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Cytotoxic T lymphocytes (CTLs) recognize peptides presented by HLA class I molecules on the cell surface. The C terminus of these CTL epitopes is considered to be produced 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 protein EBNA3C, and a clinically important epitope from the melanoma protein MART-1. TOP functioned as C-terminal trimming peptidase in antigen processing, and nardilysin contributed to both the C-terminal and N-terminal generation of CTL epitopes. By broadening the antigenic peptide repertoire, nardilysin and TOP strengthen the immune defense against intracellular pathogens and cancer.

The production of short peptides (8–12 amino acids in length) presented by HLA class I molecules, called CTL epitopes when recognized by $CD8^+$ 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[1–3]. This cleavage process is accomplished by the multicatalytic proteasome complex and cytosolic peptidases[1,3]. Tripeptidyl peptidase II (TPPII)[4], thimet oligopeptidase (TOP)[5,6] and aminopeptidases[1,2] have been linked to protein degradation, but the contribution of other (endo)peptidases has remained elusive. A few degradation products of intermediate length (8–16 amino acids, on average) escape further cytosolic destruction through translocation via the TAP transporter into the endoplasmic reticulum, where they can be trimmed at the N terminus by the endoplasmic reticulum aminopeptidases ERAP1 and ERAP2 (refs. 1,2,7) and, when they bind properly, assemble with HLA class I heterodimers before being routed to the cell surface. To bind to HLA class I, peptides need to comply with the binding motif of the particular HLA class I molecule through the presence of the proper anchor residues located mostly at the second and C-terminal positions in the peptide.

The absence of C-terminal excision of CTL epitopes in cells in which the proteasome is inhibited[8–10], together with lack of detection of C-terminal trimming activity in the cytosol or endoplasmic reticulum, has led to the idea that only the proteasome liberates the C terminus[1,2]. Cytosolic endopeptidases, however, may also produce peptides with a C terminus fit for binding HLA class I 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[11–15]. Although these compounds are known to not completely inhibit proteasome activity in cells, this finding suggests the existence of a partially proteasome-independent pathway for the generation of HLA class I ligands. So far only a CTL epitope from the human immunodeficiency virus (HIV) protein negative factor (Nef) has been shown to depend on TPPII (ref. 16), and a human tumor epitope from the melanoma antigen MAGE-A3 is reported to be produced by insulin-degrading enzyme[17], but the general importance of TPPII and insulin-degrading enzyme in the generation of CTL epitopes is still unclear[17,18]. Other peptidases that liberate the C terminus of HLA class I ligands are unknown. To define such peptidases, we delineated 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 HLA class I antigen processing.

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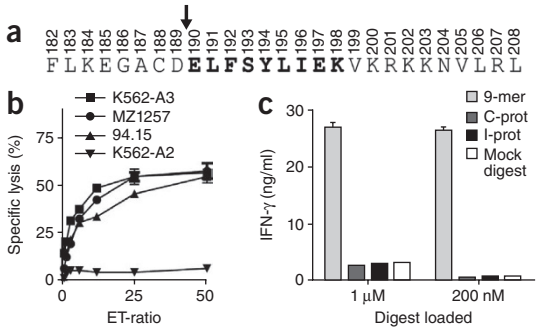

Figure 1 PRA(190–198) is an HLA-A3-presented CTL epitope with a proteasome-independent C terminus. (**a**) Proteasomal digestion site (arrow) in the 27-residue peptide PRA(182–208) (ELFSYLIEK; bold). (**b**) Specific lysis of K562-A3 cells, the renal cell carcinoma cell lines MZ1257 and 94.15 (both PRAME$^+$HLA-A3$^+$) and K562-A2 cells (K562 cells transfected with HLA-A2 (HLA-A2$^+$)) by a CTL clone (anti-ELFSYLIEK CTL) raised against the PRA(190–198) peptide (ELFSYLIEK) exogenously loaded on HLA-A3. E:T, effector/target ratio. Data are from one experiment representative of five (mean and s.e.m. of triplicate wells). (**c**) Recognition of the 25-residue peptide PRA(190–214) (ELFSYLIEKVKRKKNVLRLCCKKLK; epitope at N terminus) digested for 1 h without enzyme (Mock digest (background)) or with immunoproteasomes (I-prot) or constitutive proteasomes (C-prot), then diluted and loaded at two different concentrations (horizontal axis), onto EKR cells (HLA-A3$^+$PRAME$^-$) and coincubated with the CTL clone in **a**, then analyzed by enzyme-linked immunosorbent assay (ELISA) of the production of interferon-γ (IFN-γ) by the CTL clone (to assess recognition). 9-mer, nine-residue peptide ELFSYLIEK loaded at the same concentrations (positive control). Data are from one experiment representative of three (mean and s.e.m. of triplicate wells).

RESULTS

PRAME(190–198) has a proteasome-independent C terminus

Cleavage that yields a C-terminal lysine residue is not readily accomplished by proteasomes[19]. Therefore, we searched for a CTL epitope presented by HLA-A3, a human HLA class I molecule that binds peptides with a lysine as C-terminal anchor residue. The nine-residue peptide ELFSYLIEK derived from amino acids 190–198 of the tumor-associated protein PRAME[20] (PRA(190–198)) was selected by binding prediction and bound with high affinity to HLA-A3 (**Supplementary Fig. 1**). Digestion of the 27-residue peptide PRA(182–208) (**Fig. 1a**) with purified 20S proteasomes showed no cleavage after the epitope's C-terminal Lys198 or in the region flanking the C terminus. In contrast, its N terminus was liberated by efficient proteasomal cleavage before Glu190. Fragments containing Asp189 at the C terminus and complementary fragments with Glu190 as N terminus constituted >7% of the digested material after 1 h of digestion with proteasomes; digestion with immunoproteasomes or constitutive human 20S proteasomes gave similar results (in three experiments). We transfected the erythroleukemia cell line K562, which naturally expresses PRAME, with HLA-A3 to produce K562-A3 cells. Tandem mass spectrometry identified the sequence ELFSYLIEK (PRA(190–198)) in peptides eluted from HLA-A3 on the surface of K562-A3 cells (**Supplementary Fig. 2**). Also, a CTL clone raised against the PRA(190–198) epitope exogenously loaded onto HLA-A3 efficiently recognized K562-A3 cells and other tumor cell lines expressing PRAME and HLA-A3 in cytotoxicity assays (**Fig. 1b**), which confirmed natural presentation of the epitope. That same CTL clone (anti-ELFSYLIEK CTL) also recognized exogenously loaded variants of the epitope ELFSYLIEK extended at the C terminus to 11, 12 or 13 residues (ELFSYLIEKVK, ELFSYLIEKVKR and ELFSYLIEKVKRK), but these peptides, although they had HLA-A3-binding affinity (**Supplementary Fig. 1**), were not naturally presented (**Supplementary Fig. 2**). We used this feature

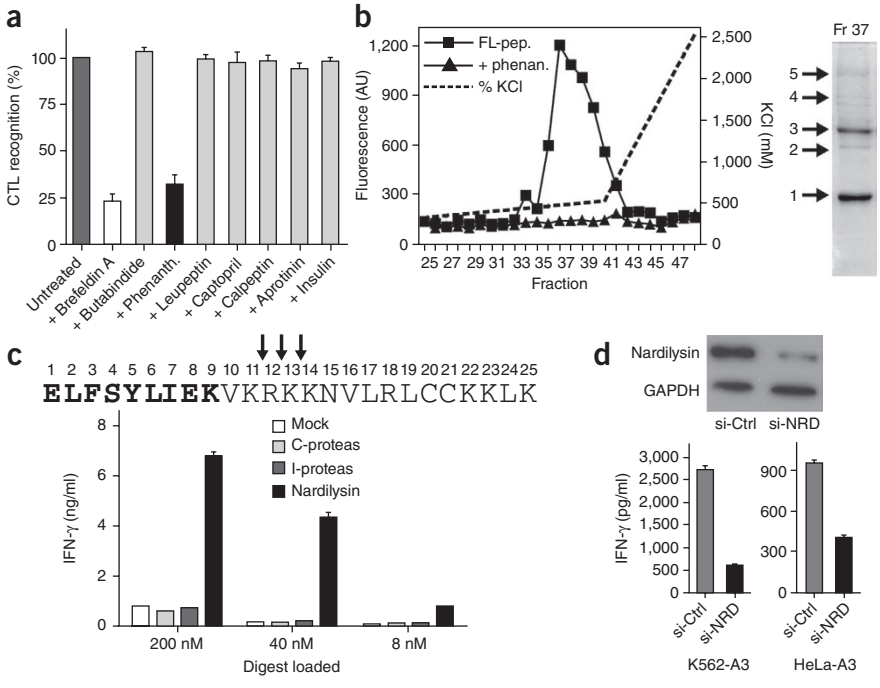

Figure 2 Nardilysin produces precursors of PRA(190–198) with C-terminal extension. (**a**) Recognition, by the anti-ELFSYLIEK CTL clone, of K562-A3 cells treated with various inhibitors (horizontal axis), assessed as intracellular IFN-γ production and presented relative to results obtained with untreated K562-A3 cells, set as 100%. Data are from three experiments (mean and s.e.m.). (**b**) Fluorescence production by the fluorogenic substrate ELFSYL(-dab)IEKVKRC(-FL)KN after digestion for 30 min by cytosolic fractions of K562-A3 cells (KCl gradient, right vertical axis) with (+ phenan) or without (FL-pep) 1 mM phenanthroline, measured at 460 nm and presented in arbitrary units (AU). The same substrate with D-amino acids at the first positions had only slightly less fluorescence (data not shown). Right, separation of fraction 37 (Fr 37) by SDS-PAGE; nardilysin was identified in band 5 (**Supplementary Fig. 4**). Data are representative of two experiments. (**c**) Nardilysin digestion sites (top) in the 25-residue peptide PRA(190–214) (epitope, bold); arrows indicate cleavage that generates the 11-, 12- and 13-residue peptides. Below, recognition of PRA(190–214) by the anti-ELFSYLIEK CTL clone after digestion for 30 min with purified nardilysin, immunoproteasomes or constitutive proteasomes and loading onto EKR cells, assessed by ELISA of IFN-γ. Data are from one experiment representative of four (s.e.m. of triplicates). (**d**) Recognition, by the anti-ELFSYLIEK CTL clone, of K562-A3 and HeLa-A3 cells expressing control siRNA (si-Ctrl) or nardilysin-specific siRNA (si-NRD), assessed by ELISA of IFN-γ (bottom); peptide-loading control experiments, **Supplementary Figure 5a**. Suppression of nardilysin did not compromise proteasome-mediated processing (**Supplementary Fig. 6**). Above, immunoblot analysis of nardilysin and GAPDH (loading control) in K562-A3 cells expressing control or nardilysin-specific siRNA. Data are from one experiment representative of five (s.e.m. of triplicates).

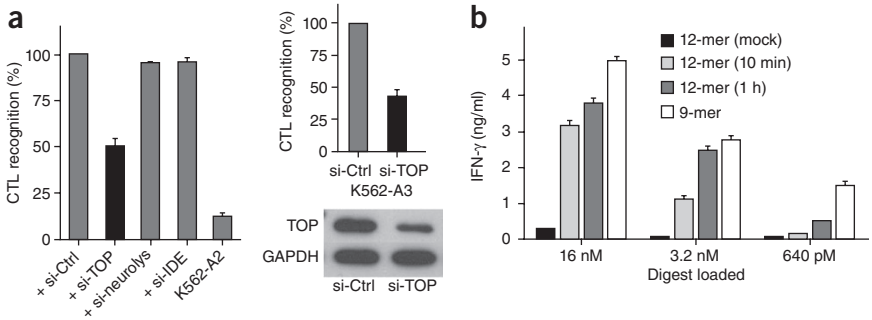

Figure 3 TOP produces the C terminus of the PRA(190–198) epitope. (**a**) Recognition, by the anti-ELFSYLIEK CTL clone, of K562-A3 cells transfected with siRNA pools targeting TOP (si-TOP), neurolysin (si-neurolys) or insulin-degrading enzyme (si-IDE) or of K562-A2 cells alone (left), or of K562-A3 cells stably expressing TOP-specific or control siRNA (top right), assessed as ELISA of IFN-γ and presented relative to results obtained with K562-A3 cells transfected with nontargeting control siRNA, set as 100% (peptide-loading control experiments, **Supplementary Fig. 5a**). TOP suppression did not compromise proteasome-mediated processing (**Supplementary Fig. 6**). Bottom right, immunoblot analysis of TOP and GAPDH in K562-A3 cells stably transfected with control or TOP-specific siRNA. Data are representative of three or four experiments (error bars, s.e.m.). (**b**) Recognition, by the anti-ELFSYLIEK CTL clone, of the 12-residue peptide PRA(190–201) (ELFSYLIEKVKR) after digestion for 10 min (12-mer (10 min)) or 1 h (12-mer (1 h)) with TOP or without TOP (12-mer (mock)), followed by 'titration' and loading onto EKR cells, assessed by ELISA of IFN-γ. 9-mer, nine-residue peptide ELFSYLIEK (positive control). Data are from one experiment representative of three (s.e.m. of triplicate wells).

of the CTL clone to monitor generation of the epitope ELFSYLIEK and its variants described above in the digestion of longer PRAME peptides. Proteasomal digestion of the 25-residue peptide PRA(190–214) (ELFSYLIEK, located at the N terminus) loaded onto EKR B-lymphoblastoid (HLA-A3$^+$PRAME$^-$) cells were not recognized by the CTL clone (**Fig. 1c**), which confirmed the proteasome independence of this epitope's C terminus.

Nardilysin produces precursors of PRAME(190–198)

We pursued identification of the peptidase(s) that generated the C terminus of the ELFSYLIEK epitope. First, recognition by the anti-ELFSYLIEK CTL was inhibited when we treated K562-A3 cells with the metallopeptidase inhibitor *o*-phenanthroline, whereas other inhibitors had no effect (**Fig. 2a**). As butabindide, an inhibitor of the serine peptidase TPPII, can be inactivated by serum[4], we further excluded the potential involvement of TPPII (refs. 16,21) by digestion analysis and RNA-mediated interference (RNAi; **Supplementary Fig. 3**). Second, to identify the peptidase, we exposed the internally quenched fluorescent 15-residue substrate ELFSYL(-dab)IEKVKRC(-FL)KN (PRA(190–204); underlining indicates the epitope, (-dab) indicates the quencher dabcyl and (-FL) indicates the fluorescein group) to digestion with cytosolic fractions obtained by anion-exchange chromatography of K562-A3 cell lysates. We identified peptidase activity in fraction 37 that cleaved, in a phenanthroline-sensitive way, between the quencher dabcyl at Lys195 and the fluorescein group at position 202, thereby releasing fluorescence (**Fig. 2b**). This fraction contained nardilysin as the only peptidase among five proteins identified by tandem mass spectrometry (**Fig. 2b** and **Supplementary Fig. 4**). Nardilysin, which has so far not been linked to HLA class I antigen processing, is a cytosolic endopeptidase of the pitrilysin family of zinc metalloproteases and is ubiquitiously expressed[22] (UniGene accession code (expressed sequence tag profile), Hs.584782).

Third, digestion of the 25-residue peptide PRA(190-214) (ELFSYLIEKVKRKKNVLRLCCKKLK) with purified nardilysin resulted in highly efficient production of variants of the epitope extended at the C terminus to 11, 12 or 13 residues by cleavage after Lys200, Arg201 or Lys202 (present for 33.9%, 41.2% and 16.9% of fragments, respectively, after 30 min of digestion; **Fig. 2c**), in accordance with the specificity of nardilysin in cleaving before or between two basic residues[23,24] in substrates of up to ~30 amino acids. Indeed, the anti-ELFSYLIEK CTL efficiently recognized the product of digestion with nardilysin when loaded onto HLA-A3$^+$ EKR cells because of the presence of the ELFSYLIEKVK, ELFSYLIEKVKR and ELFSYLIEKVKRK fragments, whereas the product of proteasomal digestion was not recognized (**Fig. 2c**). Fourth, nardilysin was needed to generate this epitope in cells, because small interfering RNA (siRNA)-mediated suppression of nardilysin in K562-A3 cells and HeLa-A3 cells (HeLa (PRAME$^+$) cervical cancer cells transfected with HLA-A3) resulted in much less recognition of these cells by the anti-ELFSYLIEK CTL (**Fig. 2d**). Overexpression of nardilysin in HeLa-A3 cells enhanced recognition by the anti-ELFSYLIEK CTL (**Supplementary Fig. 7**). Together these findings indicate that nardilysin is indispensable for the endogenous C-terminal preprocessing of the PRA(190–198) epitope by producing cleavage after Lys200, Arg201 and Lys202 but does not excise precisely at the C terminus (Lys198).

TOP liberates the C terminus of PRAME(190–198)

We searched for the peptidase that generates the C terminus of the epitope. Because of their cytosolic location, ubiquitous expression and specificity, the main candidates were TOP, neurolysin and insulin-degrading enzyme. Only the suppression of TOP, a ubiquitous endopeptidase expressed mainly in the cytosol and nucleus, by RNAi resulted in much less recognition of K562-A3 cells by anti-ELFSYLIEK CTL; suppression of the other peptidases in these cells had no effect (**Fig. 3a**), which indicated involvement of TOP in the endogenous generation of ELFYSLIEK. Purified TOP efficiently cleaved the nardilysin-dependent 12- and 13-residue precursors PRA(190–201) (ELFSYLIEKVKR) and PRA(190–202) (ELFSYLIEKVKRK) directly after the epitope's C-terminal Lys198. Thus, TOP released three or four C-terminal residues, in accordance with its cleavage preference[25,26], thereby efficiently producing the exact nine-residue epitope (**Table 1**). We digested the 12-residue peptide PRA(190–201) with TOP and loaded the digestion product onto HLA-A3$^+$ EKR cells and found this was recognized almost as efficiently as the minimal epitope was, whereas the undigested substrate was not

Table 1 C-terminal liberation of PRA(190–198) by cleavage with TOP

ELFSYLIE**K**VKRK	25.0%
ELFSYLIE**K**VKR	11.1%
ELFSYLIE**K**VK	0.8%

Production of fragments that match the ELFSYLIEK epitope (underlined) after 30 min of digestion of the 13-, 12- and 11-residue substrates PRA(190–202) (ELFSYLIEKVKRK), PRA(190–201) (ELFSYLIEKVKR) and PRA(190–200) (ELFSYLIEKVK) with purified TOP; results are presented as the percentage that match the epitope. Bolding indicates Lys198 (cleavage occurs after this residue). Data are representative of three experiments.

recognized at these concentrations (**Fig. 3b**). Consistent with TOP's preference for peptides 6–17 amino acids in length[27], longer peptides were not cleaved by TOP (**Supplementary Fig. 8** and data not shown), which indicated that preprocessing by nardilysin into the 12- and 13-residue peptides was needed to make the epitope precursors susceptible to TOP. We confirmed that prediction by digesting the 25-residue peptide PRA(190–214) with both nardilysin and TOP (together); this resulted in the production of ELFSYLIEK. Accordingly, because of the presence of the epitope, the double-digestion product was recognized more efficiently by the CTL clone than was the product of digestion with nardilysin alone, which contained only the precursors (**Supplementary Fig. 8**). Thus, the C terminus of the ELFSYLIEK epitope is released through sequential cleavage by nardilysin and TOP.

The N terminus of the ELFSYLIEK epitope was generated by efficient proteasomal cleavage between Asp189 and the N-terminal Glu190 (**Fig. 1a**) achieved by the caspase-like activity of the proteasome. This cleavage was completely blocked by a specific inhibitor of the caspase-like proteasome activity (AcAlaProNleLeuVSOH)[28], as shown by digestion of the 27-residue peptide PRA(172–198) (**Supplementary Fig. 9a**,**b**). As expected, after treatment of cells with this inhibitor, recognition of the cells by the anti-ELFSYLIEK CTL was much lower (**Supplementary Fig. 9c**); conversely, the general proteasome inhibitor epoxomicin only partially inhibited cleavage before the N terminus (**Supplementary Fig. 9a**,**b**). Notably, after treatment of cells with epoxomicin or other general proteasome inhibitors, the presentation of ELFSYLIEK was much greater (**Supplementary Fig. 9c**), probably because of less competition with epoxomicin-sensitive HLA-A3 ligands.

The findings reported above indicated that the proteasome, nardilysin and TOP together produced the minimal epitope in the cytosol (**Supplementary Fig. 10a**). As expected, presentation of the epitope was completely dependent on transport into the endoplasmic reticulum by the transporter protein TAP (**Supplementary Fig. 11a**). The ELFSYLIEK peptide was efficiently transported by TAP into the endoplasmic reticulum, whereas the 12- and 13-residue epitope precursors were translocated less efficiently (**Supplementary Fig. 11b**). Thus, precursors produced by nardilysin are available in the cytosol for processing by TOP, whereas the minimal epitope, after it is produced, can rapidly escape from cytosolic destruction by translocation into the endoplasmic reticulum. This evidence links nardilysin and TOP to the production of CTL epitopes. Do these endopeptidases, either apart or together, have a more general role in HLA 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 (**Supplementary Fig. 10b**). We analyzed all published ligands (1,620) of the most prevalent human HLA class I molecules in their flanking regions. Cumulatively, the results indicated that the dibasic cleavage motif of nardilysin[23,24] was 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 1,620 ligands, the motif was located within four amino acids of the N terminus or C terminus, respectively (**Table 2**). Thus, nardilysin can potentially liberate the precise N terminus or C terminus of many HLA class I ligands and may generate epitope precursors as well. The dibasic motif was most frequent directly at the N terminus of HLA-B27 ligands (35.2%) and at (basic) C termini of peptides presented by the HLA-A3 supertype (13%; **Table 2** and **Supplementary Table 1**).

We determined whether purified nardilysin actually excised prototypical ligands of HLA-A3 and HLA-B27 at the dibasic sites. The C termini of two overlapping immunodominant HLA-A3-restricted CTL epitopes from HIV type 1 (HIV-1) group-associated antigen (Gag p17) (ref. 29) and a naturally presented HLA-A11 ligand were

Table 2 HLA class I ligands potentially excised by nardilysin

HLA class I	Ligands (total)	C-dir (%)	C-ind (%)	N-dir (%)	N-ind (%)	Total motifs (%)
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 supertype)	(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 subtypes)	(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 (total)	(1,620)	4.8	5.7	10.4	5.2	26.1

Analysis of all ligands in the SYFPEITHI database for presence of the dibasic nardilysin motif, presented as the frequency (%) per HLA class I molecule. C-dir and N-dir, nardilysin motif at the C or N terminus, potentially leading to direct production of the C or N terminus, respectively; C-ind and N-ind, nardilysin motif within four amino acids flanking the C or N terminus, respectively, potentially leading to precursor formation. Far right, total frequency of ligands per allele potentially dependent on NRD cleavage for the generation of their N terminus or C terminus. 'Cumulative' indicates the frequency for HLA-A3, HLA-A11 and HLA-A68 (row 6) or HLA-B27 subtypes (row 18) or for all 1,620 ligands (bottom row).

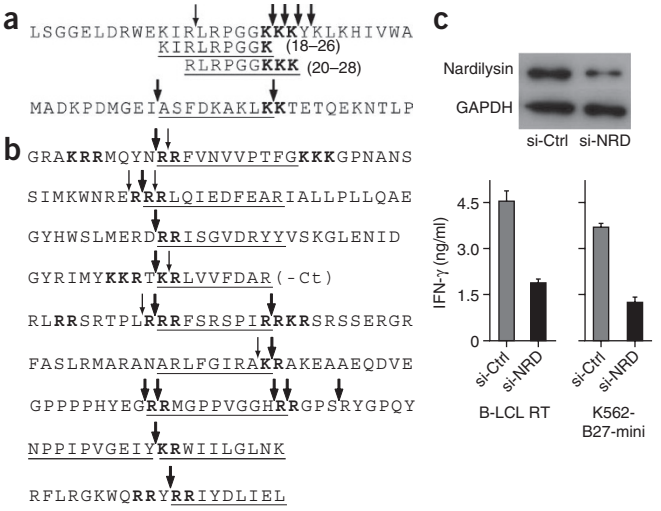

Figure 4 Role of nardilysin in HLA class I antigen processing. (**a**,**b**) Digestion sites of purified nardilysin in long peptides encompassing published ligands for HLA-A3, HLA-A11 and HLA-B27 (from the SYFPEITHI database). Bolding indicates dibasic motifs; arrows indicate cleavage present in >5% (bold arrows) or ≤5% (debold arrows) of the fragments after 30 min of digestion with nardilysin. (**a**) Top, HIV-1 gag p17 amino acids 8–37 containing two overlapping HLA-A3-presented epitopes (below; underlined, with amino acids in parentheses). Bottom, thymosin-β amino acids 1–30 containing the HLA-A11 ligand ASFDKAKLK (underlined). Proteasomes failed to produce the cleavages noted here (data not shown). (**b**) Peptides encompassing HLA-B2705 ligands (underlined). The top four ligands are reportedly insensitive to treatment with proteasome inhibitor[15] (details, **Supplementary Table 2**). (**c**) Recognition, by a CTL clone directed against EBNA3C(258–266), of RT cells (HLA-B2705$^+$EBNA3C$^+$) and of K562 cells (K562-B27-mini) that express HLA-B2705 and MFLRGKWQRRYRRIYDLIEL (EBNA3C(247–266); epitope underlined), transfected expressing control or nardilysin-specific or siRNA, assessed by ELISA of IFN-γ (exogenous peptide-loading control experiments, **Supplementary Fig. 5b**). Above, immunoblot analysis of nardilysin in RT cells transfected expressing control or nardilysin-specific siRNA. Data are from one experiment representative of four (s.e.m. of triplicate wells).

efficiently produced (**Fig. 4a**). Notably, 35.2% of all known HLA-B27 ligands contain a basic residue at position 1 (P1) next to their dominant HLA class I–binding arginine anchor at P2, which together constitute a dibasic motif (**Table 2** and **Supplementary Table 1**). Nardilysin efficiently liberated the N terminus of eight prototypical HLA-B27 ligands (of eight ligands assessed) and in three cases also produced the C terminus at a dibasic site (**Fig. 4b**). Among the peptides tested were immunodominant CTL epitopes from HIV-1 Gag p24 and Epstein-Barr virus protein EBNA3C[30] (**Supplementary Table 2**). We assessed in more detail the N-terminal generation of the HLA-B2705-presented CTL epitope EBNA3C(258–266) (RRIYDLIEL)[30]. This epitope was excised efficiently from its N-terminal flanking region by nardilysin in a digestion of the 20-residue substrate EBNA3C(247–266) (**Fig. 4b**, bottom row). We found that 35.8% and 62.5% of the fragments produced after 10 min and 30 min of digestion with nardilysin, respectively, matched the RRIYDLIEL epitope by efficient cleavage before Arg258. We next tested the nardilysin dependence of this immunodominant HLA-B2705-presented CTL epitope in live cells. Suppression of nardilysin in EBNA3C$^+$ and HLA-B2705$^+$ target cells resulted in much less recognition of the epitope by a specific CTL clone directed against EBNA3C(258–266) (ref. 30) (**Fig. 4c**). The nardilysin dependence resided in the N-terminal processing, as shown by recognition of cells expressing an epitope precursor with extension of the N terminus (**Fig. 4c**). Thus, although aminopeptidases exist in the cytosol and endoplasmic reticulum, nardilysin is required for excision of the EBNA epitope RRIYDLIEL.

TOP can do final C-terminal precursor trimming

It is believed that TOP destroys epitopes[6], thereby limiting HLA class I antigen presentation[5]; this raised the question of whether the excision of ELFSYLIEK by TOP is an exception. TOP preferentially releases, with broad sequence specificity[27,31], three to five C-terminal residues[25,26] from substrates extending to up to 17 amino acids[27], which endows 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 amino acids at the positions (P1 and P1′) surrounding the scissile bond after the C terminus of ELFSYLIEK (ELFSYLIE(P1-P1′)KRK). Substitutions at P1′ affected cleavage efficiency, but in all cases except proline at P1′, which prevented digestion, production of the epitope ELFSYLIEK was >5% after 10 min of

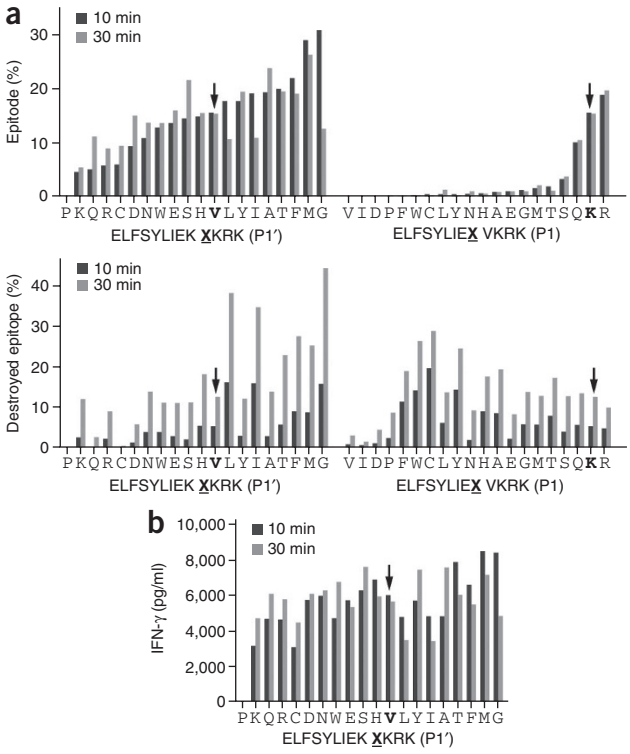

Figure 5 The epitope-generating trimming capacity of TOP. (**a**) Digestion by purified TOP of variants of the 13-residue peptide ELFSYLIEKVKRK (PRA(190–202)) with systematic substitution of P1 and P1′ residues surrounding the TOP cleavage site. Downward arrow (above) and bold V or K (horizontal axes) indicate the wild-type peptide (included twice). Above, epitope production after 10 min or 30 min of digestion, presented as percentage of total summed fragment intensities (ELFSYLIEK or ELFSYLIEX for P1′ or P1 substitution, respectively, where X is mutated); substitutions in the order of their efficiency of epitope production at 10 min. Below, epitope destruction through a subsequent cleavage in the middle of the epitope, presented as percentage of peptides in the digestion constituting fragments of the epitope after 10 min or 30 min of digestion. (**b**) Recognition, by the anti-ELFSYLIEK CTL clone, of TOP-digested P1′-substitution variants loaded onto EKR cells (HLA-A3$^+$), assessed by ELISA of IFN-γ. The P1′-proline variant, which was not digested, serves as a negative control. Data are representative of two (**a**) or three (**b**) experiments with similar results.

Table 3 CTL epitopes liberated by cleavage with TOP

	10 min	30 min	60 min
SLYSFPEPEAVKRK	5.7%	17.5%	24.6%
VLDGLDVLLVKRK	5.0%	6.3%	4.5%
SLYSFPEPEARRFV	6.6%	16.4%	25.8%
VLDGLDVLLRRFV	14.0%	19.2%	17.6%
ELFSYLIEKRRFV	2.7%	10.5%	21.5%

Liberation of the CTL epitopes (underlined) PRAME(142–151) (SLYSFPEPEA), PRAME(100–108) (VLDGLDVLL) and PRA(190–198) (ELFSYLIEK) from their non-natural C-terminal flanking sequences VKRK or RRFV after digestion for 10, 30 or 60 min with TOP, presented as the percentage of fragments matching the epitope. Data are representative of two experiments with similar results.

digestion (**Fig. 5a**). Partial destruction by subsequent cleavage in the epitope occurred but did not prevent epitope production (**Fig. 5a**), as also shown by efficient recognition of the digestion products by the anti-ELFSYLIEK CTL (**Fig. 5b**). Digestion of the variants with substitution at P1 resulted in generation of the epitope ELFSYLIEX when the amino acid at P1 was lysine (wild-type sequence) or was arginine or glutamic acid (**Fig. 5a**), but generation was inefficient or was absent when other amino acids were at this position. This was caused by inefficient cleavage after ELFSYLIEX for some amino acids at P1 (such as valine, isoleucine and aspartic acid) and/or by a rapid second cleavage in the middle of the epitope (such as phenylalanine, tryptophan, cysteine and tyrosine). The necessity for a lysine, arginine or glutamic acid at position P1 was not absolute, however. When we changed the epitope completely, TOP produced epitopes with a C-terminal alanine or leucine (SLYSFPEPEA and VLDGLDVLL)[20] (**Table 3**). These results indicate that TOP, without distinct sequence specificity, can produce CTL epitopes from a wide array of precursors.

We searched for other TOP-dependent CTL epitopes by suppressing TOP in target cells via RNAi. We assessed the clinically important melanoma-specific and HLA-A2-restricted MART-1 epitope[32], of which 9-residue and 10-residue variants (AAGIGILTV and EAAGIGILTV; amino acids 27–35 and 26–35, respectively) are considered to be naturally presented[33], for recognition by CTLs and found that recognition of melanoma cells in which TOP was suppressed was much lower (**Fig. 6a**), which indicates the dependence of presentation of the MART-1 epitope on TOP. We investigated the underlying mechanism by *in vitro* digestion of epitope precursors with extension of the C terminus. We did not find liberation of the epitope's C-terminal Val35, but purified TOP efficiently cleaved in these substrates after Leu33, Thr34 and Leu37; fragments containing those as the C terminus were each present as ≥6% of the total after 10 min of digestion (**Fig. 6b**). Therefore, the requirement for TOP may be explained if the variant epitopes EAAGIGILT (MART-1(26–34)) and/or AAGIGILTVIL (MART-1(27–37)) are among those naturally processed and recognized by CTLs. A CTL clone specific for MART-1(26–35) efficiently recognized those variant peptides when they were loaded exogenously (**Fig. 6c**), although with lower sensitivity than its recognition of the peptide EAAGIGILTV (MART-1(26–35); **Fig. 6d**).

Processing effects specific to HLA class I alleles

In addition to producing HLA class I ligands, nardilysin and TOP will sometimes also destroy other (potential) HLA class I ligands. Consequently, depending on the balance between production and destruction, the absolute quantity of ligands available for loading onto HLA class I may be affected. Therefore, we measured cell surface expression of HLA class I under conditions of overexpression and suppression of these peptidases by RNAi. Consistent with published literature[5], overexpression of TOP resulted in lower expression of HLA class I by HeLa cells (**Supplementary Fig. 12a**). Likewise, overexpression of nardilysin led to modestly attenuated expression of HLA class I molecules overall by HeLa cells, and, specifically, of HLA-A3 and HLA-B27 by various cell types (**Supplementary Fig. 12a**,**b**). These findings indicate that overexpression of both TOP and nardilysin, not unexpectedly, tilt the balance between the generation and destruction of HLA class I ligands by these peptidases. The contribution of each peptidase to the overall quantity of HLA class I ligands produced under physiological conditions is more likely to be identified by analysis of the abundance of HLA class I at the cell surface when peptidase expression is suppressed. We specifically focused on HLA-A2, HLA-A3

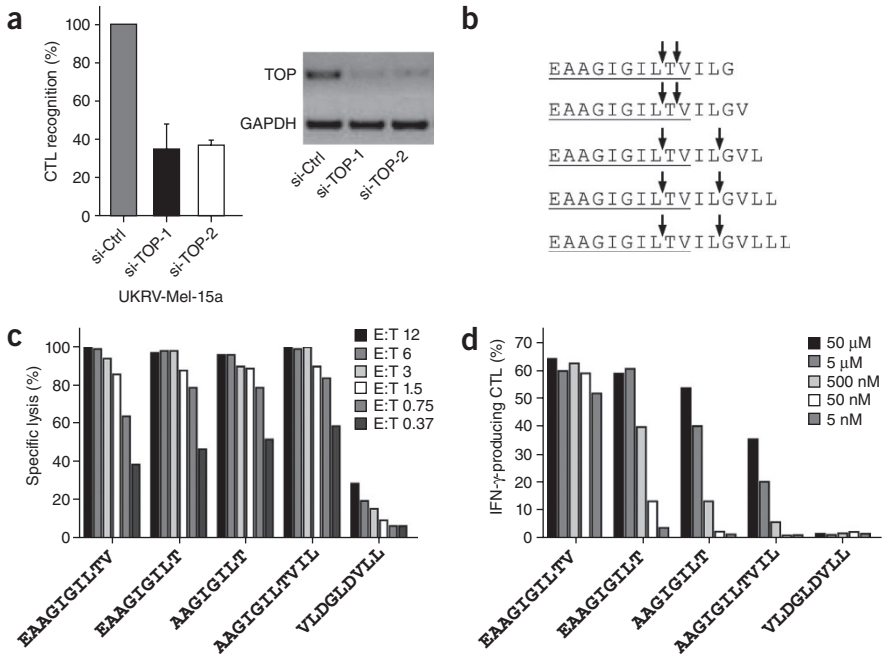

Figure 6 TOP-dependent presentation by HLA-A2 of the MART-1 CTL epitope. (**a**) Recognition, by a CTL clone directed against MART-1(26–35) (EAAGIGILTV), of UKRV-Mel-15a melanoma cells (HLA-A2$^+$MART-1$^+$) transfected with TOP-specific siRNA (si-TOP-1 or si-TOP-2), assessed by enzyme-linked immunospot assay of IFN-γ and presented relative to results obtained with cells transfected with control siRNA, set as 100% (left; peptide-loading control experiments, **Supplementary Fig. 5c**). Right, RT-PCR analysis of TOP and GAPDH in the UKRV-Mel-15a cells. (**b**) Digestion sites of purified TOP (arrows) in the 13-, 14-, 15-, 16- and 17-residue substrates (top to bottom) MART-1 (26–38), MART-1(26–39), MART-1(26–40), MART-1(26–41) and MART-1(26–42); the EAAGIGILTV epitope is underlined. (**c**) Specific lysis of JY cells (HLA-A2$^+$MART-1$^-$) loaded with various MART-1 peptides (10 μM; horizontal axis: amino acids (left to right) 26–35 (EAAGIGILTV), 26–34, 27–34 or 27–37) or the HLA-A2-restricted PRA(100–108) epitope[20] (VLDGLDVLL; negative control) by the CTL clone in **a**, after coculture at various effector/target ratios (E:T; key). (**d**) Recognition by the CTL clone in **a** of JY cells loaded with various concentrations (key) of the peptides in **c**, assessed as intracellular IFN-γ. Data are representative of two experiments (**a**; mean and s.e.m.) or are from one experiment representative of three (**c**,**d**).

and HLA-B27 (naturally expressed by the B-lymphoblastoid cell lines HAR, RT and EKR), because these were the restriction elements of the epitopes identified in our experiments reported above and also because their ligands are presumed to be either dependent on the proteasome (HLA-A2) or relatively independent of the proteasome (HLA-A3 and HLA-B27)[11,14,15]. We observed effects specific for HLA class I alleles when nardilysin expression was suppressed. Cell surface expression of HLA-A2 and HLA-A3 was slightly enhanced (4–9%), which suggests a net destructive effect of nardilysin in the production of peptides presented by these HLA class I molecules, whereas expression of HLA-B27 and HLA-B35 was slightly lower (5–11%). The latter result suggests a net productive role for nardilysin in the generation of HLA-B27 and HLA-B35 ligands (**Supplementary Fig. 12c**). When tested in the same B-lymphoblastoid cell lines, suppression of TOP led to a similar pattern of changes in the expression of HLA class I, consistently showing slightly lower expression of HLA-B27 and HLA-B35 and slightly enhanced expression of HLA-A2 and HLA-A3, whereas the expression of HLA-A24 and HLA-B7 was unaltered (**Supplementary Fig. 12d**). Both the HAR and RT cells had the pattern of lower expression of HLA-B27 and enhanced expression of HLA-A2, which excluded the possibility of general nonspecific effects induced by RNAi. These alterations in the expression of HLA class I were not changed after incubation of the cells with brefeldin A to assess the possibility of differences in the off-rate of the peptides presented by cells in which these peptidases were suppressed versus control cells (data not shown), which indicated that nardilysin- or TOP-mediated processing does not alter the overall allele-specific HLA class I–peptide stability.

DISCUSSION

Here we have shown that the cytosolic endopeptidases nardilysin and TOP, either alone or together, were indispensable for the generation of three defined CTL epitopes. Nardilysin seemed to be involved in the generation of many HLA class I ligands either directly at or within four residues of their N or C terminus. In our digestions, nardilysin efficiently produced basic C termini (for binding to HLA-A3, HLA-A11 and HLA-B27), but it can also produce non-basic C termini for binding to other HLA class I molecules (**Supplementary Fig. 10b**). The efficient cleavage of nardilysin at dibasic motifs suggests its application in the rational design of polyepitope 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 been demonstrated before[34].

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 ability in removing three to five C-terminal residues from the substrate[25–27,31]. Purified TOP, however, did not liberate the anticipated C-terminal Val35 residue of the HLA-A2-restricted MART-1 epitope, of which two N-terminal variants (AAGIGILTV (amino acids 27–35) and EAAGIGILTV (amino acids 26–35)) are considered to be naturally presented[33]. Instead, TOP cleaved after Leu33, Thr34 and Leu37. The peptide AEEAAGIGILT (MART-1(24–34)) is naturally presented by HLA-B45 (ref. 35), which indicates endogenous liberation of Thr34 as a C terminus and suggests additional presentation of EAAGIGILT (MART-1(26–34)) by HLA-A2 as the explanation for the TOP-dependent recognition by CTLs. Further experiments are needed to identify the natural presentation by HLA-A2 of a TOP-dependent variant peptide of MART-1.

A CTL epitope from the *Mycobacterium tuberculosis* protein Hsp65 with an undefined amino acid sequence has been shown to rely on TOP[36]. A possible role for TOP in the protection of some cytosolic peptides from further destruction has been documented[37]; however, this has not been confirmed[38]. In contrast, results of biochemical studies and analysis of HLA class I expression[5,6,38,39] have led to the idea that TOP has only a destructive role in HLA class I antigen processing[1,2]. However, destruction of defined epitopes by TOP, as studied by CTL recognition of target cells with suppressed expression of TOP, has thus far been shown only for the mouse H2-K^b-presented SIINFEKL epitope from ovalbumin[5]. The role of TOP in the production of the PRA(190–198) and MART-1 epitopes is in accordance with a study demonstrating that TOP in the cytosol both destroys and generates peptides of the length of HLA class I ligands[40]. Thus, the role of TOP in antigen processing seems to be twofold. TOP has a destructive role[5,38], limiting presentation of epitopes whose C terminus has already been made by, for example, the proteasome, such as SIINFEKL[5,8]. However, TOP also generates C termini fit for binding by HLA class I via trimming of epitope precursors with extension of the C terminus. Thus, TOP-mediated processing both dampens the presentation of proteasomal products by HLA class I and enlarges the HLA class I peptide repertoire. This model fits both the literature and our results.

The proportion of HLA class I ligands that depend on nardilysin or TOP for their generation is not yet known. Our results have demonstrated HLA class I allele–specific influences of processing by nardilysin and TOP. Suppression of both peptidases slightly enhanced expression of HLA-A2 and HLA-A3, which indicated a destructive effect on ligand generation, as reported for TOP in the literature[5,39]. Liberation of the aliphatic C terminus of HLA-A2 ligands is largely proteasome dependent. By trimming at the C terminus, TOP may destroy a substantial quantity of these HLA-A2 ligands. As for nardilysin, its dibasic cleavage motif was present at the C terminus (the C terminus itself and the residue at P1′) in 13% (31 of 238) of peptides presented by the HLA-A3 supertype. In our digestion studies, nardilysin produced two of these epitopes, but it may also destroy several of these peptides by cleaving in front of the dibasic motif, thereby diminishing the availability of HLA-A3 ligands. Notably, defined peptides that are presented by HLA class I molecules whose expression is quantitatively unaltered or enhanced after peptidase suppression may still depend on nardilysin or TOP. This is demonstrated by the finding that the PRA(190–198) and MART-1 epitopes, presented by HLA-A3 and HLA-A2, respectively, depended on nardilysin and/or TOP, which emphasizes the need for additional study of the processing of defined CTL epitopes.

However, suppression of nardilysin and TOP resulted in slightly lower expression of HLA-B27 and HLA-B35, which suggested a productive effect on ligand generation. Peptides presented by HLA-B27 (refs. 14,15) and HLA-B35 (ref. 11) have been found to be relatively insensitive to proteasome inhibition, consistent with the possibility of a role for cytosolic endopeptidases in their C-terminal generation. TOP can assist ligand formation for these HLA class I molecules by trimming precursors extended at the C terminus. As shown for the EBNA3 epitope and in the digestion studies, 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 the C terminus of these ligands at a dibasic site. Thus nardilysin-mediated processing probably contributes to the substantial over-representation of arginine and lysine at P1 in HLA-B27 ligands, a position that contributes only modestly 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[41]. Four of the eight HLA-B27 ligands that were excised by nardilysin at their N terminus have been found to be proteasome independent when studied with proteasome

inhibitors[15] which supports the idea of a role for an additional peptidase in their generation. Most of the proteasome-inhibitor-insensitive HLA-B27 ligands in the previous study[15] were derived from small basic proteins, which also suggests that nardilysin is involved in their processing. However, all studies showing proteasome-independent peptide presentation by HLA class I through the use of proteasome inhibitors as the main experimental tool[11,14,15] should be evaluated with consideration of the residual proteasome activity still present in inhibitor-treated cells, which leaves unanswered the question of whether completely proteasome-independent CTL epitopes are ever produced in the cytosol. This is exemplified by the ELFSYLIEK epitope, which, although it was dependent on the proteasome for its N-terminal generation, nevertheless showed enhanced presentation after proteasome inhibition.

Because of their substrate length constraints of ~30 amino acids and ~17 amino acids, respectively, nardilysin and TOP probably 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, which requires nardilysin cleavage before TOP can liberate the C terminus, again shows that both substrate length and amino acid sequence determine which peptidase acts where and at what time in cytosolic HLA class I antigen processing. With the addition of nardilysin and TOP to the list, four peptidases have now been identified in the C-terminal generation of CTL epitopes. TPPII has been shown to generate the HLA-A3-restricted Nef(73–82) epitope (amino acids 73–82 of the HIV protein negative factor)[16]. Beyond its established tripeptidyl aminopeptidase activity involved in the N-terminal generation of epitopes, a broad role for the endoproteolytic activity of TPPII in the C-terminal generation of epitopes has not yet been detected[18]. The HLA-A1-presented human tumor epitope from MAGE-A3 is reported to be produced by insulin-degrading enzyme[17], but a general role for this peptidase in antigen processing remains to be established. Together, our results here and those published findings indicate that the idea that only the proteasome is responsible for the C-terminal generation of epitopes needs reappraisal. In conclusion, by producing HLA class I–binding peptides from protein-degradation fragments that are not yet fit for antigenic presentation, especially those that lack a HLA 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 to intracellular pathogens and cancer.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/natureimmunology/.

Note: Supplementary information is available on the Nature Immunology website.

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AUTHOR CONTRIBUTIONS

J.H.K. conceived of the study, coordinated the work, designed, did and analyzed most experiments and wrote the manuscript with major input from C.J.M.M. and minor input from other authors; C.J.M.M., F.O. and P.A.v.V. provided intellectual input; S.K. did the experiments and analyses in **Figure 2b** and immunoblot analysis; K.M.C., L.B.H. and A. Prat contributed to the experiments about nardilysin; D.W.R., K.R., U.S. and A. Paschen contributed to the experiments about TOP; P.M.K., U.S. and B.T. contributed to the experiments about TPPII; H.S.O. and P.F.v.S. contributed to the experiments about the proteasome; J.N. and T.v.H. contributed to the experiments about TAP; N.v.M., U.S., A. Paschen, S.L.G. and J.M.B. contributed to CTL experiments; J.W.D., F.O., J.N., S.K. and W.E.B. contributed to substrate design and synthesis; S.A.B.-V. and K.L.M.C.F. contributed to the molecular biology; A.M. and I.I.N.D. made HLA class I mAbs and cell lines expressing a single HLA class I allele; and P.A.v.V. and A.d.R. did mass spectrometry.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cell lines and HLA class I staining. The cell lines used were K562 erythroleukemia cells (HLA class I–negative, PRAME$^+$) transduced to express HLA-A3 (HLA-A0301), HLA-A2 (HLA-0201) or HLA-B27 (HLA-B2705); HeLa cells (PRAME$^+$) transfected to express HLA-A3; MZ1257 and Le-94.15 renal cell carcinoma cell lines (PRAME$^+$HLA-A3$^+$); the B-lymphoblastoid cell lines EKR, RT and HAR; and the UKRV-Mel-15a melanoma cell line. HeLa, K562-A3 and RT cells overexpressing rat or human nardilysin were generated by transfection with the plasmid pIRES2-NRD1-eGFP or pCDNA3.1-NRD, respectively (rat and human nardilysin have similar cleavage specificity[42]). HeLa cells overexpressing human TOP were generated with the plasmid pcDNA3.1-TOP. K562-A3 cells expressing the TAP inhibitor ICP47 were generated with the plasmid pLZRS-IRES-eGFP-ICP47. K562-B27-mini cells stably expressed EBNA3C amino acids 248–266 with an extra N-terminal methionine residue. Expression of HLA-A2 and HLA-A3 was measured by flow cytometry with mouse monoclonal antibodies BB7.2 and GAP-A3, respectively. Expression of HLA-A24, HLA-B7, HLA-B27 and HLA-B35 was measured with the human monoclonal antibodies IND3H3, VTM1F11, WAR5D5 and HDG8D9, respectively[43]. Monoclonal antibody W6.32 was used to measure overall expression of HLA class I and expression of HLA-A2, HLA-A3 and HLA-B27 by K562 cells.

Synthetic peptides. Peptides were synthesized as described[20]. Fluorescein was covalently coupled to the cysteine residue with 5-(iodoacetamido)fluorescein (Fluka Chemie). The quencher residue Fmoc-L-Lys(Dabcyl)-OH was from Neosystems. A 2-naphylsulfonyl group was used for N-terminal blockade. Peptides were purified by high-performance liquid chromatography and their identities were confirmed by mass spectrometry.

HLA-A3 peptide-binding assay. The affinity of peptides for HLA-A3 was measured as described[44]. Diluted to indicated concentrations (see figures) of test peptides competed for binding to cell surface HLA-A3 with the known high-affinity fluorescein-labeled reference peptide KVFPC(FL)ALINK[44] and mean fluorescence was measured by flow cytometry. Inhibition of the binding of fluorescein-labeled reference peptide at each concentration of test peptide used was calculated with the formula: $(1 - (MF_{ref\ and\ comp} - MF_{no\ ref}) / (MF_{ref} - MF_{no\ ref})) \times 100\%$, where 'MF' is mean fluorescence, 'ref' is the reference peptide and 'comp' is the competitor peptide.

CTL clones and assays and functional peptidase-inhibition assays. CTL clone 123, raised against the PRA(190–198) epitope (ELFSYLIEK) exogenously loaded on HLA-A3, and CTL clone 313, raised against the MART-1(26–35) epitope (EAAGIGILTV), were generated by *in vitro* stimulation against synthetic peptides loaded exogenously onto antigen-presenting cells according to published procedures[20]. HLA class I–typed peripheral blood mononuclear cells obtained from an anonymous blood bank donor or a patient with melanoma (after informed consent was provided) were used for clone 123 and clone 313, respectively. CTL clone RT.c38 directed against EBNA-3C(258–266) (RRIYDLIEL) was generated before[30]. CTL recognition was determined by measurement of specific lysis in a ^{51}Cr-release cytotoxicity assay or by measurement of IFN-γ production by ELISA, enzyme-linked immunospot assay or intracellular cytokine staining. In functional peptidase-inhibition experiments, K562-A3 cells were pretreated for 1.5 h at 37 °C with the inhibitor, then were stripped from HLA class I–presented peptides (peptide stripping) by treatment for 2 min with a mild acid (300 mM glycine and 1% (vol/vol) BSA, pH 2.9) and washed (brought to a pH of 7) and then were treated for another 5 h at 37 °C with the inhibitor. Background and maximal epitope expression was obtained by treatment of cells with brefeldin A (10 μg/ml) and no treatment after stripping, respectively. After inhibitor treatment, cells were washed, plated in triplicate in 96-well plates (3×10^4 cells per well) and coincubated for 6 h with the CTL clone (3×10^4 cells per well). After 30 min, brefeldin A was added (5 μg/ml). The cocultures were then subjected to standard procedures for intracellular cytokine staining. CTLs were stained with allophycocyanin-conjugated monoclonal anti-CD8 (DK25; Dako) and phycoerythrin-conjugated monoclonal anti-IFN-γ (4S.B3; BD Pharmingen), followed by flow cytometry. The inhibitors and concentrations used were as follows: brefeldin A, 10 μg/ml (Sigma); phenanthroline (inhibitor of metallopeptidases), 400 μM (Sigma); insulin (competitive inhibitor of insulin-degrading enzyme), 50 μM (Sigma); captopril (inhibitor of angiotensin-converting enzyme), 50 μM (Sigma); leupeptin (inhibitor of trypsin-like and cysteine proteases), 100 μM (Calbiochem); calpeptin (inhibitor of calpains), 30 μM (Calbiochem); aprotinin (serine protease inhibitor), 5 μM (Calbiochem); and butabindide (inhibitor of TPPII), 200 μM (Tocris). PSI (Calbiochem), epoxomicin (Calbiochem), AdaAhx$_3$L$_3$VS[45] and AcAlaProNleLeuVSOH[28] were used for proteasome inhibition.

Peptide digestion and recognition assays. Proteasomes (20S) were purified as described[20] from HeLa cells, which contain mainly constitutive proteasomes, and from the B-lymphoblastoid cell line JY, which contains mainly immunoproteasomes. Composition was confirmed by immunoblot analysis (data not shown). TPPII was purified from erythrocyte concentrates as described[46] with minor modifications[47]. Recombinant mouse nardilysin was prepared as described[24] and was homogeneous, as judged by SDS-PAGE[24]. Long peptides were digested with mouse nardilysin and with cytosolic fraction 37 of K562 cells containing human nardilysin, with similar results. Mouse and human nardilysin have similar cleavage specificities[42]. TOP was produced by overexpression in *Escherichia coli* and was purified by nickel-resin affinity chromatography (Qiagen). Samples were digested mostly at 37 °C with 10 μM peptide and 4.3 nM enzyme; digestion with TPPII used 1 μg TPPII (molecular weight, $>10^6$) and 2 nmol peptide in a volume of 100 μl, and mock digestion was done at 37 °C without enzyme. Digestion buffers were as follows: 30 mM Tris, pH 7.5, 10 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol (proteasome and nardilysin); 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$ and 0.1 mM dithiothreitol (TOP); or 20 mM HEPES, pH 7.48, 2 mM $Mg(Ac)_2$ and 2 mM DTT dithiothreitol containing 5% (vol/vol) glycerol (TPPII). Enzymatic reactions were stopped by the addition of 5% (vol/vol) acetic acid or, for CTL assays, phenanthroline (1 mM).

For recognition by CTLs, digestion samples were diluted to indicated concentrations (see figures) and 10 μl of the digestion product was loaded onto 90 μl of EKR cells (HLA-A3$^+$PRAME$^-$) in triplicate wells of a 96-well plate (1.7×10^4 cells per well), followed by incubation for 2 h at 4 °C. Synthetic reference peptides were diluted to indicated concentrations (see figures) and loaded similarly. For improvement of loading efficiency, EKR cells were first stripped of naturally presented peptides (described above) and then were resuspended in 100 μl Iscove's modified Dulbecco's medium containing human β_2-microglublin (2 μg/ml; Sigma). After loading of target cells, 2.5×10^4 CTLs (in 50 μl) were added, followed by coincubation for 12 h and ELISA of IFN-γ.

Identification of PRA(190–198) on the surface of K562-A3 cells. K562-A3 cells (3×10^{10}) were lysed in lysis buffer (20 mM Tris, pH 8, and 5 mM $MgCl_2$ containing 0.5% (vol/vol) Zwittergent-12 (Calbiochem)). Membrane fragments were removed by ultracentrifugation and supernatants were precleared with Sepharose CL-4B (Amersham Biosciences). Monoclonal antibody W6.32 coupled to protein A beads was used for immunoaffinity purification of HLA-A3 molecules. Peptides were eluted with 10% (vol/vol) acetic acid, filtered over a filter with a cutoff of 10 kilodaltons (Amicon) and separated into 60 fractions (100 μl) by reverse-phase high-performance liquid chromatography with a SC 2.1/10 column packed with C2/C18 beads (Smart System; Amersham). Buffer A was 0.1% (vol/vol) heptafluorobutyric acid in water; buffer B was 0.1% (vol/vol) heptafluorobutyric acid in acetonitrile. Fractions were lyophilized, dissolved in water (100 μl) and used for analysis of CTL reactivity and mass spectrometry. For CTL assays, 10 μl of each fraction was loaded onto EKR cells (2×10^4 cells per well in 96-well plate), followed by coincubation with CTLs (2.5×10^3 cells per well). EKR cells were first stripped of peptides presented at the cell surface, then were resuspended in Iscove's modified Dulbecco's medium containing human β_2-microglublin (2 μg/ml; Sigma).

Isolation of nardilysin. K562-A3 cells (5×10^9) in lysis buffer (10 mM Tris-HCl, 1 mM dithiothreitol, 1 mM NaN_3, 25 mM NaCl, 10 mM $MgCl_2$, 0.1 mM EDTA, 10% (vol/vol) glycerol, 2 mM ATP, 50 mM NaF and 0.1 mM Na_8VO_4, pH 7.5) were homogenized with a Dounce glass homogenizer. Homogenates were centrifuged for 20 min at 10,000*g* for removal of nuclei and cell membrane debris. Supernatants were cleared from proteasomes and TPPII by centrifugation for 6 h at 100,000*g*. Clarified lysates were fractionated over a

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MonoQ anion-exchange column (HR 5/5; Amersham) with a linear gradient of 0–500 mM KCl in lysis buffer (20 ml). Specific peptidase activity in the fractions was assessed with the fluorogenic substrate ELFSYL(-dab)IEKVKRC(-FL)KN (0.5 μM) in substrate buffer (30 mM Tris, 10 mM KCl, 5 mM $MgCl_2$ and 1 mM dithiothreitol, pH 7.5). Fluorescence was measured at 360 nm (extinction) and 460 nm (emission). Fractions 36–40, which contained the peak proteolytic activity, were separated by 12% SDS-PAGE and protein bands were isolated, digested with trypsin and analyzed by mass spectrometry.

Suppression of peptidase expression by RNAi. Suppression of peptidase expression by RNAi was established by either stable siRNA expression with the pSUPER-puro vector or by transient transfection of pools of four interfering RNA duplexes (siGENOME SMARTpool; Dharmacon). For transient transfection, 100 nM siRNA was transfected with HiPerFect (Qiagen) or DharmaFECT-1 (Dharmacon) 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′. TOP was suppressed with siRNA targeting the sequence 5′-CCTCAACGAGGACACCACC-3′ (stable expression)[39] or an siRNA pool targeting the sequences 5′-TAGATGAGCTGGCGCAGAATT-3′, 5′-TCAAACGCATCAAGAAGAATT-3′, 5′-GCAAGGTTGGCATGGATTATT-3′ and 5′-AGACCAAGCGCGTGTATGATT-3′ (transient expression). TOP was transiently suppressed in UKRV-Mel-15a cells with the siRNA target duplex 5′-CAGCAAGGTTGGCATGGA-3′ (siTOP-1) or 5′-GCCTTCTGTGCATCGACTT-3′ (siTOP-2; Eurogentec). TPPII was suppressed with siRNA targeting the sequence 5′-GCAGTATTCACATCGCCAC-3′ (ref. 16). Neurolysin was suppressed with an siRNA pool targeting the sequences 5′-GGATAAAGCTACAGGAGAA-3′, 5′-GATAATGAATCCAGAGGTT-3′, 5′-CAATTGAGGTGGTCACTGA-3′ and 5′-GAACTCAAGTATTCCATAG-3′. Insulin-degrading enzyme was suppressed with an siRNA pool targeting the sequences 5′-TCAAAGGGCTGGGTTAATA-3′, 5′-ACACTGAGGTTGCATATTT-3′, 5′-GAACAAAGAAATACCCTAA-3′ and 5′-GTGGAGAGCATACCAATTA-3′. As nonsilencing control, the siCONTROL Non-Targeting siRNA pool (Dharmacon) was used. The nonsilencing control for siRNA expressed by pSUPER-puro was a nontargeting scrambled sequence. Suppression or overexpression of peptidases was assessed by quantitative RT-PCR and immunoblot analysis with monoclonal antibody to TOP (4D6; Santa Cruz) and either noncommercial rabbit antiserum to nardilysin or monoclonal antibody to nardilysin (A-6; Santa Cruz).

Peptide translocation by TAP. The efficiency of peptide translocation by TAP was determined with microsomes prepared from a B-lymphoblastoid cell line cell line as described[48] by measurement of competition for translocation with the fluorescein-labeled reference peptide C(-FL)VNKTERAY[49], which contains a consensus site for N-linked glycosylation in the endoplasmic reticulum. Microsomes (1.3×10^6 cell equivalents) were incubated for 10 min at 37 °C with diluted to indicated concentrations (see figures) of test peptide, 0.5 μM fluorescein-labeled reference peptide and 10 mM ATP in 100 μl transport buffer (5 mM HEPES, pH 7.3, 130 mM KCl, 10 mM NaCl, 1 mM $CaCl_2$, 2 mM EGTA and 2 mM $MgCl_2$). Thereafter, 1 ml ice-cold transport buffer with 10 mM EDTA was added and samples were washed, then pellets were resuspended in lysis buffer (50 mM Tris, pH 7.5, 500 mM NaCl, 5 mM $MgCl_2$ and 1% (vol/vol) Triton X-100) and rotated for 30 min at 4 °C. Debris were removed by centrifugation (15,000*g*) for 10 min at 4 °C and the glycosylated fluorescein-labeled reference peptide was recovered by incubation for 2 h at 4 °C with 100 μl packed concanavalin A–Sepharose 4B (GE Healthcare). This Sepharose was washed three times, and glycosylated fluorescein-labeled reference peptide was released by incubation for 1 h at 20 °C in 200 μl elution buffer (50 mM Tris-HCl, pH 8.0, 500 mM mannopyranoside and 10 mM EDTA). Fluorescence was measured at an excitation wavelength of 485 nm and emission wavelength of 535 nm. Maximum fluorescence was from a sample containing no competitor peptide, and minimum fluorescence was from a sample containing 0.5 M EDTA without ATP. The GRIDKPILK positive control competitor peptide is reported to be efficiently translocated[50].

Mass spectrometry. A Q-TOF1 mass spectrometer (Waters) equipped with an online nanoelectrospray interface with an approximate flow rate of 250 nl/min was used for electrospray ionization–mass spectrometry. Peptide-digestion samples were trapped on a precolumn (MCA-300-05-C18; Dionex) and were eluted with a steep gradient of 70–90% buffer B over 10 min (buffer A, water, acetonitrile and formic acid, 95:3:1 (vol/vol/vol); buffer B, water, acetonitrile and formic acid, 10:90:1 (vol/vol/vol)). Mass spectra were recorded from a mass of 50–2000 daltons. In tandem mass spectrometry mode, ions were selected with a window of 3 daltons. The collision gas was argon (4×10^{-5} mbar), and the collision voltage was ~30 V. For peptide digestion by purified proteasome, nardilysin, TOP and TPPII, peaks in the mass spectra were searched in source substrate peptides with BioLynx 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(190–198) epitope, peptides in the fractions eluted from K562-A3 cells by high-performance liquid chromatography were sequenced by tandem mass spectrometry essentially as described above, but were measured on an HCTplus ion trap (Bruker Daltonics) run in the data-dependent tandem mass spectrometry mode during peptide elution. Proteins in bands of cytosolic fractions of K562-A3 cells were digested with trypsin, and tandem mass spectrometry spectra were matched with those of the SwissProt database.

Statistical analysis. A two-tailed paired *t*-test was used to determine the significance of differences in HLA class I expression; *P* values of less than 0.05 were considered significant.

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