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Dynamic Antigen Presentation Patterns of Listeria monocytogenes-Derived CD8 T Cell Epitopes In Vivo

Mojca Škoberne,* Rafaela Holtappels, † Herbert Hof,* and Gernot Geginat2*

Little information exists regarding the presentation of antigenic peptides in infected tissues. In this study the in vivo presentation of four different CD8 T cell epitopes of Listeria monocytogenes was monitored. Peptide presentation was measured by a new, highly sensitive, ex vivo Ag presentation assay that was based on the testing of freshly isolated cells from infected spleens with peptide-specific CD8 T cell lines in an IFN-γ-specific ELISPOT assay. Remarkably, the peptide presentation pattern of splenocytes and that of macrophages purified from spleens of L. monocytogenes-infected mice were different from those of in vitro infected macrophage-like cell lines. The in vivo Ag presentation pattern of splenocytes also exhibited dynamic changes during the first 48 h of infection. In vivo peptide presentation at later time points postinfection was biased toward immunodominant CD8 T cell epitopes, while at an early time point, 6 h postinfection, subdominant and dominant CD8 T cell epitopes were presented with similar strength. In summary, our studies show that Ag presentation during an infection is a highly dynamic process that only can be fully appreciated by the study of cells infected in their physiological environment. The Journal of Immunology, 2001, 167: 2209–2218.

The host response against infection with a complex microorganism comprises T cells specific for a multitude of different antigenic peptides. Generally, the magnitude of the T cell response against different antigenic peptides exhibits a remarkably constant hierarchy, and the majority of the responding T cells are directed against few immunodominant T cell epitopes (1). Numerous studies in different infectious disease model systems revealed that the strength of the CD8 T cell response against a peptide is the result of the complex interplay of three major factors: the quantity and stability of peptide MHC class I complexes expressed on APC, the TCR repertoire of the responding T cell population, and the suppression of T cells specific for subdominant epitopes by T cells specific for immunodominant epitopes (reviewed in Ref. 2). Ag presentation is certainly required for the induction and expansion of CD8 T cells. However, only a few studies exist about the processing and presentation of antigenic peptides in vivo. In principle, the extraction and quantification of naturally processed antigenic peptides allow the direct analysis of Ag processing in tissues (3). Due to the relatively large number of infected cells required for this method the extraction of antigenic peptides from organs was only successful in a few model infections (4, 5). More indirectly, bacteria and viruses that express antigenic peptides in vivo were used to analyze the effects of variations in Ag presentation on the strength of the CD8 T cell response in vivo. Examples are the enhanced immunogenicity of preprocessed Ags that are directly targeted into the endoplasmic reticulum (6) or the modulation of the immunogenicity of T cell Ag by variations in the sequences flanking a T cell epitope (7, 8).

The murine infection with Listeria monocytogenes is one of the infection models where the mechanisms governing CD8 T cell induction and expansion were studied in detail. Mice infected with L. monocytogenes mount MHC class I Kβ-restricted CD8 T cell responses against peptides encompassing aa 91–99 of listeriolysin O (LLO)α (4); aa 217–225 (9), 449–457 (10), and 476–484 (11) of the p60 protein; and aa 84–92 of the listerial metalloprotease (12), respectively. In vivo the majority of CD8 T cells are specific for the immunodominant epitopes LLO91–99 and p60476–484 while relatively few T cells are directed against the subdominant epitopes p60449–457 and Mpl84–92 (13). The frequency of p60476–484-specific CD8 T cells is intermediate between the frequency of p60217–225 and that of p60449–457-specific T cells (11). Remarkably, among these four L. monocytogenes-derived peptides the immunodominant LLO91–99 is the least abundant endogenously processed peptide in infected cell lines, while the subdominant p60449–457 is the most abundant antigenic peptide in infected cell lines (14). Thus, a paradoxical inverse correlation exists between the abundance of naturally processed antigenic peptides in infected cells and the frequency of peptide-specific CD8 T cells in vivo.

It must be kept in mind that the quantitative analysis of peptide processing is based on in vitro infected cells and that it is not known to what extent this in vitro model represents the in vivo situation. Therefore, in the current study the presentation of L. monocytogenes-derived antigenic peptides was monitored in vivo. We used a novel approach for the direct measurement of Ag presentation in tissues that is based on the testing of in vivo-infected cells with peptide-specific CD8 T cell lines in a sensitive ELISPOT assay. Remarkably, the peptide presentation pattern of splenocytes infected with L. monocytogenes in vivo exhibited dynamic changes during the first 48 h of infection. In light of this new finding the possible correlation between peptide presentation and in vivo CD8 T cell expansion and function was reevaluated.

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3 Abbreviations used in this paper: LLO, listeriolysin O; AcN, acetonitrile; TFA, trifluoroacetic acid; p.i., postinfection.

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**Materials and Methods**

**Mice and infection of mice**

Female BALB/c/OlaHsd (H-2b) mice were purchased (Harlan-Winkelmann, Borchen, Germany), kept under conventional conditions and used after 8–10 wk of age. Mice were infected with *L. monocytogenes* serovar 1/2a EGD in 0.2 ml PBS either i.v. or i.p. as indicated. Infectious doses were 1 × 10⁶ and 1 × 10⁴ CFU i.v. for ex vivo peptide presentation experiments and T cell induction studies, respectively. Bacteria used for infection were in the logarithmic growth phase. The bacterial concentration was assessed from the OD at 600 nm.

**APC, in vitro infection of APC, and CD8 T cell lines**

P815 mastocytoma cells were used as targets in the cell-mediated cytotoxicity assay. For in vitro infection experiments macrophage-like J774.A1 (J774) and P388D1 (P388) cells were used as APC. Approximately 1 × 10⁵ P388 or J774 cells were infected with *L. monocytogenes* at a multiplicity of infection of 10. After 1 h at 37°C stimulation was 10°C with P815 cells transfected with the human B7.1 gene (P815/B7) (15) in BALB/c mice. CD8 T cell lines were propagated by repeated restimulation with P815 and the human B7.1 gene (P815/B7) (15) in the presence of the appropriate synthetic peptide in medium supplemented with IL-2 as described previously (16). Synthetic peptides were purchased (Jenri Biotools, Berlin, Germany). The peptide concentration used for restimulation was 10⁻⁶ M for all CD8 T cell lines. The detection limit of the CD8 T cell lines was between 10⁻¹¹ and 10⁻¹⁴ M peptide as measured using a standard chromatin release assay.

**Immunomagnetic isolation of macrophages from infected mice**

Macrophages from spleens of *L. monocytogenes*-infected mice were isolated by immunomagnetic cell sorting using paramagnetic microbeads conjugated to monoclonal hamster anti-mouse CD11b (clone M1/70.15.11.5) Abs (Miltenyi, Bergisch Gladbach, Germany). Spleens were removed 48 h after i.v. infection of mice with 1 × 10⁵ CFU *L. monocytogenes*. Spleens were injected with 500 μl of a 1 mg/ml solution of collagenase D (Roche Diagnostics, Mannheim, Germany) in HBSS. Subsequently, spleens were cut in small pieces and incubated for 30 min at 37°C in 5% CO₂ in the collagenase D buffer. Cells were collected by centrifugation and subsequently separated twice on MS-collagenase D buffer. Cells were collected by centrifugation and subsequently separated twice on MS-collagenase D buffer. Cells were collected by centrifugation and subjected to FACS analysis. The remaining cells were tested in the ELISPOT-based Ag presentation assay described below.

**Isolation of endogenously processed peptides**

Peptide extraction from infected cells and organs was performed as described previously with minor modifications (5). Spleens were removed 48 h after i.v. infection with 1 × 10⁵ CFU *L. monocytogenes*. Organs from five to eight mice were pooled and passed through a 90 μm mesh. After sonication, an aliquot for plating of bacteria, trifluoroacetic acid (TFA) was added to achieve a pH of 2.0. The lysis solution was supplemented with COMPLETE proteinase inhibitor (final concentration, 1 tablet/50 ml lysis buffer) and 1 μg/ml pepstatin (both from Roche Diagnostics). After homogenization extracts were sonicated, left for 30 min on ice, and centrifuged for 1 h at 50,000 × g, supernatants were removed and passed through a Sephadex G-25-M column with an isocratic elution flow of 1 ml 0.1% TFA/min. Low m.w. fractions were collected and passed through a Sep-Pak C18 reverse phase, solid phase extraction unit (Water, Eschborn, Germany). After washing with 5 ml 10% acetonitrile (AcN), bound material was eluted with 1.5 ml 50% AcN and 1.5 ml 100% AcN, pooled, concentrated to a final volume of 0.5 ml by vacuum centrifugation, and further fractionated by HPLC on a reverse phase C18 column (D. Pak C18–300A, 3.9 × 300 mm; Waters): 1 ml peptide extract was loaded and eluted with a flow rate of 1 ml/min on a linear AcN gradient. Solution A was 0.1% TFA; solution B was 70% AcN and 0.009% TFA. The gradient was 0–5 min of 0% B, 5–55 min linear increase to 50% B, 55–63 min linear increase to 100% B, 63–66 min of 100% B, and 66–74 min linear decrease to 0% B. One-minute fractions were collected and stored at -70°C.

Isolation of naturally processed peptides from infected cell lines was performed similarly. Six hours postinfection (p.i.) 1 × 10⁹ adherent *L. monocytogenes*-infected P388 or J774 cells were washed twice with ice-cold PBS and harvested with a cell scraper. Cell pellets were disrupted, sonicated, and lysed for 30 min in 2 ml 0.5% TFA supplemented with proteinase inhibitors as described above. Subsequently, cell lysates were centrifuged 30 min at 20,000 × g, and supernatants were further purified by ultrafiltration using MICROSEP ( Pall-Gelman, Dreieich, Germany) ultrafiltration units with a 10-kDa cutoff. The low Mᵣ fraction was further fractionated by HPLC as described above.

**Quantification of endogenously processed peptides**

For the quantification of endogenously processed antigenic peptides HPLC fractions were dried by vacuum concentration and resolved in 1 ml cell culture medium. The precise amount of antigenic peptides in HPLC fractions was determined as described previously (5). Fractions were tested in a standard chromatin release assay with 51Cr-labeled P815 cells as APC. The peptide concentration of the fractions was calculated by linear interpolation from lysis data obtained with a synthetic peptide standard. The recoveries of p60₉₁₋₂₂₀, p60₉₅₋₄₅₇, p60₄₅₇₋₄₆₆, and p60₄₇₆₋₄₇₆ were derived from the spleens of -infected mice and then eluted from the infected macrophage-like J774 cell line by immunomagnetic cell sorting.

**ELISPOT-based Ag presentation assay**

Ag presentation by APC was assessed with an ELISPOT-based Ag presentation assay. This assay applies the basic principle of the ELISPOT assay for the detection of Ag presentation by target cells infected in vitro or in vivo. Spleens were removed between 6 and 48 h after infection of mice. Spleenocytes were used as APC after passing through nylon gauze (80 mesh) and RBC lysis. Alternatively, in some experiments macrophages isolated from infected spleens or in vitro infected P388 and J774 cells were also used as APC. P388 and J774 cells were infected as described above. The setting of the assay was similar for these APC types. All APC were incubated with 10⁻⁶ M peptide for 10 days and ELISPOT plates were coated with the IFN-γ-secreting CD8 T cell lines. The specificity and sensitivity of the ELISPOT assay was assessed by the recovery of seeded (6 × 10⁶/well) CD8 T cell lines in the calculation of the total number of peptide-specific CD8 T cells or per organ or per cell the different recovery rates were counted.

**Ex vivo enumeration of peptide-specific CD8 T cells**

The frequency of peptide-specific CD8 T lymphocytes was determined in an IFN-γ-specific ELISPOT assay 10 days after i.v. infection of mice with 1 × 10⁵ CFU *L. monocytogenes* to pool previously described experiments (11). Unseeded splenocytes (6 × 10⁶/well) were stimulated for 6 h in round-bottom 96-well microtiter plates (Nunc, Wiesbaden, Germany) and aminoethylcarbazole dye solution.
Adoptive transfer

Mice were infected i.v. with $1 \times 10^3$ CFU *L. monocytogenes* and subsequently received $5 \times 10^6$ peptide-specific CD8 T cells i.v. in PBS. The number of CFU in the organ homogenates was determined 72 h later as described previously (16). The statistical significance of results was checked with the Newman-Keuls multiple comparison test at the 0.05 significance level. All tests were performed using WINKS statistical analysis software (Texasoft, Cedar Hill, TX). All experiments were repeated at least twice, with similar results.

Results

Applying the ELISPOT assay for measuring Ag presentation ex vivo

Ag presentation studies are generally performed with permanent cell lines that are cultured and infected in vitro. Ex vivo analysis of antigenic peptides from infected organs was only reported in a few instances (4, 5, 17). Acidic extraction of naturally processed peptides from tissues is a time-consuming task that involves multiple separation steps and generally requires a relatively large input of material. Another disadvantage of the quantitative peptide extraction approach is that peptides presented on the cell surface cannot be measured selectively. To overcome these restrictions we applied the principle of the ELISPOT assay for analysis of in vivo-infected APC. The ELISPOT assay is generally used for the detection of cells reactive against a defined Ag (13). For the ELISPOT-based Ag presentation assay the test principle was reversed, and the assay was used to detect the activation of peptide-specific CD8 T cells by infected APC.

To detect Ag presentation in vivo, mice were infected i.p. with a high dose ($1 \times 10^7$ or $1 \times 10^6$ CFU) of *L. monocytogenes*. Spleens were removed 48 h p.i., and splenocytes were tested in the ELISPOT-based Ag presentation assay (Fig. 1A). To quantify the strength of Ag presentation the number of spots per well was counted (Fig. 1B). In vivo infected splenocytes showed a distinct reaction pattern with CD8 T cells specific for p60$^{217-225}$, p60$^{449-457}$, p60$^{476-484}$, and LLO$_{91-99}$. The strongest response was obtained with CD8 T cells specific for LLO$_{91-99}$ and p60$^{217-225}$-CD8 T cells specific for p60$^{476-484}$ yielded an intermediate response, and p60$^{449-457}$-specific CD8 T cells yielded the weakest response.
response. The reactivity of the CD8 T cell lines was dependent on the dose used for infection of mice (Fig. 1B). Spleens of mice infected with $1 \times 10^5$ CFU revealed a ~5-fold increased load with *L. monocytogenes* ($56 \times 10^6$ and $11 \times 10^6$ CFU/spleen, respectively). Although the absolute strength of the CD8 T cell response against infected splenocytes varied in both groups of mice, the principal recognition pattern remained unchanged. The signal strength was also dependent on the number of APC added per well. A 5-fold reduction of the number of APC added per well ($1 \times 10^5$ vs $5 \times 10^4$/well) also reduced the absolute number of spots per well, but did not alter the principal recognition pattern obtained with the panel of CD8 T cell lines tested (Fig. 1B). If CD8 T cell lines were cocultivated with noninfected spleen cells or if spleen cells were cultivated in the absence of CD8 T cells, a background activity between one and four spots per well was observed (data not shown).

To enable quantitative comparison of the presentation of different peptides, CD8 T cell lines were selected that showed comparable peptide sensitivities (Fig. 1C). The peptide sensitivity of CD8 T cell lines was measured in the ELISPOT-based Ag presentation assay. Noninfected splenocytes were incubated with graded doses of synthetic p60<sub>217-225</sub>, p60<sub>449-457</sub>, p60<sub>476-484</sub>, and LLO<sub>91-99</sub> peptide, respectively, and were tested with the CD8 T cell line of corresponding specificity. As shown in Fig. 1C all CD8 T cell lines required a similar minimal peptide concentration for a significant response.

 Naturally processed antigenic peptides were detected after infection with a high dose of *L. monocytogenes*. When spleens were removed 48 h p.i., a significant bacterial load was present in the organ. Thus, the possibility exists that further replication of bacteria occurs in the in vitro phase of the assay. To address the possible effect of ongoing intracellular bacterial replication on the Ag presentation assay control experiments were performed. Graded numbers of splenocytes were tested in the ELISPOT-based Ag presentation assay either in the absence of antibiotics (Fig. 2, upper panel) or in the presence of gentamicin and tetracycline (Fig. 2, lower panel). As shown in Fig. 2 the presence of antibiotics did not influence the principle peptide presentation pattern of *L. monocytogenes*-infected splenocytes. Thus, the peptide presentation pattern of *L. monocytogenes*-infected splenocytes was not influenced by the inhibition of bacterial protein synthesis. To exclude any possible interference of viable bacteria in the Ag presentation assay, all tests were performed in the presence of gentamicin and tetracycline.

Taken together, these results demonstrate that the ELISPOT-based Ag presentation assay allows the ex vivo detection of antigenic peptides presented on the surface of *L. monocytogenes*-infected splenocytes.

### L. monocytogenes-infected cells exhibit distinct Ag presentation patterns in vivo and in vitro

Long term in vitro propagated cell lines poorly represent the characteristics of natural APC, which, e.g., are always exposed to a specific local cytokine environment. Therefore, we used the ELISPOT-based Ag presentation assay to compare in vitro and in vivo Ag presentation. Graded numbers of APC infected in vivo or in vitro were added to CD8 T cells specific for p60<sub>217-225</sub>, p60<sub>449-457</sub>, p60<sub>476-484</sub>, or LLO<sub>91-99</sub>. Fig. 3A shows the average number of spots/well obtained in the presence of $2 \times 10^4$ splenocytes, $10^3$ J774, or $10^3$ P388 cells/well, respectively. The reactivity pattern of CD8 T cell lines with in vivo-infected splenocytes (Fig. 3A, upper panel) differed consistently from the reaction pattern obtained with in vitro infected P388 (Fig. 3A, middle panel) or J774 (Fig. 3A, lower panel) cell lines. Remarkably, in comparison to *L. monocytogenes*-infected P388 or J774 cells, LLO<sub>91-99</sub> was clearly presented stronger by infected splenocytes. In contrast, the peptide presented strongest by P388 and J774 cells was p60<sub>449-457</sub>, which was barely detectable on infected splenocytes. As it is not known whether the APC type principally influences peptide recognition by CD8 T cells control experiments were performed with peptide-loaded APC. Splenocytes, P388, or J774 cells were loaded for 2 h in the presence of $10^{-9}$ M synthetic peptides, washed, and tested with peptide-specific CD8 T cells of the corresponding specificity. Fig. 3B shows the number of spots per $2 \times 10^4$ responder CD8 T cells. To directly compare the Ag presentation efficacy of different cell types, the number of APC was adjusted to 1000 APC/well. As shown in Fig. 3B splenocytes were weaker APC than P388 or J774 cells. However, different APC types did not exert a selective influence on the recognition of any of the four antigenic peptides tested. In summary, these results show that ex vivo isolated *L. monocytogenes*-infected splenocytes and in vitro infected cell lines exhibit different Ag presentation patterns.

### Ag presentation pattern of in vivo-infected macrophages

Splenocytes are not a homogenous cell population. To test whether the disparate Ag presentation patterns of whole spleen cells and in vitro infected macrophage-like cell lines are the result of a mixture of different APC types in the spleen, the Ag presentation pattern of in vivo-infected macrophages was analyzed. CD11b<sup>+</sup> cells, which are mostly macrophages, were separated from spleens 48 h after i.v. infection of mice with $1 \times 10^5$ CFU *L. monocytogenes*. The
immunomagnetic selection of CD11b+ cells yielded a highly enriched macrophage population. Approximately 90% of cells stained positively for F4/80, and all cells were negative for the dendritic cell marker CD11c (Fig. 4A). These CD11b+ cells were tested with peptide-specific CD8 T cell lines in the ELISPOT-based Ag presentation assay (Fig. 4B). The Ag presentation pattern of isolated macrophages repeated the principle peptide presentation pattern of whole spleen cells, which is characterized by strong p60217–225 and LLO 91–99 presentation. However, the direct comparison of the T cell activation by isolated macrophages and unseparated spleen cells showed that the CD11b-selected cells are much stronger Ag presenters (Fig. 4B).

Thus, isolated, in vivo-infected macrophages demonstrate the same Ag presentation pattern as unseparated spleen cells, which is clearly different from the peptide presentation pattern of in vitro infected macrophage-like cell lines.

Acidic extraction of naturally processed antigenic peptides from infected organs

The ELISPOT-mediated Ag presentation assay measures selectively cell surface presentation of peptides. To determine whether the Ag presentation pattern reflects the total peptide composition of infected cells, peptides were also quantified in whole cell extracts. Mice were infected i.v. with $1 \times 10^8$ CFU L. monocytogenes. Spleens were removed 48 h p.i., and peptides were extracted from a pool of five organs. Naturally processed antigenic peptides were extracted.
peptides were also extracted from *L. monocytogenes*-infected P388 cells. After HPLC separation, fractions containing antigenic activity were identified with peptide-specific CD8 T cells in a 51Cr release assay. Fig. 5A shows the results of a representative CTL test of HPLC fractions from a peptide extract of infected spleens. The total peptide content of positive fractions was determined by linear interpolation from a synthetic peptide standard (data not shown). In *L. monocytogenes*-infected spleens only the peptides LLO<sub>91-99</sub> and p60<sub>217-225</sub> were detectable (Fig. 5B, upper panel). Both peptides were similarly abundant, in the range between 3 × 10<sup>3</sup> and 5 × 10<sup>3</sup> peptides/spleen. The peptides p60<sub>476-484</sub> and p60<sub>449-457</sub> both ranged below the detection limit of the assay, which was ~5 × 10<sup>3</sup> peptides/spleen for both peptides. In contrast to infected splenocytes, the most abundant peptides in P388 cells were p60<sub>449-457</sub> and p60<sub>217-225</sub>, while LLO<sub>91-99</sub> was significantly less abundant than these p60-derived peptides (Fig. 5B, lower panel). The p60<sub>476-484</sub> peptide was not detected in cell extracts, indicating that less than five p60<sub>476-484</sub> peptides were presented per cell. Thus, if the peptide presentation patterns of P388 cells and spleens are compared, it is clear that p60<sub>449-457</sub> was significantly less abundant than p60<sub>217-225</sub> in infected spleens. As in infected spleens the amount of p60<sub>217-225</sub> peptides was ~10-fold greater than the detection limit of p60<sub>449-457</sub>-specific CD8 cells, it is unlikely that the presence of an equal amount of p60<sub>449-457</sub> was just overlooked. Taken together, the quantitative peptide extraction procedure confirmed the results obtained with the ELISPOT-based Ag presentation assay (Fig. 3A). Therefore, we conclude that the Ag presentation pattern displayed by infected splenocytes and P388 cells mirrors the total peptide composition of the infected cells.

**Kinetics of Ag presentation in vivo**

After sublethal *L. monocytogenes* infection of mice the bacterial load in the spleen peaks around day 3 p.i., and specific CD8 T cells can be detected 5 days p.i. and peak around day 7 p.i. (18). To study the kinetics of Ag presentation after *L. monocytogenes* infection the peptide presentation pattern of splenocytes was tested at different time points postinfection. Two different experimental approaches were chosen. To achieve a similar bacterial load in the spleen mice were infected with different doses of *L. monocytogenes*. Spleens were tested 6, 24, and 48 h after i.v. infection with 1 × 10<sup>5</sup>, 1 × 10<sup>6</sup>, and 1 × 10<sup>7</sup> CFU/mouse, respectively (Fig. 6A). Alternatively, all mice were infected with the same dose of 1 × 10<sup>6</sup> CFU *L. monocytogenes* i.v. and tested 6, 24, and 48 after infection (Fig. 6B). The number of CFU per spleen of the different experimental groups is shown in Fig. 6C. Later time points p.i. were not included due to the increasing background activity of ex vivo isolated splenocytes. Spleen cells of all mice were tested in the ELISPOT-based Ag presentation assay with a set of CD8 T cell lines. Remarkably, the relative abundance of naturally processed antigenic peptides in vivo changed dramatically during the first 48 h of *L. monocytogenes* infection. Around 6 h p.i., p60<sub>217-225</sub>, p60<sub>449-457</sub>, p60<sub>476-484</sub>, and LLO<sub>91-99</sub> were all presented with comparable strength. Later, between 24 and 48 h p.i., this relation changed. At 48 h p.i. LLO<sub>91-99</sub> and p60<sub>217-225</sub> were presented much more strongly than p60<sub>449-457</sub> and p60<sub>476-484</sub> in both experimental groups.

In summary, these data show that the Ag presentation pattern of *L. monocytogenes*-infected cells in vivo is highly dynamic, with rapid peptide-specific changes during the first 48 h p.i.

**Frequency and protective potential of peptide-specific CD8 T cells in vivo**

In previous reports a paradoxical inverse correlation between the abundance of naturally processed antigenic peptides extracted from *in vitro* infected cells and the frequency of peptide-specific CD8 T cells in vivo was noted (13, 14). We found that the Ag presentation pattern of infected cell lines differed from the pattern displayed by ex vivo isolated splenocytes. Furthermore, we found that in vivo the peptide presentation pattern of *L. monocytogenes*-infected cells changed during the course of infection. As Ag presentation is principally required for CD8 T cell induction and also for the recognition of infected target cells, we analyzed the possible correlation between in vivo peptide presentation and the frequency and effector function of peptide-specific CD8 T cells. As shown in Fig. 7A the frequency of peptide-specific CD8 T cells in primarily infected BALB/c mice on day 10 p.i. exhibited a distinct hierarchy. This hierarchy of *L. monocytogenes*-specific CD8 T

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FIGURE 5. Acidic extraction of naturally processed antigenic peptides from *L. monocytogenes*-infected cell lines and organs. Naturally processed antigenic peptides were extracted and quantified from the spleen of *L. monocytogenes*-infected mice and from infected P388 cells. After HPLC separation, fractions containing antigenic activity were identified with peptide-specific CD8 T cell lines in a 51Cr release assay. Peptides p60<sub>217-225</sub>, p60<sub>449-457</sub>, p60<sub>476-484</sub>, and LLO<sub>91-99</sub> were detected with CD8 T cells of corresponding specificity as indicated. A representative CTL test of HPLC fractions prepared from a peptide extract of infected spleens 48 h p.i. is shown (A). Arrows indicate the HPLC fraction where the indicated peptides elute. The total peptide content of positive fractions was determined by linear interpolation from a synthetic peptide standard (B). The total number of peptides per *L. monocytogenes*-infected spleen is shown (upper panel). For infected P388 cells the number of peptides per cell is indicated (lower panel). Dotted lines indicate the detection limit of the assay.
cells (LLO_91–99 > p60_217–225 > p60_476–484 > p60_449–457) corroborates results published previously by Sijts et al. (13). The recently described p60_476–484 epitope exhibited an intermediate strength among the three known CD8 T cell epitopes of p60, confirming previous results obtained after secondary L. monocytogenes infection (11). Remarkably, the immunodominant peptides LLO_91–99 and p60_217–115 were also the peptides that revealed the strongest Ag presentation 48 h p.i. (Fig. 6, A and B).

To test the possible correlation of the protective potential of peptide-specific CD8 T cells to the peptide presentation pattern in vivo, adoptive transfer experiments were performed. The CD8 T cell lines were matched for comparable peptide sensitivity and lytic potential in vitro. Mice received 5 × 10^6 CD8 T cells immediately after L. monocytogenes infection, and the bacterial load of the spleen was determined 3 days later (Fig. 7B). All CD8 T cell lines conferred significant (p < 0.05) protection against L. monocytogenes. Generally, LLO_91–99-specific CD8 T cells conferred the strongest protection, but this difference was not statistically significant (Fig. 7C). Corroborating earlier results by Dunn and North
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The high sensitivity of this assay enabled the measurement of peptide-specific CD8 T cells in a sensitive ELISPOT assay. We used a novel approach for the ex vivo measurement of Ag presentation that is based on the testing of in vivo-infected APC. Discussion

In summary, these data show that the protective capacity of adoptively transferred L. monocytogenes-specific CD8 T cells does not correlate to the immunodominance of these CD8 T cells in vivo. Further it is shown that the expansion and the effector function of CD8 T cells correlate with the in vivo Ag presentation pattern at different time points p.i.

Discussion

We used a novel approach for the ex vivo measurement of Ag presentation that is based on the testing of in vivo-infected APC with peptide-specific CD8 T cells in a sensitive ELISPOT assay. The high sensitivity of this assay enabled the measurement of peptide presentation without the technically difficult classical peptide extraction method that is generally used for the quantification of naturally processed peptides in tissues (3–5, 17). The comparison of in vivo-infected splenic macrophages and in vitro-infected macrophage-like cell lines revealed distinct peptide presentation patterns for in vivo- and in vitro-infected APC. As different APC types and also APC harvested at different time points p.i. were always directly compared in the same experiment with the same set of CD8 T cell lines, the observed differences in the peptide presentation patterns cannot be attributed to different sensitivities of the CD8 T cell lines used. In contrast to acidic peptide extraction the Ag presentation assay detects selectively cell surface peptide presentation. This is an important advantage if the relevance of Ag presentation for the induction of T cells shall be studied because some microbial immune escape mechanisms selectively prevent cell surface presentation of antigenic peptides (20). However, after infection of mice with L. monocytogenes, the peptide presentation patterns of splenocytes and infected cell lines were principally confirmed by peptide extraction of whole organs and cells, respectively. The Ag presentation pattern of in vitro infected P388 or J774 cells confirmed data reported by Pamer’s group (14). Compared with acidic peptide extraction the ELISPOT-based Ag presentation assay requires less material and is more sensitive. The ELISPOT-based assay also enables measurement of the Ag presentation pattern of defined, ex vivo isolated cell populations. This gives the assay a broad range of possible applications for the study of animal and probably also human disease states.

The quantitative aspects of Ag presentation are generally analyzed in long term cell cultures. A number of previous studies compared the abundance of naturally processed peptides in infected cell lines with the frequency of peptide-specific CD8 T cells in vivo. In the murine L. monocytogenes infection model an inverse correlation between the abundance of antigenic peptides in infected cell lines and the frequency of peptide-specific CD8 T cells has been noted (13, 14). Similarly, immunodominance among EBV-derived MHC class I-restricted epitopes does not correlate with the abundance of antigenic peptides in EBV-transformed cell lines (21). Also, a detailed study of the CD8 T cell response against influenza virus has shown that inefficient Ag processing accounts only for the poor immunogenicity of one subdominant determinant, while in other instances the limitation is located on the side of the T cell (22). Taken together, from these experiments no obvious general correlation between the frequency of peptide-specific CD8 T cells in vivo and the abundance of antigenic peptides in infected cells is evident. However, it should be noted that studies generally analyzed Ag presentation in vitro. When long-term in vitro propagated cell lines are studied, the most obvious differences to the in vivo situation include the cell type and the absence of the physiological cytokine environment. Additionally, it has to be considered that compared with an in vitro cell culture model, microorganisms in vivo generally exhibit different growth kinetics and thus also the rate and kinetics of protein expression might not be identical. Analysis of the Ag processing of p89/168–176, an antigenic peptide derived from the murine CMV, has shown that in vivo Ag processing is strongly influenced by IFN-γ (5) and also by the infected cell type (23). The important influence of the cell type on Ag presentation has been shown in a study of virus-infected dendritic and fibroblast cell lines (24). A possible explanation for these observations is that cell type-specific or IFN-γ-mediated differences in the composition of the proteasome (reviewed in Ref. 25) alter Ag presentation in vivo.

In the current study also the peptide presentation pattern of in vivo-infected macrophages was analyzed. On a per cell basis a significantly stronger Ag presentation by ex vivo isolated macrophages was observed, while the peptide presentation pattern was identical with unseparated splenocytes. As it is known that in the spleen the majority of Listeriae resides in macrophages this result could be expected (26, 27). Thus, either the peptide presentation of other infected cell types in the spleen, e.g., dendritic cells or sinusoidal lining cells is similar to macrophages or the quantitative contribution of these cell types to the overall Ag presentation pattern of unseparated splenocytes is to low to be detected.

LL091–99 and p60217–225 form relatively stable peptide/Kd complexes with a half-life of ~6 h, while p60449–457/Kd complexes have a half-life of <1 h (28, 29). The relative strength of the CD8 T cell response against these peptides correlates with the stability of the corresponding MHC class I/peptide complexes (29). Similar results were obtained with EBV-specific CD8 T cells (30) and in a CD8 T cell immunization study with a large number of synthetic peptides (31). In the context of these data it is remarkable that in L. monocytogenes-infected spleens 6 h p.i. all peptides were presented with similar strength, while at later time points p.i. the presentation of peptides that form stable MHC/peptide complexes was significantly stronger than the presentation of peptides that form unstable complexes. The remarkable correlation between the stability of MHC class I/peptide complexes, prolonged peptide presentation, and the frequency of peptide-specific CD8 T cells in vivo suggests a model for the observed changes of the in vivo Ag presentation. In infected cells p60 protein secretion is limiting for the generation of p60-derived epitopes (32). Thus, over an extended period of time it has to be expected that peptides that form stable MHC/peptide complexes outnumbers peptides that form less stable complexes. Finally, this could result in the preferential stimulation and expansion of T cells directed against the more stable peptide/MHC complexes. The importance of peptide stability for the Ag presentation pattern is obvious when protein secretion is inhibited. Sijts et al. have shown that after inhibition of p60 biosynthesis by tetracycline treatment p60449–457, that forms unstable MHC class I/peptide complexes disappears quickly, while p60217–225 that forms stable complexes persists over an extended time period (28). Accumulation of stable peptides over time could also at least in part explain the observed differences of the Ag presentation patterns of in vitro- and in vivo-infected APC. For peptide extraction macrophage-like cells were harvested 6 h p.i.,
The good protection obtained after adoptive transfer of immunity against diverse intracellular microorganisms (38 cell populations can have an important contribution to protective requirements for CD8 T cell effector cell function and expansion on priming of CD8 T cells and protective immunity. Dichotomous nonsecreted fusion protein (37). Remarkably, they have found that limiting for the expansion of p60\textsubscript{217}–225 results in a strongly enhanced CD8 T cell response after subse-

stable peptides in the spleen 48 h p.i. is clearly plausible. However, while spleens were removed 48 h p.i.. Thus, the accumulation of expressed by recombinant T cells in vivo. However, the timing requirements described by Mercado et al. are not strikingly different from the time frame of the changing Ag presentation pattern in L. monocytogenes-infected mice, as by 24 h p.i. the presentation of the subdominant epitopes p60\textsubscript{149}–457 and p60\textsubscript{276}–484 started to decrease in relation to that of the dominant CD8 T cell epitopes.

In vivo, CD8 T cells mediate protection against L. monocytogenes (35, 36). To exert their protective function CD8 T cells must recognize infected target cells. A number of observations suggest that the Ag presentation requirements for the primary stimulation of naive T cells differ from the antigenic stimulus that is necessary to stimulate the effector function of experienced T cells. Shen et al. have analyzed the CD8 T cell response against a model T cell Ag to stimulate the effector function of experienced T cells. Shen et al. have analyzed the CD8 T cell response against a model T cell Ag to stimulate the effector function of experienced T cells. Shen et al. have analyzed the CD8 T cell response against a model T cell Ag to stimulate the effector function of experienced T cells. Shen et al. have analyzed the CD8 T cell response against a model T cell Ag to stimulate the effector function of experienced T cells. 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