Single cell biochemistry to visualize antigen presentation and drug resistance

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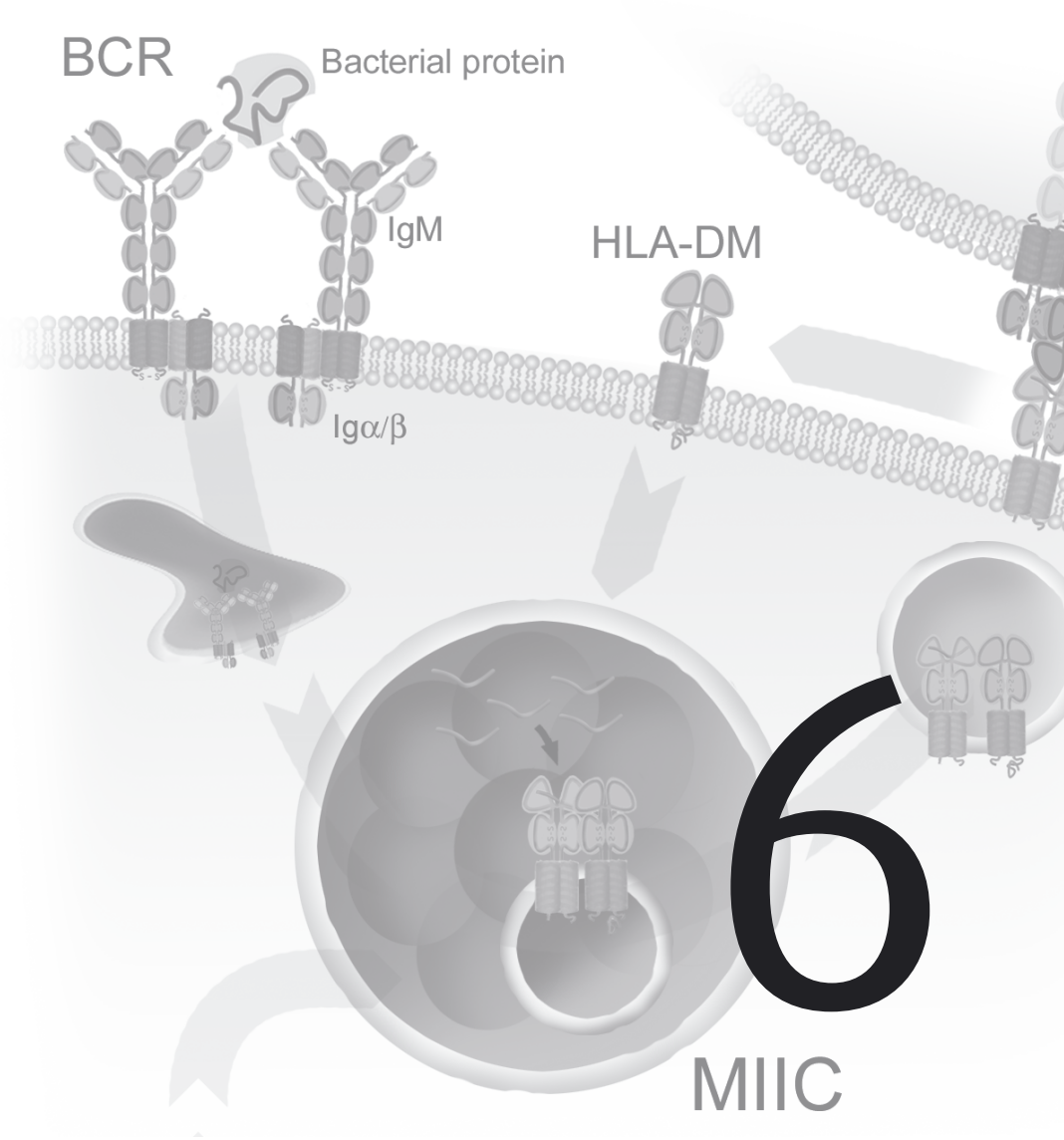
Submitted



Immune escape and spreading of Salmonella after specific B Cell Receptor-mediated uptake

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The facultative intracellular pathogen Salmonella causes significant morbidity and mortality worldwide. B cells play an important role in an effective immune response against Salmonella by generating high-affinity antibodies. Here, we studied the fate of Salmonella typhimurium upon contact with specific B cells and binding to the B cell Receptor (BCR). Upon recognition and capture by the BCR, Salmonella actively facilitates its uptake into primary B cells. Salmonella survives inside primary B cells in a dormant state that is actively maintained by the host cell. This is followed by excretion of the dormant bacteria by the B cell that then infect and propagate in secondary host cells. Salmonella specific B cells thus function as survival niche and transport vehicle to support systemic dissemination of Salmonella upon initial infection.

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Introduction

Salmonella enterica is a Gram-negative, enteric pathogen responsible for disease syndromes of significant morbidity and mortality (1, 2). After oral uptake, the bacterium crosses the intestinal epithelium and enters the Peyer's patches via M cells (3) or luminal capture by dendritic cells (4, 5), where they are internalized by macrophages, dendritic cells, and neutrophils (6-8). Entry into these cells is actively induced by the bacterium through an impressive array of effector proteins that orchestrate uptake by manipulating the host cellular machinery (9, 10). *Salmonella* resides inside the host cell within membrane-enclosed vacuoles, segregated from the normal endocytic route through the excretion of a second set of effector proteins (11-13). Here it replicates (14, 15) and escapes detection by the immune system (16, 17). From the intestine, *Salmonella* spreads via mesenteric lymph nodes to liver, bone marrow, and spleen where replication continues (18). How *Salmonella* reaches these organs is unclear. So far, especially neutrophils and CD18-expressing phagocytes have been implicated (5, 19). Here we show that *Salmonella* requires the specificity of the immune system by using antigen-specific B cells as selective transport vehicles for release at distant sites for further infection.

* A. Griekspoor and Y. Souwer contributed equally to this paper

Experimental Procedures

Antibodies, beads and fluorophores

Goat anti-mouse IgG-conjugated Dynabeads® (Dynal Biotech, Oslo, Norway) with a diameter of 5 μM were coated with monoclonal mouse anti-human IgM antibody (MH15, Sanquin, Amsterdam, the Netherlands). The same antibody or mouse anti-human IgG antibody (MH16,

Sanquin, Amsterdam, the Netherlands) was mixed with rat anti-mouse IgG1 antibody (RM161.1, Sanquin, Amsterdam, the Netherlands), and mouse monoclonal anti-*S.typhimurium* LPS (1E6, Biodesign International, Kennebunk, ME) to generate the BCR-LPS bridging tetramers used to coat the *Salmonella* bacteria. Fluorescent secondary antibodies, Texas-Red and Texas Red-phalloidin were from Molecular Probes (Leiden, The Netherlands).

DNA constructs and cell lines

The pcDNA3 DOβGFP (20) and pcDNA3 DR1β/GFP (21) fusion construct have been described before. Stable transfectants of the EBV-negative human B cell lymphoma cell line Ramos were selected and maintained in RPMI medium supplemented with 5% FCS (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine, 50 μM 2-mercaptoethanol in the presence of 2000 μg/ml G418 (Gibco, Paisley, UK). Stable expression of the GFP-tagged proteins was verified by western blotting and ensured by regular selection of positive cells by FACS sorting.

Lymphocyte Isolation

Human peripheral blood mononuclear cells were isolated from a buffycoat (Sanquin, Amsterdam, the Netherlands) by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC AS, Oslo, Norway). B and T lymphocytes were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads® and DETACHaBEAD® (Dynal Biotech, Oslo, Norway), according to the manufacturer's instructions. B lymphocytes were incubated for 30 minutes at 37°C with living coated and uncoated bacteria, and subsequently cultured in RPMI medium containing 5% FCS and supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine, 50 μM 2-mercaptoethanol and 20 μg/ml human apo-transferrin (Sigma-Aldrich, Steinheim, Germany, depleted for human IgG with prot-G sepharose).

Bacterial Strains, Growth Conditions, and Infections

The *S. typhimurium* strains SL1344, a kind gift from S. Meresse (22), GFP-*S. typhimurium* SL1344 (23), and mRFP-*S. typhimurium* SL1344 (17) were used. The *S. typhimurium* strain 14028 containing the lux operon of *P. luminescens* (luxCDABE) was a kind gift from S. Vesterlund (24). Bacteria were grown in Luria-Bertani (LB) broth overnight at 37°C while shaking, subcultured at a dilution of 1:33 in fresh LB medium, and incubated at 37°C while shaking for 3.5 h. Bacteria were washed once with PBS, incubated 1:25 with the BCR-LPS bridging tetramers in a total volume of 100μl PBS for 30 min at room temperature, and washed twice to remove unbound antibodies. Cells were grown overnight in medium without antibiotics, washed, and subsequently incubated with 2-20 coated or uncoated bacteria/cell (as indicated below) in RPMI 1640 without antibiotics for 30 min at 37°C while tumbling. Next, cells were washed four times and cultured for 1h in media containing 50 μg/ml Gentamycin (Invitrogen) to eliminate non-phagocytosed bacteria. Cells were subsequently maintained for the indicated time points in media with 10 μg/ml Gentamycin.

CLSM, wide field microscopy and electron microscopy analysis

For CLSM analysis, coverslips were coated with 1 mg/ml Poly-L Lysine (Sigma-Aldrich co., Steinheim, Germany) for 1h at room temperature and washed thoroughly. Subsequently, cells were allowed to attach on the coated coverslips for 15 min. For the visualization of the actin cytoskeleton, cells were fixed with 3.7% paraformaldehyde and stained with TexasRed-phalloidin and DAPI (Sigma-Aldrich). Confocal analysis was performed using a Leica TCS SP confocal laser scanning microscope equiped with an Argon/Krypton laser (Leica Microsystems, Heidelberg, Germany). Green fluorescence was detected at λ>515 nm after excitation at 488 nm. For dual analyses, green fluorescence was detected at 520–560 nm. Red fluorochromes were excited at 568 nm and detected at λ>585 nm. All experiments presented were repeated several times on different days, and results were consistent and reproducible. Wide field microscopy was performed using 6-well plates (coated with Poly-L Lysine), analyzed using a Zeiss Axiovert 200 M microscope equipped with a FluorArc fluorescence lamp, motorized scanning stage, 63x LD Achroplan objective, and climate chamber. GFP excitation: 470±20nm, emission: λ>515nm. TexasRed excitation: 546±12nm, emission: λ>590nm. Images were acquired using a Zeiss AxioCam MRm Rev.2 CCD in combination with the manufacturer's AxioVision software. Further image processing was performed using the ImageJ software package. For electron microscopy, cells were allowed to phagocytose beads or bacteria for 30 min, fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (0.5%), and subsequently processed for immuno-electron microscopy. After embedding in a mixture of methyl-cellulose and uranyl acetate, sections were analyzed with a Philips CM10 electron microscope (Eindhoven, the Netherlands).

Intracellular Survival and Growth Assays

Human primary B cells were incubated in a parallel experiment with either GFP- or Lux-expressing *Salmonella* as described above with 2 bacteria per cell. At various time

points cells were analyzed. The percentage of living cells and GFP levels were determined using a FACS Calibur (Becton Dickinson). Bioluminescence was measured for 5 s in a luminometer (Berthold). Bacterial growth was determined by dividing the relative bioluminescence signal by the relative number of GFP positive, living B cells, resulting in the amount of light produced per bacteria-containing B cell. For induction of apoptosis, cells were treated with 0.1 μM Edelfosine (25).

Bacterial excretion Assay

To visualize bacterial excretion, human primary B cells were incubated with uncoated GFP-expressing *Salmonella* as described above at ~20 bacteria per cell, and followed using wide field microscopy in medium containing anti-LPS antibodies precoupled to TexasRed. To quantify excretion, cells were stained at various time points with DAPI (Sigma-Aldrich) to exclude dead cells, and anti-LPS coupled to APC, and subsequently fixed with 3.7% formaldehyde before analysis using a LSR II (Becton Dickinson). For the increase in LPS levels, the initial level at timepoint 0 was set to 1. The percentage of excreted bacteria was calculated as the loss of GFP positive, LPS negative B cells compared to timepoint 0. To discriminate between bacterium and B cell-induced excretion, cells were cultured in medium containing 10μg/ml tetracycline to eliminate intracellular bacteria (bacteriostatic capacity was verified using lux-*Salmonella* in Ramos cells).

Results

Unlike other professional Antigen Presenting Cells, B cells show very limited phagocytic behavior. Antigen uptake by B cells is critically dependent on the selectivity of the B cell receptor (BCR) (26). The current view on BCR mediated antigen uptake by B cells mainly centers on soluble antigens like small foreign proteins or shed bacterial coat products (27, 28), despite observations dating back 30 years that B cells are also capable of phagocytosing larger particles like polyacrylamide beads (29). Accordingly, most B cell activation studies involve the global triggering of BCR using soluble cross-linking antigens. We opted to study the BCR-mediated recognition of particulate antigen by inducing localised clustering of the BCR using beads decorated with monoclonal antibodies directed against human BCR. When 5 μm beads contacted a Ramos B cell stably expressing the MHC class II molecule HLA-DRβ1 tagged with GFP (DR-GFP), rapid and efficient phagocytosis of the bead ensued (**Figure 1A**, Suppl. Movie S1). The Ramos B cell repositioned its microtubule organizing center (MTOC) in the direction of the contact site with the bead during phagocytosis with the nucleus ending up at the opposite side of the cell, analogous to the situation following T helper cell contact (30) or following CTL-target cell interactions (31). Phagocytosis reached completion within 10 to 20 minutes, and required a functional cytoskeleton (as the microtubule disruptive agent nocodazole prevented phagocytosis, **Suppl. Figure S2**). In addition, uptake was BCR dependent as beads coated with an irrelevant antibody were not taken up, and phagocytosis could be blocked by the addition of anti-IgM F(ab')2 fragments (data not shown). Ramos cells do not express Fcγ receptors, which excludes their involvement in bead uptake (data not shown). A detailed analysis by cryo-electron microscopy (EM) revealed some of the impressive cellular events underlying phagocytosis of such large particulate antigens. During the initial phase of contact, the Ramos B cell surrounded the bead with a surprisingly thin double membrane originating from the cell surface (**Figure 1B**). Staining with phalloidin of DR-GFP Ramos cells in the process of bead-phagocytosis revealed extensive actin fibers in the membrane protrusions surrounding the bead (**Figure 1C**, Suppl. Movie S3). Thus, different from the general concept that B cells are essentially non-phagocytic cells, they become very efficient phagocytes when particle recognition is facilitated by the BCR.

Are physiologically relevant particulate antigens like *Salmonella typhimurium* handled in a similar manner? Since the specificity of Ramos BCR is unknown, *Salmonella* was coated with anti-human IgM antibodies to achieve BCR recognition. The anti-IgM antibody was coupled via rat anti-mouse IgG1 antibodies to anti-LPS IgG bound to GFP-*Salmonella* bacteria. Ramos cells efficiently internalised GFP-*Salmonella* in a BCR dependent manner only when *Salmonella* was coated with the bridging antibodies (**Figure** 2A, Suppl. Movie S4 and quantified in **Figure 2B**). Without the bridging antibodies no GFP-positive Ramos cells were found, showing that *Salmonella* is unable to infect this cell type autonomously. To discriminate between binding of bacteria to the cell surface and actual phagocytosis, cells were labeled with anti-LPS antibodies prior to FACS analysis. GFP-positive bacteria that have been completely engulfed by the Ramos cell will not be stained, while extracellular or partially engulfed bacteria will be accessible to the anti-LPS antibody. Up to 90% of the Ramos cells were able to capture and phagocytose one or more bacteria. However, closer analysis revealed that about 25% of

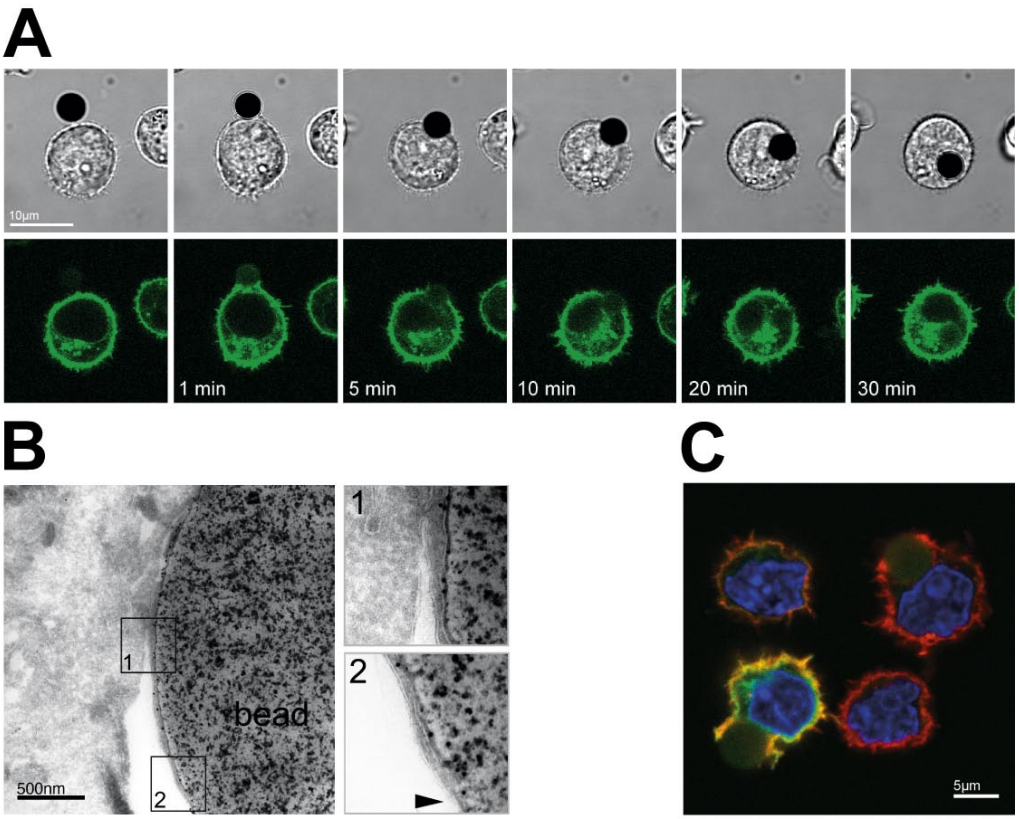

Figure 1. Efficient BCR Mediated phagocytosis of large particulate antigens. **(A)** Phagocytosis of anti-BCR coated beads by Ramos B cells stably expressing HLA-DR/GFP. Living cells were imaged every 30 seconds using confocal microscopy at 37°C. Indicated are time points after contact with the bead, top panel: transmission image, bottom panel: GFP signal. Scale bar equals 10µm. DR-GFP localizes to the plasma membrane and lysosomal vesicles. A bead coated with anti-BCR antibodies is efficiently taken up followed by reorientation of the nucleus, MTOC and vesicles. Figure represents indicated time frames taken from suppl. movie S1. **(B)** Electron micrograph of a Ramos B cell in the process of phagocytosis. Ramos cells were fixed 10 minutes after addition of anti-BCR coated beads and cryo sections analyzed by electron microscopy. Scalebar equals 500nm. Zoom-ins of the thin membrane extrusions surrounding the bead are shown of the indicated regions. The tip of the protrusion is indicated with an arrowhead in inset 2. **(C)** Actin cytoskeleton staining of Ramos B cells expressing DR-GFP during phagocytosis of anti-BCR coated beads. Cells were fixed 10 minutes after addition of anti-BCR coated beads and processed for immune-fluorescence confocal microscopy. Depicted is the overlay of the signals from DAPI nuclear staining (blue), phalloidin stained actin-cytoskeleton (red), and DR-GFP (lower left cell only). Scalebar equals 10µm. Thick actin-fibers are observed in the membrane protrusions surrounding the bead. Figure represents one slice from a Z-stack. A 3D reconstruction is provided as suppl. movie S3.

Ramos cells contained internalized GFP-*Salmonella* only, while more than 60% of Ramos cells were also positive for LPS staining. Confocal microscopy of the latter cells showed that these represented cells that had internalized some but not all bound bacteria (data not shown). Next, we repeated the above experiments using the surface IgG^+ Cess B cell line (32), now with anti-IgG/LPS bridging antibodies. Like Ramos cells, a large proportion of the Cess cells were able to phagocytose the anti-BCR coated *Salmonella* (**Figure 2B**). When uncoated bacteria were added to the Cess cells, or coated bacteria to $IgM^-/IgG^-/Fc\gamma RII(CD32)^+$ Jijoye cells (33) no bacterial uptake was observed (data not shown). Thus, B cell lines are able to phagocytose physiologically relevant particulate antigens like *Salmonella* bacteria in a strictly BCR dependent fashion.

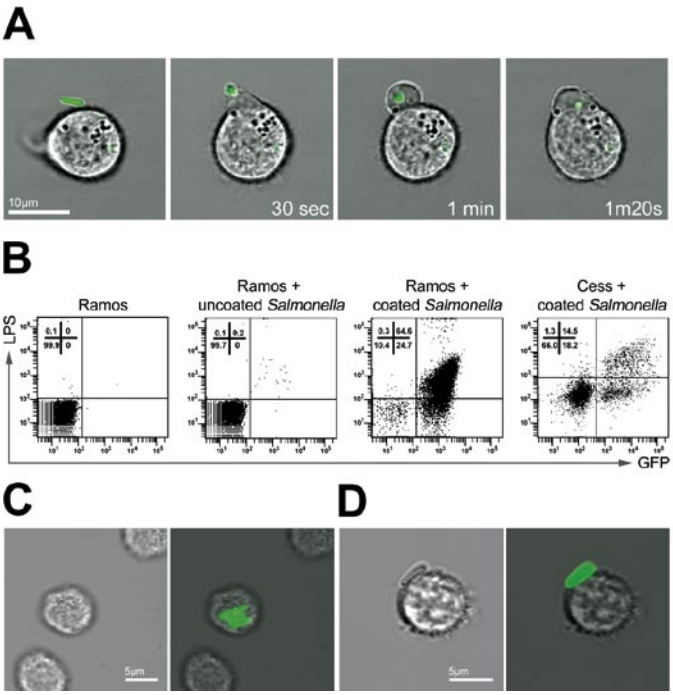

Figure 2. BCR mediated phagocytosis of Salmonella. **(A)** BCR mediated phagocytosis of anti-BCR coated *Salmonella*. Living cells and GFP-expressing *Salmonella* were imaged every 10 seconds using confocal microscopy performed at 37°C. Time points after contact with the bacterium are indicated. GFP signal is projected on top of the transmission image. Scalebar equals 10µm. A single living bacterium coated with anti-BCR antibody is efficiently phagocytosed in a BCR-dependent manner. Figure represents indicated time frames taken from suppl. movie S4.
(B) Quantification of BCR-mediated uptake of *Salmonella* by Ramos B cells using FACS. Ramos or Cess B cells were incubated with live GFP-expressing *Salmonella* for 45 minutes, and free bacteria were removed by extensive washing. Cells not incubated with *Salmonellae* were used as control. Cells were subsequently stained with an antibody against *Salmonella* LPS, before fixation and analysis by FACS. Anti-LPS-APC vs. GFP scatter plots of 50.000 events are depicted for the different conditions as indicated, and quantifications of each quadrant are given. In contrast to uncoated bacteria, anti-BCR coated *Salmonellae* are efficiently phagocytosed by Ramos B cells.
(C) Living primary B cells were incubated with live GFP-expressing *Salmonella* for 45 minutes, and imaged using confocal microscopy at 37°C, left panel: transmission image, right panel: GFP signal projected on transmission image. Scalebar equals 5µm. Three GFP-expressing bacteria have been phagocytosed by a primary B cell with a *Salmonella*-specific BCR.
(D) Living primary B cells were incubated with dead GFP-expressing *Salmonella* under identical conditions as in C, and imaged using confocal microscopy at 37°C, left panel: transmission image, right panel: GFP signal projected on transmission image. Scalebar equals 5µm. GFP-expressing bacteria bind but are not phagocytosed by primary B cell.

To test whether primary CD19$^+$ B cells isolated from human peripheral blood could also phagocytose *Salmonella*, the experiments outlined above were repeated. Analogous to Ramos cells, anti-BCR coated bacteria were efficiently phagocytosed by the primary B cells (Souwer *et al*., accompanying article). Importantly, incubation of isolated B cells with uncoated *Salmonella* consistently revealed a small but significant population of B cells able to recognize and phagocytose the native bacterium (**Figure 2C**). These B cells were CD19$^+$, IgM$^+$, CCR7$^+$, and mainly CD27+ (Souwer *et al*., accompanying article). The latter is in line with the presence of a *Salmonella*-specific memory B lymphocyte compartment. Incubation with uncoated fixed bacteria only showed binding but no phagocytosis of *Salmonella* (**Figure 2D**). *Salmonella* thus required both recognition by BCR and bacterial-mediated processes to enter human CD19$^+$ B cells.

Phagocytosed *Salmonella* grows in most cells, and can only be efficiently destroyed in specialized cells like macrophages and neutrophils in a process requiring the NADPH oxidase system (6, 34). To study the fate of phagocytosed *Salmonella* in the Ramos B cell line and in primary human CD19$^+$ B cells, we compared GFP-*Salmonella* by FACS analyses with light producing lux-*Salmonella* by luminometry. Light production requires ATP and thus forms a parameter for bacterial viability (24). We correlated the amount of light produced by the lux-*Salmonellae* to the percentage of living GFP-*Salmonella* positive B cells to assess intracellular *Salmonella* growth and viability. **Figure 3A** shows that *Salmonella* coated with anti-BCR and phagocytosed by Ramos cells can rapidly grow; over a time course of 10 hours we observed a strong increase in lux activity (**Figure 3A**, top left panel), while the number of GFP positive viable Ramos B cells remained nearly

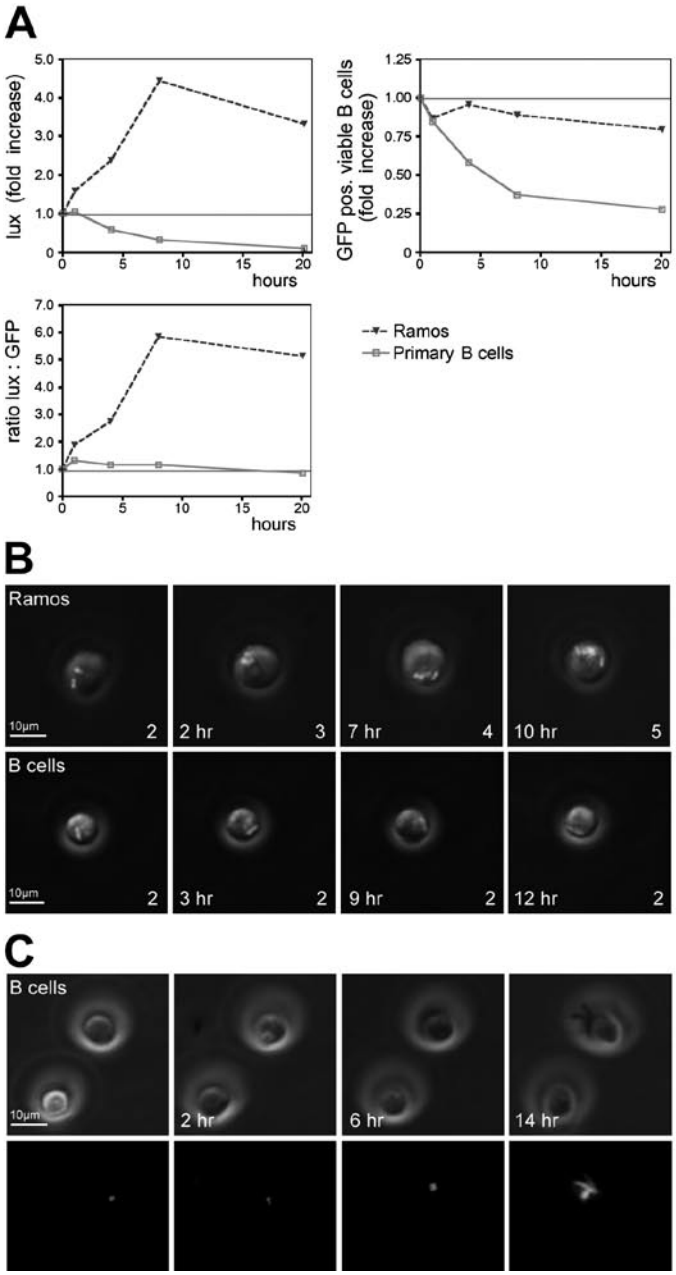

Figure 3. Primary B cells form a survival niche for intracellular *Salmonella* **(A)** Intracellular growth and survival of *Salmonella*. Analysis of either Ramos or primary human B cells incubated with living anti-BCR antibody coated lux-expressing (top left panel) or GFP-expressing (top right panel) *Salmonella*. Cells were incubated for 30 minutes, washed, and B cells with *Salmonella* followed for the time points indicated. The ratio of lux over GFP shows the amount of light produced per GFP positive B cell (bottom left panel), indicating intracellular *Salmonella* viability. The amount of light observed for Ramos B cells incubated with and positive for GFP-Salmonella increases with time, indicative of bacterial growth. In contrast, both lux activity and the fraction of GFP-*Salmonella* containing cells drop gradually in primary B cells. The ratio of GFP vs. lux did not change over time, showing that coated *Salmonellae* phagocytosed by IgM$^+$ human B cells neither grow nor die, surviving in a non-dividing state inside the cell. Shown is a representative example of three independent experiments. **(B)** *Salmonella* growth in Ramos B cell line, but not in primary B cells. Widefield fluorescence microscopy of living cells cultured at 37°C reflecting the experiment described in A. Depicted are GFP signals projected on top of the transmission image at indicated time points after incubation of the cells with the anti-BCR antibody coated bacteria and extensive washing to remove free *Salmonellae*. Scalebar equals 10µm. Number of bacteria in the cell of interest is given in the lower right corner. Upper panel: bacterial growth is observed in a Ramos cell that has phagocytosed anti-human BCR antibody coated GFP-expressing *Salmonella*. Lower panel: no bacterial growth is observed in a primary B cell that has taken up GFP-expressing *Salmonella*. Figure represents indicated time frames taken from suppl. movie S6 and S7. **(C)** Growth of *Salmonella* in primary B cells is actively suppressed. Under identical conditions as in Fig. 3B, cells were treated with the alkyl-lysophopholipid Edelfosine to induce apoptosis. No bacterial growth is observed in a primary B cell that has taken up GFP-expressing *Salmonella*, until the cell becomes apoptotic after 2h, showing characteristic nuclear morphological changes. Bacterial growth is observed after the onset of apoptosis, indicating that *Salmonella* growth is actively suppressed in living primary B cells. Top panel: transmission image, bottom panel: GFP-signal. Figure represents indicated time frames taken from suppl. movie S8.

constant (**Figure 3A**, top right panel). Consequently, the amount of light produced per GFP positive Ramos cell increased considerably (**Figure 3A**, bottom left panel), indicating that the number of bacteria per Ramos cell increased over time. In accordance, the GFP signal per B cell increased (**Suppl. Figure S5**). When isolated primary human B cells were incubated with anti-BCR coated bacteria however, lux activity sharply dropped over time (**Figure 3A**, top left panel), and the fraction of GFP-*Salmonella* containing B cells declined equally fast (**Figure 3A**, top right panel). The number of infected human primary B cells thus decreased over time. As we could rule out that this was the resultant of apoptosis of the infected B cells (**Suppl. Figure S11**), this pointed to degradation or exocytosis of ingested bacteria over time. The amount of light produced per living GFP-positive B cell however, remained constant during the course of the experiment (**Figure 3A**, bottom left panel), demonstrating that *Salmonella* did not degrade but survived in the primary human B cells, albeit under conditions of fully suppressed proliferation. To visualize this process we applied imaging of GFP-*Salmonella* positive B cells overnight using wide-field microscopy. Again rapid growth of *Salmonella* in the Ramos cells was observed (**Figure 3B**, top panel), while growth of the living bacteria was inhibited in human primary B cells (**Figure 3B**, bottom panel). Growth arrest required viable primary B cells since *Salmonella* started replicating intracellularly following induction of B cell apoptosis with Edelfosine (25) (**Figure 3C**). Apparently, factors specifically expressed in primary B cells control intracellular propagation of *Salmonella*.

How does GFP-*Salmonella* escape from B cells? Human primary B cells are highly photosensitive. To prevent apoptosis during imaging, B cells were co-cultured on a monolayer of CD40L-expressing 3T3 cells that provide a survival signal (35). Interestingly, primary B cells that had phagocytosed GFP-*Salmonella* showed extensive invasive behavior with cells continuously moving under and over the 3T3 fibroblast monolayer (**Figure 4A**). Occasionally, GFP-*Salmonella* exocytosed from the B cell at later time points. To better visualize this, GFP-*Salmonella* infected primary B cells were cultured in the presence of a low concentration of Texas-Red labeled anti-LPS antibodies. GFP-*Salmonella* will acquire this antibody upon exposure to the medium. **Figure 4B** shows a B cell with phagocytosed GFP-*Salmonella* that became accessible for anti-LPS antibodies after 8 hr of culture. *Salmonella* excretion from primary human B cells was quantified using FACS by detecting GFP-*Salmonella* and LPS-positive B cells. Strong increase in cell surface exposed LPS on cells that were initially GFP positive/LPS negative was observed (**Figure 4C**, left panel), confirming that most of the phagocytosed *Salmonella*e showed identical exocytosis behavior as the imaged representative from Figure 4B. Accordingly, the population of GFP positive/LPS negative B cells declined over time (**Figure 4C**, right panel). As expected, both processes had identical kinetics (**Suppl. Figure S11**). GFP-*Salmonella* infection of primary B cells did not affect viability or induced apoptosis (**Suppl. Figure S11**). Note that during the first phase of excretion *Salmonella* was released from phagosomes, but still remained associated to the B cells, hence the increased exposure to the anti-LPS antibodies in the first 10h. Later, the bacterium was separated from the B cell, explaining why LPS levels did not further increase. Loss of the GFP-*Salmonella* signal from infected primary B cells linearly increased for over 18 hours with more than 50% release from B cells. This suggests that GFP-*Salmonella* is slowly but constantly released from primary human B cells after initial phagocytosis.

Only viable *Salmonella* can successfully use BCR-mediated entry into primary B cells, but does it actively participate in excretion? We used Gentamycin in our experiments to prevent extracellular growth of bacteria. Gentamycin does not affect intracellular *Salmonella* replication unlike the antibiotics Tetracycline and Erythromycin that exhibit their effects also intracellularly (36). These antibiotics effectively inhibited lux-*Salmonella* growth in Ramos B cells (**Figure 4D**, left panel) by interfering with bacterial translation through binding to ribosomes. *Salmonella* secretion by primary human B cells was measured in the presence of either Gentamycin or Tetracycline. Tetracyclin did not affect excretion of GFP-*Salmonella* from primary human primary B cells, which occurred equally efficient as in the presence of Gentamycin (**Figure 4D**, right panel). Likewise, in the presence of Tetracyclin a similar increase in cell surface LPS levels was observed as that seen with Gentamycin (**Suppl. Figure S12**). Thus, *Salmonella* actively facilitates its own uptake after specific capture by the BCR, but intracellular growth and excretion of *Salmonella* is solely dependent on B cell activities.

Can *Salmonella* infect other cells after B cell exit at distant sites? We added human primary B cells with phagocytosed GFP-*Salmonella* to a fibroblast monolayer expressing CD40L and followed post-excretion

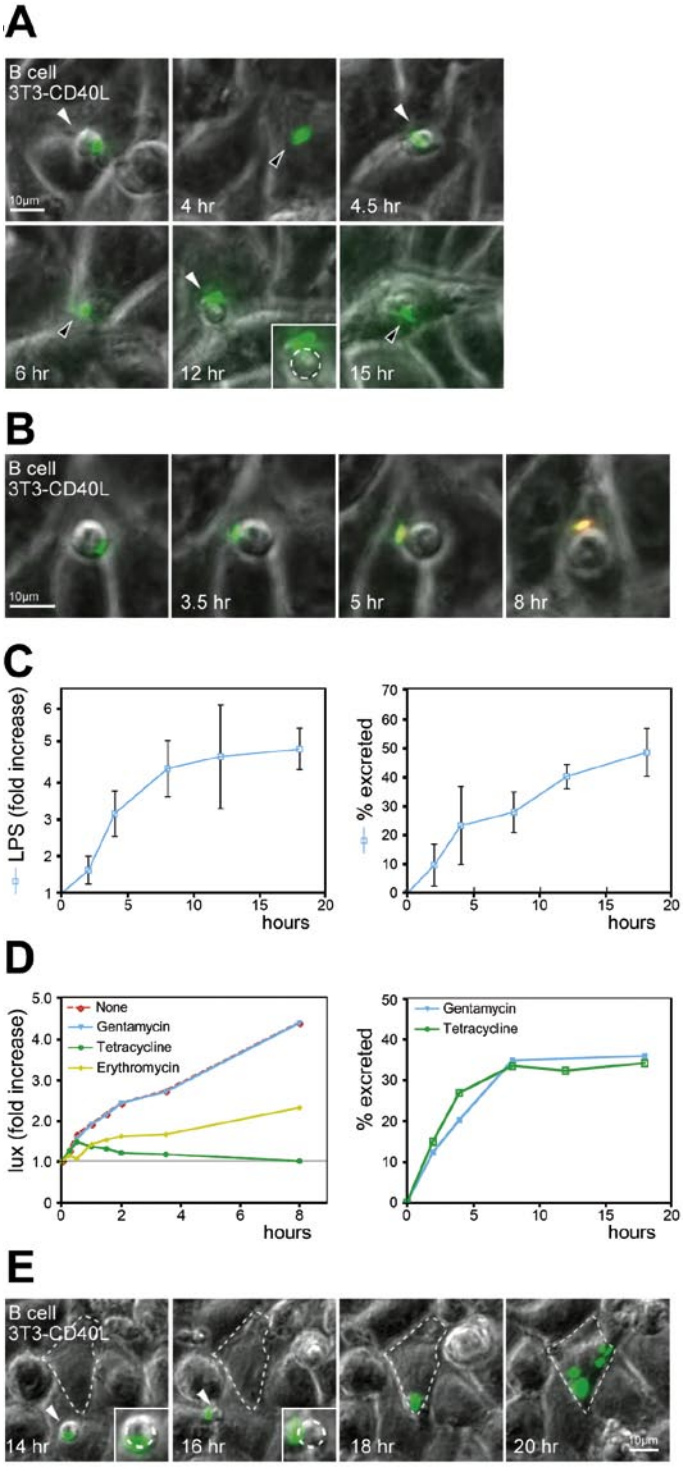

Figure 4. A. GFP-*Salmonella* containing human primary B cells show invasive behavior in a monolayer of CD40L-expressing fibroblasts. A co-culture of living primary human B cells infected with GFP-expressing *Salmonella* and CD40L-expressing 3T3 cells was imaged using widefield fluorescence microscopy at 37°C. Depicted is the GFP projected on the transmission image with images taken every 30 min. The B cell repeatedly moved under and over the monolayer. Arrows indicate the B cell, white: above the monolayer, black: below the monolayer. Also, excretion of the bacterium towards the end of the movie (best visible at 12h, zoomed in) is observed although *Salmonella* is still contacting the B cell. Figure represents indicated time frames taken from suppl. movie S9.
(B) Salmonella exocytosis from primary B cells. Living primary B cells were cocultured with CD40L-expressing 3T3 fibroblasts, and imaged using widefield fluorescence microscopy at 37°C. A single B cell that has taken up anti-BCR coated GFP-expressing *Salmonella* is imaged in medium containing a low concentration of anti-LPS antibodies labeled with Texas-Red. Depicted are GFP and Texas-Red signals projected on top of the transmission image at indicated time points after incubation of the cells with the anti-BCR coated bacteria and extensive washing to remove free bacteria. Scalebar equals 10µm. The intracellular bacterium is protected from staining by the extracellular antibodies until after 8h anti-LPS labeling is observed. Figure represents indicated time frames taken from suppl. movie S10.
(C) Quantification of *Salmonella* excretion by B cells. $CD19^+$ B cells were incubated with live uncoated GFP-expressing *Salmonellae* for 30 minutes and followed for the time points indicated. Cells were subsequently stained with anti-LPS antibodies, fixed and analyzed using FACS. Left panel: increase in cell surface exposed LPS from bacteria in the process of excretion. The level at timepoint 0 hours was arbitrarily set at 1. Right

panel: percentage of initially GFP-positive B cells that have excreted the bacterium and have become GFP negative. *Salmonella* is efficiently excreted from human primary B cells. Error bars represent standard deviations from three independent experiments. **(D)** Excretion of *Salmonella* is a B cell, rather than a *Salmonella* controlled process. Left panel: the effect of different antibiotics on the growth of lux-*Salmonella* in Ramos B cells. In the absence of antibiotics or in the presence of Gentamycin rapid bacterial growth is observed in Ramos cells, similar as in 3A. In contrast, lux activity is completely blocked by Tetracycline, and to a lesser extent Erythromycin, indicating successful elimination of lux-*Salmonella*. Right panel: the same FACS analysis as in 4C was performed in presence of either Gentamycin or Tetracycline to discriminate between host versus bacterial mediated excretion. Excretion of *Salmonella* occurred equally efficient in the presence of Tetracycline, indicating that viable *Salmonella* is not required for excretion. **(E)** Excreted *Salmonella* released from B cells can infect 3T3-CD40L cells. Imaging conditions are similar as in 4A. GFP-expressing *Salmonella* is excreted from a single primary human B cell (indicated by a white arrowhead), followed by infection of t
B cell excreting GFP-*Salmonella*. Subsequent rapid growth of *Salmonella* in a 3T3-CD40L cell but not in the B cell demonstrates that growth of the bacterium is actively suppressed inside the B cell, while its viability is maintained. Figure represents indicated time frames taken from suppl. movie S13.

events using time-lapse microscopy. **Figure 4E** shows a phagocytosed GFP-*Salmonella* that was extruded from the B cell, and subsequently infected the fibroblast monolayer. Interestingly, the bacteria rapidly replicated inside the fibroblast, demonstrating that the primary B cell had not suppressed the bacterial replication machinery in an irreversible manner. *Salmonella* can thus use B cells as a transport vehicle allowing it to escape immune attack and travel to distant locations to subsequently spread infection upon exocytosis from the B cell.

The form of immune escape reported here is unique in that *Salmonella* makes use of the specificity of the immune system to invade cells; only B cells with a BCR specific for *Salmonella* antigens phagocytose bacteria. Further analysis reveals that the bacterium targets most if not all types of BCR expressing B cells in peripheral blood, especially memory B cells (Souwer et al., accompanying article). The BCR acts as a specific receptor for *Salmonella*, and its crosslinking likely cooperates with injected bacterial effector proteins to facilitate uptake in these cells, which otherwise show very poor phagocytic behaviour. Inside B cells, *Salmonella* escapes immune attack by residing in phagosomes shielded from environmental attack. Unlike specialized phagocytes like macrophages or neutrophil, B cells are apparently less able to produce microbicidal products required to kill *Salmonella*. Primary B cells, unlike B cell lines, do however actively suppress *Salmonella* growth by a mechanism that remains elusive. It remains to be elucidated which factors are involved in the control of *Salmonella* excretion from primary B cells, except that these are host-derived. Identification and manipulation of signaling pathways to prevent excretion would potentially limit systemic spreading of *Salmonella*.

Does BCR mediated immune escape and spreading play an important role during a *Salmonella* infection? The bacterium will potentially encounter specific B cells very early during infection as it crosses the intestinal epithelium and moves into the gut-associated lymphoid tissue (GALT) sites where many B cells reside in Peyer's patches and other locations (37). About 3% of B cells have BCR against *Salmonella* antigens. Among the preferred distant sites of infection are the spleen and lymph nodes, locations *Salmonella* is thought to reach after uptake by neutrophils (19), which however also kills them (6). In the light of our experiments, a plausible alternative for dissemination of *Salmonella* would be that the bacteria use antigen-specific B cells as transport vehicles to distant sites in the body. *Salmonella* thus misuses the specificity of the adaptive immune system to evade attack from the early innate immune defenses and ensure systemic spreading of the infection.

Acknowledgments

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Supplemental Figures

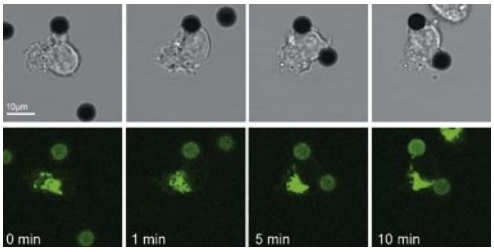


Figure S2. Treatment with nocodazole prevents bead uptake. Living Ramos B cells expressing HLA-DO/GFP were treated with nocodazole to disrupt the microtubular network, and imaged every 30 seconds using confocal microscopy at 37°C. Left panel: transmission image. Right panel: GFP signal. A single Ramos DO-GFP cell contacts two beads, neither of which it can phagocytose when the microtubule cytoskeleton is depolymerised. An intact microtubule network is required for efficient bead uptake. Note that the DO-GFP containing lysosomes form extended structures and tubules in the direction of the contact site with the bead. Total duration: 11 min.

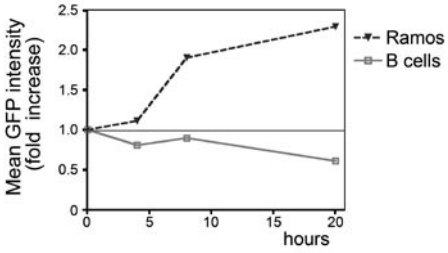


Figure S5. Intracellular growth and survival of *Salmonella*. FACS analysis of either Ramos or primary B cells incubated with GFP-expressing Salmonella. Cells were incubated for 30 minutes, washed, and B cells with *Salmonella* followed for the time points indicated. Depicted is the mean fluorescence of the GFP positive population, set arbitrarily at 1 at the beginning of the experiment. Whereas the GFP signal increases in Ramos B cells, it slightly decreases in primary human B cells.

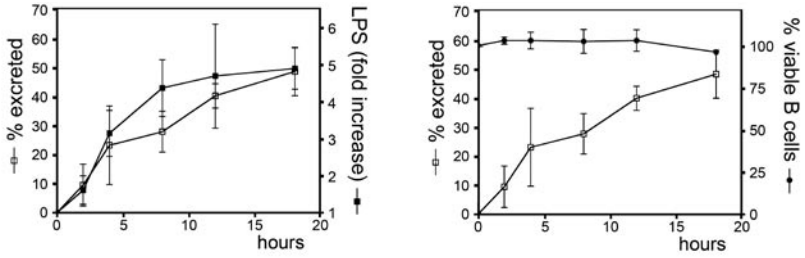
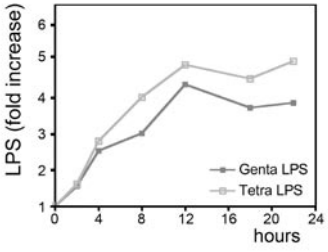


Figure S11. *Salmonella* excretion and increase in cell surface LPS levels have similar kinetics. Primary human CD19+ B cells were incubated with live uncoated GFP-expressing *Salmonella* for 30 minutes and followed up to the time points indicated. Cells were stained with antibodies against LPS, fixed and analyzed using FACS. Left panel: the derived graphs from Fig. 4C are projected on top of each other to illustrate that both processes show similar kinetics. Right panel: the fraction of living B cells is plotted to demonstrate that loss of GFP-*Salmonella* positive B cells cannot be explained by increased cell death.

Figure S12. Increase in cell surface LPS levels has similar kinetics in the presence of Gentamycin and Tetracycline, and cannot be explained by B cell death. The same FACS analysis as in Fig. 4B was performed in presence of either Gentamycin or Tetracycline to discriminate between host versus bacterial-mediated excretion. Increase in cell surface LPS levels is similar in the presence of Gentamycin and Tetracycline, indicating that viable *Salmonellae* are not required for excretion.



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B cell activation and induction of acquired immunity through BCR-mediated phagocytosis of Salmonella

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Antigen presentation by B cells is needed to generate high-affinity antibodies. Activation of B cells is triggered by binding of antigen to the B cell antigen Receptor (BCR), followed by internalisation of the BCR-complex. It is still unclear how B cells process and present particulate Ags like microbes. We studied the immunological responses induced upon encounter of B cells with Salmonella typhimurium bacteria in B cell lines expressing DR-GFP (MHC class II molecule) or DO-GFP (MHC class II chaperone) and primary B cells. We show that B cells become highly efficient phagocytes of bacteria upon ligation of the BCR by the bacteria. Phagocytosis induces immediate and extensive kiss-and-run events and fusion between the MHC class II antigen loading compartments and the phagosome. Efficient MHC class II-mediated presentation of Salmonella antigens and proliferation of antigen-specific autologous T cells is induced even while the viable bacteria are actively excreted by the B lymphocyte. BCR-mediated phagocytosis is sufficient to lead to B cell differentiation and secretion of Salmonella-specific antibodies, but is boosted by aid of specific Thelper cells. Thus, BCR-mediated phagocytosis provides a temporal immune escape niche for Salmonella, but simultaneously induces a highly efficient adoptive immune response to proficiently combat infection in the long run.

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Introduction

Defence against pathogens is essential for survival and is controlled by both the innate and acquired arms of the immune system. Antigen presentation by B lymphocytes is needed to generate high-affinity antibodies and to coordinate antigen-specific cytotoxicity (1, 2). Development of an effective humoral immune response is mediated by two subsequent actions of the BCR; 1. transmembrane signaling to induce B cell proliferation and differentiation and 2. antigen internalization for processing followed by MHC class II-mediated presentation to acquire T cell help. The proper execution of both actions requires binding of a polyvalent Ag to the BCR. Indeed, many B cell antigens are in fact polyvalent as they are bound in multiple copies to the particulate surfaces of microbes or cells (reviewed by (3)). Since B cells are not considered to be phagocytic, it is unclear how they acquire antigens from bacteria for antigen presentation.

* Y. Souwer and A. Griekspoor contributed equally to this paper

Experimental Procedures

Antibodies, beads and fluorophores

Dynabeads® goat anti-mouse IgG (Dynal Biotech, Oslo, Norway) with a diameter of

4.5 μM were coated with monoclonal mouse anti-human IgM antibody (MH15, Sanquin, Amsterdam, the Netherlands). The same antibody was mixed with the monoclonal mouse anti-*S. typhimurium* LPS antibody (1E6, Biodesign International, Kennebunk, ME) and rat anti-mouse IgG1 antibody (RM161.1, Sanquin, Amsterdam, the Netherlands) to generate BCR-LPS bridging tetramers. These tetramers were used to coat the *Salmonella* bacteria. MH15 F(ab')2 fragments were generated by pepsin digestion and complete digestion was confirmed by silver staining (Silver staining kit, Bexel, Union City, CA) of a SDS-PAGE gel (NuPage, Invitrogen, Carlsbad, CA). Phyco-erythrin (PE)-conjugated monoclonal antibody anti-CD27 was obtained from BD Biosciences (San Jose, CA) and anti-CCR7 from R&D Systems (Minneapolis, MN). Fluorescent secondary antibody goat anti-mouse Alexa Fluor 633 and Lysotracker Red were obtained from Molecular Probes (Leiden, the Netherlands.

DNA constructs and cell lines

The pcDNA3 DOβGFP (4) and pcDNA3 DR1βGFP (5) fusion constructs have been described before. DOβGFP and DR1βGFP were demonstrated to form complexes with their respective endogenous α-chain. Transfections were performed by electroporation using a Gene Pulser II with Capacitance Extender (Bio-Rad Laboratories, Hercules, CA) Stable transfectants of the EBV-negative human B cell lymphoma cell line Ramos were selected and maintained in RPMI medium supplemented with 5% FCS (Bodinco, Alkmaar, the Netherlands), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine, 50 μM 2-mercaptoethanol in the presence of 2000 μg/ml G418 (Gibco, Paisley, UK). Stable expression of the GFP-tagged proteins was verified by Western blotting and ensured by regular selection of positive cells by FACS sorting. NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) (6) were cultured in IMDM medium, supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine, 50 μM 2-mercaptoethanol and 500 μg/ml G418 (Gibco, Paisley, UK). The day before experiments, 3T3-CD40L cells were harvested, washed in medium without G418 and irradiated with 30 Gy (Gammator M38-1, MDS Nordion, Ottawa, ON, Canada). Subsequently, irradiated cells were seeded in 96 wells flat bottom plates ($5x10^3$ cells per well) and allowed to form a confluent monolayer overnight.

Bacterial Strains

The strains *Salmonella enterica* serovar *typhimurium* SL1344 (*Salmonella*) (7), GFP-*S. typhimurium* SL1344 (8) and mRFP-*S. typhimurium* SL1344 (9) were described before. Bacteria were grown in Luria-Bertani (LB) broth with ampicillin overnight at 37°C while shaking, subcultured at a dilution of 1:33 in fresh LB media, and incubated at 37°C while shaking for 3.5 h. Bacteria were washed once with PBS, incubated 1:25 with the BCR-LPS bridging tetramers in a total volume of 100 μl PBS for 30 min at room temperature, and washed twice to remove unbound antibodies.

Lymphocyte isolation and proliferation assay

Human peripheral blood mononuclear cells 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, the Netherlands). B and T cells were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads® and DETACHaBEAD® (Dynal Biotech, Oslo, Norway), according to the manufacturer's instructions. B lymphocytes were incubated for 30 minutes at 37°C with dead or living coated or uncoated *Salmonella* bacteria, and subsequently cultured with and without T lymphocytes in RPMI medium w/o phenol red, supplemented with 5% FCS (Bodinco), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-Glutamine, 50 μM 2-mercaptoethanol, 20 μg/ml human apo-transferrin ((Sigma-Aldrich, Steinheim, Germany), depleted for human IgG with protein G sepharose (Amersham, Sweden)) and 10 μg/ml gentamicin (Invitrogen) or tetracyclin (Sigma). All cells were cultured in 200 μl at 37°C in the presence of 5% CO_2 in 96 wells round bottom plates (Greiner Bio-One, Frickenhausen, Germany). The maximum proliferation capacity of T lymphocytes was established by stimulation with anti-CD3 (CLB.T3/4.E, Sanquin Reagents, Amsterdam, the Netherlands) and anti-CD28 (CLB.CD28/1, Sanquin Reagents) which were both used at 1 μg/ml. After 6 and 13 days, 150 μl of supernatant was collected for cytokine and antibody measurement and fresh medium was added. To study the kinetics of antigen presentation, B cells incubated with *Salmonellae* as indicated, were irradiated with 60 Gy at several time points before incubation with T cells. For B/T cell proliferation assays, cells were cultured for 16 hours in the presence of [^{3}H]-thymidine (GE Healthcare, Buckinghamshire, UK) at a final concentration of 1 μCi/ml (37 kBq/ml). The cells were harvested on glass fibre filters (Wallac, Turku, Finland) and radioactivity was measured with 1205 Betaplate liquid scintillation counter (Wallac, Turku, Finland).

FACS analyses

For FACS analysis, Ramos cells were grown overnight in medium without antibiotics, washed, and subsequently incubated with coated or uncoated bacteria in RPMI 1640

without antibiotics for 30 min at 37°C while tumbling. Freshly isolated primary B cells were incubated immediately after isolation. Next, cells were washed four times and cultured for 1h in media containing 50 µg/ml gentamicin (Invitrogen) or tetracycline to eliminate non-phagocytosed bacteria. Until analysis on a LSR II (Becton Dickinson), cells were maintained in medium supplemented with 10 µg/ml gentamicin or tetracyclin. FACS sorting of B cells that had phagocytosed uncoated living bacteria was performed on a MoFlo Sorter (Dakocytomation, Glostrup, Denmark).

CLSM and electronmicroscopy analyses

For CLSM analysis, coverslips were coated with 1 mg/ml Poly-L-lysine (Sigma-Aldrich co., Steinheim, Germany) for 1h and washed thoroughly with aquadest and dried on air. Cells were allowed to attach on the coated coverslips for 15 min. and subsequently beads or *Salmonella*e were added. Confocal analysis was performed using a Leica TCS SP confocal laser scanning microscope equipped with an Argon/Krypton laser (Leica Microsystems, Heidelberg, Germany). Green fluorescence was detected at λ>515 nm after excitation at 488 nm. For dual analyses, green fluorescence was detected at 520–560 nm. Red fluorochromes were excited at 568 nm and detected at λ>585 nm. All experiments presented were repeated several times on different days, and results were consistent and reproducible. Further image processing was performed using the ImageJ software package. For electron microscopy, cells were allowed to phagocytose beads or bacteria for 30 min, fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (0.5%), and subsequently processed for immuno-electron microscopy. After embedding in a mixture of methyl-cellulose and uranyl acetate, sections were analyzed with a Philips CM10 electron microscope (Eindhoven, the Netherlands).

ELISA assays

To determine IgM and IgG levels in culture supernatants, flat bottom microtiter maxisorb plates (Nunc, Roskilde, Denmark) were coated with polyclonal anti-IgM (SH15, Sanquin) or monoclonal mouse anti-human IgG (MH16-1, Sanquin), respectively, in 100 µl PBS, pH 7.4 (NPBI International BV, Emmer-Compascuum, the Netherlands) overnight at room temperature. Plates were washed with PBS/0.02%Tween-20 (Mallinckrodt Baker, Deventer, the Netherlands) and residual binding sites on plates were blocked for 1 hour at room temperature with 200 µl per well of PBS containing 2% milk (low-fat, Campina, Zaltbommel, the Netherlands). After washing, samples were incubated for 2 hours in high performance ELISA buffer (HPE, Sanquin). As a standard pooled human serum was used. Plates were washed and incubated for 1 hour with 1 µg/ml monoclonal mouse anti-human IgM horseradish peroxidase (MH15-HRP, Sanquin), or monoclonal mouse anti-human IgG horseradish peroxidase (MH16-1-HRP, Sanquin).

To detect IL-6, maxisorb plates were coated overnight at room temperature with monoclonal anti-human IL6 (IL-6/16, Sanquin) in 100 µl 0.1M Na-bicarbonate at pH 9.6. After washing, samples were incubated together with biotinylated polyclonal sheep anti-human IL6 (Sanquin) for 2 hours in high performance ELISA buffer. As a standard HGF 22.10 (Sanquin) was used. Plates were washed and incubated for 30 minutes with 0.1 µg/ml streptavidine poly-horseradish peroxidase, (M2032, Sanquin).

Whole cell-*Salmonella* ELISA was performed by coating overnight at 37°C of *Salmonellae* to maxisorb plates in 100 µl 0.1M Na-bicarbonate at pH 9.6 supplemented with 10 µg/ml tetracyclin. Plates were washed extensively with PBS/0.02%Tween-20 and supernatants were incubated in high performance ELISA buffer. Plates were washed and incubated for 1 hour with 1 µg/ml monoclonal mouse anti-human IgM horseradish peroxidase (MH15-HRP, Sanquin). After washing, peroxidase activity was visualized by incubation with 100 µl 3,5,3',5'-tetrametthylbenzidine (Merck, Darmstadt, Germany), 100 µg/ml in 0.11 M Na-acetate, pH 5.5, containing 0.003% H_2O_2 (Merck). The reaction was stopped by addition of an equal volume of 2M H_2SO_4 (Merck) and the absorbance at 450 nm and 540 nm was measured immediately in a Titertek plate reader. Results were calculated with LOGIT software (http://www.xs4all.nl/~ednieuw/Logit/logit.htm) .

Results

The ability of B cells to present particulate antigens has been noted (10-13) and presentation of particulate antigen by B cells depends critically upon the density of antigenic epitopes on the particle (14, 15). Studies on MHC-mediated presentation of BCR-specific antigens are mainly performed with soluble antigens or with pre-crosslinked anti-BCR antibodies. We focussed on the recognition of particulate, polyvalent antigens and used beads decorated with anti-IgM antibodies as a model system. We generated a stable transfectant of the Ramos B cell line with the MHC class II antigen presentation chaperone HLA-DO tagged with GFP (DO-GFP). DO-GFP localises to the MHC class II antigen loading compartments (MIICs) and thus marks the MIICs in living cells. When anti-IgM-coated beads were added to surface IgM-positive Ramos B cells the beads were rapidly

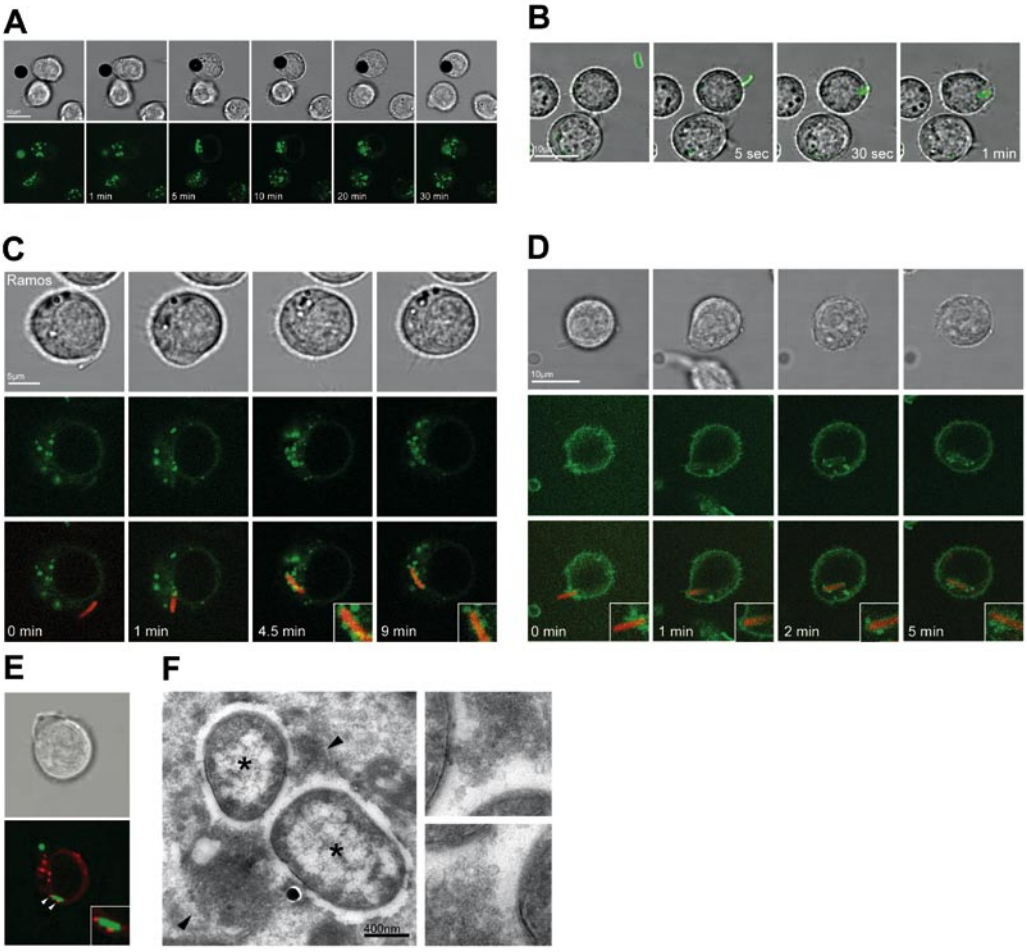

Figure 1. Efficient BCR Mediated phagocytosis of large particulate antigens. **(A)** Phagocytosis of anti-BCR coated beads by Ramos cells stably expressing DO-GFP. Living cells were imaged every 30 seconds using confocal microscopy at 37°C. Depicted are indicated timepoints after contact with the bead, top panel: transmission image, bottom panel: GFP signal. Scalebar equals 10µm. DO-GFP localizes exclusively to lysosomal vesicles which initially move rapidly towards the phagocytosed bead and interact with the phagosome and disperse again after 10 to 20 minutes. Figure represents indicated frames taken from suppl. movie S1.
(B) BCR mediated phagocytosis of anti-BCR coated *Salmonella* by Ramos cells stably expressing DO-GFP. Living cells and GFP-expressing *Salmonella* were imaged every 3 seconds using confocal microscopy at 37°C. Depicted are indicated timepoints after contact with the bacterium. GFP signal is projected on top of the transmission image. Scalebar equals 10µm. Single viable bacterium coated with anti-BCR antibody is efficiently phagocytosed in a BCR dependent manner. Figure represents indicated frames taken from suppl. movie S2.
(C) BCR mediated phagocytosis of anti-BCR coated mRFP-expressing *Salmonella* by Ramos cells stably expressing DO-GFP. Living cells and mRFP-expressing *Salmonella* were imaged every 10 seconds using confocal microscopy at 37°C. Depicted are indicated timepoints after contact with the bacterium, top panel: transmission image, middle panel: GFP signal, bottom panel: overlay of GFP and mRFP signal. Scalebar equals 5µm. Inset shows zoom-in on bacterium. Extensive docking of DO-GFP positive vesicles with the bacterial phagosome is oberved within minutes after entry. Figure represents indicated frames taken from suppl. movie S3.
(D) BCR mediated phagocytosis of anti-BCR coated mRFP-expressing *Salmonella* by Ramos cells stably expressing DR-GFP. Living cells and mRFP-expressing *Salmonella* were imaged every 10 seconds using confocal microscopy at 37°C. Depicted are indicated timepoints after contact with the bacterium, top panel: transmission image, middle panel: GFP signal, bottom panel: overlay of GFP and mRFP signal. Scalebar equals 5µm. Inset shows zoom-in on bacterium. The DR-GFP positive plasma membrane clearly forms

the major source of the phagosomal membrane surrounding the bacterium. Again, docking of DR-GFP positive vesicles with the bacterial phagosome is oberved within minutes after entry. Figure represents indicated frames taken from suppl. movie S4.
(E) Ramos cells were incubated with anti-BCR coated GFP-expressing *Salmonella* and lysotracker-Red was added to visualize the acidic MIICs. Extensive docking of the acidic vesicles and the phagosome is observed. Inset shows zoom-in on the region indicated by the arrowheads.
(F) Lysosomal fusion with *Salmonella* containing phagosome. Ramos cells were allowed to phagocytose anti-BCR coated *Salmonella* for 1hr after which they were fixed and analysed by electron microscopy. Multiple fusion events of multivesicular MIIC vesicles with the phagosome are observed. Scalebar equals 100nm. Asterisk marks the bacterium, arrowheads indicate fusion events with MIICs. Insets shows zoom-in on regions indicated by the arrowheads.

and very efficiently phagocytosed (**Figure 1A**, Suppl. Movie S1). Simultaneously, after initial contact with the bead the DO-GFP positive late endocytic/lysosomal vesicles translocated towards the phagosome. Multiple kiss-and-run events were observed between the DO-GFP positive vesicles and the phagosomal membrane, which could be an indication for fusion events of the DO-GFP positive MIICs with the phagosome in the first minutes after initial contact. Phagocytosis of the anti-IgM decorated bead reached completion after 10 to 20 minutes. DO-positive vesicles started to move away from the phagosome after 10 minutes from initial contact and returned to their normal distribution pattern in the cell. Beads coated with irrelevant antibodies remained untouched by Ramos cells (Suppl. Movie S2).

Next, we used the more physiologically relevant particle *Salmonella typhimurium*. These are Gram-negative, enteric bacteria responsible for disease syndromes of significant morbidity and mortality worldwide. Being facultative intracellular pathogens, immunity to *Salmonella* infections requires adequate humoral and cell-mediated immune responses (16, 17). *Salmonella* organisms invade host macrophages and establish a niche inside discrete vacuoles, known as *Salmonella*-containing vacuoles (18). The ability of the bacteria to establish and maintain their intracellular niche within macrophages is considered crucial for their survival and their pathogenicity (19, 20). Because the specificity of the BCR of Ramos B cells is unknown, we coated the bacteria with anti-IgM antibodies by adding tetramers of anti-human IgM antibodies and anti-*Salmonella* LPS antibodies which were coupled to each other with rat anti-mouse IgG1 antibodies. Ramos cells were capable of phagocytosing GFP-positive-*Salmonella* coated with these tetramers (**Figure 1B**, Suppl. Movie S3), while uncoated *Salmonella*e were left untouched (Suppl. Movie S4). Ramos DO-GFP cells incubated with coated mRFP positive-*Salmonella* again shows movement of the DO-positive vesicles to the phagosome (**Figure 1C**, Suppl. Movie S5). Again, kiss-and-run events were observed between the phagosome and the DO-GFP positive MIICs. The plasma membrane is the main source of the phagosomal membrane, as the phagosomal membrane is GFP-positive upon formation of the phagosome after incubation of mRFP positive coated *Salmonella* with Ramos cells stably expressing the MHC class II molecule HLA-DRβ1 tagged with GFP (DR-GFP) (**Figure 1D**, Suppl. Movie S6). Similar to the DO-positive vesicles, the intracellular DR-GFP positive vesicles move to the phagosome. To show that a part of the antigen loading compartments actually fuse with the phagosome after initial contact, we stained Ramos cells with Lysotracker Red, which accumulates in the acidic MIICs, and added anti-IgM coated GFP-*Salmonella*. The lysosomal MIIC vesicles interact with phagosomes (**Figure 1E**). Because fusion of the MIIC vesicles with the phagosome could be critical for the induction of MHC class II presentation of *Salmonella* antigens, we confirmed this observation by cryo-electron microscopy (EM) on Ramos cells incubated with the coated bacteria. Fusion events between the characteristically multivesiculair MIICs and the phagosome were observed (**Figure 1F**).

MHC class II antigen presentation requires antigen processing in the endocytic pathway and subsequent binding of antigenic peptides to MHC class II molecules. Antigen processing in B cells has two features that are not present in other types of antigen presenting cells: 1. BCR signalling ignited by antigen induces changes in the antigen processing machinery favouring MHC class II mediated antigen presentation (reviewed by (21)) and 2. expression of HLA-DO modulates peptide loading of MHC class II molecules by HLA-DM (22). These characteristics favour presentation of antigens internalized through the BCR. To address presentation of phagocytosed *Salmonella* in an autologous setting, we first studied *Salmonella* uptake in primary B cells. Primary B cells isolated from peripheral blood were incubated with anti-IgM coated *Salmonella*e for 30 minutes. After washing, B cells were incubated with anti-LPS antibodies prior to FACS analyses to discriminate between adherent bacteria (GPF^+/LPS^+) and internalized bacteria (GPF^+/LPS^-).

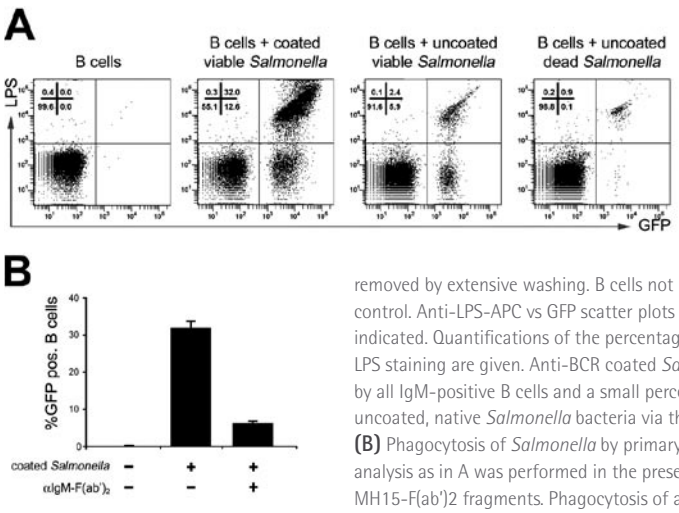

Figure 2. BCR mediated phagocytosis of *Salmonella* by primary B cells
(A) FACS analysis of BCR mediated phagocytosis of *Salmonella*, either or not coated with anti-BCR antibodies, by primary B cells isolated from human peripheral blood. $CD19^+$ B cells were isolated and incubated with live GFP-expressing *Salmonellae* for 45 minutes, and free bacteria were removed by extensive washing. B cells not incubated with *Salmonellae* were used as control. Anti-LPS-APC vs GFP scatter plots are depicted for the different conditions, as indicated. Quantifications of the percentage GFP-*Salmonella* positive B cells and anti-LPS staining are given. Anti-BCR coated *Salmonellae* are efficiently phagocytosed by all IgM-positive B cells and a small percentage of B cells are able to phagocytose uncoated, native *Salmonella* bacteria via their own, unique BCR.
(B) Phagocytosis of *Salmonella* by primary B cells is BCR mediated. The same FACS analysis as in A was performed in the presence or absence of anti-IgM heavy chain MH15-F(ab')2 fragments. Phagocytosis of anti-BCR coated GFP-expressing *Salmonella* by B cells is dependent on interaction with the BCR. Data are representative of at least 4 different donors, error bars represent SEM.

All IgM-positive primary B cells stained GFP-positive after incubation with coated GFP-*Salmonella* and on average 32% (SD=1.9, n=5) of the B cells showed a GFP^+/LPS^- phenotype demonstrating complete uptake of the bacteria (**Figure 2A**). Are primary B cells also capable of phagocytosing uncoated *Salmonella*? Primary human B cells were incubated with uncoated *Salmonella* and this consistently revealed a small but significant population of B cells (3.1%, SD =3.8, n=8) able to recognize and phagocytose the native bacterium, probably by direct recognition of *Salmonella* antigens by the B cell's BCR (**Figure 2A**). A small subset of primary B cells recognized dead uncoated GFP-*Salmonella* via their unique BCR (0.8% SD=0.63, n=4), but failed to phagocytose the dead *Salmonellae* since all B cells stained positive for LPS (**Figure 2A**). *Salmonella* thus requires both recognition by BCR and bacterial-mediated processes to enter human B cells. The BCR-dependency of the process was demonstrated as bacterial binding could be blocked by pre-incubation of the B cells with anti-IgM heavy chain MH15-F(ab')2 fragments (**Figure 2B**). Analysis by confocal microscopy showed that phagocytosed viable *Salmonellae* are completely inside the primary B cell, to the extend of one to three bacteria per cell. (Griekspoor *et al*., accompanying paper). Using wide-field fluorescence microscopy, we demonstrated that phagocytosed *Salmonellae* do not replicate inside B cells and are eventually excreted from B cells to infect other cells (Griekspoor *et al*., accompanying paper).

Does BCR-mediated phagocytosis of *Salmonella* lead to MHC class II mediated presentation of *Salmonella* antigens even though the bacteria survive within the phagosome? We incubated primary B cells with uncoated and anti-IgM-coated *Salmonellae* and cultured them for 6 days either or not in the presence of autologous primary T cells. B cells incubated with viable uncoated or coated *Salmonella* and cultured with T cells stimulated proliferation of T cells. Incubation of B cells with viable coated *Salmonella* induces proliferation of the B cells, indicating that BCR-ligation and phagocytosis of *Salmonella* leads to proliferation of B cells. Incubation with uncoated *Salmonella* did not reach proliferation levels above background levels, probably because there are too few cells that had phagocytosed *Salmonella* in the total pool of B cells (**Figure 3A**, left panel). Indeed, proliferation was independent of the antibody coat of *Salmonella* since B cells incubated with native, uncoated GFP-*Salmonella* and subsequently isolated by FACS-sorting showed proliferation. Incubation of only T cells with uncoated or coated bacteria did not lead to proliferation of the T cells (data not shown). Thus, even though *Salmonella* survives in the vacuole (Griekspoor *et al*., accompanying paper), *Salmonella* antigens are still efficiently presented. This is in line with the observed rapid fusion between MIICs and phagosome.

Is phagocytosis of the bacteria via the BCR a prerequisite for efficient antigen presentation to T cells?

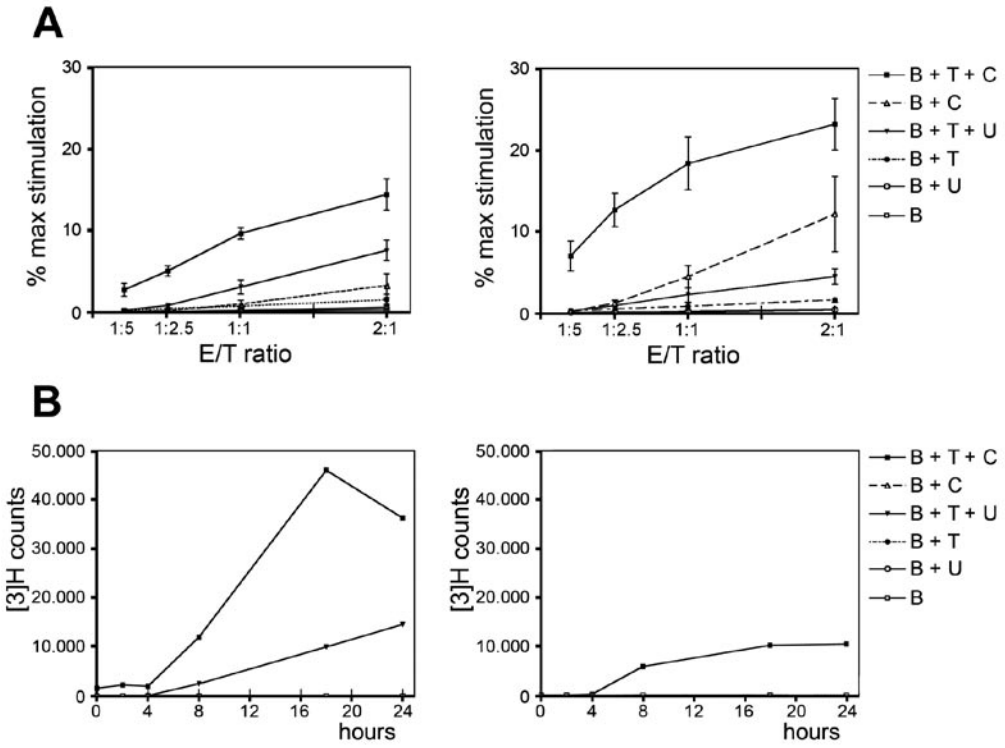

Figure 3. Phagocytosis of viable and dead *Salmonella* leads to antigen presenting B cells. **(A)** Left panel. BCR induced phagocytosis results in proliferation of B cells and antigen specific T cells. B cells (B) were either or not incubated with live uncoated (U) or anti-BCR coated (C) *Salmonella* in the presence or absence of autologous T cells (T), as indicated. After 5 days, [^{3}H]Thymidine was added and cells were harvested the next day. Results are shown as percentage of maximal stimulation of T cells with anti-CD3 and -CD28 antibodies. Uncoated *Salmonella* phagocytosed by BCR-specific B cells induce T cell proliferation. Anti-BCR coated *Salmonella* phagocytosed by IgM$^+$-B cells induce proliferation of those B cells and an antigen-specific proliferation of T cells. Right panel shows the same experiment with dead *Salmonellae* and clearly shows that phagocytosis is needed for efficient antigen presentation, as dead uncoated *Salmonellae* that stick to the BCR without being phagocytosed do not induce T cell proliferation. Data are from four independent experiments of different donors and the error bars represent SEM. E/T represents the ratio of different amount of T cells (Target) added to the fixed amount of B cells (Effector) or B cells alone. Data are from 3 independent experiments of different donors and the error bars represent SEM. **(B)** Antigen presentation by B cells starts immediately after phagocytosis of the *Salmonellae*. B cells (B) were either or not incubated with uncoated (U) or anti-BCR coated (C) *Salmonella* and irradiated with 60 Gy at different time-points before T cells (T) were added. After 5 days, [3H]-thymidine was added and cells were harvested the next day. B cells alone do not induce T cell proliferation after irradiation of the B cells. Anti-BCR coated *Salmonella* phagocytosed by IgM$^+$-B cells start to induce proliferation of T cells immediately and uncoated *Salmonella* phagocytosed by BCR-specific B cells four hours after phagocytosis of the *Salmonellae* (left panel). Incubation with fixed *Salmonellae* shows that only incubation with anti-BCR coated bacteria leads to antigen presentation (right panel).

To study this, we used dead bacteria (fixed with paraformaldehyde) in similar experiments as we demonstrated that dead uncoated bacteria are not internalised. Interestingly, dead bacteria only induced proliferation of B and T cells when coated with anti-IgM-anti-LPS tetramers for phagocytosis. Uncoated dead *Salmonella*e that are not phagocytosed but remain attached to the outside of the B cell did not induce T cell proliferation (**Figure 3A**, right panel). Thus, B lymphocytes are inefficient in presenting *Salmonella* antigens unless the bacteria are phagocytosed via the BCR.

When we irradiated B cells immediately after incubation with anti-IgM coated *Salmonella*e, no proliferation of B or T cells was found after six days. B

cells thus need to be metabolically active to process and present *Salmonella*-antigens to T cells. To study the kinetics of antigen presentation, B cells were incubated with living native and anti-IgM coated *Salmonellae* and irradiated at several time points before incubation with T cells. Antigen presentation starts immediately after BCR-induced phagocytosis and rapid fusion with the MIICs (**Figure 3B**, left panel). Incubation with dead *Salmonellae* revealed that B cells have to be viable and metabolically active for at least four hours after uptake of *Salmonella* via the BCR before antigen presentation ensues (**Figure 3B**, right panel). Primary B cells present antigens of phagocytosed *Salmonella*, even if the bacterium itself is able to survive inside a B cell.

Which peripheral B cells phagocytose particulate antigens like *Salmonella* best? Two major subsets of B cells can be identified in adult peripheral blood according to the expression of CD27. $CD27^+$ B cells comprise memory B cells and $CD27^-$ B cells comprise naive and transitional B cells (23). FACS analysis showed that predominantly memory B cells ($CD27^+$) take up *Salmonella* (**Figure 4A**), since the ratio of $CD27^+$/$CD27^-$ B cells is 3 fold higher in the GFP-*Salmonella* positive B cell population than in the B cells that were not incubated with bacteria. The majority of peripheral blood B cells is positive for CCR7 and expression of CCR7 keeps B cells for a defined period of time in close contact with T cells to allow effective B-T cell interactions (24). Analysis of the *Salmonella*-positive B cells showed that all cells were positive for CCR7 (**Figure 4B**). Memory B cells represent the major B cell population able to phagocytose *Salmonella*.

For induction of B cell differentiation towards antibody secreting cells, interleukin-6 (IL-6) is important. IL-6 is a multifunctional cytokine that regulates the growth and differentiation of various tissues, and is best known for its role in immune responses. Earlier studies showed that IL-6 production by antigen-specific B cells may play a critical role in early T cell activation (25). We investigated IL-6 secretion by B cells either or not incubated with either or not anti-IgM coated *Salmonella* either or not cultured with T cells. IL-6 was detected when the BCR was ligated with coated bacteria (**Figure 4C**), indicating that a strong BCR signal leads to an autocrine loop with production of IL-6. T cells alone or incubated with *Salmonella* do not produce IL-6 (data not shown).

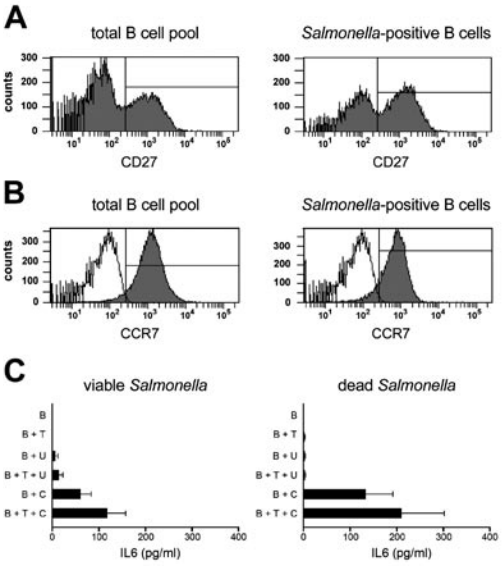

Figure 4. Preferentially memory B cells phagocytose *Salmonella* and BCR-mediated phagocytosis induces autonomous production of IL-6. **(A)** *Salmonellae* are preferentially phagocytosed by memory B cells. Primary B cells were incubated with live GFP-expressing *Salmonellae* and stained for CD27 before and after FACS-sorting. While one of every three *Salmonella*-negative B cell is CD27-positive, one of every two B cells with a BCR specific for *Salmonella* is CD27 positive. **(B)** The same cells as in 4A were analysed for expression of CCR7. All *Salmonella* positive B cells were positive for CCR7 at the cell surface. The unfilled histogram represents the IgG_1 isotype control used for both the CD27 and CCR7 antibodies. **(C)** B cells (B) were either or not incubated with dead or viable uncoated (U) or anti-BCR coated (C) *Salmonella* in the presence or absence of autologous T cells (T), as indicated. After 6 days supernatant was harvested. Supernatants were tested for IL-6 production by B cells using an IL-6 specific ELISA. IL-6 was detectable after BCR-induced phagocytosis with living (left panel) and dead (right panel) anti-BCR coated *Salmonellae*, indicating that a strong BCR signal leads to an autocrine loop with production of IL-6.

Antibodies play an important role in the acquired host defence by opsonising pathogenic organisms and subsequent complement-mediated lysis. Does BCR-specific phagocytosis of *Salmonella* drive B cells into secretion of Ig's? We tested the supernatants of B cells incubated with either or not coated bacteria for human IgM and IgG produced after six and thirteen days of culturing in the absence or presence of T cells. After incubation with viable bacteria, no strong induction of IgM secretion following phagocytosis was detectable in the supernatants, even when the *Salmonellae* were coated with anti-IgM-anti-LPS tetramers

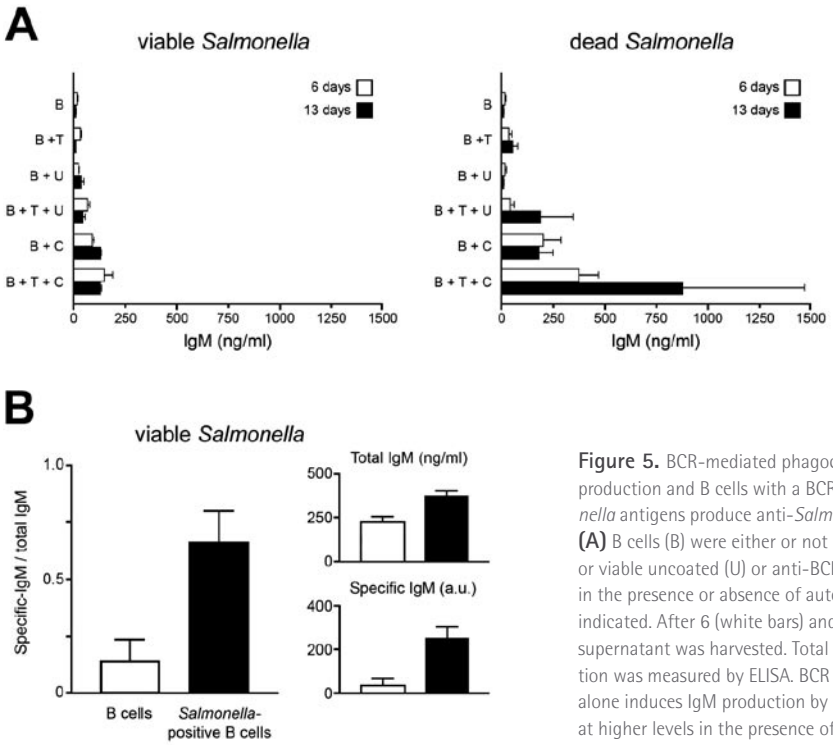

Figure 5. BCR-mediated phagocytosis induces IgM production and B cells with a BCR specific for *Salmonella* antigens produce anti-*Salmonella* IgM. **(A)** B cells (B) were either or not incubated with dead or viable uncoated (U) or anti-BCR coated C) *Salmonella* in the presence or absence of autologous T cells (T), as indicated. After 6 (white bars) and 13 days (black bars) supernatant was harvested. Total human IgM production was measured by ELISA. BCR induced phagocytosis alone induces IgM production by B cells and is produced at higher levels in the presence of T cells. **(B)** B cells either or not incubated with viable uncoated GFP-*Salmonella* were FACS-sorted and cultured on a monolayer of irradiated, CD40L-expressing fibroblasts for 13 days. Anti-*Salmonella* IgM was divided on the total amount of IgM measured in the supernatants. This clearly shows that B cells with a BCR specific for *Salmonella* antigens produce anti-*Salmonella* IgM (left panel). The top right panel shows the total IgM production and the right bottom panel the *Salmonella*-specific IgM production. Data are representative for 3 donors.

(**Figure 5A**, left panel). The few B cells that recognize dead, uncoated *Salmonella* require T cell help to produce detectable levels of IgM, while proper ligation of the BCR with anti-IgM coated bacteria is sufficient for IgM production. IgM levels are even higher when the dead, anti-IgM coated bacteria are phagocytosed and cultured in the presence of autologous T cells (**Figure 5A**, right panel). IgG production of B cells incubated with *Salmonella*e did not surpass production levels from B and T cells that were not incubated with *Salmonella*e (data not shown). Thus, *Salmonella* induces IgM production in B cells. Interestingly though, viable *Salmonella* represses the overall production of IgM in comparison to dead *Salmonella*e.

Do the *Salmonella*-specific B cells produce *Salmonella*-specific antibodies? In order to resolve this we incubated uncoated, viable GFP-*Salmonella* with primary B cells and, after incubation for 30 minutes, FACS-sorted the GFP-positive B cells. To circumvent the ability of having limited autologous *Salmonella*-specific T cells for sufficient costimulation, we cultured the sorted B cells on a monolayer of fibroblasts expressing human CD40L. After 13 days, we harvested the supernatant and measured total human IgM levels in the supernatant. Incubated and sorted B cells produce more total IgM than B cells that were not incubated with *Salmonella* and cultured on the CD40L expressing fibroblasts. The production of *Salmonella*e-specific antibodies was measured using a whole cell-*Salmonella* ELISA. Strikingly, the sorted *Salmonella*-containing B cells produce significant amounts of anti-*Salmonella*-IgM (**Figure 5B,** right panel), in contrast to B cells from the same donor, cultured on the CD40L-expressing fibroblasts, which had not been incubated with *Salmonella*. Correction of the anti-*Salmonella* specific IgM for total IgM production by the B cells revealed that the sorted B cells produce significant ($P=0.008$) more anti-*Salmonella* IgM compared to the not incubated B cells (**Figure 5B**, left panel).

B cells may encounter antigens as free antigen or delivered by Dendritic cells (DCs) (26). DCs are equipped with both nondegradative and degradative antigen uptake pathways to facilitate antigen presentation to both B and T cells. Blood DCs can capture and transport particulate Ags such as invading bacteria to the spleen, where they promote the differentiation of marginal zone B cells into IgM secreting plasma cells (27). We here show a pathway independent of DCs and macrophages. Primary B cells can phagocytose *Salmonella* via their specific BCR. B cells are able to extract proteins/peptides from internalized bacteria and process these for presentation by MHC molecules. The general dogma for antigen presentation of bacterial peptides by B cells is that B cells extract proteins from the bacterial surface or bind shedded bacterial proteins. This could occur for dead or lysed bacteria killed by antibodies and complement or after antibiotic treatment. We show that recognition of dead bacteria via the BCR induces B cell proliferation and antigen presentation to T cells which is necessary for building a protective immune response. Here we show that phagocytosis of the native, viable *Salmonella*e is the main route for antigen presentation and production of anti-*Salmonella* antibodies. B cells phagocytose *Salmonella* after BCR contact, hereby only the *Salmonella*-specific B cells are activated. This mechanism of specific BCR-mediated uptake solves the issue of selective B cell activation in response to infection.

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