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*J Immunol* 2012; 189:1868-1877; Prepublished online 6 July 2012; doi: 10.4049/jimmunol.1103592

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Why the Structure but Not the Activity of the Immunoproteasome Subunit Low Molecular Mass Polypeptide 2 Rescues Antigen Presentation

Michael Basler,*† Christoph Lauer,† Jacqueline Moebius,‡ Reinhold Weber,§ Michael Przybylski,‡ Alexei F. Kisselev,§ Christopher Tsu,* and Marcus Groettrup*†

The proteasome is responsible for the generation of most epitopes presented on MHC class I molecules. Treatment of cells with IFN-γ leads to the replacement of the constitutive catalytic subunits β1, β2, and β5 by the inducible subunits low molecular mass polypeptide (LMP) 2 (β1i), multicatalytic endopeptidase complex-like-1 (β2i), and LMP7 (β5i), respectively. The incorporation of these subunits is required for the production of numerous MHC class I-restricted T cell epitopes. The structural features rather than the proteolytic activity of an immunoproteasome subunit are needed for the generation of some epitopes, but the underlying mechanisms have remained elusive. Experiments with LMP2-deficient splenocytes revealed that the generation of the male HY-derived CTL-epitope UTY246–254 was dependent on LMP2. Treatment of male splenocytes with an LMP2-selective inhibitor did not reduce UTY246–254 presentation, whereas silencing of β1 activity increased presentation of UTY246–254. In vitro degradation experiments showed that the caspase-like activity of β1 was responsible for the destruction of this CTL epitope, whereas it was preserved when LMP2 replaced β1. Moreover, inhibition of the β5 subunit rescued the presentation of the influenza matrix 58–66 epitope, thus suggesting that a similar mechanism can apply to the exchange of β5 by LMP7. Taken together, our data provide a rationale why the structural property of an immunoproteasome subunit rather than its activity is required for the generation of a CTL epitope. The Journal of Immunology, 2012, 189: 1868–1877.

The proteasome plays a critical role in the generation of peptides from intracellular Ags that are presented to CTLs by MHC class I molecules. The proteasome is the major cytosolic endoprotease in eukaryotes consisting of α and β subunits that build a barrel-shaped complex of four rings with seven subunits each (called 20S proteasome) (1, 2). The outer two rings consist of α subunits, the inner two rings of β subunits forming the central proteolytic chamber. Three different β subunits (β1, β2, and β5) are responsible for at least three peptidase activities, as follows: caspase-like, trypsin-like, and chymotrypsin-like activities, respectively (3, 4). During an immune response in the context of IFN-γ and TNF-α, the inducible proteolytically active subunits low molecular mass polypeptide (LMP) 2 (β1i), multicatalytic endopeptidase complex-like-1 (MECL-1) (β2i), and LMP7 (β5i) are incorporated during neosynthesis into the so-called immunoproteasome. The immunological benefit gained by this energy-consuming process is attributed to minor structural changes within the proteasome and an altered cleavage pattern of the multicatalytic complex, thus optimizing quantity and quality of the generated peptides for presentation by MHC class I molecules (5–8). The immunosubunits play a pivotal role in class I ligand generation, and therefore shape the naïve CD8+ T cell repertoire in the thymus and cytotoxic T cell responses in the periphery (9–13). Recently, novel functions of immunoproteasomes in autoimmune diseases, T cell expansion, and protection from immunopathological damage in the brain have been proposed (14–17).

Replacement of β1 by LMP2 reduces the caspase-like activity to background levels (18, 19). Conversely, LMP2-deficient proteasomes derived from gene-targeted mice display an enhanced cleavage after acidic amino acids (20). In contrast, the function of the exchanges of β2 and β5 by MECL-1 or LMP7 is poorly understood. Based on structure prediction, neither the exchange of LMP7 for β5 nor that of MECL-1 for β2 is expected to grossly alter the binding characteristic of the S1 pocket (1). In accordance with this, two studies proposed that the effect of LMP7 on epitope generation might rely not only on its catalytic activity, but also on structural alterations of the proteasome caused by LMP7 (21, 22). Efficient generation of a hepatitis B virus CTL epitope relied on the structural presence of LMP7 in the proteasome, but not on the activity of LMP7 (22). Similarly, an immunodominant human CTL epitope derived from influenza virus matrix protein required the structural features of LMP7, but not its catalytic activity (21).

In this study, we investigated the mechanistic basis as to why the structural properties rather than the catalytic activity of an immunoproteasome subunit may affect Ag presentation. While investigating why the deletion but not the inhibition of LMP2...
interferes with the presentation of the male HY-derived minor CTL epitope UTY366-374. We found that the generation of this epitope relied on the replacement of the caspase-like activity of β1 by LMP2 because the β1 activity interfered with processing and presentation of this epitope. Additionally, we could demonstrate that a similar mechanism protects the influenza HLA-A0201 influenza matrix M1 58–66 epitope from degradation by β5.

Materials and Methods

Mice, viruses, cell lines, and media

C57BL/6 mice (H-2b) and BALB/c mice (H-2d) were originally purchased from Charles River, MECL-1 (10), LMP2 (20), and LMP7 (23) gene-targeted mice were provided by J. Monaco (University of Cincinnati, Cincinnati, OH), whereas PA28b targets were provided by F. Lehmann-Grube (Heinrich-Pette Institute, Hamburg, Germany) and contained GlutaMAX, 10% FCS, and 100 U/ml penicillin/streptomycin.

Synthetic peptides

The synthetic peptides UTY246–254, UTY238–262, and UTY248–253 were provided by V. Cerundolo, University of Oxford. They were propagated on BSC40 cells. Lymphocytic choriomeningitis virus (LCMV)-WE was obtained from F. Lehmann-Grube (Heinrich-Pette Institute, Hamburg, Germany) and contained GlutaMAX, 10% FCS, and 100 U/ml penicillin/streptomycin.

Purification of 20S proteasome from mouse organs and fluorogenic assays

The lysis of organ tissues, the purification of 20S proteasome from mouse liver cDNA, and the quantification of the 20S proteasome were performed, as described previously (26). Purified proteasomes were analyzed by two-dimensional gel electrophoresis. Hydrolytic assays for proteasome activity using fluorogenic substrates were performed, as described previously (26). Substrates were dissolved in DMSO and used at 12.5 μM (Ac-PAL-AMC; Millenium Pharmaceuticals) or at 100 μM (Suc-L-LYS-AMC, Bz-VRG-AMC, Z-LLE-AMC) as forward and 5′-GGATGGCTCGAGGGGAGGTGGCAACGTGGCAATG-3′ as reverse primer. The PCR product was cloned into EcoRI and XhoI sites into the plasmid pcDNA6/myc-HisA.

Transfections

B8 cells overexpressing LMP7 and H-2Db (B8-LMP7/D6) were transfected with an expression plasmid encoding β1WT-myc/His or β1T1A-myc/His, as previously described, using FuGene 6 (Roche, Basel, Switzerland) (28). Clonal and blasticidin (Invitrogen Life Technologies; 5 μg/ml) resistant cells were tested for β1WT or β1T1A expression by Western blot. For transient transfections, 2 × 106 B8-LMP7/D6 cells were transfected with either a construct encoding LMP2WT (19) or a construct encoding a mutated inactive LMP2 T1A subunit (LMP2T1A) (19) using Amaxa Nucleofector (Amaxa Biosystems), according to the manufacturer’s instructions. HEK293T cells were transfected with an expression plasmid encoding the full-length influenza matrix M1 protein (a gift of S. Ludwig, Muenster, Germany) or an expression construct encoding GFP (pEGFP-N1; Clontech) using Xect (Clontech), according to the manufacturer’s protocol.

Western blot

β1WT-myc/His– or β1T1A-myc/His–transfected and untransfected B8-LMP7/D6 cells were lysed in 150 μl lysis buffer (10 mM Tris HCl pH 7.8, 150 mM NaCl, 1% Triton X-100) on ice for 20 min. Proteins were separated by SDS-PAGE (8% gel), blotted onto nitrocellulose (Schleicher & Schuell BioSciences, Dassel, Germany), blocked (3% BSS/0.2% Tween 20) for 1 h, and agitated overnight at 4°C with an anti-polyhistidine peroxidase-labeled mouse mAb (1:4000, clone HIS-1; Sigma-Aldrich). After extensive washing with PBS/0.2% Tween 20, proteins were visualized on x-ray films by ECL.

Metabolic labeling, immunoprecipitation, and two-dimensional gel electrophoresis

B8-LMP7/D6 cells, either untransfected or stably transfected with β1WT-myc/His or β1T1A-myc/His expression constructs, were starved in cytoine/methionine-free RPMI 1640 (containing 10% dialyzed FCS) for 1 h at 37°C, then labeled with 0.2 μCi/ml Met/Cys (55S)-label (Hartmann Analytic, Braunschweig, Germany) for 2 h, and finally chased for 16 h to allow full maturation of the proteasome. Cells were washed with PBS, harvested, and lysed for 30 min in ice with 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 mM MgCl2, and 2% Triton X-100. The lysate was precleared for 1 h with protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden), followed by overnight immunoprecipitation with an anti-proteasome serum (7) bound to protein A-Sepharose at 4°C. The precipitates were washed four times with NET-TON buffer (650 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.8, 0.5% 0.05% NaN3, 1 mg/ml OVA) and twice with NET-T (150 mM NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.8, 0.05% NaN3, 0.5% OVA), separated on an equilibrium pH-gradient gel electrophoresis/SDS-PAGE, as described previously (5); and visualized by autoradiography on a Fuji BAS1500 radioimager.

Proteasome immunoprecipitation

To determine the proteasomal activity in lysates with the fluorogenic substrate Ac-PAL-AMC (Millenium Pharmaceuticals), splenocytes were incubated with inhibitors or left untreated for 2 h at 37°C. After extensive washing, equal amounts of cells were lysed (50 mM Tris HCl pH 7.8, 150 mM NaCl, 1% Triton X-100) for 20 min on ice. The lysates were centrifuged for 10 min at 20,800 × g to remove debris. Three microliters of polyclonal rabbit anti-mouse proteasome Ab (29) and 50 μl protein A-Sepharose CL-4B (Amersham Biosciences, Uppsala, Sweden) were included by guest on June 7, 2017 http://www.jimmunol.org/ Downloaded from
Proteasomal fragmentation of polypeptide and mass-spectrometric analysis of peptide products

Digestions of the 25-mer polypeptide spanning UTy residues 238–262 with purified 20S proteasomes were performed for indicated time periods, exactly as previously described (7), and analyzed by HPLC–electrospray ionization mass spectrometry (ESI-MS) to quantify the degradation peptides. The liquid chromatography-mass spectrometry systems consisted of an Agilent (Palo Alto, CA) 1100 HPLC system with a G1312A autosampler, G1312A binary pump and G1322A on-line degasser, and an ESQUIRE 3000+ (Bruker Daltonik, Bremen, Germany) ESI ion trap mass spectrometer. Control software was HyStar 3.1, and data processing and evaluation were performed using DataAnalysis 3.2 (both Bruker Daltonik).

HPLC parameters were as follows: solvent A 0.2% aqueous solution of HCOOH, solvent B 0.2% HCOOH in CH3CN, flow rate 0.05 ml/min, and the following gradient: 0 min: 0% B; 4 min: 0% B; and 44 min: 80% B. The column was a Bio WidePore (Supelco) C18, 3 μm 1.0 D, 10 cm × 1 mm. Sample injection volume was 8 μl. Mass spectrometry (MS) conditions were as follows: positive ion mode, mass range 300–1300 m/z, HV capillary 4000.0 V, capillary exit 120.0 V, skimmer 40.0 V, drying gas flow 9.0 l/min, drying gas T = 300°C, and nebulizing gas pressure 20.0 μl. MS/MS fragmentation was carried out automated using the following capillary 4000.0 V, capillary exit 120.0 V, skimmer 40.0 V, drying gas flow conditions were as follows: positive ion mode, mass range 300–1300 m/z, HV

Proteasome inhibitor

ML604440 (Millennium Pharmaceuticals) was dissolved at a concentration of 10 mM in DMSO and stored at –20°C. The β1-specific inhibitor az-NC-002 (N-Az-Gly-Ala-Pro-Leu-Leu-ek) (30) was stored at –20°C at 10 mM in DMSO. The β5 (PR-825)- and β3 (PR-957) (Onyx Pharmaceuticals)-specific inhibitors were dissolved at a concentration of 10 mM in DMSO and stored at –20°C.

Influenza Ag presentation assay

Human CTL lines to the HLA-A0201 influenza matrix M1 58–66 epitope were generated, as detailed previously (31). Shortly, PBMC from healthy HLA-A0201+ donors were restimulated with γ-irradiated (35 Gy) PBMC pulses with influenza matrix M1 58–66 peptide at a concentration of 1 μg/ml in the presence of 20 μM/ml 2-β-mercaptoethanol and 25 nM/ml 2-β-mercaptoethanol in weekly intervals over a period of 6 wk. The specificity of the CTL lines was assessed by intracellular cytokine staining for IFN-γ, as previously described (32). IFN-γ/TNF-α (200/400 U/ml)–stimulated or unstimulated HEK293T cells were transiently transfected with an expression plasmid encoding the full-length influenza matrix M1 protein. Sixteen hours later, cells were treated with 200 nM PR-825 or 300 nM ONX 0914, or were left untreated for 8 h. Cells were washed and coincubated with CTL lines at a ratio of 105 CTLs to 5 × 104 APCs in 96-well plates. After 24 h of coincubation, supernatants were analyzed for IFN-γ content using a human IFN-γ ELISA kit (BD Biosciences).

Statistical analysis

The statistical significance of the differences was determined using the Student t test. All statistical analyses were performed using GraphPad Prism Software (version 4.03; GraphPad, San Diego, CA). Statistical significance was achieved when p < 0.05. *p < 0.05, **p < 0.01, ***p < 0.001; n.s., not significant.

Results

The HY-Ag–derived MHC-I epitope UTY246–254 is LMP7 and LMP2 dependent

Generation of the CTL epitope UTY246–254 (ubiquitously transcribed tetratricopeptide repeat gene, Y-linked), derived from the endogenously expressed Y-chromosome–encoded HY-Ag, has been demonstrated to be LMP7 dependent (14, 33). To confirm this result and to investigate whether the other immunoproteasome subunits LMP2 and MECL-1 were similarly affecting this epitope, UTY246–254 presentation on the H-2Dβ class I molecule of male-derived splenocytes was determined with a UTY246–254-specific T cell hybridoma in lacZ assays (Fig. 1A). Splenocytes were used in this assay because of their constitutive expression of immunoproteasomes, independent of cytokine induction (10, 34). Presentation of UTY246–254 on splenocytes derived from male mice was reduced to background levels of C57BL/6 female mice. LMP2–/− and MECL-1–/−/LMP7–/− mice demonstrated a strong reduction in UTY246–254 presentation compared with male C57BL/6 mice. In contrast, no reduction could be observed for MECL-1–/− single-deficient mice. Investigation of mice deficient for the immunoproteasome 11S regulators PA28aβ and PA28γ revealed no altered presentation of UTY246–254 (Fig. 1B). Taken together, LMP2 and MECL-1 deficiency abrogates UTY246–254 presentation on male splenocytes.

LMP2-selective inhibition does not alter UTY246–254 presentation

Recently, we have demonstrated that LMP7 inhibition by the LMP7-selective inhibitor ONX 0914 reduces UTY246–254 presentation in a concentration-dependent manner (14). As UTY246–254 presentation is abrogated in LMP2-deficient mice (Fig. 1A), we investigated UTY246–254 presentation with the LMP2-selective inhibitor ML604440. ML604440 is a dipetide boronate bearing the same chemical warhead as the clinically employed immunoproteasome inhibitor bortezomib (Fig. 2A) (35) (U.S. Patent No. 7,838,673 B2). To demonstrate the LMP2 specificity of ML604440, the activity of purified immunoproteasomes derived from LCMV-WE–infected livers of WT or LMP2-deficient mice was analyzed. Previous studies have reported that in immunoproteasomes derived from livers of LCMV-WE–infected mice, the three catalytically active constitutive subunits, β1, β2, and β5, are almost completely replaced by the immunoproteasome subunits LMP2, MECL-1, and LMP7 (36). LMP2-deficient and WT immunoproteasomes (from the livers of LCMV-WE–infected mice) were incubated with different concentrations of ML604440 and assayed with a fluorogenic substrate specific for LMP2 activity (Ac-PAL-AMC) (37) (Fig. 2B). In contrast to WT–derived proteasome, absolutely no cleavage of this substrate could be observed with LMP2-deficient proteasome, demonstrating the LMP2 specificity of Ac-PAL-AMC. In a concentration range between 12.5 and 200 nM, ML604440 reduced the cleavage of Ac-PAL-AMC by proteasomes from WT mice in a dose-dependent manner down to background levels (Fig. 2B). The chymotrypsin-like (Suc-LLVY-AMC–hydroylizing) activity, characteristic for β5/LMP7, was not altered in a concentration range between 12.5 and 1000 nM, demonstrating the LMP2 specificity of ML604440. To investigate whether ML604440 is able to modulate UTY246–254 Ag presentation, male-derived splenocytes from C57BL/6 mice were treated with inhibitor concentrations ranging from 25 to 1000 nM and assayed with a UTY246–254–specific T cell hybridoma in lacZ assays (Fig. 2C). Unexpectedly, no change in peptide presentation could be observed, even not at high inhibitor concentrations. In contrast, UTY246–254 presentation on male LMP2-deficient and female mice was reduced to background levels. To ensure that ML604440 is cell permeable, splenocytes were incubated with 200 or 500 nM ML604440 at identical conditions as used in Fig. 2C. After extensive washing, cells were lysed and the proteasome was immunoprecipitated. The precipitated proteasome was assayed for the cleavage of the LMP2-specific substrate Ac-PAL-AMC (Fig. 2D). Whereas the substrate was converted by proteasomes from untreated cells, proteasomes derived from splenocytes incubated with 200 or 500 nM ML604440 demonstrated strongly reduced activity. To analyze whether LMP2-deficient cells are principally able to present UTY246–254, splenocytes derived from LMP2-KO mice were infected either with a recombinant VV expressing the UTY246–254 minimal epitope (VV-MG) or with a VV expressing the full-length protein (VV-FL). UTY246–254 presentation was determined with UTY246–254–specific T cell hybridomas (Fig. 2E). UTY246–254 presentation on LMP2–/− and LMP7–/− cells infec-
uted with VV-FL was strongly reduced compared with WT cells, whereas infection of LMP2−/− and LMP7−/− splenocytes with VV-MG showed similar presentation as C57BL/6 WT cells. Hence, LMP2-deficient cells are able to present UTY246–254, but the processing of this epitope from the full-length UTY protein is hampered. We conclude that ML604440 is cell permeable (Fig. 2D) and specifically inhibits LMP2 activity (Fig. 2B, 2D), but is not able to reduce UTY246–254 Ag presentation (Fig. 2C), although this CTL epitope is LMP2 dependent, as demonstrated with LMP2-deficient cells (Figs. 1A, 2E).

**UTY246–254 presentation is dependent on the structure of LMP2**

Two publications report on two different immunoproteasome-dependent epitopes, which require the structural features of LMP7,

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**FIGURE 1.** Influence of immunoproteasome subunits on the presentation of the male HY-derived epitope UTY246–254. (A) The presentation of UTY246–254 on splenocytes derived from male C57BL/6, MECL-1−/−, LMP2−/−, LMP7−/−, MECL-1−/−/LMP7−/−, or MECL-1−/−/LMP2−/− mice and from control female C57BL/6 mice (indicated on x-axis) was determined with a UTY246–254-specific T cell hybridoma in chromogenic lacZ assays. The y-axis shows absorbance of enzymatically converted chromogen at 570 nm in lacZ assays. The values are the means ± SD of three replicate cultures. (B) UTY246–254 presentation on male splenocytes derived from PA28α−/−β− or PA28β−/− mice as determined by chromogenic lacZ assays. The experiments have been performed three times, yielding similar results. ***p < 0.001.

**FIGURE 2.** UTY246–254 presentation is not altered by an LMP2-selective inhibitor. (A) Structure of ML604440. (B) Quantification of hydrolysis of fluorogenic substrates (Ac-PAL-AMC; Suc-LLYV-AMC) of 20S proteasomes from livers of LCMV-WE–infected WT or LMP2−/− mice at various ML604440 inhibitor concentrations. Data are presented as the means ± SD of relative activity from triplicate assays. The experiments were repeated three times with similar results. (C) Splenocytes derived from male C57BL/6 mice were treated with various inhibitor concentrations overnight (x-axis). Female C57BL/6- or male LMP2−/−-derived splenocytes were used as negative controls. The UTY246–254 presentation on MHC-I was analyzed with a H-2Db/UTY246–254-specific T cell hybridoma. The y-axis shows absorbance of enzymatically converted chromogen at 570 nm in lacZ assays. The values are the means ± SD of three replicate cultures. (D) ML604440 is cell permeable. Splenocytes were treated with 200 or 500 nM ML604440 overnight, or were left untreated. Proteasomes of crude lysates of these cells were immunoprecipitated and assayed for the hydrolysis of the fluorogenic substrate Ac-PAL-AMC. The y-axis shows fluorescence at 360/465 nm. The experiments have been performed three times, yielding similar results. (E) Female splenocytes derived from C57BL/6, LMP2−/−, or LMP7−/− mice infected with a VV expressing the full-length UTY protein (VV-FL) or the UTY246–254 minigene (VV-MG). The UTY246–254 presentation on MHC-I was analyzed with a H-2Dβ/UTY246–254-specific T cell hybridoma. IL-2 production in supernatants of cocultures was measured by ELISA. The values are the means ± SD of three replicate cultures. The experiments have been performed twice, yielding similar results. ***p < 0.001.
lytically inactive LMP2T1A enabled UTY246–254 presentation, we detected between the WT and mutant subunit. As even the catalytic activity of LMP2 was decreased in both LMP2WT- and LMP2T1A-transfected cells compared with nontransfected cells, but no difference could be explained our results. Structural features of LMP2, which are absent in LMP2-deficient cells, might be required for the processing of the UTY246–254 epitope. In contrast, inhibition of the catalytic activity of LMP2 by ML604440, which does not affect structural alterations caused by LMP2, has no influence on UTY246–254 presentation. To test this hypothesis, B8-LMP7/D0 were transiently transfected with either a construct encoding LMP2WT or a construct encoding a T1A mutant of LMP2 in which the catalytically active N-terminal threonine of the processed subunit was replaced by alanine (LMP2T1A). As UTY246–254 presentation is not only LMP2, but also LMP7 dependent (Fig. 1), we choose a B8 fibroblast-derived cell line as recipient that stably overexpresses LMP7 (B8-LMP7/D0) (9). The mutated LMP2T1A has been shown to become incorporated into mature proteasomes, but remains catalytically inactive (19). The transfected cells were infected with a VV expressing the full-length UTY protein, and UTY246–254 presentation was determined with a UTY246–254-specific T cell hybridoma (Fig. 3). UTY246–254 presentation was increased in both LMP2WT- and LMP2T1A-transfected cells compared with nontransfected cells, but no difference could be detected between the WT and mutant subunit. As even the catalytically inactive LMP2T1A enabled UTY246–254 presentation, we conclude that the catalytic activity is not required for the preservation of this epitope.

**UTY246–254 is preferentially cleaved by WT proteasome**

Proteasomal cleavages at the C terminus of acidic amino acids have been shown to be downregulated by the replacement of the constitutive subunit β1 by LMP2 (β1i). Overexpression of either mutated or WT LMP2 reduced the caspase-like activity of the proteasome almost to background levels (5, 19, 38). Conversely, proteasomes derived from LMP2-deficient mouse splenocytes display increased caspase-like activity compared with WT proteasomes. These observations would be consistent with an increased cleavage within the UTY246–254 epitope by WT proteasome as the UTY protein contains an aspartic acid at position 252 (Fig. 4A), thus leading to a destruction of UTY246–254. To address this hypothesis, we investigated how WT and LMP2-deficient immunoproteasomes fragment the 25-mer precursor polypeptide covering residues 238–262 of the UTY protein (Fig. 4A), which contains the 9-meric 246–254 epitope. The 20S proteasomes from the liver of LCMV-infected WT or LMP2-deficient mice (day 8 postinfection) were isolated as a source of immunoproteasomes. The subunit composition of the two proteasome populations on two-dimensional gels confirmed our previous finding that LCMV infection results in a virtually complete replacement of constitutive proteasomes by immunoproteasomes in vivo (36), with LMP2 being absent from proteasomes purified from LMP2-deficient mice (data not shown). To obtain quantitative information on how the 25-mer precursor was differentially fragmented by LMP2-deficient and WT immunoproteasomes, the produced fragments obtained after 8 h of in vitro digest were analyzed by liquid chromatography–mass spectrometry. The time period of 8 h was chosen because the 25-mer substrate was still in excess of the reaction products and none of the products generated at earlier time points appeared to be attacked by proteasomes in a second round of cleavage. A further fragmentation of primary fragments was therefore unlikely to occur (data not shown). A comparison of the peak intensities of the total ion chromatogram corresponding to selected fragments, which could be unambiguously identified by their masses, revealed that fragments originating from C-terminal cleavage of Asp252 (I253–R262; L257–N257; Q253–D252) were produced more frequently by LMP2-deficient proteasomes, resulting in a destruction of the UTY246–254 epitope.

**A β1 inhibitor rescues the UTY246–254 epitope**

In LMP2-deficient mice, the constitutive subunit β1 is incorporated into proteasomes instead of LMP2. Our ESi-MS data (Fig. 4B) suggested an enhanced cleavage after aspartic acid residue 252 by the β1 subunit. Hence, inhibition of β1 activity should rescue UTY246–254 epitope presentation. Az-NC-001 (30) is the epoxyketone analog of a β1/β1i-specific vinyl sulfone inhibitor [described as compound 4 in van Swieten et al. (39)]. To confirm the β1 specificity of Az-NC-001 in mice, purified proteasomes de-
rived from livers of WT mice were incubated with different concentrations of Az-NC-001 and assayed with a fluorogenic substrate specific for the chymotrypsin-like activity (Suc-LLVY-AMC), the trypsin-like activity (Bz-VGR-AMC), and the caspase-like activity (Z-LLE-βNA) (Fig. 5A). Az-NC-001 reduced the β1-specific caspase-like activity to background levels in a concentration-dependent manner, whereas the chymotrypsin- and trypsin-like activities were not influenced by Az-NC-001. To investigate whether UTY246–254 presentation is altered by Az-NC-001, male-derived splenocytes from C57BL/6 mice were treated with inhibitor concentrations ranging from 500 to 3000 nM and assayed with the UTY246–254-specific T cell hybridoma in lacZ assays (Fig. 5B). UTY246–254 presentation by male cells was increased in a dose-dependent manner, almost doubling at 3000 nM. Presentation of UTY246–254 on LMP2-deficient male splenocytes, which is almost reduced to background levels found in splenocytes from female mice (Fig. 1), was rescued with increasing Az-NC-001 concentrations (Fig. 5C). These results reveal that UTY246–254 is destroyed by β1 and can be rescued by a β1 inhibitor.

**Overexpression of a mutant β1 subunit increases UTY246–254 presentation**

To further confirm these experiments, we chose a genetic approach by overexpressing a mutant T1A variant of the proteasome subunit β1. B8-LMP7/D7 cells were stably transfected with either a construct encoding β1WT or a construct encoding a catalytically inactive T1A mutant of β1 (β1T1A) (Fig. 6A). To address to which extent β1WT-myc/His and β1T1A-myc/His are incorporated into the proteasome, these stably transfected cells were metabolically labeled with [35S]Met/Cys. Fully mature proteasomes were immunoprecipitated, and the subunit composition was analyzed by nonequilibrium pH-gradient gel electrophoresis/SDS-PAGE. myc/His-tagged β1WT and β1T1A (bearing 13 Met/Cys in the primary structure) were incorporated to 82% compared with endogenous β1 (bearing 12 Met/Cys in the primary structure) as determined by densitometric analyses (Fig. 6B). The transfected cells and control cells were infected with a virus expressing the full-length UTY protein, and UTY246–254 presentation was determined by a UTY246–254-specific T cell hybridoma (Fig. 6C). UTY246–254 presentation was comparable in both untransfected and β1WT-transfected cells, but strikingly increased in cells overexpressing the T1A mutant of β1. Hence, overexpression of a catalytically inactive β1 subunit rescued UTY246–254 presentation, confirming cleavage of this epitope by the constitutive proteasome subunit β1.

**β5 inhibition rescues presentation of the HLA-A0201 influenza matrix M1 58–66 epitope**

We found that the cleavage activity of LMP2 is not needed for the UTY246–254 presentation (Fig. 2C), but rather its structural presence in proteasomes (Fig. 1A). A similar phenomenon was reported for the HLA-A0201 influenza matrix M1 58–66 epitope, which is dependent on the structural presence of LMP7, but not on its catalytic activity (21). Therefore, we investigated whether LMP7 protects this influenza matrix epitope from degradation by the constitutive subunit β5. HEK293T cells were stimulated for 3 d with IFN-γ/TNF-α (Fig. 7D) to induce immunoproteasome expression or were left untreated (Fig. 7C). Stimulation of cells was confirmed by upregulation of HLA-A2 surface expression by flow cytometry (Fig. 7A). Cells were transiently transfected with a plasmid encoding the full-length influenza matrix M1 protein. Transfection efficiency 48 h posttransfection was >90% as determined by flow cytometry of cells transfected with an expression construct encoding GFP (Fig. 7B). Influenza matrix protein-transfected cells were treated with 200 nM β5-selective inhibitor PR-825 (14) or 300 nM β5i-selective inhibitor ONX 0914 (14). After coincubating these cells with influenza matrix protein-activated CTLs for 24 h, the activation of CTLs was determined by quantitative assessment of IFN-γ in the supernatant by ELISA. No presentation of the influenza matrix 58–66 epitope could be detected in unstimulated transfected cells (Fig. 7C). Whereas β5i inhibition did not alter influenza matrix 58–66 epitope presentation, β5 inhibition strongly increased stimulation of influenza matrix M1 58–66 peptide.
As negative control, nontransfected cells (Ø) were used. The arrow indicates the overexpression. Lysates of cells overexpressing myc/His-tagged influenza matrix M1 58–66 epitope from cleavage by hybridomas. IL-2 production in supernatants of cocultures was measured by ELISA. The values are the means ± SD of three replicate cultures. The experiments have been performed three times, yielding similar results.

Overexpression of a mutant β1 subunit increases UTY246–254 presentation. (A) Western blot analysis of β1WT-myc/His or β1T1A-myc/His overexpression. Lysates of cells overexpressing myc/His-tagged β1WT or β1T1A were probed with an anti-polyhistidine peroxidase-labeled mouse mAb. As negative control, nontransfected cells (Ø) were used. The arrow indicates the β1 subunit. (B) Subunit composition of proteasomes from B8-LMP7/D0 cells either left untransfected (indicated Ø) or stably transfected for β1WT-myc/His or β1T1A-myc/His. Cells were metabolically labeled with [35S] methionine/cysteine. Proteasomes were immunoprecipitated and analyzed for their subunit composition on two-dimensional gels by autoradiography. Endogenous β1, β1WT-myc/His, or β1T1A-myc/His are labeled. (C) Untransfected B8-LMP7/D0 cells or cells overexpressing β1WT or β1T1A were infected with a VV expressing the UTY protein or were left uninfected. The UTY246–254 presentation was analyzed with H-2D\textsuperscript{b}/UTY246–254-specific T cell hybridomas. IL-2 production in supernatants of cocultures was measured by ELISA. The values are the means ± SD of three replicate cultures. The experiments have been performed three times, yielding similar results.

The proteasome is centrally involved in MHC-I peptide generation and can directly produce peptides in their final form or as N-terminally extended precursors. In the context of IFN-γ or TNF-α, the three catalytically active subunits β1, β2, and β5 of the proteasome are replaced by LMP2, MECL-1, and LMP7, building the so-called immunoproteasome. These exchanges have been associated with more efficient MHC-I Ag processing. Cells expressing immunoproteasomes display a different peptide repertoire, changing the overall cytotoxic T cell specificity (6). Indeed, CTL responses to numerous viral or tumor epitopes are influenced by immunoproteasome formation (9, 11, 40, 41). How does the immunoproteasome alter the peptide repertoire presented on cells? The exchange of β1 by LMP2 leads to a reduced caspase-like activity of the proteasome, and thus to reduced generation of peptides with an acidic C terminus. As no class I molecules exist in the mouse and human that bind proteasome products with acidic C termini, LMP2 incorporation may have evolved to suppress the proteasomal caspase-like activity. This change in cleavage specificity is in perfect agreement with predictions based on the x-ray crystallographic structure of 20S proteasomes that anticipate that the S1 pocket of β1, which binds the C-terminal P1 residue of a peptide, contains a positively charged arginine as counter residue, which in β1L (LMP2) is replaced by a leucine, thus rendering the pocket more apolar and causing the observed loss of the caspase-like activity in immunoproteasomes (1, 2).

In this study, we have identified how LMP2 can exert its function in Ag processing. When we analyzed the presentation of the endogenously expressed male minor Ag UTY on mouse splenocytes, we observed that the epitope is not only LMP7 dependent, as previously reported (14, 33), but also dependent on LMP2 (Figs. 1A, 2E). In contrast, splenocytes derived from MECL-1\textsuperscript{−/−}, PA28γ\textsuperscript{−/−}, or PA28α\textsuperscript{−/−}/β\textsuperscript{−/−} mice presented the UTY epitope similarly as WT male mice (Fig. 1A, 1B). To confirm these results, we treated male splenocytes with ML604440 (Fig. 2A), a highly selective inhibitor for LMP2 (Fig. 2B). Although this inhibitor silenced LMP2 activity in splenocytes (Fig. 2D), a reduction of UTY246–254 presentation could, in contrast to LMP2-deficient cells, not be observed (Fig. 2C). Therefore, we concluded that it is not the catalytic activity of LMP2, which is silenced by the selective inhibitor, but the structural contribution of LMP2 that is crucial for the presentation of UTY246–254. Indeed, overexpression of a catalytically inactive LMP2 variant increased UTY presentation similarly to overexpression of an intact LMP2 (Fig. 3). This result confirms that the cleavage activity of LMP2 is not needed, but rather its structural presence in proteasomes. A similar phenomenon was reported for two LMP7-dependent epitopes (21, 22).
Generation of the HLA-A0201 influenza matrix 58–66 epitope was impaired by the deletion of LMP2 and LMP7. This block in Ag presentation could be relieved by transfection of LMP7 cDNA into LMP7-deficient cells. Interestingly, a mutated form of LMP7, lacking the catalytically active threonine, was equally capable of relieving the block of presentation of the influenza matrix A2 epitope (21). Similarly, presentation of the HLA-Aw68–restricted hepatitis B virus core Ag required the concerted presence of the three immunosubunits, although both catalytically active LMP7 and inactive LMP7 T1A supported CTL epitope generation (22). In contrast to our experiments, no destructive cleavage with constitutive proteasome has been observed for the HLA-Aw68–restricted hepatitis B virus core Ag epitope (22). The authors concluded that LMP7 influences the structural features of 20S proteasomes, thereby enhancing the activity of the LMP2 and MECL-1 catalytic sites, which provide cleavage specificity. Thus, LMP7 incorporation is of greater functional importance for the generation of this HBV CTL epitope than cleavage specificity. However, how an immunosubunit may exert a structural effect has remained elusive. Therefore, we investigated whether a similar mechanism as observed for the UTY epitope exists for the presentation of the influenza matrix M1 58–66 epitope. Indeed, selective inhibition of the β5 constitutive subunit rescued the presentation of the influenza matrix M1 58–66 epitope (Fig. 7C, 7D). Two publications described an improved presentation of the influenza matrix M1 58–66 epitope after proteasome inhibition with lactacystin (21, 42). Gileadi et al. (21) concluded after rescuing the influenza matrix M1 58–66 epitope presentation with the proteasome inhibitor lactacystin that LMP7 incorporation is of greater importance than the presence of the LMP7's catalytic site. Our results strongly suggest that the influenza matrix M1 58–66 epitope is destroyed by β5 and can be rescued by a β5 inhibitor and, thus, LMP7 induction protects the CTL epitope from destruction by β5. In line with our results are the data from Chapino et al. (43), which demonstrated that the MAGE-C2 336–344 peptide is produced by the immunoproteasome, but not by the constitutive proteasome, because the latter destroys the Ag by making an internal cleavage occurring after an acidic residue.

Several explanations can be proposed as to why the structure of an immunosubunit of the proteasome rather than its activity is required to preserve an epitope. Incorporation of the immunosubunits could influence the conformation of the catalytic chamber of the 20S proteasome and thereby alter substrate specificity. Thereby, a single immunosubunit could alter the specificity of the other

FIGURE 7. β5 inhibition rescues HLA-A0201 influenza matrix M1 58–66 epitope presentation. (A) Flow cytometric analysis of HLA-A2 surface expression of untreated cells (gray) and IFN-γ/TNF-α-treated (black) HEK293T cells. Unstained cells were used as negative control (white). (B) Transfection efficiency of HEK293T transfected with an expression plasmid encoding GFP (gray) or untransfected (black) cells was analyzed by flow cytometry. (C and D) HEK293T cells used as APCs were transfected with an expression plasmid encoding the influenza matrix M1 protein. Cells were treated with a β5i-specific inhibitor (indicated M1 + ONX 0914), a β5-specific inhibitor (indicated M1 + PR-825), or vehicle (indicated M1 + vehicle). Untransfected cells were used as negative control (indicated untransfected). APCs were coincubated for 24 h with influenza matrix 58-66/HLA-A0201–specific CD8+ T cells. Stimulation of CTLs was measured by determination of the IFN-γ content in the supernatant by ELISA. The values are the means ± SD of three replicate cultures. The experiments have been performed three times, yielding similar results. (D) Experiments were performed as before with unstimulated HEK293T cells (C), but, prior to transfection, HEK293T cells were stimulated for 3 d with IFN-γ/TNF-α to induce immunoproteasome expression. **p < 0.01, ***p < 0.001.
subunits irrespective of its own hydrolytic activity. Alternatively, immunosubunit incorporation could influence the substrate accessibility via the regulatory particles PA700 or PA28αβ. Indeed, mice lacking PA28β demonstrated impaired immunosubunit incorporation into 20S proteasomes (44), although these results could not be confirmed in mice lacking both PA28α and PA28β (24). Our results suggest a more simple explanation as to how the structural features of immunosubunits can influence CTL epitope generation. When we scrutinized the amino acid composition of the UTY246–254 CTL epitope, we noticed an aspartic acid at position 252 (Fig. 4A). As β1, the constitutive counterpart of LMP2, is responsible for cleavage after acidic amino acids (caspase-like activity), we hypothesized that β1 is destroying the UTY epitope. Hence, incorporation of LMP2 instead of β1 would prevent cleavage within the UTY epitope. Indeed, HPLC-ESI analysis of in vitro digests with immunoproteasome derived from WT or LMP2-deficient mice of a 25-mer polypeptide embedding the UTY246–254 epitope confirmed a reduced cleavage after Asp252 in LMP2-proficient compared with deficient immunoproteasomes (Fig. 4B), although an additional cleavage (fragment D252–R262) occurred more frequently with WT proteasome. Additionally, treatment of WT splenocytes with a β1-selective inhibitor (Fig. 5A) increased UTY246–254 presentation (Fig. 5B). It is noteworthy to mention that in naïve splenocytes ~50% of mature 20S proteasomes exist as immunoproteasomes (10). LMP2-deficient splenocytes, which incorporate β1 into immunoproteasomes and therefore cannot present UTY246–254 (Figs. 1, 2E, 5C), demonstrated enhanced presentation with increasing β1-inhibitor concentrations in a dose-dependent manner (Fig. 5C). Hence, when the catalytic β1 activity is silenced by a selective inhibitor, the caspase-like activity of β1 is no longer capable to cleave the UTY246–254 epitope. These results were corroborated by a genetic approach using an inactive β1 subunit (β1T1A). Overexpression of this subunit enhanced the presentation of the UTY246–254 CTL epitope (Fig. 6C). Taken together, mutation and inhibition of β1 preserved UTY246–254 epitope presentation, confirming our hypothesis that β1 is responsible for UTY cleavage. Interestingly, induction of the immunoproteasome had two effects on the generation of the UTY CTL epitope. First, induction of LMP2 protected the epitope from destruction by β1, and, second, the incorporation of LMP7 was essential for epitope generation. Experiments using an LMP7-selective inhibitor demonstrated that the catalytic activity of LMP7 was crucial for the production of the UTY epitope (14). As both β5 and LMP7 are responsible for the chymotrypsin-like activity, the mechanism as to how LMP7 alters UTY246–254 processing remains to be determined.

It was hypothesized that a prime function of inducing immunoproteasomes is the selective stimulation of T cells, which are specific for epitopes produced in inflammatory sites and the prevention of autoimmunity (4). Initial priming of CTLs occurs by dendritic cells constitutively expressing high levels of immunoproteasomes (45). Therefore, activation of potentially self-reactive immunoproteasome-dependent CTLs will not lead to undesired tissue destruction in noninflamed organs expressing constitutive proteasome. Our data could support such a hypothesis. Expression of β1 in organs with constitutive proteasomes leads to a destruction of the UTY epitope and therefore protects noninflamed tissues from potential self-reacting UTY-specific CTLs.

Suppressing the activity of the constitutively expressed homologous subunit can also be observed in the thymus, where the recently discovered β5T subunit is expressed exclusively in cortical thymic epithelial cells (46). Incorporation of this subunit instead of β5 reduced the chymotrypsin-like activity, leading to low affinity class I ligands used for positive selection. It has been demonstrated that immunoproteasomes determine the naïve cytotoxic T cell repertoire (10, 11, 47). Medullary thymic epithelial cells responsible for negative selection express both immunoproteasome subunits and constitutive subunits (46, 48). Our results, that an epitope is destroyed by one type of proteasome and generated by the other, support the hypothesis that the expression of both types of proteasomes in medullary thymic epithelial cells is essential to provide maximal diversity of peptides presented on these cells to eliminate all potential self-reactive T cells.

Taken together, the generation of the UTY246–254 epitope relies on the replacement of the caspase-like activity of β1 by LMP2 because the β1 activity destroys the epitope. To our knowledge, this is the first example for the structural requirement of LMP2 for the generation of a CTL epitope. Similar results were obtained for the LMP7-dependent influenza matrix 58–66 epitope. Eliminating the activity of their constitutively expressed homologous subunits may explain the requirement for immunosubunits of the proteasome also for the generation of other Ags. Thus, we have described how LMP2 and LMP7 can exert their function in Ag processing.

Acknowledgments

We thank the Millennium Pharmaceuticals Chemistry Department for providing ML60440. N. Shastri is acknowledged for providing T cell hybridomas, and V. Cerundolo for the contribution of recombinant vaccinia viruses. We thank J. Monaco and T. Chiba for providing gene-targeted mice. S. Ludwig is acknowledged for supplying the full-length influenza matrix M1 protein expression plasmid. We thank C.J. Kirk (Onyx Pharmaceuticals) for providing PR-825 and ONX 0914. Ulrike Beck is acknowledged for excellent technical assistance, and M. Mueller for taking blood samples.

Disclosures

C.T. is an employee of Millennium Pharmaceuticals, Inc., but has no other financial conflicts of interest to disclose. The other authors have no financial conflicts of interest.

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