Vaccination with a Single CD4 T Cell Peptide Epitope from a *Salmonella* Type III-Secreted Effector Protein Provides Protection against Lethal Infection

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Salmonella infections affect millions worldwide and remain a significant cause of morbidity and mortality. It is known from mouse studies that CD4 T cells are essential mediators of immunity against *Salmonella* infection, yet it is not clear whether targeting CD4 T cell responses directly with peptide vaccines against *Salmonella* can be effective in combating infection. Additionally, it is not known whether T cell responses elicited against *Salmonella* secreted effector proteins can provide protective immunity against infection. In this study, we investigated both of these possibilities using prime-boost immunization of susceptible mice with a single CD4 T cell peptide epitope from *Salmonella* secreted effector protein I (SseI), a component of the *Salmonella* type III secretion system. This immunization conferred significant protection against lethal oral infection, equivalent to that conferred by whole heat-killed *Salmonella* bacteria. Surprisingly, a well-characterized T cell epitope from the flagellar protein FliC afforded no protection compared to immunization with an irrelevant control peptide. The protective response appeared to be most associated with polyfunctional CD4 T cells raised against the SseI peptide, since no antibodies were produced against any of the peptides and very little CD8 T cell response was observed. Overall, this study demonstrates that eliciting CD4 T cell responses against components of the *Salmonella* type III secretion system can contribute to protection against infection and should be considered in the design of future *Salmonella* subunit vaccines.

Typhoid fever, caused by the facultatively intracellular bacterium *Salmonella enterica* serovar Typhi, remains a global health threat, affecting more than 20 million people worldwide annually (1–3). Increasingly, the salmonellae that cause these infections are developing resistance to first-line antibiotic therapies (4, 5). Since the current vaccines against *Salmonella* Typhi are only 50 to 80% effective and confer limited immunity (6–8), there is a real need to develop new vaccines against this pathogen. Additionally, invasive nontyphoidal *Salmonella* (iNTS) infections are increasingly common, especially among African children, and to date there are no vaccines for these infections. This may be due to the fact that the typhoidal *Salmonella* serovars (*S. Typhi* and *Salmonella enterica* serovar Paratyphi) can differ antigenically from those causing iNTS (*Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis) (9–11). One consideration that should be taken into account in designing vaccines for iNTS is that many of these types of infections arise in immunocompromised or individuals coinfecting with malaria or HIV (12, 13). The reduced CD4 T cell counts of HIV-infected patients are known to increase the incidence of *Salmonella* bacteremia (14, 15); however, it is also known that HIV-infected people mount a dysregulated humoral immune response against iNTS that prevents bacterial clearance (16). Careful consideration of the role of humoral and cell-mediated immunity is therefore important for the design of vaccines against iNTS, although it is clear that directing a CD4 T cell response against bacterial antigens will be essential.

Ideally, any vaccine against *Salmonella* infections would induce potent CD4 T cell effector responses, because *Salmonella* immunity is primarily CD4 T cell mediated (17–20). Notably, a recent study showed that the predominant protective responses against *Salmonella* infection are mediated by surface-associated antigens; however, many of the most common proteins from the *Salmonella*

type III secretion systems (T3SS), located on *Salmonella* pathogenicity island 1 (SPI1) and SPI2, have not been tested for protection (21). Many of these effector proteins are injected directly into the cytosol from within the *Salmonella*-containing vacuole (SCV) (22). It is also clear that cytosolic proteins can be shuttled into the major histocompatibility complex class II (MHC-II) pathway (23, 24). This leads to the potential outcome that peptides from these effector proteins can trigger a memory CD4 T cell response in immunized individuals. Evidence for this comes from our previous work showing a potent CD4 T cell response against peptides derived from *Salmonella* secreted effector proteins I and J (SseI and SseJ) during active infection (25).

While the development of peptide vaccines directed against infectious pathogens is unusual, there are precedents, although so far they have mostly been shown to be effective against viral pathogens. For example, a single synthetic peptide vaccine has been shown to protect swine from foot-and-mouth disease (26). Notably, that vaccine consists of a peptide that contains both T and B cell epitopes, and it is clear that neutralizing antibodies are made in response to immunization. In another instance, more pertinent to the current study, mucosal immunization with a single distinct T-helper epitope from the rotavirus Vp6 protein protects both mice and nonhuman primates against infection (27, 28). In those

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studies, B cells and CD8 T cells are dispensable, demonstrating that protection is mediated by CD4 T cells (29). While peptide vaccines such as these have been demonstrated to be effective in combating viral pathogens, the case for such vaccines directed against bacterial pathogens is less well defined. In one example, immunization with CD4 T cell epitopes from the 6-kDa early secretory antigenic target (ESAT-6) protein (30) or antigen 85 (31) of *Mycobacterium tuberculosis* induces a modest but significant decrease in bacterial burdens in the tissues of immunized individuals in a mouse model of tuberculosis infection. Importantly, multicytokine-producing memory CD4 T cells are induced by the antigen 85 vaccine (31), a finding that is correlated with protection against infection (32).

Based on the facts that a peptide vaccine that activates effector CD4 T cells during infection may be protective (27, 28) and that at least some *Salmonella* T3SS effector proteins are capable of eliciting robust CD4 T cell responses (21), we hypothesized that immunization with a single T cell epitope derived from *Salmonella* secreted effector I (SseI; also known as SrfH), a T3SS effector protein, could provide protection against lethal *Salmonella* infection via the induction of a potent effector T cell response specific for that epitope. We evaluated this hypothesis by comparing the protective capacity of a well-characterized *Salmonella* CD4 T cell epitope derived from a flagellar protein (FliC) with that of the epitope from the SseI protein, which we have described previously (25). We also investigated the immune responses elicited by this vaccination and the likely contributions of different aspects of the adaptive immune response to protection against infection.

MATERIALS AND METHODS

Ethics statement. All experimental procedures involving animals were approved and performed in compliance with the guidelines established by Tulane University Health Sciences Center's Institutional Animal Care and Use Committee.

Bacterial strains and peptides. Strain SL1344, a virulent strain of *Salmonella* Typhimurium, and strain SL3261, an avirulent Δ aroA vaccine strain, were generous gifts from Marc Jenkins (University of Minnesota, Minneapolis, MN). The SseI_{268–280} (LIYYTDFSNSSIA), FliC_{427–441} (VQNRFSNAITNLGNT), and OVA_{323–339} (ISQAVHAAHAEINEAGR) peptides were synthesized to $\geq 98\%$ purity by GenScript (Piscataway, NJ).

Mice and immunizations. C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and were maintained under specific-pathogen-free conditions. Mice (5 to 10 per group) were immunized subcutaneously at the age of 6 to 8 weeks with 200 μ g of a peptide emulsified in complete Freund's adjuvant (CFA) and were then boosted 21 days later with 200 μ g of the peptide emulsified in incomplete Freund's adjuvant (IFA). Mice were assayed or challenged 14 days after the last immunization. Vaccine control mice (5 to 10 per group) were immunized subcutaneously with 1×10^7 CFU of whole heat-killed *S. Typhimurium* strain SL1344 (emulsified in either CFA or IFA) as described above. Additional control mice were infected by oral gavage with 5×10^7 CFU of live attenuated *S. Typhimurium* strain SL3261 by oral gavage 6 weeks before challenge with the virulent strain SL1344. All mice were challenged with 1×10^5 CFU of the virulent *S. Typhimurium* strain SL1344 and were euthanized if they lost $\geq 20\%$ of their prechallenge weight or displayed signs of terminal illness (such as being hunched or lethargic, or lacking grooming). Survival curves were generated, and statistical analyses were performed using a log rank (Mantel-Cox) test.

Bacterial burdens. Tissue bacterial burdens were assessed in moribund animals after euthanasia and in survivors at the study endpoint. Organs were first weighed, then homogenized in 0.1% Triton X-100, and finally plated on LB agar containing 100 μ g/ml streptomycin for enumeration of viable CFU. The data were expressed as mean CFU per gram of

tissue. Outliers were determined using the ROUT test (confidence level, 99.5%), and statistical analysis was performed using the Student *t* test.

Antigen-specific ELISAs. A group of C57BL/6J mice was used for immune correlates (5 mice per group). Mice were bled 5 days prior to the first immunization (day –5), 5 days prior to the boost (day 16), and on the day of challenge (day 35) for the determination of serum antibody responses. Blood was drawn by submandibular vein puncture and was collected in BD Microtainer tubes with a serum separator additive (BD, Franklin Lakes, NJ). Serum was stored at -80°C until use. Peptide-specific serum IgM and IgG were analyzed by an enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates were coated with 0.5 μ g per well of a purified SseI, FliC, or ovalbumin (OVA) peptide in coating buffer (0.1 M sodium bicarbonate, 0.2 M sodium carbonate) and were incubated overnight at 4°C . The plates were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween 20 (PBST). Plates were incubated with 2-fold serial dilutions of serum samples for 2 h at room temperature. Plates were washed three times with PBST and were then incubated with alkaline phosphatase (AP)-conjugated rat anti-mouse IgM or IgG (1:300 dilution in PBST) (BD Pharmingen) for 1 h at room temperature. At the end of the incubation, the plates were washed three times with PBST and were developed with SigmaFast *p*-nitrophenyl phosphate tablets (Sigma, St. Louis, MO) dissolved in diethanolamine buffer (1 mg/ml). After 15 to 30 min of incubation, reactions were stopped with 2 M NaOH, and results were read at 405 nm using a μ Quant microplate reader and were analyzed with Gen5 software (BioTek, Winooski, VT). The results obtained were expressed as the mean reciprocal endpoint titers for total IgM and IgG. The endpoint titer was defined as the highest dilution that yielded an optical density at 405 nm (OD_{405}) more than 3 standard deviations above the mean OD_{405} for preimmune sera. For the antibody OD values, statistics were analyzed using two-way analysis of variance (ANOVA) with a Tukey multiple-comparison test, comparing the mean OD values from each vaccine group to each antigen used.

Enzyme-linked immunosorbent spot (ELISPOT) assays. A group of C57BL/6J mice was used for the analysis of T cell responses (4 mice per group). Spleens were harvested from mice 14 days after the last immunization. CD4 or CD8 T cells were purified by negative bead selection using either the CD4 or the CD8 T Cell Isolation Kit II according to the manufacturer's directions (Miltenyi Biotec, Auburn, CA). Millipore Multi-Screen-IP plates (Millipore, Billerica, MA) were coated overnight with 1 μ g/well of a purified anti-mouse gamma interferon (IFN- γ) IgG capture antibody (clone AN-18; eBioscience, San Diego, CA). Wells were blocked for 2 h with complete RPMI medium containing 10% fetal bovine serum. A total of 2.5×10^5 isolated T cells were cultured in complete RPMI medium 1:1 with naïve irradiated splenocytes for 36 h at 37°C with 1 μ g/well of each respective peptide. IFN- γ secretion was detected by using a biotin-conjugated anti-mouse IFN- γ IgG at 0.1 μ g/well (clone R4-6A2; eBioscience, San Diego, CA) incubated for 2 h, with subsequent incubation with avidin-horseradish peroxidase (HRP) at 1:1,000 (eBioscience). Freshly prepared AEC (3-amino-9-ethyl-carbazole) substrate (Sigma-Aldrich, St. Louis, MO) was added to each well. The enzymatic reaction was stopped after approximately 20 min, when visible spots appeared. Plates were allowed to dry and were analyzed with a CTL ImmunoSpot S6 Macro Analyzer (Cellular Technology, Ltd., Shaker Heights, OH). The results obtained were expressed as mean $\log_{10}$ IFN- γ -secreting cells per 1 million T cells, and images of representative wells were displayed. Statistics were analyzed using two-way ANOVA with a Dunnett multiple-comparison test, comparing each restimulation to the OVA control within each group.

In vitro intracellular cytokine staining. A separate group of vaccinated C57BL/6J mice was used for analysis of T cell responses by flow cytometry (3 mice per group). Spleens from vaccinated mice were harvested and single-cell preparations obtained. The cells were stimulated *in vitro* with 20 μ g/ml of peptides and 2 μ g/ml anti-CD28. Control samples were stimulated either with heat-killed *S. Typhimurium* SL1344 (multiplicity of infection [MOI], 10:1) and 2 μ g/ml anti-CD28 or with 2 μ g/ml concanavalin A (Sigma-Aldrich, St. Louis, MO) alone in complete RPMI

FIG 1 SseI peptide protects mice from lethal challenge. (A) Mice vaccinated with the SseI epitope showed a significantly higher percentage of survival ($P, 0.0003$) than mice vaccinated with an irrelevant peptide (OVA). (B) SseI-vaccinated mice showed levels of protection similar to those of mice vaccinated with whole heat-killed *S. Typhimurium*. In both panels A and B, the data represent two independent pooled experiments with 8 to 20 mice per group. The statistical significance of differences between survival curves was tested using the log rank (Mantel-Cox) test. (C and D) Bacterial burdens in spleens (C) and livers (D) were determined. Data are mean CFU per gram $\pm$ standard errors of the means. *, $P < 0.05$; ns, $P > 0.05$ (t test).

medium at 37°C. After 2 h of stimulation, 10 μ g/ml brefeldin A (Sigma-Aldrich) was added to prevent cytokine export, and the cells were further stimulated for an additional 4 h. The cells were washed and were blocked with Fc Block (clone 2.4G2). The cells were subsequently stained with antibodies against mouse CD19, F4/80, CD11c, CD11b (conjugated with eFluor 450), CD3e (conjugated with fluorescein isothiocyanate [FITC]), CD4 (conjugated with Brilliant Violet 605 [BV605]), CD8a (conjugated with BV510), and CD44 (conjugated with peridinin chlorophyll protein [PerCP]-Cy5.5). The cells were fixed and permeabilized by using the BD Cytofix/Cytoperm kit according to the manufacturer's instructions and were then stained overnight for IFN- γ (with phycoerythrin [PE]), interleukin 2 (IL-2) (with allophycocyanin [APC]), and tumor necrosis factor alpha (TNF- α) (with PE-Cy7). All antibodies were purchased from either BioLegend or eBioscience. Samples were washed and resuspended in sorter buffer for fluorescence-activated cell sorter (FACS) analysis. All samples were analyzed on a BD Fortessa cell analyzer equipped with 4 lasers (405 nm, 488 nm, 561 nm, and 640 nm). Data are expressed as the mean percentages of CD44^{hi} T cells producing single, double, or triple cytokines, as determined by Boolean gating using FlowJo software (Tree-Star, Ashland, OR). Statistics were analyzed using two-way ANOVA with a Dunnett multiple-comparison test, comparing each group of cytokine producers to the OVA control.

Ex vivo intracellular cytokine staining. A group of vaccinated C57BL/6J mice (2 mice per group) was analyzed for *in vivo* cytokine production by CD4 T cells. Mice were stimulated *in vivo* for 2 h via intravenous (i.v.) injection of 100 μ g of a peptide by the retro-orbital route. Spleens were harvested, and single-cell suspensions were made in the presence of 10 μ g/ml brefeldin A to prevent cytokine export. The cells were then prepared for flow cytometry as described above. Statistics were

analyzed using two-way ANOVA with a Dunnett multiple-comparison test, comparing each group of cytokine producers to the OVA control within each group.

RESULTS

A single MHC-II epitope from a secreted effector protein provides protection against *Salmonella* challenge. Only four I-A^b epitopes within *Salmonella* Typhimurium have been identified to date (25, 33, 34). While protective responses may be generated against whole protein (33), the protective efficacy of these peptide epitopes has never been demonstrated. Additionally, it has been speculated that protection against *Salmonella* Typhimurium relies on surface-associated proteins (21). We set out to determine if *Salmonella* Typhimurium MHC-II epitopes, and particularly an epitope from a type III-secreted effector protein, were sufficient to provide protection to susceptible C57BL/6J mice upon virulent *Salmonella* challenge. Mice were immunized with MHC-II epitopes against either flagellin (FliC_{427–441}) or the SPI2 T3SS secreted effector protein SseI (SseI_{268–280}). Mice immunized with the SseI peptide survived significantly longer, 2 weeks after challenge, than control mice immunized with an irrelevant peptide (median survival, 13 days postinfection compared to 8.5 days postinfection) (Fig. 1A). Surprisingly, mice immunized with the flagellar FliC peptide showed no significant increase in survival (median survival, 10 days postinfection) (Fig. 1A), although protection has been shown when mice are immunized with whole natural FliC protein (33). We next wanted to compare the single

FIG 2 Vaccination with *Salmonella*-specific peptides does not generate peptide-specific antibodies. Mice vaccinated with either SseI or FliC were screened for the production of peptide-specific IgM (A) or IgG (B) responses. The data represent the mean OD₄₀₅ values and individual replicates for each specific coating antigen. Dotted lines indicate the mean preimmune OD values, while dashed lines indicate 3 standard deviations above the mean preimmune levels. Statistics were analyzed using two-way ANOVA with Tukey's multiple-comparison test, comparing the mean ODs from each vaccine group to the mean OD for each coating antigen.

protective epitope, from SseI, to heat-killed *Salmonella* bacteria that contained multiple epitopes. For this purpose, we compared mouse survival after vaccination with the SseI peptide to that after vaccination with whole heat-killed *Salmonella*. Surprisingly, mice immunized with the SseI peptide showed levels of protection comparable to those for mice immunized with heat-killed *Salmonella* (Fig. 1B). Thus, a single MHC-II epitope against a secreted effector protein was sufficient to protect C57BL/6J mice from a

lethal *Salmonella* challenge. In order to determine whether SseI-vaccinated mice had lower bacterial loads, we isolated systemic organs (spleen and liver) to determine bacterial burdens. Mice vaccinated with the SseI epitope exhibited significantly lower bacterial burdens in both organs (P , 0.021 and 0.011, respectively) than mice vaccinated with an irrelevant peptide, while the FliC peptide showed no protection compared to the same irrelevant peptide (P , 0.279 and 0.929, respectively). As a positive control,

TABLE 1 Titers of peptide-specific antibodies for mice vaccinated with *Salmonella*-specific peptides^a

Ig type	Ig class	Stage	OVA immunization	<i>P</i> value	SseI immunization	<i>P</i> value	FliC immunization	<i>P</i> value	SL3261 immunization	<i>P</i> value
SseI specific	IgM	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	50 (50)	>0.05	50 (50–100)	>0.05	50 (50)	>0.05	50 (50)	>0.05
	IgG	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
OVA specific	IgM	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
	IgG	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	225 (50–400)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	1,650 (50–6,400)	<0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
FliC specific	IgM	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
	IgG	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
<i>M. tuberculosis</i> specific	IgM	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	62.5 (50–100)	>0.05	75 (50–100)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	92.8 (50–200)	>0.05	78 (50–100)	>0.05	114 (100–200)	<0.001	57 (50–100)	>0.05
	IgG	Preimmune	50 (50)		50 (50)		50 (50)		50 (50)	
		Prime	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05	50 (50)	>0.05
		Boost	75 (50–100)	>0.05	71 (50–100)	>0.05	114 (100–200)	<0.001	200 (200)	>0.05

^a Antibody titers were determined for mice vaccinated with either the SseI or the FliC peptide. The data are mean reciprocal endpoint titers for total IgM or IgG from each group, with the titer range given in parentheses. The endpoint titer was defined as the highest dilution that yielded an OD₄₀₅ more than 3 standard deviations above the mean OD₄₀₅ for preimmune sera. *P* values that were statistically significant when postimmunization endpoint titers were compared with preimmune titers are in boldface.

FIG 3 IFN- γ is produced by CD4 T cells from mice vaccinated with the SseI peptide. Antigen-specific CD4 (A) and CD8 (B) T cell responses from vaccinated mice were detected by IFN- γ secretion in ELISPOT assays. The data are images of representative wells from each restimulation group and mean log₁₀ levels of IFN- γ -secreting cells per 1 million T cells, with values for individual replicates shown (n , 3 to 4 mice per group). Statistics were analyzed using two-way ANOVA with Dunnett's multiple-comparison test, comparing each restimulation to the OVA control within each group. **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$; ns, $P > 0.05$.

mice vaccinated with the live attenuated strain of *Salmonella* (SL3261) also showed significantly lower burdens in both their spleens and their livers than mice immunized with the irrelevant peptide (Fig. 1C and D).

Peptide-specific CD4 T cells are associated with protection.

Next, we determined what protection against *Salmonella* Typhimurium was elicited by vaccination. Immune correlates were determined by characterizing the peptide-specific antibodies and T cells, both CD8 and CD4. In a separate subset of animals, mice were bled before each vaccination and before challenge. Initially, we assessed the OD values by antigen-specific ELISAs in order to determine if antibodies were being produced against the immunization peptides, in contrast to preimmune sera. Very little, if any, antibody response was detected against the SseI, FliC, or irrelevant peptide, but antibodies against mycobacterial proteins (present in complete Freund's adjuvant) were induced, as expected (Fig. 2A and B). We next scrutinized the sera more carefully. Titer analysis using antigen-specific ELISAs showed little evidence of IgM or IgG generated against vaccine peptides (Table 1). Thus, with this vaccine, antibodies most likely provided little, if any, protection against *Salmonella* infection in our experiment. To better understand what was contributing to protection, we analyzed T cell

responses by antigen-specific ELISPOT assays. Since IFN- γ has been shown to be an important mediator for protection against *Salmonella* (35), we chose to measure the number of IFN- γ -producing cells from each vaccine group. Either CD4 or CD8 T cells were isolated from the spleens of vaccinated mice at the time of challenge and were restimulated with their respective peptides. As expected, CD4 T cells produced significant levels of IFN- γ in response to peptide stimulation compared to the OVA control, while CD8 T cells produced little to no IFN- γ (Fig. 3A and B). T cells from mice receiving the live attenuated vaccine also responded to restimulation with both the SseI and FliC peptides (Fig. 3A and B). These data suggest that CD4 T cells contribute to protection after vaccination with MHC-II peptides and that these epitopes are also expressed during active *Salmonella* infection.

Immunization with MHC-II epitopes elicits a multifunctional Th1 cell response. Once we determined that T cell responses were stimulated after vaccination with our *Salmonella* epitopes, we next wanted to ascertain whether *Salmonella*-specific T cells were capable of producing multiple antimicrobial cytokines. Multifunctional Th1 cells have been shown to correlate with vaccine protection, specifically those that produce IFN- γ , TNF- α , and IL-2 at the same time (32). Both IFN- γ and TNF- α have been

FIG 4 Vaccination with *Salmonella*-specific peptides elicits multifunctional cytokine-producing CD4 T cells. CD4 (A) or CD8 (B) T cells were purified from vaccinated mice and were restimulated with their respective peptides *in vitro*. Cells were stained for intracellular cytokine production after 6 h of restimulation, the last 4 h in the presence of brefeldin A. The data are mean percentages ($\pm$ standard errors of the means) of antigen-experienced (CD44^{Hi}) cells producing one, two, or all three of the cytokines IFN- γ , IL-2, and TNF- α (single⁺, double⁺, or triple⁺, respectively) (n , 3 mice per group). Statistics were analyzed using two-way ANOVA with Dunnett's multiple-comparison test, comparing each group of cytokine producers to the OVA control. **, $P < 0.01$; ****, $P < 0.0001$; ns, $P > 0.05$.

shown to be important for bacterial killing (35, 36), while IL-2 helps expand and differentiate the T cell pool (37, 38). Therefore, we analyzed the multifunctional cytokine capabilities of both CD4 and CD8 T cells after vaccination with MHC-II peptides by using a Boolean gating approach. First, we analyzed T cells restimulated *in vitro* with our *Salmonella* vaccine peptides in order to determine the levels of single-, double-, and triple-cytokine-producing cells, in addition to defining the specificity of the T cell response. Splens were harvested from mice at the time of challenge, and cells were restimulated with each respective peptide. The majority of SseI- and FliC-specific CD4 T cells from each vaccinated group produced IFN- γ alone, while fewer produced TNF- α alone or IL-2 alone, when restimulated with their respective peptides. Some of these CD4 T cells produced combinations of these cytokines, making either two or, in a small percentage, all three cytokines. Notably, in all cases CD4 T cells produced cytokines only when restimulated with their respective peptides (Fig. 4A). The

percentages of double- and triple-cytokine-producing CD4 T cells from SseI- or FliC-vaccinated mice were similar to those from mice vaccinated with the live attenuated strain. The difference in the percentages of single-cytokine-producing cells could be attributed to restimulation from multiple epitopes found in heat-killed *Salmonella*. CD8 T cells showed no double or triple cytokine production, and very little single cytokine production, in response to stimulation with their respective peptides (Fig. 4B).

To determine whether CD4 T cells could produce IFN- γ , TNF- α , and IL-2 two weeks following the final vaccination, we next restimulated mice *in vivo* for 2 h with vaccine peptides injected intravenously, followed by intracellular cytokine staining directly *ex vivo*. SseI- or FliC-vaccinated mice restimulated with the SseI or FliC peptide, respectively, showed antigen-specific CD4 T cells producing IFN- γ , IL-2, or TNF- α , while the OVA peptide control did not elicit cytokine production in response to SseI or FliC peptide restimulation (Fig. 5). These cytokine profiles were similar to those seen when cells were restimulated *in vitro* (Fig. 4). Furthermore, when we analyzed their capacity for multifunctional cytokine production, SseI-vaccinated mice showed both double- and triple-cytokine-producing CD4 T cells (Fig. 6A), while CD8 T cells showed little to no cytokine production (Fig. 6B). These data suggest that immunization with a single *Salmonella* CD4 T cell epitope derived from a secreted effector protein generates multifunctional cytokine-producing cells, which may be associated with protection against lethal *Salmonella* challenge.

DISCUSSION

In the current study, we demonstrate that a single peptide from *Salmonella* secreted effector protein I (SseI) offers significant protection against lethal infection in a susceptible-mouse model. While this is not the first peptide shown to protect against infection, our findings are noteworthy for several reasons. (i) This is the first instance where a single peptide, of either MHC class I or II, has been shown to be protective against *Salmonella* bacteria, and this peptide is one of the few that demonstrate any protection against a bacterial, as opposed to a viral, pathogen (26, 39). (ii) Protection was mediated solely by immunity to a single CD4 T cell epitope from a secreted effector protein injected by the T3SS. This finding agrees with some previous studies showing that protection against *Salmonella* can be mediated by immunity to other whole proteins of the *Salmonella* T3SS, particularly *Salmonella* secreted effector B (SseB) (40, 41); however, it contrasts somewhat with a recent report showing that the predominant protective response relies on immunity to surface-associated antigens (21). (iii) Protection was antibody independent, appearing to rely entirely on CD4 T cell responses against the expressed *Salmonella* peptide. Additionally, many (approximately 1% of all of the antigen-experienced CD4 T cells in the spleen) of the peptide-specific T cells induced by immunization produced multiple Th1 cytokines in the same T cell, a finding that has been correlated with increased protection previously (32).

It is notable that no protection was provided by a previously described epitope from the flagellar protein FliC (33). This is somewhat surprising, considering that whole flagellar protein offers some protection and that vaccines derived from flagella are protective (33, 42, 43). One simple explanation for this difference is that whole protein induces a potent antibody response, while the peptide alone preferentially induces a CD4 T cell response;

FIG 5 *Salmonella*-specific CD4 T cells produce multiple cytokines *in vivo*. Vaccinated mice were restimulated with either the SseI or the FliC peptide for 2 h *in vivo*. T cells were stained for intracellular cytokine production directly *ex vivo*. The data are percentages of antigen-experienced (CD4^{Hi}) CD4 T cells positive for IFN- γ , IL-2, or TNF- α . Gates were set on naïve CD4 (CD44^{Lo}) T cells for each respective cytokine.

however, this does not explain why the SseI peptide is protective when the FliC peptide is not. One reason for this may be the differential, temporal expression of the flagella compared to that of SseI and other secreted effector proteins. It is known that *in vitro*, *Salmonella* flagella are downregulated upon entry into macrophages (44). Additionally, our previous work has shown that flagellar proteins are rapidly downregulated following i.v. infection and that the FliC-specific T cell response is nearly nonexistent later during infection (25). By comparison, the response to SseI is sustained (25). Alternatively, it is possible that because the flagella are expressed primarily during the early phase of infection in the gut, the immune response against this protein is skewed toward a more suppressive phenotype. This possibility is supported by studies showing that T cells and B cells from the gut tend to be enriched in regulatory cells that secrete suppressive cytokines, such as IL-10, that can depend on the level of inflammation there (45, 46). We show here and have shown previously that the re-

sponse to the SseI peptide is potent and sustained, possibly offering further evidence that this epitope is particularly effective at inducing a CD4 T cell response and therefore is capable of inducing the killing of intracellular bacteria (25).

Our results are also noteworthy within the larger context of the role of SseI during infection. Like many *Salmonella* T3SS effectors, SseI is encoded within *Salmonella* pathogenicity island 2 (SPI2) (47). Following the invasion and dissemination of infected macrophages, SseI is injected via the T3SS from within the *Salmonella*-containing vacuole (SCV) into the host cell cytosol, where it colocalizes with the host actin cytoskeleton and thyroid receptor-interacting protein 6 (TRIP6) or GTPase-activating protein 1 (IQGAP1) (48–50). Interestingly, these interactions appear to be important for either inhibiting or enhancing host cell migration and allow for long-term persistence of bacteria in a chronic mouse infection model (50). A naturally occurring single nucleotide polymorphism in SseI may also contribute to increased virulence

FIG 6 Multifunctional CD4 T cells are generated *in vivo* from vaccination with *Salmonella* CD4 epitopes. Boolean gating was used to determine if antigen-specific CD4 (A) or CD8 (B) T cells were producing multiple cytokines 2 h after intravenous injection of the respective peptides. Data are mean percentages ($\pm$ standard errors of the means) of antigen-experienced (CD44^{hi}) T cells positive for IFN- γ , IL-2, or TNF- α alone or for two or three of these cytokines simultaneously (n , 3 mice per group). (Insets) Total mean percentages ($\pm$ standard errors of the means) of antigen-experienced T cells producing IFN- γ , IL-2, or TNF- α . Statistics were analyzed using two-way ANOVA with Dunnett's multiple-comparison test, comparing each group of cytokine producers to the OVA control within each group. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

(51). These findings, combined with the known ability of intracellular *Salmonella* to utilize SPI2-encoded proteins that inhibit antigen presentation in the context of MHC-II (52–54), could lead to the expectation that the type of immunization we describe here would not be effective due to the inability of antigen-presenting cells to initiate a memory T cell response. However, the significant protection against lethal infection and the potent CD4 T cell responses against injected *Salmonella* epitopes that we observed indicate that, undoubtedly, these antigens were presented during the recall response to infection. This provides evidence that despite mechanisms evolved by *Salmonella* bacteria to subvert an immune response, especially with respect to non-surface-associated antigens such as SseI, priming with those antigens can actually be used to deliver protection against infection. Interestingly

SseI has also been shown in one study to be dispensable for virulence (55). This contrasts with our finding that SseI immunization can protect against infection. The most likely explanation for this is that the route of infection plays an important role in SseI expression and function. The previous study showing that SseI is not important for virulence utilized intraperitoneal infection, while studies showing an important role for SseI in virulence and dissemination used an oral infection route. Perhaps SseI has evolved specifically to initiate dissemination via the gastrointestinal (GI) tract. Since our study uses oral infection, it is possible that immunity to SseI could delay bacterial dissemination and lead to prolonged survival of the SseI-immunized mice in our study.

Another consideration for the design of vaccines against certain pathogens is the roles of CD4 T cells, CD8 T cells, and antibody-mediated immunity. In the case of *Salmonella*, it is clear that CD4 T cells are essential, whereas CD8 T cells appear to be dispensable (18–20, 56). The case for humoral immunity is somewhat more complicated. Antibodies have been shown to have a role during *Salmonella* infection in both mice and humans, especially antibodies directed against capsular polysaccharide antigens of the bacteria (57–59); however, due to the intracellular nature of the infection, it is probable that *Salmonella* immunity relies predominantly on CD4 T cell responses. Most likely, once *Salmonella* has crossed from the gut into the visceral organs, anti-*Salmonella* antibodies bind bacteria found in the extracellular space, perhaps targeting bacteria moving between phagocytic cells. Due to the reliance on CD4 T cell immunity, our results strengthen the case for providing CD4 T cell targets in future vaccines, although it will be important to determine what epitopes are most effective at combating infection. Additionally, it will be worthwhile to assess the possibility that CD4 T cell epitopes such as the one described in our study may be capable not only of providing a prophylactic intervention but also of being used to intervene during infection. Along these lines, a recent study showed that immunization with a combination of three novel MHC-II peptides from *S. Typhimurium* was able to lower the bacterial burdens of infected mice even during infection (56).

Our findings are interesting in a broader sense with regard to vaccination against different forms of *Salmonella* infection. While secreted effectors are clearly important during various phases of infection by most *Salmonella* serovars, be they typhoidal or non-typhoidal, vaccination using these proteins or peptides is likely to be less effective against acute salmonellosis, such as that seen during food-borne outbreaks in the United States. In these cases, the immune response is mostly confined to innate immunity mediated by neutrophils and macrophages at the site of infection (60). These types of infections, at least in the developed world, are often self-limiting, and adaptive immunity appears to play a minor role. Interestingly, the same serovar that causes these types of acute infections, *S. Typhimurium*, is also the most prevalent in persistent iNTS infections (13)—a fact relevant to the current study, since the SseI protein, and the peptide described here, will be expressed by *S. Typhimurium* during iNTS infection. This provides evidence that the SseI protein should be considered as a likely vaccine target for preventing iNTS infection, especially in light of a study demonstrating that antibodies from human iNTS patients directed against *Salmonella* lipopolysaccharide (LPS) were actually worse at eliminating *Salmonella* than outer membrane proteins (16). It is also notable that many of the current iNTS infections are caused by multidrug-resistant *S. Typhimu-*

rium, making a vaccine against these infections an even higher priority (61).

In summary, we demonstrate that a single peptide CD4 T cell epitope derived from a T3SS effector can provide significant protection against lethal *Salmonella* infection in mice. These results may have broader implications for investigators considering new vaccination strategies for bacterial pathogens and defining target antigens for CD4 T cell responses, especially in view of the fact that many Gram-negative bacterial pathogens express a similar T3SS. For example, several prominent bioterrorism threats, such as *Yersinia pestis* and *Burkholderia pseudomallei*, secrete effector proteins into host cells in much the same way that *Salmonella* does (62, 63). It is possible that these effectors are excellent candidates for vaccine targeting, especially with respect to the induction of CD4 T cell responses. Indeed, T3SS effectors are often highly conserved across species. For example, *Y. pestis* secreted effector proteins share a great deal of homology and function with *Salmonella* effectors (62). While ongoing research is required to better determine the most effective epitopes from *Salmonella* T3SS proteins, we believe that targeting these effectors should be considered in the design of future vaccines against this pathogen.

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