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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 Ia 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...
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-Kb-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, 23, 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 fMIGneg). 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 CB6F1 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).3 Wild-type L. monocytogenes strain 10403s was originally provided by D. Portnoy (University of California, Berkeley, CA). To generate the fMIGWII L. monocytogenes strain, the LemA region of L. monocytogenes was mutated in the fMIGWII epitope to fMIVIL 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 LemA gene region was amplified from the genomic DNA preparation of the L. monocytogenes wild-type and L. monocytogenes fMIGWII strain by PCR (PCR kit; Clontech, Palo Alto, CA) using primers outside of the 2-kb region (KecK Oligonucleotide Synthesis Facility, Yale University); 5'-LEMPAR 2 (GGACAGGCTTTCGGACT) and 3'-LEMPAR2 (GCTGATCTGATGCGGGT). The PCR products were purified (QIAQUICK PCR purification kit) and sequenced using primers for the mutational region of LemA (~300 bp up/downstream of the mutation; KecK Oligonucleotide Synthesis Facility, Yale University; 2KB-SEQ5 (CGCTACTTTCACACAGC).

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 × 108 L. monocytogenes 10403s or L. monocytogenes fMIGWII, 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 am-
1500 × 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 × 10⁷ 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 × 10<sup>−6</sup> M IMIGWII 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 × 10⁷ 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) × 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 listeriolsin 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 10⁻⁸ 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 IMIG<sup>weg</sup>). Because introduction of a new peptide sequence might inadvertantly result in the generation of a novel epitope, which might interfere with our analysis, we mutated the IMIGWII sequence to the previously described IMIVIL sequence (12), which is a subdominant H2-M3-restricted *L. monocytogenes*-derived epitope (Fig. 1A). The *L. monocytogenes* IMIG<sup>weg</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* IMIG<sup>weg</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 IMIGWII-specific CD8<sup>+</sup> T cells, we infected C57BL/6 mice with a sublethal dose (5000 bacteria, i.v.) of wild-type or IMIG<sup>weg</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 IMIGWII-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 IMIG<sup>weg</sup> *L. monocytogenes* (Fig. 2). Because no additional IMIGWII 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 IMIVIL-specific CD8<sup>+</sup> T cells following infection with *L. monocytogenes* IMIG<sup>weg</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 IMIG<sup>weg</sup> *L. monocytogenes* recognize...
fMIGWII with similar affinities, we derived CD8\(^+\)/H11001 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\(^+\)/H11001 T cells primed in the presence or absence of fMIGWII recognize this epitope with similar affinities. To determine whether in vivo cytolytic activity against fMIGWII is generated by immunization with \textit{L. monocytogenes} fMIG\textsuperscript{neg}, we measured epitope-specific cytolytic activity in mice infected with wild-type or fMIG\textsuperscript{neg} \textit{L. monocytogenes} using an in vivo cytotoxicity assay (30, 31). C57BL/6 splenocytes labeled with a high concentration of CFSE were pulsed with fMIGWII peptide and cocultured with similar numbers of control splenocytes labeled with a lower concentration of CFSE into mice 5 days following primary \textit{L. monocytogenes} infection. In vivo CTL activity, as measured by disappearance of fMIGWII-pulsed CFSE\textsuperscript{high} target cells 18 h posttransfer, was readily detectable in \textit{L. monocytogenes} 10403s-infected mice (average decrease 82%) (Fig. 4). In mice infected with \textit{L. monocytogenes} fMIG\textsuperscript{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 cell lysis in the absence of \textit{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\textsuperscript{neg} \textit{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\textsuperscript{neg} \textit{L. monocytogenes} by limiting dilution. A
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* fMIG neg 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 ligands are recognized by the CTL clones.

![FIGURE 5](http://www.jimmunol.org/)

![FIGURE 6](http://www.jimmunol.org/)
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 fMIGneg-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 and subjected them to peptidase digestion (Fig. 7, A and C). This suggests that peptides, not glycolipids, were detected by fMIGWII-specific CTL clones (data not shown). Hence, we suspected 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 fMIGneg 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 fMIGneg-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 CTLs with specificity for this epitope. 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 fMIGneg-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 fMIGneg 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 fMIGneg, we subjected HPLC fractions from L. monocytogenes fMIGneg 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 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 (fMIGYGK, fMIGPGS) 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). Thus, we suspect that other L. monocytogenes-derived peptides cross-react with fMIGWII and primed fMIGWII-specific T cells.

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47). Moreover, it was demonstrated recently that CD8
tribute to the clearance of intracellular pathogens in mice (17, 46,
eld of investigation. Clearly, MHC class Ib-restricted T cells con-
carbohydrates (44). Thus, CD1b-restricted T cells also appear to
also recognizes carbohydrate epitopes shared by a variety of self
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