a cells or downregulation of *ERAP1* by RNA interference was shown to enhance MART-$1_{26–35}$ recognition by specific $CD8^+$ T cells [13] suggesting that under these conditions, ERAP-1 destroyed the MART-$1_{26—35}$ epitope. Accordingly, recombinant ERAP1 cleaved the MART-$1_{15—40}$ substrate to generate the MART-$1_{26–35}$ epitope in vitro, but eventually destroyed it [13]. But whether eventual degradation of the MART-$1_{26—35}$ epitope was due to the absence of peptide receptive A*02;01 in the in vitro cleavage assay remains unclear.

Taken together, the report by Keller et al. [13] indicates that a combination of proteolytic activities induced by IFN-γ can result in immunoediting of tumors and promote immune evasion. This form of immunoediting might explain the curious case of a patient, identified as VMM5, who had a melanoma recur twice over a 12-year period [28]. TILs at the first recurrence were shown to react to MART-1, but TILs from the second recurrence failed to react to it but instead reacted to the epitope $_{369}$YMDGTMSQV$_{377}$ from tyrosinase [28]. As MART-1-reactive TILs were shown to attend to most of VMM5's melanoma at first recurrence [28],

soluble mediators such as IFN-γ [29] may have turned on the immunoproteasome activity in the tumor, thereby preventing the display of the MART-$1_{26—35}$ epitope. As the MART-$1_{26—35}$ epitope-reactive TILs waned in VMM5, the subdominant TILs against the $Tyr_{369—377}$ epitope became dominant at second recurrence [28], perhaps by repeated stimulation through interactions with the slowly smoldering immune escapees. Such a mechanism could be tested by determining whether $Tyr_{369—377}$ epitope generation required or is enhanced by, or alternatively resistant to, immunoproteasome activity, as is the case with the tyrosinase-related protein-2 derived epitope $TRP2_{360—368}$ [30, 31]. Nonetheless, the display of multiple $CD8^+$ T-cell epitopes derived from tumor-specific antigens [32] and neoantigens [33–36] might make immune evasion difficult.

Polymorphisms in genes that encode proteins involved in HLA class I-restricted antigen processing and presentation are linked to susceptibility to certain cancers and various immunologic diseases. How these polymorphisms impact diseases remains unclear. A recent study in human B lymphoblastoid cell lines revealed that polymorphisms impacting the enzymatic activity of ERAP-1 alter the repertoire of peptides presented by a HLA class I molecule [37]. Hence, to fully realize the protective power of T-cell-targeted immunotherapeutics against cancers, the roles of heritable polymorphisms, somatic mutations within evolving tumors, and tumor responses to immune mediators that lead to immunoediting and susceptibility to cancers needs to be fully explored.

The risk for developing a cancer increases with age and generally burdens those beyond their prime, fecund years. Hence, it is generally assumed that cancers contribute little to selection pressure for the survival of the species. Although the evolutionary implications of Keller and co-workers' findings are unclear, one could imagine how IFN-γ-induced protease activities associated with antigen processing may facilitate the control of microbial infections that cause fatal disease amongst the very young. From this evolutionary vantage, escape from tumor immunity and immunotherapy are perchance happenstance.

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Abbreviations: **ERAP**: endoplasmic reticulum-associated aminopeptidases-associated with antigen processing · **LMP**: low molecular mass polypeptide · **MECL**: multicatalytic endopeptidase complex-like · **PA**: proteasome activator · **PLC**: peptide loading complex · **TIL**: tumor-infiltrating antigen-specific T cell

Full correspondence: Dr. Sebastian Joyce, the Department of Pathology, Microbiology, and Immunology, Vanderbilt University School of Medicine, A4223 Medical Centre North, 1161 21st Avenue South, Nashville, TN 37232, USA
Fax: +1-615-343-7392
e-mail: sebastian.joyce@vanderbilt.edu

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