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H2-M3 Major Histocompatibility Complex Class Ib-Restricted CD8 T Cells Induced by *Salmonella enterica* Serovar Typhimurium Infection Recognize Proteins Released by *Salmonella* Serovar Typhimurium

S. Ugrinovic,[1]‡ C. G. Brooks,[1] J. Robson,[1] B. A. Blacklaws,[2] C. E. Hormaeche,[1,2]† and J. H. Robinson[1]*

Department of Microbiology and Immunology, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom,[1] and Centre for Veterinary Science, Department of Clinical Veterinary Medicine, University of Cambridge, Cambridge CB3 0ES, United Kingdom[2]

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***Salmonella enterica* serovar Typhimurium causes a typhoid-like disease in mice which has been studied extensively as a model for typhoid fever in humans. CD8 T cells contribute to protection against *S. enterica* serovar Typhimurium in mice, but little is known about the specificity and major histocompatibility complex (MHC) restriction of the response. We report here that CD8 T-cell lines derived from *S. enterica* serovar Typhimurium-infected BALB/c mice lysed bone marrow macrophages infected with *S. enterica* serovar Typhimurium or pulsed with proteins from *S. enterica* serovar Typhimurium culture supernatants. Cytoxicity was beta-2-microglobulin dependent and largely TAP dependent, although not MHC class Ia restricted, as target cells of several different MHC haplotypes were lysed. The data suggested the participation of class Ib MHC molecules although no evidence for the presence of Qa1-restricted T cells could be found, unlike in previous reports. Instead, the T-cell lines lysed H2-M3-transfected fibroblasts infected with *S. enterica* serovar Typhimurium SL3261 or treated with *Salmonella* culture supernatants. Thus, this report increases the number of MHC class Ib antigen-presenting molecules known for *Salmonella* antigens to three: Qa-1, HLA-E, and now H2-M3. It also expands the range of pathogens that induce H2-M3-restricted CD8 T cells to include an example of gram-negative bacteria.**

Salmonella species are intracellular gram-negative pathogens that cause a range of diseases, including typhoid fever, which, despite widespread use of antibiotics, represents a significant health problem in a number in developing nations. The recent emergence of multidrug-resistant *Salmonella* strains and the variable efficacy of existing vaccines increase the need for better understanding of the mechanisms of protective immunity to salmonellae and improvement of vaccination strategies (11, 30).

Infection of susceptible strains of inbred mice with *Salmonella enterica* serovar Typhimurium provides a murine model for typhoid fever which bears many similarities to human serovar Typhi infection (10). After oral uptake in mice, *S. enterica* serovar Typhimurium crosses the intestinal epithelium and spreads within phagocytes via the bloodstream to the spleen and liver, where the bacteria replicate (28). Innate immune mechanisms, including *Slc11a1* (formerly *Nramp1*) gene function, and production of nitric oxide and reactive oxygen intermediates are important in controlling initial bacterial growth. However, induction of the acquired immune response is necessary for clearance of infection (11).

In a primary infection, the clearance of salmonellae from infected tissues is controlled by the acquisition of specific T-cell immunity. In addition, both T and B lymphocytes are involved in protection against a secondary infection (11, 18, 19, 26). However, despite the firm evidence that T cells are indispensable in controlling *Salmonella* infection, understanding which T-cell subsets participate during distinct stages of infection is less clear. Major histocompatibility complex (MHC) class II-deficient mice fail to clear infection with an attenuated strain of *S. enterica* serovar Typhimurium and develop chronic infection, suggesting an essential role for CD4 T cells (9). However, it has also been demonstrated that beta-2-microglobulin (β2m)-deficient mice surviving primary infection with an attenuated *S. enterica* serovar Typhimurium strain suffer from impaired resistance to challenge infection with a virulent strain (16), implicating CD8 T cells in protection against *Salmonella* infection. More recently, it has been shown that CD8 T cells that secrete gamma interferon and kill *S. enterica* serovar Typhi-infected cells are elicited by immunization of volunteers with an attenuated *Salmonella* vaccine strain (27). The same group have demonstrated the concomitant induction of both CD4 and CD8 T-cell responses after vaccination with another oral typhoid vaccine candidate, CVD 908-*htrA*.

Limited information is available on the specificity and MHC restriction phenotype of T cells induced by *Salmonella* infection. *Salmonella* infection of mice has been shown to induce MHC class II-restricted CD4 T cells specific for the flagellar

* Corresponding author. Mailing address: Musculoskeletal Research Group, Clinical Medical Sciences, University of Newcastle upon Tyne, Framlington Place, Newcastle upon Tyne NE2 4HH, United Kingdom. Phone: 44 191 222 6976. Fax: 44 191 222 5455. E-mail: j.h.robinson@ncl.ac.uk.

‡ Present address: Department of Clinical Biochemistry and Immunology, Addenbrookes Hospital, Hills Road, Cambridge CB2 2QQ, United Kingdom.

† Deceased March 2005.

antigen FliC, and immunization with recombinant FliC protects mice against infection with a low dose of a virulent strain of *S. enterica* serovar Typhimurium (3, 20). CD4 T cells from infected mice also recognize the *Salmonella* invasion protein SipC, a type III secretion product (21), but a role for SipC in protection has not been reported. Similarly, the only *Salmonella* antigen so far identified that is recognized by CD8 T cells is the heat shock protein GroEL, a response that is MHC class 1b (Qa-1) restricted (16, 17). A recent report shows that a subset of human *S. enterica* serovar Typhi-specific CD8 T cells are also MHC class 1b and HLA-E restricted, but the antigen recognized has not been identified (25).

In this study, we demonstrate that cytotoxic T-cell lines from mice infected with *S. enterica* serovar Typhimurium recognize *Salmonella* proteins presented by the nonclassical MHC class Ib molecule H2-M3.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains *aro* attenuated *S. enterica* serovar Typhimurium SL3261, virulent *S. enterica* serovar Typhimurium C5, the flagellum-defective variant *S. enterica* serovar Typhimurium C5-FliA$^-$, and *Salmonella enterica* serovar Enteritidis SE795 were used in this study and grown as described elsewhere (2). *S. enterica* serovar Typhimurium C5-FliA$^-$ was grown in the presence of 25 μg/ml of tetracycline. Heat-killed *S. enterica* serovar Typhimurium SL3261 was prepared by washing bacteria in phosphate-buffered saline followed by incubation at 60°C for 3 h and washing again. Effective killing was confirmed by pour plating on LB agar plates.

Preparation of proteins released by *S. enterica* serovar Typhimurium. For the preparation of proteins, *S. enterica* serovar Typhimurium strain C5 FliA$^-$ was grown in LB medium as described above until late-exponential phase was reached. Bacteria were pelleted and proteins from filtered culture supernatant were precipitated overnight at 5°C with 40% ammonium sulfate, centrifuged at 43,000 × g, incubated at 4°C for 1 h, and dissolved in phosphate-buffered saline. The protein preparation was desalted by 10DG column passage and dialyzed according to the manufacture's protocol (Bio-Rad Laboratories Ltd., United Kingdom). Fractions were collected and assayed for protein concentration using the bicinchoninic acid protein assay kit (Pierce & Warriner, United Kingdom). Where indicated, *Salmonella* proteins were treated with 1 mg/ml of proteinase K (Sigma) for 1 h at 60°C, and dialyzed for 24 h in phosphate-buffered saline. For loading targets, cells were incubated with *S. enterica* serovar Typhimurium proteins (10 μg/ml) for 3 h at 37°C followed by washing to remove unbound proteins and proteinase K in experiments in which it was used.

Mouse strains and cell lines. We maintained 6- to 8-week-old female BALB/c (*H-2^d*), BALB.B (*H-2^b*), BALB.K (*H-2^k*), C3H/He (*H-2^k*), CBA/J (*H-2^k*), and B10.BR (*H-2^k*) mice (Harlan Olac Ltd., Bicester, United Kingdom) at the University of Newcastle upon Tyne. Cell lines J774 (*H-2^d*), IC-21 (*H-2^b*), YAC (*H-2^d*), EL-4 (*H-2^b*), RMA (*H-2^b*), and RMA-S (*H-2^b*, *TAP$^{-/-}$*) were obtained from the American Type Culture Collection. We acknowledge the kind gifts of the L cell fibroblast line (*H-2^k*) and L cells transfected with *Qa-1^b* (L-Qa-1^b) from J. Dyson, Clinical Sciences Centre, Hammersmith Hospital, London, United Kingdom; the β2m-deficient C4.4 cells (*H-2^b*) from R. Glas, Karolinska Institute, Stockholm, and the B10.CAS2 fibroblast cell line (*H-2^{w17}*) and TR8.4a, a variant of B10.CAS2 transfected with *H2-M3^{wt}* from C. R. Wang, Gwen Knapp Center for Lupus and Immunology Research, University of Chicago, Chicago, Illinois. Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 2 mM glutamine, and 50 μM 2-mercapthoethanol (RF-10). All tissue culture reagents were obtained from Sigma (United Kingdom) unless otherwise stated.

Bone marrow macrophages. Bone marrow-derived macrophages (referred to as cultured macrophages) were prepared as described elsewhere (4). Briefly, bone marrow cells were extracted from femurs and tibias and cultured for 6 days at 3 × 10^5 cells/ml in bacteriological-grade petri dishes containing RF-10 supplemented with 5% horse serum, 1 mM sodium pyruvate (Sigma), 40 μg/ml gentamicin (Sigma), and 20% conditioned medium from L929 cells as a source of macrophage colony-stimulating factor. Adherent cells were washed with phosphate-buffered saline (PBS), detached from the plastic with cold PBS containing 0.1% EDTA, washed twice with PBS, and used on days 6 to 14 of culture.

For infection cell lines were seeded at a density of 2 × 10^6 cells per well in 6 well cell culture plates in RF-10 without antibiotics. The following day the medium was removed and 1 ml of medium containing 100 × 10^6 CFU/ml of *S. enterica* serovar Typhimurium was added to each well. To facilitate infection, the plates were centrifuged at 400 × g for 10 min at room temperature, and the plates were incubated for 2 h at 37°C in 5% CO_2. The cultures were washed twice with warmed RF-10 to remove unattached bacteria and incubated for 1 h in the presence of 100 μg/ml gentamicin to kill extracellular bacteria. Cells were removed from plates by using cold PBS containing 0.1% EDTA for use in experiments.

Establishment and maintenance of T-cell lines. Female 8-week-old BALB/c mice were immunized intravenously with 10^6 SL3261 in a tail vein as described previously (19), and challenged after 5 or 8 weeks with 5 × 10^{10} CFU of *S. enterica* serovar Typhimurium C5 by the intragastric route under mild anesthesia as described previously (10). Mice were sacrificed between 1 and 5 weeks after challenge and immune splenocytes were harvested to generate T-cell lines in vitro. CD8 cells were positively selected using magnetic beads according to the manufacturer's instructions (Miltenyi Biotec, GmbH, Bergish-Gladbach, Germany). The purity of the separated cell populations was determined by flow cytometry using the following monoclonal antibodies: anti-CD3-fluorescein isothiocyanate, anti-T-cell receptor αβ-phycoerythrin, anti-T-cell receptor (TCR) γδ-phycoerythrin, anti-CD8α-fluorescein isothiocyanate, and anti-CD4-phycoerythrin (PharMingen, San Diego, CA).

We stimulated 2 × 10^6 CD8 T cells (>95% pure) with 10^5 irradiated (50 Gy) SL3261-infected J774 cells and 4 × 10^6 irradiated (20 Gy) syngeneic spleen cells in the presence of 40 μg/ml gentamicin. Recombinant human interleukin-2 (Cetus) was used at 100 IU/ml in the first week and at 10 IU/ml in the second week. Subsequent stimulations were performed fortnightly under the same conditions. Cytotoxicity assay was performed after restimulation and expansion in vitro at least three times. The phenotype of cultured T-cell lines was assessed by flow cytometry using the monoclonal antibodies described above and a mouse Vβ T-cell receptor screening panel (PharMingen). The majority of T-cell lines were CD3$^+$4$^-$8$^+$ TCRα$^+$β$^+$ Vβ8$^+$. The cytotoxic T-cell activity of lines was assayed in a standard 4-h ^{51}Cr-release assay and 19 of 25 T-cell lines were cytotoxic for J774 macrophages infected with *S. enterica* serovar Typhimurium SL3261. We selected five CD3$^+$4$^-$8$^+$ TCRα$^+$β$^+$ Vβ8$^+$ T-cell lines that were cytotoxic for SL3261-infected J774 to generate the data shown in the figures.

Cytotoxicity assay. Infected and uninfected target cells or target cells pulsed with *Salmonella* protein preparations were incubated for 1 h at 37°C with 3.7 MBq of [^{51}Cr]sodium chromate (Amersham, United Kingdom). The targets were washed, resuspended at 5 × 10^4/ml, and 100-μl aliquots were added to 100-μl serial dilutions of T cells in round-bottomed 96-well plates. After 4 h of incubation, supernatants were collected and ^{51}Cr activity was determined by gamma-scintillation spectroscopy. The percentage specific lysis was determined as follows: [(experimental release minus spontaneous release)/(maximum release minus spontaneous release)] × 100. Spontaneous release (incubation of target cells in the absence of T cells) was less than 20% of maximum release caused by Triton X detergent in all experiments except for infected J774 and cultured macrophages, in which spontaneous release was as high as 30%, presumably due to apoptosis induced by salmonellae.

RESULTS

Infection with *S. enterica* serovar Typhimurium primes cytotoxic T-cell responses in mice. Previous studies have demonstrated a role for CD8 T cells in immunity to *S. enterica* serovar Typhimurium (16, 18), but little is known about MHC restriction and specificity of the response. We generated T-cell lines from BALB/c mice that were infected with *S. enterica* serovar Typhimurium SL3261 and subsequently challenged with the virulent C5 strain. The T-cell lines used in this study were CD3$^+$4$^-$8$^+$ TCRα$^+$β$^+$ Vβ8$^+$ and cytotoxic for J774 macrophages infected with *S. enterica* serovar Typhimurium SL3261. However, T-cell lines did not lyse uninfected J774, J774 cells treated with heat-killed *S. enterica* serovar Typhimurium SL3261, or NK-sensitive YAC cells (Fig. 1a). The data are consistent with recognition of antigens synthesized and released by viable salmonellae. T-cell lines also lysed J774 macrophages infected with either *Salmonella enterica* serovar Enteritidis or *S. enterica* serovar Typhimurium with equal ef-

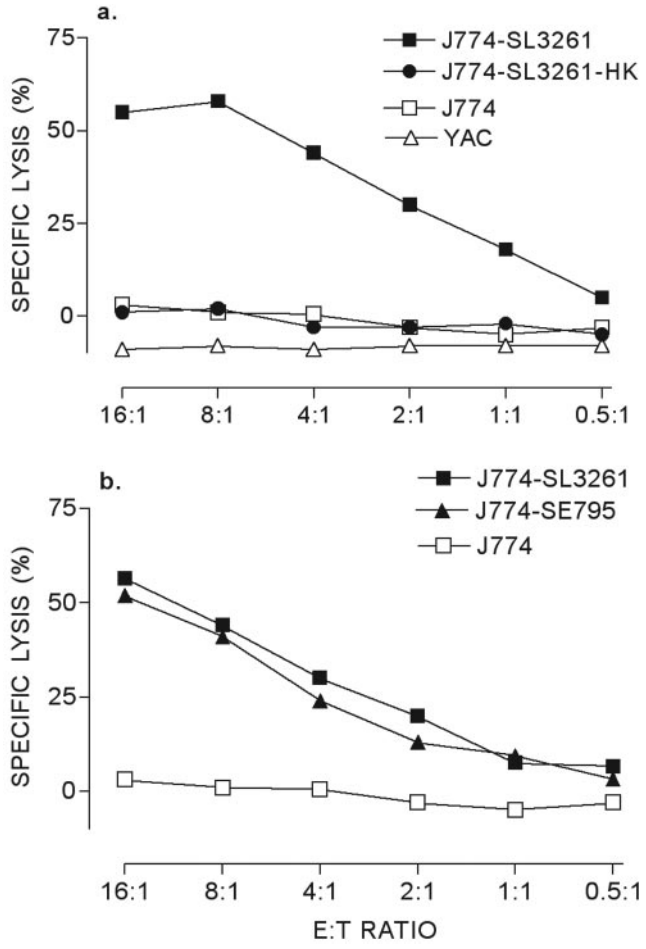

FIG. 1. CD8 T-cell lines from infected mice lyse *Salmonella enterica* serovar Typhimurium infected macrophages. BALB/c mice were infected with *S. enterica* serovar Typhimurium strain SL3261 and 4 to 8 weeks later challenged with virulent *S. enterica* serovar Typhimurium strain C5. T-cell lines from infected mice were restimulated repeatedly in vitro with SL3261-infected J774 cells and assayed for cytotoxicity against J774 untreated or infected with viable SL3261 or heat-killed SL3261 or untreated YAC (a); and J774 untreated or infected with *S. enterica* serovar Typhimurium SL3261 or *Salmonella enterica* serovar Enteritidis SE795 (b). Results from a representative of five T-cell lines are shown.

ficacy indicating shared specificity between the two *Salmonella* serovars (Fig. 1b).

***Salmonella*-induced T-cell lines recognize proteins released by salmonellae.** To assess the role of *Salmonella* secreted antigens in immunity to *Salmonella* infection, targets were sensitized with precipitated supernatants from cultured salmonellae and tested in cytotoxicity assays. T-cell lines lysed J774 cells sensitized with supernatant preparations from *Salmonella* C5FliA which lack flagellin (Fig. 2a), compared with control precipitates from LB medium alone. Furthermore, proteinase K treatment of the supernatant preparations before sensitizing target cells abrogated cytotoxicity (Fig. 2b), indicating that recognition was directed towards protein antigens.

***Salmonella* -induced T cells are not MHC class Ia-restricted.** BALB/c T-cell lines killed both J774 ($H\text{-}2^d$) and IC-21 ($H\text{-}2^b$) target cells infected with *S. enterica* serovar Typhimurium or

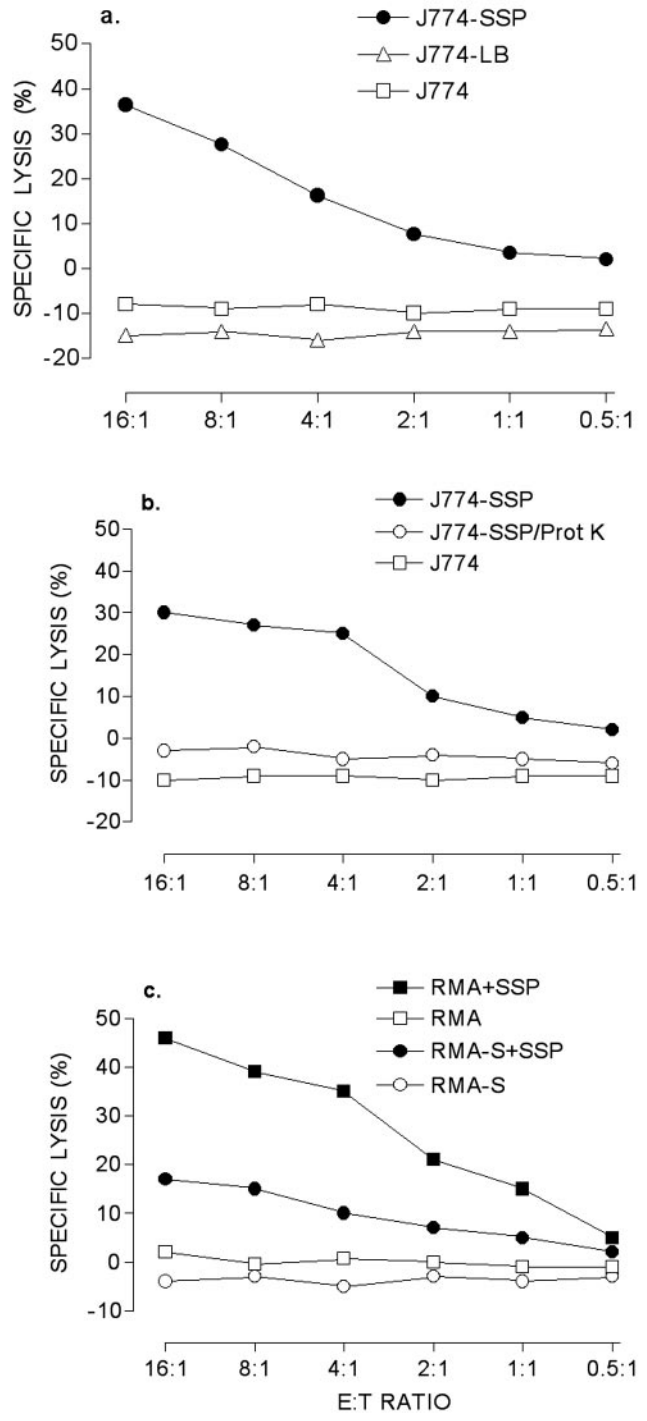

FIG. 2. CD8 T-cell lines from infected mice lyse macrophages treated *Salmonella* protein preparations. CD8 T-cell lines were assayed for cytotoxicity against J774 cells either untreated or treated with supernatant preparations from C5FliA (SSP) or LB broth (a); J774 untreated or treated with C5FliA preparations with or without proteinase K digestion (b); and RMA ($TAP^{+/+}$) and RMA-S ($TAP^{-/-}$) treated with C5FliA preparations or untreated (c). Results shown are representative of four lines tested.

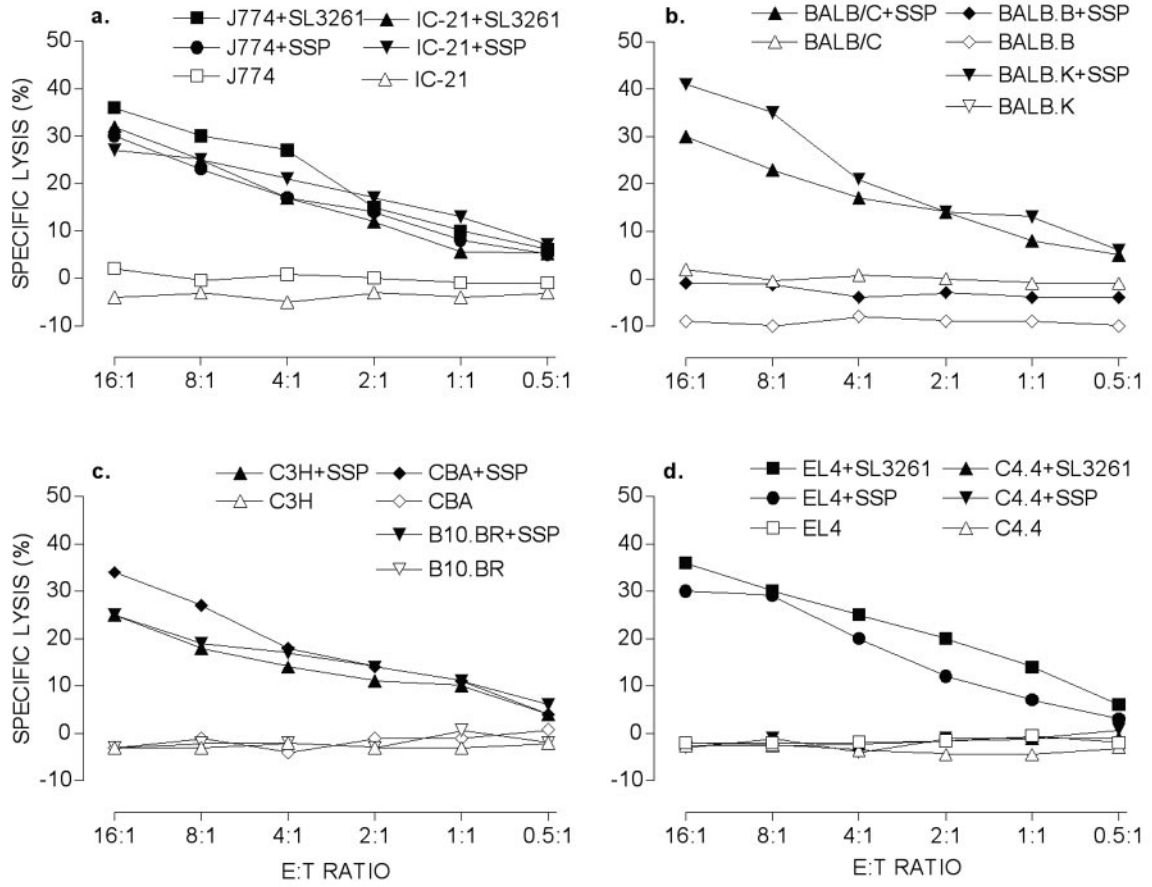

FIG. 3. MHC restriction of *Salmonella*-induced cytotoxic T cells. CD8 T-cell lines were assayed for cytotoxicity against J774 (*H-2*^{d}) or IC-21 (*H-2*^{b}) cells infected with SL3261, treated with C5FliA supernatant preparations (SSP) or untreated (a); macrophages from BALB/c, BALB.B and BALB.K mice either treated with C5FliA preparations (SSP) or untreated (b); macrophages from C3H, CBA and B10.BR mice either treated with C5FliA preparations (SSP) or untreated (c); and EL-4 ($\beta 2\ m^+$) or C4.4 ($\beta 2\ m^-$) lines either infected with SL3261, treated with C5FliA preparations (SSP) or untreated (d). Results shown are representative of four lines tested.

pulsed with *Salmonella* supernatant preparations (Fig. 3a). T-cell lines from BALB/c mice also killed antigen-pulsed bone marrow-derived macrophages from both H-2^k and *H-2*^{d} MHC haplotypes (Fig. 3b-c). Similar results were obtained when cultured macrophages were infected with SL3261 (data not shown). Antigen-pulsed BALB.B (*H-2*^{b}) macrophages were not killed for reasons that are not yet clear. However, these data clearly indicate that the cytotoxic activity of T-cell lines described here is not MHC class Ia restricted.

The TAP and β_2m dependence of antigen presentation in our system was investigated. RMA lymphoma cells pulsed with *Salmonella* supernatant preparations were lysed efficiently, whereas antigen pulsed TAP-defective RMA-S cells (17) were lysed to a much lesser extent (Fig. 2c), suggesting that presentation of *Salmonella* antigens was largely TAP dependent. The same pattern of CD8 T-cell lysis was observed when *TAP*$^{+/+}$ and *TAP*$^{-/-}$ target cells were infected with *S. enterica* serovar Typhimurium SL3261 (data not shown). T-cell lines also recognized infected or *Salmonella* antigen-pulsed EL-4 but not the β2m-deficient counterpart C4.4 (5) (Fig. 3d), showing that presentation of *Salmonella* antigens to the T-cell lines described here was β2m-dependent.

***Salmonella*-induced T cells are H2-M3 restricted.** The B10.CAS2-transfected fibroblast line was derived from the H2-M3 deficient mouse strain B10.CAS2, and TR8.4a is a variant of the B10.CAS2 line transfected with H2-*M3*^{wt} (29). Our BALB/c T-cell lines lysed antigen-pulsed H2-M3 transfected TR8.4a cells while failing to lyse antigen-pulsed H2-M3-deficient B10.CAS2 cells (Fig. 4), strongly implicating the MHC class 1b molecule H2-M3 in restriction of the responses we describe here. It has previously been reported that CD8 T cells from *Salmonella* infected mice predominantly recognized a peptide representing amino acids 192 to 200 from the *Salmonella* heat shock protein GroEL presented by Qa-1^b (16, 17). In addition, these CD8 T cells cross-reacted with peptide 216 to 224 from the endogenous mouse heat shock protein hsp60 (16, 17). The BALB/c T-cell lines generated in our system failed to lyse J774 treated with either the GroEL or hsp60 peptide whereas the a2d T-cell clone that recognizes Qa-1/Qdm (17), efficiently killed hsp peptide-pulsed J774 cells (Fig. 5a), confirming expression of functional Qa-1^b molecules. It is noteworthy that our T-cell lines lysed infected or antigen-pulsed L cells that lack Qa-1 as efficiently as antigen-pulsed Qa-1^b transfected L cells (Fig. 5b-c). Taken together, these

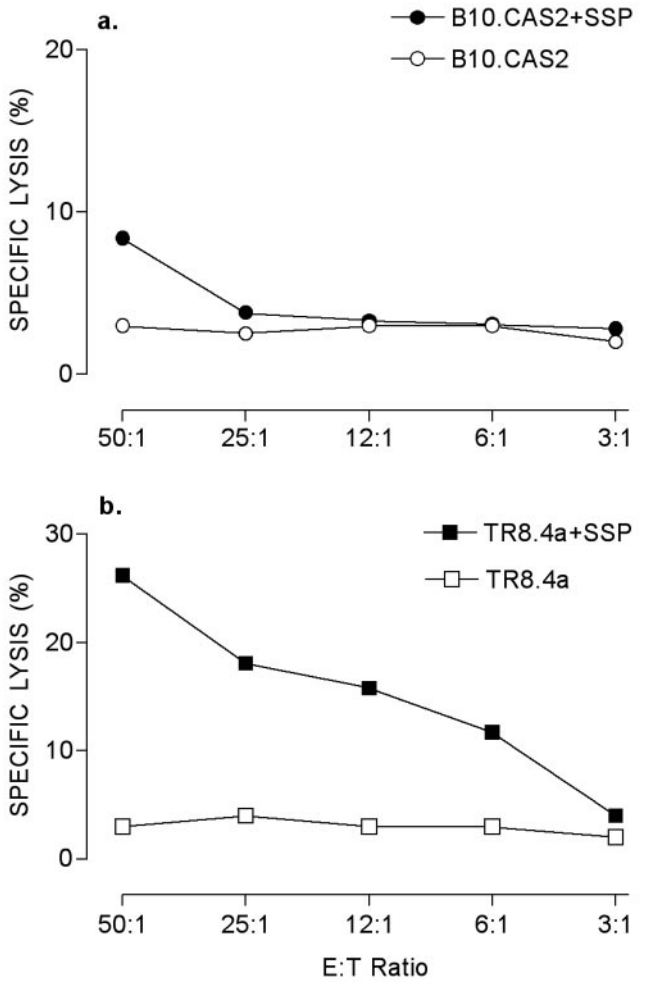

FIG. 4. *Salmonella*-induced T cytotoxic cells are H2-M3 restricted. CD8 T-cell lines were assayed for cytotoxicity against the cell line B10.CAS2 either treated with C5FliA proteins (SSP) or untreated (a); or the TR8.4a fibroblast cell line either treated with C5FliA proteins (SSP) or untreated (b). Results shown are representative of four lines tested.

data suggest that CD8 T cells in our system are H2-M3-, and not Qa-1^b-restricted.

DISCUSSION

We investigated memory CD8 T cells induced by infection and challenge of mice with *S. enterica* serovar Typhimurium. CD8 T-cell lines were generated from BALB/c mice following infection with the attenuated *S. enterica* serovar Typhimurium strain SL3261 and challenge with the virulent strain C5. T-cell lines lysed J774 macrophages infected with viable *S. enterica* serovar Typhimurium, but not J774 treated with heat-killed *S. enterica* serovar Typhimurium. Responses cross-reacted between *S. enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis. The T-cell lines also efficiently lysed macrophages treated with precipitates from *Salmonella* culture supernatants, but not supernatants digested with proteinase K. The data strongly implicated proteins synthesized and released by viable salmonellae as the principal antigens recognized by

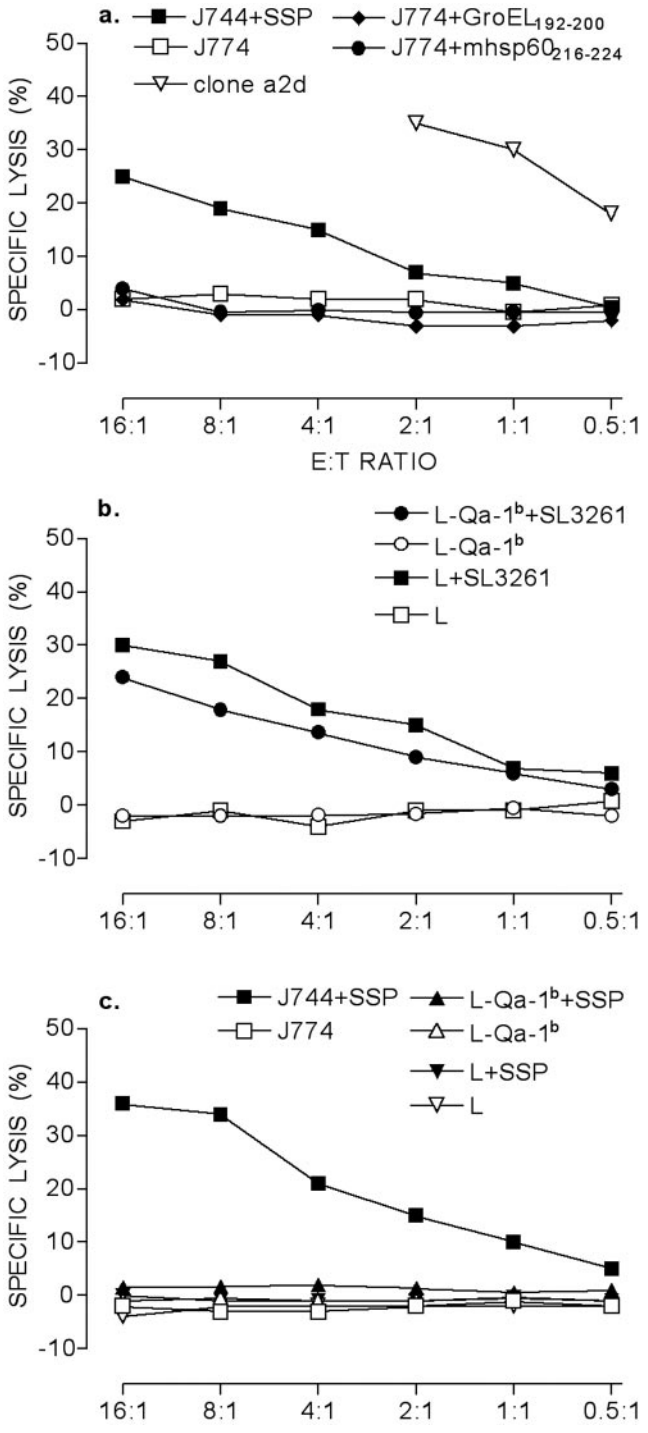

FIG. 5. *Salmonella*-induced cytotoxic T cells are not Qa-1 restricted. Representative CD8 T-cell lines were assayed for cytotoxicity against: J774 treated either with C5FliA proteins (SSP), GroEL$_{192–200}$, mhsp60$_{216–224}$ or untreated, and the Qa-1/Qdm-specific T-cell clone a2d was tested for cytotoxicity against Qa-1^b-transfected L cells treated with the mhsp60$_{216–224}$ peptide (a); Qa-1^b-transfected L cells (L-Qa-1^b) or untransfected L cells (L), either infected with SL3261 or uninfected (b); or J774, L-Qa-1^b and L cells either treated with C5FliA proteins (SSP) or untreated (c). Results shown are representative of three lines tested.

CD8 T cells in our system. Proteins released by salmonellae have not been implicated in recognition by *Salmonella*-specific CD8 T-cell responses previously, although secreted antigens have been shown to be superior carriers for the delivery of exogenous vaccine epitopes expressed by attenuated *S. enterica* serovar Typhimurium (7, 8, 23, 24).

We examined a range of wild-type and transfected target cells to determine the route of presentation and the antigen presenting molecules required for recognition of salmonellae antigens by CD8 T-cell lines. *Salmonella*-specific T-cell lines efficiently lysed the *TAP*-expressing RMA lymphoma cell line pulsed with *Salmonella* proteins or infected with *S. enterica* serovar Typhimurium, whereas similarly treated *TAP*-defective RMA-S cells were lysed only weakly. Thus, CD8 T-cell responses to viable salmonellae or proteins released from them were largely TAP dependent. However, the low level of cytotoxicity of T-cell lines against TAP-defective RMA-S targets suggest that our T-cell lines include other, TAP-independent specificities.

We further showed that presentation was β2m dependent using the paired cell lines EL-4 and the β2m-deficient counterpart C4.4 as targets. Consistent with these findings, a previous report has shown that β2m knockout mice show a dramatic increase in susceptibility to infection with *S. enterica* serovar Typhimurium (16), although an earlier report demonstrated that β2m knockout mice were resistant (9). The discrepancy is probably related to differences in both the mouse and *Salmonella* strains used in the two studies.

Our T-cell lines were cytotoxic for a variety of antigen-pulsed or infected targets of four MHC haplotypes, *H-2*^{d} (J774, IC21), *H-2*^{b} (EL-4, RMA), *H-2*^{wt17} (B10.CAS2) and *H-2*^{k} (L cells), clearly showing that the T-cell specificity is not MHC class Ia restricted. Previous studies have also uniformly failed to demonstrate a major contribution of MHC class Ia-restricted responses among the CD8 T cells induced by *S. enterica* serovar Typhimurium infection (11). One explanation is the recently demonstrated interference with peptide presentation by MHC class Ia molecules by-products of the *Salmonella yej* operon, the first such virulence mechanism to be identified in bacteria, although a common mechanism of viral immune evasion (23).

MHC class Ia-restricted T-cell responses may not be completely lacking as anti-murine MHC class Ia antibodies partially inhibited cytoxicity in the CD8 T-cell responses reported by Lo, et al. (16). Thus, MHC class Ib-restricted CD8 T-cell responses may predominate in the immune response during *S. enterica* serovar Typhimurium infection. However, *S. enterica* serovar Typhi has been shown to induce MHC-restricted cytotoxic CD8 T-cell responses in BALB/c mice that were largely inhibited by monoclonal antibodies to H-2L^{d} (22). Also, anti-HLA MHC class Ia antibodies inhibited *Salmonella enterica* serovar Typhi-specific cytotoxicity in human attenuated *S. enterica* serovar Typhi vaccinees (27). It remains to be determined whether *S. enterica* serovar Typhi lacks a homologue of the *yej*-mediated immune evasion mechanism of *S. enterica* serovar Typhimurium, which would explain the apparent difference in the predominant cytotoxic T-lymphocyte specificity between these two distinct *S. enterica* serovars.

In view of the lack of MHC class Ia restriction but β2m and TAP dependence of T-cell cytotoxicity in our system, we examined the possibility of MHC class Ib-restricted responses. We compared the lytic activity of CD8 T-cell lines for antigen-pulsed B10.CAS2 fibroblast cells with and without transfection with *M3*^{wt}, and the results clearly showed an H2-M3 dependence for the majority of the cytotoxic activity. The MHC class Ib molecule H2-M3 has been implicated in restriction of CD8 TCRαβ cytotoxic T-cell responses for a number of *N*-formylated hydrophobic peptides derived from mitochondria and two bacterial species, *Listeria monocytogenes* and *Mycobacterium tuberculosis* (13, 15). Restriction of T-cell recognition to a nonformylated peptide bound to H2-M3 has also been reported (1).

H2-M3 is associated with β2m (15), consistent with the β2m dependence of the cytotoxicity shown here. We demonstrated that cytotoxicity was TAP dependent when either viable salmonellae or *Salmonella* protein preparations were used to sensitize target cells. Previously identified H2-M3 ligands vary in their dependence on TAP-mediated transport, responses to *Listeria* antigens being TAP-independent (13, 14), whereas mitochondrial ligands for H2-M3 require TAP-dependent transport (15). Our data suggests that *Salmonella* antigens delivered in the form of viable salmonellae or as released proteins are presented via a cytosolic route requiring TAP-mediated transport into the endoplasmic reticulum, although we have not investigated the mechanisms of antigen processing and presentation any further.

We examined whether our T-cell lines exhibited Qa-1-restricted cytotoxicity. The T-cell lines we generated were equally cytotoxic for L cells whether or not transfected with the Qa-1^{b} molecule. Thus, most T cells in our lines were not Qa-1-restricted, in contrast to previous reports (16, 17). We further eliminated the possibility of Qa-1-restricted responses by showing that our lines were not cytotoxic for target cells treated with the peptide epitopes from the *Salmonella* heat shock protein GroEL or endogenous mouse hsp60 identified in the previous studies (16, 17). The discrepancy in results may be due to the different mouse strain (CB6F1) and *Salmonella* strains (SL3235 infected and C5 challenged) used in the experiments used to demonstrate Qa-1 restriction.

H2-M3-restricted, *Listeria*-specific cytotoxic T cells have been shown to provide protective immunity against listerial infection, indicating that these MHC class Ib-restricted responses can play a role in host defense against intracellular bacterial infection (13, 15). However, the antilisterial function of the H2-M3-restricted CD8 T-cell response appears to be limited to resistance against primary infection, indicating that MHC class Ib–restricted CD8 T cells serve as a bridge between the very rapid innate immune response and the slower developing MHC class Ia–restricted T-cell response (13, 15). Indeed, during *Listeria* infection it has been shown that expansion of MHC class Ib-restricted memory T cells is suppressed by the development of MHC class Ia-restricted memory T cells (6, 12).

We have generated T-cell lines from mice both infected and challenged with salmonellae, suggesting that H2-M3-restricted responses are detectable in the spleens of mice during the induction of a memory T-cell response. We suggest that MHC class Ib-restricted T cells, whether H2-M3 or Qa-1 restricted, may predominate in *Salmonella* infection due to the poor induction of MHC class Ia-restricted T cells and their conse-

quent regulatory function upon challenge. However, we do not know if H2-M3-restricted T cells play any role in the protective immune response during *Salmonella* infection.

The antigen or peptide recognized by T-cell lines in this study remains unknown. However, cytotoxicity of macrophages infected with viable, but not heat-killed, *S. enterica* serovar Typhimurium, and the lack of lysis of macrophages incubated with supernatant proteins treated with proteinase K suggest antigens that are secreted proteins that would be largely removed by washing prior to the heat treatment. Alternatively, phagocytosis and processing of heat killed bacteria may not generate sufficient amounts of antigenic peptides to enter the MHC class I pathway. Otherwise, we have no information on which *Salmonella* protein(s) is recognized by CD8 T cells in our system, although a formylated *Salmonella* peptide is a likely candidate, by analogy with the other known ligands for H2-M3. However, our demonstration of H2-M3-restricted T-cell recognition induced by *Salmonella* infection extends the range of previously reported H2-M3-restricted responses to include a member of the *Enterobacteriaceae*.

REFERENCES

1. **Byers, D. E., and K. F. Lindahl.** 1998. H2–M3 presents a nonformylated viral epitope to CTLs generated In vitro. J. Immunol. **161:**90–96.
2. **Catic, A., G. Dietrich, I. Gentschev, W. Goebel, S. H. Kaufmann, and J. Hess.** 1999. Introduction of protein or DNA delivered via recombinant *Salmonella typhimurium* into the major histocompatibility complex class I presentation pathway of macrophages. Microbes Infect. **1:**113–121.
3. **Cookson, B. T., and M. J. Bevan.** 1997. Identification of a natural epitope presented by Salmonella-infected macrophages and recognized by T cells from orally immunized mice. J. Immunol. **158:**4310–4319.
4. **Fischer, H. G., B. Opel, K. Reske, and A. B. Reske-Kunz.** 1988. Granulocyte-macrophage colony-stimulating factor-cultured bone marrow-derived macrophages reveal accessory cell function and synthesis of MHC class II determinants in the absence of external stimuli. Eur. J. Immunol. **18:**1151–1158.
5. **Glas, R., K. Sturmhöfel, G. J. Hämmerling, K. Kärre, and H.-G. Ljunggren.** 1992. Restoration of a tumorigenic phenotype by beta 2-microglobulin transfection to EL-4 mutant cells. J. Exp. Med. **175:**843–846.
6. **Hamilton, S. E., B. B. Porter, K. A. Messingham, V. P. Badovinac, and J. T. Harty.** 2004. MHC class Ia-restricted memory T cells inhibit expansion of a nonprotective MHC class Ib (H2–M3)-restricted memory response. Nat. Immunol. **5:**159–168.
7. **Hess, J., I. Gentschev, D. Miko, M. Welzel, C. Ladel, W. Goebel, and S. H. Kaufmann.** 1996. Superior efficacy of secreted over somatic antigen display in recombinant Salmonella vaccine induced protection against listeriosis. Proc. Natl. Acad. Sci. USA. **93:**1458–1463.
8. **Hess, J., L. Grode, J. Hellwig, P. Conradt, I. Gentschev, W. Goebel, C. Ladel, and S. H. Kaufmann.** 2000. Protection against murine tuberculosis by an attenuated recombinant *Salmonella typhimurium* vaccine strain that secretes the 30-kDa antigen of Mycobacterium bovis BCG. FEMS Immunol. Med. Microbiol. **27:**283–289.
9. **Hess, J., C. Ladel, D. Miko, and S. H. Kaufmann.** 1996. Salmonella typhimurium aroA-infection in gene-targeted immunodeficient mice: major role of CD4+ TCR-alpha beta cells and IFN-gamma in bacterial clearance independent of intracellular location. J. Immunol. **156:**3321–3326.
10. **Hormaeche, C. E., H. S. Joysey, L. Desilva, M. Izhar, and B. A. Stocker.** 1991. Immunity conferred by Aro-Salmonella live vaccines. Microb. Pathog. **10:**149–158.
11. **Jones, B. D., and S. Falkow.** 1996. Salmonellosis: Host immune responses and bacterial virulence determinants. Annu. Rev. Immunol. **14:**533–561.
12. **Kerksiek, K. M., A. Ploss, I. Leiner, D. H. Busch, and E. G. Pamer.** 2003. H2–M3-restricted memory T cells: persistence and activation without expansion. J. Immunol. **170:**1862–1869.
13. **Lenz, L. L., and M. J. Bevan.** 1996. H2–M3 restricted presentation of *Listeria monocytogenes* antigens. Immunol. Rev. **151:**107–121.
14. **Lenz, L. L., B. Dere, and M. J. Bevan.** 1996. Identification of an H2–M3-restricted Listeria epitope: implications for antigen presentation by H2–M3. Immunity **5:**6–72.
15. **Lindahl, K. F., D. E. Byers, V. M. Dabhi, R. Hovik, E. P. Jones, G. P. Smith, C. R. Wang, H. Xiao, and M. Yoshino.** 1997. H2–M3, a full-service class Ib histocompatability antigen. Annu. Rev. Immunol. **15:**851–879.
16. **Lo, W. F., H. Ong, E. S. Metcalf, and M. J. Soloski.** 1999. T-cell responses to Gram-negative intracellular bacterial pathogens: a role for CD8 T cells in immunity to Salmonella infection and the involvement of MHC class Ib molecules. J. Immunol. **162:**5398–5406.
17. **Lo, W. F., A. S. Woods, A. DeCloux, R. J. Cotter, E. S. Metcalf, and M. J. Soloski.** 2000. Molecular mimicry mediated by MHC class Ib molecules after infection with gram-negative pathogens. Nat. Med. **6:**215–218.
18. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche.** 1993. Adoptive transfer of immunity to oral challenge with virulent salmonellae in innately susceptible BALB/c mice requires both immune serum and T cells. Infect. Immun. **61:**3981–3984.
19. **Mastroeni, P., B. Villarreal-Ramos, and C. E. Hormaeche.** 1992. Role of T cells, TNF alpha and IFN gamma in recall of immunity to oral challenge with virulent salmonellae in mice vaccinated with live attenuated aro-Salmonella vaccines. Microb. Pathog. **13:**477–491.
20. **McSorley, S. J., B. T. Cookson, and M. K. Jenkins.** 2000. Characterization of CD4+ T-cell responses during natural infection with *Salmonella typhimurium*. J. Immunol. **164:**986–993.
21. **Musson, J. A., R. D. Hayward, A. A. Delvig, R. Hernan, C. E. Hormaeche, V. Koronakis, and J. H. Robinson.** 2002. Processing of *Salmonella typhimurium* for presentation of a CD4 T-cell epitope from *Salmonella* invasion protein C (SipC). Eur. J. Immunol. **32:**2664–2671.
22. **Pasetti, M. F., R. Salerno-Goncalves, and M. B. Sztein.** 2002. *Salmonella enterica* typhi live vector vaccines delivered intranasally elicit regional and systemic specific $CD8^+$ major histocompatibility class I-restricted cytotoxic T lymphocytes. Infect. Immun. **70:**4009–4018.
23. **Qimron, U., N. Madar, H.-W. Mittrücker, A. Zilka, I. Yosef, N. Bloushtain, S. H. E. Kaufmann, I. Rosenshine, R. N. Apte, and A. Porgador.** 2004. Identification of *Salmonella typhimurium* genes responsible for interference with peptide presentation on MHC class I molecules: D*yej Salmonella* mutants induce superior CD8+ T-cell responses. Cell. Microbiol. **6:**1057–1070.
24. **Russmann, H., H. Shams, F. Poblete, Y. Fu, J. E. Galan, and R. O. Donis.** 1998. Delivery of epitopes by the Salmonella type III secretion system for vaccine development. Science **281:**565–568.
25. **Salerno-Goncalves, R., M. Fernandez-Vina, D. M. Lewinsohn, and M. B. Sztein.** 2004. Identification of a human HLA-E-restricted CD8+ T-cell subset in volunteers immunized with Salmonella enterica serovar Typhi strain Ty21a typhoid vaccine. J. Immunol. **173:**5852–5862.
26. **Sinha, K., P. Mastroeni, J. Harrison, R. D. de Hormaeche, and C. E. Hormaeche.** 1997. *Salmonella typhimurium aroA*, *htrA*, and *aroD htrA* mutants cause progressive infections in athymic (*nu*/*nu*) BALB/c mice. Infect. Immun. **65:**1566–1569.
27. **Sztein, M. B., M. K. Tanner, Y. Polotsky, J. M. Orenstein, and M. M. Levine.** 1995. Cytotoxic T lymphocytes after oral immunization with attenuated vaccine strains of Salmonella typhi in humans. J. Immunol. **155:**3987–3993.
28. **Vazquez-Torres, A., J. Jones-Carson, A. J. Baumler, S. Falkow, R. Valdivia, W. Brown, M. Le, R. Berggren, W. T. Parks, and F. C. Fang.** 1999. Extraintestinal dissemination of Salmonella by CD18-expressing phagocytes. Nature **401:**804–808.
29. **Wang, C.-R., B. E. Loveland, and K. F. Lindahl.** 1991. H-2M3 encodes the MHC class I molecule presenting the maternally transmitted antigen of the mouse. Cell **66:**335–345.
30. **Wong, P., and E. Pamer.** 2003. CD8 T-cell responses to infectious pathogens. Annu. Rev. Immunol. **21:**29–70.

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