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# Partially TAP-Independent Protection Against *Listeria monocytogenes* by H2-M3-Restricted CD8<sup>+</sup> T Cells

Michael S. Rolph and Stefan H. E. Kaufmann<sup>1</sup>

Effective protection against *Listeria monocytogenes* requires Ag-specific CD8<sup>+</sup> T cells. A substantial proportion of CD8<sup>+</sup> T cells activated during *L. monocytogenes* infection of C57BL/6 mice are restricted by the MHC class Ib molecule H2-M3. In this study, an H2-M3-restricted CD8<sup>+</sup> T cell clone specific for a known H2-M3 epitope (fMIGWII) was generated from *L. monocytogenes*-infected mice. The clone was cytotoxic, produced IFN- $\gamma$ , and could mediate strong protection against *L. monocytogenes* when transferred to infected mice. Macrophages pulsed with heat-killed *Listeriae* presented Ag to the clone in a TAP-independent manner. Both TAP-independent and -dependent processing occurred *in vivo*, as TAP-deficient mice infected with *L. monocytogenes* were partially protected by adoptive transfer of the clone. This is the first example of CD8<sup>+</sup> T cell-mediated, TAP-independent protection against a pathogen *in vivo*, confirming the importance of alternative MHC class I processing pathways in the antibacterial immunity. *The Journal of Immunology*, 2000, 165: 4575–4580.

*Listeria monocytogenes* is a Gram-positive bacterium that has been used extensively for studying cell-mediated immunity against intracellular bacteria. Both innate and adaptive immunity are involved in the control of *Listeria* infections (1). A characteristic pathogenetic feature is the ability of *Listeria* to produce a pore-forming hemolysin (listeriolysin), which enables it to escape from the phagosome into the cytoplasm (2). One consequence of this is that pathogen-derived epitopes are readily presented by classical MHC class I to CD8<sup>+</sup> T cells, which are central to the control of infection through cytotoxicity and the production of cytokines such as IFN- $\gamma$  (3). Accordingly, mice deficient in  $\beta_2$ -microglobulin are hindered in their ability to control *L. monocytogenes* infection (4, 5).

H2-M3 is a nonclassical MHC class Ib molecule that can present pathogen-derived epitopes to CD8<sup>+</sup> T cells during the infection of mice with *L. monocytogenes* (6, 7). Several features distinguish H2-M3 from classical MHC class Ia molecules. First, H2-M3 exhibits minimal polymorphism. Only seven different forms of H2-M3 are known, and the vast majority of laboratory mice express the same form, which is designated H2-M3<sup>wt</sup> (8). Second, H2-M3 has a remarkable preference for binding formylated peptides (9), a phenomenon that is now understood at the molecular level (10). In eukaryotic cells, formyl groups are only found on 13 mitochondrial proteins, whereas all prokaryotic protein synthesis initiates with a formyl methionine group. Thus, it has been hypothesized that H2-M3 is specialized for the presentation of bacteria-derived formylated epitopes (9, 11).

Recently, several *Listeria*-derived epitopes that are presented by H2-M3 to CD8<sup>+</sup> T cells have been identified (12–14). In this study we have used one of these epitopes, fMIGWII, to investigate the protective function of H2-M3 restricted T cell responses during *L.*

*monocytogenes* infection. An H2-M3-restricted cytotoxic CD8<sup>+</sup> T cell clone was generated that could mediate protection following adoptive transfer to *Listeria*-infected mice. Partial protection was also observed following transfer to TAP1<sup>-/-</sup> recipients, indicating that an unconventional Ag processing pathway was operative *in vivo*.

## Materials and Methods

### Mice

Female C57BL/6 mice were obtained from the Bundesinstitut für gesundheitlichen Verbraucherschutz und Veterinärmedizin (Berlin, Germany), and were used between 6 and 8 wk of age. TAP1<sup>-/-</sup> mice were provided by Dr. S. Tonegawa (Massachusetts Institute of Technology, Cambridge, MA) and backcrossed six times to the C57BL/6 background. All mice were bred under specific pathogen-free conditions.

### Materials

The formylated peptide fMIGWII (12) was synthesized by Jerini BioTools (Berlin, Germany). *L. monocytogenes* strain EGD Sv1/2a was originally obtained from G. B. Mackaness (Trudeau Institute Saramac Lake, NY) and grown in tryptic soy broth (Difco, Detroit, MI). Stocks were stored at -70°C until use. Heat-killed *L. monocytogenes* (HKL)<sup>2</sup> was prepared by incubating bacteria at 70°C for 1 h. Effective killing was confirmed by incubating an aliquot of HKL overnight in tryptic soy broth. The HKL preparation did not contain peptides capable of binding directly to H2-M3 as verified using the H2-M3 peptide binding assay previously described by Vyas et al. (15) (data not shown).

### Cells

EL4 and L929 cells were cultured in RPMI medium supplemented with 10% FCS (Sigma, St. Louis, MO), 1 mM L-glutamine, 10 mM HEPES, 5 × 10<sup>-5</sup> M 2-ME, 1 mM sodium pyruvate, 100  $\mu$ g/ml penicillin, and 100 U/ml streptomycin. This medium was designated RP10. Bone marrow cells were harvested from the tibia and femur of C57BL/6 mice and seeded into 90-mm bacterial grade petri dishes (Greiner, Frickenhausen, Germany). Bone marrow-derived macrophage (BMM) medium was DMEM supplemented with 10% FCS, 5% horse serum, 1 mM glutamine, 10 mM HEPES, 1 mM sodium pyruvate, and 20% L929 cell-conditioned medium. The cells were cultured at 37°C in a humidified atmosphere containing 7% CO<sub>2</sub>. All tissue culture reagents were obtained from Biochrom (Berlin, Germany) unless otherwise stated.

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<sup>2</sup> Abbreviations used in this paper: HKL, heat-killed *L. monocytogenes*; BMM, bone marrow-derived macrophage; ELISPOT, enzyme-linked immunospot; ER, endoplasmic reticulum.

### Peptide immunization

fMIGWII (2 mg/ml in PBS) was emulsified in a 1:1 mix with IFA (Organon Teknika, Durham, NC). Mice were immunized twice at 10-day intervals with 100  $\mu$ g peptide s.c. at the base of the tail. Control mice received PBS emulsified with IFA. Ten days after the second immunization, groups of mice were either challenged with  $5 \times 10^3$  CFU *L. monocytogenes* or sacrificed to determine the efficacy of peptide immunization.

### Generation of T cell lines

To generate H2-M3-restricted CTL lines specific for fMIGWII, C57BL/6 mice were infected with  $5 \times 10^3$  CFU *L. monocytogenes* i.v., and the spleens were collected 6 days later. Single cell suspensions were prepared by passing the spleens through a metal sieve, followed by lysis of the erythrocytes with ammonium chloride lysis buffer. Forty million *L. monocytogenes*-immune spleen cells were cultured with the same number of stimulator cells in RP10. Stimulator cells were prepared by incubating irradiated (2000 rad) C57BL/6 spleen cells at 37°C for 1 h in the presence of  $10^{-7}$  M fMIGWII. The stimulator cells were washed twice before being added to the *Listeria*-immune spleen cells. The fMIGWII-specific T cell lines were cultured in RP10 and restimulated on a weekly basis by the addition of fresh stimulator cells pulsed with fMIGWII. After the second in vitro stimulation the cells were sustained by twice weekly addition of fresh RP10 containing 7% supernatant from Con A-stimulated spleen cells.

For some experiments CD8<sup>+</sup> T cell lines or clones of irrelevant Ag specificity were used as a negative control. T cell lines specific for OVA<sub>257-264</sub> (H2-K<sup>b</sup>) (16) and LLO<sub>91-99</sub> (H2-K<sup>d</sup>) (17) were cultured in the same way as 3C8 cells, using peptide-loaded irradiated spleen cells as control. Alternatively a CD8<sup>+</sup> T cell clone specific for the adenovirus type 5 E1A gene (H-2D<sup>b</sup>) was cultured as described (18).

### Cloning of fMIGWII-specific T cell lines

fMIGWII-specific T cell lines were cloned by limiting dilution. Briefly, T cells were cultured in 96-well round bottom plates (Nunc, Roskilde, Denmark) at a density of 0.3 cells/well and stimulated with  $5 \times 10^4$  irradiated, fMIGWII-coated APC per well (prepared as described above). Cultures were conducted in RP10 supplemented with 7% Con A supernatant. Clones were expanded by weekly restimulation with peptide-coated APC and by twice weekly addition of fresh Con A supernatant. One clone, 3C8, was further expanded in six-well plate (Nunc) cultures for in vivo studies.

### Cytotoxic T lymphocyte assay

Target cells were BMM infected with *L. monocytogenes* at an moi of 10 for a period of 6 h. During the final 90 min, the cells were labeled with 100  $\mu$ Ci <sup>51</sup>Cr. Alternative targets were EL4 cells labeled with 50  $\mu$ Ci <sup>51</sup>Cr and  $10^{-6}$  M fMIGWII. The targets were washed three times and  $5 \times 10^3$  targets/well were added, together with 3C8 cells at a range of E:T ratios. After a 4-h incubation, 80  $\mu$ l supernatant was collected and <sup>51</sup>Cr activity was determined. Percentage of specific lysis was determined as (experimental value - spontaneous release)/(maximum release - minimum release).

### Stimulation with HKL

BMM from C57BL/6 and TAP1<sup>-/-</sup> mice were plated out at  $5 \times 10^4$  cells per well in flat-bottom 96-well plates and allowed to adhere overnight.  $10^4$  3C8 cells/well in 100  $\mu$ l RP10 were cultured together with  $10^8$ /ml HKL. Supernatant was collected after 24 h, and IFN- $\gamma$  was measured by ELISA.

### Adoptive transfer

For adoptive transfer experiments, clone 3C8 was harvested 8–10 days following previous Ag stimulation and washed twice in PBS before i.v. administration in 200  $\mu$ l PBS. Mice were infected i.v. 30–60 min later with  $10^5$  *L. monocytogenes*. Three days following infection, the mice were killed and bacterial load in the liver and spleen were determined as described previously (4).

### Cytokine assays

IFN $\gamma$ , IL-4, IL-6, and IL-10 were measured by standard capture ELISA, using R4-6A2, 11B11, 20F3, and JES5-2A57 as capture Abs and biotinylated XMG1.2, BVD6-24G2, 32C11, and SXC-1 as detection Abs, respectively. For measurement of TNF, an L929 cell bioassay was performed. Briefly,  $5 \times 10^4$  L929 cells were cultured overnight in a flat-bottom 96-well plate (Nunc). Samples were diluted 2-fold across the plate, and actinomycin D (Sigma) was added to a final concentration of 2  $\mu$ g/ml. After a 24-h incubation the cells were fixed with 5% formaldehyde, stained

with crystal violet, and washed gently in water. After addition of 33% acetic acid, the plates were read at 595 nm. The results were measured against a recombinant TNF- $\alpha$  standard (Genzyme, Cambridge, MA).

Inguinal lymph nodes were collected from immunized mice, and peptide reactivity was determined by IFN- $\gamma$  enzyme-linked immunospot (ELISPOT). ELISPOT plates (Millipore, Bedford, MA) were coated with 4  $\mu$ g/ml anti-IFN- $\gamma$  Ab (R4-6A2) in 0.05 M carbonate buffer, pH 9.6 at 4°C overnight. The plates were washed once and blocked with PBS-1% BSA for 2 h at 37°C. After 2 washes, varying numbers of lymph node cells, together with  $10^5$  control or fMIGWII-coated ( $10^{-6}$  M; 37°C incubation for 1 h) EL4 cells were incubated in RP10 for 24 h. The plates were washed extensively, and 0.25  $\mu$ g/ml biotinylated anti-IFN $\gamma$  Ab (XMG1.2) was added. After a 2-h incubation at 37°C, the plates were washed and alkaline phosphatase-conjugated streptavidin (Dianova, Hamburg, Germany) was added for a further 1 h incubation. The plates were washed and color was developed for 15 min by addition of 5-bromo-4-chloro-3-indolylphosphate/nitro blue tetrazolium alkaline phosphatase substrate (Sigma). Spots were counted under a dissecting microscope.

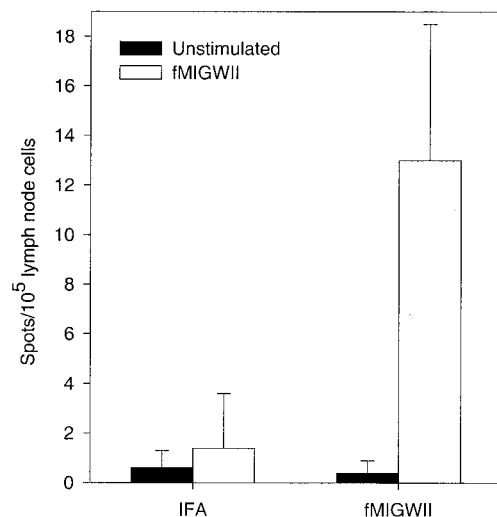
### Statistics

CFU data were analyzed using a two-tailed Mann-Whitney *U* test. Differences were considered significant when  $p < 0.05$ .

## Results

### Peptide immunization

Three *Listeria*-derived peptide epitopes that are presented by H2-M3 to CD8<sup>+</sup> T cells have been described: fMIGWII (12), fMIVIL (13), and fMIVTLF (14). To investigate the protective capacity of H2-M3-restricted T cell responses during *Listeria* infection, peptide immunization with fMIGWII was undertaken. C57BL/6 mice were immunized s.c. with 100  $\mu$ g fMIGWII, followed by a booster immunization 10 d later. After another 10 days, draining lymph nodes and spleen cells were collected from one group of mice and tested for reactivity to fMIGWII in an IFN- $\gamma$  ELISPOT. Significant T cell priming was detected in peptide-immunized mice, but not in mice receiving IFA alone (Fig. 1). The remaining immunized mice were infected i.v. with  $5 \times 10^3$  CFU *L. monocytogenes*. As a positive control, mice infected with  $10^3$  CFU *L. monocytogenes* i.v. 20 days previously were also challenged with *Listeria*. At days 2 (data not shown) and 4 (Table I) following infection, groups of four mice were sacrificed and bacterial loads in the liver and spleen were determined. In contrast to



**FIGURE 1.** Induction of fMIGWII-reactive T cells by peptide immunization. C57BL/6 mice were immunized s.c. at the base of the tail on days 0 and 10 with 100  $\mu$ g fMIGWII in IFA or with IFA alone. On day 20 the inguinal lymph nodes were collected and fMIGWII-reactive cells were quantitated by IFN- $\gamma$  ELISPOT. Data are mean  $\pm$  SD,  $n = 3$  mice per group.

Table I. *fMIGWII* immunization does not protect against *L. monocytogenes*<sup>a</sup>

Immunization	Log <sub>10</sub> CFU	
	Liver	Spleen
IFA	3.9 ± 0.3	5.6 ± 0.3
IFA + <i>fMIGWII</i>	3.5 ± 0.2	5.3 ± 0.6
<i>L. monocytogenes</i>	<2.3	2.3 ± 0.3

<sup>a</sup> C57BL/6 mice were immunized s.c. with 100 μg *fMIGWII* in IFA on days 0 and 10, or infected with 10<sup>3</sup> CFU *L. monocytogenes* i.v. on day 0. On day 20 mice were challenged with 5 × 10<sup>3</sup> CFU *L. monocytogenes* i.v. Bacterial load in the liver and spleen were determined 4 days later. Data are mean log<sub>10</sub> CFU ± SD, n = 4 mice per group.

mice that had been previously infected with *Listeria*, mice immunized with *fMIGWII* were not protected against *Listeria* challenge infection (Table I). In a subsequent experiment, we attempted to improve immunization efficacy by immunizing mice with a mix of 100 μg of both *fMIGWII* and another H2-M3 binding epitope from *L. monocytogenes*, *fMIVIL*. However, no protection was observed following challenge with *Listeria* (data not shown).

#### Generation of *fMIGWII*-specific, H2-M3-restricted T cells

Resistance to *Listeria* infection involves numerous arms of the immune response, and it is perhaps not surprising that immunization with a single H2-M3-restricted CTL epitope was not sufficient to induce protection. As an alternative approach to peptide immunization, T cells specific for *fMIGWII* were generated and characterized with respect to protective function. To generate H2-M3-restricted T cells, *Listeria*-immune spleen cells obtained 7 days following sublethal *Listeria* infection were stimulated with irradiated spleen cells pulsed with 10<sup>-7</sup>M *fMIGWII*, as described in *Materials and Methods*. The resulting cell line was cytotoxic and produced IFN-γ (data not shown). The line was cloned by limiting dilution, and one clone, 3C8, was expanded for further in vitro and in vivo analysis.

#### Characterization of the H2-M3-restricted clone 3C8

The 3C8 clone was CD8<sup>+</sup>, αβ TCR<sup>+</sup> (data not shown) and was able to lyse targets pulsed with *fMIGWII* (Fig. 2A) as well as

*Listeria*-infected targets (Fig. 2B). To characterize the cytokines produced by clone 3C8, 5 × 10<sup>4</sup> 3C8 cells (10 days after the last Ag stimulation) were cultured with 5 × 10<sup>5</sup> control or *fMIGWII*-pulsed APC, prepared as described in *Materials and Methods*. Culture supernatant was collected after 48 h and IFN-γ, TNF, IL-4, IL-6, and IL-10 were measured. Essentially no cytokine was produced by unstimulated 3C8 cells, whereas IFN-γ, TNF, and IL-6 were detected in the peptide-stimulated cultures (Table II). IL-4 and IL-10 were not detected. Neither could IL-4 be detected in the more sensitive ELISPOT assay (data not shown). Thus, 3C8 cells are associated with a typical type 1 cytokine pattern (19).

#### Adoptive transfer experiments

To investigate the role of H2-M3-restricted T cells in protection against *Listeria* infection, adoptive transfer experiments were performed. C57BL/6 mice were given 10<sup>7</sup> 3C8 cells i.v. in 200 μl PBS or PBS alone and then infected i.v. with a high dose of *L. monocytogenes* (10<sup>5</sup> CFU). Three days later, bacterial load in the liver and spleen were determined. Bacterial titers in the spleen (*p* < 0.02) and liver (*p* < 0.01) were substantially reduced in those mice that had received 3C8 cells compared with control mice (Table III). In all experiments, 3C8 cells were more active in reducing bacterial load in the liver than in the spleen. As a further control, in one experiment 10<sup>7</sup> CD8<sup>+</sup> cells specific for SIINFEKL presented by H2-K<sup>b</sup> were transferred to *Listeria*-infected C57BL/6 mice. These cells were cultured using the same conditions as 3C8 cells, but did not influence the course of *Listeria* infection (data not shown).

To further assess the antilisterial activity of 3C8 cells, their ability to protect against lethal *Listeria* infection was determined. C57BL/6 mice were given 10<sup>7</sup> 3C8 cells or PBS i.v. and were infected with 5 × 10<sup>4</sup> CFU *L. monocytogenes* i.v., a dose that results in 100% mortality in untreated mice. All PBS-treated mice rapidly died, whereas mice that received 3C8 cells survived infection (Fig. 3). Thus, H2-M3-restricted T cells can protect mice from a lethal *Listeria* infection.

#### TAP-independent processing of *Listeria*-derived Ag

The pathway by which Ag is processed and presented by H2-M3 is at least partially distinct from the conventional MHC class I

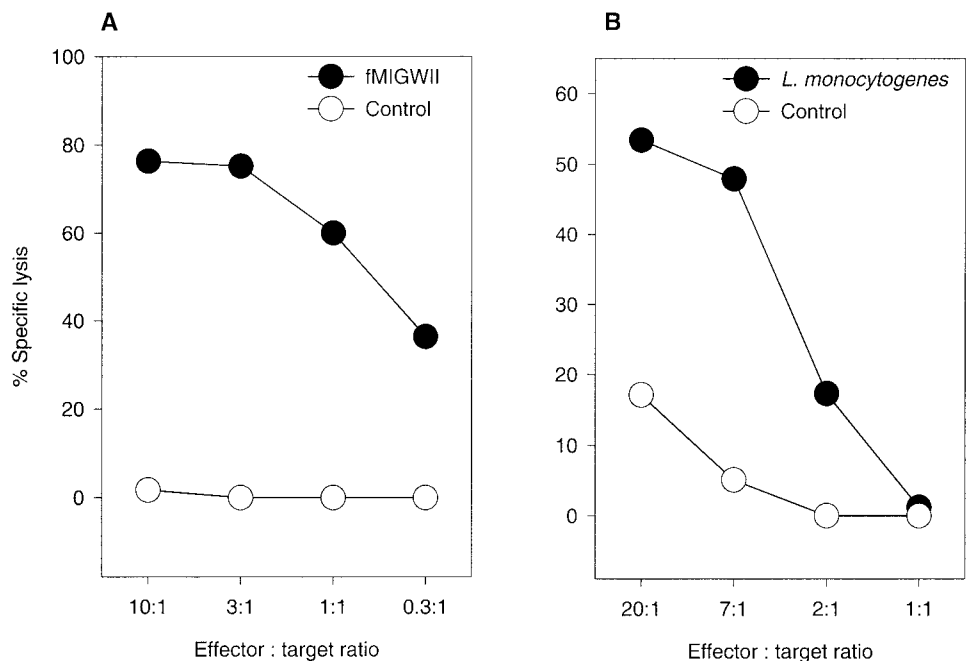


FIGURE 2. 3C8 cells are cytotoxic. Cytotoxicity of 3C8 cells against untreated or *fMIGWII*-pulsed EL4 cells (A) or uninfected or *L. monocytogenes*-infected BMM (B), was measured in a <sup>51</sup>Cr release assay.

Table II. Cytokine production in 3C8 cultures stimulated with fMIGWII<sup>a</sup>

Stimulation	IFN- $\gamma$ (U/ml)	TNF (pg/ml)	IL-4 (pg/ml)	IL-6 (pg/ml)	IL-10 (pg/ml)
Unstimulated	13 $\pm$ 3	<100	<80	<160	<500
fMIGWII	1987 $\pm$ 358	833 $\pm$ 361	<80	1751 $\pm$ 864	<500

<sup>a</sup> 3C8 cells ( $5 \times 10^4$ ) were cultured with  $5 \times 10^5$  control or fMIGWII-pulsed irradiated APC in 200  $\mu$ l in a round-bottom 96-well plate. Culture supernatant was collected after 48 h, and cytokines were measured as described in *Materials and Methods*.

processing pathway. Earlier studies indicated that exogenous HKL can be processed by macrophages and presented via H2-M3, and that such processing is at least partially TAP independent (12). Using IFN- $\gamma$  production as a readout, presentation of HKL-derived Ag by macrophages to 3C8 cells could be demonstrated (Fig. 4). 3C8 cells also recognized HKL-pulsed TAP1<sup>-/-</sup> macrophages, although IFN- $\gamma$  production was consistently reduced compared with 3C8 cells stimulated with HKL-pulsed C57BL/6 macrophages. Thus, processing of exogenous Ag by macrophages for presentation to 3C8 cells by H2-M3 was at least partially TAP independent, confirming previously published data (12). Nonspecific stimulatory effects of HKL were excluded by showing that CD8<sup>+</sup> T cell lines specific for (and capable of producing IFN- $\gamma$  in response to) the H2-K<sup>d</sup>-restricted LLO<sub>91-99</sub> epitope or the H2-K<sup>b</sup>-restricted OVA<sub>257-264</sub> epitope (16) did not produce IFN- $\gamma$  in response to HKL-pulsed macrophages (data not shown).

#### Partially TAP-independent protection against *L. monocytogenes*

To test the *in vivo* relevance of TAP-independent presentation of HKL to 3C8 cells, adoptive transfer experiments in *Listeria*-infected TAP1<sup>-/-</sup> mice were performed. Our preliminary experiments had indicated that transfer of CD8<sup>+</sup> T cells of irrelevant Ag specificity had no influence on *Listeria* infection. However, for this experiment we used an irrelevant CD8<sup>+</sup> T cell clone as an additional control. The reason for this is that the activity of the 3C8 clone in TAP1<sup>-/-</sup> mice was considerably reduced compared with C57BL/6 mice (see below), and it was considered important to include this control to rigorously confirm the findings.

Adoptive transfer of 3C8 cells to *Listeria*-infected TAP1<sup>-/-</sup> mice significantly reduced bacterial load in the liver ( $p < 0.05$ ) and spleen ( $p < 0.05$ ; Fig. 5) in comparison to mice receiving a clone of irrelevant specificity. The ability of 3C8 cells to lower bacterial burden, at least in the liver, was considerably lower in TAP1<sup>-/-</sup> recipients compared with C57BL/6 recipients ( $p < 0.05$  in the liver). The irrelevant CD8<sup>+</sup> clone, specific for the adenovirus E1A gene, did not affect bacterial load in the liver or spleen in C57BL/6 or TAP1<sup>-/-</sup> mice ( $p > 0.05$  in each case; Fig. 5). We conclude that H2-M3-restricted T cells can participate in protection and that this is, in part, TAP independent.

Table III. 3C8 cells mediate protection against *L. monocytogenes* infection<sup>a</sup>

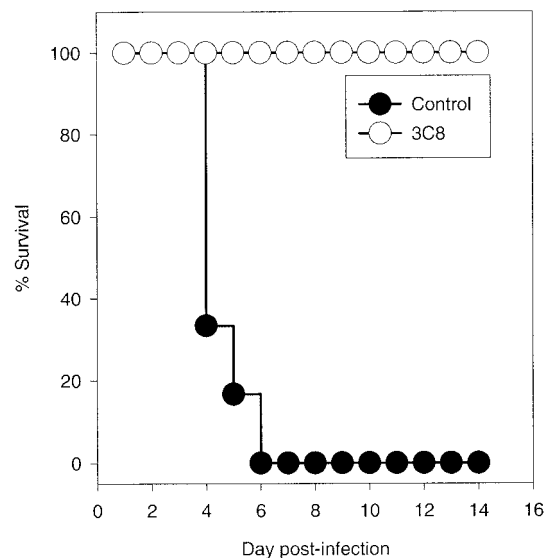
Organ	Log <sub>10</sub> CFU		$\Delta$ Log <sub>10</sub> Protection
	Control	3C8	
Spleen	7.5 $\pm$ 0.3	6.5 $\pm$ 0.5*	1.0
Liver	7.1 $\pm$ 0.9	4.9 $\pm$ 0.7†	2.2

<sup>a</sup> C57BL/6 mice received  $10^7$  3C8 cells *i.v.* in 200  $\mu$ l PBS, or PBS alone (control), and were then infected with a lethal dose of *L. monocytogenes* ( $10^5$  CFU *i.v.*). Bacterial load in the liver and spleen were determined 72 h after infection. Data are mean log<sub>10</sub> CFU  $\pm$  SD,  $n = 5$  mice per group. \*,  $p < 0.02$  vs control. †,  $p < 0.01$  vs control.

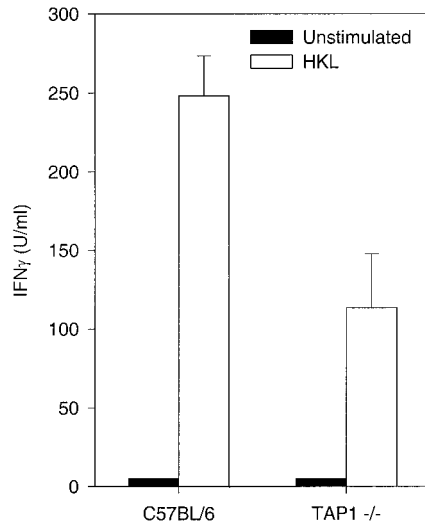
## Discussion

H2-M3 is a nonclassical MHC class I molecule with several interesting features distinguishing it from classical MHC class I molecules. It exhibits very limited polymorphism (8) and has a strong preference for binding formylated peptides (9). The importance of the formyl group in peptide binding to H2-M3 led to the hypothesis that H2-M3 may be specialized for the presentation of bacteria-derived epitopes (9, 11). This is because in eukaryotic cells, formyl methionine groups are limited to 13 mitochondrial proteins, whereas all prokaryotic protein synthesis initiates with a formyl methionine group. This hypothesis was supported by the identification of *Listeria*-derived formylated epitopes that are presented to CD8<sup>+</sup> T cells by H2-M3 (6, 7, 12–14, 20).

In this paper we have addressed the protective ability of H2-M3-restricted T cell responses during *Listeria* infection. Our first approach was to examine whether immunization with peptides restricted by H2-M3 could protect against *Listeria* infection. Peptide immunization successfully primed H2-M3-restricted responses, albeit quite weakly, but had no influence on the course of infection following challenge with *Listeria*. fMIGWII-specific responses could also be primed following transfer of irradiated syngeneic spleen cells or dendritic cells pulsed with fMIGWII, but neither protocol protected mice from subsequent challenge (M. S. Rolph and S. H. E. Kaufmann, unpublished data). The immune response to *Listeria* infection is multifaceted and it is perhaps not surprising that immunization with a single peptide epitope is not sufficient to induce protection. Indeed, previous studies aiming to demonstrate the protective capacity of a CD8<sup>+</sup> T cell response to a single



**FIGURE 3.** Adoptive transfer of 3C8 cells protects mice from lethal *L. monocytogenes* infection. Groups of six C57BL/6 mice were given  $10^7$  3C8 cells or PBS *i.v.* and infected 30–60 min later with  $5 \times 10^4$  CFU *L. monocytogenes* *i.v.* Survival of mice was monitored daily, and the experiment was terminated on day 14.



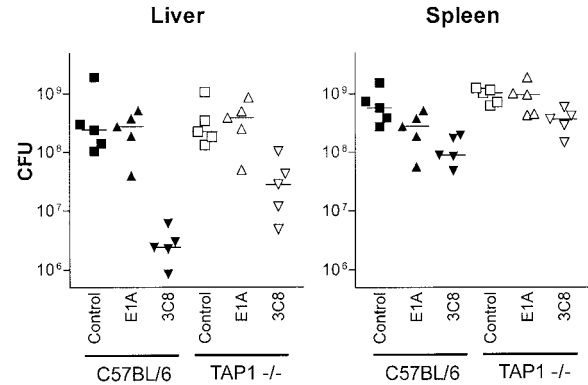
**FIGURE 4.** 3C8 cells recognize HKL-pulsed BMM in a TAP-independent manner. BMM ( $5 \times 10^4$ /well in flat-bottom 96-well plates) from C57BL/6 and TAP1<sup>-/-</sup> mice were pulsed with  $10^8$ /ml HKL, and  $10^4$  3C8 cells/well were added. Supernatant was collected 24 h later and IFN- $\gamma$  was measured by ELISA. Data are mean  $\pm$  SD from triplicate wells.

epitope have mostly resorted to adoptive transfer (21). It is also interesting to note that for *Listeria* infection, H2-M3-restricted memory responses may not be as strong as MHC class Ia-restricted memory responses (22). This phenomenon could also potentially contribute to the lack of protection observed with our immunization protocol.

To further examine the protective potential of H2-M3-restricted responses, a CD8<sup>+</sup> T cell clone specific for fMIGWII was generated. The clone was strongly cytotoxic and produced high levels of IFN- $\gamma$  and TNF. Substantial protection was observed following transfer of 3C8 cells to *Listeria*-infected mice. Similar results were obtained by Nataraj et al. using an H2-M3-restricted clone generated following *in vitro* stimulation with HKL (23). The strength of the protection mediated by 3C8 cells was further demonstrated by the ability to rescue mice from a lethal infection. Our results, using a clone of defined specificity (fMIGWII), demonstrate that H2-M3-restricted responses contribute to protection against *Listeria* infection.

A recent study has addressed the role of MHC class Ib-restricted responses during *Listeria* infection. Mice deficient for MHC class Ia molecules (H2-K<sup>b</sup>  $\times$  H2-D<sup>b</sup> double knockout mice) controlled infection as efficiently as wild-type mice, and this was associated with measurable induction of CD8<sup>+</sup> T cell responses restricted by H2-M3 and another MHC class Ib molecule, Qa1<sup>b</sup> (24). In contrast, mice deficient in  $\beta_2$ -microglobulin are more susceptible to *Listeria* infection (4, 5). Thus, CD8<sup>+</sup> T cell responses restricted by MHC class Ib molecules, at least in mice of the H2<sup>b</sup> background, are important in the control of *Listeria* infection.

Conventional MHC class I Ag processing involves degradation of polypeptides within the cytoplasm, followed by TAP-mediated transport to the endoplasmic reticulum (ER). In the ER, the peptides form a stable complex with MHC class I and  $\beta_2$ -microglobulin before being transported to the cell surface (25). Exogenous Ags do not readily gain access to the MHC class I pathway, although exceptions have been noted (26). For H2-M3, presentation of endogenous self (mitochondrial) peptides appears to follow the conventional pathway and is TAP dependent (27, 28). However, exogenous Ags can readily access the H2-M3 presentation pathway. This is illustrated by the ability of HKL-pulsed macrophages



**FIGURE 5.** Protection mediated by adoptive transfer of 3C8 cells. C57BL/6 or TAP1<sup>-/-</sup> mice were given  $10^7$  3C8 cells, adenovirus E1A-specific cells, or PBS *i.v.* and infected 30–60 min later with  $10^5$  CFU *L. monocytogenes* *i.v.* Bacterial load in the liver and spleen were measured 3 days later. Data are CFU values from individual mice with a median bar,  $n = 5$  mice per group. The irrelevant CD8<sup>+</sup> clone, specific for the adenovirus E1A gene, did not affect bacterial load in the liver or spleen in C57BL/6 or TAP1<sup>-/-</sup> mice ( $p > 0.05$  compared with control mice in each case). 3C8 cells mediated protection in TAP1<sup>-/-</sup> recipients ( $p < 0.05$  in the spleen and  $p < 0.05$  in the liver, compared with both control and E1A recipients).

to present epitopes via H2-M3 (Fig. 4; Refs. 12, 20, 23) but only minimally via MHC class Ia molecules such as H2-K<sup>d</sup> (17, 20). The mechanism by which HKL enters the H2-M3 presentation pathway is not known. Peptide regurgitation, as described by Pfeifer et al. (29) for MHC class Ia, is not likely as bystander cells are not loaded with peptide when cultured together with HKL-pulsed macrophages (30). Presentation of HKL by H2-M3 does require active processing (30, 31) but is not greatly influenced by the presence or absence of TAP (Fig. 4; Ref. 30). It is probable that formylated peptides located within the phagosome load H2-M3 molecules, which may access the phagosomal compartment either from the cell surface or en route to the cell surface from the ER (26, 30).

The extent to which TAP-independent presentation of HKL by BMM reflects events that occur *in vivo* during *L. monocytogenes* infection is not clear. To test this, 3C8 cells were transferred to *Listeria*-infected C57BL/6 and TAP1<sup>-/-</sup> mice. The results revealed that during *Listeria* infection, H2-M3-restricted, TAP-independent presentation to CD8<sup>+</sup> T cells occurs, and that this pathway is sufficient to mediate substantial protection against *Listeria* infection. However, the protective efficiency of 3C8 cells was markedly less in TAP1<sup>-/-</sup> than in C57BL/6 hosts, indicating that TAP plays a role during *in vivo* *L. monocytogenes* infection in presentation via H2-M3. This probably indicates that in C57BL/6 mice, the endogenous MHC class I presentation pathway, in which peptides are transported from the cytosol to the ER by TAP, was also operative (2, 32). The suboptimal protection observed in TAP1<sup>-/-</sup> mice could also reflect a relative paucity of H2-M3 molecules available for peptide binding. In the absence of TAP, few H2-M3 molecules would be expected to leave the ER. If peptide binding occurs after H2-M3 molecules have left the ER (e.g., in the phagosome), then the absence of TAP may influence the magnitude of presentation.

It will be important to further elucidate the processing pathway for presentation of both exogenous and endogenous foreign antigen by H2-M3. The recent development of a mAb specific for H2-M3 (28) should greatly facilitate such analysis. Here we have

shown that in vivo, in the absence of TAP, *Listeria*-derived peptides can still be presented in sufficient quantity to provide substantial protection following transfer of a specific CD8<sup>+</sup> T cell clone. Indeed, to our knowledge this is the only example of CD8<sup>+</sup> T cell-mediated, TAP-independent protection against a pathogen in vivo.

*L. monocytogenes* is the only pathogen known to induce H2-M3-restricted T cells and it will be of great interest to determine whether other bacterial pathogens can do so. Early studies identified apparently MHC-unrestricted CD8<sup>+</sup> T cell responses during infection of mice with *Mycobacterium bovis* bacille Calmette Guérin (33), which probably reflects restriction by a nonpolymorphic presenting molecule such as H2-M3. The ability of H2-M3 to present Ag via both conventional (TAP-dependent) and unconventional (TAP-independent) processing pathways suggests that it may be particularly well suited for presentation of Ag derived from vacuolar bacterial pathogens such as *Mycobacterium tuberculosis* or *Salmonella typhimurium*.

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