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## **Antigen processing by nardilysin and thimet oligopeptidase generates cytotoxic T cell epitopes**

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## ABSTRACT

Cytotoxic T lymphocytes (CTLs) recognize peptides presented by HLA class I molecules on the cell surface. The C-terminus of these CTL epitopes is predominantly generated by the proteasome. Here we demonstrate that the cytosolic endopeptidases nardilysin and thimet oligopeptidase (TOP) complemented proteasome activity. Nardilysin and TOP were required, either together or alone, for the generation of a tumor-specific CTL epitope from PRAME, an immunodominant CTL epitope from Epstein-Barr virus, and a clinically important epitope from melanoma-protein MART-1. TOP functioned as C-terminal trimming peptidase in antigen processing and nardilysin contributed to both C-terminal and N-terminal CTL epitope generation. By broadening the antigenic peptide repertoire, nardilysin and TOP strengthen the immune defense against intracellular pathogens and cancer.

## Introduction

The production of short peptides (8 – 12 aa) presented by HLA class I molecules, called CTL epitopes when recognized by CD8<sup>+</sup> cytotoxic T lymphocytes (CTLs), occurs in the cytosol during the continuous turnover of full-length and misfolded proteins into peptides and eventually free amino acids<sup>1-3</sup>. This cleavage process is accomplished by the multicatalytic proteasome complex and cytosolic peptidases<sup>1,3</sup>. Tripeptidyl peptidase II (TPPII)<sup>4</sup>, TOP<sup>5,6</sup> and aminopeptidases<sup>1,2</sup> are implicated in protein degradation, but the contribution of other (endo)peptidases has remained elusive. A few intermediate-length degradation products (on average 8 – 16 aa) escape further cytosolic destruction through translocation via the TAP transporter into the endoplasmic reticulum (ER), where they can be trimmed N-terminally by ERAP1 and ERAP2 (refs.<sup>1,2,7</sup>) and – when properly binding – assemble with HLA class I heterodimers before being routed to the cell surface. For class I binding, peptides need to comply with the binding motif of the particular class I molecule through the presence of proper anchor residues – located mostly at the second and the C-terminal positions in the peptide.

The absence of C-terminal excision of CTL epitopes in proteasome-inhibited cells<sup>8-10</sup>, together with failure to detect C-terminal trimming activities in the cytosol or ER, has led to the current notion that solely the proteasome liberates the exact C-terminus<sup>1,2</sup>. Cytosolic endopeptidases, however, may also produce peptides with a C-terminus fit for class I binding from protein degradation products lacking such an anchor. Indeed, cells treated with proteasome inhibitors still express substantial quantities of peptide-loaded HLA class I molecules<sup>11-15</sup>. Although these compounds are known to not inhibit completely, this finding suggests the existence of a partially proteasome-independent pathway of class I ligand generation. So far only a CTL epitope from HIV Nef was shown to depend on TPPII<sup>16</sup> and a human tumor epitope from MAGE-A3 was reported to be produced by insulin-degrading enzyme<sup>17</sup>, but the general importance of TPPII and insulin-degrading enzyme in CTL epitope generation is still unclear<sup>17,18</sup>. Other peptidases liberating the C-terminus of HLA class I

ligands are not known. To define such peptidases, we unraveled the precise generation of a CTL epitope with an unambiguously proteasome-independent C-terminus and subsequently assessed peptidases thus identified for their general roles in class I antigen processing.

## RESULTS

### **PRAME<sup>190-198</sup> has a proteasome-independent C-terminus**

Cleavage yielding a C-terminal lysine residue is not readily accomplished by proteasomes<sup>19</sup>. Therefore, we searched for a CTL epitope presented in HLA-A3, a human class I molecule that harbors peptides with a lysine as C-terminal anchor residue. The nonamer peptide ELFSYLIEK derived from tumor-associated protein PRAME<sup>20</sup> amino acids 190–198 (PRA<sup>190-198</sup>) was selected by binding prediction and bound with high affinity to HLA-A3 (**Supplementary Fig. 1**). Digestion of 27-mer PRA<sup>182-208</sup> with purified 20S proteasomes showed no cleavage after the epitope's C-terminal Lys-198 or in the region flanking the C-terminus. In contrast, its N-terminus was liberated by an efficient proteasomal cleavage before Glu-190 (**Fig. 1a**). The erythroleukemia cell line K562, which naturally expresses PRAME, was transfected with HLA-A3 to give K562-A3. Tandem mass spectrometry (MS/MS) identified the ELFSYLIEK peptide in peptides eluted from HLA-A3 on the surface of K562-A3 (**Supplementary Fig. 2**). Also, the CTL clone (no. 123) raised against the PRA<sup>190-198</sup> epitope exogenously loaded on HLA-A3, efficiently recognized K562-A3 and other tumor cell lines expressing PRAME and HLA-A3 in cytotoxicity assays (**Fig. 1b**), confirming natural presentation of the epitope. This CTL also recognized the exogenously loaded 11-, 12- and 13-mer C-terminally extended variants of the epitope (ELFSYLIEKVK/R/K), but these peptides - while possessing HLA-A3 binding affinity (**Supplementary Fig. 1**) - were not naturally presented (**Supplementary Fig. 2**). We used this feature of the CTL to monitor the generation of the epitope and its C-terminally extended variants in digests of longer PRAME peptides. The proteasomal digests of 25-mer PRA<sup>190-214</sup> (ELFSYLIEK located at the N-terminus) loaded onto EKR cells (HLA-A3<sup>+</sup>, PRAME-negative) were not recognized by the CTL (**Fig. 1c**), confirming proteasome-independence of this epitope's C-terminus.

### **Nardilysin produces precursors of PRAME<sup>190-198</sup>**

We pursued identification of the peptidase(s) generating the C-terminus of the ELFSYLIEK epitope. First, CTL recognition was strongly inhibited when K562-A3 cells were treated with the metallopeptidase inhibitor  $\alpha$ -phenanthroline, whereas other inhibitors had no effect (**Fig. 2a**). Since butabindide, the inhibitor of serine peptidase TPPII, can be inactivated by serum<sup>4</sup>, we further excluded the potential involvement of TPPII<sup>16, 21</sup> by digestion analysis and RNA interference (RNAi)

(**Supplementary Fig. 3**). Second, to identify the peptidase, we exposed the internally quenched fluorescent 15-mer substrate ELFSYL(-dab)IEKVKRC(-FL)KN (PRA<sup>190-204</sup>, epitope underlined) to digestion with cytosolic fractions obtained by anion exchange chromatography of K562-A3 cell lysates. Peptidase activity was identified in fraction no. 37 that cleaved, in a phenanthroline-sensitive fashion, between the quencher dabcyI at Lys-195 and the fluorescein (FL) group at position 202, thereby releasing fluorescence (**Fig. 2b**). This fraction contained nardilysin as the only peptidase among five proteins identified by MS/MS (**Fig. 2b, Supplementary Fig. 4**). Nardilysin, hitherto not implicated in class I antigen processing, is a cytosolic endopeptidase of the pitrilysin family of zinc-metalloproteases that is ubiquitously expressed<sup>22</sup> (<http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.584782>). Third, digestion of 25-mer PRA<sup>190-214</sup> (ELFSYLIEKVKRKKNVLRLCKKLK) with purified nardilysin gave highly efficient production of C-terminally extended 11-, 12- and 13-mer variants of the epitope by cleavages after Lys-200, Arg-201 and Lys-202 (**Fig. 2c**), in accordance with the specificity of nardilysin cleaving before or between two basic residues<sup>23,24</sup> in substrates up to ~30 aa. Indeed, the CTL efficiently recognized the nardilysin-digest when loaded onto HLA-A3<sup>+</sup> EKR cells, due to the presence of the ELFSYLIEKVK/R/K fragments, whereas the proteasomal digest was not recognized (**Fig. 2c**). Fourth, nardilysin was required to generate this epitope in cells because small inhibitory RNA (siRNA)-mediated suppression of nardilysin in K562-A3 and HeLa-A3 (HeLa, also PRAME<sup>+</sup> transfected with HLA-A3) strongly reduced their recognition by the CTL (**Fig. 2d**). Overexpression of nardilysin in HeLa-A3 cells enhanced CTL recognition (**Supplementary Fig. 7**). Together, these findings implicate nardilysin as indispensable for the endogenous C-terminal pre-processing of the PRA<sup>190-198</sup> epitope by producing cleavages after Lys-200, Arg-201 and Lys-202, but it does not excise the exact C-terminus (Lys-198).

### **TOP liberates the C-terminus of the PRAME<sup>190-198</sup> epitope**

We searched for the peptidase that generates the C-terminus of the epitope. Because of their cytosolic location, ubiquitous expression and specificity, prime candidates were TOP, neurolysin and insulin-degrading enzyme. Only the RNAi-mediated suppression of TOP, a ubiquitous endopeptidase predominantly expressed in the cytosol and nucleus, strongly reduced CTL recognition of K562-A3 cells whereas suppression of the other peptidases in these cells had no effect (**Fig. 3a**), indicating TOP's involvement in the endogenous generation of ELFSYLIEK. Purified TOP efficiently cleaved the nardilysin-dependent 12- and 13-mer precursors (PRA<sup>190-201/202</sup>, ELFSYLIEKVKR/K) directly after the epitope's C-terminal Lys-198. Thus, TOP released three or four C-terminal residues, in accordance with its cleavage preference<sup>25,26</sup>, thereby efficiently producing the exact nonameric epitope (**Fig. 3b**). The TOP-digest of 12-mer PRA<sup>190-201</sup> loaded on HLA-A3<sup>+</sup> EKR cells was recognized almost as efficiently as the minimal epitope (**Fig. 3c**). Consistent with its preference for peptides 6 to 17 aa in length<sup>27</sup>, longer peptides were not cleaved by TOP (**Supplementary Fig. 8** and data not shown), implying that pre-processing by nardilysin

into the 12-mer and 13-mer is required to make epitope precursors susceptible to TOP. This prediction was confirmed in the co-digestion of 25-mer PRA<sup>190-214</sup> with nardilysin and TOP, which resulted in the production of ELFSYLIEK. Accordingly, the double-digest was recognized more efficiently by the CTL - due to the presence of the epitope - than the digest with nardilysin alone which contained only the precursors (**Supplementary Fig. 8**). Thus, the C-terminus of the ELFSYLIEK epitope is released through sequential cleavages by nardilysin and TOP.

The N-terminus of the epitope was generated by an efficient proteasomal cleavage between Asp-189 and the N-terminal Glu-190 (**Fig. 1a**), which is performed by the caspase-like activity of the proteasome. This cleavage was completely blocked by the specific inhibitor AcAlaProNleLeuVSOH<sup>28</sup> of the caspase-like proteasome activity as shown in a digestion of 27-mer PRA<sup>172-198</sup> (**Supplementary Fig. 9a,b**). As expected, CTL recognition of cells treated with this inhibitor was strongly reduced (**Supplementary Fig. 9c**), conversely, the general proteasome inhibitor epoxomycin only partially inhibited cleavage before the N-terminus (**Supplementary Fig. 9a,b**). Interestingly, when cells were treated with epoxomycin or other general proteasome inhibitors, the presentation of ELFSYLIEK was markedly enhanced (**Supplementary Fig. 9c**), likely due to reduced competition with epoxomycin-sensitive HLA-A3 ligands.

These findings suggest the proteasome, nardilysin and TOP together produce the minimal epitope in the cytosol (schematic in **Supplementary Fig. 10**). As expected, presentation of the epitope was completely dependent on transport into the ER by TAP (**Supplementary Fig. 11a**). The ELFSYLIEK peptide was efficiently transported by TAP into the ER, whereas the 12- and 13-mer epitope-precursors were less efficiently translocated (**Supplementary Fig. 11b**). Thus, nardilysin-produced precursors are available in the cytosol for processing by TOP, whereas the minimal epitope - after its production - can rapidly escape from cytosolic destruction by translocation into the ER. This is the first evidence implicating nardilysin and TOP in CTL epitope production. Do these endopeptidases, either apart or together, have a more general role in class I antigen processing?

### **Nardilysin can generate both N-termini and C-termini**

Nardilysin in principle can support the generation of both the C-terminus and the N-terminus of CTL epitopes. We analyzed all published 1620 ligands of the most prevalent human class I molecules within their flanking regions. Cumulatively, the dibasic cleavage motif of nardilysin<sup>23,24</sup> is directly present at the N-terminus or C-terminus in 10.4% and 4.8% of the ligands, respectively. In another 5.2% and 5.7% of the 1620 ligands, the motif is located within four amino acids of respectively the N- or C-terminus (**Table 1**). Thus, nardilysin can potentially liberate the precise N- or C-terminus of many class I ligands and may generate precursor epitopes as well. The dibasic motif is most frequent directly at the N-terminus of HLA-B27 ligands (35.2%) and at (basic) C-termini of HLA-A3-supertype presented peptides (13%) (**Table 1** and **Supplementary Table 1**).

We examined whether purified nardilysin actually excised prototypical HLA-A3 and HLA-B27 ligands at the dibasic sites. The C-termini of two overlapping immunodominant HLA-A3-restricted CTL epitopes from HIV-1 Gag p17<sup>29</sup> and a naturally presented HLA-A11 ligand were efficiently produced (**Fig. 4a**). Importantly, 35.2% of all known HLA-B27 ligands contain a basic residue at position 1 (P1) next to their dominant class I binding Arg-anchor at P2, together constituting a dibasic motif (**Table 1** and **Supplementary Table 1**). Nardilysin efficiently liberated the N-terminus of eight (out of eight) prototypical HLA-B27 ligands and in three cases also produced the C-terminus at a dibasic site (**Fig. 4b,c**). Among the peptides tested are immunodominant CTL epitopes from HIV-1 gag p24 and Epstein-Barr virus EBNA3C<sup>30</sup>. The nardilysin dependence of the immunodominant HLA-B2705-presented EBNA3C(258-266) (RRIYDLIEL) CTL epitope<sup>30</sup> was then tested in live cells. Suppression of nardilysin in EBNA3C- and HLA-B2705-positive target cells strongly reduced the recognition of the epitope by a specific CTL<sup>30</sup> (clone RT.c38) (**Fig. 4d**). The nardilysin dependence resided in the N-terminal processing as tested by recognition of cells expressing the N-terminally extended epitope precursor (**Fig. 4d**). Thus, although aminopeptidases exist in the cytosol and ER, nardilysin is required for the excision of the EBNA epitope RRIYDLIEL.

### TOP can perform final C-terminal ligand trimming

The current notion is that TOP destroys epitopes<sup>6</sup>, thereby limiting class I antigen presentation<sup>5</sup> and raising the question whether the excision of ELFSYLIEK by TOP is an exception. TOP preferentially releases, with broad sequence specificity<sup>27,31</sup>, three to five C-terminal residues<sup>25,26</sup> from substrates extending up to 17 aa<sup>27</sup>, endowing TOP with the potential to generate the C-terminus of epitopes. We assessed the flexibility of TOP in producing different epitope sequences by systematically substituting the positions (P1 and P1') surrounding the scissile bond after the C-terminus of ELFSYLIEK (ELFSYLIEP1-P1'KRK). P1'-substitutions affected cleavage efficiency, but in all cases except for Pro at P1' which prevented digestion, production of the epitope (ELFSYLIEK) was >5% after 10 min digestion (**Fig. 5a**). Partial destruction by a subsequent cleavage in the epitope occurred, but did not prevent epitope-production (**Fig. 5a**) as also shown by efficient CTL recognition of the digests (**Fig. 5b**). Digestions of the P1-substituted variants resulted in generation of the epitope (ELFSYLIEX) when P1 is either Lys (wild-type sequence) or Arg or Glu (**Fig. 5a**), but not for other residues at this position. This result was caused by inefficient cleavage after ELFSYLIEX for some P1-residues (for example, Val, Ile, Asp) and/or by a rapid second cleavage in the middle of the epitope (for example, Phe, Trp, Cys, Tyr). The necessity for a Lys, Arg or Glu at the P1-position, however, was not absolute. When we completely changed the epitope, TOP produced epitopes with a C-terminal Ala or Leu (for example, SLYSFPEPEA and VLDGLDVLL; from ref. <sup>20</sup>) (**Fig. 5c**). These results indicate that TOP, without a distinct sequence-specificity, can produce CTL epitopes from a wide array of precursors.

We searched for other TOP-dependent CTL epitopes by RNAi-mediated suppression of TOP in target cells. CTL recognition of the clinically important melanoma-specific and HLA-A2-restricted MART-1 epitope<sup>32</sup> of which 9-mer and 10-mer variants are considered to be naturally presented<sup>33</sup> (E/AAGIGILTV, amino acids 26/27-35) was strongly reduced in TOP-suppressed melanoma cells (**Fig. 6a**), indicating its dependence on TOP. We investigated the underlying mechanism by performing *in vitro* digestions of C-terminal extended epitope precursors. Liberation of the epitope's C-terminal Val-35 was not observed, but purified TOP efficiently cleaved after Leu-33, Thr-34 and Leu-37 (**Fig. 6b**). Therefore, the requirement for TOP may be explained if variant epitopes, 26-EAAGIGILT-34 and/or 27-AAGIGILTVIL-37, are among those naturally processed and recognized by CTLs. The CTL clone specific for MART-1(26-35) efficiently recognized these variant peptides when exogenously loaded (**Fig. 6c**), although with lower sensitivity than the 26-EAAGIGILTV-35 peptide (**Fig. 6d**).

### **HLA class I allele specific processing effects**

In addition to producing class I ligands, nardilysin and TOP will sometimes also destroy other (potential) class I ligands. Consequently, dependent on the balance, the absolute quantity of ligands available for class I loading may be affected. Therefore, we measured cell surface HLA class I expression under conditions of overexpression and RNAi-mediated suppression of these peptidases. Overexpression of TOP, consistent with previous literature<sup>5</sup>, resulted in a reduced class I expression by HeLa cells (**Supplementary Fig. 12a**). Likewise, overexpression of nardilysin led to modestly attenuated expression of overall class I molecules by HeLa cells, and, specifically, of HLA-A3 and HLA-B27 by different cell types (**Supplementary Fig. 12a,b**). These findings indicate that overexpression of both TOP and nardilysin, not unexpectedly, tilt the balance between generation and destruction of class I ligands by these peptidases. The contribution of each peptidase to the overall quantity of class I ligands produced under physiological conditions is more likely to be revealed by testing cell surface class I abundance when peptidase expression is suppressed. We specifically focused on HLA-A2, -A3 and -B27 molecules (naturally expressed by three B-LCLs; HAR, RT and EKR), because these are the restriction elements of the epitopes identified in our foregoing experiments and also because their ligands are presumed either dependent (for HLA-A2) or relatively independent (for HLA-A3 and -B27) of the proteasome<sup>11,14,15</sup>. Consistently, class I allele-specific effects were observed when nardilysin expression was suppressed. Cell surface HLA-A2 and -A3 expression was slightly enhanced (4% – 9%), suggesting a net destructive effect of nardilysin in the production of peptides presented by these class I molecules, while HLA-B27 and -B35 expression was slightly reduced (5% – 11%). The latter result suggests a net productive role of nardilysin in the generation of HLA-B27-and -B35 ligands (**Supplementary Fig. 12c**). When tested in the same B-LCL, suppression of TOP led to a remarkably similar pattern of changes in class I expression, consistently showing slightly reduced expression of HLA-B27 and -B35 and slightly enhanced expression of HLA-A2 and -A3, while HLA-



A24 and -B7 expression was unaltered (**Supplementary Fig. 12d**). The pattern of reduced expression of HLA-B27 and enhanced HLA-A2 expression existed together on both the HAR and RT cells excluding general non-specific RNAi-induced effects. These alterations in class I expression were not changed after incubation of the cells with brefeldin A to test possible differences in the off-rate of the peptides presented by peptidase-suppressed cells versus control cells (data not shown), indicating that nardilysin- or TOP-mediated processing does not alter the overall allele-specific class I-peptide stability.

## DISCUSSION

The cytosolic endopeptidases nardilysin and TOP, either alone or together, are here found to be indispensable for the generation of three defined CTL epitopes. Nardilysin appears to be involved in the generation of many class I ligands either directly at or within four residues of their N- or C-terminus and can produce both basic C-termini (for binding to HLA-A3/A11/A68 and HLA-B27) or non-basic C-termini (for binding to other class I molecules). The efficient cleavage of nardilysin at dibasic motifs suggests its application in rational design of poly-epitope vaccines through the insertion of dibasic moieties between CTL epitopes to guide epitope excision and enhance vaccine immunogenicity. A positive processing effect of a dibasic motif situated adjacent to a CTL epitope has previously been demonstrated<sup>34</sup>.

TOP efficiently liberated the epitope's C-terminus in a variety of systematically substituted precursors of the ELFSYLIEK epitope and other epitopes, consistent with its known flexible capacity to remove three to five C-terminal residues from the substrate<sup>25-27,31</sup>. Purified TOP, however, did not liberate the anticipated C-terminal Val-35 residue of the HLA-A2-restricted MART-1 epitope of which two N-terminal variants – nonamer 27-AAGIGILTV-35 and decamer 26-EAAGIGILTV-35 – are considered to be naturally presented<sup>33</sup>. Instead, TOP cleaved after Leu-33, Thr-34 and Leu-37. The 24-AEEAAGIGILT-34 peptide is naturally presented by HLA-B45 (ref. <sup>35</sup>) indicating the endogenous excision of Thr-34 as C-terminus and suggesting the additional presentation of 26-EAAGIGILT-34 by HLA-A2 as the explanation for the TOP-dependent CTL recognition. Future experiments are required to identify the natural presentation by HLA-A2 of a TOP-dependent variant MART-1 peptide.

A CTL epitope from M. Tuberculosis Hsp65 with undefined aa sequence has previously been shown to rely on TOP<sup>36</sup>. A possible role for TOP in the protection of some cytosolic peptides from further destruction has been documented<sup>37</sup>, however, this could not be confirmed<sup>38</sup>. To the contrary, biochemical and class I expression results<sup>5,6,38,39</sup> led to the current notion that TOP has solely a destructive role in class I antigen processing<sup>1,2</sup>. However, destruction of defined epitopes by TOP as studied by CTL recognition of target cells with suppressed expression of TOP, has thus far only been shown for the mouse H2-K<sup>b</sup>-presented SIINFEKL epitope from ovalbumin<sup>5</sup>. The role of TOP in the production of the PRA<sup>190-198</sup> and MART-1 epitopes is in accordance with a recent study that demonstrated that TOP in the cytosol both destroys and generates peptides of the

length of class I ligands<sup>40</sup>. Thus the role of TOP in antigen processing appears to be twofold. On the one hand, TOP has a destructive role<sup>5,38</sup> – limiting presentation of epitopes whose C-terminus has already been made by e.g. the proteasome, like SIINFEKL<sup>5,8</sup>. On the other hand, TOP generates C-termini fit for class I binding by trimming of C-terminally extended epitope precursors. As such, TOP-mediated processing both dampens the class I presentation of proteasomal products and enlarges the HLA class I peptide repertoire. This new model fits with both the literature and our results.

The proportions of HLA class I ligands depending on nardilysin or TOP for their generation are not yet known. Our results show class I allele-specific influences of processing by nardilysin and TOP. Suppression of both peptidases slightly enhanced expression of HLA-A2 and -A3, indicating a destructive effect on ligand generation, as reported for TOP in the literature<sup>5,39</sup>. The liberation of the aliphatic C-terminus of HLA-A2 ligands is largely proteasome-dependent. By C-terminal trimming, TOP may destroy a substantial quantity of these HLA-A2 ligands. With respect to nardilysin, its dibasic cleavage motif is at the C-terminus (i.e. the C-terminus itself and the P1'-residue) in 13% (31 of 238) of HLA-A3-supertype presented peptides. In our digestions nardilysin produced two of these epitopes, but it may also destroy several of these peptides by cleaving in front of the dibasic motif thereby reducing HLA-A3 ligand availability. Importantly, defined peptides that are presented by class I molecules of which the expression is quantitatively unaltered or enhanced after peptidase-suppression may still depend on nardilysin or TOP. This is demonstrated by the nardilysin- and/or TOP-dependence of the PRA<sup>190-198</sup> and MART-1 epitopes presented by HLA-A3 and HLA-A2, respectively, emphasizing the need to study the processing of defined CTL epitopes.

Suppression of Nardilysin and TOP, on the other hand, slightly reduced the expression of HLA-B27 and HLA-B35, suggesting a productive effect on ligand generation. Peptides presented by HLA-B27<sup>14,15</sup> and HLA-B35<sup>11</sup> were previously found to be relatively insensitive to proteasome inhibition consistent with a role for cytosolic endopeptidases in their C-terminal generation. TOP can assist ligand formation for these class I molecules by trimming C-terminally extended precursors. As shown for the EBNA3 epitope and in the digestions, nardilysin can generate the N-terminus of many HLA-B27 ligands by virtue of their frequent dibasic N-terminal moiety (35.2%), and can also liberate these ligands C-terminally at a dibasic site. Thus nardilysin-mediated processing likely contributes to the strong over-representation of Arg and Lys at P1 in HLA-B27 ligands - a position that only modestly contributes to binding. Another factor contributing to the prevalence of the N-terminal dibasic motif of HLA-B27 ligands may be that it enforces peptide stability in the cytosol<sup>41</sup>. Four of the eight HLA-B27 ligands that were excised by nardilysin at their N-terminus were previously found to be proteasome-independent when studied with proteasome inhibitors<sup>15</sup> (see **Supplementary Table 2**), supporting a role for an additional peptidase in their generation. Most of the proteasome-inhibitor insensitive HLA-B27 ligands in that study<sup>15</sup> were derived from small basic proteins, also suggesting that nardilysin is involved in their processing. However, all studies<sup>11,14,15</sup>

that reveal proteasome-independent class I peptide presentation by the use of proteasome inhibitors as the primary experimental tool should be evaluated in the light of residual proteasome activity still present in inhibitor-treated cells, leaving unanswered whether completely proteasome-independent CTL epitopes are ever produced in the cytosol. This is exemplified by the ELFSYLIEK epitope, which although dependent on the proteasome for its N-terminal generation nevertheless showed enhanced presentation following proteasome inhibition.

Because of their substrate length constraints of ~30 aa and ~17 aa respectively, nardilysin and TOP will likely act only after hydrolysis by the proteasome of either polypeptidic defective ribosomal products or full length 'retired' proteins. The generation of the C-terminus of the ELFSYLIEK epitope, requiring nardilysin cleavage before TOP can liberate the C-terminus, again illustrates that both substrate length and aa sequence characteristics determine which peptidase acts where at what time in cytosolic class I antigen processing. With the addition of nardilysin and TOP, four peptidases have now been identified in the C-terminal generation of CTL epitopes. TPPII has been shown to generate the HLA-A3-restricted HIV Nef(73-81) epitope<sup>16</sup>. Next to its established tripeptidyl aminopeptidase activity involved in N-terminal epitope generation, a broad role for the endoproteolytic activity of TPPII in C-terminal epitope generation has not yet been detected<sup>18</sup>. Recently, the HLA-A1-presented human tumor epitope from MAGE-A3 was reported to be produced by insulin-degrading enzyme<sup>17</sup>, but a general role for this peptidase in antigen processing remains to be established. Together our results and these findings imply that the concept that solely the proteasome is responsible for C-terminal epitope generation needs reappraisal.

In conclusion, by producing novel class I binding peptides from protein degradation fragments that are not yet fit for antigenic presentation, especially those that lack a class I binding C-terminal anchor, nardilysin and TOP broaden the diversity of the antigenic peptide repertoire. Thus, both peptidases expand the options for a successful CTL response against intracellular pathogens and cancer.

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## **Author contributions**

CJMM, FO and PAvV contributed equally to this work as senior authors. JHK conceived the idea of the study, coordinated the work, designed, performed and analysed most experiments and wrote the manuscript with main input from CJMM and minor input from other authors. SK performed experiments and analyses of Fig. 2B and immunoblots. KMC, LBH and APr contributed fundamentally to the experiments on the role of nardilysin; DWR, KR, US and AP on the role of TOP; PMK, US and BT on the role of TPPII; HSO and PFvS on the role of the proteasome and JN and TvH on the role of TAP. NvM, US, AP, SLG and JMB contributed to CTL experiments. JWD, FO, JN, SK and WEB contributed to substrate design and synthesis. SABV and KLMCF contributed to the molecular biology involved. AM and IIND made HLA class I mAbs and single class I allele expressing cell lines. PAvV and AdR performed mass spectrometric analyses.

## **Competing financial interests**

The authors declare no competing financial interests.

## LEGENDS

**Figure 1. PRA<sup>190-198</sup> is an HLA-A3-presented CTL epitope with proteasome-independent C-terminus.**

**(a)** Proteasomal digestion site in 27-mer PRA<sup>182-208</sup> (ELFSYLIEK, printed bold), indicated by the arrow. Fragments containing Asp-189 at the C-terminus and complementary fragments with Glu-190 as N-terminus added up to >7% of the digested material after 1 h. Digestion by immuno- or constitutive human 20S proteasomes gave comparable results. Digestions were repeated three times with similar results.

**(b)** Specific lysis by the CTL anti-ELFSYLIEK (clone no. 123) of K562-A3, renal cell carcinoma cell lines MZ1257 and 94.15 (both PRAME<sup>+</sup>, HLA-A3<sup>+</sup>) and K562-A2 (HLA-A2<sup>+</sup>). Mean (SEM) of triplicate wells of a representative experiment (n=5).

**(c)** Absence of recognition of 25-mer PRA<sup>190-214</sup> (ELFSYLIEKVKRKKNVLRLLCCKKLK, epitope at N-terminus) digested for 1 h with immuno- or constitutive proteasomes. Mean (SEM) of triplicate wells of a representative experiment (n=3). Digests were titrated, loaded on B-LCL EKR cells (HLA-A3<sup>+</sup>; PRAME-negative), co-incubated with the CTL anti-ELFSYLIEK and IFN- $\gamma$  production was measured by ELISA. Background recognition is of the PRA<sup>190-214</sup> substrate incubated at 37 °C without enzyme (mock digest), positive control is the ELFSYLIEK 9-mer peptide loaded at the same concentrations.

**Figure 2. Nardilysin produces C-terminal extended precursors of PRA<sup>190-198</sup>.**

**(a)** Recognition of K562-A3 treated with inhibitors (concentration and activity, see Methods) by CTL anti-ELFSYLIEK relative to the recognition of untreated K562-A3 (100% recognition). Intracellular IFN- $\gamma$  production was measured. Mean (SEM) of three experiments.

**(b)** Digestion (30 min) of fluorogenic substrate ELFSYL(-dabcyI)IEKVKRC(-FL)KN by cytosolic fractions of K562-A3 cells (KCl-gradient indicated) with or without phenanthroline (1 mM). The same substrate with the first three aa being D-aa's showed only slightly reduced fluorescence (data not shown). Right, SDS-PAGE separation of fraction no. 37; nardilysin was identified in band 5, shown in **Supplementary Fig. 4**.

**(c)** Nardilysin digestion sites in 25-mer PRA<sup>190-214</sup> (epitope, bold) (30 min digestion). Arrows indicate cleavages generating the 11-, 12- and 13-mer peptides, present for 33.9%, 41.2% and 16.9% of fragments, respectively, after 30 min digestion. Graph, recognition by CTL anti-ELFSYLIEK (IFN- $\gamma$  ELISA) of PRA<sup>190-214</sup> digested (30 min) with purified nardilysin, immuno-proteasomes or constitutive proteasomes and loaded on EKR cells. SEM of triplicates of a representative experiment (n=4).

**(d)** Recognition by CTL anti-ELFSYLIEK of K562-A3 and HeLa-A3 with suppressed nardilysin (si-NRD) or control siRNA. SEM of triplicates of representative experiment (n=5). Nardilysin immunoblot of K562-A3 variants is shown. Peptide loading control experiments are shown in

**Supplementary Fig. 5a.** Nardilysin suppression did not compromise proteasome-mediated processing (see **Supplementary Fig. 6**).

**Figure 3. TOP produces the C-terminus of the PRA<sup>190-198</sup> epitope.**

**(a)** Left, recognition by CTL anti-ELFSYLIEK of K562-A3 cells transfected with siRNA pools suppressing TOP, neurolysin and insuline degrading enzyme (IDE), relative to the recognition of K562-A3 transfected with non-targeting control siRNA (100% recognition). Right, CTL recognition of K562-A3 stably expressing siRNA to suppress TOP or control siRNA. IFN- $\gamma$  production was measured by ELISA. SEM of three or four experiments is shown. TOP immunoblot of stably transfected K562-A3 variants is shown. Peptide loading control experiments are shown in **Supplementary Fig. 5a**. TOP suppression did not compromise proteasome-mediated processing (see **Supplementary Fig. 6**).

**(b)** Digestion of 13-, 12- and 11-mer substrates PRA<sup>190-202/201/200</sup> (ELFSYLIEKVK/R/K, epitope bold) with purified TOP; the substrates are shown. Arrow indicates the cleavage after Lys-198. From each substrate the percentage of fragments produced after 30 min digestion that match the ELFSYLIEK-epitope is listed.

**(c)** Recognition by CTL anti-ELFSYLIEK of 12-mer PRA<sup>190-201</sup> (ELFSYLIEKVKR) digested for 10 min or 1 h with or without TOP, titrated and loaded on EKR cells. The undigested 12-mer is not recognized at these concentrations. The ELFSYLIEK 9-mer is the positive reference. IFN- $\gamma$  measured by ELISA. SEM of triplicate wells of representative experiment (n=3).

**Figure 4. Role of nardilysin in HLA class I antigen processing.**

**(a-c)** Digestions sites of purified nardilysin in long peptides encompassing published HLA-A3-, HLA-A11- and HLA-B27-ligands, taken from the SYFPEITHI database. Dibasic motifs are bold-faced. Arrows, bold, cleavage present in >5% of the fragments after 30 min digestion; thin, cleavage present in  $\leq$  5% of fragments.

**(a)** Upper peptide, HIV-1 gag p17 aa 8-37 containing two overlapping HLA-A3-presented epitopes (separately depicted, underlined). Lower peptide, thymosin- $\beta$  aa 1-30 containing HLA-A11-ligand ASFDKAKLK (underlined). The proteasome failed to produce the indicated cleavages (data not shown).

**(b)** Peptides encompassing HLA-B2705 ligands (underlined), which are detailed in

**Supplementary Table 2.** The upper four ligands were reportedly proteasome-inhibitor treatment insensitive<sup>15</sup>. **(c)** 20-mer EBNA3C aa 246-266 (CTL epitope aa 258-266 underlined). Percentage of epitope produced after 10 and 30 min indicated. **(d)** Recognition by CTL anti-EBNA3C(258-266) of (left) B-LCL RT (HLA-B2705<sup>+</sup>, EBNA3C<sup>+</sup>) with or without RNAi-mediated suppressed nardilysin and (right) recognition of K562-B27-mini, expressing HLA-B2705 and MFLRGKWQRRYRRRIYDLIEL (EBNA3C aa 247-266, epitope underlined) with or without suppressed nardilysin. SEM of triplicate

wells of a representative experiment (n=4). Nardilysin immunoblot of RT target cells is shown. Exogenous peptide loading control experiments are shown in **Supplementary Fig. 5b**.

**Figure 5. The epitope-generating trimming capacity of TOP.**

**(a)** Digestion by purified TOP of variants of 13-mer ELFSYLIEKVKRK (PRA<sup>190-202</sup>) with systematically substituted P1- and P1'-residues surrounding the TOP cleavage site (ELFSYLIE**P**<sub>1</sub>-**P**<sub>1</sub>'KRK). Wild type peptide is included twice, indicated by arrow and bold printed residue. Top, epitope-production (ELFSYLIEK or ELFSYLIE**X** for P1'- and P1-substitutions, respectively; **X** is mutated) after 10 min and 30 min indicated as percentage of total summed fragment intensities. Substitutions ordered according to epitope-production at 10 min. Bottom, epitope-destruction through a subsequent cleavage in the middle of the epitope indicated as the percentage of peptides in the digest constituting fragments of the epitope after 10 min and 30 min digestion.

**(b)** Recognition by CTL anti-ELFSYLIEK of TOP-digested P1'-substitution variants loaded on EKR cells (HLA-A3<sup>+</sup>). Wild type peptide indicated by the arrow and bold-faced valine. The P1'-proline variant, which was not digested, served as negative control. IFN-γ measured by ELISA.

**(c)** Cleavages by TOP (arrows) liberating the CTL epitopes (underlined) SLYSFPEPEA (PRAME<sup>142-151</sup>), VLDGLDVLL (PRAME<sup>100-108</sup>) and ELFSYLIEK (PRA<sup>190-198</sup>) from their non-natural C-terminal flanking sequences VKRK or RRFV. Quantities of epitope produced after 10, 30 and 60 min digestion are listed. Digestions **(a,c)** were repeated twice and CTL experiment **(b)** three times with similar results.

**Figure 6. TOP-dependent presentation by HLA-A2 of the MART-1 CTL epitope.**

**(a)** Recognition by the CTL clone anti-EAAGIGILTV (MART-1(26-35)) of UKRV-Mel-15a melanoma cells (HLA-A2<sup>+</sup>, MART-1<sup>+</sup>) with RNAi-mediated suppressed expression of TOP, relative to recognition of control siRNA treated cells (100%). IFN-γ production was measured by ELISPOT. Mean (SEM) is of two representative experiments. TOP RT-PCR of the target cells is shown. Peptide loading control experiments are shown in **Supplementary Fig. 5c**.

**(b)** Digestion sites of purified TOP (arrows) in 13-, 14-, 15-, 16- and 17-mer substrates MART-1(26-38/39/40/41/42); the EAAGIGILTV epitope is underlined. Fragments containing Leu-33 and Thr-34 or Leu-37 as C-terminus were each present ≥ 6% after 10 min digestion.

**(c)** Recognition by the CTL anti-EAAGIGILTV (clone no. 313) of B-LCL JY cells (HLA-A2<sup>+</sup>, MART-1 negative) loaded with 10 μM of the indicated MART-1 peptides, being aa 26-35 (EAAGIGILTV), aa 26-34, aa 27-34 and aa 27-37. Negative control is VLDGLDVLL (HLA-A2-restricted PRA<sup>100-108</sup> epitope<sup>20</sup>). Specific lysis was measured and CTL were titrated at the indicated ET-ratios. A representative test of three performed is shown.

**(d)** Recognition by the CTL anti-EAAGIGILTV of JY cells loaded with titrated concentrations of the peptides indicated in **c**. Intracellular IFN- $\gamma$  production was measured. A representative test of three performed is shown.



**TABLE 1.** Proportions of HLA class I ligands potentially excised by nardilysin

HLA class I	Ligands <sup>a</sup>	C-dir. <sup>b</sup> (%)	C-ind. <sup>c</sup> (%)	N-dir. <sup>d</sup> (%)	N-ind. <sup>e</sup> (%)	Total <sup>f</sup> (%)
HLA-A1	86	5.8	7.0	0.0	3.5	16.3
HLA-A2	515	1.7	5.0	2.5	4.5	13.7
HLA-A3	121	10.7	5.8	4.1	5.0	25.6
HLA-A11	71	11.3	2.8	1.4	7.0	22.5
HLA-A68	46	21.7	17.4	2.2	0.0	41.3
Cumulative A3-type <sup>g</sup>	<b>(238)</b>	<b>13.0</b>	<b>7.1</b>	<b>2.9</b>	<b>4.6</b>	<b>27.6</b>
HLA-A24	51	2.0	3.9	5.9	9.8	21.6
HLA-B7	68	2.9	7.4	1.5	4.4	16.2
HLA-B8	52	0.0	5.8	0.0	3.8	9.6
HLA-B2701	8	0.0	12.5	37.5	25.0	75.0
HLA-B2702	18	0.0	5.6	61.1	5.6	72.3
HLA-B2703	29	3.4	3.4	62.1	0.0	68.9
HLA-B2704	52	5.8	3.8	38.5	3.8	51.9
HLA-B2705	185	7.6	5.9	31.4	4.3	49.2
HLA-B2706	38	5.3	7.9	26.3	10.5	50.0
HLA-B2707	3	0.0	0.0	66.7	0.0	66.7
HLA-B2709	56	1.8	7.1	26.8	3.6	39.3
Cumulative B27 <sup>h</sup>	<b>(389)</b>	<b>5.4</b>	<b>5.9</b>	<b>35.2</b>	<b>4.9</b>	<b>51.4</b>
HLA-B35	11	9.1	9.1	9.1	9.1	36.4
HLA-B3501	20	0.0	5.0	0.0	5.0	10.0
HLA-B44	53	3.8	1.9	1.9	15.1	22.7
HLA-B60 (B4001)	34	2.9	5.9	0.0	8.8	17.6
HLA-B61 (B4002)	7	0.0	14.3	0.0	0.0	14.3
HLA-B62 (B1501)	96	4.2	5.2	5.2	5.2	19.8
<b>Cumulative<sup>k</sup></b>	<b>1620</b>	<b>4.8</b>	<b>5.7</b>	<b>10.4</b>	<b>5.2</b>	<b>26.1</b>

<sup>a</sup> Number of ligands per class I molecule. Per allele, all ligands in SYFPEITHI database were analyzed for presence of the dibasic nardilysin (NRD)-motif at the N- or C-terminus or in the flanking regions.

<sup>b</sup> C-dir.; NRD-motif present at the C-term, potentially leading to direct production of the C-terminus.

<sup>c</sup> C-ind.; NRD-motif present within 4 aa of the C-terminus, potentially leading to precursor formation.

<sup>d</sup> N-dir.; NRD-motif present at the N-term, potentially leading to direct production of the N-terminus.

<sup>e</sup> N-ind.; NRD-motif present within 4 aa of the N-terminus, potentially leading to precursor formation.

<sup>f</sup> Total % of ligands per allele that is potentially N-terminally or C-terminally dependent on NRD-cleavage.

<sup>g</sup> Cumulative % of NRD-motif containing ligands for HLA-A3, HLA-A11 and HLA-A68.

<sup>h</sup> Cumulative % of NRD-motif containing ligands for HLA-B27 subtypes.

<sup>k</sup> Cumulative % of ligands with NRD-motif directly or indirectly at the C-term or N-term for all 1620 ligands.

## METHODS

### ***Cell lines and HLA class I staining***

Cell lines used were K562 (HLA class I neg., PRAME<sup>+</sup>) transduced to express HLA-A3 (-A0301), HLA-A2 (-0201) and HLA-B27 (-B2705); HeLa (cervical carcinoma) (PRAME<sup>+</sup>) transfected to express HLA-A3; renal cell carcinoma cell lines MZ1257 and Le-94.15 (both PRAME<sup>+</sup> and HLA-A3<sup>+</sup>); B-LCLs EKR, RT and HAR; UKRV-Mel-15a (melanoma cell line). HeLa, K562-A3 and RT cells overexpressing rat or human nardilysin were generated by transfection with plasmids pIRES2-NRD1-eGFP or plasmid pCDNA3.1-NRD, respectively (rat and human nardilysin have similar cleavage specificity<sup>42</sup>). HeLa overexpressing human TOP was generated with plasmid pcDNA3.1-TOP. K562-A3 expressing TAP inhibitor ICP47 was generated with plasmid pLZRS-IRES-eGFP-ICP47. K562-B27-mini stably expressed EBNA3C aa 247-266 with an extra N-terminal methionine. HLA-A2 and HLA-A3 expression was measured by flow cytometry using mouse mAb BB7.2 and GAP-A3, respectively. Expression of HLA-A24, HLA-B7, HLA-B27 and HLA-B35 was measured using human mAb IND3H3, VTM1F11, WAR5D5 and HDG8D9, respectively<sup>43</sup>. Pan-class I mAb W6.32 was used to measure overall HLA class I expression and HLA-A2, -A3 and -B27 expressed by K562 cells.

### ***Synthetic peptides***

Peptides were synthesized as described previously<sup>20</sup>. Fluorescein was covalently coupled to the cysteine residue using 5-(iodoacetamido)fluorescein (Fluka Chemie AG). The quencher residue Fmoc-L-Lys(Dabcyl)-OH was obtained from Neosystems. A 2-naphthylsulfonyl group was used for N-terminal blocking. Peptides were HPLC-purified and validated by mass spectrometry.

### ***HLA-A3 peptide binding assay***

Affinity of peptides for HLA-A3 was measured as described previously<sup>44</sup>. Briefly, titrated amounts of test peptides competed for binding to cell surface HLA-A3 with the known high-affinity fluorescein (FL)-labeled reference peptide KVFPC(FL)ALINK<sup>44</sup> and mean fluorescence (MF) was measured by flow cytometry. Inhibition of FL-labeled reference peptide binding at each test peptide concentration used was calculated using the formula:  $(1 - (MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}}) / (MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})) \times 100\%$ .

### ***CTL clones, CTL assays and functional peptidase inhibition assays***

The CTL clone (no. 123) anti-PRA<sup>190-198</sup> (ELFSYLIEK) and CTL clone (no. 313) anti-MART-1<sup>26-35</sup> (EAAGIGILTV) were generated by in vitro stimulation against synthetic peptides exogenously loaded on antigen presenting cells, according to procedures described previously<sup>20</sup>, using HLA class I-typed PBMCs obtained with prior informed consent from an anonymous blood bank donor or a melanoma patient, respectively. The CTL (clone RT.c38) anti-EBNA-3C (aa 258-266) (RRIYDLIEL) was generated previously<sup>30</sup>. CTL recognition was either determined in a <sup>51</sup>Cr-release cytotoxicity assay or by measuring IFN- $\gamma$  production in ELISA or ELISPOT assays or by intracellular cytokine staining (ICS). In functional peptidase inhibition experiments K562-A3 cells were pre-treated with the inhibitor for 1.5 h at 37 °C, stripped from class I-presented peptides ('peptide stripping') by mild acid treatment (300 mM glycine, 1% BSA; pH 2.9) for 2 min, washed (brought to pH 7) and treated for another 5 h at 37 °C with the inhibitor. Background and maximal

epitope expression was obtained by treating cells with brefeldin A (10 µg/ml) or leaving cells untreated after stripping, respectively. After inhibitor treatment, the cells were washed, plated in triplicate wells of a 96-well plate ( $3 \times 10^4$  cells per well) and co-incubated with the CTL clone ( $3 \times 10^4$ /well) for 6 h. After 30 min, brefeldin A was added (5 µg/ml). The co-cultures were then subjected to standard ICS procedures. CTLs were stained with CD8-APC (clone DK25, Dako) and IFN- $\gamma$ -PE (BD Pharmingen) mAbs and measured by flow cytometry. Inhibitors used were: brefeldin A (Sigma) used at 10 µg/ml; phenanthroline (Sigma), an inhibitor of metallopeptidases used at 400 µM (in cellular assays); insulin (Sigma), a competitive inhibitor of insulin degrading enzyme used at 50 µM; captopril (Sigma), an inhibitor of angiotensin converting enzyme used at 50 µM; leupeptin (Calbiochem), an inhibitor of trypsin-like and cysteine proteases used at 100 µM; calpeptin (Calbiochem), an inhibitor of calpains used at 30 µM; aprotinin (Calbiochem), a serine protease inhibitor used at 5 µM; and Butabindide (Tocris), an inhibitor of TPPII used at 200 µM. Proteasome inhibition was performed with PSI, epoxomicin (both Calbiochem), AdaAhx<sub>3</sub>L<sub>3</sub>VS<sup>45</sup> and AcAlaProNleLeuVSOH<sup>28</sup>.

### ***Peptide digestions and digest recognition assays***

Proteasomes (20S) were purified, as described<sup>20</sup>, from HeLa cells, containing mainly constitutive proteasomes, and from B-LCL JY containing mainly immunoproteasomes. Composition was confirmed by immunoblotting (data not shown). TPPII was purified from erythrocyte concentrates as described<sup>46</sup> with minor modifications<sup>47</sup>. Recombinant mouse nardilysin was prepared as described<sup>24</sup> and was homogeneous as judged by SDS-PAGE<sup>24</sup>. Digestions with long peptides were performed with mouse nardilysin and with cytosolic fraction no. 37 of K562 cells containing human nardilysin with similar results. Mouse and human nardilysin have similar cleavage specificities<sup>42</sup>. TOP was produced by overexpression in *Escherichia coli* and purified using Ni-resin (Qiagen) affinity chromatography. Digestions were performed with mostly 10 µM peptide and 4.3 nM enzyme at 37 °C. TPPII digestions were performed with 1 µg enzyme (MW > 10<sup>6</sup>) and 2 nmol peptide in 100 µl. Mock digestions were performed at 37 °C without enzyme. The digestion buffers were: for proteasome and nardilysin digestions 30 mM Tris (pH 7.5), 10 mM KCl, 5 mM MgCl<sub>2</sub> and 1 mM DTT; for TOP digestion 50 mM HEPES (pH 7.6), 2 mM MgCl<sub>2</sub>, 0.1 mM DTT and for TPPII digestion 20 mM HEPES, 2 mM Mg(Ac)<sub>2</sub>, 2 mM DTT, containing 5% glycerol. Enzymatic reactions were stopped by addition of acetic acid (5% v/v) or, when used in CTL assays, by addition of phenanthroline (1 mM). For CTL recognition, digest-samples were titrated and 10 µl digest was loaded onto 90 µl EKR cells (B-LCL: HLA-A3<sup>+</sup>, PRAME-negative) in triplicate wells of a 96-well plate ( $1.7 \times 10^4$  cells/well) for 2 h at 4 °C. Synthetic reference peptides were titrated and loaded similarly. To improve loading-efficiency, EKR cells were first stripped from naturally presented peptides (see before) and resuspended in 100 µl IMDM containing 2 µg/ml human  $\beta_2$ -microglobulin (Sigma). After loading,  $2.5 \times 10^4$  CTLs (in 50 µl) were added, co-incubated (12 h) and IFN- $\gamma$  production was measured by ELISA.

### ***Identification of PRA<sup>190-198</sup> on the surface of K562-A3 cells***

K562-A3 cells ( $3 \times 10^{10}$ ) were lysed in lysis buffer, 20 mM Tris (pH 8), 5 mM MgCl<sub>2</sub> containing 0.5% Zwittergent-12 (Calbiochem). Membrane fragments were removed by ultracentrifugation and the supernatant was precleared with Sepharose CL-4B (Amersham Biosciences). Immunoaffinity purification of HLA-A3 molecules was performed with pan-HLA class I mAb W6.32 coupled to protein A beads. Peptides were eluted with 10% HAc, filtered over a 10 kDa filter (Amicon), and separated in 60 fractions (100 µl) using

reverse-phase micro C2C18 HPLC (Smart System, Amersham). Buffer A was 0.1% heptafluorobutyric acid (HFBA) in water, buffer B 0.1% HFBA in acetonitrile. Fractions were lyophilized, dissolved in water (100  $\mu$ l) and used for testing CTL reactivity and mass spectrometric analysis. For CTL assays, 10  $\mu$ l of each fraction was loaded onto EKR cells ( $2 \times 10^4$ /well, 96-well plate) and co-incubated with CTL (25000/well). EKR cells were first stripped from cell surface presented peptides and resuspended in IMDM containing 2  $\mu$ g/ml human  $\beta_2$ -microglobulin (Sigma).

### ***Isolation of nardilysin***

K562-A3 cells ( $5 \times 10^9$ ) were homogenized in lysis buffer (10 mM Tris-HCl, 1 mM DTT, 1 mM  $\text{NaN}_3$ , 25 mM NaCl, 10 mM  $\text{MgCl}_2$ , 0.1 mM EDTA, 10% glycerol, 2 mM ATP, 50 mM NaF, 0.1 mM  $\text{Na}_2\text{VO}_4$ , pH 7.5) using a Dounce glass homogenizer. The homogenate was centrifuged for 20 min at  $10,000 \times g$  to remove nuclei and cell membrane debris. The supernatant was cleared from proteasomes and TPPII by centrifugation for 6 h at  $100,000 \times g$ . The clarified lysate was fractionated over a MonoQ anion exchange column (HR 5/5, Amersham) using a linear gradient from 0 to 500 mM KCl in lysis buffer (20 ml). Specific peptidase activity in the fractions was assessed with fluorogenic substrate ELFSYL(-dab)IEKVKRC(-FL)KN (0.5  $\mu$ M) in substrate buffer (30 mM Tris, 10 mM KCl, 5 mM  $\text{MgCl}_2$ , 1 mM DTT, pH 7.5). Fluorescence was measured at 360 nm extinction and 460 nm emission. Fractions no. 36 to 40 containing peak proteolytic activity were run on 12% SDS polyacrylamide gel and protein bands were isolated, subjected to digestion with trypsin and analyzed by mass spectrometry.

### ***Suppression of peptidase expression by RNA interference***

RNAi-mediated suppression of peptidase expression was established by either stable siRNA expression using the pSUPER-puro vector or by transient transfection of pools of four interfering RNA duplexes (siGENOME SMARTpool; Dharmacon). In transient transfections, using HiPerFect (Qiagen) or DharmaFECT-1 (Dharmacon) reagents, 100 nM siRNA was transfected and cells were used in CTL assays after 72 h. The empirically tested target sequence chosen for stable suppression of nardilysin was 5'-AGCAGACCCTTGGGTACCA-3'. Suppression of TOP by stable siRNA expression was targeted at 5'-CCTCAACGAGGACACCACC-3' (from ref. 39) and transiently by a siRNA pool targeting at: 5'-TAGATGAGCTGGCGCAGAATT-3', 5'-TCAAACGCATCAAGAAGAATT-3', 5'-GCAAGGTTGGCATGGATTATT-3' and 5'-AGACCAAGCGGTGTATGATT-3'. Transient suppression of TOP in UKRV-Mel-15a was targeted with duplexes (Eurogentec) at 5'-CAGCAAGGTTGGCATGGA-3' (siTOP-1) or 5'-GCCTTCTGTGCATCGACTT-3' (siTOP-2). Suppression of TPPII was targeted at 5'-GCAGTATTCACATCGCCAC-3' (from ref. 16). Suppression of Neurolysin by the siRNA pool was targeted at: 5'-GGATAAAGCTACAGGAGAA-3', 5'-GATAATGAATCCAGAGGTT-3', 5'-CAATTGAGGTGGTCACTGA-3' and 5'-GAACTCAAGTATTCATAG-3'. Suppression of insulin degrading enzyme by the siRNA pool was directed at: 5'-TCAAAGGGCTGGGTAAATA-3', 5'-ACACTGAGGTTGCATATTT-3', 5'-GAACAAAGAAATACCCTAA-3' and 5'-GTGGAGAGCATACCAATTA-3'. As non-silencing control, the siCONTROL Non-Targeting siRNA pool (Dharmacon) was used. The non-silencing control for the pSUPER-puro expressed siRNA's was a non-targeting scrambled sequence. Suppression of peptidases, or in other experiments overexpression, was assessed by quantitative RT-PCR and immunoblotting using mAb 4D6 (Santa Cruz) for TOP and for nardilysin either non-commercial rabbit antiserum or mAb A-6 (Santa Cruz).

### ***Peptide translocation by TAP***

TAP translocation efficiency of peptides was determined – using microsomes, prepared from a B-LCL cell line as described<sup>48</sup> – by measuring competition for translocation with the fluorescein (FL)-labeled reference peptide C-(FL)VNKTERAY<sup>49</sup>, which contains a consensus site for N-linked glycosylation in the ER. Briefly, microsomes (containing  $1.3 \times 10^6$  cell equivalents) were incubated for 10 min at 37 °C with titrated amounts of test peptide, 0.5  $\mu$ M FL-labeled reference peptide and 10 mM ATP in 100  $\mu$ l transport buffer (5 mM HEPES-pH 7.3, 130 mM KCl, 10 mM NaCl, 1 mM  $\text{CaCl}_2$ , 2 mM EGTA, 2 mM  $\text{MgCl}_2$ ). Thereafter, 1 ml ice cold transport buffer with 10 mM EDTA was added, samples were washed, pellets resuspended in lysis buffer (50 mM Tris-pH 7.5, 500 mM NaCl, 5 mM  $\text{MgCl}_2$ , 1% Triton X-100) and rotated for 30 min at 4 °C. Debris was removed by centrifugation (10 min;  $15,000 \times g$  at 4 °C) and the glycosylated FL-reference peptide was recovered by incubation for 2 h at 4 °C with 100  $\mu$ l packed concanavalin A-Sepharose 4B (GE Healthcare). Con A-Sepharose was washed three times and glycosylated FL-reference peptide was released by incubation for 1 h at 20 °C in 200  $\mu$ l elution buffer (50 mM Tris-HCl pH 8.0, 500 mM mannopyranoside, 10 mM EDTA). Fluorescence was measured at excitation 485 nm and emission 535 nm. Maximal fluorescence was from a sample containing no competitor peptide, minimal fluorescence from a sample containing 0.5 M EDTA without ATP. The GRIDKPILK positive control competitor peptide is a reported efficiently translocated peptide<sup>50</sup>.

### ***Mass spectrometric analysis***

Electrospray ionization mass spectrometry was performed on a Q-TOF1 (Waters), equipped with an on-line nanoelectrospray interface, approx. flow rate of 250 nl/min. Peptide digestion samples were trapped on a precolumn (MCA-300-05-C18; Dionex) and eluted with a steep gradient from 70% B to 90% B in 10 min (A: water-acetonitrile-formic acid (95:3:1, v/v/v); B: water-acetonitrile-formic acid (10:90:1, v/v/v)). Mass spectra were recorded from mass 50-2000 Da. In tandem mass spectrometry (MS/MS) mode, ions were selected with a window of 3 Da. The collision gas was argon ( $4 \times 10^{-5}$  mbar), and the collision voltage  $\approx 30$  V. For peptide digestions by purified proteasome, nardilysin, TOP and TPPII, peaks in the mass spectra were searched in source substrate peptides using Biolynx/proteins software (Waters) and the abundance of a specific digestion fragment was assessed quantitatively as its percentage of the total summed intensities, including undigested substrate. For identification of the PRA<sup>190-198</sup> epitope, peptides in the HPLC-fractions of peptides eluted from K562-A3 were sequenced by MS/MS, essentially as described above, but measured on an HCT<sup>plus</sup> (Bruker Daltonics) ion trap, which was run in data-dependent MS/MS mode during peptide elution. Proteins in bands of cytosolic fractions of K562-A3 were digested with trypsin and MS/MS spectra were matched against the SwissProt database.

### ***Statistical analysis***

To determine the significance of differences in the HLA class I expression experiments a two-tailed paired *t*-test was used; *p*-values of less than 0.05 were considered significant.

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**TABLE 1.** Proportions of HLA class I ligands potentially excised by nardilysin

HLA class I	Ligands <sup>a</sup>	C-dir. <sup>b</sup> (%)	C-ind. <sup>c</sup> (%)	N-dir. <sup>d</sup> (%)	N-ind. <sup>e</sup> (%)	Total <sup>f</sup> (%)
HLA-A1	86	5.8	7.0	0.0	3.5	16.3
HLA-A2	515	1.7	5.0	2.5	4.5	13.7
HLA-A3	121	10.7	5.8	4.1	5.0	25.6
HLA-A11	71	11.3	2.8	1.4	7.0	22.5
HLA-A68	46	21.7	17.4	2.2	0.0	41.3
Cumulative A3-type <sup>g</sup>	<b>(238)</b>	<b>13.0</b>	<b>7.1</b>	<b>2.9</b>	<b>4.6</b>	<b>27.6</b>
HLA-A24	51	2.0	3.9	5.9	9.8	21.6
HLA-B7	68	2.9	7.4	1.5	4.4	16.2
HLA-B8	52	0.0	5.8	0.0	3.8	9.6
HLA-B2701	8	0.0	12.5	37.5	25.0	75.0
HLA-B2702	18	0.0	5.6	61.1	5.6	72.3
HLA-B2703	29	3.4	3.4	62.1	0.0	68.9
HLA-B2704	52	5.8	3.8	38.5	3.8	51.9
HLA-B2705	185	7.6	5.9	31.4	4.3	49.2
HLA-B2706	38	5.3	7.9	26.3	10.5	50.0
HLA-B2707	3	0.0	0.0	66.7	0.0	66.7
HLA-B2709	56	1.8	7.1	26.8	3.6	39.3
Cumulative B27 <sup>h</sup>	<b>(389)</b>	<b>5.4</b>	<b>5.9</b>	<b>35.2</b>	<b>4.9</b>	<b>51.4</b>
HLA-B35	11	9.1	9.1	9.1	9.1	36.4
HLA-B3501	20	0.0	5.0	0.0	5.0	10.0
HLA-B44	53	3.8	1.9	1.9	15.1	22.7
HLA-B60 (B4001)	34	2.9	5.9	0.0	8.8	17.6
HLA-B61 (B4002)	7	0.0	14.3	0.0	0.0	14.3
HLA-B62 (B1501)	96	4.2	5.2	5.2	5.2	19.8
<b>Cumulative<sup>k</sup></b>	<b>1620</b>	<b>4.8</b>	<b>5.7</b>	<b>10.4</b>	<b>5.2</b>	<b>26.1</b>

<sup>a</sup> Number of ligands per class I molecule. Per allele, all ligands in SYFPEITHI database were analyzed for presence of the dibasic nardilysin (NRD)-motif at the N- or C-terminus or in the flanking regions.

<sup>b</sup> C-dir.; NRD-motif present at the C-term, potentially leading to direct production of the C-terminus.

<sup>c</sup> C-ind.; NRD-motif present within 4 aa of the C-terminus, potentially leading to precursor formation.

<sup>d</sup> N-dir.; NRD-motif present at the N-term, potentially leading to direct production of the N-terminus.

<sup>e</sup> N-ind.; NRD-motif present within 4 aa of the N-terminus, potentially leading to precursor formation.

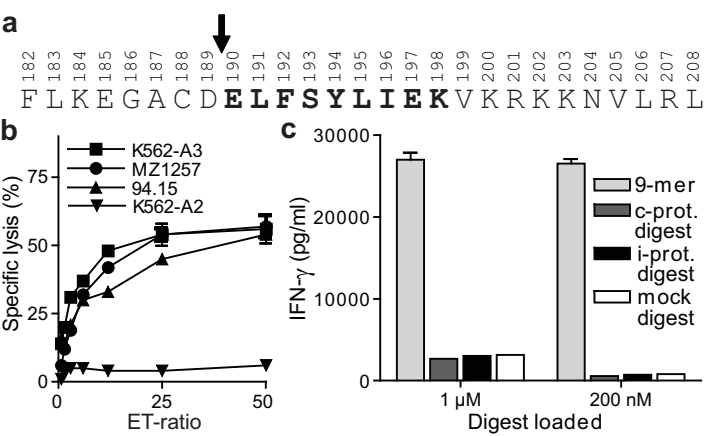
<sup>f</sup> Total % of ligands per allele that is potentially N-terminally or C-terminally dependent on NRD-cleavage.

<sup>g</sup> Cumulative % of NRD-motif containing ligands for HLA-A3, HLA-A11 and HLA-A68.

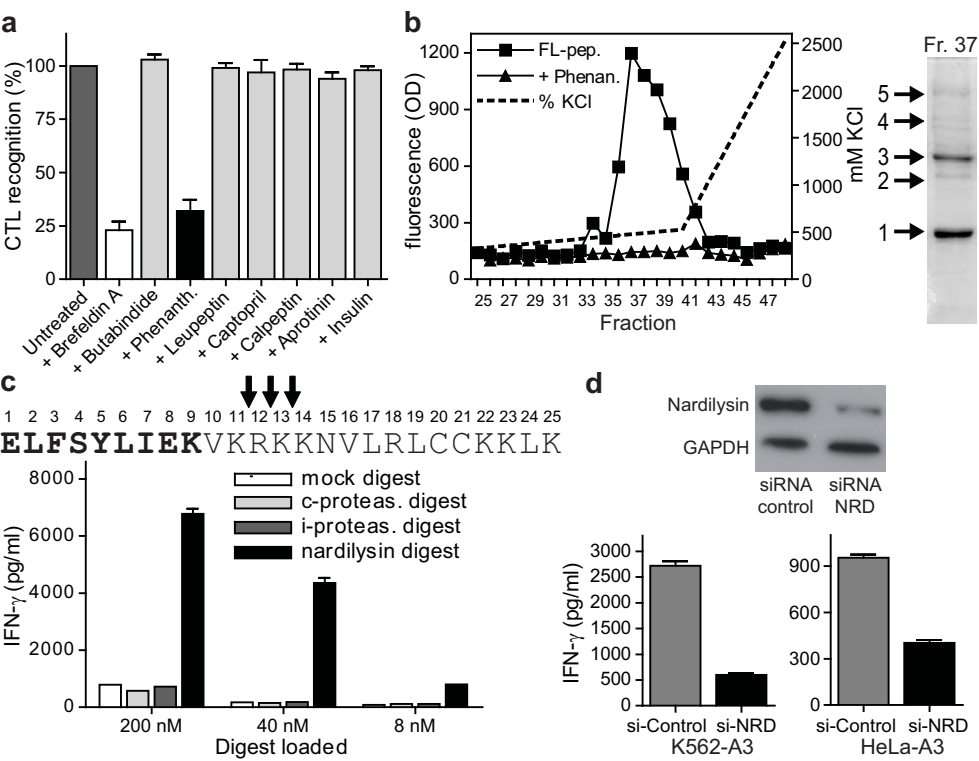
<sup>h</sup> Cumulative % of NRD-motif containing ligands for HLA-B27 subtypes.

<sup>k</sup> Cumulative % of ligands with NRD-motif directly or indirectly at the C-term or N-term for all 1620 ligands.

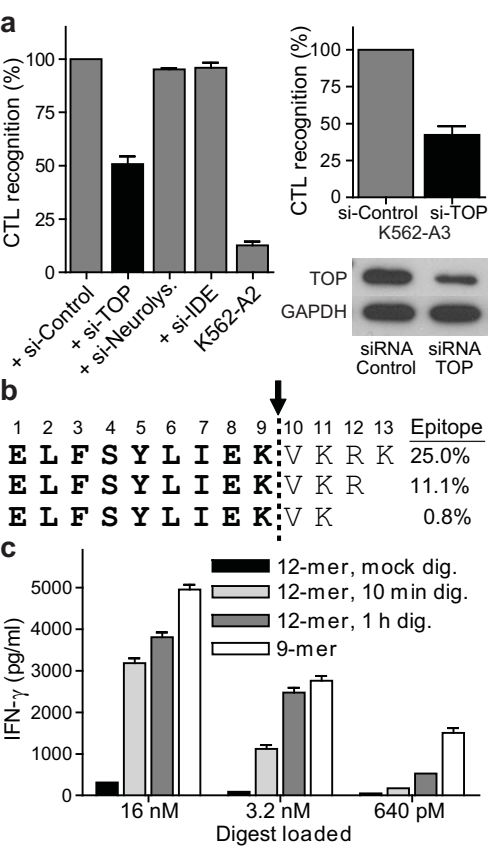
**Fig. 1**



**Fig. 2**



**Fig. 3**



**Fig. 4**

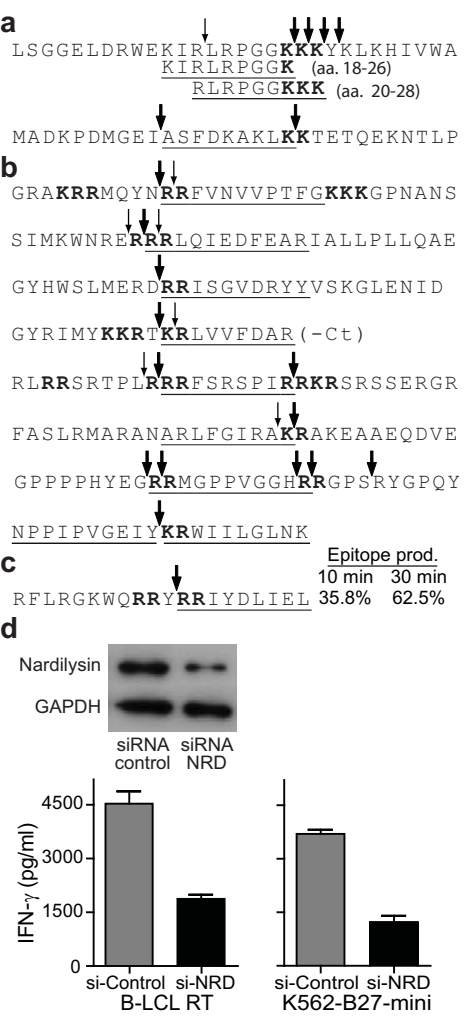
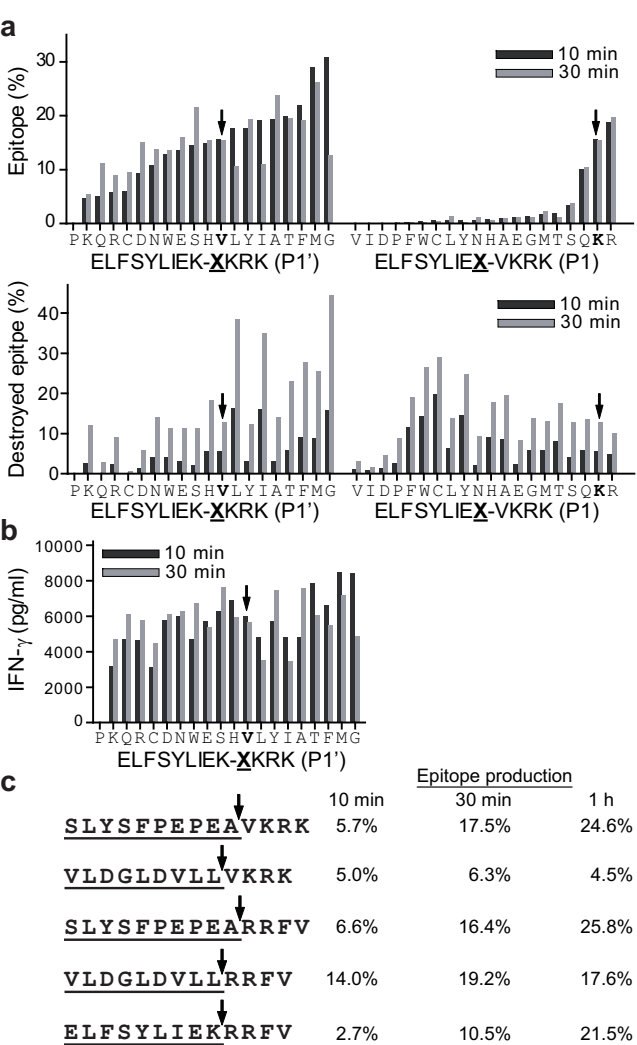


Fig. 5



**Fig. 6**

