

***Salmonella*-induced Apoptosis of Infected Macrophages Results in Presentation of a Bacteria-encoded Antigen after Uptake by Bystander Dendritic Cells**

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Abstract

Salmonella typhimurium is a gram-negative bacterium that survives and replicates inside vacuolar compartments of macrophages. Infection of macrophages with *S. typhimurium* grown under conditions allowing expression of the type III secretion system results in apoptotic death of the infected cells. Here, we show that infection of bone marrow-derived macrophages (MΦ) with wild-type *S. typhimurium* 14028 results in presentation of epitopes derived from a bacteria-encoded antigen on major histocompatibility complex (MHC) class I and MHC class II molecules after internalization of apoptotic MΦ by bystander dendritic cells (DCs). In contrast, infection of MΦ with the *phoP* constitutive mutant strain CS022, which does not induce apoptosis in infected MΦ, does not result in presentation of a bacteria-derived antigen by bystander DCs unless the infected MΦ are induced to undergo apoptosis by treatment with lipopolysaccharide and ATP. DCs appear to be unique in their ability to present antigens derived from MΦ induced to undergo apoptosis by *Salmonella*, as bystander MΦ are not capable of presenting the bacteria-derived antigen despite the fact that they efficiently internalize the apoptotic cells. These data suggest that apoptosis induction by bacterial infection of MΦ may not be a quiescent death that allows the bacteria to escape recognition by the immune system, but rather may contribute to an antimicrobial immune response upon engulfment by bystander DCs.

Key words: apoptosis • *Salmonella* • dendritic cells • macrophages • antigen processing

Introduction

Salmonella typhimurium is a gram-negative bacterium that causes a self-limiting gastroenteritis in humans and typhoid-like systemic disease in mice. The pathogen is acquired through the ingestion of contaminated food or water and crosses the epithelial barrier at the level of the ileum or the colon preferentially by invading M cells (1). This cell type, which functions to sample luminal antigens, overlies the lymphoid follicles (2). The bacteria subvert the normal function of M cells and are taken up by inducing membrane ruffling in these cells (1). After passing through M cells, the bacteria reach the subepithelial dome of the Peyer's patch (PP)¹

and encounter an extensive network of resident macrophages and dendritic cells (DCs) (3, 4). Rather than being destroyed by these phagocytes upon engulfment, *Salmonella* has evolved several mechanisms to survive in the harsh milieu of phagosomal compartments (5) and can be cytotoxic to macrophages by inducing apoptosis, as has been shown in vitro (6, 7).

Apoptosis, or programmed cell death, is a process defined by a distinct set of events characterized by expression of phosphatidylserine on the cell surface, DNA fragmentation, and release of apoptotic bodies (for a review, see reference 8). Through various receptors on the cell surface of phagocytes, apoptotic cells and bodies can be rapidly recognized and engulfed, diminishing the risk of a subsequent inflammatory response (8). It has also become evident that the interaction between several genera of bacteria and phagocytes can lead to apoptosis (6, 7, 9–11). Recently, it has been shown that *Shigella* and *Salmonella* induce programmed cell death through a similar mechanism, via a

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¹Abbreviations used in this paper: CCD, cytochalasin D; DC, dendritic cell; LB, Luria broth; MΦ, bone marrow-derived macrophage(s); MHC-I and MHC-II, MHC class I and II molecules, respectively; PI, propidium iodide; PP, Peyer's patch.

bacterial invasin that directly interacts with and activates the proapoptotic enzyme caspase 1 (12–14). In addition, DCs can acquire apoptotic material from influenza-infected monocytes and stimulate MHC class I molecule (MHC-I)-restricted CTLs (15). This suggests that apoptosis induced by microbes can lead to an immune response rather than to a “silent” nonimmunogenic death.

We have previously shown that macrophages and DCs can phagocytose and process bacteria for presentation of bacterial peptides on both MHC-I and MHC-II (16–19). In this study, we show that DCs can also acquire apoptotic material containing bacterial antigens from macrophages that have undergone apoptosis caused by *S. typhimurium* infection and present epitopes from a bacteria-derived antigen on both MHC-I and MHC-II. This indicates that bacterial induction of apoptosis in phagocytes may not necessarily result in a silent death, since both CD4⁺ and CD8⁺ T cells can be activated.

Materials and Methods

Mice. BALB/c (H-2^d) and C57BL/6 (H-2^b) mice were bred in the animal facilities at Lund University or purchased from Charles River Laboratories. Caspase 1^{-/-} mice (20; H-2^b) were provided by Manuela Baccharini, Vienna Biocenter, Vienna, Austria.

Bacterial Strains, Plasmids, and Growth Conditions. Bacterial strains used were *S. typhimurium* 14028s (American Type Culture Collection) or its rough LPS derivative 14028r (21), the *phoP* mutant CS022s (22) or its rough LPS derivative CS022r, made as described (21). *S. typhimurium* 14028r and CS022r harbored pJLP-2H-Kan (18), which encodes the fusion protein Crl-OVA containing residues 257–277 of OVA fused near the COOH terminus of the cytoplasmic bacterial protein Crl. The OVA fragment in Crl-OVA contains the K^b-binding OVA(257–264) epitope and the I-A^b-binding OVA(265–277) epitope. *S. typhimurium* 14028r and CS022r containing pJLP-2H-Kan were grown in Luria broth (LB) supplemented with 50 µg/ml kanamycin while *S. typhimurium* bacteria not containing a plasmid were grown in LB. Bacteria were grown overnight with shaking at 37°C. Logarithmically growing bacteria were obtained by diluting an overnight culture 1:20 into fresh LB and incubating until the optical density at 600 nm (OD₆₀₀) reached 1.3–1.5. Bacteria were then centrifuged at 1,700 *g* and resuspended in IMDM (without antibiotics; GIBCO BRL) to a density of 5 × 10⁸ bacteria/ml. This bacterial suspension was diluted in IMDM (without antibiotics) to obtain the desired amount of bacteria to be added to assays. Heat-killed and gentamycin-treated bacteria were prepared by incubating suspensions of bacteria (5 × 10⁸ bacteria/ml) at 65°C for 45 min or with 100 µg/ml gentamycin at 4°C for 90 min before addition to the assay.

Bone Marrow Cultures. DCs were obtained by culturing bone marrow from C57BL/6 mice (H-2^b) in IMDM supplemented with 10% culture supernatant from Ag8653 myeloma cells transfected with murine GM-CSF. DCs were separated from macrophages in the cultures by transferring nonadherent cells to new culture dishes containing IMDM plus GM-CSF and used on days 6–8 of culture as described (18). These nonadherent cells were highly enriched for DCs and were typically 60–70% pure as assessed by surface expression of MHC-II and CD11c by FACS[®]. In some experiments, the cells were then further enriched for CD11c-expressing cells (DCs) using anti-CD11c magnetic mi-

crobeads and a MiniMACS separation column (Miltenyi Biotec) following the manufacturer's protocol. The enriched population consisted of 80–90% CD11c⁺ cells as assessed using HL3 antibody (PharMingen) and FACS[®] analysis. Both of these DC populations have a heterogeneous expression of MHC-II, CD80, and CD86 (Svensson, M., C. Johansson, and M.J. Wick, manuscript in preparation) and are phagocytic (18, 19), showing that the DCs are in an intermediate stage of maturation (23). Both the bulk culture and the CD11c-purified DC populations were used in experiments and gave identical results.

Bone marrow-derived macrophages (MΦ) were prepared by culturing bone marrow from BALB/c (H-2^d) or caspase 1^{-/-} mice (H-2^b) in IMDM plus GM-CSF as above. After the nonadherent cells were removed onto new culture dishes, the strongly adherent cell population was washed with HBSS. These adherent cells, MΦ, were removed from the plastic with cold 10 mM EDTA (pH 8.0), washed twice with HBSS, and used in experiments. Alternatively, MΦ were generated by expanding bone marrow from BALB/c or caspase 1^{-/-} mice in IMDM supplemented with 30% supernatant from L929 cells. The strongly adherent population, MΦ, was removed from the plastic and washed as described for MΦ derived from IMDM plus GM-CSF cultures. MΦ cultured from bone marrow using either IMDM plus GM-CSF or IMDM plus L cell supernatant gave identical results in apoptotic antigen presentation assays (data not shown).

Induction and Detection of Apoptosis. MΦ from BALB/c mice were resuspended in IMDM containing 5% FCS (without antibiotics), and 10⁶ cells were seeded per well in 24-well cell culture-treated or low adherence plates (Corning, Inc.). Bacteria were diluted in IMDM (without antibiotics) and added to MΦ at a bacteria to MΦ ratio of 5:1, 15:1, or 50:1 as indicated in individual experiments. Bacteria were pelleted onto the cells by centrifugation at 270 *g* for 4 min at room temperature, and the plates were incubated for 30–120 min at 37°C with 5% CO₂. The cultures were then washed twice with HBSS to remove remaining bacteria and either incubated for an additional 2 h in the presence of 100 µg/ml gentamycin (a total of 4 h) or the cells were directly assessed for apoptosis by Annexin V staining at the indicated time points. Necrotic cells were excluded by staining with propidium iodide (PI) at 1 µg/ml (Sigma Chemical Co.). Incubations with Annexin V-FITC (Trevigene, Inc.) were for 20 min on ice. Flow cytometry analysis was performed on a Becton Dickinson FACS^{Sort™} flow cytometer (Becton Dickinson), and data were collected on 3 × 10⁴ PI⁻ cells. Apoptosis was further confirmed by detection of high molecular weight DNA fragments as described (24). In samples where apoptosis was chemically induced, MΦ were treated with 1 µg/ml of LPS purified from *Escherichia coli* (Sigma Chemical Co.) for 4 h at 37°C with 5% CO₂. This was followed by incubation with 5 mM ATP for 45 min (20).

Presentation of Antigens Derived from Apoptotic Material. After infection of MΦ with bacteria and washing with HBSS as described above, fresh IMDM supplemented with 5% FCS and 100 µg/ml gentamycin were added and the infected MΦ were incubated for an additional 2 h. After an additional wash with HBSS, the MΦ were resuspended in 500 µl of IMDM supplemented with 10% FCS and 50 µg/ml gentamycin. Then, 10⁶ DCs or MΦ, either live or prefixed in 1% paraformaldehyde and subsequently washed thoroughly, were added to the apoptotic MΦ in 250 µl of IMDM. This resulted in a 1:1 DC or MΦ to apoptotic MΦ ratio. Finally, 5 × 10⁵ CD8OVA (17) or OT4H.2D5 (25) T hybridoma cells, which secrete IL-2 upon specific recognition of the OVA(257–264)/K^b or OVA(265–277)/I-A^b complex, respectively, were added in 250 µl of IMDM. After a 24-h incubation

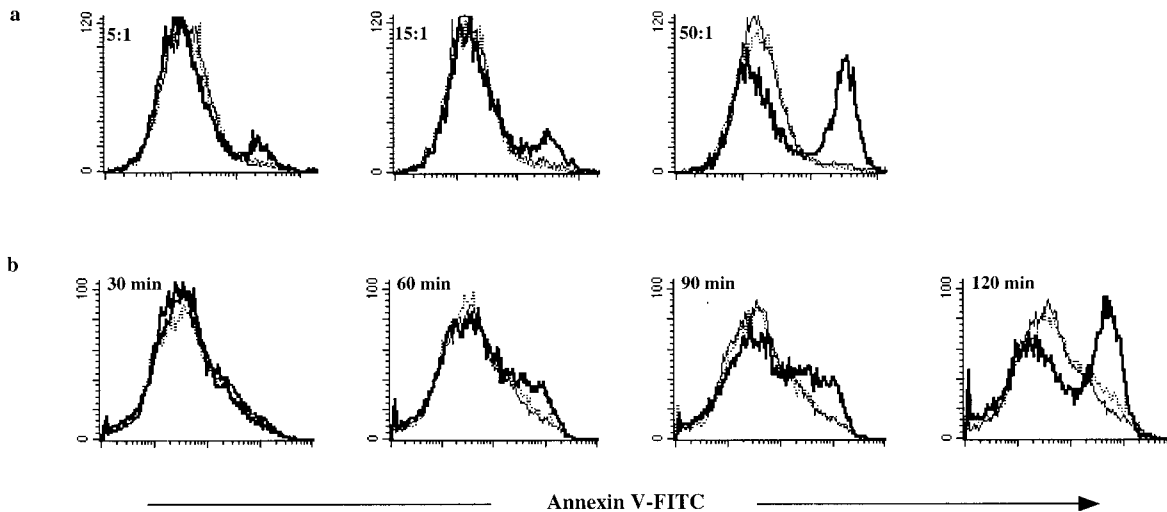


Figure 1. Logarithmically growing *S. typhimurium* 14028 induces apoptosis in MΦ, whereas the *phoP*⁻ mutant CS022 or stationary phase 14028 does not. (a) MΦ were coincubated with increasing bacteria to MΦ ratios (5:1, 15:1, or 50:1) for 2 h. Residual bacteria were washed away, and the cells were incubated for an additional 2 h in medium with gentamycin (100 μg/ml). The cells were stained with Annexin V-FITC and PI and were analyzed in a flow cytometer. Histograms show 3 × 10⁴ gated PI⁻ cells plotted against cell number of MΦ coincubated with 14028s (thick line), CS022s (dotted line), or medium (thin line). (b) MΦ were coincubated with either logarithmically growing 14028r (thick line) or stationary phase (plate-grown) 14028r (dotted line) at a bacteria to MΦ ratio of 15:1, or were incubated in medium alone (thin line). At the times indicated in the histograms, infected cells were stained and analyzed as described in a. The induction of apoptosis in the MΦ was confirmed by FACS[®] analysis or detection of high molecular weight DNA fragments in at least three independent experiments.

at 37°C, IL-2 secreted by the CD8OVA or OT4H.2D5 T hybridoma cells was quantitated using IL-2-dependent CTLL cells. In experiments where MΦ from caspase 1^{-/-} mice (H-2^b) were infected with bacteria, the assay was performed in the same way except that the T hybridoma cells were not added together with the DCs but after incubation of DCs with (apoptotic) MΦ as follows. After 20 h of DC-MΦ coculture, cells from three wells were collected and resuspended in cold PBS supplemented with 0.5% FCS and 2 mM EDTA. The suspension was then enriched for CD11c-expressing cells (DCs) using anti-CD11c magnetic microbeads (Miltenyi Biotec) following the manufacturer's protocol. The enriched population consisted of 80–90% CD11c⁺ cells as assessed using HL3 antibody (PharMingen) and FACS[®] analysis. 10⁵ purified CD11c⁺ cells were then added to duplicate wells in a 96-well plate (Corning, Inc.) together with 10⁵ T hybridoma cells. Cells were coincubated for 24 h at 37°C with 5% CO₂, and IL-2 produced by the T hybridoma cells was quantified as above. Experiments where the inhibitor cytochalasin D (CCD, 5 μg/ml; Sigma Chemical Co.) was used were also performed such that the DCs were coincubated with MΦ for 20 h in the presence of CCD followed by enrichment of CD11c⁺ DCs by magnetic sorting. The enriched DCs were subsequently added to T hybridoma cells in fresh wells. This was done to retain viability of the DCs and to assure that equal amounts of viable DCs were added to the T hybridoma cells.

Direct Presentation of Bacterial Antigens by Infected DCs. Experiments to assess direct presentation of bacterial antigens by DCs were conducted in 24-well or 96-well plates as described previously (18), except that DCs were not fixed after coincubation with the bacteria. Instead, bacteria were coincubated with DCs at the indicated bacteria to DC ratio for 2 h and the cells were washed three times with HBSS. Then, fresh IMDM containing 5% FCS and 50 μg/ml gentamycin was added. This was followed by addition of T hybridoma cells and quantitation of IL-2 production as described for the other antigen presentation assays.

Phagocytosis of Apoptotic Cells. MΦ were dyed red using PKH26-GL (Sigma Biosciences), and 10⁶ dyed cells were coincubated with 14028r or CS022r for 90 min and washed as described above. After incubation for an additional 2 h in IMDM supplemented with 50 μg/ml gentamycin, 2.5 × 10⁵ MACS-purified DCs or MΦ dyed green using PKH67-GL (Sigma Biosciences) were added to the red MΦ. After 2 h of coincubation, flow cytometry was performed and the percentage of double positive cells among the green cells was determined. In samples where CCD was present, the green cells were first pretreated for 60 min with CCD at 10 μg/ml and the concentration of CCD was 5 μg/ml during the coincubation with the red MΦ.

Results

DCs Acquire Antigens from MΦ Induced to Undergo Apoptosis by Salmonella Infection. Infection of MΦ and DCs with *S. typhimurium* using conditions that do not alter the viability of the infected cells, such as with bacteria in the stationary phase of growth, results in processing of the bacteria for peptide presentation on MHC-I and MHC-II (16–19, 21). It has also been shown that infection of MΦ with *S. typhimurium* grown under conditions resulting in an invasive phenotype and expression of the type III secretion apparatus (6, 26, 27), such as during the logarithmic growth phase, results in apoptotic death of infected MΦ (6, 7). Thus, we sought to determine whether *S. typhimurium*-induced apoptosis of infected MΦ was a quiescent death that did not result in presentation of bacterial antigens to T cells, or whether bystander APCs could acquire bacterial antigens from MΦ induced to undergo apoptosis by *S. typhimurium* infection. MΦ were infected with logarithmically growing wild-type *S. typhimurium* 14028r, which in-

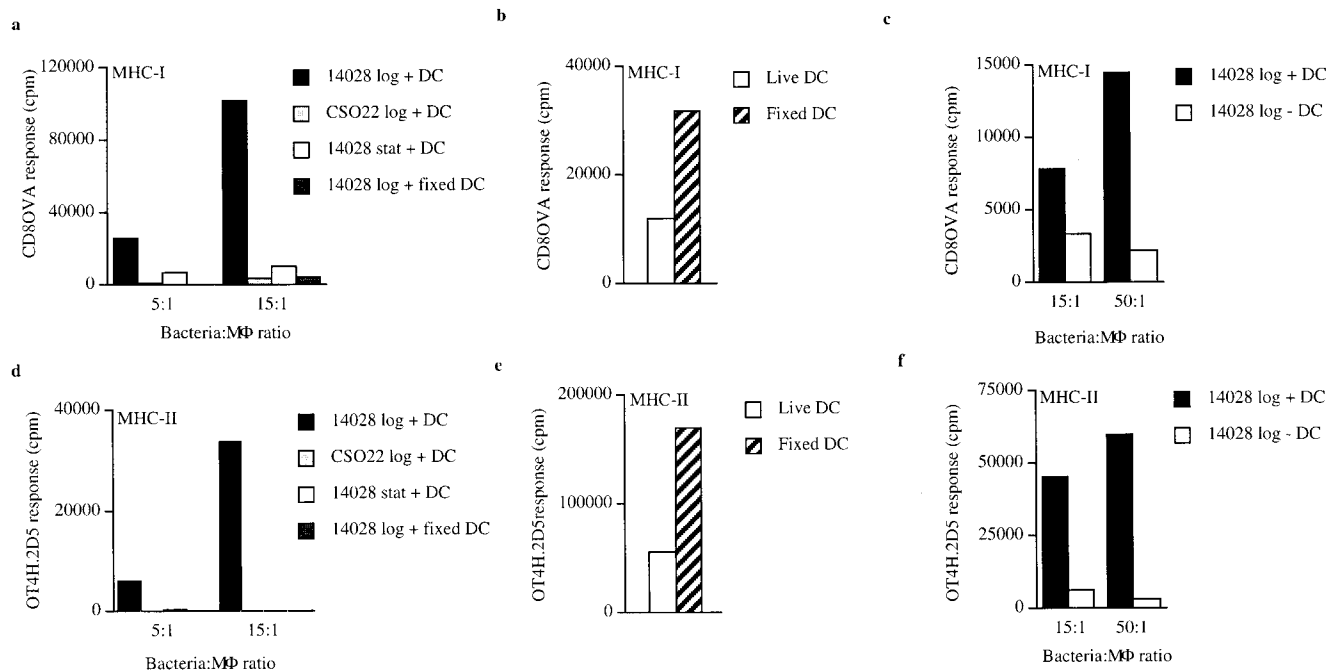


Figure 2. Viable bystander DCs acquire antigenic material from MΦ induced to undergo apoptosis by infection with wild-type *S. typhimurium*. (a and c) OVA peptide presentation on MHC-I (K^b) and (d and f) MHC-II ($I-A^b$) by bystander DCs quantitated using the OVA(257–264)/ K^b - or the OVA(265–277)/ $I-A^b$ -specific T cell hybridoma CD8OVA or OT4H.2D5, respectively. MΦ (H-2^d) were coincubated for 90 min with either stationary phase (stat) 14028r, logarithmically growing (log) 14028r, or logarithmically growing CS022r expressing Crl-OVA as indicated. After washing and addition of gentamycin, live (+ DC) or paraformaldehyde-fixed (+ fixed DC) (H-2^b) or no DCs (– DC) and T hybridoma cells were added as indicated. (b and e) The CD8OVA (b) or OT4H.2D5 (e) response to live or fixed DCs (H-2^b) loaded with 1 nM OVA(257–264) or 100 μ M OVA(265–280) peptide, respectively. Counts per minute in wells where bystander DCs were omitted or where DCs or MΦ were incubated in medium only along with the appropriate T cell hybridoma were typically 250–2,500. The results are representative of three to five independent experiments.

duces apoptosis in the infected cells, or with logarithmically growing *phoP* constitutive (*phoP*^c) strain CS022r, which does not induce apoptosis upon infection of MΦ as assessed by Annexin V staining (Fig. 1) or DNA fragmentation (data not shown). Both bacterial strains expressed the model antigen Crl-OVA, which is expressed in the cytosol of the bacteria (16) and encodes the MHC-I-binding OVA(257–264) as well as the MHC-II-binding OVA(265–277) epitope. Conditions that resulted in Annexin V⁺ PI⁻ MΦ after infection with 14028r but not with CS022r (Fig. 1) resulted in OVA(257–264) presentation on the MHC-I molecule K^b as well as OVA(265–277) presentation on the MHC-II molecule $I-A^b$ by bystander DCs (Fig. 2, a and d). This presentation was observed when MΦ were infected with logarithmically growing 14028r but not with logarithmically growing CS022r, bacteria which can and cannot induce apoptosis in infected MΦ, respectively (Fig. 1). In addition, no presentation of the OVA peptides on either MHC-I or MHC-II by bystander DCs was observed when MΦ were infected with 14028r in the stationary phase of growth (grown on agar plates for 20 h; Fig. 2, a and d). Stationary phase wild-type *S. typhimurium* does not induce apoptosis in infected MΦ (Fig. 1 b; references 6, 7). The presentation observed when MΦ were infected with logarithmically growing 14028r was not due to release of peptides into the culture that bound preformed surface MHC molecules: addition of prefixed DCs instead of viable DCs

as the bystander APCs did not result in stimulation of the OVA(257–264)/ K^b - or the OVA(265–277)/ $I-A^b$ -specific T cell hybridomas (Fig. 2, a and d). This was the case in spite of the enhanced ability of prefixed DCs to bind exogenously added OVA(257–264) or OVA(265–280) peptide compared with viable DCs (Fig. 2, b and e). The observed presentation was not due to presentation of the bacteria-derived OVA peptides by infected MΦ. This was ensured by using MΦ from a haplotype of mouse (BALB/c; H-2^d) different from that recognized by the H-2^b-specific CD8OVA and OT4H.2D5 T cell hybridomas (17, 25). Finally, omitting addition of bystander DCs (H-2^b) to the infected MΦ (H-2^d) resulted in a similar background level of stimulation of the T cell hybridomas as when DCs (H-2^b) or MΦ (H-2^b) were grown in medium only (Fig. 2, c and f, and see Fig. 5, a–c).

OVA Peptide Presentation Is Due to Uptake of Apoptotic Material and Not to Direct Presentation of Residual Bacteria. Several lines of data support that the MHC-I and MHC-II presentation shown in Fig. 2, a and d, was due to uptake of apoptotic material and not to phagocytic processing of remaining bacteria by the added DCs. First, addition of stationary phase 14028r, which does not induce apoptosis in the infected MΦ (Fig. 1 b; references 6 and 7), did not result in MHC-I or MHC-II presentation in the apoptotic antigen presentation assay (Fig. 2, a and d). This is the case despite the fact that stationary phase *S. typhimurium* express-

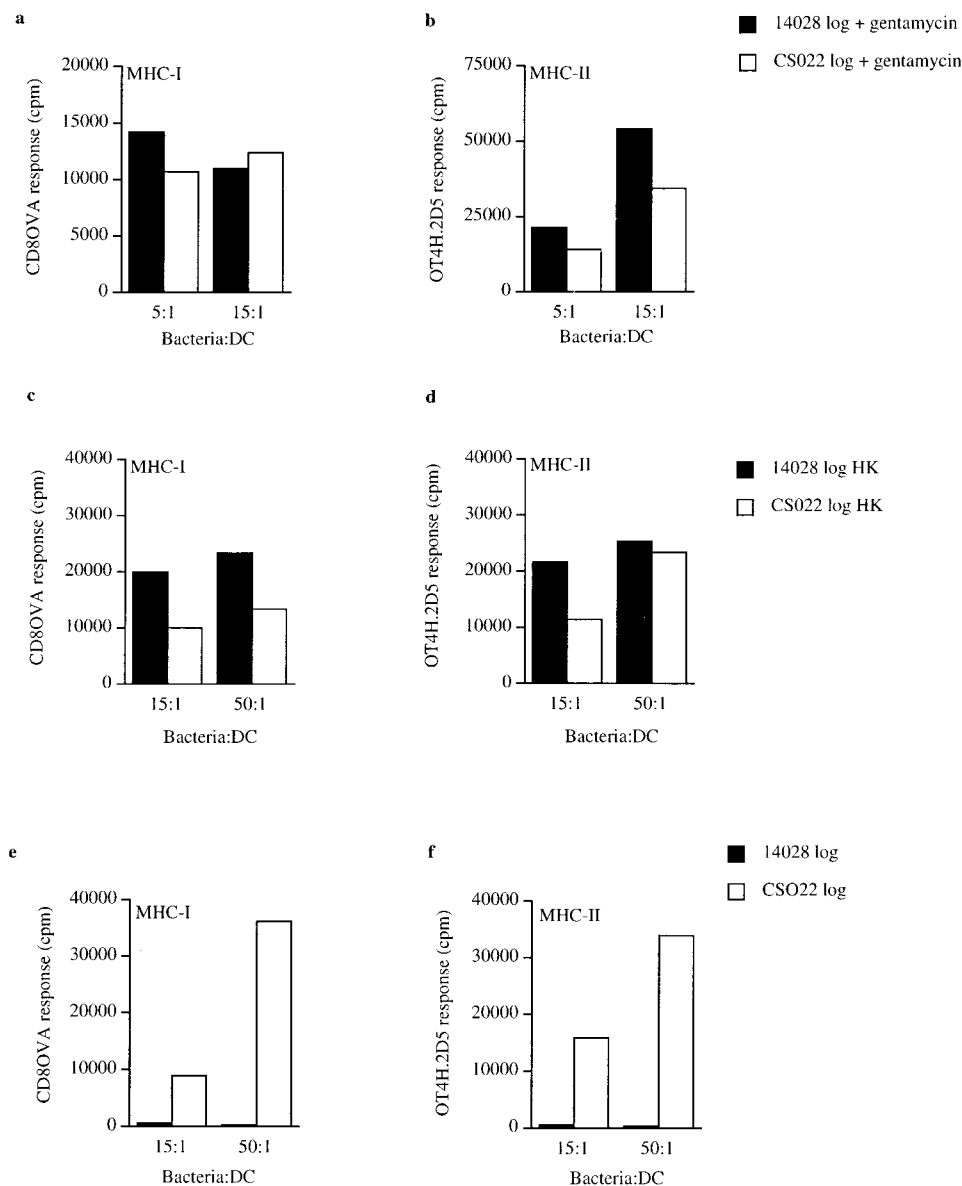


Figure 3. DCs process residual 14028 and CS022 expressing CrI-OVA for OVA peptide presentation on MHC-I and MHC-II equally well. Logarithmically growing ($OD_{600} = 1.3-1.5$) 14028r CrI-OVA or CS022r CrI-OVA bacteria were treated with 100 $\mu\text{g/ml}$ of gentamycin (a and b), heat killed (HK) at 65°C (c and d), or were left untreated (e and f) before coincubation with DCs for 90 min. After several washes, OVA(257-264)/K^b (a, c, and e) and OVA(265-277)/I-A^b (b, d, and f) were quantitated using CD8OVA and OT4H.2D5 T hybridoma cells, respectively. The results are representative of three independent experiments.

ing CrI-OVA is efficiently presented when phagocytosed by DCs (18, 19). In addition, similar amounts of the bacteria were internalized by the M Φ . This was confirmed by coincubating M Φ with logarithmically growing or stationary phase 14028s or logarithmically growing CS022s expressing green fluorescent protein followed by flow cytometry analysis (data not shown) as described elsewhere (19). Second, we reasoned that if the presentation observed in the apoptotic presentation assay was due to remaining bacteria that were phagocytosed by the added DCs, it must be due to gentamycin-killed bacteria remaining in the wells, as no bacterial growth was observed during the 24-h incubation time. To directly test this possibility, bacterial suspensions of 14028r and CS022r were treated with gentamycin, equal numbers of these bacteria were added to viable DCs, and presentation of the OVA peptides was quantitated. This resulted in equal levels of bacterial processing by DCs

for MHC-I presentation of OVA(257-264) (Fig. 3 a) and MHC-II presentation of OVA(265-277) (Fig. 3 b). Likewise, similar levels of presentation of the OVA peptides on MHC-I and MHC-II derived from the bacteria expressing CrI-OVA was observed if the bacteria were heat-killed before addition to DCs (Fig. 3, c and d). The similar level of MHC-I and MHC-II presentation of OVA peptides derived from gentamycin-treated or heat-killed 14028r and CS022r expressing CrI-OVA makes it unlikely that the presentation observed in Fig. 2, a and d, was due to phagocytic processing of bacteria remaining in the wells. Furthermore, viable bacterial counts from samples removed from wells of apoptotic presentation experiments where M Φ were infected with either 14028r or CS022r consistently showed that similar quantities of 14028r and CS022r were recovered (data not shown). Such data were obtained from >10 individual experiments that gave apoptotic presenta-

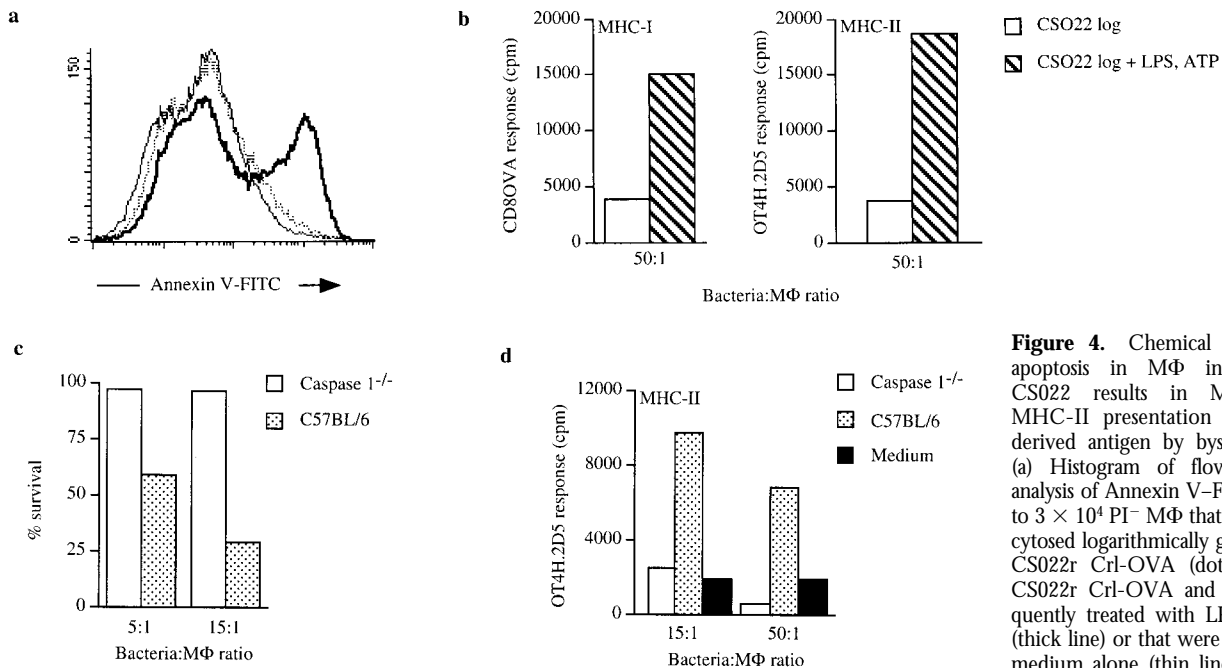


Figure 4. Chemical induction of apoptosis in MΦ infected with CS022 results in MHC-I and MHC-II presentation of bacteria-derived antigen by bystander DCs. (a) Histogram of flow cytometry analysis of Annexin V-FITC binding to 3×10^4 PI⁻ MΦ that have phagocytosed logarithmically growing (log) CS022r CrI-OVA (dotted line) or CS022r CrI-OVA and were subsequently treated with LPS and ATP (thick line) or that were incubated in medium alone (thin line). Flow cytometry analysis was carried out 20 h

after the apoptosis induction. (b) OVA(257–264)/K^b and OVA(265–277)/I-A^b presentation on MHC-I and MHC-II by bystander DCs (H-2^b) after co-incubation with MΦ (H-2^d) previously co-incubated with logarithmically growing CS022r CrI-OVA and left untreated or treated with LPS plus ATP as indicated. OVA(257–264)/K^b and OVA(265–277)/I-A^b presentation was quantitated using CD8OVA and OT4H.2D5 T hybridoma cells, respectively. (c) Viability of MΦ from wild-type and caspase 1^{-/-} mice after coincubation with logarithmically growing 14028r CrI-OVA for 90 min measured as exclusion of PI staining. (d) MΦ derived from C57BL/6 or caspase 1^{-/-} mice were coincubated with logarithmically growing 14028r CrI-OVA or medium only for 90 min. After washing and addition of gentamycin, DCs were added for 24 h. The DCs were then MACS-purified using anti-CD11c magnetic beads, and presentation of OVA(265–277) on MHC-II by the DCs was quantitated by addition of OT4H.2D5 T hybridoma cells to the MACS-purified DCs.

tion results similar to that shown in Fig. 2, a and d, despite the fact that the absolute number of bacteria recovered varied between individual experiments. In addition, OVA-specific ELISA was performed on lysates prepared from bacteria used in apoptotic antigen presentation assays that gave results similar to those shown in Fig. 2, a and d, to quantitate the level of CrI-OVA expressed in logarithmically growing 14028r and CS022r. These data consistently showed similar levels of CrI-OVA expression in the two bacterial strains and never exceeded a twofold difference in relative expression, sometimes slightly greater in 14028r and sometimes greater in CS022r. Despite this, we never observed significant presentation of the bacteria-encoded antigens by DCs in our apoptotic bystander assay when MΦ were infected with CS022r. Finally, no presentation of either OVA(257–264) on K^b or OVA(265–277) on I-A^b was detected when logarithmic 14028r CrI-OVA was co-incubated with the DCs (Fig. 3, e and f), as logarithmically growing 14028r is cytotoxic to these cells (our unpublished results). In contrast, logarithmically growing CS022r expressing CrI-OVA, which does not induce apoptosis in infected cells (Fig. 1 a), was processed for OVA peptide presentation on both MHC-I and MHC-II by DCs (Fig. 3, e and f). Together, these data support the notion that the presentation observed in Fig. 2, a and d, is due to uptake of material from MΦ that were induced to undergo apoptosis by infection with wild-type *S. typhimurium* by bystander

DCs rather than to direct presentation of residual bacteria in the wells by bystander DCs.

Chemical Induction of Apoptosis in MΦ Infected with CS022r Results in Presentation of Bacterial Antigens by Bystander DCs. To determine if inducing MΦ to undergo apoptosis upon phagocytosis of *S. typhimurium* was sufficient to result in presentation of apoptotic material by bystander DCs, MΦ infected with CS022r expressing CrI-OVA were induced to undergo apoptosis by addition of LPS and ATP (20). This treatment induced apoptosis as assessed by Annexin V staining (Fig. 4 a). Presentation of OVA(257–264) on MHC-I as well as OVA(265–277) on MHC-II by bystander DCs was significantly enhanced when MΦ (MHC-mismatched) co-incubated with logarithmically growing CS022r were induced to undergo apoptosis by treatment with LPS and ATP (Fig. 4 b).

To further investigate the role of apoptosis induction in generating material for presentation by bystander DCs, MΦ from caspase 1^{-/-} mice (20), which were previously shown to be resistant to the cytotoxic effects of *Salmonella* infection (14), were used in the apoptotic antigen presentation assay. Although a 2-h co-incubation of caspase 1^{-/-} MΦ with 14028r CrI-OVA resulted in no detectable cell death, significant death occurred upon co-incubation of 14028r with C57BL/6 MΦ (Fig. 4 c). Apoptotic antigen presentation assays were performed by adding DCs to MΦ from either caspase 1^{-/-} or C57BL/6 mice that had been coincu-

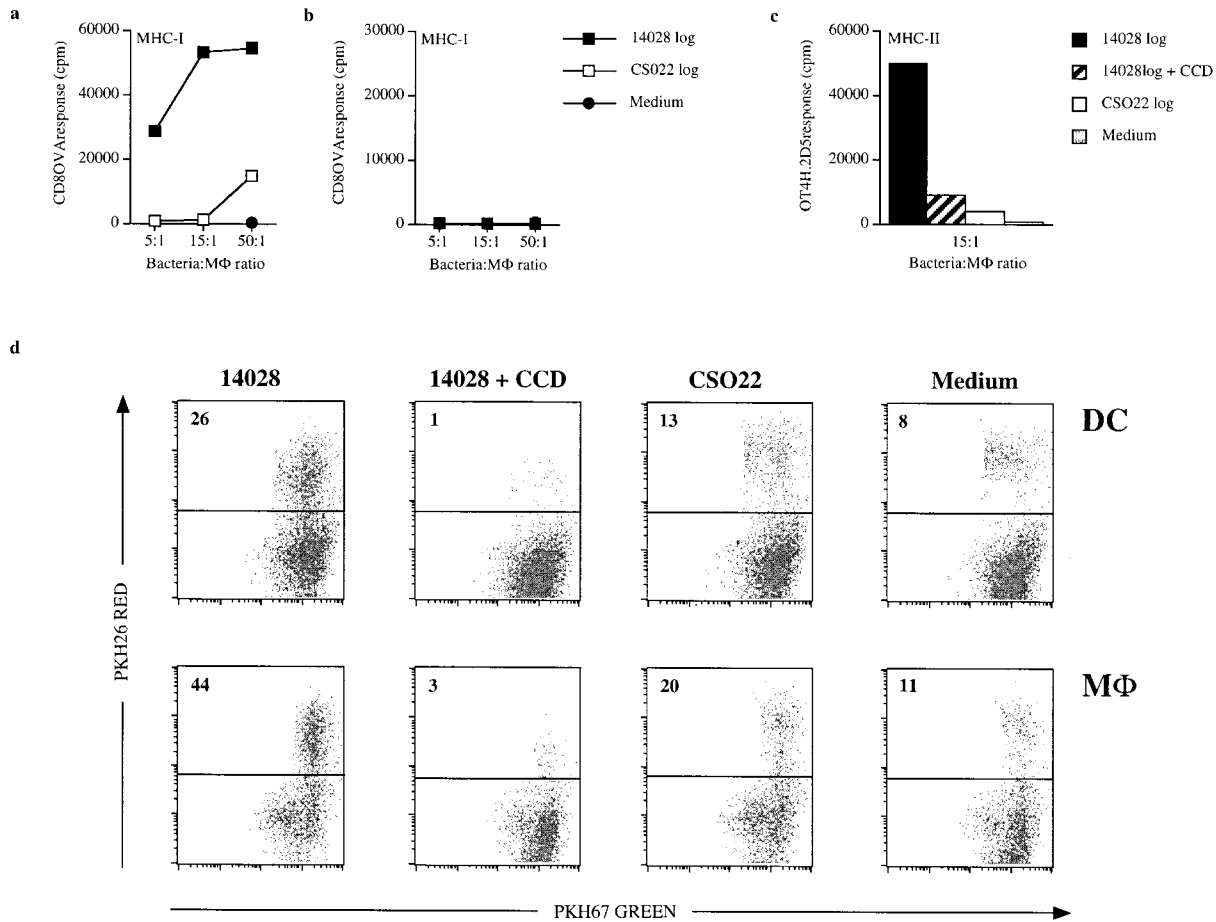


Figure 5. Both MΦ and DCs engulf apoptotic material by a process that requires cytoskeletal rearrangement despite the fact that OVA(257–264) presentation on MHC-I is restricted to DCs. OVA(257–264) presentation on MHC-I by bystander DCs (H-2^b) (a) or MΦ (H-2^b) (b) after coincubation with MΦ (H-2^d) that previously phagocytosed logarithmically growing 14028r Crl-OVA or CS022r Crl-OVA. After 90 min of coincubation with bacteria, cells were washed, gentamycin was added, and OVA(257–264) presentation by added bystander cells was quantitated using CD8OVA T hybridoma cells. The response of MΦ (H-2^d) and bystander cells incubated in medium only is also shown. (c) MΦ (H-2^d) were coincubated with logarithmically growing 14028r Crl-OVA, CS022r Crl-OVA, or were incubated in medium only for 90 min. After washing and addition of gentamycin, DCs were added in the absence or presence of CCD (+ CCD) for 20 h. The DCs were then MACS-purified using anti-CD11c magnetic beads, and presentation of OVA(265–277) on MHC-II by the DCs was quantitated by addition of OT4H.2D5 T hybridoma cells to the MACS-purified DCs. The response of MΦ (H-2^d) and bystander DCs incubated in medium only is also shown. (d) MΦ dyed red were either incubated in medium only or were coincubated with logarithmically growing 14028r or CS022r as indicated above the dot plots. These red MΦ were then coincubated with DCs (top) or MΦ (bottom) dyed green. This coincubation was done in the absence or presence (+ CCD) of CCD as indicated. Flow cytometry analysis was then performed on 3×10^4 cells. The percentage of double positive cells among green cells is indicated in each dot plot.

bated with 14028r expressing Crl-OVA. In contrast to the experiments presented above, which use MHC-mismatched MΦ for bacterial infections, MΦ from caspase 1^{-/-} mice are of the correct haplotype (H-2^b) to be recognized by the OT4H.2D5 T cell hybridoma. To avoid detecting direct presentation by caspase 1^{-/-} or C57BL/6 MΦ, the infected MΦ were first coincubated with the bystander DCs (also H-2^b) for 20 h. After this incubation, CD11c⁺ cells, i.e., the added bystander DCs, were positively selected from the cultures using anti-CD11c-coated magnetic beads. These purified DCs were then added to wells containing OT4H.2D5 T hybridoma cells, and presentation of OVA(265–277) on I-A^b was subsequently quantitated in the absence of the caspase 1^{-/-} or C57BL/6 MΦ. These experiments showed that DCs coincubated with

MΦ from wild-type but not caspase 1^{-/-} mice could sensitize the MHC-II-restricted T cell hybridoma (Fig. 4 d).

DCs but Not MΦ Present Antigens from Apoptotic Material on MHC-I Despite the Fact that Both Cell Types Internalize Apoptotic MΦ. To investigate if MΦ also had the capacity to acquire antigens from bacteria-induced apoptotic MΦ, experiments were performed where MΦ or DCs were added as the bystander APCs. Thus, MΦ (MHC-mismatched, H-2^d) were coincubated with 14028r or CS022r expressing Crl-OVA, and either MΦ or DCs from C57BL/6 mice (H-2^b) were added as the bystander cells. Quantification of OVA(257–264)/K^b presentation showed that DCs (Fig. 5 a) but not MΦ (Fig. 5 b) could present the OVA epitope on MHC-I from bacteria-encoded Crl-

OVA. No presentation by bystander M Φ was observed even at a higher bacteria to M Φ infection ratio (50:1). However, the M Φ could present bacterial antigens on MHC-I when stationary phase 14028r or CS022r expressing Crl-OVA was added directly to the cells (data not shown).

We next investigated if the lack of presentation of antigens from apoptotic material on MHC-I by bystander M Φ was due to an inability of M Φ to engulf apoptotic material. To test this, M Φ were dyed red and were coincubated with logarithmically growing 14028r or CS022r expressing Crl-OVA. These red M Φ were then coincubated with M Φ or DCs that were dyed green. Flow cytometry was subsequently used to analyze the number of double positive cells among the green cells. These data showed that M Φ and DCs efficiently internalized apoptotic material from M Φ that were coincubated with 14028r. In contrast, significantly less uptake of apoptotic material was apparent when the red M Φ were coincubated with CS022r or when they were incubated in medium alone before coincubation with green bystander cells (Fig. 5 d). Furthermore, the uptake of apoptotic material was inhibited when CCD was present during the coculture (Fig. 5 d). This demonstrates that cytoskeletal rearrangements are required for the uptake of apoptotic material. In addition, CCD also abolished presentation of OVA(265–277) on MHC-II by bystander DCs when it was present during the coincubation of the bystander DCs and the M Φ that had previously phagocytosed 14028r expressing Crl-OVA (Fig. 5 c). Finally, the uptake of apoptotic material by bystander DCs after coincubation with bacterially induced apoptotic M Φ was further confirmed by electron microscopy, showing apoptotic bodies within DCs (data not shown).

Discussion

An increasing number of bacterial species have been shown to induce apoptosis in phagocytes, including *Yersinia pseudotuberculosis*, *Shigella flexneri*, and *S. typhimurium* (6, 7, 9, 10). Although these studies have examined the mechanism underlying the observed apoptosis, none of them have investigated if this process generates antigenic material. Thus, this is the first demonstration that bacteria-induced apoptosis of infected cells results in presentation of a bacteria-encoded antigen on both MHC-I and MHC-II by bystander DCs. Apoptosis induction in infected cells is critical for presentation of bacteria-derived material by bystander APCs. This was demonstrated here using bacterial strains that can or cannot induce apoptosis in infected M Φ , and by chemically inducing apoptosis in M Φ infected with bacteria that cannot themselves induce apoptosis. Interestingly, it has been suggested that *Salmonella* also can induce cells to undergo a necrotic type of cell death (28). However, conditions inducing such death in infected cells, for example using a very high bacteria to macrophage ratio, resulted in drastic reduction of the presentation of bacteria-encoded antigens on MHC-I by bystander DCs, whereas the presentation on MHC-II was not as dramatically affected (our

unpublished observations). These observations support the important role of apoptosis induction for presentation of antigenic material by bystander DCs and suggest a different role for MHC-I versus MHC-II presentation of apoptotic material by bystander DCs.

We also showed that the observed presentation of epitopes to T cells after bacterial induction of apoptosis in infected M Φ occurred when live DCs but not M Φ were present as bystander cells, and furthermore that this presentation involved active phagocytosis of antigenic material. The mechanism underlying the ability of DCs but not M Φ to present antigens derived from apoptotic material, despite the fact that both cell types actively internalize material from neighboring apoptotic cells, remains to be clarified. In preliminary experiments where bystander M Φ were added to bystander DCs coincubated with bacteria-induced apoptotic M Φ , presentation of the bacteria-encoded antigens on MHC-I and MHC-II was diminished. Whether the observed reduction in presentation by bystander DCs in the presence of simultaneously added bystander M Φ was due to a soluble factor secreted by the macrophages or a result of competition for the antigenic material between the DCs and the M Φ remains to be determined. Interestingly, a similar ability of bystander DCs but not M Φ to present antigenic material from monocytes induced to undergo apoptosis by influenza virus infection to CD8⁺ cytotoxic T cells, as well as reduced presentation by bystander DCs in the presence of added bystander M Φ , has also been observed (15). Such differences in the ability of these different APCs to present antigens from apoptotic material may be due to different functions they may have in initiating an immune response. For example, DCs are the most efficient stimulators of naive T cells due to their extensive antigen capture capacity before they have been exposed to antigenic stimuli and their enhanced antigen presentation capacity after encountering antigenic stimuli or after exposure to immunomodulatory factors such as LPS or TNF- α (for a review, see reference 29). M Φ , on the other hand, are not as efficient stimulators of naive T cells as DCs. This is due, at least in part, to low or absent expression of MHC-II and costimulatory molecules, unless the cells are activated by, for example, IFN- γ or LPS (30, 31). M Φ also have extensive antigen degradative capacity, which is important in their role in innate protection against microbes. This latter property of M Φ may result in extensive degradation of phagocytosed apoptotic material and concomitant lack of antigenic peptide presentation for T cell recognition.

S. typhimurium infection naturally occurs by the oral route. After reaching the intestine, the bacteria penetrate into deeper tissue through M cells by inducing membrane ruffling (1). The bacteria then pass through the epithelium of the PP and enter the subepithelial dome. This consists of a network of M Φ and DCs intermingled with CD4⁺ T cells and B cells from the underlying follicle (4). The potential interaction of *S. typhimurium* with M Φ and DCs in the follicle dome after oral infection, combined with the bacteria's ability to survive inside phagocytic cells (32) and

its ability to induce apoptosis in infected M Φ (Fig. 1; references 6, 7), raises the question of the significance of bacteria-induced apoptosis of infected M Φ and its influence on the immune response to oral *Salmonella* infection. Indeed, it has been shown that apoptosis occurs in vivo during infection not only with *S. typhimurium*, but also with *Y. pseudotuberculosis* and *S. flexneri* (33, 34). Although apoptotic CD18⁺ cells have been identified in the liver of mice infected with *S. typhimurium* (35), apoptotic M Φ were present in the lymphoid follicles of the PP in a rabbit ligated ileal loop model of *S. flexneri* infection (34). Furthermore, Zychlinsky et al. (34) showed that apoptotic M Φ were present when virulent (apoptosis-inducing) *S. flexneri* was used, whereas no increase in the frequency of apoptotic cells above background levels was apparent using avirulent bacteria not capable of inducing apoptosis. Not only were apoptotic M Φ present after infection with virulent *S. flexneri*, but also large lymphoid follicle cells containing multiple apoptotic nuclei were demonstrated (34). This suggests that these cells had phagocytosed apoptotic cells, presumably apoptotic M Φ . Orogastric inoculation with *Y. pseudotuberculosis* revealed Mac1⁺ cells in spleen and mesenteric lymph nodes that were terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling assay-positive (TUNEL⁺) upon infection with wild-type bacteria (33). In contrast, no increase in Mac1⁺ TUNEL⁺ cells relative to uninfected mice was apparent when the mice were infected with a *yopJ* mutant (33). Furthermore, a *yopJ* mutant strain was more readily cleared from infected mice (33). These data suggest that apoptosis induction may contribute to spread of the bacteria and severity of the infection. However, the *yopJ* locus is also required for *Yersinia*-mediated inhibition of IL-8 and TNF- α expression (36), which could affect clearance of the bacteria from the host. Thus, although three genera of bacteria that infect hosts by penetrating the intestinal epithelium and accessing the lymphoid follicles of the PPs via M cells (37) induce apoptosis in vivo, the relative contribution of apoptosis by itself or apoptosis induction in combination with bacteria-mediated effects on cytokine production has not clearly been established. However, the ability of bacterial antigens to be presented to T cells from bacteria-induced apoptotic material may be yet another factor to consider when evaluating the influence of bacteria-induced apoptosis on the host immune response.

Immature DCs have an extensive capacity to internalize antigens (38), including apoptotic material (15, 39–41), for presentation on MHC-I and MHC-II. DCs in the subepithelial dome are endocytic and can process protein in vivo for presentation to naive T cells (42). It could be envisioned that when bacteria reach M Φ underlying the epithelial layer, the cells undergo apoptosis after phagocytosing the bacteria; such a scenario has been suggested for *Shigella* infection (34). In this way, M Φ neutralize the internalized bacteria and generate apoptotic material containing bacterial antigens in a “package” that does not induce apoptosis in a subsequently phagocytosing cell. In this study, we have shown that apoptotic material generated by *Salmonella* infection can be efficiently phagocytosed by DCs and M Φ . In the case of uptake by DCs, these cells can

then present bacterial antigens on MHC-I and MHC-II. LPS, TNF- α , IL-1 β (43, 44), and uptake of apoptotic material (40) have all been shown to induce DC maturation. This in turn triggers enhanced surface expression of costimulatory molecules and DC migration (for a review, see reference 29). In the case of *Salmonella*, DCs containing apoptotic material may migrate to lymphoid organs and activate naive T cells.

Indeed, apoptotic bodies can affect the immune system. For example, apoptotic bodies can have an immunosuppressive effect by reducing secretion of the proinflammatory cytokines TNF- α , IL-1 β , and IL-12 by LPS-stimulated monocytes (45, 46). Apoptotic cells have also been shown to induce autoantibody production when injected into syngeneic mice (47). The recent report by Ronchetti et al. (48) also demonstrated that injection of apoptotic RMA cells primes a functional tumor-specific immune response in vivo. Furthermore, DCs but not M Φ pulsed with apoptotic material prime a specific antitumor CTL response despite the fact that M Φ engulf apoptotic cells in vivo (48). M Φ and DCs have been suggested to use different integrin receptors, $\alpha_v\beta_3$ and $\alpha_v\beta_5$, respectively, to phagocytose apoptotic material (39). This may result in differences in the intracellular trafficking of the phagocytosed apoptotic material and concomitant differences in ability to present the antigens to T cells, as has been shown for M Φ here and elsewhere (15, 39). The cytokine production profile of M Φ and DCs after uptake of apoptotic material has also been shown to differ. For example, M Φ synthesize TGF- β (45) and downregulate production of the proinflammatory cytokines TNF- α , IL-1 β , and IL-12 (46), whereas DCs increase secretion of IL-10, TNF- α , and IL-1 β (40). The differences in cytokine profiles produced by the different APCs as well as the differential use of receptors to phagocytose apoptotic material may suggest that DCs and M Φ have different functions in the clearance of apoptotic material. Interestingly, *S. flexneri* apoptosis of infected M Φ also results in release of IL-1 β in its biologically active form (49). Although IL-1 β production by M Φ induced to undergo apoptosis by *Salmonella* infection has not yet been assessed, it is interesting to note that a similar mechanism of apoptosis induction, direct binding of a homologous effector protein to caspase 1 (12–14), is responsible for *Salmonella*- and *Shigella*-induced apoptosis of infected M Φ . Given the ability of apoptotic material to influence the immune response and the vast number of different bacteria that induce apoptosis in infected cells, the ability to combat microbial infections and develop effective recombinant bacterial vaccines must include understanding the relationship between microbe-induced apoptosis and antimicrobial immunity.

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References

1. Jones, B.D., N. Ghorri, and S. Falkow. 1994. *Salmonella typhimurium* initiates murine infection by penetrating and destroying the specialized epithelial M cells of the Peyer's patches. *J. Exp. Med.* 180:15–23.
2. Neutra, M.R., A. Frey, and J.-P. Kraehenbuhl. 1996. Epithelial M cells: gateways for mucosal infection and immunization. *Cell.* 86:345–348.
3. Jones, B.D., and S. Falkow. 1996. Salmonellosis: host immune responses and bacterial virulence determinants. *Annu. Rev. Immunol.* 14:533–561.
4. Neutra, M.R., E. Pringault, and J.-P. Kraehenbuhl. 1996. Antigen sampling across epithelial barriers and induction of mucosal immune responses. *Annu. Rev. Immunol.* 14: 275–300.
5. Foster, J.W., and M.P. Spector. 1995. How *Salmonella* survive against the odds. *Annu. Rev. Microbiol.* 49:145–174.
6. Chen, L.M., K. Kaniga, and J.E. Galán. 1996. *Salmonella* spp. are cytotoxic for cultured macrophages. *Mol. Microbiol.* 21: 1101–1115.
7. Monack, D.M., B. Raupach, A.E. Hromockyj, and S. Falkow. 1996. *Salmonella typhimurium* invasion induces apoptosis in infected macrophages. *Proc. Natl. Acad. Sci. USA.* 93: 9833–9838.
8. Cohen, J.J., R.C. Duke, V.A. Fadok, and K.S. Sellins. 1992. Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* 10:267–293.
9. Zychlinsky, A., M.C. Prevost, and P.J. Sansonetti. 1992. *Shigella flexneri* induces apoptosis in infected macrophages. *Nature.* 358:167–169.
10. Monack, D.M., J. Mecsas, N. Ghorri, and S. Falkow. 1997. *Yersinia* signals macrophages to undergo apoptosis and YopJ is necessary for this cell death. *Proc. Natl. Acad. Sci. USA.* 94: 10385–10390.
11. Muller, A., D. Gunther, F. Dux, M. Naumann, T.F. Meyer, and T. Rudel. 1999. Neisserial porin (PorB) causes rapid calcium influx in target cells and induces apoptosis by the activation of cysteine proteases. *EMBO (Eur. Mol. Biol. Organ.) J.* 18:339–352.
12. Chen, Y., M.R. Smith, K. Thirumalai, and A. Zychlinsky. 1996. A bacterial invasin induces macrophage apoptosis by binding directly to ICE. *EMBO (Eur. Mol. Biol. Organ.) J.* 15:3853–3860.
13. Hilbi, H., J.E. Moss, D. Hersh, Y. Chen, J. Arondel, S. Banerjee, R.A. Flavell, J. Yuan, P.J. Sansonetti, and A. Zychlinsky. 1998. *Shigella*-induced apoptosis is dependent on caspase-1 which binds to IpaB. *J. Biol. Chem.* 273:32895–32900.
14. Hersh, D., D.M. Monack, M.R. Smith, N. Ghorri, S. Falkow, and A. Zychlinsky. 1999. The *Salmonella* invasin SipB induces macrophage apoptosis by binding to caspase-1. *Proc. Natl. Acad. Sci. USA.* 96:2396–2401.
15. Albert, M.L., B. Sauter, and N. Bhardwaj. 1998. Dendritic cells acquire antigen from apoptotic cells and induce class I-restricted CTLs. *Nature.* 392:86–89.
16. Pfeifer, J.D., M.J. Wick, D.G. Russell, S.J. Normark, and C.V. Harding. 1992. Recombinant *Escherichia coli* express a defined, cytoplasmic epitope that is efficiently processed in macrophage phagolysosomes for class II MHC presentation to T lymphocytes. *J. Immunol.* 149:2576–2584.
17. Pfeifer, J.D., M.J. Wick, R.L. Roberts, K. Findlay, S.J. Normark, and C.V. Harding. 1993. Phagocytic processing of bacterial antigens for class I MHC presentation to T cells. *Nature.* 361:359–362.
18. Svensson, M., B. Stockinger, and M.J. Wick. 1997. Bone marrow-derived dendritic cells can process bacteria for MHC-I and MHC-II presentation to T cells. *J. Immunol.* 158:4229–4236.
19. Svensson, M., and M.J. Wick. 1999. Classical MHC class I peptide presentation of a bacterial fusion protein by bone marrow-derived dendritic cells. *Eur. J. Immunol.* 29:180–188.
20. Li, P., H. Allen, S. Banerjee, S. Franklin, L. Herzog, C. Johnston, J. McDowell, M. Paskind, L. Rodman, J. Salfeld, et al. 1995. Mice deficient in IL-1 β -converting enzyme are defective in production of mature IL-1 β and resistant to endotoxic shock. *Cell.* 80:401–411.
21. Wick, M.J., C.V. Harding, S.J. Normark, and J.D. Pfeifer. 1994. Parameters that influence the efficiency of processing antigenic epitopes expressed in *Salmonella typhimurium*. *Infect. Immun.* 62:4542–4548.
22. Miller, S.I., and J.J. Mekalanos. 1990. Constitutive expression of the PhoP regulon attenuates *Salmonella* virulence and survival within macrophages. *J. Bacteriol.* 172:2485–2490.
23. Pierre, P., S.J. Turley, E. Gatti, M. Hull, J. Meltzer, A. Mirza, K. Inaba, R.M. Steinman, and I. Mellman. 1997. Developmental regulation of MHC class II transport in mouse dendritic cells. *Nature.* 388:787–792.
24. Håkansson, A., J. Andreasson, B. Zhivotovsky, D. Karpman, S. Orrenius, and C. Svanborg. 1999. Multimeric α -lactalbumin from human milk induces apoptosis through a direct effect on cell nuclei. *Exp. Cell Res.* 246:451–460.
25. Li, Y., Y. Ke, P.D. Gottlieb, and J.A. Kapp. 1994. Delivery of exogenous antigen into the major histocompatibility complex class I and class II pathways by electroporation. *J. Leukoc. Biol.* 56:616–624.
26. Lee, C.A., and S. Falkow. 1990. The ability of *Salmonella* to enter mammalian cells is affected by bacterial growth state. *Proc. Natl. Acad. Sci. USA.* 87:4304–4308.
27. Collazo, C.M., and J.E. Galán. 1997. The invasion-associated type-III protein secretion system in *Salmonella*—a review. *Gene.* 192:51–59.
28. Lindgren, S.W., and F. Heffron. 1997. To sting or be stung: bacteria-induced apoptosis. *Trends Microbiol.* 5:263–264.
29. Banchereau, J., and R.M. Steinman. 1998. Dendritic cells and the control of immunity. *Nature.* 392:245–252.
30. Beller, D.I. 1984. Functional significance of the regulation of macrophage Ia expression. *Eur. J. Immunol.* 14:138–143.
31. Hathcock, K.S., G. Laszlo, C. Pucillo, P. Linsley, and R.J. Hodes. 1994. Comparative analysis of B7-1 and B7-2 costimulatory ligands: expression and function. *J. Exp. Med.* 180:631–640.
32. Fields, P.I., R.V. Swanson, C.G. Haidaris, and F. Heffron. 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. *Proc. Natl. Acad. Sci. USA.* 83:5189–5193.
33. Monack, D.M., J. Mecsas, D. Bouley, and S. Falkow. 1998. *Yersinia*-induced apoptosis in vivo aids in the establishment of

- a systemic infection of mice. *J. Exp. Med.* 188:2127–2137.
34. Zychlinsky, A., K. Thirumalai, J. Arondel, J.R. Cantey, A.O. Aliprantis, and P.J. Sansonetti. 1996. In vivo apoptosis in *Shigella flexneri* infections. *Infect. Immun.* 64:5357–5365.
 35. Richter-Dahlfors, A., A.M.J. Buchan, and B.B. Finlay. 1997. Murine salmonellosis studied by confocal microscopy: *Salmonella typhimurium* resides intracellularly inside macrophages and exerts a cytotoxic effect on phagocytes in vivo. *J. Exp. Med.* 186:569–580.
 36. Schesser, K., A.-K. Spiik, J.-M. Dukuzumuremyi, M.F. Neurath, S. Pettersson, and H. Wolf-Watz. 1998. The *yopJ* locus is required for *Yersinia*-mediated inhibition of NF- κ B activation and cytokine expression: YopJ contains a eukaryotic SH2-like domain that is essential for its repressive activity. *Mol. Microbiol.* 28:1067–1079.
 37. Jepsen, M.A., and M.A. Clark. 1998. Studying M cells and their role in infection. *Trends Microbiol.* 6:359–365.
 38. Austyn, J.M. 1996. New insights into the mobilization and phagocytic activity of dendritic cells. *J. Exp. Med.* 183:1287–1292.
 39. Albert, M.L., S.F. Pearce, L.M. Francisco, B. Sauter, P. Roy, R.L. Silverstein, and N. Bhardwaj. 1998. Immature dendritic cells phagocytose apoptotic cells via $\alpha_v\beta_3$ and CD36, and cross-present antigens to cytotoxic T lymphocytes. *J. Exp. Med.* 188:1359–1368.
 40. Rovere, P., C. Vallinoto, A. Bondanza, M.C. Crosti, M. Rescigno, P. Ricciardi-Castagnoli, C. Rugarli, and A.A. Manfredi. 1998. Bystander apoptosis triggers dendritic cell maturation and antigen-presenting function. *J. Immunol.* 161:4467–4471.
 41. Inaba, K., S. Turley, F. Yamaide, T. Iyoda, K. Mahnke, M. Inaba, M. Pack, M. Subklewe, B. Sauter, D. Sheff, et al. 1998. Efficient presentation of phagocytosed cellular fragments on the major histocompatibility complex class II products by dendritic cells. *J. Exp. Med.* 188:2163–2173.
 42. Kelsall, B.L., and W. Strober. 1996. Distinct populations of dendritic cells are present in the subepithelial dome and T cell regions of the murine Peyer's patch. *J. Exp. Med.* 183:237–247.
 43. Sallusto, F., M. Cella, C. Danieli, and A. Lanzavecchia. 1995. Dendritic cells use macropinocytosis and the mannose receptor to concentrate macromolecules in the major histocompatibility complex class II compartment: downregulation by cytokines and bacterial products. *J. Exp. Med.* 182:389–400.
 44. Winzler, C., P. Rovere, M. Rescigno, F. Granucci, G. Penna, L. Adorini, V.S. Zimmermann, J. Davoust, and P. Ricciardi-Castagnoli. 1997. Maturation stages of mouse dendritic cells in growth factor-dependent long-term cultures. *J. Exp. Med.* 185:317–328.
 45. Fadok, V.A., D.L. Bratton, A. Konowal, P.W. Freed, J.Y. Westcott, and P.M. Henson. 1998. Macrophages that have ingested apoptotic cells in vitro inhibit proinflammatory cytokine production through autocrine/paracrine mechanisms involving TGF- β , PGE2, and PAF. *J. Clin. Invest.* 101:890–898.
 46. Voll, R.E., M. Herrmann, E.A. Roth, C. Stach, J.R. Kalden, and I. Girkontaite. 1997. Immunosuppressive effects of apoptotic cells. *Nature.* 390:350–351.
 47. Mevorach, D., J.L. Zhou, X. Song, and K.B. Elkon. 1998. Systemic exposure to irradiated apoptotic cells induces autoantibody production. *J. Exp. Med.* 188:387–392.
 48. Ronchetti, A., P. Rovere, G. Iezzi, G. Galati, S. Heltai, M.P. Protti, M.P. Garancini, A.A. Manfredi, C. Rugarli, and M. Bellone. 1999. Immunogenicity of apoptotic cells in vivo: role of antigen load, antigen-presenting cells, and cytokines. *J. Immunol.* 163:130–136.
 49. Zychlinsky, A., C. Fitting, J.-M. Cavaillon, and P.J. Sansonetti. 1994. Interleukin 1 is released by murine macrophages during apoptosis induced by *Shigella flexneri*. *J. Clin. Invest.* 94:1328–1332.