MHC class II antigen presentation by B cells in health and disease

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Chapter 4

Phagocytosis of *Salmonella* by B cells generates an effective cytotoxic T cell response via cross-presentation of *Salmonella*-antigens

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Abstract

The eradication of facultative intracellular bacterial pathogens, like *Salmonella typhi*, requires the concerted action of both the humoral immune response and the cytotoxic $CD8^+$ T cell response. Dendritic cells (DCs) are considered to orchestrate the cytotoxic $CD8^+$ T cell response via cross-presentation of bacterial antigens onto MHC class I molecules. Cross-presentation of *Salmonella* by DCs however, is accompanied by the induction of apoptosis in the DCs. Besides antibody production, B cells are required to clear *Salmonella* infection for other unknown reasons. Here we show that *Salmonella*-specific B cells that phagocytose *Salmonella* upon BCR-ligation are able to activate human $CD8^+$ memory T cells via cross-presentation yielding a *Salmonella*-specific cytotoxic T cell response. Unlike the DCs, B cell-mediated cross-presentation of *Salmonella* does not coincide with apoptosis. Thus, B cells are a viable alternative for DC in the activation of the cytotoxic effector arm and the generation of effective adaptive immunity against *Salmonella* reinfection.

Introduction

Salmonella is a pathogenic bacterium that causes severe disease in mice and man. *Salmonella typhi* (*Salmonella enterica* serovar Typhi) causes invasive diseases in human, which has many features in common with *Salmonella typhimurium* in mice. The gastrointestinal tract is the major site of primary infection of the host and has to be passed before systemic infection can occur. One way to infect the host cells is via sampling of bacteria by DCs in the intestine. *In vitro* studies showed that DCs located in the lamina propria under the gut epithelium of the small bowel extend processes across the tight junctions between the epithelial cells and capture bacteria from the luminal side of the gut (1,2). The major route of infection however, is via microfold cells or M cells (3,4). The specialized antigen-sampling M cells are located in the dome region of the Peyer's Patches and are efficient in transportation of macromolecules and microorganisms to the underlying immune cells (2,5). Like other Gram-negative bacteria, *Salmonella* uses specific virulence factors to invade other cell types, called the Type III Secretion System (TTSS). Many *Salmonella* virulence genes are clustered in *Salmonella* pathogenicity islands (SPIs). SPI-1 and SPI-2 encode TTSSs that mediate the injection of effector proteins into the host cell cytoplasm via sophisticated secretion devices (6). SPI-1 is associated with invasion of intestinal epithelia and enhanced intestinal inflammation in the infected host (7,8). SPI-2 modulates intracellular trafficking and enables replication within a modified vacuolar compartment, called the *Salmonella*-containing vacuole (SCV) (9-11) and enhances inflammation during enteric phase (12,13). *Salmonella* activates the PKB/Akt1 pathway to prevent maturation of SCV into destructive phagolysosomes, thus manipulating the host for its own survival (14).

After transcytosis by M cells, *Salmonella* reaches the subepithelial dome of the Peyer's patches and encounters an extensive network of resident macrophages, DCs and great numbers of B cells (15,16). Instead of being immediately destroyed by these cells, *Salmonella* have evolved several mechanisms to survive in the harsh milieu of phagosomal compartments (17) and can be cytotoxic to macrophages by inducing apoptosis *in vitro* (18,19).

Recently, we showed that recognition of *Salmonella* via the specific B cell receptor (BCR) on B cells results in internalization of *Salmonella*. *Salmonella* is able to survive intracellularly in primary B cells in a non-replicative state (20). Following uptake of *Salmonella*, B cells do not go into apoptosis, but differentiate and start to

produce *Salmonella*-specific antibodies. In addition, BCR-mediated phagocytosis of *Salmonella* by B cells leads to antigen presentation via MHC class II and subsequent $CD4^+$ T cell activation, which in turn boosts antibody production by the infected B cell.

Antibody transfer studies have shown that the requirement for B cells in the clearance of *Salmonella* does not solely depend on antibody formation (21). Which additional immune responses need B cell involvement remains unclear. For clearance of *Salmonella*, not only the humoral immune response is required, but also the activation of cytotoxic $CD8^+$ T cells is needed to eliminate *Salmonella*-infected cells. Recently, DCs have been shown to prime *Salmonella*-specific $CD8^+$ memory T cells after direct uptake of bacteria or via suicide cross-presentation after uptake of *S. typhi*-infected human cells (22). As the generation of *Salmonella* antigens for MHC class II molecules is an efficient process in infected B cells, we tested whether BCR-mediated phagocytosis also leads to cross-presentation of *Salmonella* antigens via the MHC class I pathway of B cells and whether this elicits a cytotoxic T cell response against *Salmonella*-infected cells.

Here we show that *Salmonella*-specific primary B cells that have internalized *Salmonella* do cross-present *Salmonella* antigens via MHC class I molecules in a proteasome-dependent manner. Subsequently, we demonstrate that cross-presentation of *Salmonella* antigens by B cells activates *Salmonella*-specific $CD8^+$ memory cells that acquire a cytotoxic phenotype and are efficient in the killing of *Salmonella*-infected cells. Thus, B cells are an unappreciated source of cells cross-presenting bacterial antigens for T cell stimulation.

Materials and methods

Antibodies and fluorophores

mAb anti-human IgM (MH15, Sanquin, Amsterdam, The Netherlands) was mixed with rat anti-mouse IgG1 antibody (RM161.1, Sanquin) and mAb anti-*S. typhimurium* LPS (1E6, Biodesign International, Kennebunk, ME) to generate BCR-LPS tetrameric antibody complexes, used to coat bacteria as previously described (20).

The following labeled anti-human mAbs were obtained from BD Biosciences (San Jose, CA): anti-IFN-γ-FITC, anti-CD27-PE, anti-CD107a-PE, anti-CD8-PerCP-Cy5.5, anti-CD4-APC, anti-CD45RO-PE, AnnexinV-APC and IgG1-PerCP-Cy5.5 isotype control. FITC-conjugated antibody IgG1, IgG2a and IgG2b, IgG1-PE and IgG-APC

isotype controls were obtained from DAKO (Glostrup, Denmark). Anti-CD45RA-FITC and anti-CD45RO-FITC were obtained from Sanquin and DAPI from Sigma-Aldrich (Steinheim, Germany). CFSE (Invitrogen, Paisley, UK) labeling was used in proliferation assays.

Bacterial growth conditions

GFP expressing-*S. typhimurium* SL1344 was described before (23). GFP-*Salmonella* defective in SPI-1 (invA mutant) or SPI-2 (ssrA mutant) were a kind gift of M. Rescigno (European Institute of Oncology, Milan, Italy). *Staphylococcus aureus* expressing GFP (RN4220 with pWVW189GFP) was kindly provided by S. A. J. Zaat (Academic Medical Center, Amsterdam, The Netherlands). All bacteria strains were grown overnight at 37ºC in Luria-Bertani (LB) broth with carbenicillin or chloramphenicol (Sigma-Aldrich, St Louis, MO) to maintain GFP expression while shaking, subcultured at a dilution of 1:33 in fresh LB medium and incubated while shaking at 37ºC for 3 to 4 hours to obtain exponentially growing bacteria. For coating, bacteria were washed twice with PBS and incubated with BCR-LPS tetrameric antibody complexes for 30 minutes at room temperature and washed twice with PBS to remove unbound antibodies. For experiments with dead *Salmonella*, bacteria were heat killed by incubation at 65°C for 15 minutes.

***Lymphocyte isolation and B lymphocyte infection with* Salmonella**

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS, Oslo, Norway) from a buffycoat obtained from healthy donors after informed consent (Sanquin). B and T cells were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads and DETACHaBEAD (Invitrogen), according to the manufacturer's instructions.

B lymphocytes were incubated for 45 minutes at 37°C with *Salmonella* without antibiotics. Next, cells were washed to remove unbound bacteria four times and cultured for 1 hour in medium containing 100 µg/ml gentamycin (Invitrogen) to eliminate non-phagocytosed bacteria. Cells were washed and cultured in RPMI 1640 medium w/o phenol red (Lonza, Basel, Switserland), supplemented with 5% FCS (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-Glutamine, 50 µM 2-ME, 20 µg/ml human apo-transferrin ((Sigma-Aldrich), depleted for human IgG with protein G sepharose (Amersham, Uppsala, Sweden)) and 10 µg/ml gentamycin.

Flow cytometry

$1*10^5$ B cells, or *Salmonella*-infected B cells were cultured with $5*10^4$ CFSE-labeled T cells ($CD8^+$ T cells alone or in 1:1 ratio with $CD4^+$ T cells) for 6 days to activate and expand *Salmonella*-specific T cells. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD. To test plasma membrane markers, $1*10^5$ B cells with $5*10^4$ T cells were cultured for 6 days and after addition of 10 IU/ml IL-2 (Chiron, Emeryville) for another 6 days. All plasma membrane stainings were performed for 15 minutes at room temperature and washed after each incubation with PBS containing 0.1% BSA. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD). Lymphocytes were gated by forward and side scatter. Dead cells were excluded based on their positive reaction to DAPI staining.

For some experiments, naïve T cells were sorted as $CD8^+CD45RA^+CD45RO^-$ (T_N) and memory T cells as $CD8^+CD45RA^-CD45RO^+$ (T_{MEM}), and $CD8^+CD45RO^+CD27^-$ (T_{EM}) and $CD8^+CD45RO^+CD27^+$ (T_{CM}) cells. Populations were >98% purified.

Intracellular cytokine staining

B cells and T cells were cultured for 12 days and cytokine production was measured by intracellular staining after restimulation with 0.1 µg/ml PMA, 1 µg/ml ionomycin and 10 µg/ml brefeldin A (Sigma-Aldrich) for 5 hours. Cells were washed twice with PBS, fixed with 1% formaldehyde (Merck, Darmstadt, Germany) for 15 minutes and after washing twice with PBS, permeabilized with 0.5% saponin (Calbiochem, CA) in PBS containing 1% BSA (Sigma-Aldrich) and incubated with fluorescent antibodies for 30 minutes at room temperature. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD).

$CD8^+$ degranulation assay

$CD8^+$ T cells were primed by 6 day incubation of $1*10^5$ *Salmonella*-infected B cells with $2.5*10^5$ $CD4^+$ T cells and $2.5*10^5$ $CD8^+$ T cells. The dividing T cells ($CD8^+CFSE^{low}$) were FACS sorted after 6 days on a MoFlo Sorter (Dakocytomation, Glostrup, Denmark) and cultured with 50 IU/ml IL-2 for 6 more days. Next, isolated autologous B cells were thawed and infected with *Salmonella*. For proteasome inhibition, MG-132 (Sigma-Aldrich) was added at a concentration of 20 µM before incubation with bacteria. *Salmonella*-infected B cells were incubated in medium containing 10 µg/ml gentamycin for 15 hours at 37ºC to allow processing and

presentation of *Salmonella* antigens. Subsequently, the B cells were incubated at 37ºC for 5 hours together with the primed *Salmonella*-specific $CD8^+$ T cells in a ratio of 4:1, in the presence of anti-CD107a-PE labeled antibodies. Cells were washed twice with wash buffer (1 mM HEPES, 0.15 M NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 M $MgCl_2$, 0.1% BSA) and stained for CD8 and AnnexinV. After washing twice with washing buffer, DAPI was added and $CD8^+$ T cells were analyzed for CD107a expression. 50,000 events were acquired on a LSR II (BD) and analyzed with FACSDiva software (BD).

^{51}Cr release assay

Salmonella-specific $CD8^+$ T cells were activated and FACS sorted as described and expanded with 50 U/ml IL-2. Autologous B cells were thawed and infected with *Salmonella*. After 15h, B cells were labeled with ^{51}Cr (185MBq/ml; Perkin Elmer, Boston, MA) for 45 minutes at 37°C. After washing, the B cells were incubated in a 96-wells U-bottom plate (Costar Corning Inc., NY) with primed $CD8^+$ T cells in a 1:2 ratio. Incubation in medium or in Triton X-100 (1% final concentration; Merck) was used to determine spontaneous and maximum ^{51}Cr release, respectively. ^{51}Cr release was measured in the supernatant using filters with a gamma counter (Cobra II, Canberra Packard, Mississauga, Canada). The percentage of specific cell lysis was calculated using the following formula:

$$\%\ \text{specific lysis} = \frac{[\text{experimental release (cpm)} - \text{spontaneous release (cpm)}]}{[\text{maximal release (cpm)} - \text{spontaneous release (cpm)}] \times 100\%}$$

Statistical analysis

Statistical differences were determined by a paired Student's t test, using GraphPad Prism (version 4.03, GraphPad Software, San Diego, CA)

Results

Salmonella*-infected B cells initiate a $CD8^+$ T cell response*

To study MHC class I antigen presentation by B cells, we used *Salmonella typhimurium* as a model for cross-presentation against facultative intracellular bacteria. Previously, we showed that about 4% of the B cells recognize *Salmonella* by their BCR, and subsequently initiate a $CD4^+$ T cell response (20). To study T cell

responses in detail, we enhanced the uptake of *Salmonella* by B cells by coating *Salmonella* with a tetrameric antibody complex, consisting of anti-LPS antibodies and anti-IgM-BCR antibodies. As a result, all B cells expressing an IgM-BCR, recognize *Salmonella* and are able to phagocytose the bacterium via their BCR. This resulted in an uptake of *Salmonella* by 30% to 60% of the B cells (data not shown). Next, we investigated whether *Salmonella*-infected B cells were able to initiate proliferation of $CD8^+$ T cells in addition to the *Salmonella*-specific $CD4^+$ T cell response. Therefore we cultured B cells that had phagocytosed *Salmonella* with CFSE-labeled $CD4^+$ and $CD8^+$ T cells. As observed before, B cells that had phagocytosed *Salmonella* induced proliferation of the $CD4^+$ T cells (20). Interestingly, a considerable amount of $CD8^+$ T cells had proliferated as well (Fig. 1*A*). To investigate the requirement of $CD4^+$ T cell help for the proliferation of the $CD8^+$ T cells *Salmonella*-infected B cells were cultured with $CD8^+$ T cells in the absence of $CD4^+$ T cells. This situation almost completely abolished proliferation of the $CD8^+$ T cells (Fig. 1*B* and 1*C*). Thus, B cells infected by *Salmonella* act as antigen presenting cells and induce $CD8^+$ T cell proliferation, but activation of $CD8^+$ T cells requires the simultaneous activation of *Salmonella*-specific $CD4^+$ T cells to enable T cell help.

To exclude a role for the tetrameric antibody complex in cross-presentation, we analyzed the induction of a $CD8^+$ T cell response by *Salmonella*-specific primary B cells that had taken up *Salmonella* via their specific BCR. Although the percentage of *Salmonella*-infected B cells was lower compared to B cells that had phagocyosed *Salmonella* via the tetrameric antibody complex, with appropriate $CD4^+$ T cell help *Salmonella*-specific B cells also induce $CD8^+$ T cell activation (Fig. 1*D*).

Salmonella-*infected B cells activate both the central memory and effector memory* $CD8^+$ *compartment*

Do B cells elicit a naïve or a memory $CD8^+$ cell response? To study this, we cultured the total $CD8^+$ T cell population with or without *Salmonella*-infected B cells, in presence of $CD4^+$ T cells for help, and determined the naïve ($CD45RO^-CD27^+$; T_N), central memory ($CD45RO^+CD27^+$; T_{CM}), effector memory ($CD45RO^+CD27^-$; T_{EM}) or terminal effector ($CD45RO^-CD27^-$; T_E) phenotype of the $CD8^+$ T cells after prolonged culture. Upon 11 days of culture, purified $CD8^+$ T cells show mainly a naïve phenotype. In contrast, following activation with *Salmonella*-infected B cells, the $CD8^+$ population shifts towards the T_{EM} phenotype (Fig. 2*A*). $CD8^+$ T cells that are

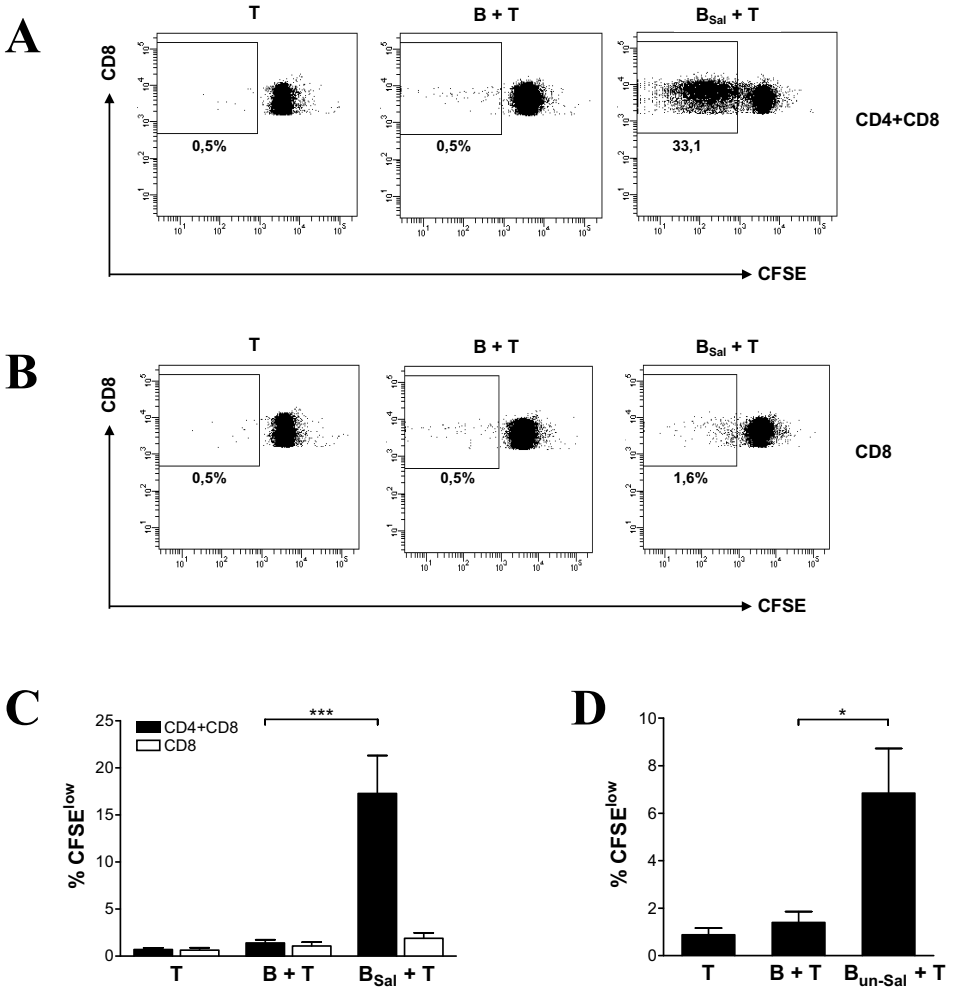


Figure 1. *Salmonella*-infected B cells induce $CD8^+$ T cell proliferation with help of $CD4^+$ T cells. (*A*) CFSE labeled $CD8^+$ T cells were cultured alone (T), together with B cells (B + T) or together with B cells that had phagocytosed *Salmonella* (B_{Sal} + T), in presence of $CD4^+$ T cells. Proliferation was measured after 6 days. (*B*) $CD8^+$ T cell proliferation was measured in absence of $CD4^+$ T cells. (*C*) $CD8^+$ proliferation (shown in *A* and *B*) of multiple independent experiments combined. Percentages are $CD8^+CFSE^{low}$ cells, in the presence of $CD4^+$ T cells (black bars) or not (open bars). The data are expressed as mean ± SEM, of twenty-two independent experiments of different donors and *** $p < 0.001$. *D*, $CD8^+$ T cell proliferation was also measured after culture with B cells that had phagocytosed uncoated Salmonella (B_{un-Sal}) in presence of $CD4^+$ T cell help. The data are expressed as mean ± SEM, of five independent experiments of different donors, and * $p < 0.05$.

activated with *Salmonella*-infected B cells in the absence of help by $CD4^+$ T cells, do not differentiate to a T_{EM} phenotype (Fig. 2*B*). As these data indicate that *Salmonella*-infected B cells mainly induce a $CD8^+$ memory response, we investigated if *Salmonella*-infected B cells can prime naïve T cells at all. Therefore

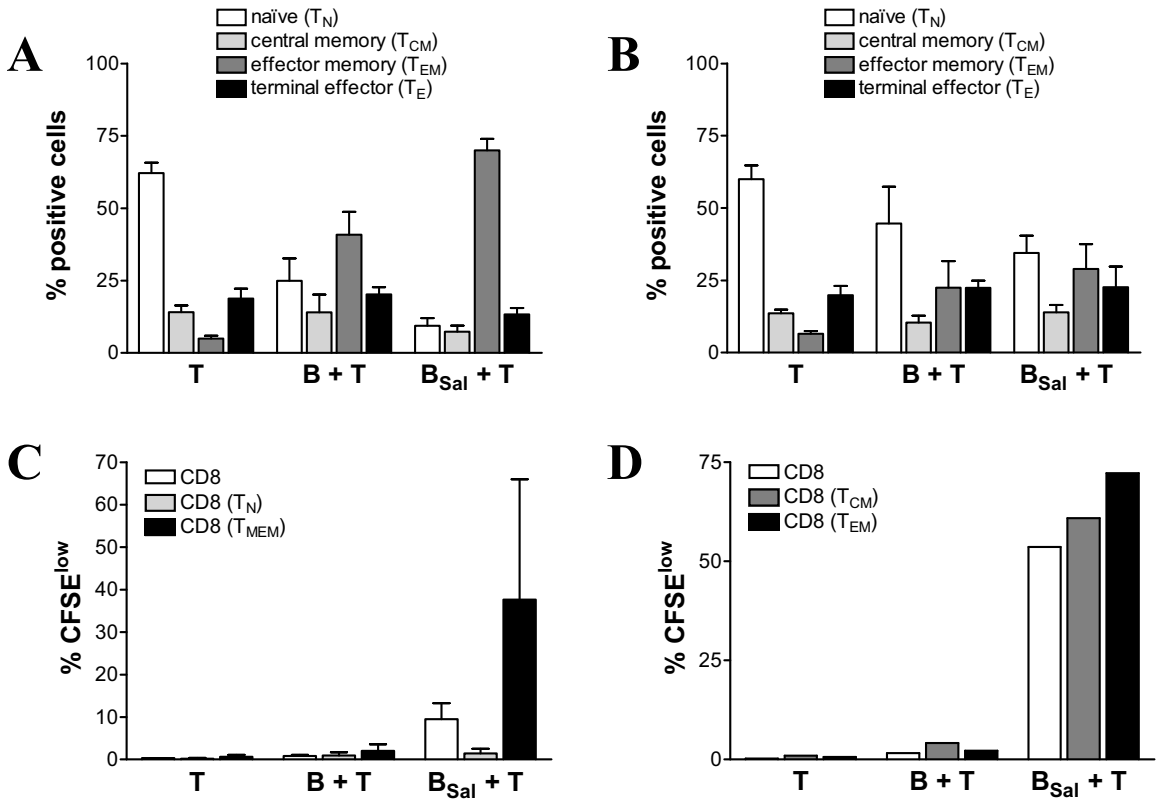


Figure 2. *Salmonella*-infected B cells elicit a $CD8^+$ memory response.
(*A*) $CD4^+$ and $CD8^+$ T cells were cultured alone (T), together with B cells (B + T) or with *Salmonella*-infected B cells (B_{Sal} + T). After 11 days, cells were stained for CD45RO and CD27 to discriminate between the different T cell populations: naïve (T_N; $CD45RA^+CD27^+$), central memory (T_{CM}; $CD45RO^+CD27^+$), effector memory (T_{EM}; $CD45RO^+CD27^-$) and terminal effector (T_E; $CD45RA^+CD27^-$) T cells. Gated $CD8^+$ cells were analyzed. (*B*) Without $CD4^+$ T cells, the differentiation of $CD8^+$ T cells to effector memory is attenuated. Data are mean ± SEM from five independent experiments. (*C*) *Salmonella*-infected B cells activate proliferation of sorted $CD8^+CD45RO^+$ cells (T_{MEM}), but not of purified $CD8^+CD45RA^+$ cells (T_N) in the presence of $CD4^+$ help. Data are mean ± SEM from three independent experiments. *D*, *Salmonella*-infected B cells activate proliferation of sorted $CD8^+CD45RO^+CD27^-$ (T_{EM}) and $CD8^+CD45RO^+CD27^+$ (T_{CM}) cells. Data are of one experiment.

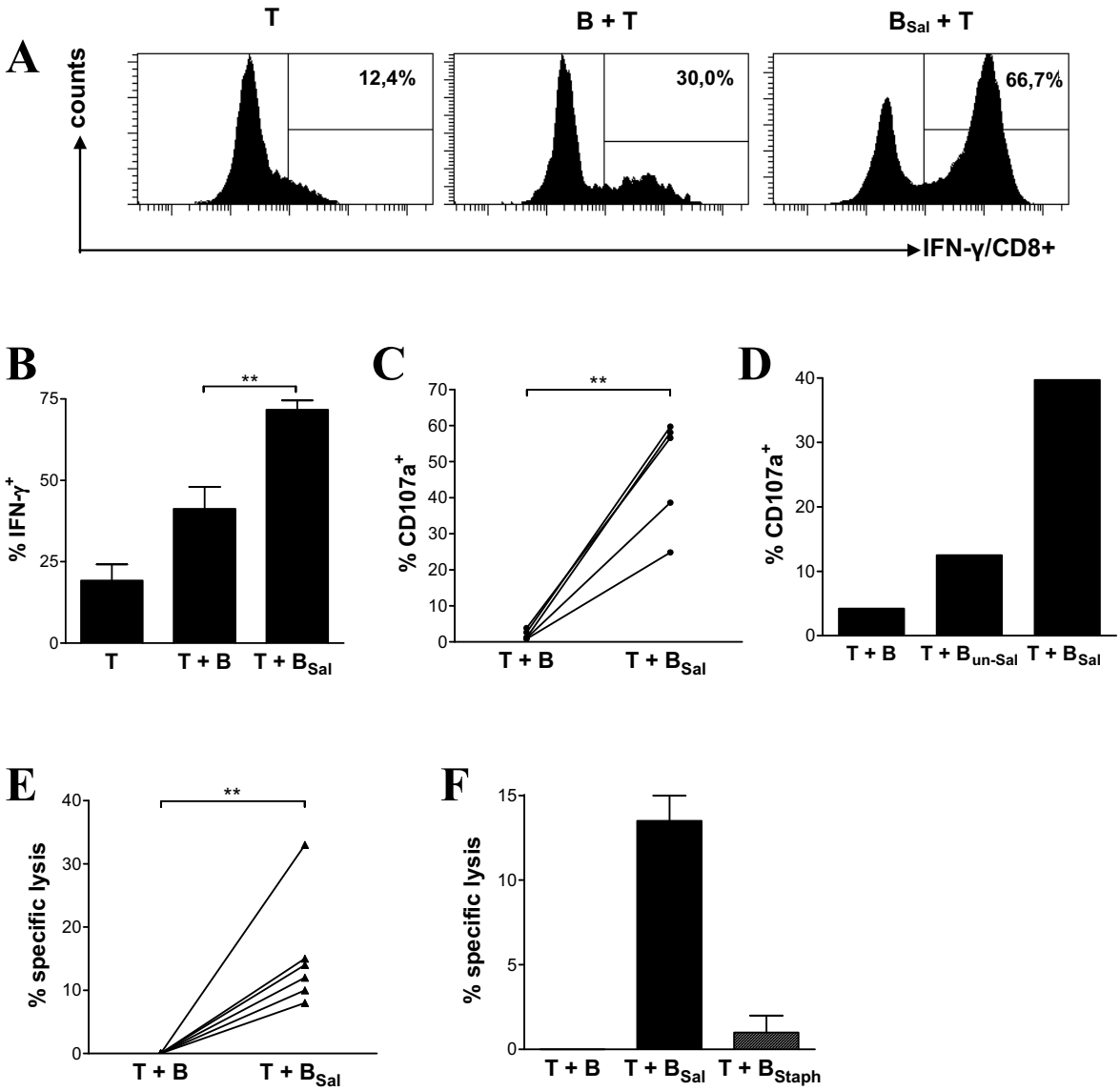
we studied proliferation of sorted naïve ($CD45RA^+CD45RO^-$; T_N) or memory ($CD45RA^-CD45RO^+$; T_{MEM}) $CD8^+$ T cells in response to *Salmonella*-infected B cells. Figure 2*C* shows that the naïve $CD8^+$ population does not proliferate upon stimulation with *Salmonella*-infected B cells, whereas the memory $CD8^+$ T cells proliferated vigorously. Sorting of T_{CM} ($CD45RO^+CD27^+$) and T_{EM} ($CD45RO^+CD27^-$) $CD8^+$ T cells before coculture with *Salmonella*-infected B cells, showed that both T_{CM} and T_{EM} can be activated by *Salmonella*-infected B cells. (Fig. 2*D*). Recent experiments in mice show that both T_{CM} and T_{EM} cells can arise from activation and proliferation of the T_{CM} compartment, whereas T_{EM} cells are more terminally

differentiated and therefore proliferate poorly and only give rise to T_{EM} progeny (24). Indeed, expansion of the sorted T_{CM} by *Salmonella*-infected B cells yielded offspring with both a T_{CM} and a T_{EM} phenotype, whereas T_{EM} activation yielded mainly T_{EM} progeny. Together, these data show that *Salmonella*-infected B cells activate a recall response of memory $CD8^+$ T cells, yielding expansion of both the "memory stem cell" containing T_{CM} compartment and the memory effector cell containing T_{EM} compartments.

Activated* Salmonella*-specific CTLs are able to kill* Salmonella*-infected cells

Activation of cytotoxic $CD8^+$ T cells by B cells is controversial. Earlier reports showed that B cells induce tolerance or anergy in $CD8^+$ T cells. In contrast, we showed proliferation of $CD8^+$ T cells upon B cell activation when B cells had phagocytosed *Salmonella*. Although this proliferation is unlikely to yield tolerance, proliferation itself does not guarantee that these B cells activate functional, cytotoxic $CD8^+$ T cells. $CD8^+$ T cells kill intracellular pathogens either via secretion of IFN-γ or via direct killing of the infected target cell (25). To investigate if *Salmonella*-containing B cells induce a functional $CD8^+$ T cell response, the IFN-γ secretion of $CD8^+$ T cells was measured using an intracellular IFN-γ staining. This showed that, after culture with *Salmonella*-infected B cells, the number of $CD8^+$ T cells producing IFN-γ is increased to more than 65% (Fig. 3*A* and 3*B*). Thus, cross-presentation of *Salmonella* antigens by B cells induces not only proliferation of the $CD8^+$ T cells but also renders the cells functional in that it initiates IFN-γ secretion.

As these data demonstrate that B cells that had taken up *Salmonella* are able to functionally activate $CD8^+$ T cells, the question remained if the activated $CD8^+$ T cells were *Salmonella*-specific and whether $CD8^+$ T cells can acquire a cytotoxic phenotype through B cell-mediated activation. First, we investigated if the $CD8^+$ degranulate their cytotoxic granules upon recognition of *Salmonella*-infected target cells. For this we analyzed expression of the marker CD107a, which is expressed at the plasma membrane of $CD8^+$ T cells upon degranulation. Anti-IgM-coated *Salmonella*-infected B cells were cultured together with CFSE labeled $CD4^+$ and $CD8^+$ T cells and the proliferating *Salmonella*-primed $CD8^+$ T cells were sorted after 6 days. After expansion with IL-2 for another 6 days, the $CD8^+$ T cells were re-exposed to autologous, *Salmonella*-infected B cells and CD107a expression was measured. Upon re-exposure to *Salmonella*-infected B cells, the *Salmonella*-primed $CD8^+$ T cells show an increase of CD107a expression at the plasma membrane.



Since the proliferating CD8$^+$ T cells did not show degranulation when re-exposed to autologous, non-infected B cells, the CD8$^+$ T cells specifically degranulated upon recognition of *Salmonella*-infected cells (Fig. 3*C*). To confirm our results without anti-IgM coating of the bacteria, we also primed CD8$^+$ T cells with *Salmonella*-specific B cells that had phagocytosed uncoated *Salmonella* via the *Salmonella*-specific BCR. These primed CD8$^+$ T cells degranulated specifically upon recognition of autologous B cells infected with uncoated *Salmonella*, of which a relative small percentage (5%, data not shown) contained *Salmonella*-infected antigen-specific B cells (Fig. 3*D*; middle bar). In addition, the *Salmonella*-primed CD8$^+$ T cells very efficiently degranulated upon contact with autologous B cells of which a higher percentage (26%, data not shown) of cells had been infected with anti-IgM coated *Salmonella* (Fig. 3*D*; right bar).

Figure 3. *Salmonella* infected B cells induce *Salmonella*-specific $CD8^+$ T cells to secrete IFN-γ and are cytotoxic**.**

(*A*) $CD4^+$ and $CD8^+$ T cells were cultured alone (T), with B cells (B + T) or with *Salmonella*-infected B cells (B_{Sal} + T). After 11 days, $CD8^+$ T cells were analyzed for IFN-γ production by intracellular FACS staining. A representative experiment of five independent experiments using cells from different healthy donors is shown. (*B*) *Salmonella*-infected B cells induce IFN-γ-expression by $CD8^+$ T cells compared to non-infected B cells. Data are the mean ± SEM from five independent experiments of different donors and ** $p < 0.01$. (*C*) $CD8^+$ T cells were primed with *Salmonella*-infected B cells and the proliferating $CD8^+$ T cells were sorted after 6 days. Sorted $CD8^+$ T cells were restimulated with autologous B cells that had either or not phagocytosed anti-IgM-coated *Salmonella*. *Salmonella*-specific degranulation was measured by CD107a expression at the plasma membrane $CD8^+$ T cells. Data are means ± SEM of five independent experiments of different donors, and ** $p < 0.01$. (*D*) Proliferating $CD8^+$ T cells primed with B cells that had phagocytosed uncoated *Salmonella* were sorted and CD107a expression was measured upon re-encounter of B cells (T + B), B cells infected with uncoated (T + $B_{un\text{-}Sal}$) or anti-IgM-coated *Salmonella* (T + B_{Sal}). Data are of one experiment. *E*, Sorted *Salmonella*-primed $CD8^+$ T cells (see *C*) specifically kill *Salmonella*-infected B cells as measured by ^{51}Cr release of autologous B cells that were either or not infected with *Salmonella.* Data are expressed as mean ± SEM, of six independent experiments of different donors, and ** $p < 0.01$. *F*, Sorted *Salmonella*-primed $CD8^+$ T cells (see *C*) do not kill *Staphylococcus*-infected B cells as measured by ^{51}Cr release of autologous B cells that were either infected with *Salmonella* or with *Staphylococcus*. The data are expressed as mean ± SEM, of two independent experiments of different donors.

Finally, we investigated if degranulation of the *Salmonella*-specific $CD8^+$ T cells also leads to death of the *Salmonella*-infected cells to determine the true cytotoxic potential of the lytic granules. CFSE labeled $CD8^+$ T cells were primed by incubation with *Salmonella*-infected B cells and proliferating $CD8^+$ cells were sorted and expanded with IL-2 for 6 days. Re-exposure of the *Salmonella*-primed T cells to *Salmonella*-infected, ^{51}Cr-labeled autologous B cells demonstrated that the primed $CD8^+$ T cells were able to kill *Salmonella*-infected B cells but not non-infected B cells (Fig. 3*E*). In addition, the *Salmonella*-primed CD8+ T cells were not able to kill B cells that had phagocytosed *Staphyloccocus* via BCR ligation (Fig. 3*F*), demonstrating that the $CD8^+$ cells are indeed *Salmonella*-specific and do not recognize autologous B cells that are activated via BCR-mediated uptake of other bacteria. Thus, activation of $CD8^+$ T cells by B cells cross-presenting *Salmonella* antigens induces a cytotoxic phenotype in the $CD8^+$ T cells that specifically mediates killing of *Salmonella*-infected cells.

Cross-presentation of* Salmonella *antigens is partly proteasome dependent

We showed that after phagocytosis of *Salmonella*, B cells are able to cross-present antigens to $CD8^+$ T cells and thereby initiate a *Salmonella*-specific cytotoxic T cell response. The mechanism of cross-presentation of *Salmonella* antigens by B cells is unknown. One possible mechanism is that after phagocytosis, antigens are translocated from the *Salmonella*-containing vacuole (SCV) into the cytoplasm of B cells. Next, the antigens are degraded in the cytoplasm by proteasomes into small peptides, which are presented via the classical MHC class I antigen presentation route. To investigate this mechanism of cross-presentation, we used a chemical compound that specifically blocks the proteasome (MG-132). To study the effect of cross-presentation when blocking the proteasome, we used the CD107a degranulation assay. Re-exposure of *Salmonella*-primed $CD8^+$ T cell to autologous, *Salmonella*-infected B cells showed that by blocking of the proteasome, a *Salmonella*-infected B cell is much less efficient in inducing $CD8^+$ T cell degranulation when proteasomes are inhibited (Fig. 4). This observation implies that the proteasome is somehow involved in cross-presentation of *Salmonella* antigens by B cells. How the proteasome is involved is unclear as proteasome inhibition has many secondary effects on the ubiquitin cycle, the modification of histones and the formation of multivesicular bodies (26).

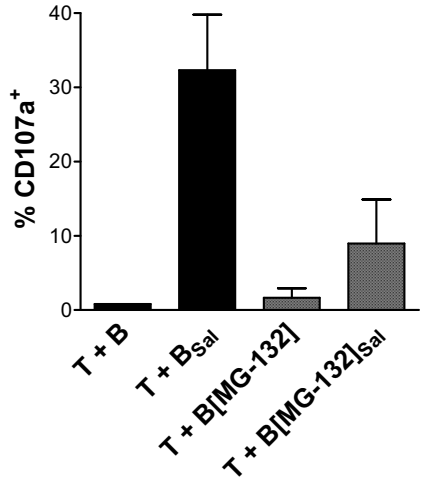


Figure 4. Proteasome inhibition of *Salmonella*-infected B cells diminishes degranulation of *Salmonella*-specific $CD8^+$ T cells.

Salmonella-specific $CD8^+$ T cells were sorted and co-cultured with B cells (T + B) or with *Salmonella*-infected B cells (T + B_{Sal}). Treatment of B cells with proteasome inhibitor MG-132 (20 μM) leads to a decrease in degranulation of the *Salmonella*-specific $CD8^+$ T cells (T + B[MG-132]$_{Sal}$), as measured by CD107a expression.

***B cells do not cross-present heat-killed* Salmonella**

Salmonella survives inside a cell via expression of the TTSS that create an intracellular environment that neutralizes the destructive forces of the host cell (27). The TTSS components SPI-1 and SPI-2 play a role in this neutralization by exporting proteins into the host cell. Because of the capacity of *Salmonella* to

invade cells and to control its maintenance inside the cell via SPI-1 and SPI-2, it is possible that *Salmonella* might directly play a role in the cross-presentation by B cells. To determine the role of SPI-1 and SPI-2 in cross-presentation of *Salmonella* by B cells, we analysed $CD8^+$ T cells proliferation via co-culture with $CD4^+$ T cells and B cells that had phagocytosed wild type *Salmonella*, or *Salmonella* with a mutation in SPI-1 ($invA^-$) or SPI-2 ($ssrA^-$). Both *Salmonella* mutants were still able to elicit a $CD8^+$ T cell response, albeit at lower percentages of T cell proliferation compared to wild type *Salmonella* (Fig. 5*A*). Thus SPI-1 and SPI-2 each contribute to cross-presentation of *Salmonella* antigens by B cells, but are not essential.

By creating an environment in which *Salmonella* itself cannot be killed, it is possible that the intracellular survival plays a role in the efficacy of cross-presentation of *Salmonella* antigens by B cells. To study the contribution of *Salmonella* on the efficacy of cross-presentation of *Salmonella* antigens, we compared the efficiency of living and dead *Salmonella* to induce $CD8^+$ T cell activation. Living and heat-killed *Salmonella* were coated with the LPS-BCR tetrameric antibody complexes to ensure similar levels of bacterial phagocytosis by B cells (data not shown). B cells that have phagocytosed dead *Salmonella* less initiate $CD8^+$ T cell proliferation (Fig. 5*B*), indicating that *Salmonella* contributes to the process of cross-presentation in B cells.

However, another part of the response continued with dead *Salmonella*. To confirm that *Salmonella* antigens of dead bacteria are inefficiently cross-presented to $CD8^+$ T cells by B cells, we primed $CD8^+$ T cells with B cells infected with living or heat-killed *Salmonella*, sorted and expanded the primed T cells followed by re-exposure to autologous B cells that had not phagocytosed *Salmonella*, or had phagocytosed either living or dead *Salmonella*. Cross-presentation of *Salmonella* antigen was measured by the extent of degranulation of the primed T cells using CD107a expression.

$CD8^+$ T cells that had been primed by B cells infected with living *Salmonella* efficiently degranulated upon recognition of autologous B cells that had phagocytosed living *Salmonella* (upregulation of CD107a to 60%), whereas the cells did not degranulate upon contact with B cells that had phagocytosed dead *Salmonella* (Fig. 5*C*; left panel). This indicates either that B cells present different *Salmonella* antigens to $CD8^+$ T cells from live or dead bacteria or that B cells cannot cross-present *Salmonella* antigens when the intracellular bacterium is not alive. Furthermore, $CD8^+$ T cells that had been primed with B cells that had taken up

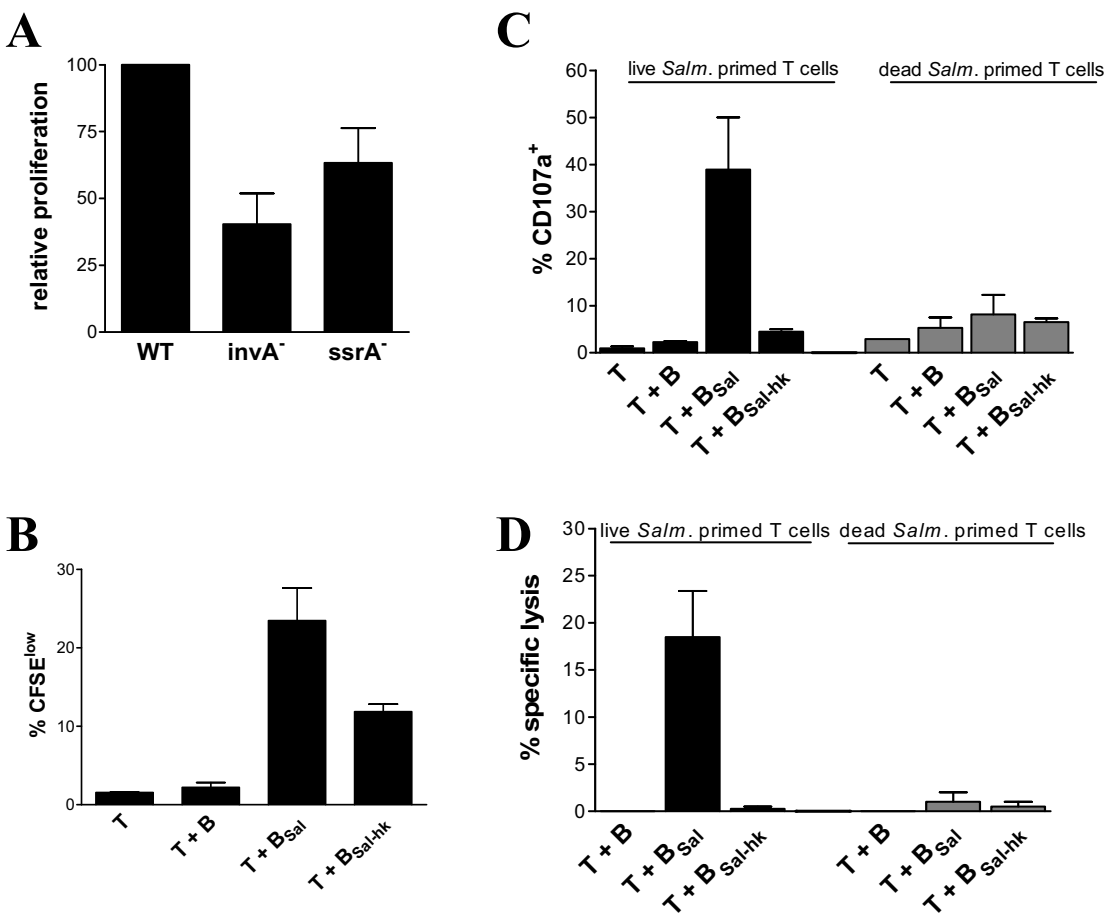


Figure 5. Induction of the cytotoxic T cell response against *Salmonella* by B cells requires uptake of living *Salmonella*.

(*A*) $CD8^+$ T cells were labeled with CFSE and cultured with B cells that had phagocytosed either wild type *Salmonella* (WT), mutant for SPI-1 ($invA^-$) or SPI-2 ($ssrA^-$). Proliferation was measured after 6 days. Data shown are proliferation of $CD8^+$ cells relative to wild type proliferation and are of two different donors; error bars are SEM. (*B*) $CD8^+$ T cells were labeled with CFSE and cultured with B cells (T + B), or with B cells that have internalized either living *Salmonella* (T + B_{Sal}) of heat killed *Salmonella* (T + $B_{Sal\text{-}hk}$). Proliferation was measured after 6 days. *C, Salmonella*-specific T cells that were primed with B cells that had either internalized living (left panel) or dead *Salmonella* (right panel) were sorted and restimulated with B cells (T + B), B cells that had internalized living *Salmonella* (T + B_{Sal}) or dead *Salmonella* (T + $B_{Sal\text{-}hk}$). Degranulation was analyzed as CD107a expression by $CD8^+$ T cells. *D*, $CD8^+$ T cell mediated kill was measured as the release of ^{51}Cr by B cells. Data are expressed as mean ± SEM, from four (live *Salmonella* primed) or two (dead *Salmonella* primed) independent experiments of using material from different healthy donors.

dead *Salmonella* showed poor degranulation to B cells that had taken up either live or heat-killed *Salmonella* (Fig. 5*C*; right panel), demonstrating that B cells indeed do not induce a cytotoxic $CD8^+$ T cell response via cross-presentation of *Salmonella* antigens from dead intracellular bacteria. This observation was confirmed by studying elimination of *Salmonella*-infected B cells by ^{51}Cr-release (Fig. 5*D*).

Together, these results show that B cells are not able to cross-present antigens of dead *Salmonella* and that *Salmonella* contributes to the activation of a *Salmonella*-specific cytotoxic $CD8^+$ memory response.

Discussion

Studies in B-cell-deficient mice showed that protective immunity to *Salmonella* strongly depends on B cells (28). This dependency does not only result from antibody generation, as passive transfer of *Salmonella*-immune serum cannot transfer resistance to *Salmonella* (29). In addition, B cells are involved in the generation of a profound $CD4^+$ and $CD8^+$ T cell response after *Salmonella* infection (21), but the precise role of B cells remained unclear. We previously showed that human antigen-specific B cells that have internalized *Salmonella* via their BCR are able to induce a *Salmonella*-specific $CD4^+$ T cell response (20). The data described here may explain the role of B cells in immune response against *Salmonella* infection other than antibody formation. In contrast to other data, in which uptake of *Salmonella* does not lead to antigen cross-presentation via MHC class I in mouse B cell lines (30), we now demonstrate that *Salmonella*-specific human B cells that have phagocytosed *Salmonella* via their BCR are capable to induce a strong recall response of cytotoxic $CD8^+$ T cells after cross-presentation of *Salmonella* antigens.

We previously showed that in the human Ramos B cell line, *Salmonella* is not only capable to survive, but also to replicates intracellularly. In contrast, in primary human B cells *Salmonella* survives, but is unable to replicate inside the B cell (20). The incapability to replicate in primary human B cells may play a role in cross-presentation and activation of $CD8^+$ T cells. A recent report shows that the most functional $CD8^+$ T cell responses are induced against *Salmonella* that do not replicate intracellularly (31). These observations may explain our finding why *Salmonella*-specific primary B cells are efficient in cross-presentation of *Salmonella* antigens from the non-replicating bacteria and activate *Salmonella*-specific $CD8^+$ cells that show a functional cytotoxic T cell response. Thus, inhibition of *Salmonella* replication by primary human B cells may be beneficial for cross-presentation to $CD8^+$ T cells.

After internalization by the B cell, *Salmonella* survives in the SCV. For cross-presentation, *Salmonella*-antigens should be loaded onto MHC class I. The TTSS of *Salmonella* could play a role in delivery of antigens in the cytosol by injecting proteins directly into the cytosol (32). These proteins can be degraded by the

proteasome and after transportation into the ER, loaded onto MHC class I molecules. We demonstrated that SPI-1 and SPI-2 contribute to cross-presentation, but both are not required separately. It is therefore likely that antigens excreted in the host cytosol by SPI-1 and SPI-2 are both used for antigen processing to MHC class I. In addition, we showed that B cells were not able to cross-present *Salmonella*-specific antigens of heat killed *Salmonella*. Thus, *Salmonella* itself appears to be involved in the generation of an effective cytotoxic $CD8^+$ T cell response against the bacteria, a phenomenon that points to the occurrence of co-evolution of bacterial immune evasion and the generation of effective anti-bacterial immunity.

Cross-presentation of *Salmonella* antigens by B cells leads to activation of a $CD8^+$ cytotoxic T cell response, but help of $CD4^+$ T cells is required. The exact kind of $CD4^+$ T cells help could be via cell-cell contact or cytokine mediated and is presently under investigation.

Various reports have described that B cells play a role in the expansion of *Salmonella*-specific T cells, but how specific T cell responses are induced is not understood. This has been attributed to the fact that antigen-specific B cells are probably the main B cell population with an antigen presenting function in *Salmonella* infection and that the frequency of antigen-specific B cells is elevated during a secondary infection (reviewed in (33)). Indeed, our data concur with these observations as we show that the antigen-specific B cells are the cells that are responsible for efficient cross-presentation of *Salmonella* antigens. In addition, our data provide an additional explanation why B cells are mainly involved in the recall response; *Salmonella*-infected B cells do not prime naïve $CD8^+$ T cells, but are very efficient in inducing a potent recall response of cytotoxic $CD8^+$ memory T cells. This implies that other APCs (e.g. DCs) are still needed to prime naïve $CD8^+$ T cells. In secondary infections however, DCs can induce $CD8^+$ proliferation via direct infection or via suicide cross-presentation upon ingestion of infected apoptotic cells (22), but also B cells are very efficient in generating an anti-*Salmonella* cytotoxic $CD8^+$ responses. In addition, DC were shown to mainly activate the T_{EM} compartment (22), whereas *Salmonella*-infected B cells activate both the T_{CM} and T_{EM} compartment. This ensures not only the direct terminal differentiation of effector memory cells, but also the expansion of the *Salmonella*-specific $CD8^+$ memory T cell compartment, which may both amplify the anti-*Salmonella* immune response

and simultaneously ensure generation of anti-*Salmonella* memory for further reinfections.

In summary, we propose a model on the role of B cells in the generation of the humoral and cellular immune response against *Salmonella*. After infection, *Salmonella* enters the body via DCs that activate $CD4^+$ and $CD8^+$ T cells or via M cells after which it encounters B cells, which are situated in the Peyer's Patches, directly under the M cells. *Salmonella*-specific B cells internalize *Salmonella* via their BCR and are activated. Next, internalization of *Salmonella* leads to presentation of *Salmonella* antigens MHC class II molecules and activates *Salmonella*-specific $CD4^+$ T-helper cells that stimulate *Salmonella*-infected B cells to secrete *Salmonella*-specific antibodies (20), either locally or upon arrival or the infected B cells in the mesenteric lymph node. Upon re-infection, infection of *Salmonella*-specific memory B cells by *Salmonella* ensures rapid antibody production but also mediates a strong cytotoxic $CD8^+$ recall response to eliminate infected cells. The *Salmonella*-specific $CD4^+$ response that aided antibody production in early stages of the immune response is now also required for the reactivation of the cytotoxic T cell response against *Salmonella* in later stages of the immune response or upon reinfection. Thus, uptake of *Salmonella* by antigen-specific B cells may generate a survival niche for *Salmonella*, but at the same time strongly contributes to the generation of effective anti-*Salmonella* immunity at multiple levels of the adaptive immune response. The combined attack of pathogens by various members of the adaptive immune system will allow efficient eradiation of the infection.

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