CD8+ T Cells Specific for a Single Nonamer Epitope of Listeria monocytogenes Are Protective In Vivo
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Summary
Class I major histocompatibility complex (MHC)-restricted CD8+ T cells have been demonstrated to be effective mediators of both acquired and adoptive immunity to the intracellular bacterium Listeria monocytogenes. We have recently determined that L. monocytogenes-infected H-2a mice recognize a nonamer peptide, residues 91-99, of the secreted protein listeriolysin O (LLO), in a H-2Kd-restricted fashion. In this report we have generated CD8+ T cell lines with specificity for LLO 91-99 in the context of H-2Kd by in vitro stimulation with P815 (H-2b) cells transfected with LLO. These CD8+ lines have been generated from immune donors after sublethal infection with L. monocytogenes, or after in vivo immunization with syngeneic spleen cells coated with synthetic LLO 91-99 peptide. LLO-specific CD8+ T cells derived from either protocol were capable of significant protection against L. monocytogenes infection. The in vivo protection by these CD8+ T cell lines has been shown to be solely due to recognition of LLO 91-99 in the context of H-2Kd. These studies demonstrate that CD8+ T cell immunity to a single, naturally produced peptide epitope has the potential for significant protection in a bacterial infection. Thus, the allele-specific motif approach to epitope prediction has identified a naturally produced bacterial epitope with biological relevance.

The resolution of an acute, sublethal infection with the facultative intracellular bacterium Listeria monocytogenes, and the subsequent long-lasting immunity to lethal infection have been characterized as classic examples of cell-mediated immunity (1). A number of studies have clearly demonstrated that adoptive transfer of T cells from immune donors, before or shortly after L. monocytogenes infection of mice, can result in protection against bacterial replication in target organs such as spleen or liver (2-4). CD8+ T cells are the most effective T cell subset in these protection assays (5-10). As first proposed by Mackaness (11), T cells are thought to protect against intracellular bacteria by a mechanism involving activation of the microbicidal activities of macrophages after antigen-driven lymphokine release. Evidence for this mechanism in L. monocytogenes infection is based primarily on macrophage accumulation in target organs (12), the effectiveness of in vivo γIFN treatment against L. monocytogenes infections (13, 14), and the exacerbation of disease in mice treated with neutralizing mAb to γIFN (15). However, recent reports suggest that γIFN secretion by CD4-8- cells may also play a role in resistance to L. monocytogenes infection (16, 17). The role of cytokine release by CD8+ T cells in resolution of listeriosis has not been directly addressed, and a recent study questions the requirement for γIFN in resolution of infection (18). Thus, CD8+ T cell–mediated cytotoxicity against L. monocytogenes infected cells may function as a mechanism of protection in vivo, although in vivo lysis by CD8+ T cells has not been directly demonstrated.

In contrast to the body of evidence supporting a role for T cells in immunity to L. monocytogenes, several recent reports involving T cell subset depletion in vivo have questioned the role of CD8+ T cells in resolution of acute listeriosis and the relevance of the adoptive transfer assay within this context (16, 17, 19). In these studies, bacteria were cleared from the livers of infected mice with only slightly delayed kinetics in animals depleted of CD4+ and CD8+ T cells, suggesting that early γIFN production by CD4-8- NK cells or γ/δ T cells may be critical in clearance of bacteria from this organ. However, similar studies by other investigators have shown that T cell–depleted mice (14) and mice with the SCID mutation (20) fail to completely resolve L. monocytogenes infection, suggesting a role for T cells in this process. In the absence of further information, the simplest interpretation of these data suggests that many facets of the immune response may contribute to host defense against acute, sublethal L. monocytogenes infection, and that natural immunity clearly plays a role in resolution of sublethal infection. Despite these conflicting results regarding the cells responsible for resolution of acute listeriosis, it is clear that previously sensitized T cells are capable of enhancing the protective capacity of natural immunity to L. monocytogenes infection in vivo.

To better understand protective CD8+ T cell immunity
to intracellular bacteria, further information is needed regarding the nature and number of bacterial proteins recognized through the endogenous antigen processing and presentation pathway. *L. monocytogenes* is a complex organism, producing perhaps 4,000 protein products with differentially regulated expression. Under these circumstances, identification of the proteins contributing to class I MHC-restricted, immunogenic peptide epitopes is a complex undertaking. The use of gene transfection technology allowed us to identify listeriolysin O (LLO) as a target molecule for H-2K\(^{d}\) restricted CD8\(^{+}\) T cells derived from *L. monocytogenes*-infected mice (21). LLO is a sulfhydryl (SH)-activated cytotoxin secreted by virulent *L. monocytogenes* that permits entry of the organism into the cytoplasm of eukaryotic cells from the endocytic pathway (reviewed in 22). LLO secretion is necessary for *L. monocytogenes* to induce protective immunity in vivo (10), and to elicit γIFN production from specific CD8\(^{+}\) T cells in vitro (23). Heat-killed organisms and organisms mutant in LLO production are incapable of inducing these responses (10, 23, 24). These observations have suggested a model in which the requirement for LLO secretion for the induction of protective immunity is based on access of the bacterium to the cytoplasm of the infected cell (23). Cytoplasmic localization is sufficient to introduce bacterial proteins into the class I MHC antigen processing pathway (25), resulting in the potential for CD8\(^{+}\) T cell recognition.

Recent advances in biochemical characterization of class I MHC bound peptides have determined that individual class I alleles bind peptides of defined length that contain anchor residues of defined composition and spacing (26–28). The motif identified for H-2K\(^{d}\) (27, 29) has allowed identification of a naturally produced nonamer epitope recognized by LLO-specific CD8\(^{+}\) T cells (21). Since this epitope is presented by H-2K\(^{d}\) molecules in infected mice, it has the potential for relevance in immunity to *L. monocytogenes*. In this study, we report the derivation of CD8\(^{+}\) T cell lines with specificity for the identified LLO epitope and the in vivo biological activity of these class I T cell lines.

**Materials and Methods**

**Mice.** Young female adult DBA/2J and Balb/cByJ mice were obtained from The Jackson Laboratories (Bar Harbor, ME).

**Bacteria.** *L. monocytogenes* strain 43251 was grown in trypticase soy broth (TSB, Becton Dickinson & Co., Cockeysville, MD) to log phase, and aliquots were frozen and stored at -70°C. Freshly thawed bacterial stocks were allowed to return to log phase by incubation at 37°C for 3–4 h, and the bacteria were adjusted to 5 × 10\(^{8}\) CFU/ml.

**Culture Conditions.** *L. monocytogenes* strain 43251 was grown in TSB at 37°C for 3–4 h, and the bacteria were adjusted to the appropriate dilution for intravenous injection in 0.2 ml PBS. The LD\(_{50}\) of this strain of *L. monocytogenes* for DBA/2J mice is <5 × 10\(^{5}\) CFU/animal.

**mAbs.** SF1-1.1 is a mouse IgG2a mAb specific for H-2K\(^{d}\) obtained from American Type Culture Collection (ATCC, Rockville, MD). Culture supernatants and protein G (Pharmacia Fine Chemicals, Piscataway, NJ) purified mAb were used in blocking experiments. 34-4-20s is a mouse IgG2a mAb, and 34-4-21s a mouse IgM mAb, both of which are specific for H-2D\(^{e}\). These mAbs were obtained from ATCC, and both were used in the form of culture supernatant in blocking experiments. 3.168 is a rat anti-mouse mAb specific for CD8\(^{+}\) T cell lines.

**Acid Extraction of MHC Class I Bound Peptides from *L. monocytogenes*-infected Spleens.** Acid extraction of class I MHC bound peptides from 44-h *L. monocytogenes*-infected spleens was performed as described previously, except that DBA/2 mice were infected with 50 LD\(_{50}\) (21). Briefly, spleens were harvested from infected animals and homogenized in 0.1% TFA. After 30-min incubation at 4°C, insoluble material was removed by ultracentrifugation for 35 min at 180,000 g. Soluble material <10,000 molecular weight was recovered after lyophilization and centrifugation of the resuspended material through centricon 10 columns (Amicon, Beverly, MA), and was subjected to reverse phase HPLC using the previously described conditions. Fractions were recovered, and prepared for analysis in CTL assays as described (21).

**In Vitro Stimulation of Effectors and Long-Term CD8\(^{+}\) T Cell Lines.** Primary in vitro stimulation of CD8\(^{+}\) T cell lines was performed by incubating 35–40 × 10\(^{6}\) immune spleen cells with 3 × 10\(^{7}\) irradiated (20,000 rad) PHem3 or PHem3.3 for 7 d in 20 ml RPMI 1640 containing 10% FCS and 50 μM 2-ME (RP10), and then washed three times in RPMI.

**Tumor Cells and Target Cells for CTL Assays.** The target cells used in this study were the class II MHC-negative P815 (DBA/2, H-2\(^{b}\)) mastocytoma and various transfectants of P815. Derivation of the LLO transfectant of P815, PHem3 has been described (21). PHem3.3 is a subclone of PHem3 derived by limit dilution, while PBa-1 is P815 transfected with the parental pH4APr-1-neo vector (31) without insert. P13.4 is P815 transfected with the Escherichia coli β-galactosidase gene (32).

**In Vivo Stimulation of Effectors and Long-Term CD8\(^{+}\) T Cell Lines.** Primary in vitro stimulation of CD8\(^{+}\) T cell lines was performed by incubating 35–40 × 10\(^{6}\) immune spleen cells with 3 × 10\(^{8}\) irradiated (20,000 rad) PHem3 or PHem3.3 for 7 d in 20 ml RPMI in upright 25-cm² flasks (50313, Falcon, Becton Dickinson & Co.) at 37°C in 7% CO\(_2)/air. Subsequent weekly restimulations were carried out with 2.5 × 10\(^{5}\) responder cells, 2 × 10\(^{5}\) irradiated PHem3.3 cells, and 35 × 10\(^{6}\) irradiated (2,000 rad) syngeneic spleen cells in 20 ml RPMI containing 5% supernatant from Con A-stimulated spleen cells and 50 mM α-methyl mannoside in upright 25-cm² flasks. The 0805B CD8\(^{+}\) T cell line was a gift of H.-G. Rammensee and is specific for H-2L\(^{d}\). The 0805B was maintained in analogous fashion to the LLO specific lines except that the stimulators were P13.4 cells.

**CTL Assay.** Target cells (10\(^6\)) in 300 μl RPMI were labeled with 100 μCi [\(^{3}H\)]Cr]sodium chromate for 1 h at 37°C. After washing, 10\(^5\) target cells were incubated with serial dilutions of effector cells in 200 μl of RPMI in round bottomed 96-well plates. The synthetic peptide representing LLO 91–99 (single letter code GYKDGNHEY) was added to the target cells just before their addition to the wells. In vitro targeting activity of HPLC fractions was assessed using 50 μl of each resuspended fraction, 10\(^5\) target cells, and the indicated number of effector CTL in 200 μl RPMI. After 3–4 h incubation at 37°C, 100 μl supernatant was collected and specific lysis was determined as: percent specific lysis = 100 × [(release by CTL – spontaneous release)/maximum release –}]

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*Abbreviations used in this paper: LLO, listeriolysin O; SH, sulfhydryl; TSB, trypticase soy broth.*
In Vivo Protection Assay. CD8+ T cell lines were harvested at day eight or nine after stimulation and were washed twice in PBS before intravenous injection in 0.2 ml. Animals were infected 30 min later by intravenous injection of 5 x 10^5 L. monocytogenes (>1 LD50) in 0.2 ml PBS. Organs were harvested 48 or 72 h after infection and were homogenized (Ultra-Turrax; Tekmar Co., Cincinnati, OH) in sterile distilled water containing 0.2% NP-40. Splenic homogenates were performed in a 5 ml volume, while liver homogenates were performed in 10 ml. Homogenates were incubated at room temperature for 30 min and serial 10-fold dilutions were made in 0.2% NP-40. Aliquots of relevant dilutions were plated on TSB-agar, incubated 18-24 h, and the colonies enumerated. Colony counts from each series were corrected for dilution and averaged to yield CFU/organ. Detection limits were 50 CFU/spleen and 100 CFU/liver. Student's t test was employed in the analysis of results. Each group consisted of three animals, and each experiment was performed at least twice with similar results.

Results

Generation of LLO-specific CD8+ T Cells from Infected Mice. The LLO-expressing P815 transfectant, PHem3, is recognized by CD8+ T cells derived from L. monocytogenes-infected H-2d mice, and a nonamer peptide presented by H-2Kd has been identified as a target epitope (21). To assess the biological relevance of a CD8+ T cell response to this protein, we sought to derive CD8+ T cell lines with specificity directed solely against LLO. DBA/2 (H-2d) mice were sublethally infected with 5 x 10^2 L. monocytogenes via intravenous injection, and their spleen cells were harvested 7 d later and stimulated in vitro with the MHC class II-negative PHem3 transfectant, or a subclone designated PHem3.3. After several rounds of in vitro stimulation, a CD8+ T cell line designated 479-2 was found to specifically lyse the LLO transfectant PHem3.3 and not the P815 cells transfected with the parental vector (Fig. 1). In addition, Fig. 1 demonstrates that the 479-2 line is capable of lysing the control transfectant when incubated in the presence of the previously identified H-2Kd-restricted natural epitope, LLO 91-99. Thus, CD8+ T cell line 479-2 is specific for LLO in the context of H-2d class I molecules and recognizes LLO 91-99 in the context of H-2Kd.

In Vivo Protection by the LLO-Specific CD8+ T Cells. LLO is a secreted protein required for L. monocytogenes entry into the cytoplasm of the infected cell (reviewed in 22). As such, it is likely to be one of the first bacterial proteins accessible to the class I MHC antigen processing pathway and thus, one of the first bacterial proteins to provide epitopes presented at the cell surface during infection. In addition, class I MHC presentation of LLO epitopes should be independent of the need for bacterial destruction in the infected cells. Under these circumstances, a CD8+ T cell response to this protein might be effective at an early stage of L. monocytogenes infection. We addressed this question by infusing line 479-2 into syngeneic DBA/2 mice just before infection of the mice with >1 LD50 of L. monocytogenes. Using this type of protocol, previous experiments have shown that CD8+ T cells from immunized mice (5-10) or a MHC-unrestricted CD8+ T cell clone specific for L. monocytogenes (34) are effective in protection from bacterial replication in spleen and liver. However, these studies have not provided information regarding the nature of the bacterial protein(s) contributing the relevant epitopes in the protective response. Fig. 2 illustrates the results of such an experiment with the LLO-specific line in which injection of 15 x 10^4 479-2 CD8+ T cells just before infection of DBA/2 mice with >1 LD50 of L. monocytogenes reduced the bacterial load by >3 log_10 in spleens.
Figure 3. In vivo protection by the 479-2 CD8+ T cell line is dose dependent and specific (A). Various numbers of 479-2 CD8+ T cells were injected intravenously followed by infection with 5 x 10^3 L. monocytogenes. Spleens were harvested and the numbers of bacteria per spleen determined 72 h after infection. Bacterial numbers from animals infected with 5 x 10^3 L. monocytogenes were determined in spleens (B) and livers (C) after no injection ( ), injection of 10 x 10^6 of the E. coli β-galactosidase-specific CD8+ T cell line 0805B ( ), or injection of 5 x 10^6 479-2 CD8+ T cells ( ).

and by >1.5 log10 in livers, with respect to control mice. The protective ability of the 479-2 CD8+ T cells was dose dependent (Fig. 3 A) and specific since the H-2d-derived CD8+ T cell line 0805B, which is specific for E. coli β-galactosidase in the context of H-2Ld (33), had no protective effect (Fig. 3, B and C). This CD8+ T cell line is maintained in an analogous fashion to the 479-2 line by periodic stimulation with an irradiated P815 transfectant expressing β-galactosidase and irradiated syngeneic spleen cells. Line 479-2 is >97% CD8+ by FACS® analysis, and anti-CD8+ complement treatment completely removed the protective activity of the line (data not shown). These data demonstrate that a CD8+ T cell response to the secreted LLO molecule is capable of inhibiting bacterial replication in mice infected with >1 LD50 of L. monocytogenes. CD8+ T Cell Line 479-2 Is Specific for LLO 91-99. CD8+ T cell line 479-2 clearly has specificity for LLO 91-99 (Fig. 1). However, additional specificities for other epitopes have not been completely ruled out. Antibody blocking of line 479-2 lysis of the LLO transfectant indicates that lysis is inhibited only by anti-H-2Kd antibodies as was demonstrated with recognition of LLO 91-99 (data not shown) (21).

To determine whether other epitopes might be recognized by this line, peptides were acid extracted from DBA/2 spleens 44 h after infection with 50 LD50 of L. monocytogenes and separated by reverse phase HPLC as previously described (21). Line 479-2 reactivity was confined to a single peak with maximal targeting activity in HPLC fraction 28, which has previously been identified as the fraction containing LLO 91-99 (21) (Fig. 4). While these data do not rule out reactivity to other LLO peptides that may have identical elution characteristics, they do strongly suggest, in conjunction with the ability of line 479-2 to very efficiently lyse P815 cells incubated and by >1.5 log10 in livers, with respect to control mice. The protective ability of the 479-2 CD8+ T cells was dose dependent (Fig. 3 A) and specific since the H-2d-derived CD8+ T cell line 0805B, which is specific for E. coli β-galactosidase in the context of H-2Ld (33), had no protective effect (Fig. 3, B and C). This CD8+ T cell line is maintained in an analogous fashion to the 479-2 line by periodic stimulation with an irradiated P815 transfectant expressing β-galactosidase and irradiated syngeneic spleen cells. Line 479-2 is >97% CD8+ by FACS® analysis, and anti-CD8+ complement treatment completely removed the protective activity of the line (data not shown). These data demonstrate that a CD8+ T cell response to the secreted LLO molecule is capable of inhibiting bacterial replication in mice infected with >1 LD50 of L. monocytogenes. CD8+ T Cell Line 479-2 Is Specific for LLO 91-99. CD8+ T cell line 479-2 clearly has specificity for LLO 91-99 (Fig. 1). However, additional specificities for other epitopes have not been completely ruled out. Antibody blocking of line 479-2 lysis of the LLO transfectant indicates that lysis is inhibited only by anti-H-2Kd antibodies as was demonstrated with recognition of LLO 91-99 (data not shown) (21).

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with LLO 91-99 peptide (Figs. 1 and 5), that the in vivo biological activity of the line is due to recognition of the LLO 91-99 epitope in the context of H-2Kd.

**Generation of LLO 91-99-Specific CD8^+ T Cells by Peptide Immunization.** To further determine whether the protective activity of LLO-specific CD8^+ T cells in vivo is due to recognition of LLO 91-99, CD8^+ T cell lines were derived from peptide-immunized mice. Responder cells were spleen cells obtained from DBA/2 mice that had been immunized 10 d earlier by intravenous injection of irradiated syngeneic spleen cells that had been incubated with 1 μM LLO 91-99 at 37°C followed by extensive washing. Line 603-1-2 was derived from peptide-immunized mice stimulated in vitro with the LLO transfectant, and specifically lysed the PHem3.3 cells, as well as the transfectant control cells in the presence of synthetic LLO 91-99, but did not recognize the transfectant control cells alone (Fig. 6). Line 603-1-2 was >97% CD8^+ by FACS® analysis (data not shown).

**In Vivo Protection by CD8^+ T Cells Derived from Peptide-immunized Mice.** CD8^+ T cell line 603-1-2 should have specificity only for the LLO 91-99 epitope because of the in vivo peptide immunization protocol used. Naive spleen cells stimulated in vitro with PHem3.3 exhibited no response to the transfectant or the transfectant control cells (data not shown). It has been observed that CD8^+ T cells derived by in vitro stimulation with peptides often will not recognize endogenously processed and presented antigen despite their ability to recognize peptide-coated targets (35, 36). Stimulation of the LLO 91-99 peptide-immune spleen cells in vitro with the LLO transfectant was carried out to expand CD8^+ T cells with the ability to recognize endogenously presented antigens.

The LLO 91-99-specific CD8^+ T cell line 603-1-2 is capable of protecting DBA/2 mice from *L. monocytogenes* replication in both spleens and livers of mice infected with >1 LD_{50} when the CD8^+ T cells are administered as late as 24 h after infection, reducing bacterial titers by >1 log_{10} and >1.5 log_{10}, respectively (Fig. 8). In contrast, multi-epitope-specific T cells obtained from immune donors 6 d after infection have been found ineffective when injected 24 h postinfection (19). These data confirm that in vivo protection against *L. monocytogenes* can be mediated by CD8^+ T cells with...
specificity for LLO 91-99, and clearly demonstrate that pep-tide immunization in vivo can elicit cells with protective ca-pacity after in vitro stimulation with endogenously processed and presented antigen.

Discussion

CD8⁺ T cell–mediated immunity to L. monocytogenes has been hypothesized to result from antigen–specific release of T cell–derived lymphokines, resulting in activation of mac-rophages (37, 38). However, the target protein(s) for this re-sponse have not been defined. In this report, we have demon-strated that a single, naturally produced epitope of nine amino acids in the LLO molecule can elicit CD8⁺ T cell responses capable of inhibiting L. monocytogenes replication in H-2d mice. In addition, we have shown that LLO 91-99-specific CD8⁺ T cells can reduce bacterial replication in target organs when injected 24 h after infection. This result is in contrast to the reported inability of T cells obtained from 6-d infected animals to affect L. monocytogenes replication when injected 24 h after infection (19). The latter experiments were performed in H-2b mice that have been shown to recog-nize LLO 91-99 (21). It is likely that the differences in these data result from differences in the number of LLO 91-99-specific CD8⁺ T cells injected. These data do not imply that the in vitro expanded CD8⁺ T cells described in this study are more efficient in protection than multi-epitope–specific immune spleen cells, but do suggest that a substantial immune re-sponse to a single nonamer epitope can affect L. monocytogenes replication even after 24 h of infection.

The protective CD8⁺ T cell responses were obtained after in vitro stimulation of immune spleen cells with a transfectant expressing the LLO molecule that is a secreted protein produced early in the intracellular infection by virulent L. monocytogenes (reviewed in 22). Thus, these experiments identify a class of bacterial proteins, those secreted by the or-ganism during intracellular growth, as target proteins for pro-ective CD8⁺ T cell–mediated immunity. The results are con-sistent with the view that any protein reaching the cytoplasm of an eukaryotic cell may be accessible to the class I MHC antigen processing pathway (25). In addition, a poten-tially important feature of epitopes derived from secreted bacterial proteins is that they are likely to be presented early in the infection of a cell. While our experiments do not address the mechanism of protection by the LLO 91-99-specific CD8⁺ T cells, early recognition of the infected cell by CD8⁺ T cells could possibly expand the spectrum of effective immunity to include lytic events, as well as the role for γIFN/lymphokine production and macrophage activation. Under both of these scenarios, the immune CD8⁺ T cells could function to effectively convert a lethal infection into a sublethal infection. This situation could then be dealt with via the components of the natural immune response to L. monocytogenes that are intact and contributing to the resolu-tion of infection in the protected animals. In contrast, class I MHC–restricted presentation of epitopes derived from non-secreted proteins may require intracellular death and degra-dation of the organisms after their entry into the cytoplasm.

These events may be relatively rare since γIFN-activated macrophages apparently prevent L. monocytogenes from exiting the endocytic vacuole (39), and the organism may be degraded without access to the cytoplasm in these cells. One testable hypothesis generated by these suggestions is that protective CD8⁺ T cell immunity may be specific for epitopes found only in secreted proteins from L. monocytogenes.

The ability of single epitope-specific CD8⁺ T cell re-sponses to protect against intracellular pathogens other than viruses is somewhat controversial. In the L. monocytogenes system, a MHC unrestricted CD8⁺ T cell clone has been shown to have some protective effect in vivo, but the single-epitope specificity of the clone was not rigorously demon-strated, and this clone required in vivo administration of rIL-2 for effectiveness (34). Thus, the nature of the recognition event in this protection experiment was undefined. In the mouse model of Plasmodium berghei infection, some circum-sporozite-coat protein–specific CD8⁺ T cell clones ob-tained by in vitro stimulation with peptides were effective in vivo and some were not, despite having reactivity to the same 12 amino acid peptide (40), a finding that may be ex-plained by downmodulation of appropriate homing receptors on some clones (41), or by isolation of peptide–specific clones that could not recognize endogenously presented antigen (35, 36). In addition, repetitive immunization of mice with a trans-fectant expressing a single Plasmodium falciparum coat pro-tein yielded only partial protection, whereas immunization with two transfectants expressing different coat proteins resulted in complete protection (42). The failure to generate a protective response after immunization can result either from the failure to elicit specific T cells, or from the inability of the elicited T cells to affect the outcome of the infection. Our results indicate that LLO 91-99-specific CD8⁺ T cell lines generated from infected mice, or mice immunized with a synthetic peptide derived from the identified natural epi-topo are capable of mediating protection against L. monocyto-genes replication in infected mice. Analysis of the response of single epitope–specific T cell lines composed of oligoclonal populations of responder cells may better reflect the in vivo response to antigen and its biological consequences than the response of individual T cell clones. As we have shown, it is clearly feasible to generate single epitope–specific, high-affinity CD8⁺ T cell lines using a combination of peptide immunization and in vitro stimulation with endogenously processed and presented antigen. This approach overcomes problems with peptide restimulations in vitro that can result in CD8⁺ T cells capable of lysing peptide-coated cells, but that often fail to recognize endogenously presented antigen (35, 36), although exceptions exist at the clonal level (43). Using this immunization/stimulation approach we have de-termined that recognition of LLO 91–99 is relevant to pro-tection against L. monocytogenes, and that LLO 91–99 may be a candidate epitope for analysis of the conditions required for efficient peptide vaccination against intracellular pathogens.

LLO 91–99 was identified using the allele-specific motif for H-2Kd (21). In this report, we have extended this ap-proach to determine that the identified epitope has the potential to elicit CD8⁺ T cell responses capable of significantly
affecting the outcome of infection with the intracellular bacterium *L. monocytogenes*. Thus, this approach to epitope determination, which is based on the identification of allele-specific anchor residues required for class I MHC binding (27, 28), has allowed the prediction of a T cell epitope with biological relevance in the infected animal.

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