CD8 T-cells of *Listeria monocytogenes*-infected mice recognize both linear and spliced proteasome products

Anouk C.M. Platteel¹, Michele Mishto²,³, Kathrin Textoris-Taube², Christin Keller², Juliane Liepe⁴, Dirk H. Busch⁵, Peter M. Kloetzel² and Alice J.A.M. Sijts¹,⁶

¹ Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands.
² Institut für Biochemie, Charité - Universitätsmedizin Berlin, Berlin, Germany.
³ Interdepartmental Centre “Luigi Galvani” for Bioinformatics, Biophysics and Biocomplexity (CIG), Alma Mater Studiorum, University of Bologna, Bologna, Italy.
⁴ Centre for Integrative Systems Biology and Bioinformatics, Department of Life Sciences, Imperial College London, London, UK.
⁵ Institute for Medical Microbiology, Immunology and Hygiene, TU Munich, Munich, Germany.

# To whom the correspondence should be addressed:
Dr. Alice Sijts, Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands.
Phone: +31 (0)30 253 2471; Email: e.j.a.m.sijts@uu.nl.
Abstract

CD8 T-cells responding to infection recognize pathogen-derived epitopes presented by MHC class-I molecules. While most of such epitopes are generated by proteasome-mediated antigen cleavage, analysis of tumor antigen processing has revealed that epitopes may also derive from proteasome-catalyzed peptide splicing (PCPS). To determine whether PCPS contributes to epitope processing during infection, we analyzed the fragments produced by purified proteasomes from a *Listeria monocytogenes* polypeptide. Mass spectrometry identified a known H-2K\(^b\)-presented linear epitope (LLO\(_{296-304}\)) in the digests, as well as four spliced peptides that were trimmed by ERAP into peptides with *in silico* predicted H-2K\(^b\) binding affinity. These spliced peptides, which displayed sequence similarity with LLO\(_{296-304}\), bound to H-2K\(^b\) molecules in cellular assays and one of the peptides was recognized by CD8 T-cells of infected mice. This spliced epitope differed by one amino acid from LLO\(_{296-304}\) and double staining with LLO\(_{296-304}\) and spliced peptide-folded MHC multimers showed that LLO\(_{296-304}\) and its spliced variant were recognized by the same CD8 T-cells. Thus, PCPS multiplies the variety of peptides that is processed from an antigen and leads to the production of epitope variants that can be recognized by cross-reacting pathogen-specific CD8 T-cells. Such mechanism may reduce the chances for pathogen immune evasion.

Keywords

Proteasome, proteasome-catalyzed peptide splicing, MHC class I antigen processing, CD8 T-cell, *Listeria monocytogenes*.

Abbreviations

PCPS  Proteasome-catalyzed peptide splicing

LLO  Listeriolysin O
Introduction

CD8 T-cells recognize antigenic peptides that are presented by MHC class I molecules on the cell surface. These epitopes mainly derive from proteasome-mediated processing of intracellular proteins, which produces both final sized epitopes and N-terminally extended epitope precursor fragments that are trimmed to final size by cytosolic or ER-localized amino- and endopeptidases [1, 2]. Epitopes or their precursors are translocated into the ER by TAP and there, if containing a binding motif, are loaded into the antigen binding cleft of MHC class I molecules [3].

Although proteasomes are present in all eukaryotic cells, their subunit composition may vary. Proteasomes consist of four stacked rings, formed of seven subunits each. The two inner rings are composed of β-subunits, of which the three subunits β1, β2 and β5 are constitutively expressed and display catalytic activity. Exposure of cells to inflammatory cytokines, such as IFNγ, induces the expression of the facultative subunits β1i/LMP2, β2i/MECL-1 and β5i/LMP7 that are preferentially incorporated by newly assembled proteasome complexes, leading to the formation of immunoproteasomes [4]. Cells of the immune system express different combinations of the facultative subunits in a constitutive manner.

Proteasomes generate epitopes by simple peptide-bond cleavage as well as by proteasome-catalysed peptide splicing (PCPS), which involves the linkage of fragments originally distant in the parental protein [5-9]. Cleavage by the proteasome is the result of a nucleophilic attack on peptide bonds by the catalytic threonines of the β1, β2 and β5 subunits in constitutive proteasomes, or of the β1i, β2i and β5i subunits in immunoproteasomes. This attack results in the formation of an acyl-enzyme intermediate. These peptides are released from the proteasome by rapid hydrolysis, giving rise to linear proteasome-generated products. However when the acyl-enzyme intermediate is stabilized at the active site for an extended time span, the N-termini of released peptide fragments may outcompete hydrolysis and make a nucleophilic attack on the ester bond of the acyl-enzyme intermediate, forming a new peptide bond and producing a proteasome-catalysed spliced product [10]. Quantitative differences in catalytic activity between proteasome isoforms have been shown to strongly affect antigen presentation of both linear- and spliced epitopes [10-18].
One theoretical outcome of PCPS is to enhance the variety of antigenic epitopes that is presented during infection, which would have implications for the ability of our immune system to tackle pathogens [19, 20]. While PCPS has been proven to generate MHC class I-presented tumor epitopes [5-7, 9, 12], its relevance in immunity is still controversial and no evidence for its involvement in pathogen-specific immune responses has been found so far. In this study, applying a SpliceMet-facilitated reverse immunology approach [21], we identified both spliced and linear H-2Kb-presented epitopes generated by mouse proteasomes from a Listeria monocytogenes-derived model antigen, Listeriolysin O (LLO)291-317. Our studies show that PCPS broadens the peptide repertoire derived from a single antigen.

Results

Digestion of LLO291-317 by purified proteasomes produces LLO296-304 and spliced epitope variants. In previous in vitro studies, digestion of polypeptide substrates by purified 20S proteasomes was shown to lead to the production of both linear- and spliced peptide products [10, 21, 23]. To determine whether PCPS contributes to proteasome-mediated production of MHC class I-presented antigenic peptides, we analysed the products generated in vitro by mouse 20S proteasomes from the polypeptide LLO291-317 by mass spectrometry [10] and evaluated them for their MHC class I binding affinity using the online available NetMHC3.4/ANN prediction software. As expected, this approach identified LLO296-304, a well-known H-2Kb-presented epitope [22] (Table 1). By applying the mass spectrometry-based method (SpliceMet) [21] we further identified four putative epitope precursors in the in vitro digests that were generated by PCPS (Table 1), hereby confirming our previous identifications obtained with 20S human and yeast proteasomes [10]. From these precursors, six spliced peptides (Fig. 1), with in silico predicted binding affinity for H-2Kb, were generated by N-terminal trimming with recombinant ERAP1 in vitro (Fig. 2A and Fig. S1). The predicted IC50 values showed that two of these spliced peptides might bind the MHC class I H-2Kb with similar affinity as LLO296-304, while the other four spliced peptides were predicted to bind the H-2Kb complex with an IC50 > 100 nM (Table 1).

Listeria monocytogenes infection evokes vigorous innate immune responses, leading to the release of large quantities of proinflammatory cytokines and, consequently, to different degrees of modification
of proteasome subunit composition in the infected tissues, in favor of larger contents of immunoproteasomes. Because such differences may strongly affect the generation of specific linear epitopes [25-30], we determined whether the increased expression of immunosubunits in spleens of mice infected by *Listeria monocytogenes* (Fig 2B) affected the *in vitro* generation kinetics of the studied potential spliced epitope precursors, derived from the polypeptide LLO_{291-317}. The quantitative method QME was applied, which estimates the absolute content of spliced and non-spliced peptide products based on their mass spectrometry ion strength measured in the digestion probe, as used before [10, 15, 23]. As shown in Fig 2C, 20S proteasomes derived from spleens of *Listeria monocytogenes* infected or from uninfected control mice produced similar amounts of the four spliced peptides, and only a marginal alteration in frequency of cleavages along the polypeptide was observed (data not shown). Thus, the increased immunosubunit content in infected mouse spleens compared to regular spleen tissue (Fig. 2B) did not lead to a change in quantities of spliced peptides generated. These observations can be explained by the fact that for example dendritic cells, macrophages and B cells in the spleen constitutively express large amounts of proteasomes containing immunosubunits [15], thus, uninfected spleen tissue already contains considerable quantities of immunoproteasomes (Fig. 2B).

To understand the specific role of the individual immunosubunits in the generation of the spliced LLO peptide products, we further compared the digests of proteasomes purified from *wt* mouse spleens with those from spleens of gene-deficient mice that lacked either β1i or β5i [10, 15]. We found that both immunosubunit-deficient proteasomes generated the spliced peptides LLO_{291-298/300-304} and LLO_{291-300/302-304} less efficiently than proteasomes of *wt* mice (Fig. 2D). These two spliced products are the N-terminally extended precursors of the H-2K^{b}-restricted LLO_{295-298/300-304}, LLO_{296-298/300-304} and LLO_{296-300/302-304} epitope candidates (Fig. 2A and Table 1). The generation efficiency of the other spliced peptides LLO_{291-294/297-304} and LLO_{291-298/291-293} was not affected by the complete depletion of immunosubunits (Fig. 2D). No significant variation in the LLO_{291-317} degradation rate was observed comparing digests of proteasomes of *wt*, β1i- and β5i-deficient mice (data not shown), as shown before [15]. Of note, the average amount of LLO_{296-304} generated *in vitro* by *wt* spleen 20S proteasomes over time was higher (89.3 pmol per nmol of substrate processed [15]) than that of the spliced peptides LLO_{291-294/297-304} (1.7 pmol
per nmol of substrate processed), LLO_{291-298/291-293} (0.8 pmol per nmol of substrate processed), LLO_{291-298/300-304} (4.7 pmol per nmol of substrate processed) and LLO_{291-300/302-304} (1.5 pmol per nmol of substrate processed) (Fig. 2D). Taken together, while the absence of specific immunosubunits diminished the generation of two spliced peptide products, all proteasome types generated all four spliced, potential epitope precursors, with no significant differences observed between proteasome preparations that varied in immunosubunit contents. Furthermore, the amount of linear epitope generated over time was larger than those of spliced peptide products, for all 20S proteasomes used, independent of subunit composition (data not shown).

Six putative spliced LLO epitopes bind the H-2K^{b} molecule.

LLO_{296-304} is presented to CD8 T-cells by the MHC class I H-2K^{b} molecule [22]. To verify that the predicted spliced epitope candidates are MHC class I H-2K^{b} binders, their ability to stabilize MHC class I molecules on RMA-S cells was tested. Incubation with the spliced epitope candidates LLO_{294/297-304}, LLO_{293-298/291-293}, LLO_{294-298/291-293}, LLO_{296-298/300-304} upregulated H-2K^{b} levels on RMA-S cells to a similar extent as incubation with LLO_{296-304}, while LLO_{295-298/300-304} and LLO_{296-300/302-304} stabilized H-2K^{b} to a lesser extent (Fig. 3A). During a subsequent chase in the absence of peptide, three spliced epitope candidates LLO_{296-298/300-304}, LLO_{295-298/300-304} and LLO_{296-300/302-304} rapidly dissociated from H-2K^{b} molecules while the decay of the other spliced epitope candidates and LLO_{296-304} was substantially slower (Fig. 3B, Fig. S2). Corresponding half-lives ranged from 14 to 120 min for the linear epitope, which displayed the highest binding affinity (Table 1). Comparing the IC50 values predicted by the MHC3.4/ANN (Table 1) with the measured ability of the spliced epitope candidates to upregulate H-2K^{b} expression (Fig. 3A) and their dissociation rates (Fig. 3B, Table 1), we observed similar patterns, illustrating the accuracy of *in silico* prediction by NetMHC3.4/ANN. We conclude that not only LLO_{296-304}, but also the six putative spliced antigenic peptides can bind to H-2K^{b} molecules and thus could be presented by infected cells *in vivo*.

CD8 T-cells of *Listeria monocytogenes* infected mice recognize a spliced epitope candidate.
To test whether the spliced epitope candidates are targeted during infection, CD8 T-cell responses to the putative LLO\textsubscript{291-317}-derived epitopes were measured in the spleens of \textit{Listeria monocytogenes} infected C57BL/6 mice \textit{ex vivo}, at the peak of the response after primary infection and secondary infection [31]. Intracellular IFN\(\gamma\) staining (Fig. S3) showed that both LLO\textsubscript{296-304} and the spliced LLO\textsubscript{294/297-304} epitope candidate were recognized by CD8 T-cells stimulated \textit{ex vivo} with these peptides, in both primary (Fig. 4A) and secondary infection (Fig. 4B). None of the other spliced LLO\textsubscript{291-317} -derived epitope candidates induced IFN\(\gamma\) production in CD8 T-cells, exceeding the background measured upon incubation in the absence of peptide (Fig. 4A, Fig. 4B).

Because LLO\textsubscript{296-304} and LLO\textsubscript{294/297-304} share 8 out of 9 residues, the detected CD8 T-cell response to LLO\textsubscript{294/297-304} could be due to cross-reactivity of CD8 T-cells recognizing LLO\textsubscript{296-304}. To test this possibility, we stained splenocytes of infected mice and uninfected controls \textit{ex vivo} with PE-labelled MHC class I H-2K\(^b\)/LLO\textsubscript{294/297-304} and APC-labelled MHC class I H-2K\(^b\)/LLO\textsubscript{296-304} multimers. CD8 T-cells specific for the linear epitope were significantly \((p = 0.007)\) detected in all infected mice (Fig. 5A, Fig. 5D). In contrast, the percentages of LLO\textsubscript{294/297-304}–specific CD8 T-cells were above the background in only 5 out of 9 infected mice (Fig. 5B, Fig. 5D). In double staining with the two MHC/peptide multimers, CD8 T-cells specific for both LLO\textsubscript{294/297-304} and LLO\textsubscript{296-304} were significantly \((p = 0.025)\) detected in all mice (Fig. 5C, Fig. 5D). While a small population of single, MHC H-2K\(^b\)/LLO\textsubscript{296-304} multimer-positive CD8 T-cells were detected in double stained samples (Fig. 5D, right panel), no solely LLO\textsubscript{294/297-304} positive cells were detected any more (Fig. 5D, right panel). Thus, all CD8 T-cells specific for the spliced epitope candidate also recognize the linear epitope LLO\textsubscript{296-304}. These data suggest that LLO\textsubscript{296-304}–specific CD8 T-cells cross-react with LLO\textsubscript{294/297-304}–presented on H-2K\(^b\) molecules, demonstrating that spliced epitopes could be recognized by pathogen-specific CD8 T-cells during infection.

**Discussion**

CD8 T-cell responses to complex pathogens often target a relatively small number of antigenic peptides only. The parameters that influence whether a pathogen-derived peptide will elicit an immune response...
are still not fully understood. Nevertheless, the requirements to be fulfilled by antigenic peptides to be recognized by T-cells, e.g. successful proteolytic liberation, TAP transport, MHC class I binding and TCR recognition, are numerous [32]. Thus, the number of epitopes that is processed from an antigen and presented by a specific MHC class I molecule is limited. It has been hypothesized that PCPS could expand the pool of antigenic peptides [19, 20] and here we report first evidence that this may happen during an infection in vivo. We demonstrate that, from a small portion of the Listeria monocytogenes LLO antigen only, proteasomes generate one linear epitope along with four spliced epitope precursors from which, by ERAP-mediated N-terminal trimming, six spliced antigenic peptides that bind to H-2Kb molecules can be generated. The generation efficiency of the spliced peptides was smaller than that of the linear epitope, as described by quantitative comparison of several linear and spliced peptides produced in vitro by 20S proteasomes [10]. These findings are in line with a publication that appeared during the revision of this manuscript [33]. It is worth to note however that spliced epitopes can be presented in similar amounts as linear epitopes on the cell surface (Ebstein et al., personal communication).

Of the six spliced epitope candidates, one spliced peptide, LLO204/297-304, along with the linear epitope LLO296-304, was recognized by CD8 T-cells of mice infected with Listeria monocytogenes, in both primary- and secondary infection (Fig. 4). Double staining with MHC/peptide multimers folded with either LLO204/297-304 or LLO296-304 (Fig. 5D) showed most but not all CD8 T-cells stained with LLO296-304-folded multimers were stained by the multimer folded with the spliced epitope candidate, indicating that LLO296-304 presented during Listeria monocytogenes infection primes both LLO296-304-specific and cross reactive CD8 T cells, recognizing also LLO204/297-304. Conversely, all CD8 T-cells specific for the spliced epitope were detected also by the multimer folded with the linear epitope. Thus, it is unclear whether LLO204/297-304 participates in CD8 T cell priming during infection.

Notwithstanding the above notion, the H-2Kb off rate of LLO204/297-304, although higher than that of LLO296-304 (Table 1), is within the range of off rates that we detect for CD8 T-cell epitopes in RMA-S based assays [26]. In addition, in vivo antigen processing of Listeria monocytogenes derived antigens has been shown to be highly efficient [34-36] LLO296-304 is the immunodominant known epitope in C57BL/6 mice [22] and LLO91-99 is immunodominant in BALB/c mice [22, 31] which most likely is due to the
rapid degradation of LLO antigen and the very efficient liberation of these two peptides in combination with high MHC class I binding affinity [35]. Because the spliced antigenic peptides are derived from the LLO antigen, they are processed with similar rates as the described dominant epitopes (although with a lower efficiency) and therefore will appear early on MHC class I molecules. Thus, LLO294/297-304 may well be presented over the course of *Listeria monocytogenes* infection, albeit in smaller quantities than LLO296-304, and be recognized by CD8 T-cells primed against the linear epitope, thereby facilitating bacterial clearance.

Remarkably, it has been argued that the ability of any given TCR to recognize multiple epitopes is a strict necessity, because if every T-cell recognized one epitope only, the breadth of the T-cell response needed to eradicate a pathogen could not be achieved [37]. Moreover, if multiple TCR recognize multiple peptides, displaying a high degree of similarity, the chances of a diverse, broad T-cell response are higher, which would make it substantially more difficult for pathogens to escape the immune system. It was shown that amino acid exchanges in the epitope- or epitope flanking residues can diminish the ability of proteasomes to efficiently liberate epitope’ C-termini [38-40]. In case of presentation and concurrent recognition of proteasome cleavage and proteasome spliced products by (cross reactive) CD8 T-cells, the impact of such mutations in epitope or epitope flanking sequences of pathogens would decrease substantially.

Thus, it might be advantageous for infected cells to present linear epitopes as well as spliced epitope variants simultaneously. Such mechanism may help the host by tackling mutation-driven escape mechanisms of pathogens, which might have been one of the reasons for the preservation of PCPS along evolution.
**Materials and methods**

**Peptides and peptide synthesis.**

The *Listeria monocytogenes* Listeriolysin O polypeptide LLO\textsubscript{291-317} (AYISSVAYGRQVYLKLSTNSHSTKVKA) and epitope candidates derived from this sequence were synthesized using Fmoc solid phase chemistry as previously described [41]. The purity of synthetic peptides was tested by amino acid analysis [10].

**20S proteasome purification.**

20S proteasomes were purified from spleens of: i. control or *Listeria monocytogenes*-infected C57BL/6 mice kept in animal facilities of the Utrecht University and used at 8 weeks of age (Fig. 2C); ii. wild type or β1i (N2 C57BL/6x129SvJ) [42] or β5i (N6 C57BL/6) [43] gene-targeted C57BL/6 mice, which had been kept in animal facilities of the Charité and were used at 8 weeks of age (Fig. 2D). To purify the 20S proteasome we modified the earlier described protocol [44] as follows: mouse spleens were homogenized and centrifuged, the supernatant was fractionated by ammonium sulphate precipitation, chromatography on DEAE-Sephacel, 10-40 % sucrose gradient and anion exchange chromatography on Mono Q. In each step the fractions were monitored by degradation assays of standard short fluorogenic peptides. Proteasome concentration was measured by Bradford staining and verified by coomassie staining in a SDS-Page gel as previously shown [45]. Spleens from 5 mice were pooled prior to proteasome purification. The purity of these proteasome preparations has been shown elsewhere [21].

**In vitro digestion of synthetic polypeptide by proteasomes and peptide quantification by QME.**

The synthetic LLO\textsubscript{291-317} polypeptide (20 μM) was digested with 2 μg 20S proteasomes in 100 μl TEAD buffer (Tris 20 mM, EDTA 1 mM, NaN\textsubscript{3} 1 mM, DTT 1 mM, pH 7.2) for the time intervals specified in the figure legends, at 37°C. Liquid-chromatography mass spectrometry analyses of polypeptide digestion products were performed as previously described [21] with the ESI-ion trap instrument DECA XP MAX (ThermoFisher Scientific, USA). Database searching was performed using the SpliceMet’s ProteaJ algorithm [21]. Quantification of proteasome-generated linear and spliced peptides was carried out by
applying the QME method to the liquid-chromatography-mass-spectrometry analyses as previously described [10].

**ERAP1-mediated N-terminal trimming of the epitope precursors and product identification.**

*In vitro* digestion of the spliced peptides LLO_{291-298/300-304}, LLO_{291-300/302-304}, LLO_{291-294/297-304} and LLO_{291-298/291-293} by recombinant ERAP1 was carried out as described elsewhere [23]. Briefly, 50 µM peptide was digested *in vitro* by 3 ng recombinant ERAP1 (R&D systems) in 20 µl buffer (25 mM Tris pH 7.5, 150 mM NaCl, 0.5 µg/ml albumin) at 37°C. Reactions were stopped by addition of 0.5% TFA after 4 hours. As a control, to test whether observed trimming was mediated by ERAP1, 3 ng ERAP1 was incubated with 30 µM leucinethiol [46] at room temperature for 20 min and then used in the *in vitro* experiments as described above. No production of trimmed peptides was observed (data not shown) thereby confirming the specific trimming by recombinant ERAP1.

For identification of the trimming products, 10 µl samples of ERAP1-mediated digestion were analyzed directly by nanoscale LC-MS/MS using an Ultimate 3000 and LTQ Orbitrap XL mass spectrometer (both Thermo Fisher Scientific). The system comprises a 5 mm × 300 µm, 100 Å trapping column (PepMap C18, 5 µm; Dionex) and a PicoChip analytical column (Reprosil-pur, 3 µm; New Objective). The mobile phase (A) was 0.1% (v/v) formic acid in water, and (B) was 80:20 (v/v) acetonitrile/water containing 0.1% (v/v) formic acid. The elution was carried out using a gradient 15-50% B in 34′ with a flow rate of 300 nl / min. Full MS spectra (m/z 200–2000) were acquired in an Orbitrap instrument at a resolution of 60000 (FWHM). The 10 most abundant precursor ions were selected for either data-dependent CID fragmentation (TOP10) with parent list (1+ charge state included). Fragment ions were detected in an ion trap instrument. Dynamic exclusion was enabled with a repeat count of 1- and 60-s exclusion duration. The maximum ion accumulation time for MS scans was set to 200 ms and for MS/MS scans to 500 ms. Background ions at m/z 391.2843 and 445.1200 act as lock mass. Trimmed products were identified by comparing retention time and tandem mass spectra (MS/MS) of their synthetic counterparts (Fig. S1).

**Western blot assay.**
Protein homogenates were extracted from one spleen or liver of mice not infected or infected by *Listeria monocytogenes* as previously described [47]. Proteasome subunits were revealed by Western blot assays as follows: 20 µg protein homogenates were separated in a 12.5% polyacrilamide SDS gel and transferred on a polyvinylidene difluoride filter (Immobilon-P Transfer Membrane, Millipore). Unspecific sites on the membrane were blocked for 1 h in a 5% non-fat dry milk (AppliChem) - PBS solution. Mouse proteasome subunits were detected by staining the membrane overnight at 4°C with the following primary antibodies: anti-β1 (1:10000; custom), anti-β2 (1:1000; Enzo Life Science), anti-β5 (1:1000; Abcam), anti-β1i (1:10000; Thermo Fisher Scientific), anti-β2i (1:2000; custom) and anti-β5i (1:1000; Thermo Fisher Scientific). As secondary anti-mouse or anti-rabbit HRP-conjugated Ab (1:5000; Calbiochem) was used for 2 h at room temperature followed by ECL detection (Amersham).

**Cell culture.**

RMA-S cells were cultured in IMDM (Invitrogen Life Technologies), supplemented with 10% FBS (LONZA), 2 mM L-glutamine, 30 µM 2-mercaptoethanol, and penicillin/streptomycin.

**MHC class I stability assays.**

RMA-S off rate assays were performed in serum-free medium as described [25]. In short, RMA-S cells were incubated overnight in the presence or absence of 100 µM synthetic peptide, at 37 °C. The next day, cells were harvested, washed 3 times with PBS, and chased in the absence of peptide, at 37 °C. Samples of the cells were taken at t: 0, 0.5, 1 and 2 hours, stained for H-2Kb class I expression with a conformation-sensitive, biotin-conjugated mouse antibody (AF6-88.5; BD Bioscience) and PE-conjugated streptavidin (eBioscience). FACS Canto II (BD Bioscience) and FlowJo software (Tree Star) were used for the analysis.

**Mice and infection.**

*Listeria monocytogenes* strain 10403S was grown in brain-heart infusion medium (Sigma-Aldrich) and harvested while in log phase. Six to eight weeks old female C57BL/6 J mice were purchased from
Charles River. For primary infection, mice were inoculated intravenously in the tail vain with 2000 bacteria in 200 µl PBS. Re-infection was performed 21 days later with 2x10⁵ CFU. All in vivo animal experiments were approved by the Animal Ethics Committee of Utrecht University (DEC 2014.II.11.081 and DEC 2014.II.01.003).

**Analysis of specific CD8 T-cell responses.**

*Intracellular cytokine staining*

2.5 x 10⁶ erythrocyte depleted splenocytes were incubated with or without 100 ng/ml synthetic peptide for 6 h in 1 ml RPMI Medium 1640 (Life Technologies) containing 50 µg/ml gentamycin (GIBCO) and 10 µM monensin (eBioscience), at 37 °C. Subsequently, cells were stained with an APC-conjugated anti-mouse CD8 antibody (53-6.7; eBioscience) in the presence of anti-mouse CD16/CD32 (clone 2.4G2). Cells were fixed with 2% paraformaldehyde and then stained with PE-conjugated anti-mouse IFNγ mAb (XMG1.2; eBioscience) in the presence of 0.5% saponin and analyzed on a FACS Canto II (BD Biosciences) using FlowJo software (Tree Star).

*Multimer staining*

PE or APC labelled MHC class I multimers were prepared by refolding H-2Kᵇ heavy chains and murine β2m in the presence of synthetic *Listeria monocytogenes*-derived LLO₂⁹⁶-₃₀₄ or LLO₂⁹⁴/₂⁹⁷-₃₀₄. Splenocytes were incubated with either one or both tetramers simultaneously for 20 minutes at 4 °C. Cells were washed, cell surface stained with a V500 conjugated anti-mouse CD8 antibody (53 - 6.7; eBioscience) and analyzed on a FACS Canto II (BD Biosciences) using FlowJo software (Tree Star).

**Statistical Analysis.**

Data was tested for normality by Levene’s test and for homoscedasticity by Shapiro-Wilk’s test. Independent *in vitro* experiments were performed with different proteasome preparations, therefore the SD also represents variability between different proteasome preparations. To compare responses to different peptides within infected mice a paired One-way ANOVA followed by Dunnet’s post hoc test.
was used. Independent student t-test was used to compare differences between infected and non-infected mice. P values < 0.05 were considered significant.

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Disclosure
The authors declare that they have no conflict of interest.

References


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<td>14</td>
<td>LLO&lt;sub&gt;291-300/302-304&lt;/sub&gt;</td>
<td>[AYISSVAYGR][VYL]</td>
</tr>
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<sup>a</sup> epitope candidates, identified by in silico analysis of proteasome-generated peptides products for MHC class I binding affinity.

<sup>b</sup> NetMHC 3.4/ANN predicted H-2K<sup>b</sup> binding affinities (IC50). Only peptides with predicted IC50 smaller than 500 nM were included in this study [48].

<sup>c</sup> half live of H-2K<sup>b</sup>-peptide complexes on the cell surface of RMA-S cells, calculated from Fig. 3B.

<sup>d</sup> linear and spliced peptides identified by SpliceMet [21] in 20S proteasome digests of the synthetic substrate LLO<sub>291-317</sub>. 

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Table 1. LLO<sub>291-317</sub> - derived spliced epitope candidates.
Figure Legends.

Figure 1. Overview of the different peptides derived by PCPS from LLO\textsubscript{291-317} and used in this study. All common residues between spliced antigenic peptides and the linear epitope LLO\textsubscript{296-304} (VAYGRQVYL) are depicted in white whereas the amino acids that originate from flanking residues are grey. Dashes indicate that the original residues are not present in the spliced peptides.

Figure 2. Generation of LLO\textsubscript{291-317}-derived spliced epitopes and epitope precursors by mouse spleen 20S proteasomes and recombinant ERAP1. (A) Products of the \textit{in vitro} degradation of the spliced peptides LLO\textsubscript{291-294/297-304}, LLO\textsubscript{291-298/291-293}, LLO\textsubscript{291-298/300-304}, LLO\textsubscript{291-300/302-304} by recombinant ERAP1. Educts and enzymatically processed products were separated by reversed phase HPLC (lower panels) and identified by MS/MS (Fig. S1). Extracted ion chromatograms of the single and double protonated peptides versus time are displayed. The synthetic counterparts are shown in the upper panels. All peptides are detected by mass spectrometry with almost the same ionization products [M+H]\textsuperscript{+} and by chromatography with similar retention times of their synthetic analogous. The spliced peptides produced by recombinant ERAP1 are reported in Table1. (B) Western blot assays performed on spleen and liver of mice infected with \textit{Listeria monocytogenes} compared to not infected mice. The three standard- and three immuno-subunits are shown together with the α4 subunit, which is present on every proteasome isoform. (C) Degradation kinetics of the synthetic substrate LLO\textsubscript{291-317} and the generation kinetics of the spliced peptides LLO\textsubscript{291-294/297-304}, LLO\textsubscript{291-298/291-293}, LLO\textsubscript{291-298/300-304} and LLO\textsubscript{291-300/302-304}. (D) The efficiency of generation of the spliced peptides LLO\textsubscript{291-294/297-304}, LLO\textsubscript{291-298/291-293}, LLO\textsubscript{291-298/300-304} and LLO\textsubscript{291-300/302-304} is here depicted as pmol peptide produced \textit{per} nmol of cleaved substrate over time. Digestions of the synthetic substrate LLO\textsubscript{291-317} were carried out by wild type, β\textsubscript{l}i subunit\textsuperscript{−/−} or β\textsubscript{5}i subunit\textsuperscript{−/−} mouse spleen 20S proteasomes. In (C-D) quantitation of the digestion products was carried out by applying the QME method. Values are the mean and bars the SD of two independent experiments measured in triplicate.

Figure 3. Binding of spliced LLO epitope candidates to H-2K\textsuperscript{b} molecules. RMA-S cells were incubated overnight with or without 100 µM synthetic peptide at 37 °C. (A) H-2K\textsuperscript{b} complexes (t = 0)
were stained with a conformation-sensitive, biotin-conjugated anti-H-2K\textsuperscript{b} mAb (AF6-88.5; BD Biosciences) and with PE-conjugated streptavidin (eBiosciences) and detected by FACS analysis. Filled circles represent stabilization of H-2K\textsuperscript{b} molecules measured in independent experiments in MFI±SEM. (B) RMA-S cells loaded with peptide overnight were chased in the absence of peptide and at the indicated time points samples were stained for H-2K\textsuperscript{b} expression. Depicted are mean percentages of remaining MHC class I complexes (t=0 is 100%) ± SEM of three independent experiments. Gating strategy is shown in Fig. S2.

Figure 4. Recognition of LLO-derived linear and spliced peptides by CD8 T-cells of Listeria

\textit{monocytogenes} infected mice. C57BL/6 mice were infected \textit{i.v.} with (A) a primary dose of 2000 CFU \textit{Listeria monocytogenes}, and (B) challenged 21 days later with a 100 fold higher bacterial dose. At day 7 following primary infection and day 26 following secondary infection, percentages of LLO peptide-specific CD8 T-cells in the spleen were measured \textit{ex vivo} by restimulation of splenocytes with the indicated peptides, followed by staining of cell surface CD8 with APC-anti-CD8 (53-6.7; eBioscience) and intracellular IFN\textgreek{y} staining with PE-anti-IFN\textgreek{y} (XMG1.2; eBioscience) antibodies. Every dot represents an individual mouse, bars ± SEM per peptide are indicated. Data are representative of three independent experiments (n > 5 animals per experiment), tested for normality using Levene’s test and analyzed using a paired One-way ANOVA, comparing responses per peptide to the no peptide control per mouse, followed by Dunnet’s post hoc test. At both time points, a significant difference (p < 0.05) was found between the CD8 T-cell responses specific for LLO\textsubscript{296-304} and LLO\textsubscript{294/297-304} compared to background responses measured in samples stimulated without peptide. Uninfected control animals did not show any response (data not shown). Gating strategy is shown in Fig. S3.

Figure 5. Recognition of LLO\textsubscript{296-304} and its spliced epitope variant by CD8 T-cells of Listeria

\textit{monocytogenes} infected mice. LLO\textsubscript{296-304} - and LLO\textsubscript{294/297-304} - specific CD8 T-cell responses in the spleens of infected and control mice were measured \textit{ex vivo} at day 7 post primary infection with 2000 CFU \textit{Listeria monocytogenes} using V500-anti-CD8 mAb (53-6.7; eBioscience) and (A) APC-labeled
MHC multimers folded with LLO$_{296-304}$, (B) PE-labeled MHC multimers folded with LLO$_{294/297-304}$ or (C) by double staining with the two MHC multimers. Dots indicate percentages of specific CD8 T-cells in individual mice ± SEM per group of infected mice (circles) compared to uninfected (squares) control mice. Data are representative of two independent experiments (n = 15 animals in total), tested for normality using Levene’s test and are analyzed using an independent student’s T test comparing infected with non-infected mice. A significant difference (p < 0.05) was found between the CD8 T-cell response of infected versus non-infected mice in case of APC-labeled MHC multimers folded with LLO$_{296-304}$ and double staining with both tetramers. (D) Representative FACS plots of CD8 T-cells from the spleens of infected mice stained with V500-anti-CD8 in combination with APC conjugated MHC I H-2K$^b$/LLO$_{296-304}$ multimers only (left), PE conjugated MHC I H-2K$^b$/LLO$_{294/297-304}$ multimers only (middle) or both multimers (right).