Necrosis-like cell death induced by bacteria in mouse macrophages

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The death of individual cells is a frequent and physiological event in the mammalian immune system and most often occurs by apoptosis. It is becoming increasingly clear that cell death is also induced during bacterial infections. Here we report that, in addition to the apoptotic form already established, a necrosis-like form of cell death is induced by pyogenic bacteria (Enterobacteriaceae, *Pseudomonas*, enterococci) in mouse macrophages. Necrosis could be separated from apoptosis as it did not require phagocytosis of bacteria and occurred when apoptosis was inhibited by caspase blockade or by Bcl-2. Furthermore, ligands that stimulate Toll-like receptors were also found to have the capacity to induce necrosis. Strikingly, this form of cell death was sufficient for the uptake of dead cells by either mouse bone marrow-derived DC or a cell line derived from DC, possibly by virtue of the externalization of phosphatidylserine. Since the loading with bacteria-carrying cells is likely to impact on DC function, this form of necrosis may have a previously unsuspected role in the development of an immune response.

Key words: Apoptosis / Macrophages / Bacterial / Phagocytosis

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1 Introduction

Cell death is a frequent and physiological event in mammalian organisms. Cell death *in vivo* probably in most cases occurs by apoptosis, *i.e.* as the result of the activation of a specialized intracellular signal transduction pathway. Apoptosis can be triggered by a great variety of stimuli, as extensively studied *in vitro*. Such stimuli, although very different in nature, all lead to the activation of the same central effector system and to similar morphology. In an intact organism, dead cells are rapidly cleared through uptake and intracellular digestion by phagocytes, probably largely macrophages or DC [1].

Apoptotic cell death is implemented by proteases of the caspase family. A great number of details are known about caspase physiology [2]. Notably, during apoptosis cytochrome c is released from cellular mitochondria and triggers caspase activation [3]. Cytochrome c release itself is regulated by the concerted action of apoptosis-inducing and –inhibiting members of the Bcl-2 family of cellular proteins [4].

Separate from apoptosis (although in some cases overlapping), a distinct process of cell death has been recognized; here, we will refer to this process as 'necrosis'. Necrosis can be defined as cell death in the absence of apoptosis, and recent work indicates that cells actively contribute to the induction of necrosis [5]. However, no specialized (or even uniform) pathway to necrosis has been characterized. One form of necrosis occurs when cells are exposed to gross physical injury (for instance freeze-thawing). Necrosis has further been described in situations where apoptosis was induced but at the same time inhibited (usually by chemical caspase inhibition). A growing number of reports further suggest that stimuli that have been well characterized as inducers of apoptosis have also the capacity to induce necrosis. This has been most clearly demonstrated for signaling through death receptors like TNFR1 [6] and CD95 [7].

The occurrence of cell death in the encounter with bacteria has been noted in many settings. Well-investigated examples are especially the cell death induction by vari-

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The first two authors contributed equally to this work.

Abbreviations: TLR: Toll-like receptor **BMDM:** Bone marrow-derived macrophage **BMDDC:** Bone marrow-derived DC **PI:** Propidium iodide **PS:** Phosphatidylserine **zVAD-fmk:** Benzyloxymethyl-Val-Ala-Asp-fluoromethylketone ous virulent bacteria: *Shigella* can cause cell death in infected macrophages by secreting bacterial factors into the cytosol [8], *Yersinia* induces rapid apoptosis in macrophages which requires secreted virulence factors [9], and *Legionella* triggers apoptosis in myeloid cell lines [10]. While these bacteria have a number of recognized virulence factors and a high potential to cause infections in humans, there is evidence that apoptosis can also occur when cells of the innate immune system encounter pyogenic bacteria (*i.e.* bacteria such as *E. coli,* staphylococci and streptococci that are often found as commensals and that can cause opportunistic infections). In this context, it has been shown that apoptosis can be triggered by bacterial products such as LPS [11, 12] and bacterial lipopeptides [13], at least predominantly through toll-like receptors (TLR) [13, 14]. Furthermore, we have recently reported that phagocytosis of *E. coli* bacteria can be a strong stimulus for macrophages to undergo apoptosis. Upon phagocytosis and following intracellular digestion of the bacteria, macrophages activate their apoptotic pathway as evident by a number of biochemical parameters and the typical apoptotic nuclear morphology [15].

In our further investigations of the molecular events during this form of apoptosis, we noticed that necrosis is also induced when macrophages encounter pyogenic bacteria. Necrosis appeared to be a process separate from apoptosis as it occurred under conditions when apoptosis was blocked. Not only intact bacteria but also bacterial components were able to induce necrosis in macrophages, probably through TLR. Since necrotic cells were taken up avidly by DC, this process likely has implications for the development of an immune response.

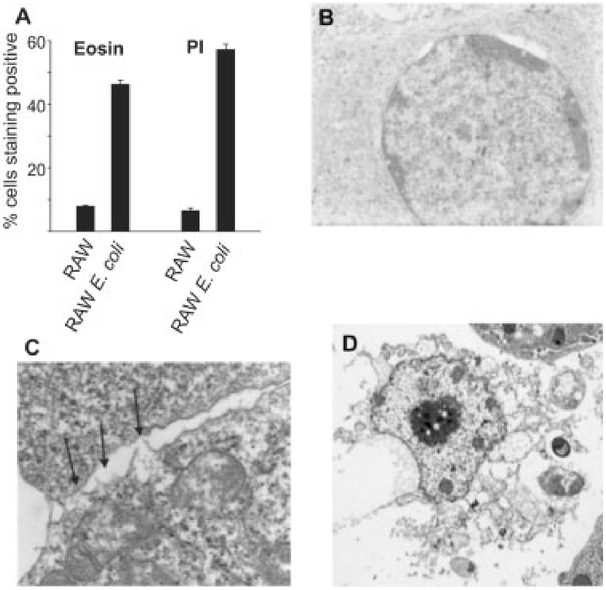


Fig. 1. Loss of cell membrane integrity in RAW264.7 cells upon incubation with bacteria. (A) RAW264.7 cells (5×10^5) were co-incubated with a suspension of *E. coli* for 1 h in 5-ml tubes with 2.5 ml medium. Then cells were washed once with PBS and incubated for 6 h under normal culture conditions in 12-well culture plates. Cells were analyzed for plasma membrane integrity either by staining with eosin and visual microscopic scoring or by staining with PI and flow cytometry. Values are means ± SD of three wells. (B–D) Morphology of cells as seen by electron microscopy. RAW264.7 cells were either left untreated (B) or treated as above (C, D). After 8 h, cells were processed for electron microscopy. (C) Disintegration of the plasma membrane seen in one of two adjacent cells (arrows). (D) Nearly complete disintegration of a RAW264.7 cell with release of cytoplasma and organelles.

2 Results

2.1 Contact with bacteria induces necrosis in mouse macrophages

Upon uptake and digestion of bacteria, macrophages die by apoptosis. While studying the effect of inhibitors of apoptosis on this process we noticed that, although apoptosis could be profoundly inhibited, cell death still occurred. We will here refer to this cell death as necrosis and define necrosis as cell death that (i) occurs in the absence of apoptotic changes and (ii) cannot be inhibited by either Bcl-2 or by inhibition of caspases. Cell death again will be defined as the loss of plasma membrane integrity as measured by the uptake of vital dyes. In RAW264.7 cells, phagocytosis of *E. coli* bacteria induces apoptosis at time points later than 8 h after uptake. However, already 6 h after incubation with bacteria a high percentage of RAW264.7 cells had lost plasma membrane integrity, indicating that cell death in the absence of apoptosis had occurred (Fig. 1A). Electron microscopy showed that cell death was accompanied by disintegration of the cells, resulting in the release of cytoplasmic contents and organelles without the appearance of apoptotic changes such as nuclear condensation (Fig. 1B–D).

2.2 Necrosis occurs independently of apoptosis

Similar experiments were performed under conditions when apoptosis was blocked. Two ways of blocking the apoptosis pathway were employed: caspase inhibition and expression of the apoptosis-inhibiting protein Bcl-2. The broad-spectrum caspase inhibitor benzyloxymethyl-Val-Ala-Asp-fluoromethylketone (zVAD-fmk) targets

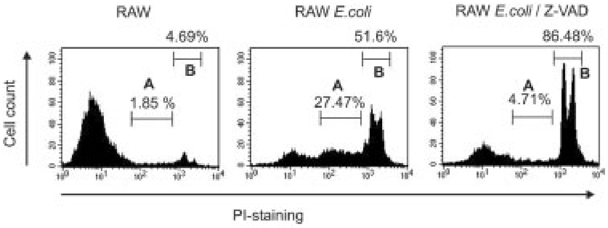


Fig. 2. Nuclear apoptosis but not loss of plasma membrane integrity is blocked by caspase inhibition. RAW264.7 cells were exposed to a suspension of *E. coli* for 1 h as described in Fig. 1. Bacteria were then removed by washing and cells were cultured in 12-well plates in the presence or absence of the caspase inhibitor zVAD-fmk (25 μM). After 22 hours, cells were collected, stained with PI and analyzed by flow cytometry (without fixation). Intact cells stain negative for PI (left peak; this peak may also contain early apoptotic cells that have not yet lost their membrane integrity). (Late) Apoptotic cells stain with intermediate integrity ('sub-G1') (A). Cells that have lost their plasma membrane intensity but are not apoptotic ('necrotic cells') appear in the double peak to the right (B).

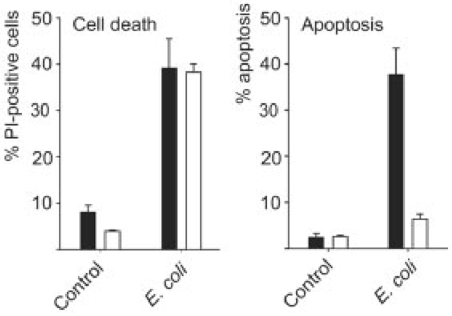


Fig. 3. Cell death but not apoptosis proceeds normally in primary macrophages overexpressing Bcl-2. BMDM from C57BL/6 wild-type or C57BL/6 vav-Bcl-2-transgenic mice were seeded in 12-well plates (2×10^5 cells/well). After overnight culture, cells were incubated with *E. coli* bacteria for 1 h and washed. Culture was then continued under normal conditions. Left panel: Cell death was quantified 6 h later by PI staining of unfixed cells and flow cytometry. Right panel: Apoptosis was measured after 24 h by Hoechst staining and visual scoring of nuclear morphology. Data are presented as means ± SD of duplicate wells. Filled bars, wild-type mice; open bars, vav-Bcl-2-transgenic mice. Data are representative of a total of two vav-Bcl-2-transgenic mice and three wild-type mice.

active caspases and thereby blocks apoptosis. To prevent mitochondrial apoptotic changes, we further used cells that overexpress Bcl-2. As expected [15], zVAD-fmk potently blocked the appearance of apoptosis as assessed by nuclear morphology and 'sub-G1 staining' by flow cytometry (Fig. 2). The assay for 'sub-G1 staining' takes advantage of the finding that apoptotic cells take up less propidium iodide (PI) than non-apoptotic cells and therefore appear to the left of the non-apoptotic cells when assayed by flow cytometry [16]. In a modification of this test (which usually employs fixation to permeabilize the cells) we added PI to the cells without prior fixation. This allowed us to distinguish, within the population of dead (PI-positive) cells, between cells with low PI-uptake (apoptosis) and cells that had lost their plasma membrane integrity but were taking up normal amounts of PI. As shown in Fig. 2, zVAD-fmk almost completely blocked the appearance of apoptotic cells, but the percentage of cells taking up PI was not substantially altered, suggesting that the process of cell death was independent of caspase activity.

The effect of Bcl-2 expression was investigated in both primary mouse bone marrow-derived macrophages (BMDM) and RAW264.7 cells. Although Bcl-2 effectively blocked phagocytosis-induced apoptosis in RAW264.7 cells, loss of membrane integrity proceeded normally (data not shown). Similarly, in BMDM from transgenic mice expressing high levels of human Bcl-2 [17], the percentage of cells that had taken up PI was nearly identical to wild type controls, although the percentage of apoptotic cells was strongly reduced (Fig. 3). Thus, a process of necrosis could be separated from apoptosis. Most experiments were done using either RAW264.7 macrophages or BMDM. To investigate the broader applicability, we also tested granulocytes that had been freshly isolated from mouse bone marrow by cell sorting as described [18]. These cells also underwent accelerated apoptosis upon phagocytosis of bacteria, and this process was inhibited in granulocytes from Bcl-2-transgenic animals. However, cell death still occurred in Bcl-2-expressing granulocytes (unpublished observations). These data indicate that the basic principles of apoptosis and necrosis induction upon bacterial exposure are the same in mouse macrophages and granulocytes.

The original experiments were done using the laboratory strain *E. coli* DH5α. A number of clinical isolates of other gram-negative bacteria and the gram-positive organism *Enterococcus faecalis* were also tested. All species and isolates tested induced necrosis (measured as eosin uptake after 6 h, Fig. 4A). All isolates tested also induced apoptosis after 20 h (data not shown). Titration experiments were further performed with *E. coli* DH5α bacteria. These experiments showed that a bacteria: macrophage ratio of about 2–10:1 was sufficient for necrosis induction (Fig. 4B). Heat treatment failed to destroy the necrosis-inducing potential of the bacteria.

The mechanisms leading to necrosis are not well defined, and necrosis may be caused by a variety of

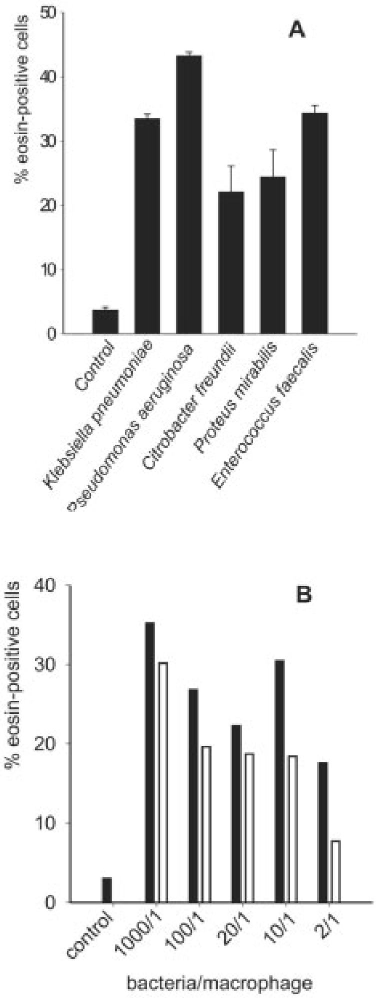


Fig. 4. Induction of necrosis by pyogenic bacteria in RAW264.7 cells. (A) Clinical isolates of a number of bacterial species induce necrosis. RAW264.7 cells were incubated with suspensions of the indicated bacterial species (gram-negative bacteria and the gram-positive bacterium *E. faecalis*). After 1 h bacteria were washed away, and cells were incubated for a further 6 h. Cell death was quantified by eosin staining of unfixed cells and visual analysis by light microscopy. Data are presented as means ± SD of two replicates. (B) RAW264.7 cells were either left untreated or were incubated for 6 h with live or heat-inactivated (65°C for 30 min) *E. coli* bacteria (DH5α) at various bacteria/macrophage ratios (dilutions from 1000 bacteria/cell to 2 bacteria/cell). Cell death was quantified by eosin staining and light microscopy. Filled bars, normal bacteria; open bars, heat-inactivated bacteria. Control, untreated RAW264.7 cells.

(undefined) intracellular disturbances. A number of agents have been described to be capable of protecting against necrosis. Such substances are butylated hydroxyanisole (BHA) and pyrrolidine dithiocarbamate (PDTC). BHA has been found to protect against TNF-induced cytotoxicity [19] and against CD95-induced necrotic death (in the presence of caspase inhibitors [6]). PDTC inhibited caspase-independent cell death in CD95 signaling [20]. Similarly, glycine which can act as an ion channel blocker inhibited necrosis induced by hypoxia or ATP depletion [21, 22] and has also been found effective in blocking *Salmonella*-induced caspase-1-dependent necrosis [23]. We tested the above-mentioned agents, but none of them gave an appreciable protection against bacteria-induced necrosis (data not shown).

2.3 TLR ligands induce necrosis in RAW264.7 cells, and necrosis occurs *in vivo*

We next sought to determine the active components for necrosis induction by bacteria. As established earlier, heat-treated *E. coli* bacteria are taken up poorly by RAW264.7 macrophages and fail to induce apoptosis [15]. However, as shown in Fig. 5A and B, they retain the capacity to induce necrosis in RAW264.7 cells. Uptake of the bacteria appears therefore not required for the induction of necrosis.

To investigate whether necrosis induction was only an *in vitro* phenomenon or also occurred *in vivo*, mice were injected with heat-killed bacteria. After 24 h peritoneal exsudate cells were collected and assayed for necrosis and apoptosis. As shown in Fig. 5C, very little apoptosis was seen in the wash-out cells (probably largely granulocytes by morphology and as judged from [24]) but a substantial proportion was necrotic. As dead cells are cleared rapidly *in vivo*, we believe that the 15–20% of necrosis induced under these circumstances is significant.

A large part of signaling into macrophages upon contact with bacteria is conveyed by TLR. We therefore tested whether TLR ligands were able to induce necrosis in RAW264.7 macrophages. The lipopeptide Pam$_3$Cys (a TLR2 ligand), LPS (a TLR4 ligand) and CpG oligonucleotides (CpG-ODN, a TLR9 ligand) were used to stimulate RAW264.7 cells. In a series of experiments, cell death induction by CpG was only marginal or not detectable while Pam$_3$Cys weakly but reproducibly and LPS most potently induced cell death. The combination of the three TLR ligands showed a greater effect than each ligand by itself (Fig. 6A). We found very little induction of apoptosis in RAW264.7 cells under these circumstances (Fig. 6B). This again suggests that the two processes, apoptosis and necrosis, are the result of separate signaling events. To investigate the initial necrotic signaling through TLR, BMDM from mice deficient for the adapter molecule MyD88 were investigated. These cells still underwent necrosis in a way very similar to wild-type macrophages suggesting that MyD88 is at least not indispensable for

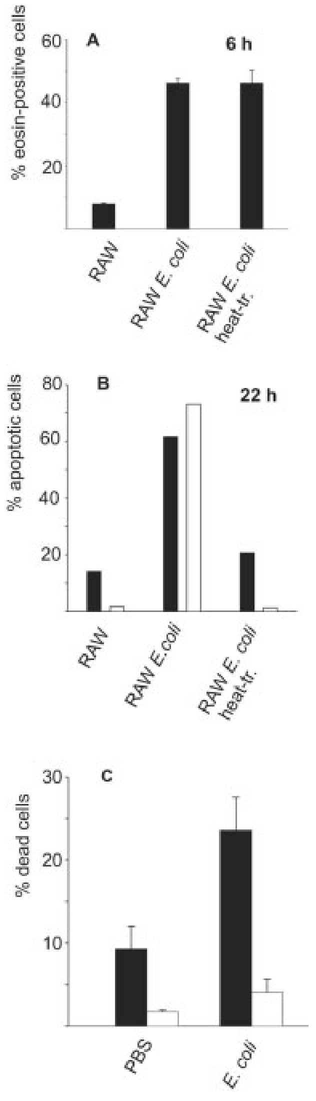


Fig. 5. Heat-treated bacteria induce necrosis but not apoptosis in RAW264.7 macrophages and induce necrosis *in vivo*. (A) RAW264.7 macrophages were seeded into 12-well plates and exposed to a suspension of either untreated or heat-inactivated (at 65°C for 30 min) *E. coli* bacteria for 1 h and cultured under normal conditions. After 6 h, aliquots were stained with eosin and necrosis was assessed visually as eosin uptake under the microscope. Values are means ± SEM of two wells. (B) After 22 h of culture, cells were stained with Hoechst dye and analyzed for apoptosis by assessing nuclear morphology. Filled and open bars represent separate experiments. Data shown are representative of at least three independent experiments. (C) C57BL/6 mice (three per group) were injected with either PBS or a suspension of 10^8 heat-treated *E. coli* bacteria (DH5α). After 12 h, peritoneal wash-out cells were collected and analyzed for apoptosis by Hoechst staining (white bars) and necrosis by assessing eosin exclusion (black bars). Data are shown as means ± SD of three individual mice.

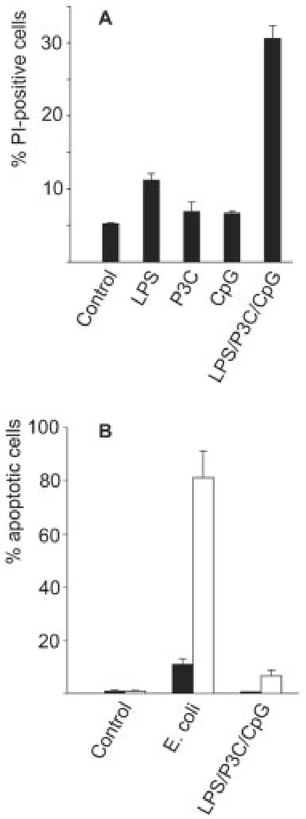
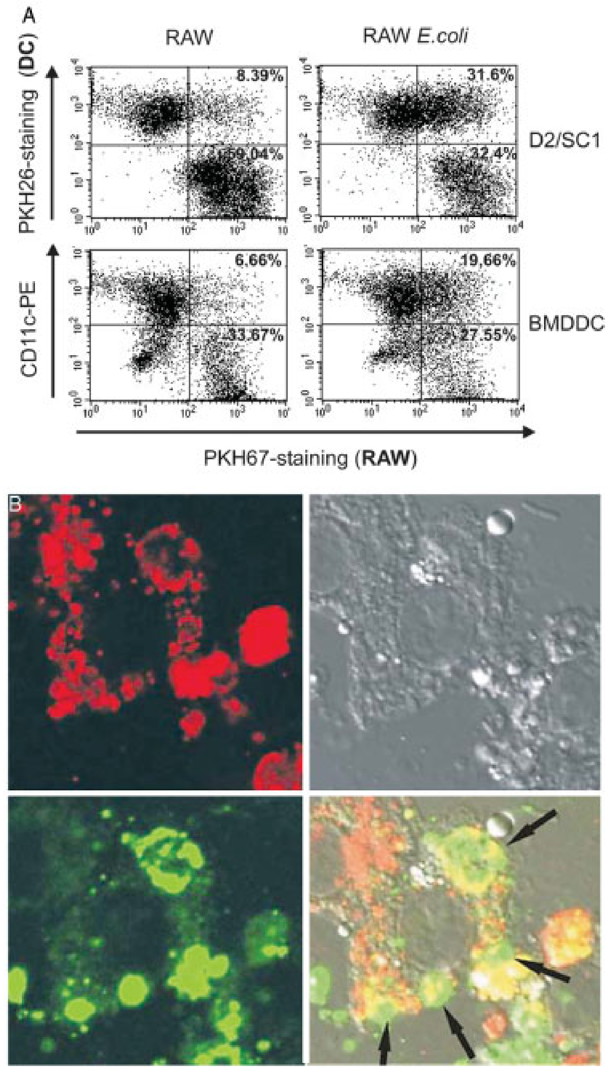


Fig. 6. Induction of cell death in RAW264.7 cells by TLR ligands. RAW264.7 cells were seeded at 2×10^5 cells/well in 12-well plates overnight. The next day, cells were treated with the TLR ligands LPS (1 μg/ml), Pam$_3$Cys (P3C; 1 μg/ml) or CpG-DNA (CpG; 1 μM) alone or in combination or stimulated with *E. coli* bacteria (B). (A) Cell death was quantified after 6 h by measuring PI uptake. (B) Apoptosis was quantified after 9 h (filled bars) and after 24 h (open bars) by Hoechst staining and assessment of nuclear morphology. Data are means ± SD of duplicate wells.

the induction of necrosis. It has recently been shown that LPS-dependent apoptosis requires the adapter molecule TRIF [25]. TRIF may thus also be an important mediator of necrosis signaling.

A possibility was that necrosis induction was mediated in an auto/paracrine fashion by TNF secreted upon stimulation by TLR ligands. We tested this by first measuring the levels of TNF induced under circumstances of stimulation when necrosis occurred or not, *i.e.* during stimulation with either intact or heat-killed bacteria or with LPS, Pam$_3$Cys, CpG DNA (or the combination of these ligands). Bacteria and all individual ligands and their combination caused secretion of similar amounts of TNF (although necrosis induction was different, see above; the highest level of TNF detected was about 6 ng/ml after 6 h, *i.e.* when necrosis starts to occur). This makes



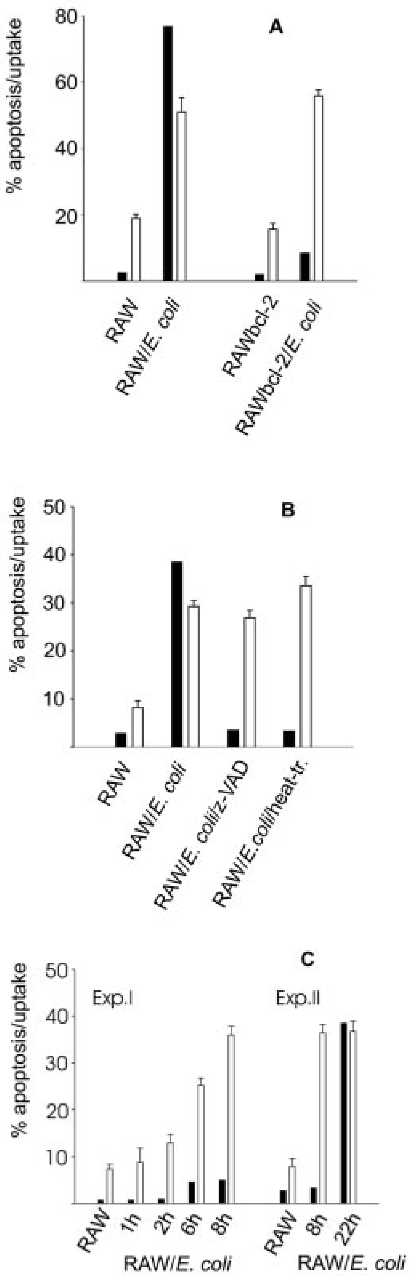
◀ *Fig. 7.* Uptake of RAW264.7 cells by DC upon contact with *E. coli* bacteria. (A) RAW264.7 cells were stained green (PKH67) and either left untreated or treated with *E. coli* bacteria (5×10^5 cells/well in a 12-well plate). Top panel: After 22 h, RAW264.7 cells were added to the culture of DC from the red-stained (PKH26) cell line D2SC/1 (2.5×10^5 cells/well of a 12-well plate), and cultures were incubated for 2 h. Cells were then fixed and analyzed by flow cytometry. Bottom panel: After 22 h, RAW264.7 cells were added to 2.5×10^5 BMDDC cultured in a 12-well plate. After 2 h co-incubation, cultures were stained with anti-CD11c-PE to identify BMDDC, fixed and analyzed by flow cytometry. (B) Confocal microscopy. RAW264.7 cells stained with the green fluorescence dye PKH67 were co-incubated with *E. coli* bacteria as described above for 1 h. Cells were then seeded onto coverslips placed into 12-well plates and incubated under normal culture conditions (5×10^5 cells/well). After 24 h, red-stained (PKH26) D2SC/1 cells (2.5×10^5 cells/well) were added to the RAW264.7 macrophages into the 12-well plate. After 2 h of co-incubation, cells were fixed and photographed under a confocal microscope. Black arrows indicate green material from dead green RAW264.7 cells inside a DC.

it unlikely that TNF is a critical mediator of necrosis in this setting. Furthermore, when murine TNF (6 ng/ml) was added directly to cultures of RAW264.7 cells, neither necrosis nor apoptosis was induced over a period of 6 h (9.33±0.63% *vs.* 9.53±0.82% dead cells [PI-positive cells, mean ± SD of two wells] in control *vs.* TNF-treated cells; apoptosis was under 2% in both cases). TNF therefore does not appear to be centrally involved.

2.4 Bacteria-induced necrosis is sufficient for uptake by DC

It is thought that cell death *in vivo* leads to the rapid uptake of the dead cells by phagocytes such as macrophages and DC. *In vitro* uptake of dead cells has also been demonstrated numerous times, and it appears that apoptotic and necrotic cells are both taken up efficiently by phagocytes [26]. However, in these studies, necrosis was almost invariably induced by freeze-thawing of a suspension of cells. We thought it therefore important to test whether the form of necrosis we observed here is sufficient for uptake of the dead cells by DC. RAW264.7 macrophages were subjected to conditions under which bacteria induce either apoptosis or necrosis, and uptake by DC was studied upon co-incubation of the cells. A mouse DC cell line, D2SC/1, as well as bone marrow-derived DC (BMDDC) were used for these studies.

Cells were stained with two different fluorescent dyes and uptake of cells was investigated by flow cytometry. When untreated RAW264.7 cells were co-incubated with DC for 2 h, few double-positive cells were observed. However, when RAW264.7 cells had been exposed to *E. coli* bacteria for 20 h (the apoptosis-inducing protocol), a high number of cells became double positive indicative of efficient uptake of apoptotic RAW264.7 cells by DC (Fig. 7A). Laser-scanning microscopy showed particles of presumably apoptotic RAW264.7 cells inside DC (Fig. 7B). RAW264.7-Bcl-2 cells or RAW264.7 cells treated in the presence of zVAD-fmk to inhibit apoptosis were now used. Then RAW264.7 cells were incubated with heat-treated *E. coli* bacteria. Under all of these conditions very little apoptosis occurred (Fig. 8A, B, and above). However, RAW264.7 cells were still taken up efficiently by DC, indicating that bacteria-induced necrosis was sufficient for uptake (Fig. 8). Time course studies further showed that, for uptake, stimulation of RAW264.7 cells with bacteria for about 6–8 h is



◀ *Fig. 8.* Necrosis is sufficient for uptake of RAW264.7 cells by DC. (A) Bcl-2 prevents bacteria-induced apoptosis but not uptake by DC. RAW264.7 cells or RAW264.7 cells stably expressing Bcl-2 were incubated with *E. coli* bacteria for 1 h, washed and seeded into 12-well culture plates (5×10^5 cells/well). After 22 h, apoptosis was assessed as nuclear morphology in one aliquot (filled bars). For uptake experiments, RAW264.7 cells were stained with the fluorescent dye PKH67 before exposure to bacteria. After 22 h, RAW264.7 cells were collected and added to a culture of DC (D2SC/1 cells, 2.5×10^5 cells/well of a 12-well plate) stained with PKH26. Another 2 h later, cells were fixed and uptake was assessed by flow cytometry (open bars). Results are given as percentage of RAW264.7 cell uptake calculated as percentage of PKH67/PKH26 double-positive cells of all PKH67-positive (*i.e.* RAW264.7) cells (means ± SD of three individual wells). (B) Necrosis induced in the presence of caspase inhibition or by heat-treated bacteria is sufficient for uptake. RAW264.7 cells were stained with the dye PKH67 and treated with normal or heat-treated bacteria as indicated (as above). To some wells, the caspase inhibitor zVAD-fmk was added following exposure to bacteria. After 22 h, apoptosis was assessed by scoring nuclear morphology. For uptake, RAW264.7 cells were added to DC for 2 h, and cultures were analyzed as under (A). Filled bars, apoptosis; open bars, uptake. (C) Time course of apoptosis and uptake. RAW264.7 cells (5×10^5 cells/well) stained with PKH67 were incubated with *E. coli* bacteria for 1 h, washed and cultured in 12-well plates. At the indicated time points, cells were collected, stained with Hoechst dye and assessed for apoptosis as above (filled bars). At the same time points, RAW264.7 cells were harvested and added to a culture of D2SC/1 cells stained with PKH26, and culture was continued for 2 h. Cells were then harvested, fixed, and uptake of RAW264.7 cells by DC was analyzed by flow cytometry (open bars). Data are taken from two separate experiments. Values are means ± SEM of three individual wells.

sufficient (Fig. 8C). Exposure to bacteria for longer times did not greatly increase the number of cells taken up.

For the uptake of apoptotic cells, the exposure of phosphatidylserine (PS) on the outer plasma membrane leaflet appears to be an important step; this exposure can be measured by testing the binding of annexin V to the cell surface. We investigated whether necrotic cells would also bind annexin V and found that a proportion of RAW264.7 cells exposed to bacteria for 6 h did indeed stain positive for annexin V (Fig. 9). Exposure of PS may thus be a signal for uptake by DC, not only in apoptotic but also in necrotic cells.

3 Discussion

The interaction between phagocytes and pyogenic bacteria is a central event in the defense against these pathogens. Such contact between bacterial organisms and macrophages has the potential to induce necrosis, *i.e.* cell death in the absence of apoptosis, in the macrophage. TLR ligands were also shown to induce necrosis. Together with the finding that this form of bacteria-induced necrosis is sufficient for consecutive uptake by DC, these data show a chain of events that may impact on the development of an immune response.

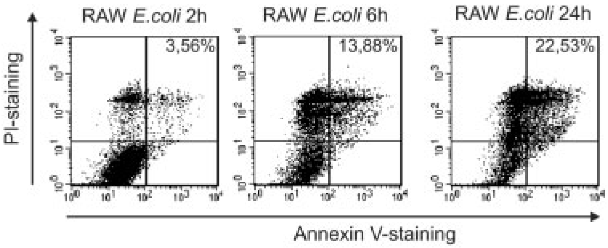


Fig. 9. Binding of annexin V to necrotic cells. RAW264.7 cells were treated with bacteria as described in Sect. 4.4. At the indicated time points after incubation, cells were collected, stained with annexin V-FITC and PI and analyzed by flow cytometry. Percentages of cells staining positive for annexin V are given.

Phagocytosis is a strong stimulus to macrophages to undergo apoptosis [15]. The observation that necrosis is also induced has probably the greatest significance in the finding that necrosis, which occurs earlier, is sufficient for uptake of the dead cells by DC. Necrosis induction is a relatively rapid process and starts after 4–6 h; the intracellular killing of bacteria, however, is more rapid and appears to be concluded at about 2–4 h [15]. Necrosis would therefore probably not interfere with macrophage function.

It is still unclear how, on a molecular level, cell death by necrosis is caused and implemented. The initial steps of death receptor-induced necrosis have been found to involve signaling though the kinase RIP [7]. TNF signaling did not seem to be involved in bacteria/TLR-induced necrosis (see above), and CD95 is an efficient inducer of necrosis only when caspases are inhibited [6, 27], making its involvement here very unlikely. It is worth noting that signaling of death receptors and signaling through TLR share some downstream events, in particular the induction of NF-κB activity and the potential to induce apoptosis. That both groups of receptors can also induce necrosis may therefore be seen as an additional common feature. The RIP-related kinase RIP2 has a role in TLR2/3/4-dependent activation of NF-κB [28]. It is therefore conceivable that RIP2 has a similar function in TLR-dependent necrosis as RIP has in necrosis triggered by death receptors.

The events that eventually effect necrosis are not understood. Some further details are known in a case of necrosis in the nematode *Caenorhabditis elegans* where a mutation in an ion channel results in an elevated cytosolic calcium concentration which in turn causes cell death by necrosis [29] with the participation of calpain-like proteases [30]. TLR ligation was shown to lead to the elevation of intracellular calcium in DC [31], and this also occurs in macrophages (unpublished observations). Calcium is therefore a possible mediator of TLR-mediated necrosis.

Cells necrotic upon exposure to bacteria bound annexin V, indicating PS exposure. Although a number of receptors have been described that potentially recognize dead cells, PS seems to play an important role as ligand on the dead cell [1]. PS then is bound by at least one receptor on the phagocyte. A specialized PS receptor has been described that can mediate the uptake of an apoptotic cell [32]. Alternatively, a bridging molecule named milk fat globulin-E8 can bind PS on the apoptotic cell and can stimulate its uptake by macrophages [33]. Further candidate molecules are integrins and CD36 receptors [1].

Alternatively, it would also appear conceivable that annexin V binds ‘non-specifically’ to the plasma membrane of dead cells. Such ’stickiness’ could confer non-specific binding of the dead cell to the DC, which might again signal uptake. Upon permeabilization of the plasma membrane, annexin V may also diffuse into the cells and stain the membranes from the inside.

That apoptotic cells ‘go quietly’ and necrotic cells cause inflammation is a long-held view that has over recent years been substantiated on a cellular and molecular level. Necrotic cells but not apoptotic cells were able to activate DC in one study [34], although this was not reproduced by others [35]. Proteins (perhaps heat-shock proteins [36]) and the cellular DNA [37] have been implicated as factors from necrotic cells that can stimulate DC. Given the situation that large numbers of dead cells are generated and die by apoptosis, it is probably important that the ingesting phagocytes are not stimulated by the apoptotic material. However, when whole bacteria are present (as during bacteria-induced necrosis) it may well be argued that stimulation of DC by cellular material released from necrotic cells would be irrelevant as bacterial molecules will already have exerted their strong stimulatory effect on DC.

Our data suggest that the mechanism of necrosis induction by bacteria is part of the cellular response of phagocytes to contact to bacteria or bacterial components. As the dead cells are efficiently taken up by DC, bacterial antigens will end up in a potent antigen-presenting cell, and this process may thus impact on the generation of an adaptive immune response. That necrosis can be triggered by defined signals appears clear and means that the organism will have to deal with it. Whether beneficial or not, necrosis may well affect the organism, for instance through a modulation of the immune response.

4 Materials and methods

4.1 Cell lines, bacteria and stimulation of macrophages

RAW264.7 mouse macrophages were cultured in Low-Tox Click's RPMI 1640 (Biochrom, Berlin, Germany) supplemented with 10% FCS, 50 μM 2-mercaptoethanol and antibiotics (100 IU/ml penicillin G and 100 IU/ml streptomycin sulfate). The immortalized DC cell line D2SC/1 [38] was maintained under the same conditions. RAW264.7 cells were grown in non-culture-coated petri dishes, D2SC/1 in tissue culture dishes, and only for experiments seeded into 12-well culture-coated plates. *Escherichia coli* K12 strain DH5α was grown overnight at 37°C with shaking. Clinical isolates from various bacterial species were grown under the same conditions and used the same way. Cells were collected, passed through a 5-μM disposable filter and resuspended in PBS to an optical density of 2 at 600 nm. For heat inactivation, bacteria were then incubated for 30 min at 65°C, cooled to room temperature and used for experiments. In some experiments, RAW264.7 cells were stimulated with LPS (1 μg/ml), Pam$_3$Cys-pentapeptide (1 μg/ml) or CpG-DNA (ODN 1668 [39], 1 μM).

4.2 Generation of mouse BMDM

For generation of BMDM, MyD88$^{-/-}$ and vav-Bcl-2-transgenic mice on a C57BL/6 background (generously provided by Dr. S. Akira and Dr. J. M. Adams) were used. As wild-type controls, age-matched C57BL/6 mice were used for the MyD88$^{-/-}$ and vav-Bcl-2 transgenic mice, and 129/C57BL/6 wild-type mice for the TLR2$^{-/-}$TLR4$^{-/-}$mice. BMDM were grown according to standard protocols. Briefly, mouse bone marrow was harvested by rinsing the femora and tibiae. Bone marrow cells (2×10^7/10 ml) in a non-culture-coated petri dish were cultured in complete medium supplemented with 10 ng/ml recombinant mouse M-CSF (R&D Systems, Wiesbaden, Germany). On day 3, another 10 ng/ml M-CSF was added. Adherent cells were harvested on day 8 or 9 by accutase treatment according to the manufacturer's protocol (PAA Laboratories, Cöelbe, Germany), seeded in 12-well cell culture plates (2×10^5 cells/well), and used after overnight culture for experiments as described for RAW264.7 cells.

4.3 Generation of BMDDC

BMDDC were differentiated from bone marrow according to standard protocols. Bone marrow cells (5×10^6) were cultured in medium supplemented with supernatant from a GM-CSF-producing cell line [40] containing ~4 ng/ml GM-CSF. On day 3 of culture, another 10 ml of medium containing GM-CSF was added. On day 6 or 7, cells were harvested and used for experiments.

4.4 Co-culture of macrophages and bacteria

RAW264.7 cells (5×10^5) were placed into a 5-ml tube in 2.5 ml complete medium. Bacterial suspension (250 μl) was added and cells were cultured for 1 h at 37°C. Cells were then washed once with PBS, seeded in 12-well culture plates in 1 ml complete medium and cultured for the indicated periods of time.

4.5 Cell death assays

Cell death was measured as loss of membrane integrity by staining of cells with eosin or PI. Cells were harvested by pipetting, washed once with PBS, stained with eosin, and at least 300 cells per sample were counted by light microscopy. When using primary macrophages, and also in some experiments with RAW264.7 cells, cells were harvested by accutase treatment to avoid vigorous pipetting. In some experiments, cells were seeded onto coverslips, and eosin uptake was determined in cells without further manipulation. Alternatively, cells were stained with PI in PBS and analyzed by flow cytometry. For assessment of nuclear morphology, cells were stained with Hoechst dye for 30 min at 37°C, harvested and scored as apoptotic or non-apoptotic in UV light under a fluorescence microscope. At least 300 cells were counted per sample. For electron microscopy, cells were harvested after the appropriate treatment, fixed, and pictures were taken with a Zeiss EM 10CR electron microscope.

4.6 Annexin V/PI staining

After the indicated periods of time of co-incubation with bacteria, RAW264.7 cells were harvested, washed with PBS and transferred into 1.2-ml FACS microtubes in a volume of 100 μl annexin V binding buffer. Annexin V-FITC (5 μl; PharMingen, Hamburg, Germany) and PI (5 μg/ml; Sigma-Aldrich, Munich, Germany) were added and the tubes were incubated for 15 min at 4°C in the dark. Within 30 min cells were analyzed by flow cytometry.

4.7 Fluorescent labeling of cells

Before exposure to bacteria, RAW264.7 cells were stained with the green fluorescent cell linker PKH67, and D2SC/1 cells were stained with the red fluorescent cell linker PKH26 (Sigma), according to the manufacturer's instructions. To identify BMDDC, cultures containing BMDDC and RAW264.7 cells were washed in PBS containing 2% FCS and incubated for 30 min on ice with FcγRII/III antibody (PharMingen) to block unspecific antibody binding. Then cells were incubated for another 30 min with a PE-labeled anti-CD11c antibody (PharMingen). Cells were then washed twice with PBS and fixed with 2% formaldehyde.

4.8 Assay for uptake of macrophages by DC

DC (2.5×10^5 cells/well red-stained D2SC/1 or unstained BMDDC) were seeded into 12-well plates in 1 ml complete medium. Green-stained RAW264.7 cells (5×10^5) were exposed to *E. coli* bacteria as described above. After the indicated periods of time, macrophages were harvested and added (in a volume of 50 μl medium) to the DC into the 12-well plate. After 2 h of co-culture, cells were collected. Uptake was measured by flow cytometry in a FACSCalibur (BD Biosciences, Mountain View, CA). For microscopy studies, D2SC/1 cells and RAW264.7 cells were initially seeded onto coverslips, fixed after 2 h of co-incubation with 2% formaldehyde and analyzed with a Zeiss confocal microscope.

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