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Yersinia pestis YopE Contains a Dominant CD8 T Cell Epitope that Confers Protection in a Mouse Model of Pneumonic Plague

Jr-Shiuan Lin,* Frank M. Szaba,* Lawrence W. Kummer,* Brett A. Chromy,† and Stephen T. Smiley*  

Septic bacterial pneumonias are a major cause of death worldwide. Several of the highest priority bioterror concerns, including anthrax, tularemia, and plague, are caused by bacteria that acutely infect the lung. Bacterial resistance to multiple antibiotics is increasingly common. Although vaccines may be our best defense against antibiotic-resistant bacteria, there has been little progress in the development of safe and effective vaccines for pulmonary bacterial pathogens. The Gram-negative bacterium Yersinia pestis causes pneumonic plague, an acutely lethal septic pneumonia. Historic pandemics of plague caused millions of deaths, and the plague bacilli’s potential for weaponization sustains an ongoing quest for effective countermeasures. Subunit vaccines have failed, to date, to fully protect nonhuman primates. In mice, they induce the production of Abs that act in concert with type 1 cytokines to deliver high-level protection; however, the Y. pestis Ags recognized by cytokine-producing T cells have yet to be defined. In this study, we report that Y. pestis YopE is a dominant Ag recognized by CD8 T cells in C57BL/6 mice. After vaccinating with live attenuated Y. pestis and challenging intranasally with virulent plague, nearly 20% of pulmonary CD8 T cells recognize this single, highly conserved Ag. Moreover, immunizing mice with a single peptide, YopE69–77, suffices to confer significant protection from lethal pulmonary challenge. These findings suggest YopE could be a valuable addition to subunit plague vaccines and provide a new animal model in which sensitive, pathogen-specific assays can be used to study CD8 T cell-mediated defense against acutely lethal bacterial infections of the lung. The Journal of Immunology, 2011, 187: 897–904.

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of the bacilli’s capsule-like surface, and LcrV, a component of its plasmid-encoded type III secretion system, demonstrate considerable efficacy in several animal models of pneumonic plague (11, 14), but they confer little protection in others, most notably in African green monkey models (15). Mechanistic studies in the mouse have established that subunit vaccines protect by inducing production of IFI/LcrV-specific Abs; however, optimal protection also requires the type 1 cytokines IFN-γ and TNF-α (16–18).

These findings suggest that subunit vaccines might demonstrate improved efficacy if they prime Y. pestis-specific memory T cells capable of producing type 1 cytokines, in addition to inducing production of IFI/LcrV-specific Ab.

To facilitate the development of such vaccines, we sought to identify protective T cell Ags. In this study, we demonstrate that Y. pestis YopE is a dominant Ag recognized by CD8 T cells in mice immunized with live attenuated Y. pestis. Moreover, we found that immunization with a single peptide, YopE ϵ69–77, suffices to confer remarkable CD8-mediated protection against lethal pulmonary challenge. To our knowledge, we report the first model in which sensitive, quantitative, pathogen-specific assays can be applied to the study of CD8 T cell-mediated defense against acutely lethal bacterial infections of the lung.

Materials and Methods

Mice

C57BL/6 wild-type mice and B cell-deficient μMT mice (6–8 wk old) were purchased from The Jackson Laboratory (Bar Harbor, ME) and then bred at the Trudeau Institute Animal Breeding Facility after embryo recovery. Experimental mice were matched for age and sex and cared for according to Trudeau Institute Animal Care and Use Committee guidelines.

Bacteria

Strains D27 (pCD1+, pPCP+, pMT+) and D27 (pCD1−, pPCP−, pMT−), two pigment locus (pgm)-deficient variants of Y. pestis strain KIM, were provided by Dr. Robert Brubaker (Michigan State University, East Lansing, MI). A pgm-deficient variant of Y. pestis strain CD92 (pCD1−, pPCP−, pMT−) was provided by Dr. James B. Bliska (State University of New York, Stony Brook, NY). Attenuated strain D27-pLpxL was prepared by transforming strain D27 with plasmid pLpxL (10), which was provided by Drs. Egil Lien and Jon Goguen (University of Massachusetts Medical School, Worcester, MA). For challenge infections, strain D27 was grown overnight at 26°C in Bacto heart infusion broth supplemented with 2.5 mM CaCl2, diluted to an OD of 0.1 at 620 nm, regrown for 3–4 h at 26°C, quantified by measuring the OD, and resuspended in saline at the desired concentration. The number of bacteria in the inoculating dose was confirmed by plating. For immunizations, strain D27-pLpxL, was prepared as described for strain D27, except the broth was supplemented with 100 μg/ml ampicillin (10). To prepare heat-killed bacteria, Y. pestis strains were grown overnight at 26°C, diluted to an OD of 0.1 at 620 nm, regrown for 3–4 h at 26°C or for 4.5 h at 37°C, quantified by measuring the OD, resuspended in saline, and then inactivated by heating to 60°C for 1 h. Escherichia coli strain 018: K1 was grown at 37°C and then inactivated by heating to 60°C for 1 h.

Generation of Y. pestis-specific CD8 T cell clones

APC pulsed with Y. pestis were prepared by harvesting splenocytes from naïve C57BL/6 mice, treating with 50 μg/ml mitomycin C (Sigma-Aldrich) for 30 min at 37°C in complete medium (DMEM supplemented with 10% heat-inactivated FBS, 2 mM l-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin-streptomycin, and 55 μM 2-ME), washing with complete medium, and then incubating at 37°C for 2 h with heat-killed Y. pestis strain D27 grown at 37°C, using an APC/bacterium ratio of 1:5. The D27-pulsed APC were used to stimulate immune splenocytes harvested from C57BL/6 mice that were vaccinated intranasally with live attenuated Y. pestis strain D27-pLpxL (2 × 10⁶ CFU), and then boosted 62 and 92 d later by challenging intranasally with Y. pestis strain D27 (2 × 10⁶ CFU). Thirty-five days after the final boost, the splenocytes were harvested and cultured with the D27-pulsed APC in complete medium at an APC/splenocyte ratio of 1:1. After 48 h, human rIL-2 (PeproTech, Rocky Hill, NJ) was added to the culture at a final concentration of 20 U/ml. Culture medium was replenished every other day with fresh IL-2–containing medium. After 2 wk, cells were cloned by limiting dilution in 96-well flat-bottom plates containing D27-pulsed APC, prepared as described above. The resultant T cell clones were maintained by re-exposure to D27-pulsed APC every 2 wk, followed by expansion in IL-2–containing medium. Clone phenotypes were determined by flow cytometry using fluorochrome-conjugated Abs specific for mouse CD3, CD4, and CD8.

T cell assays

Cloned T cells (1 × 10⁶/well) were cultured in 96-well flat-bottom plates containing mitomycin C-treated splenic APC (1 × 10⁶/well) and the indicated peptides or heat-killed bacteria in a total volume of 200 μl complete medium. Culture supernatants (100 μl) were collected after 48 h of culture, and IFN-γ levels were measured by ELISA using an OptEIA kit (BD Biosciences, San Diego, CA). The supernatant removed from the cultures was replaced with fresh complete medium and 0.2 μg/ml [3H]thy midine. Cells were incubated for another 24 h before harvesting DNA and measuring [3H]thymidine incorporation using a Luminescence Counter (PerkinElmer, Waltham, MA). The stimulation index was calculated as follows: (cpm in culture with Ag)/(cpm in culture without Ag).

E. coli expressing recombinant Y. pestis proteins

A set of 4021 individual E. coli clones containing the open reading frames from Y. pestis strain KIM was obtained from the Pathogen Functional Genomics Resource Center. Selected clones were converted to expression format in plasmid pDEST17 using Gateway Technology (Invitrogen, Carlsbad, CA), following the manufacturer’s instructions. This plasmid enables the expression of recombinant proteins under the transcriptional control of the bacteriophage T7 promoter in frame with an N-terminal 6×His tag. The plasmid was transferred to E. coli strain BL21-AI, and the bacteria were grown in Luria broth containing 100 μg/ml ampicillin and 0.2% l-arabinose to induce production of recombinant proteins. Heat-killed bacteria were obtained by heating cultures to 60°C for 1 h. Expression of recombinant protein was confirmed by Western blotting using anti-His tag Ab.

Synthetic peptides

All peptides were synthesized by New England Peptide (Gardner, MA). A set of 42 overlapping Y. pestis YopE peptides was generated; each peptide contained 15 contiguous amino acids of the YopE protein and overlapped its neighbors by 10 aa. In addition, peptides predicted to bind H2-Kb MHC class I molecules were identified using RANKPEP (http://bio.dfci.harvard.edu/RANKPEP/), SYFPEITHI (http://www.syfpeithi.de/home.htm), and BIMAS (http://www-bimas.cit.nih.gov/molbio/hla_bind/) algorithms. For immunizations, YopE ϵ69–77 (H-N-SVIGFIQRM-OH) and control peptide OA257–264 (H-N-SIINFEKL-OH) were synthesized and purified (≥95%) at larger scale.

Intracellular cytokine staining

Lungs were perfused with saline containing heparin, miniced, and digested with collagenase and DNase, as described previously (10). Cells were stimulated with plate-bound anti-CD3 (clone 145-2C11; 2 μg/ml) or peptides (10 nM) for 5 h in complete medium containing brefeldin A (Sigma-Aldrich; 12.5 μg/ml) and stained with anti-CD4 allophycocyanin (clone RM4-5) and anti-CD8 PerCP (clone 53-6.7) for 30 min at 4°C. Lungs were minced and digested with protease and DNase, and then filtered through a 70-μm cell strainer. The lungs were treated with 0.85% ammonium chloride solution prior to use. A single-cell suspension was prepared by gentle pipetting. Lungs were collected on a BD Biosciences FACSCanto II and analyzed using FlowJo software (Tree Star). The TNF-α and IFN-γ specific mAb were purchased from eBioscience, and all other staining mAbs were purchased from BD Biosciences.

MHC class I tetramer reagents and staining

Allophycocyanin-conjugated MHC class I peptide tetramers K ϵYopE ϵ69–77 were generated by the Trudeau Institute Molecular Biology Core Facility. Lungs were prepared as described above. PBL were obtained from blood collected by cardiac puncture using heparin-treated syringes. RBCs were lysed by treatment with 0.85% ammonium chloride solution prior to tetramer staining. To stain with tetramers, cells were treated with Fc block (clone 2.4G2, 1 μg/ml) for 15 min at 4°C, washed, and incubated with tetramers for 1 h at room temperature. Each tetramer concentration was optimized empirically. After washing again, cells were stained with anti-CD4 FITC (clone RM4-5) and anti-CD8 PerCP (clone 53-6.7) for 30 min at 4°C. Data were collected on a BD Biosciences FACSCanto II and
analyzed using FlowJo software. For each sample, at least 10,000 CD8-positive events were collected.

**Immunizations using bone marrow-derived dendritic cells**

Bone marrow was harvested from the femurs of C57BL/6 mice. RBCs were lysed, as described above, and 2 × 10^6 cells were cultured in bacteriological petri dishes containing 10 ml complete medium supplemented with 20 ng/ml recombinant murine GM-CSF (PeproTech). On day 3, an additional 10 ml supplemented medium was added to the cultures. On day 6, 10 ml medium was removed and replaced with 10 ml fresh, supplemented medium. On day 8, the nonadherent cells were collected, centrifuged, resuspended at 2 × 10^6/ml in complete medium containing 1 μM peptide, and cultured for another 24 h. Then the pulsed dendritic cells (DC) were washed, resuspended in neart DMEM, and injected i.v. into mice (5 × 10^6 cells/mouse in 200 μl). The mice were challenged with *Y. pestis* strain D27 14 d later.

**Immunizations using cholera toxin adjuvant**

Mice were lightly anesthetized by isoflurane and immunized intranasally with a 15 μl saline solution containing 1 or 10 μg peptide and 1 μg cholera toxin (CT; List Biological Laboratory, Campbell, CA). Mice were immunized on days 0, 7, and 21, and challenged with *Y. pestis* strain D27 on day 37 or day 56.

**Challenge infections**

Mice were lightly anesthetized by isoflurane and infected intranasally with 20 or 200 median lethal doses (MLD) *Y. pestis* strain D27 in 30 μl saline. The intranasal MLD of *Y. pestis* strain D27 is ∼1 × 10^4 CFU when the bacteria are grown and administered, as described above. For all survival studies, recipient mice were considered moribund and euthanized. For measurement of bacterial burden, mice were euthanized at the indicated day postinfection. Liver and lung tissues were harvested and plated for CFU determination, as described previously (19).

**CD8 T cell depletion**

CD8 T cells were depleted by treating mice with 1 mg rat IgG2b mAb specific for mouse CD8 (clone 2.43). The mAb was diluted in saline and administered as two i.p. doses of 500 μg each on the day before and the day after challenge. Control mice received an equal quantity of isotype-matched rat IgG2b mAb (clone LTF-2). All mAbs were supplied by Bio X Cell (West Lebanon, NH).

**Statistics**

Statistical analyses were performed using the program Prism 4.0 (GraphPad Software). Survival data were analyzed by log-rank tests, and cell numbers/percentages were analyzed by Student's t test. Bacterial burden was analyzed by nonparametric test (Kruskal–Wallis); CFU less than the detection limit were assigned values 0.2 log below the detection limit.

**Results**

**YopE is a dominant Ag recognized by the *Y. pestis*-specific CD8 T cells**

To facilitate the identification of *Y. pestis* Ags recognized by protective T cells, we generated T cell clones from C57BL/6 mice that were immunized with live attenuated *Y. pestis* strain D27-PLpxL and then challenged 2 and 3 mo later with virulent *Y. pestis* strain D27. After another month, the T cells from surviving mice were expanded in vitro using splenocytes as APC and heat-killed *Y. pestis* strain D27 as a source of Ag. The resulting T cell line was cloned by limiting dilution.

To assess the Ag specificity of the T cell clones, we measured their response to various *Y. pestis* strains. The strains included D27, a ppg-deficient member of biovar Medivialis; D28, a derivative of D27 that lacks the pCD1 virulence plasmid; and CO92 pgm−, a ppg-deficient member of biovar Orientalis. All of the CD8 T cell clones produced IFN-γ and proliferated in response to strains D27 and CO92 pgm−, but not to strain D28, suggesting that they recognized a pCD1-dependent Ag (Fig. 1A). Because temperature regulates the expression of many *Y. pestis* genes (20), we also grew each strain at either 26°C or 37°C before assaying T cell responses. The CD8 T cell clones only produced IFN-γ and proliferated in response to *Y. pestis* bacilli grown at 37°C (Fig. 1A). We also observed that supplementing cultures with Abs reactive with mouse MHC class I H-2K^b^, but not H-2D^b^, suppressed the response to strain D27 (data not shown). Together, these findings indicated that the CD8 T cell clones recognize a temperature-regulated, H-2-K^b^-restricted Ag encoded by, or dependent upon, the pCD1 plasmid.

To identify the protein Ag, we measured the response of the CD8 T cell clones to individual pCD1-encoded proteins expressed in *E. coli*.
coli. We focused initially on proteins whose expression is known to be up-regulated when *Y. pestis* bacilli are grown at 37°C (20). As shown in Fig. 1B, the CD8 T cell clones responded specifically to *E. coli* expressing *Y. pestis* YopE. To decisively demonstrate that the clones recognized YopE and to identify the specific epitope(s) recognized by the clones, we synthesized overlapping peptides encompassing the entire sequence of the *Y. pestis* YopE protein. Each peptide was 15 aa in length and overlapped its neighboring peptides by 10 aa. The CD8 T cell clones all responded specifically to YopE<sub>66–80</sub> (VAVHSVIGFIQRMFSE) (Fig. 1C). We used computer algorithms to predict peptides within YopE<sub>66–80</sub> that were likely to bind H2-K<sup>e</sup>. As shown in Fig. 1D, the CD8 T cell clones responded to YopE<sub>69–77</sub> (SVIGFIQR), but not YopE<sub>96–97</sub> (SVIGFIQR), and the response to YopE<sub>96–97</sub> remained strong at very low concentrations (10 nM). Notably, the CD8 T cell clones did not recognize YopE<sub>66–80</sub> of *Yersinia enterocolitica* strain WA:08 (MARSAIEFIKRMFSE), which contains multiple sequence substitutions between aa 69 and 77 (Fig. 1C). Together, these results established that the CD8 T cell clones specifically recognized YopE<sub>96–97</sub>.

To identify and quantify YopE-specific CD8 T cells in vivo, we generated a MHC class I H2-K<sup>e</sup> tetramer containing the *Y. pestis* YopE<sub>96–97</sub> peptide (K<sub>b</sub>YopE<sub>96–97</sub>). Whereas this tetramer stained very few cells in naive mice, we detected a small, but readily measurable increase in the percentage of tetramer-positive CD8 T cells in the lungs, spleens, and peripheral blood of mice that had been immunized 3 mo earlier with attenuated *Y. pestis* strain D27-pLpxL (Fig. 2A). Moreover, the percentage and number of tetramer-positive CD8 T cells in the lung increased dramatically when the immunized mice were challenged intranasally with virulent strain D27. By day 4 after challenge, nearly 20% of pulmonary CD8 T cells stained positive for the tetramer (Fig. 2A).

The specificity of CD8 T cells for YopE<sub>96–97</sub> was further confirmed by intracellular cytokine staining. After challenging the immunized mice with virulent *Y. pestis*, 11% of CD8 cells in the lung could be induced to produce TNF-α and IFN-γ upon ex vivo stimulation with anti-CD3 mAb, a polyclonal stimulus for all effector T cells. Remarkably, nearly the same percentage of CD8 cells could be induced to produce these cytokines by culture with YopE<sub>96–97</sub>, whereas few CD8 T cells responded to a control peptide, OVA<sub>257–264</sub> (Fig. 2B). CD4 T cells did not respond to YopE<sub>96–97</sub>, further confirming the specificity of the CD8 T cell response to YopE. Together, these data indicate that YopE<sub>96–97</sub> is a dominant Ag recognized by CD8 T cells in this C57BL/6 mouse model of pneumonic plague.

**Immunization with YopE<sub>96–97</sub> primes CD8 T cells that confer protection against pulmonary *Y. pestis* infection**

To assess whether YopE-specific CD8 T cells could suffice to protect against *Y. pestis* infection, we vaccinated mice with the YopE<sub>96–97</sub> epitope. Initially, we employed a vaccination strategy in which bone marrow-derived DC were pulsed with peptide and injected i.v. into mice. Specifically, C57BL/6 mice were vaccinated with DC that had been pulsed with either YopE<sub>96–97</sub> or OVA<sub>257–264</sub>. Two weeks later, we confirmed that the DC vaccination primed peptide-specific T cells by measuring the frequency of cells in lung, spleen, and peripheral blood that stained with MHC class I tetramers. After vaccination with OVA<sub>257–264</sub>-pulsed DC, <1% of the lung CD8 T cells stained positive for the K<sub>b</sub>YopE<sub>96–97</sub> tetramer (Fig. 3A). In contrast, >20% of lung CD8 T cells stained with K<sub>b</sub>YopE<sub>96–97</sub> tetramer after vaccination with YopE<sub>96–97</sub>-pulsed DC. Vaccination with YopE<sub>96–97</sub>-pulsed DC also increased the frequency of YopE<sub>96–97</sub>-specific CD8 T cells significantly in the spleen and peripheral blood, and increased the absolute number of these cells >1000-fold in the lung (Fig. 3A). In parallel with the tetramer analysis, additional cohorts of vaccinated mice were challenged intranasally with 20 MLD of *Y. pestis* strain D27. In comparison with mice vaccinated with OVA<sub>257–264</sub>, mice vaccinated with YopE<sub>96–97</sub> displayed modest, but significant protection (p < 0.0001), as evidenced by both a

**FIGURE 2.** Detection of YopE<sub>96–97</sub>-specific CD8 T cells in vaccinated mice challenged with *Y. pestis*. C57BL/6 mice were immunized twice with attenuated *Y. pestis* strain D27-pLpxL and then challenged intranasally with 200 MLD virulent *Y. pestis* strain D27 3 mo after the last immunization. At day 4 after challenge, cells were analyzed by flow cytometry. A, Lung cells, peripheral blood leukocytes (PBL), and splenocytes were stained immediately with Abs to CD4 and CD8 and a MHC class I H2-K<sup>e</sup> tetramer loaded with YopE<sub>96–97</sub> (K<sub>b</sub>YopE<sub>96–97</sub>). Plots are representative of five individual mice for each condition. The percentages of lymphocytes staining positive for CD8 and tetramer are shown, with parentheses depicting the percentage of tetramer-positive cells within the CD8 T cell population. YopE tetramer-positive CD8 T cells were detectable in the lungs, blood, and spleens of vaccinated mice, and their frequency increased dramatically in the lung after *Y. pestis* challenge. B, Lung cells were stimulated ex vivo with plate-bound anti-CD3 mAb, *Y. pestis* YopE<sub>96–97</sub> peptide, or control peptide OVA<sub>257–264</sub> and then stained for intracellular cytokines. Data depict results from one representative mouse that was vaccinated and challenged. The numbers depict the percentage of cells in the indicated quadrant. The YopE<sub>96–97</sub> peptide specifically stimulated CD8 T cells to produce TNF-α and IFN-γ.
D27, the YopE 69–77-immunized mice demonstrated remarkably significantly increased the frequency and number of CD8 T cells specific for Y. pestis challenge. C57BL/6 mice were immunized i.v. with bone marrow-derived DC loaded with YopE69–77 or control OVA257–264 peptides. On day 14 after DC transfer, lung cells were stained with Abs to CD4 and CD8 and MHC class I tetramers KbYopE69–77. Left panel shows the percentages of lymphocytes staining positive for CD8 and KbYopE69–77 tetramer in lung (left axis), spleen (right axis), or PBL (right axis); right panel shows the numbers of these cells in lung and spleen at day 14 after DC transfer. The data depict the mean and SD of five mice per group. Vaccination significantly increased the frequency and number of CD8 T cells specific for YopE69–77 (*p < 0.0001). B, At day 14 after DC transfer, mice were challenged intranasally with 20 MLD Y. pestis strain D27. In comparison with mice immunized with DC loaded with OVA257–264 peptide, mice that received DC loaded with YopE69–77 peptide displayed significantly improved survival (p < 0.0001 by log-rank test; n = 37 mice/group; data pooled from three independent experiments).

FIGURE 3. Vaccination with DC loaded with YopE69–77 peptide primes CD8 T cells and protects against lethal pulmonary Y. pestis challenge. C57BL/6 mice were immunized i.v. with bone marrow-derived DC loaded with YopE69–77 or control OVA257–264 peptides. A, On day 14 after DC transfer, lung cells were stained with Abs to CD4 and CD8 and MHC class I tetramers KbYopE69–77. Left panel shows the percentages of lymphocytes staining positive for CD8 and KbYopE69–77 tetramer in lung (left axis), spleen (right axis), or PBL (right axis); right panel shows the numbers of these cells in lung and spleen at day 14 after DC transfer. The data depict the mean and SD of five mice per group. Vaccination significantly increased the frequency and number of CD8 T cells specific for YopE69–77 (*p < 0.0001). B, At day 14 after DC transfer, mice were challenged intranasally with 20 MLD Y. pestis strain D27. In comparison with mice immunized with DC loaded with OVA257–264 peptide, mice that received DC loaded with YopE69–77 peptide displayed significantly improved survival (p < 0.0001 by log-rank test; n = 37 mice/group; data pooled from three independent experiments).

2-d prolongation in median survival time and an increase in the overall survival rate to 25% (Fig. 3B).

To investigate whether the protection conferred by immunization with YopE69–77 could be improved further, we immunized mice intranasally with 10 μg YopE69–77 and CT, a mucosal adjuvant known to induce peptide-specific CD8 T cell responses (21). Control mice received CT adjuvant alone or CT and OVA257–264. Two weeks after the last of three immunizations, >50% of the lung CD8 T cells stained specifically with the KbYopE69–77 tetramer (Fig. 4A). The absolute number of YopE69–77-specific CD8 T cells in the lung increased >1000-fold after immunization (Fig. 4A). When challenged intranasally with 20 MLD Y. pestis strain D27, the YopE69–77-immunized mice demonstrated remarkably improved protection (p < 0.0001), with 83% surviving the lethal challenge (Fig. 4B). Parallel cohorts of mice vaccinated with 10-fold less YopE69–77 (i.e., 1 μg) showed fewer tetramer-positive cells, but still displayed robust protection (75%, Fig. 4B). When mice were challenged 35 d after the last immunization, lower percentages and numbers of YopE-specific CD8 T cells were measured (Fig. 4C), but similar levels of protection were observed (Fig. 4D), suggesting that a memory response to YopE69–77 can suffice to protect against Y. pestis infection.

The YopE-specific T cells in the mice immunized 35 d earlier with YopE69–77 and CT possessed a memory/effector phenotype; almost all of the YopE-specific CD8 T cells in the lung and spleen were CD44highCD62Llow and many of them also expressed CD43 (data not shown). In the spleen, very few of the YopE-specific cells were CD44highCD62Llow and many of them also expressed CD43

FIGURE 4. Intranasal immunization with YopE69–77 confers protection against lethal pulmonary Y. pestis challenge. On days 0, 7, and 21, C57BL/6 mice were immunized intranasally with either 1 μg (●) or 10 μg (●) YopE69–77 mixed with CT adjuvant. Control mice received CT alone (△), 1 μg (○), or 10 μg (□) OVA257–264 mixed with CT. Mice were either harvested without challenge (A, C) or challenged with Y. pestis (B, D) at day 37 (A, B) or day 56 (C, D). A, Left panel shows the percentages of lymphocytes staining positive for CD8 and KbYopE69–77 tetramer in lung (left axis), spleen (right axis), or PBL (right axis); right panel shows the numbers of these cells in lung and spleen at day 37. The data depict the mean and SD of six to eight mice per group. In comparison with CT alone, both doses of YopE69–77 significantly primed tetramer-positive cells (hp < 0.01, *p < 0.0001). B, On day 37, parallel cohorts of mice were challenged intranasally with 20 MLD Y. pestis strain D27. In comparison with mice immunized with CT alone or CT and OVA257–264, mice immunized with CT and YopE69–77 displayed significantly increased survival (p < 0.0001 for both 10 and 1 μg by log-rank test; n = 30 mice for CT alone and YopE69–77 groups; n = 20 for OVA257–264 groups). Data are pooled from three independent experiments. C, Left panel shows the percentages of lymphocytes staining positive for CD8 and KbYopE69–77 tetramer in lung (left axis), spleen (right axis), or PBL (right axis); right panel shows the numbers of these cells in lung and spleen at day 56. The data depict the mean and SD of 10 mice per group. D, On day 56, parallel cohorts of mice were challenged intranasally with 20 MLD Y. pestis strain D27. In comparison with mice immunized with CT alone or CT and OVA257–264, mice immunized with CT and YopE69–77 displayed significantly increased survival (p < 0.0001 by log-rank test; n = 30 mice for YopE69–77 group; n = 10 for CT alone and OVA257–264 groups). Data are pooled from two independent experiments.
expressed the classical early activation marker CD69, consistent with a resting memory phenotype. Interestingly, more than half of the YopE-specific cells in the lung expressed CD69 (data not shown), consistent with prior observations that lung airway memory CD8 T cells possess a highly activated phenotype (CD44highCD62LlowCD43+CD69+) long after viral clearance (22, 23).

Measurements of bacterial CFU revealed that YopE69–77 immunization helps mice control bacterial burden. Mice immunized with CT and YopE69–77 exhibited significantly reduced numbers of bacterial CFU in both lung and liver tissues at days 3, 4, and 5 after Y. pestis challenge, as compared with control mice immunized with CT alone or CT and OVA257–264 (Fig. 5, p < 0.05 for days 3 and 5, and p < 0.01 for day 4). The burden had fallen below the detection limit of our assay in most of the mice immunized with YopE69–77 by day 7 after challenge (8 of 10 mice for lung; 6 of 10 mice for liver), whereas the control mice had succumbed to infection by that time.

Depleting CD8 T cells at the time of challenge fully abrogated the protective effect of immunization (Fig. 6A), further confirming that CD8 T cells mediated the protection. The protection conferred by immunization with YopE69–77 did not require Ab, as it was also evident in B cell-deficient μMT mice (Fig. 6B). Taken together, these findings demonstrate that YopE is not only a dominant CD8 T cell epitope of natural Y. pestis infection, but also a protective Ag in the C57BL/6 mouse model of pneumonic plague.

**FIGURE 5.** Intranasal immunization with YopE69–77 decreases bacterial burden after pulmonary Y. pestis challenge. On days 0, 7, and 21, C57BL/6 mice were challenged intranasally with 10 plaque-forming units YopE69–77 mixed with CT adjuvant (■). Control mice received CT alone (△) or 1 μg OVA257–264 (○) mixed with CT. On day 37, mice were challenged intranasally with 20 MLD Y. pestis strain D27. A. At the time of challenge, the wild-type mice that were immunized with CT and 10 μg YopE69–77 were subdivided into two groups. One group of mice was treated with depleting mAb specific for CD8 (aCD8, ▴), and the other was treated with an isotype-matched mAb (ctrl Ig, ■). Mice treated with mAb specific for CD8 displayed significantly reduced survival compared with mice treated with control mAb (p < 0.0001 by log-rank test; n = 20 mice/group). Data are pooled from two independent experiments. B. In comparison with μMT mice immunized with CT alone, μMT mice immunized with CT and YopE69–77 displayed significantly increased survival (p < 0.0001 for both 10 and 1 μg by log-rank test; n = 20 mice/group). Data are pooled from two independent experiments.

**FIGURE 6.** Protection conferred by intranasal immunization with YopE69–77 is mediated by CD8 T cells. On days 0, 7, and 21, C57BL/6 wild-type (A) or μMT (B) mice were immunized intranasally with either 10 μg (●) or 1 μg (▲) YopE69–77 mixed with CT adjuvant. Control mice received CT alone (△) or 10 μg OVA257–264 mixed with CT (○). On day 37, mice were challenged intranasally with 20 MLD Y. pestis strain D27. A. Wild-type mice that were immunized with YopE69–77 were subdivided into two groups. One group of mice was treated with depleting mAb specific for CD8 (aCD8, ▴), and the other was treated with an isotype-matched mAb (ctrl Ig, ■). Mice treated with mAb specific for CD8 displayed significantly reduced survival compared with mice treated with control mAb (p < 0.0001 by log-rank test; n = 20 mice/group). Data are pooled from two independent experiments. B. In comparison with μMT mice immunized with CT alone, μMT mice immunized with CT and YopE69–77 displayed significantly increased survival (p < 0.0001 for both 10 and 1 μg by log-rank test; n = 20 mice/group). Data are pooled from two independent experiments.

**Discussion**

Y. pestis, *Yersinia pseudotuberculosis*, and *Y. enterocolitica*, the three *Yersinia* species that cause human disease, all descend from a common ancestor (8). The sequence of YopE is identical in most strains of *Y. pestis* and *Y. pseudotuberculosis*. Although the YopE69–77 sequence is altered in the 0:8 serotype of *Y. enterocolitica*, it is conserved in many other *Y. enterocolitica* strains. Thus, we anticipate that *Y. pestis* YopE69–77 will be a dominant and protective CD8 T cell epitope in many H2-Kb mouse models of disease caused by pathogenic *Yersinia* spp.

YopE was never previously shown to serve as a protective Ag for any *Yersinia* spp. One prior study of mutant *Y. pseudotuberculosis* strains suggested that YopE may be a target of rat CD8 T cells (24). However, subsequent mouse studies reported that immunization with YopE does not confer significant protection from plague (25, 26). Moreover, an ELISPOT-based screen using recombinant proteins did not identify YopE as an Ag recognized by *Yersinia* T cells in BALB/c mice immunized with attenuated *Y. pestis* EV76 (27). The methods used in these prior studies differed significantly from those reported in this work, which focused specifically on Ags recognized by CD8 T cells and immunization strategies known to prime CD8 T cells. Further studies are required to determine whether *Yersinia* YopE is a dominant Ag recognized by CD8 T cells in all mammals, or only subsets thereof.

The extent to which the biology of *Y. pestis* YopE accounts for its immunodominance in the C57BL/6 mouse model of plague is
presently unclear. *Yersinia* spp. use a plasmid-encoded type III secretion system to translocate YopE into the cytosol of eukaryotic cells (28), where it functions as a GTPase-activating protein (29). A series of studies have established that attenuated strains of *Yersinia* and *Salmonella* can translocate recombinant YopE-fusion proteins, thereby priming CD8 T cell responses to heterologous Ags (30–33). Although these prior studies used YopE as a molecular carrier to deliver heterologous Ags and did not report that YopE itself was an Ag, they suggest that translocation facilitates the presentation of Ags to CD8 T cells. However, our data indicate that heat-killed *Y. pestis* bacilli, which presumably lack the capacity to translocate YopE, also effectively deliver YopE_{275–77} to APC (Fig. 1). In addition, several epitope prediction algorithms predicted that many peptides encoded by other translocated Yops should bind H2-Kb very much more efficiently than YopE_{275–77}. Indeed, initially we attempted, but failed, to identify the CD8 T cell Ag by screening 90 high-scoring pCD1-encoded peptides predicted by the BIMAS and SYFPEITHI algorithms. Clearly, further studies are required to understand why YopE_{275–77} dominates the CD8 T cell response to plague.

Wang et al. (34) recently identified epitopes recognized by CD8 T cells in BALB/c mice immunized with a DNA vaccine encoding LcrV, a leading vaccine candidate. They also demonstrated that CD8 T cells may play a role in protection elicited by LcrV-based vaccines. However, Wang et al. did not investigate whether LcrV is a dominant T cell Ag during *Y. pestis* infection. We know remarkably little about the function of T cells during acute lethal bacterial pneumonia. Given that bacterial infections of the lung are a leading cause of death worldwide, and given growing concerns about antibiotic-resistant and weaponized bacteria, further studies of the mouse model of pneumonic plague will lead to improved vaccines and immunotherapeutics not only against *Y. pestis*, but also for the many other bacterial pathogens that infect the lung.

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**Disclosures**

The authors have no financial conflicts of interest.

**References**


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