Systemic CD8 T-Cell Memory Response to a *Salmonella* Pathogenicity Island 2 Effector Is Restricted to *Salmonella enterica* Encountered in the Gastrointestinal Mucosa[∇]

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To better understand the evolution of a systemic memory response to a mucosal pathogen, we monitored antigen-specific OT1 CD8 T-cell responses to a fusion of the SspH2 protein and the peptide SIINFEKL stably expressed from the chromosome of *Salmonella enterica* and loaded into the class I pathway of antigen presentation of professional phagocytes through the *Salmonella* pathogenicity island 2 type III secretion system (TTSS). This strategy has revealed that effector memory CD8 T cells with low levels of CD62L expression (CD62L^{low}) are maintained in systemic sites months after vaccination in response to low-grade infections with *Salmonella*. However, the CD8 T-cell pool eventually declines. Low numbers of central memory cells surviving after prolonged resting from an antigen encounter can nevertheless reconstitute the systemic effector memory pool in a route-specific recall response to cognate antigens encountered in the gut. Accordingly, populations of CD62L^{high} interleukin-7 receptor-positive progenitor central memory cells grafted into naïve mice expand in response to orally administered *Salmonella* expressing the chromosomal translational fusion of *sspH2* and the sequence encoding the SIINFEKL peptide but fail to proliferate following systemic stimulation. Moreover, populations of systemic memory CD8 T cells restricted to *Salmonella* in oral vaccines selectively expand in response to cognate antigens presented by cells isolated from mesenteric lymph nodes (MLN). Together, these findings have revealed the imprinting of systemic CD8 central memory T-cell recall responses against enteropathogens by MLN. MLN restriction represents a novel mechanism by which systemic CD8 T-cell immunity is confined to periods of high risk for extraintestinal dissemination.

The gastrointestinal mucosa hosts the largest and most varied population of indigenous microbiota in the body and serves as a residence for a plethora of transient microorganisms and frank enteropathogens (18, 48). Some enteric pathogens such as *Shigella* species do not usually progress beyond the intestinal mucosa, whereas others, such as *Salmonella* and *Yersinia* species, not only invade the mucosal surface but can also be recovered from extraintestinal sites. The systemic dissemination of *Salmonella enterica* in humans is frequently associated with serovars Typhi and Paratyphi, which account for 22 million cases of bacteremia and 220,000 deaths a year worldwide (7). The extraintestinal dissemination of *Salmonella*, however, is not limited to typhoidal strains, as demonstrated by the fact that nontyphoidal salmonellae are the most common cause of hospital admission due to bacteremia in sub-Saharan Africa (16). Therefore, successful immunization against this class of disseminating enteropathogens must evoke long-lasting immunity in both local and systemic compartments of the immune system. Here we have studied long-term systemic memory responses to an oral vaccine containing *Salmonella* with early tropism for ileal phagocytes by using CD8 T-cell responses to

an effector protein delivered into the host cell cytosol as an indicator of immunity.

CD8 T cells are an important component of the multiple cellular lineages required for optimal protective immunity against the intracellular pathogen *Salmonella* (13, 19, 25, 29). The differentiation of CD8 T cells has proven to be an excellent indicator of memory. Memory CD8 T cells expressing high levels of CD44 (the CD44^{high} phenotype) have been categorized into effector and central populations according to their patterns of L-selectin CD62L expression (38). In contrast to effector memory cells, long-lived central memory T cells capable of antigen-independent or homeostatic proliferation express high levels of the CD62L receptor. The patterns of CD62L expression by CD8 T cells have shown, for instance, that acute systemic *Salmonella* infections induce a protracted effector memory response (20). To study the type of systemic immunity induced by pathogenic microorganisms encountered in the gastrointestinal tract, we measured the CD8 T-cell memory response elicited by invasion-deficient *Salmonella* strains capable of extraintestinal dissemination (34, 44, 45). An oral vaccine containing an invasion-deficient *Salmonella* strain was engineered to inject the SIINFEKL model peptide into the cytosol of professional phagocytes as a chimera with the *Salmonella* pathogenicity island 2 (SPI2) SspH2 effector that is selectively expressed within endosomes. Systemic OT1 CD8 T cells specific to the SspH2::SIINFEKL chimera stably expressed from the *Salmonella* chromosome were used to mon-

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FIG. 1. A model for studying CD8 systemic responses to oral *Salmonella* vaccines. (A) Gel showing PCR fragments of wild-type *sspH2* and the *sspH2::SIINFEKL* translational fusion. Below, the OVA₂₅₂₋₂₆₇ peptide sequence is shown in bold, flanking *SspH2* domains lie within the green boxes, and the scar left after the excision of pKD13 is underlined. (B) The SIINFEKL peptide (black rectangle) is injected into the cytosol of APC via the SPI2 TTSS as an *SspH2* chimera. After digestion, the SIINFEKL peptide is transported by TAP-1 into the endoplasmic reticulum (ER) for binding with major histocompatibility complex class I molecules (red). OT1 CD8 $\alpha\beta$ T-cell responses to the SIINFEKL peptide serve as surrogate markers of *Salmonella*-specific immunity. (C) Ly5.1⁺ C57BL/6 mice were orally immunized with an invasion-deficient *Salmonella* vaccine strain (*aroA invA sspH2::SIINFEKL*⁺) 1 to 3 days after the grafting of naive Ly5.2⁺ CD8⁺ OT1 cells. The expression of Ly5.2 by CD8⁺ splenocytes was measured by flow cytometry. The SPI2 dependency of cytosolic translocation was studied by expressing the chimera in *sseB* mutant *Salmonella* cells. (D and F) Levels of IFN- γ in the sera of orally immunized mice (D) and in the supernatants of splenocytes cultured with the SIINFEKL peptide or *Salmonella* parboiled antigens (F) were measured by ELISAs. Because *Salmonella* cells disseminating extraintestinally reach the spleen and liver in identical numbers (45), the bacterial burden was quantified by plating all tissue from livers macerated in a stomacher. (E) The role of the cytosolic pathway in the *SspH2::SIINFEKL*-dependent stimulation of Ly5.1⁺ OT1 cells was tested with Ly5.2⁺ TAP-1 knockout (KO) mice. All responses were measured 7 days after oral boosting. The data are representative of five to eight independent observations from at least two separate experiments.

itor the progression of long-term systemic CD8 memory responses to mucosal stimulation.

MATERIALS AND METHODS

Bacterial strains. *S. enterica* serovar Typhimurium strain ATCC 14028s was used as the background for the construction of isogenic vaccine strains. The *sspH2::SIINFEKL* chromosomal translational fusion was constructed by following the one-step mutation procedure originally described by Datsenko and Wanner (9). The forward primer contained 60 bp of DNA homologous to the *sspH2* target gene, followed by a sequence encoding amino acids 257 to 264 of the ovalbumin peptide (OVA₂₅₇₋₂₆₄; SIINFEKL) and the upstream FLP recombination target site of the pKD13 plasmid. DNA sequencing verified the insertion of the sequence encoding the OVA₂₅₇₋₂₆₄ peptide SIINFEKL in frame between nucleotides encoding residues 453 and 455 of *SspH2* (Fig. 1A). The *sspH2::SIINFEKL* allele was inserted into *Salmonella* strains CL1509 (*aroA*) (40) and AJB82 (*aroA invA*) (4) to yield strains AV2199 (*aroA sspH2::SIINFEKL*⁺) and AV2200 (*aroA invA sspH2::SIINFEKL*⁺). The $\Delta sseB::aphT$ mutation that inactivates the SPI2 translocase was moved from strain HH102 (12) into strain AV2200, yielding strain AV2201 (*aroA invA sseB sspH2::SIINFEKL*⁺).

Mice. Wild-type Ly5.1⁺ C57BL/6 and Ly5.2⁺ TAP-1-deficient mice (43) and OVA transgenic Ly5.2⁺ OT1 mice expressing the V β 5.1 T-cell receptor, which was originally derived from the K^b-restricted OVA₂₅₇₋₂₆₄-specific clone 149.42

(14), were purchased from Jackson Laboratories (Bar Harbor, ME). The OT1 transgenic allele in a Ly5.2⁺ background was backcrossed into wild-type Ly5.1⁺ C57BL/6 mice in our animal facility. Mice were housed and treated in accordance with National Institutes of Health guidelines and Institutional Animal Care and Use Committee protocols. Oral immunization was initiated with 6- to 8-week-old mice.

Adoptive transfers of OT1 cells and oral immunization. About 2×10^6 nylon wool column-purified T cells isolated as described previously (33) from among splenocytes of OT1 transgenic mice were inoculated intravenously into naive or immunized C57BL/6 mice. Mice receiving OT1 cell grafts were immunized orally with approximately 5×10^9 CFU of *Salmonella* strain AJB82, AV2200, or AV2201 and were given a booster dose 2 weeks later. Selected groups of naive or immunized mice received grafts of memory cells isolated from immunized animals 40 to 110 days after oral immunization. Memory CD8 T cells were isolated from splenocytes by nylon wool column purification, and the cell population was further enriched with memory CD8 T cells by positive magnetic-bead sorting using biotinylated anti-CD8 monoclonal antibodies and magnetic bead-labeled streptavidin (Miltenyi Biotec, Auburn, CA). CD8 memory cells were adoptively transferred into naive controls or mice immunized 40 to 50 days earlier. Selected groups of mice were orally immunized a few days after grafting, whereas the water of some orally immunized mice was treated during the last 3 weeks with nalidixic acid before memory cells were grafted.

Immunoassays. SIINFEKL peptide-specific CD8 splenic responses were analyzed by flow cytometry by dually labeling CD8 and Ly5.1 or Ly5.2 in a lymphocytic gate designed according to forward and side scattering. Memory cells, identified on the basis of CD44^{high} expression, were classified further as central or effector memory lymphocytes according to their high or low levels of CD62L expression, respectively (37). The production of gamma interferon (IFN- γ) by splenocytes cultured in RPMI medium supplemented with 10% fetal calf serum (BioWhittaker, Walkersville, MD), 2 mM L-glutamine, 15 mM HEPES, 1 mM Na pyruvate (Sigma-Aldrich, St. Louis, MO), 5 μ M 2-mercaptoethanol, 100 U of penicillin/ml, and 100 mg of streptomycin (Cellgro, Herndon, VA)/ml in the presence or absence of 0.50 μ g of the SIINFEKL peptide/ml was measured by sandwich enzyme-linked immunosorbent assays (ELISAs). All the reagents for flow cytometry and the ELISAs were purchased from Caltag (Burlingame, CA), BD Pharmingen (San Diego, CA), or eBiosciences (San Diego, CA).

Isolation of APC. Antigen-presenting cells (APC) were isolated from the spleens, Peyer's patches, or mesenteric lymph nodes (MLN) of C57BL/6 mice. Spleens were macerated and the red blood cells were eliminated by using Gey's lysis buffer. MLN and Peyer's patches were resected aseptically and digested in Dulbecco's phosphate-buffered saline, without Ca²⁺ or Mg²⁺ (Sigma-Aldrich), containing 0.2% collagenase I and IV (Sigma-Aldrich) and 2% heat-inactivated fetal calf serum for 5 min at 37°C in a 5% CO₂ incubator. The specimens were placed on ice for 10 min in 5 mM EDTA diluted in Dulbecco's phosphate-buffered saline supplemented with 2% heat-inactivated fetal calf serum. Cells from MLN were pelleted and resuspended in RPMI medium as described above for splenocytes.

Antigen presentation. Cells (4×10^5) isolated from spleens, Peyer's patches, and MLN were added to 24-well plates containing 10^6 OT1 cells. OT1 cells were isolated from mice immunized orally between 70 and 110 days earlier with an *aroA invA sspH2::SIINFEKL*⁺ *Salmonella* vaccine strain. OT1 cells contained in the splenocyte samples were nylon wool-purified, and the samples were further enriched with OT1 cells by positive selection for CD8 T cells by magnetic-bead cell sorting according to the indications of the bead manufacturer (Miltenyi Biotec). APC and OT1 cells were cultured for 3 days with 0.50 μ g of the SIINFEKL peptide/ml at 37°C in a 5% CO₂ incubator. The percentages of CD8⁺ (fluorescence parameter FL1) and Ly5.1⁺ (fluorescence parameter FL3) OT1 T cells contained in a lymphocyte gate were determined by flow cytometry after Topro-3⁺ (fluorescence parameter FL4) dead cells were eliminated from the analysis.

CD8 T-cell proliferation. Selected groups of naïve or memory OT1 cells were labeled as previously described (21) with 5 μ M carboxyfluorescein diacetate succinimidyl ester (CFSE) prior to the adoptive transfer. Levels of proliferating CD8⁺ Ly5.1⁺ T cells were estimated by using a four-color FACSCalibur flow cytometer (Becton Dickinson, Mountain View, CA) to monitor the loss of CFSE 5 to 21 days after transfer. Dead cells were eliminated from the analysis after the gating out of Topro-3⁺ (FL4) cells.

RESULTS

Stimulation of antigen-specific CD8 systemic T-cell responses by an orally administered *Salmonella* vaccine strain expressing the *sspH2::SIINFEKL* translational fusion. The acquired response against *Salmonella* involves B cells and $\gamma\delta$ T cells as well as CD4 and CD8 $\alpha\beta$ T cells (13, 19, 23–25, 29, 30). Several investigators have successfully measured acute CD8 T-cell responses to *Salmonella* expressing OVA from a plasmid (11, 39, 49). However, in the absence of selective pressure, plasmids are frequently lost and thus are less reliable for long-term studies. To examine long-term systemic responses to oral *Salmonella* vaccines, we engineered a stable OVA_{257–264} SIINFEKL construct expressed from the *Salmonella* chromosome as a chimera with an SPI2 effector that allows long-term expression in vivo (Fig. 1A). Because substrates of the type III secretion system (TTSS) are frequently poor immunogens, SPI2 TTSS effectors were evaluated for homology to the SPI1 SptP effector that is known to stimulate T-cell responses (35). Protein sequence alignments to determine homology to SptP domains identified the SspH2 effector, which is secreted into the host cell cytosol in an SPI2-dependent fashion (27, 28). The

SIINFEKL peptide inserted in frame in a predicted flexible loop of SspH2 (Fig. 1B) was expressed in an *aroA invA Salmonella* vaccine strain (45).

Similar to CD8 T cells involved in the acute responses triggered by *Salmonella* expressing high levels of OVA (49), antigen-specific Ly5.2⁺ CD8⁺ T cells accumulated in the spleen shortly after oral immunization with the *Salmonella* vaccine strain expressing the *sspH2::SIINFEKL* chromosomal translational fusion (Fig. 1C). Further analysis verified that the Ly5.2⁺ CD8⁺ T cells expressed the V β 5.1 allele characteristic of OT1 transgenic cells (data not shown). To demonstrate dependency on the SPI2 TTSS for the delivery of the SspH2::SIINFEKL chimera into the cytosol, the *sspH2::SIINFEKL* allele was expressed in an isogenic *aroA invA* vaccine strain carrying the *sseB::aphT* mutation that inactivates all intracellular SPI2-mediated translocation. The *sseB* mutant strain triggered significant ($P < 0.01$) *Salmonella*-specific IFN- γ systemic responses, although it was less immunogenic than the corresponding SPI2-proficient control (Fig. 1D, left panel). In sharp contrast to its IFN- γ -stimulating capability, the *sseB* mutant vaccine strain failed to trigger any significant ($P = 0.32$) expansion of the population of Ly5.2⁺ CD8⁺ cells (Fig. 1C). The lack of a CD8 T-cell response cannot be explained by reduced survival and extraintestinal dissemination of the *sseB*-deficient vaccine strain, since mice inoculated orally with either *sseB*-proficient or *sseB*-deficient vaccine strains harbored identical hepatic bacterial loads ($P = 0.87$) (Fig. 1D, right panel). The lack of SIINFEKL peptide-specific CD8 T-cell responses in mice immunized orally with the *aroA invA sseB sspH2::SIINFEKL*⁺ vaccine strain is consistent with the SPI2-dependent translocation of SspH2 into the cytosol (27, 28).

TAP-1-deficient mice were used to test whether CD8 T-cell responses elicited by the *Salmonella* vaccine require the translocation of the SIINFEKL peptide from the cytosol into the endoplasmic reticulum. Because TAP-1-deficient mice express the Ly5.2 allele, the OT1 transgene was backcrossed into an Ly5.1⁺ C57BL/6 background. Ly5.1⁺ OT1 transgenic mice were used for this and all subsequent experiments. Figure 1E shows that the TAP-1 transporter is critical for the expansion of populations of antigen-specific CD8⁺ T cells derived in response to the orally administered *sspH2::SIINFEKL*⁺ *Salmonella* vaccine strain. In agreement with the results of other studies (1, 41), an endogenous Ly5.1⁺ CD8⁺ population consisting mainly of $\gamma\delta$ T cells was nevertheless observed in the spleens of immunized TAP-1-deficient mice. In contrast, $\alpha\beta$ T cells represented most of the Ly5.1⁺ CD8⁺ lymphocytes in wild-type mice. Splenocytes isolated from orally vaccinated TAP-1-deficient mice did not secrete IFN- γ in response to the SIINFEKL peptide but synthesized amounts of IFN- γ similar to those synthesized by TAP-1-competent controls in response to parboiled *Salmonella* antigens (Fig. 1F). Together, these findings are consistent with a model in which the SIINFEKL peptide is injected into the cytosol via the SPI2 TTSS as a chimera with SspH2, translocates into the endoplasmic reticulum for loading into major histocompatibility complex class I molecules, and is highly immunogenic. This strategy was used to evaluate long-term systemic CD8 T-cell memory responses evoked by an oral *Salmonella* vaccine.

Oral immunization elicits systemic effector memory CD8 T-cell responses. The kinetics of systemic CD8 T-cell responses to oral

FIG. 2. Effector memory CD8⁺ T-cell responses to an oral *Salmonella* vaccine. (A) The percentage of CD8⁺ Ly5.1⁺ SIINFEKL peptide-specific T cells recovered from the spleens of mice receiving grafts of OT1 cells was determined at different time points after oral immunization with *aroA invA sspH2::SIINFEKL*⁺ *Salmonella*. Naïve mice and mice inoculated orally with *aroA invA* *Salmonella* were used as controls. 7d, 7 days. (B) The proliferation of lymphocytes in the CD8⁺ Ly5.1⁺ gate was visualized as the loss of CFSE among cells from naïve or orally immunized (immune) mice 21 days after immunization. (C and D) The abundance of central memory cells was expressed as the percentages of CD8⁺ Ly5.1⁺ splenocytes that were CD44^{high} (C) and CD62L^{high} (D). Regions were drawn by using isotype controls (data not shown) according to the CD44 and CD62L expression patterns of naïve OT1 cells. The mice in these experiments were immunized orally with the *aroA invA sspH2::SIINFEKL*⁺ *Salmonella* vaccine strain. The data are representative of nine independent observations from three separate experiments.

immunization with *Salmonella* were studied. In contrast to the relatively slow responses seen when OVA is delivered into the phagosome by systemically administered wild-type *Salmonella* (20), a six- to sevenfold increase in antigen-specific CD8 T cells in mice receiving OT1 cell grafts was already detected 7 days after oral exposure to *sspH2::SIINFEKL*⁺ *Salmonella* (Fig. 2A). The percentage of SIINFEKL peptide-specific T cells increased 15-fold 2 weeks after oral immunization and had increased about 65-fold by 7 days after oral boosting. The increase in Ly5.1⁺ CD8⁺ T cells was antigen specific and not the product of the expansion of populations of bystander T cells, because populations of OT1 T cells did not expand in mice

immunized orally with an isogenic vaccine strain (i.e., an *aroA invA* strain) expressing the *sspH2* wild-type allele (Fig. 2A). To determine whether the expansion of populations of antigen-specific CD8 T cells is a consequence of T-cell proliferation, donor OT1 cells were labeled with CFSE before grafting. As shown in Fig. 2B, all the grafted cells proliferated 3 weeks after oral immunization. Over 90% of the cells that proliferated in the spleens exhibited the CD44^{high} memory phenotype, whereas 80% of them were CD62L^{low} (Fig. 2C and D). Predominantly CD62L^{low} expression by CD44⁺ cells suggests that oral *Salmonella* vaccines preferentially trigger early effector memory T-cell responses.

FIG. 3. Effector memory responses to oral *Salmonella* vaccines. (A) The frequencies of CD8⁺ Ly5.1⁺ cells in naïve mice (N) and orally immunized mice 40 days postimmunization (I) and those in orally immunized mice after intraperitoneal boosting with the *aroA invA sspH2::SIINFELK*⁺ *Salmonella* vaccine strain (I-B) were determined. (B and D) The percentages of CD62L^{high} central memory (CM) and CD62L^{low} effector memory (EM) cells (B) and the hepatic bacterial burdens (D) in animals 40 days postimmunization after intraperitoneal boosting with ~100 CFU were determined. (C) Splenocytes from immunized mice were pulsed in vitro with the SIINFELK peptide and analyzed for their IFN- γ -secreting capacities. The data in all panels are representative of six independent observations from two separate experiments.

An oral *Salmonella* vaccine triggers a sustained effector memory systemic response. A large population of antigen-specific CD8 T cells remained in orally immunized mice 40 days after boosting (Fig. 3A). Systemic boosting with the

SIINFELK peptide-expressing *Salmonella* vaccine strain did not lead to further increases in the CD8⁺ Ly5.1⁺ T-cell pool, which remained at a level of around 1 to 3% of the total lymphocyte population for at least 3 weeks postchallenge. Further analysis indicated that CD62L^{low} effector memory T cells were predominant, accounting for about 75% of the CD8⁺ memory pool (Fig. 3B). Splenocytes isolated from immune mice secreted IFN- γ in response to the SIINFELK peptide (Fig. 3C). Since *Salmonella* reaches similar burdens in livers and spleens (45), the hepatic *Salmonella* burden was determined as an indicator of systemic infection. The *Salmonella* burden in livers peaked 7 days after the intraperitoneal challenge of orally immunized mice but dropped to very low levels after 21 days.

Memory T cells responding to *Salmonella* are capable of homeostatic and antigen-driven proliferation. To test whether memory cells are maintained by foci of infection, Ly5.1⁺ splenocytes isolated from orally vaccinated mice 40 days after immunization were grafted into orally immunized mice or naïve controls. About 20% of immunized mice that received grafts of memory OT1 cells harbored CD8⁺ Ly5.1⁺ cells at levels of approximately 1% of the lymphocytic populations in the spleens (Fig. 4A). Mice with large numbers of CD8⁺ Ly5.1⁺ cells (i.e., those with CD8⁺ Ly5.1⁺ cells accounting for about 1% of the CD8 $\alpha\beta$ T cells) were classified as high responders. The observations that antibiotic treatment abolished the occurrence of high CD8⁺ Ly5.1⁺ responders and that populations of naïve T cells expanded in mice that had been immunized 50 days before grafting (Fig. 4B) suggest the existence of foci of infection. Exposure to antigenic stimulation for extended periods of time may lead to lymphocyte exhaustion, a condition in which antigen-specific T cells are eventually depleted (46). Therefore, the ability of memory cells to sustain homeostatic and antigen-driven proliferation was studied (Fig. 4C). The proliferation of CFSE-labeled Ly5.1⁺ memory cells in naïve mice revealed that oral *Salmonella* vaccines elicit a population of memory cells capable of homeostatic turnover. When results were corrected for absolute numbers, memory cells were found to proliferate to a fivefold-higher level when

FIG. 4. Memory CD8 T cells are capable of both homeostatic and antigen-driven proliferation. (A and B) Ly5.1⁺ splenocytes isolated 40 days after animals were orally immunized (mem; panel A) or Ly5.1⁺ cells isolated from naïve controls (naïve; panel B) were grafted into naïve mice or mice that had been orally immunized 45 days earlier (immune). Some immune animals were treated with nalidixic acid in drinking water 3 weeks before grafting (immune+ATB). The frequency of SIINFELK peptide-specific Ly5.1⁺ CD8⁺ splenic T cells was estimated by flow cytometric analysis. (C) CFSE-labeled naïve splenocytes or Ly5.1⁺ splenocytes taken from immune mice 45 days postimmunization (mem) were grafted into either orally immunized mice 45 days postimmunization (imm) or naïve recipients. Selected groups of mice were orally inoculated with the *sspH2::SIINFELK*⁺ *Salmonella* vaccine strain 2 days after grafting (imm+boost). Cell division was evaluated by flow cytometry as the loss of the CFSE label among the CD8⁺ Ly5.1⁺ population. All responses were measured 7 days after grafting. The data are representative of 5 to 10 independent observations from at least two separate experiments.

FIG. 5. The antigen-driven expansion of CD8 memory cells is restricted to *Salmonella* cells encountered in the oral mucosa. (A) The percentages of CD8⁺ Ly5.1⁺ splenocytes in mice receiving grafts of OT1 cells were determined 50 days (50d) and 110 days (110d) after oral vaccination. An analysis was also performed 7 days after mice received oral (110d+po) or parenteral (110d+ip) booster vaccines at 110 days postimmunization. (B) The IFN- γ production in splenocytes isolated 50 or 110 days after oral immunization was measured. The SIINFEKL peptide (SIIN) was added to selected groups of splenocytes. The right panel shows the IFN- γ response 7 days after animals received oral booster doses at 110 days postimmunization. (C) Cells isolated from high responders (immune) were analyzed by flow cytometry for intracellular IFN- γ . OT1 cells transferred into naïve mice were used as controls. (D) CD8⁺ Ly5.1⁺ cells isolated from mice with elevated or reduced numbers of OT1 cells (i.e., high responders [H.R.] and low responders [L.R.], respectively) were examined for the expression of the interleukin-7 receptor (IL7R) and CD62L 110 days postimmunization. The data are representative of four to seven independent observations from two independent experiments.

recipients were immunized orally shortly after grafting. These findings rule out the possibility that *Salmonella* vaccines elicit oral tolerance by either depletion or anergy of antigen-specific cells and suggest that the suppression recently attributed to *Salmonella* (42) does not appear to block antigen-specific CD8 T-cell recall responses.

Oral *Salmonella* vaccines elicit CD8 T-cell memory responses restricted to antigens encountered in the gastrointestinal tract. We found that 80% of orally immunized animals maintained a robust CD8 T-cell memory pool (i.e., they were high responders) 50 days postimmunization (Fig. 5A); however, the frequency of high responders dropped to 40% by 110 days. The administration of oral booster doses increased ($P < 0.01$) the number of high responders among mice at 110 days postimmunization (Fig. 5A). In contrast, the number of high responders did not significantly increase ($P = 0.65$) after the administration of parenteral booster doses to mice at late time points postimmunization. Collectively, these data suggest that the reconstitution of the pool of antigen-specific splenic CD8 T cells in orally immunized mice is restricted to antigens encountered in the gastrointestinal tract. The patterns of IFN- γ production by splenocytes isolated from mice at late time points postimmunization (Fig. 5B) further support the enteric restriction of memory CD8 T-cell responses elicited by oral *Salmonella* vaccines. The early ability of splenocytes to secrete IFN- γ in response to the SIINFEKL peptide was dramatically lessened at later time points (e.g., 50 versus 110 days post-oral immunization). However, treating mice with oral booster doses at late time points postimmunization effectively increased the incidence of IFN- γ producers (Fig. 5B, right panel). Although both effector and central memory cells synthesized IFN- γ , the majority of the memory cells expressed a CD62L^{low} effector phenotype (Fig. 5C). By 110 days postimmunization, the majority of the high responders expressed a CD62L^{low} effector memory phenotype, whereas a central memory CD62L^{high} cell population predominated in low responders (Fig. 5D). Of note, both populations of memory cells expressed high levels of the interleukin-7 receptor.

Central memory cells restricted to MLN drive systemic effector memory cells in response to oral immunization. The selective expansion of populations of OT1 memory cells in response to *sppH2::SIINFEKL*⁺ *Salmonella* encountered orally but not systemically suggests that memory CD8 T cells selected by oral vaccines are restricted to cognate antigens encountered in the gastrointestinal tract. To test this idea, the capacity of memory grafts to expand in response to oral or systemic re-stimulation was studied. Consistent with the data presented in Fig. 5, populations of memory CD8 T cells in naïve mice expanded after oral but not parenteral immunization with *sppH2::SIINFEKL*⁺ *Salmonella* (*aroA invA*) (Fig. 6A). CD62L^{high} central and CD62L^{low} effector memory cells were isolated by flow cytometric sorting from vaccinated mice 110 days after oral immunization. Grafts consisting of CD62L^{low} effector memory CD8 T cells survived in low numbers in mice that received oral booster doses (Fig. 6B, left panel); however, these cells disappeared in naïve hosts (Fig. 6B). Conversely, central memory cells survived after grafting into naïve mice (Fig. 6B, central panel), possibly reflecting the capacity of this population of memory cells to undergo homeostatic turnover (15, 47). In contrast to effector memory cells, central memory grafts (i.e., CD62L^{high}) expanded in response to *SppH2::SIINFEKL*⁺ *Salmonella* administered through the oral mucosa (Fig. 6B, right panel). Central memory cells restricted to orally administered antigens repopulated both CD62L^{low} and CD62L^{high} memory subsets.

The compartmentalization of the recall response to the oral mucosa was further examined by comparing the expansion of populations of memory CD8⁺ Ly5.1⁺ T cells in response to APC from Peyer's patches, MLN, or spleens. As shown in Fig. 7, the greatest expansion of Ly5.1⁺ CD8⁺ transgenic cells isolated from orally immunized mice was seen upon coculture with SIINFEKL peptide-primed APC from MLN. In contrast, APC from spleens, Peyer's patches, and MLN supported the expansion of populations of naïve OT1 cells (data not shown). About 2% of the SIINFEKL peptide-specific OT1 cells selected at 110 days after oral immunization expressed $\alpha 4\beta 7$

FIG. 6. Central memory CD8 T cells restricted to orally administered antigens drive systemic effector memory responses. (A) Ly5.1⁺ cells isolated from mice orally immunized with *Salmonella* 70 to 110 days earlier were grafted into naïve mice after positive selection over a magnetic bead column. The expansion of the Ly5.1⁺ CD8⁺ T-cell population was tested 7 days after the intraperitoneal (ip; middle panel) or oral (po; right panel) administration of an *aroA invA sspH2::SIINFEKL*⁺ *Salmonella* vaccine strain. (B) CD62L^{low} and CD62L^{high} CD8⁺ Ly5.1⁺ cells isolated by flow cytometric sorting from mice orally immunized 110 days earlier with *aroA invA sspH2::SIINFEKL*⁺ *Salmonella* were grafted into naïve mice. A day after grafting, selected groups of mice were either intraperitoneally or orally immunized with *aroA invA sspH2::SIINFEKL* *Salmonella*. The abundance of CD62L CD8⁺ T cells in a Ly5.1⁺ gate was analyzed by flow cytometry 7 days after *Salmonella* challenge. The data are representative of five independent observations from two separate experiments.

gut-homing receptors, whereas at late time points after oral immunization with *S. enterica* serovar Typhi, most of the CD8 T cells coexpress this receptor (36).

DISCUSSION

The findings presented herein reveal that the imprinting of a central memory CD8 T-cell response specific to an oral pathogen capable of disseminating extraintestinally occurs in the MLN. Although central memory CD8 T cells elicited in response to oral stimulation undergo antigen-independent, homeostatic turnover, their ability to replenish the systemic effector memory pool is not only antigen-driven but also restricted by cognate antigens encountered in MLN. These data are consistent with the idea that long-lasting CD8 T-cell surveillance is established in the oral mucosa, where the enteropathogen was originally encountered. MLN restriction ensures the temporal confinement of systemic memory CD8 T-cell responses to periods in which the cognate pathogen is

present in the gut and during which extraintestinal dissemination is a considerable risk.

The selective expansion of populations of SspH2::SIINFEKL-specific CD8 T cells following oral stimulation indicates that the SspH2 SPI2 effector is expressed in cells populating the gut mucosa. In vitro cultures of gut APC have identified MLN as the anatomical location where the restriction of SspH2::SIINFEKL-specific memory CD8 T cells to orally encountered antigens takes place. MLN restriction of CD8⁺ memory responses to the SspH2::SIINFEKL chimera is analogous to but different from the selective imprinting for gut-homing T cells by Peyer's patches (32), since these SspH2::SIINFEKL-specific memory CD8 T cells do not appear to be restricted by Peyer's patches. Differences in the anatomical restriction of memory cells to selected lymphoid tissues of the intestinal mucosa exemplify the functional compartmentalization of the inductive sites of the gut. Imprinting by Peyer's patches may be critical for directing recall effector and memory responses to the gas-

FIG. 7. Selective expansion of central memory cells restricted to antigen administered orally in response to cells isolated from MLN. APC from Peyer's patches, MLN, or spleens were cocultured for 7 days with OT1 memory CD8 T cells isolated from spleens of mice immunized orally 85 to 90 days earlier. The T-cell population was enriched with OT1 cells by CD8 magnetic-bead sorting of nylon wool-purified T cells. The expansion of transgenic cells was quantified by flow cytometric analysis by monitoring the frequency of CD8⁺ Ly5.1⁺ cells. The data are representative of 12 independent observations from three separate experiments.

trointestinal mucosa, whereas MLN restriction may orchestrate temporal responses in distal sites during times of intestinal infection.

It should be noted, however, that not all systemic responses elicited in response to oral immunization are necessarily limited to antigens encountered in intestinal lymphoid tissues. In fact, a population of CD8⁺ Ly5.1⁺ donor cells primed during oral vaccination expanded following systemic boosting. Moreover, polyclonal populations of splenocytes selected by oral immunization do indeed proliferate in response to *Salmonella* antigens presented by APC from spleens (24). The recently described delayed extraintestinal route of dissemination that proceeds without previous colonization of Peyer's patches and MLN (3) may evoke systemic responses that are not restricted by intestinal lymphoid organs. The segregation of APC in defined anatomical compartments resembles the distribution of populations of *Salmonella*-specific, polyclonal memory CD4⁺ and CD8⁺ T cells at distinct anatomical locations according to functional capabilities (17). Together, these data illustrate the complexity in the mucosal and systemic compartments of the immune system. Immunity to enteropathogens involves local and distal inductive sites, which together with distinct T-cell migration patterns provide temporal and spatial diversification in the immune response required to effectively defend against enteropathogens with tropism for both mucosal and systemic organs.

An effector memory response was maintained in a few animals over 110 days. Most of these effector cells expressed the interleukin-7 receptor, suggesting that at late time points effector cells are committed to becoming long-lived memory cells (15). The inhibition of CD8 population expansion after antibiotic therapy and the expansion of populations of naïve cells in hosts that were immunized orally 50 days earlier (Fig. 4) suggest that a focus of infection maintains the lingering effector response. MLN phagocytes, which have been shown to facilitate the prolonged survival of enteric commensals and wild-type *Salmonella* (22, 31), may keep *Salmonella* vaccine cells viable for extended periods of time. These observations consequently indicate that the duration of systemic CD8 memory responses elicited by enteropathogens depends on microbial persistence in the host. Efficacious live-pathogen vaccines must therefore balance virulence potential and the capacity of vectors to cause chronic infections with the development of long-lasting immunity.

Populations of systemic memory CD8 T cells, nonetheless, decline overtime. The loss of effector memory may explain the advised reimmunization of seasonal travelers visiting areas of endemicity as well as the progressive fading of immunity in residents that depart from such areas for extended periods of time. The smoldering *Salmonella* infection does not appear, however, to induce the partial exhaustion of antigen-specific lymphocytes, because memory cells are capable of homeostatic and antigen-driven proliferation. The persistence of T-cell function suggests that the level of antigenic stimulation associated with *Salmonella* must be significantly lower than that associated with pathogenic microorganisms linked to T-cell deletion (46). In addition, the IFN- γ response seen here and elsewhere (23, 25) may inhibit SPI2 transcription via nitric oxide (26), thus limiting the expression of the SspH2::SIINFEKL chimera at later times. The adjuvanticity

of *Salmonella*'s outer membrane may also trigger inflammatory signals that preserve T-cell function despite enduring antigenic stimulation.

Several properties associated with the SspH2::SIINFEKL chimera have made it possible to elucidate the anatomical restriction of long-term central memory CD8 T cells elicited by the oral *Salmonella* vaccines used in this study. First, the SIINFEKL epitope is transcribed from a single *sspH2* locus in the *Salmonella* chromosome, so CD8 OT1 responses in this system are sensitive surrogate markers of *Salmonella*-specific immunity to an SPI2 TTSS effector. Second, the chromosomal location of this translational fusion guarantees high stability, allowing studies of long-term immunity in vivo. Third, the cytosolic secretion of SPI2 substrates such as SspH2 is selectively induced within the *Salmonella*-containing vacuole (5, 10). SPI2-based systems, therefore, provide significant advantages for studying systemic responses compared to delivery systems based on a flagellar TTSS that is downregulated extraintestinally (8). And fourth, translation-secretion coupling of TTSS effectors such as SspH2 ensures the exclusive translocation of the chimera into the cytosol (2, 6), minimizing both antigenic build-up in phagosomes and cross-presentation from endocytic vacuoles. The direct loading of an antigen into the cytosolic pathway of antigen presentation may initiate faster CD8 T-cell priming and recall responses than those elicited by the intraphagosomal delivery of OVA (20). The SspH2 delivery system described herein can be used for the detailed characterization of mucosal and systemic responses to *Salmonella* and can be easily adopted to deliver a variety of heterologous antigens to the inductive sites of both the mucosal and systemic compartments of the immune system.

In summary, our findings have exposed the previously unrecognized MLN restriction of central memory CD8 T cells to *Salmonella* encountered in the gastrointestinal tract. MLN restriction may limit rapid and focused systemic memory CD8 T-cell recall responses to periods during which enteropathogens populate their natural niches in the host. The robust contraction of populations of CD8 effector memory cells that occurs over time may be advantageous in the context of intestinal immunity. Effector cells may wane with the disappearance of luminal cognate antigens, freeing space and resources for rising clones with specificity for the latest pathogenic challenges encountered in the intestinal mucosa. Central memory CD8 T cells restricted to orally encountered antigens that survive the contraction phase are nonetheless long lived and selectively reconstitute a labile pool of effector memory cells in systemic compartments of the immune system in response to cognate pathogens reaching MLN.

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