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Promiscuity of MHC Class Ib-Restricted T Cell Responses

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Murine infection with the Gram-positive intracellular bacterium Listeria monocytogenes activates CD8+ T cells that recognize bacterially derived N-formyl methionine peptides in the context of H2-M3 MHC class Ib molecules. Three peptides, fMIGWII, fMIVIL, and fMIVTLF, are targets of L. monocytogenes-specific CD8+ T cells. To investigate epitope cross-recognition by H2-M3-restricted CD8+ T cells, we deleted the sequence encoding fMIGWII from a virulent strain of L. monocytogenes. Infection with fMIGWII-deficient L. monocytogenes unexpectedly primed CD8+ T cells that stain with fMIGWII/H2-M3 tetramers and lyse fMIGWII-coated target cells in vivo. Because the fMIGWII sequence is nonredundant, we speculated that other bacterially derived Ags are priming these responses. HPLC peptide fractionation of bacterial culture supernatants revealed several distinct L. monocytogenes-derived peptides that are recognized by fMIGWII-specific T cells. Our results demonstrate that the dominant H2-M3-restricted CD8+ T cell population, although reactive with fMIGWII, is primed by other, non-fMIGWII peptides derived from L. monocytogenes. Although this degree of Ag receptor promiscuity is unusual for the adaptive immune system, it may be a more common feature of T cell responses restricted by nonpolymorphic MHC class Ib molecules. The Journal of Immunology, 2003, 171: 5948–5955.

To provide effective defense and immunity against a plethora of pathogens, mammals have developed rapid innate and relatively delayed adaptive immune responses to infection. Innate immune responses are triggered by engagement of germline-encoded receptors, such as Toll-like receptors (1) and nucleotide-binding oligomerization domain proteins (2), with pathogen-associated molecular patterns. Signals mediated by these receptors initiate early antimicrobial defense mechanisms and fortify the developing adaptive immune response. During the adaptive immune response, pathogen-specific B and T cell clones expand and differentiate following recognition of specific Ags.

CD8+ T cells recognize pathogen-derived peptides presented on MHC class I molecules and are important for clearance of many invading pathogens (3). MHC class I molecules can be divided into two groups. MHC class Ia molecules in mice are encoded by the highly polymorphic H-2K, D, and L loci, and their principal function is to present pathogen-derived peptides to CD8+ T cells. MHC class Ib molecules, in contrast, are much less polymorphic, and, in mice, are encoded by genes in the Q, T, and M region of the MHC. Of these, the H2-M3 MHC class Ib molecule has the most completely defined role in antimicrobial immunity. H2-M3 was originally discovered through studies of a minor histocompatibility Ag, maternally transmitted Ag (4), which is a mitochondrial peptide derived from the N terminus of ND1, a subunit of NADH dehydrogenase (5). Mitochondrial protein synthesis initiates with N-formyl methionine, and H2-M3 selectively binds peptides containing this modified amino acid (6–8). The crystal structure of H2-M3 revealed a hydrophobic peptide-binding groove that specifically accommodates N-formyl methionine as an essential anchor residue (9). The specificity for N-formylated peptides positions H2-M3 especially well for presentation of bacterial ligands, because prokaryotic protein synthesis initiates with N-formyl methionine. Several peptides derived from Mycobacterium tuberculosis, for example, can be bound by H2-M3 and presented to CD8+ T cells (10). In the setting of murine infection with Listeria monocytogenes, three H2-M3-restricted peptides, fMIGWII (11), fMIVIL (Fr38) (12), and fMIVTLF (13), have been identified (11, 14). Although most recent studies have demonstrated peptide presentation, one study suggested that bacterial glycolipids may be presented by H2-M3 to L. monocytogenes-specific CD8+ T cells (15).

H2-M3-restricted CD8+ T cells differ in several respects from their MHC class Ia-restricted counterparts. During thymic development, H2-M3-restricted CD8+ T cells are predominantly selected on bone marrow-derived cells rather than thymic cortical epithelial cells (16), resulting in naive T cells with a partially activated (CD44+) phenotype (17). In addition, positive selection of H2-M3-restricted T cells is mediated by a very small subset of mitochondrially encoded peptides, some of which were recently defined as weak agonists (18–20). Thus, compared with MHC class Ia-restricted T cells, the number of selecting ligands is very limited (21).

Distinct thymic selection may have consequences for H2-M3-restricted T cell responses in the peripheral immune compartment. For example, H2-M3-restricted T cells are distinguished by their

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relative peptide promiscuity. Although most MHC class Ia-restricted T cells are specific to a single pathogen-derived epitope, H2-M3-restricted CD8\(^+\) T cells display a much higher degree of peptide cross-reactivity (22). H2-M3-restricted CD8\(^+\) T cells also manifest distinct primary and memory responses during bacterial infection. Following primary infection with \(L.\) monocytogenes, H2-M3-restricted CD8\(^+\) T cells expand and contract more rapidly than H2-K\(^d\)-restricted CD8\(^+\) T cells (23). Upon rechallenge with \(L.\) monocytogenes, H2-M3-restricted memory CD8\(^+\) T cells undergo very limited expansion, in contrast to the explosive expansion of MHC class Ia-restricted T cells (17, 22, 24).

In this study, we explored the extent of H2-M3-restricted promiscuity by generating a mutant strain of \(L.\) monocytogenes lacking the immunodominant fMIGWII epitope (\(L.\) monocytogenes fMIG\(^{neg}\)). Surprisingly, immunization with fMIGWII-deficient \(L.\) monocytogenes primes fMIGWII-specific CD8\(^+\) T cells, as determined by H2-M3/fMIGWII-tetramer staining and in vivo CTL assays. This suggests that other cross-reacting ligands activate CD8\(^+\) T cells during \(L.\) monocytogenes infection. Indeed, HPLC fractionation of \(L.\) monocytogenes culture supernatants revealed several N-formylated peptides that activate fMIGWII-specific CD8\(^+\) T cells. Our results suggest that H2-M3-restricted CD8\(^+\) T cell responses are primed by a complex mixture of N-formylated bacterial peptides that induce multiple cross-reactive T cell clones. Promiscuous recognition by clonal, MHC class Ib-restricted CD8\(^+\) T cells of peptides sharing a similar molecular pattern is an example of the adaptive immune system pirating a fundamental innate immune strategy for antimicrobial defense.

Materials and Methods

Cell lines and mice

C57BL/6 and C57BL/6F, mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Eight- to 10-wk-old females were used in all experiments and maintained under specific pathogen-free conditions.

The P815 mastocytoma cell line, which expresses H2-M3, was obtained from the American Type Culture Center (Manassas, VA) and maintained in RPMI/10\% FCS.

Bacterial strains

Bacteria were cultured in brain heart infusion (BHI). Wild-type \(L.\) monocytogenes strain 10403s was originally provided by D. Portnoy (University of California, Berkeley, CA). To generate the fMIG\(^{neg}\) \(L.\) monocytogenes strain, the LmaA region of \(L.\) monocytogenes was mutated in the fMIGWII epitope to fMIVTLF by the PCR overlap extension method. The mutation was then incorporated into the chromosome of \(L.\) monocytogenes 10403s by homologous recombination, as described previously (25). To confirm the presence of the mutation, the LmaA gene region was amplified from the genomic DNA preparation of the \(L.\) monocytogenes wild-type and \(L.\) monocytogenes fMIG\(^{neg}\) strain by PCR (PCR kit; Clontech, Palo Alto, CA) using primers outside of the 2-kb region (Keck Oligonucleotide Synthesis Facility, Yale University): 5'-LEMFAR 2 (GGACAGGCTTTCGGACT) and 3'-LEMFAR 2 (CCTGATCTAGTGGCCGT). The PCR products were purified (QIAQUICK PCR purification kit) and sequenced using primers for the mutational region of the LmaA (~300 bp up/downstream of the mutation; Keck Oligonucleotide Synthesis Facility, Yale University; 2K-SEQ5 (CGCTACTTTACAACAGC).

Viable bacterial counts within spleen and liver of infected mice were determined by homogenizing the tissue in PBS containing 0.1% Triton X-100 and plating on BHI agar plates.

Immunization with Listeria

Mice were immunized by i.v. injection of \(5 \times 10^8\) \(L.\) monocytogenes 10403s or fMIGWII-deficient \(L.\) monocytogenes fMIG\(^{neg}\), respectively, into the lateral tail vein. Spleens were harvested 6 days after immunization, and splenocytes were dissociated through a wire mesh. Erythrocytes were lysed with ammonium chloride, and splenocytes were resuspended in RPMI/10\% FCS (Life Technologies, Gaithersburg, MD).

Abs, tetramers, and flow cytometric analysis

PE-conjugated streptavidin tetramers of H2-M3 class Ib MHC complexed with various H2-M3 peptide ligands (fMIGWII, fMIVTLF, fMIVIL) for detecting epitope-specific T cell populations were generated, as previously described (26). For flow cytometric analysis, ~5 \times 10^6 cells were ali-quoted per staining well of a 96-well plate. After incubation at 4°C for 20 min with unconjugated streptavidin (0.5 mg/ml; Molecular Probes, Eugene, OR) and Fc-block (BD Pharmingen, San Diego, CA) in FACS staining buffer (SB; PBS, pH 7.4, 0.5% BSA, and 0.02% sodium azide), cells were triple stained with FITC-conjugated anti-CD45R 1 (BD Pharmingen), or double stained with FITC-conjugated mAbs specific for TCR-β (clone H57-597; BD Pharmingen), or with 15 different TCR Vβ segments (Vβ2, 3, 4, 5/5.2, 6, 7, 8.1/8.2, 8.1–3 (pan), 9, 10, 11, 12, 13, 14, 15, and 17 (all obtained from BD Pharmingen)) in SB for 60 min at 4°C. Subsequently, cells were washed three times in SB and then fixed in 10% paraformaldehyde/PBS (pH 7.4). Flow cytometric analysis was performed using a BD LSR flow cytometer, and data were further analyzed with CellQuest software (BD Biosciences, Mountain View, CA).

Generation of H2-M3-restricted CTL lines and clones

C57BL/6 mice were infected i.v. with \(5 \times 10^7\) \(L.\) monocytogenes 10403s or fMIG\(^{neg}\) strain. Six days after infection, spleenocytes were harvested and stimulated with syngeneic fMIGWII-pulsed irradiated (3000 rad) splenocytes for 14 days. CTL clones were prepared using a limiting dilution technique. A total of 30, 300, 3,000, and 30,000 CTLs, respectively, was cultured with \(2 \times 10^6\) syngeneic fMIGWII-pulsed irradiated splenocytes in RPMI/10\% FCS containing IL-7 (5 ng/ml) and 5% T-Stim (rat Con A supernatant). Twelve distinct, H2-M3-restricted CTL clones were derived.

In vitro restimulation of CTL lines and clones

The CTL cell line and CTL clones were restimulated in vitro with naive, irradiated (3000 rad) syngeneic splenocytes (1/2 spleen/clone) that had been coated with \(1 \times 10^{-6}\) M fMIGWII for 1 h and washed twice in RPMI/10\% FCS (27). For maintenance of the CTL cultures, the medium was additionally supplemented with recombinant mouse IL-7 (5 ng/ml; R&D Systems), rat Con A supernatant (5% v/v; Collaborative Biomedical Products, Bedford, MA), and α-methyl-mannoside (5% v/v; Calbiochem).

Synthetic peptides

Synthetic peptides for the generation of CTL lines and clones, CTL assays, and tetramer generation were obtained from Research Genetics (Huntsville, AL).

Bacterial supernatant preparation (14)

\(L.\) monocytogenes 10403s and the fMIGWII epitope-deficient strain (fMIG\(^{neg}\)) were grown overnight in 5 ml BHI medium. Ten liters of 10403s were harvested, as described previously (27). Bacteria were pelleted and the supernatants were first passed through a 0.22-μm filter and then through a YM-10 membrane (molecular mass cutoff 5 kDa). The resulting filtrate was applied to a 20-ml preparative C18 column at a rate of 1 mL/day. The columns were washed with 40 ml water (HPLC grade), and peptides were eluted with 30 ml of 10–40% acetonitrile. The eluates were lyophilized (Speedvac SPDU11V; Savant/E-C Apparatus, Holbrook, NY), resuspended in 1 mL water (HPLC grade), and passed through 0.22-μm Millex filter (Millipore, Bedford, MA). These filtrates were applied to a C18 300Å reversed-phase HPLC column and fractionated on a 0–60% acetonitrile gradient. One-milliliter fractions were collected, lyophilized, and resuspended in 500 μl of PBS + 5% DMSO. The fractions were tested for activity in CTL assays.

CTL assays

A total of \(1 \times 10^6\) P815 target cells was labeled with 3.7 MBq of \(^{51}Cr\) sodium chromate in 200 μl RPMI/10% FCS for 1–1.5 h. Cells were washed twice with RPMI + 10% FCS and resuspended at \(5 \times 10^5\) per 50 μl. To assay the HPLC fractions, 50 μl of each fraction and 50 μl of the target cells were placed in the wells of a 96-well tissue culture plate. CTLs were added to the wells in a volume of 100 μl of RPMI/10% FCS at an E:T ratio of 10:1. After 4-h incubation at 37°C, the plates were centrifuged at

\[95\]
1500 x g for 5 min, and 50 μl of the supernatant from each well was assayed for 51Cr release with a gamma counter (Top Count NXT; Packard Instruments, Meriden, CT). The percentage of specific lysis was determined, as previously described (29).

**CFSE labeling**

Cells were washed with PBS and resuspended at 5 x 10^6 cells/ml in PBS containing CFSE (Molecular Probes). After incubating at 37°C for 10 min in the dark, cells were immediately washed with cold RPMI/10% FCS before resuspension in PBS for i.v. injection into mice.

**In vivo cytotoxicity assay**

Analysis of in vivo cytolytic activity was conducted, as described previously (30, 31). Splenocytes from C57BL/6 mice were divided into two populations and labeled with either a high concentration (4 μM) or a low concentration (0.25 μM) of CFSE. Next, CFSE<sub>high</sub> cells were pulsed with 1 x 10<sup>-6</sup> M fMIGWII peptide for 1 h at 37°C in the dark, while CFSE<sub>low</sub> cells remained nonpulsed. After washing, CFSE<sub>high</sub> cells were mixed with equal numbers of CFSE<sub>low</sub> cells and 2 x 10<sup>2</sup> cells were injected i.v. into individual mice. Recipient spleens were harvested 18 h later for flow cytometric analysis to measure in vivo killing, as indicated by loss of the CFSE<sub>high</sub> Ag-pulsed population relative to the control CFSE<sub>low</sub> population.

Percentage of specific lysis was calculated according to the formula: (1 – (ratio unprimed/ratio primed) x 100), in which the ratio unprimed = percentage of CFSE<sub>low</sub>/percentage of CFSE<sub>high</sub> cells remaining in noninfected recipients, and ratio primed = percentage of CFSE<sub>low</sub>/percentage of CFSE<sub>high</sub> cells remaining in infected recipients.

**Peptidase digestion of listeriolysin O<sub>91.99</sub> (LLO<sub>91.99</sub>) and MWB fractions**

LLO<sub>91.99</sub> peptide was resuspended in PBS at a concentration of 10<sup>-3</sup> M. The HPLC fractions from MWB bacterial cultures were prepared, as described above. Aminopeptidase M was used at an enzyme concentration of 0.2 U/ml, and carboxypeptidase Y was used at 400 μg/ml. Peptides were digested at 37°C for 1 h and then heated to 55°C for 5 min. The digest was used directly in CTL assays. The concentrations of aminopeptidase M and carboxypeptidase Y used in these experiments did not interfere with CTL function, as shown previously (32).

**Results**

**Generation and characterization of the fMIGWII knockout strain**

To test degree of cross-reactivity of MHC class Ib-restricted CD8<sup>+</sup> T cells, we generated a mutant strain of *L. monocytogenes*, which lacks the major H2-M3-restricted epitope fMIGWII (*L. monocytogenes* fMIG<sup>neg</sup>). Because introduction of a new peptide sequence might inadvertently result in the generation of a novel epitope, which might interfere with our analysis, we mutated the fMIGWII sequence to the previously described MIVIL sequence (12), which is a subdominant H2-M3-restricted *L. monocytogenes*-derived epitope (Fig. 1A). The *L. monocytogenes* fMIG<sup>neg</sup> strain was passaged twice through mice, and the mutation was confirmed by DNA sequencing of bacterial colonies isolated from the spleen. The mutation did not affect the growth of *L. monocytogenes* fMIG<sup>neg</sup> because it grew with similar kinetics as parental *L. monocytogenes* 10403S (Fig. 1B). Virulence was marginally affected by deletion of the fMIGWII epitope, with slightly lower bacterial loads in liver, but equivalent bacterial numbers in spleen (Fig. 1C).

**Deletion of the fMIGWII epitope does not abrogate priming of fMIGWII-specific CD8<sup>+</sup> T cells**

Conventional MHC class Ia-restricted T cells are not primed by *L. monocytogenes* strains lacking their cognate epitope (25). To test whether deletion of an MHC class Ib-restricted epitope also leads to abrogation of priming of fMIGWII-specific CD8<sup>+</sup> T cells, we infected C57BL/6 mice with a sublethal dose (5000 bacteria, i.v.) of wild-type or fMIG<sup>neg</sup> *L. monocytogenes*. H2-M3-restricted CD8<sup>+</sup> T cell responses were measured by tetramer staining and flow cytometry 6 days following primary infection. Surprisingly, the fMIGWII-specific CD8<sup>+</sup> T cell population was equivalent, as measured by staining with H2-M3/fMIGWII tetramers, in mice infected with either wild-type or fMIG<sup>neg</sup> *L. monocytogenes* (Fig. 2). Because no additional fMIGWII sequences can be found in the *L. monocytogenes* proteome (33), we speculated that other H2-M3-restricted epitopes must exist that prime CD8<sup>+</sup> T cells in vivo and cross-react with fMIGWII. Introduction of a second fMIVIL sequence did not detectably increase the frequency of fMIVIL-specific CD8<sup>+</sup> T cells following infection with *L. monocytogenes* fMIG<sup>neg</sup> (Fig. 2).

**CD8<sup>+</sup> T cells primed in the presence or absence of fMIGWII recognize fMIGWII with similar sensitivity in vitro and in vivo**

To test whether fMIGWII-specific CD8<sup>+</sup> T cells derived from mice immunized with wild-type or fMIG<sup>neg</sup> *L. monocytogenes* recognize
fMIGWII with similar affinities, we derived CD8\(^+\) T cell lines from mice immunized with these two strains. After two rounds of in vitro restimulation with fMIGWII peptide, both CTL lines lysed fMIGWII-coated target cells equivalently, as measured using a titration of peptide (Fig. 3). Remarkably, CD8\(^+\) T cells primed in the presence or absence of fMIGWII recognize this epitope with similar affinities. To determine whether in vivo cytolysis against fMIGWII is generated by immunization with L. monocytogenes fMIG\(^{neg}\), we measured epitope-specific cytolytic activity in mice infected with wild-type or fMIG\(^{neg}\) L. monocytogenes using an in vivo cytotoxicity assay (30, 31). C57BL/6 spleenocytes labeled with a high concentration of CFSE were pulsed with fMIGWII peptide and coinjected with similar numbers of control spleenocytes labeled with a lower concentration of CFSE into mice 5 days following primary L. monocytogenes infection. In vivo CTL activity, as measured by disappearance of fMIGWII-pulsed CFSE\(^{high}\) target cells 18 h posttransfer, was readily detectable in L. monocytogenes 10403s-infected mice (average decrease 82%) (Fig. 4). In mice infected with L. monocytogenes fMIG\(^{neg}\), in vivo cytolysis of fMIGWII-bearing target cells was also detected; however, the degree of lysis was diminished compared with mice infected with wild-type bacteria (average decrease 59%). The slight reduction in peptide-coated targets in uninfected mice resulted from a slight inequity of the transferred cells. In repeat experiments, there was no evidence for lysis of peptide-coated target cells in the absence of L. monocytogenes infection. This result confirms that fMIGWII-specific cells are primed in the absence of fMIGWII, but also suggests that fMIGWII presentation enhances the magnitude of the fMIGWII-specific response.

**Generation and characterization of CTL clones derived from mice infected with fMIG\(^{neg}\) L. monocytogenes**

To characterize the bacterial Ags that prime fMIGWII cross-reactive CD8\(^+\) T cells, we generated 12 CTL clones from mice infected with fMIG\(^{neg}\) L. monocytogenes by limiting dilution.

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**FIGURE 2.** Deletion of the fMIGWII epitope does not abrogate priming of fMIGWII-specific T cells. Female C57BL/6 mice were infected with 5 \(\times\) 10\(^3\) L. monocytogenes 10403s (A) or L. monocytogenes fMIG\(^{neg}\) (B), and spleenocytes were harvested 6 days later. Spleenocytes were stained with anti-CD62L, anti-CD8, ethidium monoazide, and one of the three H2-M3/N-formyl peptide tetramers noted on the left. Dot plots are gated on live (ethidium monoazide-negative) CD8\(^+\) T cells. The percentages of Ag-specific, activated (CD62L low) CD8\(^+\) T cells are shown in the upper left quadrant. The background staining in uninfected control mice is negligible (<0.1%). Because of the mouse to mouse variability in H2-M3-restricted T cell responses, dot plots for three different mice are shown.

**FIGURE 3.** CTL lines derived from L. monocytogenes fMIG\(^{neg}\) recognize fMIGWII peptide with similar affinity in vitro. CTL lines derived from C57BL/6 mice infected with 5 \(\times\) 10\(^3\) L. monocytogenes 10403s or L. monocytogenes fMIG\(^{neg}\). Seven days after the second restimulation with fMIGWII peptide-pulsed, irradiated syngeneic splenocytes, \(^{31}\)Cr release assays were performed. The percentage of specific lysis (x-axis) is plotted against the concentration of fMIGWII peptide used in the CTL assays.

**FIGURE 4.** fMIGWII-specific, in vivo cytolysis is induced by immunization with L. monocytogenes fMIG\(^{neg}\). Similar numbers of CFSE high, fMIGWII peptide-pulsed and 1 \(\times\) 10\(^7\) CFSE low, nonpulsed C57BL/6 spleenocytes were coinjected i.v. into C57BL/6 mice that were uninfected (upper panels) or infected 5 days previously with 5000 L. monocytogenes 10403s (middle panels) or L. monocytogenes fMIG\(^{neg}\) (lower panels). In vivo cytolysis of donor cells was assessed 18 h later. Histograms are gated on CFSE\(^{low}\) splenocytes in recipient mice, and are shown for three different mice in each group. Numbers at the top of each plot represent the percentage of CFSE\(^{low}\) or CFSE\(^{high}\) cells of total CFSE\(^{+}\) donor cells recovered.
CD8+ T cell line from *L. monocytogenes* 10403s-infected mice was also generated. All T cell clones and the T cell line stained with fMIGWII:H2-M3 tetramers (Fig. 5, A and B) and recognized fMIGWII with similar affinities, as determined by chromium release assays using a peptide titration (Fig. 5, C and D). Analysis of the TCR Vβ repertoire by staining with Vβ-specific mAbs demonstrated the complexity of the CTL line (Fig. 5E) and supported the clonality of the T cell clones (Fig. 5, F and G).

Multiple ligands are recognized by H2-M3-restricted fMIGWII-specific T cells

The fMIGWII peptide was originally identified by expression cloning (11), but can also be purified from bacterial supernatants. fMIVIL was identified by mass spectrometric sequencing of HPLC-fractioned *Listeria* supernatants (12). To further characterize the ligand(s) which primes fMIGWII-specific T cells in the absence of fMIGWII, we HPLC fractionated bacterial culture supernatants and assayed fractions for recognition by fMIGWII-specific CTL clones. *L. monocytogenes* fMIGneg was grown in peptide-free medium, and filtered culture supernatants were applied to C-18 reverse-phase column. The bound material was eluted with increasing concentrations of acetonitrile into four fractions (10, 20, 30, 40%). These bulk eluates were lyophilized and subjected to reverse-phase HPLC fractionation. The HPLC fractions were tested in CTL assays with all 12 CTL clones and demonstrated complex and distinct recognition pattern for each clone. Multiple

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**FIGURE 5.** Generation and characterization of CTL clones from mice infected with *L. monocytogenes* fMIGneg. A fMIGWII-specific CTL line was derived from C57BL/6 mice infected with *L. monocytogenes* 10403s, and 12 CTL clones were generated by limiting dilution from C57BL/6 mice infected with *L. monocytogenes* fMIGneg. Staining with fMIGWII/H2-M3 tetramers is shown for the CTL line and clone 11.148 (A, B). C and D, show the cytolytic activities; E and F, show TCR Vβ staining of the CTL line and clone 11.148. The table in G provides the Vβ expression of the other clones.

**FIGURE 6.** Several ligands in the supernatant of *L. monocytogenes* fMIGneg are recognized in the absence of the fMIGWII epitope. *L. monocytogenes* fMIGneg was grown in MWB medium, applied to C-18 resin columns, and eluted with water containing acetonitrile at increasing concentrations (10, 20, 30, 40%). These bulk eluates were lyophilized and subjected to reverse-phase HPLC fractionation. The HPLC fractions were tested in CTL assays with all 12 CTL clones and demonstrated complex and distinct recognition pattern for each clone. Multiple
fractions derived from the 20 and 30% bulk eluates contained fMIGWII cross-reactive ligands and induced cytolytic activity in three representative CTL clones derived from L. monocytogenes fMIGWII–infected mice (Fig. 6).

fMIGWII–specific CD8+ T cells are also primed by peptides with a blocked amino terminus

All identified L. monocytogenes–derived, H2-M3–restricted epitopes are N-formyl methionine–containing peptides (21). However, the heat-killed L. monocytogenes–associated Ag was reported to be highly protease resistant, but periodate sensitive, suggesting that it contains a carbohydrate component (15). Subsequently, it was shown that the heat-killed L. monocytogenes–associated Ag consists of a complex of immunogenic fMIGWII associated with bacterial cardiolipin A (34). To determine whether the antigenic HPLC fractions recognized by fMIGWII–specific CTL clones are peptides, as opposed to glycolipids, we selected HPLC fractions derived from the 20 and 30% bulk eluates that are narrow and more hydrophobic than that of most MHC class Ia molecules. This can be explained in part by the distinct structural groove of H2-M3 and other MHC class Ib molecules such as CD1. The N-formyl group of the amino terminus renders fMIGWII (Fig. 7B), but not LLO91–99 (Fig. 7A), resistant to aminopeptidase M digestion. Interestingly, the peptides in the HPLC fraction are similarly resistant to degradation from the N terminus, which indicates that their N termini are also protected from peptidase digestion (Fig. 7, C and D). Given the formyl-methionine specificity of H2-M3, it is likely that the HPLC fractions recognized by fMIGWII–specific CD8+ T cell clones contain other N-formyl methionine-containing peptides that are secreted by L. monocytogenes.

Discussion

In this work, we describe the generation of a strain of L. monocytogenes lacking the dominant, fMIGWII/H2-M3 CTL epitope. To our surprise, murine infection with this epitope-deficient strain primed a large, dominant CD8+ T cell population that stains with MIGWII/H2-M3 tetramers. A database search proved that there are no additional N-terminal MIGWII sequences in the L. monocytogenes proteome. Only two other N-terminal peptides (MIGYGK, MIGPGS) are present in the L. monocytogenes proteome that share identity with the first three residues of fMIGWII. Despite this sequence similarity, neither peptide induced detectable cytolytic response by chromium release assay with the H2-M3–restricted CTL clones (data not shown). Hence, we suspect that other L. monocytogenes–derived peptides cross-react with fMIGWII and primed fMIGWII–specific T cells.

fMIGWII–specific CD8+ T cell clones generated from mice following infection with L. monocytogenes fMIGWII recognize the fMIGWII peptide in vitro with similar affinities as T cell lines generated from mice immunized with wild-type bacteria. Because it is possible that even a few rounds of in vitro restimulation with fMIGWII peptide provide a growth advantage for high affinity clones, we assessed the cytolytic activity of fMIGWII–specific T cells directly in vivo. Using this sensitive assay, we found that fMIGWII–coated target cells were killed slightly less effectively in L. monocytogenes fMIGWII–infected mice than in mice infected with wild-type L. monocytogenes. This suggests that fMIGWII is a bona fide epitope that primes a subset of T cells specific for fMIGWII, and that this epitope is expressed after infection with wild-type bacteria. However, our results also indicate that a substantial fraction of T cells, specific for fMIGWII, is primed by other L. monocytogenes–derived Ags.

Generation of CTL clones from L. monocytogenes fMIGWII–infected mice allowed us to distinguish several secreted peptides that could be fractionated from supernatants of wild-type L. monocytogenes cultures (results not shown). Interestingly, most of the targeting activity was also detected in L. monocytogenes fMIGWII culture supernatants, demonstrating that deletion of fMIGWII removes only one of many potential ligands. The large number of targeting fractions can be attributed to either several distinct ligands or a series of truncation variants of one or several Ags.

Glycolipid bacterial Ags may induce cytolytic activity by H2-M3–restricted CD8+ T cells (15). To prove that other peptides, rather than lipids or glycolipids, primed fMIGWII–specific CD8+ T cells during infection with L. monocytogenes fMIGWII, we subjected HPLC fractions from L. monocytogenes fMIGWII culture supernatants that induced the strongest cytolytic activity to enzymatic digestion. Upon incubation with carboxy-, but not aminopeptidases, cytolytic activity was markedly reduced, demonstrating that peptides are responsible for the cytolytic activity and that their amino terminus is blocked.

H2-M3 and several other MHC class Ib molecules are known to bind a narrower range of peptides than conventional MHC class Ia molecules. This can be explained in part by the distinct structural features of MHC class Ib molecules. For example, the binding groove of H2-M3 and other MHC class I-like molecules such as CD1 is narrower and more hydrophobic than that of most MHC class Ia molecules (9, 35). Only a few of the 13 mitochondrial proteins, the only source for endogenous H2-M3 ligands, and some bacterially derived N-terminal sequences fulfill the requirements
for this molecular pattern (21, 36). A restricted ligand specificity for endogenous peptides was also reported for the nonclassical MHC class Ib molecule Qa-2 (37), which is encoded in the Q region of the H-2 gene locus. The GPI-anchored Qa-2 molecule, implicated in innate and adaptive responses, specifically appears to be a resistance gene for murine cyssticercosis (38). Qa-2 can still associate with a substantially more diverse array of peptides than other nonclassical MHC class Ib molecules, but due to special constraints in the hydrophobic binding groove, the number of ligands is more limited compared with MHC class Ia molecules (39).

Interactions between H2-M3 and its peptide ligands, crucial for assembling a stable complex, have been investigated carefully. To identify those peptides that bind best to H2-M3, phage display libraries were screened. Amino acids with hydrophobic side chains preferentially occupied positions P2, P3, P4, and P6, which is consistent with the very hydrophobic pocket and the sequences of the natural ligands for H2-M3 (40). Furthermore, ~70% of the hydrophobic interactions are formed between the first four residues of the ligand and residues in the H2-M3-binding groove. Taking this into consideration, the requirements that are essential for binding to H2-M3, namely hydrophobic amino acid composition and interactions with essentially only four N-terminal residues, the number of N-terminal sequence permutations for H2-M3 ligands is limited. Of those hydrophobic peptides that do bind to H2-M3, the amino acid side chains are buried within the H2-M3-binding groove and therefore inaccessible to TCR (9, 41). Hence, similar surface characteristics that are formed by the H2-M3 molecule with different ligands might result in cross-reactive recognition by a given TCR.

Cross-reactive recognition of multiple ligands has also been reported for T cells specific for other MHC class Ib molecules. Following Salmonella typhimurium infection, Qa-1-restricted CTLs specific for an epitope derived from the bacterial GroEL molecule also cross-react with and expand in response to a peptide derived from self heat shock protein 60 (42). CD1-restricted CD8 T cells recognize glycolipids derived from endogenous and bacterial sources. Various ubiquitous lipid structures in mammalian cells can be presented by CD1 to T cells, resulting in a considerable degree of autoreactivity in the CD1-restricted T cell pool (43). For example, CD1b presents ganglioside self Ags with complex carbohydrate structures to CD8 T cells. Remarkably, the same TCR also recognizes carbohydrate epitopes shared by a variety of self glycosphingolipids. The GM1 ganglioside epitope that is recognized by autoreactive T cells can also recognize carbohydrate epitopes shared by a variety of self pathogens (44). Thus, CD1b-restricted T cells also appear to cross-react with several distinct Ags, in this case both self and microbial glycolipid structures (45).

MHC class Ib-restricted T cell responses remain an important field of investigation. Clearly, MHC class Ib-restricted T cells contribute to the clearance of intracellular pathogens in mice (17, 46, 47). Moreover, it was demonstrated recently that CD8 T cells in humans recognize a M. tuberculosis-derived Ag in the context of the nonclassical, monomorphic MHC class I molecule HLA-E (48). Eliciting MHC class Ib-restricted T cells against infectious pathogens remains an exciting option for vaccine development.

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References


