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

Yuri Souwer,²† Alexander Griekspoor,²† Tineke Jorritsma,*, Jelle de Wit,*, Hans Janssen,† Jacques Neefjes,³† and S. Marieke van Ham³*

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.

Defense 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 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 phagocytes of Salmonella. 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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2 Y.S. and A.G. contributed equally to this work.

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 (Dynal 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; Biodesing 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-phenylnilidole (DAPI) from Sigma-Aldrich.

#### Transfectant cell lines

The pDNA3 DO\(_{67}GFP\) (22) and DR\(_{1}GFP\) (23) constructs have been described before. DO\(_{67}GFP\) and DR\(_{1}GFP\) 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 (Bodinco), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, and 50 μM 2-ME in the presence of 2000 μg/ml G418 (Life Technologies). Stable expression of the GFP-tagged proteins was verified by Western blotting and ensured by regular selection of positive cells by FACS sorting.

#### FACS analyses

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 cells that had internalized Salmonella were analyzed. Two hundred thousand events were acquired on an LSR II (BD Biosciences) and analyzed using FACSDiva software (BD Biosciences).

#### Live cell imaging and FACS analyses

For confocal laser scanning microscopy analysis, coverslips were coated with 1 mg/ml poly-L-lysine (Sigma-Aldrich) for 1 h 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 were added. For visualization of the actin cytoskeleton, cells were fixed with 3.7% parafomaldehyde 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 ×63 objective and 1.4 aperture (Leica Microsystems). Green fluorescence was detected at < 515 nm with 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 > 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.

#### Plating assay

For enumeration of intracellular surviving bacteria, freshly isolated primary B cells were incubated with anti-IgM coated Salmonella and Ramos cells with uncoated Salmonella as a control, washed, and cultured in medium with 10 μg/ml gentamicin as described above. At the indicated time points, cells were washed with PBS and lysed in 0.1% Triton X-100 (Merck) for 10 min on ice, washed with PBS, and a dilution series was plated onto LBagar plates. Plates were incubated overnight at 37°C and colonies were counted.

#### 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; NBPI International) overnight at room temperature. Plates were washed with PBS/0.02% Tween 20 (Malinkcrodt 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). The maximum proliferation capacity of T lymphocytes (varying between 35 and 60 × 10\(^3\) cpm) was established by stimulation with anti-CD3 (CLB.T3/4.E; Sanquin) and anti-CD28 (CLB.CD28/1; Sanquin), which were both used at 1 μg/ml. After 5 and 12 days, 150 μl of supernant was collected for Ab measurement and fresh medium was added. To study the kinetics of Ag presentation, B cells incubated with Salmonella were irradiated with 60 Gy at indicated time points before incubation with T cells. PBMCs/T cell proliferation assays, after 5 days of culturing, [\(^{3}H\)]thymidine (GE Healthcare) was added at a final concentration of 1 μCi/ml (37 KBq/ml) for 16 h. Cells were harvested on glass fiber filters (Wallac) and radioactivity was measured with a 1205 Betaplate liquid scintillation counter (Wallac). For blocking experiments, B cells were preincubated with 5 μg/ml anti-HLA-DR (L243) for 30 min before T cells were added. For the Ag-specificity assay, CD4\(^+\) T cells were CFSE labeled and cocultured with B cells that had taken up Salmonella. The dividing T cells were sorted after 6 days and cultured with 10 IU/ml IL-2 (Chiron) for 6 more days. PBMCs were labeled with CFSE and incubated with tetanus toxoid (7.5 μg/ml; Statens Serum Institut, Copenhagen, Denmark) for 11 days, with 10 IU/ml IL-2 added on day 6, and proliferating CD4\(^+\) T cells were sorted. B cells from the same donor were incubated with Salmonella and PBMCs from the same donor with tetanus toxoid, irradiated after 18 h, and then the sorted T cells were added for 2 days before [\(^{3}H\)]thymidine was added for 16 h.
FIGURE 1. Efficient BCR-mediated phagocytosis of large particulate Ags. A. Living Ramos B cells expressing DR-GFP and anti-IgM-coated beads were imaged every 30 s. Depicted are time points after initial contact: top panel, transmission image; bottom panel, GFP signal. Scale bar, 10 μm. Images are frames from supplemental movie 1. B. Ramos cells were fixed 10 min after the addition of anti-IgM-coated beads and analyzed by cryoelectron microscopy. Scale bar, 500 nm. Zoom-ins of the thin membrane protrusions surrounding the bead are shown for the indicated regions. The tip of the protrusion is indicated with an arrowhead in insert 2. C. Cells were fixed 10 min after the addition of anti-IgM-coated beads and processed for immunofluorescence 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). Scale bar, 10 μm. Figure represents one section from a Z-stack. A three-dimensional reconstruction is provided as supplemental movie 4.

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 3,3’,5,5’-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 activity by incubation with 100 μg/ml of 3,5,3’,5’-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.

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 MHCs 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
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+ and extracellular indicates GFP+ LPS+ B cells. Data are from two independent experiments with cells from four different donors and error bars indicate SEM. E, B cells were incubated with anti-IgM-coated Salmonella and Ramos cells with uncoated Salmonella, lysed, and plated onto LBagar plates. Data are from duplicates of experiments performed with B cells from two individual donors and error bars indicate SEM.

FIGURE 3. BCR-mediated internalization and survival of Salmonella in primary B cells. A, CD19+ B cells were incubated with viable GFP-Salmonella for 40 min and FACS analyzed. Anti-LPS-allophycocyanin vs GFP scatter plots are depicted. B, Living primary human B cells were incubated with viable (left) or dead (right) GFP-Salmonella for 40 min, stained with an Ab against Salmonella LPS, and analyzed by FACS. Depicted are anti-LPS-allophycocyanin vs GFP scatter plots of 50,000 events. C, CD19+ B cells were incubated with viable wild-type GFP-Salmonella (Salm) or mutant for SPI-1 (invA−) or SPI-2 (ssrA−) for 40 min and analyzed by FACS. Intracellular indicates B cells that are GFP+ LPS+ and extracellular indicates GFP+ LPS+ B cells. Data are from two independent experiments with cells from four different donors and error bars indicate SEM.

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 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 secretion 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 vital 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-*Salmonella* LPS 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 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 Ags to T cells. To study the kinetics of Ag presentation, B cells were either or not incubated with viable uncoated or anti-BCR-coated *Salmonella* and were irradiated at several time points before incubation with T cells. Data are from two independent experiments with cells from different donors and error bars indicate SEM. D, *Salmonella*-primed or *Staphylococcus*-primed T cells were restimulated for 2 days with autologous B cells that were incubated with viable *Salmonella* or *Staphylococcus* or restimulated with PBMCs incubated with tetanus toxoid. Data are representative of three independent experiments with different donors and error bars indicate SEM. E, T cells (T) were cultured with B cells (B) that had taken up uncoated (U) or anti-BCR-coated (C) *Salmonella* either or not in the presence of the MHC class II Ag presentation blocking Ab L243. F, Ag presentation by B cells starts immediately after internalization of the *Salmonella*. 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. Data are representative for four independent experiments of different donors.

**FIGURE 5.** BCR-mediated uptake of *Salmonella* induces Ag presentation by B cells. A, BCR-induced internalization results in proliferation of B cells and Ag-specific T cells. B cells (B) were either or not incubated with viable anti-BCR-coated (C) *Salmonella* in the presence or absence of autologous T cells (T) as indicated. Results are shown as a percentage of maximal stimulation of T cells with anti-CD3 and -CD28 Abs. B. The same experimental setup as in Fig. 4A was performed with uncoated (U) viable and dead *Salmonella*. Data are from four independent experiments of different donors and error bars indicate SEM. B:T represents the ratio of different amounts of B cells added to a fixed amount of T cells. Experiments with uncoated and coated *Salmonella* were performed in parallel using the same donor. C, B cells incubated with viable uncoated GFP-*Salmonella* were FACS sorted as indicated and incubated for 6 days with T cells. White bar, B cells without bacteria; black bar, B cells before sorting; green bar, GFP-*Salmonella*; blue bar, GFP-*Salmonella* LPS-sorted B cells; and gray bar, GFP-*Salmonella* LPS-sorted B cells. Data are from two independent experiments with cells from different donors and error bars indicate SEM. D, *Salmonella*-primed or *Staphylococcus*-primed T cells were restimulated for 2 days with autologous B cells that were incubated with viable *Salmonella* or *Staphylococcus* or restimulated with PBMCs incubated with tetanus toxoid. Data are representative of three independent experiments with different donors and error bars indicate SEM. E, T cells (T) were cultured with B cells (B) that had taken up uncoated (U) or anti-BCR-coated (C) *Salmonella* either or not in the presence of the MHC class II Ag presentation blocking Ab L243. F, Ag presentation by B cells starts immediately after internalization of the *Salmonella*. 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. Data are representative for four independent experiments of different donors.

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**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*-reactive 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 Salmonella in B cells is a random process (39), primary B cells can internalize Salmonella via their specific BCR. So far, the general concept for Ag presentation of bacterial peptides by B cells was that B cells extract proteins from the surface of DCs or bacteria or bind shedded bacterial proteins (15). Indeed, this may occur for dead or lysed bacteria killed by Abs and complement or after antibiotic treatment. Our observation that recognition via the BCR of dead bacteria without internalization induces Ag presentation to T cells is in line with this concept. However, internalization of viable bacteria leads to superior CD4+ T cell activation and instantaneous generation of anti-Salmonella Abs by autonomous activation of the Salmonella-reactive B cells.

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 salmoneiosis. 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 secrion 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 bacteriocal 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


13. Zhang, Y. P., S. J. Tzartos, and H. Wekerle. 1988. B-T lymphocyte interactions and activation alone, albeit less efficient than observed with addi...


