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).

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

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
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 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-2b) and C57BL/6 (H-2d) mice were bred in the animal facilities at Lund University or purchased from Charles River Laboratories. Bacterial strains containing the plasmid pJLP-2H-Kan were grown in Luria broth or its rough LPS derivative 14028r (21), the phoP mutants CS022s (22) or its rough LPS derivative CS2022r, made as described (21). S. typhimurium 14028r and CS022s 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 Kb-binding ova epitope (257–264) and the I-Ab-binding ova epitope (265–277). S. typhimurium 14028r and CS022s 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 1:10 into fresh LB and incubating until the optical density at 600 nm (OD600) reached 1.3–1.5. Bacteria were then centrifuged at 8,000 g for 90 min before addition to the assay.

Bone marrow–derived macrophages (Mφ) were prepared by culturing bone marrow from BALB/c (H-2b) or caspase 1Δ/Δ mice (H-2d) in IM DM plus GM-CSF as described (22). The OD600 of cultures was monitored for 8 h, washed twice with HBSS and used in experiments. Alternatively, Mφ were generated by expanding bone marrow from BALB/c or caspase 1Δ/Δ mice in IM DM 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 IM DM plus GM-CSF. Mφ cultures from bone marrow using either IM DM plus GM-CSF or IM DM plus GM-CSF cultures were monitored for 8 h and 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 (Trevigen, Inc.) were for 20 min on ice. Flow cytometry analysis was performed on a Becton Dickinson FACSort™ flow cytometer (Becton Dickinson), and data were collected on 3×105 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 E. 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. Bacteria in 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 to Mφ to apoptotic Mφ ratio. Finally, 5×105 CD80VA (17) or OT4H.2DS (25) T hybridomas, which secrete IL-2 upon specific recognition of the Ova(257–264)/Kb or Ova(265–277)/I-Ab complex, respectively, were added in 250 µl of IMDM. After a 24-h incubation,

614 Salmonella-induced Apoptosis and Antigen Presentation
CCD followed by enrichment of CD11c+ DCs were coincubated with MΦs. Cells were coincubated for 24 h at 37°C, IL-2 secreted by the CD8OVA or OT4H.2D5 T hybridoma cells was quantitated using IL-2–dependent CTLL cells. Instead, bacteria were coincubated with DCs at 37°C, except that the T hybridoma cells were not added together with the bacteria. Thus, we sought to determine whether MΦs induced apoptosis of infected MΦs 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. MΦs were infected with logarithmically growing wild-type S. typhimurium 14028r, which induces apoptosis as described for the other antigen presentation assays.

Figure 1. Logarithmically growing S. typhimurium 14028 induces apoptosis in MΦs, whereas the phoP- mutant CS022 or stationary phase 14028 does not. (a) MΦs 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 × 10^4 gated PI– cells plotted against cell number of MΦ coincubated with 14028s (thick line), CS022s (dotted line), or medium (thin line). (b) MΦs 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.

Results

DCs acquire antigens from MΦs induced to undergo apoptosis by Salmonella infection. Infection of MΦs 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 apoptosis of infected MΦs 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. MΦs were dyed red using PKH26-GL (Sigma Biosciences), and 10^6 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 × 10^5 MACS-purified DCs or MΦs dyed green using PKH67-GL (Sigma Biosciences) were added to the red MΦs. 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.
duces apoptosis in the infected cells, or with logarithmically growing phoP (phoP<sup>c</sup>) strain CS022<sup>r</sup>, which does not induce apoptosis upon infection of M<sub>F</sub> 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<sup>+</sup>PI<sup>+</sup>M<sub>F</sub> after infection with 14028<sup>r</sup> but not with CS022<sup>r</sup> (Fig. 1) resulted in OVA(257–264) presentation on the MHC-I molecule K<sup>b</sup> as well as OVA(265–277) presentation on the MHC-II molecule I-Ab by bystander DCs (Fig. 2, a and d). This presentation was observed when M<sub>F</sub> were infected with logarithmically growing 14028<sup>r</sup> but not with logarithmically growing CS022<sup>r</sup> bacteria, which can and cannot induce apoptosis in infected M<sub>F</sub>, respectively (Fig. 1). In addition, no presentation of the OVA peptides on either MHC-I or MHC-II by bystander DCs was observed when M<sub>F</sub> were infected with stationary 14028<sup>r</sup>, which does not induce apoptosis in the infected cells (Fig. 1 b; references 6, 7). The observed presentation was not due to presentation of the bacteria-derived OVA peptides by infected M<sub>F</sub>. This was ensured by using M<sub>F</sub> from a haplotype of mouse (BALB/c; H-2<sup>d</sup>) different from that recognized by the H-2<sup>b</sup>–specific CD8<sub>OVA</sub> and OT4H.2D5 T cell hybridomas (17, 25). Finally, omitting addition of bystander DCs (H-2<sup>b</sup>) to the infected M<sub>F</sub> (H-2<sup>d</sup>) resulted in a similar background level of stimulation of the T cell hybridomas as when DCs (H-2<sup>b</sup>) or M<sub>F</sub> 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, is due to uptake of apoptotic material and not to phagocytic processing of remaining bacteria by the added DCs. First, addition of stationary phase 14028<sup>r</sup>, which does not induce apoptosis in infected M<sub>F</sub>, did not result in stimulation of the OVA(257–264)/K<sup>b</sup>– or the OVA(265–277)/I-Ab–specific T cell hybridomas (Fig. 2, a and d). This was the case despite 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 M<sub>F</sub>. This was ensured by using M<sub>F</sub> from a haplotype of mouse (BALB/c; H-2<sup>d</sup>) different from that recognized by the H-2<sup>b</sup>–specific OVA(257–264) and OT4H.2D5 T cell hybridomas (17, 25). Finally, omitting addition of bystander DCs (H-2<sup>d</sup>) to the infected M<sub>F</sub> (H-2<sup>b</sup>) resulted in a similar background level of stimulation of the T cell hybridomas as when DCs (H-2<sup>d</sup>) or M<sub>F</sub> (H-2<sup>b</sup>) were grown in medium only (Fig. 2, c and f, and see Fig. 5, a–c).

**Figure 2.** Viable bystander DCs acquire antigenic material from M<sub>F</sub> induced to undergo apoptosis by infection with wild-type S. typhimurium. (a and c) OVA peptide presentation on MHC-I (K<sup>b</sup>) and (d and f) MHC-II (I-Ab) by bystander DCs quantitated using the OVA(257–264)/K<sup>b</sup>– or the OVA(265–277)/I-Ab–specific T cell hybridomas CD8<sub>OVA</sub> or OT4H.2D5, respectively. M<sub>F</sub> (H-2<sup>b</sup>) were coincubated for 90 min with either stationary phase (stat) 14028<sup>r</sup>, logarithmically growing (log) 14028<sup>r</sup>, or logarithmically growing CS022<sup>r</sup> expressing Crl-OVA as indicated. After washing and addition of gentamycin, live (+ DC) or paraformaldehyde-fixed (+ fixed DC) (H-2<sup>b</sup>) or no DCs (− DC) and T hybridoma cells were added as indicated. (b and e) The CD8<sub>OVA</sub> (b) or OT4H.2D5 (e) response to live or prefixed DCs (H-2<sup>b</sup>) loaded with 1 nM OVA(257–264) or 100 μM OVA(265–280) peptide, respectively. Counts per minute in wells where bystander DCs were omitted or where DCs or M<sub>F</sub> were incubated in medium only along with the appropriate T cell hybridomas were typically 250–2,500. The results are representative of three to five independent experiments.

616  Salmonella-induced Apoptosis and Antigen Presentation
ing Crl-OVA is efficiently presented when phagocytosed by DCs (18, 19). In addition, similar amounts of the bacteria were internalized by the MΦ. This was confirmed by coincubating MΦ 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 100 μg/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)/Kb (a, c, and e) and OVA(265-277)/I-Ab (b, d, and f) were quantitated using CD8OVA and OT-4H.2D5 T hybridoma cells, respectively. The results are representative of three independent experiments.

Figure 3. DCs process residual 14028 and CS022 expressing Crl-OVA for OVA peptide presentation on MHC-I and MHC-II equally well. Logarithmically growing (OD_600 = 1.3–1.5) 14028r Crl-OVA or CS022r Crl-OVA bacteria were treated with 100 μg/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)/Kb (a, c, and e) and OVA(265-277)/I-Ab (b, d, and f) were quantitated using CD8OVA and OT-4H.2D5 T hybridoma cells, respectively. The results are representative of three independent experiments.
after the apoptosis induction. (b) OVA(257–264)/Kb and OVA(265–277)/I-Ab presentation on MHC-I and MHC-II by bystander DCs (H-2b) after co-incubation with Mφ indicated. OVA(257–264)/Kb and OVA(265–277)/I-Ab presentation was quantitated using CD8OVA and OT4H.2D5 T hybridoma cells, respectively.

Material from Mφ presentation observed in Fig. 2, a and d, is due to uptake of...CD8OVA, which does not induce apoptosis in in-fected cells (Fig. 1 a), was processed for OVA peptide pre-
sessing Crl-OVA, which does not induce apoptosis in in-
fecttos CS022r ex-
growing 14028r is cytotoxic to these cells (our unpublished
data). 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.

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 × 10⁶ Mφ that have phago-cytosed logarithmically growing (log) CS022r Crl-OVA (dotted line) or CS022r Crl-OVA and were subse-
quently treated with LPS and ATP (thick line) or that were incubated in medium alone (thin line). Flow cy-

tometry analysis was carried out 20 h...
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-2d) 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-2d) 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-Ab 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 a apoptotic MΦ 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-2d) (a) or MΦ (H-2b) (b) after coincubation with MΦ (H-2b) 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 CD80 VA T hybridoma cells. The response of MΦ (H-2b) and bystander cells incubated in medium only is also shown. (c) MΦ (H-2b) 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-I by the DCs was quantitated by addition of OT4H.2D5 T hybridoma cells to the MACS-purified DCs. The response of MΦ (H-2b) 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.
that can or cannot induce apoptosis in infected Mφs. This was demonstrated here using bacterial strains for presentation of bacteria-derived material by bystander DCs. Apoptosis of infected cells results in presentation of a bacterial antigenic material.

We next investigated if the lack of presentation of antigens from apoptotic material on MHC-I by bystander Mφs was due to an inability of Mφs to engulf apoptotic material. To test this, Mφs were dyed red and were coincubated with logarithmically growing 14028r or CS022r expressing Crl-OVA. These red Mφs were then coincubated with Mφs 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 Mφs and DCs efficiently internalized apoptotic material from Mφs that were coincubated with 14028r. In contrast, significantly less uptake of apoptotic material was apparent when the red Mφs were coincubated with CS022r or when they were incubated in medium alone before coincubation with green bystander cells (Fig. 5d). Furthermore, the uptake of apoptotic material was inhibited when 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 Mφ that had previously phagocytosed 14028r expressing Crl-OVA (Fig. 5c). Finally, the uptake of apoptotic material by bystander DCs after coincubation with bacterially induced apoptotic Mφs 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 studies of 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 bacterial 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 Mφs, and by chemically inducing apoptosis in Mφs 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 Mφs occurred when live DCs but not Mφs 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 Mφs 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 Mφs were added to bystander DCs coincubated with bacteria-induced apoptotic Mφs, 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 Mφs was due to a soluble factor secreted by the macrophages or a result of competition for the antigenic material between the DCs and the Mφ remains to be determined. Interestingly, a similar ability of bystander DCs but not Mφs to present antigenic material from monocytoid 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 Mφs, 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). Mφs, 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). Mφs also have extensive antigen degradative capacity, which is important in their role in innate protection against microbes. This latter property of Mφs 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 Mφs and DCs intermingled with CD4+ T cells and B cells from the underlying follicle (4). The potential interaction of S. typhimurium with Mφs and DCs in the follicle dome after oral infection, combined with the bacteria's ability to survive inside phagocytic cells (32) and

620 Salmonella-induced Apoptosis and Antigen Presentation
its ability to induce apoptosis in infected MΦ (Fig. 1; references 6, 7), raises the question of the significance of bacteria-induced apoptosis of infected MΦ 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 DCs have been identified in the liver of mice infected with S. typhimurium (35), apoptotic MΦ 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 MΦ 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 MΦ 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 phagocyted apoptotic cells, presumably apoptotic MΦ. Orogenic 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 yop mutant (33). Furthermore, a yop 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 yop locus is also required for Yersinia-mediated inhibition of IL-1b 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 MΦ 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, MΦ 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 MΦ. In the case of uptake by DCs, these cells can then present bacterial antigens on MHC-I and MHC-II. LPS, TNF-α, IL-1b (43, 44), and uptake of apoptotic material (40) have all been shown to induce DC maturation. This in turn enhances 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 naïve 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-1b, 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 MΦ pulsed with apoptotic material prime a specific antitumor CTL response despite the fact that MΦ engulf apoptotic cells in vivo (48). MΦ and DCs have been suggested to use different integrin receptors, α5β1 and α5β2, 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 MΦ here and elsewhere. The cytokine production profile of MΦ and DCs after uptake of apoptotic material has also been shown to differ. For example, MΦ synthesize TGF-β (45) and downregulate production of the proinflammatory cytokines TNF-α, IL-1b, and IL-12 (46), whereas DCs increase secretion of IL-10, TNF-α, and IL-1b (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 MΦ have different functions in the clearance of apoptotic material. Interestingly, S. flexneri apoptosis of infected MΦ also results in release of IL-1b in its biologically active form (49). Although IL-1b production by MΦ 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 MΦ. 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. The authors acknowledge Dr. Anders Häkanson (Lund University) for assistance with analysis of high and low molecular weight fragments in apoptotic cells, Dr. Manuela Baccarini and Veronika Jesenberger (Vienna Biocenter) for providing caspase 1+/− mice, and Dr. Judith A. Kapp (Emory University School of Medicine, Atlanta, GA) for providing the OT4.2D5 T hybridoma cells. This work was supported by the Swedish Natural Sciences Research Council (project 10610-306), Lund University Medical Fac-
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