Effector CD8⁺ T Cells Are Generated in Response to an Immunodominant Epitope in Type III Effector YopE during Primary *Yersinia pseudotuberculosis* Infection

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YopE is a virulence factor that is secreted into host cells infected by *Yersinia* species. The YopE C-terminal domain has GTPase-activating protein (GAP) activity. The YopE N-terminal domain contains an epitope that is an immunodominant CD8⁺ T cell antigen during primary infection of C57BL/6 mice with *Yersinia pseudotuberculosis*. The characteristics of the CD8⁺ T cells generated in response to the epitope, which comprises YopE amino acid residues 69 to 77 (YopE_{69–77}), and the features of YopE that are important for antigenicity during primary infection, are unknown. Following intravenous infection of naïve C57BL/6 mice with a *yopE* GAP mutant (the R144A mutant), flow cytometry analysis of splenocytes by tetramer and intracellular cytokine staining over a time course showed that YopE_{69–77}-specific CD8⁺ T cells producing gamma interferon (IFN- γ) and tumor necrosis factor alpha (TNF- α) were generated by day 7, with a peak at day 14. In addition, ~80% of YopE_{69–77}-specific CD8⁺ T cells were positive for KLRG1, a memory phenotype marker, at day 21. To determine if residues that regulate YopE activity by ubiquitination or membrane localization affect the antigenicity of YopE_{69–77}, mice were infected with a *yopE* ubiquitination or membrane localization mutant (the R62K or L55N I59N L63N mutant, respectively). These mutants elicited YopE_{69–77}-specific CD8⁺ T cells producing IFN- γ and TNF- α with kinetics and magnitudes similar to those of the parental R144A strain, indicating that primary infection primes effector CD8⁺ T cells independently of the ubiquitination or membrane localization of YopE. Additionally, at day 7, there was an unexpected positive correlation between the numbers of YopE_{69–77}-specific CD8⁺ T cells and CD11b⁺ cells, but not between the numbers of YopE_{69–77}-specific CD8⁺ T cells and bacterial cells, in spleens, suggesting that the innate immune response contributes to the immunodominance of YopE_{69–77}.

Effector CD8⁺ T cells produce cytokines and have cytotoxic activity; as such, they are invaluable in the host defense against a variety of infectious diseases and cancer (1). Antigen-presenting cells (APC) activate naïve CD8⁺ T cells using at least two sets of signals: peptide antigens presented on MHC class I (MHC-I) molecules that are recognized by specific CD8⁺ T cells and costimulatory factors expressed on the surfaces of APCs. The classical pathway for antigen presentation on MHC-I molecules depends on the processing of cytosolic proteins by the proteasome after polyubiquitination. The resulting peptides are transported to the endoplasmic reticulum (ER) to be further trimmed, loaded onto MHC-I molecules, and finally presented on the plasma membrane to CD8⁺ T cells (2, 3). In addition to viral proteins synthesized inside the cytosol, bacterial factors that gain access to the cytosol of APCs are potential sources of epitopes for presentation to CD8⁺ T cells by the MHC-I pathway. For example, many bacterial pathogens translocate virulence factors across plasma or vacuolar membranes using the type III secretion system (T3SS). T3SSs are required by these Gram-negative bacterial pathogens for virulence (4–7). T3SSs are activated upon contact of the bacteria with host cells and function to deliver effector proteins into or across the eukaryotic plasma membrane (8–12). Because of their ability to deliver proteins into the cytosol of host cells and to stimulate a strong innate response, T3SS-containing bacteria are being considered for use as live vaccine vectors to induce protective CD8⁺ T cell responses against heterologous antigens (13–15). However, among other limitations, the lack of advantage over conventional methods in inducing a strong effector response prevents the widespread use of T3SS-containing bacterium-based vaccine vectors (13, 15).

Recent studies have demonstrated a dominant CD8⁺ T cell response to the T3SS effector YopE in C57BL/6 mice infected with attenuated *Yersinia pestis* (16) or *Yersinia pseudotuberculosis* (17). This H-2K^b-restricted epitope, SVIGFIQRM, corresponds to amino acid residues 69 to 77 of YopE (YopE_{69–77}) in *Y. pestis* and *Y. pseudotuberculosis*. Importantly, *Y. pseudotuberculosis* serogroup O1 strain 32777 induces an unusually large CD8⁺ T cell response to YopE during primary infection of C57BL/6 mice; at the peak of the response, at day 14 postinfection, an average of 30% and as many as 50% of splenic CD8⁺ T cells recognize YopE_{69–77} (17). This high level of response is unprecedented. In a typical virus infection, about 5 to 10% of CD8⁺ T cells respond to a dominant epitope. For example, in mice infected with murine cytomegalovirus, at the initial peak of response a few days postinfection, around 6% of blood or splenic CD8⁺ T cells respond to the dominant epitope encoded in IE1, and after about 1 year of continuous accumulation, ~20% of CD8⁺ T cells respond to this

Received 10 March 2014 Returned for modification 29 March 2014

Accepted 25 April 2014

Published ahead of print 5 May 2014

Editor: A. J. Bäuml

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Supplemental material for this article may be found at <http://dx.doi.org/10.1128/IAI.01687-14>.

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doi:10.1128/IAI.01687-14

antigen (18). During the primary response to *Listeria monocytogenes* infection in mice, approximately 2 to 3% of splenic CD8⁺ T cells are specific for listeriolysin O amino acid residues 91 to 99 (LLO₉₁₋₉₉) at the peak of response (19); even at the peak of a recall response, about 17% of all CD8⁺ T cells in the spleen recognize LLO₉₁₋₉₉ (20). Not only is the YopE₆₉₋₇₇ response large in terms of the number of CD8⁺ T cells recognizing the epitope, but these YopE-specific CD8⁺ T cells potentially exhibit effector functions, since C57BL/6 mice vaccinated with the YopE₆₉₋₇₇ peptide are protected from lethal challenge with *Y. pestis* (16) or *Y. pseudotuberculosis* (17). Moreover, the CD8⁺ T cells from *Y. pestis*-immunized mice can, after secondary challenge with virulent *Y. pestis*, be induced to produce tumor necrosis factor alpha (TNF-α) and gamma interferon (IFN-γ) upon *ex vivo* stimulation with YopE₆₉₋₇₇ (16). However, the characteristics of the CD8⁺ T cell response to YopE₆₉₋₇₇ during primary infection of mice with *Y. pseudotuberculosis* have not been investigated.

Y. pseudotuberculosis causes intestinal infections (yersiniosis) in humans and a variety of animals and is genetically highly similar to *Yersinia pestis*, the agent of plague (21). *Yersinia enterocolitica* also causes intestinal yersiniosis but is genetically distantly related to *Y. pseudotuberculosis* and *Y. pestis*. Rodents are natural hosts for pathogenic *Yersinia* species, and therefore, mice have been used extensively for the study of pathogenesis and host responses to virulent or vaccine carrier strains of these bacteria.

YopE has GTPase-activating protein (GAP) activity that is critical for *Yersinia* virulence. YopE functions as an antiphagocytic factor (22) and also as an inhibitor of the generation of reactive oxygen species (ROS) (23). In mice infected with *Y. pseudotuberculosis*, YopE is translocated preferentially into neutrophils but also into APCs such as dendritic cells, macrophages, and B cells (24). The eukaryotic host cell targets of YopE include the small GTPases RhoA, Rac1, and Rac2 (22, 23, 25, 26).

Besides the C-terminal GAP domain (residues 100 to 219), YopE also contains two other functional domains: a signal sequence (residues 1 to 15) and a chaperone-binding domain (Cb) (residues 23 to 78) (see Fig. 1A). The SVIGFIQRM epitope is located within the Cb. The Cb regulates the translocation of YopE through the binding of the chaperone SycE (27–31). When *Y. pseudotuberculosis* was tested as a vaccine carrier, epitopes from heterologous antigens (e.g., LLO₉₁₋₉₉ or ovalbumin amino acid residues 257 to 264 [OVA₂₅₇₋₂₆₄]) fused to the N-terminal 138 amino acids of YopE were presented by the MHC-I pathway after APCs were infected with *Y. pseudotuberculosis* carriers (32–34). Furthermore, the level of presentation of LLO₉₁₋₉₉ by APCs infected with a *Y. pseudotuberculosis* carrier was reduced in the presence of proteasome inhibitors (34), indicating that translocated YopE-LLO is processed by the conventional MHC-I pathway.

Besides facilitating translocation through the T3SS, the Cb also contains other residues that regulate YopE activity in the host cell. The membrane localization domain (MLD; residues 53 to 79) is required for the translocated YopE to associate with membranes in host cells, enhancing its access to GTPase substrates (35–37). Lysine (K) residues at positions 62 and 75 of YopE function as ubiquitination recipients, and these residues are subject to allelic variation (38–40). YopE proteins in *Y. enterocolitica* serogroup O8 strains contain K residues at these positions, are subject to increased ubiquitination and degradation, and consequently have reduced GAP activity in host cells (38, 40). YopE proteins in *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica* serogroup O3

TABLE 1 Bacterial strains used in this study

<i>Yersinia</i> strain	Relevant characteristic(s)	Reference or source
32777	Serogroup O:1; pYV ⁺	54
mE	32777 pYV yopE ^{R144A}	17
M77A mutant	32777 pYV yopE ^{M77A}	This study
V70A-G72E mutant	32777 pYV yopE ^{V70A-G72E}	This study
R62K mutant	32777 pYV yopE ^{R62K}	This study
MLD mutant	32777 pYV yopE ^{L55N-I59N-L63N}	This study
mE-M77A	32777 pYV yopE ^{M77A-R144A}	This study
mE-V70A-G72E	32777 pYV yopE ^{V70A-G72E-R144A}	This study
mE-R62K	32777 pYV yopE ^{R62K-R144A}	This study
mE-MLD	32777 pYV yopE ^{L55N-I59N-L63N-R144A}	This study
mEΔB	32777 pYV yopE ^{R144A} yopB (internal deletion of yopB)	This study

and O9 strains contain arginine (R) and glutamine (Q) at positions 62 and 75, respectively, resulting in lower ubiquitination, greater stability, and more-effective GAP activity in host cells (38, 40). Ubiquitination and degradation through the proteasome are steps in classical antigen presentation, and therefore, it is important to investigate their contribution to the antigenicity of a given antigen.

YopE is the first type III effector recognized as a dominant CD8⁺ T cell antigen during bacterial infection. It is therefore of interest to determine the characteristics of the YopE-specific CD8⁺ T cell response during primary infection and to identify features of this protein that lead to its efficient recognition by the adaptive immune system. We demonstrated previously that GAP activity in YopE is not required for the dominant CD8⁺ T cell response to the SVIGFIQRM epitope in C57BL/6 mice infected with *Y. pseudotuberculosis* (17). In this study, C57BL/6 mice were infected with a *Y. pseudotuberculosis* yopE R144A mutant lacking GAP activity, and the production of effector CD8⁺ T cells was measured using flow cytometry in conjunction with staining for surface markers or intracellular cytokines and an H-2K^b MHC class I YopE₆₉₋₇₇ tetramer. The other goal of this study was to determine if ubiquitination or membrane localization of YopE affects its antigenicity. We hypothesized that increased ubiquitination would enhance the degradation of YopE by the proteasome, thereby enhancing the kinetics and/or magnitude of the CD8⁺ T cell response during primary infection; on the other hand, membrane binding could sequester YopE from the proteasome and therefore reduce the YopE-specific CD8⁺ response. To test these hypotheses, codon changes were introduced into yopE to increase the ubiquitination or reduce the membrane localization of YopE without changing the sequence of the SVIGFIQRM epitope.

MATERIALS AND METHODS

Bacterial strains. The *Y. pseudotuberculosis* strains used in this study are derived from the serogroup O1 strain 32777 (Table 1) (41). Strain mE encodes the catalytically inactive YopE(R144A) protein, which has been described previously (42). To create the R62K and MLD (L55N I59N L63N) mutants, codon changes were first introduced into plasmid pSB890-yopEplus or pSB890-yopE R144A using a QuikChange kit, and then standard allelic exchange procedures (42) were followed to introduce the mutations into strain 32777 or mE. For the R62K mutant, primers 5'-TCATCAGTGGCCCACTCTGTG-3' and 5' CTTAGCCAGCCGTAT CATTGAGA 3' were used to introduce the codon change and to incor-

porate a HindIII restriction site, which was used diagnostically to determine the presence of the mutation. For the MLD mutant, primers 5'-GCCTCAGGGTTCCAGCAACGCCA GCCGTAACATTGAGAGGAATT CATCAGTGGCCCACTC-TG-3' and 5'-CAGAGTGGGCCACTGATG AATTCCTCTCAATGTTACGGCTGGCGTTGCTGGAACCTGAGGG C-3' were used to introduce the codon changes and to create a diagnostic EcoRI restriction site. The M77A and V70A-G72E epitope mutants were generated as described above. For the M77A mutant, the primers used were 5'-TTCTCGGAGGGGAGCCATA-3' and 5'-TGTGATTGGGTTT ATCCAACGC-3', which incorporated a diagnostic BsmI site. For the V70A-G72E mutant, the primers were 5'-GTTTATCCAACGCATGTTC TCGG-3' and 5'-GTTATCATCAGTGGCCCACTCTG-3', which created a diagnostic MfeI site. Strain mEΔB was created by introducing the YopE(R144A) mutation into 32777ΔB as described previously (42).

Preparation of BMDMs and infection of mice. Eight-week-old female C57BL/6 mice were obtained from The Jackson Laboratory. Bone marrow-derived macrophages (BMDMs) were prepared as described previously (42). For intravenous (i.v.) infection, overnight bacterial cultures grown in Luria-Bertani (LB) medium at 28°C were washed once and were resuspended in phosphate-buffered saline (PBS) to achieve the desired CFU/ml. Then 100-μl volumes were delivered via the lateral tail vein. For intragastric (i.g.) infection, mice were fasted 16 h before the inoculation of 200 μl of bacteria diluted in PBS. At 7, 14, or 21 days postinfection, or when death was imminent, mice were euthanized by CO₂ asphyxiation. Mouse spleens and livers were dissected aseptically and were weighed. The spleen was homogenized with a 5-ml syringe plunger in 5 ml of Dulbecco's modified Eagle medium (DMEM); the liver was homogenized in 4 ml of PBS using a Stomacher 80 instrument (Seward Lab System). Serial dilutions of 100 μl of LB broth were plated on LB agar in order to determine levels of bacterial colonization by CFU assays, and the limit of detection was 50 CFU or a log₁₀ CFU value of 1.7. All animal procedures were approved by the Stony Brook University Institutional Animal Care and Use Committee.

Detergent solubility assay and immunoblot analysis. A detergent solubility assay was used to determine the amounts of different YopE proteins translocated into the cytosol of BMDMs infected with *Y. pseudotuberculosis* as described previously (47). Briefly, overnight cultures of *Y. pseudotuberculosis* were diluted to an optical density at 600 nm (OD₆₀₀) of 0.1 into LB medium containing 20 mM magnesium chloride and 20 mM sodium oxalate and were grown first at 28°C for 1 h and then at 37°C for 2 h, with shaking. Then bacteria were washed once in Hanks' balanced salt solution (HBSS), mixed into 1 ml of bone marrow macrophage-low medium (Dulbecco modified Eagle medium containing 15% L-cell conditioned medium, 10% fetal bovine serum, and 1 mM sodium pyruvate), and applied at a multiplicity of infection (MOI) of 50 to C57BL/6-derived BMDMs at 8 × 10⁵ cells/well on a 6-well plate. After incubation for 1.5 h, the monolayer was washed with PBS and was scraped into 50 μl of 1% Triton X-100 buffer (10 mM Tris [pH 7.6], 150 mM NaCl, 10% glycerol, 1% Triton X-100) containing a protease inhibitor cocktail (Roche). Then the lysate was centrifuged for 10 min at 12,000 × g and 4°C to separate the supernatants (soluble fractions) from the pellet (insoluble fractions), and these fractions were mixed with or resuspended in Laemmli sample buffer.

Macrophage fractions were boiled, resolved by SDS-PAGE, transferred to a nitrocellulose membrane, and analyzed by immunoblotting with a cocktail of three monoclonal antibodies specific for YopE (unpublished data). Immunoblot analysis and quantification were carried out, and images were acquired with an Odyssey VI scanner (LI-COR Biosciences), as described previously (43).

To determine the steady-state expression levels of different YopE proteins in *Y. pseudotuberculosis* mutants, overnight bacterial cultures were diluted to an OD₆₀₀ of 0.1 and were grown for 2 h in LB medium with 2.5 mM CaCl₂ at 37°C with shaking. Cultures were adjusted to an OD₆₀₀ of 0.4. Equal volumes of cultures were centrifuged to obtain pellets. Lysates were prepared from the pellets by the addition of Laemmli sample buffer.

Samples of the lysates were analyzed by SDS-PAGE followed by immunoblotting as described above.

Flow cytometry. Single-cell suspensions of spleens were prepared as described previously (41). Briefly, splenocytes in suspension were incubated in an additional 20 ml of DMEM containing penicillin-streptomycin for 20 min. Then red blood cells (RBC) were lysed, and viable cells were counted using trypan blue exclusion with the Countess automated cell counter (Invitrogen). Suspended cells (1 × 10⁶) were blocked using anti-mouse CD16/CD32 (FcγIII/II receptor) clone 2.4G2 (BD Pharmingen) and were labeled with a YopE₆₉₋₇₇-specific tetramer at room temperature for 1 h and/or with fluorophore-conjugated antibodies on ice for 20 min. The antibodies used were Alexa Fluor 488- or phycoerythrin (PE)-conjugated anti-mouse CD8α (clone 53-6.7; BD, BioLegend), PE-conjugated anti-mouse KLRG1 (clone 2F1; BioLegend), and peridinin chlorophyll protein (PerCP)-conjugated anti-mouse CD3e (clone 145-2C11; PharMingen). The allophycocyanin-conjugated MHC class I tetramer K^bYopE₆₉₋₇₇ was provided by the NIH Tetramer Core Facility (Emory University, Atlanta, GA). To stain for intracellular cytokines, suspended splenocytes (1 × 10⁶ cells) were incubated in 200 μl of complete T cell medium (Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated fetal bovine serum, 12.5 mM HEPES, 2 mM L-glutamine, 1 mM sodium pyruvate, 1 mM penicillin-streptomycin, and 55 μM β-mercaptoethanol) containing 10 nM OVA₂₅₇₋₂₆₄ (H₂N-SIINFEL L-OH) or YopE₆₉₋₇₇ peptide and brefeldin A (Sigma, 5 μg/ml) for 4.5 h at 37°C. The cells were then washed and stained for the tetramer and CD8α as described above; then the cells were fixed and permeabilized with a BD Cytofix/Cytoperm kit according to the manufacturer's instructions. Finally, the cells were stained with PE-conjugated anti-mouse IFN-γ (clone XMGI.2) and with PerCP/Cy5.5-conjugated anti-mouse TNF-α (clone MP6-XT22) (both from BioLegend). Isotype-matched antibodies were used to control for nonspecific binding. Labeled cells were analyzed using a BD FACSCalibur flow cytometer. Gating on side scatter and forward scatter was used to focus on intact splenocytes. Data were processed with FlowJo software.

Statistical analysis. Statistical analysis was performed with GraphPad Prism software, version 4.0. The tests used are indicated in the figure legends or the text. *P* values of <0.05 were considered significant.

RESULTS

Analysis of the SVIGFIQRM epitope and generation of *Y. pseudotuberculosis* mutants encoding modified YopE proteins. The primary goal of this study was to characterize the dominant CD8⁺ T cell response to YopE₆₉₋₇₇ during primary infection. The secondary goal was to determine if structural features of YopE in the vicinity of the epitope are important for its antigenicity. We begin by analyzing the epitope and describing the generation of *Y. pseudotuberculosis* mutants encoding modified YopE proteins that were used to complete the second goal of the study.

The YopE₆₉₋₇₇ (SVIGFIQRM) epitope is located within the MLD of YopE (Fig. 1A) and is fully conserved in *Y. pseudotuberculosis*, *Y. pestis*, and *Y. enterocolitica* serogroup O3 and O9 strains (Fig. 1B). As shown in Table 2, SVIGFIQRM is predicted (44, 45) to bind with high affinity to murine H-2K^b (50% inhibitory concentration [IC₅₀], 20 nM), as well as to human HLA-A26:01 (12 nM), encoded by one of the most common MHC-I alleles among Asians. The model MHC-I epitope SIINFELK (OVA₂₅₇₋₂₆₄) is predicted to bind H-2K^b with similar affinity (17 nM [Table 2]). The corresponding YopE peptide in *Y. enterocolitica* serogroup O8 strains has three amino acid differences (SAIEFIKRM [different amino acids are underlined]) (Fig. 1B) and is predicted to bind weakly to H-2K^b (82 nM) and HLA-A26:01 (224 nM) (Table 2).

To determine if structural features of YopE in the vicinity of the epitope affect antigenicity, amino acid substitutions were used to

FIG 1 Primary sequence model of YopE showing the location of the YopE₆₉₋₇₇ epitope and comparison of the sequence with those of other *Yersinia* strains. (A) Primary sequence model of YopE. The signal sequence (Ss), chaperone-binding domain (Cb), and Rho GTPase activation protein (RhoGAP) domain are indicated. The positions of amino acid residues at domain boundaries and the inactivating R144A codon change are indicated above the model. Beneath it is the sequence of the membrane localization domain (MLD), with the CD8⁺ T cell epitope shown in blue. The positions of codon changes that either disrupt the MLD (L55N, I59N, L63N) (black letters), increase ubiquitination (R62K) (red letters), or alter the epitope (V70A, G72E, M77A) (blue letters) are indicated. (B) Clustal alignment of MLD sequences from the YopE proteins encoded by the indicated *Yersinia* strains. *Y. enterocolitica* O9 and O3 YopE proteins have the same sequence. *Y. enterocolitica* 8081, A127/90, and WA-314 are O8 strains. The location of the epitope is indicated above the sequence alignment, and the residues that make up the symmetrical L- and I-rich MLD motif are indicated by asterisks below the sequences.

create a ubiquitination site or to disrupt the MLD motif without affecting the epitope. The R62K substitution (Fig. 1A) was used to create one ubiquitination site N-terminal to the epitope. By following the strategy of Zhang and Barbieri (35), the codon changes L55N, I59N, and L63N were used to inactivate three MLD motif positions N-terminal to the epitope (Fig. 1A).

Site-directed mutagenesis was used to introduce the codon changes described above into *yopE* carried on the virulence plasmid of the *Y. pseudotuberculosis* wild-type (WT) serogroup O1 strain 32777. The mutant with the L55N, I59N, and L63N substitutions is referred to as the MLD mutant, and the other mutants are designated by the codon changes they carry; for example, the gain-of-ubiquitination mutant is called the R62K mutant. The codon changes were also introduced into the *yopE*^{R144A} mutant strain mE, which encodes a catalytically inactive YopE^{R144A} protein (Fig. 1A). As shown previously, YopE GAP activity is not

required *per se* for the CD8⁺ T cell response to YopE (17); however, the mE strain is attenuated, and as such, it can be used for i.v. infections. The i.v. route of infection allows for the delivery of equivalent loads of mutant bacterial strains to systemic sites and thus avoids the possibility that attenuated strains may not colonize deep tissues efficiently during mouse infection. An additional advantage of using the mE strain background for these experiments is related to the known negative effect of GAP activity on YopE translocation (22, 46). Substitutions (e.g., in the MLD) that indirectly decrease GAP activity could increase YopE translocation, potentially increasing antigen presentation. The use of the mE background avoided this complication, because catalytically inactive YopE (YopE^{R144A}) would be maximally translocated irrespective of additional amino acid substitutions. Strains constructed in the mE background are designated “mE” followed by the mutation (e.g., mE-MLD or mE-R62K).

TABLE 2 Summary of predicted affinities of YopE peptides for MHC-I alleles^a

Peptide	Strain(s) or source	Allele	Log score	IC ₅₀ (nM)	Affinity
SVIGFIQRM	<i>Y. pseudotuberculosis</i> , <i>Y. pestis</i> , <i>Y. enterocolitica</i> serogroups O9 and O3	Mouse <i>H</i> -2K ^b	0.720	20	Strong
SAIEFIKRM	<i>Y. enterocolitica</i> serogroup O8	Mouse <i>H</i> -2K ^b	0.592	82	Weak
SAIEFIQRM	<i>Y. pseudotuberculosis</i> V70A-G72E mutant	Mouse <i>H</i> -2K ^b	0.708	23	Strong
SVIGFIQRA	<i>Y. pseudotuberculosis</i> M77A mutant	Mouse <i>H</i> -2K ^b	0.424	506	Weak
SVIGFIQRM	<i>Y. pseudotuberculosis</i> , <i>Y. pestis</i> , <i>Y. enterocolitica</i> serogroups O9 and O3	Human <i>HLA</i> -A26:01	0.768	12	Strong
SAIEFIKRM	<i>Y. enterocolitica</i> serogroup O8	Human <i>HLA</i> -A26:01	0.500	224	Weak
SIINFEKL ^b	OVA ₂₅₇₋₂₆₄	Mouse <i>H</i> -2K ^b	0.734	17	Strong

^a Data were obtained by entering peptide sequences into the NetMHC 3.4 server (<http://www.cbs.dtu.dk/services/NetMHC/>).

^b Tested for comparison with YopE peptides.

FIG 2 Characterization of YopE proteins from different mutant strains of *Y. pseudotuberculosis* for expression and translocation to the cytosol of BMDMs. (A) The indicated *Y. pseudotuberculosis* strains were grown in LB medium containing 2.5 mM Ca^{2+} at 37°C. Lysates prepared from the bacteria were analyzed by immunoblotting using antibodies specific for YopE. The positions of molecular weight markers (in thousands) are shown on the left. (B) BMDMs either were left uninfected (UI) or were infected with the indicated strains at an MOI of 50 for 1.5 h. The BMDMs were lysed in a nonionic detergent and were separated into soluble and insoluble fractions. Samples of the soluble cytosolic portion (top) and the insoluble portion (bottom) were analyzed by immunoblotting with antibodies against YopE. The positions of bands representing YopE or ubiquitinated YopE (Ub-YopE) are indicated on the right. (C) Bar graph showing quantified YopE signals in soluble and insoluble fractions of BMDMs from the experiment described in the legend to panel B. The values shown have been normalized to 32777 YopE signals. For the R62K and mE-R62K mutants, the YopE and Ub-YopE signals in the soluble fraction were combined. The data shown are representative results from three experiments performed.

As part of this study, we determined whether YopE_{69–77} is important for the virulence of *Y. pseudotuberculosis*. Amino acid substitutions were designed to change the epitope sequence without disrupting the MLD motif or creating ubiquitination sites. As shown in Table 2, changing the C-terminal anchor residue of SV IGFIQRM from M to A (M77A) is predicted to decrease affinity for MHC-I by 25-fold (IC_{50} , 506 nM [Table 2]). Therefore, an M77A substitution was used to inhibit the presentation of the epitope (Fig. 1A). Residues V70 and G72 were changed to the corresponding residues in *Y. enterocolitica* O8 YopE, resulting in the V70A-G72E mutant (Fig. 1A), which was expected to change the repertoire of T cell receptors that recognize the epitope without decreasing affinity for MHC-I significantly (IC_{50} , 23 nM [Table 2]).

Characterization of expression and translocation properties of modified YopE proteins. When the wild-type and mutant strains were cultured in LB medium containing 2.5 mM Ca^{2+} at 37°C (growth conditions that induce the expression of the T3SS but not Yop effector secretion), the amounts of the different YopE proteins produced at steady state were similar, as determined by anti-YopE immunoblot analysis of bacterial-cell lysates (Fig. 2A). A detergent solubility assay (47) was used to measure the translocation of the wild-type or mutant YopE proteins into the cytosol of BMDMs infected with *Y. pseudotuberculosis*. BMDMs were either left uninfected or infected with a translocation-defective *yopB* mutant (mEΔB) as a control. Anti-YopE immunoblotting of non-detergent-soluble fractions (containing bacterium-associated protein) showed that the different mutant proteins were produced at

FIG 3 Survival of mice infected i.v. with different mutant strains of *Y. pseudotuberculosis*. Groups of C57BL/6 mice were infected with the indicated strains at 2×10^4 CFU/mouse. Survival was monitored for 28 days. The results shown are combined from 2 to 3 experiments. Each bacterial strain was used to infect 3 mice in each experiment. The total number of mice infected with each strain is given in parentheses after the strain name. The survival of mice infected with mE-V70A-G72E was significantly different from that of mice infected with mE (P value obtained by the log rank test).

similar levels, except for the YopE^{MLD} proteins, which were present in larger amounts (Fig. 2B, bottom, compare lanes 10 and 14 to lanes 5 to 9 and lanes 11 to 13, respectively; Fig. 2C). As shown in Fig. 2B, top, analysis of the soluble fractions (containing protein in BMDM cytosol) by anti-YopE immunoblotting showed that all the wild-type and mutant YopE proteins were translocated, except in BMDMs infected with the *yopB* mutant (lane 2). The amounts of the effector protein in the soluble fractions of BMDMs infected by the different mutants differed, and consistently larger amounts of YopE^{R144A} were translocated (Fig. 2B, compare lanes 1 and 3 and lanes 5 and 6; Fig. 2C). In contrast, smaller amounts of the YopE^{R62K} proteins were present in the soluble fractions of BMDMs infected with the ubiquitination mutants (Fig. 2C), and additional bands consistent with the molecular size of ubiquitinated YopE were present in these samples (Fig. 2, top, Ub-YopE). The decreased stability of ubiquitinated YopE was expected to result in lower steady-state levels of the translocated protein (38).

Virulence of *Y. pseudotuberculosis* strains encoding modified YopE proteins. In order to identify appropriate doses of the strains to be used for the immunization of mice, the relative degrees of virulence of the different mutants were determined. When the mutant strains in the mE background were used for i.v. infection of naïve C57BL/6 mice at an inoculation dose of 2×10^4 CFU/mouse, the difference in mouse survival between mE and mE-MLD, mE-R62K, or mE-M77A was not significant (Fig. 3). The difference in survival between mice infected with mE and mice infected with mE-V70A-G72E was significant (P , 0.049 by the log rank test) (Fig. 3), suggesting that the latter mutant was slightly attenuated in this infection model. The virulence of mE was further compared with those of mE-MLD and mE-R62K in i.v. infection with three different doses (2,000, 1,000, or 500 CFU). At all three infection doses, the difference in survival between mE and mE-MLD or mE-R62K was not significant (Fig. 4). From these results, we decided to use i.v. infections at 1,000 CFU/mouse to carry out the studies, described below, that assessed the abilities of the mE, mE-MLD, and mE-R62K mutants to colonize tissues and elicit a YopE-specific CD8⁺ T cell response.

For completeness, the virulence of the mutant strains encoding catalytically active YopE proteins was also tested by i.g. infection of naïve C57BL/6 mice. Gaus et al. (38) showed that the presence

FIG 4 Survival of mice infected i.v. with different doses of mutant *Y. pseudotuberculosis* strains. Groups of C57BL/6 mice were infected with the indicated strains at 2,000 (A), 1,000 (B), or 500 (C) CFU/mouse. Survival was monitored for 28 days. The results shown are combined from 2 experiments, with 3 mice in each group in each experiment, giving a total of 6 mice under each condition.

of K residues at positions 62 and 75 in YopE was associated with increased colonization of the spleen and liver by *Y. enterocolitica* after i.g., but not i.v., infection of mice. In agreement with their finding, we found that the *Y. pseudotuberculosis* R62K mutant was more virulent following i.g. infection: with an inoculation dose of 5×10^8 CFU/mouse, 33% of the C57BL/6 mice infected with the WT strain, 32777, survived, while all the mice infected with the R62K mutant at 5×10^7 CFU/mouse died (see Fig. S1A in the supplemental material). When the mice were inoculated with 5×10^8 CFU of 32777, the M77A mutant, or the V70A-G72E mutant, the differences in survival were not significant by the log rank test (see Fig. S1B in the supplemental material). These results indicate that the loss of the dominant YopE₆₉₋₇₇ epitope or a change in the repertoire of T cell receptors that recognize the epitope did not significantly change virulence. In a preliminary test in which 3

FIG 5 Levels of colonization of mouse spleens and livers by mutant *Y. pseudotuberculosis* strains. C57BL/6 mice were infected i.v. with 1,000 CFU of mE, mE-R62K, or mE-MLD. At 7, 14, or 21 days postinfection, cohorts of mice were euthanized, and the levels of bacterial colonization of their spleens (A) and livers (B) were determined by a CFU assay. Each symbol represents the value obtained from one mouse (log₁₀ CFU/organ). The limit of detection was 50 CFU, as indicated by the horizontal dashed lines. For days 7 and 14, the data shown are combined from two independent experiments, with cohorts of 3 mice in each experiment. For day 21, the data shown are combined from three (mE) or four (mE-MLD and mE-R62K) experiments with cohorts of 1 to 3 mice. Means and standard errors of the means are plotted. Circled or boxed symbols, or symbols indicated by arrows, represent data points from mice that are discussed in relation to Fig. 6.

mice were infected with the MLD mutant at 5×10^8 CFU, loss of MLD activity did not fully attenuate virulence (see Fig. S1B in the supplemental material). Therefore, in the i.g. model of infection, the R62K mutant displayed increased virulence while the pathogenicity of other mutant strains remained relatively unchanged.

Characterization of YopE₆₉₋₇₇-specific CD8⁺ T cell responses in mice during primary infection with *Y. pseudotuberculosis* strains encoding modified YopE proteins. We next characterized the YopE₆₉₋₇₇-specific CD8⁺ T cell response induced by mE, mE-MLD, or mE-R62K over a 21-day time course. Groups of naïve C57BL/6 mice were infected i.v. with 1,000 CFU of mE, mE-MLD, or mE-R62K, and spleens and livers were collected on days 7, 14, and 21 for a CFU assay or flow cytometry. From 7 to 21 days postinfection, the mean levels of bacterial colonization of the spleens and livers decreased gradually, and at each time point there was no significant difference in colonization levels between the three strains (Fig. 5A and B). Flow cytometric analysis of splenocytes in conjunction with tetramer staining showed that at day 7 postinfection, mE, mE-MLD, and mE-R62K induced significant increases in the numbers of YopE₆₉₋₇₇-specific CD8⁺ T cells in the spleen over those for uninfected control mice (Fig. 6). Although the data for the mE and mE-MLD groups showed considerable spreading at day 7, there was no significant difference in the

FIG 6 Determination of the numbers of YopE₆₉₋₇₇-specific CD8⁺ T cells from mice infected with different *Y. pseudotuberculosis* mutants. C57BL/6 mice either were infected as described in the legend to Fig. 5 or were left uninfected (UI). The numbers of YopE₆₉₋₇₇-specific CD8⁺ T cells in the spleens of mice were determined by tetramer staining, followed by surface staining and flow cytometry. Means and standard errors of the means are plotted. The differences between the UI group and the other groups were significant by the Mann-Whitney test ($P < 0.01$, except for mE-R62K at 14 days postinfection [$P, 0.04$]). Circled or boxed symbols, or symbols indicated by an arrow, represent data points from mice that are discussed in relation to Fig. 5.

mean number of YopE₆₉₋₇₇-specific CD8⁺ T cells among the three infection conditions (Fig. 6). Compared collectively to the response at day 7, the average numbers of YopE₆₉₋₇₇-specific CD8⁺ T cells in the spleen at days 14 and 21 did not increase significantly; however, there was less spreading in the mE and mE-MLD groups (Fig. 6). Overall, the numbers of YopE₆₉₋₇₇-specific CD8⁺ T cells at days 14 and 21 were similar for all three infection groups (Fig. 6). Therefore, primary i.v. infection with mE induces a dominant YopE₆₉₋₇₇-specific CD8⁺ T cell response, and mutations enhancing ubiquitination or disrupting the MLD of YopE did not significantly alter this response.

We next characterized the quality of the response by determining if the YopE₆₉₋₇₇-specific cells produced IFN- γ and TNF- α , which are markers of effector CD8⁺ T cells (48). Total splenocytes from mice infected with mE, mE-MLD, or mE-R62K were stimulated *ex vivo* with the YopE₆₉₋₇₇ peptide or a control peptide, OVA₂₅₇₋₂₆₄. IFN- γ and TNF- α production by CD8⁺ T cells was assessed by flow cytometry after intracellular cytokine staining. Figure 7A and B show representative results obtained with cells from mE-infected mice at day 14. Among the cells stimulated with YopE₆₉₋₇₇, a percentage of CD8⁺ T cells produced either IFN- γ or TNF- α , while the majority produced both (Fig. 7A). In contrast, the response of cells stimulated with the control OVA peptide was at background levels (Fig. 7B). Figure 7C shows the numbers of IFN- γ - and TNF- α -producing (double-positive) CD8⁺ T cells in the three infection groups on days 7, 14, and 21. For all three strains, infection induced an effector CD8 T cell response that peaked on day 14. There was no statistically significant difference between infection groups in the number (Fig. 7C) or percentage (data not shown) of IFN- γ ⁺ TNF- α ⁺ double-positive CD8⁺ T cells. Similar results were obtained when the analysis was performed for single-positive cells producing either IFN- γ or TNF- α (see Fig. S2 in the supplemental material). Therefore, primary i.v. infection with mE induces an effector CD8 T cell response to YopE₆₉₋₇₇, independently of ubiquitination or membrane localization of YopE.

FIG 7 *Ex vivo* production of IFN- γ and TNF- α by YopE₆₉₋₇₇-specific CD8⁺ T cells from mice infected with different *Y. pseudotuberculosis* mutants. Total splenocytes from mice infected as described in the legend to Fig. 5 were first stimulated *ex vivo* with the YopE₆₉₋₇₇ peptide or the control peptide OVA₂₅₇₋₂₆₄ for 4.5 h and then stained for the intracellular cytokines IFN- γ and TNF- α after surface labeling with an anti-CD8 antibody, followed by fixation and permeabilization. (A and B) Scatter plots obtained by flow cytometry of CD8⁺ T cells obtained from one mouse infected with mE at 14 days postinfection and stimulated with YopE₆₉₋₇₇ (A) or OVA₂₅₇₋₂₆₄ (B). (C) The number of CD8⁺ T cells from mice infected with the indicated *Y. pseudotuberculosis* strain for 7, 14, or 21 days that produced both IFN- γ and TNF- α following stimulation with YopE₆₉₋₇₇ was calculated based on the number of splenocytes and the percentage of CD8⁺ T cells producing both cytokines. Means and standard errors of the means are plotted. The data shown, combined from two to four experiments, were obtained from the same mice as those described in the legend to Fig. 5.

Expression of KLRG1 on the surfaces of CD8 T cells is used as a marker of a memory phenotype (49). As shown in Fig. 8A and B, approximately 80% of YopE₆₉₋₇₇-specific CD8 T cells were positive for KLRG1 in mice infected for 21 days with mE, while uninfected mice had few such cells. Similar results were obtained with mice infected with mE-R62K or mE-MLD (Fig. 8A). These results indicate that primary i.v. infection with mE, mE-R62K, or mE-MLD results in the production of YopE₆₉₋₇₇-specific CD8 T cells with the potential to develop into a memory population.

Correlation between the number of YopE₆₉₋₇₇-specific CD8⁺ T cells and the number of CD11b⁺ cells in mice at an early stage of primary infection. During the course of these studies to characterize the dominant CD8⁺ T cell response to YopE during primary infection, the CFU values from tissues (Fig. 5) were compared to numbers of splenic YopE₆₉₋₇₇-specific CD8⁺ T cells (Fig. 6) in order to determine if a positive correlation existed. At 7 days postinfection, one mouse infected with mE-MLD carried the lowest number of bacteria in the liver, had no detectable bacteria in its spleen (Fig. 5A and B, arrows), and had the lowest number of

YopE₆₉₋₇₇-specific CD8⁺ T cells (Fig. 6, arrow). However, aside from this animal, the number of YopE₆₉₋₇₇-specific CD8⁺ T cells was not correlated with colonization levels. For example, 7 days postinfection, the mouse that carried the fewest YopE₆₉₋₇₇-specific CD8⁺ T cells in the mE-infected group actually had the highest colonization levels in both the spleen and the liver (Fig. 6 and 5A and B, respectively, circled symbols). This lack of positive correlation is obvious at later time points as well. One mouse infected with mE-R62K was well colonized at 14 days postinfection, yet it harbored only 0.15 million YopE₆₉₋₇₇-specific CD8⁺ T cells, barely above the mean number of such cells found in mice left uninfected (Fig. 5A and B and 6, respectively, boxed symbols). When a linear correlation between the log₁₀ CFU values and the number of YopE₆₉₋₇₇-specific CD8⁺ T cells in the spleens of mice infected with mE, mE-R62K, or mE-MLD was calculated, the value of R^2 , which is the indicator of the goodness of fit, ranged from 0.0007 to 0.21, far away from the value of a perfect line of 1, and the slope was not significantly different from zero at day 7 (Fig. 9A) and at days 14 and 21 (data not shown). Therefore, the

FIG 8 Determination of the percentages of YopE₆₉₋₇₇-specific CD8⁺ T cells positive for the memory marker KLRG1 in mice infected with different *Y. pseudotuberculosis* mutants. Splenocytes from mice left uninfected (UI) or infected with the indicated strains for 21 days as described in the legend to Fig. 5 were analyzed by flow cytometry. (A) Representative scatter plots of total CD8⁺ cells for the expression of KLRG1 and the YopE₆₉₋₇₇ tetramer. (B) Percentage of YopE₆₉₋₇₇-specific CD8⁺ T cells that expressed KLRG1. The data shown are combined from three (mE) or four (mE-MLD and mE-R62K) experiments with cohorts of 1 to 3 mice, as described in the legend to Fig. 5. Means and standard errors of the means are plotted. The difference between each infection group and the UI group was significant ($P < 0.05$) as determined by the Mann-Whitney test.

number of YopE₆₉₋₇₇-specific CD8⁺ T cells in the spleen did not correlate directly with bacterial colonization levels during primary infection.

In contrast to the comparison presented above, there was an excellent correlation between the number of splenocytes and the number of YopE₆₉₋₇₇-specific CD8⁺ T cells: on day 7, R^2 values of 0.96, 0.76, and 0.75 were obtained for the groups of mice infected with mE, mE-MLD, and mE-R62K, respectively (Fig. 9B). However, the level of correlation decreased thereafter. The R^2 value for the same comparison for mE was 0.42 at 14 days postinfection and

0.32 at 21 days postinfection (data not shown). These results suggested that the YopE₆₉₋₇₇-specific CD8⁺ T cell response is correlated with the number of cells in the spleen in the initial stage of infection.

In the initial stage of infection of C57BL/6 mice with *Y. pseudotuberculosis* strain 32777 through the intragastric route, a large number of CD11b⁺ cells were recruited to the spleens, such that they become the major component of the splenocytes (17, 41). CD11b is found on the surfaces of phagocytes such as neutrophils, macrophages, monocytes, and some dendritic cells. The number of phagocytes determines the number of host cells targeted for Yop translocation during infection (50). Since translocated YopE is likely to be a major source of antigen presented to CD8⁺ T cells, we next investigated whether there was a correlation between the number of CD11b⁺ cells and the number of the YopE₆₉₋₇₇-specific CD8⁺ T cells in the spleen. As in the intragastric infection model, i.v. infection also resulted in the rapid accumulation of CD11b⁺ cells in the spleens. Seven days after the infection of mice with mE, mE-MLD, or mE-R62K, an average of 25% of the splenocytes were CD11b⁺ (data not shown). More importantly, a good linear correlation was observed between the number of CD11b⁺ cells and the number of YopE₆₉₋₇₇-specific CD8⁺ T cells (Fig. 9C) in the groups infected with mE or mE-MLD, and a moderate correlation existed in the group infected with mE-R62K. The R^2 values were 0.97, 0.71, and 0.50 for the three infection groups, respectively. These results are consistent with the idea that CD11b⁺ cells recruited into the spleen during the innate immune response are a determining factor in the immunodominance of YopE₆₉₋₇₇ during primary infection.

DISCUSSION

Previously we have shown that primary i.g. infection of C57BL/6 mice with the *Y. pseudotuberculosis* serogroup O1 strain 32777 resulted in an exceptionally dominant CD8⁺ T cell response to YopE₆₉₋₇₇ (17). We extend these results here by demonstrating that primary infection through the i.v. route brought about a similarly strong YopE₆₉₋₇₇-specific CD8⁺ T cell response and that the CD8⁺ T cells that were elicited exhibited effector function in producing TNF- α and IFN- γ . Based on our analysis of the R62K and MLD mutants, ubiquitination and membrane localization features in YopE do not appear to be important in framing the large scale of the effector CD8⁺ T cell response. We also found that bacterial colonization levels did not correlate with the number of YopE₆₉₋₇₇-specific CD8⁺ T cells generated; instead, we discovered that a good correlation existed between the numbers of CD8 T cells specific for YopE₆₉₋₇₇ and the numbers of CD11b⁺ cells in spleens at the early stage of infection.

A previous study that demonstrated the production of effector CD8 T cells specific for YopE₆₉₋₇₇ utilized mice that were vaccinated intranasally twice with attenuated *Y. pestis* (D27-pLpxL), followed by intranasal challenge with a virulent strain of the bacterium (16). At day 4 postchallenge, 10% of YopE₆₉₋₇₇-specific CD8 T cells in the lungs produced TNF- α and IFN- γ (16). A major finding in our study is that TNF- α ⁺ IFN- γ ⁺ bifunctional CD8⁺ T cells specific for YopE₆₉₋₇₇ are produced in the spleen during primary i.v. infection with the *Y. pseudotuberculosis* mE strain at day 7 postinfection, with a peak in the response at day 14 (Fig. 7C). In addition, ~15% of all CD8⁺ T cells, or ~60 to 70% of all YopE₆₉₋₇₇-specific CD8⁺ T cells, were TNF- α ⁺ IFN- γ ⁺ at the

FIG 9 Correlation between the number of YopE₆₉₋₇₇-specific CD8⁺ T cells and the number of CFU, splenocytes, or CD11b⁺ cells in spleens at 7 days postinfection. C57BL/6 mice either were infected for 7 days as described in the legend to Fig. 5 or were left uninfected (UI). The number of CD11b⁺ cells was determined by surface staining and flow cytometry. The numbers of YopE₆₉₋₇₇-specific CD8⁺ T cells in spleens were determined as described in the legend to Fig. 6 and were plotted against the log₁₀ CFU values in the spleen (A), the total number of splenocytes (B), and the number of CD11b⁺ cells (C). Each symbol represents the value obtained for one mouse. The results of linear regression are plotted. The differences in slopes and intercepts among the three infection groups were not significant.

peak of the response (Fig. 7A), indicative of a dominant effector response. Furthermore, at day 21 postinfection, ~90% of YopE₆₉₋₇₇-specific CD8 T cells were positive for KLRG1 (Fig. 8), indicating that these cells had the potential to develop into a memory population. These findings are important because they demonstrate that the dominant response to YopE₆₉₋₇₇ during primary infection results in the generation of effector CD8⁺ T cells producing TNF- α and IFN- γ , two cytokines that are key for the protective immune defense against *Yersinia* (51). Thus, our model provides a unique opportunity to dissect the bacterial and host factors that contribute to a dominant effector CD8 T cell response to a type III-secreted antigen during primary infection.

Y. pseudotuberculosis strains with substitution mutations designed to create a ubiquitination site (R62K) or reduce membrane localization (MLD) in YopE were studied in order to gain insight into the antigenicity of YopE. Infecting mice i.v. with the R62K and MLD mutants in the mE genetic background resulted in equivalent levels of colonization of the liver and spleen (Fig. 5), and under these conditions, the kinetics and magnitudes of the YopE₆₉₋₇₇-specific CD8⁺ T cell responses to mE, mE-R62K, and mE-MLD were similar (Fig. 6). In addition, infection with mE, mE-R62K, and mE-MLD elicited similar numbers of TNF- α ⁺ IFN- γ ⁺ bifunctional CD8⁺ T cells (Fig. 7C). At day 7, there were >50% more TNF- α ⁺ IFN- γ ⁺ CD8⁺ T cells in the spleens of mice infected with mE-R62K than in those of mice infected with mE (an average of 1.6 million versus 0.9 million per mouse spleen); however, this difference was not significant (P , 0.065), and this trend was not seen at the two later time points. Overall, it is concluded that increased ubiquitination and degradation of YopE by the proteasome and reduced localization of this effector to host membranes does not enhance the YopE₆₉₋₇₇-specific CD8 T cell response. It may not be surprising that the MLD does not affect YopE antigenicity; however, increased ubiquitination and degradation have been shown to enhance MHC-I presentation of epitopes in other proteins (52). It is possible that translocated YopE follows a recently identified high-efficiency pathway of MHC-I presentation (53). This pathway, identified using recombinant proteins secreted into APCs from *L. monocytogenes*, results in efficient MHC-I presentation of epitopes from a subset of cytosolic antigens, regardless of the half-life of the protein (53).

Interestingly, our results pointed to a correlation between the number of CD11b⁺ phagocytes and the magnitude of the CD8⁺ T cell response to YopE₆₉₋₇₇ at an early (day 7) time point (Fig. 9).

Previously, Busch et al. concluded from a study of a *Listeria* infection model that the multiplication of antigen-specific T cells is regulated independently of the quantity of antigen (20). In agreement with their conclusion, we show here that the number of YopE₆₉₋₇₇-specific CD8⁺ T cells did not correlate with bacterial colonization levels during infection (Fig. 9A). In searching for a determining factor for the large scale of the CD8⁺ T cell response, we found that the magnitude of the YopE₆₉₋₇₇-specific CD8⁺ T cell response correlates with the numbers of splenocytes and the numbers of CD11b⁺ cells in the spleen (Fig. 9B and C). As we have shown before, CD11b⁺ cells are recruited in large numbers to the spleens of mice infected with 32777 (41), and potentially to the livers as well. In fact, CD11b⁺ cells are the most prominent population of cells in the spleens of 32777-infected mice, increasing from <5% of total splenocytes in uninfected mice to an average of ~25% of splenocytes at 7 days postinfection (data not shown). Based on their surface markers, the CD11b⁺ population can be subdivided into neutrophils, macrophages, monocytes, and dendritic cells; the latter three of these professional phagocytes can function as APCs. Durand et al. had suggested that these phagocytes determine the number of host cells targeted for Yop translocation (50). It is plausible that the amount of YopE translocated into the cytosol of these APCs is proportional to the amount of peptide presented to CD8⁺ T cells in the early stage of infection with *Y. pseudotuberculosis*. Furthermore, it is possible that the large amount of YopE available to the APCs saturates the antigen presentation pathway and that additional regulation of YopE localization within host cells or of its degradation through the proteasome does not affect the amount of YopE₆₉₋₇₇ presented to CD8⁺ T cells. It will be important in the future to investigate whether a specific CD11b⁺ APC population plays a decisive role in the formation of the dominant YopE₆₉₋₇₇-specific CD8⁺ T cell response.

ACKNOWLEDGMENTS

We thank the NIH Tetramer Core Facility for providing tetramer reagents, Jr-Shiuan Lin at Trudeau Institute for assistance with the intracellular cytokine staining method, and Ando van der Velden and Jason Tam for critical reading of the manuscript.

This work is supported by grants from the National Institutes of Health (R01-AI099222) and the Northeast Biodefense Center (U54-AI057158-Lipkin). awarded to J.B.B.

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