Vaccination and the TAP-independent Antigen Processing Pathways

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Daniel López *, Elena Lorente, Alejandro Barriga, Carolina Johnstone, and Carmen Mir

From Centro Nacional de Microbiología, Instituto de Salud Carlos III, 28220 Majadahonda (Madrid), Spain.

* Correspondence to: Dr. Daniel López. Unidad de Procesamiento Antigénico-Inmunología Viral. Centro Nacional de Microbiología. Instituto de Salud Carlos III. 28220 Majadahonda (Madrid), Spain. Tel: +34 91 822 37 08, FAX: +34 91 509 79 19, E-mail: dlopez@isciii.es

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ABSTRACT

The cytotoxic $CD8^+$ T lymphocyte (CTL)-mediated cellular response is important for the elimination of virus-infected cells and requires the prior recognition of short viral peptide antigens previously translocated to the endoplasmic reticulum by the transporter associated with antigen processing (TAP). However, individuals with non-functional TAP complexes or infected cells with TAP molecules blocked by specific viral proteins, such as the cowpoxvirus, a component of the first source of early empirical vaccination against smallpox, are still able to present several HLA class I ligands generated by the TAP-independent antigen processing pathways to specific CTLs. Currently, bioterrorism and emerging infectious diseases have renewed interest in poxviruses. Recent works that have identified HLA class I ligands and epitopes in virus-infected TAP-deficient cells have implications for the study of both the effectiveness of early empirical vaccination and the analysis of HLA class I antigen processing in TAP-deficient subjects.

HISTORICAL VIEW OF SMALLPOX VACCINATION

Among the multiple pandemic contagious diseases that have plagued the human race since time immemorial, most likely none of them has had such a universal and continual impact on the human population as smallpox. Since its emergence, most likely in the first irrigated agricultural settlements, it is estimated that variola virus has been responsible for nearly one billion deaths and severely altered the course of history in different times, even contributing to the decline of several human civilizations [(1)]. Early efforts to control this pandemic disease by inoculation with smallpox pus or scabs have been historically documented from ancient times in several oriental cultures, and the technique of variolation gradually spread from these cultures to south-western Asia, the Ottoman Empire and later to Europe and the various European colonies [(2)].

Although in general, variolation was a relatively effective and preventive measure against smallpox, unfortunately, some subjects inoculated with scabs that contained infectious variola virus died, and others transmitted the disease to the neighboring susceptible population. The low vulnerability of milkmaids to smallpox was widely known in several rural areas of various European countries. This observation was used by Edward Jenner and other English and Dutch physicians and even some concerned farmers to inoculate volunteers with fluid extracted from pustules on the hands of cowpox-infected milkmaids [(2)]. Later, the use of arm-to-arm pustule fluid as a means of vaccination was quickly accepted in Europe (including the Ottoman Empire), overseas European colonies and the newly independent United States of America, leading to the era of prophylactic vaccines.

These old smallpox vaccines and others obtained later in different European countries when the Jenner technique was accepted world-wide were randomly mixed during the nineteenth century to propagate complex mixtures of vaccine viruses similar

to quasispecies [3]. Two centuries after this early empirical cross-protective vaccination with cowpox and horsepox virus, health efforts culminated with a massive worldwide vaccination program coordinated by the World Health Organization that eradicated smallpox [4]. In this global program, vaccinia virus (VACV), another poxvirus that is related to smallpox, horsepox and cowpox [5], was used as the active principle. Currently, the origin of VACV remains uncertain. A reasonable explanation is that it is derived from either horsepox [6,3] or cowpox [7,8], and cowpox could be the most ancient of all poxviruses [9]. Vaccination induces a strong humoral response, leading to viral clearance, and the role of cellular responses in this cross-protection is well documented [10,11].

ORTHOPOXVIRUSES AND VACCINES

Orthopoxviruses have large and complex virions that are visible by light microscopy. Their genome is a double-stranded DNA molecule of 130–375 kbp that varies between genera. Thus, variola major has a genome of 190 kbp and encodes approximately 200 proteins, whereas the VACV and cowpox genomes are somewhat larger, with a size of 200 and 224 kbp encoding approximately 220 and 240 proteins, respectively [5]. Poxvirus proteomes include numerous immunomodulatory proteins that disrupt the host immune response (reviewed in [12,13]), including secreted proteins directed toward altering the function of chemokines, complement, cytokines, and interferons and different intracellular immunomodulators that could disrupt the antiviral effects of interferons, apoptosis, host gene transcription and innate immune signalling. Particularly, orthopoxvirus immune evasion mechanisms involve blocking the trafficking of assembled MHC class I proteins in the endoplasmic reticulum to the plasma membrane by a KDEL-mediated retention pathway [14] or decreasing MHC class II expression on antigen-presenting cells after infection [15]. Many of these immune evasion strategies are common among different members of the poxvirus family, but some are specific to individual poxviruses.

Of particular interest is the cowpox protein CPXV12, which has no homologs in VACV or variola viruses. CPXV12 interferes with the human leukocyte antigen (HLA) class I/peptide complex folding by inhibiting peptide translocation by the transporter associated with antigen processing (TAP) [16]. These TAP heterodimers play a pivotal role in the classical antigen presentation pathway. The inhibition of these heterodimers prevents the translocation of most viral epitopes, which are mainly generated by the proteolytic degradation of viral proteins by the proteasome and other cytosol proteases, to the endoplasmic reticulum. There, these epitopes must be folded into HLA class I structures prior to their specific recognition on the surface of the infected cells by $CD8^+$ cytolytic T lymphocytes (CTLs) [17].

TAP-INDEPENDENT RESPONSES AND TAP-DEFICIENT INDIVIDUALS

Thus, the TAP-independent antigen processing and presentation pathways must be important to generate the crossreactive HLA class I cowpox epitopes that could be subsequently protective in subjects exposed to variola virus and contributed with other immune system layers to the successful early empirical vaccination against the smallpox pandemic. In addition, mutations in the TAP genes that generate non-functional TAP complexes have been described in both humans [18] and mice [19]. The few dozens of human patients with this HLA class I deficiency show a reduced functional $CD8^+$ T cell population, but they may appear asymptomatic for long periods of their lives. TAP-deficient patients do not seem particularly susceptible to neoplasms or viral infections (largely and highly dependent on the T cell immune responses) but show susceptibility to some chronic respiratory bacterial infections [18]. Therefore, the immune system of these TAP-deficient individuals must be reasonably competent, and NK cells, antibodies from B cells, $CD8^+$ γδ T cells, and the decreased cytotoxic $CD8^+$ αβ T subpopulation that is specific for TAP-independent antigens may all generate competent immune defenses that protect against severe viral infections in these individuals. These data reinforce the relevance of the TAP-independent antigen

processing and presentation pathways (in addition to other immune mechanisms such as humoral responses [20]) in the effective control of viral pathogens.

IMMUNOPROTEOMICS OF TAP-INDEPENDENT HLA CLASS I LIGANDS FROM ORTHOPOXVIRUS

In recent years, various studies in either humanized HLA-transgenic mouse models or vaccinated humans have allowed the identification of more than 170 or 120 epitopes presented by different HLA class I or class II molecules respectively, from multiple VACV proteins (reviewed in [21,22] and Immunoepitope Database) in TAP-sufficient models. In addition, several recent studies carried out in our laboratory have tried to further investigate two issues: TAP blocking by the cowpox virus and human TAP deficiency using the same viral model but with two different experimental approaches. First, a TAP-deficient human cell line that expresses four common HLA class I molecules on its surface was used to identify TAP-independent viral ligands that were presented simultaneously in the same infected TAP-deficient cells. The HLA-bound peptide pools were sequentially isolated from large numbers of TAP-deficient VACV-infected cells using specific antibodies. Mass spectrometry analysis identified eleven viral ligands bound to the four HLA class I molecules expressed by the TAP-deficient cells (summary in Table 1). Although most ligands were restricted by a single HLA class I allele, VACV ligands $K2L_{16-30}$ and $C11R_{101-110}$ were found in association with HLA class I molecules HLA–Cw1 and either HLA-B27 or -B51, respectively. Therefore in the same infected TAP-deficient cells a total of thirteen different natural peptide-HLA class I complexes were formed simultaneously [23]. A similar number of ligands (3 or 4) associated to HLA class I molecules previously described to have high (HLA-B27) [24], low (HLA-A2) [25,26] or unknown (HLA-B51 and –Cw1) TAP dependency were identified. In a more recent study, the $D8L_{112-119}$ viral ligand previously described to be presented on the classical HLA-Cw1 class I allele was also physiologically presented by the non-classical HLA-E class I molecule in TAP-deficient cells [27]. In

summary, these data indicate that fourteen different natural peptide-HLA class I complexes of five HLA class I molecules (HLA-A2, -B27, -B51, -Cw1, and -E) were simultaneously presented in the same infected TAP-deficient cells (Table 1).

ANTIVIRAL T CELL RESPONSES IN HLA CLASS I-TRANSGENIC MICE MODELS

Second, to quantify the TAP-independent immune response that is conserved among orthopoxviruses, a TAP-independent polyclonal VACV-polyspecific $CD8^+$ T cell line from vaccinated HLA-B7 transgenic mice was generated [28]. Two out of the seven peptides previously reported as HLA-B7 epitopes were identified from the HLA-B7 transgenic mice ($A34R_{82-90}$, $D1R_{808-817}$, and $J2R_{116-124}$) [29] or the human vaccines ($AC1L_{97-106}$, $D1R_{686-694}$, $F4L_{6-14}$, and $J6R_{303-311}$) [30,31] and were recognized by the TAP-independent CTL cell line. Thus these peptides were presented by TAP-independent pathways (Table 1). Notably, the $D1R_{808-817}$ peptide identified as a TAP-independent peptide [28] was the immunodominant epitope in the standard antiviral response from these H-2 class I double-knockout HLA-B7-transgenic mice. Thus, TAP-independent HLA-B7 antigen presentation could contribute to controlling VACV infection in the absence of a functional TAP complex. If the data obtained with the HLA-B7 allele were representative of most HLA class I molecules, this may help to explain why individuals with nonfunctional TAP proteins do not seem to be particularly susceptible to viral infections and may appear asymptomatic for much of their lives (reviewed in [18]).

TAP-INDEPENDENT LIGANDS AND HIDROPHOBICITY

Although TAP-independent viral epitopes are known (reviewed in [32,33,25]), no methodical studies on TAP-independent antigen presentation with a single virus and different HLA molecules have been reported, with the exception of Epstein-Barr Virus (EBV). Different studies (reviewed in [34]) have shown that CTLs from several donors

recognize eight EBV epitopes from only three different viral proteins and are restricted by four HLA class I molecules in TAP-deficient cells. These epitopes are always located in the hydrophobic regions of the respective EBV proteins, and they are highly hydrophobic, unlike the TAP-dependent EBV epitopes (Figure 1). Thus, the global picture of these data with regard to EBV could suggest a limited TAP-independent antigen-processing ability, focused on the hydrophobic regions of some proteins independently of the large genomic size of this herpesvirus (approximately 200 Kbp, similar to poxviruses).

In contrast, TAP-independent antigen processing in TAP-negative cell backgrounds generates thirteen ligands from ten unrelated VACV proteins (expressed in the three gene expression temporality clusters of the viral life cycle) presented by six different HLA class I classical and non-classical molecules (Table 1). No obvious protein patterns, such as gene expression, function, viral life cycle, localization, and domains, or ligand characteristics, such as position and sequence (data not shown), including their specific hydrophobicity (shown in Figure 1), were found. Therefore, hydrophobicity is not a necessary condition for the TAP-independent presentation of ligands/epitopes for all viruses. Furthermore, VACV TAP-independent antigen processing appears to be less restricted than EBV TAP-independent presentation.

EXPERT COMMENTARY

Only the HLA class I epitopes conserved between the vaccine virus (cowpox or VACV) and the pathogenic variola virus were responsible for the crossreactive protection in individuals exposed to variola virus. Variola virus shows 72% and 82% amino acid homology with cowpox and VACV proteomes, respectively (Poxvirus Bioinformatics Resource Center, http://www.poxvirus.org). As the amino acid differences are not equally distributed in the viral proteome, the conservation analysis of the 79 TAP^+ VACV epitopes previously described [31,35] between both poxviruses

shows that 60% of these epitopes are conserved in the variola proteome. When a similar analysis was performed with the sequences of thirteen TAP-independent VACV ligands that were previously identified [23,27,28], the ligands were found to be highly conserved among orthopoxviruses (Table 2). Only a minor substitution at the P7 position of one of the thirteen ligands (sequence $C11R_{101-110}$) was found and thus twelve ligands (92%) are fully conserved in variola major, variola minor, and cowpox viruses. These data show TAP-independent VACV ligands are more conserved than TAP-dependent epitopes between pathologic and immunogenic poxviruses, which could explain the effectiveness of early empirical vaccination with cowpox virus against smallpox disease.

So far, different proteases have been implicated in the processing of endogenously synthesized antigens independent of the classical proteasome pathway: signal peptidase [36,37], furin [38,39], tripeptidyl peptidase II [40,41,42], lysosomal chloroquine-sensitive enzymes [43,44,45], and caspases [46,47]. In our recent studies [23,28], a systematic analysis of the antigen processing pathways involved in the endogenous generation of the HLA-A2 and –B7 TAP-independent viral epitopes generated in VACV-infected cells was performed. This analysis was performed using polyclonal $CD8^+$ T cell lines that were monospecific for each viral epitope produced from the HLA transgenic mouse models in the presence of diverse protease inhibitors in VACV-infected TAP-proficient cells. As shown in Figure 2, in normal cells the presentation of four HLA-A2 and –B7 epitopes occurs via complex pathways involving antigen-processing by the proteasome and/or by diverse subsets of metalloproteinases (amino-, carboxy-, and endoproteases) acting in parallel or sequentially. These data support the fact that peptides recognized by the antiviral cellular immune response are supplied by different cellular proteolytic systems that are therefore contributing and facilitating immunosurveillance.

FIVE-YEAR VIEW

In summary, the study of the HLA class I-mediated response in VACV-infected TAP-deficient cells results in a new and intricate picture of the TAP-independent antigen processing pathways, which could explain both the effectiveness of early empirical vaccination with cowpox virus against smallpox and why TAP-deficient individuals live for long periods without enhanced susceptibility to viral infections. In addition, the existence of these multiple TAP-independent VACV ligands generated by multiple antigen processing and presentation routes, which operate in the absence of the TAP-dependent classical pathway, suggests that these pathways could be a secondary but extended and relevant mechanism in addition to the multiple components of immune protection against viral infection, and this hypothesis requires further investigation including:

- Immunoproteomics analysis of human cowpox-infected cells.
- Analysis and quantification of the T cell immune responses using cowpox-vaccinated HLA class I transgenic mice models.
- Identification of proteases that may contribute to the processing of TAP-independent HLA-class I epitopes from cowpox-infected cells.
- Role of viral TAP-independent HLA-class I ligands in NK cell recognition.

KEY ISSUES

- The transporter associated with antigen processing (TAP) delivers the viral proteolytic products generated by the proteasome in the cytosol to the endoplasmic reticulum lumen that are subsequently recognized by cytotoxic T lymphocytes (CTL).
- Individuals with mutations in the TAP gene that generate non-functional TAP complexes do not seem particularly susceptible to viral infections. Thus, the reduced cytolytic CTL subpopulation that is specific for TAP-independent antigens may contribute to immune defenses that protect against severe viral infections in these individuals.
- The eradication of smallpox, a disease caused by variola major virus, was made possible by early empirical, cross-protective vaccination with both cow and horse orthopoxvirus.
- Cowpoxvirus specifically inhibits TAP-dependent peptide translocation; therefore, TAP-independent epitopes conserved between variola and this virus probably contributed to for the initial cross-protection.
- Using mass spectrometry to analyze complex human leukocyte antigen (HLA)-bound peptide pools isolated from large numbers of TAP-deficient, multiple viral ligands naturally presented by different HLA-A, -B, -C and -E class I molecules and conserved among the *Orthopoxviridae* family were identified.
- Two of four epitopes (including the immunodominant epitope) detected in the standard antiviral response from the H-2 class I double-knockout HLA-B*07-transgenic mice were presented by TAP-independent pathways. Thus, TAP-independent HLA-B*07 antigen presentation could be sufficient to control orthopoxvirus infection in the absence of a functional TAP complex.
- The existence of multiple TAP-independent orthopoxvirus ligands generated by multiple proteases in complex antigen processing and presentation routes, which operate in the absence of the TAP-dependent classical pathway,

suggests that these pathways could be a secondary but extended and relevant mechanism of immune protection against viral infection, and this hypothesis requires further investigation *in vivo*.

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FOOTNOTES

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The abbreviations used are as follows: CTLs, cytotoxic T lymphocytes; ER, endoplasmic reticulum; ERAP, endoplasmic reticulum aminopeptidase; HLA, human leukocyte antigen; TAP, transporters associated with antigen processing; VACV, vaccinia virus.

FIGURE LEGENDS

Figure 1. TAP-independent ligands/epitopes and their hydrophobicity.

Comparison of hydrophobicity measured on the grand average of hydropathicity (GRAVY) scale (ProtParam tool, ExPASy Proteomics Server, http://www.expasy.ch) of 8 TAP-independent (circles) and 9 TAP-dependent (squares) epitopes from EBV [34] versus 13 TAP-independent ligands/epitopes (triangles) [23,27,28] and 79 TAP-dependent epitopes (diamonds) [31,35] from VACV. The respective means are indicated as lines. *** Significant P values ($P < 0.001$).

Figure 2. Diversity of proteases and processing pathways involved in TAP-independent VACV epitope presentation.

The models show the components involved in each of the proposed pathways for both HLA-A2-restricted ($A17L_{9-17}$, and $A10L_{688-696}$ (upper panel)) and HLA-B7-restricted ($J6R_{303-311}$ (middle panel) or $D1R_{808-817}$ (lower panel)) epitopes. The role of different proteases was deduced from the sensitivity of the respective $CD8^+$ T cells to the various protease inhibitors (see references [23,28]).

Table 1

Summary of HLA molecules bound by vaccinia virus TAP-independent ligands

Ligand	Sequence	HLA restriction	Detection method	$CD8^+$ T cell response	Reference
A17L $_{9-17}$	MLDDFSAGA	-A2	IP [a]	Yes	(23)
A10L $_{614-623}$	SPEGEETII	-A2	IP	Yes	(23)
A10L $_{688-696}$	ILDRIITNA	-A2	IP	Yes	(23)
A10L $_{867-876}$	SRGYFEHMKK	-B27	IP	ND [b]	(23)
B8R $_{53-59}$	WQTMYTN	-B27	IP	ND	(23)
K2L $_{16-30}$	YRLQGFTNAGIVAYK	-B27, -Cw1	IP	ND	(23)
D5R $_{148-157}$	IAMKRTLLEL	-B51	IP	ND	(23)
A50R $_{294-301}$	LPFGSLGI	-B51	IP	ND	(23)
C11R $_{101-110}$	IPSPGIMLV	-B51, -Cw1	IP	ND	(23)
A17L $_{9-25}$	MLDDFSAGAGVLDKDL	-Cw1	IP	ND	(23)
D8L $_{112-119}$	DGLIIISI	-Cw1, -E	IP	Yes (in HLA-E)	(23,27)
D1R $_{808-817}$	RPSTRNFFEL	-B7	T Cell line [a]	Yes	(28)
J6R $_{303-311}$	MPAYIRNTL	-B7	T Cell line	Yes	(28)

[a] IP, sequential immunoprecipitation with specific antibodies; T cell line, TAP-independent HLA-B7-restricted polyclonal VACV-polyspecific $CD8^+$ T cell line. [b] ND, not determined.

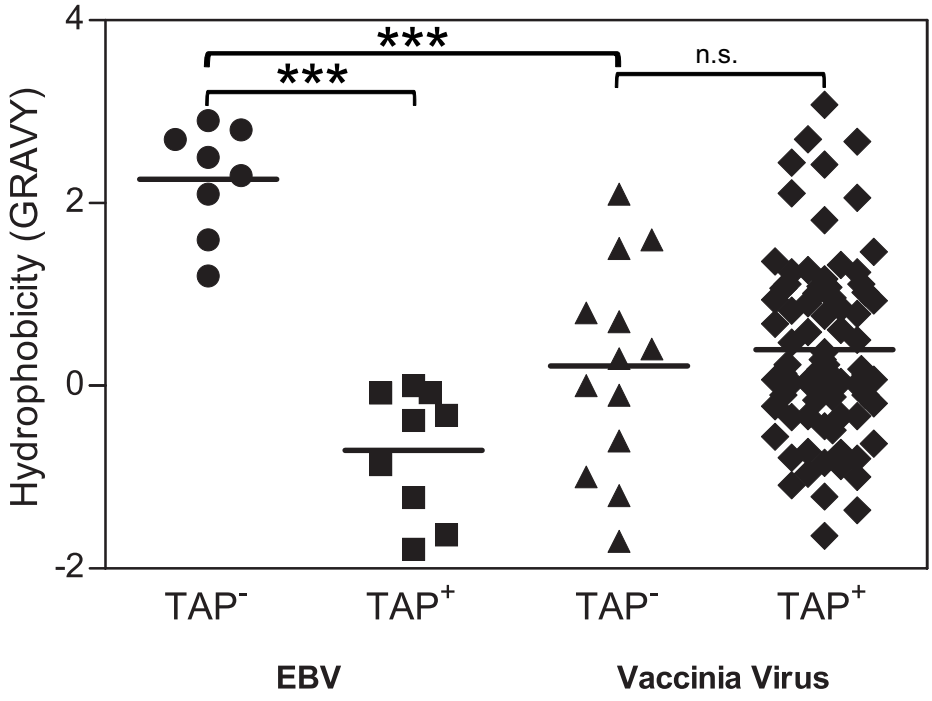
Table 2

Conservation of TAP-independent viral HLA ligands in several orthopoxviruses

Poxvirus[a]	$A17L_{9-17}$ (HLA-A2)	$A10L_{614-623}$ (HLA-A2)	$A10L_{688-696}$ (HLA-A2)	$D1R_{808-817}$ (HLA-B7)	$J6R_{303-311}$ (HLA-B7)	$A10L_{867-876}$ (HLA-B27)	$B8R_{53-59}$ (HLA-B27)
Vaccinia virus	MLDDFSAGA	SPEGEETII	ILDRIITNA	RPSTRNFFEL	MPAYIRNTL	SRGYFEHMKK	WQTMYTN
Variola major	---------	---------	---------	----------	---------	----------	-------
Variola minor	---------	---------	---------	----------	---------	----------	-------
Cowpox	---------	---------	---------	----------	---------	----------	-------

Poxvirus[a]	$K2L_{16-30}$ (HLA-B27, -Cw1)	$D5R_{148-157}$ (HLA-B51)	$A50R_{294-301}$ (HLA-B51)	$C11R_{101-110}$ (HLA-B51, -Cw1)	$A17L_{9-25}$ (HLA-Cw1)	$D8L_{112-119}$ (HLA-Cw1, -E)
Vaccinia virus	YRLQGFTNAGIVAYK	IAMKRTLLEL	LPFGSLGI	IPSPGIMLV	MLDDFSAGAGVLDKDL	DGLIIISI
Variola major	---------------	----------	--------	------V--	----------------	--------
Variola minor	---------------	----------	--------	------V--	----------------	--------
Cowpox	---------------	----------	--------	------V--	----------------	--------

[a] The sequences used were obtained from the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi).



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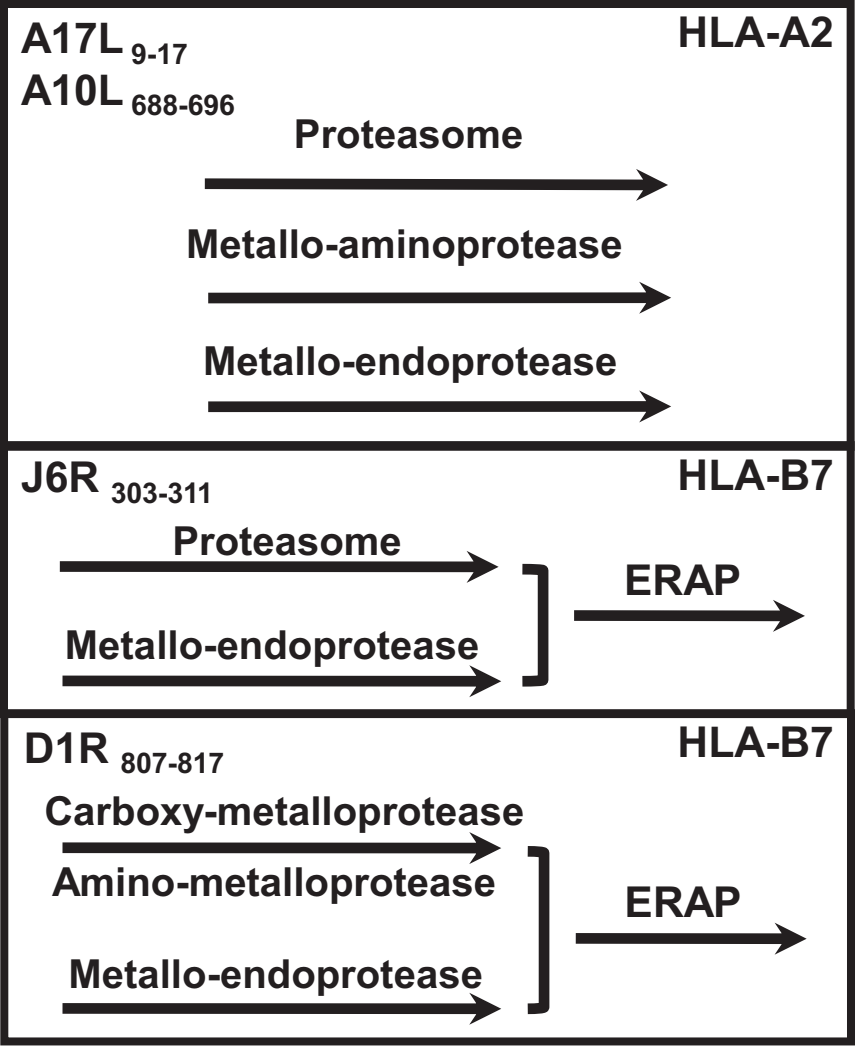

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