ProductionAutonomous B Cell Activation and Antibody Production

Yuri Souwer, Alexander Griekspoor, Tineke Jorritsma, Jelle de Wit, Hans Janssen, Jacques Neefjes and S. Marieke van Ham

J Immunol 2009; 182:7473-7481; ;
doi: 10.4049/jimmunol.0802831
http://www.jimmunol.org/content/182/12/7473

Supplementary Material

http://www.jimmunol.org/content/suppl/2009/06/02/182.12.7473.DC1

References

This article cites 46 articles, 23 of which you can access for free at:
http://www.jimmunol.org/content/182/12/7473.full#ref-list-1

Subscription

Information about subscribing to The Journal of Immunology is online at:
http://jimmunol.org/subscription

Permissions

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

Email Alerts

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
B Cell Receptor-Mediated Internalization of \textit{Salmonella}: A Novel Pathway for Autonomous B Cell Activation and Antibody Production\textsuperscript{1}

Yuri Souwer,\textsuperscript{2*†} Alexander Griekspoor,\textsuperscript{2†} Tineke Jorritsma,\textsuperscript{*} Jelle de Wit,\textsuperscript{*} Hans Janssen,\textsuperscript{†} Jacques Neefjes,\textsuperscript{3†} and S. Marieke van Ham\textsuperscript{3*}

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 \textit{Salmonella typhimurium} results in B cell differentiation and secretion of anti-\textit{Salmonella} Ab by the \textit{Salmonella}-specific B cells. In addition, BCR-mediated internalization leads to efficient Ag delivery to the MHC class II Ag-loading compartments, even though \textit{Salmonella} remains vital intracellularly in primary B cells. Consequently, BCR-mediated bacterial uptake induces efficient CD4\textsuperscript{+} T cell help, which boosts \textit{Salmonella}-specific Ab production. BCR-mediated internalization of \textit{Salmonella} by B cells is superior over extracellular Ag extraction to induce rapid and specific humoral immune responses and efficiently combat infection. 


The role of CD4\textsuperscript{+} T cells in the induction of protective immunity against pathogens is well established (4, 5). CD4\textsuperscript{+} 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\textsuperscript{4} 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\textsuperscript{+} T cells.

Since primary B cells are considered to be not phagocytic, 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 \textit{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 \textit{Salmonella} requires adequate humoral and cell-mediated immune responses (16, 17). \textit{Salmonella} invades host macrophages, but also many other cells and establishes an intracellular niche inside discrete vacuoles, known as \textit{Salmonella}-containing vacuoles or SCVs (18). This feature of \textit{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 \textit{Salmonella} generates autonomous B cell activation and rapid anti-\textit{Salmonella} Ab secretion. Immediate intimate contact and fusion occurs between MIICs and SCVs. Consequently, Ag presentation and proliferation of \textit{Salmonella}-specific CD4\textsuperscript{+} T cells is induced.

\textsuperscript{1}Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Amsterdam, The Netherlands; and \textsuperscript{2}Division of Tumor Biology, The Netherlands Cancer Institute, Amsterdam, The Netherlands

Received for publication August 27, 2008. Accepted for publication April 3, 2009.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{*}This work was supported by grants from the Dutch Cancer Society (Koningin Wilhelmina Fonds) (Grant NKI 2001-2415), the Landsteiner Foundation for Blood Research (Grant 0533), and The Netherlands Organization for Scientific Research (De Nederlandsche Organisatie voor Wetenschappelijk Onderzoek).

\textsuperscript{†}Y.S. and A.G. contributed equally to this work.

\textsuperscript{5}Address correspondence and reprint requests to Dr. Jacques Neefjes, Division of Tumor Biology, The Netherlands Cancer Institute, Plesmanlaan 121, 1066 CX, Amsterdam, The Netherlands and Dr. S. Marieke van Ham, Department of Immunopathology, Sanquin Research and Landsteiner Laboratory, Academic Medical Center, University of Amsterdam, Plesmanlaan 125, 1066 CX, Amsterdam, The Netherlands. E-mail addresses: j.neefjes@nki.nl and m.vanham@sanquin.nl

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

Copyright © 2009 by The American Association of Immunologists, Inc. 0022-1767/09/$2.00

www.jimmunol.org/cgi/doi/10.4049/jimmunol.0802831
Although BCR-mediated internalization suffices to drive T cell 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 IgG Ab (MH15) was mixed with mAb anti-S. *typhimurium* LPS (16E6; Biosign Design 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 (455; 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-conjugated goat anti-mouse IgG1 were from Sigma-Aldrich.

**Transfectant cell lines**

The pDNA3 DO6/GFP (22) and DR1/GFP (23) constructs have been described before. DO6/GFP and DR1/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 (Bodino), 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 cell lines by FACS sorting.

NIH3T3 fibroblasts expressing human CD40L (3T3-CD40L) (24) were cultured in IMDM supplemented with 5% FCS (Bodino), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 50 μM 2-ME, and 500 μg/ml G418 (Life Technologies). 3T3-CD40L cells were harvested, irradiated with 30 Gy (Gammator M38–1; MDS Nordion), and seeded without antibiotics in 96-well flat-bottom plates (2 x 10⁵ cells/well) to form confluent monolayer overnight.

**Bacterial strains**

*S. typhimurium* SL1344 (*Salmonella*) (25), GFP-Salmonella (26), and mRFP-Salmonella (27) were described before. GFP-Salmonella defective in SPI-1 (invA mutant) or SPI-2 (srfA mutant) were a gift from M. Rescigno (European Institute of Oncology, Milan, Italy). *Staphylococcus aureus* expressing GFP (RN4220 with pWVW189GFP) was a gift from S. A. J. Zaat (Academic Medical Center, Amsterdam, The Netherlands). Bacteria were grown in Luria-Bertani (LB) broth with antibiotics 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 twice with PBS, incubated 1/25 with the BCR-LPS tetrameric Ab complexes for 30 min at room temperature, and washed twice to remove unbound Abs. Dead Salmonella were bacteria fixed with paraformaldehyde (3.7% in PBS).

**Lymphocyte isolation and proliferation assay**

Human PBMCs were isolated by centrifugation on a Ficoll-Hypaque gradient (Axis-Shield PoC) from a buffy coat obtained from healthy donors after informed consent (Sanquin). B and T cells were subsequently purified using anti-CD19 and anti-CD4, anti-CD8 Dynabeads, and DETACHA-BEAD (Dynal Biotech) according to the manufacturer’s instructions.

Lymphocytes were incubated for 40 min at 37°C with *Salmonella* without antibiotics. Next, cells were washed four times and cultured for 1 h in medium containing 100 μg/ml gentamicin (Invitrogen) to eliminate non-internalized bacteria. Cells were washed and cultured in RPMI 1640 medium without phenol red, supplemented with 5% FCS (Bodino), 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM l-glutamine, 50 μM 2-ME, and 500 μg/ml G418 (Life Technologies). 3T3-CD40L cells were harvested, irradiated with 30 Gy (Gammator M38–1; MSD Nordin), and seeded without antibiotics in 96-well flat-bottom plates (2 x 10⁴ cells/well) to form a confluent monolayer overnight.

**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 properties. 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 confocal laser scanning microscope 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 to 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% 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 63 x/63 objective and 1.4 aperture (Leica Microsystems). Green fluorescence was detected at 520–560 nm. Red fluorochromes were excited at 568 nm and detected at 630–660 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 or 4 h (to study MiCSCV fusion) and fixed in a mixture of paraformaldehyde (4%) and glutaraldehyde (0.5%). After embedding in a mixture of methyl cellulose and uranyl acetate, ultrathin sections were stained and analyzed with a Philips CM10 electron microscope.

**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 LB-agar 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; NPBI International) overnight at room temperature. Plates were washed with PBS/0.02% 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). The maximum proliferation capacity of T lymphocytes (varying between 35 and 60 x 10⁶ cpm) was established by stimulation with anti-CD3 (CLB.T3.4E; Sanquin) and anti-CD28 (CLB.CD28/1; Sanquin), which were both used at 1 μg/ml. After 5 and 12 days, 150 μl of supernatant 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. B/T cell proliferation assays, after 5 days of culturing, [3H]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 3 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 [3H]thymidine was added for 16 h.
FIGURE 1. Efficient BCR-mediated phagocytosis of large particulate Ags by B cells. 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 for the indicated regions. The tip of the protrusion is indicated with an arrowhead for the indicated regions. 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 0.11 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 MHC 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-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 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

The online version of this article contains supplemental material.
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− 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 LB-agar plates. Data are from duplicates of experiments performed with B cells from two individual donors and error bars indicate SEM.

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, lysed, and plated onto LB-agar plates. Data are from duplicates of experiments performed with B cells from two individual 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 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 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 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). T cell help did occur within 12 days, leading to a strong increase in IgM production (Fig. 5A). 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

---

**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 +sorted B cells; blue bar, GFP-Salmonella LPS-sorted B cells; and gray bar, GFP-Salmonella -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.
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 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 bacterial uptake via the BCR by B cells forms a highly efficient pathway to generate an immediate antimicrobial humoral immune response.

Acknowledgments

We are grateful to Lauran Oomen and Lenny Brocks for support with confocal laser scanning microscopy imaging, Erik Mul, Floris van Alphen, Anita Pi-auth, and Frank van Diepen for excellent FACS sorting, Hanny Klaasse Bos for technical support, and Nico Ong for photography. We thank S. A. J. Zaat (Department of Medical Microbiology, Academic Medical Center, University of Amsterdam, Amsterdam, the Netherlands) and W. van Wamel (Department of Medical Microbiology, Erasmus University Medical Center, Rotterdam, the Netherlands) for S. aureus RN4220 pWWV189, and A. Cheung (Departments of Microbiology and Immunology, Dartmouth Medical School, Hanover, NH) for pALC1484 used to construct pWVV189. We thank M. Rescigno (European Institute of Oncology, Milan, Italy) for the Salmonella mutant strains.

Disclosures

The authors have no financial conflict of interest.

References