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B Cell Receptor-Mediated Internalization of Salmonella: A Novel Pathway for Autonomous B Cell Activation and Antibody Production

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The present paradigm is that primary B cells are nonphagocytosing cells. In this study, we demonstrate that human primary B cells are able to internalize bacteria when the bacteria are recognized by the BCR. BCR-mediated internalization of Salmonella typhimurium results in B cell differentiation and secretion of anti-Salmonella Ab by the Salmonella-specific B cells. In addition, BCR-mediated internalization leads to efficient Ag delivery to the MHC class II Ag-loading compartments, even though Salmonella remains vital intracellularly in primary B cells. Consequently, BCR-mediated bacterial uptake induces efficient CD4+ T cell help, which boosts Salmonella-specific Ab production. BCR-mediated internalization of Salmonella by B cells is superior over extracellular Ag extraction to induce rapid and specific humoral immune responses and efficiently combat infection. The Journal of Immunology, 2009, 182: 7473–7481.

D efense against pathogens is essential for survival and is controlled by the innate and acquired arms of the immune system. Ag presentation by B lymphocytes is needed to generate high-affinity Abs (1, 2). Development of an effective humoral immune response is mediated by two actions of the BCR: transmembrane signaling through BCR complexes to induce B cell differentiation and Ag 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 multiple BCR molecules. Indeed, many B cell Ags are polyvalent as they are bound in multiple copies to the particulate surfaces of microbes or cells (3).

The role of CD4+ T cells in the induction of protective immunity against pathogens is well established (4, 5). CD4+ T cell activation requires MHC class II Ag presentation after Ag processing in the endocytic pathway and subsequent binding of antigenic peptides to MHC class II molecules, a process controlled by the MHC class II chaperones HLA-DM and HLA-DO (6–8). B cells use their BCR to concentrate specific Ag to the Ag-loading compartments (termed MIIC for MHC class II-containing compartment) for loading of Ag onto newly synthesized MHC class II molecules (3). Besides internalization of Ag, the BCR drives intracellular targeting by accelerating the delivery of Ag to MIICs (9). Furthermore, BCR signaling ignited by Ag induces acidification of the MIICs, which favors Ag loading onto newly synthesized MHC class II molecules (10). Together, these cellular adaptations enable B cells to preferentially present specific Ags that have been internalized via the BCR to CD4+ T cells.

Since primary B cells are considered to be nonphagocytic, it is unclear how they acquire Ags from bacteria for Ag presentation. B cells can present particulate Ags in the context of MHC class II (11–14) and are able to extract Ag from a non-internalizable surface (15). Studies on MHC-mediated presentation of BCR-specific Ags are mainly performed with soluble Ags or with pre-cross-linked anti-BCR Abs. We used Salmonella typhimurium as a model system to study MHC class II Ag presentation of particulate, polyvalent Ags, and B cell activation. Being facultative intracellular pathogens, immunity to Salmonella requires adequate humoral and cell-mediated immune responses (16, 17). Salmonella invades host macrophages, but also many other cells and establishes an intracellular niche inside discrete vacuoles, known as Salmonella-containing vacuoles or SCVs (18). This feature of Salmonella is considered crucial for their survival and pathogenicity (19, 20).

In this report, we show that B cells are highly efficient phagocytes of inert particles, like beads, when these particles are recognized by the BCR. B cells are thus ligand-selective phagocytic cells. BCR-mediated internalization of Salmonella generates autonomous B cell activation and rapid anti-Salmonella Ab secretion. Immediate intimate contact and fusion occurs between MIICs and SCVs. Consequently, Ag presentation and proliferation of Salmonella-specific CD4+ T cells is induced.

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4 Abbreviations used in this paper: MIIC, MHC class II-containing compartment; SCV, Salmonella-containing vacuole; EM, electron microscopy; DAPI, 4′,6-diamidino-2-phenylindole; LB, Luria-Bertani; SPI, Salmonella pathogenicity island; DC, dendritic cell.

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Although BCR-mediated internalization suffices to drive Ab production, T cell help further improves the response. The observation that B cells can proliferate and differentiate autonomously after Salmonella uptake is important in light of the remaining Ab responses to pathogens when CD4⁺ T cell help fails, as is the case in HIV patients.

Materials and Methods

Abs, beads, and fluorophores

Goat anti-mouse IgG-conjugated Dynabeads M-450 (Dyna Biotech) were coated with mAb anti-human IgM (MH15; Sanquin). The anti-human IgM Ab (MH15) was mixed with mAb anti-S. typhimurium LPS (1E6; Biodesign International) and rat anti-mouse IgG1 (RM161.1; Sanquin) to generate BCR-LPS tetrameric Ab complexes. The mAb anti-human HLA-DR (L243) (21) was used to block MHC class II-TCR interaction in T cell proliferation assays. For immunoelectron microscopy (EM), mAb anti-human CD63 (435; Sanquin), rabbit anti-mouse (Nordic), and gold (10 nm)-conjugated protein A (EM Laboratory, Utrecht University, Utrecht, The Netherlands) were used. F(ab')₂ of MH15 were generated by standard pepsin digestion.

PE-conjugated anti-IgM was obtained from Sanquin (MH15-PE) and anti-CD27-PE and IgG1-PE isotype control from BD Biosciences. Fluorescent secondary Ab goat anti-mouse Alexa Fluor 633 and Texas Red-phalloidin were obtained from Molecular Probes and 4,6-diamidino-2-phenylindole (DAPI) from Sigma-Aldrich.

Transfectant cell lines

The pcDNA3 D0JGFP (22) and DR1JGFP (23) constructs have been described before. D0JGFP and DR1JGFP 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). Stable transfectants of the human B cell line Ramos were selected and maintained in RPMI 1640 supplemented with 5% FCS (Biotrend, Germany) and rabbit anti-mouse IgG1 (NM161.1; Sanquin) or 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 50 μM 2-ME, and gold (10 nm)-conjugated protein A (EM Laboratory, Utrecht University, Utrecht, The Netherlands) were used. F(ab')₂ of MH15 were generated by standard pepsin digestion.

For confocal laser scanning microscopy analysis, coverslips were coated with 1 mg/ml poly-l-lysine (Sigma-Aldrich; Sigma-Aldrich) for 1 h and washed thoroughly with PBS, and then incubated with 5% FCS. The coverslips were washed twice to remove any remaining FCS. After 5 min of washing, the coverslips were incubated with 50 μM of 10 nm gold (EM Laboratory, Utrecht University, Utrecht, The Netherlands) were used. F(ab')₂ of MH15 were generated by standard pepsin digestion.

FACS analyses

FACS sorting of B cells that had internalized uncoated living bacteria was performed on a MoFlo Sorter (DakoCytomation); populations were >75% purified.

Live cell imaging and EM analyses

For EM, cells were allowed to take up beads or bacteria for 40 min at 37°C and then washed with PBS containing 100 μg/ml gentamicin. B cells that had taken up bacteria were fixed for 15 min at 4°C and then washed with PBS containing 0.1% Triton X-100. After embedding in a mixture of 70% glycerol and 30% Pluronic F127, the samples were stored at −80°C overnight before being freeze-fractured and stained with 90% poly-L-lysine for 1 h. The samples were then incubated with 0.1% glutaraldehyde in PBS containing 0.8% Formaldehyde, dehydrated with 96% ethyl alcohol, and critical point dried. After critical point drying, the samples were gold-coated with mAb anti-human IgM (MH15; Sanquin). Gold (10 nm)-conjugated protein A (EM Laboratory, Utrecht University, Utrecht, The Netherlands) were used. F(ab')₂ of MH15 were generated by standard pepsin digestion.

Plate assay

For enumeration of intracellular surviving bacteria, freshly isolated primary B cells were incubated with Salmonella, washed four times, and cultured for 1 h in medium containing 100 μg/ml gentamicin. Cells were incubated with directly labeled Abs and for LPS staining cells were incubated with anti-LPS Ab and subsequently with Alexa Fluor 633-conjugated goat-anti-mouse Ab. Cells were washed with PBS containing 0.1% BSA. Lymphocytes were gated by forward and side scatter and gated as lymphocytes. The anti-human IgM Ab was used to block Fcγ interaction in T cell proliferation assays. For visualization of the actin cytoskeleton, cells were fixed with 3.7% paraformaldehyde and stained with Texas Red-phalloidin and DAPI. Confocal analysis was performed at 37°C using a Leica TCS SP confocal laser scanning microscope equipped with an argon/krypton laser, with excitation at 488 nm and detected at 515 nm after emission at 488 nm. For dual analyses, green fluorescence was detected at 515 nm after emission at 488 nm. For visualization of the actin cytoskeleton, cells were fixed with 3.7% paraformaldehyde and stained with Texas Red-phalloidin and DAPI. Confocal analysis was performed at 37°C using a Leica TCS SP confocal laser scanning microscope equipped with an argon/krypton laser, with excitation at 488 nm and detection at >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 EM, cells were allowed to take up beads or bacteria for 40 min at 37°C and then washed with PBS containing 100 μg/ml gentamicin. B cells that had internalized living bacteria were fixed for 15 min at 4°C and then washed with PBS containing 0.1% Triton X-100. After embedding in a mixture of 70% glycerol and 30% Pluronic F127, the samples were stored at −80°C overnight before being freeze-fractured and stained with 90% poly-L-lysine for 1 h. The samples were then incubated with 0.1% glutaraldehyde in PBS containing 0.8% Formaldehyde, dehydrated with 96% ethyl alcohol, and critical point dried. After critical point drying, the samples were gold-coated with mAb anti-human IgM (MH15; Sanquin). Gold (10 nm)-conjugated protein A (EM Laboratory, Utrecht University, Utrecht, The Netherlands) were used. F(ab')₂ of MH15 were generated by standard pepsin digestion.

ELISAs

To determine IgM levels in culture supernatants, flat-bottom Maxisorb plates (Nunc) were coated with polyclonal anti-IgM (SH15; Sanquin) in 100 μl of PBS (pH 7.4; NPFI International) overnight at room temperature. Plates were washed with PBS containing 0.05% Tween 20 (Mallinkrodt Baker) and samples were incubated for 2 h in high-performance ELISA buffer (Sanquin). As a standard, pooled human serum was used. Plates were washed and incubated for 1 h with 1 μg/ml mAb anti-human IgM-HRP (MH15-HRP; Sanquin).
Whole-cell Salmonella ELISA was performed by coating overnight at 37°C of Salmonella to Maxisorb plates in 100 μl of 0.1 M sodium bicarbonate at pH 9.6 supplemented with 10 µg/ml gentamicin. 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 h with 1 µg/ml mAb anti-human IgM-HRP (MH15-HRP; Sanquin).

After washing, peroxidase activity was visualized by incubation with 100 µl of 3,5,3'-tetramethylbenzidine (Merck), 100 µg/ml in 0.1 M sodium acetate (pH 5.5) containing 0.003% H2O2 (Merck). The reaction was stopped by addition of an equal volume of 2 M H2SO4 (Merck) and the absorbance at 450 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).

Statistical analysis

Statistical significance was determined using the Mann-Whitney U test.

Results

Efficient BCR-mediated phagocytosis of large particulate Ags by B cells

Unlike other professional APCs, primary B cells show very limited phagocytic capacity. Ag uptake by B cells is critically dependent on the selectivity of the BCR (10, 28). The current view on BCR-mediated Ag uptake by B cells mainly centers on soluble Ags like small foreign proteins or shedded bacterial coat products (29). Accordingly, most B cell activation studies involve the global triggering of BCR using soluble cross-linking Ags rather than pathogen-associated Ags. We opted to study BCR-mediated recognition of particulate Ags in B cells by inducing localized clustering of the BCR using beads decorated with mAb against the BCR. When anti-IgM coated beads contacted a Ramos B cell stably expressing MHC class II HLA-DR1 tagged with GFP (DR-GFP), which localizes to the plasma membrane and lysosomal MIIC vesicles, rapid and efficient internalization of the bead ensued (Fig. 1A and supplemental movie 1). Ramos cells polarized themselves toward the contact site during uptake of the beads such that the nucleus moved to the side opposite of the bead, analogous to the situation following Th cell contact (30) or following CTL-target cell interactions (31). Internalization reached completion within 10–20 min and required an intact cytoskeleton (as the microtubule-disruptive agent nocodazole prevented phagocytosis, supplemental movie 2). In addition, uptake was BCR dependent because beads coated with an irrelevant Ab were not internalized (supplemental movie 3). Ramos cells do not express FcγRs, which excludes their involvement in bead uptake. A detailed analysis by cryoelectron microscopy revealed some of the impressive cellular events underlying uptake of large particulate Ags. During the initial phase of contact, Ramos cells surrounded the bead with a surprisingly thin double membrane originating from the cell surface (Fig. 1B). Staining with phalloidin of Ramos cells in the process of bead phagocytosis revealed extensive actin fibers in the membrane protrusions surrounding the bead (Fig. 1C and supplemental movie 4). Together, these data show that B cells are able to internalize inert particles in a process that fulfills the criteria of phagocytosis. Thus, different from the general concept that primary B cells are essentially nonphagocytic cells, B cells are very efficient phagocytes when particle recognition is facilitated by the BCR.

B cell lines can internalize Salmonella via their BCR

We generated a stable transfectant of the Ramos B cell line with the MHC class II Ag presentation chaperone HLA-DO tagged with GFP (DO-GFP). DO-GFP localizes to the MIICs in living cells. Since the Ag specificity of the BCR of Ramos cells is unknown, we coated the bacteria with anti-IgM anti-LPS tetrameric Ab complexes. Within 1 min after first contact, Ramos cells efficiently internalized GFP-Salmonella coated with tetrameric Ab complexes (Fig. 2A and supplemental movie 5). Uncoated Salmonella were ignored by Ramos B cells (supplemental movie 6), showing that Salmonella by itself is not able to invade the Ramos B cell line. To investigate the internalization process in more detail, we used cryoelectron microscopy on Ramos cells shortly after encounter of the coated bacteria. This showed the formation of a phagocytic cup and the extension of pseudopodia around the bacteria, demonstrating that B cells actually seem to
phagocytose the *Salmonella* bacteria (Fig. 2B). To study the interaction between the green MIICs in Ramos DO-GFP with the SCVs, we used a red mRFP-*Salmonella*. Ramos DO-GFP cells incubated with coated mRFP-*Salmonella* showed bacterial uptake and rapid translocation of the MIICs to the SCVs (Fig. 2C and supplemental movie 7). Multiple intimate contact events were observed between the membrane of the SCVs and the DO-GFP-positive MIICs, suggesting fusion events of the MIICs with the SCVs immediately after bacterial uptake.

To study the acquisition of MHC class II molecules on the SCV membrane, we used Ramos cells expressing DR-GFP. MHC class II molecules already localized to the membrane of the SCVs during the actual process of BCR-mediated uptake of coated mRFP-*Salmonella* (Fig. 2D and supplemental movie 8). Similar to the DO⁺ vesicles, we observed extensive docking of DR-GFP⁺ vesicles with the SCV membrane within minutes after entry. Because molecular exchange between the MIIC vesicles and the SCVs is critical for generation of MHC class II molecules complexed with *Salmonella* Ags, we visualized MIIC-SCV fusion by EM. Indeed, fusion between the characteristic multivesicular MIICs and the SCVs was frequently observed. Immunostaining showed that, next to the MIICs, the SCV membrane stained positive for CD63 (Fig. 2E). Quantification of the fusion events between MIICs and 100 SCVs showed that in 10% of the SCVs the actual process of MIIC-SCV fusion was captured in the time frame of cell fixation. Thus, the combined acquisition of MHC class II on the SCVs and the frequent fusion events between SCVs and MIICs generates all conditions required for Ag presentation of *Salmonella* Ags.

**BCR-mediated internalization of Salmonella by primary human B cells**

Since Ramos cells are at least 1.5 times larger than primary B cells, we tested whether primary B cells could internalize *Salmonella* as well. To distinguish between binding of bacteria to the BCR and actual uptake, we used a mAb against *Salmonella*-LPS. Completely internalized GFP-positive bacteria will not be stained, while extracellular and partially engulfed bacteria will be accessible to the anti-LPS Ab. Incubation of primary human B cells with uncoated GFP-*Salmonella* consistently revealed a small but significant population of B cells (4.3%, SD = 1.1, n = 6) that recognized and internalized the native bacterium via direct recognition of *Salmonella* Ags by the B cell’s BCR (Fig. 3A). A similar proportion of primary B cells recognized and captured dead uncoated GFP-*Salmonella* via their BCR (4.1%, SD = 1.5, n = 4), but failed to internalize dead *Salmonella* since these B cells stained all positive for LPS (Fig. 3A). Analysis by confocal microscopy confirmed that internalized viable *Salmonella* are completely taken up by primary B cells, resulting in one to three intracellular bacteria per B cell (Fig. 3B, left panel). Incubation with

**FIGURE 2.** Efficient BCR-mediated internalization of *Salmonella*. A, Living Ramos cells, expressing DO-GFP and GFP-expressing *Salmonella* were imaged every 3 s. Depicted are time points after initial contact. GFP signal is projected on top of the transmission image. Scale bar, 10 μm. Images are frames from supplemental movie 5. B, Electron microscopic analysis of Ramos cells in the process of phagocytosing anti-BCR-coated *Salmonella*. Scale bar, 800 nm. Note the cup-shaped pseudopodia of the B cell at contact places with the bacteria. C, Living Ramos cells expressing DO-GFP and mRFP-*Salmonella* were imaged every 10 s. Depicted are time points after initial contact: top panel, transmission image; middle panel, GFP signal; and bottom panel, overlay of GFP and mRFP signal. Scale bar, 5 μm. Inset shows zoom-in on bacterium. Images are frames from supplemental movie 7. D, Living Ramos cells expressing DR-GFP and mRFP-*Salmonella* were imaged every 10 s. Depicted are indicated time points after initial contact: top panel, transmission image; middle panel, GFP signal; and bottom panel, overlay of GFP and mRFP signal. Scale bar, 5 μm. Inset shows zoom-in on bacterium. Images are frames from supplemental movie 8. E, Immunoelectron microscopic analysis of primary B cells with internalized anti-BCR-coated *Salmonella* and CD63 (10-nm gold particles). Black asterisks mark bacteria, the white asterisk marks an MIIC; and arrows indicate fusion events with MIICs.
fixed bacteria only showed binding but no uptake of *Salmonella* (Fig. 3B, right panel). To address the possible involvement of FcRs on primary B cells, we preincubated primary B cells with F(ab′)2 of the anti-IgM Ab MH15 to block the internalization of anti-IgM-coated *Salmonella* by primary B cells. This resulted in inhibition of 80%, illustrating that internalization is indeed BCR-mediated (supplemental Fig. 1). Ideally, we would also like to block BCR-mediated internalization of *Salmonella* via direct recognition of *Salmonella* Ags by the BCR. However, since blocking of the Ag binding site of the BCR is impossible due to lack of anti-Id Abs, we studied the effect of BCR internalization before addition of the bacteria on the efficiency of bacterial uptake. We combined Abs against the H chain of IgM with cross-linking goat-anti-mouse Abs. This resulted in a partial internalization of the BCR (mean fluorescence intensity for membrane-bound IgM dropped from 3767 to 2275) and a concomitant reduction in GFP⁺/LPS⁻ B cells that had completely internalized *Salmonella* (supplemental Fig. 2). In addition, Ramos cells efficiently internalized GFP-*Salmonella* in a BCR-dependent manner only when *Salmonella* was coated with anti-IgM (Fig. 3C). No GFP-positive Ramos cells were found without the anti-IgM coat, showing that *Salmonella* is unable to infect Ramos cells autonomously. As a control for IgM-type BCR-mediated uptake of Ramos, *Salmonella* coated with anti-IgG were incubated with Ramos cells, and (similar to uncoated bacteria) these were not internalized. Incubation of the IgG-type BCR-expressing B cell line Cess with anti-IgG-coated bacteria showed that the anti-IgG-coated bacteria were efficiently taken up by Cess (data not shown). Up to 90% of the Ramos cells acquired one or more anti-IgM-coated *Salmonella* and 25% of Ramos cells contained internalized GFP-*Salmonella* only, while 60% of Ramos cells were also positive for LPS staining. Confocal microscopy of cells labeled by anti-LPS Abs showed that these represented Ramos cells that had internalized some but not all bound bacteria (data not shown). Since dead bacteria were not internalized, *Salmonella* may be requiring both recognition by BCR and bacterial-mediated processes to enter primary human B cells. *Salmonella* can invade host cells by expressing type III secretion systems encoded either by *Salmonella* pathogenicity island (SPI)-1 to translocate effector proteins into host cell cytoplasm that triggers internalization of the bacteria or by SPI-2 to modulate intracellular trafficking and replication of *Salmonella* within a modified vacuolar compartment. Recent studies however have modified this concept to some extent because they show a partial overlap in SPI-1 and SPI-2 functions (32). To address involvement of these type III secretions systems, we used *Salmonella* defective in SPI-1 (invA mutant) or SPI-2 (ssrA mutant). Incubation of primary B
cells with SPI-1-defective Salmonella showed a reduction in internalized bacteria and no bacteria adhering to primary B cells. Incubation with SPI-2-defective Salmonella showed a milder reduction in internalized bacteria and a minimal reduction in adhering bacteria (Fig. 3D). This indicates that SPI-1 is involved in attachment to primary B cells in conjunction with the BCR. Components of SPI-1, and to a lesser extent SPI-2, are involved in BCR-mediated uptake of Salmonella by primary B cells.

Phagocytosed Salmonella grow in many cell types but can efficiently be destroyed in specialized cells like macrophages and neutrophils (33). To examine survival of internalized Salmonella in primary B cells, we performed plating assays. B cells were incubated with anti-IgM-coated Salmonella and washed and cells with internalized bacteria were followed in time. At different time points, cells were lysed and intracellular bacteria were plated onto agar. Internalized Salmonella remained viable for the 18 h that we tested (Fig. 3E). Ramos cells incubated with uncoated Salmonella were used as a control, since uncoated Salmonella are not taken up by Ramos cells (supplemental movie 6). Indeed, hardly any Salmonella were recovered after incubation with Ramos (Fig. 3E). Thus, Salmonella survives intracellularly after BCR-mediated internalization by primary B cells.

Which peripheral B cell type is able to internalize Salmonella? Most peripheral B cells express the IgM surface Ig receptor (34). To confirm that internalization of Salmonella occurs via the BCR, we analyzed IgM expression on Salmonella-containing B cells. This showed that B cells that have internalized Salmonella expressed surface IgM (Fig. 4A). The mean fluorescence intensity of IgM for the total B cell pool is 478 and for the Salmonella-containing B cells 825. Two major subsets of B cells can be identified in adult peripheral blood according to the expression of CD27. CD27-expressing B cells comprise memory B cells while CD27-negative B cells represent naive and transitional B cells (35). FACS analysis showed that IgM memory B cells (CD27+) internalized Salmonella more efficiently than IgM+CD27− naive B cells (Fig. 4B). Although a proportion of the naive IgM+B cells are able to take up Salmonella, Salmonella is preferentially internalized by the circulating IgM− memory B cells.

Presentation of BCR-internalized Ags by B cells to T cells

When Salmonella survives within the phagosome (see Fig. 3E) following BCR-mediated internalization, does this result in MHC class II-mediated presentation of Salmonella Ags? To test this, primary human B cells were incubated with anti-IgM-coated Salmonella to achieve BCR-mediated uptake by all IgM+ B cells and to maximize the number of Ag-presenting B cells. These cells were subsequently cultured in the presence or absence of autologous primary T cells. After 5 days, [3H]thymidine was added and cells were harvested after 18 h. Incubation of B cells with anti-BCR-coated Salmonella induces proliferation of the B cells (green line, Fig. 5A), demonstrating that BCR ligation and BCR-mediated internalization of Salmonella effectively activated B cells. B cells incubated with coated Salmonella and cultured with autologous T cells result in an Ag-specific proliferation of T cells (red line, Fig. 5A).

To study whether primary B cells with a BCR directed against Salmonella also induce T cell proliferation, we incubated uncoated Salmonella with primary B cells (Fig. 5B, left panel). Addition of autologous T cells yielded a Salmonella-specific T cell proliferation response (Fig. 5B, blue vs pink line). B cells incubated with uncoated dead Salmonella that could not be internalized were able to induce T cell proliferation (Fig. 5B, right panel). However, T cell proliferation is optimal when viable Salmonella have been internalized by B cells (Fig. 5B, left vs right panel). Incubation of only T cells with uncoated or coated bacteria did not result in B cell-independent proliferation of the T cells (data not shown).

To demonstrate that the T cell proliferation in Fig. 5B was indeed induced by the fraction of B cells that had captured Salmonella, we FACS sorted the GFP-Salmonella-positive B cells in fractions positive and negative for anti-LPS staining and cultured these with autologous T cells. T cells only proliferated when cultured with B cells that had captured GFP-Salmonella (Fig. 5C). It may be that complete internalization is not required for Ag presentation but that only capturing of the bacteria by the BCR suffices. We can however not exclude that GFP-SalmonellaLPS+B cells also contain completely internalized bacteria as we have shown that B cells are able to take up more than one Salmonella. Moreover, even though Salmonella survives in the vacuole and suppresses MHC class II Ag presentation in phagosomes (27), Salmonella Ags are still efficiently presented by B cells. This probably reflects Ag degradation and loading on MHC class II molecules in normal MIICs after content exchange between phagosome and MIICs due to the observed intimate contact and extensive fusion events.

To demonstrate the Ag specificity of the proliferating T cells, we performed restimulation assays in which we sorted the T cells that proliferated in response to B cells that had taken up Salmonella and restimulated these Salmonella-primed T cells with autologous B cells that had taken up Salmonella or Staphylococcus. This showed that the Salmonella-primed T cells are indeed for the large part Ag specific, as they proliferate better in response to B cells that had internalized Salmonella than B cells that had internalized Staphylococcus or control B cells without bacteria. As a control, T cells primed against Staphylococcus did not proliferate in response to B cells that had internalized Staphylococcus, demonstrating that these B cells did present Staphylococcus Ags to CD4+ T cells (Fig. 5D, left panel). In addition, we restimulated Salmonella-primed T cells with PBMCs presenting tetanus toxoid Ags. This showed no response of the Salmonella-primed T cells, while tetanus toxoid-primed T cells from the same donor proliferated after restimulation (Fig. 5D, right panel).

Furthermore, we performed blocking assays to show that the T cell response depends on the MHC class II Ag presentation pathway with L243, an Ab that blocks the MHC class II-TCR interaction (Fig. 5E). The induction of T cell activation depended on presentation of Salmonella Ags via MHC class II (HLA-DR), as the T cells failed to respond after blocking of MHC class II with L243.
The observation that fusion of MIICs with the phagosome occurs swiftly prompted us to examine whether this had consequences for Ag presentation. When we irradiated B cells immediately after incubation with anti-IgM-coated *Salmonella*, no proliferation of B or T cells was observed after 6 days. B cells apparently need to be viable to process and present *Salmonella* Ags to T cells. To study the kinetics of Ag presentation, B cells were incubated with native or anti-IgM-coated *Salmonella* and B cells were irradiated at several time points before incubation with T cells. After 5 days, [³H]thymidine was added and cells were harvested after 18 h. Noninfected B cells did not induce T cell proliferation after irradiation. Anti-BCR-coated *Salmonella* internalized by IgM⁺ B cells start to induce proliferation of T cells immediately (red line) and uncoated *Salmonella* internalized by BCR-reactive B cells 4 h after uptake of the *Salmonella* (blue line, Fig. 5F). Ag presentation thus starts at times corresponding to the earliest phases of BCR-mediated internalization and rapid fusion with the MIICs. Primary B cells rapidly present Ags of internalized *Salmonella*, even if the bacterium survives inside a B cell.

**BCR-mediated internalization induces IgM secretion**

To test whether BCR-mediated internalization of *Salmonella* leads to differentiation of B cells into Ab-secreting cells, supernatants of B cells that internalized bacteria were tested for the presence of human IgM after culture. After 5 days of incubation with viable uncoated bacteria, no strong induction of IgM secretion following BCR-mediated internalization was detectable (Fig. 6A, left panel). When the *Salmonella* were coated with anti-IgM Abs, B cells produced four times more IgM than uncoated bacteria. Addition of T cells did not increase IgM production in the first 5 days, indicating that IgM production resulted from a T cell-independent activation of B cells (Fig. 6A, right panel). T cell help did occur within 12 days, leading to a strong increase in IgM production (Fig. 5A, right panel). Thus, BCR-mediated internalization of *Salmonella* induces autonomous IgM secretion by B cells, whereas T cell help is required during the late stage of Ig secretion by B cells. IgG production of B cells incubated with *Salmonella* did not significantly surpass IgG production levels from B and T cells that were not incubated with *Salmonella* (data not shown). This is in line with the observation that the B cells that take up *Salmonella* are IgM⁺ memory B cells. It also indicates that BCR-mediated internalization of *Salmonella* by the naive IgM⁺ B cell pool does not induce Ig class switching under our culture conditions.

When B cells that internalize *Salmonella* through the BCR are activated, they might produce *Salmonella*-specific Abs. We incubated uncoated, viable GFP-*Salmonella* with primary B cells and FACS sorted the GFP⁺ B cells. We cultured the sorted B cells on a monolayer of fibroblasts expressing human CD40L to provide costimulation. After 12 days, total human IgM as well as *Salmonella*-specific Abs were quantified. B cells that internalized *Salmonella* produced more total IgM than B cells that did not take up *Salmonella* (Fig. 6B, upper left panel). The production of *Salmonella*-reactive Abs was measured using a whole-cell *Salmonella* ELISA. Strikingly, the sorted *Salmonella*-containing B cells produced significant amounts of...
anti-Salmonella IgM (Fig. 6B, lower left panel), unlike control B cells from the same donor. Correction of the anti-Salmonella-reactive IgM for total IgM production by the B cells revealed that the sorted B cells produced significantly higher levels of anti-Salmonella IgM compared with control B cells (Fig. 6B, right panel). The production of significantly higher levels of anti-Salmonella IgM clearly shows the involvement of the BCR in internalization of Salmonella. If the BCR would not be involved, but bacterial internalization and subsequent B cell activation would solely depend on TLR stimulation and/or co-stimulation, the B cells that had taken up Salmonella would not be able to produce Salmonella-specific IgM. Thus, BCR-mediated internalization of Salmonella forms an efficient pathway to induce differentiation of Salmonella-specific B cells and production of Salmonella-reactive IgM Abs.

Discussion

B cells may encounter Ags as free Ag or delivered by dendritic cells (DCs) (36). DCs are equipped with both nondegradative and degradative Ag uptake pathways to facilitate Ag 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 differentiation of marginal zone B cells into IgM-secreting plasma cells (37). We here show a pathway independent of DCs and macrophages. In contrast to the dogma that mammalian B cells lack the ability to ingest pathogens and are only involved in the adaptive phase of the immune response (38) or that entry of autologous T cells (T). After 5 (□, left panel) and 12 days (□, right panel) total secreted human IgM was determined. 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 12 days and supernatant was analyzed for total IgM production and Salmonella-reactive IgM production. □ B cells and □, Salmonella-positive B cells. Anti-Salmonella IgM was divided on the total amount of IgM measured in the supernatants (right panel, p = 0.006). Data are representative of three independent experiments of different donors.

It has been proposed that Abs made by IgM memory B cells are the first-line defense mechanism against all infections and that Abs produced by IgM memory B cells are the only B cell defense against T-independent Ags (40). IgM+ memory B cells in peripheral blood represent circulating splenic marginal zone B cells in charge of T-independent responses (41). Since marginal zone B cells express a BCR of a polyreactive nature (42), this could explain the relatively high numbers of CD27+ B cells that take up Salmonella. As for IgM+ memory B cells, a subset of mature naive B cells in peripheral blood are polyreactive (43). Combined, the primary B cells that we found to internalize Salmonella seem to represent naive and IgM+ memory B cells with a polyreactive BCR.

How do these findings relate in the involvement of B cells in Salmonella infection? Studies in B cell-deficient mice show that B cells are necessary for efficient protection against both primary and secondary infection with Salmonella (44). Passive transfer of Salmonella-immune serum could not restore resistance of mice to Salmonella (45), demonstrating that high-affinity Ab production alone is not the only function of B cells in salmonellosis. Moreover, at the early stage of primary infection class-switched high-affinity Abs against Salmonella are not yet available and cannot explain the importance of B cells at this stage. Therefore, polyreactive, IgM+ memory B cells may well be involved in protection against primary infection via BCR-mediated internalization of Salmonella and rapid generation of protecting Salmonella-reactive IgM Abs. B cell-deficient Igh-6-/- mice have impaired Th1 T cell responses from the early stage of Salmonella infection, showing that B cells play an essential role in the initiation of T cell-mediated protection as well (46). The importance of B cells in this line of immune defense may relate to their property to present Ags to T cells. It remained unclear how Ag presentation was achieved since processing and presentation of Ags by naive B cells was not observed. In this study, we provide a missing link in these observations by showing that the IgM+ B cells can internalize viable bacteria and very efficiently induce Th activation. However, IgM secretion can also be induced by BCR-mediated Salmonella uptake.
and activation alone, albeit less efficient than observed with additional CD4+ T cell help. The rapid secretion of IgM before B cells encounter CD4+ T cells represents a first line of specific immune responses to pathogens and may represent the remaining humoral response when CD4+ T cell help fails, as is the case in HIV patients. In conclusion, we demonstrate for the first time that bacte- rial uptake via the BCR by B cells forms a highly efficient pathway to generate an immediate antimicrobial humoral immune response.

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References

Supplemental Data Legends

**Supplemental Figure S1.**
**Blocking of the BCR with F(ab)\(_2\) fragments.** Primary B cells were pre-incubated with F(ab)\(_2\) fragments of the anti-IgM antibody before incubation with anti-IgM coated *Salmonella*. Data are from five different donors, error bars represent SEM.

**Supplemental Figure S2.**
**BCR internalization before incubation with bacteria.** Primary B cells were pre-incubated with an anti-IgM antibody followed by incubation with a goat-anti-mouse antibody to achieve partial BCR internalization before incubation with uncoated *Salmonella*. Data are one representative set of experiments from four different donors.

**Movie 1.**
**Phagocytosis of anti-IgM coated beads by Ramos B cells stably expressing DR-GFP.** Living cells were imaged every 30 seconds by confocal microscopy at 37°C. Left panel: GFP signal, right panel: transmission image. Single bead coated with an anti-IgM antibody is efficiently taken up followed by repositioning of the nucleus to the opposite site of the contact region and repositioning of the DR-GFP vesicles. Total duration: 42 min.

**Movie 2.**
**Treatment with nocodazole prevents bead uptake.** Living Ramos B cells expressing 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 fluorescent signal. A single Ramos DO-GFP cell contacts two beads, neither of which are phagocytosed 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.

**Movie 3.**
**Ramos DO-GFP cells are inert to beads coated with an irrelevant antibody.** Living cells were imaged every 30 seconds by confocal microscopy at 37°C. Shown is a transmission image. Total duration: 7.5 min.

**Movie 4.**

3D reconstruction of the actin cytoskeleton staining in Ramos DR-GFP B cells during phagocytosis of anti-BCR coated beads. Cells were fixed 10 minutes after addition of anti-IgM coated beads and processed for confocal microscopy. Depicted is the overlay of the signals from DAPI nuclear staining (blue), phalloidin stained actin fibers (red), and DR-GFP (lower left cell only). The signature of the bead was derived from the transmission image and is depicted in purple. Thick actin-fibers are observed in the membrane protrusions surrounding the bead.

**Movie 5.**

BCR-mediated phagocytosis of anti-BCR coated *Salmonella* by Ramos DO-GFP cells. Living cells and GFP-expressing *Salmonellae* were imaged every 10 seconds using confocal microscopy at 37°C. GFP signal is projected on top of the transmission image. Single living bacterium coated with anti-BCR tetrameric antibody complexes is efficiently phagocytosed in a BCR-dependent manner. Left panel: transmission image, middle panel: GFP signal, right panel: merge. Total duration: 6 min.

**Movie 6.**

Ramos DO-GFP cells are inert to native, uncoated *Salmonellae*. Living cells and bacteria were imaged every 20 seconds by confocal microscopy at 37°C. Merge image. Total duration: 9 min.

**Movie 7.**

Recruitment of MIIC vesicles to the phagosome after BCR-mediated uptake of *Salmonella*. Living cells and bacteria were imaged every 30 seconds by confocal microscopy at 37°C. Ramos DO-GFP cells incubated with anti-BCR coated mRFP-expressing *Salmonella* shows movement of the DO-positive vesicles to the phagosome. Numerous kiss-and-run events were observed
between the phagosome and the DO-GFP positive MIICs. Left panel: GFP signal, middle panel: GFP and mRFP merge, right panel: transmission image. Total duration: 31 min.

Movie 8.
The plasma membrane is the main source of the phagosomal membrane. Living cells and bacteria were imaged every 30 seconds by confocal microscopy at 37°C. Phagocytosis of anti-BCR coated mRFP-expressing *Salmonella* by Ramos DR-GFP cells clearly shows that the phagosomal membrane is GFP-positive upon formation of the phagosome. Similar to the DO-positive vesicles, the intracellular DR-GFP positive vesicles moved to the phagosome. Left panel: GFP signal, middle panel: GFP and mRFP merge, right panel: transmission image. Total duration: 20 min.
Supplementary Fig. S1

- B + coated Salmonella
- B + F(ab)2 + coated Salmonella
Supplementary Fig. S2