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Salmonella enterica Serovar Typhi Live Vector Vaccines Delivered Intranasally Elicit Regional and Systemic Specific CD8$^+$ Major Histocompatibility Class I-Restricted Cytotoxic T Lymphocytes

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We investigated the ability of live attenuated *Salmonella enterica* serovar Typhi strains delivered to mice intranasally to induce specific cytotoxic T-lymphocyte (CTL) responses at regional and systemic levels. Mice immunized with two doses (28 days apart) of *Salmonella* serovar Typhi strain Ty21a, the licensed oral typhoid vaccine, and genetically attenuated mutants CVD 908 (Δ*aroC* Δ*aroD*), CVD 915 (Δ*guaBA*), and CVD 908-*htrA* (Δ*aroC* Δ*aroD* Δ*htrA*) induced CTL specific for *Salmonella* serovar Typhi-infected cells in spleens and cervical lymph nodes. CTL were detected in effector T cells that had been expanded in vitro for 7 days in the presence of *Salmonella*-infected syngeneic splenocytes. A second round of stimulation further enhanced the levels of specific cytotoxicity. CTL activity was observed in sorted αβ$^+$ CD8$^+$ T cells, which were remarkably increased after expansion, but not in CD4$^+$ T cells. CTL from both cervical lymph nodes and spleens failed to recognize *Salmonella*-infected major histocompatibility complex (MHC)-mismatched cells, indicating that the responses were MHC restricted. Studies in which MHC blocking antibodies were used showed that H-2L^d was the restriction element. This is the first demonstration that *Salmonella* serovar Typhi vaccines delivered intranasally elicit CD8$^+$ MHC class I-restricted CTL. The results further support the usefulness of the murine intranasal model for evaluating the immunogenicity of typhoid vaccine candidates at the preclinical level.

Attenuated *Salmonella enterica* serovar Typhi strains have been intensively investigated as oral typhoid vaccines and as live vectors to deliver heterologous antigens because of their ability to induce humoral and cell-mediated immunity (CMI) (21–23). The only licensed live oral typhoid vaccine, Ty21a, is well tolerated but modestly immunogenic, requiring three or four consecutive doses to achieve moderate levels of protection (20). Investigators have therefore undertaken the development of new genetically defined attenuated *Salmonella* serovar Typhi strains that would be safe yet robust and immunogenic, to serve as a single-dose mucosal live-vector vaccines (21–23). Results from phase 1 and phase 2 clinical trials in volunteers vaccinated orally with *Salmonella* serovar Typhi strain CVD 908, which harbors deletion mutations in genes involved in aromatic amino acid synthesis (Δ*aroC* Δ*aroD*) (12, 42), and *Salmonella* serovar Typhi strain CVD 908-*htrA*, a derivative of CVD 908 with an additional mutation in *htrA*, which encodes a heat shock protease (41, 43, 44), advanced the field by showing that these strains are well tolerated yet broadly immunogenic. Serum antibodies, mucosal immunoglobulin A-secreting cells, and CMI, including cytokine production and cytotoxic T-lymphocyte (CTL) activity, against bacterial and foreign antigens have been demonstrated (12, 23, 39–44), emphasizing the potential of these organisms as typhoid vaccines and mucosal live vectors for humans. A new attenuated strain, CVD 915, which has a deletion mutation in the *guaBA* locus that interrupts the synthesis of guanine nucleotides, has been developed recently (48). This strain has proven to be highly immunogenic as a mucosal live vector in preclinical studies and is regarded as a promising vaccine candidate to enter phase 1 clinical evaluation (32, 48).

Demonstration that new *Salmonella* serovar Typhi engineered strains are immunogenic in a reliable animal model is required before clinical testing can be initiated. Because of the narrow restriction of *Salmonella* serovar Typhi for human hosts, researchers have used *Salmonella enterica* serovar Typhimurium infection in mice, which results in typhoid-like disease, as an experimental model to study typhoid fever pathogenesis. Although the *Salmonella* serovar Typhimurium model has proved to be useful for assessing the attenuation and immunogenicity of novel recombinant strains (7), the performance of *Salmonella* serovar Typhi vaccines in humans cannot be predicted from results obtained with *Salmonella* serovar Typhimurium in mice (22).

To assess the immunogenicity of new *Salmonella* serovar Typhi vaccine candidates at a preclinical level, our group established and characterized a murine model of intranasal (i.n.) immunization (3, 10, 33, 34). We showed that the i.n. route of immunization was remarkably better for inducing immune responses to *Salmonella* vaccine strains than the traditionally used orogastric route (10, 33). Mice inoculated i.n. with different attenuated *Salmonella* serovar Typhi strains alone or carrying prokaryotic or eukaryotic antigen expression systems induced specific antibodies and CMI responses, including T-cell proliferation and production of Th1 type cytokines to bacterial and foreign antigens, at mucosal and systemic levels (3, 10, 32–34, 48) that were consistent with those observed in volunteers vaccinated with some of these vaccine strains (12, 39–44, 50).

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CMI, particularly CTL, has proven to be critical for effective clearance of intracellular pathogens. Studies performed with *Salmonella* serovar Typhimurium and mice showed that macrophages and natural killer (NK) cells are involved in the preliminary stages of infection, mainly by interfering with bacterial growth through the production of tumor necrosis factor alpha, gamma interferon (IFN-γ), and interleukin-12 (IL-12) (7, 29). *Salmonella* infection also induces specific $CD4^+$ and $CD8^+$ T cells which contribute to protection during primary and secondary responses (15, 16, 26–28, 31, 35, 37; for reviews see references 7 and 29). It has been shown that $CD4^+$ T cells that secrete tumor necrosis factor alpha and IFN-γ are required to resolve infection (27) and that mice lacking $CD4^+$ $\alpha\beta^+$ T cells (16) and athymic (*nu/nu*) mice (31, 37) fail to clear *Salmonella*.

There is mounting evidence that $CD8^+$ T cells also participate in protection (15, 24, 26). Depletion of $CD8^+$ T cells from donor mice reduced their ability to transfer protection against virulent *Salmonella* serovar Typhimurium (26). $\beta 2m^{-/-}$ mice deficient in $CD8^+$ T cells were found to be more susceptible to infection with *Salmonella* serovar Typhimurium and exhibited impaired protection when they were challenged with a virulent strain (24). The mechanisms underlying *Salmonella*-specific $CD8^+$ effector function, however, are poorly understood. Because *Salmonella* is a facultatively intracellular pathogen, CTL-mediated lysis of infected cells could be one of the mechanisms likely to contribute to clearing infections, by releasing the bacteria from their protective habitat and thus rendering them accessible to activated macrophages and specific antibodies. It has been shown that mice infected with *Salmonella* serovar Typhimurium elicit $CD8^+$ CTL that recognize *Salmonella*-infected cells (24) or target cells loaded with bacterial peptides (25). *Salmonella* serovar Typhimurium live vector strains have also been shown to induce CTL responses against a variety of foreign antigens expressed from prokaryotic (1, 9, 46, 47) or eukaryotic plasmid systems (4, 6). Recently, dendritic cells that phagocytosed *Salmonella* serovar Typhimurium expressing ovalbumin (OVA) in vitro were shown to prime OVA-specific cytolytic effector cells as well as specific IFN-γ-producing $CD4^+$ and $CD8^+$ T cells when they were administered to naive mice (52).

With respect to *Salmonella* serovar Typhi live vectors, two reports showed the ability of *Salmonella* serovar Typhi to elicit CTL to foreign viral and protozoan antigens in mice (8, 14). Strain Ty21a carrying a DNA vaccine plasmid encoding measles virus nucleoprotein delivered to BALB/c mice intraperitoneally (i.p.) induced virus-specific $CD8^+$ CTL responses (8), and *Salmonella* serovar Typhi strain CVD 908 expressing gp63 of *Leishmania mexicana* elicited gp-specific CTL in BALB/c mice immunized orogastrically (14). However, no information is available on the ability of *Salmonella* serovar Typhi strains to induce CTL specific for cells expressing bacterial antigens. Also unexplored is the contribution of regional lymphoid tissues as reservoirs of specific effector T cells primed by mucosal vaccination, which can potentially move to effector sites or spread to nonmucosal tissues to prevent reinfection.

We have demonstrated that volunteers who ingested *Salmonella* serovar Typhi strains CVD 908 and CVD 908 expressing the circumsporozoite protein of *Plasmodium falciparum* elicited circulating $CD8^+$ cells, major histocompatibility complex (MHC) class I-restricted CTL specific to *Salmonella* serovar Typhi-infected autologous Epstein-Barr virus-transformed cells (39). These findings suggest that CTL may play an important role in protection against typhoid infection.

In the present study, we evaluated the abilities of attenuated *Salmonella* serovar Typhi vaccine strains Ty21a, CVD 908, CVD 915, and CVD 908-*htrA* to induce CTL responses in mice following i.n. immunization. We demonstrated for the first time that attenuated *Salmonella* serovar Typhi vaccine strains induced $CD8^+$ MHC class I-restricted CTL responses in cervical lymph nodes (CLN) and spleens.

MATERIALS AND METHODS

Bacterial vaccine strains and immunization. The following attenuated strains, derived from wild-type *Salmonella* serovar Typhi strain Ty2, were used in this study: Ty21a, the licensed attenuated live vector typhoid vaccine (20, 21, 23); CVD 915, a Δ*guaBA* deletion mutant (48); CVD 908, a Δ*aroC* Δ*aroD* mutant (42); and CVD 908-*htrA*, a Δ*aroC* Δ*aroD* Δ*htrA* mutant (43). Female BALB/c and C57BL/6 mice (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) that were 6 to 8 weeks old (10 per group) were immunized i.n. with each vaccine strain. A 10-μl portion (5 μl/nostril) containing 2×10^9 to 4×10^9 CFU was administered on days 0 and 28, as previously described (33, 34). A control group received 10 μl of phosphate-buffered saline (PBS) i.n. No anesthesia was used during the immunization procedures. Mice were sacrificed 56 to 60 days after immunization. Experiments with animals were authorized by the University of Maryland Institutional Animal Care and Use Committee under protocol 1100005.

Preparation of *Salmonella* serovar Typhi-infected cells. Splenocytes from BALB/c mice ($H\text{-}2^d$) and C57BL/6 mice ($H\text{-}2^b$), as well as MHC-matched cell lines P815 [a murine mastocytoma ($H\text{-}2^d$)] and EL4 [a mouse T lymphoma ($H\text{-}2^b$)], obtained from the American Type Culture Collection (Rockville, Md.), were infected with wild-type *Salmonella* serovar Typhi strain ISP1820 (as described below) and used as stimulators to expand *Salmonella* serovar Typhi-specific CTL effectors in vitro and as targets to measure CTL responses in ^{51}Cr release assays, respectively. In vitro infection was performed as follows: cells were resuspended in RPMI 1640 (Life Technologies, Gaithersburg, Md.) supplemented with 10% fetal calf serum, 2 mM glutamine, and 10 mM HEPES without antibiotic at a final concentration of 1×10^6 to 2×10^6 cells/ml and incubated with bacteria at a ratio of 70:1 to 80:1 for 3 h at 37°C in the presence of 5% CO_2. After infection, cells were washed and incubated overnight in supplemented medium containing 100 μg of gentamicin per ml. The next day, cells were washed twice with RPMI 1640. The absence of live bacteria was demonstrated by plating serial dilutions of the infected cell suspension in Luria-Bertani agar. Mock-infected cells were included as controls. In order to assess the efficiency of infection, the presence of *Salmonella* serovar Typhi antigens present intracellularly and expressed in the cell surface was evaluated by flow cytometry by using fluorescein isothiocyanate (FITC)-labeled antibodies against common *Salmonella* structural antigens (CSA-1) from Kirkegaard & Perry Laboratories (Gaithersburg, Md.). Anti-CSA-1 is an antibody broadly reactive to *Salmonella* that was purified by affinity chromatography from a pool of serum from goats immunized sequentially with different *Salmonella* strains, including *Salmonella* serovar Typhi. Infected and mock-infected cells were also stained with FITC-labeled control isotype antibodies. Representative histograms showing surface bacterial antigen expression on infected splenocytes and cell line cells are shown in Fig. 1. Infected splenocytes used as stimulators for in vitro expansion of CTL effectors were irradiated (dose, 2,000 rads) before being added to the cultures.

In vitro expansion of CTL effectors. Single-cell suspensions were prepared from spleens and CLN as previously described (32). Effector cells from immunized and control mice (~4.5×10^7 cells) were incubated with irradiated *Salmonella* serovar Typhi-infected splenocytes at a ratio of 3:1 to 5:1 in 25-ml flasks for 7 days (one round of stimulation) or 14 days (two rounds of stimulation [7 days each]) at 37°C in the presence of 5% CO_2. Murine recombinant IL-7 (330 U/ml; R&D Systems Inc., Minneapolis, Minn.) was added to the cultures on the day that each expansion was started (17), and recombinant IL-2 (20 U/ml; R&D Systems) was added 2 days later. The addition of these cytokines was shown to optimize the survival and expansion of specific CTL effector cell populations (17, 45). When two rounds of stimulation were performed, cells were washed and counted after the first 7 days of stimulation and cultured for another 7 days with freshly infected splenocytes and cytokines, as described above.

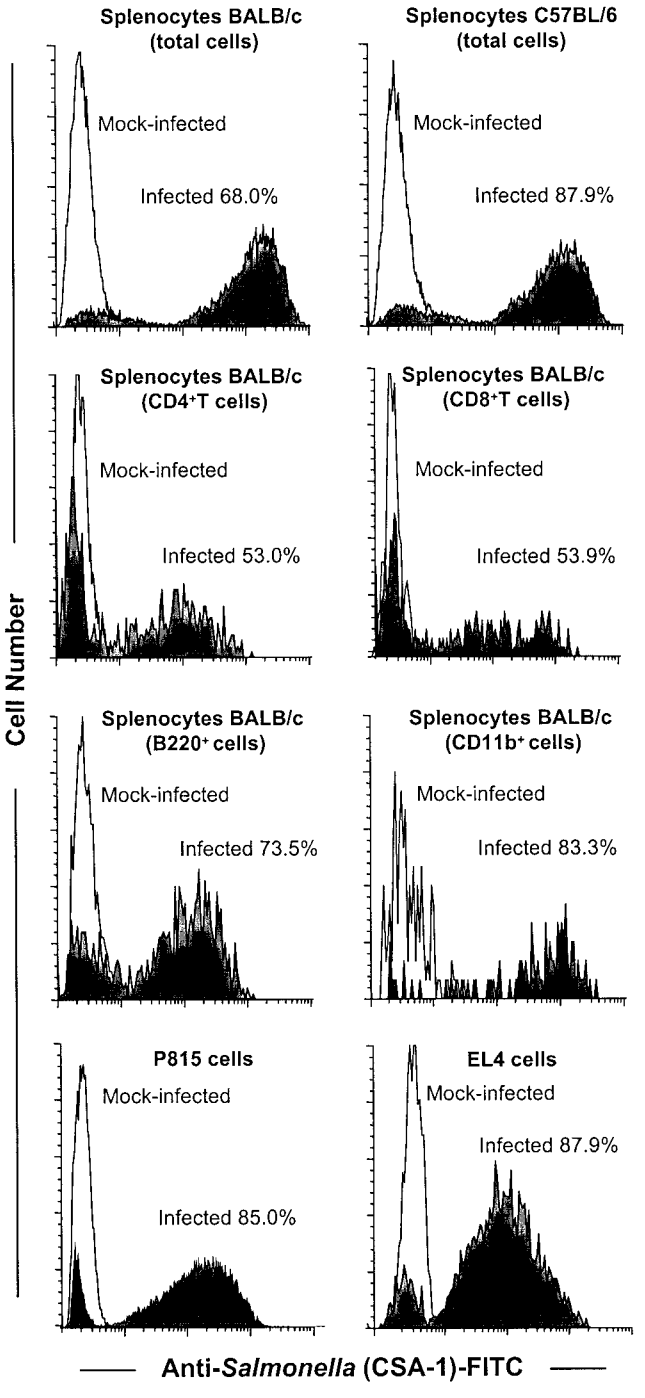

FIG. 1. Surface expression of *Salmonella* serovar Typhi antigens on infected splenocytes and cell lines. Splenocytes from BALB/c (H-2^d) and C57BL/6 (H-2^b) mice and MHC-matched cell lines P815 (H-2^d) and EL4 (H-2^b) were infected in vitro with *Salmonella* serovar Typhi strain ISP1820 as described in Materials and Methods. Mock-infected cells were included as controls. Surface expression of bacterial antigens was measured in infected and mock-infected cells by multicolor flow cytometry by using FITC-conjugated anti-*Salmonella* CSA-1 antibodies. Cells were also incubated with FITC-labeled isotype control antibodies to determine nonspecific staining. Expression of bacterial antigens in BALB/c splenocytes was analyzed in the total cell population or gated on a particular cell phenotype (i.e., $CD4^+$, $CD8^+$, $B220^+$, and $CD11b^+$), as indicated. The percentage of specific staining is shown on each histogram. The data are representative of the data from four separate experiments in which similar results were obtained.

CTL assays. Serial twofold dilutions of expanded effector cells (1.56×10^4 to 5×10^5 cells/well) were incubated with *Salmonella* serovar Typhi-infected and mock-infected P815 and EL4 targets cells (5×10^3 cells/well) previously labeled with 200 μCi of $^{51}CrO_4Na_2$ (Amersham Pharmacia Biotech, Piscataway, N.J.) as described elsewhere (39). Cultures were centrifuged at $50 \times g$ for 5 min and incubated for 4 h at 37°C in the presence of 5% CO_2. Supernatants were then collected and transferred to 96-well plates containing scintillation liquid (OptiPhase; LKB Wallac, Gaithersburg, Md.). The amount of ^{51}Cr released was measured with a Wallac Trilux β-counter (LKB Wallac). Cultures were tested in triplicate or quadruplicate wells. The percentage of cytotoxicity was calculated as follows: (experimental release − spontaneous release)/(maximal release − spontaneous release) ×100, where spontaneous release is the counts per minute released by target cells in the absence of effector cells and maximal release is the counts per minute released in the presence of 5% Triton X-100. Data are presented below as percentages of specific cytotoxicity, determined as follows: (percentage of cytotoxicity by effector cells incubated in the presence of *Salmonella* serovar Typhi-infected targets) − (mean percentage of cytotoxicity by effector cells incubated in the presence of mock-infected targets). The values are means ± standard deviations based on data for replicate cultures.

Flow cytometric analysis and sorting of $CD8^+$ and $CD4^+$ expanded T cells. Phenotypic analysis of the *Salmonella* serovar Typhi-infected splenocytes and effector populations was performed by multicolor flow cytometry by using an appropriate combination of the following monoclonal antibodies (MAbs): Red 613-conjugated anti-CD4, phycoerythrin- and FITC-conjugated anti-CD8, phycoerythrin-conjugated anti-DX5, peridinin chlorophyll *a* protein-conjugated anti-B220, allophycocianine-conjugated anti-CD11b, anti-CD3, and anti-T-cell receptor αβ (TCRαβ), and FITC-conjugated anti-TCRγδ. The isotype-matched controls included mouse immunoglobulins with irrelevant specificities labeled with the appropriate fluorochrome. Most MAbs were purchased from PharMingen, San Diego, Calif.; the only exception was Red 613-conjugated anti-CD4, which was purchased from Life Technologies. Immunofluorescence surface staining and flow cytometry analysis were performed as previously described (39). Intracellular staining was performed as follows: 1×10^6 cells prestained with antibodies to surface molecules were fixed for 20 min with 100 μl of 4% formaldehyde in PBS. After incubation, the cells were washed with staining buffer (2% fetal calf serum and 0.01% NaN_3 in PBS) and incubated with 50 μl of permeabilizing buffer (0.1% saponin in staining buffer) containing 4 μl of anti-CSA antibodies for 20 min at 4°C. The cells were then washed twice with permeabilizing buffer and fixed with 1% formaldehyde. For phenotype analysis, 10,000 to 20,000 cells were analyzed. $CD4^+$ and $CD8^+$ T cells were positively sorted from expanded effector cell cultures by using charge droplet deflection with an Epics Elite ESP flow cytometer-cell sorter system (Beckman-Coulter, Miami, Fla.). Multicolor flow cytometric analysis was performed by using the WinList software package (Verity Software House, Topsham, Minn.) or the Beckman-Coulter Elite software package.

MAbs blocking studies. *Salmonella* serovar Typhi-infected and mock-infected targets (~1.5×10^5 cells) labeled with $^{51}CrO_4Na_2$ were resuspended in 500 μl of complete medium and incubated with 90 μg of the appropriate blocking MAbs for 40 min at 37°C in the presence of 5% CO_2. After incubation and without washing, 2.5 ml of complete medium was added to obtain a suspension of 5×10^4 cells/ml in the presence of 30 μg of blocking antibodies (target cells) per ml. Serial dilutions of effector cells were incubated with 100 μl of the target cell suspension in the presence of blocking antibodies. The MAbs used in this study were directed to the BALB/c mice class I classical molecules H-$2D^d$ (clones 34-5-8S and 34-2-12; PharMingen), H-$2K^d$ (clone A4C8.1-Do9; Leinco Technologies, St. Louis, Mo.), and H-$2L^d$ (clone 30-5-7S; Cedarlane, Hornby, Ontario, Canada), as well as to the nonclassical class I molecule Qa-1b (clone 6A8.6F10.1A6; PharMingen) and the anti-class II molecule Ia (clone 34-5-3; PharMingen). Purified mouse anti-trinitrophenol MAb [immunoglobulin G1(κ), clone 107.3; PharMingen] was used as a negative control in these studies.

RESULTS

***Salmonella* serovar Typhi infection of syngeneic splenocytes and MHC-matched cell lines.** Prior to measuring CTL responses, we established an in vitro infection procedure that allowed us to obtain large numbers of viable *Salmonella* serovar Typhi-infected murine splenocytes that could be used as stimulators to expand effector T cells in vitro, as well as infected cell lines that were used as CTL targets in ^{51}Cr release assays. The efficiency of infection was evaluated by flow cytom-

etry by measuring surface and intracellular expression of *Salmonella* serovar Typhi antigens on infected and mock-infected cells stained with specific anti-*Salmonella* antibodies. Representative histograms showing surface expression of *Salmonella* serovar Typhi antigens by infected splenocytes from BALB/c (H-2^d) and C57BL/6 (H-2^b) mice, as well as MHC-matched P815 (H-2^d) and EL4 (H-2^b) cell lines, are shown in Fig. 1. The number of cells displaying *Salmonella* serovar Typhi antigens intracellularly was consistent with the number of cells showing surface expression; therefore, the latter was used to routinely screen bacterial infection in subsequent experiments. A phenotypic analysis of the cell populations displaying bacterial antigens within infected BALB/c splenocytes (i.e., $CD4^+$ and $CD8^+$ T cells, B cells [$B220^+$], and monocytes, macrophages, and dendritic cells [$CD11b^+$]) revealed that the $CD11b^+$ population contained the highest percentage of cells expressing *Salmonella* antigens (83.3%), followed by B cells (73.5%) (Fig. 1). Interestingly, about one-half of the $CD4^+$ and $CD8^+$ T cells also displayed specific *Salmonella* surface staining. MHC-matched cell lines P815 and EL4 were also effectively infected by *Salmonella* serovar Typhi and expressed bacterial antigens following infection (Fig. 1).

Specific CTL responses in mice following i.n. immunization with attenuated *Salmonella* serovar Typhi vaccine strains. We investigated the presence of CTL activity in spleen cells from BALB/c mice immunized i.n. with *Salmonella* serovar Typhi vaccine strains Ty21a, CVD 915, CVD 908, and CVD 908-*htrA*. Effector cells were expanded in vitro in the presence of *Salmonella* serovar Typhi-infected syngeneic splenocytes, IL-7, and IL-2 for 7 days. All vaccine strains were able to induce specific CTL responses, as indicated by the curves of specific lysis against *Salmonella* serovar Typhi-infected MHC-matched P815 cells (H-2^d) at different effector cell/target cell (E/T) ratios (Fig. 2A). The highest CTL activity was observed at E/T ratios of 60:1 to 80:1, and the level of cytotoxic activity declined as the E/T ratio decreased. The greatest responses were consistently observed in mice immunized with vaccine strains CVD 908-*htrA* and CVD 915. No significant CTL responses were detected in mice that received PBS (control) or when splenocytes from any group were assessed immediately after sacrifice (i.e., without expansion of specific effector cells).

CTL responses are induced at both regional and systemic sites in BALB/c mice. Because T cells primed in the nasal tissues have the ability to migrate and expand in the mucosal associated regional lymph nodes, we also investigated the presence of specific *Salmonella* serovar Typhi CTL responses in CLN. CTL responses were observed both in CLN and spleen cells in mice immunized with CVD 908-*htrA* following in vitro expansion with *Salmonella* serovar Typhi-infected syngeneic cells (Fig. 2B). Although the intensity was lower, similar results were observed in CLN from mice immunized with CVD 915 (data not shown).

Expansion of effector cells in the presence of *Salmonella* serovar Typhi-infected syngeneic splenocytes and cytokines enhances levels of specific cytotoxic activity. To further study the effect of expansion on the CTL responses in our system, we selected the most potent vaccine strains, CVD 915 and CVD 908-*htrA*, and measured CTL activity against *Salmonella* serovar Typhi-infected targets using effector cells obtained immediately after sacrifice or effector cells that had been expanded with *Salmonella* serovar Typhi-infected splenocytes for 7 days (one round of stimulation) or 14 days (two consecutive rounds of stimulation, 7 days each). The results are shown in Fig. 3. Confirming what we have previously observed, no responses were detected with fresh cells. In contrast, CTL activity was evident in T-cell cultures expanded with infected syngeneic antigen-presenting cells (APCs) for 7 days. CTL levels increased remarkably when cells were further stimulated for a total of 14 days. About 70% of the effector cells were viable

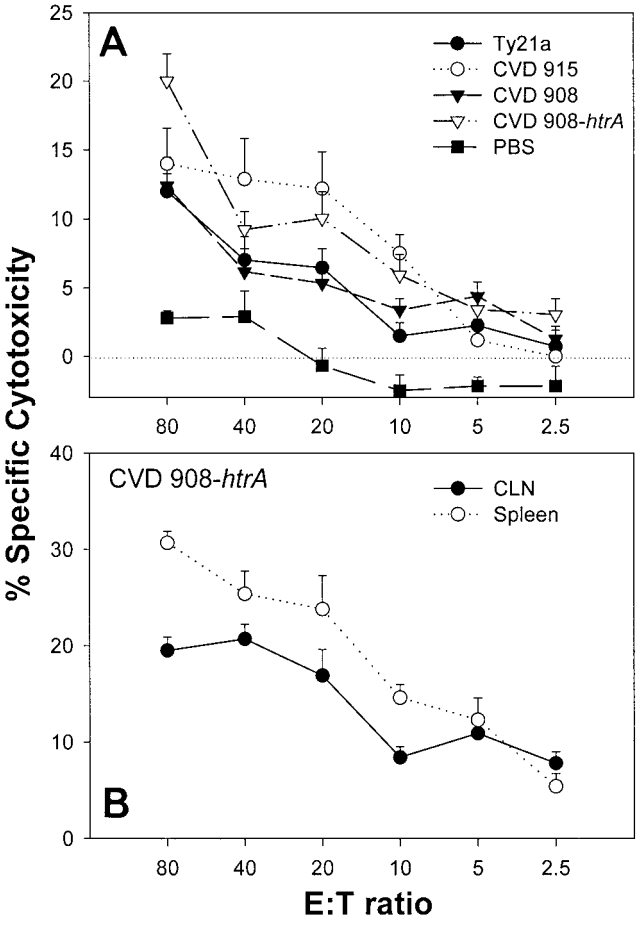

FIG. 2. Detection of *Salmonella* serovar Typhi-specific CTL responses in mice immunized i.n. with attenuated *Salmonella* serovar Typhi vaccine strains. BALB/c mice were immunized i.n. with two doses (28 days apart) of *Salmonella* serovar Typhi vaccine strains Ty21a, CVD 915, CVD 908, and CVD 908-*htrA* or PBS (control) as described in Materials and Methods. Spleens and CLN were collected 56 to 60 days after the primary immunization. Effector cells were stimulated in vitro for 7 days with *Salmonella* serovar Typhi-infected syngeneic splenocytes in the presence of IL-7 and IL-2 and assayed for CTL activity against *Salmonella* serovar Typhi-infected and mock-infected P815 cells in ^{51}Cr release assays. (A) CTL responses in splenocytes from mice inoculated with different vaccine strains or PBS (control). (B) CTL responses in CLN and spleens of mice that received CVD 908-*htrA*. All curves show the mean percentages of specific cytotoxicity at different E/T ratios for triplicate or quadruplicate wells; the error bars indicate standard deviations. The percentage of background lysis of mock-infected P815 target cells was subtracted from the percentage of *Salmonella* serovar Typhi-infected P815 lysis. The data are representative of the data from two complete, separate experiments in which similar results were obtained.

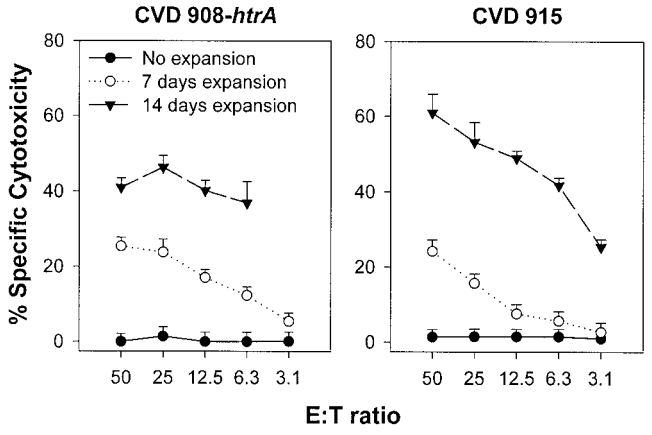

FIG. 3. In vitro expansion of CTL effectors with *Salmonella* serovar Typhi-infected cells is required to detect cytotoxic activity. Splenocytes from mice immunized with *Salmonella* serovar Typhi strains CVD 908-*htrA* and CVD 915 were examined for CTL activity without expansion, after in vitro expansion for 7 days in the presence of *Salmonella* serovar Typhi-infected syngeneic splenocytes, or after two consecutive rounds of expansion for a total of 14 days. The curves show the mean percentages of specific cytotoxicity for replicate wells; the error bars indicate standard deviations. The data are representative of the data from four different experiments in which similar results were obtained.

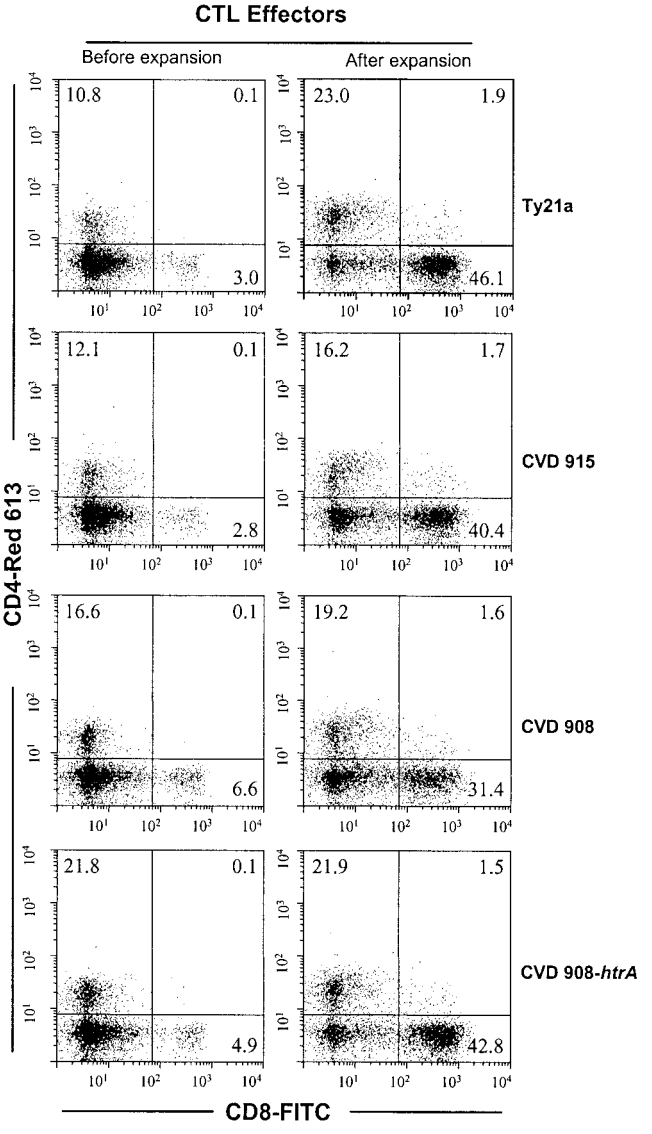

FIG. 4. Increased proportion of $CD8^+$ T cells in effector cell populations after expansion with *Salmonella* serovar Typhi-infected cells. The distribution of $CD4^+$ and $CD8^+$ T cells was measured by flow cytometry for splenocytes from immunized mice before and after expansion in the presence of *Salmonella* serovar Typhi-infected cells. The data are representative of the data from two complete, separate experiments in which similar results were obtained.

after each round of expansion, and as cultures proceeded, the cells appeared to be noticeably enlarged, with dense cytoplasmic granulation. The presence of expanded T cells with these characteristics was generally associated with detectable CTL activity.

To obtain a large and homogeneous expanded effector CTL population to perform biological and functional studies of CTL responses (e.g., phenotypic characterization, MHC restriction, evaluation of cytokine production, antigen presentation, etc.), we investigated whether expanded cells could be frozen and used in future assays without losing their functional activity. We found that the CTL responses measured in splenocytes from BALB/c mice immunized i.n. with CVD 908-*htrA* that was freshly harvested after expansion were very similar to those measured in effector cells from the same group that had been previously expanded and cryopreserved in liquid N_2 for up to 2 months (data not shown).

Identification of the CTL effector cell population. To characterize the effector CTL population, we analyzed the phenotypes of effector T cells from immunized mice before and after expansion with *Salmonella* serovar Typhi-infected syngeneic APCs. As shown in Fig. 4, increased proportions of $CD8^+$ T cells were consistently observed in splenocyte cultures after 7 days of expansion in all vaccinated groups. Similar increases in the proportions of $CD8^+$ T cells were observed in expanded CLN cultures. To determine whether the increased proportions of $CD8^+$ T cells were responsible for the observed CTL activity, both $CD4^+$ and $CD8^+$ populations were positively sorted from expanded splenocytes by using a flow cytometry-cell sorter system. The purity of sorted cell populations in repeat assays was always more than 92%. Serial dilutions of purified $CD4^+$ and $CD8^+$ T cells were incubated in the presence of *Salmonella* serovar Typhi-infected target cells in ^{51}Cr release assays. As shown in Fig. 5, $CD8^+$ T cells mediated lysis of infected cells, whereas purified $CD4^+$ T cells failed to produce significant CTL activity. A similar pattern was observed in mice immunized with all *Salmonella* serovar Typhi vaccine strains. The greatest responses were observed in $CD8^+$ T cells obtained from mice immunized with CVD 908-*htrA*. A multicolor phenotypic analysis of this cell population indicated that the vast majority of expanded $CD3^+$ $CD8^+$ T cells bore TCR$\alpha\beta$ (Fig. 6A). Interestingly, sorted $CD8^+$ T-cell populations exhibited significantly lower nonspecific lysis in ^{51}Cr release assays than unfractionated splenocytes, suggesting that nonspecific cytotoxic activity appeared to be mediated by cells other than $CD4^+$ or $CD8^+$ T lymphocytes. To determine whether this nonspecific activity was mediated by NK cells, we

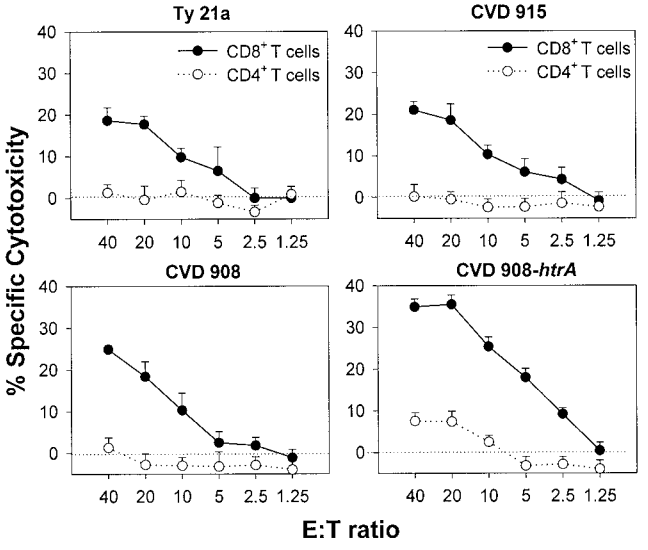

FIG. 5. Cytotoxic activity is mediated by $CD8^+$ T cells. $CD4^+$ and $CD8^+$ T cells sorted by flow cytometry from expanded splenocyte cultures from mice immunized with the different vaccine strains were assayed for cytotoxicity in the presence of *Salmonella* serovar Typhi-infected and mock-infected targets. The purity of sorted populations in different experiments was between 92 and 98%. No CTL activity was observed in PBS (control) mice. The curves show the mean percentages of specific cytotoxicity for replicate wells; the error bars indicate standard deviations. The data are representative of the data from two complete, separate experiments in which similar results were obtained.

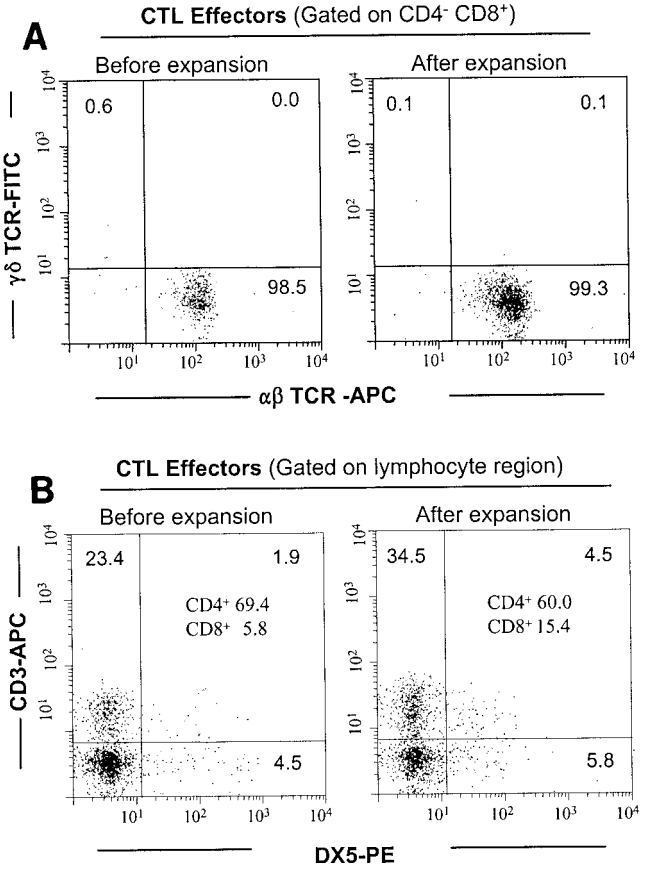

FIG. 6. CTL activity is mediated by $CD8^+$ $\alpha\beta^+$. Splenocytes from immunized mice were phenotypically evaluated for expression of CD4, CD8, TCR$\alpha\beta$, TCR$\gamma\delta$, CD3, and NK markers before and after expansion with *Salmonella* serovar Typhi-infected cells by multicolor flow cytometry. The values represent the percentages of positive cells in each quadrant. The percentages of $CD4^+$ and $CD8^+$ cells within the NK-T cell population ($CD3^+$ $DX5^+$) are also shown. The data are representative of the data from four different experiments performed with effector cells from mice immunized with CVD 908-*htrA*. PE, phycoerythrin.

measured the percentages of total NK cells ($DX5^+$ $CD3^-$) before and after expansion with infected APCs and found that in repeated experiments they were around 5% (4.5 and 5.8%, respectively, for the representative experiment shown in Fig. 6B). We also determined the percentages of NK T cells ($DX5^+$ $CD3^+$) in these populations and found that they were <2% before expansion (1.9% for the experiment shown) and <5% after expansion (4.5% for the experiment shown); the majority of these cells (~60 to 70%) were TCR$\alpha\beta^+$ $CD4^+$ T cells (Fig. 6B).

MHC restriction studies. To determine whether the observed CTL responses were MHC restricted, we measured cytotoxic activity using expanded CLN and spleen cells from BALB/c mice immunized with vaccine strains CVD 908-*htrA* and CVD 915 against two different infected target cells, the syngeneic P815 cell line ($H\text{-}2^d$) and the allogeneic EL4 cell line ($H\text{-}2^b$). Figure 7 shows representative results for mice immunized with CVD 908-*htrA*. Strong CTL activity against *Salmonella* serovar Typhi-infected P815 cells ($H2^d$) but not against EL4 cells ($H\text{-}2^b$) was demonstrated for both CLN and spleen cells. These results were confirmed by using effector splenocytes that had been expanded in vitro with two rounds of stimulation (Fig. 7).

To determine the MHC molecules involved in the restriction of CTL responses, *Salmonella* serovar Typhi-infected target cells were pretreated with MAbs to class I classical ($H\text{-}2D^d$, $H\text{-}2K^d$, $H\text{-}2L^d$) and nonclassical ($Qa\text{-}1^b$) molecules, as well as with MAbs to class II (Ia) molecules before exposure to expanded CTL effectors. In preliminary experiments we assessed the expression of each of these molecules with target P815 cell line and found that 98.9% of the cells expressed $Qa\text{-}1^b$, 100% expressed $H\text{-}2D^d$, 100% expressed $H\text{-}2K^d$, and 100% expressed $H\text{-}2L^d$, whereas 7% expressed Ia^d. Antibodies to the class I MHC molecules, but not antibodies to the class II MHC molecules, inhibited the CTL activity of expanded effector cells from immunized mice. The presence of MAbs blocking the classical $H\text{-}2D^d$ and $H\text{-}2K^d$ molecules and the nonclassical class Ib molecule $Qa\text{-}1^b$ did not affect $CD8^+$-mediated CTL activity (Fig. 8). Repeated experiments with anti-$H\text{-}2D^d$ and anti-$H\text{-}2L^d$ blocking MAbs in which CTL effectors generated by two rounds of in vitro expansion were used confirmed that the CTL activity was restricted by $H\text{-}2L^d$ molecules (Fig. 8). Taken together, these studies clearly demonstrate that the CTL effector population in BALB/c mice vaccinated with attenuated *Salmonella* serovar Typhi strains is a classic $CD8^+$ MHC I-restricted population.

CTL responses were elicited in BALB/c but not C57BL/6 mice immunized with attenuated *Salmonella* serovar Typhi

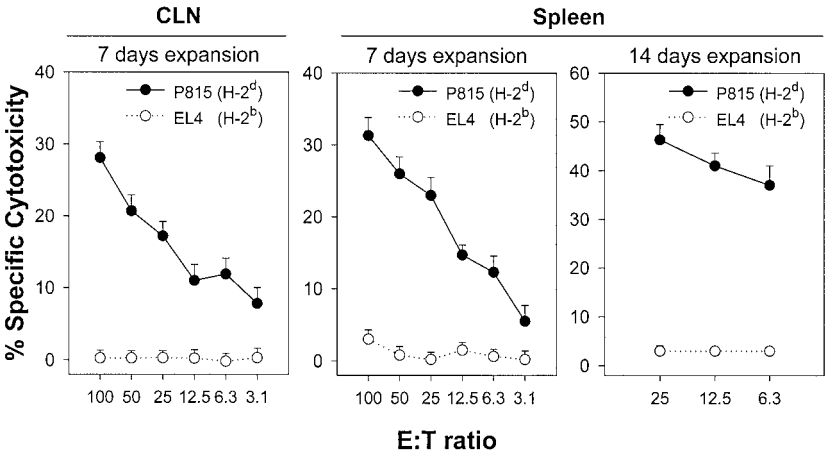

FIG. 7. CTL responses in spleens and CLN are MHC restricted. Effector cells from CLN and spleens of mice immunized with CVD 908-*htrA* were expanded for 7 days or 14 days (two rounds of 7 days each) and tested for cytotoxic activity in the presence of *Salmonella* serovar Typhi-infected and mock-infected MHC-matched P815 cells and mismatched EL4 cells. The curves show the mean percentages of specific cytotoxicity for replicate wells; the error bars indicate standard deviations. The data are representative of the data from three separate experiments in which similar results were obtained.

vaccine strains. We also evaluated the presence of CTL responses in C57BL/6 mice, a strain known to be susceptible to infection with *Salmonella* serovar Typhimurium (51). In contrast to what we observed in BALB/c mice, no CTL activity could be detected in spleens or CLN from C57BL/6 mice immunized with either CVD 908-*htrA* or CVD 915 (data not shown). Cells were assayed fresh and after 7 days of in vitro T-cell expansion. To determine whether the absence of CTL activity was due to a low frequency of CTL effectors, we also assessed cells stimulated in vitro with two rounds of expansion, which had previously been shown to markedly increase the level of CTL activity in BALB/c mice. No CTL were observed either in CLN or splenocytes. Effector cells from immunized BALB/c mice tested against *Salmonella* serovar Typhi-infected P815 cells were included as a positive control in these assays. Despite the lack of CTL responses, we observed that C57BL/6 mice immunized i.n. with attenuated *Salmonella* serovar Typhi strains induced high levels of antibodies against *Salmonella* serovar Typhi lipopolysaccharide (LPS) (data not shown).

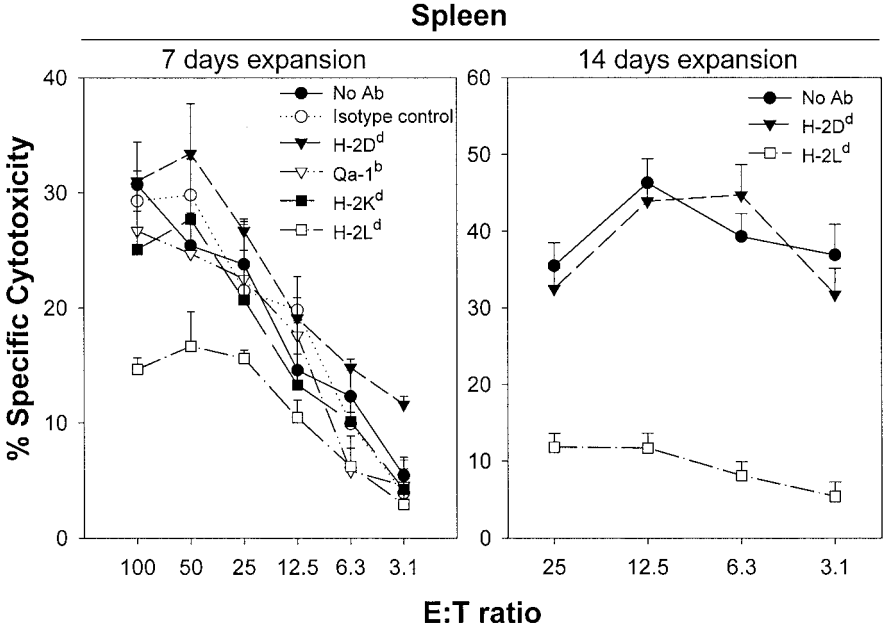

FIG. 8. Inhibition of CTL activity by MAbs to MHC class I molecules. *Salmonella* serovar Typhi-infected target cells were treated with medium (No Ab), an isotype control, or MAbs to the class I molecules H-2D^d, Qa-1^b, H-2K^d, and H-2L^d, and CTL assays were performed. Effector cells had been expanded in vitro with *Salmonella* serovar Typhi-infected splenocytes for 7 or 14 days. The curves show the mean percentages of specific cytotoxicity for replicate wells; the error bars indicate standard deviations. The data are representative of the data for mice immunized with CVD 908-*htrA* in three separate experiments.

DISCUSSION

Following our initial discoveries that attenuated *Salmonella* serovar Typhi vaccine strains were able to induce strong antibody and CMI when they were delivered to mice through the i.n. route (32, 33, 48), we investigated their ability to elicit CTL responses in regional CLN and spleens. Here we demonstrate, for the first time, that BALB/c mice immunized i.n. with a variety of attenuated *Salmonella* serovar Typhi strains, including the licensed typhoid vaccine Ty21a and a new generation of genetically defined attenuated mutants (i.e., CVD 908, CVD 915, and CVD 908-*htrA*) induced specific CTL at both regional and systemic levels. Moreover, we demonstrated that CTL responses were mediated by $CD8^+$ T lymphocytes and restricted by MHC class I H-$2L^d$ molecules. Importantly, these results resemble those observed in volunteers vaccinated orally with CVD 908 (39), Ty21a, and CVD 908-*htrA* (Sztein, unpublished observations), which also showed induction of circulating CTL, which appeared to be CD8 mediated and MHC class I restricted (39).

We selected the i.n. route of immunization because we have previously found it to be highly effective at inducing mucosal and systemic immunity to bacterial and foreign antigens delivered by *Salmonella* serovar Typhi live-vector vaccines, in contrast to orogastric immunization, which failed to result in significant immune responses (10, 33). The i.n. route has also been proposed as an attractive alternative to oral vaccination with live vectors in humans (19, 30).

The presence of CTL in CLN, a lymphoid organ that is not directly colonized by *Salmonella* serovar Typhi in this model (34), suggests that *Salmonella* serovar Typhi-specific $CD8^+$ T cells primed in the nasal tissue-associated lymphoid tissue (NALT) migrated to the regional lymph nodes. It is also possible that antigen-loaded APCs from the NALT could have migrated to the CLN after immunization and stimulated a different pool of naive T cells. Sensitized T cells in the CLN are likely to serve as a reservoir of effector and memory T cells that disseminate to mucosal and nonmucosal effector lymphoid tissues to protect them from reinfection (49).

In vitro expansion of CTL effectors in the presence of *Salmonella* serovar Typhi-infected syngeneic APCs was required to detect cytotoxic activity. We observed the same phenomenon when measuring CTL responses in vaccinated volunteers (39). Other groups have reported detection of CTL in mice infected with *Salmonella* serovar Typhimurium following expansion of effector cells with concanavalin A (24, 25), a mixture of *Salmonella* peptides (25), or *Salmonella* serovar Typhimurium-infected cells (24). This was likely due to a low frequency of CTL precursors in the effector cell population, a hypothesis further supported in our studies by the impressive increase in specific CTL activity observed after a second round of stimulation.

Experiments directed toward determining the spleen cell populations infected by *Salmonella* serovar Typhi that served as APCs for the expansion of effector T cells in vitro showed that *Salmonella* serovar Typhi was able to infect not only cells from the macrophage/monocyte lineage ($CD11b^+$) but also B ($B220^+$) cells and $CD4^+$ and $CD8^+$ T cells. Moreover, these cells contained bacterial antigens both intracellularly and expressed on the cell surface. We observed a similar phenomenon in vivo. Studies with cells isolated from the NALT of mice immunized i.n. with CVD 908-*htrA*, collected 12 to 16 h after infection, revealed primarily macrophages ($CD11b^+$) and B cells but also $CD4^+$ and $CD8^+$ T cells displaying *Salmonella* antigens intracellularly or expressed on the cell surface, as measured by flow cytometry and confirmed by confocal microscopy (Pasetti, unpublished observations). To the best of our knowledge, this is the first evidence of the ability of *Salmonella* serovar Typhi to infect murine T and B lymphocytes. These results are in agreement with our previous observations that wild-type *Salmonella* serovar Typhi could efficiently infect Epstein Barr virus-transformed lymphoblastic B-cell lines, express bacterial antigens on the cell membrane, and stimulate specific $CD8^+$ CTL in peripheral blood mononuclear cells from vaccinated volunteers (39). If our findings with the murine model could be extended to humans, they might contribute to our understanding of typhoid pathogenesis. Infected lymphocytes not only might help disseminate bacteria or antigens to distant sites but also might play a critical role in modulation of the host immune response.

The phenotypic analysis of effector cells from immunized mice before and after expansion with *Salmonella* serovar Typhi-infected cells showed that there was a consistent increase in $\alpha\beta^+$ $CD8^+$ T cells. In subsequent experiments, these cells were sorted and identified as the CTL effector population in all immunized groups. Similarly, we found a significant increase in $CD8^+$ T cells in peripheral blood mononuclear cells from volunteers vaccinated with *Salmonella* serovar Typhi strains after in vitro expansion with *Salmonella*-infected autologous blast cells (R. Salerno-Gonçalves, M. F. Pasetti, and M. B. Sztein, submitted for publication).

Interestingly, we observed that when CTL assays were performed with sorted $CD8^+$ and $CD4^+$ T cells, the levels of nonspecific lysis of mock-infected targets were significantly lower than the levels in assays performed with unfractioned expanded populations, suggesting that nonspecific cytolytic activity might be mediated by NK or NK-T cells. However, similar amounts (~5%) of NK cells ($CD3^-$ $DX5^+$) were found within the effector cell population before expansion (when no CTL was detected) and after expansion. It is therefore unlikely that they could be a major source of nonspecific killing in our system. This phenomenon does not appear to be mediated by NK-T cells ($CD3^+$ $DX5^+$) either, since although small increases in the numbers of NK-T cells were observed following expansion, most of these cells were $CD4^+$. However, we cannot rule out the possibility that NK and/or $CD4^-CD8^-$ NK-T cells, although present at low percentages, could have become activated during expansion, contributing to the nonspecific lysis observed in the total expanded population. The role of NK-T cells in specific acquired immunity against other infectious pathogens, such as *Listeria* and mycobacteria, is becoming increasingly evident (11). These cells appear to be primarily $CD4^+$ and CD1 dependent, producing high levels of IL-4 and IFN-γ (11). However, the role of NK-T cells in the mechanisms of defense against salmonellosis is unknown.

The strong cytotoxic activity observed against *Salmonella* serovar Typhi-infected P815 cells, but not against *Salmonella* serovar Typhi-infected EL4 cells, demonstrated that recognition of bacterial antigens by *Salmonella* serovar Typhi-induced CTL was MHC restricted. Subsequent experiments performed

with MHC blocking antibodies demonstrated that cytotoxic effector cells recognized *Salmonella* serovar Typhi antigens presented in the context of MHC class I molecules, with H-2L^d molecules acting as the restricting element. Our findings showing that class I-restricted *Salmonella* serovar Typhi-specific CTL were present are consistent with the findings reported by Pope et al., who described specific cytotoxic activity to bacterial antigens restricted to the Lyt-2$^+$ T-cell subset after i.p. inoculation with *Salmonella* serovar Typhimurium (35), and the findings of of Lo et al., who showed that class I-deficient B-6 $\beta 2m^{-/-}$ mice had increased susceptibility to infection with *Salmonella* serovar Typhimurium strain compared with normal B6 mice (24). In a recent publication Yrlid et al. also described induction of MHC class I-restricted IFN-γ-producing CD8$^+$ T cells in mice given a single i.p. dose of attenuated *Salmonella* serovar Typhimurium (OVA) (52).

Studies described by Lo et al. showed induction of specific CTL responses in mice immunized i.p. with attenuated *Aro Salmonella* serovar Typhimurium and then challenged with the virulent C5 strain (24). In our studies, we observed induction of CTL activity after two i.n. doses of *Salmonella* serovar Typhi vaccine strains. Lo et al. also reported that CTL activity against *Salmonella* serovar Typhimurium-infected cells was restricted by both classical and nonclassical MHC class Ia molecules. In fact, they showed that a considerable proportion of the CTL activity against P815-infected targets (~56%) was restricted by nonclassical class Ib Qa-1^b MHC molecules (24). We found that *Salmonella* serovar Typhi-specific CTL were restricted solely by classical class I molecules and that there was no evidence of restriction by Qa-1^b molecules. In several repeat experiments we failed to block the observed CTL activity using concentrations of the anti-Qa-1^b MAb similar to those used in the studies of Lo et al. (24). This observation was supported by the fact that *Salmonella* serovar Typhi-infected allogeneic EL4 cells, which express levels of Qa-1^b similar to those expressed by P815, were not killed by expanded CTL effectors. These discrepancies are probably the result of differences between the types and/or potencies of the immune responses obtained with the different experimental approaches, our approach employing attenuated *Salmonella* serovar Typhi delivered to mice through the i.n. route and the other approach using a combination of attenuated and wild-type *Salmonella* serovar Typhimurium inoculated i.p.

A few reports have shown that H-2L molecules play a major role in restricting antigen-specific recognition to viral (5, 18, 36) and tumor antigens (2). To the best of our knowledge, our findings provide the first demonstration of a CTL response against an intracellular bacterial pathogen restricted by H-2L^d.

Surprisingly, we failed to detect CTL responses in the C57BL/6 mice despite the fact that this mouse strain is known to be susceptible to *Salmonella* infection (51) and the fact that several studies have described protective T-cell responses against *Salmonella* serovar Typhimurium in this strain (24, 27). Since it was shown that H-2L^d appears to be the major restriction element in BALB/c mice, the lack of CTL responses in C57BL/6 could be explained by the absence of the class I H-2L product gene in the H-2 haplotype in this mouse strain (38). Despite the lack of CTL responses, these mice exhibited strong antibody responses to *Salmonella* serovar Typhi LPS, indicating that they were still able to mount an immune response to *Salmonella* serovar Typhi. Induction of antibody responses to *Salmonella* serovar Typhi LPS and outer membrane proteins in C57BL/6 mice has also been reported by others (13).

The most vigorous CTL responses were observed in mice immunized with strains CVD 908-*htrA* and CVD 915. These results are consistent with our previous observations that these strains elicited the most potent serum and CMI responses against bacterial and foreign antigens in preclinical studies (32–34, 48). Phase 1 and 2 clinical trials confirmed the safety and strong immunogenicity in humans of CVD 908-*htrA* when it is given to volunteers alone or carrying a foreign antigen, and they identified this *Salmonella* serovar Typhi strain as a leading vaccine candidate (41, 43, 44). Although we cannot conclude from these observations that the murine i.n. model can predict the effectiveness of typhoid vaccines in humans, we strongly believe that the data presented here further support the use of this model as a suitable preclinical alternative to assess immunogenicity of potential *Salmonella* serovar Typhi vaccine candidates in route to human trials.

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