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Polyubiquitination of lysine-48 is an essential but indirect signal for MHC class I antigen processing

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Peptides presented on major histocompatibility complex (MHC) class I molecules are generated via cytosolic proteolysis. However, the nature of the endogenous peptide precursors and the intracellular processing steps preceding protein degradation remain poorly defined. Here, we assessed whether ubiquitination is an essential signal for proteasomal cleavage of antigen substrates in human cells. Conversion into antigenic peptides occurred in the absence of any detectable N-terminal ubiquitination of the model antigens, and did not require the presence of any of the four types, nor a minimum number of ubiquitinatable amino acids within the antigen substrate. However, the knockdown of ubiquitin, expression of a lysine 48 (K48) ubiquitin mutant, or inhibition of proteasome-associated deubiquitinases significantly impaired antigen presentation. The results presented here are consistent with a model in which the binding of the antigen substrate by an adaptor protein leads to its K48-polyubiquitination and the subsequent delivery of the antigen cargo for degradation by the 26S proteasome. Altogether, these findings show an important but indirect role of K48-polyubiquitination in preproteasomal antigen sampling.

Keywords: Antigen presentation · Antigen processing · MHC class I · Proteasome · Ubiquitin

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Introduction

The continuous display of peptides derived from endogenous proteins on major histocompatibility complex class I molecules (MHC I) facilitates the detection of invading pathogens and cellular transformation by $CD8^+$ T cells. While it is well established that the end products of proteolysis by the 26S proteasome form the principal source of peptides loaded on MHC class I, the intracellular processing steps that precede protein degradation into peptides are still poorly defined [1–4]. The absence of a clear relationship between the abundance of MHC I-bound peptides and the abundance or half-live of the proteins from which they derive, implies that access to the antigen presentation pathway is restricted [3, 5]. How this process of antigen selection is regulated and which molecules are involved, is still unknown.

Proteins destined for degradation by the 26S proteasome are usually marked with ubiquitin, which requires the coordinated activities of ubiquitin-activating (E1), -conjugating (E2), and -ligating (E3) enzymes. Although primarily conjugated via isopeptide bonds to lysine (K) residues, ubiquitin can also be attached to the amino terminus of the substrate [6], or to cysteine (C), serine (S), and threonine (T) residues of the target protein [7–10].

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In addition to proteasome-dependent proteolysis, ubiquitin controls a variety of biological processes, including modulation of protein function, structure, assembly, and localization [11]. Specificity of the ubiquitin signaling is achieved by alternative conjugation signals and the interactions with ubiquitin-binding proteins that decode ubiquitinated target signals into biochemical cascades. Besides ubiquitin monomers, target proteins may carry polyubiquitin chains that are formed by conjugating additional ubiquitin moieties to the N-terminus or to any of the seven lysine residues of an ubiquitin molecule. Polyubiquitination via lysine at position 48 (K48) is the canonical signal for the degradation of target proteins by the 26S proteasome [11, 12].

By recognizing target proteins, substrate specificity is conferred by E3 ligases. Although the human genome codes for approximately 600 E3 ligases [13], and their substrate specificity may be broadened by engaging in large multimeric complexes [14], it remained elusive how a large but limited number of enzymes can recognize and ubiquitinate all possible intracellular proteins, including those from newly emerging pathogens that the host has never adapted to during evolution. Either some E3 ligases recognize common features present in all/many proteins, e.g. the N-terminus, or the consensus view that the conversion of proteins into MHC class I peptides requires ubiquitination of the antigen is incorrect. Ubiquitin-independent proteasomal degradation has indeed been described for a number of protooncoproteins and oncosuppressive proteins [15].

Earlier studies have provided evidence in support of both, ubiquitin-dependent and -independent pathways in antigen processing. Using cell lines expressing temperature-sensitive E1 protein, antigen presentation was reduced after thermal inactivation of E1 in one, but remained unaffected in another study [5, 16, 17]. In more recent experiments with a dominant-negative ubiquitin mutant, presentation of ER-resident, but not cytosolic antigens, was dependent on polyubiquitination [18]. Moreover, chemical inhibition of polyubiquitin chain disassembly was reported to decrease presentation of a cytosolic model antigen [19]. While these results implied a potential role for ubiquitination and ubiquitin hydrolases in antigen processing, the knockdown of a component of the 19S proteasome regulator that recognizes ubiquitinated substrates was found to have no effect on antigen presentation [20].

Given the numerous cellular processes regulated by ubiquitin, interfering with its function might have affected antigen presentation indirectly and added to these conflicting results. To more directly investigate the role of ubiquitin in antigen selection, we generated mutants of model antigens that lack ubiquitinatable amino acids. Antigen presentation on MHC I remained unaffected by these mutations, demonstrating that direct ubiquitination of the antigen protein is not required, but was reduced when polyubiquitination on lysine-48 (K48) or proteasome-associated deubiquitination was prevented. These results implicate an important but indirect role of ubiquitin in antigen presentation.

Results

Presentation of BZLF1 and MART-1 on MHC class I depends on the proteasome

For assessing the role of ubiquitin in antigen processing, the Epstein-Barr virus protein BZLF1 and the cellular protein MART-1 were chosen as model antigens. Both are well characterized $CD8^+$ T-cell targets and their localization to the nucleus (BZLF1) and to the cell surface (MART-1) allowed to compare antigen presentation from different subcellular compartments. To test whether presentation of these antigens requires proteasomal processing, DG75 cells were transiently transfected with expression plasmids for the fusion proteins BZLF1-YVL-GFP and MART-1-YVL-GFP (Fig. 1A). After overnight culture, the cells were treated for 6 h with the proteasome inhibitor MG132 and then analyzed by flow cytometry. MG132 treatment resulted in an approximately 2.5-fold increase in GFP-positive cells (Fig. 1B). In addition, proteasome-dependent antigen presentation was tested by coculturing the transfected cells with $CD8^+$ T cells specific for the inserted YVL epitope (Fig. 1C), as well as the BZLF1-derived RAK epitope and the MART-1 derived AAG epitope (Supporting Information Fig. 1A, B). In order to keep the treatment period with proteasomal inhibitors at a minimum, the cells were incubated with stripping buffer 24 h post transfection to remove peptides from MHC class I molecules on the cell surface. This treatment resulted in an almost complete loss of T-cell recognition (Fig. 1C). T-cell recognition was restored when the stripped cells were cultured for additional 6 h in the absence, but was greatly reduced when cultured in the presence of the proteasomal inhibitor MG132 (Fig. 1C). To control for nonspecific toxic effects of MG132, the same cells were cotransfected with plasmids coding for the EBNA1-derived HPV-epitope and the restricting HLA-B*35.01 molecule. Minimal peptide determinants initiated by an ATG translation start site do not require proteasomal activity for presentation on MHC class I [21, 22]. Accordingly, MG132 treatment had no effect on the presentation of the peptide epitope, even when plasmid concentrations were decreased to exclude saturation effects (Supporting Information Fig. 1C). The stabilization of the fusion proteins and the significantly reduced presentation of two peptides on two different MHC class I molecules in the presence of MG132 demonstrated that the presentation of BZLF1- and MART-1-derived peptides depends on the proteasome.

Mutating single ubiquitinatable amino acids in the antigen does not affect MHC class I presentation

Peptides presented on MHC I are thought to derive from proteins degraded by the 26S proteasome in an ubiquitin-dependent manner [1, 3]. Although lysines are considered as the prime targets of ubiquitination, recent findings have also identified C, S, and T residues as potential ubiquitin conjugation sites [11]. Therefore, these amino acids were separately mutated to arginine (R; in the

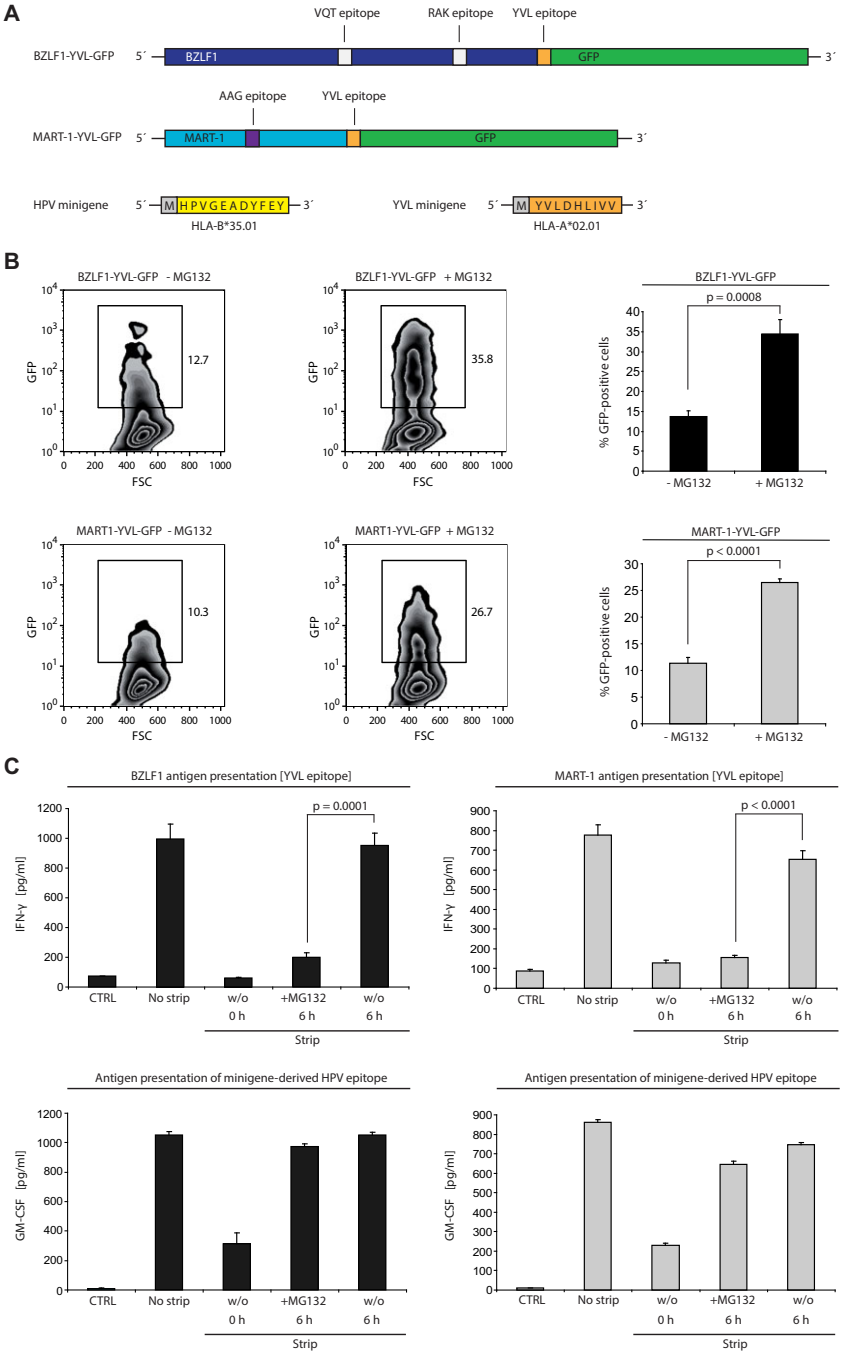

Figure 1. Proteasome-dependent presentation of BZLF1 and MART-1 on MHC class I (A) Illustration of the expression plasmids coding for BZLF1-YVL-GFP and MART-1-YVL-GFP fusion proteins and the location of the T-cell epitopes VQT (presented on HLA-B*1501), RAK (HLA-B*0801), AAG (HLA-A*0201), and YVL (HLA-A*0201). Shown beneath are the minigene constructs coding for the ATG-initiated EBNA1-derived HPV-epitope (yellow box), which is recognized by specific $CD8^+$ T cells on HLA-B*3501, and for the BRLF1-derived YVL-epitope. (B) DG75 cells were transiently transfected with expression plasmids for BZLF1-YVL-GFP (top) or MART-1-YVL-GFP (bottom). After 24 h of incubation, the cells were treated for 6 h with the proteasome inhibitor MG132 and the percentage of GFP-positive cells measured by flow cytometry. Representative histograms showing the percentage of GFP-positive cells are depicted. Quantification of the flow cytometry results is also shown (right). (C) DG75 cells were transfected with plasmids coding for either BZLF1-YVL-GFP (left) or MART-1-YVL-GFP (right). In both cases, expression plasmids for the EBNA1-derived HPV epitope and the restricting HLA-B*3501 molecule were cotransfected. 24 h posttransfection, the cells were either probed directly with $CD8^+$ T cells specific for the YVL- or the HPV-epitope, or stripped and then assayed with T cells, or stripped and then cultured for additional 6 h in the presence or absence of the proteasomal inhibitor MG132 before addition of T cells. In all cases, cells were fixed with paraformaldehyde before T-cell coculture. Cytokine secretion, IFN-γ (top) and GM-CSF (bottom), by T cells was measured by ELISA. (B and C) Statistical significance was evaluated with the unpaired *t*-test. Data are shown as mean ±SD of $n = 3$ and are from single experiments representative of three independent experiments.

case of lysine) and alanine (A; in the case of C, S, or T). As shown for BZLF1, these mutants were expressed at similar levels as the wild-type protein, excluding that the amino acid exchange affected protein expression (Fig. 2A). Although the exchange of K and T residues resulted in T-cell epitope destruction and ablated recognition by the RAK- and VQT-specific T cells, respectively, antigen presentation could still be assessed with at least one of the T-cell lines because these two epitopes contain different ubiquitinatable amino acids. Except for these instances of T-cell epitope destruction, recognition of transfected DG75 cells was neither impaired by the separate exchange of the four amino acids, nor by the mutation of all K > R and C > A in the mutant BZLF1ΔCK (Fig. 2B). In order to verify these findings with a natural protein that does not contain any K residues, sarcolipin (SLN) was C-terminally fused with the BRLF1-derived YVL-epitope (SLN-YVL) that is devoid of any ubiquitinatable C, K, S, or T amino acids. DG75 cells transfected for 24 h with the SLN-YVL expression construct were readily recognized by YVL-specific $CD8^+$ T cells (Fig. 2C). Again, T-cell recognition was almost completely abrogated when proteasomal degradation was inhibited.

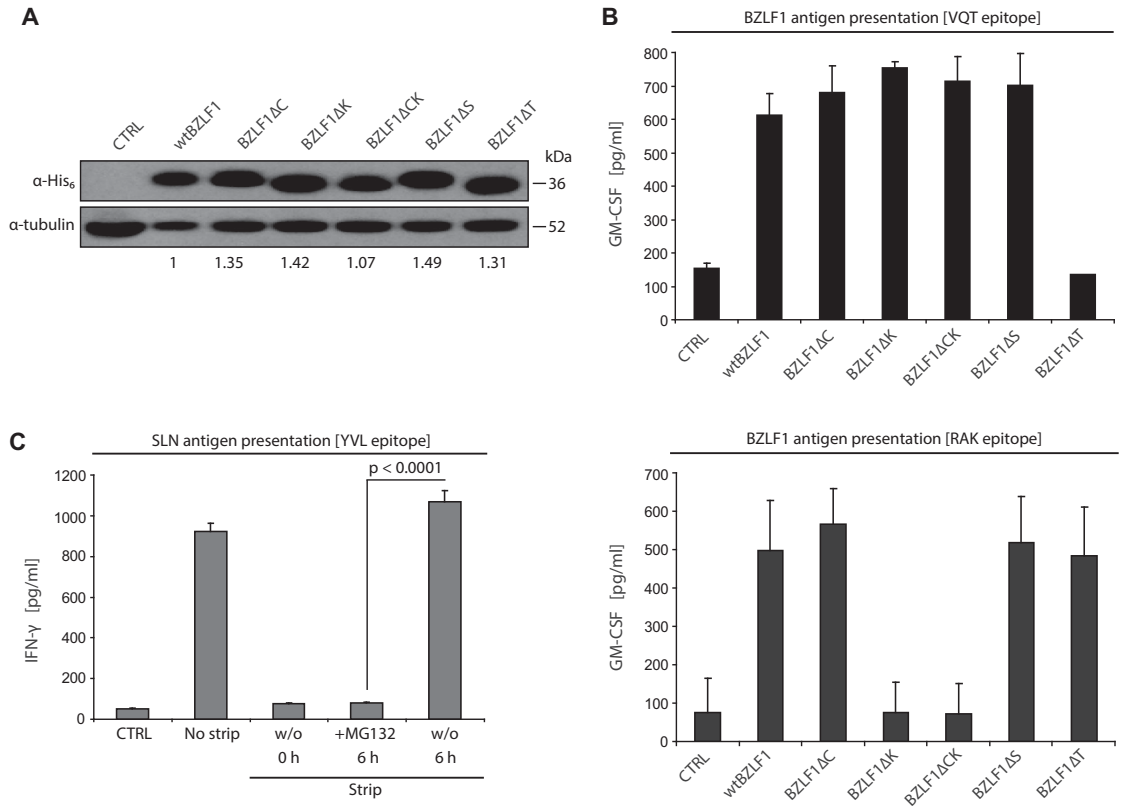

Figure 2. Mutation of ubiquitinatable amino acids does not abrogate antigen presentation (A) DG75 cells were transiently transfected with an empty vector or expression plasmids for wild-type BZLF1 (wtBZLF1), or BZLF1 mutants in which all cysteines (BZLF1ΔC), serines (BZLF1ΔS), or threonines (BZLF1ΔT) were exchanged for alanines, and lysines (BZLF1ΔK) for arginines. In addition, a BZLF1 mutant was used in which all lysines were substituted by arginines and all cysteines by alanines (BZLF1ΔCK). All BZLF1 proteins carried a His-tag (His_6) at the C-terminus. 24 h posttransfection, protein expression was analyzed by Western blot using a His_6-specific antibody (α-His_6). Tubulin served as loading control. The numbers underneath denote the expression levels of the different BZLF1 variants (adjusted to tubulin) relative to wtBZLF1. A representative blot (out of three) is shown. (B) DG75 cells were transiently transfected with the expression plasmids described in (A) together with the restricting HLA-B*0801 and HLA-B*1501 molecules and cultured for 24 h. The cells were then probed with $CD8^+$ T cells recognizing the VQT- (top) or the RAK-(bottom) epitope. GM-CSF secretion by T cells was measured by ELISA. (C) The open reading frame of sarcolipin (SLN), which does not encode any lysine, was C-terminally fused with the BRLF1-derived YVL-epitope, that lacks C, K, S, or T residues. DG75 cells were transfected with the SLN-YVL expression plasmid and probed with YVL-specific $CD8^+$ T cells 24 h later. In addition, a fraction of the cells was treated with an acid stripping buffer and then either cultured directly with the YVL-specific T cells, or first cultured for 6 h in the presence or absence of the proteasomal inhibitor MG132 and then assayed with T cells. All target cells were fixed before T cells were added. IFN-γ secretion by T cells was measured by ELISA. (B and C) Statistical significance was evaluated with the unpaired *t*-test. Data are shown as mean ± SD of $n = 3$. Representative results of at least three independent experiments are shown.

These results showed that proteasomal antigen processing was not dependent on the presence of lysines or any of the other ubiquitinatable amino acids within the antigens. Thus, antigen ubiquitination may either occur at C, K, S, or T residues in a redundant manner, or processing of these model antigens is not dependent on ubiquitination.

Mutation of all ubiquitinatable amino acids within the antigens does not impair their presentation

To investigate whether a minimal number of ubiquitin conjugation sites was required for antigen presentation, mutants of BZLF1 and MART-1 were generated in which all four ubiquitinatable amino acids were progressively exchanged for R (in the case of K) or A (in the case of C, S, and T). The full-mutants BZLF1-ΔCKST and MART-1-ΔCKST were completely devoid of C, K, S, or T residues. To facilitate analyses of antigen expression and presentation, the YVL-epitope as well as a His_6-tag, that both do not contain any C, K, S, or T amino acids, were C-terminally fused to all proteins (Fig. 3A). Irrespective of experimental variations, the different mutants were expressed at similar levels as the wild-type proteins in DG75 cells (Fig. 3B). Importantly, DG75 cells transfected with the different expression constructs were equally well recognized by the YVL-epitope-specific $CD8^+$ T cells (Fig. 3C). Identical results were obtained in LCL and in HeLa cells, excluding that this was a cell line-specific phenomenon (Supporting Information Fig. 2A). Because transient transfection of antigen

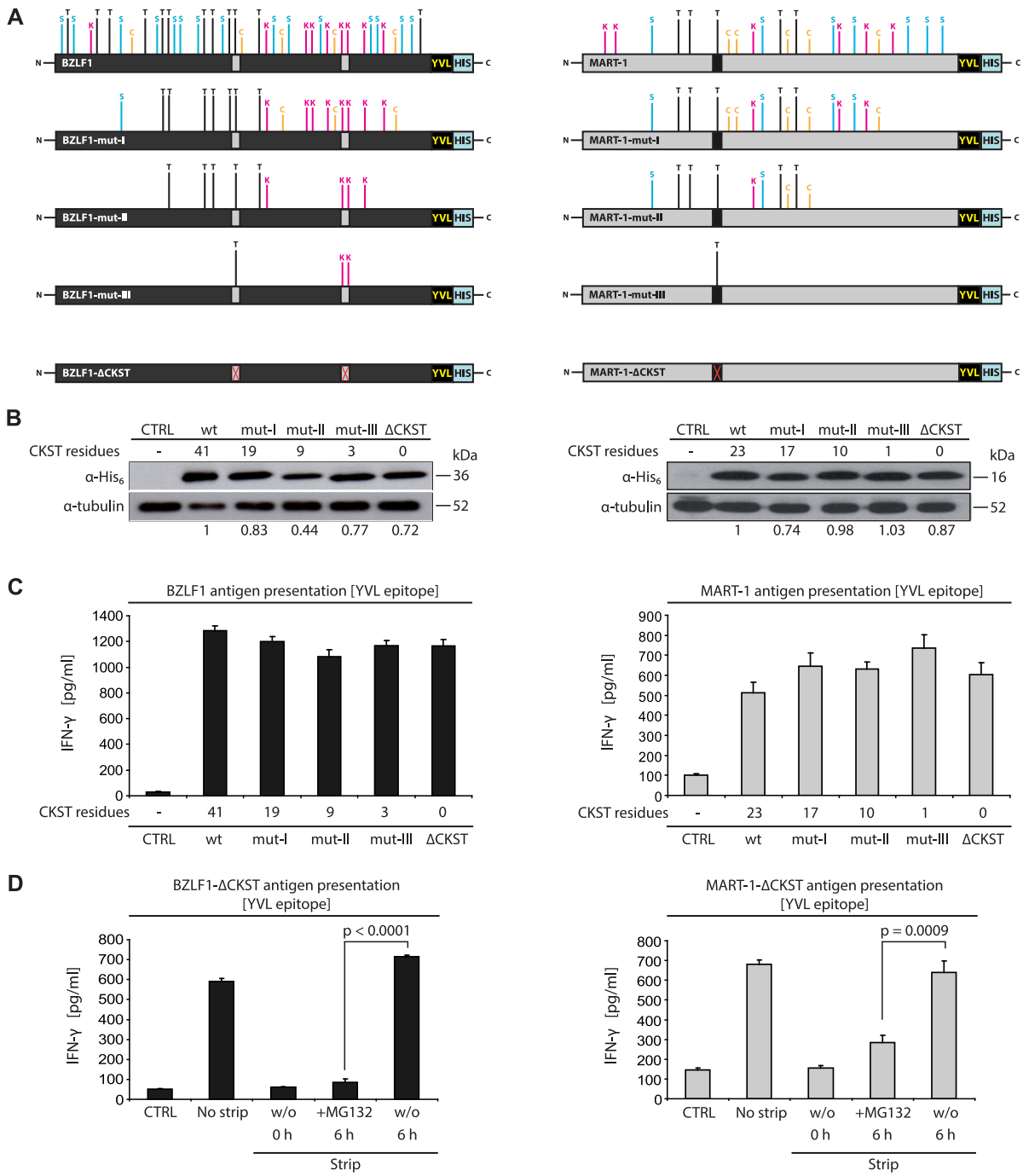

Figure 3. Antigen processing and presentation is not affected by deletion of potential ubiquitin conjugation sites (A) Schematic depiction of the cysteine (C), lysine (K), serine (S), and threonine (T) amino acid positions within the BZLF1 (left) and MART-1 (right) proteins, and the respective protein mutants in which C, S, and T were exchanged for alanine, and K for arginine. All open reading frames carried at the 3′ end sequences coding for the BRLF1-derived YVL-epitope as well as the His_6-tag that both do not carry any CKST residues. The YVL-epitope allowed assessing antigen presentation from all protein variants, including the full-mutants BZLF1-ΔCKST and MART-1-ΔCKST in which the T-cell epitopes (depicted as boxes) were destroyed by the introduced mutations (crossed boxes). (B) DG75 cells were transiently transfected with the expression plasmids for the BZLF1 (left) and MART-1 (right) variants and protein expression analyzed by Western blot using a His_6-specific antibody. Tubulin served as loading control. Values underneath blots denote the expression levels of the different mutants (adjusted to tubulin) relative to the respective wild-type protein. A representative blot (out of five) is shown. (C) The same transfectants as in (B) were cocultured with YVL-epitope-specific $CD8^+$ T cells and cytokine secretion measured 20 h later. (D) DG75 cells were cotransfected with the full-mutant constructs BZLF1-ΔCKST or MART-1-ΔCKST. After 24 h, the cells were probed with YVL- or HPV epitope-specific $CD8^+$ T cells. The transfected cells were either directly cocultured with T cells (no strip), or first stripped and then used as T-cell targets (strip 0 h). In addition, stripped cells were cultured for further 6 h in the presence or absence of the proteasomal inhibitor MG132 and then cocultured with T cells. In all cases, target cells were fixed with paraformaldehyde before incubation with T cells. (C and D) Statistical significance was evaluated with the unpaired *t*-test. Data are shown as mean ±SD of $n = 3$. Shown are representative data from one out of five independent experiments.

expression vectors might have led to saturating levels of antigen presented on the cell surface, and thereby might have masked differences in the efficiency of antigen processing, T-cell recognition of target cells transfected with titrated amounts of BZLF1 and BZLF1-ΔCKST plasmids was compared. No differences in antigen presentation were detected at any of the plasmid concentrations tested (Supporting Information Fig. 2B). Furthermore, proteasome-dependent processing of the full-mutant proteins was verified by MG132 treatment of transiently transfected DG75 cells (Fig. 3D). As observed for the wild-type proteins, inhibition of proteasomal degradation strongly decreased antigen presentation from BZLF1-ΔCKST and MART-1-ΔCKST. By contrast, presentation of the cotransfected minigene-derived HPV epitope was not affected by MG132 treatment (data not shown), excluding nonspecific toxic side effects of the inhibitor. These results demonstrate that antigen processing is dependent on the proteasome, but does not depend on antigen ubiquitination at C, K, S, or T residues.

BZLF1 and MART-1 mutants lacking C, K, S, and T residues are not ubiquitinated

In addition to ubiquitinatable amino acids within a protein, the free amino group of the N-terminal methionine (M) also functions as ubiquitin conjugation site [6]. To address whether the model antigens were N-terminally ubiquitinated, His_6-tagged wild-type and ΔCKST proteins of BZLF1 and MART-1 were purified from HEK293T cells using nickel beads and analyzed by MS. In accordance with the N-end rule pathway, loss of the first of the two consecutive methionines in BZLF1 was noted in some instances, but neither of the proteins was found to be N-terminally conjugated with ubiquitin. By contrast, ubiquitin-modifications were detected at several lysine residues within the wild type, but not the BZLF1-ΔCKST protein. Likewise, ubiquitin-conjugates were found on all cytoplasmic lysine residues of MART-1, but not on MART-1-ΔCKST and not on the N-termini of the proteins (Supporting Information Fig. 3, and data not shown). To corroborate these findings, His_6-tagged wild type and ΔCKST mutant proteins were expressed in HEK293T cells together with an N-terminally E1 antibody epitope-tagged ubiquitin (E1-Ub), purified via nickel beads and analyzed by Western blot (Fig. 4). To control for precipitation efficiency, blots were first hybridized with an α-His_6 antibody. Similar protein levels were detected for the respective wild type and ΔCKST variants of BZLF1 (Fig. 4A) and MART-1 (Fig. 4B). Note that due to the amino acid exchanges, MART-1-ΔCKST migrated faster than wild-type MART-1. After longer exposure, additional bands with increments of approximately 8 kDa were detected for the wild type, but not the ΔCKST proteins. This became even more apparent, when the same blots were stripped and then reprobed with an antibody directed against E1-tagged ubiquitin, exposing protein ladders probably corresponding to mono- and polyubiquitinated proteins (right panel). Importantly, no such ubiquitin conjugates were detected for the ΔCKST mutants (Fig. 4). High molecular weight proteins detected by the α-E1 antibody most likely represent E1-Ub-conjugated cellular proteins that nonspecifically bound to Ni-NTA beads. Taken together, these results argued against N-terminal ubiquitination of the model antigens and suggested that ubiquitination is not essential for antigen processing.

Antigen presentation depends on K48-linked polyubiquitination and proteasomalde ubiquitination

To more directly address the role of the ubiquitin signal in the presentation of our model antigens, DG75 cells were cotransfected with expression plasmids for BZLF1 and shRNA targeting ubiquitin (shUb; Supporting Information Fig. 4). Knockdown of endogenous ubiquitin resulted in a decrease in antigen presentation of wild type as well as full-mutant MART-1 (Fig. 5A, B) and BZLF1 (data not shown). By contrast, presentation of the minigene-encoded HPV epitope remained unaffected (Fig. 5C), indicating that the downregulation of ubiquitin directly interferes with proteasomal degradation of the antigen.

To corroborate this potential role of ubiquitin in antigen presentation, two additional experimental approaches were used. First, wtBZLF1 and BZLF1-ΔCKST were coexpressed with either empty vector control, wild-type ubiquitin (Ub), or the ubiquitin mutants Ub-K33R or Ub-K48R. The latter mutant should prevent K48-linked polyubiquitination that targets proteins for proteasomal degradation, whereas lysine at position 33 has not been associated with protein degradation and served as control. In fact, antigen presentation was not affected by Ub or Ub-K33R. However, Ub-K48R coexpression significantly decreased presentation of the model antigens, but not of the minigene-derived peptides (Fig. 5D–F), suggesting that antigen processing depends on K48-linked polyubiquitination. Second, inhibition of the deubiquitinating activity of the 26S proteasome in transfected cells by b-AP15 [23] strongly reduced presentation of epitopes derived from the model antigens, but not from minigene constructs (Fig. 5G, and data not shown). Of note, antigen presentation of endogenously expressed MART-1 and BZLF1 also depended on proteasomal cleavage and deubiquitination (Supporting Information Fig. 5), indicating that the transfected antigens were processed as their natural counterparts. Taken together, these experiments implicated a critical role of K48-linked polyubiquitin in proteasomal antigen processing. Because wild type and ΔCKST mutants were equally affected, ubiquitin either provided an indirect signal, or was attached to the N-terminus of the proteins. In the latter case, the failure to detect N-terminal ubiquitination in mass spectrometric and Western blot analyses implied that this was at most a rare modification and, consequently, that proteins carrying this modification would enter the antigen processing pathway very efficiently. However, T-cell recognition of target cells expressing stable N-terminal in-frame Ub-fusions of the model antigens was only moderately increased over cells expressing unmodified model antigens (Fig. 5H, I). These results argue against N-terminal ubiquitination as crucial signal for antigen processing and hint toward an indirect role of ubiquitin in proteasomal antigen processing.

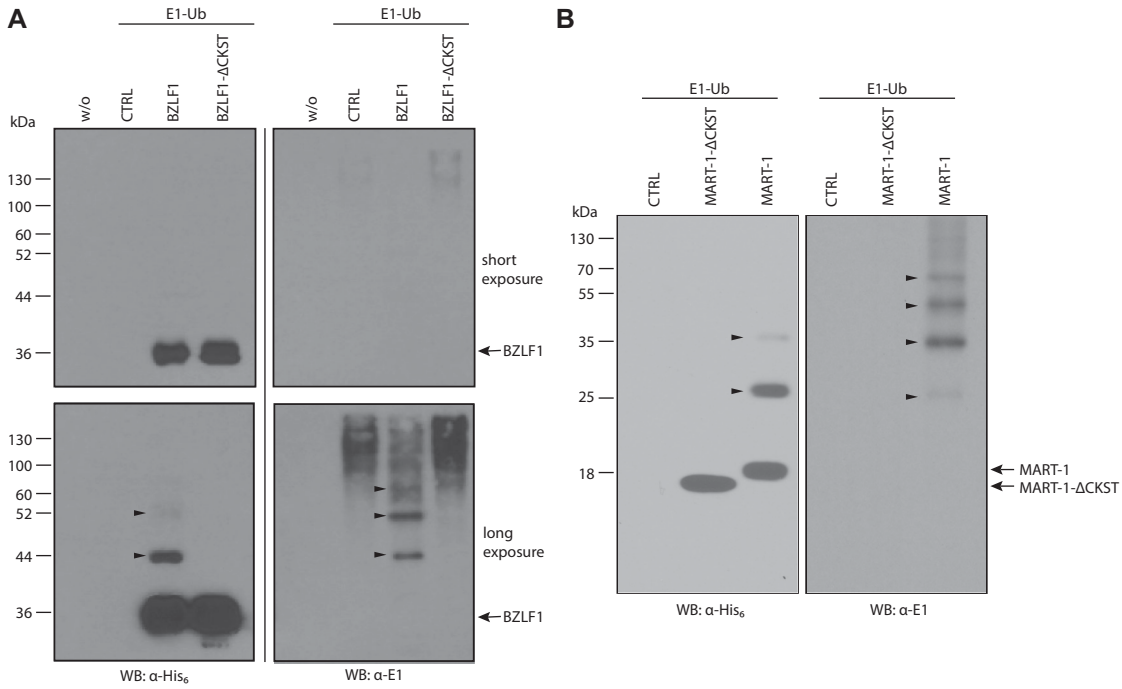

Figure 4. Ubiquitination occurs on wild-type BZLF1 and MART-1, but not on ΔCKST mutants (A) HEK293T cells were left untransfected (w/o), or transfected with expression plasmids for E1-tagged ubiquitin (E1-Ub) together with either empty vector control (CTRL), or His_6-tagged wild-type BZLF1 or BZLF1-ΔCKST. After 24 h of incubation, the proteasome inhibitor MG132 was added and recombinant proteins isolated 6 h later via their His_6-tag using Ni-NTA beads. Following SDS-PAGE, the proteins were immunoblotted using His_6- and E1-tag-specific antibodies and detected by chemiluminescence. Short (top) and long (bottom) exposures of the same blots are shown. The α-His_6 antibody was used to verify that similar amounts of wild type and mutant protein were precipitated (left panel). With increments of approximately 8 kDa, additional proteins (denoted by arrowheads), were detected in the BZLF1, but not in the BZLF1-ΔCKST lane. The same ladder of larger proteins was also detected when the membranes were stripped and reprobed with an α-E1 antibody (right panel) and thus probably represent mono/multiubiquitinated BZLF1 proteins. (B) HEK293T cells were transfected with expression plasmids for E1-tagged ubiquitin (E1-Ub) together with either empty vector control (CTRL), or His_6-tagged wild-type MART-1 or MART-1-ΔCKST and then analyzed as in (A). Data are representative of one out of (A) three or (B) two independent experiments.

Discussion

Given that the 26S proteasome primarily degrades ubiquitin-conjugated substrates [12, 24], it has been generally assumed that ubiquitination is the common and essential signal for the conversion of proteins into MHC I-restricted peptides [1, 17]. Experimental evidence in support of such an association, however, is sparse and mostly circumstantial [5].

By employing various strategies to thwart the ubiquitin system, previous studies have indicated the existence of functionally distinct antigen processing pathways that are dependent or independent of ubiquitination, and contingent on the subcellular location and the nature of the antigen [5, 25]. Here, we sought to assess the role of ubiquitin in proteasome-dependent antigen processing without interfering with its many signaling functions by modifying the substrate.

Initial experiments with lysineless variants of our model antigens or SLN, a small protein that per se does not contain any lysine residues, demonstrated that lysine residues are not essential for proteasome-dependent antigen processing. Although lysines are considered primary targets, ubiquitin can also be attached to C, S, and T residues of target proteins [6–9, 12]. By extensive mutagenesis of the model antigens, antigen presentation was found to neither require the presence of any of the four types of ubiquitinatable amino acids, nor a minimum number of ubiquitinatable amino acids within the antigen substrate. In fact, antigen presentation was unaltered when all C, K, S, and T residues within the antigen protein were mutated. Although proteasome-dependent, the processing of the model antigens thus appeared to be ubiquitin-independent. Unexpectedly, knockdown of ubiquitin, coexpression of the ubiquitin K48R mutant, or inhibition of proteasome-associated deubiquitination significantly reduced presentation of peptides derived from wild-type as well as ΔCKST-proteins, but not of epitope peptides derived from minigene constructs. Because ubiquitin conjugation to the α-NH_2 group of the N-terminal residue may also regulate the fate of the target protein [6, 12, 26], mass spectrometric and immunoblot analyses of the model antigens were performed. Within the detection limits of these experiments, no evidence for N-terminal ubiquitination was obtained. Even after long exposures of the immunoblots, bands corresponding to mono-, di-, or triubiquitinated variants were only detected for the wild type, but not for the ΔCKST mutant proteins. Since ubiquitination and deubiquitination are highly dynamic and parallel processes, it appears very unlikely

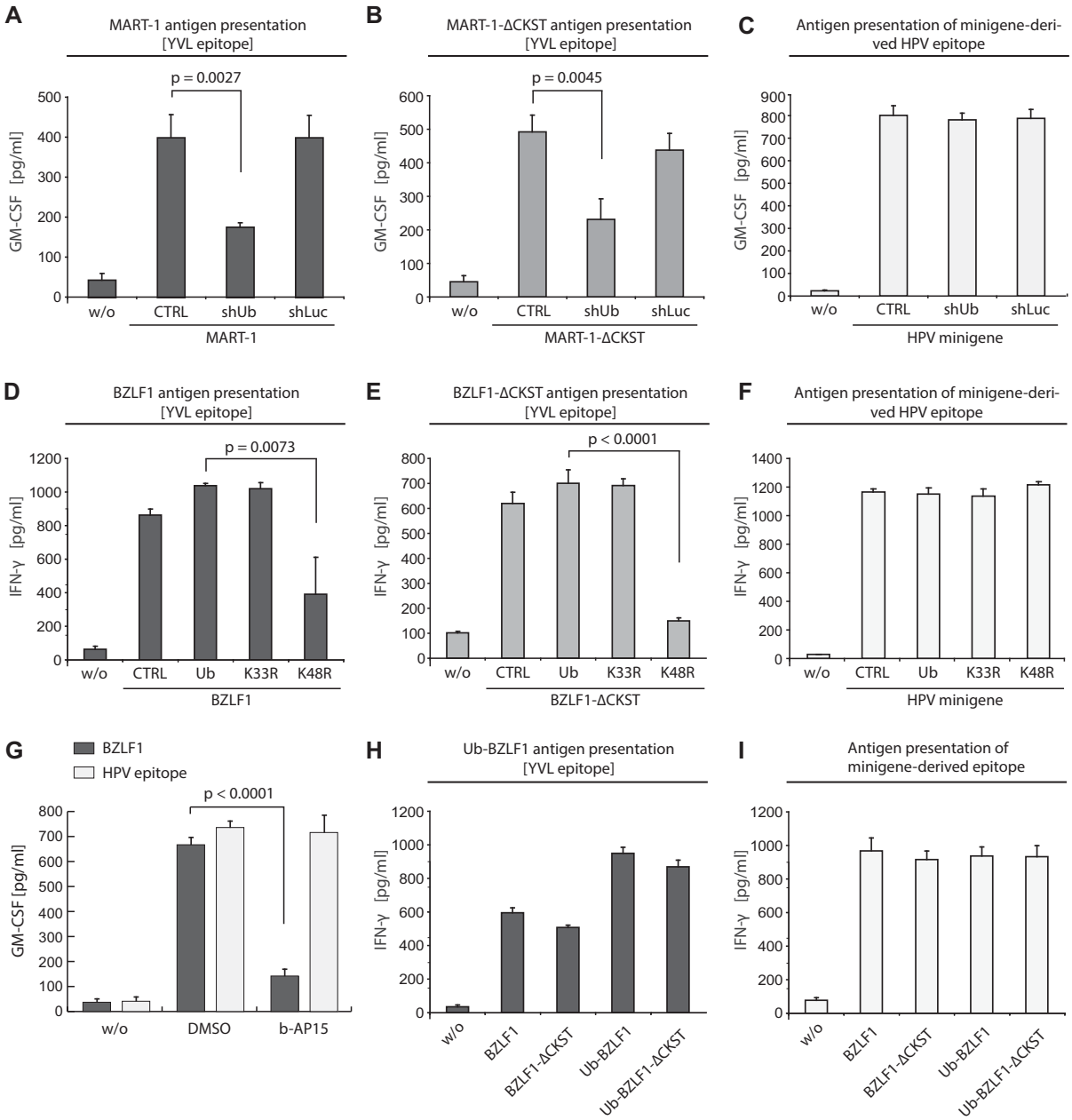

Figure 5. Inhibition of ubiquitination or proteasome-associated deubiquitinases diminishes antigen presentation (A–C) DG75 cells were either left untransfected (w/o), or transfected with empty vector control (CTRL), or expression plasmids for (A) MART-1, (B) MART-1- ΔCKST or (C) the HPV minigene and the restricting HLA-B*3501 molecule together with plasmids coding for shRNA against ubiquitin (shUb) or luciferase (shLuc). Twenty four hours post-transfection the cells were cocultured with antigen-specific $CD8^+$ T cells. (D–F) DG75 cells were either left untransfected (w/o), or transfected with (D) BZLF1 or (E) BZLF1-ΔCKST expression plasmids, together with (F) the HPV minigene plus the restriction element. In addition, empty vector (CTRL), or vectors coding for wild-type ubiquitin (Ub), the ubiquitin mutants Ub-K33R, or Ub-K48R were cotransfected. Twenty four hours posttransfection the cells were probed with (D and E) YVL- or (F) HPV- specific $CD8^+$ T cells (G) DG75 cells, either left untransfected (w/o), or transfected with a BZLF1 expression plasmid, the HPV minigene, and the restricting HLA-B*3501 molecule, were cultivated for 24 h in the presence of 0.5 μM b-AP15 or an equal volume of the solvent (DMSO). Following extensive washing, the cells were probed with BZLF1 or HPV-specific $CD8^+$ T cells. (H and I) DG75 cells were either left untreated (w/o), or transfected with expression plasmids for BZLF1, BZLF1-ΔCKST, or the in-frame N-terminal ubiquitin fusion constructs Ub-BZLF1 and Ub-BZLF1-ΔCKST. In all cases, a minigene construct and the restriction element were cotransfected. After 24 h, the cells were cocultured with $CD8^+$ T cells specific for (H) the YVL epitope or (I) the minigene-derived epitope. (A–I) Cytokine secretion, IFN-γ and GM-CSF, by T cells was measured by ELISA. The unpaired *t*-test was used for evaluating statistical significance. Data are shown as mean + SD of $n = 3$ and are from single experiments representative of three independent experiments.

that a given protein should only contain four or more ubiquitin moieties. Thus, the background in the high molecular weight range of the immunoblots most likely stemmed from contaminating cellular proteins that had bound to the Ni-NTA beads.

However, one has to keep in mind that the efficiency of antigen processing is low with estimates ranging from one peptide/MHC complex generated per 10^3–10^4 proteins degraded [27]. Since the conversion of proteins into antigenic peptides is a selective rather than a stochastic process, only a small fraction of proteins destined for antigen processing might have been ubiquitinated at the N-terminus, which might have remained undetected with the methods applied. In addition, stable in-frame fusion of Ub to the N-terminus of proteins has been shown to enhance their proteasomal degradation and to augment their presentation on MHC I [28, 29], albeit to variable degrees [30].

Nevertheless, N-terminal ubiquitination as principal signal for proteasomal processing of antigens appears unlikely for two reasons. First, this N-terminal modification has been detected on only a limited number of proteins [6, 12, 26]. Moreover, approximately 90% of human proteins are cotranslationally acetylated at the N-terminus, which is incompatible with N-terminal ubiquitination [26, 31]. Second, the engineered expression of in-frame ubiquitin-antigen fusion proteins only moderately increased presentation of the model antigens on MHC I. If N-terminal ubiquitination was the signal that directs a small proportion of a given protein into the antigen processing pathway, then much stronger effects on antigen presentation would have been expected when all proteins were stably fused to ubiquitin.

Collectively, these results indicate that the generation of antigenic precursor peptides from these model antigens is dependent on ubiquitin-mediated proteasomal degradation, but the ubiquitin signal is not attached directly to the substrate protein. Furthermore, the strong reduction in antigen presentation following inhibition of the 26S proteasome-associated deubiquitinylating enzymes might indicate that the model antigens are cleaved by the 26S proteasome in an indirectly ubiquitin-dependent manner, rather than by the 20S proteasome in an ubiquitin-independent manner, as described for a growing number of targets [32].

Our findings are consistent with a model in which the antigen proteins are bound by adaptor protein(s), which deliver the cargo to the proteasome for degradation in an ubiquitin-dependent manner. It is intriguing to speculate that these adaptors may recognize features present in all proteins, e.g. the N-terminus or the exposure of hydrophobic areas in misfolded or newly synthesized proteins. Consistent with this, hydrophobicity has recently been identified as a signal for immediate degradation of proteins and MHC I peptide generation [33]. Such a model might provide an explanation for how the antigen processing machinery manages to recognize and select antigen substrates from innumerable self as well as non-self-proteins with different amino acid compositions, biochemical characteristics, and tertiary structures. Besides dispensing with the need for E3 ligases for the whole intracellular antigenome, ubiquitinylation of the adaptor rather than the antigen substrate would also preclude immune evasion by removing/masking ubiquitinatable amino acids in antigen proteins.

While the identity of these putative adaptor protein(s) is not known, cytosolic precursors of MHC I binding peptides have previously been found in complex with chaperones, most importantly Hsp90α [34]. Moreover, shRNA-mediated knockdown of Hsp90α prevented intracellular accumulation of these precursors, as did the knockdown of CHIP (carboxyl terminus of Hsc70-interacting protein), an E3 ligase that ubiquitinates Hsc70 or Hsp90α-associated proteins for destruction by the proteasome. In other studies, however, antigen presentation was not diminished when Hsc70 activity was modulated by the expression of a dominant negative mutant [20], and peptides bound by Hsp90 were reported to be proteasomal cleavage products [35]. Of note, degradation of chaperone clients is not impaired in CHIP-deficient cells [36, 37], suggesting that CHIP is functionally redundant with other chaperone-assisted ubiquitin-protein ligases.

Conceivably, the task of directing proteins into the antigen processing pathway is distributed among several members of the chaperone family to ensure a comprehensive display of all potential antigens and to minimize the risk of evasion by pathogens. Defining these molecules and the responsible E3 ligases involved in this process of antigen selection is expected to provide new insights into the nature of endogenous peptide precursors and the molecular mechanisms underlying immunogenicity and immunodominance.

Materials and methods

Antibodies and reagents

Monoclonal antibodies against the E1-tag (derived from EBNA1 and recognized by clone 1H4) and a histidine-tag consisting of six consecutive histidines (His_6; clone 3D9, both provided by Dr. E. Kremmer; Helmholtz ZentrumMünchen), BZLF1 (Argene), and α-tubulin (Argene) were used. HRP-conjugated secondary antibodies against mouse (GE-Healthcare), rat (Jackson ImmunoResearch) and rabbit antibodies (Jackson ImmunoReasearch) were used. Unless otherwise indicated MG132 (Santa Cruz) was used at a final concentration of 10 μM. b-AP15 (UBPBio), which inhibits the deubiquitinating activity of the 26S proteasome, was used at 0.5 μM.

Cell culture

The adherent human cells lines HEK293T, HeLa, and Mel-93.04A12 were grown in DMEM medium supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 1.25 μg/mL fungizone and 50 μg/mL gentamycin. The HLA-A*0201 positive Burkitt-Lymphoma cell line DG75 and the EBV-immortalized human B cell lines FL and EBV1.11 were grown as suspension cultures in RPMI-1640 medium supplemented with 10% FCS, 1% nonessential amino acids, 1 mM sodium pyruvate, 2 mM L-glutamine, 1.25 μg/mL fungizone, and 50 μg/mL gentamycin.

The $CD8^+$ T cells LG-BZLF1, recognizing the BZLF1 peptide $AA_{122–130}$VQTAAAVVF on HLA-B*1501, the FL-BZLF1 $CD8^+$ T cells specific for the BZLF1-derived peptide $AA_{190–197}$RAKFKQLL presented on HLA-B*0801, the BRLF1-specific T cells JM-BRLF1 specific for the $AA_{109–117}$ YVLDHLIVV peptide presented on HLA-A*0201, the MART-1-specific T cells (kindly provided by Dr. E. Nössner, Helmholtz Zentrum München) recognizing the epitope $AA_{27–35}$ AAGIGILTV on HLA-A*0201 [38], as well as the EBNA1-specific T cells that recognize the $AA_{407–417}$ HPVGEADYFEY peptide on HLA-B*3501 [39] were cultured in T-cell media consisting of AIM-V medium supplemented with 10% heat-inactivated human serum, 2 mM L-glutamine, 1.25 μg/mL fungizone and 10 Mm Hepes.

T cells were restimulated every 2 weeks with irradiated (80 Gy) autologous lymphoblastoid B cells that had been pulsed with cognate peptide (1 μg/mL) as described [40]. 50 U/ml IL-2 (Novartis) was added the next day and subsequently every third day until the cells had to be restimulated.

For assessing antigen presentation, DG75, EBV1.11, Mel-93.04A12, and HeLa cells were transiently transfected using the GenPulser II electroporator (BioRad) with the indicated expression plasmids for 24 h and then cocultivated for 20 h with antigen-specific $CD8^+$ T cells. In some experiments, peptides were removed from MHC class I molecules by incubating the cells in stripping buffer as described [39]. Cytokine secretion by the T cells as a means of T cells activation was measured by ELISA (R&D System). Plotted data represent the mean plus standard deviation (SD) of triplicates.

Construction of expression vectors

All expression constructs are derivatives of the pCMV/cyto vector (Invitrogen). The restricting HLA-B*0801, B*1501, and HLA-B*3501 molecules were cloned from cell lines positive for these alleles using PCR-based techniques. The genomic sequence of BZLF1 was amplified by PCR from B95.8 virus. The open reading frame of MART-1 was cloned from a melanoma-derived cDNA library. To generate BZLF1 and MART-1 mutants, PCR-based site-directed mutagenesis and gene assembly were used. The sequence coding for the BRLF1-derived epitope YVLDHLIVV was fused C-terminally to the open reading frames using overlapping PCR.

Overlapping PCR was used to generate the BZLF1-GFP and MART-1-GFP fusion genes. Expression plasmids coding for wild-type ubiquitin (Ub) and the mutants Ub-K33R and Ub-K48R were generated using PCR-based techniques. To enable detection of ubiquitin by Western blot, the epitope recognized by the EBNA1-specific antibody 1H4 (EQGPADDPGEG) was N-terminally fused to ubiquitin (E1-Ub). To prevent E1-Ub from knockdown, seven conservative exchanges were introduced into the ubiquitin sequence (E1-Ub-mut) that is targeted by the siRNA. SLN was amplified from genomic human DNA and cloned into the pCMV/cyto vector. The expression plasmids coding for methionine-initiated T-cell epitopes HPV and YVL were generated by ligating the corresponding DNA-linkers into the pCMV/cyto vector (Invitrogen). The short-hairpin RNA expression constructs specific for ubiquitin (shUb) and luciferase (shLuc) were obtained by ligating DNA-linkers for selected siRNA sequences, designed by the siRNA design algorithm (BLOCK-IT RNAi Designer, https://rnaidesigner.invitrogen.com/rnaiexpress/; Invitrogen) into the pMIRTOP construct [41]. Stable in-frame fusions of ubiquitin, in which the C-terminal glycine-76 was mutated to valine, to the N-termini of the BZLF1 proteins were engineered by overlap PCR [42]. Sequences of all used primers are available upon request. Identity and integrity of the created plasmids were verified by restriction enzyme digestion and sequence analysis of the modified regions.

Protein expression analysis

RIPA buffer containing *Protease Inhibitor Cocktail* (Roche) was used to prepare whole cell extracts for Western blot analysis. The samples were separated on a 10.5% SDS-PAA gel, blotted onto PVDF membranes (GE-Healthcare) and incubated with antibodies. Visualization of proteins was accomplished by chemiluminescence using the ECL plus detection kit (GE-Healthcare). Relative expression levels were determined using ImageJ software.

For flow cytometric detection of GFP expression, the cells were washed in FACS buffer (PBS with 1% FCS), resuspended in 500 μL FACS buffer containing 0.5 mg/mL propidium iodide, and acquired in a FACSCalibur™ (BD Biosciences) using CellQuest software. Sample analysis was performed using FlowJo (Tri Star) software.

Ubiquitination of proteins was analyzed following transfection of plasmids coding for the protein of interest into HEK293T cells for 48 h. MG132 (10 μM) was added to the cells 6 h prior to cell harvest to stabilize possible ubiquitin-modifications. The cells were lysed using urea lysis buffer (8M Urea, 0.1M NaH_2PO_4, 0.01M Tris, 0.05% Tween 20, 20mM imidazole; pH 8.0) supplemented with 10 μM MG132 and the C-terminally His_6-tagged recombinant proteins were purified via Ni-NTA beads (Qiagen). Isolated proteins were separated in a 10.5% SDS-PAA gel, the resolved proteins visualized with Coomassie blue staining solution and cut out of the gel. LC-MS/MS analysis was performed by trypsin digestion and protein fragments detected using a LTQ Orbitrap XL. Acquired MS/MS spectra were used for peptide identification with Mascot. An extended Swissprot database was generated by adding the various BZLF1 and MART-1 variants. Results were imported into Scaffold software and protein identification probability threshold was set to 95%.

Statistical analysis

Significances of the experiments were calculated by using the log-rank test (unpaired *t*-test). All *p*-values were based on a two-sided testing using the GraphPad Prism 5 program, and *p*-values of 0.05 or less were considered significant.

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References

1 **Sijts, E. J. and Kloetzel, P. M.**, The role of the proteasome in the generation of MHC class I ligands and immune responses. *Cell. Mol. Life Sci.* 2011. **68**: 1491–1502.

2 **Rock, K. L. and Goldberg, A. L.**, Degradation of cell proteins and the generation of MHC class I-presented peptides. *Ann. Rev. Immunol.* 1999. **17**: 739–779.

3 **Blum, J. S., Wearsch, P. A. and Cresswell, P.**, Pathways of antigen processing. *Ann. Review Immunol.* 2013. **31**: 443–473.

4 **van Endert, P.**, Post-proteasomal and proteasome-independent generation of MHC class I ligands. *Cell. Mol. Life Sci.* 2011. **68**:1553–1567.

5 **Yewdell, J. W.**, DRiPs solidify: progress in understanding endogenous MHC class I antigen processing. *Trends Immunol.* 2011. **32**: 548–558.

6 **Ciechanover, A. and Ben-Saadon, R.**, N-terminal ubiquitination: more protein substrates join in. *Trends Cell Biol.* 2004. **14**: 103–106.

7 **Cadwell, K. and Coscoy, L.**, Ubiquitination on nonlysine residues by a viral E3 ubiquitin ligase. *Science* 2005. **309**: 127–130.

8 **Ravid, T. and Hochstrasser, M.**, Autoregulation of an E2 enzyme by ubiquitin-chain assembly on its catalytic residue. *Nat. Cell Biol.* 2007. **9**: 422–427.

9 **Wang, X., Herr, R. A., Chua, W. J., Lybarger, L., Wiertz, E. J. and Hansen, T. H.**, Ubiquitination of serine, threonine, or lysine residues on the cytoplasmic tail can induce ERAD of MHC-I by viral E3 ligase mK3. *J. Cell Biol.* 2007. **177**: 613–624.

10 **Shimizu, Y., Okuda-Shimizu, Y. and Hendershot, L. M.**, Ubiquitylation of an ERAD substrate occurs on multiple types of amino acids. *Mol. Cell.* 2010. **40**: 917–926.

11 **Husnjak, K. and Dikic, I.**, Ubiquitin-binding proteins: decoders of ubiquitin-mediated cellular functions. *Ann. Rev. Biochem.* 2012. **81**: 291–322.

12 **Ciechanover, A. and Stanhill, A.**, The Complexity of Recognition of Ubiquitinated Substrates by the 26S Proteasome. *Biochim. et Biophys. Acta* 2014.**1843**: 86–96.

13 **Metzger, M. B., Hristova, V. A. and Weissman, A. M.**, HECT and RING finger families of E3 ubiquitin ligases at a glance. *J. Cell Sci.* 2012. **125**: 531–537.

14 **Nagy, V. and Dikic, I.**, Ubiquitin ligase complexes: from substrate selectivity to conjugational specificity. *Biol. Chem.* 2010. **391**: 163–169.

15 **Jariel-Encontre, I., Bossis, G. and Piechaczyk, M.**, Ubiquitin-independent degradation of proteins by the proteasome. *Biochim. Biophys. Acta.* 2008. **1786**: 153–177.

16 **Cox, J. H., Galardy, P., Bennink, J. R. and Yewdell, J. W.**, Presentation of endogenous and exogenous antigens is not affected by inactivation of E1 ubiquitin-activating enzyme in temperature-sensitive cell lines. *J. Immunol.* 1995. **154**: 511–519.

17 **Michalek, M. T., Grant, E. P., Gramm, C., Goldberg, A. L. and Rock, K. L.**, A role for the ubiquitin-dependent proteolytic pathway in MHC class I-restricted antigen presentation. *Nature* 1993. **363**: 552–554.

18 **Huang, L., Marvin, J. M., Tatsis, N. and Eisenlohr, L. C.**, Cutting edge: selective role of ubiquitin in MHC class I antigen presentation. *J. Immunol.* 2011. **186**:1904–1908.

19 **Dolan, B. P., Li, L., Veltri, C. A., Ireland, C. M., Bennink, J. R. and Yewdell, J. W.**, Distinct pathways generate peptides from defective ribosomal products for CD8+ T cell immunosurveillance. *J. Immunol.* 2011. **186**:2065–2072.

20 **Qian, S. B., Princiotta, M. F., Bennink, J. R. and Yewdell, J. W.**, Characterization of rapidly degraded polypeptides in mammalian cells reveals a novel layer of nascent protein quality control. *J. Biol. Chem.* 2006. **281**: 392–400.

21 **Fruci, D., Lauvau, G., Saveanu, L., Amicosante, M., Butler, R. H., Polack, A., Ginhoux, F. et al.**, Quantifying recruitment of cytosolic peptides for HLA class I presentation: impact of TAP transport. *J. Immunol.* 2003. **170**:2977–2984.

22 **Princiotta, M. F., Finzi, D., Qian, S. B., Gibbs, J., Schuchmann, S., Buttgereit, F., Bennink, J. R. et al.**, Quantitating protein synthesis, degradation, and endogenous antigen processing. *Immunity* 2003. **18**: 343–354.

23 **D'Arcy, P., Brnjic, S., Olofsson, M. H., Fryknas, M., Lindsten, K., De Cesare, M., Perego, P. et al.**, Inhibition of proteasome deubiquitinating activity as a new cancer therapy. *Nat. Med.* 2011. **17**:1636–1640.

24 **Finley, D.**, Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Ann. Rev. Biochem.* 2009. **78**: 477–513.

25 **Dolan, B. P., Bennink, J. R. and Yewdell, J. W.**, Translating DRiPs: progress in understanding viral and cellular sources of MHC class I peptide ligands. *Cell. Mol. Life Sci.* 2011. **68**:1481–1489.

26 **McDowell, G. S. and Philpott, A.**, Non-canonical ubiquitylation: mechanisms and consequences. *Int. J. Biochem. Cell Biol.* 2013. **45**:1833–1842.

27 **Yewdell, J. W., Reits, E. and Neefjes, J.**, Making sense of mass destruction: quantitating MHC class I antigen presentation. *Nat. Rev. Immunol.* 2003. **3**: 952–961.

28 **Stack, J. H., Whitney, M., Rodems, S. M. and Pollok, B. A.**, A ubiquitin-based tagging system for controlled modulation of protein stability. *Nat. Biotechnol.* 2000. **18**: 1298–1302.

29 **Setz, C., Friedrich, M., Hahn, S., Dorrie, J., Schaft, N., Schuler, G. and Schubert, U.**, Just one position-independent lysine residue can direct MelanA into proteasomal degradation following N-terminal fusion of ubiquitin. *PLos One* 2013. **8**: e55567.

30 **Andersson, H. A. and Barry, M. A.**, Maximizing antigen targeting to the proteasome for gene-based vaccines. *Mol. Ther.* 2004. **10**: 432–446.

31 **Varshavsky, A.**, The N-end rule pathway and regulation by proteolysis. *Protein Sci.* 2011. **20**: 1298–1345.

32 **Orlowski, M. and Wilk, S.**, Ubiquitin-independent proteolytic functions of the proteasome. *Arch. Biochem. Biophys.* 2003. **415**: 1–5.

33 **Huang, L., Kuhls, M. C. and Eisenlohr, L. C.**, Hydrophobicity as a driver of MHC class I antigen processing. *EMBO J.* 2011. **30**: 1634–1644.

34 **Kunisawa, J. and Shastri, N.**, Hsp90alpha chaperones large C-terminally extended proteolytic intermediates in the MHC class I antigen processing pathway. *Immunity* 2006. **24**: 523–534.

35 **Callahan, M. K., Garg, M. and Srivastava, P. K.**, Heat-shock protein 90 associates with N-terminal extended peptides and is required for direct

and indirect antigen presentation. *Proc. Natl. Acad. Sci. USA* 2008. **105**: 1662–1667.

36 **Xu, P., Duong, D. M., Seyfried, N. T., Cheng, D., Xie, Y., Robert, J., Rush, J. et al.**, Quantitative proteomics reveals the function of unconventional ubiquitin chains in proteasomal degradation. *Cell* 2009. **137**: 133–145.

37 **Kriegenburg, F., Ellgaard, L. and Hartmann-Petersen, R.**, Molecular chaperones in targeting misfolded proteins for ubiquitin-dependent degradation. *FEBS J.* 2012. **279**: 532–542.

38 **Rivoltini, L., Kawakami, Y., Sakaguchi, K., Southwood, S., Sette, A., Robbins, P. F., Marincola, F. M. et al.**, Induction of tumor-reactive CTL from peripheral blood and tumor-infiltrating lymphocytes of melanoma patients by in vitro stimulation with an immunodominant peptide of the human melanoma antigen MART-1. *J. Immunol.* 1995. **154**: 2257–2265.

39 **Fiebiger, B. M., Moosmann, A., Behrends, U. and Mautner, J.**, Mature proteins derived from Epstein-Barr virus fail to feed into the MHC class I antigenic pool. *Eur. J. Immunol.* 2012. **42**: 3167–3173.

40 **Nimmerjahn, F., Milosevic, S., Behrends, U., Jaffee, E. M., Pardoll, D. M., Bornkamm, G. W. and Mautner, J.**, Major histocompatibility complex class II-restricted presentation of a cytosolic antigen by autophagy. *Eur. J. Immunol.* 2003. **33**: 1250–1259.

41 **Holzel, M., Rohrmoser, M., Orban, M., Homig, C., Harasim, T., Malamoussi, A., Gruber-Eber, A. et al.**, Rapid conditional knock-down-knock-in system for mammalian cells. *Nucleic Acids Res.* 2007. **35**: e17.

42 **Johnson, E. S., Bartel, B., Seufert, W. and Varshavsky, A.**, Ubiquitin as a degradation signal. *Embo. J.* 1992. **11**: 497–505.

Abbreviations: **FCS**: fetal calf serum · **SLN**: sarcolipin

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