Published in final edited form as:

J Immunol Methods. 2007 December 1; 328(1-2): 45–52.

Use of the CD107 mobilization assay reveals that cytotoxic T lymphocytes with novel MHC-Ib restriction are activated during *Listeria monocytogenes* infection

Denise S. McElroy, Adina M. Badstibner, and Sarah E.F. D'Orazio*

Department of Microbiology, Immunology, & Molecular Genetics, University of Kentucky College of Medicine, Lexington, KY

Abstract

Detection of cytotoxic activity by pathogen-specific T cells of unknown antigenic specificity is difficult due to the limitations of using infected cells, instead of peptide-pulsed cells, as targets. We report here that the recently described CD107 mobilization assay readily allowed for the ex vivo detection of cytotoxic T lymphocytes (CTL) with a novel MHC-Ib restriction that specifically recognized *Listeria monocytogenes*-infected macrophages. The CD107 mobilization assay is likely to be a useful tool for detection of CD8⁺ T cells that recognize a wide variety of intracellular pathogens.

Keywords

CD107 mobilization assay; Cytotoxic T lymphocytes; *Listeria monocytogenes*; MHC-Ib; CD8⁺ T cells

1. Introduction

In recent years, many investigators interested in the dynamic relationship between intracellular pathogens and the immune system have chosen to study pathogen-specific T cells rather than T cells that recognize model antigens such as ovalbumin. Initially, most pathogen-specific CD8⁺ T cells can be identified only by means of their ability to acquire effector functions (cytotoxicity and cytokine secretion) following exposure to infected target cells. Flow cytometric assays can be used for sensitive detection of cytokine secretion by individual T cells (Serbina and Pamer, 2003; Suni et al., 2005), while cytotoxic potential has traditionally been identified using the chromium (⁵¹Cr) release assay, which assesses the viability of target cells after incubation with bulk populations of T cells (Brunner et al., 1968). Each of these assays can easily be performed with T cell clones of known antigenic specificity using target cells coated with synthetic peptide. Unfortunately, the identification of antigenic epitopes is a challenging and time-consuming process, therefore, many pathogen-specific CD8⁺ T cell lines currently exist for which the peptide antigen is unknown (Starnbach et al., 1995; D'Orazio et al., 2003).

*Corresponding Author. Mailing address: University of Kentucky, Department of Microbiology, Immunology, & Molecular Genetics, 800 Rose Street – MS415, Lexington, KY 40536. Phone: (859) 323-8701. Fax: (859) 257-8994. Email: sarah.dorazio@uky.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

In vitro assays of pathogen-specific CD8⁺ T cell effector function are further complicated by the fact that many intracellular bacterial pathogens, including *Listeria monocytogenes*, infect adherent cells more efficiently than non-adherent cells. Infected adherent target cells do not pose a problem for use in intracellular cytokine staining assays, since the adherent target cells are not collected for flow cytometric analysis. However, ⁵¹Cr release assays rely on the ability of target cells that have not been specifically lysed by cytotoxic T lymphocytes (CTL) to remain intact throughout the duration of the assay. Manipulation of infected target cells during ⁵¹Cr release assays typically results in high background levels of lysis due to the inherent fragility of heavily infected cells. High non-specific lysis of infected cells severely restricts the ability to detect specific lysis by CTL in these assays, and because of this limitation, most studies of pathogen-specific CD8⁺ T cell effector function have assessed only the secretion of cytokines such as IFN γ or TNF α .

Betts et al. recently described a sensitive flow cytometric assay for the detection of cytotoxic activity that could be used as an alternative to the standard ⁵¹Cr release assay (Betts et al., 2003; Betts and Koup, 2004). The CD107 mobilization assay detects the transient surface expression of CD107a and CD107b (LAMP-1/LAMP-2) that occurs during T cell degranulation following antigenic stimulation. Previous studies have shown that the presence of CD107⁺ T cells correlated well with cytotoxic activity for either CD8⁺ T cells exposed to peptide-pulsed target cells or NK cells exposed to tumor targets (Betts and Koup, 2004; Penack et al., 2005). Since the CD107 mobilization assay measures changes in the T cells themselves, and does not require target cells to remain intact throughout the duration of the experiment, we reasoned that it would be useful for the detection of pathogen-specific CTL following exposure to infected target cells.

2. Materials and methods

2.1. Mice

C57BL/6(J) mice were obtained from the Jackson Laboratory. A colony of C.B10 MHC-Ia deficient mice (D'Orazio et al., 2003) was maintained in a specific pathogen free facility at the University of Kentucky. All animal procedures were performed in accordance with the UK IACUC guidelines.

2.2. *L. monocytogenes* infection

L. monocytogenes JIL-OVA (*Lm* OVA) is a wildtype strain that expresses ovalbumin (Pope et al., 2001). *Lm* SDA1 is a *fnt* mutant strain derived from *L. monocytogenes* 10403s that lacks formylated peptides and therefore does not activate M3-restricted T cells (D'Orazio et al., 2006). Each *L. monocytogenes* strain was grown to early stationary phase at 37°C with aeration in Brain Heart Infusion (BHI) broth (Difco), aliquoted, titered and stored at -80°C. Prior to infection, frozen aliquots were thawed on ice and then incubated in BHI broth shaking at 37°C until early exponential log phase. Dilutions prepared in phosphate buffered saline (PBS) were used to infect tissue culture cells or mice. For infection of macrophages, bacterial contact was facilitated by centrifugation of culture dishes at 600 $\times$ g for 5 min. at 25-35°C. The cells were incubated at 37°C in 7% CO₂ for either 30 (BMM ϕ) or 60 (BM12) mins., and then washed three times with prewarmed PBS, and suspended in media containing 25 μ g/ml gentamicin to kill extracellular bacteria. Mice were injected intravenously in the lateral tail vein using a total volume of 200 μ l.

2.3. Cell culture

The BM12.11.C2 macrophage-like cell line (hereafter referred to as BM12 cells; generously provided by Ken Rock, Worcester, MA) was grown in RP-10 media, consisting of RPMI 1640 (21870; Invitrogen Life Technologies) supplemented with L-glutamine, HEPES, 50 mM 2-

mercaptoethanol, and 10% FCS. To generate bone marrow-derived macrophages (BMM ϕ), bone marrow cells were harvested from femurs of mice and plated in BMM-20 medium, consisting of DMEM (11960; Invitrogen Life Technologies) supplemented with L-glutamine, 20% FCS and 20% L929 supernatant (harvested after 10 days in culture). For assays performed in 24-well dishes, 5×10^5 macrophages were seeded per well. For assays performed in 96-well dishes, 1×10^5 macrophages were seeded per well. On day 4, cells were washed once with PBS and replenished with BMM-20. BMM ϕ were fed fresh media every 3-4 days and maintained in culture for up to 6 weeks.

The OVA₂₅₇₋₂₆₄-specific CD8⁺ T cell line DM102 was isolated from the spleen of a C57BL/6 mouse infected 3 weeks earlier with 2×10^3 CFU of *Lm* OVA. Splenocytes were co-cultured with irradiated (2000 rads) syngeneic splenocytes coated with 50 nM OVA₂₅₇₋₂₆₄ synthetic peptide (Bio-Synthesis, Lewisville, TX). The T cell line was maintained by weekly in vitro re-stimulation in RP-10 supplemented with supernatant from concanavalin A-stimulated rat splenocytes and 50 mM α -methylmannoside.

2.4. CD107 mobilization assay

BMM ϕ seeded in either 24-well or 96-well dishes were infected (MOI = 0.1 – 1.0) with *L. monocytogenes* for 4 hours and then effector T cells (either whole splenocytes, CD8-enriched splenocytes, or DM102 cells) were added at an effector to target ratio (E:T) of 1:2. CD8-enriched splenocytes were isolated by negative selection using IMagTM antibody-coated magnetic beads according to the manufacturer's instructions (BD Biosciences). FITC-conjugated anti-CD107a and anti-CD107b monoclonal antibodies were added at a concentration of 4 μ l/ml each and then monensin (1 μ l/ml) was added to each well (all from BD Biosciences). In pilot experiments, we showed that the addition of Golgi Plug (BD Biosciences) at this time at a final concentration of 1 μ l/ml allowed for simultaneous detection of IFN γ production by intracellular cytokine staining without affecting the CD107 assay. Cells were harvested 4-6 hours later, and stained with anti-CD16 antibodies (Fc Block; BD Biosciences) to inhibit non-specific binding of antibodies. The cells were then stained with fluorescently conjugated monoclonal antibodies specific for the following murine cell surface molecules: CD4, CD8, TCR β , and B220 (eBioscience) and fluorescence intensities were determined using a FACSCalibur flow cytometer. Data (25,000-50,000 events gated as lymphocytes based on FSC and SSC) were analyzed using CellQuest software (BD Biosciences). In some experiments, the infected BMM ϕ were pre-treated for 30 mins. with rat anti-mouse IL-12 (Peprotech, Rocky Hill, NJ) and rat anti-mouse IL-18 (clone 93-10C; Medical & Biological Laboratories, Woburn, MA) monoclonal antibodies (final conc. of 2 μ g/ml). The antibodies were maintained in the sample wells at a final concentration of 1 μ g/ml for the remainder of the assay. For experiments that utilized Transwell filter inserts (Corning; 0.4 μ m pore size), monolayers of macrophages were seeded on the bottom of the wells of a 24-well dish in a total volume of 600 μ l. CD8⁺-enriched splenocytes were added to the top chamber in a total volume of 100 μ l.

2.5. Chromium release assay

BM12 target cells (10^6) were prepared by either pre-treating with synthetic OVA₂₅₇₋₂₆₄ peptide (final conc. 1 μ M) for 1 h or infecting (MOI = 0.5) with *Lm* OVA for 3 h at 37°C in 7% CO₂. Sodium [⁵¹Cr] chromate (100 μ Ci; PerkinElmer) was added during the final hour of incubation. Target cells were washed three times with RPMI 1640, suspended in RP-10 at 1×10^5 cells/ml, and distributed into 96-well round bottom plates (100 μ l/well). Serial dilutions of effector cells (either whole splenocytes or DM102 T cells) were added, and the plates were incubated for 4 h at 37°C in 7% CO₂. The assay plates were centrifuged at 500 $\times$ g for 5 min, and 100 μ l of the supernatant from each well was applied to a Luma Plate (PerkinElmer) and allowed to dry overnight at room temperature. The amount of ⁵¹Cr released into the supernatant

of each well was evaluated (without the addition of scintillation fluid) using a TopCount gamma counter (PerkinElmer). Spontaneous release (SR) was determined by incubating target cells in RP-10 alone (no effector cells); maximum release (MR) was determined by incubating target cells in RP-10 with 0.1% Triton X-100. Percent specific lysis (SL) was calculated using the following formula: $\%SL = 100 \times (\text{release by effector T cells} - SR) / (\text{release by effector T cells} - MR)$.

3. Results

3.1 Adherent *L. monocytogenes*-infected cells can be used as targets in a CD107 assay

To determine whether the CD107 mobilization assay could be used to detect infected adherent target cells, we first tested the ability of an OVA₂₅₇₋₂₆₄-specific T cell line (DM102) to recognize macrophages that were either infected with *Lm* OVA or coated with synthetic peptide (Fig. 1). BMM ϕ isolated from a C57BL/6 mouse were either pre-incubated for one hour with varying concentrations of peptide or infected for 3 hours with *Lm* OVA. DM102 CD8⁺ T cells that had been re-stimulated in vitro 5 days earlier were added at an E:T of 1:2. If the T cells recognized their cognate peptide:MHC complex on the target cells, CD107a and CD107b molecules would be transiently expressed on the T cell surface during degranulation, and the T cells would be fluorescently tagged following binding by the anti-CD107a/b antibodies included in the media.

As shown in Fig. 1A, CD107 staining of the DM102 T cells was dose dependent, with a majority of the T cells responding to high concentrations of OVA₂₅₇₋₂₆₄ peptide, and detectable degranulation occurring even with picomolar concentrations of peptide. Approximately 50% of the DM102 T cells expressed CD107 on their surface after incubation with *Lm* OVA-infected (MOI=1) BMM ϕ (Fig. 1B), a result that was comparable to macrophages incubated with 10 nM peptide. These data indicate that adherent cells, such as BMM ϕ infected with an intracellular bacterial pathogen, can readily be used as targets in the CD107 assay.

3.2 The CD107 mobilization assay detects CTL activity ex vivo

Since ⁵¹Cr release assays measure the total loss of viability for a population of target cells, they are most sensitive when the effector T cells are present in large excess compared to the targets. This means that ex vivo detection of CTL activity in splenocyte preparations can be challenging because the number of pathogen-specific T cells in the spleen following most infections is not high enough to easily achieve E:T ratios of at least 20-50:1 in a given sample well. A notable exception occurs during lymphocytic choriomeningitis virus (LCMV) infection where, at the peak of the response, more than 50% of the CD8⁺ T cells in the spleen are specific for one of the dominant LCMV-derived peptide epitopes (Butz and Bevan, 1998; Masopust et al., 2007). In contrast, seven days following *L. monocytogenes* infection, only 1-2% of the splenic CD8⁺ T cells recognize the dominant LLO₉₁₋₉₉ epitope (Vijh and Pamer, 1997; Kerksiek et al., 1999). Unlike ⁵¹Cr release assays, optimal results for the CD107 assay occur when the E:T ratio is low, to ensure that every T cell has adequate access to a target cell presenting the appropriate peptide:MHC complex. Therefore, we predicted that pathogen-specific CTL would be more readily detected ex vivo using a CD107 mobilization assay than a ⁵¹Cr release assay.

C57BL/6 mice were either infected with *Lm* OVA or given PBS as a control and spleens were harvested from these mice 7 days post-infection for use as effector cells in both ⁵¹Cr release and CD107 mobilization assays. For the ⁵¹Cr release assay, we used BM12 cells, a macrophage-like cell line that only loosely adheres to tissue culture-treated plastic, as targets. As expected, weak cytotoxic activity was detected in splenocytes harvested from *Lm* OVA-infected mice using the ⁵¹Cr release assay, and the naïve splenocyte effectors did not lyse the target cells

(Fig. 2A). Enhanced cytotoxicity was observed for the positive control OVA-specific T cell line, however, even these T cells only recognized the peptide-coated targets and did not cause significant specific lysis of the *Lm* OVA-infected BM12 cells. Infection of BM12 macrophages occurred with ~60% efficiency and was confirmed by lysing the cells in sterile water and plating serial dilutions to recover intracellular bacteria (data not shown). The results confirm that infected cells cannot readily be used as targets in a ^{51}Cr release assay, and was likely due to the high degree of background lysis we observed with the infected targets.

In contrast, CTL that recognized both *Lm* OVA-infected and OVA₂₅₇₋₂₆₄ peptide-coated target cells were detected ex vivo using the CD107 assay (Fig. 2B). Approximately 5% of the CD8⁺ T cells in 7 day immune mice expressed CD107 on the surface following exposure to peptide-coated target cells and approximately 2% of the CD8⁺ T cells exposed to *Lm* OVA-infected target cells stained positively with anti-CD107 antibodies. These results are consistent with previous reports showing that T cell recognition of infected target cells occurs at lower levels than recognition of peptide-pulsed cells (Rolph and Kaufmann, 2000; Skoberne et al., 2001). The most likely explanation for these results is that exogenous treatment with high concentrations of a single synthetic peptide results in significantly more epitope:MHC complexes being present on the cell surface than occurs naturally during infection.

3.3 CTL with novel MHC-Ib restriction were activated during *L. monocytogenes* infection

We recently showed that CD8⁺ T cells with a novel MHC-Ib restriction can play an important role in the clearance of *L. monocytogenes* infection (Seaman et al., 2000; D'Orazio et al., 2003; D'Orazio et al., 2006). To date, we have primarily used IFN γ secretion as an indicator of antigen-specific effector function to characterize these T cells because of the limitations described above. However, IFN γ secretion can be triggered by both antigen-dependent and independent pathways and one of the major roles for CD8⁺ T cells in the clearance of *Listeria* infection is thought to be the ability to lyse infected cells, releasing bacteria from their protected intracellular niche. The only *L. monocytogenes*-specific MHC-Ib restricted CD8⁺ T cells that have been studied in great detail are M3-restricted cells that recognize the formylated peptides fMIVIL, fMIGWII, and fMIVTLF (Pamer et al., 1992; Lenz et al., 1996; Princiotta et al., 1998). Hamilton et al. showed that fMIGWII-specific T cells were deficient in rapid perforin-mediated cytotoxicity and caused lysis of target cells primarily by a slower-acting FasL-mediated mechanism (Hamilton et al., 2004). Thus, it is not yet clear whether any MHC-Ib restricted T cells can effectively lyse infected target cells in vivo.

To determine if other T cells with a novel (not M3) MHC-Ib restriction pattern could display *Listeria*-specific cytotoxic activity, we infected MHC-Ia deficient mice with a mutant strain of *L. monocytogenes* (*Lm* SDA1) that lacks formyltransferase activity and, therefore, does not produce formylated peptides (D'Orazio et al., 2006). We previously showed that during infection of mice, *Lm* SDA1 had only a slight growth defect in the liver, and no difference in the spleen was observed compared to wildtype *L. monocytogenes* (D'Orazio et al., 2006). Importantly, M3-restricted T cells were not activated during *Lm* SDA1 infection of mice. Since CD8⁺ T cells comprise only 1-3% of total splenocytes in MHC-Ia deficient mice (Vugmeyster et al., 1998) we used antibody-coated magnetic beads to enrich for CD8⁺ T cells by negative selection prior to use of the immune splenocyte effectors in a CD107 mobilization assay.

As shown in Fig. 3, *Lm* SDA1-immune mice contained significantly higher numbers of splenic CD107⁺ CD8⁺ cells than did naïve mice. Approximately 2.2% of the total CD8⁺ T cell population in the spleen was specific for *L. monocytogenes* at this time point. We further showed that this CTL activity was not induced by cytokines released from the infected BMM ϕ in vitro by adding antibodies specific for IL-12 and IL-18 to the assay at a final concentration of 1 $\mu\text{g}/\text{ml}$. Antibody treatment only slightly reduced the number of CD107⁺ CD8⁺ T cells detected, suggesting that the presence of IL-12 and IL-18 enhances, but is not

required for *L. monocytogenes*-specific cytotoxic activity. Furthermore, when the CD8⁺-enriched splenocytes were separated from the *L. monocytogenes*-infected BMMφ by incubation in a Transwell filter insert, an increase in CD107⁺ CD8⁺ T cells was not observed (Fig. 3). Thus, contact between the infected macrophage and the CD8⁺ T cell was required for degranulation to occur, suggesting that this was a TCR-dependent event that was not mediated by local cytokine release. Together, these results indicated that CTL with a novel MHC-Ib restriction pattern were activated during *Listeria* infection and suggest that M3-restricted T cells are not the only murine MHC-Ib restricted T cells that have the potential to lyse *L. monocytogenes*-infected cells in vivo.

4. Discussion

The “gold standard” method for detection of CTL activity has been the ⁵¹Cr release assay. However, this assay reveals very little about the effector cell population, and has technical limitations that prevent widespread use with infected target cells. We demonstrate in this report that the CD107 mobilization assay can readily be used with adherent and/or infected target cell populations for the detection of CTL. The CD107 mobilization assay is quantitative and can be used to assess other phenotypic markers on the surface of CD8⁺ T cells with multicolor flow cytometric analysis. Other alternative methods for detection of antigen-specific CD8⁺ T cells have recently been described, including the TRAP assay, which detects transfer of label from surface biotinylated antigen-presenting cells to T cells (Beadling and Slifka, 2006). However, like the ⁵¹Cr release assay, the TRAP assay also requires intact, viable target cells for maximal sensitivity.

In vitro studies suggest that cytotoxic activity can be triggered at lower peptide concentrations than are required for IFNγ production (Betts et al., 2004). This raises the possibility that some bacterial antigens may not be present on the infected cell surface at high enough levels to trigger IFNγ production. If this were true, it would mean that we may be missing some T cell clones specific for epitopes that are present at much lower concentrations on the cell surface by relying solely on in vitro cytokine secretion assays to detect T cells that can recognize infected targets. Additionally, since cell fixation is not necessary in a CD107 mobilization assay, one could easily sort for CD107⁺ cells that respond to infected targets as a means of enriching for subdominant T cell populations contained within immune spleen or lymph node. Future use of the CD107 mobilization assay to detect CTL that specifically recognize infected adherent cells is likely to yield many new pathogen-specific T cell clones.

Acknowledgements

We thank Charlie Lutz for helpful discussions, Greg Baumann for technical assistance, and Wendy Loomis for critical review of this manuscript.

This work was supported by a grant from the National Center for Research Resources (P20 RR20171) to S.E.F.D.

References

- Beadling C, Slifka MK. Quantifying viable virus-specific T cells without a prior knowledge of fine epitope specificity. *Nat Med* 2006;12:1208–12. [PubMed: 17013384]
- Betts MR, Brenchley JM, Price DA, De Rosa SC, Douek DC, Roederer M, Koup RA. Sensitive and viable identification of antigen-specific CD8⁺ T cells by a flow cytometric assay for degranulation. *Journal of Immunological Methods* 2003;281:65–78. [PubMed: 14580882]
- Betts MR, Koup RA. Detection of T-cell degranulation: CD107a and b. *Methods in Cell Biology* 2004;75:497–512. [PubMed: 15603439]
- Betts MR, Price DA, Brenchley JM, Lore K, Guenaga FJ, Smed-Sorensen A, Ambrozak DR, Migueles SA, Connors M, Roederer M, Douek DC, Koup RA. The functional profile of primary human antiviral

- CD8+ T cell effector activity is dictated by cognate peptide concentration. *Journal of Immunology* 2004;172:6407–17.
- Brunner KT, Mauel J, Cerottini JC, Chapuis B. Quantitative assay of the lytic action of immune lymphoid cells on ⁵¹Cr-labelled allogeneic target cells in vitro; inhibition by isoantibody and by drugs. *Immunology* 1968;14:181–96. [PubMed: 4966657]
- Butz EA, Bevan MJ. Massive expansion of antigen-specific CD8+ T cells during an acute virus infection. *Immunity* 1998;8:167–75. [PubMed: 9491998]
- D’Orazio SEF, Halme DG, Ploegh HL, Starnbach MN. Class Ia MHC-deficient BALB/c mice generate CD8+ T cell-mediated protective immunity against *Listeria monocytogenes* infection. *Journal of Immunology* 2003;171:291–8.
- D’Orazio SEF, Shaw CA, Starnbach MN. H2-M3-restricted CD8+ T cells are not required for MHC class Ib-restricted immunity against *Listeria monocytogenes*. *Journal of Experimental Medicine* 2006;203:383–391. [PubMed: 16461341]
- Hamilton SE, Porter BB, Messingham KAN, Badinovac VP, Harty JT. MHC class Ia-restricted memory T cells inhibit expansion of a nonprotective MHC class Ib (H2-M3)-restricted memory response. *Nature Immunology* 2004;5:159–168. [PubMed: 14745446]
- Kerksiek KM, Busch DH, Pilip IM, Allen SE, Pamer EG. H2-M3-restricted T cells in bacterial infection: rapid primary but diminished memory responses. *Journal of Experimental Medicine* 1999;190:195–204. [PubMed: 10432283]
- Lenz LL, Dere B, Bevan MJ. Identification of an H2-M3-restricted *Listeria* epitope: implications for antigen presentation by M3. *Immunity* 1996;5:63–72. [PubMed: 8758895]
- Masopust D, Murali-Krishna K, Ahmed R. Quantitating the magnitude of the lymphocytic choriomeningitis virus-specific CD8 T-cell response: it is even bigger than we thought. *J Virol* 2007;81:2002–11. [PubMed: 17151096]
- Pamer EG, Wang C-R, Flaherty L, Lindahl KF, Bevan MJ. H2-M3 presents a *Listeria monocytogenes* peptide to cytotoxic T lymphocytes. *Cell* 1992;70:215–223. [PubMed: 1353418]
- Penack O, Gentilini C, Fischer L, Asemussen AM, Scheibenbogen C, Thiel E, Uharek L. CD56dimCD16neg cells are responsible for natural cytotoxicity against tumor targets. *Leukemia* 2005;19:835–40. [PubMed: 15744340]
- Pope C, Kim S-K, Marzo A, Masopust D, Williams K, Jiang J, Shen H, LeFrancois L. Regulation of the CD8 T cell response to *Listeria monocytogenes* infection. *Journal of Immunology* 2001;166:3402–3409.
- Princiotta MF, Lenz LL, Bevan MJ, Staerz UD. H2-M3 restricted presentation of a *Listeria*-derived leader peptide. *Journal of Experimental Medicine* 1998;187:1711–1719. [PubMed: 9584149]
- Rolph MS, Kaufmann SHE. Partially TAP-independent protection against *Listeria monocytogenes* by H2-M3-restricted CD8⁺ T cells. *Journal of Immunology* 2000;165:4575–4580.
- Seaman MS, Wang C-R, Forman J. MHC Class Ib-restricted CTL provide protection against primary and secondary *Listeria monocytogenes* infection. *Journal of Immunology* 2000;165:5192–5201.
- Serbina N, Pamer EG. Quantitative studies of CD8+ T-cell responses during microbial infection. *Curr Opin Immunol* 2003;15:436–42. [PubMed: 12900276]
- Skoberne M, Holtappels R, Hof H, Geginat G. Dynamic antigen presentation patterns of *Listeria monocytogenes*-derived CD8 T cell epitopes in vivo. *J Immunol* 2001;167:2209–18. [PubMed: 11490007]
- Starnbach MN, Bevan MJ, Lampe MF. Murine cytotoxic T lymphocytes induced following *Chlamydia trachomatis* intraperitoneal or genital tract infection respond to cells infected with multiple serovars. *Infect Immun* 1995;63:3527–30. [PubMed: 7642287]
- Suni MA, Maino VC, Maecker HT. Ex vivo analysis of T-cell function. *Curr Opin Immunol* 2005;17:434–40. [PubMed: 15950444]
- Vijh S, Pamer EG. Immunodominant and subdominant CTL responses to *Listeria monocytogenes* infection. *Journal of Immunology* 1997;158:3366–3371.
- Vugmeyster Y, Glas R, Perarnau B, Lemmonier FA, Eisen H, Ploegh H. Major histocompatibility complex (MHC) class I K^bD^b -/- deficient mice possess functional CD8⁺ T cells and natural killer cells. *Proceedings of the National Academy of Sciences* 1998;95:12492–12497.

Abbreviations

CTL	cytotoxic T lymphocytes
BMMΦ	bone marrow-derived macrophages
<i>Lm</i> OVA	ovalbumin-expressing strain of <i>Listeria monocytogenes</i>
FCS	fetal calf serum
PBS	phosphate buffered saline
BHI	Brain Heart Infusion
E:T	effector to target ratio
⁵¹Cr	chromium

Fig. 1.

OVA₂₅₇₋₂₆₄ specific T cells express CD107 on the cell surface following exposure to peptide-coated or *Lm* OVA-infected BMMφ. The cytotoxic potential of line DM102 CD8⁺ T cells was assessed by CD107 mobilization assay. (A) C57BL/6 BMMφ were incubated for 1 h in RP-10 media containing the indicated final concentration of synthetic OVA₂₅₇₋₂₆₄ peptide. (B) BMMφ were infected with *Lm* OVA for 3 h at the MOI indicated. DM102 CD8⁺ T cells were added at an E:T ratio of 1:2. The percentage (A) or total number (B) of CD107a/b⁺ cells (gated on CD8⁺ cells) is shown.

Fig. 2.

Listeria-infected cells can readily be used as targets in a CD107 mobilization assay, but not in a ^{51}Cr release assay, for ex vivo detection of CTL activity in the spleen. C57BL/6 mice were infected with 2×10^3 CFU of *Lm* OVA (immune) or given PBS (naïve) and splenocyte effectors were harvested 7 d later. The OVA₂₅₇₋₂₆₄ specific T cell line DM102 was used as a positive control. (A) BM12 macrophages were incubated with OVA₂₅₇₋₂₆₄ peptide (final conc. of 1 μM) for 1 h or infected for 3 h with *Lm* OVA (MOI = 0.5). CD8⁺ T cells were added at the given effector:target ratios (for splenocyte effectors, CD8⁺ T cells were assumed to be ~10% of the total population) and the % specific lysis was determined 4 h later. (B) C57BL/6 BMM ϕ were infected (MOI=0.2) for 4 h with *Lm* OVA or incubated with OVA₂₅₇₋₂₆₄ peptide (final conc. of 1 μM) for 1 hr prior to the addition of either splenocyte effectors or DM102 T cells (E:T = 1:2). The % OVA-specific CD8⁺ T cells present in the spleen was determined by subtracting the number of CD107⁺ cells exposed to untreated BMM ϕ (range = 0.5 – 1.5%) from the number of CD107⁺ cells exposed to either *Lm*-infected or peptide-coated BMM ϕ . Average values $\pm$ SD for groups of mice (n=3) from one of three separate experiments are shown. For the DM102 cell line, the mean $\pm$ SD for pooled data obtained from all three experiments is given.

Fig. 3.

Listeria-specific CTL with a novel MHC-Ib restriction were activated during *L. monocytogenes* infection. MHC-Ia deficient mice were either infected with 1×10^3 CFU of *Lm* SDA1 (7 dpi) or given PBS (naïve). Splenocytes were harvested 7 d later, enriched for CD8⁺ T cells, and added (E:T= 1:2) to BMM ϕ infected for 4 h with *Lm* SDA1 (MOI=0.1-1.0) for analysis in a CD107 mobilization assay. The % *Lm*-specific CD8⁺ T cells present in the spleen was determined by subtracting the number of CD107⁺ cells exposed to uninfected BMM ϕ (typically ~1.0%) from the number of CD107⁺ cells exposed to *Lm*-infected BMM ϕ . In some experiments, the infected BMM ϕ were either pre-treated with anti-IL-12 + anti-IL-18 monoclonal Abs (final conc. 1 μ g/ml) prior to the addition of splenocytes or separated from

the CD8⁺-enriched immune splenocytes by incubation in a Transwell filter (0.4 μ M pore size) insert. (A) Representative FACS plots for splenocytes from individual mice exposed to *Listeria*-infected macrophages. (B) Data for individual mice pooled from 6 separate experiments (n=2-3 per group for each exp.) is shown; mean values are indicated by thick horizontal lines. *P* values for the results of paired T test analyses are shown at the top of the graph.