The generation of cytotoxic T cell epitopes and their generation for cancer immunotherapy

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CHAPTER 6

CHAPTER 9

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Novel antigen-processing pathways for cytotoxic T cell recognition

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ABSTRACT

Cytotoxic T lymphocytes (CTL) kill cells through recognition of microbial or cancer-associated antigenic peptides presented by HLA class I molecules on the cell surface. For binding to class I molecules these peptides need to have an appropriate C-terminal anchor residue, which is classically generated by the proteasome complex.

Here we identify two cytosolic endopeptidases that complement the proteasome. Nardilysin contributes not only to C-terminal but also to N-terminal generation of HLA class I presented peptides. Thimet oligopeptidase (TOP) acts as a C-terminal trimming endopeptidase in antigen processing. We define a novel mechanism by which these peptidases cooperatively produce the C-terminus of a tumor-specific CTL epitope.

The actions of nardilysin and TOP broaden the antigenic peptide repertoire by shaping non-functional protein degradation products into class I ligands, thereby strengthening the immune defense against intracellular pathogens and cancer.

INTRODUCTION

Short peptides (8–12 aa) presented by HLA class I molecules, called CTL epitopes when recognized by $CD8^+$ cytotoxic T lymphocytes (CTL), are produced during the continuous turnover in the cytosol of full length and misfolded proteins into peptides and eventually free amino acids [1-3]. This degradation process is accomplished by the multicatalytic proteasome complex and by cytosolic peptidases [1,3]. Tripeptidyl peptidase II (TPPII) [4], TOP [5,6] and aminopeptidases [1,2] have been implicated in 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 ERAAP/ERAP1 [1,2] and assemble with HLA class I heterodimers before being routed to the cell surface.

The absence of C-terminal excision of CTL epitopes in proteasome-inhibited cells [7,8], together with a 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 [1,2]. 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 significant levels of peptide-loaded HLA class I molecules [9-13]. Although these compounds are known to not inhibit completely, this suggests the existence of a partially proteasome-independent pathway of class I ligand generation. So far only TPPII reportedly produces the C-terminus of a defined CTL epitope (from HIV Nef) [14], but a broad role of TPPII in the generation of class I ligands is considered unlikely [15]. Other peptidases liberating the C-terminus of HLA class I ligands are not known. To define such peptidases, we began an in-depth investigation of the generation of a CTL epitope with an unambiguously proteasome-independent C-terminus.

RESULTS

Cleavage C-terminally to a lysine is not readily accomplished by proteasomes [16]. In line with this, expression of HLA-A3, a class I molecule that binds peptides with a C-terminal lysine, is particularly insensitive to proteasome inhibition [9,12]. Therefore, we searched for a CTL epitope with a proteasome-independent C-terminus presented in HLA-A3. The nonamer ELFSYLIEK ($PRA^{190-198}$) from tumor-associated protein PRAME [17] was selected by binding prediction and bound with high affinity to HLA-A3 (Suppl. Fig. 1). Digestion of 27-mer $PRA^{182-208}$ 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). Tandem mass spectrometry (MS/MS) identified the ELFSYLIEK peptide in peptides eluted from HLA-A3 on the surface of cell line K562-A3 (K562, $PRAME^+$, transfected with HLA-A3) (Suppl. Fig. 2). Also, the CTL clone raised against the $PRA^{190-198}$ epitope exogenously loaded on HLA-A3, efficiently recognized K562-A3 and other tumor cell lines expressing PRAME and HLA-A3 (Fig. 1B) confirming natural presentation of the epitope. The CTL also recognized the exogenously loaded 11-, 12- and 13-meric C-terminally extended variants of the epitope (ELFSYLIEKVK/R/K), but these peptides – while possessing HLA-A3 binding affinity (Suppl. Fig. 1) – are not naturally presented (Suppl. Fig. 2). We used this feature of the CTL

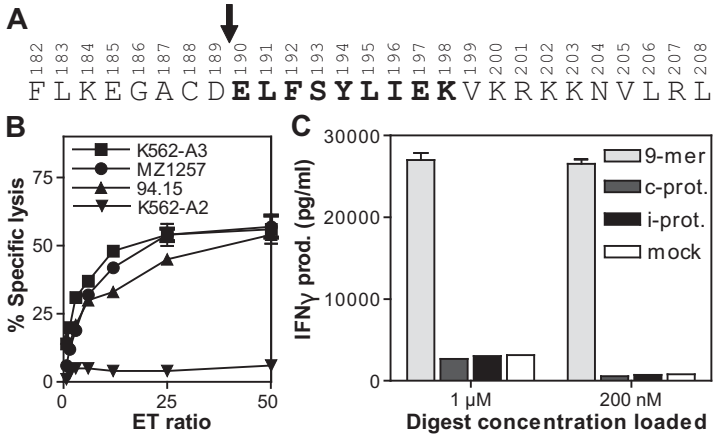

Figure 1. PRA$^{190-198}$ is an HLA-A3 presented CTL epitope with proteasome-independent C-terminus.

A: Proteasomal digestion of 27-mer PRA$^{182-208}$ (ELFSYLIEK, bold). Digestion (1 h incubation) by immuno- or constitutive human 20S proteasomes gave comparable results. Bold arrow indicates the efficient cleavage before Glu-190. Fragments containing Asp-189 at the C-terminus or complementary fragments added up to >7% of the digested material (1 h digest) as determined by mass spectrometrical analysis. Digestions were repeated three times with similar results.
B: Recognition by CTL anti-ELFSYLIEK of erythroleukemia cell line K562-A3 and renal cell carcinoma (RCC) lines MZ1257 and 94.15 (all PRAME$^+$ and HLA-A3$^+$). K562-A2 (HLA-A2$^+$) was not recognized.
C: Absence of recognition of 25-mer PRA$^{190-214}$ (ELFSYLIEKVKRKKNVLRLCCKKLK, epitope at N-terminus) digested for 1 h with immuno- or constitutive proteasomes (SEM of triplicate wells). Digests were titrated, loaded on EKR cells (HLA-A3$^+$) and co-incubated with CTL anti-ELFSYLIEK. Background recognition is 25-mer substrate incubated at 37°C without enzyme (mock digest), positive control is the equally titrated ELFSYLIEK peptide (9-mer).

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$^{190-214}$ (ELFSYLIEK at its N-terminus) loaded onto PRAME-negative HLA-A3$^+$ cells were not recognized by the CTL (Fig. 1C), confirming proteasome-independence of the epitope's C-terminus.

Identification of nardilysin

Therefore, we searched for 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 o-phenanthroline, whereas other inhibitors had no effect (Fig. 2A). Since the TPPII inhibitor butabindide can be inactivated by serum [4], we further excluded the potential involvement of TPPII [14,18] by digestion analysis and RNA interference (RNAi) (Suppl. Fig. 3).
Second, in order to identify the peptidase, we exposed the internally quenched fluorescent 15-mer substrate **ELFSYL(-dab)IEK**VKRC(-FL)KN (PRA$^{190-204}$, epitope in bold) to digestion with cytosolic fractions (separated by anion exchange chromatography) of K562-A3 cells. Fraction #37 cleaved, in a phenanthroline-sensitive fashion, between the quencher dabcyl 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, Suppl. Fig. 4). Nardi-

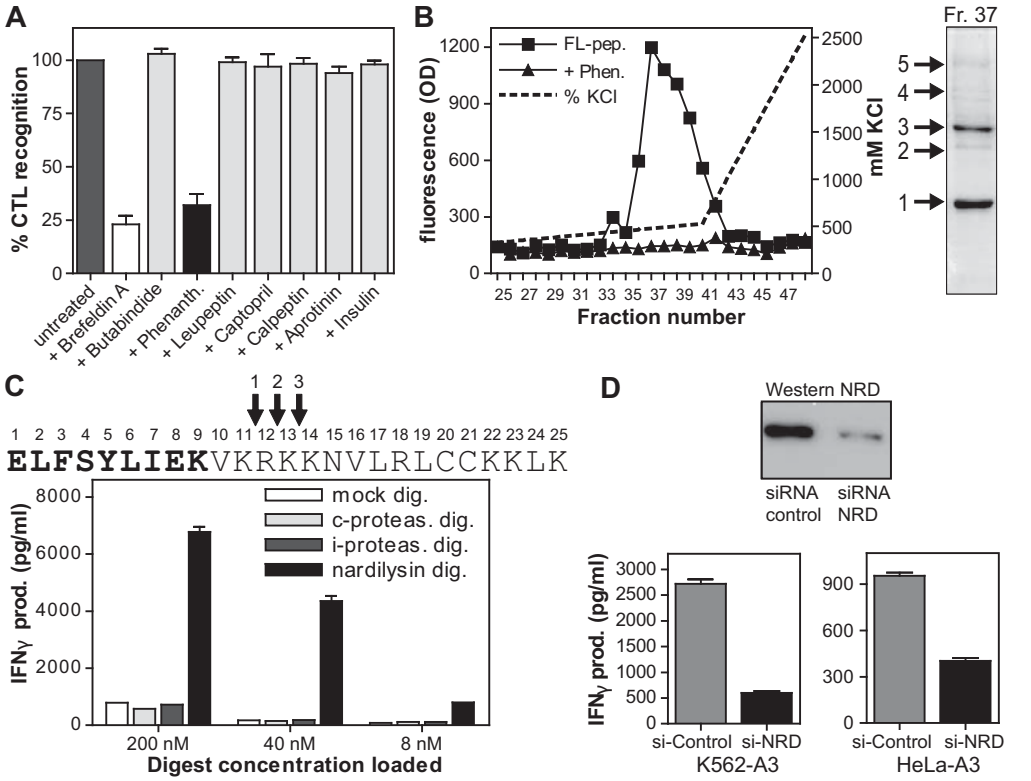

Figure 2. Nardilysin produces C-terminal extended precursors of the PRA$^{190-198}$ epitope.

A: K562-A3 was stripped and untreated (100% recognition) or treated with indicated inhibitors (concentration and activity, see methods section) and co-incubated with the CTL clone (SEM of three experiments).

B: Cytosolic fractions eluted from an anion exchange column (KCl-gradient indicated; see Methods for details) of K562-A3 were co-incubated for 30 min with fluorogenic substrate ELFSYL(-dabcyl)IEKVKRC(-FL)KN in the absence (square symbol) or presence (triangle) of phenanthroline (1 mM). The same substrate with the first three aa being D-aa's to block N-terminal trimming showed only slightly reduced fluorescence (data not shown). Fraction no. 37 was separated by SDS-PAGE and the bands were isolated. Proteins were identified by MS/MS as: (band 1) high mobility protein, (2) keratin, (3) α- & β-tubulin, (4) no matches and (5) nardilysin (see Suppl. Fig. 4).

C: Digestion of 25-mer PRA$^{190-214}$ (epitope, bold) with purified nardilysin, immuno-proteasomes or constitutive proteasomes for 30 min. Nardilysin cleavages are shown: efficient cleavage behind the 11-mer was present in 33.9% of digestion-fragments (arrow 1), behind the 12-mer in 41.2% of the fragments (arrow 2) and behind the 13-mer in 16.9% of the fragments (arrow 3). Digests (nardilysin, proteasome, mock) were titrated, loaded on HLA-A3$^+$ cells and co-incubated with the CTL to assess generation of epitope-precursors.

D: CTL recognition of K562-A3 and HeLa-A3 cells stably transfected to express siRNA suppressing nardilysin (NRD) or non-targeting control siRNA (SEM of triplicate wells of representative experiment). Immunoblot with nardilysin antibody of K562-A3 transfectants is shown.

lysin, hitherto not implicated in class I antigen processing, is a cytosolic endopeptidase of the pitrilysin family of zinc-metalloproteases and is ubiquitiously expressed (see ref. 19 and the UniGene database at: http://www.ncbi.nlm.nih.gov/UniGene/ESTProfileViewer.cgi?uglist=Hs.584782).

Third, the digest of 25-mer PRA$^{190-214}$ (**ELFSYLIEK**VKRKKNVLRLCCKKLK) with purified nardilysin showed highly efficient production of C-terminally extended 11-, 12- and 13-mer variants of the epitope by efficient cleavages after Lys-200, Arg-201 and Lys-202 (Fig. 2C), in accordance with the cleavage specificity of nardilysin before or between two basic residues [20,21] in substrates up to ~30 aa in length. Indeed, the CTL efficiently recognized the nardilysin-digest, due to the presence of the ELFSYLIEKVK/R/K fragments, whereas the proteasomal digest was not recognized (Fig. 2C). Fourth, nardilysin was required in cells for epitope generation because small inhibitory RNA (siRNA)-mediated suppression of nardilysin in K562-A3 and HeLa-A3 (HeLa, PRAME$^+$, with HLA-A3) strongly reduced their recognition by the CTL (Fig. 2D), and overexpression of nardilysin in HeLa-A3 cells enhanced CTL recognition (Suppl. Fig. 5). Together, these findings implicate nardilysin as indispensable for the endogenous C-terminal pre-processing of the PRA$^{190-198}$ epitope by producing cleavages after Lys-200, Arg-201 and Lys-202, but it does not excise the exact C-terminus (Lys-198).

Identification of thimet oligopeptidase

Next we pursued identification of the peptidase that generates the correct 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 in K562-A3 strongly reduced CTL recognition, whereas suppression of the other peptidases had no effect (Fig. 3A), indicating TOP's involvement in the endogenous generation of ELFYSLIEK. Moreover, purified TOP cleaved efficiently directly behind the epitope's C-terminal Lys-198 in the nardilysin-dependent 12- and 13-mer precursors (PRA$^{190-201/202}$; ELFSYLIEKVKR/K). Thus, TOP released three or four C-terminal residues, in accordance with its cleavage preference [22,23], thereby efficiently producing the exact nonameric epitope (Fig. 3B). Indeed, the TOP-digest of 12-mer PRA$^{190-201}$ loaded on target cells was recognized almost as efficiently as the minimal epitope (Fig. 3C). In line with its preference for peptides 6 to 17 aa in length [24], longer peptides were not cleaved by TOP (Suppl. Fig. 6B, and data not shown), indicating that pre-processing by nardilysin into the 12-mer and 13-mer is required to make epitope-precursors susceptible to TOP. This was confirmed in the co-digestion of 25-mer PRA$^{190-214}$ with nardilysin and TOP, which resulted in the production of ELFSYLIEK (identified by mass spectrometry, data not shown). 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 (Suppl. Fig. 6B). Thus, the C-terminus of the ELFSYLIEK epitope is released through sequential cleavages by nardilysin and TOP.

Generation of the N-terminus of the PRA$^{190-198}$-epitope

The N-terminus of the epitope is generated by an efficient caspase-like proteasomal cleavage before Glu-190 (Fig. 1A). This cleavage was completely blocked by the specific inhibitor AcAlaProNleLeuVSOH [25] of the caspase-like proteasome activity as shown in a digestion of

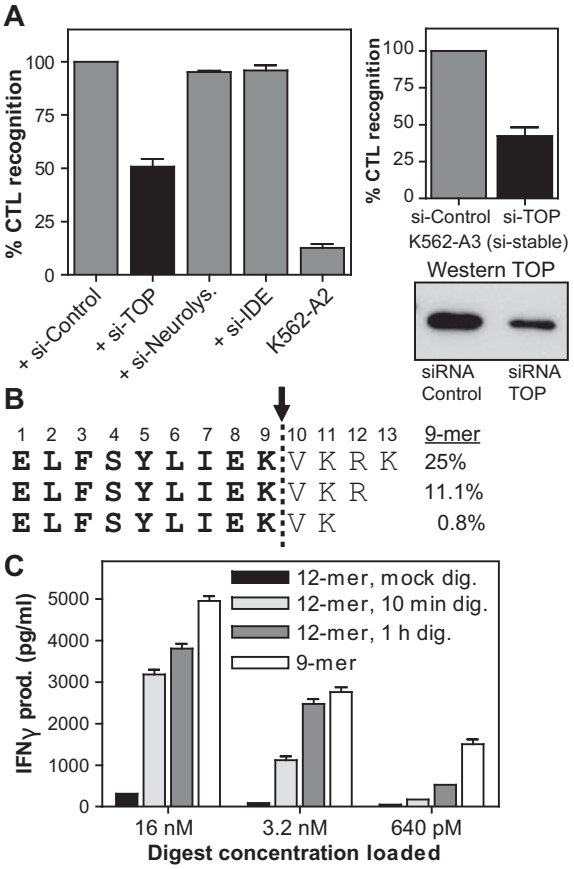

Figure 3. TOP produces the C-terminus Lys-198 of the epitope.

A: Left: CTL recognition of K562-A3 transiently transfected with pools of siRNA duplexes suppressing TOP, neurolysin and insuline degrading enzyme (IDE) or non-targeting control siRNA (100% reference recognition). Right: CTL recognition of K562-A3 stably transfected to express siRNA suppressing TOP or non-targeting control siRNA. SEM of three or four experiments. Immunoblot with TOP antibody of stably transfected K562-A3 variants is shown.

B: Digestion of 13-, 12- and 11-mer substrates PRA$^{190\text{-}202/201/200}$ (ELFSYLIEKVK/R/K, epitope bold) with purified TOP; the substrates are shown. Bold arrow indicates the efficient cleavage behind Lys-198. From each substrate the % of fragments in the digest (including non-digested substrate) that match the ELFSYLIEK-epitope after 30 min digestion are listed.

C: CTL recognition of 12-mer PRA$^{190\text{-}201}$ (ELFSYLIEKVKR) digested by TOP for 10 min or 1 h. The digests were titrated and loaded on HLA-A3^{+} EKR cells. The non-digested 12-mer (mock dig.) is not recognized at these concentrations. Recognition of equally titrated ELFSYLIEK peptide (9-mer) is the positive reference.

27-mer PRA$^{172\text{-}198}$ (Suppl. Fig. 7A,B). Indeed, CTL recognition of cells treated with this inhibitor was strongly reduced (Suppl. Fig. 7C). The general proteasome inhibitor epoxomycin only partially inhibited cleavage before the N-terminus (Suppl. Fig. 7A,B). Interestingly, when cells were treated with epoxomycin the presentation of ELFSYLIEK was markedly enhanced (Suppl. Fig. 7C), likely due to reduced competition with epoxomycin-sensitive HLA-A3 ligands.

TAP-dependence of PRA$^{190\text{-}198}$

Thus, together, the proteasome, nardilysin and TOP produce the minimal epitope in the cytosol (schematic in Suppl. Fig. 8A). As expected, presentation of the epitope was completely dependent on transport into the ER by TAP (Suppl. Fig. 9A). The ELFSYLIEK peptide was efficiently transported by TAP into the ER, whereas the 12-, and 13-mer epitope-precursors were less efficiently translocated (Suppl. Fig. 9B). Therefore, these 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.

General roles of nardilysin and TOP in class I antigen processing

This is the first evidence implicating nardilysin and TOP in CTL epitope production. Do these enzymes, either apart or together, have a more general role in class I antigen processing?

Nardilysin in principle can support the generation of both the C-terminus and the N-terminus of epitopes (Suppl. Fig. 8B). A bioinformatics analysis of all 1620 published ligands of the most prevalent class I molecules with their flanking regions revealed the dibasic cleavage motif of nardilysin [20,21] directly at the N-terminus or C-terminus in 15% of the ligands (Table 1). The motif is especially frequent directly at the (basic) C-terminus of ligands presented by the HLA-A3 supertype (-A3, -A11, -A68) and at the N-terminus of HLA-B27 ligands (Tables 1 and 2). We then examined whether purified nardilysin actually excised prototypical HLA-A3 and -B27 ligands at the motif sites. Indeed, the C-termini of two HLA-A3-presented overlapping immunodominant epitopes from HIV-1 Gag p17 [26] were efficiently produced (Fig. 4A) whereas the proteasome failed to do so (data not shown). Importantly, over 35% of all 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 nardilysin cleavage-site (Tables 1 and 2). Nardilysin efficiently cleaved directly before the N-terminus of eight out of eight HLA-B27 ligands tested and in three cases produced the epitope's C-terminus; among these are immunodominant viral epitopes from HIV-1 gag p24 and EBNA3C [27] (Fig. 4B,C). Noteworthy, four of these ligands were identified previously to be proteasome-independent as studied with proteasome inhibitors (the others were not tested) [13].

To demonstrate nardilysin-dependence of these epitopes during processing in cells, nardilysin was suppressed by RNAi in EBNA3C-positive target cells resulting in strongly reduced recognition by CTL against the HLA-B2705-presented EBNA3C$^{258\text{-}266}$ (RRIYDLIEL) epitope [27] (Fig. 4D). Endogenous processing of the minimal epitope with its N-terminal flanking region confirmed the nardilysin-dependence to reside in the N-terminal epitope processing (Fig. 4D). Thus, although several aminoeptidases exist in the cytosol and ER, nardilysin is required for the N-terminal liberation of the EBNA3C$^{258\text{-}266}$ epitope. Our results help to explain the strong over-representation of Arg and Lys at P1 in HLA-B27 ligands, residues that contribute at that position only modestly to binding capacity but enforce peptide stability in the cytosol [28].When nardilysin cleaves before a basic doublet, it may also liberate ligands with a non-basic C-terminus presented by e.g. HLA-A1, -A2, -B8 and HLA-B35 (Suppl. Fig. 8B). In addition, the dibasic motif is often present within 4 aa of the N- or C-terminus (Table 1), suggesting a multi-step mechanism of epitope-production. The motif is present directly at or within 4 aa of the N- or C-terminus in 26% of the 1620 ligands analyzed (Table 1). Because nardilysin can cleave sometimes before mono-basic sites [21] or at atypical sites (see e.g. Fig. 4A: ASFDKAKLK-epitope), its influence may reach beyond the dibasic motif. Nardilysin may also destroy certain potential class I binding peptides. In vaccine design, the deliberate insertion of the nardilysin cleavage motif between CTL epitopes in poly-epitope sequences may be useful to promote vaccine efficacy.

TOP has been considered to destroy epitopes [6], thereby limiting class I antigen presentation [5] and raising the question whether the excision of ELFSYLIEK by TOP is an exception. The preferred action of TOP is the release of three to five C-terminal residues [22,23] with a broad sequence specificity [24,29] from substrates up to 17 aa long [24], endowing TOP with potential C-terminus generating capacity. Furthermore, an undefined epitope-promoting effect of TOP has been reported [30].

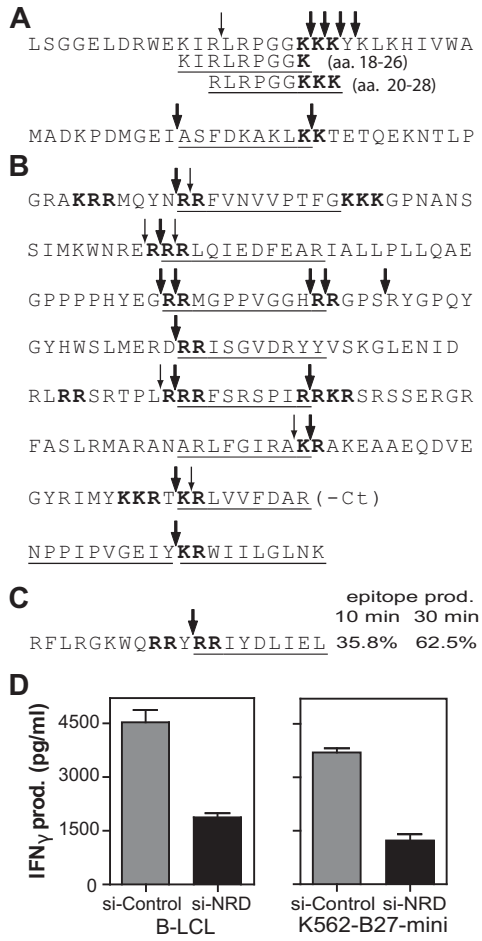

Figure 4. The role of nardilysin in HLA class I antigen processing.

Digestions by purified nardilysin of prototypical HLA-A3, -A11 and -B27 presented peptides. Bold residues constitute nardilysin-cleavage motif. Digestion time was 30 min. Bold arrow, cleavage present in >5% of the digestion-fragments; thin arrow, cleavage present in ≤ 5% of fragments.
A: Digestion by purified nardilysin of (upper) 30-mer HIV-1 gag p17 aa 8-37 encompassing two overlapping HLA-A3-presented CTL epitopes (separately depicted, underlined) and (lower) 30-mer thymosin-β aa 1-30 encompassing HLA-A11-presented ligand ASFDKAKLK (underlined). These examples were taken from the SYFPEITHI database (www.syfpeithi.de).
B: Digestion by purified nardilysin (30 min) of long peptides encompassing eight published HLA-B2705 presented ligands (underlined) from the SYFPEITHI database in their natural flanking regions. Ligands are (top to bottom): 40S ribosomal protein S30 aa 40-51; Cell death-regulatory protein GRIM19 aa 57-67; Ribonucleoprotein aa 312-322; NADH-ubiquinone oxidoreductase aa 52-61; Son3 DNA-binding protein BASS 1 aa 2026-2034; 60S ribosomal protein L13 aa 188-196; DNA-directed RNA polymerases 7 kDa polypeptide aa 50-58; and HIV-1 gag p24 aa 265-274, which is an immunodominant CTL epitope presented in HLA-B2705 (KRWIILGLNK) adjacent to the HLA-B35 presented CTL epitope NPPIPVGEIY (at the left side).
C: Digestion by purified nardilysin of 20-mer RFLRGKWQRRYRRIYDLIEL (EBNA3C aa 246-266, HLA-B2705 presented epitope underlined). Production of the epitope after 10 and 30 min incubation is indicated.
D: Left panel: recognition by CTL anti-RRIYDLIEL of autologous B-LCL (HLA-B2705$^+$) expressing EBNA3C, stably expressing siRNA suppressing nardilysin or control siRNA. Right panel: CTL recognition of K562-B27-mini cell line, which is K562 expressing HLA-B2705 and minigene MFLRGKWQRRYRRIYDLIEL (EBNA3C aa 247-266, epitope underlined), with or without suppressed nardilysin by stable siRNA expression.

Therefore, we assessed the flexibility of TOP in producing different potential epitopes by systematically substituting the positions (P1 and P1') surrounding the site of TOP's cleavage creating the C-terminus of ELFSYLIEK. 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 of digestion (Suppl. Fig. 10A). Partial destruction by a subsequent cleavage in the middle of the epitope occurred, but did not prevent epitope-production (Suppl. Fig. 10A) as also shown by efficient CTL recognition of these digests (Suppl. Fig. 10B). Digestions of the P1-substituted variants,

on the other hand, resulted in generation of the 'epitope' (ELFSYLIE**X**) when P1 is either Lys (wild type sequence), Arg or Glu (Suppl. Fig. 10A), but not for other residues at this position. This was caused by inefficient cleavage after ELFSYLIE**X** for some P1-residues (e.g. Val, Ile, Asp) and/or by rapid efficient cleavage in the middle of the epitope (for e.g. Phe, Trp, Cys, Tyr). The necessity for a Lys, Arg or Glu at the P1-position, however, is not absolute. When we changed the epitope-sequence completely, TOP produced epitopes with e.g. a C-terminal Ala or Leu (SLYSFPEPEA and VLDGLDVLL [17]) (Suppl. Fig. 10C). These data indicate that TOP can produce epitopes from a wide array of C-terminally extended precursors.

Therefore, TOP's role in class I antigen processing is likely balanced between its anti-epitopic and pro-epitopic actions: on the one hand limiting presentation – by partial destruction – of epitopes whose correct C-terminus has already been made by (e.g.) the proteasome (like SIINFEKL [5,7]), and, on the other hand, producing correct C-termini fit for class I binding by trimming C-terminally extended epitope precursors that lack such an anchor. Importantly, this new model is in accordance with a recent analysis of cytosolic substrates and products of TOP, demonstrating that TOP both destroys and generates peptides of the length of class I ligands [31]. This study also showed, in line with our results, that many TOP-products have a Lys at the C-terminus.

The general implication of our study is that protein degradation products that are not fit for antigenic presentation due to lack of an appropriate C-terminal anchor residue can be processed by nardilysin and/or TOP to suit the binding requirements of HLA class I molecules. Because of the substrate length constraints of nardilysin and TOP, 30 aa and 17 aa respectively, prior to their action, protein hydrolysis by the proteasome or other proteolytic systems is likely required in most cases. The proportions of class I ligands that are dependent on nardilysin and TOP are not definitively determined, but our data indicate that both enzymes are capable of shaping and broadening the antigenic peptide repertoire, thereby expanding the options for successful immune responses to intracellular pathogens and cancer.

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TABLES

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

HLA class I	Ligands[a]	C-dir.[b] (%)	C-ind.[c] (%)	N-dir.[d] (%)	N-ind.[e] (%)	Total[f] (%)
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[g]	***(238)***	***13.0***	***7.1***	***2.9***	***4.6***	***27.6***
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[h]	***(389)***	***5.4***	***5.9***	***35.2***	***4.9***	***51.4***
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
Cumulative	**1620**	**4.8**	**5.7**	**10.4**	**5.2**	**26.1**

[a] Number of ligands per class I molecule. For each allele, all ligands in the SYFPEITHI database were analysed for presence of NRD-motifs in the ligand and its N-term. and C-term. flanking regions.

[b] C-dir.; NRD-motif present leading to direct production of the C-terminus.

[c] C-ind.; indirect production of C-terminus: NRD-motif present within 4 aa of the C-terminus.

[d] N-dir.; NRD-motif present leading to direct production of the N-terminus.

[c] N-ind.; indirect production of N-terminus: NRD-motif present within 4 aa of the N-terminus.

[f] Percentage of ligands per allele that may be either N-term. or C-term. dependent on NRD cleavage.

[g] Cumulative percentages of NRD-motif containing ligands for HLA-A3, HLA-A11 and HLA-A68.

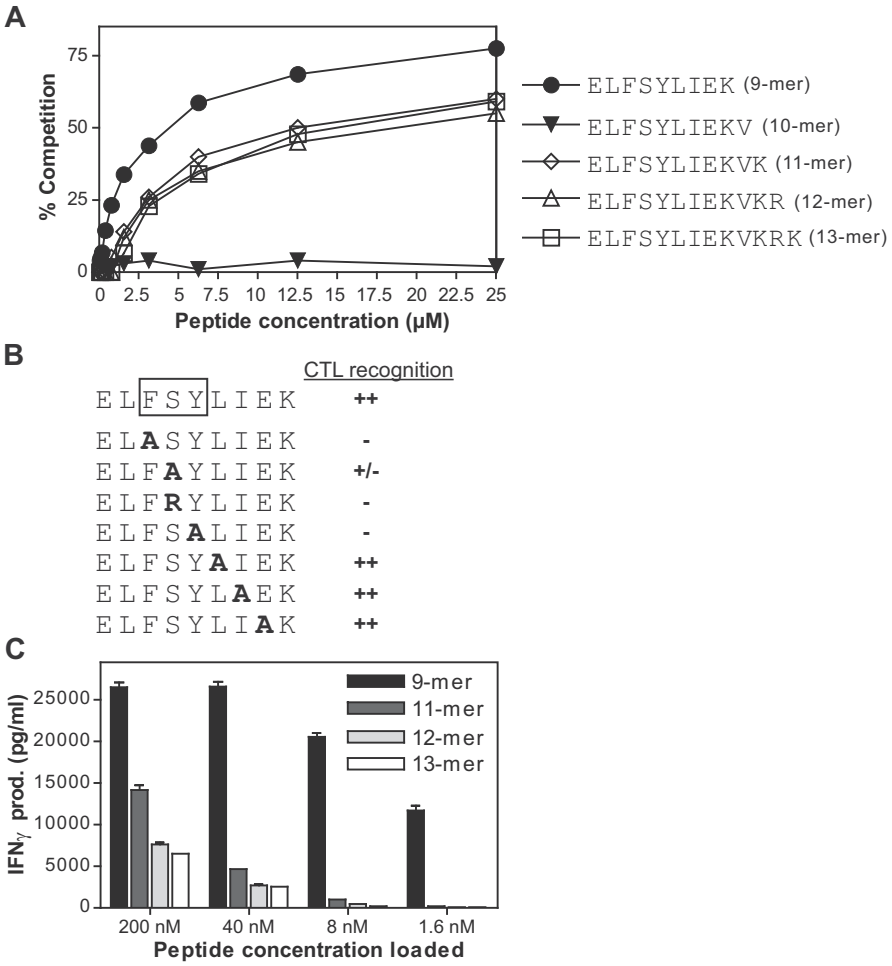
[h] Cumulative percentages of NRD-motif containing ligands for HLA-B27 subtypes.

TABLE 2. HLA-B27 ligands and nardilysin-cleavage motifs, exemplified for HLA-B2703.

Ligands in flanking regions and dibasic-motifs[a]	Source protein and position of ligand
KLQEEERE**RR**DNYVPEVSALDQEIIE	40S ribosomal protein S17 aa.79-87
VAEVDKVTGRFNGQFKTYAICGAI**RR**	40S ribosomal protein S21 aa.44-53
GLIKLVSKHRAQVIYTRNTKGGDAPA	40S ribosomal protein S25 aa.103-111
KNYDPQKD**KR**FSGTVRLKSTPRPKFS	60S ribosomal protein L10A (Csa-19) aa.46-54
NNLKARNSFRYNGLIH**RK**TVGVEPAA	60S ribosomal protein L28 aa.37-45
LFESWCTD**KR**NGVIIAGYCVEGTLAK	Cleavage and polyadenylation specif. Fact. 73kDa aa.348-357
AIRPQIDL**KR**YAVPSAGLRLFALHAS	DNA dep. protein kinase catal. subunit Q13 aa.263-272
DIPHALRE**KR**TTVVAQLKQLQAETEP	EIF-3 protein 6 aa.73-81
IPDVITYLSRLRNQSVFNFCAIPQVM	Farnesyl-diphosphate farneysltransferase 1 aa.278-286
VDLVRFTE**KR**YKSIVKYKTAFYSFYL	Farnesyl pyrophosphate synthetase aa.191-199
LEKIIQVGNRIKFVI**KR**PELLTHSTT	General transcription factor II aa.532-540
GTVALREI**RR**YQKSTELLI**RK**LPFQR	Histone H3.3 aa.52-60
EVECATQL**RR**FGDKLNFRQKLLNLIS	Immediate early response protein APR aa.30-38
VGEKIALS**RR**VEKHWRLIGWGQI**RR**G	Initiation factor eIF-2 gamma aa.444-452
VHFKDSQN**KR**IDIIHNLKLDRTYTGL	KIAA1197 aa.399-407
IWQLSSSL**KR**FDDKYTLKLTFISGRT	Microsomal sign. peptidase 25kDa Su aa.164-172
HWSLMERD**RR**ISGVDRYYVSKGLENI	NADH-ubiquinone oxidoreductase aa.52-60
SPDDKYSRHRITI**KKR**FKVLMTQQPR	Nop10p aa.44-52
DFDWNLKHGRVFIIKSYSEDDIHRSI	Ny-Ren antigen aa.410-418
PLLLTEEE**KR**TLIAEGYPIPTKLPLT	OASIS protein aa.267-275
MLSTILYS**RR**FFPYYVYNIIGGLDEE	Proteasome Su C5 aa.99-107
VKGPRGTL**RR**DFNHINVELSLLG**KKK**	Ribosomal protein L9 aa.35-43
SLVKGGLC**RR**VLVQVSYAIGVSHPLS	S-adenosylmethionine synthetase gamma aa.312-320
RGDSVIVVLRNPLIAGK *(*C-term. protein)	Small nuclear ribonucleoprotein SM D2 aa.110-118
KDSKTDRL**KR**FGPNVPALLEAIDDAY	SMC6 protein aa.478-487
PRLAILYA**KR**ASVFVKLQKPNAAIRD	ST13-like tumor suppressor aa.149-157
KLEAINELIRFDHIYTKPLVLEIPSE	XBP1 aa.281-289

[a] All HLA-B2703 ligands in the SYFPEITHI database (at: www.syfpeithi.de) are listed and were analysed for the presence of dibasic nardilysin cleavage motifs. Ligand is underlined and nardilysin cleavage motifs are printed bold.

SUPPLEMENTARY FIGURES

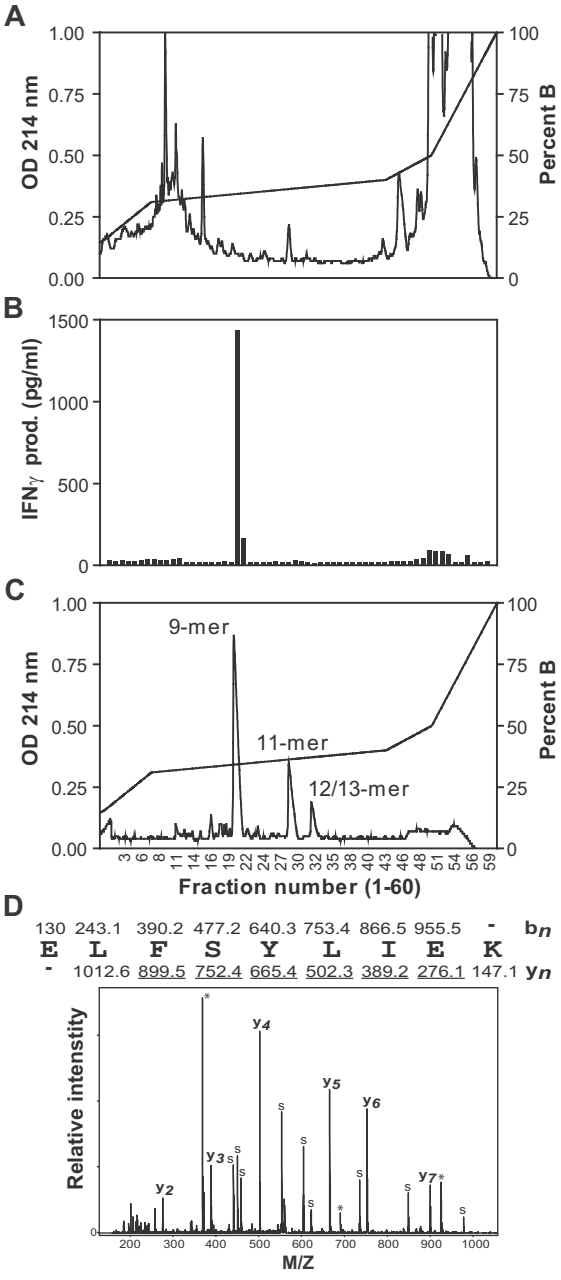


Suppl. Figure 1. HLA-A3 binding and CTL recognition of the ELFSYLIEK epitope and its C-terminally extended precursors.

A: Binding affinity for HLA-A3 of the nonameric (PRA$^{190-198}$) ELFSYLIEK epitope and its C-terminally elongated 10-, 11-, 12- and 13-meric variants as determined in a competition-based binding assay. As shown, the 10-mer has no binding capacity due to its lack of a C-terminal HLA-A3 binding anchor.

B: Alanine-scan to identify T cell receptor (TCR)-contact residues in the ELFSYLIEK peptide. Substitution-variants of the ELFSYLIEK peptide, each with a single amino acid replaced by Ala, were loaded on B-LCL EKR (HLA-A3$^+$) and co-incubated with the CTL clone that was raised against the exogenously-loaded ELFSYLIEK peptide. T cell recognition of the variants is indicated as (++), (+/-) or (-) (all variants did bind to HLA-A3; data not shown). The results imply that the ^{192}Phe-Ser-Tyr^{194} sequence (indicated as box) is required for TCR recognition, allowing the recognition of C-terminally extended variants of ELFSYLIEK by the CTL clone.

C: CTL recognition of C-terminally extended variants of the ELFSYLIEK peptide. The 11-mer, 12-mer and 13-mer PRA$^{190-200/201/202}$ (ELFSYLIEKVK/R/K) were titrated and separately loaded on B-LCL EKR cells (HLA-A3$^+$). The targets were co-incubated with the CTL for 14 h and the IFNγ-production was measured.



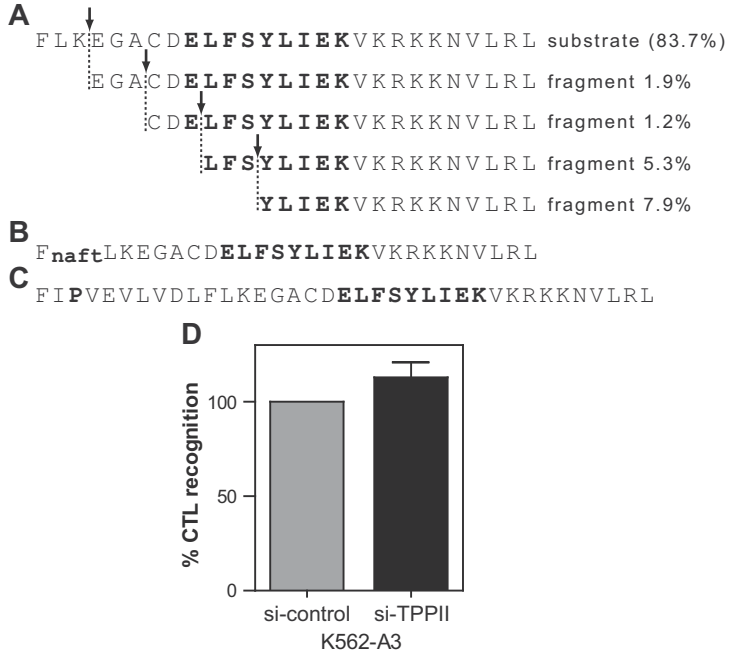
Suppl. Figure 2. Identification of PRA$^{190-198}$ as HLA-A3 presented peptide.

A: HPLC-profile of HLA-A3 presented peptides purified from K562-A3 cells (2x10^{10}). HLA-A3 molecules were immunoaffinity purified from the cell lysate, and acid-eluted peptides were separated in 60 fractions by reverse-phase HPLC, applying a gradient (depicted) of 31% to 40% buffer B in 36 min (left axis, OD214; right axis, % B).

B: Recognition by CTL anti-ELFSYLIEK of 60 HPLC-fractions loaded on B-LCL EKR (HLA-A3$^+$) to detect the fraction containing the eluted peptide-epitope. Fraction no. 21 sensitized the CTL.

C: HPLC-profile of the separation of a mixture of the synthetic 9-mer ELFSYLIEK peptide and its C-terminal extended 11-, 12-, and 13-mer variants ELFSYLIEKVK/R/K (PRA$^{190-200/201/202}$). The separation was conducted identical to the separation of cell surface expressed peptides (in panel A). The 9-mer, 11-mer, 12-mer and 13-mer peptides eluted in fractions 21, 29, and 32, respectively (confirmed by mass spectrometry; not shown).

D: The MS/MS spectrum of m/z 571.3 (2+) present in fraction 21 of the eluted peptides was identified as ELFSYLIEK; matching b- an y-ions are underlined. The MS/MS spectrum of the eluted peptide was identical to the MS/MS spectrum of the synthetic ELFSYLIEK peptide (not shown). Peaks indicated with 'S' could not be explained by 'regular' peptide fragment-ions, however, were also present in the MS/MS spectrum of the synthetic peptide. The peaks indicated with an asterisk are unrelated background peaks.



Suppl. Figure 3. TPPII does not generate the ELFSYLIEK epitope.

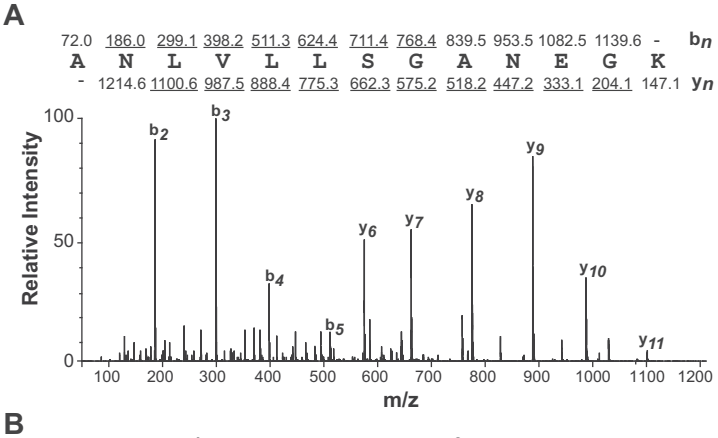
A: In vitro digestion with purified TPPII of 27-mer PRA$^{182-208}$ containing the ELFSYLIEK peptide (bold) in the middle. The fragments obtained after 1 h digestion are shown and quantified as the percentage of the total summed intensities measured by mass spectrometry. Arrows indicate cleavage-sites in the substrate and in the subsequently generated fragments.

B: A similar digestion with purified TPPII of 27-mer PRA$^{182-208}$, however with a modified substrate. At the N-terminal phenylalanine a 2-naphylsulfonyl group is attached to protect the substrate from N-terminal exo-peptidase activity. No cleavage products were detected (1 h digestion).

C: In vitro digestion with purified TPPII of 37-mer PRA$^{172-208}$. The third position in this peptide is a proline which is known to block TPPII-mediated N-terminal exo-peptidase activity when present at this position. No cleavage products were detected (1 h digestion). Together, the results point to a sequential removal of 3 aa from the N-terminus of the non-modified PRA$^{182-208}$ substrate and its subsequently generated fragments, which is in accordance with the known exo-peptidase trimming-activity of TPPII.

D: CTL recognition of K562-A3 stably transfected to express siRNA suppressing TPPII or non-targeting control siRNA (SEM of triplicate wells of representative experiment).

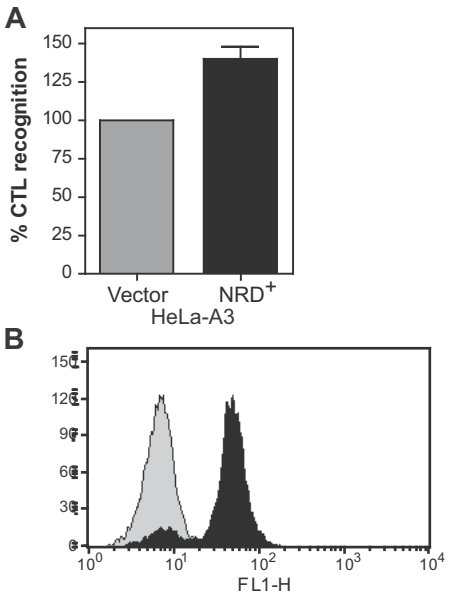
A



B

Band[a]	MS/MS[b]	Sequence identified[c]	Source protein
1	507.6 (3+)	IKGEHPGLSIGDVAK	High mobility group protein
	531.6 (3+)	KHPDASVNFSEFSK	High mobility group protein
	796.9 (2+)	KHPDASVNFSEFSK	High mobility group protein
2	536.3 (2+)	LAGLEEALQK	Keratin[d]
	739.8 (2+)	DSLENTLTETEAR	Keratin
	426.2 (2+)	GLTGGFGSR	Keratin
	813.4 (2+)	LNVEVDAAPTVDLNR	Keratin
	519.7 (2+)	AQYDDIASR	Keratin
3	514.6 (2+)	TAVCDIPPR	β-tubulin
	508.3 (2+)	DVNAAIATIK	α-tubulin
	912.9 (2+)	VGINYQPPTVVPGGDLAK	α-tubulin
	470.9 (3+)	QLFHPEQLITGK	α-tubulin
4	no precursors found		
5	643.3 (2+)	ANLVLLSGANEGK	Nardilysin[e]

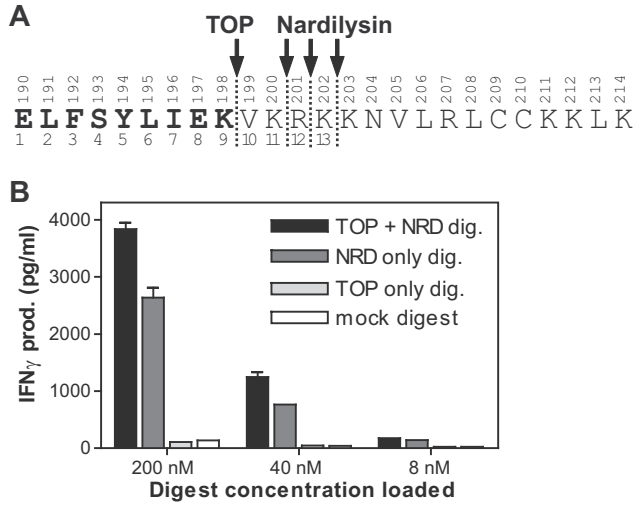
Suppl. Figure 4. Identification of nardilysin in cytosolic fraction no. 37 of K562-A3 cells.
Anion-exchange fraction no. 37 of K562-A3 cell lysate, which showed the highest digestive capacity of fluorogenic substrate ELFSYL(-dabcyl)IEKVKRC(-FL)KN, was separated by SDS-PAGE and protein bands were isolated (as shown in Fig. 2B). The proteins were identified by tandem mass spectrometry (MS/MS).
A: The MS/MS spectrum of m/z 643.3 (2+) that was found in band 5 (see panel B) is depicted. It was identified as peptide ANLVLLSGANEGK from nardilysin; matching b- and y-ions are underlined.
B: Identification by MS/MS of proteins in bands 1–3 as high mobility protein (band 1), keratin (band 2), and α- & β-tubulin (band 3). Notes: (a) Protein bands isolated from SDS-PAGE gel; (b) Mass (charge) that was chosen for MS/MS; (c) aa sequence as identified by MS/MS; (d) keratin is a contamination that is often found is this kind of samples; (e) from band 5, nardilysin was further confirmed by mass only of fragments 536.27 (2+), being aa seq. YIATTDFTLK and 679.32 (2+) which is aa seq. IEEFLSS-FEEK (data not shown).



Suppl. Figure 5. CTL recognition of nardilysin over-expressing HeLa-A3 cells.

A: Recognition by CTL anti-ELFSYLIEK of HeLa-A3 cells over-expressing nardilysin (HeLa-A3-NRD$^+$) as measured by IFNγ ELISA. Nardilysin over-expressing HeLa-A3 cells were selected by high EGFP expression. The population with low EGFP expression with natural nardilysin expression was used as reference population. Mean ± SEM of four experiments is shown.

B: Overlay histogram of HeLa-A3 cells transfected with pIRES-NRD-EGFP and sorted after transfection for either EGFP-low or EGFP-high expression, the latter population over-expressing nardilysin.

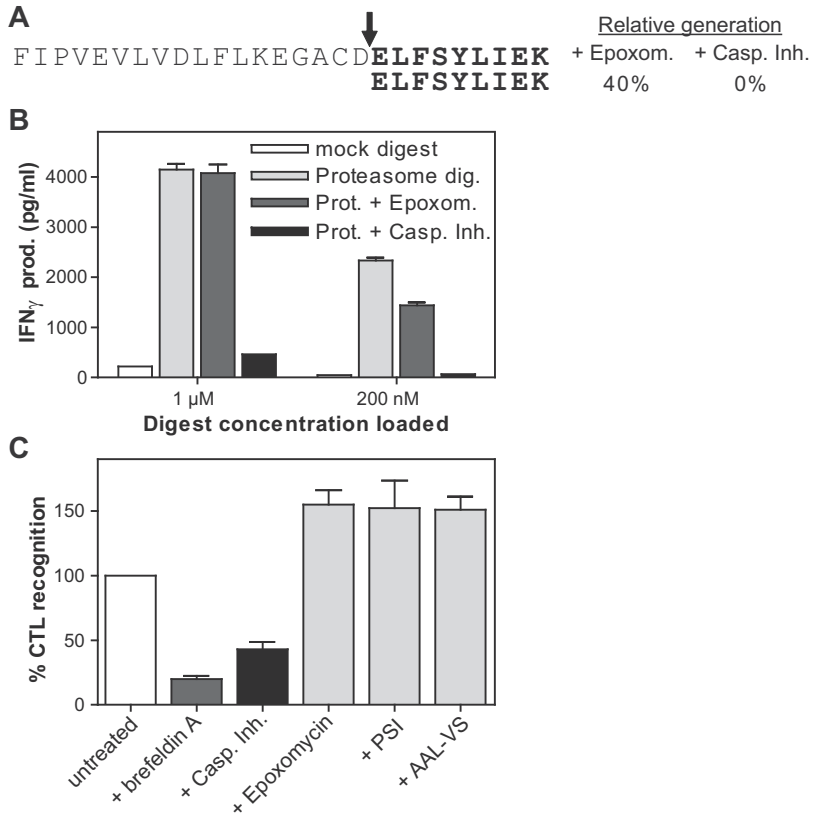


Suppl. Figure 6. CTL recognition of PRA$^{190-214}$ co-digested with nardilysin and TOP.

A: Substrate 25-mer PRA$^{190-214}$ (ELFSYLIEKVKRKKNVLRLCCKKLK, epitope boldfaced) with cleavage sites of nardilysin and TOP indicated as arrows. In the co-digest with nardilysin and TOP (2 h), approximately 3% of the digestion fragments were the minimal nonameric ELFSYLIEK epitope, as analysed by mass spectrometry.

B: CTL recognition of 25-mer PRA$^{190-214}$ digested for 2 h by nardilysin (NRD), TOP, or both peptidases together. SEM of triplicate wells of a representative experiment is indicated.

Importantly, the 25-mer incubated with TOP alone did not result in the minimal epitope (or other digestion fragments), as is shown here from the absence of CTL recognition. A similar digest of 17-mer PRA$^{190-206}$ with TOP also showed absence of (C-terminal) epitope generation (data not shown).

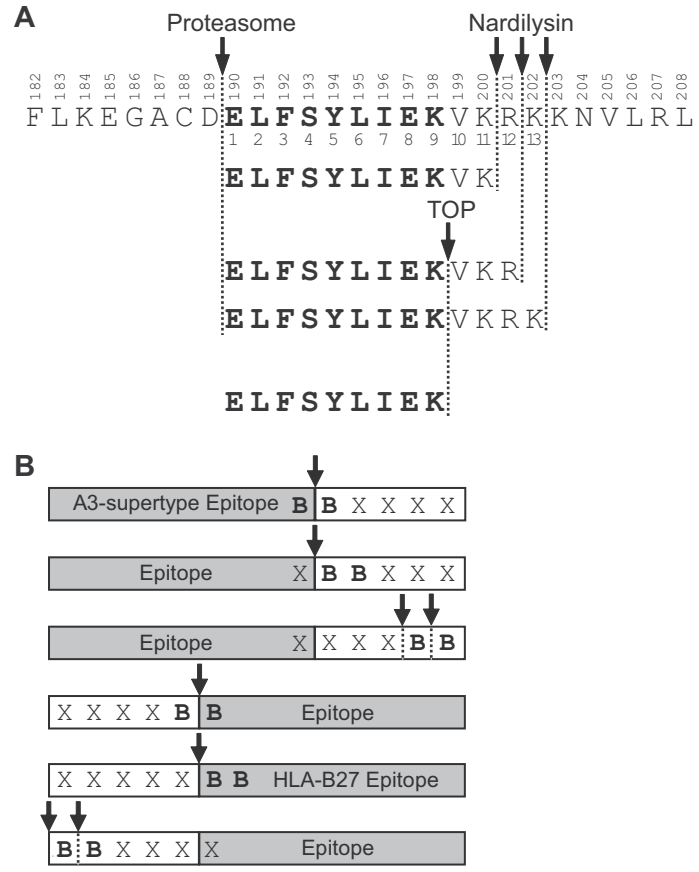


Suppl. Figure 7. The N-terminus of ELFSYLIEK is produced by the caspase-like cleavage activity of the proteasome.

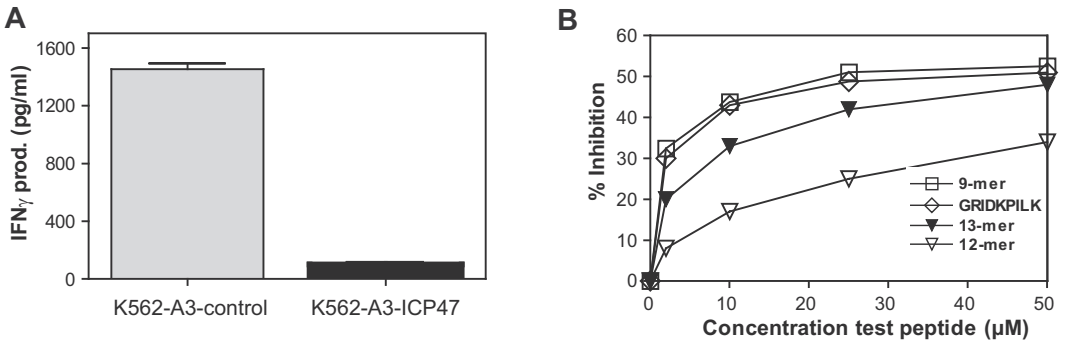
A: Proteasomal cleavages in 27-mer PRA$^{172-198}$ (epitope at C-terminus). Digestions were performed with immuno- and constitutive human 20S proteasomes for 1 h in the absence of inhibitors or in the presence of epoxomycin (10 µM) or AcAlaProNleLeuVSOH (Casp. Inh., used at 50 µM), a specific inhibitor of the caspase-like proteasomal activity. Arrow indicates the abundant cleavage before Glu-190 (liberating the epitope): the precise epitope-fragment and complementary fragments (containing Asp-189 as C-terminus) added up to >5% of the digested material after 1 h. Results for immuno- and constitutive proteasomes were comparable. The efficiency of epitope generation in the presence of the inhibitors is indicated as percentage of the epitope generated without inhibitor. The experiment was repeated three times with similar results.

B: CTL recognition of the digests of 27-mer PRA$^{172-198}$. The substrate was digested with constitutive proteasomes (1 h) in the absence of inhibitors or in the presence of epoxomycin or AcAlaProNleLeuVSOH (Casp. Inh.). Digests were titrated, loaded on HLA-A3^{+} cells and co-incubated with the CTL anti-ELFSYLIEK. Negative control is the 27-mer incubated at 37°C without proteasome (mock digest).

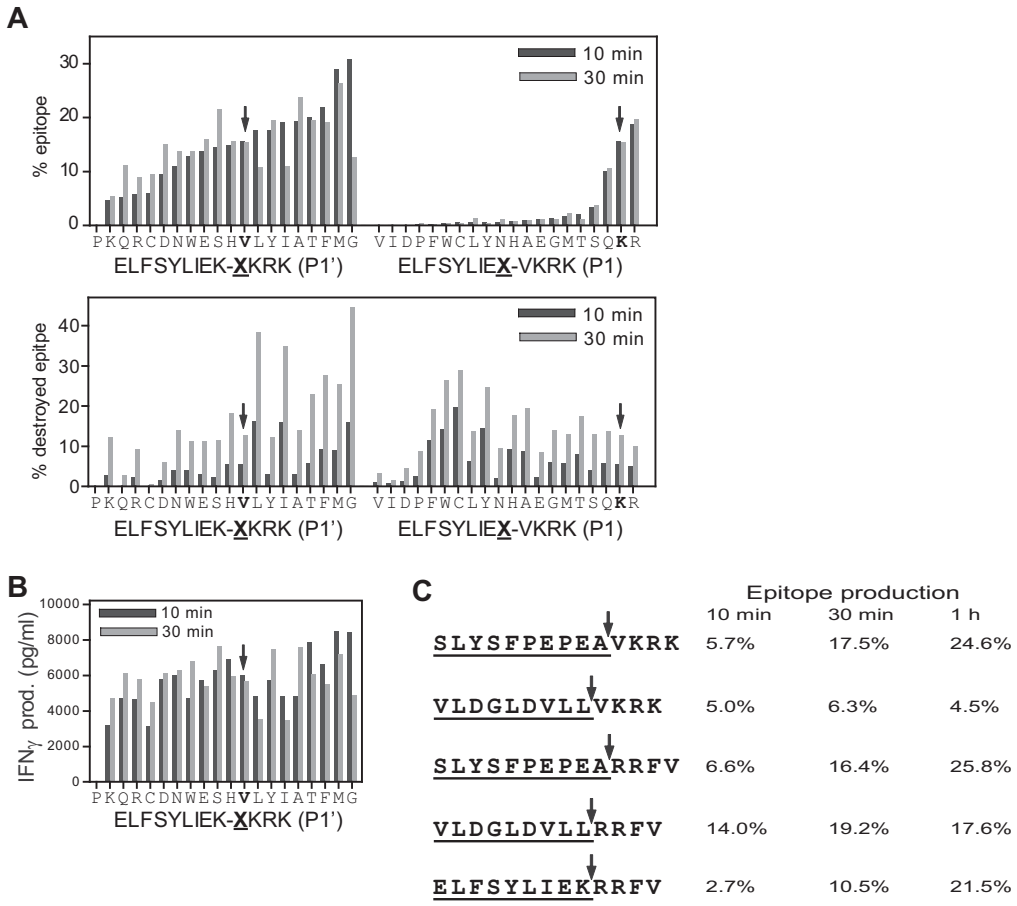
C: Relative CTL recognition of K562-A3 cells treated with general proteasome inhibitors epoxomycin (1 µM), PSI (10 µM) and AdaAhx3L3VS (AAL-VS; 50 µM) or the inhibitor of the caspase activity AcAlaProNleLeuVSOH (Casp. Inh., at 50 µM) as measured with intracellular IFNγ-staining. Reference 100% recognition is K562-A3 stripped (see methods), but untreated; background recognition is K562-A3 stripped and immediately treated with brefeldin A to block class I re-expression. SEM of three experiments are indicated.



Suppl. Figure 8. Mechanism of epitope generation and role of nardilysin in class I processing.
A: The proteasome, nardilysin and TOP co-operate in the generation of the PRA$^{190-198}$ epitope. The arrows depict the efficient cleavages of (1) the proteasome before Glu-190, (2) of nardilysin behind Lys-200, Arg-201 and Lys-202 and (3) the cleavage of TOP behind Lys-198 in the nardilysin-dependent 12- and 13-mer precursors together generating the epitope's C-terminus.
B: Sites where nardilysin can aid in the generation of HLA class I presented peptides depending on the position of the dibasic motif relative to the epitope (B, basic aa: Arg or Lys).



Suppl. Figure 9. TAP-dependency and translocation of PRA$^{190-198}$ epitope and its precursors.
A: TAP-dependent presentation of the ELFSYLIEK epitope. K562-A3 cells were transduced with the viral TAP-inhibitor ICP47 (K562-A3-ICP47) to block TAP-mediated peptide translocation, and used as target for the CTL anti-ELFSYLIEK. Reference K562-A3 cells were transduced with the empty construct (K562-A3-control). SEM of triplicate wells of representative experiment.
B: Efficiency of TAP translocation of 9-mer ELFSYLIEK and its 12-mer and 13-meric C-terminal extended length variants (ELFSYLIEKVKR/K). The peptides were tested as competitors in a TAP-translocation assay with a fluorescently labeled reference peptide that is known to be translocated efficiently. Peptide GRIDKPILK was used as positive control; this peptide is an HLA-B7 presented ligand also known to be efficiently translocated by TAP.



Suppl. Figure 10. The epitope-generating capacity of TOP.
A: Digestion by TOP of variants of 13-mer ELFSYLIEKVKRK (PRA$^{190-202}$), where the residues at the P1- and P1'-positions surrounding the TOP cleavage site (ELFSYLIEP1-P1'KRK) were substituted for all possible residues. The wild type 13-mer sequence is included two times (at both the P1'- and P1- substitutions) and is indicated by an arrow and bold printed residue. Upper panel: epitope-production (ELFSYLIEK or ELFSYLIEX for P1'- and P1-substitutions, respectively) after 10 min and 30 min is indicated and quantified as its percentage of the total summed intensities measured by mass spectrometry. Substitutions are ordered according to efficiency of epitope-production at 10 min. Lower panel: epitope-destruction (i.e. peptides in the digest constituting fragments of the epitope) after 10 min and 30 min is indicated as percentage of total summed intensities.
B: CTL recognition of TOP-digested 13-mer variants of PRA$^{190-202}$ with substitutions at P1' (ELFSYLIEK-P1'KRK). The digests were titrated, loaded on HLA-A3^{+} EKR target cells, co-incubated with the CTL anti-ELFSYLIEK and IFNγ-release was measured. Recognition of the wild type 13-mer sequence is indicated by an arrow and bold printed residue.
C: Capacity of purified TOP to generate the C-terminus of different CTL epitopes from precursors with two artificial C-terminal extended regions. Epitopes tested are SLYSFPEPEA (PRA$^{142-151}$), VLDGLDVLL (PRA$^{100-108}$) with flanking sequence VKRK or RRFV and ELFSYLIEK (PRA$^{190-198}$) with flanking sequence RRFV. Epitope production after 10, 30 and 60 min incubation of the substrate with TOP is indicated and expressed as percentage of the total summed intensities measured by mass spectrometry.

METHODS

Cell lines

Cell lines used were erythroleukemia cell line K562 (naturally expressing PRAME), cervical carcinoma cell line HeLa (PRAME$^+$), renal cell carcinoma (RCC) lines MZ1257, Le-94.15 (both lines, PRAME$^+$ and HLA-A3$^+$) and MZ1851 (HLA-A3$^+$, PRAME-negative) (RCC lines provided by Dr. E. Verdegaal, LUMC, The Netherlands) and B-LCL EKR (HLA-A3$^+$ and PRAME-negative). PRAME-expression was assessed by rtPCR. K562, which is naturally class I negative, and HeLa cell lines were transduced retrovirally to express HLA-A3, HLA-A2 or HLA-B2705 (designated K562-A3, K562-A2, K562-B27 and HeLa-A3) using HLA-A2 and -A3 encoding retroviral LZRS plasmids [32] (provided by dr. M. Heemskerk, dept. Experimental Hematology, Leiden University Medical Center) or an HLA-B2705 encoding plasmid. Cell surface expression of HLA-A3, -A2 and -B2705 was measured by flow cytometry using monoclonal antibodies GAP-A3, BB7.2 and W6.32 respectively (data not shown). HeLa-A3 cells overexpressing nardilysin were made by transfection with the pIRES2-EGFP vector containing rat NRD1 cDNA (pIRES2-NRD1-EGFP) (rat NRD1 has similar cleavage specificity as human NRD1). After transfection cells were sorted in EGFP-low and EGFP-high populations, the latter population over-expressing nardilysin. K562-A3 cells expressing viral TAP inhibitor ICP47 and control cells (K562-A3-ICP47 and K562-A3-vector) were made by retroviral transduction using the pLZRS-IRES-eGFP plasmid with or without the ICP47 insert. K562-B2705 was stably transfected with a plasmid encoding the minigene MFLRGKWQRRY**RRIYDLIEL** (EBNA3C aa 247-266, with an extra N-terminal methionine; CTL epitope in bold), transfected cells were selected with hygromycine. All cell lines were cultured in complete culture medium consisting of IMDM (Biowithaker, Verviers, Belgium) supplemented with 8% fetal calf serum (FCS; PAA, Linz, Austria), 100 IU/ml penicillin and 2 mM L-glutamine.

Synthetic peptides

Peptides were synthesized by solid-phase strategies on an automated multiple peptide synthesizer (Syro II, MultiSyntech, Witten, Germany) using Fmoc-chemistry. For fluorescent peptides, fluorescein was covalently coupled to the cysteine residue using 5-(iodoacetamido)fluorescein (Fluka Chemie AG, Buchs, Switzerland). The quencher residue, Fmoc-L-Lys(Dabcyl)-OH was obtained from Neosystems (France). For blocking of N-terminal trimming in digestion experiments, the N-terminus of the peptide was blocked with a 2-naphylsulfonyl group. All peptides were HPLC-purified and validated by mass spectrometry.

Isolation and identification of nardilysin in cytosolic fractions

K562-A3 cells (5×10^9) were homogenized in lysis buffer (10 mM Tris-HCl, 1 mM DTT, 1 mM NaN_3, 25 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol, 2 mM ATP, 50 mM NaF, 0.1 mM Na_8VO_4, pH 7.5) using a Dounce glass homogenizer. The homogenate was centrifuged for 20 min at 10,000 × *g*, and the supernatant was centrifuged for 6 h at 100,000 × *g* thereby removing proteasomes and TPPII. The clarified lysate was filtered over a 0.2 µm filter. The proteins in the lysate were separated (60 fractions of 0.5 ml) by anion exhange chromatography using a mono Q column (HR 5/5, Amersham) with a linear gradient (20 ml) from 0 to 500 mM KCl in lysis buffer at a flow rate of 1 ml/min. Specific peptidase activity in the fractions was assessed with in-

ternally quenched fluorogenic 15-mer substrate ELFSYL(-dab)IEKVKRC(-FL)KN ($PRA^{190-204}$). For technical reasons the fluorescein (FL)-group was attached to a cysteine in stead of the natural lysine at this position. The assay was performed with substrate at a final concentration of 0.5 µM in substrate buffer (30 mM Tris, 10 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, pH 7.5) either in the absence or presence of 1 mM phenathroline. Appearance of fluorescence, due to spatial separation of the quencher from the fluorophore, was measured using 360 nm extinction and 460 nm emission settings. Fractions (20 µg) containing peak proteolytic activity (fraction #36 to 40) were run on 12% SDS polyacrylamide gel and stained with Coomassie Brilliant Blue according to standard procedure. Selected protein bands were isolated from fraction #37, subjected to digestion with trypsin as described [33] and analysed using mass spectrometry.

HLA-A3 peptide binding assay

Affinity of peptides for HLA-A3 was measured with a competition-based cellular peptide binding assays as described previously [34]. In this assay the capacity of test peptides to compete with the cell surface expressed HLA class I binding of a fluorescein (FL)-labeled reference peptide that is known to bind with high affinity in the HLA molecule of interest was tested. The percentage inhibition of fluoresceine labeled reference peptide binding by the competitor test peptide was calculated using the following formula: $(1-(MF_{\text{reference and competitor peptide}} - MF_{\text{no reference peptide}}) / (MF_{\text{reference peptide}} - MF_{\text{no reference peptide}})) \times 100\%$. The binding affinity of the test peptide is expressed as the concentration needed to inhibit 50% binding of the FL-labeled reference peptide (IC_{50}). An $IC_{50} \leq 5$ mM was considered high affinity binding and 5 mM < $IC_{50} \leq 15$ mM was considered intermediate affinity binding. The FL-labeled reference peptide used for HLA-A3 was KVFPC(FL)ALINK [34].

RNA interference mediated suppression of peptidases

RNA interference mediated suppression of peptidase expression in K562-A3, K562-B27 and HeLa-A3 was established by transient transfection with pools of four interfering oligonucleotide RNA duplexes for each peptidase (siGENOME SMARTpool from Dharmacon) and/or by stable transfection of the pSUPER-puro vector (a gift from dr. R. Bernards, Netherlands Cancer Institute) encoding siRNA sequences. Transfection of pSUPER-puro plasmids in K562-A3 and K562-B27 was performed by electroporation, and in HeLa-A3 with reagent FuGENE (Roche Molecular Biochemicals, Indianapolis) according to the instructions of the manufacturer. Cells stably expressing siRNA were obtained by culturing in the presence of puromycine (2 µg/ml for K562-A3 and –B27 and 0.2 µg/ml for HeLa-A3). Transient transfection of siRNA duplexes in K562-A3 was performed with HiPerFect (Qiagen) and transfection in HeLa-A3 with DharmaFECT 1 (Dharmacon), according to instructions of the manufacturers. Stable suppression of TPPII was targeted at GCAGTATTCACATCGCCAC (from ref. 14). The target sequence chosen for stable suppression of nardilysin was AGCAGACCCTTGGGTACCA (resulting in the best suppression from five newly designed sequences tested). Suppression of TOP by stable expression was targeted at CCTCAACGAGGACACCACC (from ref. 35) and the transiently transfected pool of duplexes was directed at: (1) TAGATGAGCTGGCGCAGAATT, (2) TCAAACGCATCAAGAAGAATT, (3) GCAAGGTTGGCATGGATTATT, and (4) AGACCAAGCGCGTGTAT-

GATT (in accession number NM_003249). Suppression of Neurolysin by the pool of duplexes was targeted at: (1) GGATAAAGCTACAGGAGAA, (2) GATAATGAATCCAGAGGTT, (3) CAATTGAGGTGGTCACTGA, and (4) GAACTCAAGTATTCCATAG (in accession number NM_020726). Suppression of insulin degrading enzyme by the pool of duplexes was directed at: (1) TCAAAGGGCTGGGTTAATA, (2) ACACTGAGGTTGCATATTT, (3) GAACAAAGAAATACCCTAA, and (4) GTGGAGAGCATACCAATTA (in accession number NM_004969). As non-silencing control for the SMARTpool duplexes we used the si*CONTROL* Non-Targeting siRNA pool (Dharmacon). The non-silencing control for the pSUPER-puro expressed siRNA's was a non-targeting scrambled sequence. The level of suppression of the peptidases was assessed by quantitative RT-PCR and western blotting. For detection of TOP by immunoblotting, we used monoclonal antibody IVD6 (Upstate), and Western blotting of nardilysin was performed with an affinity-purified rabbit antiserum.

Peptide translocation

TAP translocation of PRAME peptide was tested using microsomes that were prepared from a B-LCL cell line as described previously [36]. Microsomes were thawed from frozen aliquots and resuspended in transport buffer (5 mM Hepes-pH 7.3, 130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA, 2 mM $MgCl_2$). Translocation efficiency of test peptides was measured by competition for translocation with a fluorescein (FL)-labeled reference peptide that contains a consensus site for N-linked glycosylation in the ER. Briefly, samples (60 µl) were pepared containing titrated amounts of the competing test peptide (50, 25, 10 and 2 µM – final concentrations), 0.5 µM FL-labeled reference peptide C(-FL)VNKTERAY [37] and 10 mM ATP in transport buffer. Per sample, 40 µl of microsomes (containing 1.3×10^6 cell equivalents) was added and samples were incubated for 10 min at 37°C. Peptide translocation was stopped by adding 1 ml ice cold transport buffer with 10 mM EDTA, samples were washed, pellets resuspended in lysis buffer (50 mM Tris-pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$, 1% Triton X-100) and rotated for 30 min at 4°C. Debris was removed by centrifugation (10 min, 13000 rpm at 4°C) and the glycosylated FL-reference peptide was recovered by incubation for 2 h at 4°C with 100 µl packed concanavalin A-Sepharose 4B (GE Healthcare, Uppsala, Sweden). Con A-Sepharose was then washed three times in lysis buffer and glycosylated FL-reference peptide was released in the supernatant by incubation for 1 h at RT on a shaker in 200 ul elution buffer (50 mM Tris-HCl pH 8.0, 500 mM mannopyranoside, 10 mM EDTA). Fluorescence in the supernatant was measured using excitation 485 nm and emission 535 nm settings. Maximal fluorescence was obtained from a sample containing no competitor peptide, minimal fluorescence from a sample without ATP and containing 0.5 M EDTA. The GRIDKPILK positive control competitor peptide is a reported efficiently translocated peptide [38].

Identification of the ELFSYLIEK peptide on the cell surface in HLA-A3

K562-A3 cells (30×10^9) were lysed in lysis buffer, 20 mM Tris/pH 8, 5 mM $MgCl_2$ 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 antibody W6.32 coupled to protein A beads. Peptides were eluted with 10% HAc, filtered

over a 10 kD 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 and a gradient of 31% to 40% buffer B in 36 min was applied. Fractions were lyophilized to remove acetonitrile, dissolved in water (100 µl end volume) and 10 µl was used for testing CTL reactivity and 90 µl for mass spectrometry (see mass spectrometry). For CTL assays, the fractions were loaded on EKR cells (20000/well, 96 well plate) and co-incubated with CTL (25000/well). EKR cells were pre-treated with mild acid buffer to remove cell surface expressed peptides, and resuspended in IMDM containing 2 µg/ml human β_2-microglublin (Sigma). CTL reactivity was measured with IFNγ ELISA.

CTL assays and functional peptidase inhibitor studies

CTL recognition was measured by the ^{51}Cr-release assay according to standard procedures [17] or by IFNγ ELISA (Sanquin Reagents, Amsterdam, The Netherlands) according to manufacturer's instruction. In case of peptidase inhibitor studies, intracellulair IFNγ-staining of CD8$^+$ T cells was used as readout. Briefly, target K562-A3 cells were first treated with the indicated inhibitors for 1.5 h at 37°C then stripped by mild acid treatment (300 mM glycine, pH 2.9, 1% BSA) for 90 sec to remove cell surface class I presented peptides, washed, and treated with the same inhibitor for 5 h at 37°C. Background epitope expression was measured by treating a sample with brefeldin A (10 µg/ml) immediately after stripping, and the maximal epitope expression sample was stripped but left untreated to allow full reconstitution of class I molecules. After inhibitor treatment, the samples were washed in PBS, plated as target cells in triplicate wells of a 96-well plate (30000/well) and co-incubated with the CTL clone (30000/well) for 6 h. After 30 min co-incubation brefeldin A (Sigma) was added at 5 µg/ml. The co-cultures were harvested, washed, fixed and permeabilized with Cytofix/Cytoperm (BD Pharmingen, San Diego, CA) according the manufacturer's instructions and T cells were stained intracellularly with CD8-APC (Dako) and IFNγ-PE (BD Pharmingen) to measure T cell activation by flow cytometry. Inhibitors used were: Brefeldin A (Sigma), used at 10 µg/ml; Phenanthroline (Sigma), inhibitor of metallopeptidases used at 400 µM (in cellular assays); Insulin (Sigma), competitive inhibitor of insulin degrading enzyme used at 50 µM; Captopril (Sigma), inhibitor of angiotensin converting enzyme used at 50 µM; Leupeptin (Calbiochem) inhibitor of trypsin-like proteases and cysteine proteinases used at 100 µM; Calpeptin (Calbiochem) inhibitor of calpains used at 30 µM; Aprotinin (Calbiochem) a serine proteases inhibitor used at 5 µM; and Butabindide (Tocris), inhibitor of TPPII used at 200 µM. For inhibition of the proteasome we used PSI, epoxomycine (both Calbiochem), AdaAhx3L3VS [39] and AcAla-ProNleLeuVSOH [25].

In vitro induction of CTL

The in vitro CD8$^+$ T cell induction against the exogenously loaded synthetic PRA$^{190-198}$ peptide (ELFSYLIEK) was performed as described previously [17]. Briefly, PBMC of an HLA-A3 positive healthy donor were used for the generation of antigen presenting cells (DC's and activated B cells) and autologous CD8$^+$ T cells. T cells were stimulated with peptide-pulsed DC's and restimulated three times with peptide-pulsed activated B cells. Peptide specificity of the bulk CTL culture was tested at day 28 and the culture was cloned by limiting dilution

at day 29 to obtain CTL clones specifically recognizing the ELFSYLIEK peptide. One specific CTL clone was selected for functional CTL assays based on T cell sensitivity and growth characteristics. The CTL clone specific for the HLA-B2705 presented immunodominant CTL epitope RRIYDLIEL from EBNA-3C (aa 258-266) was generated as reported previously [27].

Digestions with proteasomes, TPPII, nardilysin and TOP and digest recognition assays

Proteasomes (20S) were purified as described [40] from HeLa cells, which contain mainly constitutive proteasomes, and B-LCL line JY that expresses mainly immunoproteasomes. Proteasome composition was confirmed by Western blotting (data not shown). TPPII was purified to homogeneity from erythrocyte concentrates as described [41] with minor modifications [42]. Purified TPPII (0.05 mg/ml) in 100 mM potassium phosphate buffer, pH 7.5, containing 30% glycerol and 1 mM DTT was stored at –80°C before usage. Recombinant mouse nardilysin was prepared as previously described [21] using the Bac-to-Bac system (Invitrogen, Carlsbad, Calif.) and purified using Ni-resin (Qiagen) affinity chromatography. The nardilysin preparation was homogeneous as judged by SDS-PAGE [21]. Long peptide digestions were performed with purified mouse nardilysin and with cytosolic fraction #37 of K562 cells containing human nardilysin as enzyme (see for details the section *'Isolation and identification of nardilysin in cytosolic fractions'*) with similar results. Mouse and human nardilysin have identical cleavage specificities [43]. TOP was produced by overexpression in Escherichia coli and purified using Ni-resin (Qiagen) affinity chromatography as previously described [44].

Digestions were performed in a volume of 300 µl with mostly 10 µM peptide and 4.3 nM enzyme at 37°C. TPPII digestions were performed with 1 µg enzyme (MW $>10^6$) and 2 nmol peptide in 100 µl. A mock digestion of the substrate at 37°C without enzyme was always included. For proteasome and nardilysin, the digestion buffer was 30 mM Tris (pH 7.5), 10 mM KCl, 5 mM $MgCl_2$ and 1 mM DTT. For TOP digestions the buffer was 50 mM Hepes (pH 7.6), 2 mM $MgCl_2$, 0.1 mM DTT. TPPII digestions were performed in a buffer of 20 mM Hepes, 2 mM $Mg(Ac)_2$, 2 mM DTT, containing 5% glycerol. At different time points (dependent on the digest at 10 min, 30 min, 1 h, 2 h, and 4 h) samples of 50 µl or 75 µl were drawn and the enzymatic reaction was stopped by addition of 5% (v/v) acetic acid, or when digest were used for CTL recognition assays by addition of 1 mM phenanthroline. Samples were diluted four times before measuring with mass spectrometry. For functional recognition assays the digest-samples were first titrated and then 10 µl digest was loaded onto 90 µl B-LCL EKR cells (HLA-$A3^+$, PRAME-negative) in triplicate wells of a 96-well plate (17000 cells/well) for 2 h at 4°C. Synthetic reference PRAME peptides were titrated at equal concentrations and loaded similarly. Prior to loading, to improve loading-efficiency, EKR cells were stripped from naturally expressed peptides by acid treatment and resuspended in IMDM containing 2 µg/ml human β_2-microglublin (Sigma). After loading, CTL (25000) were added in 50 µl/well, co-incubated for 12 h, and IFNγ in the supernatant was measured by ELISA.

Mass spectrometry

Electrospray ionization mass spectrometry was performed on a Q-TOF1 (Micromass), equipped with an on-line nanoelectrospray interface, approx. flow rate of 250 nL/min.

This flow was obtained by splitting of the 0.4 ml/min flow of a conventional high pressure gradient system, using an Acurate flow splitter (LC Packings). Injections were done with a dedicated micro/nano HPLC autosampler, the FAMOS (LC Packings). Digestion solutions were diluted five times in water-acetonitrile-formic acid (95:3:1, v/v/v), and 1 µl was trapped on a precolumn (MCA-300-05-C18; LC Packings) in water-acetonitrile-formic acid (95:3:1, v/v/v). The precolumn was washed for 3 min to remove the buffers in the digests and the trapped analytes were eluted with a steep gradient from 70% B to 90% B in 10 min, with a flow of 250 nl/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 MS/MS mode ions were selected with a window of 3 Da. The collision gas was argon (4×10^5 mbar), and the collision voltage 30V.

For proteasome, nardilysin, TOP and TPPII digestions peaks in the mass spectra were searched in source substrate peptides using Biolynx/proteins software (Micromass). The intensity of the peaks was used for the relative quantification of the digestion fragments as percentage of the total amount of peptide digested at indicated time points. For identification of the proteins in fraction no. 37 of the K562-A3 cytosolic preparation, MS/MS spectra were interpreted against the SwissProt database using the PeptideSearch software.

For identification of the $PRA^{190-198}$ epitope, peptides present in the HPLC-fractions of eluted peptides were sequenced by tandem mass spectrometry. To this end, the fractions were first lyophilized, resuspended in 95/3/0.1 v/v/v water/acetonitrile/formic acid (solvent A) and analyzed by online nanoHPLC-MS. The nanoHPLC system consisted of a conventional gradient HPLC system (Agilent 1100), the flow of which was reduced to 300 nl/min by an in-house constructed splitter. Two-microliter injections were done onto a precolumn (10 mm × 100 µm; AQUA-C18, 5-µm particle size (Phenomenex)) and eluted via an analytical nano-HPLC column (15 cm × 75 um; AQUA-C18, 5-µm particle size. HPLC columns were packed in-house. The gradient was run from 0 to 50% solvent B (10/90/0.1 v/v/v water/acetonitrile/formic acid) in 90 min. The mass spectrometer was an HCT^{plus} (Bruker Daltonics), which was run in data-dependent MS/MS mode during peptide elution. The ELFSYLIEK peptide was identified by its mass and MS/MS spectrum.

Supplementary references in methods

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