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Nonsecreted Bacterial Proteins Induce Recall CD8 T Cell Responses But Do Not Serve as Protective Antigens

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Secreted or nonsecreted Ag expressed by recombinant Listeria monocytogenes can prime CD8 T cells. However, Ag-specific memory CD8 T cells confer protection against bacteria secreting Ag, but not against bacteria expressing the nonsecreted form of the same Ag. This dichotomy may be explained by a long-standing hypothesis that nonsecreted Ags are less effective than secreted Ags at inducing a protective immune response at the onset of infection. We tested this hypothesis by examining whether these two different forms of Ag induce different primary and secondary CD8 T cell responses. The primary responses to secreted and nonsecreted Ags expanded and contracted almost synchronously, although the responses to nonsecreted Ags were of lower magnitude. These results demonstrate that the kinetics of the CD8 T cell response are similar regardless of whether Ag is accessible to the endogenous MHC class I pathway or can only be presented through cross-presentation. No differences were detected in the CD8 T cell recall response to L. monocytogenes expressing secreted or nonsecreted Ags. Nonsecreted Ags are as effective as secreted Ags at the induction of a rapid recall response by memory CD8 T cells. Thus, the inability of nonsecreted bacterial proteins to serve as protective Ags cannot be attributed to a defective CD8 T cell response. The Journal of Immunology, 2002, 169: 5805–5812.

Listeria monocytogenes is a Gram-positive facultative intracellular bacterium that has been extensively studied as a model pathogen and has aided in the elucidation of both innate and cell-mediated immune responses. L. monocytogenes invades both phagocytic and nonphagocytic cells, escapes from the endosome, replicates in the cytosol, and spreads into neighboring cells without encountering the extracellular milieu (1, 2). L. monocytogenes induces a strong CD8 T cell response that plays an important role in the clearance of primary infection and in protective immunity against secondary infection. Secreted proteins from bacteria growing in the host cell cytosol are readily sampled by the endogenous MHC class I processing pathway for presentation to CD8 T cells (3). All known CD8 T cell epitopes are derived from secreted virulence proteins (4–7), and the immune response to these epitopes has been well characterized (8). Little is known about the CD8 T cell response to nonsecreted bacterial Ags. Nonsecreted proteins are compartmentalized within bacteria and are not readily accessible to the classical endogenous MHC class I pathway. Nonsecreted Ags are presumably presented by the MHC class I pathway to CD8 T cells via cross-presentation (9, 10).

Previously, we reported that a dichotomy exists in the CD8 T cell response between L. monocytogenes expressing a secreted or nonsecreted fusion protein containing a H-2Kd-restricted epitope, nucleoprotein (NP)18–1263 derived from lymphocytic choriomeningitis virus (LCMV) (11). Both forms of Ag are capable of priming NP18–126-specific CD8 T cells and establishing functional memory. However, infection of LCMV-immunized mice with L. monocytogenes expressing either the secreted or nonsecreted LCMV-derived Ag produces unexpected results. L. monocytogenes expressing the secreted Ag is rapidly cleared from the mice, whereas L. monocytogenes expressing the nonsecreted Ag is cleared at the same rate as in naive mice. These results indicate that both secreted and nonsecreted Ag can induce CD8 T cell responses, while only secreted Ag can serve as protective Ag for CD8 T cell-mediated immunity. This dichotomy as a result of Ag compartmentalization reveals an intriguing aspect of CD8 T cell surveillance during L. monocytogenes infection. Several critical questions remain. Does this phenomenon hold true for other Ags and therefore does it apply to bacterial Ags in general? What is the underlying mechanism for the dichotomy between priming and protective immunity?

Two possible mechanisms have been postulated to explain why nonsecreted bacterial Ag induces CD8 T cell responses, yet cannot serve as a protective target (11). One mechanism relates to the presentation of nonsecreted Ag by only a subset of infected cells. L. monocytogenes can infect both phagocytic and nonphagocytic cells. Secreted bacterial Ag in the host cell cytosol is presented by the endogenous MHC class I pathway, which is operative in most cell types. On the other hand, presentation of nonsecreted Ag may be limited to cross-presentation, which, in turn, is restricted to professional APCs, such as dendritic cells. Thus, Ag-specific memory CD8 T cells specific for a nonsecreted epitope may be unable to control infection, since they cannot recognize most infected cells, including hepatocytes, which are a major site of L. monocytogenes replication (2). The second possibility is suggested by the long-standing hypothesis that nonsecreted Ag does not induce a strong CD8 T cell response during the pivotal early stages of infection. While our previous results have shown that nonsecreted Ag induces CD8 T cell memory to a level similar (only 3- to 5-fold lower) to that induced by its secreted counterpart, it is not

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Abbreviations used in this paper: NP, nucleoprotein; BHI, brain heart infusion; DHFR, dihydrofolate reductase; LCMV, lymphocytic choriomeningitis virus; p.i., postinfection; rLM-sAg, recombinant L. monocytogenes expressing nonsecreted Ag; rLM-nAg, recombinant L. monocytogenes expressing secreted Ag; LLO, listeriolysin O.
known whether similar levels of CD8 T cell responses are induced by secreted and nonsecreted Ags at early time points during infection. Furthermore, protective immunity against L. monocytogenes is mediated by the recall response of memory CD8 T cells, which was not examined in our previous study. Thus, it is possible that the lack of protection against recombinant L. monocytogenes expressing nonsecreted Ag is due to the absence of a strong recall response to nonsecreted Ag.

In this study we extend our analysis of Ag compartmentalization to include another epitope that is recognized in a different haplotype, the H-2k-restricted gp33–41 epitope of LCMV. The results of this study confirmed our previous observations, thus demonstrating that the dichotomy between CD8 T cell priming and protective immunity as a result of Ag compartmentalization is not limited to a particular epitope or MHC haplotype. In addition, we further examined whether secreted and nonsecreted Ags induce similar levels of primary and secondary CD8 T cell responses during the early stages of infection. Our results showed that secreted Ag induced a much more robust response than nonsecreted Ag at the onset of a primary CD8 T cell response, supporting the hypothesis that secreted Ags induce a more effective early immune response than nonsecreted Ags. However, memory CD8 T cells mounted rapid recall responses similar in magnitude and kinetics to both secreted and nonsecreted Ags. Therefore, the lack of protection against L. monocytogenes expressing nonsecreted Ag cannot be attributed to the absence of a strong recall response by memory CD8 T cells.

Materials and Methods

Mice

BALB/c and C57BL/6 female mice (National Cancer Institute, Frederick, MD), aged 6–10 wk, were housed in insulator cages and cared for in accordance with institutional animal care and use committee-approved protocols at University of Pennsylvania School of Medicine animal facility. Mice within experiments were age-matched. P14 TCR transgenic mice (specific for H-2D b -gp33–41) (12) and C57BL/6 Thy1.1 mice (The Jackson Laboratory, Bar Harbor, ME) were bred to produce an F1 generation (P14 Thy1.1/Thy1.2), which was used as donors in adoptive transfer experiments.

Bacteria and virus

Recombinant L. monocytogenes expressing secreted (XFL703) or nonsecreted (XFL704) fusion proteins containing CD8 T cell epitopes were constructed as previously described (11). Briefly, DNA sequences encoding dihydrofolate reductase (DHFR), the hly promoter, and the hly signal sequence and five additional amino acids (only in XFL703) were PCR amplified from pH118 (13) and L. monocytogenes chromosomal DNA, respectively. Two complementary oligonucleotides were synthesized for sequences encoding the gp33–41 and NP118–128 epitopes and the mAb HA epitope, derived from influenza virus hemagglutinin (14) or the vesicular stomatitis virus epitope, derived from vesicular stomatitis virus glycoprotein (15). Ag cassettes were cloned into delivery vector pHS-LV and introduced into the genome of wild-type L. monocytogenes 10403S as previously described (16). Strains were maintained as −80°C stocks in brain heart infusion (BHI)/50% glycerol. Before each experiment each strain was inoculated onto BHI agar. A single colony was inoculated in BHI, and the culture was grown overnight at 30°C with aeration. For clarity, XFL703 and XFL704 will be referred to as rLM-sAg and rLM-nsAg, respectively, throughout this paper. LCMV Armstrong was propagated as previously described (17).

Western blot

Overnight cultures of 10403S, rLM-sAg, and rLM-nsAg were subcultured 1/200 in BHI with 100 mM MOPS in duplicate. Cultures were incubated at 37°C with aeration until an OD 600 of 1.0 was reached. To isolate secreted proteins, TCA was added to one set of cultures to a final concentration of 10%. After incubation on ice, the samples were centrifuged and the supernatant was collected. For surface staining, 1% BSA/PBS was added to splenocytes to block nonspecific mAb binding. Cells were then washed once with PBS, serially diluted in PBS to 10 5,3×10 4,3×10 3, and 3×10 2 CFU (0.1 of the LD 50 ), and BALB/c mice were infected with 5×10 4 CFU (0.1 of the LD 50 ). Bacterial load was determined by plating 10-fold serial dilutions of spleen and liver homogenates in sterile 1% Tryptone-X100/PBS. To generate LCMV-immunized mice, mice were injected i.p. with 2×10 5 PFU LCMV Armstrong strain. Challenges with L. monocytogenes were performed at least 21 days after LCMV immunization.

Splenocyte preparation

Spleens were aseptically removed from sacrificed mice, placed into cold RPMI 1640, and passed through a wire-mesh screen. RBC were lysed with 0.83% ammonium chloride. Splenocytes were resuspended in complete RPMI 1640 medium containing 5% FCS, 4 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Aliquots were diluted in 0.1% trypan blue/PBS to calculate the number of viable cells per spleen.

Surface and intracellular staining

For surface staining, 1% BSA/PBS was added to splenocytes to block nonspecific mAb binding. All mAb and reagents were purchased from BD Pharmingen (San Diego, CA). mAbs to one of several activation markers, including anti-CD44 (clone IM7) and anti-CD43 (clone B111) in 1% BSA/PBS. After several washes in 1% BSA/PBS, splenocytes were fixed with 2% paraformaldehyde. For intracellular staining to analyze IFN-γ production, splenocytes were incubated with 50 U/ml recombinant human IL-2 and Golgistop and with or without 1 μM synthetic peptide in complete RPMI 1640 medium. Peptides included gp33–41 (KAVYNFATM), listeriolysin O (LLO190–201 (GYKDGNEYI), LLO190–201 (NEKYQAYNVPS), and p60121–123 (KYGVSGQDI). After 5-h incubation at 37°C with 5% CO 2 , the cells were surface-stained as described above. According to the manufacturer’s protocol, splenocytes were then permeabilized with Cytofix/Cytoperm solution, then stained with α-IFN-γ–allophycocyanin mAb (clone XMG1.2) and fixed with paraformaldehyde. Splenocytes were analyzed with a FACSCalibur (BD Biosciences, San Jose, CA), and data were analyzed using Flow-Jo version 3.4 (TreeStar, San Carlos, CA).
were stained for intracellular IFN-γ mAb (clone XMG1.2), and developed with HRP-streptavidin conjugate (Vector, Burlingame, CA) and aminoethylcarbazole dye solution (Sigma-Aldrich, St. Louis, MO).

**Adoptive transfer**

Spleens from female P14 Thy1.1/Thy1.2 transgenic mice (at least 8 wk old) were aseptically removed and prepared as described above. Spleenocytes were resuspended in PBS, labeled with 5 μM CFSE (Molecular Probes, Eugene, OR), and quenched with FCS. After washing, cells were resuspended in PBS, and 1–2 × 10^7 spleenocytes/mouse were injected i.v. into C57BL/6 (Thy1.2) female mice. Mice were injected i.v. 18 h later with either 1 × 10^6 CFU rLM-sAg or rLM-nsAg. Mice were sacrificed at days 2, 3, and 4 postinfection (p.i.). Cells were stained with mAb s-CD8 and w-Thy.1.1-PE (clone OX-7) to identify transferred Thy1.1+ T cells and then were stained for intracellular IFN-γ as described above.

**Results**

Nonsecreted bacterial Ags prime CD8 T cells, but do not serve as protective targets

To extend our previous studies on the effect of Ag compartmentalization to the CD8 T cell response, we constructed new isogenic strains of *L. monocytogenes*, rLM-sAg and rLM-nsAg, that express two CD8 T cell epitopes as secreted or nonsecreted fusion proteins, respectively (Fig. 1A). Both rLM-sAg and rLM-nsAg expressed gp33–41 and NP_{118–126} epitopes from LCMV within a DHFR fusion protein under the control of a virulence gene promoter (P_{lep}). The fusion proteins in rLM-sAg and rLM-nsAg were identical, with the exception of an N-terminal signal sequence in rLM-sAg that is cleaved upon secretion from the bacterium. The cassettes encoding the fusion proteins were integrated into the chromosome of wild-type *L. monocytogenes* 10403S between the lecithinase and lactate dehydrogenase operons as previously described (11). gp33–41 and NP_{118–126} are recognized in C57BL/6 and BALB/c mice, respectively. Inclusion of both epitopes within the same recombinant *L. monocytogenes* strain makes it possible to examine the CD8 T cell response in two mouse strains, thus providing cross-validation of our results. Addition of the gp33–41 epitope also allows the use of P14 TCR transgenic cells in an adoptive transfer model to visualize in vivo proliferation of Ag-specific CD8 T cells.

Examination of *L. monocytogenes* proteins from in vitro cultures by Western blot analysis demonstrated proper compartmentalization of the fusion proteins in the two strains (Fig. 1B). The fusion protein of rLM-sAg was present only in the supernatant, demonstrating efficient secretion of the fusion protein from the bacterium. On the other hand, the nonsecreted fusion protein of strain rLM-nsAg was present only in the bacterial pellet, as expected. Fusion proteins expressed by rLM-nsAg and secreted by rLM-sAg had an apparent size of ~28 kDa on the Western blot, consistent with their predicted Mr. The fusion protein expressed by rLM-sAg appeared slightly larger than the protein expressed by rLM-nsAg due to the addition of five amino acid residues after the secretion signal cleavage site that were included to ensure cleavage efficiency. The expression of foreign proteins can affect bacterial in vitro and in vivo growth, particularly when they are fused to a signal sequence but cannot be efficiently translocated (19). It is hypothesized that these proteins congest bacterial secretory pathways, thus blocking the secretion of virulence factors. While DHFR is efficiently secreted in many organisms, including *L. monocytogenes* (11), we nevertheless assayed the in vitro and in vivo virulence of the recombinant strains. In J774 cells, a murine macrophage-like cell line, rLM-sAg and rLM-nsAg invaded with equal efficiency and replicated to similar numbers (Fig. 1C). After infection of mice with a sublethal dose of rLM-sAg or rLM-nsAg, similar levels of bacteria were recovered from spleens in both C57BL/6 (Fig. 1D) and BALB/c (data not shown) mice on days 2 and 3 p.i. Thus, rLM-sAg and rLM-nsAg were equally virulent, and therefore any differences observed in the immune responses to these strains were not due to differences in levels of infection.

To determine whether secreted or nonsecreted gp33–41 can prime naive CD8 T cells, C57BL/6 mice were infected with wild-type *L. monocytogenes*, rLM-sAg or rLM-nsAg. At least 20 days p.i., the number of gp33–41-specific CD8 T cells in the spleen was quantitated by intracellular IFN-γ staining after in vitro peptide stimulation. Both rLM-sAg and rLM-nsAg generated gp33–41-specific CD8 T cell populations (Fig. 2A). The rLM-sAg generated a 5- to 10-fold greater number of Ag-specific cells than rLM-nsAg (Fig. 2B). The parental strain, wild-type *L. monocytogenes*, did not induce gp33–41-specific CD8 T cells above background levels (Fig. 2A).
We next examined whether either of our fusion proteins could serve as protective Ag in C57BL/6 mice. LCMV-immunized or naive age-matched C57BL/6 mice were infected with $1 \times 10^4$ CFU rLM-sAg or rLM-nsAg. On day 3 p.i., bacterial loads in spleens were similar in naive mice infected with either strain (Fig. 2C). In LCMV-immunized mice, rLM-sAg was cleared by day 3 p.i.; however, LCMV-immunized mice challenged with rLM-nsAg had bacterial loads equal to those seen in naive challenged mice (Fig. 2C). Thus, nonsecreted gp33–41 was not protective, although it was capable of priming CD8 T cells. These results extend our previous findings to include another Ag in a different mouse strain, thus confirming our observation of a dichotomy between CD8 T cell priming and protective immunity as a result of Ag compartmentalization (11).

**Secrected bacterial Ags induce more robust primary CD8 T cell responses at early time points than nonsecreted Ags**

As a first step toward understanding why nonsecreted bacterial Ags are not protective, we examined the kinetics of the primary immune response to secreted and nonsecreted Ag. C57BL/6 mice were infected with rLM-sAg or rLM-nsAg, and at different days p.i., the numbers of gp33–41-specific CD8 T cells per spleen were quantitated by intracellular IFN-γ-staining assay (Fig. 3A). Ag-specific CD8 T cells were first detected on day 5 p.i. in the spleens of mice infected with either rLM-sAg or rLM-nsAg. In mice infected with rLM-sAg, the number of Ag-specific cells peaked on day 8 p.i. with $\sim 10^6$ cells/spleen and then contracted to $\sim 10^5$ cells per day 14 p.i. Interestingly, the response to nonsecreted gp33–41 expanded and contracted almost synchronously with the response.

**FIGURE 2.** Nonsecreted bacterial Ag primes CD8 T cells, but does not serve as a protective Ag. A, Induction of a gp33–41-specific response by rLM-sAg and rLM-nsAg immunization. C57BL/6 mice were infected with $1 \times 10^4$ CFU wild-type *L. monocytogenes* (LM wt), rLM-sAg, or rLM-nsAg, and gp33–41-specific CD8 T cells on day 20 p.i. were determined by intracellular IFN-γ staining assay. Cells are gated on the CD8+ population. The number indicates the percentage of CD8 T cells that are specific to the indicated Ag. B, Splenocytes that were not stimulated with gp33–41 did not produce IFN-γ (data not shown). C, gp33–41-specific CD8 T cells in spleens 20 days after priming with rLM-sAg or rLM-nsAg. The total number of gp33–41-specific CD8 T cells per spleen was calculated based on the FACs data analyzed in A and the total number of splenocytes recovered from each mouse. Bars represent the mean ± SD from three mice from one of three similar independent experiments. C, Protection against rLM-sAg and rLM-nsAg in LCMV-immunized mice. LCMV-immunized and age-matched naive mice were challenged with $1 \times 10^5$ CFU rLM-sAg (⧫) or rLM-nsAg (●), and bacterial loads in the spleen were determined on day 3 p.i. Bars represent the mean ± SD from spleens of three mice from one of three independent experiments with similar results. The limit of detection is depicted as a dotted line.

**FIGURE 3.** Kinetics of primary CD8 T cell responses to secreted or nonsecreted bacterial Ag. A, Kinetics of the gp33–41 response in C57BL/6 mice. Total numbers of gp33–41-specific CD8 T cells in the spleens of C57BL/6 mice following infection with $1 \times 10^4$ CFU rLM-sAg (○) or rLM-nsAg (□). The chart shows gp33–41-specific IFN-γ-producing CD8 T cells determined by intracellular IFN-γ staining. Each data point represents the mean of three mice; bars are the SD. Data are representative of two independent experiments with three mice per group. Similar results were obtained from ELISPOT assays (data not shown). B, Kinetics of CD8 T cell responses specific to recombinant NP118–126, endogenous LLO91–99, and p60225–225 epitopes. BALB/c mice were infected with $5 \times 10^5$ CFU rLM-sAg (⧫) or rLM-nsAg (●). The chart shows the total number of Ag-specific IFN-γ-producing cells per spleen as determined by intracellular IFN-γ staining after in vitro stimulation with different peptides: NP118–126 (top panel), LLO91–99 (middle panel), or p60225–225 (bottom panel). Each symbol represents the mean of three mice, bars depict the SD, and dotted lines show the limit of detection. Similar results were obtained by ELISPOT assay (data not shown).
to secreted gp33–41 (Fig. 3A), although the magnitude of the response to nonsecreted Ag was ~5- to 10-fold less throughout the expansion and contraction phases. This difference was not due to variation in the levels of *L. monocytogenes* infection, since we observed that the CD4 T cell response to the endogenous epitope LLO<sub>100–201</sub> (7) was similar in mice infected with rLM-sAg and those infected with rLM-nsAg (data not shown).

We also examined the kinetics of the primary CD8 T cell response to secreted or nonsecreted NP<sub>118–126</sub> in BALB/c mice (Fig. 3B). In mice infected with rLM-sAg, NP<sub>118–126</sub>-specific CD8 T cells were first detected on day 5 p.i., expanded to a peak of 3 × 10<sup>6</sup> cells/spleen on day 7 p.i., and then contracted to a smaller population of 5 × 10<sup>4</sup> cells/spleen by day 14 p.i. The kinetics of the response to nonsecreted NP<sub>118–126</sub> were similar, although the magnitude was 5- to 10-fold less at each of the time points examined. These results are consistent with the gp33–41-specific response observed in C57BL/6 mice (Fig. 3A) and with our previous findings that secreted NP<sub>118–126</sub> induces a 3- to 5-fold higher level of memory (11). As internal controls, we examined the kinetics of the CD8 T cell response to endogenous secreted *L. monocytogenes* proteins. LLO<sub>91–99</sub> and p60<sub>217–225</sub>-specific responses in mice infected with rLM-sAg or rLM-nsAg were similar in magnitude and synchronous in kinetics (Fig. 3B). Thus, these two bacterial strains induced similar overall immune responses, and any differences observed in the response to recombinant secreted or nonsecreted Ag were not due to differences in the virulence of the two bacterial strains. Furthermore, responses to secreted and nonsecreted NP<sub>118–126</sub> were nearly synchronous with the responses to endogenous LLO<sub>91–99</sub> and p60<sub>217–225</sub> epitopes.

The earliest time point at which Ag-specific CD8 T cells could be detected by IFN-γ intracellular staining or ELISPOT was day 5 following *L. monocytogenes* infection of normal mice. To further examine earlier time points, we employed an adoptive transfer model using CFSE-labeled TCR transgenic P14 cells that are specific to the H-2D<sup>d</sup>-restricted gp33–41 epitope. CFSE-labeled splenocytes from P14 mice (Thy1.1/Thy1.2) were adoptively transferred into congenic C57BL/6 (Thy1.2) mice, which were then infected with rLM-sAg or rLM-nsAg (Fig. 4A). On day 2 p.i., transferred P14 cells had not divided significantly in mice infected with either rLM-sAg or rLM-nsAg. By day 3, transferred P14 cells began to respond to both secreted and nonsecreted gp33–41, although there was substantially more proliferation of P14 cells in rLM-sAg infected mice, with 85% of P14 cells having divided compared with 30% in mice infected with rLM-nsAg. By day 4 p.i., the population of P14 cells in rLM-sAg infected mice was comprised of mostly (97%) daughter cells, while only 57% of P14 cells had divided in mice infected with rLM-nsAg. P14 cells that had divided in response to infection with either rLM-sAg or rLM-nsAg produced IFN-γ when stimulated with gp33–41 peptide in vitro (Fig. 4B). Thus, both secreted and nonsecreted Ags prime functional CD8 T cells, but secreted Ag induces a stronger early response than nonsecreted Ag. In summary, our results show that CD8 T cells begin to respond to both secreted and nonsecreted bacterial Ags at about the same time (between days 2 and 3 p.i.). These responding populations of Ag-specific cells then expand and contract almost synchronously, although the magnitude of the response to secreted Ags is 5- to 10-fold greater.

**Nonsecreted bacterial proteins induce recall CD8 T cell responses, but do not serve as protective Ags**

Infection of mice with rLM-nsAg induced less proliferation of Ag-specific CD8 T cells during the primary immune response, suggesting that the lack of protective immunity may be due to nonsecreted Ag inducing a less robust response early during infection. However, protective immunity is mediated by memory CD8 T cells, which are present at a greater frequency and respond faster than naive CD8 T cells (20). The recall responses to secreted and nonsecreted Ags by memory CD8 T cells have not been examined, and it is possible that the lack of protection against rLM-nsAg is due to the absence or delay of a recall response to nonsecreted Ag. To determine whether memory CD8 T cells mount a response against nonsecreted Ag, we examined the secondary immune response on days 1 and 2 after challenge. Effective recall responses by memory CD8 T cells at these early time points are critical for conferring protective immunity. LCMV-immunized and age-matched naive C57BL/6 mice were infected with rLM-sAg or rLM-nsAg. Similar numbers of rLM-sAg or rLM-nsAg were detected in the spleens of naive mice, while LCMV-immunized mice quickly cleared infection by rLM-sAg, but not by rLM-nsAg (Fig. 4B).
Total numbers of gp33–41-specific CD8 T cells were comparable in LCMV-immunized mice challenged with rLM-sAg or rLM-nsAg (Fig. 5B). Therefore, LCMV-immunized mice were protected from rLM-sAg, but not rLM-nsAg, infection. This disparity in protective immunity was not due to differences in the number of Ag-specific CD8 T cells in the spleen that were capable of deploying effector functions, such as IFN-γ secretion. To extend our analysis, we distinguished between memory and effector Ag-specific CD8 T cells by using the mAb 1B11 that recognizes two core O-glycans on CD43. Glycosylation of mucin-type glycoproteins is up-regulated on primary and secondary effector cells, but down-regulated on memory cells, and mAb 1B11 epitope expression has been demonstrated to directly correlate with effector function (21). In LCMV-immunized mice, most of the gp33–41-specific CD8 T cells had low levels of O-glycan CD43 expression, consistent with their memory phenotype. Upon challenge with either rLM-sAg or rLM-nsAg, a substantial number of gp33–41-specific CD8 T cells up-regulated O-glycan CD43 expression and thus had become effectors (Fig. 5A). Interestingly, similar levels of gp33–41-specific effector cells were generated in mice challenged with either rLM-sAg or rLM-nsAg. Thus, secreted and nonsecreted gp33–41 induced similar levels of differentiation of gp33–41-specific memory CD8 T cells to effector cells. Taken together, these data shown that the lack of protective immunity was not due to the inability of Ag-specific memory CD8 T cells to respond to nonsecreted Ag.

The early responses of Ag-specific memory CD8 T cells to rLM-sAg and rLM-nsAg were not significantly different. Extending our analysis of the recall immune response kinetics beyond day 2 p.i. verified that there was no significant difference between the magnitudes of the recall responses to rLM-sAg and rLM-nsAg (Fig. 6). Infection with both rLM-sAg and rLM-nsAg increased the total number of Ag-specific CD8 T cells in LCMV-immunized mice compared with unchallenged immune mice, from $2 \times 10^5$ to $\sim 1 \times 10^6$ cells/spleen. Thus, although rLM-sAg was rapidly cleared, and rLM-nsAg persisted in the mice, there were no detectable differences in the recall responses induced by these bacteria.

Discussion

All known *L. monocytogenes* CD8 T cell epitopes are derived from secreted proteins, even though secreted proteins comprise only 1–2% of the total proteins expressed (22). The response to these known epitopes accounts for only ~20% of activated CD8 T cells during *L. monocytogenes* infection (23). In contrast, in viral infections such as LCMV, influenza, and EBV, known epitopes account for >70% of the activated CD8 T cells (24–26). These data suggest that many CD8 T cell epitopes in *L. monocytogenes* have yet to be identified, some of which may be derived from nonsecreted proteins. While T cells that proliferate in response to nonsecreted Ags have been shown to exist by in vitro culturing with bacterial lysate (27), CD8 epitopes from nonsecreted proteins have not yet been identified due to an experimental bias toward secreted proteins. Early studies using in vitro stimulation assays to identify *L. monocytogenes* epitopes relied on isolation of CD8 T cell clones specific to *L. monocytogenes* (4, 5). In these studies splenocytes from *L. monocytogenes*-immunized mice were generated and maintained by stimulation with *L. monocytogenes*-infected J774 cells. Since J774 cells are nonbactericidal, nonsecreted proteins are unlikely to be presented in this in vitro culture to stimulate and maintain CD8 T cells. A recent study employed overlapping synthetic dodecamer peptides to screen for epitopes using the ELISPOT assay with splenocytes from *L. monocytogenes*-immunized mice (7). While this approach should allow identification of epitopes from nonsecreted *L. monocytogenes* proteins, it is costly and was only applied to secreted proteins, which are thought to be more likely vaccine targets because of their localization in the host cell cytosol. However, recent advances in our understanding of T cell activation in vivo have unveiled the importance of dendritic cells in priming a CD8 T cell response through cross-presentation (28). While nonsecreted bacterial Ags are not accessible to the classical endogenous MHC class I pathway, our study using two model Ags has clearly demonstrated that nonsecreted proteins can induce a CD8 T cell response. Our results therefore support the role of cross-presentation in initiating a CD8 T cell response to infection.

A long-standing hypothesis postulates that secreted proteins induce a more robust response than nonsecreted proteins at the onset of infection and therefore are more relevant vaccine targets. While

**FIGURE 5.** Rapid generation of effectors from gp33–41 memory CD8 T cells upon challenge of LCMV-immunized mice with *L. monocytogenes* expressing secreted or nonsecreted gp33–41. Naive and LCMV-immunized C57BL/6 mice were challenged with $1 \times 10^6$ CFU rLM-sAg or rLM-nsAg. On days 1 and 2 after challenge, gp33–41-specific cells in the spleens of LCMV-immunized mice were examined by intracellular IFN-γ staining, and the memory and effector cells were differentiated by staining with mAb 1B11 that recognizes O-glycans on CD43 (A; gated on CD8+ cells). Numbers within the left gate are the percentage of IFN-γ-producing memory cells (CD43low), and numbers within the right gate are the percentage of IFN-γ-producing effector cells (CD43high). Plots are from one representative mouse of three in each group. Total numbers of gp33–31-specific CD8 T cells per spleen were calculated based on the data of intracellular IFN-γ staining and the number of splenocytes recovered from each mouse (B). Bacterial loads in spleens of naive (□) and LCMV-immunized mice (□) on days 1 and 2 p.i. were determined (C). Bars represent the mean ± SD from three mice in each group. The limit of detection is depicted as a dotted line.
A direct test of this hypothesis has been difficult because available assays for measuring Ag-specific responses are not sensitive enough to measure the extremely low levels of Ag-specific CD8 T cells at the onset of the immune response. To overcome this limitation, we employed an adoptive transfer model using CFSE-labeled TCR transgenic cells. Our results show that Ag-specific CD8 T cells did not respond to secreted or nonsecreted Ags by day 2 p.i. However, on days 3 and 4 there was substantially more proliferation of Ag-specific CD8 T cells responding to secreted than nonsecreted Ag. While it was difficult to precisely quantitate the number of P14 cells recruited and the number of times they divided due to limited resolution of CFSE peaks in our in vivo experiments, the results suggest that 1) the timing of recognition and the extent of proliferation of recruited cells were similar during infection with rLM-sAg and rLM-nsAg; and 2) the difference in the numbers of Ag-specific CD8 T cells was mostly due to a difference in the number of precursors recruited into division. These results from early time points in adoptive transfer experiments are consistent with data from later time points taken from normal mice and show that secreted Ag induced a relatively greater CD8 T cell response during primary infection compared with nonsecreted Ag. Furthermore, CD8 T cell populations responding to secreted and nonsecreted recombinant Ags expanded and contracted synchronously with those specific to epitopes from endogenous secreted bacterial proteins. These results provide a direct comparison of the responses induced by two MHC class I pathways and demonstrate that the kinetics are similar regardless of whether Ag is accessible to the endogenous MHC class I pathway or is restricted to cross-presentation. The difference in the magnitudes of the responses is probably due to different numbers of APC presenting the Ag and the amount of Ag presented on APC. We compared the amounts of secreted and nonsecreted recombinant Ag presented during the early stages of L. monocytogenes infection using a modified direct ex vivo Ag detection assay (29, 30). Antigenic peptides were readily measurable in splenocytes of mice infected with rLM-sAg, but were barely detectable in splenocytes of rLM-nsAg-infected mice (data not shown). These differences in Ag availability and in the magnitude of the responses to secreted and nonsecreted Ag probably reflect distinct features of cross-presentation in inducing the activation and expansion of CD8 T cells, which are currently under investigation.

Protective immunity against L. monocytogenes is mediated by the recall response of memory CD8 T cells. Bacteria will gain a foothold and multiply rapidly within their intracellular niche if an effective immune response is not initiated soon after the onset of infection. Thus, the decisive battle between pathogen and host is fought at the onset of infection. Ineffective induction of a recall response has been hypothesized to contribute to the lack of protection against L. monocytogenes expressing nonsecreted Ag (11). However, our results demonstrate that both L. monocytogenes expressing secreted or nonsecreted Ag induced differentiation of memory CD8 T cells by day 1 p.i., and Ag-specific CD8 T cells had similar expansion kinetics during a recall response. Thus, less effective induction of an immune response by nonsecreted Ag is unlikely to be a critical factor contributing to the lack of protective immunity. Why do memory CD8 T cells not provide protection despite the fact that they are activated to produce cytokines such as IFN-γ? We are currently investigating the possibility that the lack of protective immunity relates to the presentation of nonsecreted Ag by only a subset of infected cells and/or dendritic cells that have acquired exogenous Ags. L. monocytogenes infects many different cell types that are incapable of presenting Ag through cross-presentation. These infected cells will not display peptides from nonsecreted bacterial proteins on their surface and therefore will not be recognized. These intracellular bacteria will continue to multiply and disseminate by direct cell-to-cell spread despite an ongoing CD8 T cell response to nonsecreted Ags presented by APCs. This is true even when CD8 T cells are activated to produce IFN-γ, since activated CD8 T cells exert little bystander control of intracellular bacteria. Memory CD8 T cells provided protection only against rLM-sAg, but not wild-type L. monocytogenes, when LCMV-immunized mice were challenged with a mix of rLM-sAg and wild-type bacteria (J. Jiang and H. Shen, unpublished observations).

In summary, we have extended our previous findings to another Ag restricted by a different MHC haplotype, thus validating the conclusion that protein secretion in bacteria is a major factor in determining whether an Ag can serve as a protective target. While both secreted and nonsecreted bacterial Ags can prime CD8 T cell responses, only recognition of secreted Ags by memory CD8 T cells results in protective immunity against L. monocytogenes. We show that the kinetics of the primary responses to secreted and nonsecreted bacterial Ags are similar, although the magnitude of
the response to secreted Ags is relatively greater. We further demonstrate that nonsecreted bacterial proteins induce recall CD8 \(^+\) T cell responses, but do not serve as protective Ags. Understanding how different determinants of bacterial Ags influence the nature and magnitude of host defense is critical for designing effective vaccines that induce potent responses and for selecting candidate Ags that can serve as protective targets.

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References